The Sesquiterpene Botrydial Produced by *Botrytis cinerea* Induces the Hypersensitive Response on Plant Tissues and Its Action Is Modulated by Salicylic Acid and Jasmonic Acid Signaling

Franco Rubén Rossi,¹ Andrés Gárriz,¹ María Marina,¹ Fernando Matías Romero,¹ María Elisa Gonzalez,¹ Isidro González Collado,² and Fernando Luis Pieckenstein¹

¹Instituto de Investigaciones Biotecnológicas-Instituto Tecnológico de Chascomús/Universidad Nacional de General San Martín-Consejo Nacional de Investigaciones Científicas y Técnicas (IIB-INTECH/UNSAM-CONICET), Camino Circunvalación Laguna, Km. 6 CC 164 (B7130IWA) Chascomús, Argentina; ²Departamento de Química Orgánica, Universidad de Cádiz, Campus Ciencia y Tecnología, E-11510 Puerto Real, Spain

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Botrytis cinerea, as a necrotrophic fungus, kills host tissues and feeds on the remains. This fungus is able to induce the hypersensitive response (HR) on its hosts, thus taking advantage on the host's defense machinery for generating necrotic tissues. However, the identity of HR effectors produced by *B. cinerea* is not clear. The aim of this work was to determine whether botrydial, a phytotoxic sesquiterpene produced by *B. cinerea*, is able to induce the HR on plant hosts, using *Arabidopsis thaliana* as a model. Botrydial induced the expression of the HR marker *HSR3*, callose deposition, and the accumulation of reactive oxygen species and phenolic compounds. Botrydial also induced the expression of *PR1* and *PDF1.2*, two pathogenesis-related proteins involved in defense responses regulated by salicylic acid (SA) and jasmonic acid (JA), respectively. *A. thaliana* and tobacco plants defective in SA signaling were more resistant to botrydial than wild-type plants, as opposed to *A. thaliana* plants defective in JA signaling, which were more sensitive. It can be concluded that botrydial induces the HR on its hosts and its effects are modulated by host signaling pathways mediated by SA and JA.

Plant pathogens are often classified as biotrophs and necrotrophs, according to their lifestyles. The former feed on living host tissue, whereas the latter kill host tissue and feed on the remains (Glazebrook 2005). *Botrytis cinerea* Pers. (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel) is a classical example of a polyphagous necrotrophic fungus, which causes serious plant diseases in more than 200 crops worldwide (Williamson et al. 2007). Diseases caused by *Botrytis cinerea* are usually manifested by necrotic areas with extensive fungal growth, giving the characteristic appearance of gray mold. A variety of

nonspecific phytotoxic metabolites are secreted by this fungus, which contribute to cell death on different plant hosts (Choquer et al. 2007). Among them, the best known is the sesquiterpene botrydial, initially identified in liquid cultures (Collado et al. 2000, 2007) but later demonstrated to be produced during infection of pepper (*Capsicum annuum*), common bean (*Phaseolus vulgaris*), and *Arabidopsis thaliana* (Deighton et al. 2001). Botrydial concentrations that oscillated between 0.08 and 260 nmol per gram of infected tissue were found during an analysis of the levels of this toxin in infected plants of several cultivars of the abovementioned plant species. Botrydial induces chlorosis and collapse of French bean tissues (Colmenares et al. 2002) in a way similar to infection by *B. cinerea* (Collado et al. 2000). In addition to botrydial and its derivatives, virulent *B. cinerea* strains also produce other phytotoxic metabolites such as the polyketide botcinic acid and related botcinins (Reino et al. 2004; Tani et al. 2006). *B. cinerea* also synthesizes several phytotoxic proteins (Kunz et al. 2006; Staats et al. 2007) that induce cell death, whose role in pathogenesis is currently under investigation. *B. cinerea* has also been reported to produce oxalic acid both in vitro (Germeier et al. 1994) and on tomato (*Lycopersicon esculentum*) petioles (Verhoeff et al. 1988), although this compound seems to act as a cofactor in pathogenesis rather than as a direct phytotoxic agent.

In addition to the abovementioned phytotoxic metabolites and proteins, *B. cinerea* produces reactive oxygen species (ROS) (Schouten et al. 2002), such as hydrogen peroxide, which are accumulated in germinating conidia during early steps of infection of French bean leaves (Choquer et al. 2007). Inactivation of the *B. cinerea* superoxide dismutase (SOD) gene demonstrated that this hydrogen peroxide-generating enzyme acts as a virulence factor, leading to the accumulation of phytotoxic levels of hydrogen peroxide in French bean tissues (Rolke et al. 2004). Thus, hydrogen peroxide derived from *B. cinerea* contributes to host cell death and favors fungal infection. However, ROS are not only derived from the fungus itself but also from host tissues, as part of the defense responses (Apel and Hirt 2004).

The hypersensitive response (HR) is part of the array of defense mechanisms mounted by plants in response to pathogen attack. This response is now almost universally accepted as a form of programmed cell death (PCD) characterized by the rapid death of plant cells at the site of pathogen infection

F. R. Rossi and A. Gárriz contributed equally to this work.

Corresponding authors: F. L. Pieckenstein; Telephone: +54-2241-424049; Fax: +54-2241-424048; E-mail: pieckenstein@intech.gov.ar; and I. González Collado; Telephone: +34-956 016 368; Fax: +34-956-016193; E-mail: isidro.gonzalez@uca.es

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(Heath 2000a and b; Lam 2004) and is typically triggered upon recognition of a pathogen-encoded avirulence protein by a resistance (R) protein (Martin et al. 2003). The HR involves changes in ion fluxes, ROS and nitric oxide production, activation of defense-related genes, and cell wall reinforcement (Heath 2000a). Salicylic acid (SA) plays a key role in the activation of disease resistance mechanisms frequently associated with the HR (Dangl et al. 1996) and seems to be part of a feedback loop both upstream and downstream of cell death (Alvarez 2000). In addition to R gene-mediated cell death, plants also induce cell death in response to pathogen-associated molecular patterns (Schwessinger and Zipfel 2008).

The R protein-mediated HR and SA-mediated basal resistance, although effective against biotrophs, are normally ineffective against necrotrophic pathogens. Instead, plant resistance to necrotrophs is often mediated by jasmonic acid (JA) signaling, a process also involved in plant responses to wounding (Spoel et al. 2007). During the HR, JA seems to restrict cell death processes elicited by SA, ethylene (ET), and ROS (Overmyer et al. 2003), thus being part of the machinery that prevents excessive damage to host tissues. Evidence for a cross-talk between SA- and JA-mediated signaling pathways through a complex regulatory network has been provided (Koornneef and Pieterse 2008; Spoel et al. 2007).

In spite of contributing to resistance to biotrophic pathogens, the HR increases the virulence of necrotrophic ones (Dickman et al. 2001; Govrin and Levine 2000; Kliebenstein and Rowe 2008; Mayer et al. 2001), which is not surprising taking into account the dependence of necrotrophic pathogens on death tissues for pathogenesis. In fact, necrotrophic pathogens were demonstrated to actively stimulate the HR in their own benefit (Govrin and Levine 2000; Kliebenstein and Rowe 2008; van Kan 2006). In particular, *Botrytis* spp. are known to induce cell death with molecular features of the HR (Govrin and Levine 2000; van Baarlen et al. 2007). As an example, penetration of host tissues by *B. cinerea* leads to an oxidative burst that involves the simultaneous production of hydrogen peroxide and nitric oxide, as well as the formation of proteolytic vesicles at the host-pathogen interface and lipid peroxidation (van Baarlen et al. 2007). Interestingly, evidence was recently provided that *B. cinerea* does not suffer hydrogen peroxide-induced oxidative stress in planta (Temme and Tudzynski 2009). Thus, HR-related alterations of the redox status seem to promote, rather than restrict, host infection by *B. cinerea* (van Kan 2006; Williamson et al. 2007). Additional evidence regarding the contribution of the HR to enhanced virulence of *B. cinerea* derives from the observation that growth of this fungus was suppressed in the *A. thaliana* HR-deficient mutant *dnd1* whereas, on the contrary, growth was stimulated by simultaneous inoculation with an HR-inducing avirulent bacterium (Govrin and Levine 2000). In spite of the amount of evidence that demonstrates that infection by *B. cinerea* induces the HR in host tissues, the nature of the fungal effectors involved in this induction process has not been clearly established (van Kan 2006). Evidence for the presence of an HR elicitor in intercellular fluids of *A. thaliana* plants infected by *B. cinerea* was provided by Govrin and associates (2006) but its identity was not established. Despite the abovementioned ability of *B. cinerea* to produce a variety of phytotoxic compounds, the involvement of these molecules in the induction of the HR has not been documented. Thus, it remains to be established if toxic compounds produced by *B. cinerea* merely act as "plain toxins" or if, on the contrary, they require the active participation of the host cell machinery in order to induce cell death (van Kan 2006). Therefore, the aim of this work was to evaluate whether the sesquiterpene botrydial, one of the best-known phytotoxic compounds produced by *B. cinerea*, induces the

HR in plant tissues and depends on host metabolism for causing cell death.

For this purpose, the model plant *A. thaliana* was used to evaluate whether botrydial induces biochemical and molecular processes typical of the HR. In addition, the dependence of botrydial toxicity on host defense signaling pathways was investigated by using genetically modified *A. thaliana* and tobacco (*Nicotiana tabacum*) plants impaired in SA and JA signaling.

RESULTS

Botrydial toxicity on *A. thaliana* leaves.

Botrydial toxicity to *A. thaliana* was evaluated after the application of 5- μ l aliquots containing different toxin concentrations on the adaxial surface of leaves. Botrydial caused necrotic lesions on *A. thaliana* leaves in a dose-dependent manner, as evaluated 48 h after treatment under the naked eye (Fig. 1A). In this way, botrydial exerted toxic effects on *A. thaliana* similar to those previously described for other plant species (Colmenares et al. 2002). Leaf staining with trypan blue demonstrated that tissue necrosis started as early as 3 h after botrydial application to *A. thaliana* leaves, which was more evident in leaves treated for 24 h (Fig. 1B).

Molecular and biochemical indicators of the HR in *A. thaliana* leaves treated with botrydial.

As an initial step to evaluate whether botrydial induces the HR on host tissues, the effect of this toxin on *HYPER-SENSITIVITY-RELATED 3* (*HSR3*) expression was analyzed. This gene, considered to be an HR marker, has previously been shown to be induced early and transiently during the HR of *A. thaliana* to *Xanthomonas campestris* pv. *campestris* (Lacomme and Roby 1999), and was also found in the present work to be upregulated in *A. thaliana* leaves infected by *B. cinerea* (Supplementary Fig. S1). Steady-state *HSR3* mRNA levels were threefold higher in *A. thaliana* leaves treated with botrydial than in controls, as evaluated 3 h after treatment. When evaluated 24 h after treatment, *HSR3* mRNA levels in leaves treated with botrydial were similar to control leaves (Fig. 2).

Biochemical indicators of HR induction such as callose deposition, ROS accumulation, and autofluorescence due to the accumulation of phenolic compounds were also evaluated in leaf tissues treated with botrydial. Aniline-blue staining and fluorescence microscopy revealed callose deposition as isolated spots detected in leaf tissues exposed to botrydial for 3 h and in intercellular spaces of more extended areas of leaves treated for 24 h (Fig. 3A). Similar fluorescent signals were not detected in control leaves, thus demonstrating that callose deposition in these tissues was induced by botrydial. A fluorescent background not related to botrydial application and restricted to trichomes and leaf veins was detected in control leaves, as previously reported by Stone and associates (2000).

ROS accumulation was made evident by dihydrofluorescein diacetate staining of leaves treated with botrydial for 3 h, with this effect being stronger in leaves treated for 24 h. ROS accumulation was not evident in control leaves at 3 or 24 h (Fig. 3B). In addition, accumulation of phenolic compounds was evident 3 h after botrydial application and further increased 24 h after application compared with controls (Fig. 3C). As occurred during the analysis of callose deposition, a fluorescent background not related to botrydial application was detected in leaf veins and trichomes of both botrydial-treated leaves and controls (Fig. 3C).

Botrydial induces pathogenesis-related gene expression.

PDF1.2 and PR1 are two pathogenesis-related (PR) proteins well known to be involved in defense responses mediated by

SA and JA, respectively. The effect of botrydial on the expression of the genes that encode these two proteins was analyzed. *A. thaliana* leaves treated with botrydial for 3 h exhibited 40-fold higher steady-state *PDF1.2* mRNA levels than controls, while *PR1* mRNA levels were not affected (Fig. 4). When leaves were treated for 24 h, the effect of botrydial on *PDF1.2* expression was even more pronounced, mRNA levels being three orders of magnitude higher than controls. At this time, leaves treated with botrydial also exhibited an increase in *PR1* mRNA levels, which were approximately 700-fold higher than controls (Fig. 4).

Botrydial toxicity depends on host signaling pathways mediated by SA and JA.

Results depicted in previous sections demonstrated that several processes typical of the HR are induced by botrydial in *A. thaliana* leaves. Moreover, botrydial was found to induce *PR-1*

and *PDF1.2* expression, thus suggesting that defense responses mediated by these hormones were activated by botrydial. In order to evaluate whether botrydial toxicity depends on host defense signaling pathways mediated by these hormones, genetically modified *A. thaliana* plants showing alterations in SA and JA signaling were exposed to botrydial. Transgenic *nahG* plants, which overexpress a bacterial salicylate hydroxylase gene that degrades SA, as well as the *eds5-1* mutant, which has been shown to accumulate very little SA in response to pathogen infection, were treated with botrydial. In addition, the JA-insensitive *coi1* mutant was also treated with botrydial, and the sensitivity of all the genetically modified lines to the toxin was compared with wild-type (WT) Col-0 *A. thaliana* plants. Botrydial induced cell death in a dose-dependent manner in all the abovementioned lines (Fig. 5). However, the magnitude of lesion development in genetically modified lines was different from WT plants. In this way, lesions developed

Fig. 1. Botrydial toxicity on *Arabidopsis thaliana* leaves. Aliquots (5 μl) of botrydial solutions (161 to 3,220 μM) in acetone/water (4:6) were dispensed on both sides of the central vein of the adaxial surface of detached *A. thaliana* Col-0 leaves, which were placed in petri dishes containing 0.8% water agar and incubated in a plant growth chamber. Leaves treated with acetone/water (4:6) were used as controls. **A**, Development of necrotic lesions 48 h after botrydial application, as observed with the naked eye. **B**, Arrows indicate spots of incipient (3 h after botrydial application) and advanced (24 h after botrydial application) tissue necrosis, as made evident by staining with Trypan Blue and observation under a binocular microscope. **A** and **B**, Bars represent 8 and 4 mm, respectively.

Fig. 2. *HYPER-SENSITIVITY-RELATED 3 (HSR3)* gene expression in *Arabidopsis thaliana* leaves treated with botrydial. Quantitative real-time polymerase chain reaction was used to analyze the abundance of *HSR3* transcripts in tissues surrounding the lesions developed on *A. thaliana* Col-0 leaves 3 and 24 h after treatment with 5- μ l aliquots of a 805- μ M botrydial solution in acetone/water (4:6). Tissues obtained from leaves treated with acetone/water (4:6) were used as controls. For each time after treatment, transcript levels were normalized to the average signal intensity of controls, which was assigned to 1 and is represented by a dotted line. Results are the mean of three replicates $\pm$ standard error, and statistically significant differences in gene expression between leaves treated with botrydial and controls, as analyzed with the REST software, are shown as * ($P \leq 0.05$).

in *A. thaliana nahG* and *eds5-1* after botrydial application were smaller than those of WT plants for the entire concentration range evaluated (Fig. 5). In contrast, the size of the necrotic area developed in response to botrydial application was higher in leaves of the *coi-1* mutant than in leaves of WT plants, for botrydial concentrations $\geq 805 \mu\text{M}$ (Fig. 5). Thus, lesion size in leaves of the *coi1* mutant treated with 805 and 1,610 μM botrydial was 1.2- and 0.7-fold higher, respectively, than in WT plants.

The dependence of botrydial toxicity on SA signaling was also evaluated in tobacco plants by testing the sensitivity of the *nahG-10* line to this toxin. As was evident for *A. thaliana*, lesions developed on tobacco *nahG* leaves were smaller (40 to 72%) than on WT ones, in the whole range of botrydial concentrations tested (Fig. 6).

DISCUSSION

The HR, usually triggered after R protein-mediated recognition of pathogens that harbor an avirulence protein (Martin et al. 2003), is effective in protecting plants against biotrophs, whose growth and propagation depends on the availability of living tissues. However, this response does not protect plants against necrotrophic pathogens, which feed on necrotic tissue (Glazebrook 2005). As a consequence, the virulence of necrotrophs can be increased in plant hosts that activate the HR, consistently with the pathogenesis strategy of these organisms. In this sense, the polyphagous and ubiquitous necrotrophic fungus *B. cinerea*, which causes extensive damage on host tissues, has been shown to induce the HR on *A. thaliana* plants and, thus, benefit from increased cell death triggered by the host defense machinery (Govrin and Levine 2000). However, the identity of the fungal effector involved in HR induction has not been established (Govrin et al. 2006).

Fig. 3. Botrydial-mediated induction of biochemical responses associated to the hypersensitive response. *Arabidopsis thaliana* Col-0 leaves were treated with 5- μ l aliquots of a 805- μ M botrydial solution in acetone/water (4:6) for **B, F, and J**, 3 h and **D, H, and L**, 24 h. Leaves treated with acetone/water (4:6) were used as controls for **A, E, and I**, 3 h and **C, G, and K**, 24 h. Fluorescence microscopy was used to analyze the leaves in **A to D**, callose deposition after aniline-blue staining; **E to H**, accumulation of reactive oxygen species after 2',7'-dichlorofluorescein diacetate staining; and **I to L**, accumulation of phenolic compounds (autofluorescence). Bars = 500 μm .

B. cinerea produces several toxins that are known to kill cells from a large spectrum of plants but the mechanisms underlying their toxicity are not completely clear. *B. cinerea* toxins probably exert a direct toxic effect on a precise host target not identified thus far, such as the inhibition of a particular metabolic process or enzymatic activity, as occurs with several toxins produced by different plant pathogens (Mobius and Hertweck 2009). Examples of metabolic and structural targets of fungal phytotoxins are membrane integrity, iron acquisition, and energy breakdown. Beyond its ability to affect a particular host target, some fungal toxins are able to activate cell death programs on their hosts (Mobius and Hertweck 2009). In this way, the cyclic pentapeptide victorin produced by *Cochliobolus victoriae* triggers PCD after entering the mitochondria and binding to a subunit of the glycine decarboxylase complex (Navarre and Wolpert 1999). Similarly, the sphingoid-like AAL toxin secreted by the tomato pathogen *Alternaria alternata* f sp *lycopersici*, and the structurally related fumonisin

FB1 produced by the maize pathogen *Fusarium moniliforme*, are able to induce PCD on tomato leaves (Moore et al. 1999).

Thus, taking into account the abovementioned ability of *B. cinerea* to induce the HR on plant hosts, the possibility that such an eliciting effect is mediated by the sesquiterpene botrydial was explored in the present work, by using *Arabidopsis thaliana* as a host. Evidence was obtained that botrydial is able to induce cell death as soon as 3 h after its application on host tissues, and lesion development continued thereafter. Several biochemical events typical of the HR, such as callose deposition and the accumulation of ROS and phenolic compounds, were also induced in plant tissues treated with botrydial. Moreover, a transient increase in the expression of the *HSR3* gene, similar to that elicited by infection by avirulent *X. campestris* pv. *campestris* (Lacomme and Roby 1999) and *B. cinerea* (present work), was also detected in *A. thaliana* leaves treated with botrydial. Thus, several pieces of evidence demonstrate that this sesquiterpene exerts its toxic effects on *A. thaliana* tissues, at least in part, by triggering the HR. In this way, the present work demonstrates that the previously reported ability of *B. cinerea* to induce the HR on its hosts (Govrin and Levine 2000; Govrin et al. 2006) can be mediated by botrydial. However, botrydial is not expected to be the sole effector involved in triggering the HR on plant hosts. This view is supported by the fact that some botrydial-deficient strains are still virulent (Pinedo et al. 2008; Siewers et al. 2005). Moreover, some botrydial-deficient strains have been found to overproduce botcinic acid derivatives (Pinedo et al. 2008) and, more recently, a redundant role of botrydial and botcinic acid in the virulence of *B. cinerea* has been demonstrated (Dalmás et al. 2011). Moreover, in addition to the phytotoxic proteins mentioned in the introduction, two cell-wall-degrading enzymes produced by *B. cinerea* are related to virulence. In this sense, the endopolygalacturonase 1 has been shown to elicit diverse defense reactions on grapevine (Poinssot et al. 2003) and the xylanase Xyn11A exhibits necrotizing activity on tomato and tobacco leaves (Noda et al. 2010). Interestingly, the elicitor activity of these two proteins seems to be independent of their catalytic activities.

The finding that botrydial induces the HR demonstrates that the toxic effect of this compound depends, at least in part, on the defense machinery of its hosts. Thus, the relationship between defense-response signaling and botrydial toxicity was further analyzed by using *A. thaliana* mutants impaired in defense responses regulated by SA and JA. Interestingly, disruption of SA and JA signaling exhibited contrasting effects on cell death caused by botrydial. In this way, *A. thaliana* and tobacco plants impaired in SA signaling showed a reduction of the necrosis caused by botrydial compared with WT plants, while the opposite picture was found when botrydial toxicity toward *A. thaliana* plants deficient in JA signaling was evaluated. Thus, defense responses regulated by SA contribute to botrydial toxicity on host tissues. This phenomenon could be related to the cell-death-promoting effects exerted by SA in conjunction with ET and ROS during the HR (Overmyer et al. 2003). On the other hand, the increased level of tissue necrosis observed after botrydial application to *A. thaliana* plants deficient in JA signaling, compared with the WT, indicates that JA somehow attenuates the cell death process triggered by botrydial. The enhanced sensitivity of JA-deficient plants to botrydial could be related to a previous finding by Thomma and associates (1998), in that JA-deficient plants are more susceptible to *B. cinerea*.

It is worth pointing out that botrydial induced the expression of both *pr1* and *pdf1.2*, thus suggesting that both SA and JA signaling are activated in response to this toxin. Interestingly, these genes are also induced during plant responses to infection by *B. cinerea* (AbuQamar et al. 2006; Thomma et al.

Fig. 4. Pathogenesis-related (PR) gene expression in *Arabidopsis thaliana* leaves treated with botrydial. Quantitative real-time polymerase chain reaction was used to analyze the abundance of *PR1* and *PDF1.2* transcripts in tissues surrounding the lesions developed on *A. thaliana* Col-0 leaves **A**, 3 and **B**, 24 h after treatment with 5- μ l aliquots of a 805- μ M botrydial solution in acetone/water (4:6). Tissues obtained from mock-inoculated leaves were used as controls. For each time after treatment, transcript levels were normalized to the average signal intensities of controls, which were assigned to 1 and are represented by a horizontal dotted line. Results are means of three replicates $\pm$ standard error, and statistically significant differences in gene expression between leaves treated with botrydial and controls, as analyzed with the REST software, are shown as * ($P \leq 0.05$).

2001). The HR is often associated with the activation of SA-regulated defense mechanisms in both local and distal parts of the plant, which results in the activation of systemic acquired resistance (Ryals et al. 1996). Thus, it is not surprising that *PR1* expression was enhanced in tissues treated with botrydial, in that this compound was found to induce the HR in the present work. A complex interplay between SA, JA, nitric oxide, and ET levels regulates the extent of the cell death process associated with the HR triggered by pathogen infection. Thus, the relative levels of each of these signal molecules and ROS in cells around the attempted site of penetration determine the number of cells committed to die as part of the HR. In this sense, while SA promotes HR-related cell death, JA suppresses superoxide-mediated cell death, thus restricting cell death (Overmyer et al. 2003; Rao et al. 2000). In this way, it is possible that the induction of JA-signaling detected in *A. thaliana* tissues treated with botrydial results from the activation of metabolic responses addressed by the host to control the cell-death process triggered by botrydial. In this regard, it should be kept in mind that the samples of host tissues used for the analysis of PR gene expression included both the necrotic cells within the lesion developed at the spot of botrydial application and the surrounding healthy cells. On the other hand, synergis-

tic effects between SA- and JA-mediated defense pathways have been reported. In this way, some components of the JA/ET resistance pathway are required for SA-mediated, NPR-1 independent resistance (Clarke et al. 2000; Shah et al. 1999; van Wees et al. 2000). Thus, it is not surprising that both SA- and JA-mediated signaling pathways were activated by botrydial application. Further research about the mechanisms of SA and JA pathway activation by botrydial would contribute to understanding the contribution of both pathways to plant responses to this fungal compound.

In summary, the present work demonstrated that the sesquiterpene botrydial produced by *B. cinerea* induces the HR on its hosts and its toxicity is modulated by host signaling pathways mediated by SA and JA. In this way, botrydial is probably one of the effectors that allows *B. cinerea* to manipulate host defenses for its own benefit by promoting cell death and, thus, enabling it to feed on necrotic tissues.

MATERIALS AND METHODS

Chemicals.

Standard chemicals were purchased from Sigma-Aldrich (St. Louis). Molecular biology reagents were obtained from Bio-

Fig. 5. Botrydial toxicity on *Arabidopsis thaliana* lines affected in salicylic acid and jasmonic acid signaling. Transgenic *A. thaliana nahG* and the *eds5-1* mutant, both impaired in salicylic acid signaling, as well as the jasmonate-insensitive *coi1* mutant, were treated with 5-μl aliquots of botrydial solutions (161 to 1,610 μM) in acetone/water (4:6), and the sensitivity of these lines to botrydial was compared with wild-type (WT) Col-0 plants. Lesion size was determined 48 h after treatment. For each plant line, controls (no botrydial) were treated with acetone/water (4:6) and exhibited no damage (data not shown). Results are the mean of five to seven replicate leaves $\pm$ standard deviation, and statistically significant differences between genetically modified and WT plants according to one-way analysis of variance and posthoc comparisons by Dunnett's test are indicated as *, **, and *** ($P \leq 0.05$, 0.01, or 0.001, respectively).

dynamics SRL, with the exception of *Moloney murine leukemia virus* retro-transcriptase enzyme and FastStart Universal SYBR Green Master (Rox), which were from Promega Corporation (Madison, WI, U.S.A.) and Roche (Basel, Switzerland), respectively. Botrydial was isolated and purified as described previously (Durán-Patrón 1999). Basically, the fungus was grown at 25°C in Czapek-Dox medium on an orbital shaker at 250 rpm. After 4 days, the culture broth was acidified to pH 2.0 with HCl, saturated with NaCl, and extracted with ethyl-acetate. The organic extract was washed with an NaHCO₃ solution before drying over anhydrous Na₂SO₄. The resultant oil was separated by column chromatography on silica gel, with an increasing gradient of ethyl acetate in petroleum ether, followed by a final purification using high-performance liquid chromatography.

Plant material, growth conditions, and fungal inoculation.

Transgenic *nahG* *A. thaliana* B-15 and tobacco *nahG*-10 were kindly provided by Syngenta Biotechnology, Inc. (Research Triangle Park, NC, U.S.A.). The *A. thaliana* mutants *coi1* (N535548) and *eds5-1* (N3735) were obtained from the Nottingham *Arabidopsis* Stock Centre. *A. thaliana* Col-0 and tobacco Xanthi-Nc were used as WT lines.

A. thaliana seed were planted in pots containing a vermiculite/soil/sand mix (1:1:1) and were stratified at 4°C for 2 days. Subsequently, pots were transferred to a growth chamber with a photoperiod of 16 and 8 h (day and night, respectively) and a photon flux density of 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ provided by cool-white fluorescent and incandescent lamps. Day and night temperature and relative humidity were set at 24 and 21 $\pm$ 2°C and 55 and 65 $\pm$ 5%, respectively. Plants with 8 to 10 fully developed leaves were used for all the experiments.

Tobacco plants were grown in pots containing a mixture of vermiculite/soil/sand (1:1:1) irrigated with half-strength Hoagland's nutrient solution (Hoagland and Arnon 1950). When required, disks were cut from leaves of 2-month-old plants.

Fig. 6. Botrydial toxicity on tobacco plants affected in salicylic acid signaling. Leaves of transgenic tobacco *nahG* plants impaired in salicylic acid signaling were treated with 5- μl aliquots of botrydial solutions (161 to 3,220 μM) in acetone/water (4:6), and the sensitivity of these lines to botrydial was compared with wild-type (WT) Xanthi nc plants. Lesion size was determined 48 h after treatment. For each plant line, controls (no botrydial) were treated with acetone/water (4:6) and exhibited no damage (data not shown). Results are the mean of four replicate leaves $\pm$ standard deviation, and statistically significant differences between genetically modified and WT plants according to Student's *t* test are indicated as *, **, and *** ($P \leq 0.05$, 0.01, or 0.001, respectively).

For the analysis of defense gene expression in response to infection, leaves of *A. thaliana* Col-0 plants were inoculated with conidial suspensions of *B. cinerea* isolate B05.10. Inoculum consisted of 5- μl droplets of a conidial suspension (5×10^5 conidia/ml) prepared as described by Flors and associates (2007). The adaxial surface of fully expanded 30-day-old leaves were inoculated with two droplets on both sides of the central vein and incubated in petri dishes at high humidity during 24 h.

Botrydial application.

In order to explore its toxic effect on *A. thaliana* leaves, botrydial was dissolved in acetone/water (4:6) and two droplets (5 μl each) of this solution were spotted on each side of the central vein in the adaxial surface of detached leaves placed in petri dishes containing 0.8% water agar. Plates were incubated in a growth chamber under the conditions mentioned in previous sections and pictures recorded at indicated times. When evaluated on tobacco, a similar procedure was employed except that, instead of whole detached leaves, 2-cm-disks cut from fully developed leaves obtained from 2-month-old plants were used. The size of the necrotic area developed in response to botrydial application was determined with the Image-Pro Plus V 4.1 software (Media Cybernetics LP, Bethesda, MD, U.S.A.). Treatments consisted of five to seven replicate leaves for *A. thaliana* and four leaf disks for tobacco. For gene expression analysis, botrydial solutions were applied on nondetached fully expanded leaves. Plants treated in this way were incubated for the time periods indicated elsewhere, until harvesting for RNA extraction.

Microscopic analysis of cell death, callose deposition, ROS, and phenolic compound accumulation.

For cell viability analysis, leaves were submerged in staining solution (3.75 ml of distilled water; 3.75 ml of lactic acid, 3.75 ml of glycerol, 3.75 ml of equilibrated phenol, and 0.15 ml of a 25-mg/ml solution of Trypan Blue in water) and incubated for 6 h at 37°C. Afterwards, leaves were rinsed in water and cleared with chloral hydrate (2.5 g/ml). After rinsing twice with water, samples were equilibrated in 50% glycerol before visualization under a binocular microscope.

For autofluorescence analysis due to the accumulation of phenolic compounds, samples were submerged in fixing solution (10% formaldehyde, 5% acetic acid, and 45% ethanol in H₂O) for 15 min, followed by a sequential treatment in 50% ethanol for 20 min and an overnight incubation in 95% ethanol. After this period, ethanol was removed and samples equilibrated in 70% glycerol. Autofluorescence was observed by epifluorescence (excitation filter, 330 to 380 nm; emission filter, 435 to 485 nm) as described by Stone and associates (2000).

For callose staining, leaves were vacuum infiltrated with an ethanol/lactophenol solution (2:1 [vol/vol]) and incubated at 60°C for 30 min. Samples were then thoroughly rinsed in water and incubated overnight in staining solution (0.01% aniline blue powder in 150 mM K₂HPO₄, pH 9.5). Before microscopic analysis, leaves were equilibrated in 50% glycerol. Callose deposition was visualized by epifluorescence using the same filter set as for autofluorescence.

ROS production was evidenced with the redox-sensitive dye 2',7'-dichlorofluorescein diacetate (HFD). For this purpose, leaves were detached and their petioles submerged in 15 μM HFD for 30 min before botrydial treatment. Green fluorescence was visualized by epifluorescence (excitation filter, 460 nm; emission filter, >515 nm).

RNA isolation and quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR).

Leaf disks (1 cm in diameter) containing necrotic spots as well as surrounding healthy tissues were cut from *A. thaliana*

leaves treated with botrydial and used for RNA extraction. Each replicate consisted of a pool of disks obtained from three treated leaves, and each treatment consisted of three replicates. Discs obtained from mock-inoculated leaves were used as controls. Total RNA was isolated and purified using the TRI reagent (Sigma-Aldrich) according to the manufacturer's instructions. First-strand cDNA was generated by adding 2 µg of total RNA to the following mixture: 1.6 mM dNTPs, 1 µl of *Moloney murine leukemia virus* retrotranscriptase enzyme (200 units µl⁻¹), 5 µl of 5× reaction buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM MgCl₂, and 50 mM dithiothreitol, pH 8.3), 10 pmol oligo(dT) primer, and distilled water up to a final volume of 25 µl. The reaction mixture was incubated at 37°C for 1 h. For each RNA sample, an identical reaction without reverse transcriptase was performed as a control to discriminate genomic DNA contamination.

To analyze steady-state mRNA levels by qRT-PCR, 1 µl of synthesized first-strand cDNA (1:5 dilution) was further diluted to 7.5 µl with water and the same volume of FastStart Universal SYBR Green Master (Rox) was added to complete a final volume of 15 µl. Primers used in these reactions are listed in Supplementary Table 1. Reactions were performed in an Mx3005P qPCR System with the help of the MxPro qPCR Software 4.0 (Stratagene, La Jolla, CA, U.S.A.). Relative quantification was performed by the comparative cycle threshold method with the ubiquitin-encoding gene *UBQ10* as an endogenous control. For comparative purposes, relative gene expression in control plants was defined as 1. The REST software tool was used to calculate the relative expression ratios on the basis of group means for target gene transcripts versus the reference gene transcript (Pfaffl et al. 2002).

Statistics.

Each experiment was conducted at least two times with similar results, and representative experiments are shown. Results are presented as means ± standard deviation or standard error, as indicated in the corresponding figures. Lesion size data were analyzed by Student's *t* test or analysis of variance followed by posthoc comparisons by Dunnet's test. qRT-PCR results were analyzed with REST software v. 2.0.7 (Pfaffl et al. 2002).

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