Necrotrophic Pathogens Use the Salicylic Acid Signaling Pathway to Promote Disease Development in Tomato

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Plants use different immune pathways to combat pathogens. The activation of the jasmonic acid (JA)-signaling pathway is required for resistance against necrotrophic pathogens; however, to combat biotrophic pathogens, the plants activate mainly the salicylic acid (SA)-signaling pathway. SA can antagonize JA signaling and vice versa. NPR1 (non-inducible pathogenesis-related 1) is considered a master regulator of SA signaling. NPR1 interacts with TGA transcription factors, ultimately leading to the activation of SA-dependent responses. SA has been shown to promote disease development caused by the necrotrophic pathogen *Botrytis cinerea* through NPR1, by suppressing the expression of two JA-dependent defense genes, *proteinase inhibitors I and II*. We show here that the transcription factor TGA1.a contributes to disease development caused by *B. cinerea* in tomato by suppressing the expression of *proteinase inhibitors I and II*. Finally, we present evidence that the SA-signaling pathway contributes to disease development caused by another necrotrophic pathogen, *Alternaria solani*, in tomato. Disease development promoted by SA through NPR1 requires the TGA1.a transcription factor. These data highlight how necrotrophs manipulate the SA-signaling pathway to promote their disease in tomato.

Plants are able to defend themselves against attack by a variety of potential pathogens through the deployment of both constitutive and induced defenses (Jones and Dangl 2006). The plant hormones salicylic acid (SA) and jasmonic acid (JA) are major players in the regulation of signaling networks that are involved in induced defense responses against pathogens (Ryan and Moura 2002; van Loon et al. 2006). Some plant defense responses are associated with the activation of an SA-dependent signaling pathway which leads to the expression of certain pathogenesis-related (PR) proteins thought to contribute to resistance (van Loon et al. 2006). SA is required for both local and systemic resistance (Durrant and Dong 2004). SA effects occur through the activity of the transcription coactivator “nonexpressor of *PR* genes” (NPR1), one of the well-known regulators of plant immunity (Després et al. 2003; Glazebrook 2005). *Arabidopsis* plants affected in the accumulation of NPR1 are unable to respond to SA, are defective in local and systemic resistance, and, consequently, are suscepti-

ble to several pathogens (Cao et al. 1994; Delaney et al. 1995; Wang et al. 2006). NPR1 and its orthologous, NPR3 and NPR4, have been shown recently to act as receptors of SA (Fu et al. 2012; Moreau et al. 2012; Wu et al. 2012). In *Arabidopsis*, NPR1 interacts with several TGA transcription factors which bind to the promoter of the SA-dependent defense gene *PR1* and activate its expression (Durrant and Dong 2004). Biochemical studies and genetic analysis showed that NPR1 interacts with TGA1, TGA2, and TGA3 in planta, in which SA plays a key role (Beckers and Spoel 2006; Boyle and Després 2010). In tomato, Pto-mediated resistance against the hemibiotrophic pathogen *Pseudomonas syringae* carrying AvrPto is SA and NPR1 dependent (Ekengren et al. 2003), and it is compromised by silencing of TGA1.a and TGA2.2 genes (Ekengren et al. 2003).

Other plant defense responses are controlled by mechanisms dependent on JA leading to the expression of, for example, *defensin* (*PDF1*) in *Arabidopsis* or *proteinase inhibitors I and II* (*PI I* and *PI II*) in tomato (Doares et al. 1995; Manners et al. 1998; Ryan and Moura 2002).

It has become clear that different defensive pathways are differentially effective against specific types of attackers. In general, biotrophic pathogens that absolutely depend on nutrient supply from host live cells are more sensitive to SA-dependent responses, whereas necrotrophic pathogens that benefit from host cell death are better resisted by a JA-dependent defense (Grant and Lamb 2006). It is also well documented that SA- and JA-dependent pathways are reciprocally antagonistic (Devoto and Turner 2003; Koornneef and Pieterse 2008; Koornneef et al. 2008; Kunkel and Brooks 2002; Lorenzo and Solano 2005; Mur et al. 2006; Spoel et al. 2007). Thus, JA-responsive genes are strongly suppressed by induction of the SA response, either by exogenous application of SA or by pathogen infection. Moreover, Spoel and associates (2007) showed that inoculation of *Arabidopsis* plants with the biotroph renders them susceptible to the necrotroph through the activation of SA, which suppressed the JA pathway. Conversely, JA signaling can act antagonistically on SA-dependent defenses (Glazebrook 2005). *P. syringae* produces the phytotoxin coronatine, which functions as a JA mimic and suppresses effectual SA-dependent defenses, thereby promoting susceptibility of the plant to this pathogen (Brooks et al. 2005; Cui et al. 2005; Glazebrook 2005; Laurie-Berry et al. 2006). On the other hand, synergistic effects between SA and JA had been described previously (Beckers and Spoel 2006; Mur et al. 2006). Simultaneous activation of SA- and JA-dependent defense pathways results in enhanced resistance to pathogenic *P. syringae* pv. *tomato* DC3000 compared with either defense

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response alone (O'Donnell et al. 2003; van Wees et al. 2000). Expression of genes associated with either JA or SA signaling was enhanced by application of low concentrations of both SA and JA (Mur et al. 2006). SA and JA cross-talk presumably reduces fitness costs of inappropriate resistance and provides the plant with a regulatory potential to fine-tune the defense reaction depending on the type of attacker encountered.

In *Arabidopsis*, the antagonism between SA and JA is modulated through NPR1 (Spoel et al. 2003). Also, it has been shown recently that glutaredoxin 480 induced by SA through NPR1 interacts with a TGA transcription factor, which is already bound to the *PDF1* promoter region and suppresses its expression (Ndamukong et al. 2007). Glutaredoxins are small redox proteins which act on ribonucleotides through electron transfer from NADPH via its disulfide (–SH) groups to deoxyribonucleotides, a process required for DNA synthesis (Holmgren 1989; Höög et al. 1983). Recently, La Camera and associates (2011) showed that the glutaredoxin ATGRXS13 is required to facilitate *Botrytis cinerea* infection of *Arabidopsis* plants.

Necrotrophic fungi are one of the most destructive pathogens of plant crops. *B. cinerea* and *Alternaria solani* are two necrotrophic plant pathogens that cause gray mold and early blight diseases, respectively, in several crops, including tomato and potato (Mansfield 1980; Rotem 1994; Thomma 2003). Necrotrophic pathogens produce several common virulence factors to invade their hosts, which include toxins and cell-wall-degrading enzymes (Choquer et al. 2007). They penetrate through wounds or natural openings in the plant tissue and colonize senescent or dead plant tissues and cause softening in fruit (Choquer et al. 2007; El Oirdi and Bouarab 2007). *B. cinerea* and *A. solani* cause considerable economic losses in both pre- and post-harvest crops (Mansfield 1980; Rotem 1994). However, the regulation of defense responses against the previous pathogens is much less understood in economically important crops than in the model plant *Arabidopsis*.

Pathogens use different, sophisticated strategies to boost their virulence, overcome plant immune responses, and invade their hosts (Bouarab et al. 2002; Chisholm et al. 2006; El Oirdi et al. 2011; González-Lamothe et al. 2012; Rigano et al. 2007; Yun et al. 2006). We showed recently that *B. cinerea* manipulates the antagonistic effect between SA and JA to promote its disease development in tomato (El Oirdi et al. 2011). Indeed, the fungus produces an exopolysaccharide (EPS) known as β -(1,3)(1,6)-D-glucan, which acts as an elicitor of SA accumulation. In turn, SA suppresses the expression of two JA-dependent genes, *PI I* and *PI II*, through NPR1, thereby allowing the fungus to develop its disease in tomato (El Oirdi et al. 2011). Here, we further investigate the molecular basis of the antagonism between SA and JA in tomato. We present evidence showing that two necrotrophic pathogens, *B. cinerea* and *A. solani*, use the SA-signaling pathway component TGA1.a to promote their disease in tomato. Thus, necrotrophs use a common strategy by manipulating a plant signaling pathway to develop their disease.

RESULTS

TGA1.a transcription factor contributes to disease caused by *B. cinerea* in tomato.

In addition to the importance of NPR1 in the SA-signaling pathway, TGA transcription factors are also involved in the regulation of the expression of SA-dependent genes, which led us to test whether TGA proteins are involved in the SA-contributed disease development caused by *B. cinerea* in tomato. We choose to investigate TGA1.a and TGA2.2 because they have been shown to be important for the SA- and NPR1-de-

pendent Pto-mediated resistance to *P. syringae* carrying AvrPto (Ekengren et al. 2003). We used two isolates of *B. cinerea*, B191 and B8403, as described previously (El Oirdi et al. 2011). Detached leaves from 5-week-old tomato plants (*Solanum lycopersicum* 'Moneymaker') were infected with mycelium plugs (3 mm in diameter) of both isolates. Expression of the disease was analyzed 4 days postinoculation (dpi). Inoculations of tomato leaves with isolate B191 consistently yielded expanding disease lesions (Fig. 1A). In contrast, lesion expansion was not observed following inoculation of tomato leaves with the B8403 isolate (Fig. 1A). The severity of disease was quantified by measuring the area of the necrotic lesions. Isolate B191 but not B8403 induced highly necrotic lesions after 4 days (Fig. 1B). Disease severity observed in detached leaves is comparable with that observed in plants sprayed with spore suspension (Supplementary Fig. S1). We then tested whether *B. cinerea* regulates the expression of *TGA1.a* and *TGA2.2* in tomato. Tomato plants were sprayed with either 10^6 spores of *B. cinerea* isolate B191 or B8403 and, at various later times, samples were harvested for quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) experiments to investigate the kinetic of expression of the two *TGA* genes. Both isolates of *B. cinerea* induced the expression of *TGA2.2* in tomato at 6 and 12 h postinoculation (hpi); however, the levels of expression were higher in tomato inoculated with the virulent isolate B191 compared with the one inoculated with the isolate B8403 (Fig. 2A). In contrast, only the virulent isolate of *B. cinerea* (B191) induced the accumulation of mRNA of *TGA1.a* at 6 and 12 hpi (Fig. 2B). To test whether this regulation has a biological significance in the interaction between *B. cinerea* and tomato, virus-induced gene silencing (VIGS) experiments were carried out in which tomato plants were inoculated with a *Tobacco rattle virus* (TRV) vector containing a fragment of 300 bp of the cDNA of either tomato *TGA2.2* (TRV:*TGA2.2*) or tomato *TGA1.a* (TRV:*TGA1.a*). Control plants were inoculated with the TRV vector without cDNA insert (TRV:00). After 3 weeks, transcript levels of *TGA1.a* and *TGA2.2* were analyzed in the silenced plants. qRT-PCR results showed that both genes *TGA1.a* and *TGA2.2* were silenced in tomato TRV *TGA1.a* and TRV:*TGA2.2* plants, respectively, compared with TRV:00 plants (Fig. 2C). The level of *TGA2.2* and *TGA1.a* was not affected in TRV *TGA1.a* and TRV:*TGA2.2* plants compared with TRV:00 plants, respectively. Detached leaves from TRV-infected plants were then challenged with plugs (3 mm in diameter) of the virulent isolate of *B. cinerea*, B191. The disease was scored by the surface of the necrotic area 4 dpi (Fig. 2D and E). TRV:00-inoculated plants that had subsequently been challenged with plugs of the isolate B191 showed disease symptoms resembling those produced in wild-type plants (Fig. 1). Interestingly, leaves from TRV:*TGA1.a*-silenced plants were significantly less susceptible to the virulent isolate B191 compared with leaves from TRV:00-silenced plants (Fig. 2D and E). However, the disease caused in the leaves from TRV:*TGA2.2*-silenced plants was comparable with that observed in TRV:00-silenced plants (Fig. 2D and E). These data suggest that, in contrast to *TGA2.2*, *TGA1.a* transcription factor contributes to disease development caused by *B. cinerea* in tomato.

Disease development promoted by SA requires TGA1.a transcription factor.

We showed previously that *B. cinerea* secretes an EPS which induces the accumulation of SA. In turn, SA promotes disease development caused by *B. cinerea* in tomato by suppressing the expression of *PI I* and *PI II* through NPR1 (El Oirdi et al. 2011). Our results described above showed clearly that *TGA1.a* promotes disease development caused by *B. cinerea*

in tomato (Fig. 2). However, we do not know whether SA-enhanced susceptibility to *B. cinerea* occurs through TGA1.a. To answer this question, we sprayed TRV:00- and TRV:TGA1.a-silenced plants with SA (0.5 mM) or EPS (50 µg/ml) and, 24 h later, detached leaves were inoculated with plugs of isolate B8403. The concentrations of SA and EPS used in this study were already shown able to promote disease development caused by *B. cinerea* in tomato (El Oirdi et al. 2011). As controls, TRV-infected plants were sprayed with the buffer and water that was used to dissolve SA and EPS, respectively. As expected, B8403 was not able to infect leaves from TRV:00 plants but the infection was significantly higher after pretreatment with either SA or EPS compared with mock-treated plants (Fig. 3A and B). Remarkably, either SA or EPS were not able to induce susceptibility to B8403 in TRV:TGA1.a-silenced plants (Fig. 3A and B). These results clearly suggest that disease development promoted by either EPS or SA occurs through TGA1.a.

TGA1.a negatively regulates the expression of JA-dependent genes *PI I* and *PI II*.

We showed previously that *PI I* and *PI II* are important for resistance against *B. cinerea* and that the EPS produced by this pathogen leads to the inhibition of the expression of these two defense genes through NPR1 (El Oirdi et al. 2011). We then tested whether TGA1.a affects the expression of *PI I* and *PI II*. We first showed that B8403 induced high expression of *PI I* and *PI II* at 0, 24, and 48 hpi compared with mock treatment and B191 inoculation (Fig. 4A and B); this is consistent with previous report published by El Oirdi and associates (2011). Then, TRV:00- and TRV:TGA1.a-silenced plants were sprayed with 10^6 spores of *B. cinerea* isolate B191 and samples were harvested for RNA extraction at 0, 24, and 48 hpi. Effects of TGA1.a knockdowns on *PI I* and *PI II* expression was analyzed by qPCR. In contrast with TRV:00, activation of *PI I* was already observed at 0 hpi in TRV:TGA1.a knockdown (Fig. 4C). Moreover, the expression of both genes *PI I* and *PI II* was significantly induced in TGA1.a knockdown in response to B191 inoculation compared with the control (TRV:00) (Fig. 4C and D). These results suggest that TGA1.a suppresses the expression of the JA-dependent genes *PI I* and *PI II*.

SA-signaling pathway enhances tomato susceptibility to another necrotrophic pathogen *A. solani*.

Because the SA-signaling pathway is used by the necrotrophic pathogen *B. cinerea* to develop its disease in tomato (El

Oirdi et al. 2011) (Fig. 3), we tested whether this strategy is specific to *B. cinerea* or whether it can be exploited by other necrotrophs. To test this hypothesis, we used *A. solani*, which causes early blight in several plant crops, including tomato and potato (Rotem 1994; Thomma 2003). Five-week-old tomato plants were treated with 0.5 mM SA as described below and, 24 h afterward, detached leaves were inoculated with mycelia plugs of *A. solani* (3 mm in diameter). Plants pretreated with SA became more susceptible to *A. solani* (Fig. 5A, left and middle panels). The lesion sizes recovered from SA-pretreated leaves infected with *A. solani* were significantly larger than those recovered from mock-treated leaves. SA-deficient NahG plants were also used to test the importance of SA in disease development caused by *A. solani* (El Oirdi et al. 2011). Interestingly, leaves from SA-deficient NahG plants were less susceptible to *A. solani* compared with wild-type plants (Fig. 5A, right panel). This suggests that SA is used by *A. solani* to promote its disease development in tomato. We then tested whether NPR1 and TGA1a are important for disease development caused by this fungus in tomato. Detached leaves from TRV:00-, TRV:NPR1-, and TRV:TGA1.a-silenced plants were used for infection, as described below and previously (El Oirdi et al. 2011). Disease caused by *A. solani* in leaves from TRV:NPR1- and TRV:TGA1.a-silenced plants was significantly lower than that observed in leaves from TRV:00-silenced plants (Fig. 5B). Tomato plants overexpressing *Arabidopsis* NPR1 were also tested and, as previously described for *B. cinerea* (El Oirdi et al. 2011), lesion areas caused by *A. solani* were significantly higher in the transgenic plants compared with the corresponding wild type (Fig. 5C). We also tested whether disease development promoted by SA occurs through NPR1 and TGA1a, as shown with *B. cinerea* (El Oirdi et al. 2011) (Fig. 3). Infection experiments were made, as described below, by using plugs of *A. solani* for leaf inoculations. Again, as in the *B. cinerea*–tomato model, SA was not able to promote disease development caused by *A. solani* in NPR1 and TGA1.a knockdowns (Fig. 5D). These results suggest that, as in the *B. cinerea*–tomato interaction, SA contributes to disease development caused by *A. solani* in tomato through NPR1 and TGA1a. Interestingly, TRV:*PI I*- and TRV:*PI II*-silenced plants are more susceptible than TRV:00-silenced plants to this fungus (Fig. 5E), which suggest that *PI I* and *PI II* contribute to less susceptibility of tomato to *A. solani*. Thus, our results show that the SA-signaling pathway contributes to disease development caused by two necrotrophic pathogens, *B. cinerea* and *A. solani*.

Fig. 1. Infection of tomato leaves with two *Botrytis cinerea* isolates. Detached leaves from 5-week-old plants were inoculated with 3-mm-diameter plugs of *B. cinerea* isolate B8403 or B191. **A**, Photographs were taken 4 days postinoculation (dpi) and **B**, lesion areas were analyzed 4 dpi. Error bars represent the standard deviation ($n = 60$) from three independent experiments. Data sets marked with an asterisk are significantly different from B8403-infected leaves as assessed by Student's t test; *** indicates $P < 0.001$.

DISCUSSION

The intimate relationship between eukaryotes and microbial pathogens has led to the co-evolution of a number of complex combat necrotrophic pathogens, plants need to activate the JA pathway. Cross-talk between SA- and JA-dependent signaling pathways allows a plant to finely tune its response to the attacker encountered. In this article, we are interested in under-

strategies for attack and defense. Plants have evolved a powerful and multi-layered immune system to defend against infection by most microbial organisms. Resistance against biotrophic and hemibiotrophic pathogens requires SA signaling but, to standing how the antagonism between SA- and JA-dependent responses regulates tomato resistance to necrotroph pathogens.

Antagonism between SA and JA has been well documented over the last 10 years. Thus, *P. syringae* was shown to be able

Fig. 2. TGA1.a enhances tomato susceptibility to the virulent isolate of *Botrytis cinerea*. Expression patterns of tomato **A**, *TGA2.2* and **B**, *TGA1.a* transcripts after *B. cinerea* infection. Five-week-old plants were sprayed with *B. cinerea* isolate B191 or B8403 at 10^6 spores/ml; samples were harvested at 0, 6, and 12 h after infection for RNA extraction. Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) was carried out with specific primers for tomato *TGA2.2*, *TGA1.a*, and *Actin* (control). **C**, *TGA2.2* and *TGA1.a* transcript levels in silenced plants. VIGS was carried out by using *Tobacco rattle virus* (TRV) vector. Seedlings were inoculated with either TRV:*Sl TGA2.2*, TRV:*Sl TGA1.a*, or empty vector as a control (TRV:00). Three weeks after inoculation, RNA was isolated and qRT-PCR was carried out as in A and B. **D** and **E**, Detached leaves from silenced plants were infected with *B. cinerea* B191; **D**, photographs were taken and **E**, disease diameter analyzed 4 days postinoculation. Error bars represent the standard deviation ($n = 60$) from three independent experiments. Data sets marked with an asterisk are significantly different from TRV:00-infected leaves as assessed by Student's *t* test; *** indicates $P < 0.001$.

to manipulate the crosstalk between SA and JA in order to cause its disease in plants (Brooks et al. 2005). Indeed, the phytotoxin coronatine (COR) produced by *P. syringae* is required for full susceptibility to these bacteria in both *Arabidopsis* and tomato (Uppalapati et al. 2007). It is believed that one of the roles of COR is to mimic JA and promote disease symptom development of the *P. syringae* hemibiotrophic pathogen by downregulating SA-inducible defense response pathways via an SA-JA antagonism (Ausubel 2005). On the other hand, infection with biotrophic *P. syringae*, which induces SA-mediated defense, rendered plants more susceptible to the necrotrophic pathogen *A. brassicicola* by suppression of the JA-signaling pathway (Spoel et al. 2007).

Pharmacological experiments with *Arabidopsis* revealed that transcription of JA-responsive marker genes, such as *PDF1.2*,

is highly sensitive to suppression by exogenous application of SA. NPR1 was shown to be a key regulator of SA-mediated suppression of JA signaling (Spoel et al. 2003). Nuclear localization of NPR1, which is essential for SA-mediated defense gene expression (Kinkema et al. 2000), is not required for the suppression of JA-responsive genes, indicating that the antagonistic effect of SA on JA signaling is modulated through a function of NPR1 in the cytoplasm (Spoel et al. 2003). In tomato, we have recently shown that *B. cinerea* manipulates the NPR1-dependent SA-JA antagonism through production of an EPS (El Oirdi et al. 2011). In addition to NPR1, other molecular actors of this antagonism have been revealed. Thus, the glutaredoxin GRX480 and the transcription factor TGA1 are important players in SA- or NPR1-dependent suppression of JA-responsive *PDF1* gene expression (Ndamukong et al. 2007). However, the biological relevance of these two proteins in the suppression of JA responses was not investigated. Interestingly, a recent work showed that the glutaredoxin ATGRXS13 is induced by production of SA after *B. cinerea* infection in *Arabidopsis*, and its mutation enhances resistance in *B. cinerea* (La Camera et al. 2011). Our work shows for the first time that, in tomato, a TGA transcription factor is biologically relevant for the resistance to *B. cinerea*. A role of TGA transcription factors in the SA-dependent defense response in tomato has been observed (Ekengren et al. 2003) but, to our knowledge, this is the first time that a biological role in plant susceptibility to necrotrophs is shown for a TGA transcription factor. From the two TGA factors analyzed, TGA1.a but not TGA2.2 is involved in disease caused by the necrotroph, suggesting specific roles of each of these transcription factors in the SA-signaling pathway, because the transcript level of *TGA2.2* in *TGA2.2*-knockdowns is not highly reduced compared with the transcript level of *TGA1.a* in *TGA1.a*-silenced plants, we cannot exclude completely the role of *TGA2.2* in SA-mediated susceptibility. The effect of SA in disease caused by both *B. cinerea* and *A. solani* is highly affected in *TGA1.a*-silenced plants, while the transcript level of *TGA2.2* is not modified in these VIGSed plants (Figs. 2 and 3). This supports our conclusion that *TGA2.2* is not involved in SA-mediated susceptibility or its effect is minimal and not significant compared with *TGA1.a*. Therefore, our results complement previous work about the role of TGA transcription factors in the antagonism between SA and JA pathways and accentuate the relevance of *TGA1.a* in the control of such antagonism and in the defense response against *B. cinerea*.

We also show that another necrotrophic pathogen, *A. solani*, uses the SA-signaling pathway to develop disease in tomato and, as for *B. cinerea*, this mechanism is dependent on NPR1 and *TGA1.a* (Fig. 5). As we did for *B. cinerea* (El Oirdi et al. 2011), we also demonstrated that silencing of *PI I* and *PI II* results in increased sensibility to *A. solani*. Our results suggest that the role of *TGA1.a* in the inhibition of JA-dependent responses via SA signaling could be a general mechanism of necrotrophic pathogens.

Our results indicate that *TGA1.a* directly or indirectly regulates the expression of the two JA-dependent *PI I* and *II* genes. TGA transcription factors recognize TGACT, TACGTA, AACGA, AACGTG, GACTTTTGAGT, and ACGTAT elements in the promoters of a variety of plant genes, including those regulating the expression of *Arabidopsis* and tobacco *PR-I* (Beckers and Conrath 2007; Dong 2004; Fobert and Després, 2005; Izawa et al. 1993; Spoel et al. 2003). Within approximately 700 bp of the *S. lycopersicum PI I* (accession number M13938.1) and 1,000 bp of the *S. lycopersicum PI II* (accession number AY129402.1) sequences upstream of the transcriptional start site, several TGA-binding motifs are found in both promoters. Thus, *TGA1.a* might directly regulate nega-

Fig. 3. Salicylic acid (SA)- and exopolysaccharide (EPS)-induced tomato susceptibility to *Botrytis cinerea* B8403 occurs through *TGA1.a*. Five-week-old TRV:00- and TRV:SI *TGA1.a*-silenced plants were sprayed either with **A**, SA (0.05 mM) or **B**, *B. cinerea* EPS (50 µg/ml) and then, 24 h later, leaves were detached and inoculated with 3-mm-diameter plugs of *B. cinerea* B8403. Water and sodium phosphate buffer were used as mocks for the EPS and SA treatment, respectively. Lesion areas were assessed 4 days postinoculation. Error bars represent the standard deviation ($n = 60$) from three independent experiments. Within each figure, letters above bars indicate statistical significance; bars not sharing letters represent significant mean differences assayed by Duncan's multiple range test at $P < 0.05$.

tively the expression of *PI I* and *II* through its binding to their promoters and NPR1 might have a role in this binding.

We and other groups showed previously that SA promotes disease development caused by *B. cinerea* through NPR1 in tomato and *Arabidopsis* (El Oirdi et al. 2011; La Camera et al. 2011). The data in this work clearly show that two necrotrophic pathogens, *B. cinerea* and *A. solani*, use a TGA1.a-dependent SA-signaling pathway to promote their disease development in tomato by suppressing the expression of the two JA-dependent defense genes, *PI I* and *II*, which are important for resistance against these two pathogens (Fig. 6). It is interesting to notice that potato (*S. tuberosum*) calcium-dependent kinase 5 (CDPK5) increases susceptibility to *A. solani* (Kobayashi et al. 2012). CDPK proteins are plant-specific proteins that sense changes in [Ca²⁺] to activate downstream responses. StCDPK4 and StCDPK5 play a critical role in stress-induced oxidative burst and cell death (Kobayashi et al. 2007, 2012). CDPK5 negatively controls JA accumulations in *Nicotiana attenuata* in response to wounding and herbivore attack (Yang et al. 2012). Tobacco CDPK2 and CDPK3 function in pathogen-induced hypersensitive response (Romeis et al. 2000, 2001). It is also known

that SA contributes to plant hypersensitive cell death development (Alvarez 2000). Thus, necrotrophs hijack host signaling leading to cell death and SA signaling to cause plant disease development.

Our data present an important advance in understanding the strategies used by necrotrophic pathogens to overcome the plant immune system and, subsequently, establish disease in an economically important crop such as tomato. This is a prerequisite for further development of a solid knowledge-based strategy toward management of diseases caused by necrotrophic pathogens.

MATERIALS AND METHODS

Pathogen growth conditions.

The *B. cinerea* wild-type isolates MEE B191 and MEE B8403 that were used for this study were provided by the Canadian Collection of Fungal Cultures (Agriculture and Agri-Food Canada, Ottawa, ON, Canada). *A. solani* was provided by MAPAQ (Laboratoire de diagnostic en phytoprotection, Complexe scientifique du Québec, Sainte-Foy, QC, Canada).

Fig. 4. TGA1.a modulates negatively the expression of *proteinase inhibitors I* and *II* (*PI I* and *PI II*). Expression levels of **A**, *PI I* and **B**, *PI II* after *Botrytis cinerea* inoculation of tomato plants. Five-week-old plants were sprayed with *B. cinerea* isolate B191 or B8403 at 10⁶ spores/ml or H₂O; samples were harvested for RNA extraction 0, 24, and 48 h after infection. Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) was carried out with specific primers for tomato *PI I*, *PI II*, and *Actin* (control). Error bars represent the standard deviation from three biological replicates. **C**, Expression levels of *PI I* and **D**, *PI II* after *B. cinerea* inoculation of tomato-silenced plants. TRV:00- and TRV:*Sl TGA1.a*-silenced plants were sprayed with *B. cinerea* B191 at 10⁶ spores/ml; samples were harvested for RNA extraction 0, 24, and 48 h after inoculation. qRT-PCR was carried out as described in A. Error bars represent the standard deviation from three biological replicates.

Fig. 5. Salicylic acid (SA) signaling pathway enhances disease caused by *Alternaria solani* in tomato. **A**, Left and middle panels: 5-week-old tomato plants were sprayed with 0.05 mM SA and then, 24 h later, leaves were detached and inoculated with 3-mm-diameter plugs of *A. solani*. Sodium phosphate buffer was used as a mock. Photographs were taken (left panel) and disease area was analyzed (middle panel) 7 days postinoculation (dpi). Right panel: Detached leaves from wild-type (WT) or SA-deficient NahG plants were inoculated with 3-mm-diameter plugs of *A. solani* and disease size was analyzed 7 dpi. Error bars represent the standard deviation ($n = 60$) from three independent experiments. Data sets marked with an asterisk are significantly different from infected-mock treated leaves as assessed by Student's t test; *** indicates $P < 0.001$. **B**, Detached leaves from TRV:00-, TRV:*Sl NPR1*-, and TRV:*Sl TGA1a*-silenced plants were inoculated with 3-mm-diameter plugs of *A. solani*. Lesion areas were assessed 7 dpi. Error bars represent the standard deviation ($n = 45$) from three independent experiments. Data sets marked with an asterisk are significantly different from TRV:00-infected leaves as assessed by Student's t test; *** indicates $P < 0.001$. **C**, Detached leaves from WT and tomato overexpressing *Arabidopsis NPR1* plants were inoculated with 3-mm-diameter plugs of *A. solani*. Lesion areas were assessed 7 dpi. Error bars represent the standard deviation ($n = 45$) from three independent experiments. Data sets marked with an asterisk are significantly different from WT-infected leaves as assessed by Student's t test; *** indicates $P < 0.001$. **D**, TRV:00-, TRV:*Sl NPR1*-, and TRV:*Sl TGA1a*-silenced plants were sprayed with 0.05 mM SA and then, 24 h later, leaves were detached and inoculated with 3-mm-diameter plugs of *A. solani*. Sodium phosphate buffer was used as a mock. Lesion areas were assessed 7 dpi. Error bars represent the standard deviation ($n = 45$) from three independent experiments. Within each figure, letters above bars indicate statistical significance; bars not sharing letters represent significant mean differences assayed by Duncan's multiple range test at $P < 0.05$. **E**, *Proteinase inhibitors (PI) I* and *II* contribute to less susceptibility of tomato to *A. solani*. Detached leaves from TRV:00-, TRV:*Sl PI I*-, and TRV:*Sl PI II*-silenced plants were inoculated with 3-mm-diameter plugs of *A. solani*. Lesion areas were assessed 7 dpi for *A. solani*. Error bars represent the standard deviation ($n = 60$) from three independent experiments. Data sets marked with an asterisk are significantly different from TRV:00-infected leaves as assessed by Student's t test; *** indicates $P < 0.001$.

The fungus isolates were subcultured as previously described (El Oirdi and Bouarab 2007; El Oirdi et al. 2011; González-Lamothe et al. 2012).

Plant growth and inoculation conditions.

S. lycopersicum MoneyMaker and SA-deficient NahG seed were germinated and grown seed were grown as previously described (El Oirdi et al. 2010, 2011). Wild-type and transgenic tomato plants (*S. lycopersicum* line CL5915-93D4-1-0-3) expressing the *Arabidopsis* NPR1 have been described previously (Lin et al. 2004) and were grown in the same manner.

For pathogenicity tests, detached leaves from 5-week-old tomato plants were laid on petri dishes containing two blotting filter papers (Whatman, Schleicher & Schuell, Ottawa, ON, Canada) wetted with sterile water, then spotted with 3-mm-diameter agar plugs containing actively growing hyphae of *B. cinerea* or *A. solani* (3 days old). Petri dishes were then incubated at 22°C at high humidity in glass trays covered with polyethylene wrap. The disease caused by *B. cinerea* and *A. solani* was assessed in detached leaves 4 and 7 dpi, respectively, by measuring the necrosis area induced by the mycelium plugs. All plant inoculations involved a minimum of 30 leaves and each experiment was carried out at least three times. Disease lesion sizes which corresponded to the area of the lesion (in square millimeters) were made by using Axio-Vision software (V 4.6.3, 2007).

For quantitative RT-PCR experiments, the infection tests were carried out in planta; wild type and silenced plants were sprayed with spores (10^6 spores/ml in Tween 0.01%) of either *B. cinerea* isolate B191 or B8403. Plants were then incubated in a growth chamber with high humidity and samples were then harvested for RNA extractions.

EPS and SA treatments.

For EPS (50 µg/ml) and SA (0.5 mM) treatments, silenced plants were sprayed as previously described (El Oirdi et al. 2011). Twenty-four hours later, detached leaves from mock-, SA-, and glucan-pretreated plants were infected with either *B. cinerea* or *A. solani* as described below and previously (El Oirdi et al. 2011).

TRV-based VIGS in *S. lycopersicum*.

A 300-bp fragment from tomato *NPR1*, *TGA1a*, *PI I*, and *PI II* cDNAs were PCR-amplified using the primers described in Supplementary Table S1. The fragments were cloned in the *SmaI* site of pTRV-RNA2 vector (Liu et al. 2002). As a control, the pTRV-RNA2 empty vector was used (TRV:00). *Agrobacterium* cultures (optical density [OD] = 2) containing either TRV:00, TRV:*NPR1*, TRV:*TGA1a*, TRV:*PI I*, or TRV:*PI II* were mixed with those containing pTRV-RNA1 (OD = 2) in a 1:1 ratio before agroinfiltration. *S. lycopersicum* seedlings were infiltrated with the mixture and, 3 weeks later, the level of targeted transcripts was analyzed by qRT-PCR.

qRT-PCR experiments.

Total RNA was extracted from leaves of three plants in each experiment using the RNeasy plant mini kit according to the manufacturer's recommendation (Qiagen, Gaithersburg, MD, U.S.A.). RNAs were treated with DNAase-free RNAase. First-strand cDNA was synthesized from 2 µg of total RNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, U.S.A.). Primers were used for the amplification of the targets. qPCR was performed using the SYBR Green method. SYBR Green PCR experiments were performed using 2 µl of cDNA samples (50 ng), 5 µl of the Fast SYBR Green Master Mix (Applied Biosystem), and 10 pmol of each primer in a total volume of 10 µl. Melting curves were determined using the

Fig. 6. Proposed model showing how *Botrytis cinerea* and *Alternaria solani* use salicylic acid (SA)-signaling pathway to promote their disease development in tomato. Two necrotrophic pathogens, *B. cinerea* and *A. solani*, use the SA-signaling pathway through NPR1 (noninducible pathogenesis-related 1) and TGA1a to promote their disease development in tomato. NPR1 and TGA1a suppress the expression of *proteinase inhibitors I and II* (*PI I* and *PI II*), which are important for resistance against these two pathogens.

dissociation curve software SDS 2.2.2 to ensure that only a single product was amplified. The ABI PRISM7500HT sequence detection system (Applied Biosystems, Foster City, CA, U.S.A.) was used to detect the amplification level and was programmed with an initial step of 10 min at 95°C followed by 40 cycles alternating between 15 s at 95°C and 1 min at 60°C. All reactions were run in technical triplicates for each biological replicate, and the average values were used for quantification. The relative quantification of target genes was determined using the $\Delta\Delta C_T$ method. Briefly, the threshold cycle (C_t) values of target genes were normalized to an endogenous control gene (actin) ($\Delta C_T = C_{t_{\text{target}}} - C_{t_{\text{actin}}}$) and compared with a calibrator ($\Delta\Delta C_T = \Delta C_{t_{\text{sample}}} - \Delta C_{t_{\text{calibrator}}}$). Relative expression (RQ) was calculated using the sequence detection system SDS 2.2.2 software (Applied Biosystems) and the formula $RQ = 2^{-\Delta\Delta C_T}$. All qPCR analyses were made with three biological replicates.

Statistical analysis.

Statistical analysis was carried out using the GLM procedure of the SAS 9.1 statistical package. Our data were subjected to either one- or two-way analysis of variance, followed by a comparison of the means according to Duncan's multiple range test at $P < 0.05$, unless otherwise stated. Pairwise comparisons with lower numbers of treatments were conducted according to Student's *t* test.

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