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FULL RESEARCH PAPER

Botrytis cinerea induces senescence and is inhibited by autoregulated expression of the *IPT* gene

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Abstract *Botrytis cinerea* is a non-specific, necrotrophic pathogen that attacks many plant species, including *Arabidopsis* and tomato. Since senescing leaves are particularly susceptible to infection by *B. cinerea*, we hypothesized that the fungus might induce senescence as part of its mode of action and that delaying leaf senescence might reduce the severity of *B. cinerea* infections. To examine these hypotheses, we followed the expression of *Arabidopsis SAG12*, a senescence-specific gene, upon infection with *B. cinerea*. Expression of *SAG12* is induced by *B. cinerea* infection, indicating that *B. cinerea* induces senescence. The promoter of *SAG12*, as well as that of a second *Arabidopsis* senescence-associated gene, *SAG13*, whose expression is not specific to senescence, were previously analyzed in tomato plants and were found to be expressed in a similar manner in the two species. These promoters were previously used in tomato plants to drive the expression of isopentenyl transferase (*IPT*) from *Agrobacterium* to suppress leaf senescence (Swartzberg et al. in Plant Biology 8:579–586, 2006). In this study, we examined the expression of these promoters following infection of tomato plants with *B. cinerea*. Both promoters exhibit high expression levels upon *B. cinerea* infection of non-senescing leaves of tomato plants, supporting our conclusion that *B. cinerea* induces senescence as part of its mode of action. In contrast to *B. cinerea*, *Trichoderma harzianum* T39, a saprophytic fungus that is used as a biocontrol agent against *B. cinerea*, induces expression of *SAG13* only. Expression of *IPT*, under the control of the *SAG12* and *SAG13* promoters in response to infection with *B. cinerea* resulted in suppression of *B. cinerea*-induced disease symptoms, substantiating our prediction that delaying leaf senescence might reduce susceptibility to *B. cinerea*.

Keywords *Botrytis cinerea* · Cytokinin · Promoter · Senescence · Tomato · *Trichoderma harzianum* T39

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Introduction

Senescence is one of the most important factors affecting tissue susceptibility to *B. cinerea*, but whether *B. cinerea* induces senescence as part of its mode of action is not known (Elad and Evensen

1995). The *Arabidopsis SAG12* and *SAG13* genes are senescence-associated genes (*SAG*s) expressed in senescing leaves (Noh and Amasino 1999, Weaver et al. 1998, Lohman et al. 1994). *SAG12* is expressed exclusively in senescing leaves while *SAG13* is expressed at basal levels in non-senescing leaves and at increasing levels as senescence progresses (Weaver et al. 1998). In addition, *SAG13* is also expressed under various stress conditions (Noh and Amasino 1999, Weaver et al. 1998). Gan and Amasino (1995) exploited the highly senescence-specific promoter of the *SAG12* gene to create transgenic tobacco plants with an autoregulated cytokinin production system that delayed leaf senescence. The *SAG12* promoter (P_{SAG12}) was fused to the *IPT* gene from the Ti plasmid of *Agrobacterium tumefaciens* which encodes isopentenyl transferase, a key enzyme in cytokinin biosynthesis in plants. The senescence-inhibiting effects of cytokinins have been well documented (Gan and Amasino 1996). The fused P_{SAG12}*::IPT* gene was activated only at the onset of senescence, resulting in cytokinin biosynthesis upon initiation of senescence in tobacco leaves. This inhibited leaf senescence and, consequently, repressed further expression of the promoter; thus preventing cytokinin overproduction (Gan and Amasino 1995).

We have recently produced tomato plants that express either *GUS* or *IPT* under the control of *SAG12* and *SAG13* promoters (P_{SAG12}*::GUS*, P_{SAG13}*::GUS*, P_{SAG12}*::IPT* and P_{SAG13}*::IPT*, respectively). Expression of the *Arabidopsis SAG12* and *SAG13* promoters in tomato plants was analyzed using the reporter gene *GUS*. Both promoters are expressed in tomato leaves in a manner similar to their expression in *Arabidopsis* plants. That is, *SAG12* is expressed only in senescing leaves while *SAG13* is expressed at basal levels in non-senescing leaves and at increasing levels as the senescence process progresses (Swartzberg et al. 2006). Expression of *IPT* under the control of either promoter provided autoregulation inhibition of leaf senescence in tomato plants (Swartzberg et al. 2006).

The primary aim of this study was to examine whether *B. cinerea* induces senescence as part of its mode of pathogenicity. To that end, we investigated the expression of the senescence specific *SAG12* promoter in *Arabidopsis* and tomato plants upon infection with *B. cinerea*. A second aim of this study was to examine whether delaying senescence through the expression of *IPT* under the control of the *SAG12* and *SAG13* promoters would reduce the susceptibility of plants to *B. cinerea* infection. For that purpose, we used the tomato plants expressing P_{SAG12}*::IPT* and P_{SAG13}*::IPT* which exhibit autoregulated inhibition of senescence.

Materials and methods

Plant material

Arabidopsis thaliana (*Landsberg erecta*) plants were grown in a climate-controlled growth room which was kept at 18°C, with 8 h of illumination each day. After infection, they were moved to another growth room which was kept at 20°C, with 12 h of illumination each day. Independent isogenic transgenic tomato (*Lycopersicon esculentum*) lines expressing P_{SAG12}*::GUS* (lines 514-5, 514-14, 514-17), P_{SAG13}*::GUS* (lines 763-3, 763-8), P_{SAG12}*::IPT* (lines 529–9, 529–19) and P_{SAG13}*::IPT* (lines 766-20, 766-25), described in Swartzberg et al. (2006), were used in this study. The parental isogenic line MP-1 (Barg et al. 1997) was used as a control.

Microorganisms, culture conditions, infection and scoring of disease severity

Botrytis cinerea (isolate BcI16) was cultured on potato dextrose agar (PDA, Difco, Detroit, MI) in Petri dishes incubated at 20°C. Conidia were harvested from 10- to 14-day-old cultures by agitating small pieces of agar, bearing mycelium and conidia, in a glass tube. The suspension was filtered through cheesecloth. The concentration of conidia was determined using a hemocytometer and adjusted to either 5×10^5 or 1×10^6 cells ml^{-1}. Since *B. cinerea* conidia need carbon and phosphate for germination and penetration (Cole et al. 1996), 0.1% glucose or 0.1% sucrose was added to the final aqueous conidial suspension, together with 0.1% KH_2PO_4. These supplements have been shown to enable the germination of *B. cinerea* conidia and subsequent leaf infection (Elad et al. 1994). At least five *Arabidopsis* plants, 10 leaves on each plant were inoculated with 10 µl drops of the conidial suspension (1×10^6 cells ml^{-1}), one drop per leaf. Following inoculation, the plants were covered with plastic and incubated at 20°C, 95% RH and 12 h of illumination each day.

In tomato, detached and attached leaves were tested. Detached leaves were inoculated immediately after harvest and kept in a humidity chamber at 20°C and 95% RH. For attached leaves, >5 leaves per plant from at least four plants were inoculated with 20 µl drops of the conidial suspension (5×10^5 cells ml^{-1}), 2 drops per leaf. These experiments were performed three times. Following inoculation, the plants were incubated at 20°C and 95% RH. The severity of the resultant necrotic lesions was determined according to the scale described below. The 20 µl drop on a tomato leaf was 5 mm diam, corresponding to an area of 19.6 mm^2. Disease developed gradually and the first symptoms were visible 3–5 days after inoculation. The initial symptoms were small necrotic lesions covering only part of the area originally covered by the *B. cinerea* suspension drop. These small lesions eventually merged to form a single lesion of approx. 9 mm diam on a typical control tomato leaf at 9–10 days after inoculation, corresponding to an area of 63.5 mm^2. This area was used as a base size and assigned a value of 100%. A pictorial scale of lesions, similar to that developed for use in strawberry (Guetsky et al. 2001), was used to determine the relative size of lesions (a measure of disease severity) from each inoculation; 0, 1, 2, 5, 10, 20, 40, 75 and 100% of the base lesion size.

Trichoderma harzianum T39 was cultured on PDA in Petri dishes incubated at 25°C. Conidia were harvested from 10- to 14-day-old cultures by agitating small pieces of agar, bearing mycelium and conidia, in a glass tube. The suspension was filtered through cheesecloth. The concentration of conidia was determined using a hemocytometer and adjusted to 5×10^5 cells ml^{-1}. Application of *T. harzianum* T39 was carried out by spraying each plant with 2 ml of an aqueous suspension containing 5×10^5 conidia ml^{-1}. Control plants were sprayed with water only. Two days later, the plants were inoculated with *B. cinerea* and disease severity was scored 3–9 days later, as described above.

RNA extraction and cDNA preparation

Samples were collected from both young and mature leaves from the infected and control *Arabidopsis* plants. The samples were taken 24 and 48 h after infection, frozen immediately in liquid nitrogen and stored at −80°C. Total RNA was isolated from the leaves using the Gen Elute-Mammalian Total RNA Miniprep kit (RTN-70, Sigma, USA), according to the manufacturer's protocol. To prepare cDNA for real-time PCR, the RNA was treated with DNase (RQI-RNase free DNase, Promega Corporation, Madison, WI) and tested as a template in a PCR-amplification as a negative control. First strand cDNA synthesis was carried out for each sample by reverse transcription using the Superscript II pre-amplification system (Gibco BRL Life Technologies, Glasgow, UK) according to the manufacturer's instructions.

Real-time PCR analysis

The primers used for the expression analysis of *SAG12* (GenBank accession no. 123957.2) were: Forward primer-148F AGGCACATCGAGTGGATGAC and

Reverse primer-248R TCAATGCGTTCGACGTT GTT. The primers were designed to yield fragments of approximately 100 bp. Quantitative RT-PCR reactions contained the first strand cDNA of each tissue as a template, specific primers and qPCR Core kit for SYBR Green (Eurogentec, Leige, Belgium) in a final volume of 25 µl. Fluorescence increments of each reaction were simultaneously monitored using the GeneAmp 5700 Sequence Detection System (version 1.3, PE BioSystems, CA, USA). Amplifications were performed for 40 cycles, consisting of 30 sec at 95°C, 30 sec at 60°C and 30 sec at 72°C, and an initial preheating at 95°C for 15 min. The reference gene was GAPDH (GenBank accession no. NM_101214). The primers used were: Forward primer F-GCAACA TACGACGAAATCAAGAAG and Reverse primer: R-CGGTAGACACAA CATCATCCTCAG. They were used under the same conditions in the quantitative RT-PCR reactions.

Reporter gene assays

Quantitative *GUS* (*β*-glucuronidase) activity was determined fluorometrically, as described by Jefferson et al. (1987). Histochemical *GUS* analysis was performed as follows: Leaves were incubated at room temperature for 24–48 h in approximately 3 ml of incubation solution (100 mM sodium phosphate buffer, pH 7.2, 10 mM ethylenediamine tetraacetic acid (EDTA), 2 mM potassium ferricyanide, 2 mM potassium ferricyanate and 2 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc)). Following this incubation, the leaves were washed several times with 70% EtOH to remove the chlorophyll.

Results

Expression analysis of SAG12 upon infection of *Arabidopsis* plants with *B. cinerea*

To examine whether *B. cinerea* induces senescence in the course of its attack, we inoculated *Arabidopsis* plants with either *B. cinerea* or water. Expression of *SAG12*, a senescence-specific molecular marker, was examined in leaves from the infected and control plants. No visible senescence symptoms were observed until 72 h after infection, at which point plants infected with *B. cinerea* exhibited yellowing of the leaf tissue adjacent to the infection spots (Fig. 1). Expression of *SAG12* was observed 24 h after infection and was even greater at 48 h after infection

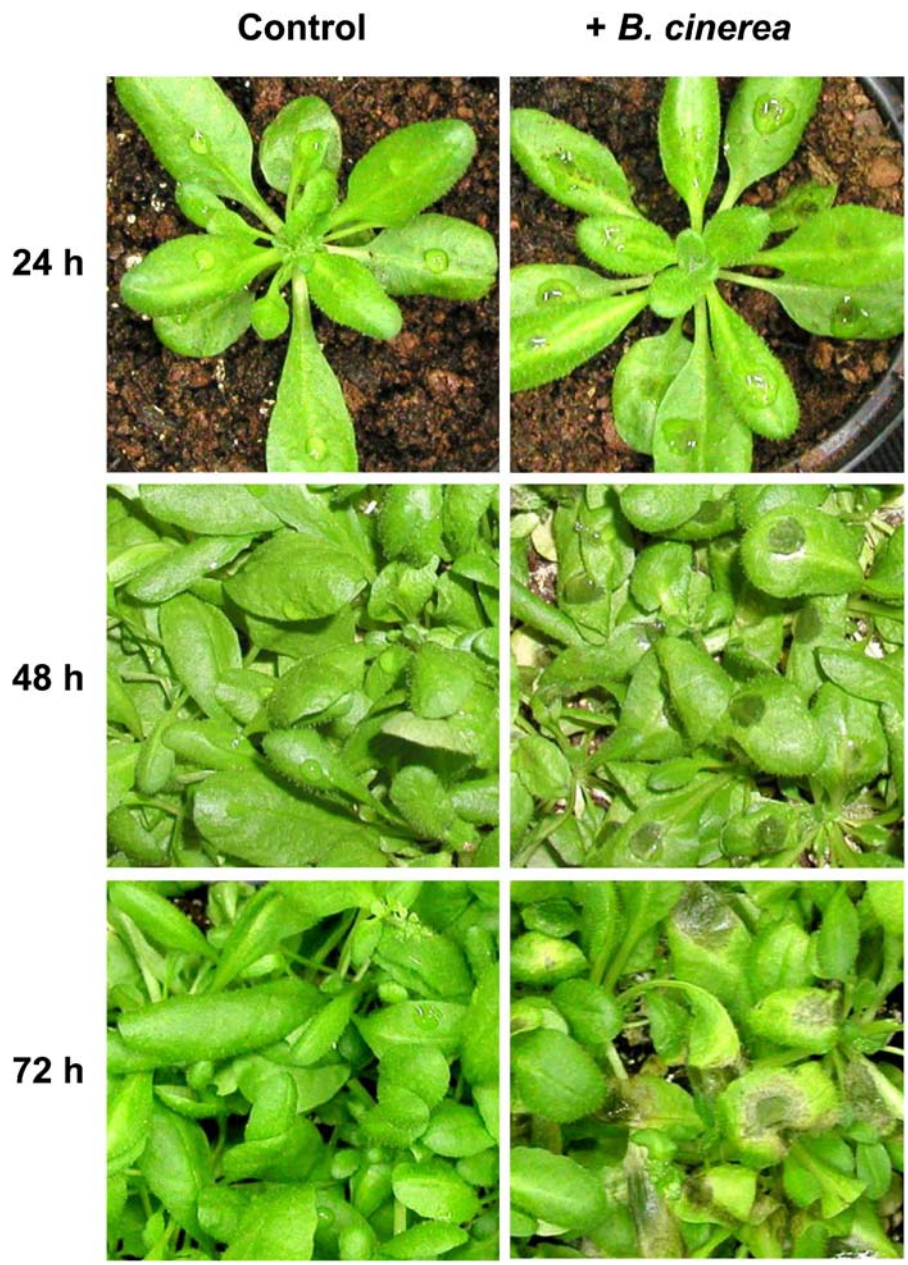

Fig. 1 *Arabidopsis* plants infected with *B. cinerea*. *Arabidopsis* plants were inoculated with 10 μl drops of *B. cinerea* or water (as a control). Pictures were taken 24, 48 and 72 h after infection

(Fig. 2), suggesting that *B. cinerea* induces precocious senescence in *Arabidopsis* leaves that precedes the visible leaf-yellowing.

Analysis of the expression of SAG12 and SAG13 promoters in detached tomato leaves in response to *B. cinerea* infection

To analyze the induction of senescence by *B. cinerea* in tomato plants, transgenic plants with P_{SAG12}::*GUS* and P_{SAG13}::*GUS* were tested. In many studies, detached leaves have been used to study leaf senescence (Park et al. 1998, Weaver et al. 1998). We therefore first inoculated non-senescing leaves of the transgenic tomato plants with *B. cinerea*, immediately after detaching them from the plants. In these samples, P_{SAG12}::*GUS* was expressed 48 h after infection, prior to the expression of P_{SAG12}::*GUS* in the control (uninfected) leaves, suggesting acceleration of senescence by *B. cinerea* (Fig. 3). One week after leaf detachment, both P_{SAG12}::*GUS* and P_{SAG13}::*GUS* were expressed in control, uninfected leaves, but infected leaves (infected upon detachment), expressed these genes at levels twice those seen in the uninfected leaves.

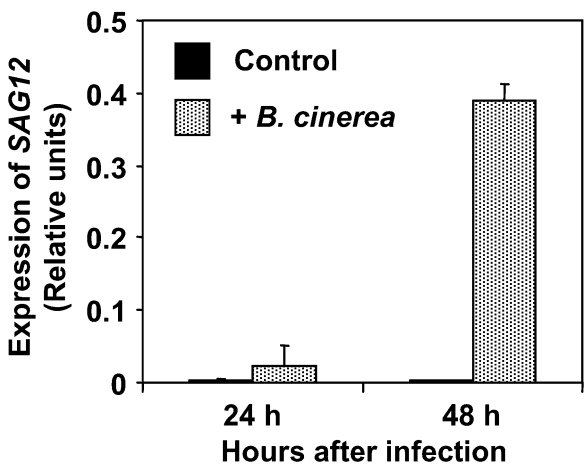

Fig. 2 Expression of *SAG12* in *Arabidopsis* plants infected with *B. cinerea*. Expression levels of *SAG12* were determined by real-time PCR analysis of leaf tissue from *Arabidopsis* plants inoculated with either *B. cinerea* or water. Data was normalized to that of the reference gene GAPDH (GenBank accession no. NM_101214). The results are the means of three plants per treatment from two independent treatments ± SE

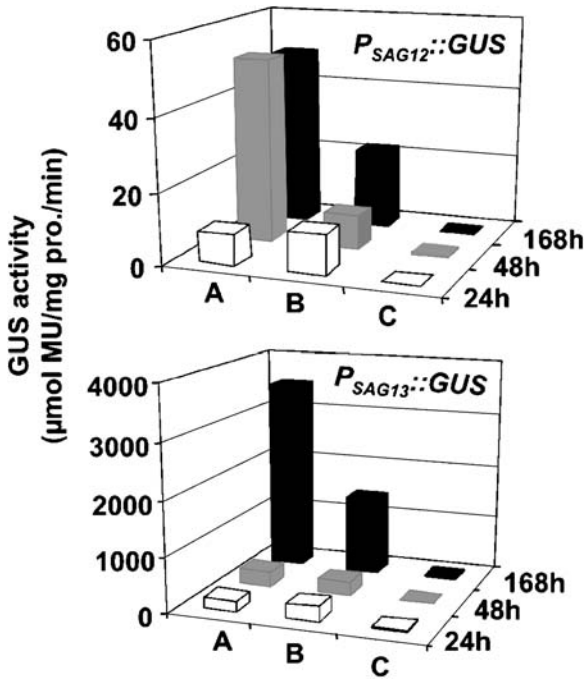

Fig. 3 Expression of P_{SAG12}::*GUS* and P_{SAG13}::*GUS* in detached tomato leaves infected with *B. cinerea*. Detached mature, non-senescing leaves were infected immediately after harvest and kept in a humidity chamber at 20°C and 95% RH. Expression of GUS was determined by assaying GUS activity 24 h, 48 h and 168 h (1 week) after infection. The examination was performed on three different tissues: **A**. leaf tissue adjacent to the point of infection; **B**. tissue from an uninfected leaf of the same age from the same line; and **C**. leaf tissue from uninfected control (non-transformed) plants. Similar results were obtained in three independent experiments in three independent lines from P_{SAG12}::*GUS* (lines 514-5, 514-14, 514-17) and two independent lines of P_{SAG13}::*GUS* (lines 763-3 and 763-8)

Analysis of the expression of SAG12 and SAG13 promoters in attached tomato leaves in response to *B. cinerea* infection

The expression of P_{SAG12}::*GUS* and P_{SAG13}::*GUS* was also tested upon infection of attached, non-senescing leaves. In control plants, no expression of P_{SAG12}::*GUS* and basal expression of P_{SAG13}::*GUS* were observed in leaves of the same age (Fig. 4). However, infection with *B. cinerea* induced expression of both P_{SAG12}::*GUS* and P_{SAG13}::*GUS* both within and a few mm away from the infection area at 48 h after treatment (Fig. 4), indicating induction of senescence. To test the specificity of this induction by *B. cinerea*, we checked the expression of P_{SAG12}::*GUS* and P_{SAG13}::*GUS* by the saprophytic fungus *T. harzianum* T39, which confers resistance in tomato

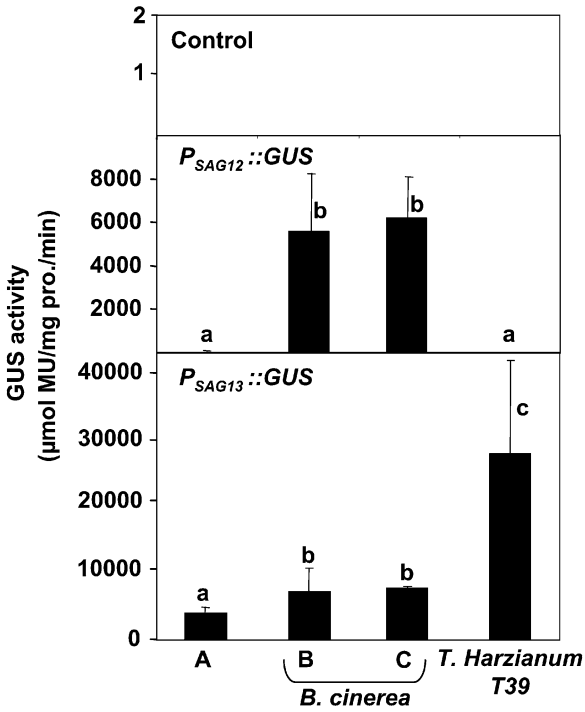

Fig. 4 *GUS* activity in attached leaves from P_{SAG12}::*GUS* and P_{SAG13}::*GUS* of plants infected with *B. cinerea* and *T. harzianum*. The examination was performed 48 h after infection with *B. cinerea*. Three different tissues were examined: **A**. tissue from an uninfected leaf of the same age from the same line; **B**. tissue from within the infection spot; **C**. leaf tissue a few mm from the infection spot. Leaves from plants that had been sprayed with *T. harzianum* were also examined. The results are the means of three replicates ± SD. Means not connected by the same letter are significantly different (*t* test; alpha = 0.05)

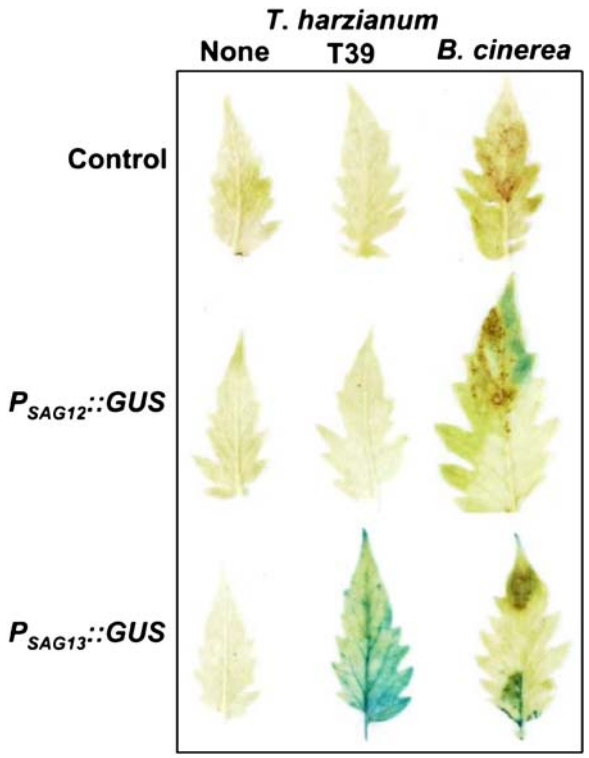

Fig. 5 *GUS* staining of control and infected leaves. Uninfected and infected leaves of control, P_{SAG12}::*GUS* and P_{SAG13}::*GUS* plants were stained with X-Gluc 48 h after infection. The pictures were taken following chlorophyll bleaching

plants against *B. cinerea* and is used as a biocontrol agent (De Meyer et al. 1998). *T. harzianum* T39 induced expression of P_{SAG13}::*GUS*, but not of the senescence-specific promoter P_{SAG12}::*GUS* (Fig. 4 and 5), emphasizing the specific induction of *SAG12* and hence of senescence by *B. cinerea*.

Expression of P_{SAG12}::IPT and P_{SAG13}::IPT attenuates symptoms of *B. cinerea* infection

If *B. cinerea* induces senescence as part of its mode of action, inhibition of senescence might reduce susceptibility to *B. cinerea*. Autoregulated inhibition of senescence has been previously achieved in leaves of various plant species, including tomato, through autoregulated expression of the *Agrobacterium IPT* gene under the control of the *SAG12* and *SAG13* promoters (P_{SAG12}::*IPT* and P_{SAG13}::*IPT*, respectively) (Swartzberg et al. 2006, Chang et al. 2003, Gapper et al. 2002, McCabe et al. 2001, Gan and Amasino 1995). We therefore monitored the severity of disease symptoms of tomato plants containing P_{SAG12}::*IPT* and P_{SAG13}::*IPT* genes upon infection with *B. cinerea*. As expected, disease symptoms developed significantly more slowly in the plants expressing either P_{SAG12}::*IPT* or P_{SAG13}::*IPT* than in the control plants (Fig. 6). We also examined the severity of symptoms caused by *B. cinerea* in plants pre-treated with *T. harzianum* T39. P_{SAG12}::*IPT* and P_{SAG13}::*IPT* plants pre-treated with *T. harzianum* T39 exhibited a further significant reduction in the severity of their disease symptoms suggesting an additive effect of senescence inhibition to the *T. harzianum* T39 conferred resistance.

Discussion

In this study, we demonstrated that the *Arabidopsis* senescence-specific gene *SAG12* is induced by *B.*

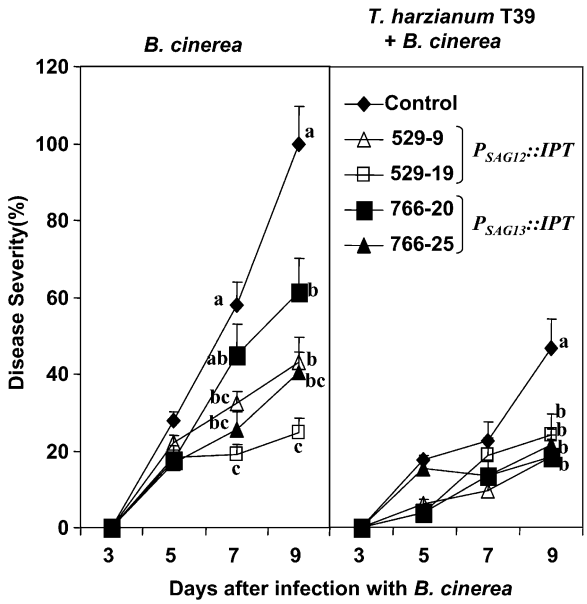

Fig. 6 Disease severity in control, P_{SAG12}::*IPT* and P_{SAG13}::*IPT* plants infected with *B. cinerea*, with and without pre-treatment with *T. harzianum* T39. Disease severity was evaluated on a scale of 0–100% (Guetsky et al. 2001) at 3, 5, 7 and 9 days after infection with *B. cinerea* in plants from two independent lines, P_{SAG12}::*IPT* (529-9 and 529-19) and P_{SAG13}::*IPT* (766-20 and 766-25). Plants with P_{SAG12}::*GUS* or P_{SAG13}::*GUS* were used as controls, respectively. Half of the plants were sprayed with *T. harzianum* T39 two days before being infected with *B. cinerea*; the other plants did not receive this pre-treatment. The results are the means of evaluations of at least 10 infection spots (two spots on five leaves) of each treatment ± SE. The experiments were performed three times with similar results each time. Means not connected by the same letter are significantly different (*t* test; alpha = 0.05)

cinerea in *Arabidopsis* and tomato plants. In previous studies, expression of *SAG12* throughout natural senescence was observed only in older leaves that had already become about 20% yellow (Weaver et al. 1998). Interestingly, *B. cinerea* induced expression of *SAG12* in *Arabidopsis* 24–48 h after infection, about 24 h before visible senescence symptoms were observed (Figs. 1 and 2), indicating that the induction of precocious senescence by *B. cinerea* might be part of its mode of action.

Tomato homologs of the *A. thaliana SAG12* gene have not yet been characterized. Therefore, we followed the expression of the *Arabidopsis SAG12* promoter in tomato plants, previously shown to be a reliable senescence marker in tomato plants (Swartzberg et al. 2006). *Botrytis cinerea* induced the expression of the *Arabidopsis SAG12* promoter in tomato plants while *T. harzianum* T39, a saprophytic fungus, did not induce the expression of the *SAG12* promoter, emphasizing the specificity of the induction of senescence by *B. cinerea*. Unlike *SAG12*, both pathogens induced expression of the senescence-associated *Arabidopsis SAG13* promoter which is not a senescence-specific gene in either *Arabidopsis* or tomato (Swartzberg et al. 2006, Weaver and Amasino 2001). Since, unlike *SAG12*, *SAG13* is expressed not only during senescence, but also under various stress conditions (Noh and Amasino 1999, Weaver et al. 1998), *T. harzianum* T39 might induce a stress response that could be related to its biocontrol activity. Nevertheless, the expression of the *SAG12* promoter by *B. cinerea* in both *Arabidopsis* and tomato suggests that induction of senescence is a general feature of this pathogen's mode of action.

The hypothesis that *B. cinerea* induces senescence as part of its mode of action is supported by previous observations that *B. cinerea* produces ABA (Siewers et al. 2004, 2006, Sharon et al. 2004, Tapani et al. 1993, Hirai et al. 1986, Marumo et al. 1982), a known inducer of senescence (Yang et al. 2003). However, the production of ABA by *B. cinerea* throughout infection has not yet been shown. Nevertheless, this hypothesis is also in agreement with the work of Shaul et al. (1996), which demonstrated that external applications of ABA can enhance the development of disease symptoms caused by *B. cinerea*. In addition, the ABA-deficient tomato *Sitiens* mutant was more resistant to *B. cinerea* and exogenous ABA resulted in increased susceptibility of the *Sitiens* mutant to *B. cinerea* (Achuo et al. 2006, Audenaert et al. 2002).

It has been shown that infection by *B. cinerea* induces programmed cell death (PCD) in the host (Govrin and Levine 2000, reviewed also in Van Kan 2006). Furthermore, mutations in the type 1 metacaspase genes *MCA7* and *MCA8*, which probably participate in suppression of cell death, rendered *Arabidopsis* plants more susceptible to *B. cinerea* (Van Baarlen et al. 2007). Senescence is also considered a PCD process, which involves the regulated disintegration of cells and tissues that allows for nutrient recycling (Van Doorn and Woltering 2004). Indeed, *mca7* and *mca8* mutants showed accelerated senescence even in non-inoculated leaves, with symptoms of yellowing, leaf discolouration and spontaneous necrotic spots (Van Baarlen et al. 2007).

It is likely therefore that *B. cinerea* induces senescence and harnesses host senescence PCD machinery to facilitate its own infection. P_{SAG12}::*GUS* and P_{SAG13}::*GUS* are expressed not only at the site of *Botrytis* infection, but also in tissues located a few mm away from the infection spot (Fig. 4). This suggests that *B. cinerea* not only induces senescence in the infected area, but also evokes a senescence signal that spreads to the adjacent tissue.

Botrytis cinerea induces stronger expression of P_{SAG12}::*GUS* and P_{SAG13}::*GUS* in attached leaves than in detached leaves. It has been shown that nutrient availability enhances *B. cinerea* pathogenicity (Holz et al. 2004). It has also been shown that sugars may play a role in the onset of leaf senescence in tomato and *Arabidopsis* (Pourtau et al. 2006, Dai et al. 1999) and that, in *Arabidopsis*, the expression of *SAG12* is directly correlated with the availability of sugars (Pourtau et al. 2006, Dai et al. 1999). It is therefore likely that *B. cinerea* may accelerate senescence better in attached leaves, due to their high levels of available carbohydrates.

The expression of *IPT* under *SAG12* and *SAG13* promoters delayed leaf senescence in tomato plants (Swartzberg et al. 2006) and reduced the severity of the disease symptoms following infection with *B. cinerea* (Fig. 6). It has been shown in various plant species that expression of *IPT* increases the level of cytokinin *in planta* (Cowan et al. 2005, Chang et al. 2003, McCabe et al. 2001, Jordi et al. 2000). Spraying cut rose flowers with cytokinin (benzyladenine) has been shown to significantly delay the development of disease symptoms caused by *B. cinerea* (Elad 1993). We suggest plants that express P_{SAG12}::*IPT* and P_{SAG13}::*IPT* are less susceptible to *B. cinerea*, due to the inhibition of pathogen-induced senescence by the *IPT*-produced cytokinin.

Pre-treatment of tomato plants with *T. harzianum* T39 provides protection against *B. cinerea*. *Trichoderma harzianum* T39 acts as a biocontrol agent by interfering with *B. cinerea* pathogenicity enzymes (Kapat et al. 1998) and by inducing plant resistance (Lapsker and Elad 2001, Elad 2000). Treatment of P_{SAG12}::*IPT* and P_{SAG13}::*IPT* plants with *T. harzianum* T39 had a significant additive protective effect (Fig. 6). This implies that the mechanism by which *T. harzianum* T39 confers protection is probably unrelated to senescence inhibition. A combination of *T. harzianum* T39 treatments and senescence-inhibition might prove to be effective against *B. cinerea*.

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