Running head: Loss of ALC1/THFL results in Pst DC3000 induced necrosis

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A virus-induced gene silencing screen identifies a role for *Thylakoid Formation1* in *Pseudomonas syringae* pv. *tomato* symptom development in tomato and Arabidopsis

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Summary

*Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000), which causes disease in tomato and Arabidopsis, 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 MeJA identified several potential targets for COR and MeJA. In this study, we utilized *Nicotiana benthamiana* and virus-induced gene silencing (VIGS) to individually reduce the expression of over 4,000 genes. The silenced lines of *N. benthamiana* were then screened for altered response to purified COR. Using this forward genetics approach several genes were identified with altered responses to COR. These were designated as altered COR response (*ALC*) genes. When silenced, one of the identified genes, *ALC1*, produced a hypersensitive/necrosis-like phenotype upon COR application in a Coronatine insensitive 1 (*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 chloroplast in a *COI1*-dependent manner. In conclusion, our results show that VIGS-based forward genetic screen has potential to identify new players in COR signaling and disease associated necrotic cell death.
INTRODUCTION

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 (ET) pathways (Kunkel and Brooks, 2002; Glazebrook, 2005). Pathogens, in turn, have co-evolved 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, phaseleotoxin, and tabtoxin; Mitchell, 1976; Gnanamanickam, 1982; Levi, 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; Uppalapati *et al*., 2008).

Coronatine (COR), a phytotoxin produced by *P. syringae* pv. *tomato* (*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 (Li *et al*., 1998). COR contributes to the virulence of *Pst DC3000* in Arabidopsis, tomato, collard and turnip (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-phytadienonic acid (12-OPDA), methyl jasmonate (MeJA), and related derivatives known as the jasmonates (JAs) (Feys *et al*., 1994; Weiler *et al*., 1994; Uppalapati *et al*.,
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 salicylic acid (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 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 ROS in modulating necrotic cell death during bacterial speck disease of tomato (Ishiga et al., 2009). 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 (Kastir 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, b; Anand et al., 2007a) to screen a Nicotiana benthamiana cDNA library for genes that are involved in response to COR. We identified a N. benthamiana gene, ALC1, that when silenced displayed an unexpected
hypersensitive/necrosis-like phenotype rather than a typical chlorotic phenotype in response to COR application. *ALCI* has homology to an Arabidopsis gene, *Thylakoid Formation1* (*THF1*; Wang et al., 2004). The pathogenicity assays performed in this study indicate that loss of *ALCI/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* and therefore this host is suitable for large-scale fast-forward screening studies (Lu et al., 2003; del Pozo et al., 2004; Anand et al., 2007a). Purified COR, when spotted onto *N. benthamiana* leaves at different concentrations (0.002–2 nmol in 2 μl aliquots), produced a visible, confined chlorosis in a dose-dependent manner (Fig. 1A). 0.2 nmol 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 *A. tumefaciens* GV2260 was used
(Anand *et. al*, 2007a). *N. benthamiana* plants were individually inoculated with *Agrobacterium* containing TRV2 cDNA clones, along with an *Agrobacterium* strain containing TRV1, in duplicates, to silence their corresponding genes in *N. benthamiana* (Anand *et al*., 2007a). Two weeks after TRV inoculation, COR (0.2 nmol) was spotted on the leaves of silenced plants, and the phenotypes were recorded 5-7 days after COR application.

After screening ~4,000 cDNA clones, we identified five non-redundant cDNA clones that when silenced resulted in altered COR response (ALC) phenotype upon exogenous application of COR (Fig. 1D-H). The application of COR to wild-type (Fig. 1B) or TRV::GFP (mock control, Fig. 1C; the GFP sequence does not have any homology to plant DNA and therefore will not cause gene silencing) inoculated *N. benthamiana* plants resulted in a defined chlorotic halo. The silenced lines with ALC phenotype exhibited either hypersensitive (HR)-like necrosis (Fig. 1D-F) or increased chlorosis (Fig. 1G, 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. 1D, E), whereas *Nb37G8* (Fig. 1F) silenced lines displayed a less severe necrotic phenotype. *Nb37B3*- and *Nb39H9*-silenced plants displayed slightly diffused chlorosis (Fig. 1G, 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 Thylakoid Formation1 protein**

Since the silencing of *Nb28C12* produced the most dramatic phenotype in response to COR application, *Nb28C12*-silenced line was selected for further study. The phenotype of *Nb28C12*-silenced plants was similar to control plants (TRV::GFP) up to four weeks post-silencing. However, after the fifth week, leaves of the *Nb28C12*-silenced plants turned slightly pale green in color (Supplemental Fig. S1a). At five weeks post-inoculation, portions of some leaves showed a variegated gray coloration (Fig. S1A). To confirm the suppression of the *Nb28C12* mRNA in the silenced plants, quantitative real-time RT-PCR (qRT-PCR) was performed. The relative expression ratio of *Nb28C12* gene in the silenced line was 0.023, indicating that the transcript level was approximately forty-fold lower than in the control plant (Supplemental Fig. S2A).

We termed *Nb28C12* as altered COR response 1 (*ALC1*). The sequence information was then analyzed to predict gene function. A BLASTn search against the TIGR database using the *NbALC1* sequence revealed 77% identity to an Arabidopsis gene named *THF1* (Genbank ID AY899908); 92% identity to a potato gene that encodes a light-regulated chloroplast localized protein (*Solanum tuberosum THF1*, GenBank ID AY342161); 81% identity to a rice (*Oryza sativa*) gene encoding inositol phosphatase-like protein (GenBank ID AY224446); and 79% identity to a wheat (*Triticum aestivum*) gene encoding Ptr ToxA binding protein (GenBank ID 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 tobacco (*N. tabacum*) ortholog (TC10126). The cloned gene was then sequenced and the translated amino acid sequence was then aligned with orthologous plant protein sequences using ClustalW.
As shown in supplemental Fig. 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 to 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) when compared to the vector control (47% reduction over mock) (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 COI11-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 (TC162724), we cloned the tomato *ALC1* (see Experimental Procedures) 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 five-fold lower in the silenced plants as compared to control plants (Supplemental Fig. S2B). However, majority of the leaves in *ALC1* silenced tomatoes did not exhibit any obvious phenotype (Fig. S1B, middle panel), some
of the older leaves (six weeks post TRV-inoculation) showed variegated coloration on the leaf surface (Fig. S1B, right panel). When purified COR (2 nmol) was exogenously applied, the silenced line showed a necrosis-like phenotype, whereas the control plants showed a typical confined chlorosis as expected (Fig. 3A).

Although we were fairly successful in transiently silencing the tomato \textit{ALC1} gene, a uniform and pronounced silencing, such as that observed in \textit{N. benthamiana}, is often difficult to achieve in tomato (Ekengren \textit{et al.}, 2003; Ryu \textit{et al.}, 2004). Therefore, to achieve stable and uniform silencing and to confirm the necrosis phenotype induced by COR and \textit{Pst} DC3000 on \textit{SlALC1}-silenced tomato lines, we generated \textit{SlALC1} RNAi lines. We assayed three independent transgenic RNAi lines and all responded similarly to COR application and \textit{Pst} DC3000 infection. Here, we discuss the data for one of the transgenic lines, 3-2. Results obtained from qRT-PCR indicated the transcript levels of \textit{SlALC1} were twenty-fold less in RNAi line 3-2 when compared to wild-type tomato plants (Supplemental Fig. S2B). When COR (2 nmol) was spotted on the leaves of the RNAi line 3-2, necrosis appeared five days post inoculation (Fig. 3B, second panel from left).

\textit{Coronatine insensitive 1/jasmonic acid insensitive 1} (COI1/JAI1), a F-box protein is shown to be required for COR signaling in tomato and Arabidopsis (Feys \textit{et al.}, 1995; Zhao \textit{et al.}, 2003; Katsir \textit{et al.}, 2008). By using VIGS, we transiently silenced \textit{SlALC1} in \textit{jai1} mutant tomato plants (Fig. S4) to know whether the necrosis we observed in \textit{SlALC1}-silenced tomato plants upon COR treatment (Fig. 3A, B) is \textit{COI1/JAI1} dependent. When purified COR (2 nmol) was exogenously applied, the \textit{SlALC1}-silenced \textit{jai1} plants showed no visible necrosis (Fig. 3C). Furthermore, transient double silencing
of COII and NbALC1 in N. benthamiana abolished ALC1-mediated COR induced necrosis (Fig. S9). There results confirmed that JAI1/COII is required for altered chlorosis phenotype in ALC1-silenced tomato and N. benthamina 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 (10^8 CFU/ml). Control (TRV::GFP) plants showed typical bacterial speck symptoms at 5 dpi, which consisted of necrotic lesions surrounded by chlorotic halo (Fig. 4A, left panel). At 5 dpi, the leaves of *SlALC1*-silenced plants showed necrosis with little or no chlorosis (Fig. 4A, right panel). At 10 dpi, the necrosis observed on the silenced plants was severe (Fig. 4A, right panel). Similarly, when line 3-2 was spray inoculated with *Pst* DC3000 (10^8 CFU/ml), leaves developed severe coalescing necrotic lesions without visible chlorotic halos surrounding the necrotic lesions as seen on the wild-type (Fig. 4B, right panel), further confirming that *ALC1* plays a role or is required 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. 5A, B). 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.
Arabidopsis thf1 mutant displays severe necrosis upon Pst DC3000 inoculation

As mentioned above, ALC1 is closely related to an Arabidopsis gene called THF1 (Fig. S3A). An Arabidopsis thf1 mutant was previously identified and shown to have variegated leaves (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 Col-0, thf1 mutant line, the complemented line, and the THF1 overexpressing line on half-strength MS medium containing 2 nmol COR (Laurie-Berry et al., 2006). As expected, Col-0 seedlings showed anthocyanin accumulation within ten days 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 phenotype of the thf1-complemented and the overexpressing lines were similar to 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) or syringe-infiltrated (10^6 CFU/ml) 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, 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). We therefore 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 coronatine 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 *tabaci* multiplied to a significant level on Col-0 or *thf1* plants (Supplemental Fig. S5A).

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* subsp. *carotovora*. *P. syringae* pv.
maculicola induced chlorosis on Col-0 but not on thf1 mutant line (Supplemental Fig. S5B). Neither Col-0 nor thf1 plants developed visible symptoms in response to P. syringae pvs. tabaci or glycinea (Fig. S5B). E. carotovora subsp. 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 (Fig. S5B). Infiltration of leaves with cell death inducing agents such as NaCl (500 mM) or H₂O₂ (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 to Col-0. This response was similar to the one induced by Pst DC3000. The 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 mimic of JAs and mediates signaling via JA perception machinery in tomato and Arabidopsis (Feys et al., 1995; 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 thf1 mutant line, especially at 4 dpi, the JA pathway appears to be functional (Fig. 8A, B) at the time points analyzed.
Chlorosis occurs due to the degradation of proteins in the chloroplast (Quirino et al., 2000) and the Arabidopsis CORII 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 thfl could be due to repression of CORII as a result of loss of THF1 function. Thus, we analyzed CORII transcript levels in Pst DC3000 inoculated Col-0 and thfl plants. CORII expression in Col-0 and thfl was upregulated ~175 fold and ~75 respectively, 1dpi (Fig. 8D). These results further suggest that the chlorophyllase activity and JA dependent pathway are not severely affected in the thfl 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 thfl plants. Similarly, significant levels COR-induced chlorophyll degradation was observed in NbALC1-silenced plants (Fig. 2). Taken together, these results suggest that THF1 may be operating down-stream of COR-induce JA signaling and chlorosis.

**Coronatine affects the localization of ALC1 in a COII-dependent manner in N. benthamiana plants**

To determine if coronatine directly affected ALC1, we monitored the effect of coronatine treatment on the localization/stability of ALC1. 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 Experimental Procedure section. Coronatine 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 post-COR inoculation (Fig. 9). As previously reported for THF1, GFP-ALC1 localized to the chloroplast (Fig. 9A). Interestingly, within four hours upon COR application, ALC1 was destabilized/degraded as shown by the loss of GFP fluorescence (Fig. 9A). ALC1 fluorescence was not detected even after 24 (Fig. 9A), 48 hours and 72 hours (time at which chlorosis is visible on the leaf; data not shown). It also noteworthy that the destabilization/degradation of GFP-ALC1 is seen only at the site of application of COR and nearby region but the leaf areas away from the region of coronatine application remains unaffected even after 24 and 48 hours (Supplemental Fig. S6). To rule out the possibility that COR application is leading to alterations in chloroplast structure, therefore resulting in non-specific effects on ALC1, we tested COR effects on GFP-RecA (Kohler et al., 1997), another chloroplast localizing protein (Fig. S7). Interestingly, COR application did not result in destabilization/degradation of GFP-RecA or GFP alone (Fig. S7; Fig. 9C).

The COII-dependent nature of COR induced alterations in ALC1 localization were further confirmed in COII-silenced N. benthamiana plants (Fig. 9B). Interestingly, in COII-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 COII-silencing or a COI1-independent activity of COR on ALC1. Silencing of COII did not affect the expression levels of 35S::ALC1 GFP florescence (Fig. S8). However, silencing of COII abolished COR-induced destabilization/degradation of 35S::ALC1 GFP florescence (Fig. 9B). Consistent with these results, COII and NbALC1 double silenced plants showed no chlorosis/necrosis phenotypes upon COR application (Fig. S9). Taken together, these results suggested that direct effects of COR or COR-
induced effects on chloroplast/ALC1 directly alters 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 the loss of *N. benthamiana* gene *NbALC1* and its orthologs, *SlALC1* in tomato and *AtTHF1* in Arabidopsis results in 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 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 previously identified (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 accumulation of membrane vesicles, but no thylakoids within the intact chloroplast membrane in the white sector of leaves (Wang et al., 2004).

The exogenous application of COR (2 nmol) 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 \textit{thf1} mutant as compared to the wild-type plants. Therefore, the \textit{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 \textit{ALC1}-silenced tomato plants, inoculation of \textit{thf1} with \textit{Pst} DC3000 did not result in a typical chlorotic phenotype around the water-soaked lesion. Analysis of the \textit{Pst} DC3000 population dynamics in both \textit{ALC1}-silenced tomato plants and the \textit{thf1} mutant line indicated that there was no difference in the bacterial population dynamics when compared to wild-type plants. Interestingly, \textit{Pst} DC3000-inoculated leaves of \textit{ALC1}-silenced tomato and the Arabidopsis \textit{thf1} mutant line died earlier than corresponding wild-type lines. These results suggested that \textit{Pst} DC3000 during infection 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 results in light-dependent reactive oxygen species (ROS) generation in tomato seedlings (Ishiga et al.,
We speculate that in COR-treated or *Pst* DC3000-inoculated *ALC1* silenced tomatoes 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 biogenesis of photosystem II and sugar signaling (Keren *et al*., 2005; Huang *et al*., 2006). *THF1* is also identified as an interactor of G-protein, GPA1 in Arabidopsis and 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; Fig. S2), directly interacts with *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 *P. syringae* pv. *tomato*-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 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 presently screening a COR-responsive N. benthamiana cDNA library to identify components involved in COR-induced chlorosis. Although the precise role of THF1 in COR signaling pathway could be argued and need 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

N. benthamiana plants were maintained in the greenhouse with conditions as described previously (Senthil-Kumar et al., 2006). Seeds of tomato (Solanum lycopersicum) cv. Glamour were obtained from Stokes Seeds, Inc. (Buffalo, NY, U.S.A.). Seeds of the Arabidopsis thfl T-DNA mutant, its complemented lines, and overexpression lines were kindly provided by Dr. Ken Korth, University of Arkansas, U.S.A. Tomato jai1 mutants (cv Castlemart) were obtained from Dr. Gregg Howe, Michigan State University, U.S.A. Agar and broth cultures of P. syringae pvs. tomato (DC3000), glycinea, maculicola and tabaci were grown on King’s B medium with appropriate antibiotics (King et al., 1954). A. tumefaciens and Escherichia coli cultures were grown on Luria Bertani (LB) medium (1% yeast extract, 0.5% Bacto-tryptone, 1% NaCl, 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 Inc., Danbury, CT, USA), and sprayed on plants using a Paasche VL airbrush (Paasche Airbrush Co. Chicago, IL,
USA). To infect Arabidopsis, 4-week old plants were either infiltrated (OD$_{600} = 0.2$) with bacterial suspensions into the leaves using needle-less syringe, or the plants were dipped upside down into the bacterial suspension (OD$_{600} = 0.002$). The plants were then placed in trays and covered with transparent lids and incubated in growth chambers for the rest of the experimental period. Inoculated leaves were harvested, ground in 10mM MgCl$_2$ and CFU/cm$^2$ was determined by serial dilution of leaf extracts. The bacterial growth data was subjected to ANOVA analysis using Biostat 2008 (Analystsoft Inc.). Significant difference in the means of the treatments were obtained based Fisher's LSD set at p a value of <0.005.

**VIGS-mediated forward genetic screening**

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

**Cloning a full length ALC1 gene**

To clone the full length *N. benthamiana ALC1* gene, a tobacco (*N. tabacum*) full length sequence of *ALC1* (226 bp) homolog was obtained from J. C. Venter Institute’s plant genome database (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, Madison WI) and then 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, U.S.A. An antisense sequence of *SlALC1* consisting of a 324 bp fragment (TIGR accession no. TC162724, currently http://compbio.dfci.harvard.edu/tgi/ accession no. 178313) was PCR-amplified from tomato (cv. Glamour) by reverse transcriptase-PCR (RT-PCR), using primers *SlALC1*attB1: 5’-ggggacaagtttgtacaaa aagcaggctTTCCACCTCTCGCTTTGTCG-3’ and *SlALC1*attB2: 5’-gggg accactttgtacaagaaagctgggtGCATCAGCTCTGTATTGCTC-3’ (the small 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 acetoxyringone and incubated at room temperature for 3-4 h. Two-week old tomato seedlings with fully
expanded cotyledons were removed from the pots and were completely submerged in *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, Bellevue, WA). 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 days with a 12 h photoperiod (22°C, day; 18°C, night). Then the plants were moved to greenhouse and maintained at 14 h daylight at 25°C and 22°C at night for the next 10-14 days.

**Generation of tomato ALC1 transgenic RNAi lines**

For the generation of a tomato ALC1 RNAi line, the SlALC1 fragment (described above) was introduced into a GATEWAY-ready binary RNAi vector pK7GWIWG2(I) (Karimi et al., 2002) to generate 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 7-8 day-old tomato seedlings (cv. Glamour) were dissected and maintained on KCMS (KC Biological MS medium; Frary and Van Eck, 2005) for 24 h. Tomato cotyledons were then co-cultivated 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 in Arnon (1949) and Ishiga et al. (2009). Two leaf discs (0.78 cm² each) were isolated six days post-inoculation from leaves treated with water (mock control), 2µl of COR (0.2 nmol) 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 and b were quantified spectrophotometrically using the formula described by Arnon (1949).

RNA isolation and reverse transcription-PCR (RT-PCR) analysis

Total RNA was isolated from leaves of *N. benthamiana*, tomato and Arabidopsis plants using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). The first strand cDNA was synthesized using oligo (dT) 15 primer and Omniscript RT kit (Qiagen, Valencia, CA, USA). For quantitative analysis of transcripts, primer pairs were designed using the Primer Express software (Applied Biosystems Inc., Foster City, CA) to amplify the target sequences. qRT-PCR was performed with ABI HT7900 machine using the SYBR Green method (Applied Biosystems Inc., Foster City, CA). PCR efficiency was determined using linear regression software LinRegPCR (Ramakers *et al.*, 2003). In order to normalize the data, parallel reactions were run using the elongation factor-alpha (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 \textit{ALC1} in \textit{N. benthamiana}, the GATEWAY-ready pMDCC83 was used as a vector to generate a GFP fusion (Curtis and Grossniklaus, 2003). Full-length \textit{ALC1} sequence was amplified from \textit{N. benthamiana} cDNA using the following gene specific primers: \textit{ALC1}attB1: 5′-ggg gac aag ttt gta caa aaa agc agg ctt c ATG GCG GCA GTT ACT TCG-3′; and \textit{ALC1}attB2: 5′-ggg gac cac ttt gta caa gaa agc tgg gtc CCT CCC AGC ATA TTG GT AAT CT-3′ (small letters indicate the GATEWAY adapters). The amplified sequence was cloned into the donor vector pDONR 207 (Invitrogen, Carlsbad, CA, USA), and the resulting clone was then transformed into \textit{E. coli} DH5\textasciicircum competent cells (Invitrogen, Carlsbad, CA, USA). The full length gene was further sub-cloned into pMDC83, and pMDC83-\textit{ALC1} was then introduced into \textit{A. tumefaciens} GV2260 by electroporation. To generate pMDC83 empty vector that can replicate in \textit{A. tumefaciens} GV2260 without killing the host (Dao-Thi \textit{et al.}, 2005), the vector was restriction digested with \textit{KpnI} to remove the \textit{ccdB9} (controller of cell division or death) region. The open ends were then ligated with T4 DNA ligase. pMDC83:\textit{ΔccdB} was then introduced into \textit{A. tumefaciens} GV2260 by electroporation. pCAMBIA1390-RecA in \textit{Agrobacterium} C58C1 (Kohler \textit{et al.}, 1997) was obtained from Dr. Elison Blancaflor, Noble Foundation.

To test the \textit{COI}-dependent ALC1 localization, \textit{N. benthamiana} leaves were syringe inoculated with 1:1 mixture of TRV1 and \textit{pTRV2::NbCOI1} (Ekengren \textit{et al.}, 2003) as described by Ryu \textit{et al.} (2004). Wild-type and \textit{COI1}-silenced leaves after 2-weeks post inoculation were used for ALC1-GFP localization studies described, below. Purified COR at a concentration of 2.0 nmol was spotted on the leaves of wild-type or
COI1-sienced *N. benthamiana* plants that were infiltrated, 3 days 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 disk confocal system coupled to a Zeiss Observer D1 inverted microscope equipped with a 63 X water immersion objective (Numerical Aperture 1.2). GFP was excited with the 488 nm line of the Argon-Kyrpton laser and emission detected at 510 nm. To image chloroplast autofluorescence, leaf samples were excited with the 647 nm line of the Argon-Kyrpton laser and emission detected at 680 nm.

SUPPLEMENTAL DATA

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

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

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

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

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

**Figure S6.** Effect of coronatine on localization or stability of *ALC1* near and away from the site of inoculation zone in *N. benthamiana* leaf samples.
**Figure S7:** Effect of coronatine on localization of a chloroplast localized RecA protein in *N. benthamiana* leaf samples.

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

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

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Figure Legends:

Figure 1. Coronatine (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, 2 nmol, from lower to upper parts of the leaf); a visible chlorotic zone was observed at 4 dpi. B. Chlorosis induced by 0.2 nmol COR (per inoculation site) on *N. benthamiana*. (C-H) Response of silenced lines of *N. benthamiana* leaves to 2 nmol COR. COR was applied three weeks post Agro-inoculation to silenced lines of *N. benthamiana*. In response to COR, leaves of silenced lines showed necrosis (C) or a necrosis-like phenotype (D and E). Some lines exhibited an enhanced chlorosis (F and H). Photos were taken 7 days after COR application.

Figure 2. Coronatine (COR) induces chlorophyll degradation in vector control (TRV::GFP) and *NbALC1*-silenced (TRV::NbALC1) *N. benthamiana* plants. Two leaf discs (0.78 cm²) were collected 6 days post-inoculation with 0.2 nmol of COR and were analyzed for Chlrophyll a (Chl a) content to monitor chlorosis.

Figure 3. Silencing of *ALC1* in tomato displays a necrosis-like phenotype in a *COI1/JAI1* dependent manner in response to (2 nmol) COR seven days post-treatment. Transient (TRV::SlALC1) (A) and stably silenced (3-2) (B) tomato (cv. Glamour) show necrosis in response to COR; whereas vector control (TRV::GFP) and transiently silenced (TRV::SlALC1) *jai1* mutants (cv. Casselmart) (C) showed no chlorosis or necrosis. Photographs were taken seven days after COR application.
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 x 10^6 CFU/ml) was spray-inoculated on control (*TRV::GFP*; left panels) and *SlALC1* silenced (by VIGS; right panels) tomato plants. Photographs were taken after 5 and 10 days post inoculation (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 wild-type; described in Fig. 3) were collected at various days post inoculation (dpi), homogenized in water, and dilutions were plated onto KB medium to determine colony forming units (CFU). The error bars represent the standard deviation. All experiments were repeated at least twice with several biological replicates, and the data shown are representative of the experiments. Growth measurements with same letters showed no significant differences based on Fisher LSD values (p <0.005)

Figure 6. 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 nmol COR (upper panels). These lines were also germinated on half strength MS medium alone without COR (controls, lower panels). Pictures were taken 10 days after
germination. Each scale bar indicates 3 mm. B. Foliar parts of 4-weeks old Arabidopsis lines mentioned above either dipped in a Pst DC3000 culture suspension (10^8 CFU/ml; upper panel) or infiltrated with 10^6 CFU/ml of Pst DC3000 using a needle-less syringe (lower panel). Pictures were taken 6 days post inoculation.

**Figure 7.** The mutation in Arabidopsis *THF1* has no effect on the growth of *Pst* DC3000. Arabidopsis leaves of the lines described in Fig. 5 were syringe-infiltrated with *Pst* DC3000 (10^6 CFU/ml), collected at intervals after inoculation, homogenized in water, and plated on KB medium to determine colony forming units (CFU). The error bars represent the standard deviation. The experiments were conducted at least three times with several replicates, and the data shown are representative of each experiment. Growth measurements with same letters showed no significant differences based on Fisher LSD values (p <0.005)

**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 (open bars) after *Pst* DC3000 inoculation. Four-week old Col-0 and *thf1* mutant line were syringe-infiltrated with either *Pst* DC3000 (10^6 CFU/ml) 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 analyses. The transcript levels were normalized against the elongation factor EF1α that was used as endogenous control as described by Pfaffl (2001). The transcript levels were quantified relative to the transcript levels on mock control which was assigned as 1. A,B.
JA pathway genes (represented by *LOX2* and *PDF1.2*); and (C) the chlorophyllase encoding gene *CORII* were activated in *Pst* DC3000 infected Col-0 and the *thf1* mutant. All experiments were repeated at least three times. The data shown here represent the average of three biological replicates and three technical replicates with the standard deviation values shown as the error bars.

**Figure 9.** COII-dependnet effect of coronatine on localization or stability of ALC1 in *N. benthamiana*. Localization of ALC1-GFP in the wild-type (A), and COII-silenced (TRV::COII) (B) *N. benthamiana* leaves treated with COR and observed by fluorescence microscopy at 0, 4 and 24 hours post treatment. C. Localization of GFP in *N. benthamiana* leaves treated with COR. RED, autofluorescence of the chloroplast by excitation at 647 nm and emission in Cyan/Far red channel; GFP, GFP-fluorescence of the tagged protein by excitation at 488nm and emission at Green channel. All images were magnified using 63X water immersion objective. Scale bars–5 μM.
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