Ethylene Sensing and Gene Activation in *Botrytis cinerea*: A Missing Link in Ethylene Regulation of Fungus-Plant Interactions?

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Ethylene production by infected plants is an early resistance response leading to activation of plant defense pathways. However, plant pathogens also are capable of producing ethylene, and ethylene might have an effect not only on the plant but on the pathogen as well. Therefore, ethylene may play a dual role in fungus–plant interactions by affecting the plant as well as the pathogen. To address this question, we studied the effects of ethylene on the gray mold fungus *Botrytis cinerea* and the disease it causes on *Nicotiana benthamiana* plants. Exposure of *B. cinerea* to ethylene inhibited mycelium growth in vitro and caused transcriptional changes in a large number of fungal genes. A screen of fungal signaling mutants revealed a $G\alpha$ null mutant ($\Delta bcl1$) which was ethylene insensitive, overproduced ethylene in vitro, and showed considerable transcriptional changes in response to ethylene compared with the wild type. Aminoethoxyvinylglycine (AVG)-treated, ethylene-nonproducing *N. benthamiana* plants developed much larger necroses than ethylene-producing plants, whereas addition of ethylene to AVG-treated leaves restricted disease spreading. Ethylene also affected fungal gene expression in planta. Expression of a putative pathogenicity fungal gene, *bcspl1*, was enhanced 24 h after inoculation in ethylene-producing plants but only 48 h after inoculation in ethylene-nonproducing plants. Our results show that the responses of *B. cinerea* to ethylene are partly mediated by a G protein signaling pathway, and that ethylene-induced plant resistance might involve effects of plant ethylene on both the plant and the fungus.

Additional keywords: *SnodProt1*.

Infected plants release large amounts of the plant hormone ethylene. Although many microorganisms, including plant pathogens, are capable of synthesizing ethylene in culture, it is unclear whether plant pathogens synthesize ethylene in planta and whether or not they contribute to or are affected by “disease” ethylene (ethylene which is released following infection of plants by pathogens) (Tudzynski and Sharon 2002). In plants, ethylene is synthesized from the amino acid methionine through the intermediate aminocyclopropane-1-carboxylic acid (ACC pathway). Two additional ethylene metabolic pathways are known in microorganisms; the ethylene-forming enzyme

(EFE) and α -keto- γ -methylthiobutyric acid (KMBA) pathways. The plant ACC pathway is rare in eukaryotic microorganisms and has been reported only in the slime mould *Dictyostelium mucoroides* (Amagai and Maeda 1992) and in *Penicillium citrinum* (Jia et al. 1999). Most commonly, ethylene is synthesized in fungi from 2-oxoglutarate. The reaction is catalyzed by the multifunction enzyme EFE and requires additional amino acids, arginine, or histidine as cofactors (Fukuda et al. 1986; Hottiger and Boller 1991; Pazout and Pazoutova 1989). The third ethylene pathway is the bacterial pathway (Primrose and Dilworth 1976; Yang 1969), in which ethylene is produced from methionine through KMBA. This pathway has been reported in *Botrytis cinerea* (Chagué et al. 2002; Cristescu et al. 2002).

Depending on the nature of plant–microbe interaction, ethylene might have a reducing, enhancing, or no effect on disease development (Tudzynski and Sharon 2002). In the case of necrotrophic interactions, ethylene seems to act predominantly as a plant defense factor. For example, constitutive expression of the ethylene response factor *Erf1* conferred resistance to several necrotrophic fungi in *Arabidopsis* (Berrocal-Lobo et al. 2002). A similar result was obtained when tomato leaves were pretreated with ethylene, whereas pretreatment of leaves with the ethylene perception inhibitor methylcyclopropene (MCP) enhanced disease development (Diaz et al. 2002). In addition to the activation of plant defense, ethylene also might have a direct effect on the pathogen (Flaishman and Kolattukudy 1994; Kepczynska 1989; Kepczynski and Kepczynska 1977; Tudzynski and Sharon 2002). As with other phytohormones, the response of fungi to ethylene is highly dependent on the experimental conditions, especially ethylene concentration. For example, exposure to ethylene (released from ethephone) at 10 and 100 μ l/liter enhanced conidial germination in *B. cinerea* and *P. expansum* in water (Kepczynski and Kepczynska 1977), but these or higher concentrations of ethylene had no effect when the fungi were grown on potato dextrose agar (PDA) (El-Kazzaz et al. 1983). Similarly, ethylene enhanced spore germination of *P. digitatum* under some conditions (El-Kazzaz et al. 1983), whereas no effect was observed in other studies (Eckert and Ratnayake 1994). Light, CO₂, temperature, and growth medium composition altered the effect of ethylene on *Aspergillus* spp. (Roze et al. 2004; Sharma et al. 1985). These results suggest that ethylene might have a direct effect on both the plant and the fungus during fungus–plant interactions.

In plants, ethylene biosynthesis and downstream sensing or signaling pathways are closely interconnected. It has been shown recently that an MPK signaling pathway modulates the ethylene biosynthesis in plants by accumulation of ACC syn-

thase (ACS), the rate-limiting enzyme of this biosynthetic pathway (Liu and Zhang 2004). In this model, stress stimuli are activating the *Arabidopsis* MAP kinase MPK6, an ortholog of the stress-responsive *Nicotiana tabacum* SIPK. MPK6 activation leads to ACS protein accumulation and cellular activity by phosphorylation stabilization of ACS2 and ACS6 isoforms, resulting in elevated ethylene production. Then, ethylene is sensed by a family of membrane-associated histidine-kinase receptors that includes Etr1, Etr2, Ers1, Ers2, and Ein4 (Guo and Ecker 2004; Klee 2004). In the absence of ethylene, Etr1 activates Ctr1, a Raf-like serine/threonine kinase (Hua and Meyerowitz 1998; Kieber et al. 1993). Ctr1 is a negative regulator of ethylene signaling and its MAPKKK feature suggests that ethylene signaling in plants might involve a MAP kinase cascade (Ecker 2004). Downstream of these are the positive regulators of the Ein3 DNA-binding transcription factors that accumulate in the presence of ethylene, leading to regulation of ethylene response genes (Guo and Ecker 2004).

Although molecular evidence is still lacking, the various effects of ethylene on fungi indicate that fungi might sense and respond to ethylene. Roze and associates (2004) recently showed that aflatoxin production by *Aspergillus* spp. was repressed in the presence of ethylene. In addition, the expression level of the nor-1 promoter (an early aflatoxin biosynthetic gene) was reduced to nondetectable levels by similar quantities of ethylene, suggesting that the inhibitory effect on toxin synthesis occurred, at least partially, at the level of transcription. These results support the suggestion that specific ethylene sensor molecules mediate the function of ethylene-responsive signaling pathways in fungi.

We previously showed that *B. cinerea* produces large amounts of ethylene in vitro through the KMBA pathway (Chagué et al. 2002). In the current study, we investigated the effect of ethylene on *B. cinerea* and the disease it causes on *N. benthamiana* plants. We show that ethylene causes specific gene activation in the fungus, and that a mutant in the α subunit-encoding gene *Bcg1* (Schulze-Gronover et al. 2001) is specifically impaired in ethylene production and in the responses to ethylene at physiological and molecular levels.

RESULTS

Effect of exogenous ethylene on fungal growth and development.

The effect of ethylene on colony area was determined in both the wild-type B05.10 and the α 1 null mutant $\Delta bcg1$. In order to avoid possible side effects by ethylene-releasing or ethylene-inhibiting compounds, we kept the cultures in a sealed container (vacuum bell) that was injected with 100% ethylene to a final concentration of 200 ppm. Under these conditions, ethylene caused between 24 and 30% growth retardation in the wild-type after 48 and 72 h, respectively (Table 1). We also tested the effect of ethylene on growth rate of the $\Delta bcg1$ mutant. As previously shown, $\Delta bcg1$ had a lower growth rate compared with the B05.10 wild-type strain

Table 1. Effect of ethylene on mycelium growth

Treatment	Colony area (cm ² ± sd) ^a	
	48 h	72 h
B05.10		
Control	13.4 ± 1.5	38.6 ± 5.7
Ethylene	10.2 ± 0.8	27.7 ± 2.1
$\Delta bcg1$		
Control	2.4 ± 0.8	11.8 ± 1.6
Ethylene	2.6 ± 0.6	11.4 ± 1.2

^a sd = Standard deviation.

(Schulze-Gronover et al. 2001). Unlike the growth inhibition that was observed in the wild type, $\Delta bcg1$ retained the same growth rate with and without ethylene (Table 1). These results suggest that *B. cinerea* can sense and respond to ethylene and that this function might be impaired in the $\Delta bcg1$ mutant strain.

Some plant ethylene-sensing mutants also are altered in ethylene biosynthesis; therefore, we compared ethylene production in *B. cinerea* wild-type and signaling mutants with deletions in one of the α subunit-encoding genes, *Bcg1* or *Bcg2* (Schulze-Gronover et al. 2001), or the adenylate cyclase-encoding gene, *Bcac* (Klimpel et al. 2002). Isolates were cultured in potato dextrose broth (PDB) supplemented with methionine and ethylene production was determined after 24 and 48 h, which correspond to the peak of ethylene biosynthesis (Chagué et al. 2002). The mutant strains $\Delta bcg2$ and $\Delta bcac$ produced ethylene levels similar to the wild-type isolate B05.10 (data not shown). In contrast, the $\Delta bcg1$ mutant produced approximately 2.5 times more ethylene than the wild type in light-grown cultures (Table 2). Only a low level of ethylene was detected in $\Delta bcg1$ dark-grown cultures, which was similar to ethylene levels produced by the wild type under these conditions. Thus, ethylene biosynthesis in $\Delta bcg1$ proceeds through KMBA as in the wild type, but regulation of ethylene biosynthesis is impaired in $\Delta bcg1$.

Fungal ethylene is not detected during the plant-fungus interaction.

B. cinerea produces ethylene through the KMBA pathway, which is insensitive to the ACC pathway inhibitor AVG (Chagué et al. 2002). Therefore, production of ethylene by the plant can be inhibited by AVG without affecting the biosynthesis of fungal ethylene. Detached *N. benthamiana* leaves were treated with AVG, the leaves were inoculated with isolate B05.10, and ethylene levels were measured at different time points thereafter. Infected, non-AVG-treated leaves produced large amounts of ethylene after 24 h and ethylene levels continued to increase, reaching a plateau 72 h postinoculation (Fig. 1A). Treating leaves with AVG prior to fungal inoculation completely blocked ethylene production (Fig. 1A). Thus, no fungal ethylene could be detected during plant infection by the B05.10 wild-type isolate under these conditions. We also tested ethylene production by $\Delta bcg1$ in planta. *N. benthamiana* leaves inoculated with $\Delta bcg1$ produced significant amounts of ethylene only during the first 24 h after inoculation, and then ethylene levels dropped back to basal level (Fig. 1B). Similar to the results with B05.10, no fungal ethylene could be detected in $\Delta bcg1$ -infected plants, as determined by infection of AVG-treated leaves.

It previously has been shown that $\Delta bcg1$ causes small lesions after 24 h and then disease is arrested (Schulze-Gronover et al. 2001). This might explain the production of ethylene during the first 24 h after inoculation of plants with the mutant and

Table 2. Ethylene production in vitro by the wild type and $\Delta bcg1$

Treatment	Ethylene (nl/h/g ± sd) ^a	
	24 h	48 h
B05.10		
Control	0.06 ± 0.01	0.00 ± –
Met. light	5.37 ± 1.32	3.39 ± 1.00
Met. dark	0.72 ± 0.15	0.18 ± 0.05
$\Delta bcg1$		
Control	0.08 ± 0.00	0.02 ± –
Met. light	13.92 ± 0.97	9.32 ± 1.48
Met. dark	0.54 ± 0.28	0.24 ± 0.06

^a sd = Standard deviation.

then the drop in ethylene, which correlates with arrest of disease progression. These results also suggest that the reduced pathogenicity of $\Delta bcl1$ is unrelated to ethylene overproduction because, although $\Delta bcl1$ produced almost 2.5 times more ethylene in axenic culture, it did not produce any ethylene in planta.

Ethylene-reduced disease progression.

Effect of ethylene on disease development was assessed in the same leaves in which ethylene production was measured. Disease developed much faster in AVG-treated leaves compared with infection of AVG-untreated leaves (Fig. 2A). When AVG-treated leaves were inoculated with the fungus and then placed under ethylene atmosphere, disease development was similar to the control (Fig. 2B). These results support previous studies by Diaz and associates (2002), which showed that plant ethylene antagonizes the disease caused by *B. cinerea*. AVG-treated leaves that were inoculated with $\Delta bcl1$ developed the same small, 1- to 2-mm-diameter necroses that normally are formed by this mutant (Schulze-Gronover et al. 2001). Furthermore, addition of cAMP did not cause any change in disease development with or without AVG (data not shown). Taken together, these results suggest that ethylene only affected disease spreading but had no effect on the initial infection phase.

Isolation of ethylene-induced *B. cinerea* genes.

We employed three different screening methods in search of putative ethylene responsive genes. i) Differential screening of a cDNA library was performed with probes generated from mycelium that was grown for 24 h with methionine in light or dark. Under these conditions, ethylene production by the fungus is maximal in light, whereas no ethylene is produced in the dark (Chagué et al. 2002). From this analysis, 30 putative differential clones were isolated and sequenced. ii) Suppressive subtraction hybridization (SSH) followed by mirror orientation selection (MOS) screening was performed on mycelium that was grown for 24 h and then placed in closed chambers for 3.5 h with or without ethylene at 200 ppm. The SSH libraries resulted in 9 and 26% putative differential clones for the forward and reverse libraries, respectively. The MOS libraries yielded 42 and 47% putative differential clones for the forward and reverse libraries, respectively. In all, 81 clones that produced a strong signal with only one of the probes were sequenced, leading to 43 contigs. iii) Macroarrays containing 5,000 known *B. cinerea* sequences (Siewers et al. 2004) were differentially screened with cDNA probes generated from mycelium that was grown for 24 h without ethylene; then, ethylene at 200 ppm was added and the cultures were left for another 24 h under ethylene atmosphere.

Sequences were analyzed by BLASTX and selected clones were subjected to Northern blot analysis in order to verify the differential expression patterns obtained in the initial screening experiments (Table 3). Northern blots confirmed differential expression of 4 of 14 clones from the cDNA library, 5 of 12 for the SSH-MOS clones, and the 3 genes found by the macroarray screening. Several clones were isolated by more than one method: *Bcpg1*, encoding endopolygalacturonase 1 (ten Have et al. 2002) (contigs SSH21 and DF3), a gene encoding the elongation factor *Efl* (contigs DF11 and MA5), and a gene encoding the stress response element heat shock protein *bchsp30* (contigs SSH18 and MA1).

We further characterized expression of two clones, the *bchsp30* gene, which is a general stress response element, and the *bcspl1* (*B. cinerea* snod-prot-like) gene, which is a putative pathogenicity factor. The *bchsp30* gene, which is a homolog of *Saccharomyces cerevisiae* *HSP30*, was chosen among several other stress-related genes (Table 3) because it is considered a

general stress element induced by heat shock, ethanol, and weak acids in *S. cerevisiae* (Seymour and Piper 1999). It also was identified by two different methods, suggesting that it was elevated at different time points following ethylene treatment. The *bcspl1* gene that was identified by differential hybridization of the macroarrays encodes a new *B. cinerea* putative pathogenicity factor and was chosen as a representative of ethylene-responsive, pathogenicity-related genes. It shows 75% similarity and 62% identity at amino acid level to the *Snodprot1* gene (accession number 07238) from *Phaeosphaeria nodorum*, the causal agent of glum blotch disease of wheat. The *Snodprot1* protein is produced by *P. nodorum* during wheat infection and has been suggested as a pathogenicity factor in this fungus (Hall et al. 1999). In addition, a *Snodprot1* homolog protein in *Ceratocystis fimbriata* induced necrosis on tobacco leaves and the release of phytoalexins, suggesting a role as elicitor of plant defense (Pazzagli et al. 1999). Homologues of the *Snodprot1* and *bcspl1* genes are present in additional fungi (Fig. 3) and have been suggested to represent a family of fungal proteins that possess biological activity (Pazzagli et al. 1999).

Induction of *bchsp30* and *bcspl1* genes by ethylene in axenic culture.

We studied the expression level of the *bchsp30* and *bcspl1* genes after 24 and 48 h with and without ethylene in the wild-type B05.10 and $\Delta bcl1$ isolates. Experiments were conducted with ethylene at 200 ppm, although significant induction was observed with ethylene at as low as 2 ppm (data not shown). The *bchsp30* expression level was induced by ethylene in B05.10

Fig. 1. Ethylene production by *Botrytis cinerea*-infected leaves. Detached *Nicotiana benthamiana* leaves were inoculated with *B. cinerea* isolates **A**, B05.10 or **B**, $\Delta bcl1$. Ethylene production rates were recorded during 5 days following inoculation of the leaves. Symbols: ■ = infected untreated leaves, ○ = infected leaves pretreated with aminoethoxyvinylglycine, and ◆ = uninfected control leaves.

and $\Delta bcl1$ at both time points (Fig. 4). The *bcspl1* expression also was detected at both time points, but significant differences were noted between B05.10 and $\Delta bcl1$ (Fig. 4). In $\Delta bcl1$ background, no *bcspl1* transcript was detected without ethylene after 24 h; whereas, in B05.10, ethylene caused strong induction of *bcspl1* at both time points. A low level of gene expression was detected without ethylene after 48 h in $\Delta bcl1$ and there was no noticeable induction by ethylene at this time point in the mutant.

Thus, Bcl1 is necessary for ethylene activation of *bcspl1* at the late (48-h) time point (Fig. 4, right panel). It should be pointed out that *bcspl1* expression also is Bcl1 dependent in the absence of ethylene, because the basal level was much lower in $\Delta bcl1$ compared with B05.10. Taken together, the gene expression results suggest that Bcl1 is involved in ethylene activation of *bcspl1* but that additional cascades also are involved in ethylene signaling. The *bchsp30* seems not to be regulated by this G protein, because there was no difference in induction of *bchsp30* by ethylene in B05.10 and in $\Delta bcl1$.

Induction of *bchsp30* and *bcspl1* by heat and oxidative stresses.

We compared the transcriptional response of the *bchsp30* and *bcspl1* genes to high temperature (28°C) and hydrogen peroxide stresses. At 24 h, the basal level of *bchsp30* was low and it was elevated at 28°C in both B05.10 and $\Delta bcl1$ (Fig. 5). H_2O_2 treatment also activated *bchsp30* in both isolates, although to a lower level than the heat treatment. Induction of *bchsp30* by both conditions was stronger in the mutant than in the wild type. After 48 h, *bchsp30* expression was enhanced at 28°C in both the wild type and $\Delta bcl1$, whereas H_2O_2 had no effect on *bchsp30* expression at this time point. Thus, induction of *bchsp30* by heat and oxidative stress is stage specific and it does not depend on Bcl1. The effect of 28°C on *bcspl1* transcriptional activation was similar to ethylene effect. It was activated in both the wild type and the mutant at 24 h whereas, at 48 h, expression was enhanced only in the wild type. H_2O_2 had no effect on the *bcspl1* expression (Fig. 5). These results further demonstrate the requirement of Bcl1 for activation of the *bcspl1* gene in mature (48 h) but not in young (24 h) cultures.

In planta expression of *bcspl1* and *bchsp30*.

AVG-treated and untreated *N. benthamiana* leaves were infected with B05.10 and expression of *bcspl1* and *bchsp30* was tested at 24, 48, and 72 h postinoculation. A high level of *bcspl1* expression was detected 24 h after inoculation in the AVG-untreated leaves (Fig. 6). In AVG-treated, ethylene-non-producing leaves, expression of *bcspl1* remained low at 24 h, similar to expression levels in conidia, and it was elevated only 48 h postinoculation. These results show that, at an early stage of the disease, *bcspl1* is induced by plant ethylene whereas, during plant colonization, this gene might be induced by additional factors. In planta expression of the *bchsp30* gene did not correlate with ethylene production: only low-level expression was detected 72 h postinoculation in AVG-untreated leaves whereas, in AVG-treated leaves, it already could be detected after 48 h, suggesting that in planta expression of this gene is not regulated by ethylene (Fig. 6).

DISCUSSION

Plant hormones have long been suspected of affecting fungal growth and of mediating fungus-plant interactions. Here,

Fig. 2. Ethylene restricts disease spreading. **A**, Untreated and aminoethoxyvinylglycine (AVG)-treated *Nicotiana benthamiana* leaves inoculated with *Botrytis cinerea*. Numbers denote the necrotic area in square centimeters $\pm$ standard deviation. **B**, Inoculated leaves were placed in a closed vacuum bell and atmosphere was refreshed every day. The necrotic area was measured after 72 h. Cont. = leaves were placed in ethylene-free atmosphere, AVG = leaves were pretreated with AVG before inoculation and placed in ethylene-free atmosphere, Ethyl. = leaves were placed in atmosphere containing ethylene at 200 ppm, and Ethyl. + AVG = leaves were pretreated with AVG and then placed in ethylene-containing atmosphere.

Table 3 *Botrytis cinerea* putative ethylene responsive genes

Contig ^a	Accession no. ^b	Sequence homology
Stress response		
DF1#	XM387023*	14.3.3 protein (U24158)
DF2	CNS0191N*	FKBP21 (AJ006297)
SSH18, MA1#	CNS01BLI*	HSP30 (S50131)
SSH20	PAU81165*	HSP90 (CAC28765.1)
SSH16	CNS01DFM*	HSP82 (HS82 ASPFU)
SSH27	CNS018ZT*	HSP100 (NP596503.1)
SSH40	CNS01CVM*	Sti35 (A37767)
Pathogenicity		
SSH13, SSH30#	CNS10BNG*	Cyanide hydratase (CAC69666.1)
SSH21, DF3	CNS01DMH*	Endopolygalacturonase 1 (AAC64374.1)
SSH36	CNS01CCL*	Trichodiene oxygenase P450 (CAB99378.1)
MA2#	CNS01CE1*	Snodprot1, phytotoxin (07238)
Signal transduction		
SSH5#	CNS01AZ6*	NADPH dehydrogenase (OYE1 YEAST)
SSH25#	CV102355	Aspartyl proteinase (AF522873.1)
Transcription, translation regulation		
DF10	CV102352	RNA binding protein (AL356192)
DF11, MA5#	CNS01DQG*	EF1 (AF054510)
DF12	CV102362	SUD1 regulation factor (L24435)
DF22	CNS01D8K*	rRNA 5s binding protein (XP390186.1)
DF17	CNS01C7N*	Ribosomal protein 60sL3 (AF198447)
DF18	CNS01DQG*	Ribosomal protein 60sP2 (AF331715)
DF20	CNSDQW*	Ribosomal protein 60sL29 (RL29 YEAST)
SSH12	CNS01C8D*	Ribosomal protein 30s (NP595969.1)
SSH45	CNS01B68*	Ribosomal protein 60sL10 (CAB10813.1)
Translocation		
DF6	CNS01ATD*	Proteasome 20s subunit α (AB026561)
DF7#	CV102351	ER translocation complex Sec61 (AJ277554)
SSH39	CNS01DQC*	Glucose regulated protein 78 (T50464)
SSH43	CNS01DMP*	ER ATPase (CAB11085.1)
Metabolism		
DF4#	CNS01951*	Phosphoglycerate kinase (AF072893)
DF9	CNS01AVD*	ATP/ADP carrier (X00363)
SSH23	AF327711.1*	Peroxisomal copper oxidase (NP593985.1)
SSH32	CNS01C52*	Formate dehydrogenase (FDH NEUCR)
SSH1#	CNS01C7H*	Thiamine synthase (AAC64375.1)
SSH19, SSH26	CNS01C12*	GPDH (AAD46569.1)
SSH28	CNS01C62*	Plasma membrane H ⁺ /ATPase (AF406807.1)
SSH44	CNS01CNE*	Pyruvate carboxylase (CAC19838.1)
MA3	CNS01AHA	Homogentisate dioxygenase
MA8	CNS01AT6	6-Phosphogluconate dehydrogenase (CAD80254.1)
Other		
DF5	AY230415.1*	Kinesin (U86521)
DF8	CNS0192M*	Ubiquitin (AL7390)
SSH15	CV102354	Carboxy extension protein (AAC24705.1)
DF13	CNS01962*	Histone H2B (X55547)
SSH11	CNS01DPS*	Histone H3 (H3 EMENI)
SSH22	CNS01DMS*	Histone H4 (CAD21509.1)
Unidentified function		
DF15	CNS01CUE*	...
SSH6	CNS018WZ*	...
SSH17	AY277723.1*	...
SSH46	CNS01BCS*	...
SS37	CV102360	...
MA6	CNS01AHF	...
DF16	CV102353	...
SSH34	CNS01CM1	...
SSH8	CNS01CI5*	...
SSH38	CNS01D0S*	...
SSH33	CV102358	...
SSH29	CV102356	...
MA7	CNS0197L	...
SSH31	CV102357	...
SSH4	CNS01C7J*	...
SSH9	CNS01DAN*	...
SSH41	CNS019X1*	...
SSH35	CV102359	...
SSH42	CV102361	...

^a Contig identification: DF = differential screening; SSH = suppressive subtraction hybridization analysis; MA = macroarray analysis. Clones that were confirmed by Northern blots are marked with #.

^b Accession number of the most homologous gene identified by BLASTX analysis. Numbers followed by an asterisk (*) were identified with BLAST. Other clones did not have a match in the databases.

we showed that the plant hormone ethylene activates transcriptional changes of fungal genes, some of which may play a role in fungus-plant interaction. We also presented evidence that ethylene signaling is partially mediated by a specific fungal G protein α subunit, Bcg1, and showed that *bcspl1*, a putative pathogenicity factor, is induced in planta by plant ethylene in an early phase of the interaction.

We used the ethylene growth retardation assay to identify ethylene-insensitive *B. cinerea* mutants. By screening *B. cinerea* signaling mutants, we were able to identify two ethylene-insensitive mutants, $\Delta bcg1-1$ and $\Delta bcg1-2$, both of which carry a deletion of the G α 1 protein Bcg1 (Schulze-Gronover et al. 2001). In plants, ethylene-sensing mutants also frequently are modified in ethylene production. For example, *Arabidopsis ctrl-3* mutant shows decreased levels of ethylene production compared with wild-type plants (Cancel and Larsen 2002), whereas the *N. tabacum etr1-1* line overproduces ethylene (Knoester et al. 1998). Tomato fruit of the LeETR4 antisense line that are hypersensitive to ethylene also produce more ethylene during ripening, whereas fruit of the ethylene-insensitive never ripe (NR) antisense line produce half the amount of ethylene compared with wild-type fruit (Tieman et al. 2000). Similarly, the *B. cinerea* $\Delta bcg1-1$ and $\Delta bcg1-2$ mutants, which were ethylene insensitive, also produced 2- to 2.5-fold more ethylene in culture compared with the wild type, further supporting the suggestion that Bcg1 is involved in ethylene sensing. Because both mutants had the same phenotypes, we used only one of them, $\Delta bcg1-1$ (referred to as $\Delta bcg1$) for more detailed analysis. This $\Delta bcg1$ was the only mutant affected in ethylene response and ethylene production among the signaling mutants that we analyzed, including a mutant in another G α protein (Bcg2), suggesting a specific role for Bcg1 in ethylene sensing. The ethylene signal cascade probably does not involve the cAMP pathway, as the adenylate cyclase mutant Δbac was unaffected in ethylene response. Furthermore, addition of cAMP to $\Delta bcg1$ cultures had no effect on ethylene production rates or on growth rate of the mutant in response to ethylene (*unpublished results*). This result is in agreement with previous findings by Schulze-Gronover and associates (2004): comparison of in planta transcription profiles between $\Delta bcg1$ and the wild type revealed 20 transcripts that were downregulated in the $\Delta bcg1$ mutant compared with the wild type. Only four of these genes also were downregulated in the Δbac adenylate cyclase mutant, suggesting that Bcg1 controls at least one more signaling cascade in addition to the cAMP pathway. Because cAMP did not restore ethylene sensing in the $\Delta bcg1$ mutant, we suggest that a cAMP-independent Bcg1-regulated pathway controls ethylene signaling.

We took advantage of the two different ethylene biosynthesis pathways in the plant and fungus to determine the source of ethylene and its role in infected plants by specifically blocking

only the plant pathway using AVG. These experiments showed that all of the measured ethylene in infected plants originated from the plant, providing the first conclusive evidence that the fungus does not produce significant amounts of ethylene during plant colonization. This result is surprising given the very high amounts of ethylene that *B. cinerea* produces in cultures. Addition of methionine to spores during plant inoculation did not stimulate ethylene production, suggesting that the lack of ethylene production by the fungus during plant colonization was not due to substrate limitations, but rather to suppression of ethylene biosynthesis under these conditions.

AVG-treated leaves were far more sensitive to infection than ethylene-producing leaves. Furthermore, when ethylene was added to AVG-treated leaves, disease was reduced to normal levels. Previous reports showed that ethylene pretreatment reduced susceptibility of tomato leaves to *B. cinerea*, whereas treatment with an inhibitor of ethylene perception increased plant susceptibility to the fungus (Diaz et al. 2002). Thus, plant ethylene reduces the spreading of *B. cinerea*. This might explain the lack of in planta ethylene production by the fungus, because early production of ethylene by the pathogen might cause early activation of the plant defense.

The $\Delta bcg1$ isolate causes small necroses after 24 h (primary lesions) and then disease development is arrested before appearance of spreading lesions (Schulze-Gronover et al. 2001). The mutant produces very high levels of ethylene in culture compared with the wild-type strain; therefore, we analyzed ethylene production by the mutant in planta and tested the effect of AVG on its pathogenicity. However, the $\Delta bcg1$ mutant did not

Fig. 4. Induction of *bchsp30* and *bcspl1* by ethylene. RNA was extracted from mycelia of the wild-type BO5.10 and G α mutant $\Delta bcg1$ after 24 and 48 h of growth without (–) or with (+) ethylene. The relative transcript levels of the *bchsp30* and *bcspl1* were determined by Northern blot analysis. Total RNA loading was evaluated by hybridization with 18S ribosomal RNA.

Fig. 3. Amino acid sequence alignment of the *Botrytis cinerea* *bcspl1* gene. Sequence alignment was performed using ClustalW. Black shading with white letters represents identical amino acids and gray shading with black letters represents similar amino acids. Percentages at the end of the alignment represent amino acids identical and similar, respectively, to the *B. cinerea* *bcspl1* gene. Accession numbers: *Phaeosphaeria nodorum*: 07238; *Magnaporthe grisea*: EAA54552; *Neurospora crassa*: XP328493; *Gibberella zeae*: XP391381; *Ceratocystis fimbriata*: CAC84090.

produce any ethylene on leaves and there was no enhancement of infection by $\Delta bcl1$ in AVG-treated leaves, further suggesting different regulation of fungal ethylene biosynthesis in vitro and in planta. This result also shows that the reduced pathogenicity of $\Delta bcl1$ is not due to ethylene overproduction or ethylene insensitivity.

We used several conditions and transcription profiling screening methods to study the possible effect of ethylene on transcription of fungal genes. These analyses revealed that ethylene affects transcription of a large number of fungal genes (up- or down-regulation), similar to the situation in plants. To better evaluate the specificity of the ethylene signal and to study the possible involvement of Bcl1 in ethylene signal transduction, we compared the effect of ethylene and stress conditions on expression of two genes; *bchsp30*, a general stress response element, and *bcspl1*, which is a putative pathogenicity factor.

Although both genes were upregulated by ethylene, the specific gene expression patterns differed significantly under various conditions. The gene *bchsp30* was highly induced by ethylene at 200 ppm and high temperature at the 24- and 48-h time points, whereas hydrogen peroxide caused only a slight increase in *bchsp30* expression at 24 h and had no effect at 48 h, with similar induction patterns in the wild-type and $\Delta bcl1$ mutant (Figs. 4 and 5). Thus, Bcl1 does not seem to be involved in regulation of *bchsp30*, suggesting that other pathways might mediate the effect of ethylene (and other signals) on expression of this gene. A different situation was revealed with the *bcspl1* gene. In the wild type, it was only slightly expressed without ethylene and was upregulated by ethylene at 24 and 48 h in a dose-dependent manner. However, in the $\Delta bcl1$ mutant, a *bcspl1* transcript was undetectable at 24 h and was highly induced by ethylene (Fig. 4); whereas, at 48 h, only

Fig. 5. Induction of *bchsp30* and *bcspl1* by high temperature and oxidative stresses. RNA was extracted from mycelia of BO5.10 and $\Delta bcl1$ grown for 24 or 48 h at 22°C and then exposed for 4 h at 28°C or incubated for 1 h in the presence of 20 mM H₂O₂. The relative transcript levels of the *bchsp30* and *bcspl1* genes in each condition were determined by Northern blot analysis. Total RNA loading was evaluated by hybridization with 18S ribosomal RNA and the relative amount was normalized accordingly.

Fig. 6. In planta expression of *bchsp30* and *bcspl1* genes. Aminoethoxyvinylglycine (AVG)-untreated or AVG-treated detached *Nicotiana benthamiana* leaves were inoculated with *Botrytis cinerea*. The necrotic tissue was collected every day during 4 days postinoculation and RNA was extracted. The relative transcript level of the *bchsp30* and *bcspl1* genes was determined by Northern blot analysis. Expression levels of both genes also were compared with the expression level of the more constitutively expressed actin gene. Total RNA loading was evaluated by hybridization with actin A, a *B. cinerea*-specific probe.

a low level of *bcspl1* transcript was detected regardless of ethylene treatment. As with the *bchsp30* gene, a highly similar expression pattern was obtained by exposure to a high temperature, whereas hydrogen peroxide had no effect on *bcspl1* expression in wild-type or $\Delta bcl1$ strains (Fig. 5). The differences in *bchsp30* and *bcspl1* response to ethylene in the wild-type and $\Delta bcl1$ strains further suggest that at least two different signaling cascades regulate ethylene sensing in *B. cinerea*. One of these pathways involves the G α subunit protein Bcl1, whereas components of other pathways are as yet undefined.

We previously showed that ethylene biosynthesis in *B. cinerea* does not proceed through the plant pathway, but rather through the KMBA bacterial pathway (Chagué et al. 2002). The current results show that not only biosynthesis but also ethylene sensing and signal transduction are different in the fungus from those known in plants. These findings suggest that the production of and response to ethylene (and possibly other phytohormones) in *B. cinerea* are more closely related to prokaryotic microorganisms than to plants (Sharon et al. 2004).

Disease caused by *B. cinerea* can be divided into two phases. An early phase lasts up to 24 h, during which conidia germinate, penetrate the leaf, and form primary lesions. This is the stage at which infection by $\Delta bcl1$ is arrested. The second infection phase is characterized by intense mycelial growth in planta and the spreading of necrotic lesions. Notably, *bcspl1* expression in planta was high compared with expression in an ethylene-free liquid culture already after 24 h, and it remained high during the first 72 h after leaf inoculation. However, when production of plant ethylene was blocked (by AVG), *bcspl1* expression was delayed by 24 h and was induced only 48 h postinoculation (Fig. 6). This result suggests that *bcspl1* is specifically induced by the plant ethylene during early infection stages, whereas other signals might stimulate it during later infection stages.

It is well established that microbial proteins and secondary metabolites might have dual function as elicitors of plant defense in some situations and as virulence factors in other (Tudzynski and Sharon 2003; Wolpert et al. 2002). Accordingly, the protein encoded by *bcspl1* might act as an elicitor of plant defense in the early infection phase (penetration and primary lesion formation) whereas, at a later phase (plant colonization), it might act as a virulence factor. In this scenario, the induction of high expression of *bcspl1* (and possibly of additional fungal pathogenicity genes) by plant ethylene may result in secretion of fungal compounds, such as the Bcspl1 protein, which activate the plant defense, thereby limiting disease spreading. In the absence of ethylene (e.g., in AVG-treated leaves), these fungal genes are not turned on at the beginning of the interaction and, therefore, triggering of plant defense is delayed, allowing faster disease development by the fungus. According to this model, disease ethylene can stimulate a “ping-pong” response, which is initiated by sensing and responding of the fungus to plant ethylene during early stages of plant infection.

MATERIALS AND METHODS

Fungi.

B. cinerea wild-type isolate B05.10 (Quidde et al. 1999) and the $\Delta bcl1$ null mutant strain (Schulze-Gronover et al. 2001) were used in all experiments. Isolates were cultured and maintained on PDA at 22°C, under continuous fluorescent light supplemented with near-UV light for conidiation. Liquid cultures were produced in 250-ml flasks with 50 ml of PDB and grown under similar conditions with agitation at 190 rpm. Liquid media were inoculated with fresh conidia (10^5 conidia/ml) that

were washed off 6-day-old plates as described previously (Chagué et al. 2002).

Plant inoculation.

Inoculation experiments were performed with detached leaves of 4- to 6-week-old *N. benthamiana* plants. Only leaves that had just reached full size were used. Leaves were inoculated with two 5-by-5-mm mycelium cubes that were cut from the edge of 6-day-old *B. cinerea* cultures. One mycelial cube each was placed on the left and right half of the leaf between the midvein and the edge of the leaf. Treated leaves then were placed on a wet filter paper in germination trays with 100% relative humidity and incubated at 22°C under continuous fluorescent light. When indicated, leaves were pretreated with AVG, which was administered by placing the cut petiole in flasks containing 1 mM AVG for 2 h before inoculation. This procedure was necessary to avoid possible side effects of AVG on the fungus. Control leaves were treated with water in a similar way. Disease level was estimated by measuring the necrotic area around the inoculation sites.

Ethylene production.

Ethylene production by *B. cinerea* liquid cultures was performed as described by Chagué and associates (2002). Ethylene production by detached leaves was checked after incubation of leaves for 4 h in 120-ml air-space flasks sealed with a rubber cap.

Effect of ethylene on mycelium growth.

The effect of ethylene on mycelium growth was tested by placing cultures in a 7.5-liter desiccator. Ethylene was injected through a rubber cap seal to a final concentration of 200 ppm. Control treatments were conducted in an ethylene-free atmosphere under similar conditions. External control was always added, consisting of samples placed in the growth chamber out of the vacuum bell to detect any possible effect by the change of atmosphere inside the vacuum bell. Ethylene atmosphere was monitored by gas chromatography before each measurement of colony diameter. Fresh ethylene was injected after each measurement.

Construction of a cDNA library.

Isolate B05.10 was cultured for 24 h in PDB supplemented with 10 mM methionine under continuous light (Chagué et al. 2002). Mycelium was harvested and DNA-free mRNA was extracted using an SV RNA extraction kit (Promega, Madison, WI, U.S.A.). A cDNA library was constructed in a λ Screen-1 phage vector using the OrientExpress cDNA synthesis kit (Novagen Inc., Madison, WI, U.S.A.) and the λ Screen-1 construction library kit (Novagen Inc.) according to the manufacturer's instructions.

Differential screening of cDNA library.

cDNA probes were prepared from mycelium that was grown for 24 h in PDB supplemented with 10 mM methionine in dark (no ethylene production, “minus” probe) or light (high ethylene production, “plus” probe). mRNA samples (1 μ g) were used to synthesize radiolabeled cDNA with 5 μ Ci 32 P-dCTP using Superscript II MMLV reverse transcriptase (Stratagene, La Jolla, CA, U.S.A.) according to the manufacturer's instructions. Probes were purified using the ProbeQuant G50 columns (Amersham, Tokyo). Nitrocellulose membranes (Whatmann, Clifton, NJ, U.S.A.) of the cDNA library were hybridized sequentially, first with the minus and then with the plus probes. Clones that gave a strong differential signal were purified by two rounds of phage dilution and monitored by hybridization with the same probe that was used to identify the clones.

Construction of SSH and SSH-MOS libraries.

Isolate B05.10 was cultured for 24 h in PDB without methionine in the dark (no ethylene) and then the culture was placed for 3.5 h in a vacuum bell with or without ethylene at 200 ppm. Mycelium was harvested and RNA extracted using an SV RNA extraction kit (Promega). A polymerase chain reaction (PCR)-Select cDNA subtraction kit (Clontech, Palo Alto, CA, U.S.A.) was used to produce SSH forward and reverse libraries using the ethylene conditions as tester. The MOS procedure was performed in order to increase the number of true differential clones in the final expressed sequence tag pools (Rebrikov et al. 2000). In all, four cDNA libraries were constructed: forward SSH and MOS libraries, containing putative ethylene induced clones, and reverse SSH and MOS libraries, containing putative ethylene-repressed clones. Libraries were cloned in pUC57 vector (Fermentas, Vilnius, Lithuania), and 192 clones from each library were analyzed by differential hybridization. PCR products of each clone were amplified using the -20 and -21 vector primers. Amplification products were dot-blotted onto Hybond-N membranes (Amersham) and hybridized with ³²P-labeled cDNA probes that were generated from ethylene-exposed and ethylene-unexposed mycelium. Clones that strongly hybridized with only one of the probes were propagated and sequenced.

Differential probing of macroarrays.

Cultures were grown for 24 h and then transferred for another 24 h to vacuum bells with or without ethylene at 200 ppm. ³³P-labeled cDNA probes were prepared and used to hybridize nylon membranes containing 5,000 *B. cinerea* genes (Siewers et al. 2004). Differential clones were identified by comparing the reaction on two identical membranes, each hybridized with a different probe.

Northern blot analysis.

RNA samples were extracted using the Sigma Total Mammalian RNA Miniprep kit. Total RNA (1 to 2.5 µg) was used for Northern blot analysis. Samples were separated on a formaldehyde denaturing gel and blotted onto a Hybond N+ membrane (Amersham). DNA fragments of *B. cinerea* ribosomal rRNA18s and actin A genes were amplified by PCR from *B. cinerea* genomic DNA using primer pairs CGGAGAA GGAGCCTGAG/GGCCCAAGGTTCAACTACGAG and CCC AATCAACCCAAAGTCCAACAG/CCACCGCTCTCAAGAC CCAAGA for the rRNA18s gene and the actin A gene, respectively. cDNA probes were radiolabeled with ³²P-dCTP and hybridized with membranes according to standard protocols.

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