Abscisic Acid Determines Basal Susceptibility of Tomato to *Botrytis cinerea* and Suppresses Salicylic Acid-Dependent Signaling Mechanisms[1]

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Abscisic acid (ABA) is one of the plant hormones involved in the interaction between plants and pathogens. In this work, we show that tomato (*Lycopersicon esculentum* Mill. cv Moneymaker) mutants with reduced ABA levels (*sitiens* plants) are much more resistant to the necrotrophic fungus *Botrytis cinerea* than wild-type (WT) plants. Exogenous application of ABA restored susceptibility to *B. cinerea* in *sitiens* plants and increased susceptibility in WT plants. These results indicate that ABA plays a major role in the susceptibility of tomato to *B. cinerea*. ABA appeared to interact with a functional plant defense response against *B. cinerea*. Experiments with transgenic *NahG* tomato plants and benzo(1,2,3)thiadiazole-7-carbothioic acid demonstrated the importance of salicylic acid in the tomato-*B. cinerea* interaction. In addition, upon infection with *B. cinerea*, *sitiens* plants showed a clear increase in phenylalanine ammonia lyase activity, which was not observed in infected WT plants, indicating that the ABA levels in healthy WT tomato plants partly repress phenylalanine ammonia lyase activity. In addition, *sitiens* plants became more sensitive to benzo(1,2,3)thiadiazole-7-carbothioic acid root treatment. The threshold values for PR1a gene expression declined with a factor 10 to 100 in *sitiens* compared with WT plants. Thus, ABA appears to negatively modulate the salicylic acid-dependent defense pathway in tomato, which may be one of the mechanisms by which ABA levels determine susceptibility to *B. cinerea*.

Upon pathogen attack, infected plant cells generate signaling molecules to initiate defense mechanisms in surrounding cells to limit pathogen spread. The role of the plant hormones salicylic acid (SA), jasmonic acid (JA), and ethylene in this process is supported by well-documented observations and molecular characterization (Hammond-Kosack and Jones, 1996). This kind of information is not available for another plant hormone, abscisic acid (ABA), which participates in several processes. The role of ABA in developmental programs, such as seed dormancy, root geotropism, opening of stomata through stomatal guard cells, and dormancy of buds, has been most extensively documented (Walton, 1980). Furthermore, ABA is involved in the wound response (WR) activated upon insect feeding (Birkenmeier and Ryan, 1998).

Regarding plant-pathogen interactions, information on ABA involvement is mainly based on indirect observations. Increased endogenous ABA levels were observed in response to infection with viruses, bacteria, and fungi (Whenham et al., 1986; Steadman and Sequeira, 1970; Kettner and Dörffling, 1995). In addition, it is generally found that application of exogenous ABA increases the susceptibility of plants to fungal pathogens (Henfling et al., 1980; Ward et al., 1989; McDonald and Cahill, 1999). ABA also seems to interact with pathogen associated plant defense. In soybean (*Glycine max*), ABA suppressed Phe ammonia lyase (PAL) activity and transcription of PAL mRNA in hypocotyls inoculated with the incompatible pathogen *Phytophthora megasperma* f.sp. *glycinea* (Ward et al., 1989). Moreover, physiological ABA concentrations down-regulate β-1,3-glucanase at the level of transcription in tobacco (*Nicotiana tabacum*) cell cultures. β-1,3-Glucanases have been implicated in responses to stress, wounding, and pathogen infection (Rezzonico et al., 1998). However, these observations only give a fragmentary picture and provide few or indirect clues for the mechanistic basis of the involvement of ABA in plant defense toward pathogens.

To study the role of plant hormones such as SA, JA, and ethylene in plant defense to pathogens, mutants impaired in the perception or biosynthesis of these hormones have been successfully used in Arabidopsis and tomato (*Lycopersicon esculentum* Mill. cv Moneymaker; Lund et al., 1998; Thomma et al., 1998). To our knowledge, ABA mutants have not previously been used in plant-pathogen interaction studies, with the exception of the work by Kettner and Dörffling (1995). The ABA-deficient *flacca* tomato mutant was used in their study on biosynthesis and metabolism of ABA in tomato leaves infected with *Botrytis cine-*

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rea. To further elucidate the role of ABA in plant-pathogen interactions, we used ABA-negative *sitiens* tomato mutants. *Sitiens* mutants have a residual ABA level of 8% of the WT plants and are unable to increase their ABA levels upon elicitation by wounding, heat, or electrical current (Herde et al., 1999). *Sitiens* tomato mutants are defective in the structural gene for ABA-aldehyde oxidase, the enzyme that converts ABA-aldehyde to ABA.

The present study shows that ABA-negative *sitiens* tomato plants are much more resistant to *B. cinerea* than WT plants, indicating that ABA increases susceptibility of tomato to *B. cinerea.* In a first attempt to elucidate the mechanistic basis for this observation, we studied the potential cross talk between the plant hormone ABA- and SA-associated plant defenses. Results suggest that ABA negatively regulates SA-dependent defense signaling, which in turn appears to be an effective plant defense mechanism against *B. cinerea*.

RESULTS

ABA Increases Susceptibility of Tomato to *B. cinerea*

To analyze the role of ABA in the interaction between tomato and *B. cinerea*, leaves of *sitiens* tomato mutants (Linforth et al., 1987), impaired in the biosynthesis of ABA, and wild-type (WT) leaves (tomato cv Moneymaker) were infected in a comparative assay. Because *B. cinerea* typically needs a nutrient supply to initiate an infection (Van Den Heuvel, 1981), several inoculation solutions composed of different Glc and phosphate concentrations were tested on detached WT tomato leaves (Fig. 1A) based on the infection methods described by Van Den Heuvel (1981) and Von Tiedemann (1997).

When using the infection solution composed of 10^6 spores mL^{-1}, 0.1 M Glc, and 67 mM KH_2PO_4 (pH 5), all inoculations resulted in brownish spreading lesions colonizing the whole leaf area (Fig. 1A). When a very mild infection solution was used (10^6 spores mL^{-1} + 0.01 M Glc), none of the infection droplets resulted in a spreading lesion. This interaction is considered to be resistant because further fungal spread was not observed and the fungus did not colonize the leaf. In this resistant interaction, *B. cinerea* development was restricted to a few black spots under the inoculation droplet, indicating a clear reduction of pathogen growth. Because we wanted to use assay conditions that would result in a moderately aggressive infection, an inoculation solution containing 10^6 spores mL^{-1}, 0.01 M Glc, and 6.7 mM KH_2PO_4 (pH 5) was selected. This solution produced a moderate number of spreading *B. cinerea* lesions in WT detached leaves (Fig. 1A). This infection allowed us to detect both increases and decreases in disease severity.

To eliminate an effect of leaf detachment, infection solutions were tested on intact plants (Fig. 1B). These

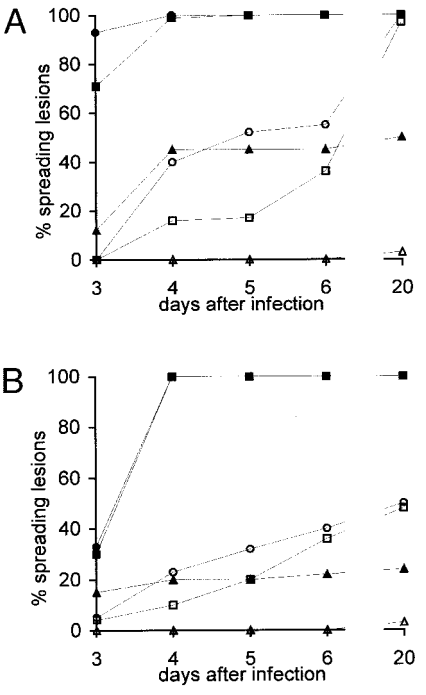

Figure 1. Effect of Glc and phosphate concentrations on the infection of *B. cinerea* on tomato. Detached tertiary leaves (A) or tertiary leaves of intact plants (B) were infected by a droplet solution. Ten droplets each containing 4 μL of spore suspension were placed on a tomato leaf surface. The infection was evaluated at several time points after infection by counting the number of *B. cinerea* lesions spreading out of the initial inoculation droplets on each leaf. The inoculation solutions tested in this work were: ●, 0.1 M Glc, 67 mM KH_2PO_4 (pH 5), and 10^6 spores mL^{-1}; ■, 0.05 M Glc, 33 mM KH_2PO_4 (pH 5), and 10^6 spores mL^{-1}; and ▲, 0.01 M Glc, 6.7 mM KH_2PO_4 (pH 5), and 10^6 spores mL^{-1}. White signs show the infection development without adding phosphate to the inoculation solutions. Data are means of three experiments containing 12 leaves per treatment.

experiments showed that the infection developed similarly on intact plants and on detached leaves, although fewer lesions spread out of the initial inoculum droplet when the infection was carried out on intact plants. Because the uniformity of infection was higher on detached leaves than on intact plants, it was decided to perform further infections using detached leaves.

In the comparative assay, *sitiens* leaves appeared to be much more resistant to *B. cinerea* than WT leaves because a considerable decrease in the number of spreading *B. cinerea* lesions was observed (Fig. 2, A–C). Experiments were performed subsequently to determine whether exogenous ABA applied to petioles of *sitiens* leaves could restore the susceptibility observed in WT plants. Results presented in Figure 3A clearly illustrate that concentrations from 10 to 100 μM ± cis-trans ABA increased the susceptibility of *sitiens* leaves to *B. cinerea*. These results also demonstrate that a threshold concentration of ABA is necessary to induce the susceptible response in *sitiens* tomato to *B. cinerea* because 1 μM ABA did not induce susceptibility in *sitiens* leaves. Finally, applying ABA

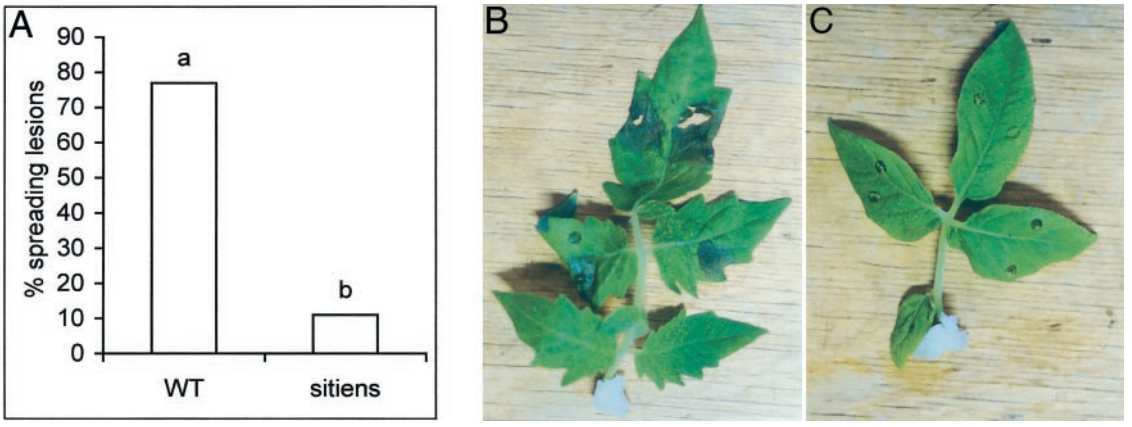

Figure 2. A, Influence of endogenous ABA concentrations on spreading of *B. cinerea* in tomato 4 d after infection. Tertiary WT (tomato cv Moneymaker) and *sitiens* leaves (tomato cv Moneymaker) were detached from 5-week-old tomato plants and infected with 10 droplets of a 4-μL spore suspension containing 10^6 spores mL^{-1}, 0.01 M Glc, and 6.7 mM KH_2PO_4. Infection was evaluated 4 d after infection by counting the number of spreading lesions on each leaf. Data are means of three experiments containing 12 leaves per treatment. B shows an infected WT leaf (tomato cv Moneymaker) with four spreading lesions of six lesions. C shows a resistant *sitiens* leaf (tomato cv Moneymaker) with no spreading lesions. Bars with different letters are significantly different with $P = 0.05$ after a logistic regression.

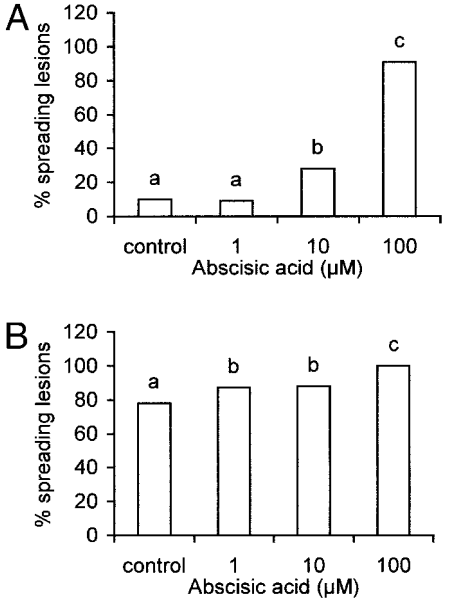

Figure 3. Effect of exogenous ABA fed to petioles of 5-week-old of *sitiens* (A) and WT (B) leaves (tomato cv Moneymaker) on an infection with *B. cinerea*. Tertiary leaves were detached and placed for 16 h in ABA solutions varying from 1 to 100 μM. ABA solutions were prepared from 1 mL of 10 mM stock solution of ABA in ethanol. Ethanol concentrations varied from 0.1% to 0.001% (v/v), respectively, in the final solutions. Control leaves were fed with water containing 0.1% (v/v) ethanol. Inoculation solutions contained 0.01 M Glc, 6.7 mM KH_2PO_4, and 10^6 spores mL^{-1}. Infection was scored 4 d after inoculation by counting the number of spreading *B. cinerea* lesions on each leaf. Data are means of three experiments containing 12 leaves per treatment. Bars with different letters are significantly different with $P = 0.05$ after a logistic regression.

to WT leaves further increased the susceptibility to *B. cinerea*, although in these experiments no threshold concentration to increase susceptibility was observed (Fig. 3B). These experiments clearly illustrate the importance of ABA, at levels present in WT tomato plants, in the interaction with *B. cinerea*.

Basis of Increased Resistance in *sitiens* Plants to *B. cinerea*

Several hypotheses were tested in search of an explanation for the altered resistance of *sitiens* plants to *B. cinerea*. First, we had to exclude the possibility that the increased resistance of *sitiens* mutants to *B. cinerea* was an artifact resulting from the use of detached leaves in our infection assays. It is known that endogenous ABA levels can increase in WT plants upon wounding, whereas *sitiens* mutants do not show this increase (Herde et al., 1999). Therefore, we infected intact *sitiens* and WT with *B. cinerea* and still observed increased resistance in *sitiens* plants compared with WT plants (Fig. 4).

To investigate a potential direct effect of ABA on fungal growth, *B. cinerea* was plated on potato dextrose agar (Oxoid, Drongen, Belgium) medium containing 1 to 100 μM ± cis-trans ABA. None of the ABA concentrations stimulated fungal growth. Moreover, in vivo experiments illustrated that lesions in *sitiens* plants grew at the same rate in WT and *sitiens* plants, indicating that ABA present in WT plants did not stimulate directly the growth rate of *B. cinerea* (data not shown). These results suggest that ABA does not directly influence the pathogen or its interaction with the host but rather modulates the defense mechanism of the host to the pathogen.

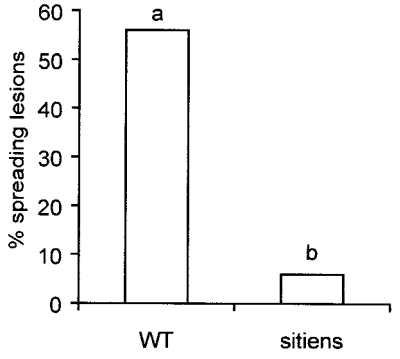

Figure 4. Influence of endogenous ABA concentrations on spreading of *B. cinerea* in intact tomato plants. Tertiary leaves of WT (tomato cv Moneymaker) and *sitiens* (tomato cv Moneymaker) plants were infected with 10 droplets of 4 μL of spore suspension containing 10^6 spores mL^{-1}, 0.01 M Glc, and 6.7 mM KH_2PO_4. Data are means of two experiments containing five plants (10 leaves) per treatment. Infections were evaluated 4 d after inoculation by counting the number of spreading *B. cinerea* lesions on each leaf. Bars with different letters are significantly different with $P = 0.05$ after a logistic regression.

JA-Dependent Plant Signaling Defense Is Not Involved in Defense of Tomato to *B. cinerea*

Because we wanted to investigate further the possible involvement of ABA in the defense response of tomato to *B. cinerea*, we first had to know which defense signaling pathways play a role in the tomato-*B. cinerea* interaction. Because it is known that JA-dependent defense is important in the Arabidopsis-*B. cinerea* interaction (Thomma et al., 1998, 1999), we investigated whether the same was true in tomato. For this purpose, the JA biosynthesis tomato mutant *def 1* was used (Howe et al., 1996). *Def 1* is mutated in the conversion of hydroxyperoxylinolenic acid to oxy-phytodieonic acid. We infected WT plants (tomato cv Castlemart) and *def1* mutants (tomato cv Castlemart) with *B. cinerea* and found no effect of JA on the resistance level of tomato to *B. cinerea* (Fig. 5A). Moreover, applying concentrations of exogenous JA from 5 up to 100 μM to the tomato leaf petiole did not affect the resistance level of WT tomato to *B. cinerea* (Fig. 5B). In tomato, JA apparently does not play an important role in the basal defense to *B. cinerea*.

SA-Dependent Resistance Controls *B. cinerea* in Tomato

Because De Meyer et al. (1999a, 1999b) showed that resistance to *B. cinerea* in bean (*Phaseolus vulgaris*) could be induced via the SA-dependent defense pathway, the role of this pathway in the tomato-*B. cinerea* interaction was investigated. For this purpose, we used transgenic *NahG* tomato plants that cannot accumulate SA because they express a bacterial SA hydroxylase that converts SA to catechol (Brading et al., 2000). *NahG* leaves were slightly more susceptible to *B. cinerea* than WT leaves (Fig. 6A), suggesting a role for SA in the basal defense of tomato to *B. cinerea*. In addition, PAL activity in *sitiens* leaves increased severalfold 16 h after infection, whereas in WT leaves, only a small increase could be observed (Fig. 6B). Basal PAL activity, however, was lower in *sitiens* leaves than in WT leaves (118 μkats kg^{-1} protein for WT and 18 μkats kg^{-1} protein for *sitiens*). Mock-inoculated *sitiens* and WT leaves did not show an increased PAL activity. Because PAL is a major enzyme in the phenylpropanoid pathway, which is also involved in SA synthesis (Pallas et al., 1996; Mauch-Mani and Slusarenko, 1996; Smith-Becker et al., 1998), these results indicate that SA-dependent defense in *sitiens* plants was activated to a higher extent than in WT plants.

To confirm the role of SA-dependent defense in the control of *B. cinerea*, the plant defense activator and SA-analog benzo(1,2,3)thiadiazole-7-carbothioic acid (BTH, BION; Novartis, Basel) was applied to the soil at the time of sowing and 10 d later when seedlings

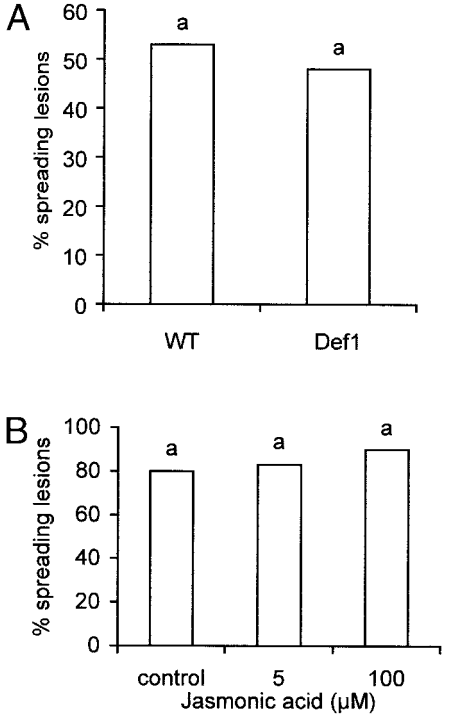

Figure 5. A, Effect of endogenous JA concentrations on the infection of *B. cinerea* in tomato. Tertiary leaves of WT (tomato cv Castlemart) and *def1* tomato mutants (tomato cv Castlemart) were infected with 10 droplets of 4 μL of spore suspension containing 10^6 spores mL^{-1}, 0.01 M Glc, and 6.7 mM KH_2PO_4. Data are means of two experiments containing 10 leaves per treatment. Infections were evaluated 4 d after inoculation by counting the number of spreading *B. cinerea* lesions on each leaf. Bars with different letters are significantly different with $P = 0.05$ after a logistic regression. B, Effect of exogenous ± JA feeding through petioles of tomato leaves from 5-week-old plants. Leaves were infected with 10 droplets of 4 μL of spore suspension containing 10^6 spores mL^{-1}, 0.01 M Glc, and 6.7 mM KH_2PO_4. Data are means of two experiments containing 10 leaves per treatment. Bars with different letters are significantly different with $P = 0.05$ after a logistic regression.

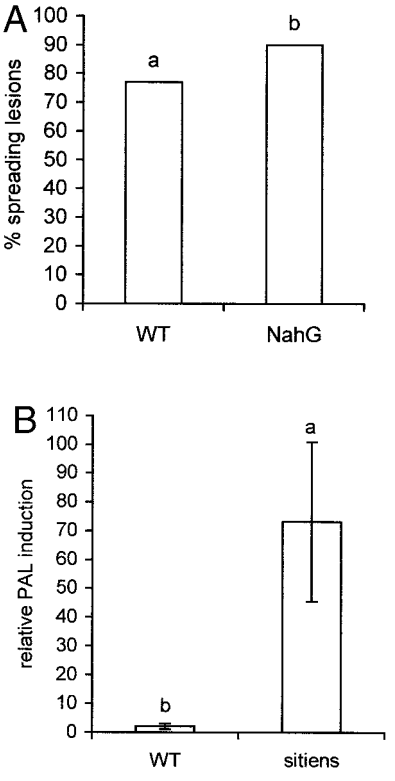

Figure 6. A, Influence of endogenous SA on spreading of *B. cinerea* in the detached leaf assay. Tertiary WT (tomato cv Moneymaker) and *NahG* leaves (tomato cv Moneymaker) were infected with 10 droplets of 4 μL of spore suspension containing 10^6 spores mL^{-1}, 0.01 M Glc, and 6.7 mM KH_2PO_4. Data are means of two experiments each composed of 10 leaves per treatment. Infection was evaluated 4 d after inoculation by counting the number of spreading *B. cinerea* lesions on each leaf. Bars with different letters are significantly different with $P = 0.05$ after a logistic regression. B, Relative induction of PAL activity in 5-week-old WT (tomato cv Moneymaker) and *sitiens* leaves (tomato cv Moneymaker) 16 h after infection with *B. cinerea* with an infection solution of 10^6 spores mL^{-1}, 0.01 M Glc, and 6.7 mM KH_2PO_4. Values for PAL activity were obtained by dividing values for PAL activity 16 h after infection by values for PAL activity of control leaves. Bars represent the average of 10 individual tomato leaves of *sitiens* and WT plants. Data represent two independent experiments. Data were analyzed by an ANOVA analysis. Bars with different letters are significantly different with $P = 0.05$.

were transferred. Application of BTH at several concentrations (0.01, 0.1, 1, and 10 mg kg^{-1} soil) induced resistance to *B. cinerea* (Fig. 7A) in WT leaves, whereas PR1a gene expression was detected only at 10 mg kg^{-1} soil (50 μmol kg^{-1} soil; Fig. 7B). However, when applying higher concentrations of BTH, e.g. 100 mg kg^{-1} soil, resistance declined, whereas PR1a was still expressed. Applying such high concentrations was rather dramatic with regard to the morphology of tomato plants. Leaves turned dark green and became shrunken compared with control leaves (not shown). In addition, applying 100 mg kg^{-1} BTH to roots of tomato plants probably induced plant defense to a high extent because this concentration resulted in the development of spontaneous necrotic lesions. Although 10 mg kg^{-1} did not induce spontaneous lesions, minor changes in morphology, resulting in lengthening of internodes and shrinking of leaves, were observed. Applying 10 mg kg^{-1} BTH not only induces PR1a gene expression but initiates changes in plant morphology and plant resistance. As a consequence,10 mg kg^{-1} could be considered as a threshold concentration.

There is no clear link between PR1 gene expression and induced resistance by BTH to *B. cinerea* in tomato. Moreover, activating plant defense too extensively leads to necrotic lesions, which may serve as a nutritional source for *B. cinerea*. From these results, it can be concluded that inducing SA-dependent plant defense mechanisms in tomato can lead to an enhanced resistance to the necrotrophic fungus *B. cinerea*.

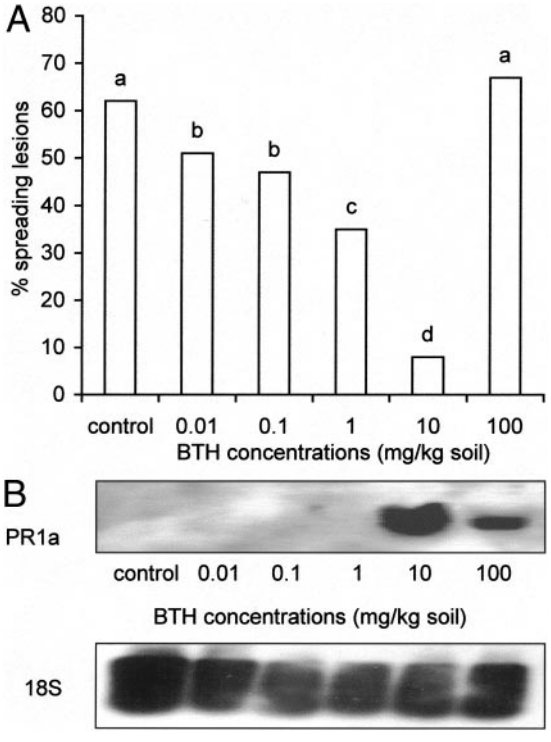

Figure 7. A. Effect of BTH (BION) root application on spreading of *B. cinerea* lesions on the third pair of 5-week-old WT tomato leaves (tomato cv Moneymaker). Leaves were infected with 10 droplets of 4 μL of spore suspension containing 10^6 spores mL^{-1}, 0.01 M Glc, and 6.7 mM KH_2PO_4 25 d after the last BTH treatment. Data are means of three experiments with each 12 leaves per treatment. Infections were evaluated 4 d after inoculation by counting the number of spreading *B. cinerea* lesions on each leaf. Bars with different letters are significantly different with $P = 0.05$ after a logistic regression. B, Induction of PR1a gene expression in 5-week-old leaves of WT tomato plants after BTH root application. Each lane contains 30 μg of total RNA and PR1a was detected via digoxygenin (DIG)-labeled cDNA probes. BTH concentrations are represented in milligrams per kilogram. Detection limit of PR1a was determined to be 0.5 pg. An 18S rRNA probe was used as a constitutive probe to verify for equal RNA loading and transfer.

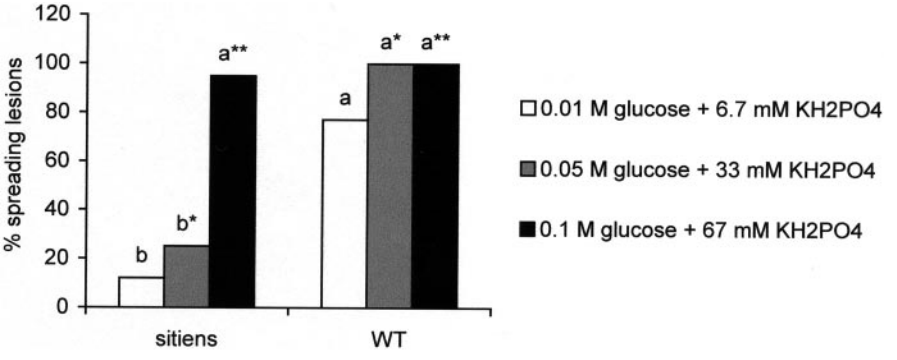

Figure 8. Influence of inoculum aggressiveness on the resistance of *sitiens* leaves (tomato cv Moneymaker) to *B. cinerea*. Tertiary leaves of WT and *sitiens* plants were infected with 10 droplets of 4 μL of spore suspension containing different Glc and phosphate concentrations. Data are means of three experiments with each 12 leaves per treatment. Infections were evaluated 4 d after infection by counting the number of spreading *B. cinerea* lesions on each leaf. Bars with different letters are significantly different with $P = 0.05$ after a logistic regression.

SA-Dependent Resistance Induced by BTH Is Modulated by Endogenous Plant-ABA

Because it was shown that BTH, which activates the SA-dependent pathway, induced resistance to the necrotrophic fungus *B. cinerea* in WT plants, we wanted to test whether the SA-dependent response was altered or enhanced in *sitiens* plants. BTH was applied at several concentrations to *sitiens* roots as described above to assay for induction of resistance. Because *sitiens* leaves displayed a high basal level of resistance (Fig. 2), more aggressive inoculation solutions were tested on *sitiens* leaves (Fig. 8). The infection solution with 0.05 M Glc and 33 mM KH_2PO_4 resulted in 100% spreading lesions in WT leaves (Fig. 1, A and B), whereas in *sitiens* leaves the infection was only moderately aggressive (Fig. 8). This solution was used in the subsequent study.

Results shown in Figure 9A clearly demonstrate the induction of resistance by applying BTH to roots of *sitiens* tomato plants. However, in *sitiens* plants, PR1a expression was induced at a BTH concentration of 1 mg kg^{-1} (Fig. 9B), in contrast to the 10 mg kg^{-1} BTH, which was needed to induce these effects in WT plants. In addition, spontaneous necroses and stimulation of *B. cinerea* infection were already observed at a concentration of 1 mg kg^{-1}, whereas 100 mg kg^{-1} was needed to induce these effects on WT plants. These results indicate that *sitiens* plants are sensitized to respond to the chemical plant activator BTH and suggest that ABA levels in WT plants negatively interfere with the SA-dependent defense pathway in tomato.

DISCUSSION

In this study, we developed a reliable and reproducible infection method to study the *B. cinerea*-tomato interaction. By using this method we have shown that ABA-negative *sitiens* plants are much less susceptible to *B. cinerea* infection than WT plants. Moreover, in *sitiens* leaves, susceptibility could be restored by exogenous application of ABA at concentrations above 10 μM. Applying ABA also increased the level of susceptibility of WT tomato leaves to *B. cinerea*.

Several studies have shown that exogenously applied ABA can increase susceptibility of various plants species toward various pathogens such as *Phytophthora infestans* and *Cladosporium cucumerinum* on potato (*Solanum tuberosum*) slices (Henfling et al., 1980), *P. megasperma* f. sp. *glycinea (P. sojae)* on soy-

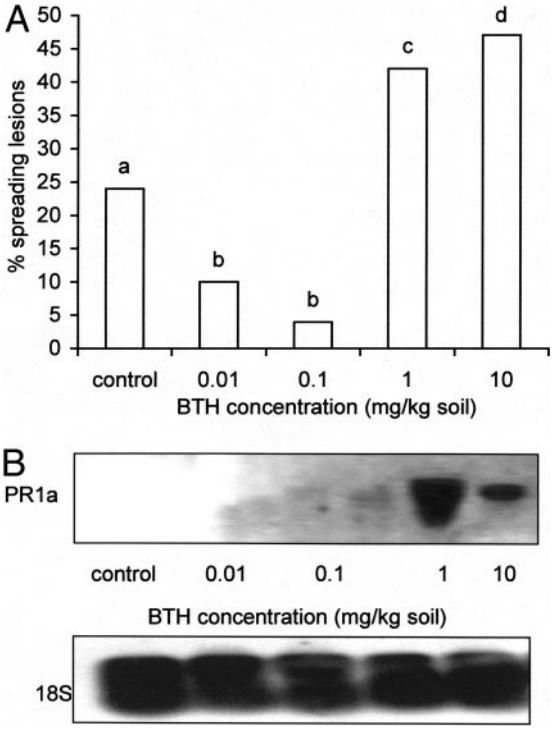

Figure 9. A, Effect of BTH root application on spreading of *B. cinerea* lesions on the third pair of *sitiens* tomato leaves (tomato cv Moneymaker). Leaves were infected with 10 droplets of 4 μL of spore suspension containing 10^6 spores mL^{-1}, 0.05 M Glc, and 33 mM KH_2PO_4 25 d after the last BTH treatment. Data are means of three experiments with each 12 leaves per treatment. Infections were evaluated 4 d after infection by counting the number of spreading *B. cinerea* lesions on each leaf. Bars with different letters are significantly different with $P = 0.05$ after a logistic regression. B, Induction of PR1a gene expression in 5-week-old leaves of *sitiens* tomato plants after BTH root application. Each lane contains 30 μg of total RNA and PR1a was detected via DIG-labeled cDNA probes. An 18S rRNA probe was used as a constitutive probe to verify for equal RNA loading and transfer.

bean (Ward et al., 1989), *Peronospora tabacina* on tobacco (Salt et al., 1986), and *B. cinerea* on tomato (Kettner and Dörffling, 1995). In addition, Kettner and Dörffling (1995) showed that *B. cinerea* infection resulted in increased ABA levels in infected tomato plants by at least four processes: stimulation of fungal ABA biosynthesis by the host, release of ABA or its precursor by the fungus, stimulation of biosynthesis of plant ABA by the fungus, and inhibition of its metabolism by the fungus. However, they did not mention a decline in susceptibility of ABA-negative *flacca* plants toward *B. cinerea* compared with WT plants like we observed in *sitiens* plants. We inoculated *flacca* tomato mutants (Marin and Marion-Poll, 1997) with *B. cinerea* using a moderately aggressive infection solution (0.01 M Glc and 6.7 mM KH_2PO_4) and found the same level of resistance as for *sitiens* plants (data not shown). The infection assay used by Kettner and Dörffling (1995) may have been too aggressive to visualize changes in resistance levels. Infecting *sitiens* plants with a very aggressive infection solution (0.1 M Glc and 67 mM KH_2PO_4) resulted in almost 100% spreading lesions, which was comparable with the number of spreading lesions in the WT plants (Fig. 8). This indicates that an aggressive infection method masks a possible defense mechanism against *B. cinerea*.

Because at least 10 μM exogenous ABA is needed to induce susceptibility to *B. cinerea* in *sitiens* tomato, a threshold ABA concentration appears to be necessary for a susceptible response of tomato toward *B. cinerea*. This threshold concentration is apparently higher than ABA levels present in *sitiens* (ABA levels 8% of WT) and *flacca* (ABA levels 21% of WT) plants (Herde et al., 1999). Herde et al. (1999) also suggested the presence of a threshold level of ABA within the plant must be reached for early events in electrical signaling and for proteinase inhibitor II gene expression upon wounding.

We subsequently studied how ABA induces susceptibility to *B. cinerea*. ABA did not have an effect on the growth of *B. cinerea* on plates or on the plant, indicating that ABA interferes with the plant response and not with the pathogen. Based on other plant hormone studies, two major possibilities for the interaction of ABA with plant defense could be proposed. One consists of an ABA-dependent defense signal transduction pathway. Alternatively, ABA could modulate one of the well-described plant defense responses dependent on the plant hormones SA, ethylene, or JA (Sticher et al., 1997; Thomma et al., 1998).

The presence of an ABA-dependent defense-signaling pathway has not been documented yet, to our knowledge. Dammann et al. (1997) illustrated the presence of an organ-specific ABA signal transduction pathway distinct from the classical JA-dependent WR signaling in potato, but its function and physiological relevance is not clear. Therefore, we suggest that the effects observed in the present study are more likely because of a modulation of a functional plant defense pathway.

Because it is not known which plant defense pathways are involved in the tomato-*B. cinerea* interaction, we initially tried to characterize a functional defense response. Based on previous work by Thomma et al. (1998) in Arabidopsis, we investigated the role of JA in defense of tomato to *B. cinerea*. However, exogenous JA application or elimination of JA in *def1* mutants did not affect the response of tomato to *B. cinerea*. These results appear to be contradictory to results obtained by Thomma et al. (1998, 1999) who demonstrated a clear role for JA in resistance of Arabidopsis against *B. cinerea* using JA-insensitive *coi1–1* plants. However, WT Arabidopsis has a very strong basal level of resistance to various isolates of *B. cinerea* (Thomma et al., 1998, 1999) including the *B. cinerea* isolate used in the present study (K. Audenaert, unpublished data). The most aggressive infection solution used in the present study (0.1 M Glc and 67 mM KH_2PO_4) did not give any spreading lesions in Arabidopsis. This indicates that the *B. cinerea*-Arabidopsis interaction approaches a non-host response that is completely different from the highly susceptible response of tomato to *B. cinerea*. In the Arabidopsis response, both JA and ethylene play an important role (Thomma et al., 1998). The strong basal level of resistance to *B. cinerea* observed in Arabidopsis is based on different mechanisms than the compatible interaction between tomato and *B. cinerea.*

In the present work, we show that in tomato, SA-dependent defense is a potential defense mechanism against *B. cinerea. NahG* tomato plants were more susceptible than WT plants. Furthermore, BTH treatment rendered WT plants more resistant than control plants. Again, this appears to be in contradiction with results obtained by Thomma et al. (1998) in Arabidopsis, where *NahG* plants were not more susceptible to *B. cinerea* than WT plants. However, Zimmerli et al. (2001) recently showed that *NahG* Arabidopsis plants were more susceptible to *B. cinerea* than WT Arabidopsis Columbia-0 plants and that a soil drench application of BTH drastically slowed down the *B. cinerea* infection on Arabidopsis, which is in agreement with our observations on tomato. Zimmerli et al. (2001) explained the discrepancy in their results and the results obtained by Thomma et al. (1998) by the fact that in their experiments the Arabidopsis plants were kept in constant high air humidity, which strongly favored the infection process. This indicates that in conditions that favor infection, SA-dependent signaling also contributes to restrict *B. cinerea* infection in Arabidopsis.

In addition, we could observe a dual modulation of the SA-dependent defense response by ABA. First, *sitiens* leaves showed a clear increase in PAL activity 16 h after infection with *B. cinerea*, which was not

observed in WT leaves. These results suggest that PAL activity is partially repressed by ABA levels present in WT tomato leaves. A correlation between PAL and resistance to *B. cinerea* was previously described in bean plants (De Meyer et al., 1999a). Moreover, in soybean, exogenously applied ABA suppressed PAL activity and synthesis of PAL mRNA in the incompatible interaction of soybeans with *P. megasperma* f. sp. *glycinea* (Ward et al., 1989). Second, *sitiens* turned out to be hyper-responsive to BTH treatment. Threshold values for induction of PR1a gene expression and toxicity declined by a factor of 10 to 100 in *sitiens* leaves compared with WT leaves (Figs. 7 and 9).

It is possible that the higher BTH concentrations needed to induce PR1a in WT are because of the fact that ABA, at levels present in WT leaves, can directly influence expression of the PR1a gene promoter, which contains a negative-acting ABA-responsive element TAACAAA (for review, see Giraudat et al., 1994). This could lead to a transcriptional down-regulation of PR1a. Earlier studies by Rezzonico et al. (1998) illustrated down-regulation by ABA of β-1,3-glucanase (βGLU I) genes but not of chitinase (CHN) genes in cultured tobacco pith cells. It was suggested that the differential effect of ABA on βGLU I and CHN expression could be because of the absence of the ABA-responsive element in the CHN I gene, whereas distal and proximal copies of the TAACAAA box were present in the promoter of βGLU I (Rezzonico et al., 1998). We therefore suggest that the presence of the ABA-responsive element could result in transcriptional down-regulation of PR1a. However, expression of PR1a was not correlated with resistance of tomato to *B. cinerea* in our model system.

An attractive hypothesis to explain the interference of ABA with SA-dependent defense signaling originates from results in NIM1 overexpressing Arabidopsis mutants. NIM1, also called NPR1, is one of the first characterized proteins in SA-signaling downstream of SA (Cao et al., 1994). NIM1 overexpressing lines became more responsive to SA and were hyper-responsive to BTH, suggesting a direct or indirect interaction of BTH with the NIM1 protein (Friedrich et al., 2001). Because ABA-negative *sitiens* leaves show the same type of responsiveness to BTH as NIM1-overexpressing Arabidopsis lines, it is possible that ABA levels present in WT plants suppress NPR1 activity either directly or indirectly.

Because we have shown that ABA negatively modulates SA-dependent defense responses, it is interesting to notice that some *B. cinerea* strains produce high amounts of ABA in culture and that in vitro ABA production has also been observed for several other phytopathogenic and mycorrhizal fungi (Dörffling et al., 1984; Crocoll et al., 1991; Danneberg et al., 1993). In addition, it is known that endogenous ABA levels can rise upon pathogen infection (Kettner and Dörffling, 1995). ABA was also found at a considerably higher level in maize plants colonized with arbuscular mycorrhiza than in control plants (Bothe et al., 1994). It is tempting to speculate that these fungi produce ABA and/or induce endogenous ABA production in the plant to suppress SA-dependent defense mechanisms. Zimmerli et al. (2001) observed that *B. cinerea* fails to induce a strong SAR response in Arabidopsis and the suppression of plant defense responses seems to be a widespread phenomenon in associations between plants and endomycorrhizal fungi (Harrison, 1999). It remains to be investigated, however, whether the *B. cinerea* strains used in this study and the study of Zimmerli et al. (2001) produce ABA and/or stimulate ABA biosynthesis by the plant and whether increased endogenous ABA levels lead to a stronger suppression of SA-dependent defense mechanisms.

Although our results show an interaction of ABA with the SA-dependent disease response, which is functional in the control of *B. cinerea*, one cannot exclude an involvement of other plant hormones in the increased resistance of *sitiens* to *B. cinerea*. Where JA levels are unchanged in ABA-negative tomato plants (Herde et al., 1999), aminocyclopropane carboxylate levels (the direct precursor of ethylene) were 2-fold higher in ABA-negative tomato plants compared with WT plants (Sharp et al., 2000). The role of ethylene with respect to resistance of plants to *B. cinerea* is not well established. Thomma et al. (1999) reported the participation of ethylene in the defense of Arabidopsis against *B. cinerea*. In tomato, however, ethylene production during a *B. cinerea* infection was correlated with the development of necrosis (Elad, 1990). In addition, exposure of strawberries (*Fragaria ananassa*) to ethylene increased *B. cinerea* development (El Kazzaz et al., 1983). Recently, the role of ethylene in the tomato-*B. cinerea* interaction was extensively studied by using ethylene perception blockers and tomato mutants impaired in the biosynthesis or perception of ethylene (A.T. Have, J. Díaz, and J.A.L. van Kan, personal communication).

In conclusion, we have shown that ABA-negative *sitiens* tomato plants are more resistant to *B. cinerea* than WT plants, indicating that endogenous ABA levels present in WT plants increase susceptibility of tomato to *B. cinerea.* In a first attempt to elucidate the mechanistic basis for this observation, our results suggest that cross talk occurs between the plant hormone ABA- and SA-induced defenses. Hence, we suggest that negative modulation of SA-dependent signaling is probably one of the mechanisms by which ABA determines susceptibility of tomato to *B. cinerea*.

MATERIALS AND METHODS

Plant Material

Tomato (*Lycopersicon esculentum* Mill.) *sitiens* mutants (Taylor et al., 1988; Taylor et al., 2000), tomato cv Moneymaker NahG transgenes, and tomato cv Castlemart *def1*

mutants (Howe et al., 1996) were grown in potting compost soil (Klassmann Substrat 4, Hesepe, Germany). Maarten Koornneef (University of Wageningen, The Netherlands) provided seeds of tomato *sitiens* plants, Gregg Howe (Washington State University, Pullman) provided *def1* seeds, and Jonathan Jones (John Innes Centre, Norwich, UK) provided seeds of NahG-tomato plants. Plant material was thereafter propagated by seed multiplication. Plants were grown for 5 weeks under greenhouse conditions (24°C ± 3) with a 16-h-light photoperiod and high humidity to prevent the *sitiens* plants from wilting.

Chemical Treatment of Plant Material

Tomato seeds were treated with BTH, or BION (Novartis), before planting by dipping them in a BTH solution of 0.01, 0.1, 1, or 10 mg L^{-1}. Seeds were sown subsequently in soil containing already 0.1, 1, and 10 mg kg^{-1} BTH. Ten days after sowing, roots of seedlings were dipped in BTH solutions and transferred to pots containing BTH-treated soil in concentrations as mentioned above.

Treatments with ABA were performed by dipping petioles of 5-week-old tomato leaves in a solution containing 1 to 100 μM of cis,trans-ABA (Sigma, Bornem, Belgium) during 16 h before infection with *B. cinerea*. ABA solutions were prepared from a 1 mM stock solution containing 1% (v/v) ethanol. In accordance, final ethanol concentrations were, respectively, 0.1 to 0.001 in the 100 to 1 μM ABA solutions. Control leaves were dipped in water containing 0.1% (v/v) ethanol.

Treatments with JA were performed by dipping petioles of 5-week-old tomato leaves in a solution of 5 to 100 μM of ± JA (Sigma) during 16 h before infection with *B. cinerea*.

B. cinerea Infection

B. cinerea isolate R16 resulting from the cross SAS56 × SAS405 (Faretra and Pollastro, 1991) was grown on tomato leaf agar (Salinas and Schot, 1987) under a light regime of UV/dark (12 h/12 h). After 10 d, spores were washed from the plates with distilled water containing 0.01% (v/v) Tween 20. After removing mycelial debris, spores were counted and added to the inoculation solution in the proper concentration.

Tertiary leaves of 5-week-old tomato plants were excised by cutting the petioles near the stem. The petiole was immediately wrapped in wet absorbing paper. Leaves were transferred to trays and placed on a plastic lattice supported by glass rods. The wrapped petioles were put through the lattice to touch several layers of wet absorbing paper on the bottom of the trays. Finally, each tomato leaf composed of five leaflets was infected by putting 10 droplets of 4 μL of inoculation solution containing 10^6 spores mL^{-1}, Glc, and KH_2PO_4 (pH 5) on the leaf surface. The amount of Glc and phosphate was dependent on the inoculation conditions used in the experiment. In experiments on induced resistance, WT leaves were infected with a solution containing 10^6 spores mL^{-1}, 0.01 M Glc, and 6.7 mM KH_2PO_4 unless mentioned otherwise. *Sitiens* leaves were infected using 10^6 spores mL^{-1}, 0.05 M Glc, and 33 mM KH_2PO_4 unless mentioned otherwise. Trays were covered with plastic folium to guarantee a relative humidity of 95% to 100%. Four days after inoculation, infection was evaluated by counting the number of spreading lesions on each leaf. Data were statistically analyzed as a dichotomous variable by logistic regression.

RNA Extraction, Gel-Blot Hybridization, and Enzyme Activity

Leaf material of 5-week-old plants was frozen in liquid N_2 and ground to a fine powder with a mortar and a pestle. Total RNA was extracted by the phenol-SDS method as described by Ausubel et al. (1993). Fifteen micrograms of total RNA was loaded to a formaldehyde-denatured 1% (w/v) agarose gel and then transferred to a nylon membrane (Hybond N^+, Amersham Pharmacia Biotech, Antwerpen, Belgium). Hybridization took place at 65°C. Nonradioactive labeled DIG probes (Boehringer Mannheim, Brussels) were prepared by random labeling using DIG-High-Prime (Boehringer Mannheim). The PR1a-probe was kindly provided by Pierre de Wit (University of Wageningen, Wageningen, The Netherlands). Stringency washes were performed for 1 h at room temperature in 2× SSC and for 1 h in 0.5× SSC at 65°C with 0.1% (w/v) SDS each. All RNA samples for WT and *sitiens* plants were loaded on the same gel, blotted on the same membrane, and hybridized in the same tube to eliminate differences in the gene expression pattern in WT and *sitiens* plants. To verify for equal amounts of RNA, hybridization was performed with an 18S rRNA probe.

PAL activity was measured as described by Edwards and Kessmann (1992). Three leaves, infected with 30 droplets each containing 2 μL of *B. cinerea* inoculum, were ground to powder in liquid nitrogen and extracted with 50 mM Tris-HCl (pH 8.5) containing 14 mM mercapto-ethanol and 5% (w/v) polyvinylpyrollidone. After centrifugation, protein levels were measured with bovine serum albumin as a standard. PAL activity was determined in 50 mM Tris-HCl (pH 8.5 containing 10 mM L-Phe) at 40°C with a UVIKON922 spectrometer (Kontron, B.R.S., Anderlecht, Belgium; A_{290}), indicating the conversion of L-Phe to trans cinnamic acid. D-Phe was used as a blank. The increase in PAL activity upon *B. cinerea* was measured 16 h after infection by dividing PAL-activity in infected leaves by PAL activity in noninfected leaves. Data were statistically analyzed by an ANOVA analysis (Posthoc: Duncan).

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