SGT1 positively regulates the process of plant cell death during both compatible and incompatible plant–pathogen interactions

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SUMMARY

SGT1 (suppressor of G2 allele of Skp1), an interactor of SCF (Skp1-Cullin-F-box) ubiquitin ligase complexes that mediate protein degradation, plays an important role at both G1–S and G2–M cell cycle transitions in yeast, and is highly conserved throughout eukaryotes. Plant SGT1 is required for both resistance (*R*) gene-mediated disease resistance and nonhost resistance to certain pathogens. Using virus-induced gene silencing (VIGS) in *Nicotiana benthamiana*, we demonstrate that *SGT1* positively regulates the process of cell death during both host and nonhost interactions with various pathovars of *Pseudomonas syringae*. Silencing of *NbSGT1* in *N. benthamiana* plants delays the induction of hypersensitive response (HR)-mediated cell death against nonhost pathogens and the development of disease-associated cell death caused by the host pathogen *P. syringae* pv. *tabaci*. Our results further demonstrate that *NbSGT1* is required for *Erwinia carotovora*- and *Sclerotinia sclerotiorum*-induced disease-associated cell death. Overexpression of *NbSGT1* in *N. benthamiana* accelerates the development of HR during *R* gene-mediated disease resistance and nonhost resistance. Our data also indicate that SGT1 is required for pathogen-induced cell death, but is not always necessary for the restriction of bacterial multiplication *in planta*. Therefore, we conclude that SGT1 is an essential component affecting the process of cell death during both compatible and incompatible plant–pathogen interactions.

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INTRODUCTION

Plants have evolved intricate defence mechanisms against a variety of environmental stresses, including attacks from a vast number of potential pathogens. There are at least two classes of plant innate immune responses (Chisholm *et al*., 2006; Jones and Dangl, 2006). The primary immune response of plants to pathogen perception is the recognition of pathogen molecules, called pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs), such as flagellin (Gomez-Gomez and Boller, 2002) and elongation factor *Tu* (Kunze *et al*., 2004), by plant pattern recognition receptors (PRRs), resulting in PAMP-triggered immunity (PTI). This class of immune response is called basal resistance. The second class of plant immunity involves direct or indirect recognition of the specific effector(s) secreted from pathogens by the host surveillance system, resulting in so-called 'gene-for-gene resistance' or effector-triggered immunity (ETI), which occurs, for example, during AvrPto–Pto and AvrRpt2–RIN4 interactions (Mudgett, 2005; Pedley and Martin, 2003). Plant resistance and pathogen virulence have co-evolved as a 'zigzag' system (Chisholm *et al*., 2006; Jones and Dangl, 2006).

Generally, plant immunity triggered by ETI involves rapid plant cell death, known as the hypersensitive response (HR), resulting in an unsuccessful infection of the host plant (Greenberg, 1997; Greenberg and Yao, 2004). As the process of HR-mediated cell death involves signal transduction pathways similar to those of programmed cell death (PCD), it is generally considered as a form of PCD (Lam *et al*., 2001). Significant progress has been made in our understanding of the signal transduction pathways leading to HR. Many resistance (*R*) genes in plants and corresponding avirulence (*Avr*) genes or specific effectors in pathogens have been identified, and their direct or indirect interactions often lead to HR, which limits the spread of the pathogen from the initial infection site (Martin *et al*., 2003).

Some effectors contributing to pathogen virulence serve as suppressors of PTI or ETI, so that the pathogens can successfully colonize the host plants and cause disease (Bent and Mackey,

2007). For example, in the absence of resistance protein Pto, the avirulence effector avrPtoB functions to promote bacterial virulence and cause plant disease by the inhibition of PCD through host E3 ubiquitin ligase activity (Abramovitch *et al.*, 2006; Jamir *et al.*, 2004; Rosebrock *et al.*, 2007). *Pseudomonas syringae* virulence effector HopM1 suppresses host immunity by destroying an immunity-associated protein, AtMIN7, in *Arabidopsis thaliana* via the host proteasome to cause infection in plants (Nomura *et al.*, 2006). The disease symptoms developed during compatible interactions of various pathovars of *P. syringae* with their respective hosts are often associated with cell death. The molecular basis of cell death derived from compatible host–pathogen interactions is still poorly understood and has been suggested to be genetically controlled (Greenberg and Yao, 2004). A MAPKKKα has been shown to function as a positive regulator of bacterial speck disease-associated cell death in tomato (del Pozo *et al.*, 2004).

SGT1 (suppressor of G2 allele of Skp1) physically associates with SCF (Skp1-Cullin-F-box) ubiquitin ligase complex and plays an essential role in yeast kinetochore function (Kitagawa *et al.*, 1999). SGT1 is a critical signalling component required for *R* gene-mediated resistance in several plant species against various plant pathogens, including fungi, bacteria and viruses (Austin *et al.*, 2002; Azevedo *et al.*, 2002; Liu *et al.*, 2004; Tör *et al.*, 2002). In *Nicotiana benthamiana*, it has been shown that SGT1 is required for nonhost resistance against various bacterial pathogens (Peart *et al.*, 2002). Taken together, these studies indicate that SGT1 plays an important role in both PTI- and ETI-mediated PCD during the plant resistance response. However, it is also important to note that SGT1 has also been shown to be involved in cell death that promotes the pathogenesis of the necrotrophic fungal pathogen, *Botrytis cinerea* (El Oirdi and Bouarab, 2007), and the hemibiotrophic fungal pathogen, *Fusarium culmorum* (Cuzick *et al.*, 2009).

In this study, we used virus-induced gene silencing (VIGS) in *N. benthamiana* to investigate the involvement of SGT1 in the process of cell death during compatible interaction, and further characterized the involvement of SGT1a and SGT1b in cell death in *A. thaliana* using nonhost pathogens. We demonstrated that SGT1-mediated cell death during plant–bacterial interactions, at least in *N. benthamiana* and *A. thaliana*, did not always correlate with the bacterial population *in planta*, and therefore a delay in SGT1-mediated cell death did not always result in increased accumulation of all nonhost bacterial pathogens. Furthermore, we showed that overexpression of SGT1 expedited cell death in *N. benthamiana* during ETI and PTI as well as compatible and incompatible (nonhost) plant–bacterial interactions. Therefore, we conclude that SGT1 is a critical component that positively regulates the process of PCD during both compatible and incompatible plant–pathogen interactions.

RESULTS

SGT1 is required for plant cell death during incompatible interaction, but does not always limit *in planta* bacterial growth

Nicotiana benthamiana is an ideal plant species for VIGS to assess the functions of candidate genes in development and environmental stress (Lu *et al.*, 2003b). To examine the role of *SGT1* in pathogen-induced PCD, we silenced *NbSGT1* in *N. benthamiana* by VIGS using the *Tobacco rattle virus* (TRV) (Liu *et al.*, 2002). *NbSGT1* gene silencing efficiency was examined by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) 2 weeks post-inoculation with *Agrobacterium* containing TRV::*NbSGT1*. The transcript level of *NbSGT1* in the gene-silenced plants was at least 10-fold lower than that in nonsilenced control plants (Fig. 1A), indicating that *NbSGT1* was efficiently silenced in *N. benthamiana* by VIGS.

Previous studies have shown that nonhost resistance-associated HR develops in *N. benthamiana* 1–2 days after inoculation with nonhost pathogens, such as *P. syringae* pv. *maculicola*, at a concentration of 10^8 colony-forming units (cfu)/mL (Kang *et al.*, 2004; Peart *et al.*, 2002). To investigate the general role of SGT1 in PCD (HR or disease-associated cell death) and *in planta* bacterial growth, we first examined the effects of *SGT1* silencing on the development of PCD during nonhost resistance against pathovars of *P. syringae* that do not cause disease on *N. benthamiana*. As compatible pathogens also cause rapid cell death in *N. benthamiana* when inoculated at high levels of inoculum, low levels of inoculum are preferred for the differentiation of compatible and incompatible interactions (Wei *et al.*, 2007). We used low concentrations of two different incompatible pathogens, including *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *maculicola*, to challenge *NbSGT1*-silenced *N. benthamiana* plants. Two to three weeks after *Agrobacterium* inoculation, the plants were vacuum infiltrated with the nonhost pathogens at a concentration of 1×10^5 cfu/mL. HR was observed in the leaves of non-silenced control plants, but not in *NbSGT1*-silenced plants, at 3 days post-inoculation (dpi) with *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *maculicola* (Fig. 1B). The bacterial population *in planta* was determined by plating serial dilutions of plant leaf extracts every 24 h after inoculation. During the first 24 h after infiltration, there was no significant difference in the number of nonhost bacteria tested in both *SGT1*-silenced and control plants (Fig. 1C, D). However, by 3 dpi, there was a significant reduction in the bacterial populations of *P. syringae* pv. *maculicola* in control plants, but a rapid increase in *NbSGT1*-silenced plants (Fig. 1D). This result demonstrates that *NbSGT1* is required for cell death, which, in turn, may limit the growth of *P. syringae* pv. *maculicola* in *N. benthamiana*.
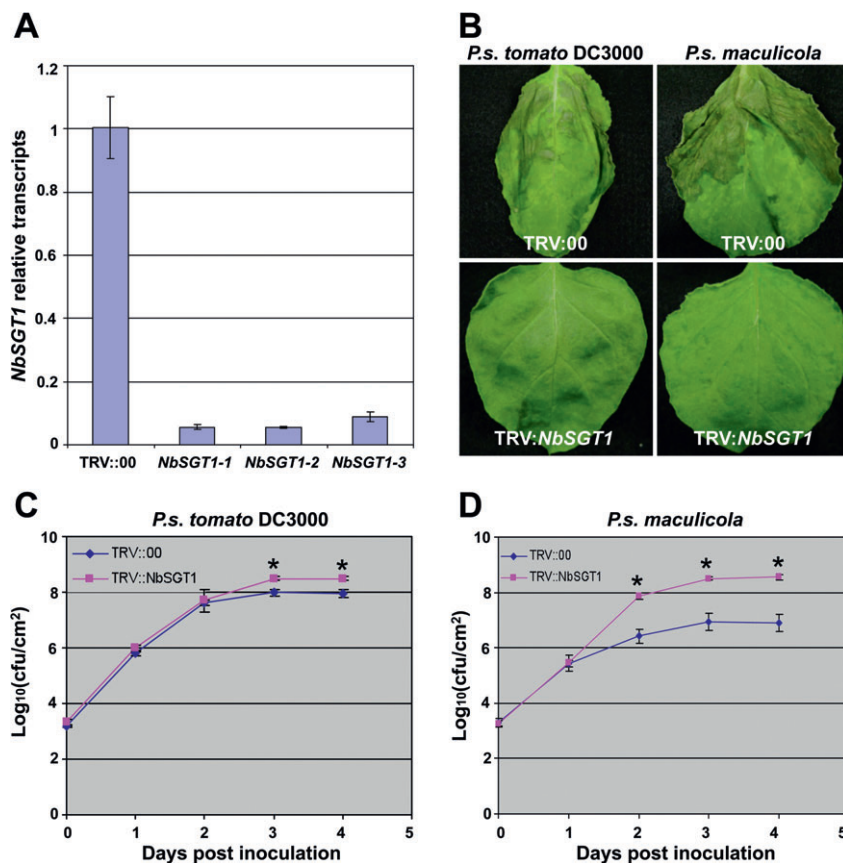
Fig. 1 Silencing of *NbSGT1* in *Nicotiana benthamiana* results in delayed cell death induced by nonhost bacterial pathogens. Three-week-old *N. benthamiana* plants were inoculated with disarmed *Agrobacterium* containing TRV::*NbSGT1* or TRV::00 (vector control). (A) Relative expression of *NbSGT1* determined by real-time reverse transcription-polymerase chain reaction (RT-PCR) in *N. benthamiana* plants 2 weeks after *Agrobacterium* inoculation. Three plants (NbSGT1-1, NbSGT1-2, NbSGT1-3) silenced with TRV::*NbSGT1* were used to examine the silencing effect. The plants silenced by TRV:00 were used as controls. The error bars were derived from three technical replicates. (B) Cell death phenotypes in *NbSGT1*-silenced and control *N. benthamiana* plants induced by nonhost pathogenic bacteria. The *NbSGT1*-silenced and control plants were vacuum infiltrated with the nonhost pathogens *Pseudomonas syringae* pv. *tomato* DC3000 and *P. syringae* pv. *maculicola* at a concentration of 10^5 colony-forming units/mL. Photographs were taken at 3 days post-inoculation (dpi). (C, D) Growth of bacteria in *NbSGT*-silenced and control *N. benthamiana* plants. Bacterial numbers were examined by plating serial dilutions of leaf extracts every 24 h after inoculation. Error bars represent standard deviations of four replicates. Asterisk indicates significant difference at $\alpha = 0.01$ by Student's *t*-test.

Interestingly, *P. syringae* pv. *tomato* DC3000 multiplied rapidly (close to 10^8 cfu/cm^2 by 3 and 4 dpi) in control *N. benthamiana* plants (Fig. 1C). This result was consistent with those of a previous study (Wei *et al*., 2007). As this pathogen did not cause disease on *N. benthamiana* when inoculated at 10^4 cfu/cm^2, whereas the host pathogen *P. syringae* pv. *tabaci* did cause disease under the same conditions, *P. syringae* pv. *tomato* DC3000 was considered to be an incompatible pathogen (Wei *et al*., 2007). However, the population of *P. syringae* pv. *tomato* DC3000 in *NbSGT1*-silenced plants was only about three-fold greater than that in control plants at 3 dpi and later (Fig. 1C), although the development of cell death in *NbSGT1*-silenced plants was significantly slower than that in nonsilenced control plants inoculated with *P. syringae* pv. *tomato* DC3000 (Fig. 1B). These data demonstrate that *NbSGT1* is a

critical component for cell death induction during incompatible interaction with *P. syringae* pv. *tomato* DC3000. However, HR associated with incompatible interaction with *P. syringae* pv. *tomato* DC3000 was not effective in controlling *in planta* bacterial growth (Fig. 1B). Furthermore, silencing of SGT1 did not result in enhanced bacterial growth (Fig. 1C), suggesting that HR and bacterial growth can be delineated during this specific incompatible interaction. However, the population of nonhost pathogen *P. syringae* pv. *maculicola* in *SGT1*-silenced plants increased to 100-fold greater than that in the vector control (Fig. 1D), which was consistent with a previous study (Peart *et al*., 2002). Taken together, these results indicate that *NbSGT1* is required for nonhost pathogen-associated HR/PCD, but may not function as a major factor limiting the growth of all nonhost pathogens.
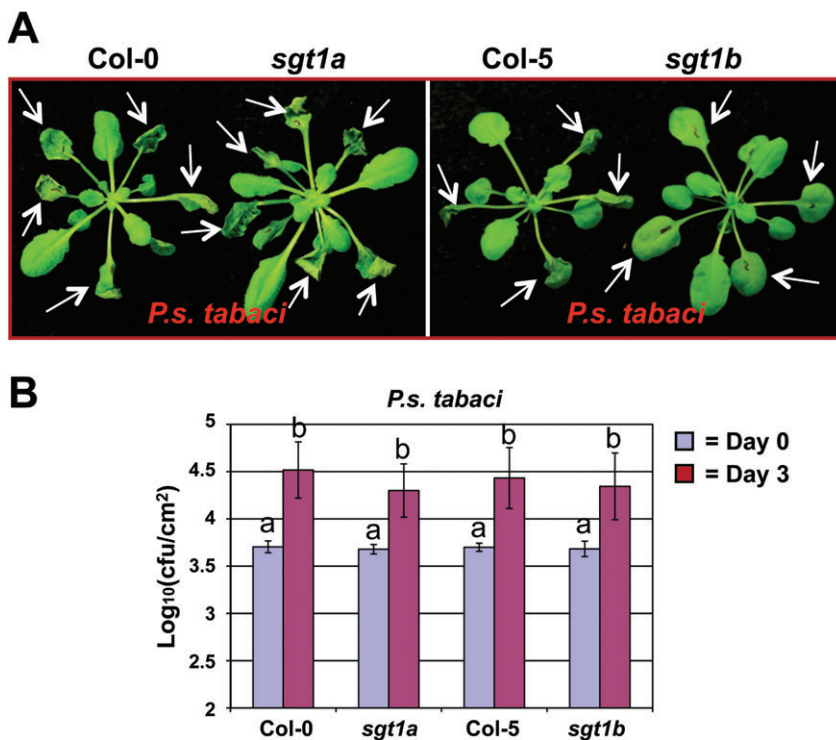
Fig. 2 Cell death induced by the nonhost pathogen *Pseudomonas syringae* pv. *tabaci* in *sgt1* mutants and wild-type Arabidopsis. (A) Leaves of 4-week-old plants were infiltrated with the nonhost pathogen *P. syringae* pv. *tabaci* at a concentration of 2×10^7 colony-forming units/mL. White arrows indicate infiltrated leaves. Photographs were taken at 2 days post-inoculation (dpi). (B) Growth of bacteria in *sgt1* mutants and corresponding wild-type Arabidopsis plants. Leaves of 4-week-old plants were infiltrated with the nonhost pathogen *P. syringae* pv. *tabaci* at a concentration of 1×10^6 cfu/mL. Bacterial cell numbers were examined by plating serial dilutions of leaf extracts at 3 dpi. Error bars represent the standard deviations of four replicates. Letters indicate a significant difference at $\alpha = 0.01$ using Student's *t*-test.

To further test whether nonhost bacterial-induced cell death and *in planta* bacterial multiplication were correlated, we used Arabidopsis *sgt1* mutants. Arabidopsis has two SGT1 paralogues, AtSGT1a and AtSGT1b, which share 87% similarity at the amino acid level. However, previous studies have shown that only AtSGT1b is required for certain *R* gene-mediated resistance responses because of greater expression of AtSGT1b, although AtSGT1a and AtSGT1b are functionally redundant during embryo development (Austin *et al*., 2002; Azevedo *et al*., 2006; Holt *et al*., 2005; Tör *et al*., 2002). We examined the requirement of these two SGT1 paralogues in PCD on pathogen infection. We first examined the effect of *AtSGT1a* and *AtSGT1b* on plant cell death during the development of HR induced by nonhost pathogens. Arabidopsis *sgt1a* and *sgt1b* mutants, as well as wild-type plants, were inoculated with the nonhost pathogen *P. syringae* pv. *tabaci* by leaf infiltration at a concentration of 2.0×10^7 cfu/mL. As the mutants *sgt1a* and *sgt1b* have a different genetic background, *A. thaliana* ecotype Col-0 was used as control for the *sgt1a* mutant and Col-5 for the *sgt1b* mutant. As shown in Fig. 2A, the leaves of wild-type *A. thaliana* Col-5 inoculated with *P. syringae* pv. *tabaci* showed cell death (HR) by 1 dpi, whereas the Arabidopsis *sgt1b* mutant did not show any HR by 1 dpi. However, the Arabidopsis *sgt1a* mutant showed a similar degree of plant cell death (HR) as its wild-type counterpart by 1 dpi. These data indicate an important role for *AtSGT1b* in the process of HR (PCD) during nonhost plant–bacterial interactions.

We then measured bacterial growth to determine whether or not there was a correlation between cell death and bacterial growth in *sgt1* mutants. Interestingly, the bacterial number of nonhost pathogen *P. syringae* pv. *tabaci* did not show a significant difference in mutants and their wild-types, although the development of cell death in the *sgt1b* mutant was delayed as indicated above (Fig. 2B). It has been reported that Arabidopsis *sgt1a* and *sgt1b* mutants do not enhance the growth of the host bacterial pathogen *P. syringae* pv *tomato* DC3000 (Holt *et al*., 2005). These data further confirm our conclusion that AtSGT1b-mediated cell death induced by nonhost pathogens is independent of *in planta* bacterial populations.

SGT1 is required for cell death in *N. benthamiana* during compatible interaction with bacterial pathogens

Plant cell death during a compatible host–pathogen interaction is considered to be a genetically programmed control process (Greenberg and Yao, 2004; del Pozo *et al*., 2004). Although our results and previous studies have shown that SGT1 is required for HR development during nonhost resistance and *R* gene-mediated resistance (Peart *et al*., 2002), the function of SGT1 in cell death during compatible plant–bacterial pathogen interactions has not been studied. It is believed that both resistant and susceptible plant responses share signalling components that lead to PCD (Greenberg and Yao, 2004; Lincoln *et al*., 2002; del

Pozo and Lam, 2003; del Pozo *et al.*, 2004). To determine whether SGT1 is required for PCD during compatible plant–pathogen interactions, the host pathogen *P. syringae* pv. *tabaci* was used to inoculate *NbSGT1*-silenced *N. benthamiana* plants and control plants by vacuum infiltration. As it has been demonstrated that *P. syringae* pv. *syringae* B728a is highly virulent on *N. benthamiana* (Vinatzer *et al.*, 2006), we also included this strain to investigate compatible plant–pathogen interactions. At 4 dpi, disease-associated cell death developed in the leaves of non-silenced control plants, but no cell death was observed in *NbSGT1*-silenced plants inoculated with *P. syringae* pv. *tabaci* (Fig. 3A). The occurrence of disease-associated cell death in
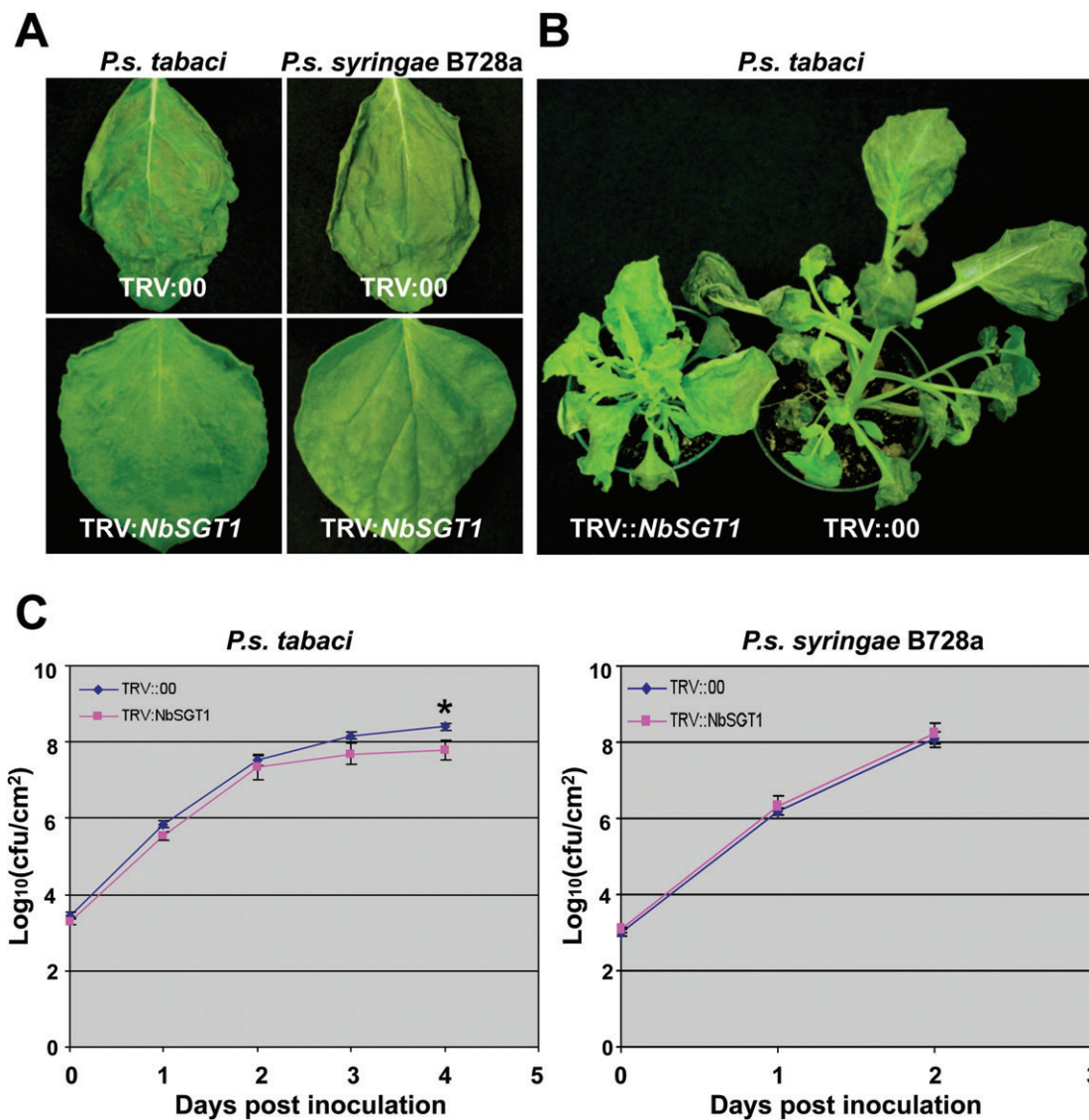

Fig. 3 Silencing of *NbSGT1* in *Nicotiana benthamiana* results in delayed disease-associated cell death induced by *Pseudomonas syringae* pv. *tabaci* and *P. syringae* pv. *syringae* B728a. (A) Cell death phenotypes in *NbSGT1*-silenced and control *N. benthamiana* plants induced by the virulent pathogens *P. syringae* pv. *tabaci* and *P. syringae* pv. *syringae* B728a. Two weeks after inoculation with *Agrobacterium* containing TRV::*NbSGT1* or empty vector, the plants were vacuum infiltrated with the virulent pathogens *P. syringae* pv. *tabaci* and *P. syringae* pv. *syringae* B728a at a concentration of 10^5 colony-forming units (cfu)/mL. Photographs were taken at 4 days post-inoculation (dpi). (B) Disease symptoms (cell death) of *NbSGT1*-silenced and control *N. benthamiana* plants. Two weeks after *Tobacco rattle virus* (TRV) inoculation, plants were vacuum infiltrated with *P. syringae* pv. *tabaci* at a concentration of 10^5 cfu/mL. Photographs were taken at 14 dpi. (C) Growth of bacteria in *NbSGT1*-silenced and control *N. benthamiana* plants. Bacterial numbers were examined by plating serial dilutions of leaf extracts every 24 h after inoculation. The bacterial population in plant leaves inoculated with *P. syringae pv. syringae* B728a from 3 dpi was not examined as all inoculated wild-type plant leaves were completely dead. Error bars represent the standard deviations of four replicates. Asterisk indicates a significant difference at $\alpha = 0.01$ by Student's *t*-test.

NbSGT1-silenced *N. benthamiana* inoculated with *P. syringae* pv. *tabaci* was delayed for up to 2 weeks when compared with control plants (Fig. 3B). To further demonstrate the involvement of SGT1 in the compatible interaction, we also checked the effect of *NbSGT1* silencing on cell death caused by two other compatible bacterial pathogens, *P. syringae* pv. *syringae* B728a and a broad host range pathogen *Erwinia carotovora*. Cell death was observed in the leaves of the nonsilenced control plants, but not in *NbSGT1*-silenced plants, at 2 and 5 dpi with *P. syringae* pv. *syringae* B728a and *E. carotovora*, respectively (Figs 3A and 4A). These data indicate that SGT1 also plays a critical role in the development of cell death during more than one compatible plant–bacterial interaction.

To examine the relationship between cell death and bacterial population during compatible interactions, we monitored bacterial growth after vacuum inoculation. The populations of *P. syringae* pv. *tabaci* in *NbSGT1*-silenced plants were similar at 2 dpi and only slightly (two- to five-fold) less than that in control plants at 3 and 4 dpi (Fig. 3C). *Pseudomonas syringae* pv. *syringae* B728a grew rapidly in both *NbSGT1*-silenced and control plants (Fig. 3C), in which the population of *P. syringae* pv. *syringae* B728a reached 10^8 cfu/cm^2 by 2 dpi after vacuum inoculation (Fig. 3C), although no cell death was observed in *NbSGT1*-silenced plants at that time (Fig. 3A). As wild-type plant leaves inoculated with *P. syringae pv. syringae* B728a were completely dead, bacterial numbers *in planta* from 3 dpi were not examined. The multiplication of *E. carotovora* was also not severely compromised in *NbSGT1*-silenced plants when compared with control plants (Fig. 4B). These data confirm that *NbSGT1* is required for cell death during *N. benthamiana* responses to all tested host and nonhost bacterial pathogens, but not necessarily required to restrict bacterial growth.

To confirm the above results from vacuum/syringe inoculations and to mimic the natural infection and disease development (cell death), *NbSGT1*-silenced and control *N. benthamiana* plants were spray inoculated with *P. syringae* pv. *tabaci* at a concentration of 2×10^8 cfu/mL. The control plants developed typical wild fire disease symptoms at 9 dpi, whereas, at the same time point, *NbSGT1*-silenced plants did not show any symptoms (Fig. 5A). The small visible spots of cell death were not observed in *NbSGT1*-silenced plants until 15 dpi, during which time the control plants showed severe cell death (Fig. 5B). At 32 dpi, the control *N. benthamiana* plants were completely dead, whereas *SGT1*-silenced plants were still alive (Fig. 5D). Bacterial growth *in planta* was also monitored by bacterial plating assay. The bacterial cell numbers of *P. syringae* pv. *tabaci* in *NbSGT1*-silenced plants (1.60×10^8 cfu/cm^2) were not significantly different from those of control plants (1.63×10^8 cfu/cm^2) at 9 dpi. Interestingly, at 15 dpi, the bacterial populations in *NbSGT1*-silenced plants were
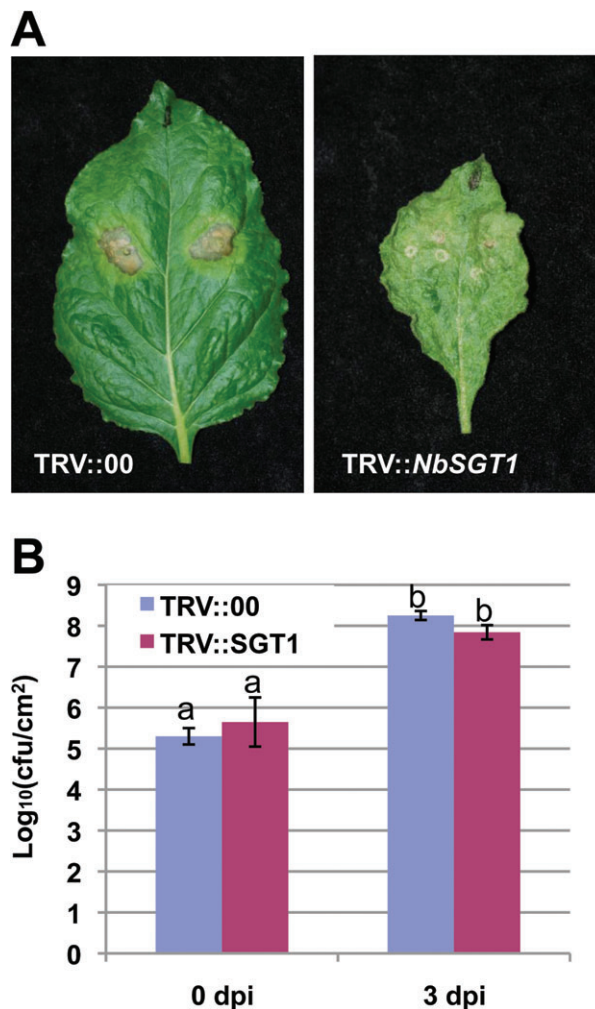

Fig. 4 *Erwinia carotovora* disease symptoms (cell death) and bacterial growth in *NbSGT1*-silenced (TRV::*NbSGT1*) and control (TRV::00) *Nicotiana benthamiana* plants. (A) Disease (cell death) phenotypes were monitored following infiltration with *E. carotovora* at a concentration of 1×10^5 colony-forming units/mL. The inoculated plants were incubated in a glasshouse at 26 °C and 16 h daylight. Photographs were taken at 5 days post-inoculation (dpi). (B) Bacterial populations in *NbSGT1*-silenced and control plants. Error bars represent the standard deviations of three replicates. Letters indicate a significant difference at $\alpha = 0.01$ using Student's *t*-test.

increased two-fold to 3.27×10^8 cfu/cm^2, whereas the bacterial numbers in control plants were decreased significantly by nearly four-fold to 4.28×10^7 cfu/cm^2. Bacterial populations in plant leaves at 24 and 32 dpi were not examined for comparison as the inoculated control *N. benthamiana* leaves were completely dead. The decrease in bacterial cell number in control plants could be caused by cell death. These results further demonstrate that *NbSGT1* is required for cell death during compatible plant–bacterial interactions, but is not
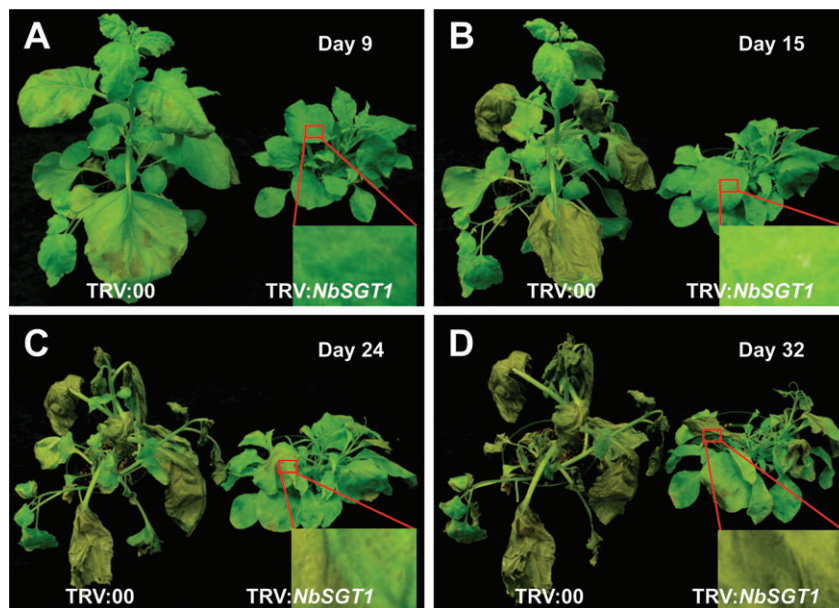
Fig. 5 Cell death-associated disease symptoms developed in *NbSGT1*-silenced and control *Nicotiana benthamiana* plants. The plants were spray inoculated with *Pseudomonas syringae* pv. *tabaci* at a concentration of 2×10^8 colony-forming units/mL (Silwet, 0.02%). The inoculated plants were incubated at room temperature. Photographs were taken 9 (A), 15 (B), 24 (C) and 32 days post-inoculation (dpi) (D).

involved in resistance (bacterial multiplication) to the host bacterial pathogen.

SGT1 is required for cell death during the development of disease symptoms in *N. benthamiana* during compatible interactions with fungal pathogens

To test whether *NbSGT1* plays a general role in disease-associated cell death, we monitored disease development in control and *NbSGT1*-silenced plants on inoculation with *Sclerotinia sclerotiorum* (Fig. 6) and *Phytophthora infestans* (Fig. S2, see Supporting Information). Silencing of *NbSGT1* significantly reduced cell death and disease symptom development by the necrotrophic fungal pathogen *S. sclerotiorum* (Fig. 6). To further understand whether *NbSGT1* is required for cell death associated with hemibiotrophic fungal pathogens, we used an isolate of *Ph. infestans* that has been reported previously to be pathogenic on *N. benthamiana* (Becktell *et al*., 2006). When the sporangial suspensions were inoculated on *N. benthamiana* leaves, disease-associated cell death was observed on control but not on *NbSGT1*-silenced plants (Fig. S2). However, it is important to note that, in some of the experiments, *Ph. infestans* sporangial suspensions induced HR-like cell death on control plants which was not associated with *in planta* mycelial growth (Fig. S2). *Nicotiana benthamiana* is generally considered to be a nonhost to *Ph. infestans* (Kamoun *et al*., 1998). In our hands, the results were variable and both compatible and incompatible interactions of *Ph. infestans* on *N. benthamiana* were observed (Fig. S2). It is possible that the sporangial concentration or plant age might alter
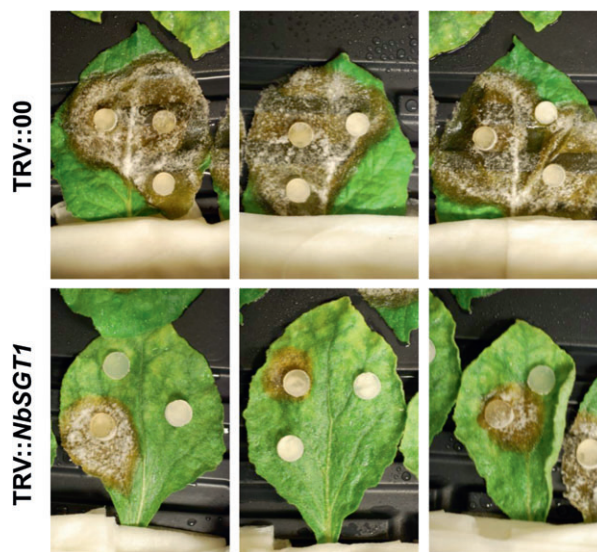

Fig. 6 *Sclerotinia sclerotiorum* disease symptoms on *NbSGT1*-silenced (TRV::*NbSGT1*) and control (TRV::00) *Nicotiana benthamiana* plants. Control and *NbSGT1*-silenced plants were inoculated with potato dextrose agar (PDA) plugs with actively growing *S. sclerotiorum* cultures. The inoculated plants were incubated at room temperature. Photographs were taken 4 days post-inoculation.

the outcome of the interaction. In either case, *NbSGT1*-silenced plants showed no cell death (Fig. S2). These data, taken together with the results from *S. sclerotiorum* (Fig. 6), suggest that NbSGT1 may play a general role in disease-associated cell death during plant–fungal interactions.

Overexpression of *NbSGT1* in *N. benthamiana* expedites PCD induced by compatible and incompatible pathovars of *P. syringae* pathogens

We have shown that SGT1 is required for PCD during compatible and incompatible plant–bacterial interactions. To explore further the function of SGT1 in PCD, we examined the effects of SGT1 overexpression on plant cell death during plant responses to bacterial infection. The full-length *NbSGT1* gene was cloned under the control of the double 35S promoter in a binary vector. *Agrobacterium tumefaciens* strain GV2260 carrying the construct 35S:*NbSGT1* was used to transform *N. benthamiana*. We selected three homozygous transgenic lines, SGT6521, SGT633 and SGT662, for further characterization. The phenotype of these transgenic lines was similar to that of wild-type *N. benthamiana* in terms of development (data not shown). The transcription levels of *NbSGT1* were determined by real-time RT-PCR. As shown in Fig. 7A, *NbSGT1* transcripts in the transgenic lines SGT6521 and SGT662 were at least three-fold higher than that in wild-type plants. However, the transgenic line SGT633 did not show a significant increase in *NbSGT1* transcript level compared with wild-type plants. Therefore, the transgenic lines SGT6521 and SGT662 were selected for further analyses.

We challenged the transgenic lines overexpressing *NbSGT1* with the host pathogen *P. syringae* pv. *tabaci*, virulent pathogen *P. syringae* pv. *syringae* and nonhost pathogens *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *maculicola* by vacuum infiltration at low concentration (1×10^5 cfu/mL). Notably, HR/cell death on the leaves of transgenic plants was observed 30 h after inoculation with nonhost and host pathogens, whereas wild-type *N. benthamiana* did not show cell death until 48 h after inoculation with *P. syringae* pv. *syringae*, and at 3 days following infiltration with *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *maculicola* (Fig. 7B). The cell death-associated disease symptoms on the transgenic lines caused by the host pathogen *P. syringae* pv. *tabaci* were observed about 12 h earlier than those on wild-type plants (data not shown). However, the populations of host and nonhost bacterial pathogens tested in this study did not show significant differences between the *NbSGT1*-overexpressing lines and the wild-type control (Fig. S1, see Supporting Information). These data indicate that increased expression of *NbSGT1* in *N. benthamiana* accelerates cell death on infection with both host and nonhost bacterial pathogens when compared with wild-type plants, but does not affect the growth of host and nonhost pathogens.

It has been shown that NbSGT1 is required for HR induced by multiple gene-for-gene interactions (Peart *et al.*, 2002). Therefore, we hypothesized that *NbSGT1* overexpression might expedite the development of HR associated with ETI and PTI. The *R* gene *Cf-9* and its cognate avirulence gene *Avr9* were transiently co-expressed, using *Agrobacterium*, in fully expanded young leaves of *NbSGT1* overexpressors and control plants. As hypothesized, HR due to ETI was observed 5 days after *Agrobacterium* inoculation in *NbSGT1*-overexpressing lines, whereas cell death was not observed in control plant leaves until 7 dpi (Fig. 8A). Similarly, when the *Ph. infestans* gene encoding elicitor protein INF1 (PAMP) was transiently expressed, HR developed at least 1 day earlier in *NbSGT1*-overexpressing lines (Fig. 8B). Overall, these data suggest that NbSGT1 positively regulates the process of cell death during both ETI and PTI.

DISCUSSION

Plant cell death can occur in both resistant and susceptible plant–pathogen interactions. HR-associated cell death, a form of PCD, results from plant immunity reactions triggered by host receptor-mediated perception of pathogen nonrace-specific elicitors or by the recognition of race-specific elicitors (Jones and Dangl, 2006; Zipfel and Felix, 2005). It has also been reported that SGT1 is required for cell death mediated by several pairs of Avr–R interactions and other effector-induced cell death (Figs 1 and 4) (Peart *et al.*, 2002). However, we have just begun to understand the molecular events involved in plant cell death associated with diseases caused by hemibiotrophs. In this study, we have clearly shown that SGT1 is involved in the process of PCD for both compatible and incompatible plant–pathogen interactions (Figs 1–6). This strongly suggests that cell death during plant responses to resistant and susceptible interactions shares a common step mediated by SGT1. The existence of common steps during HR and susceptible host resistance is also supported by previous studies. del Pozo *et al.* (2004) identified a MAPKKKα that positively regulates cell death associated with both gene-for-gene-mediated resistance and disease. The expression of the anticaspase baculovirus protein p35 also attenuates cell death during both susceptible and resistance responses to a number of pathogens (Lincoln *et al.*, 2002; del Pozo and Lam, 2003).

Wang *et al.* (2008) have reported that the overexpression of OsSGT1 in rice increases the basal resistance to *Xanthomonas oryzae* pv. *oryzae* PXO99, but not to *X. oryzae* pv. *oryzae* strain DY89031. Furthermore, in rice, the overexpression of SGT1 does not result in the acceleration of disease-associated cell death (Wang *et al.*, 2008). In our study, overexpression of NbSGT1 accelerated cell death induced by nonhost pathogens (Fig. 7B), host pathogens, during ETI and PTI (Fig. 8). However, overexpression of NbSGT1 did not result in cell death in the absence of the pathogen (Fig. 7B). These results suggest that NbSGT1 is a component of the signalling cascade that mechanistically controls cell death associated with compatible and incompatible interactions. It is also important to note that SGT1-mediated pathways may also vary in different plants and in response to a specific pathogen. It is not clear how increased levels of SGT1 expedite cell
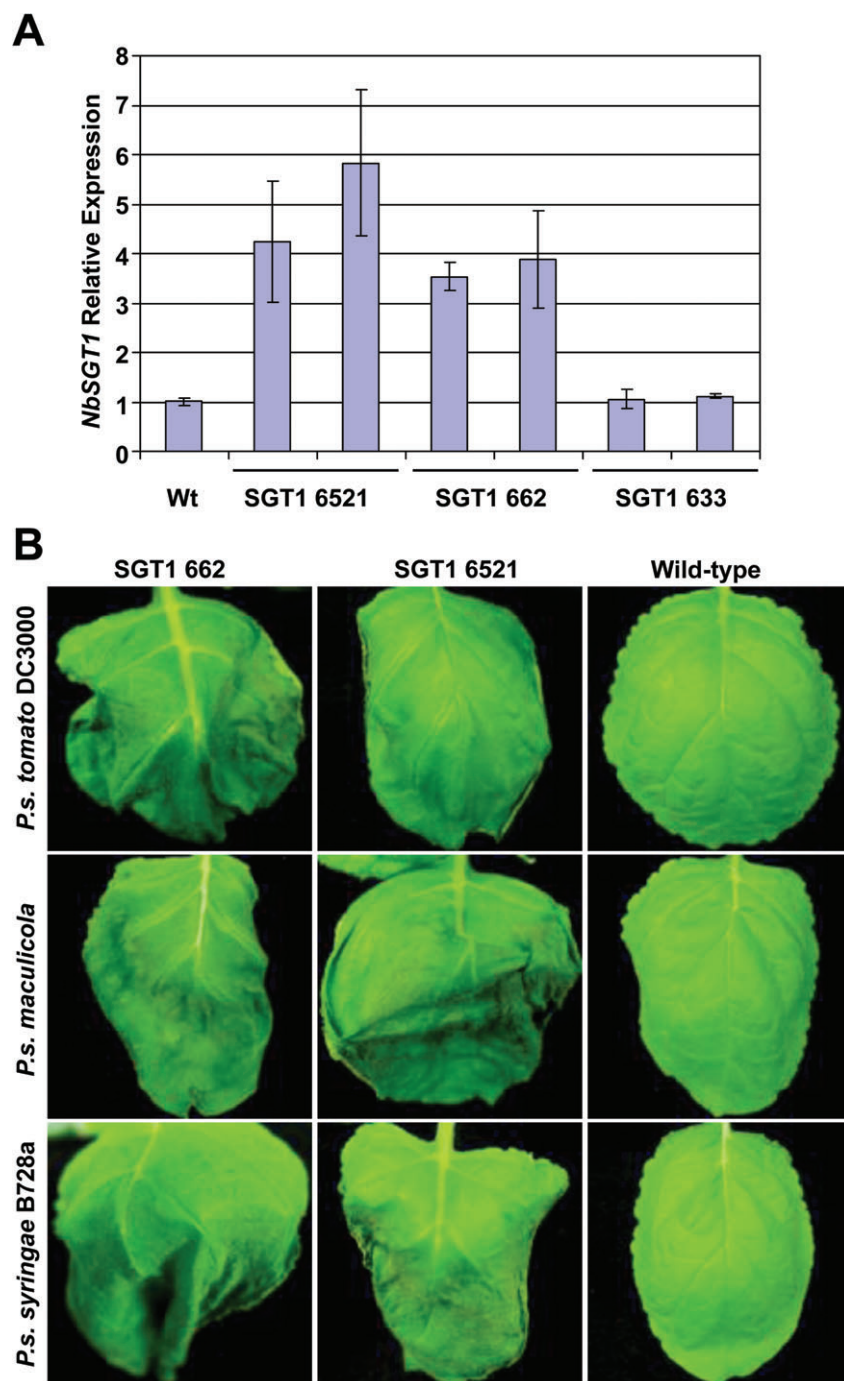
Fig. 7 Overexpression of *NbSGT1* in *Nicotiana benthamiana* expedites the cell death induced by various pathogens. (A) Relative expression of *NbSGT1* examined by real-time reverse transcription-polymerase chain reaction (RT-PCR) in transgenic *NbSGT1*-overexpressing lines of *N. benthamiana*. Error bars were derived from three technical replicates. (B) Early cell death response of *N. benthamiana NbSGT1* overexpressors on challenge with various pathogens. Plants were vacuum infiltrated with *Pseudomonas syringae* pv. *tomato* DC3000, *P. syringae* pv. *maculicola* and *P. syringae* pv. *syringae* B728a at a concentration of 10^5 colony-forming units/mL. Photographs were taken 30 h post-inoculation.
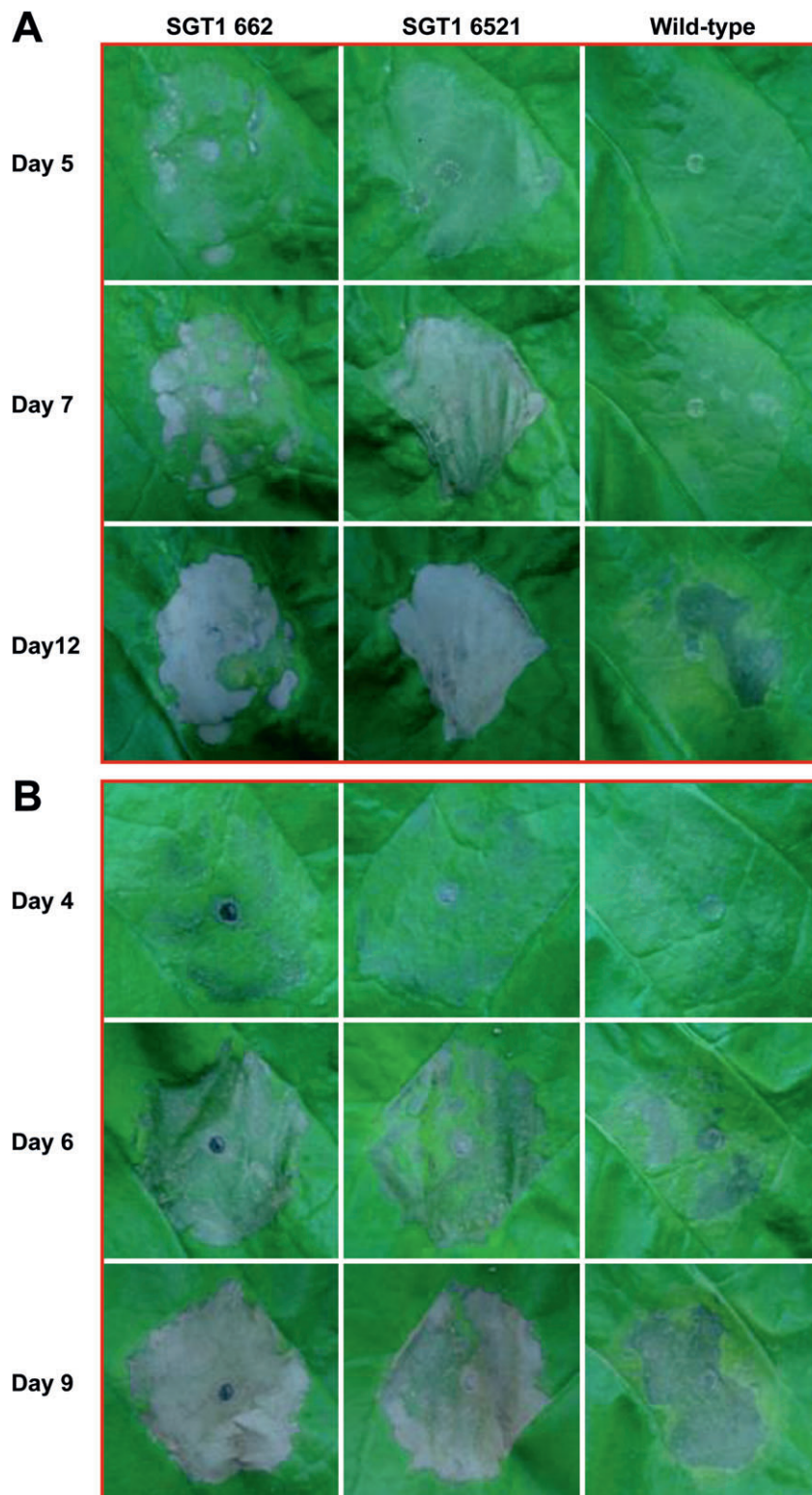
Fig. 8 ETI and PTI-mediated cell death in *NbSGT1*-overexpressing lines and wild-type *Nicotiana benthamiana* plants. Cell death was elicited by *Agrobacterium*-mediated transient co-expression of a resistance protein Cf9 and corresponding avirulence protein Avr9 (A) or expression of elicitor Inf1 (B). Seven-week-old *N. benthamiana* plant leaves were infiltrated with virulence-induced *Agrobacterium* carrying constructs 35S : *Inf1* or the mixed bacterial suspension of *Agrobacterium* with 35S : *Cf9* and 35S : *Avr9*. Photographs were taken on different days as indicated after *Agrobacterium* infiltration.

death and whether SGT1 is part of the previously reported MAPKKKα-mediated cell death pathway (del Pozo *et al*., 2004).

Recently, it has been shown that SGT1b is required for fungal disease development in Arabidopsis and *N. benthamiana* (Cuzick *et al*., 2009; El Oirdi and Bouarab, 2007). *Botrytis cinerea* has been shown to exploit the SGT1-mediated HR cell death pathway to initiate its necrotrophic life style (El Oirdi and Bouarab, 2007). Furthermore, *Fusarium culmorum* has been shown to require *SGT1b* to cause full disease symptoms associated with cell death and tissue dehydration (Cuzick *et al*., 2009). These results suggest that cell death associated with either necrotrophic or hemibiotrophic fungal pathogens may require SGT1. Our results showing the requirement of SGT1 for cell death during susceptible interactions in *N. benthamiana* to several bacterial and fungal pathogens indicate that cell death during disease development is, at least partially, genetically programmed (Greenberg, 1997). Interestingly, very little or no difference in the virulent bacterial population (*P. syringae* pv. *tabaci*, *P. syringae* pv. *syringae* B728a and *E. carotovora*) between *NbSGT1*-silenced and nonsilenced control *N. benthamiana* plants was observed, in contrast with a dramatic difference in the development of cell death-associated disease symptoms in *NbSGT1*-silenced plants (Figs 3–5 and Fig. S1). It is unlikely that the slightly reduced bacterial population in *NbSGT1*-silenced plants contributed to a significant delay (1–2 weeks) in the development of disease-associated cell death. Therefore, our data support the earlier suggestion (Greenberg and Yao, 2004) that pathogen-induced cell death during disease development is a result of several events, and not solely dependent on the pathogenic bacterial population. Our data also support a number of findings which indicate that apoptotic-like cell death does not always correlate with pathogen growth (Bent *et al*., 1992; Liang *et al*., 2003; Pilloff *et al*., 2002; Yao *et al*., 2002). Similarly, HR is considered to be a secondary feature of Rx-mediated viral resistance, and cell death has been shown to strictly correlate with viral growth (Bendahmane *et al*., 1999). However, when the viral coat protein was overexpressed *in planta*, Rx was shown to mediate SGT1-dependent HR cell death (Bendahmane *et al*., 1999; Peart *et al*., 2002). These results suggest that resistance-associated cell death may or may not correlate with the pathogen load. As HR occurs during incompatible plant–pathogen interactions, it has long been speculated that cell death is directly responsible for restricting pathogen growth and development. Our study shows that the attenuation of pathogen-induced cell death (HR) by the silencing of *SGT1* in *N. benthamiana* only slightly increases the growth of *P. syringae* pv. *tomato* DC3000 (Fig. 1). This is in contrast with the dramatic growth of the nonhost pathogen *P. syringae* pv. *maculicola* (Fig. 1B) and *P. syringae* pv. *tomato* strain T1 (Peart *et al*., 2002; Wang *et al*., 2007), which increased almost 100-fold in *SGT1*-silenced *N. benthamiana* when compared with nonsilenced control plants. In addition, the acceleration of cell death by the

overexpression of *SGT1* in *N. benthamiana* does not confer resistance by reducing the bacterial growth of the nonhost pathogen (Fig. S1). Our results support the earlier observation that cell death is not an obligatory step in achieving the resistance observed in plant–fungus interactions, in which the arrest of fungal development may occur before cell death in heterozygous plants with one copy of the *Mlg* resistance gene (Görg *et al*., 1993). In addition, Arabidopsis *dnd1* (defence, no death) exhibits strong gene-for-gene resistance to avirulent *P. syringae* without the HR phenotype, and shows a certain degree of constitutive resistance to some virulent bacterial pathogens (Yu *et al*., 1998, 2000). The majority (90%) of VIGS-identified genes involved in HR associated with *ArvPto-Pto* resistance against *P. syringae* strains failed to suppress bacterial growth in *N. benthamiana* plants (Lu *et al*., 2003a). Therefore, it is possible that cell death is a secondary feature of disease resistance and not always necessary for the suppression of pathogen growth. In some plant–pathogen interactions, PCD even promotes pathogen growth, so that pathogen-secreted toxins, such as AAL toxin secreted from *Alternaria alternata* f.sp. *lycopersici*, can kill host cells rapidly (Akamatsu *et al*., 1997; Greenberg and Yao, 2004). This is also suggested indirectly by the fact that the population of the host pathogen *P. syringae* pv. *tabaci* was reduced slightly in *NbSGT1*-silenced *N. benthamiana* plants in which pathogen-induced cell death was delayed significantly when compared with control plants (Fig. 2).

As SGT1 is required for much *R* gene-mediated disease resistance and/or HR (Austin *et al*., 2002; Azevedo *et al*., 2002; Peart *et al*., 2002), it has been proposed that SGT1 might be required for defence signalling (Muskett and Parker, 2003; Tör *et al*., 2002). However, a recent study has indicated that AtSGT1b is not required for disease resistance signalling *per se*, as *RPS5*-, *RPP4*-, *RPP*8- and *RPP31*-mediated resistance can be restored in the Arabidopsis *rar1 sgt1b* double mutant (Holt *et al*., 2005). It was speculated that SGT1b is required for efficient HR development to limit pathogen spread. Our data indicate that SGT1 is essentially required for PCD during both compatible and incompatible plant–pathogen interactions for all the tested pathogens in this study. However, the requirement of SGT1 for resistance is specific to a particular plant–pathogen interaction. Therefore, our findings support the previous proposal that the function of SGT1 in plant resistance is particularly relevant in cases in which HR plays a key role in limiting pathogen spread (Holt *et al*., 2005). For resistance in which cell death does not correlate with pathogen growth, SGT1 may have little effect on resistance. This model can explain why *SGT1* mutation or silencing results in an alteration in cell death, but little change in pathogen growth or resistance to some pathogens.

On the basis of our experimental results and previous findings, SGT1 appears to positively regulate the process of cell death during nonhost, ETI- and PTI-mediated resistance. In addition, our results have identified a new role for SGT1 in the process of

cell death associated with disease development of compatible bacterial and fungal pathogens in *N. benthamiana*. Therefore, we propose a common role for SGT1 as a shared signalling component of cell death during both immunity (gene-for-gene and nonhost) and susceptibility. Furthermore, *SGT1*, especially *SGT1b*, might associate with shared or distinct complexes that trigger the cell death pathway leading to HR, necrosis or trailing necrosis associated with a broad range of plant–pathogen interactions. Our identification of a role for SGT1 in disease-associated cell death will facilitate future studies aimed at the identification of shared and unique molecular events during cell death associated with host and nonhost resistance and disease. The identification of corresponding nonhost pathogen elicitors or host effectors that trigger SGT1-mediated cell death in response to pathogen attack may provide new insights into the pathways that control cell death during these processes.

EXPERIMENTAL PROCEDURES

Bacterial strains and plant materials

Agrobacterium tumefaciens GV2260 was routinely grown in Luria–Bertani (LB) medium at 28 °C. *Pseudomonas syringae* pv. *tomato* strain DC3000, *P. syringae* pv. *maculicola*, *P. syringae* pv. *syringae* B728a and *P. syringae* pv. *tabaci* were grown in King's B (KB) medium at 28 °C. The following concentrations of antibiotics (Sigma, St. Louis, MO, USA) were used individually or combined for selection when necessary, unless otherwise indicated: rifampicin (100 μg/mL), kanamycin (50 μg/mL). Cultures of *E. carotovora* ssp. *carotovora* were maintained on KB medium at 28 °C without any supplementary antibiotics.

Bacterial growth was monitored by measuring the optical density at 600 nm (OD_{600}) for the preparation of the inoculum, and the number of viable cells was determined by counting the colonies grown on appropriate plates supplemented with the appropriate antibiotics.

Nicotiana benthamiana seeds were germinated in Metro Mix 350 (SUNGRO Horticulture Distribution Inc., Bellevue, WA, USA) in a growth chamber at 26 °C and 16 h daylight. Fertilizer (20–12–20), together with soluble trace element mix (The Scotts Co., Marysville, OH, USA), was applied to 2-week-old seedlings with water in the tray. Three-week-old seedlings were transplanted to 4-in pots containing Professional Blend soil (SUNGRO Horticulture Distribution Inc.) and grown in a glasshouse under the following conditions: 24 $\pm$ 2 °C, 70% humidity and 16 h daylight supplemented with 50–100 μE/s/m^2 light intensity. The VIGS experiment was conducted 2 days after transplanting.

The T-DNA knockout lines *sgt1a* and *sgt1b* were kindly provided by B.F. Holt (Holt *et al.*, 2005). The seeds of *A. thaliana* were cold treated for 3–4 days at 4 °C and germinated in Professional Blend soil in a growth chamber at 20–22 °C under short-day

conditions (8 h light). Two-week-old seedlings were transplanted into 4-in pots containing Professional Blend soil and grown under the same conditions as seed germination. Four-week-old plants were used for inoculation in all experiments.

TRV-based VIGS in *N. benthamiana*

The TRV vector system was used for gene silencing in *N. benthamiana* as described previously (Liu *et al.*, 2002). Briefly, *A. tumefaciens* strain GV2260 containing construct TRV2::*SGT1* or TRV::00 was mixed with *A. tumefaciens* strain GV2260 containing TRV1 at a 1:1 ratio and infiltrated into cotyledons and the first two leaves of 3-week-old *N. benthamiana* plants. The transcript levels were examined by real-time RT-PCR and the plants were used for bacterial challenge 2–3 weeks after TRV infection.

Agrobacterium-mediated transient expression in *N. benthamiana*

The expression of *Cf-9*, *Avr9* and *Inf1* was driven by the cauliflower mosaic virus (CaMV) 35S promoter in the pBtex vector. Transient expression in *N. benthamiana* mediated by *Agrobacterium* was performed as described previously (Peart *et al.*, 2002). Briefly, bacterial cells of *A. tumefaciens* GV2260 containing constructs were harvested by centrifugation, washed twice and resuspended in an induction medium containing acetosyringone (200 μM) at $OD_{600} = 1$. The virulence genes of *A. tumefaciens* were induced for at least 4 h at room temperature by shaking at 100 r.p.m. The bacterial cells were collected by centrifugation and resuspended in 10 mM 2-(*N*-morpholino)ethanesulphonic acid (MES) with acetosyringone (200 μM). The concentrations of *A. tumefaciens* containing *Cf-9* and *avr9* were adjusted to $OD_{600} = 0.4$, respectively, and mixed at a 1:1 ratio, whereas the bacterial suspension of *Agrobacterium* containing *Inf1* was adjusted to a final concentration of $OD_{600} = 0.2$ for the infiltration of leaves of *N. benthamiana*.

NbSGT1 overexpression in *N. benthamiana*

Full-length *NbSGT1* cDNA was amplified by RT-PCR from *N. benthamiana* and cloned into the donor vector pDONR207. *NbSGT1* in pDONR207 was subcloned into the plant expression vector pYL436 (Rubio *et al.*, 2005) using the GATEWAY cloning system (Invitrogen, Carlsbad, CA, USA), resulting in the plasmid pYL436-NbSGT1. Transgenic *N. benthamiana* plants expressing *NbSGT1* were created by introducing pYL436-NbSGT1 into *N. benthamiana* plants using standard *Agrobacterium*-mediated leaf disc transformation (Horsch *et al.*, 1985).

RNA isolation and real-time RT-PCR

Total RNA was extracted from the leaves of *N. benthamiana* using the RNAeasy Kit (Qiagen, Valencia, CA, USA) following the manu-

facturer's instructions. The RNA concentration was measured using a NanoDrop® ND-1000 UV–visible spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) and adjusted to 1 μg/μL. Reverse transcription was carried out by using 2 μg of total RNA and 0.5 μg of poly(dT)$_{16}$ primer in a final volume of 20 μL for 1 h at 37 °C employing an Omniscript RT Kit (Qiagen) and RNAase inhibitor (Promega, Madison, WI, USA). The following gene-specific primers for PCR were designed using PrimerExpress software (Applied Biosystems, ABI, Foster City, CA, USA) and synthesized by Integrated DNA Technologies (Coralville, IA, USA): for SGT1 silencing: NbSGT1F, 5′-TTTGCCAAGGGAATACC AGCCAA-3′; NbSGT1R, 5′-TTCCCATTTCTTCAGCTCCATGC-3′; for SGT1 overexpression: LeSGT1F, 5′-CACATCCTGCATCTGAG TTACC-3′; LeSGT1R, 5′-CTCTGAATTACAACAGGTTCCGTT-3′. Primer concentrations were optimized on an ABI PRISM® 7000 Sequence Detection System (Applied Biosystems) and specific amplification was confirmed by melting curve assay and agarose gel electrophoresis. Real-time quantitative PCR was performed with the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems) using Power SYBR Green PCR Master Mix (ABI) under the following conditions: 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The quality of amplification was determined using dissociation curve analysis of the 7900HT Real-Time PCR system. Three biological replicates and three technical replicates were used for each line or treatment. Threshold cycle (C_T) values reported by the 7900HT Real-Time PCR system were used for data analysis. The relative quantification of PCR products was calculated by the comparative C_T method ($\Delta\Delta C_T$) using *N. benthamiana Actin* gene as endogenous control.

Plant inoculation and bacterial population measurement

Pseudomonas syringae pathovars were grown in KB broth with the appropriate antibiotics. Bacterial cells were collected by centrifugation of an overnight culture at 3000 **g** for 10 min, washed twice, resuspended in 10 mM MgCl$_2$ and diluted to the desired concentration in 10 mM MgCl$_2$ with 0.01% (v/v) Silwet L-77 (Osi Specialties, Friendship, WV, USA) to facilitate infiltration. The bacterial suspension was used to inoculate gene-silenced *N. benthamiana* plants 2 weeks after TRV infection by vacuum infiltration, or to inoculate 4-week-old *Arabidopsis* plants by leaf infiltration with needle-less syringes. The fully expanded leaves were used for disease assays. The inoculated plants were kept in a growth chamber at 20–22 °C for disease development. Two leaf discs (0.5 cm^2 each) of each plant were sampled, homogenized and serially diluted in 10 mM MgCl$_2$ for the measurement of bacterial growth *in planta*. The bacterial population was calculated as cfu/cm^2. Experiments were performed with at least four replicates.

Erwinia carotovora was grown on KB medium, suspended in distilled water and diluted to the desired concentration. The inoculum was infiltrated using a needle-less syringe into fully expanded leaves of control (TRV::00) and gene-silenced *N. benthamiana* plants 2 weeks after TRV infection. The bacterial populations were quantified as described above for *P. syringae* pathovars.

Fungal inoculum preparation and plant inoculation

Agar plugs (diameter, 5 mm) from growing regions of *Sclerotinia sclerotiarum* cultures on potato dextrose agar (PDA; Difco, Sparks, MD, USA) medium were used as inoculum. Control and *NbSGT1*-silenced *N. benthamiana* leaves were inoculated with two to three agar plugs per leaf, and fungal inoculated leaves were placed on moist filter paper, sealed and incubated at 22 °C/ 19 °C for a 16-h photoperiod at a photon flux density of 150-200 μmol/m^2/s. The disease symptoms were recorded at 5 dpi.

Phytopthora infestans (Becktell *et al.*, 2006) was maintained on Rye A agar (Caten and Jinks, 1968), and then transferred onto Rye B agar supplemented with β-sitosterol (Sigma Aldrich Inc.) to induce sporulation for 14 days in a dark chamber maintained at 15 °C. Sporangia were collected in distilled water, counted using a haematocytometer and adjusted to 2×10^4 sporangia/ mL. An aliquot of 25 μL of the sporangial suspension was spot inoculated onto control and *NbSGT1*-silenced *N. benthamiana* leaves. The leaves were incubated at 15 °C (16-h light period) and maintained at 100% humidity using moist filter paper. Disease symptoms were monitored periodically until 10 dpi.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Growth of bacteria in *Nicotiana benthamiana* leaves.
Fig. S2 *Phytopthora infestans*-induced cell death in control (*TRV::00*) and SGT1-silenced (*TRV::NbSGT1*) *Nicotiana benthamiana* leaves.

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