NTRC and Chloroplast-Generated Reactive Oxygen Species Regulate *Pseudomonas syringae* pv. *tomato* Disease Development in Tomato and *Arabidopsis*

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Coronatine (COR)-producing pathovars of *Pseudomonas syringae*, including pvs. *tomato*, *maculicola*, and *glycinea*, cause important diseases on tomato, crucifers, and soybean, respectively, and produce symptoms with necrotic lesions surrounded by chlorosis. The chlorosis is mainly attributed to COR. However, the significance of COR-induced chlorosis in localized lesion development and the molecular basis of disease-associated cell death is largely unknown. To identify host (chloroplast) genes that play a role in COR-mediated chlorosis, we used a forward genetics approach using *Nicotiana benthamiana* and virus-induced gene silencing and identified a gene which encodes 2-Cys peroxidoreductase (*Prxs*) that, when silenced, produced a spreading hypersensitive or necrosis-like phenotype instead of chlorosis after COR application in a *COII*-dependent manner. Loss-of-function analysis of *Prx* and NADPH-dependent thioredoxin reductase C (*NTRC*), the central players of a chloroplast redox detoxification system, resulted in spreading accelerated *P. syringae* pv. *tomato* DC3000 disease-associated cell death with enhanced reactive oxygen species (ROS) accumulation in a COR-dependent manner in tomato and *Arabidopsis*. Consistent with these results, virulent strain DC3000 suppressed the expression of *Prx* and *NTRC* in *Arabidopsis* and tomato during pathogenesis. However, interestingly, authentic COR suppressed the expression of *Prx* and *NTRC* in tomato but not in *Arabidopsis*, suggesting that COR in conjunction with other effectors may modulate ROS and cell death in different host species. Taken together, these results indicated that *NTRC* or *Prx* function as a negative regulator of pathogen-induced cell death in the healthy tissues that surround the lesions, and COR-induced chloroplast-localized ROS play a role in enhancing the disease-associated cell death.

Plants are constantly exposed to a wide-range of microorganisms, including potential pathogens in their natural habitats. Therefore, they have evolved innate immune systems that

sense invading pathogens and activate an array of defense responses to protect themselves. The plant innate immune system consists of two main branches. One is called effector-triggered immunity (ETI) or host-specific resistance based on the gene-for-gene theory, which is governed by individual plant resistance genes and their corresponding avirulence (*Avr*) genes (Ellis et al. 2000). In addition, plants also have a general resistance to almost all host or nonhost pathogens potentially mediated by a resistance mechanism called basal resistance or nonhost resistance, respectively. The primary response of nonhost or basal resistance is the recognition of conserved molecules from microorganisms, called microbe-associated molecular patterns or pathogen-associated molecular patterns (PAMPs), such as flagellin, elongation factor Tu, lipopolysaccharides, peptidoglycans (Gomez-Gomez and Boller 2002; Gust et al. 2007; Segonzac and Zipfel 2011), or chitin (Wan et al. 2008a and b) by plant pattern-recognition receptors, resulting in PAMP-triggered immunity (PTI) (Zipfel 2008). ETI and PTI induce similar innate immune responses, including rapid oxidative burst, callose (β -1,3-glucan) deposition in the cell wall, and the activation of hormone-mediated signaling pathways leading to defense responses (Zhang and Zhou 2010; Zipfel 2008). However, ETI-triggered immunity generally includes the induction of a rapid programmed cell death (PCD) known as the hypersensitive response (HR) cell death (Heath 2000).

The HR cell death induced by ETI is the most well-characterized form of plant PCD (Greenberg and Yao 2004). Although the precise role of secondary messengers in HR cell death is still unclear, the most intensively investigated signal for HR cell death is reactive oxygen species (ROS) (Mur et al. 2008). Apoplastic ROS derived from plasma membrane-bound NADPH oxidase is well known to regulate PCD associated with the HR (Mur et al. 2008). In contrast to HR cell death associated with incompatible interactions, the molecular basis of disease-associated cell death during compatible interactions is largely unknown. Localized cell death and tissue chlorosis are often associated with disease symptom development during compatible plant–pathogen interactions. Most of our present understanding of the disease-associated cell death is obtained from the genetic analysis of compatible interactions of *Pseudomonas syringae* pv. *tomato* DC3000 with *Arabidopsis* and tomato. It has been shown that a mitogen-activated protein kinase kinase (MAPKKK α) plays a role in the regulation of disease-associated cell death induced by strain DC3000 in tomato (del Pozo et al. 2004). Furthermore, a gene involved in autophagy, ATG6, was shown to be required for the limitation of disease-associated cell death induced by strain DC3000 in *Arabidopsis* (Liu et al. 2005; Patel and Dinesh-Kumar 2008). The bacterial

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speck disease caused by pathovars of *P. syringae* pv. *tomato* includes the formation of necrotic lesions (cell death) surrounded by diffuse chlorotic halos (Bender et al. 1999; Preston 2000). Disease-associated chlorosis has been attributed mainly to the phytotoxin coronatine (COR) produced by several pathovars of *P. syringae* (Bender et al. 1987, 1998; Uppalapati et al. 2007; Zhao et al. 2003) and a less-characterized chlorosis-inducing factor, PSPTO4723 (Munkvold et al. 2009). However, our understanding of the host signaling components exploited by pathogen effectors to cause chlorosis and disease-associated cell death is limited (del Pozo et al. 2004; Greenberg and Yao 2004; Ishiga et al. 2009a and b; Wangdi et al. 2010a). Also, a molecular link between COR-induced chlorosis and disease-associated cell death during the necrogenic phase of bacterial speck disease development has not been determined.

COR has structural and functional similarity to jasmonates, including jasmonic acid (JA)-isoleucine (Katsir et al. 2008; Staswick and Tiryaki 2004; Uppalapati et al. 2005; Weiler et al. 1994). Furthermore, the F-box protein COR-insensitive 1/JA-insensitive 1 (COI1/JAI1) was shown to be required for COR signaling in tomato and *Arabidopsis* (Feys et al. 1994; Katsir et al. 2008; Kloeck et al. 2001; Zhao et al. 2003). F-box proteins belonging to an E₃ ubiquitin ligase family assemble SCF (Skp1/Cul1/F-box) complexes and play a major role in controlled protein degradation through a ubiquitin/26S proteasome pathway during plant hormone signaling and development (Santner and Estelle, 2009). By mimicking jasmonates, COR stimulates the JA pathway in *Arabidopsis* and tomato and, thereby, functions to suppress the salicylic acid pathway or closure of stomata, allowing bacteria to reach higher densities in planta (Kloeck et al. 2001; Melotto et al. 2006; Uppalapati et al. 2007; Zhao et al. 2003). However, the physiological significance of COR-induced chlorosis in disease development is not completely understood. Recently, we demonstrated that silencing of *SGT1* (suppressor of G2 allele of *skp1*) abolished COR-induced chlorosis. Furthermore, in *Arabidopsis*, *AtSGT1b* but not *AtSGT1a* was required for COR responses and *P. syringae* pv. *tomato* DC3000 symptom (water-soaked lesion) development, and silencing of *SGT1* in tomato resulted in reduction of disease-associated cell death and chlorosis, suggesting a connection between COR-induced chlorosis and cell death (Uppalapati et al. 2010). In addition, COR targets photosynthetic machinery to modulate chloroplast ROS homeostasis in promoting disease-associated cell death during bacterial speck disease of tomato (Ishiga et al. 2009a and b). However, the precise plant targets for COR and a physiological and molecular understanding of localized cell death (bacterial speck) during disease development is not known. Understanding of this process is important for engineering bacterial speck resistance in tomato.

To identify the targets for COR and molecular interaction between COR-induced chlorosis and necrosis, we utilized *Nicotiana benthamiana* and virus-induced gene silencing (VIGS) to individually reduce the expression of over 4,000 genes, and identified several altered COR response (*ALC*) genes that caused altered responses to COR when silenced (Wangdi et al. 2010b). One of these sequences (Nb2F10; *ALC2*) showed 94% identity to tobacco (*N. tabacum*) thioredoxin peroxidase (GenBank accession number AJ309009) (Wangdi et al. 2010b). In this study, we further investigated the role of peroxiredoxin (Prx) or thioredoxin peroxidase and NADPH-dependent thioredoxin reductase C (NTRC) in COR signaling and disease-associated cell death. Our results showed that the loss-of-function of *NTRC*, an electron donor for Prxs in the NADPH-dependent thioredoxin (Trx) system, leads to accelerated ROS accumulation and *P. syringae* pv. *tomato* DC3000 disease-associated cell death in tomato and *Arabidopsis*

in a COR-dependent manner, and further indicated that NTRC or Prxs functions as a negative regulator of pathogen-induced cell death and, thereby, limits runaway cell death. Furthermore, *NTRC*-silenced tomato plants showed accelerated disease-associated cell death in response to the fungal pathogen *Sclerotinia sclerotiorum*. Therefore, our results indicate that chloroplast-generated ROS plays a common role in enhancing the cell death associated with hemibiotrophic and necrotrophic pathogens.

RESULTS

Prx- and *NTRC*-silenced *N. benthamiana* and tomato plants show necrosis in response to COR in a *COI1*-dependent manner.

The application of COR to vector control (TRV2::GFP; the green fluorescent protein [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 (Fig. 1A). However, *NbALC2/NbPrx*-silenced *N. benthamiana* plants which show a very high efficiency of silencing, as confirmed using real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR) (Supplementary Fig. S1A), produced necrosis upon COR application (Fig. 1A). The necrotic phenotype was associated with early induction and accumulation of higher levels of ROS when compared with the mock control plants (Fig. 1B). These results indicate that COR-induced ROS (H₂O₂) overaccumulate in *NbALC2/NbPrx*-silenced plants. Consistent with this hypothesis, externally applied H₂O₂ also induced more severe necrotic cell death at lower concentrations in *NbPrx*-silenced plants compared with the vector control plants (Supplementary Fig. S2). To further test the role of chloroplast-generated ROS in COR signaling, we cloned a fragment of tomato gene encoding *SlPrx* (SGN-U579709) and *NTRC*, a more efficient reductant of Prx, *SINTRC* (SGN U572332), with a very high amount of nucleotide sequence identity between tomato and *N. benthamiana* sequences encoding *NTRC* (Supplementary Figs. S3 and S4). It has been suggested that a 21-nucleotide stretch of 100% identity between the heterologous and endogenous gene sequences is not absolutely required to achieve efficient gene silencing using VIGS (Senthil-Kumar et al. 2007); therefore, we used *NTRC/Prx* coding sequences from tomato to silence ortholog genes in *N. benthamiana* or vice versa. Silencing of *SINTRC* in *N. benthamiana* resulted in efficient silencing of the endogenous *NbNTRC*. Application of COR to *NTRC*-silenced *N. benthamiana* resulted in a necrosis-like phenotype (Fig. 1C). These results suggested that COR induces chloroplastic ROS and, in *Prx*- and *NTRC*-silenced plants that are compromised in an efficient redox detoxification or homeostasis, COR-induced ROS overaccumulates, leading to necrotic cell death. Interestingly, no runaway cell death phenotype was observed in *Prx*- or *NTRC*-silenced *N. benthamiana* plants in response to HR-inducing nonhost pathogens (*P. syringae* pv. *tomato* strains DC3000 and T1), ETI (AvrPto), and a PAMP (INF1) (Supplementary Fig. S5).

However, it is important to note that silencing of *Prx* or *NTRC* in *N. benthamiana* resulted in a light-green leaf phenotype. Therefore, one could argue that the COR-induced necrotic phenotype in these plants may result from photobleaching or degradation products of chlorophyll rather than from the direct effects of COR-induced ROS. To rule out this possibility, we applied COR to *N. benthamiana* plants silenced for *ChlH*, the gene encoding the H subunit of the magnesium chelatase enzyme. Although *ChlH*-silenced *N. benthamiana* leaves had very low levels of their respective transcripts and showed reduced chlorophyll content and photobleaching, they failed to

show necrotic phenotype in response to COR (Fig. 1C). These results confirmed that the yellowish coloration or photobleaching per se does not affect the COR responses, and specific elimination of *Prx* or *NTRC* is required for induction of necrosis by COR in *N. benthamiana*.

To better understand the role of chloroplast-generated ROS in bacterial speck disease, we first tested whether COR induces the necrotic phenotype in *SlPrx*-silenced tomato plants. Consistent with our observations in *N. benthamiana*, COR induced necrosis phenotype on *Prx*-silenced tomato plants (Fig. 2A). Because *COI1/JAI1* is shown to be required for COR signaling in tomato and *Arabidopsis* (Feys et al. 1994; Katsir et al. 2008; Wangdi et al. 2010b), we transiently silenced *SlPrx* in *jai1* mutant plants to determine whether or not the necrosis we observed in *Prx*-silenced tomato plants upon COR treatment is *COI1/JAI1* dependent. The *Prx*-silenced *jai1* tomato plants showed no visible chlorosis or necrosis in response to exogenously applied COR (Fig. 2B), indicating that a COR-elicited necrosis phenotype in *Prx*-

silenced tomato and *N. benthamiana* plants is dependent on *COI1/JAI1*-mediated signaling.

***Prx*-silenced tomato plants show accelerated necrosis in response to *P. syringae* pv. *tomato* DC3000.**

To determine whether *SlPrx* affects disease symptoms induced by strain DC3000, we spray-inoculated vector control (TRV2::GFP) and *Prx*-silenced tomato plants with strain DC3000. The *Prx*-silenced tomato plants showed yellowish leaf coloration, which helped us select the plants with high efficiency of silencing, as further confirmed by the quantification of the endogenous transcript levels using qRT-PCR. Vector control plants developed typical bacterial specks with necrotic lesions surrounded by chlorotic halos (Fig. 3A). However, *Prx*-silenced plants developed accelerated (coalescing) necrotic lesions (Fig. 3A). Furthermore, the increased necrosis in strain DC3000-inoculated *Prx*-silenced plants was accompanied by a significant increase in ROS accumulation compared with the control (TRV2::GFP) plants (Fig. 3B). Interestingly, no sig-

Fig. 1. Coronatine (COR) induces necrosis and reactive oxygen species (H_2O_2) on peroxiredoxin (*Prx*)- and NADPH-dependent thioredoxin reductase C (*NTRC*)-silenced *Nicotiana benthamiana* leaves. **A**, Purified COR was applied to vector control (TRV2::GFP) or *Prx*-silenced (TRV2::NbPrx) *N. benthamiana* leaves in 2- μ l aliquots (2 nM) and a visible chlorotic zone or hypersensitive/necrotic cell death phenotype was recorded 4 days postinoculation (dpi). Phenotypes from three replicate treatments (marked circles) on each leaf from two independent silenced plants are shown. **B**, H_2O_2 production in vector control (TRV2::GFP) or *Prx*-silenced (TRV2::NbPrx) *N. benthamiana* leaves in response to COR at 12, 24, 48, and 72 h postinoculation (hpi). Three leaf discs from independently silenced plants from two biological replicates are shown. **C**, Chlorosis or necrosis phenotypes induced by COR on vector control (TRV2::GFP), *NTRC* (TRV2::NbNTRC)-, or *ChlH* (TRV2::NbChlH)-silenced *N. benthamiana* leaves in response to COR. Distilled water (2- μ l aliquots) was used as mock control. Photographs were taken 4 dpi with COR.

nificant differences in bacterial multiplication were observed between the control and *Prx*-silenced plants at 3 and 6 days postinoculation (dpi) (Fig. 3C). However, higher bacterial multiplication was observed in *Prx*-silenced plants at 1 dpi, suggesting that *Prx* may function in an early step of basal defense (Fig. 3C). These results indicated that chloroplast-localized ROS contributes to the necrotic symptom development without altering the pathogen multiplication in tomato, and *Prx* may function as a negative regulator of disease-associated cell death.

Loss-of-function of *NTRC* in *Arabidopsis* and tomato results in accelerated necrotic cell death in response to *P. syringae* pv. *tomato* DC3000.

Previous results indicated that COR functions as an important virulence factor in tomato and *Arabidopsis*, although it functions differently in these hosts (Bent et al. 1992; Brooks et al. 2005; Uppalapati et al. 2007). To investigate the role of chloroplast ROS homeostasis in strain DC3000 disease development in *Arabidopsis*, we analyzed four individual T-DNA

Fig. 2. Coronatine (COR)-insensitive 1 (COI1) is required for a COR-inducible necrosis phenotype on peroxiredoxin (*Prx*)-silenced tomato leaves. **A**, Purified COR elicits chlorosis on vector control (TRV2::GFP) plants and a necrosis phenotype on *Prx*-silenced (TRV2::SIPrx) tomato leaves. **B**, COR failed to induce chlorosis in *jai1/coi1* mutants (TRV2::GFP) or necrosis on *Prx*-silenced jasmonic acid-insensitive 1 (*jai1/coi1*) plants. COR was applied in 2- μ l aliquots (2 nM) and a visible chlorotic zone or necrotic cell death phenotype was recorded 4 days postinoculation. Phenotypes from two replicates (marked circles) on each leaf from two independently silenced plants are shown.

insertional mutants deficient in chloroplast-localized *peroxiredoxins* (*PrxA*, *PrxB*, *PrxQ*, and *PrxIIIE*) and a T-DNA insertion mutant of *NTRC*, an electron donor for Prxs in the Trx system (Spinola et al. 2008). To test whether these *prx* and *ntrc* *Arabidopsis* mutants are loss-of-function alleles, the expression levels of respective genes were confirmed by semiquantitative

RT-PCR (Supplementary Fig. S6). Two-week-old *Arabidopsis* plants, including wild-type (Col-0), *prx*, and *ntrc* mutants grown on Murashige and Skoog (MS) agar, were dip-inoculated with strain DC3000 (5×10^6 CFU/ml) and monitored for disease development. The wild-type Col-0 plants showed typical disease symptoms, consisting of chlorosis with water-soaked

Fig. 3. Silencing of peroxiredoxin (*Prx*) (TRV2::SIP_{Prx}) in tomato leads to severe cell death with coalescing lesions in response to *Pseudomonas syringae* pv. *tomato* DC3000. **A**, Bacterial speck disease development; **B**, reactive oxygen species (ROS) accumulation visualized by staining hydrogen peroxide using 3,3'-diaminobenzidine; and **C**, in planta bacterial population numbers in vector control (TRV2::GFP) and *Prx*-silenced tomato (TRV2::SIP_{Prx}). Plants were spray inoculated to runoff with strain DC3000 at 2 to 5×10^7 CFU/ml in sterile distilled water containing 0.025% Silwet L-77. Inoculated plants were observed for 7 days postinoculation (dpi) for symptom development. Bacterial growth was evaluated in three independent experiments at 0, 1, 3, and 6 dpi. Disease phenotypes from three independent silenced plants are shown. Vertical bars indicate the standard error for three independent experiments.

Fig. 4. Response of *Arabidopsis* peroxiredoxin (*prx*) and NADPH-dependent thioredoxin reductase C (*ntrc*) mutant to *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000). **A**, Disease phenotype of *Arabidopsis* chloroplast-localized *prx* and *ntrc* mutants after dip-inoculation with strain DC3000 at 5×10^6 CFU/ml. Photographs were taken 2 days postinoculation (dpi). **B**, Hydrogen peroxide production in *Arabidopsis* chloroplast-localized *prx* and *ntrc* mutants in response to strain DC3000. Reactive oxygen species (ROS) were visualized by staining hydrogen peroxide using 3,3'-diaminobenzidine, at 2 dpi. **C**, The intensity of DAB staining shown in panel B is quantified and expressed as percentage of coloration, where the color intensity of mock-treated wild-type (WT) Col-0 leaves was set at 100%. Asterisks indicate a significant difference from WT Col-0 in a Student *t* test (* < 0.01). **D**, Ion leakage from *Arabidopsis* chloroplast-localized *prx* and *ntrc* mutants after treatment with water (mock) or inoculation with strain DC3000 at 5×10^6 CFU/ml; measurements were taken 2 dpi. Values show the percentage of total ions. Vertical bars indicate the standard error for three independent experiments. Asterisks indicate a significant difference from WT Col-0 in a Student *t* test (* < 0.01). **E**, Growth of strain DC3000 in *Arabidopsis* chloroplast-localized *prx* and *ntrc* mutants. Bacterial populations were obtained by surface sterilizing inoculated leaves, then plating dilutions to selective media 2 dpi. Vertical bars indicate the standard error for three independent experiments.

lesions in response to strain DC3000 (Fig. 4A). Interestingly, individual mutations in any of the four chloroplastic *prx* genes (*prxa*, *prxb*, *prxq*, and *prxllc*) did not significantly impact disease symptom development. These plants showed disease symp-

toms typical of wild-type Col-0 plants (Fig. 4A). However, the *ntrc* mutant plants showed severe chlorosis and necrotic cell death in response to strain DC3000 when compared with the wild-type Col-0 plants (Fig. 4A). Furthermore, *NTRC*-overex-

Fig. 5. *Pseudomonas syringae* pv. *tomato* DC3000 induces cell death in a coronatine (COR)-dependent manner in NADPH-dependent thiredoxin reductase (*NTRC*)-silenced tomato and *ntrc* mutants of *Arabidopsis*. **A**, Disease phenotype of vector control (TRV2::GFP) and *NTRC*-silenced (TRV2::SINTRC) tomato plants spray-inoculated to runoff with strain DC3000 (*Pst* DC3000) or COR mutant *P. syringae* pv. *tomato* DB29 (*Pst* DB29) at 2×10^7 to 5×10^7 CFU/ml or with distilled water (Mock) containing 0.025% Silwet L-77. Photographs were taken 6 days postinoculation (dpi). **B**, In planta bacterial population numbers in vector control (TRV2::GFP) and *NTRC*-silenced tomato (TRV2::SINTRC) plants spray-inoculated with strain DC3000 or DB29 in sterile distilled water. Bacterial growth was evaluated in three independent experiments at 0, 2, and 4 dpi. Vertical bars indicate the standard error for three independent experiments. Asterisks indicate a significant difference between TRV2::GFP and TRV2::SINTRC in a Student *t* test (* < 0.01). **C**, Disease phenotype of wild-type Col-0 and *ntrc* mutant plants after flood-inoculation with distilled water (Mock), strain DC3000, or DB29. Photographs were taken at 3 dpi. Phenotypes from three independent plants are shown. **D**, Growth of strain DC3000 or DB29 in *Arabidopsis ntrc* mutants evaluated at 0, 2, and 4 dpi. Vertical bars indicate the standard error for three independent experiments.

pressing plants displayed typical disease symptoms similar to the wild-type Col-0 plants after inoculation with strain DC3000 (Supplementary Fig. S7).

To further investigate whether strain DC3000-induced necrotic cell death is associated with accelerated ROS, 3,3'-diaminobenzidine (DAB) staining was carried out to detect H₂O₂ in wild-type, *prx*, and *ntrc* mutants after dip inoculation with strain DC3000. The accumulation of H₂O₂ was detected in wild-type, *prx*, and *ntrc* mutants in response to strain DC3000 (Fig. 4B and C). No significant differences in H₂O₂ accumulation were observed between wild-type and *prx* mutants. However, a significantly higher level of ROS accumulation was observed in *ntrc* mutant plants compared with wild-type Col-0 and *prx* plants (Fig. 4B and C). Furthermore, consistent with increased necrosis, a higher level of ion leakage (an indicator of cell death) was observed in *ntrc* plants inoculated with strain DC3000 compared with the wild-type Col-0 and *prx* mutants plants (Fig. 4D). These results indicated that the chloroplast-generated ROS functions as a positive signal leading to cell death in *ntrc* mutants.

To determine whether the more severe disease symptoms in the *ntrc* mutant plants could be caused by increased bacterial populations, the bacterial populations were evaluated at 1, 2, and 4 dpi with strain DC3000. No significant differences were observed in the populations of strain DC3000 in *prx* mutants, the *ntrc* mutant, and wild-type Col-0 plants (Fig. 4E). Taken together, these results suggested that chloroplast-generated ROS play a role in disease-induced cell death development in *Arabidopsis*, and NTRC functions as a negative regulator of pathogen-induced cell death by increased scavenging of the ROS in the healthy tissue surrounding the lesions.

We further investigated whether the accelerated disease-associated cell death in plants with altered ROS homeostasis is dependent on COR-induced effects on the chloroplast. To this end, we inoculated NTRC-silenced tomato plants and *ntrc* mutant *Arabidopsis* plants with strain DC3000 and *P. syringae* pv. *tomato* DB29, a COR-defective mutant of strain DC3000. *SINTRC* silenced plants with a high level of silencing of endogenous *SINTRC* (as confirmed using qRT-PCR, which was used for inoculation assays). The vector control plant showed typical bacterial speck symptoms, consisting of necrotic lesions surrounded by chlorosis, within 7 days after spray inoculation with strain DC3000 (Fig. 5A), whereas NTRC-silenced plants showed severe necrotic cell death with coalescing lesions (Fig. 5A). Inoculation with COR-deficient strain DB29 failed to induce higher levels of cell death (necrosis) on NTRC-silenced plants. However, strain DB29 caused sporadic cell death (Fig. 5A) and multiplied higher in *SINTRC*-silenced plants compared with the vector control plants (Fig. 5B), indicating that NTRC has more generic effects on cellular redox associated with many biotic stresses. Furthermore, following flood inoculation, strain DB29 also failed to induce severe disease symptoms in wild-type and *ntrc* mutant seedlings of *Arabidopsis* (Fig. 5C). Interestingly, unlike tomato, strain DB29 did not multiply on *ntrc* mutant plants (Fig. 5D). These results further demonstrated a role for COR-induced chloroplast-generated ROS in promoting disease-associated cell death in tomato and *Arabidopsis*.

***P. syringae* pv. *tomato* DC3000 suppresses the expression of chloroplast-localized *Prxs* and NTRC in tomato and *Arabidopsis*.**

To further investigate the expression profiles of chloroplast-localized *Prxs* and NTRC during strain DC3000–host interactions, we carried out the Northern blot analysis of these genes in tomato and *Arabidopsis* after inoculation with *P. syringae* pv. *tomato* DC3000 and COR-defective mutant strain DB29,

TTSS-defective *hrcC* mutant (Huang et al. 1992), or the exogenous application with 2 nM of COR. In tomato plants, the expression of chloroplast-localized 2-Cys *Prx*, *PrxQ*, *PrxIIIE*, and NTRC was downregulated by strain DC3000 but not by strain DB29 (Fig. 6A). In addition, exogenous COR application strongly suppressed the expression of these genes in tomato (Fig. 6A), further confirming that strain DC3000 may manipulate chloroplast ROS homeostasis in a COR-dependent manner. The expression of NTRC was not suppressed in *hrcC* mutant-inoculated tissue (Fig. 6B); however, slight suppression of 2-Cys *Prx*, *PrxQ*, and *PrxIIIE* transcripts was observed at 24 h postinoculation (hpi) in *hrcC* mutant-inoculated tissues compared with the mock-inoculated control (Fig. 6A). These results suggested that virulent (symptomatic) strain DC3000 but not strain DB29 or *hrcC* downregulates NTRC and *Prx* genes.

In *Arabidopsis* plants, similar to tomato plants, strain DC3000 but not strain DB29 and *hrcC* mutants suppressed the expression of chloroplast-localized 2-Cys *Prx*, *PrxQ*, *PrxIIIE*, and NTRC

Fig. 6. Expression profiles of chloroplast-localized 2-Cys peroxiredoxin (*Prxs*), peroxiredoxin IIE (*PrxIIIE*), *PrxQ*, and NADPH-dependent thioredoxin reductase C (*NTRC*) in **A**, tomato and **B**, *Arabidopsis* plants treated with water (control), coronatine (COR) (2 nM), or inoculated with *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000), COR mutant *P. syringae* pv. *tomato* strain DB29 (*Pst* DB29), or *hrcC* mutant. Total RNA (10 µg) was analyzed, and ethidium bromide staining was used to show that the quality and equal quantity of RNA loaded on different blots.

(Fig. 6B). Furthermore, unlike in tomato, authentic COR did not suppress the expression of *2-Cys Prx*, *PrxQ*, *PrxIIIE*, and *NTRC* at 12 and 24 hpi in *Arabidopsis* (Fig. 6B). Taken together, these results suggest that strain DC3000 may modulate chloroplast ROS homeostasis by downregulating the expression of genes encoding ROS-quenching enzymes, such as *Prxs* and *NTRC*.

***NTRC*-silenced tomato plant shows accelerated necrotic cell death in response to *S. sclerotiorum*.**

Oxalic acid produced by *S. sclerotiorum* has been shown to induce ROS accumulation and elicit PCD during disease development (Kim et al. 2008). To further examine whether the chloroplast-generated ROS plays a general role in disease-associated necrotic cell death, we inoculated vector control and *NTRC*-silenced tomato plants with a necrotrophic fungal pathogen, *S. sclerotiorum*, and monitored disease symptom development. The *NTRC*-silenced plants showed accelerated necrotic cell death and disease symptoms compared with vector-control plants (Fig. 7), indicating that chloroplast ROS may play a role in disease-associated cell death during tomato–*S. sclerotiorum* interactions.

DISCUSSION

In this study, altered chlorosis in response to COR led to the identification of a role for chloroplast ROS and a detoxification mechanism based on *Prxs* and *NTRC* in regulation of *P. syringae* pv. *tomato* DC3000 disease-associated cell death (Figs. 1 and 2). Furthermore, necrosis and ROS (H_2O_2) spread beyond the region of COR application (Fig. 1A and B), and spray inoculation of strain DC3000 on *Prx*- or *NTRC*-silenced tomato plants induced accelerated necrotic lesions similar to the runaway cell death phenotype reported earlier in the *Arabidopsis* *acd1*, *acd2*, and *thf1* mutants (Greenberg and Ausubel 1993; Mach et al. 2001; Wangdi et al. 2010a). However, *Prx*- or *NTRC*-silenced *N. benthamiana* plants showed no runaway

cell death phenotype in response to HR-inducing nonhost pathogens and gene-for-gene interactions.

To further investigate the role of chloroplast ROS and a detoxification mechanism based on *Prx* and *NTRC* during host interactions, we chose *Arabidopsis*, because it is genetically tractable and is a host of strain DC3000. Mutations in any of the four individual chloroplast *prx* genes did not result in altered disease symptom development in response to host pathogen strain DC3000 (Fig. 4), suggesting that *Arabidopsis* *Prxs* function as redundant regulators of ROS homeostasis in the chloroplast. Consistently, Kirchsteiger and associates (2009) reported no phenotypic changes associated with the single T-DNA insertional mutants of *Prx A* and *Prx B*. Furthermore, a double mutant of *Prx A* and *Prx B* ($\Delta 2cp$) was also shown to have wild-type growth, suggesting that redox imbalance in these mutants can be compensated for by alternative chloroplast antioxidant systems or other members of the *Prx* family (Pulido et al. 2010). However, silencing of *NbPrx* (closest homologue of *PrxA* and *PrxB*) via VIGS in *N. benthamiana* and tomato resulted in pale green leaves and necrosis phenotype upon COR application (Figs. 1 and 2), and strain DC3000-inoculated, *Prx*-silenced tomato plants also showed accelerated cell death and higher levels of H_2O_2 accumulation compared with the control plants (Fig. 3). Deficiencies of multiple members of *Prx* were required to see any significant effects of *Prx* in pathogen-associated cell death. Furthermore, based on VIGS analysis, we could not determine which members of *Prx* are specifically involved in chloroplast ROS homeostasis. Therefore, in this study, we focused on *NTRC*, an NADPH thioredoxin reductase that plays a central role in redox regulation (Pulido et al. 2010). Interestingly, an *Arabidopsis* knockout mutant of *NTRC* showed accelerated necrotic cell death in response to strain DC3000 (Fig. 4). *NTRC* contains an NADP-thioredoxin reductase (NTR) and a Trx domain in a single polypeptide chain and uses NADPH in detoxifying H_2O_2 via *Prx* (Pulido et al. 2010; Spinola et al. 2008). An *Arabidopsis* *ntrc* mutant has been shown to have pale green leaves associated with reduced chlorophyll contents, abnormal chloroplast structure, and unbalanced redox state (Lepisto et al. 2009; Perez-Ruiz et al. 2006). Furthermore, *NTRC* was demonstrated to be required for protection against oxidative damage, especially in low light and prolonged darkness (Perez-Ruiz et al. 2006), and was shown to play a more important role in chloroplast *Prx* reduction than *Trx* (Pulido et al. 2010). Consistent with the previous studies that show *NTRC* to be critical for redox regulation, here, we demonstrated a role for *NTRC* in regulation of pathogen-associated cell death, possibly via increased accumulation of chloroplast-generated ROS (H_2O_2) in *ntrc* mutants (Fig. 4). Overexpression of *NTRC* did not alter bacterial multiplication or disease symptom development in *Arabidopsis*. Consistent with our findings, overexpression of *NTRC* did not alter sensitivity to prolonged darkness (Perez-Ruiz et al. 2006). However, unlike the phenotype shown by the overexpression of *NTRC* in this study, the enhancement of oxidative stress tolerance in transgenic plants overexpressing *ascorbate peroxidase* (*APX*) has been reported (Badawi et al. 2004; Murgia et al. 2004). Furthermore, overexpression of *Gentiana triflora* *PrxQ* was shown to impart resistance against both oxidative stress and fungal disease (Kiba et al. 2005). It is important to note that, unlike *APX* and *PrxQ*, *NTRC* functions as a central redox regulator in *Prx*-based detoxification system. Moreover, Pérez-Ruiz and associates (2006) reported that, although more transcripts of *NTRC* were detected in *NTRC*-overexpressing *Arabidopsis* plants, the amount of *NTRC* proteins was similar with the wild type (Perez-Ruiz et al. 2006). Therefore, it is tempting to speculate that the protein level of *NTRC* might be tightly controlled by posttranscriptional regulation.

Fig. 7. *Sclerotinia sclerotiorum* disease symptoms on NADPH-dependent thioredoxin reductase C (*NTRC*)-silenced tomato plants. Control (TRV2::GFP) and *NTRC*-silenced (TRV2::SINTRC) plants were inoculated with potato dextrose agar plugs with actively growing *S. sclerotiorum* cultures. Inoculated plants were incubated at room temperature. Photographs were taken 24, 48, and 72 h postinoculation. Phenotypes from two replicate treatments (agar discs) on each leaf from two independent silenced plants are shown.

Taken together with this study, there is increasing evidence for the role of chloroplast-generated ROS in HR or disease-associated cell death. In support of our results, a recent study demonstrated the requirement of chloroplast-generated ROS for HR cell death using tobacco plants expressing a chloroplast-targeted cyanobacterial flavodoxin, which functions as a general antioxidant to prevent the formation of ROS in chloroplasts (Zurbriggen et al. 2009). A chloroplastic ROS detoxification mechanism based on ferredoxin:thioredoxin reductase (FTR) was also shown to regulate PCD in tomato (Lim et al. 2010). Interestingly, unlike the phenotypes shown by the *NTRC*- or *Prx*-silenced plants in this study, Lim and associates (2010) showed that silencing of *SIFTR-c* results in spontaneous necrosis development even without the pathogen inoculation. Moreover, *SIFTR-c*-silenced plants showed enhanced resistance to *P. syringae* pv. *tomato* DC3000 when inoculated at a lower concentration of initial inoculum (1×10^5 CFU/ml) (Lim et al. 2010). In the absence of FTR, O_2 is the major acceptor of electrons from the photosynthetic electron transport. Thus, *SIFTR-c*-silenced plants overaccumulate chloroplast-generated ROS during general oxygenic photosynthesis, and these ROS serve as triggers of cell death and defense response before the onset of pathogen invasion. However, COR-induced effects on the photosynthetic machinery were required to create a metabolic imbalance in *Prx*- or *NTRC*-silenced plants to generate higher levels of ROS that are sufficient to trigger disease-associated cell death. These observations suggested that chloroplast-generated ROS play a crucial role in localized lesion (cell death) development in a COR-dependent manner. In support of these observations, pretreatment of tomato seedlings with 3-(3,4-dichlorophenyl)-1,1-dimethylurea, which inhibits photosynthetic electron transport and subsequent ROS generation, decreased the strain DC3000-induced necrotic symptoms (Ishiga et al. 2009a). It is important to note that reduction of Prx or NTRC by VIGS resulted in significantly accelerated disease-associated cell death accompanied by increased production of ROS in a COR-dependent manner, suggesting that the NTRC-mediated detoxification system functions as a crucial negative regulator of pathogen-induced cell death in the healthy tissue surrounding the lesions. Based on these results, it is tempting to speculate that cells that are in direct contact with COR produced by bacterial colonies in the infected leaves may produce sufficiently high rates of chloroplast-generated (sourced) ROS, resulting in localized cell death leading to lesion development. Furthermore, ROS are shown to play a role in regulating programmed cell death via MAPK pathways (Ren et al. 2002). Therefore, it remains to be tested whether the chloroplast-generated ROS may also serve as a signal to activate MAPKKK α , a positive regulator of cell death induced by strain DC3000 in tomato (del Pozo et al. 2004).

In addition, *SGT1/SGT1b* is shown to be required for COR-induced chlorosis and subsequent necrotic disease development in tomato and *Arabidopsis* (Uppalapati et al. 2010). However, *SGT1* was found to be a component of the COR/JA-mediated signal transduction pathway but not a component of the COI1 complex (Uppalapati et al. 2010). Taken together, these studies suggest that COI1/SGT1-dependent COR-induced chlorosis generates ROS that trigger targeted cell death leading to lesion formation. Moreover, several effector proteins and PAMPs function through COI1- and JA-dependent pathways to promote virulence but not necessarily cell death during disease- or HR-associated PCD (Chang et al. 2000; DebRoy et al. 2004; Katou et al. 2005). In a previous report, it was demonstrated that the *N. benthamiana* jasmonate ZIM domain (JAZ) protein was related to PAMP-mediated signaling pathways leading to cell death. *N. benthamiana* JAZ proteins NtPPS1 and NtPPS3 were shown to be phosphorylated by PAMP-me-

diated MAPK. NtPPS3 was also demonstrated to be efficient for cell death activated by an MAPK cascade (Katou et al. 2005). It is well-known that JAZ proteins are direct targets of the SCF^{COI1} E3 ubiquitin-ligase, and JA or COR treatment induces their proteasome-mediated degradation, leading to release of the key transcriptional activator of JA responses, such as AtMYC2 (Chini et al. 2007; Thines et al. 2007). Furthermore, the COI1-JAZ complex was demonstrated to function as a receptor for JA or COR (Katsir et al. 2008). Based on these reports, we speculate that the COI1-JAZ complex or other components of JA or COR signaling pathways are related to both disease-associated cell death and HR cell death. Further characterization of targets for the chloroplast-generated ROS involved in disease-associated cell death and identification of plant signaling components targeted by COR and PAMPs are needed to understand the mechanisms of chloroplast-generated ROS-mediated redox signaling pathways leading to cell death.

In addition to COR, other virulence determinants, including effector proteins, are shown to be involved in necrotic cell death associated with disease (Abramovitch and Martin 2004; Block and Alfano 2011; Chang et al. 2000; Cohn and Martin 2005; DebRoy et al. 2004; del Pozo et al. 2004; Munkvold et al. 2009). Interestingly, silencing of LeMAPKKK α , a positive regulator of disease-associated cell death, is shown to reduce bacterial growth, suggesting that host-mediated cell death during the compatible interaction plays an important role in allowing full growth of *P. syringae* pv. *tomato* DC3000 (del Pozo et al. 2004). Therefore, our results, taken together with previous reports (Badel et al. 2006; Cohn and Martin 2005; del Pozo et al. 2004), raise some important questions on the mechanism or mechanisms of cell death elicitation during bacterial speck disease and the significance of host-mediated cell death during strain DC3000-associated cell death. It appears that host-cell (chloroplast)-death pathways may serve to remobilize nutrients into the apoplast by inducing senescence. It is tempting to speculate that several of the effectors may target chloroplast and hijack components of host-mediated cell death pathways to favor virulence, whereas chloroplast-generated ROS may serve as one of the components in triggering PCD associated with compatible interactions and leaf senescence (Quirino et al. 2000; Zapata et al. 2005). Therefore, it is tempting to speculate that chlorosis (or senescence) or localized cell death induced by COR may help to remobilize nutrients into the apoplast to help bacteria achieve full growth during the necrogenic phase of the hemibiotrophic pathogen, strain DC3000. In support of our hypothesis, it has been shown that *P. syringae* has acquired genes or pathways to assimilate a wide range of metabolites corresponding to those abundantly present in the apoplast of healthy plants and those that accumulate in plants during biotic and abiotic stress and senescence (Rico and Preston 2008).

Furthermore, taken together with previous reports, our results further support a central role for chloroplasts in disease development and as a major target for virulence determinants (e.g., COR and Hop1) of strain DC3000 (Guttman et al. 2002; Ishiga et al. 2009b; Jelenska et al. 2007, 2010; Munkvold et al. 2009; Uppalapati et al. 2010; Wangdi et al. 2010b). Further identification of other effectors that target chloroplasts and characterization of their mode of action in conjunction with COR and targets for the chloroplast-generated ROS are needed to understand the mechanisms of chloroplast-generated ROS in disease-associated cell death.

MATERIALS AND METHODS

Plant materials, bacterial strains, and inoculation methods.

Nicotiana benthamiana and tomato (*Solanum lycopersicum* ‘Glamour’) plants were maintained in the greenhouse under a

photoperiod with 14 h of light and 10 h of darkness at $25 \pm 2^\circ\text{C}$. *Arabidopsis thaliana* homozygous T-DNA insertion mutants deficient in *PrxA* (At3g11630; CS875813), *PrxB* (At5g06290; SALK_017213), *PrxQ* (At3g26060; SALK_070860), and *PrxIII* (At3g52960; SALK_064512) were identified from the SALK Institute's collection by PCR analysis of genomic DNA according to the recommended protocols, and the expression levels of respective genes were confirmed by semiquantitative RT-PCR. Seed of the *Arabidopsis ntrc* T-DNA mutant, its complemented lines, and overexpression lines (Perez-Ruiz et al. 2006) were kindly provided by F. J. Cejudo (Instituto de Bioquímica Vegetal y Fotosíntesis, Spain). The homozygous lines of *NTRC* were confirmed by PCR analysis of the genomic DNA and *NTRC* expression analysis using semiquantitative RT-PCR. Seeds of *Arabidopsis* lines were germinated and maintained on half-strength MS medium (0.3% phytigel) with Gamborg vitamins (PhytoTechnologies Laboratories, Shawnee Mission, KS, U.S.A.) and used for inoculation assay 2 weeks after germination at 25°C with a light intensity of $200 \mu\text{E m}^{-2} \text{s}^{-1}$ and a photoperiod with 12 h of light and 12 h of darkness.

P. syringae pv. *tomato* DC3000 was used as the wild-type strain for virulence assays on tomato and *Arabidopsis*. The COR-defective mutant strain DB29 was previously described (Brooks et al. 2004) and was kindly provided by B. Kunkel (Washington University, St. Louis). The *P. syringae* pv. *tomato* strains DC3000 and DB29 were grown at 28°C on mannitol-glutamate (MG) medium (Keane et al. 1970) containing antibiotics in the following concentrations: rifampicin, $50 \mu\text{g ml}^{-1}$; kanamycin, $25 \mu\text{g ml}^{-1}$; and spectinomycin, $25 \mu\text{g ml}^{-1}$ for 36 to 48 h. Prior to inoculation, bacteria were suspended in 1 ml of sterile distilled H_2O .

Pathogen inoculation assays on vector control and VIGS-silenced tomato were conducted as described (Uppalapati et al. 2007). Briefly, bacterial suspensions (optical density at 600 nm [OD_{600}] = $0.1 = 2$ to 5×10^7 CFU/ml) were prepared in sterile distilled water containing 0.025% Silwet L-77 (OSI Specialties Inc., Danbury, CT, U.S.A.) and spray-inoculated to runoff. The inoculated plants were then incubated in growth chambers at approximately 100% relative humidity (RH) for the first 24 h and at approximately 70% RH for the rest of the experimental period. High humidity was achieved by placing a cool mist humidifier (ReliOn, 2 gallons/day) in the growth chamber. The inoculated plants were observed for 7 dpi for symptom development. Bacterial growth in leaves was measured by determining the internal bacterial populations. Prior to sampling, leaves were surface sterilized with 15% H_2O_2 for 3 min to eliminate epiphytic bacteria and then washed with sterile distilled water. The leaves were then homogenized in sterile distilled water, and serial dilutions were plated onto MG medium containing antibiotics. The bacterial population at 0 dpi was estimated from leaves harvested 1 hpi. Bacterial growth was evaluated in three independent experiments.

Arabidopsis seedlings were inoculated by a modified method in which plants grown on solid media plates, prepared with half-strength MS medium with Gamborg vitamins and 1% sucrose (pH 5.7 to 5.8) and solidified with 0.3% phytigel (Sigma-Aldrich, St. Louis) in deep petri plates (100 mm $\times$ 25 mm), were flooded with bacterial cells as described for tomato (Ishiga et al. 2011; Uppalapati et al. 2010). Briefly, *Arabidopsis* seedlings grown on half-strength MS solid media were flooded with bacterial suspension with an $\text{OD}_{600} = 0.02$ (5×10^6 CFU/ml) that consisted of sterile distilled H_2O containing 0.025% Silwet L-77 (OSI Specialties Inc.). To achieve uniform inoculation, the bacterial suspension was dispensed into the petri dishes until the seedlings were completely submerged, and the seedlings were exposed to bacterial cells for 2 to 3 min with gentle mixing. After the inoculum was discarded, petri

dishes containing inoculated seedlings were sealed with 3M micropore 2.5-cm surgical tape (3M, St. Paul, MN, U.S.A.) and were incubated for 2 to 3 days at 24°C with light intensity of 100 to $150 \mu\text{E m}^{-2} \text{s}^{-1}$ and 12-h light-and-dark photoperiod. Another set of seedlings was flooded with sterile water that served as a mock control. In each experiment, 36 to 48 seedlings were evaluated, and each experiment was repeated at least three times. For bacterial growth in leaves, *Arabidopsis* seedlings were surface sterilized with 5% H_2O_2 . The bacterial populations were evaluated using the methods described above for tomato.

VIGS and *Agrobacterium*-mediated transient expression assay.

Agrobacterium tumefaciens GV2260 containing pTRV1 and pTRV2 with gene of interest (e.g., pTRV2::*GFP*, pTRV2::*Prx*, pTRV2::*NTRC*, pTRV2::*ChlH*, and pTRV2::*PDS*) was grown overnight on Luria-Bertani (LB) medium containing antibiotics (rifampicin, $25 \mu\text{g/ml}$ and kanamycin, $50 \mu\text{g/ml}$) at 28°C . Bacterial cells were harvested and resuspended in induction medium (10 mM MES, pH 5.5, and $200 \mu\text{M}$ acetosyringone) and incubated at room temperature on an orbital shaker for 5 h. The bacterial cultures containing pTRV1 and pTRV2 with the gene of interest were mixed in equal ratios ($\text{OD}_{600} = 0.5$) and infiltrated into lower leaves using a 1-ml needleless syringe (Liu et al. 2002; Ryu et al. 2004). The infiltrated plants were maintained in the greenhouse and used for studies 15 to 21 days postinfiltration.

To analyze the impact of gene-for-gene or PAMP-mediated HR in wild-type, *Prx*, and *NTRC*-silenced plants, *A. tumefaciens* GV2260 containing 35S::*Pto* and 35S::*AvrPto* or 35S::*INFL* were grown overnight on LB medium containing antibiotics (rifampicin, $25 \mu\text{g/ml}$ and kanamycin, $50 \mu\text{g/ml}$) at 28°C . Bacterial cells were harvested and resuspended in induction medium and incubated at room temperature in an orbital shaker for 12 h. For experiments in which *Pto* and *AvrPto* were coexpressed, *Agrobacterium* cultures carrying these plasmids were mixed in equal ratios ($\text{OD}_{600} = 0.8$) and infiltrated into leaves using a 1-ml needleless syringe. Four days after infiltration, gene-for-gene mediated HR cell death was observed.

Northern blot, real-time qRT-PCR and semiquantitative RT-PCR analyses.

Total RNA extraction and real-time qRT-PCR were done as described previously (Uppalapati et al. 2008). Total RNA was treated with Turbo DNase (Ambion, Austin, TX, U.S.A.) to eliminate genomic DNA, and $5 \mu\text{g}$ of DNase-treated RNA was reverse transcribed using Superscript III reverse transcriptase (Invitrogen Corp., Carlsbad, CA, U.S.A.) with oligo d(T)15-20 primers. The cDNA (1:20) was then used for RT-PCR or qRT-PCR. qRT-PCR was performed using primers shown in Supplementary Table S1 with Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, U.S.A.) with an ABI Prism 7900 HT sequence detection system (Applied Biosystems). The *N. benthamiana* and tomato *Actin* was used as internal control. To study the downregulation of transcripts in silenced plants, qRT-PCR primers that anneal outside the region targeted for silencing were used. Average cycle threshold values calculated using Sequence Detection Systems (version 2.2.2; Applied Biosystems) from triplicate samples were used to determine the fold expression relative to controls, whereas semiquantitative RT-PCR was performed using a thermal cycler using gene-specific primers. *AtActin* was used as internal control.

Northern blot analysis was carried out as described previously with slight modifications (Ishiga et al. 2009b). Total RNA was extracted using TRIzol (Sigma) according to the manufacturer's

protocol. Total RNA (10 µg) was denatured, fractionated by electrophoresis on a 1% agarose gel, and then blotted onto nylon membranes (Amersham BioScience, Uppsala, Sweden). Digoxigenin (DIG)-labeled DNA probes were made with DIG PCR Labeling Mix (Boehringer Mannheim, Mannheim, Germany) with gene-specific primer sets. Hybridization was performed at 65°C overnight in a solution containing 50 mM Church buffer (pH. 7.2), 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% laurylsarcosine, 7% sodium dodecyl sulfate (SDS), and 1% blocking solution (Boehringer Mannheim). Final washes were at 65°C in a solution containing 0.5X SSC and 0.1% SDS. Hybridized mRNAs were detected with anti-DIG antibody conjugated with alkaline phosphatase (Boehringer Mannheim) and its color detection substrate, BCIP/NBT (Promega Corp.).

Detection of hydrogen peroxide.

The generation of hydrogen peroxide was detected using DAB staining as described previously (Ishiga et al. 2009b; Ueno et al. 2003). After application with 2 µl of COR (1 mM) or inoculation with *P. syringae*, the leaves were placed in DAB-HCl (pH 3.8) at 1 mg/ml. After incubation for 6 h at room temperature, chlorophyll was removed with 95% ethanol. DAB staining in the leaves was quantified using Image J software (version 1.44). The intensity of staining was expressed as percentage of coloration, where the intensity of coloration of mock-inoculated wild-type Col-0 was set to 100%.

Ion leakage measurements.

Ion leakage was used as an indirect quantitative measure for disease-associated cell death. Ion leakage was measured as described previously (Ishiga et al. 2009b). *Arabidopsis* seedlings grown on MS phytigel plates inoculated with *P. syringae* were gently removed and agitated in 30 ml of distilled water for 3 h, and electroconductivity was measured using an ion conductivity meter (Orion 555A; Thermo Electron Corp., Marietta, OH, U.S.A.). Plants were then autoclaved for 20 min to kill the cells and release total ions into the medium. Values relative to the whole ion content after autoclaving were used to express the percent ion leakage.

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