A Virus-Induced Gene Silencing Screen Identifies a Role for *Thylakoid Formation1* in *Pseudomonas syringae* pv *tomato* Symptom Development in Tomato and Arabidopsis[1][W][OA]

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Pseudomonas syringae pv *tomato* DC3000 (*Pst* DC3000), which causes disease in tomato (*Solanum lycopersicum*) and Arabidopsis (*Arabidopsis thaliana*), produces coronatine (COR), a non-host-specific phytotoxin. COR, which functions as a jasmonate mimic, is required for full virulence of *Pst* DC3000 and for the induction of chlorosis in host plants. Previous genetic screens based on insensitivity to COR and/or methyl jasmonate identified several potential targets for COR and methyl jasmonate. In this study, we utilized *Nicotiana benthamiana* and virus-induced gene silencing to individually reduce the expression of over 4,000 genes. The silenced lines of *N. benthamiana* were then screened for altered responses to purified COR. Using this forward genetics approach, several genes were identified with altered responses to COR. These were designated as *ALC* (for altered COR response) genes. When silenced, one of the identified genes, *ALC1*, produced a hypersensitive/necrosis-like phenotype upon COR application in a *Coronatine-Insensitive1* (*COI1*)-dependent manner. To understand the involvement of *ALC1* during the *Pst* DC3000-host interaction, we used the nucleotide sequence of *ALC1* and identified its ortholog in Arabidopsis (*Thylakoid Formation1* [*THF1*]) and tomato (*SlALC1*). In pathogenicity assays performed on Arabidopsis *thf1* mutant and *SlALC1*-silenced tomato plants, *Pst* DC3000 induced accelerated coalescing necrotic lesions. Furthermore, we showed that COR affects ALC1 localization in chloroplasts in a *COI1*-dependent manner. In conclusion, our results show that the virus-induced gene silencing-based forward genetic screen has the potential to identify new players in COR signaling and disease-associated necrotic cell death.

In nature, plants come in contact with numerous microbes that are potential pathogens. Active plant defense mechanisms, in general, involve a complex network of three genetically distinct signaling pathways, known as the salicylic acid (SA), jasmonic acid (JA), and ethylene pathways (Kunkel and Brooks, 2002; Glazebrook, 2005). Pathogens, in turn, have coevolved by developing mechanisms that suppress plant defense pathways by secreting virulence factors. Several pathovars of *Pseudomonas syringae* produce phytotoxins. In plants, these phytotoxins generally induce chlorosis (e.g. coronatine [COR], phaseolotoxin, and tabtoxin; Mitchell, 1976; Gnanamanickam et al., 1982; Levi and Durbin, 1986) or necrosis (e.g. syringomycin and syringopeptin; Paynter and Alconero, 1979; Iacobellis et al., 1992). Bacterial toxins act as virulence factors and contribute to increased disease severity by facilitating bacterial movement in planta (Patil et al., 1974), lesion size (Bender et al., 1987; Xu and Gross, 1988), pathogen multiplication (Bender et al., 1987; Feys et al., 1994; Mittal and Davis, 1995), and suppression of plant defense (Uppalapati et al., 2007, 2008).

COR, a phytotoxin produced by *P. syringae* pv *tomato* DC3000 (*Pst* DC3000), is induced in the presence of the plant host metabolites such as malic, citric, shikimic, and quinic acids, which are present in leaf extracts and apoplastic fluids of tomato (*Solanum lycopersicum*; Li et al., 1998). COR contributes to the virulence of *Pst* DC3000 in Arabidopsis (*Arabidopsis thaliana*), tomato,

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collard (*Brassica oleracea* var *viridis*), and turnip (*Brassica rapa* var *utilis*; Brooks et al., 2004; Elizabeth and Bender, 2007; Uppalapati et al., 2007). It has been shown that COR has structural and functional resemblance to 12-oxo-phytodienoic acid, methyl jasmonate (MeJA), and related derivatives known as the jasmonates (JAs; Feys et al., 1994; Weiler et al., 1994; Uppalapati et al., 2005). MeJA is a plant growth hormone that plays a key role in plant defense response to biotic and abiotic stress (Howe et al., 1996; McConn et al., 1997; Vijayan et al., 1998; Truman et al., 2007).

During a compatible interaction with a host, *Pst* DC3000 infection results in the activation of the JA signaling pathway (Zhao et al., 2003; Laurie-Berry et al., 2006; Uppalapati et al., 2007). This causes the suppression of the SA pathway owing to its antagonistic relation with the JA pathway (Kloek et al., 2001; Kunkel and Brooks, 2002; Zhao et al., 2003; Uppalapati et al., 2007). The suppression of the SA pathway during the *Pst* DC3000-host interaction is thought to be caused by COR, which functions as a molecular mimic of JAs (Feys et al., 1994; Bender et al., 1999; Staswick and Tiryaki, 2004).

Pst DC3000 causes disease on several plant species including tomato and Arabidopsis. A typical symptom on tomato leaves is bacterial speck, which includes necrosis surrounded by a chlorotic halo (Mittal and Davis, 1995; Zhao et al., 2003). In Arabidopsis, the infected area exhibits water-soaked lesions accompanied by diffused chlorosis (Mittal and Davis, 1995; Brooks et al., 2004). *Pst* DC3000 infection also causes chlorosis in other plants belonging to the Brassicaceae family, such as collard and turnip (Elizabeth and Bender, 2007). In addition to chlorosis, *Pst* DC3000-infected collard plants exhibit water-soaked lesions and anthocyanin, suggesting that *Pst* DC3000 elicits unique responses in different plants. Studies have shown that tomato plants inoculated with a COR-defective mutant of *Pst* DC3000 did not develop typical chlorotic symptoms; furthermore, COR contributed to pathogen fitness and disease development in a SA-independent manner (Uppalapati et al., 2007). Tomato leaf tissues treated with purified COR show chlorosis (Gnanamanickam et al., 1982; Uppalapati et al., 2005, 2007). Unlike tomato, purified COR does not elicit chlorosis on Arabidopsis leaves (Bent et al., 1992; Mach et al., 2001). However, in Arabidopsis, COR is required for full disease symptom development and pathogen fitness in a SA-dependent manner (Kloek et al., 2001; Brooks et al., 2004). These results suggest that COR functions as an important virulence factor in tomato and Arabidopsis, although it functions differently in these hosts.

More recently, we have demonstrated a role for COR-induced effects on photosynthetic machinery and reactive oxygen species (ROS) in modulating necrotic cell death during bacterial speck disease of tomato (Ishiga et al., 2009a). Despite our present understanding of COR function, it is not clear how chlorosis impacts or benefits pathogen virulence. Furthermore, the identity of host molecular targets for COR and the downstream signaling cascades that ensue are not well understood. Based on similarities between COR and JAs in terms of structure and function (Feys et al., 1994; Uppalapati et al., 2005), it seems likely that COR and JA interact with at least one common host receptor (Katsir et al., 2008). Thus, in addition to furthering our understanding of disease development, studies aimed at understanding the molecular mechanism of COR may provide information on JA-mediated plant defense.

In an effort to identify plant proteins that are the molecular targets of COR, we used a tobacco rattle virus (TRV)-based virus-induced gene silencing (VIGS) as a fast-forward genetics tool (Liu et al., 2001a, 2001b; Anand et al., 2007) to screen a *Nicotiana benthamiana* cDNA library for genes that are involved in the response to COR. We identified a *N. benthamiana* gene, *ALC1* (for altered COR response), that when silenced displayed an unexpected hypersensitive/necrosis-like phenotype rather than a typical chlorotic phenotype in response to COR application. *ALC1* has homology to an Arabidopsis gene, *Thylakoid Formation1* (*THF1*; Wang et al., 2004). The pathogenicity assays performed in this study indicate that loss of *ALC1/THF1* leads to accelerated cell death in response to *Pst* DC3000 infection in both tomato and Arabidopsis.

RESULTS

Application of Purified COR on *N. benthamiana* Leaves Results in Chlorosis

Unlike tomato, the efficiency of VIGS is quite uniform in *N. benthamiana*; therefore, this host is suitable for large-scale fast-forward screening studies (Lu et al., 2003; del Pozo et al., 2004; Anand et al., 2007). Purified COR, when spotted onto *N. benthamiana* leaves at different concentrations (0.002–2 nM in 2-μL aliquots), produced a visible, confined chlorosis in a dose-dependent manner (Fig. 1A). The 0.2 nM concentration, which produces a confined chlorosis phenotype, was used for screening (Fig. 1B). Based on these results, we concluded that a VIGS-based approach in *N. benthamiana* was suitable for screening silenced plants that show an altered chlorosis phenotype upon COR application; therefore, this approach was used to identify genes involved in COR-mediated signaling.

VIGS-Based Screening Identifies Several *N. benthamiana* Genes with Altered COR-Induced Response

To identify plant genes that are involved in COR signaling, a normalized *N. benthamiana* cDNA library cloned in pTRV2 and transformed into *Agrobacterium tumefaciens* GV2260 was used (Anand et al., 2007). *N. benthamiana* plants were individually inoculated with *Agrobacterium* containing TRV2 cDNA clones, along

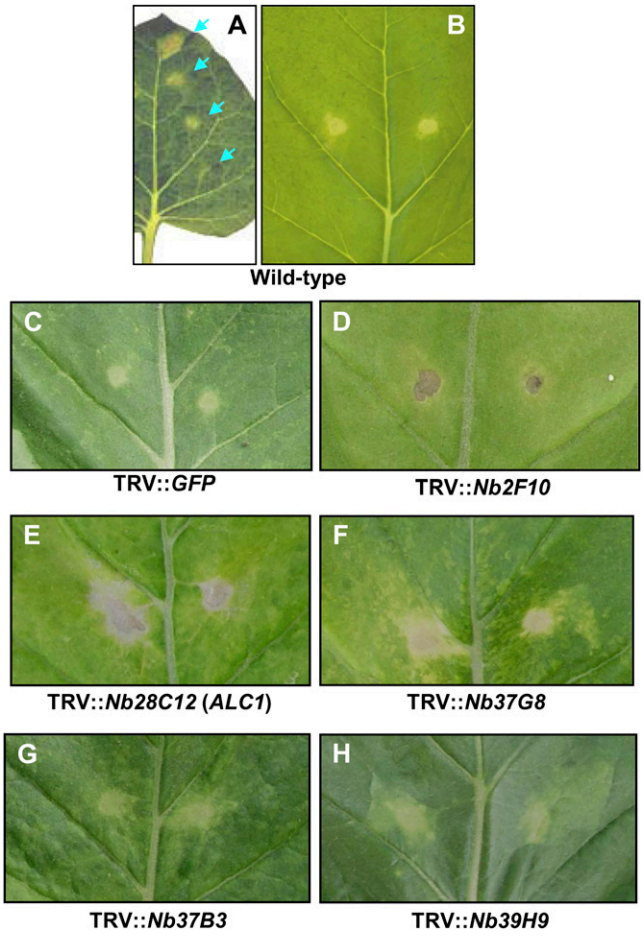

Figure 1. COR induces visible chlorosis on *N. benthamiana* leaves. A, Purified COR when applied to *N. benthamiana* leaves in 2-μL aliquots (arrows) at different concentrations (0.002, 0.02, 0.2, and 2 nM; from bottom to top part of the leaf); a visible chlorotic zone was observed at 4 dpi. B, Chlorosis induced by 0.2 nM COR (per inoculation site) on *N. benthamiana*. C to H, Responses of silenced lines of *N. benthamiana* leaves to 2 nM COR. COR was applied 3 weeks post agroinoculation to silenced lines of *N. benthamiana*. In response to COR, leaves of silenced lines showed necrosis (D) or a necrosis-like phenotype (E and F). Some lines exhibited an enhanced chlorosis (G and H). Photographs were taken 7 d after COR application.

with an *Agrobacterium* strain containing TRV1, in duplicate, to silence their corresponding genes in *N. benthamiana* (Anand et al., 2007). Two weeks after TRV inoculation, COR (0.2 nM) was spotted on the leaves of silenced plants, and the phenotypes were recorded 5 to 7 d after COR application.

After screening approximately 4,000 cDNA clones, we identified five nonredundant cDNA clones that when silenced resulted in the ALC phenotype upon exogenous application of COR (Fig. 1, D–H). The application of COR to wild-type (Fig. 1B) or TRV::*GFP*-inoculated (mock control [Fig. 1C]; the *GFP* sequence does not have any homology to plant DNA and therefore will not cause gene silencing) *N. benthamiana* plants resulted in a defined chlorotic halo. The silenced lines with the ALC phenotype exhibited either hypersensitive response (HR)-like necrosis (Fig. 1, D–F) or increased chlorosis (Fig. 1, G and H) in response to COR. For example, *Nb28C12*- and *Nb2F10*-silenced plants exhibited a severe HR-like necrosis that is confined or extended beyond the area of COR application (Fig. 1, D and E), whereas *Nb37G8*-silenced lines (Fig. 1F) displayed a less severe necrotic phenotype. *Nb37B3*- and *Nb39H9*-silenced plants displayed slightly diffused chlorosis (Fig. 1, G and H). In brief, the VIGS-mediated fast-forward genetic approach proved to be an effective tool to identify plant genes involved in COR-induced responses in *N. benthamiana*.

ALC1 Is an Ortholog of a Gene Encoding THF1 Protein

Since the silencing of *Nb28C12* produced the most dramatic phenotype in response to COR application, the *Nb28C12*-silenced line was selected for further study. The phenotype of *Nb28C12*-silenced plants was similar to that of control plants (TRV::*GFP*) up to 4 weeks postsilencing. However, after the 5th week, leaves of the *Nb28C12*-silenced plants turned slightly pale green in color (Supplemental Fig. S1A). At 5 weeks postinoculation, portions of some leaves showed a variegated gray coloration (Supplemental Fig. S1A). To confirm the suppression of the *Nb28C12* mRNA in the silenced plants, quantitative real-time reverse transcription (qRT)-PCR was performed. The relative expression ratio of the *Nb28C12* gene in the silenced line versus the control plant was 0.023, indicating that the transcript level was approximately 40-fold lower than in the control plant (Supplemental Fig. S2A).

We termed *Nb28C12* as *ALC1*. The sequence information was then analyzed to predict gene function. A BLASTn search against The Institute for Genomic Research database using the *NbALC1* sequence revealed 77% identity to an Arabidopsis gene named *THF1* (GenBank accession no. AY899908); 92% identity to a potato (*Solanum tuberosum*) gene that encodes a light-regulated chloroplast-localized protein (*StTHF1*; GenBank accession no. AY342161); 81% identity to a rice (*Oryza sativa*) gene encoding inositol phosphatase-like protein (GenBank accession no. AY224446); and 79% identity to a wheat (*Triticum aestivum*) gene encoding Ptr ToxA-binding protein (GenBank accession no. AY377991). To facilitate a more comprehensive comparative analysis of *ALC1*, we designed a primer pair to clone the full-length *ALC1* gene based on the sequence of a tobacco (*Nicotiana tabacum*) ortholog (GenBank accession no. TC10126). The cloned gene was then sequenced and the translated amino acid sequence was aligned with orthologous plant protein sequences using ClustalW (http://www.ebi.ac.uk/clustalw/). As shown in Supplemental Figure S3A, *N. benthamiana* ALC1 shows strong sequence identity with orthologs from other species. *N. benthamiana* ALC1 also displays a higher degree of evolutionary relatedness with the tobacco ortholog when compared

with other plant orthologs that were analyzed (Supplemental Fig. S3B).

The silencing of *Nb28C12* resulted in a necrotic phenotype upon COR treatment without a visible chlorosis (Fig. 1D). To test if the COR-induced altered necrotic phenotype is associated with chlorophyll degradation, we quantified chlorophyll *a* (Chl *a*) levels in vector control and *NbALC1*-silenced plants. Application of COR resulted in greater reduction of Chl *a* levels in *NbALC1*-silenced plants (65% reduction over mock treatment) when compared with the vector control (47% reduction over mock treatment; Fig. 2). These results suggested that the necrotic phenotype in *NbALC1*-silenced plants is associated with COR-induced chlorophyll degradation.

ALC1-Silenced Tomato Plants Show Accelerated Necrosis in Response to COR in a *COI1*-Dependent Manner

To understand the potential role of *ALC1* in *Pst* DC3000-mediated disease development, we used the host plant tomato. Using primers specific to the tomato ortholog (The Institute for Genomic Research accession no. TC162724), we cloned the tomato *ALC1* (see "Materials and Methods"), which we refer to as *SlALC1*. A fragment of *SlALC1* was subcloned into pTRV2 and used for VIGS in tomato. qRT-PCR analysis of silenced tomato plants revealed that the expression of *SlALC1* in the silenced plant was about 5-fold lower than control plants (Supplemental Fig. S2B). However, the majority of the leaves in *ALC1*-silenced tomatoes did not exhibit any obvious phenotype (Supplemental Fig. S1B, middle), and some of the older leaves (6 weeks after TRV inoculation) showed variegated coloration on the leaf surface (Supplemental Fig. S1B, right). When purified COR (2 nM) was exogenously applied, the silenced line showed a necrosis-like phenotype, whereas the control plants showed a typical confined chlorosis as expected (Fig. 3A).

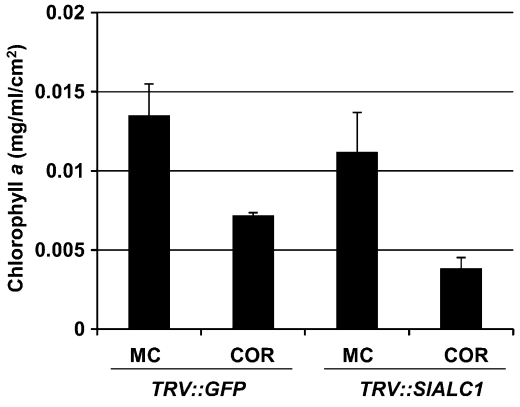

Figure 2. COR induces chlorophyll degradation in vector control (TRV::*GFP*) and *NbALC1*-silenced (TRV::*NbALC1*) *N. benthamiana* plants. Two leaf discs (0.78 cm^2) were collected 6 dpi with 2 nM COR and were analyzed for Chl *a* content to monitor chlorosis. MC, Mock control.

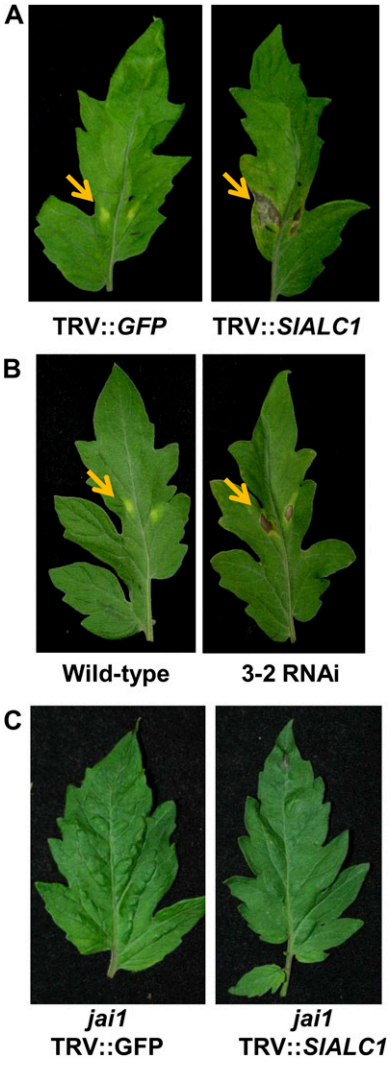

Figure 3. Silencing of *ALC1* in tomato displays a necrosis-like phenotype in a *COI1/JAI1*-dependent manner in response to (2 nM) COR at 7 d posttreatment. Transient (TRV::*SlALC1*; A) and stably silenced (3-2; B) tomato (cv Glamour) showed necrosis in response to COR, whereas vector control (TRV::*GFP*) and transiently silenced (TRV::*SlALC1*) *jai1* mutants (cv Castlemart; C) showed no chlorosis or necrosis. Photographs were taken 7 d after COR application. Arrows indicate COR response.

Although we were fairly successful in transiently silencing the tomato *ALC1* gene, a uniform and pronounced silencing, such as that observed in *N. benthamiana*, is often difficult to achieve in tomato (Ekengren et al., 2003; Ryu et al., 2004). Therefore, to achieve stable and uniform silencing and to confirm the necrosis phenotype induced by COR and *Pst* DC3000 on *SlALC1*-silenced tomato lines, we generated *SlALC1* RNA interference (RNAi) lines. We assayed three independent transgenic RNAi lines, and all responded similarly to COR application and *Pst* DC3000 infection. Here, we discuss the data for one of the transgenic lines, 3-2. Results obtained from

qRT-PCR indicated that the transcript levels of *SlALC1* were 20-fold less in RNAi line 3-2 when compared with wild-type tomato plants (Supplemental Fig. S2B). When COR (2 nM) was spotted on the leaves of the RNAi line 3-2, necrosis appeared 5 d post inoculation (dpi; Fig. 3B, right).

COI1/Jasmonic Acid Insensitive1 (JAI1), an F-box protein, is shown to be required for COR signaling in tomato and Arabidopsis (Feys et al., 1994; Zhao et al., 2003; Katsir et al., 2008). Using VIGS, we transiently silenced *SlALC1* in *jai1* mutant tomato plants (Supplemental Fig. S4) to know whether the necrosis we observed in *SlALC1*-silenced tomato plants upon COR treatment (Fig. 3, A and B) is *COI1/JAI1* dependent. When purified COR (2 nM) was exogenously applied, the *SlALC1*-silenced *jai1* plants showed no visible necrosis (Fig. 3C). Furthermore, silencing of *COI1* and *NbALC1* in *N. benthamiana* abolished *ALC1*-mediated COR-induced necrosis (Supplemental Fig. S9). These results confirmed that *COI1/JAI1* is required for the altered chlorosis phenotype in *ALC1*-silenced tomato and *N. benthamiana* plants.

ALC1-Silenced Tomato Plants Show Accelerated Necrosis in Response to *Pst* DC3000

To study the influence of *SlALC1* on the virulence of *Pst* DC3000 in tomato, *SlALC1*-silenced and control (TRV::*GFP*) tomato plants were spray inoculated with *Pst* DC3000 (5×10^6 colony-forming units [cfu] mL^{-1}). Control (TRV::*GFP*) plants showed typical bacterial speck symptoms at 5 dpi, which consisted of necrotic lesions surrounded by chlorotic halo (Fig. 4A, left). At 5 dpi, the leaves of *SlALC1*-silenced plants showed necrosis with little or no chlorosis (Fig. 4A, right). At 10 dpi, the necrosis observed on the silenced plants was severe (Fig. 4A, right). Similarly, when line 3-2 was spray inoculated with *Pst* DC3000 (5×10^6 cfu mL^{-1}), leaves developed severe coalescing necrotic lesions without the visible chlorotic halos surrounding the necrotic lesions as seen on the wild type (Fig. 4B, right), further confirming that *ALC1* plays a role in bacterial speck symptom development.

To determine if the severe necrosis could be explained by a higher amount of bacterial growth in the silenced lines, the population of *Pst* DC3000 was monitored at 1, 3, and 5 dpi. Interestingly, the bacterial population on the transiently or stably silenced *SlALC1* plants was not significantly different from that on the inoculated control plants (Fig. 5). These results suggest that silencing of *ALC1* does not have a significant effect on the growth of the bacteria in tomato plants during the early stage of infection.

The Arabidopsis *thf1* Mutant Displays Severe Necrosis upon *Pst* DC3000 Inoculation

As mentioned above, *ALC1* is closely related to an Arabidopsis gene called *THF1* (Supplemental Fig. S3A). An Arabidopsis *thf1* mutant was previously identified and shown to have variegated leaves (Supplemental Fig. S1C) that lacked normal chloroplast development in the variegated regions (Wang et al., 2004). We obtained the Arabidopsis *thf1* mutant and reconfirmed the mutation by ascertaining the insertion of T-DNA in *THF1* (data not shown). Unlike *N. benthamiana* and tomato, exogenous application of COR on Arabidopsis leaves does not induce chlorosis. Instead, Arabidopsis seedlings are shown to respond to COR by displaying a strong purple hue indicative of anthocyanin accumulation (Bent et al., 1992; Laurie-Berry et al., 2006). To further characterize the *thf1* mutant line, we germinated seeds of Arabidopsis ecotype Columbia (Col-0), the *thf1* mutant line, the complemented line, and the *THF1*-overexpressing line on half-strength Murashige and Skoog (MS) medium containing 2 nM COR (Laurie-Berry et al., 2006). As expected, Col-0 seedlings showed anthocyanin accumulation within 10 d after germination (Fig. 6A). Strikingly, the *thf1* mutant showed hypersensitivity to COR by displaying a severe growth defect and more anthocyanin accumulation than Col-0 (Fig. 4A). The growth phenotypes of the *thf1*-complemented and the overexpressing lines were similar to that of the wild type (Fig. 6A).

To determine whether *THF1* has an effect on *Pst* DC3000-induced disease symptoms on Arabidopsis, we dip inoculated (10^8 cfu mL^{-1}) or syringe infiltrated

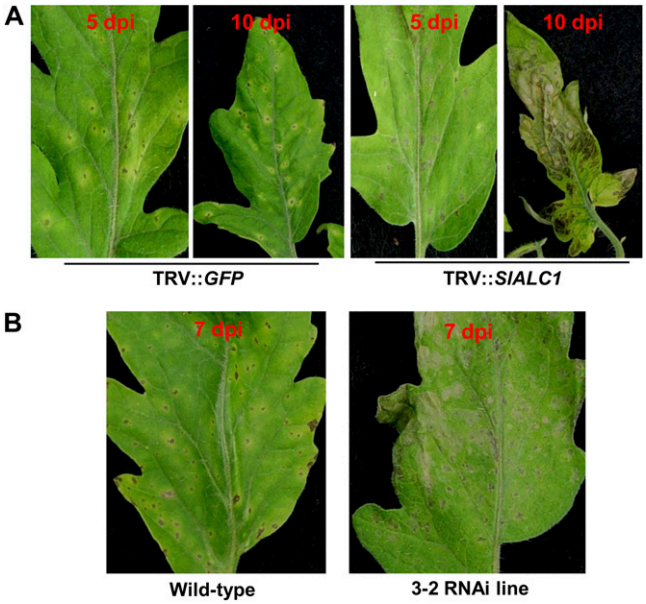

Figure 4. Response of *SlALC1*-silenced tomato lines to *Pst* DC3000. A, Response of control and transiently silenced tomato lines to *Pst* DC3000. *Pst* DC3000 (5×10^6 cfu mL^{-1}) was spray inoculated on control (*TRV::GFP*; left panels) and *SlALC1*-silenced (by VIGS; right panels) tomato plants. Photographs were taken 5 and 10 dpi. B, *SlALC1*-silenced transgenic RNAi line 3-2 and wild-type tomato plants were also spray inoculated with *Pst* DC3000. Photographs were taken 7 dpi.

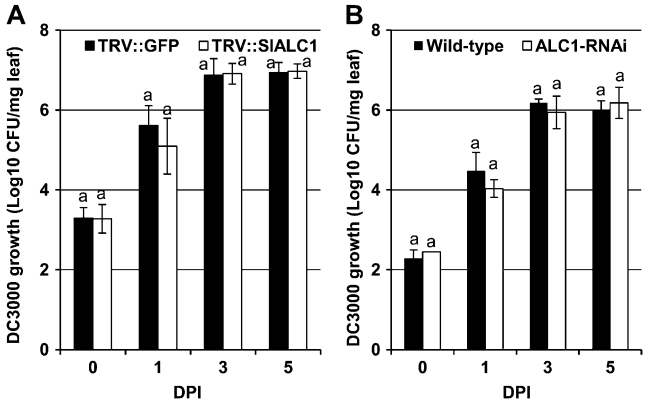

Figure 5. Silencing of *ALC1* in tomato has no effect on the growth of *Pst* DC3000. Leaf samples from *Pst* DC3000-inoculated *SlALC1*-silenced VIGS plants (A) and *SlALC1*-silenced RNAi line 3-2 (B) and their corresponding control plants (*TRV*::*GFP* and the wild type; described in Fig. 3) were collected at various days after inoculation and homogenized in water, and dilutions were plated onto King's B medium to determine cfu. Error bars represent SD. All experiments were repeated at least twice with several biological replicates, and the data shown are representative of the experiments. Growth measurements with the same letters showed no significant differences based on Fisher LSD values ($P < 0.005$).

(10^6 cfu mL^{-1}) the wild-type Col-0 and *thf1* mutant with *Pst* DC3000. As expected, Col-0 showed water-soaked necrotic lesions accompanied by chlorosis (Fig. 6B). However, the *thf1* mutant plants exhibited accelerated necrotic lesions without visible chlorosis (Fig. 6B). Complemented lines of the *thf1* mutant and *THF1*-overexpressing plants displayed disease symptoms similar to the wild-type Col-0 after inoculation with *Pst* DC3000 (Fig. 6B). Interestingly, when the growth of *Pst* DC3000 was monitored at 0, 1, 2, and 4 dpi, no significant fold differences in the bacterial growth were observed between the wild-type Col-0, the *thf1* mutant, the complemented line of *thf1*, and the *THF1* overexpression line (Fig. 7). However, unlike in tomato, *thf1* mutants supported slightly increased (1.5-fold) bacterial growth than the wild type (Fig. 7). These results suggest that THF1 does not significantly contribute to the pathogen growth in Arabidopsis, at least for the duration of time the bacterial growth was monitored.

Necrosis occurred much earlier on *Pst* DC3000-infected *thf1* leaves than on leaves of the wild-type Col-0 (data not shown). Therefore, we investigated whether the *thf1* mutant had a weaker defense response and was more susceptible to biotic and abiotic stress because of defects in thylakoid formation (Wang et al., 2004). To investigate this, leaves of Col-0 and the *thf1* mutant were infiltrated with two nonhost pathogens that do not infect Arabidopsis, *P. syringae* pv *tabaci* and *P. syringae* pv *glycinea*, and growth and symptoms were compared with a COR-producing *P. syringae* pv *maculicola*, which is pathogenic to Arabidopsis (Dong et al., 1991; Cuppels and Ainsworth, 1995; Mishina and Zeier, 2006). As expected, the population of *P. syringae* pv *maculicola* increased approximately 100-fold on both Col-0 and *thf1* leaves by 3 dpi; however, neither *P. syringae* pv *glycinea* nor *P. syringae* pv *tabaci* multiplied to a significant level on Col-0 or *thf1* plants (Supplemental Fig. S5A).

Figure 6. The Arabidopsis *thf1* mutant exhibits hypersensitivity in the presence of COR and produces necrosis with no chlorosis upon *Pst* DC3000 inoculation. A, Arabidopsis wild-type Col-0, the *thf1* mutant line, the complemented line of *thf1* (Comp), and the *THF1* overexpression line (35S-*Thf1*) were germinated on half-strength MS medium containing 2 nM COR (top row). These lines were also germinated on half-strength MS medium alone without COR (controls; bottom row). Photographs were taken 10 d after germination. B, Foliar parts of 4-week-old Arabidopsis lines mentioned above either dipped in a *Pst* DC3000 culture suspension (10^8 cfu mL^{-1}; top row) or infiltrated with 10^6 cfu mL^{-1} *Pst* DC3000 using a needleless syringe (bottom row). Photographs were taken 6 dpi.

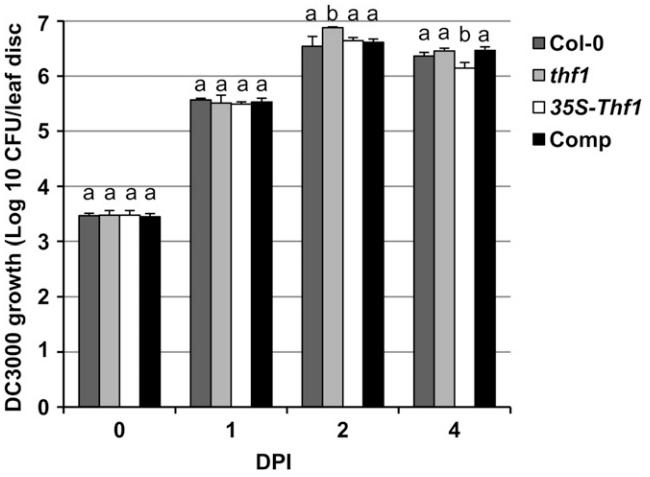

Figure 7. The mutation in Arabidopsis *THF1* has no effect on the growth of *Pst* DC3000. Arabidopsis leaves of the lines described in Figure 5 were syringe infiltrated with *Pst* DC3000 (10^6 cfu mL^{-1}), collected at intervals after inoculation, homogenized in water, and plated on KB medium to determine cfu. Error bars represent SD. The experiments were conducted at least three times, and the data shown are representative of each experiment. Growth measurements with the same letters showed no significant differences based on Fisher LSD values ($P < 0.005$). Comp, Complemented line of *thf1*.

Arabidopsis Col-0 and the *thf1* mutant were also monitored for symptom development in response to inoculation with *P. syringae* pvs *maculicola*, *glycinea*, and *tabaci* and the soft rot pathogen *Erwinia carotovora* subspecies *carotovora*. *P. syringae* pv *maculicola* induced chlorosis on Col-0 but not on the *thf1* mutant line (Supplemental Fig. S5B). Neither Col-0 nor *thf1* plants developed visible symptoms in response to *P. syringae* pvs *tabaci* or *glycinea* (Supplemental Fig. S5B). *E. carotovora* subspecies *carotovora* induced soft rot on both Col-0 and *thf1*, with no apparent difference in phenotypic response between the wild-type and the mutant line (Supplemental Fig. S5B). Infiltration of leaves with cell death-inducing agents such as NaCl (500 mM) or hydrogen peroxide (3%; Peart et al., 2002; Kang et al., 2004) caused similar cell death responses on both Col-0 and the *thf1* mutant line (data not shown). In the above experiments, only *P. syringae* pv *maculicola* induced a unique response on *thf1* when compared with Col-0. This response was similar to the one induced by *Pst* DC3000. These results indicate that the early necrotic cell death of infected leaves in the *thf1* mutant is specific to the COR-producing pathogens of Arabidopsis, *Pst* DC3000, and *P. syringae* pv *maculicola*.

The JA Pathway Appears Intact in Arabidopsis *thf1* Mutant Plants after *Pst* DC3000 Inoculation

COR functions as a mimic of JAs and mediates signaling via JA perception machinery in tomato and Arabidopsis (Feys et al., 1994; Zhao et al., 2003). Thus, it remained possible that the absence of chlorosis was due to disruption of the JA-dependent signaling pathway. Therefore, we used qRT-PCR to analyze transcript levels of *Lipoxygenase2* (*LOX2*) and *Plant Defensin1.2* (*PDF1.2*). Transcripts of *LOX2* and *PDF1.2* were induced in both Col-0 and *thf1* in response to *Pst* DC3000. Although expression of both genes was lower in the *thf1* mutant line, especially at 4 dpi, the JA pathway appears to be functional (Fig. 8, A and B) at the time points analyzed.

Chlorosis occurs due to the degradation of proteins in the chloroplast (Quirino et al., 2000), and the Arabidopsis *CORI1* gene (encoding chlorophyllase) is induced upon COR or MeJA application (Benedetti et al., 1998), resulting in chlorophyll degradation (Benedetti and Arruda, 2002). The lack of chlorosis in *thf1* could be due to repression of *CORI1* as a result of loss of *THF1* function. Thus, we analyzed *CORI1* transcript levels in *Pst* DC3000-inoculated Col-0 and *thf1* plants. *CORI1* expression in Col-0 and *thf1* was upregulated approximately 175-fold and approximately 75-fold, respectively, at 1 dpi (Fig. 8C). These results further suggest that the chlorophyllase activity and JA-dependent pathway are not severely affected in the *thf1* mutant. Although we did not notice any visible chlorosis in COR- or *Pst* DC3000-inoculated tissues (Fig. 6B), the COR-induced chlorophyllase activity suggests some degree of chlorophyll degradation in COR- or *Pst* DC3000-inoculated *thf1* plants. Similarly, significant levels of COR-induced chlorophyll degradation were observed in *NbALC1*-silenced plants (Fig. 2). Taken together, these results suggest that THF1 may be operating downstream of COR-induced JA signaling and chlorosis.

COR Affects the Localization of ALC1 in a *COI1*-Dependent Manner in *N. benthamiana* Plants

To determine if COR directly affected ALC1, we monitored the effect of COR treatment on the localization/stability of ALC1. An *Agrobacterium* strain containing a binary plasmid that included 35S::*ALC1* fused to *GFP* within its T-DNA was infiltrated into *N. benthamiana* plants as described in "Materials and Methods." COR was spotted on four to five marked regions on the *Agrobacterium*-infiltrated leaf, and leaf samples were monitored for localization of GFP-ALC1 at various time intervals after COR inoculation (Fig. 9). As reported previously for THF1, GFP-ALC1 localized to the chloroplast (Fig. 9A). Interestingly, within 4 h of COR application, ALC1 was destabilized/degraded, as shown by the loss of GFP fluorescence (Fig. 9A). ALC1 florescence was not detected even after 24 (Fig. 9A), 48, and 72 h (the time at which chlorosis is visible on the leaf; data not shown). It is also noteworthy that the destabilization/degradation of GFP-ALC1 is seen only at the site of application of COR and the nearby region, but the leaf areas away from the region of COR application remain unaffected even after 24 and 48 h (Supplemental Fig. S6). To rule out the possibility that

COR application is leading to alterations in chloroplast structure, therefore resulting in nonspecific effects on ALC1, we tested COR effects on GFP-RecA (Kohler et al., 1997), another chloroplast-localizing protein (Supplemental Fig. S7). Interestingly, COR application did not result in destabilization/degradation of GFP-RecA or GFP alone (Fig. 9C; Supplemental Fig. S7).

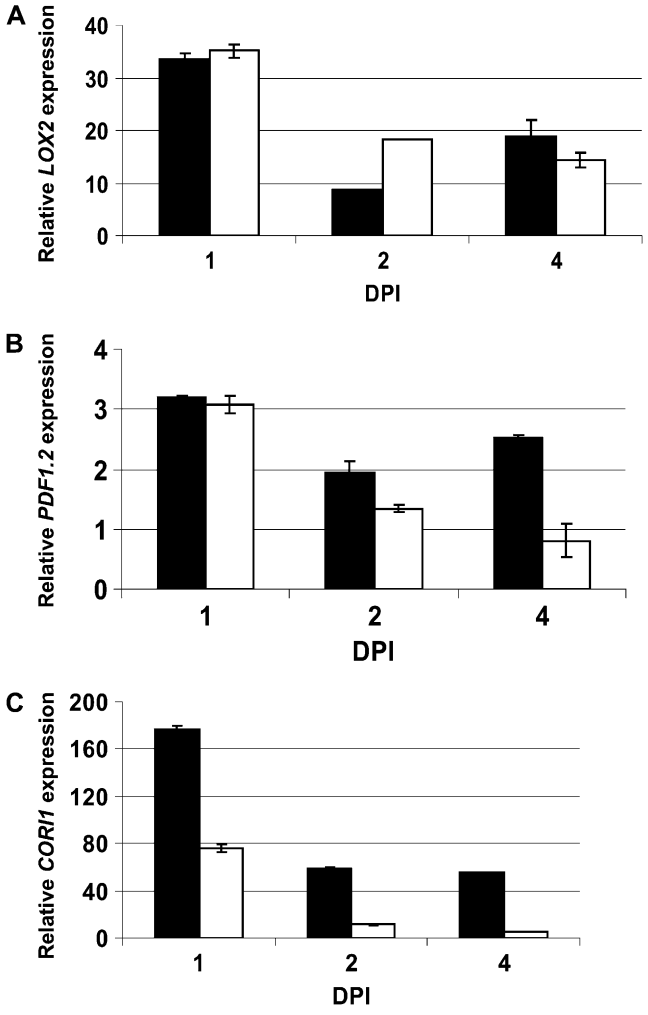

Figure 8. JA-dependent signaling in *thf1* mutants in response to *Pst* DC3000. Transcripts of *LOX2*, *PDF1.2*, and *CORI1* were quantified by real-time qRT-PCR in both wild-type Col-0 (black bars) and *thf1* mutant (white bars) after *Pst* DC3000 inoculation. Four-week-old Col-0 and the *thf1* mutant line were syringe infiltrated with either *Pst* DC3000 (10^6 cfu mL^{-1}) or buffer (mock control). Total RNA was extracted from the leaves of the infected plants collected 1, 2, and 4 dpi, and cDNA was synthesized for qRT-PCR analysis. The transcript levels were normalized against EF1α, which was used as an endogenous control as described by Pfaffl (2001). The transcript levels were quantified relative to the transcript levels on the mock control, which was assigned a value of 1. JA pathway genes (represented by *LOX2* and *PDF1.2*; A and B) and the chlorophyllase-encoding gene *CORI1* (C) were activated in *Pst* DC3000-infected Col-0 and the *thf1* mutant. All experiments were repeated at least three times. The data shown represent averages of three biological replicates and three technical replicates, with the SD values shown as error bars.

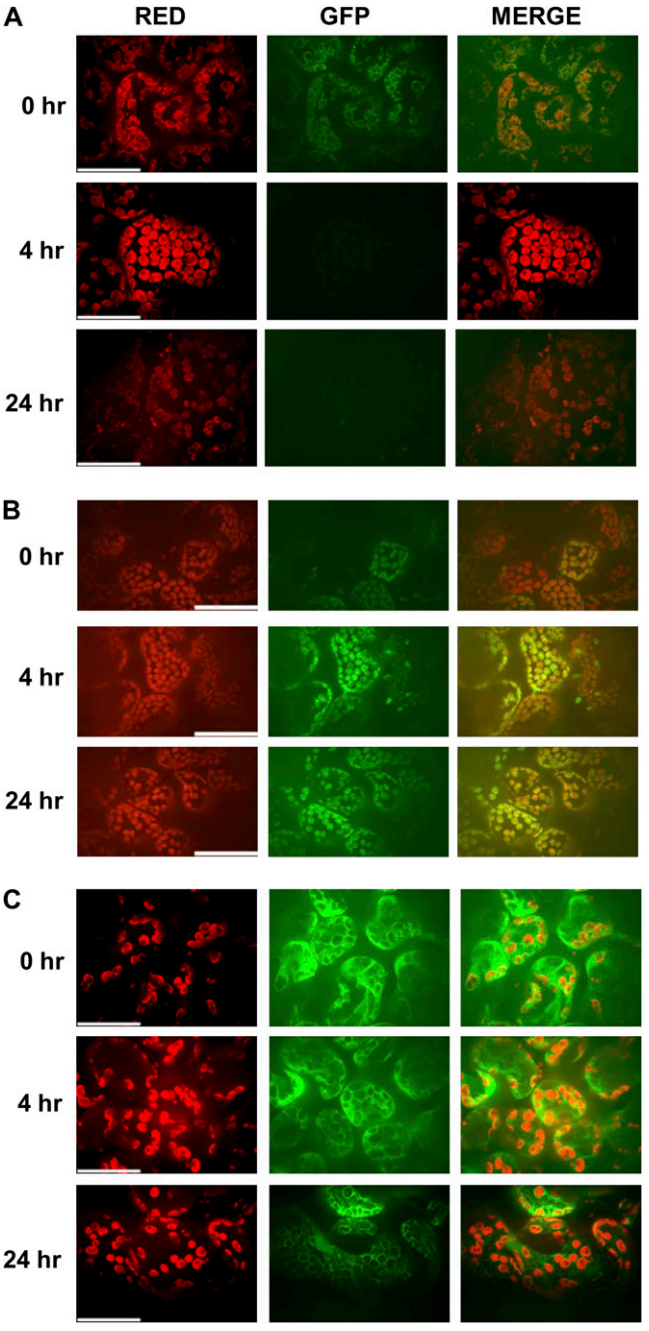

Figure 9. COI1-dependent effect of COR on localization or stability of ALC1in *N. benthamiana*. A and B, Localization of ALC1-GFP in the wild-type (A) and *COI1*-silenced (TRV::*COI1*; B) *N. benthamiana* leaves treated with COR was observed by fluorescence microscopy at 0, 4, and 24 h posttreatment. C, Localization of GFP in *N. benthamiana* leaves treated with COR. Red, Autofluorescence of the chloroplast by excitation at 647 nm and emission in the cyan/far-red channel; GFP, GFP fluorescence of the tagged protein by excitation at 488 nm and emission in the green channel. All images were magnified using a 63× water-immersion objective. Bars = 5 μm.

The *COI1*-dependent nature of COR-induced alterations in ALC1 localization was further confirmed in *COI1*-silenced *N. benthamiana* plants (Fig. 9B). Inter-

estingly, in *COI1*-silenced plants, an increased signal intensity of 35S::*ALC1* GFP was observed following COR application (Fig. 9B). It is not clear if this is due to lack of COR activity upon *COI1* silencing or a COI1-independent activity of COR on ALC1. Silencing of *COI1* did not affect the expression levels of 35S::*ALC1* GFP florescence (Supplemental Fig. S8). However, silencing of *COI1* abolished COR-induced destabilization/degradation of 35S::*ALC1* GFP florescence (Fig. 9B). Consistent with these results, *COI1* and *NbALC1* double-silenced plants showed no chlorosis/necrosis phenotypes upon COR application (Supplemental Fig. S9). Taken together, these results suggested that direct effects of COR or COR-induced effects on chloroplast/ALC1 directly alter ALC1 localization or stability in a *COI1*-dependent manner and therefore may affect its function.

DISCUSSION

Our observation that COR could induce chlorosis on *N. benthamiana* and our ability to do VIGS in *N. benthamiana* provided an excellent strategy to identify plant genes that play a role in COR signaling. Here, we have shown that loss of the *N. benthamiana* gene *NbALC1* and its orthologs, *SlALC1* in tomato and *AtTHF1* in Arabidopsis, results in a necrotic phenotype in response to COR and/or *Pst* DC3000. Spray inoculation of *Pst* DC3000 on *ALC1*-silenced tomato plants induced accelerated necrotic lesions without visible chlorosis on the majority of the leaves instead of typical bacterial speck symptoms with a chlorotic halo (Mittal and Davis, 1995; Zhao et al., 2003). Furthermore, necrosis spread beyond the region where COR was applied as early as 10 dpi, which is similar to the runaway cell death phenotype reported earlier in the Arabidopsis *lsd1* mutant (Fig. 2B; Jabs et al., 1996).

To determine the role of *ALC1* in the development of symptoms in response to COR or *Pst* DC3000, we chose Arabidopsis, since it is genetically tractable and is a host of *Pst* DC3000. The ortholog of *ALC1* in Arabidopsis, known as *THF1*, is a single-copy gene with no closely related sequences in the Arabidopsis genome (Wang et al., 2004). Expression of the Arabidopsis *THF1* gene is positively regulated by light, and the gene product is a chloroplast-localized protein. A T-DNA knockout mutant of *THF1* has been identified previously (Wang et al., 2004). The mutant line, *thf1*, is stunted and has variegated leaves. The chloroplasts in the *thf1* mutant contain shorter stacks of thylakoids in the green sector of the leaves and show accumulation of membrane vesicles but no thylakoids within the intact chloroplast membrane in the white sector of the leaves (Wang et al., 2004).

The exogenous application of COR (2 nM) on Arabidopsis leaves did not produce any chlorosis. However, consistent with earlier observations (Bent et al., 1992; Feys et al., 1994), our study showed that Arabidopsis seedlings grown on MS medium supplemented with COR accumulated anthocyanin. Interestingly, anthocyanin accumulation was significantly elevated in the *thf1* mutant as compared with the wild-type plants. Therefore, the *THF1* mutation has a positive effect on anthocyanin accumulation in Arabidopsis. These results are consistent with an earlier observation that COR induces different phenotypes in Arabidopsis and tomato (Mach et al., 2001; Uppalapati et al., 2005).

Similar to *ALC1*-silenced tomato plants, inoculation of *thf1* with *Pst* DC3000 did not result in a typical chlorotic phenotype around the water-soaked lesion. Analysis of the *Pst* DC3000 population dynamics in both *ALC1*-silenced tomato plants and the *thf1* mutant line indicated that there was no difference in the bacterial population dynamics when compared with wild-type plants. Interestingly, *Pst* DC3000-inoculated leaves of *ALC1*-silenced tomato and the Arabidopsis *thf1* mutant line died earlier than corresponding wild-type lines. These results suggested that *Pst* DC3000 may tightly regulate the chloroplast homeostasis and the levels of THF1 for controlled necrosis during infection to help pathogen dissemination and spread. We have recently demonstrated that COR-induced effects on the photosynthetic machinery result in light-dependent ROS generation in tomato seedlings (Ishiga et al., 2009a, 2009b). We speculate that in COR-treated or *Pst* DC3000-inoculated *ALC1*-silenced tomato and in the *Pst* DC3000-inoculated Arabidopsis *thf1* mutant, the necrosis/HR-like cell death phenotype may appear because the effect of ROS supersedes the detoxifying capacity of antioxidants. Current evidence suggests that *THF1* may have multiple functions in the biogenesis of PSII and sugar signaling (Keren et al., 2005; Huang et al., 2006). *THF1* was also identified as an interactor of the G-protein GPA1 in Arabidopsis and was shown to play a role in far-red irradiation-preconditioned cell death (Huang et al., 2006; Wei et al., 2008). Our results using GFP-tagged ALC1 suggest that COR has direct effects on ALC1 and might target ALC1 to degradation in a *COI1*-dependent manner (Fig. 8). Based on these results, it is tempting to speculate if ALC1/THF1, localized in the chloroplast membrane, may directly interact with COR. Interestingly, a chloroplast protein in wheat, ToxABP1 (an ortholog of THF1; Supplemental Fig. S2), directly interacts with the *Pyrenophora tritici-repentis* protein ToxA (Manning et al., 2007). ToxA is a determinant of virulence in *P. tritici-repentis*, a pathogen that causes the tan spot of wheat. Therefore, it is possible that COR may interact directly with ALC1/THF1 during the *Pst*-host interactions. Thus, we speculate that ALC1 and THF1 (Wang et al., 2004), which is localized on the chloroplast, is somehow involved in the maintenance of ROS homeostasis and, therefore, that Arabidopsis *thf1* mutants and *ALC1*-silenced tomato leaves are more sensitive to COR/pathogen-induced ROS, leading to accelerated cell death (necrosis) in tomato and Arabidopsis.

In conclusion, we have developed a VIGS-based forward genetic screen for identification of new targets involved in COR signaling. Although we set out to identify genes involved in COR-induced chlorosis, we identified a gene, *THF1*, that when silenced causes necrosis upon COR application. We are now screening a COR-responsive *N. benthamiana* cDNA library to identify components involved in COR-induced chlorosis. Although the precise role of THF1 in the COR signaling pathway could be argued and needs further confirmation, our results present a new role for chloroplast-localized THF1 in bacterial speck disease development.

MATERIALS AND METHODS

Plant Materials, Bacterial Cultures, and Plant Infections

Nicotiana benthamiana plants were maintained in the greenhouse with conditions as described previously (Senthil-Kumar et al., 2007). Seeds of tomato (*Solanum lycopersicum* 'Glamour') were obtained from Stokes Seeds. Seeds of the Arabidopsis (*Arabidopsis thaliana*) *thf1* T-DNA mutant, its complemented lines, and overexpression lines were kindly provided by Dr. Ken Korth (University of Arkansas). Tomato *jai1* mutants (cv Castlemart) were obtained from Dr. Gregg Howe (Michigan State University). Agar and broth cultures of *Pseudomonas syringae* pvs *tomato* (DC3000), *glycinea*, *maculicola*, and *tabaci* were grown on King's B medium with appropriate antibiotics (King et al., 1954). *Agrobacterium tumefaciens* and *Escherichia coli* cultures were grown on Luria-Bertani medium (1% yeast extract, 0.5% bacto-tryptone, 1% NaCl, and 1.5% agar for plates) with appropriate antibiotics. For pathogen infection assays on silenced tomato lines, plants were inoculated with a bacterial suspension as described (Uppalapati et al., 2007). Bacterial suspensions (optical density at 600 nm [OD_{600}] = 0.1) were prepared in sterile distilled water containing 0.0025% Silwet L-77 (OSI Specialties) and sprayed on plants using a Paasche VL airbrush (Paasche Airbrush Co.). To infect Arabidopsis, 4-week-old plants were either infiltrated (OD_{600} = 0.2) with bacterial suspensions into the leaves using a needleless syringe or were dipped into the bacterial suspension (OD_{600} = 0.002). The plants were then placed in trays, covered with transparent lids, and incubated in growth chambers for the rest of the experimental period. Inoculated leaves were harvested, ground in 10 mM $MgCl_2$, and cfu per leaf disc (1-cm diameter) was determined by serial dilution of leaf extracts. The bacterial growth data were subjected to ANOVA using Biostat 2008 (Analystsoft). Significant differences in the means of the treatments were obtained based on Fisher's LSD set at $P < 0.005$.

VIGS-Mediated Forward Genetic Screening

A *N. benthamiana* cDNA library cloned in a TRV-VIGS vector (Anand et al., 2007) was used to screen and identify plant genes involved in COR responses. Agroinoculation for VIGS was performed using the toothpick method as described previously (Anand et al., 2007). About 3 weeks postinoculation, 2 μL of COR (0.2 nM) was placed on either side of the midrib of two fully expanded leaves per plant. Using *N. benthamiana* inoculated with *TRV::GFP* as a control, altered phenotypes in response to COR were recorded 5 to 7 d after COR application.

Cloning a Full-Length *ALC1* Gene

To clone the full-length *N. benthamiana ALC1* gene, a tobacco (*Nicotiana tabacum*) full-length sequence of *ALC1* (226 bp) homolog was obtained from the J.C. Venter Institute's plant genome database (accession no. TC10126; www.tigr.org). Based on the tobacco sequence, PCR primers were designed (forward, 5′-CAACTCCATTCTCTAAAGCAAC-3′; reverse, 5′-GTCAATGAGGTCCAAGCAGG-3′) at approximately 70 bp upstream and 40 bp downstream of the putative coding region. PCR was performed using the above primer sets on a cDNA mixture obtained from *N. benthamiana* leaves to amplify a full-length *N. benthamiana ALC1*. The PCR product obtained was then cloned into the pGEMT Easy vector (Promega) and transformed into JM109-competent cells. The insert was confirmed by sequencing.

Construction of pTRV::*SlALC1* and VIGS in Tomato

The vectors pTRV1 and Gateway-ready pTRV2 (Liu et al., 2002b) were kindly provided by Dr. S.P. Dinesh-Kumar (Yale University). An antisense sequence of *SlALC1* consisting of a 324-bp fragment (The Institute for Genomic Research accession no. TC212458) was PCR amplified from tomato (Glamour) by RT-PCR using primers *SlALC1*attB1 (5′-ggggacaagtttgtacaaaaaagcaggctTTCCACCTCTCGCTTTGTCG-3′) and *SlALC1*attB2 (5′-ggggaccactttgtacaagaaagctgggtGCATCAGCTCTGTATTGCTC-3′; lowercase letters indicate the Gateway adapters) and was cloned into Gateway-ready pTRV2 (Liu et al., 2002b). The construct, pTRV2-*SlALC1*, was then introduced into *A. tumefaciens* strain GV2260 by electroporation.

For gene silencing in wild-type or *jai1* mutant tomato plants, *A. tumefaciens* strains containing pTRV1 and pTRV2::*SlALC1* were mixed in a 1:1 ratio (OD_{600} = 1.0) in a buffer containing 10 mM MES, 10 mM $MgCl_2$, and 100 μM acetosyringone and incubated at room temperature for 3 to 4 h. Two-week-old tomato seedlings with fully expanded cotyledons were removed from the pots and were completely submerged in an *Agrobacterium* mixture and vacuum infiltrated for 2 min as described earlier by Uppalapati et al. (2007). The seedlings were then transplanted into Professional Blend potting mixture (Sun Gro). To improve the silencing efficiency, the remaining *Agrobacterium* culture was dispensed around the seedlings using the Agrodrench method (Ryu et al., 2004). Inoculated potted seedlings were then maintained in growth chambers for 2 d with a 12-h photoperiod (22°C day, 18°C night). Then the plants were moved to a greenhouse and maintained at 14 h of daylight at 25°C and 22°C at night for the next 10 to 14 d.

Generation of Tomato *ALC1* Transgenic RNAi Lines

For the generation of a tomato *ALC1* RNAi line, the *SlALC1* fragment (described above) was introduced into the Gateway-ready binary RNAi vector pK7GWIWG2(I) (Karimi et al., 2002) to generate the *SlALC1* RNAi construct that was later transformed into *A. tumefaciens* strain GV2260 by electroporation. For the transformation of tomato plants, a tomato tissue culture method developed by Frary and Van Eck (2005) was followed with a slight modification in that no tobacco feeder cells were used. Cotyledons of 7- to 8-d-old tomato seedlings (Glamour) were dissected and maintained on KCMS (KC Biological MS medium; Frary and Van Eck, 2005) for 24 h. Tomato cotyledons were then cocultivated with *A. tumefaciens* cultures carrying the RNAi construct and maintained in darkness for 48 h. For all subsequent steps, the transformation protocol described by Frary and Van Eck (2005) was followed.

Measurement of Chlorophyll Content

The chlorophyll content of leaf discs was measured as described by Arnon (1949) and Ishiga et al. (2009a). Two leaf discs (0.78 cm^2 each) were isolated 6 dpi from leaves treated with water (mock control) or 2 μL of COR (2 nM) and then macerated in liquid nitrogen, placed in 6 mL of acetone, and incubated at 4°C in the dark for 12 h. Aliquots of total chlorophyll dissolved in acetone were mixed with hexane and 10 mM KOH at a ratio of 4:6:1 (v/v). Chl *a* was quantified spectrophotometrically using the formula described by Arnon (1949).

RNA Isolation and RT-PCR Analysis

Total RNA was isolated from leaves of *N. benthamiana*, tomato, and Arabidopsis plants using TRIZOL reagent (Invitrogen). The first-strand cDNA was synthesized using oligo(dT)15 primer and the Omniscript RT kit (Qiagen). For quantitative analysis of transcripts, primer pairs were designed using the Primer Express software (Applied Biosystems) to amplify the target sequences. qRT-PCR was performed with an ABI HT7900 machine using the SYBR Green method (Applied Biosystems). PCR efficiency was determined using the linear regression software LinRegPCR (Ramakers et al., 2003). In order to normalize the data, parallel reactions were run using the Elongation Factor-α (EF1α) primers as the endogenous control for Arabidopsis and actin

or tubulin primers as the endogenous control for *N. benthamiana* and tomato (Supplemental Table S1). The relative transcript levels were quantified as described previously (Pfaffl, 2001).

Subcellular Localization of ALC1

To transiently express *ALC1* in *N. benthamiana*, the Gateway-ready pMDCC83 was used as a vector to generate a GFP fusion (Curtis and Grossniklaus, 2003). Full-length *ALC1* sequence was amplified from *N. benthamiana* cDNA using the following gene-specific primers: *ALC1*attB1 (5′-ggggacaagtttgtacaaaaaagcaggcttcATGGCGGCAGTTACTTCG-3′) and *ALC1*attB2 (5′-ggggaccactttgtacaagaaagctgggtcCCTCCCAGCATATTGGTAA-TCT-3′; lowercase letters indicate the Gateway adapters). The amplified sequence was cloned into the donor vector pDONR 207 (Invitrogen), and the resulting clone was then transformed into *E. coli* DH5α-competent cells (Invitrogen). The full-length gene was further subcloned into pMDC83, and pmDC83-*ALC1* was then introduced into *A. tumefaciens* GV2260 by electroporation. To generate pMDC83 empty vector that can replicate in *A. tumefaciens* GV2260 without killing the host (Dao-Thi et al., 2005), the vector was restriction digested with *Kpn*I to remove the *ccd*B9 (for controller of cell division or death) region. The open ends were then ligated with T4 DNA ligase. pMDC83:ΔccdB was then introduced into *A. tumefaciens* GV2260 by electroporation. pCAMBIA1390-RecA in *Agrobacterium* C58C1 (Kohler et al., 1997) was obtained from Dr. Elison Blancaflor (Noble Foundation).

To test the *COI*-dependent ALC1 localization, *N. benthamiana* leaves were syringe inoculated with a 1:1 mixture of TRV1 and pTRV2::*NbCOI1* (Ekengren et al., 2003**)** as described by Ryu et al. (2004). Wild-type and *COI1*-silenced leaves at 2 weeks postinoculation were used for ALC1-GFP localization studies. Purified COR at a concentration of 2.0 nM was spotted on the leaves of wild-type or *COI1*-silenced *N. benthamiana* plants that were infiltrated, 3 d prior, with *Agrobacterium* containing *ALC1-GFP, RecA-GFP,* or *GFP* alone. Imaging of COR-treated or untreated cells was conducted using a Perkin-Elmer UltraView ERS spinning-disc confocal system coupled to a Zeiss Observer D1 inverted microscope equipped with a 63× water-immersion objective (numerical aperture 1.2). GFP was excited with the 488-nm line of the argon-kyrpton laser, and emission was detected at 510 nm. To image chloroplast autofluorescence, leaf samples were excited with the 647-nm line of the argon-kyrpton laser, and emission was detected at 680 nm.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number EU106046.1.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Phenotypes of the Arabidopsis *thf1* line, *ALC1*-silenced *N. benthamiana*, and *ALC1*-silenced tomato share similarities.

Supplemental Figure S2. Determination of silencing efficiency of *ALC1*.

Supplemental Figure S3. *ALC1* has homologs in several plants.

Supplemental Figure S4. Determination of silencing efficiency of *SlALC1* in COR-treated mock and *jai1* tomato mutants.

Supplemental Figure S5. Response of the Arabidopsis *thf1* mutant line to pathogens (*P. syringae* pv *maculicola* and *Erwinia carotovora* subspecies *carotovora*) and nonhost pathogens (*P. syringae* pvs *tabaci* and *glycinea*).

Supplemental Figure S6. Effect of COR on localization or stability of ALC1 near and away from the site of inoculation zone in *N. benthamiana* leaf samples.

Supplemental Figure S7. Effect of COR on localization of a chloroplast-localized RecA protein in *N. benthamiana* leaf samples.

Supplemental Figure S8. Effect of *COI1* silencing on expression and localization of ALC1 in *N. benthamiana* leaf samples.

Supplemental Figure S9. COR-induced chlorosis and *NbALC1*-mediated necrotic phenotype are *COI1* dependent in *N. benthamiana*.

Supplemental Table S1. Primers used for qRT-PCR.

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