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Functional Analysis of the Cytochrome P450 Monooxygenase Gene *bcbot1* of *Botrytis cinerea* Indicates That Botrydial Is a Strain-Specific Virulence Factor

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The micrographic phytopathogen *Botrytis cinerea* causes gray mold diseases in a large number of dicotyledonous crop plants and ornamentals. Colonization of host tissue is accompanied by rapid killing of plant cells ahead of the growing hyphen, probably caused by secretion of nonspecific phytotoxins, e.g., the sesquiterpene botrydial. Although all pathogenic strains tested so far had been shown to secrete botrydial and although the toxin causes comparable necrotic lesions as infection by the fungus, the role of botrydial in the infection process has not been elucidated so far. Here, we describe the functional characterization of *bcbot1*, encoding a P450 monooxygenase and provide evidence that it is involved in the botrydial pathway, i.e., it represents the first botrydial biosynthetic gene identified. We show that *bcbot1* is expressed in planta and that expression in vitro and in planta is controlled by an α-subunit of a heterotrimeric GTP-binding protein, BCG1. Deletion of *bcbot1* in three standard strains of *B. cinerea* shows that the effect on virulence (on several host plants) is strain-dependent; only deletion in one of the strains (T4) led to reduced virulence.

Additional keywords: secondary metabolism, signaling.

Botrytis cinerea (teleomorph: *Botryotinia fuckeliana*) is the causal agent of gray mold diseases in a broad range of dicotyledonous plants. *B. cinerea* is a typical necrotroph, i.e., its infection strategy includes killing of host cells and feeding on dead tissue and it secretes cell wall degrading enzymes and toxic metabolites that induce cell death in advance of the invading hyphae (Clark and Lorbeer 1976; Govrin and Levine 2000; Kars and van Kan 2004). Several secondary metabolites showing phytotoxic activity have been identified in culture filtrates of *B. cinerea* (Fig. 1) (Collado et al. 2000), among them botcinolide, a highly substituted lactone (Cutler et al. 1993), botrydial, a bicyclic sesquiterpene (Colmenares et al. 2002), and several related compounds (intermediates, derivatives) with lower phytotoxic potential. All these compounds are unspecific phytotoxins, i.e., so far, no host-selective toxins have been identified in *B. cinerea*. This fits well to the broad host range of this pathogen. Most of the analyses of toxin secretion in *B. cinerea* are restricted to axenic cultures; accumulation in planta has been demonstrated so far only for botrydial (Deighton et al. 2001). An interesting feature of this toxin is also the light-dependence of its toxic effect (Colmenares et al. 2002).

Though the necrotic lesions caused by botrydial application are similar to those caused by *B. cinerea* infection—indicating a vital role in the generation of disease symptoms—no unequivocal proof for an essential role of these toxins in the pathogenicity of *B. cinerea* has been found so far. No tox$^-$ mutants are available that would allow a functional analysis. Here, we present evidence that a P450 monooxygenase, BcBOT1, is involved in botrydial biosynthesis, that the corresponding gene is controlled by a signal chain including the Gα subunit BCG1, and that the toxin effect on virulence is strain dependent and is less pronounced than expected.

RESULTS

Characterization of a putative secondary metabolite gene cluster.

During the screening of a population of P450 monooxygenase genes of strain SAS56 of *B. cinerea* for genes involved in biosynthesis of the phytohormone abscisic acid (ABA), a gene, *bcP450-12*, was identified that was transiently induced under ABA production conditions but was not involved in its biosynthesis (Siewers et al. 2004). The same gene was identified by Viaud and associates (2003) within a group of calcineurin-dependent (*CND*) genes of *B. cinerea* T4 and was named *CND5*. *CND5* was shown to be closely linked to two other genes identified in the same cDNA macroarray analysis, *CND15*, encoding a putative terpene cyclase, and *CND11*, also encoding a P450 monooxygenase like *CND5*; this arrangement indicated a gene cluster typical for secondary metabolite pathways in filamentous fungi (Fig. 2). Indeed, the best matches in BLAST analyses were genes of the trichothecene cluster of the phytopathogen *Fusarium sporotrichioides* (Viaud et al. 2003). Since the strains T4 and SAS56 used in these analyses are not closely related and differ in their original host plant—T4 was iso-

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lated from *Lycopersicon esculentum* (Levis et al. 1997a) and SAS56 is an ascospore isolate of a strain isolated from *Vitis vinifera* (Faretra et al. 1988)—and in their aggressiveness on various host plants (i.e., SAS56 is more aggressive than T4 on most plants and plant organs tested), the genomic region of *bcP450-12*/*CND5* was characterized in both strains.

As shown in Figure 2, chromosome walking analysis with strains SAS56 and T4 revealed that there is an additional putative P450 monooxygenase gene (*bcP450-13*) downstream of *bcP450-12* (*CND5*). Using a polymerase chain reaction (PCR) approach with primers bridging the intergenic regions, the physical organization of the "cluster" has been shown to be similar in four *B. cinerea* strains, T4, SAS56, ATCC 58025 (a nonsporulating, ABA overproducing mutant [Siewers et al. 2004]) and B05.10 (an aggressive isolate used in most molecular-focused *B. cinerea* labs as standard recipient strain [Quidde et al. 1999]) (data not shown). Obviously, this putative gene cluster is conserved in *B. cinerea* strains, suggesting that the secondary metabolite synthesized by the encoded enzymes is common in all the strains tested. Since botrydial is a sesquiterpene, the biosynthesis of which includes a cyclase reaction and several oxidation steps (Fig. 3, compounds 3 through 7), which, in fungi, normally are catalyzed by P450 monooxygenases, botrydial would be a possible candidate for a product of this cluster of genes. Preliminary biochemical analysis of a cytochrome P450 oxidoreductase mutant (ΔBccpr1-8; Siewers et al. 2004), which lacks the electron-donating partner essential for all P450 monooxygenase catalyzed reactions, substantiated this idea; thin-layer chromatography (TLC) analysis showed the lack of botrydial in this mutant (data not shown). Prior to a functional analysis by gene inactivation, we performed a detailed expression analysis to see if there is indeed a link between *bcP450-12*/*CND5* (and the cluster genes) and botrydial biosynthesis.

Expression studies.

The expression of *CND5* (strain T4) in axenic culture had been shown by Northern analysis (Viaud et al. 2003). We tested whether the corresponding gene, *bcP450-12*, is expressed under conditions favoring botrydial biosynthesis (discussed below) and if (and when) the gene is expressed in planta. As shown in Figure 4A, *bcP450-12* is highly expressed in a botrydial producing culture (Fig. 4A, bottom, shown by TLC) in a timecourse-dependent manner. The same is true for *bcP450-13* (data not shown) and *CND15* (Fig. 4A), the expression of which is coregulated with *bcP450-12* in axenic culture. An in planta experiment showed that *bcP450-12/CND5* is expressed in all wild strains used in this study; a maximum of expression is around 44 hours postinoculation (hpi), concomitant with the development of spreading lesions (Fig. 4C).

To get a first impression of the signal cascades involved in regulation of this putative toxin gene, we used a mutant lacking an α-subunit of a heterotrimeric GTP-binding protein (BCG1), which had been shown to be blocked after primary lesion development (Schulze Gronover et al. 2001) and which, according to preliminary TLC analysis, seemed to lack botry-

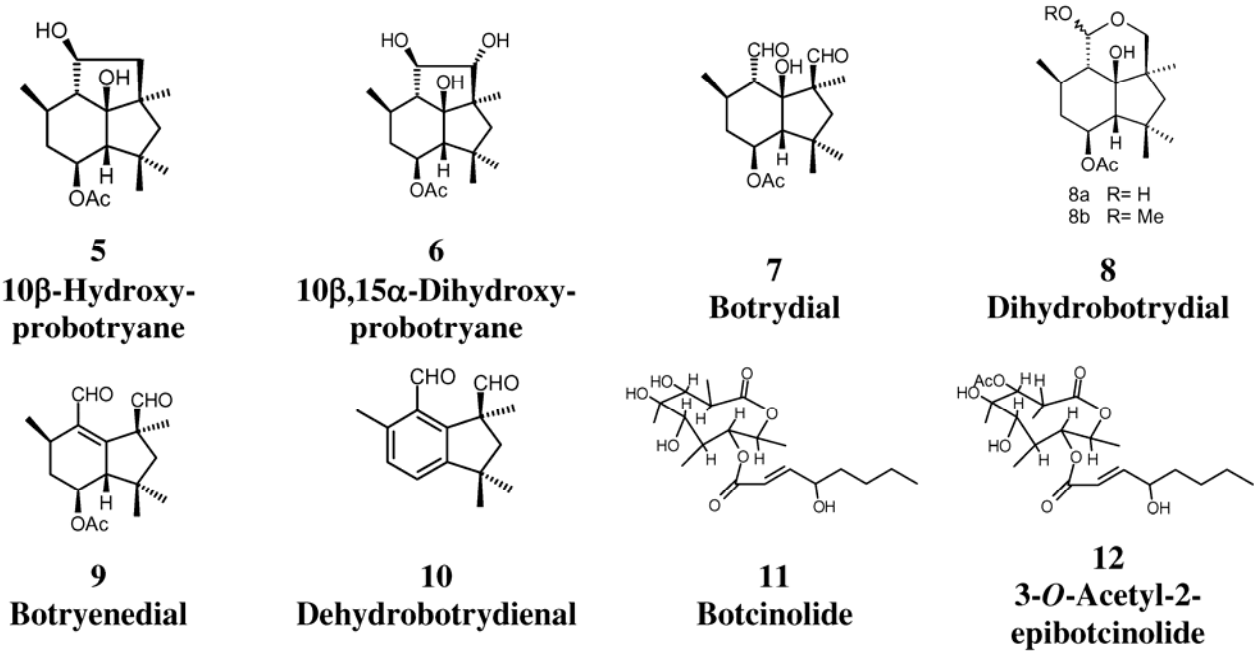

Fig. 1. Structures of *Botrytis cinerea* toxins and derivatives (Collado et al. 1996; Durán-Patrón et al. 2001).

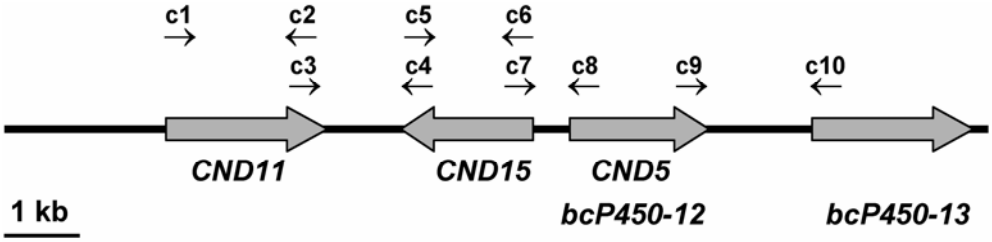

Fig. 2. Scheme of the putative secondary metabolite gene cluster of *Botrytis cinerea* (composed from data of strains SAS56 and T4) in the neighborhood of the *CND5/bcP450-12* gene. Location of primers c1 to c10 used for polymerase chain reaction analysis is indicated by arrowheads.

dial (data not shown). The Northern data (Fig. 4) confirm that the expression of *bcP450-12* is under control of BCG1; there is no *bcP450-12* transcript detectable in axenic culture (Fig. 4A) and also, in planta 48 hpi, there is no expression of *bcP450-12* (Fig. 4B), although the lesions caused by both strains at this early stage are comparable.

In conclusion, *bcP450-12* is expressed concomitantly with botrydial biosynthesis and is controlled by BCG1, as is botrydial biosynthesis. This suggests a role of BcP450-12 in botrydial biosynthesis. For an unequivocal testing of this hypothesis, a targeted inactivation approach was used.

Functional analysis of *CND5/bcP450-12* by inactivation.

For the generation of knock-out mutants, three *B. cinerea* strains were used as recipients, two sporulating "wild-type" strains with different aggressiveness (T4, SAS56) and a nonsporulating strain optimized for production of secondary metabolites (ATCC 58025). In these three strains, *bcP450-12/CND5* was deleted by the gene replacement approaches presented in Figure 5.

Transformants of strains T4 and SAS56 showing homologous integration of vector DNA (tested by PCR; data not shown) were genetically purified by one round of single-spore isolation, since

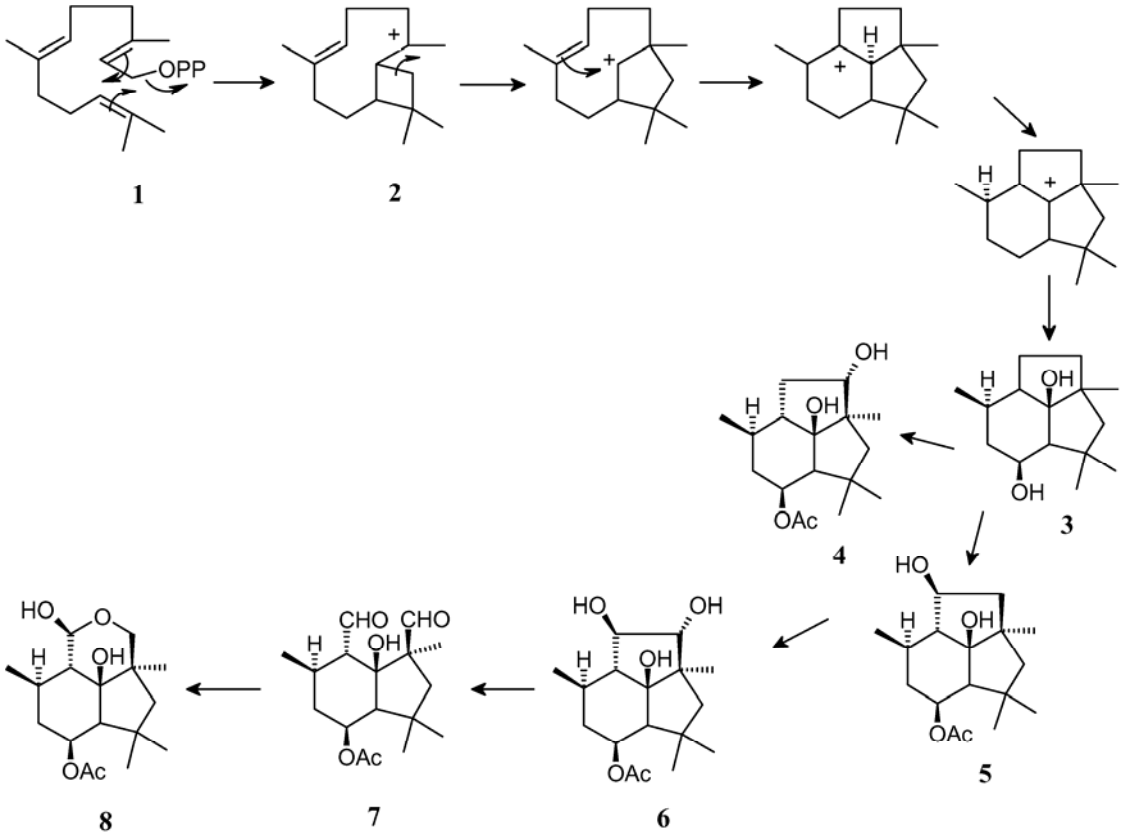

Fig. 3. Biosynthetic pathway to botrydial (7) and related compounds (established by isotopic labeling and isolation of intermediates (Durán-Patrón et al. 2001) 1, Farnesyl pyrophosphate; 2, proposed carbocation intermediate; 3, probotryane-4β,9β-diol; 4, 4β-acetoxyprobotryane-9β,15α-diol; 5, 4β-acetoxy-probotryane-9β,10β-diol (10-hydroxyprobotryane); 6, 4β-acetoxy-9β,10β,15α-trihydroxyprobotrydial; and 8, dihydrobotrydial.

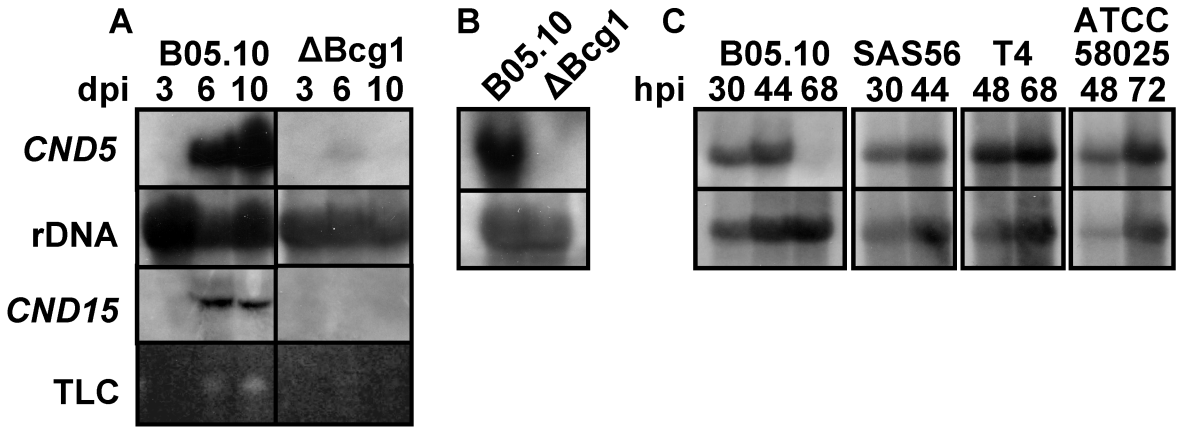

Fig. 4. A, Expression of *bcP450-12* in the wild-type strain B05.10 and mutant Δbcg1. Timecourse experiment in axenic culture under conditions of botrydial production. Bottom: thin-layer chromatogram showing botrydial formation at the indicated timepoints (days after inoculation [dpi]). **B,** Expression of *bcP450-12* in wild-type strain B05.10 and mutant Δbcg1 in planta (48 h postinoculation [hpi]). **C,** Expression of *bcP450-12, CND5* in wild-type strains B05.10, SAS56, T4, and ATCC 58025 in planta in a timecourse experiment.

they still contained wild-type nuclei, i.e., they were heterokaryotic. It was not necessary to purify the primary ATCC 58025 transformants, because they already lacked the wild-type PCR fragment and, hence, were homokaryotic. Identity of the mutants was confirmed by Southern analysis and PCR, as shown for three transformants of strain SAS56 and two transformants of ATCC 58025 in Figure 6; the mutants lack the wild-type hybridizing band (3.9 kb) and have one new band (1.1 kb) (due to the integration of the replacement fragment) (Fig. 6), whereas the ectopic transformants BcP12-S1 and BcP12-G2 show the wild-type fragment and an additional fragment differing in size from the replacement fragment. The deletion mutants give rise to diagnostic PCR fragments of both replacement flanks while lacking the wild-type fragment (data not shown; Figure 5B shows localization of primers). Altogether, six homokaryotic mutants were obtained: one from strain T4 (ΔCND5-1), three from strain SAS56 (ΔBcP12-S5, ΔBcP12-S9, ΔBcP12S21), and two from ATCC 58025 (ΔBcP12-G12, ΔBcP12-G14).

Chemical characterization.

The culture filtrates from all isolates were initially studied directly by TLC and gas chromatography-mass spectrometry

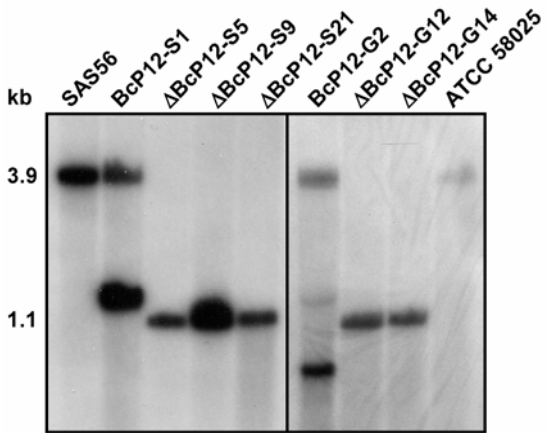

Fig. 6. Southern blot analysis of transformants. DNA of the recipient strains (SAS56, ATCC 58025), replacement mutants (ΔBcP12-S5, ΔBcP12-S9, ΔBcP12-S21, ΔBcP12-G12, ΔBcP12-G14), and ectopic transformants (BcP12-S1, BcP12-G2) was digested with *Pst*I, was transferred to a nylon membrane, and was hybridized with the 5′ flank of the *bcP450-12*-replacement vector. Sizes of wild-type and replacement fragments, respectively, are indicated.

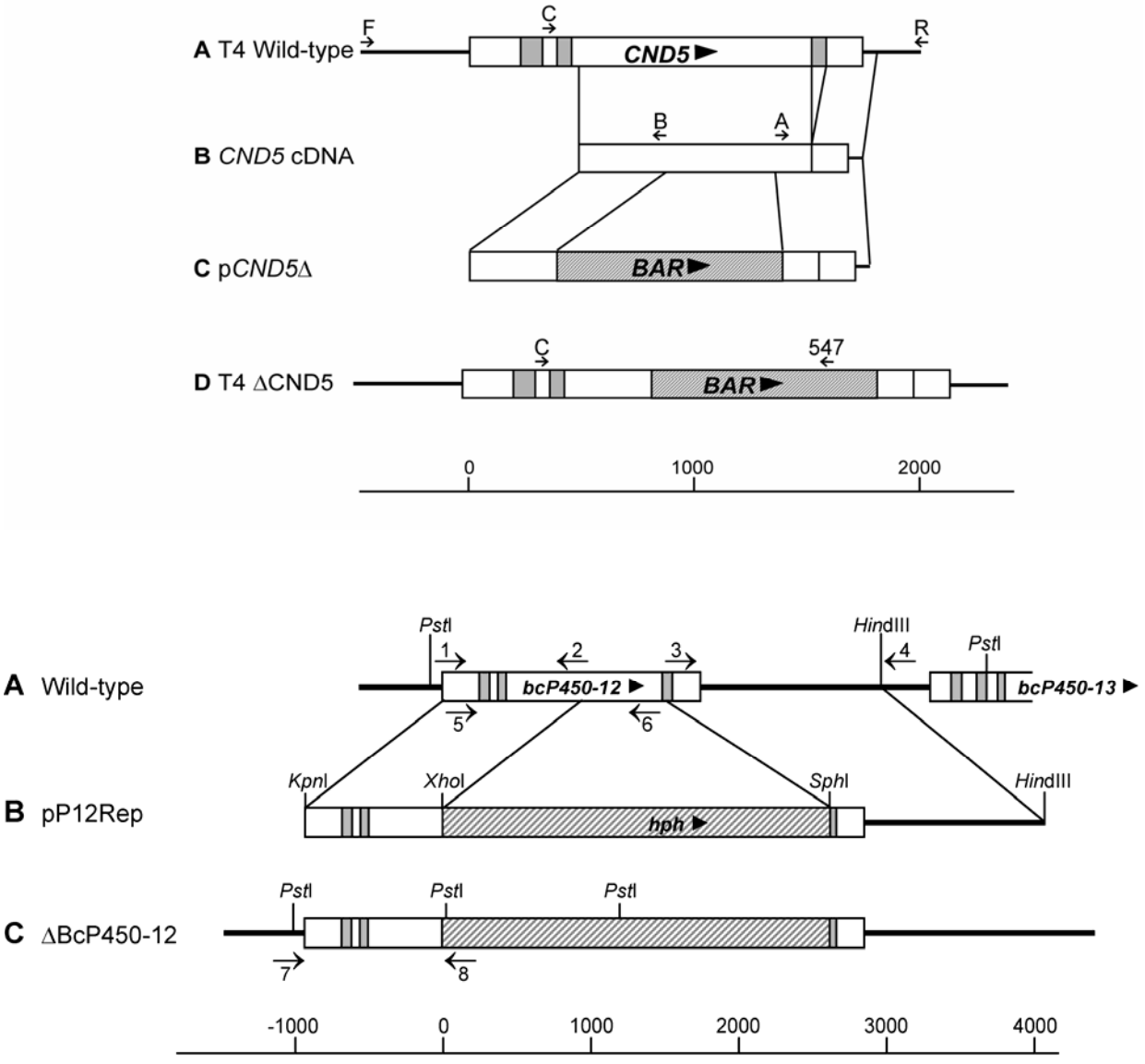

Fig. 5. Replacement strategies for *CND5* (top) and *bcP450-12* (bottom). The location of primers used for amplification of flanks and for identification of transformants is indicated by arrows. Hatched areas: hygromycin- and and bialaphos resistance cassettes, respectively. Gray areas: introns.

(GC-MS). However, the presence of metabolites with structures and retention times in GC similar to those of botrydial (Collado et al. 2000) led to unreproducible results. Therefore, the metabolites from all strains studied were purified first by column chromatography and high-pressure liquid chromatography (HPLC). Extensive spectroscopic methods, specifically ^{1}H-NMR (nuclear magnetic resonance) and ^{13}C-NMR, were then employed to study the structures of the toxins, which were compared with authentic samples. The structures of compounds identified by spectroscopic methods are shown in Figure 1. The data are summarized in Table 1. Whereas all four reference strains (T4, SAS56, ATCC 58025, B05.10) contain botrydial and some of its precursors and derivatives, the mutants analyzed, including ΔBccpr1-8 (lacking the single partner of all P450 monooxygenases, the cytochrome P450 oxidoreductase [Siewers et al. 2004]) do not all contain botrydial (Table 1). In two of the deletion strains analyzed (ΔBcP12-G12 and ΔCND5-1), enhanced levels of the precursor 10-hydroxyprobotryane (Fig. 3, number 5) (Durán-Patrón et al. 2001) were found, whereas in mutants ΔBcP12-S5 and ΔBcP12-S9 (derivatives of strain SAS56), no intermediates could be detected. The enhanced level of compound 5 for mutant ΔCND5-1, in comparison with that for strain T4, is exemplified in the chromatograms of fraction 40% etylacetate (Fig. 7). These data show that *CND5/bcP450-12* encodes an enzyme involved in botrydial biosynthesis; the gene was therefore named *bcbot1*. Also, the lack of botrydial in Δbcg1-1 proves that botrydial synthesis is indeed controlled by this

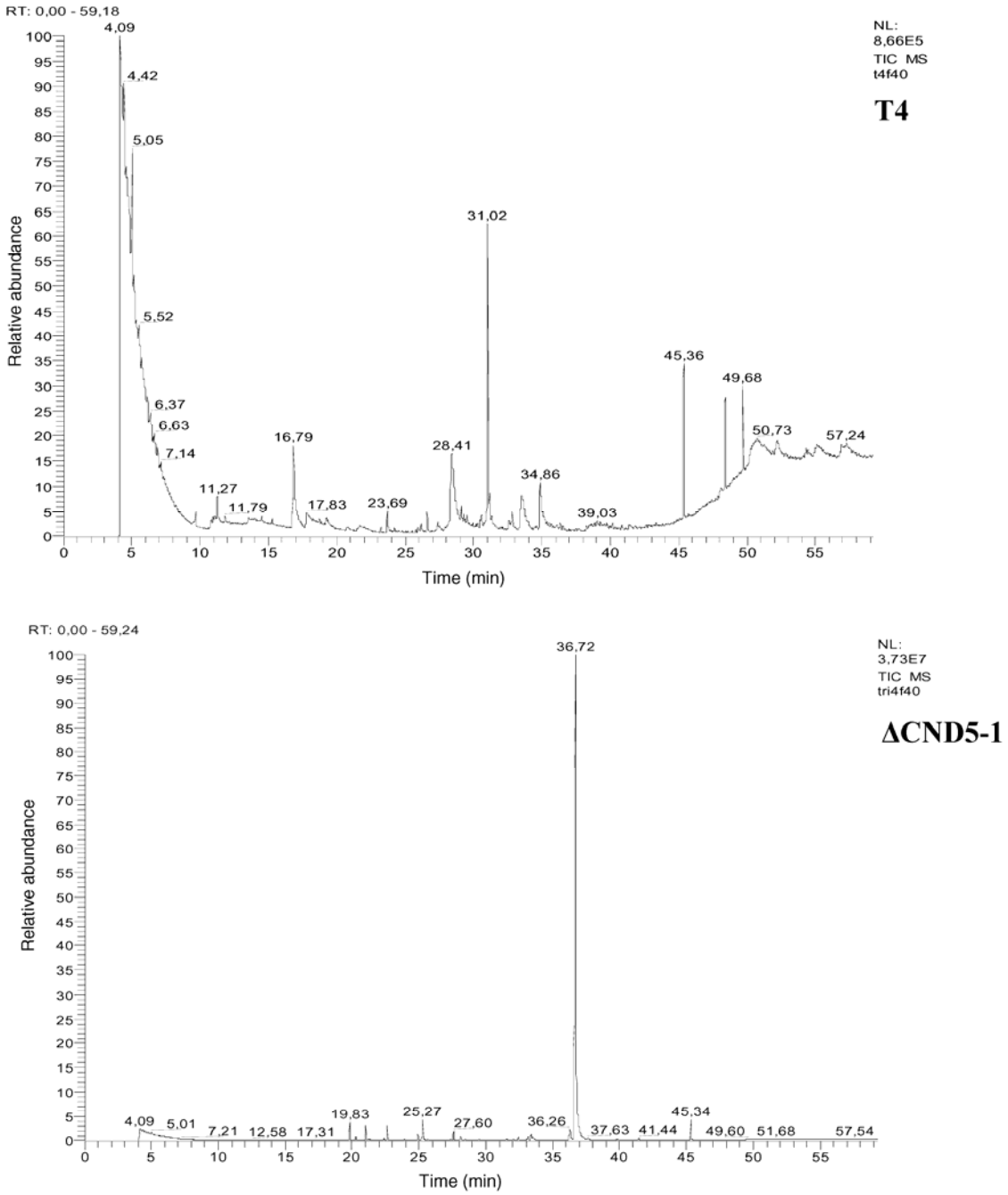

Fig. 7. Chemical analysis of strain T4 (top) and its mutant ΔCND5-1 (bottom). Total ion chromatogram of equivalent quantities of fraction 40% etylacetate, indicating relative abundance (peak with highest concentration = 100%). The signals at 31.02 and 28.41 correspond to compounds 7 and 8, respectively (top). The signal at 36.72 corresponds to compound 5 (bottom).

signal pathway. As a control, the Δbcg2-1 mutant, which lacks another Gα-subunit and has only limited reduction in virulence (Schulze Gronover et al. 2001) was analyzed and was still found to produce botrydial (Table 1).

The analyses revealed another interesting difference between the strains. Strains SAS56 and ATCC 58025 and the mutants derived from them contained the second major *Botrytis* toxin, the hydroxylated lactone botcinolide or some of its derivatives (Table 1; Fig. 1), whereas T4 and its derivative ΔCND5-1 lacked these toxins.

Effect of BcBOT1 on virulence.

The availability of Δ*bcbot1* mutants allowed an analysis of the impact of the ability to produce botrydial on virulence and aggressiveness. All mutants were tested for their pathogenic potential on various pathosystems. Figure 8 shows results obtained with the standard bean infection test using primary leaves of young, whole plants. It should be noted that the reference strains differed significantly in their virulence. SAS56 caused large lesions after only three days (after seven days, the whole plant is dead), comparable to those of T4 and ATTC 58025 after seven days. Interestingly, the Δ*bcbot1* mutants differ in virulence depending on the recipient strain. Whereas mutants of SAS56 (ΔBcP12-S5) and ATTC 58025 (ΔBcP12-G12) show the same degree of virulence as their corresponding recipient strains, the T4 mutant (ΔCND5-1) is significantly less pathogenic than the (already less pathogenic) strain T4. To confirm that this reduction in virulence is indeed caused by the knock-out of the *bcbot1* gene in strain T4, the mutant was transformed with a complementation vector containing the wild-type gene copy of *bcbot1*. Altogether six transformants were obtained and analyzed by Southern blot analysis. The presence of a single copy of the *bcbot1* gene was observed for one transformant. Pathogenicity tests on tomato and bean leaves showed that this transformant has the same degree of virulence as the original T4 strain (data not shown), which indicated that the wild-type phenotype was restored. These data demonstrate that the role of BcBOT1 (and hence botrydial)

Table 1. Metabolites identified in *Botrytis cinerea* strains and mutants

Strain	Metabolites identified	
	Botryanes[a]	Botcinolides
SAS 56	6, 7, 8, 10	11
ΔBcP12- S5	–	12
ΔBcP12-S9	–	12
ATCC 58025	7, 8	11, 12
ΔBc P12-G12	5	11, 12
ΔBccpr1-8	–	–
T4	7, 8	–
ΔCND5-1	5	–
B05.10	6, 7, 8, 9	traces
Δbcg1-1	–	–
Δbcg2-1	7, 9	–

[a] Numbers refer to formulas presented in Figure 1.

Table 2. Pathogenicity tests of *Botrytis cinerea* strains and mutants[a]

Strain	Host plant system	Lesion diameter (mm)	SD
T4	Tomato leaves, 3 dpi	9.30	1.30
ΔCND5-1	Tomato leaves, 3 dpi	3.45	1.64
SAS56	Tomato leaves, 3 dpi	14.20	3.17
ΔBcP12-S5	Tomato leaves, 3 dpi	14.35	2.52
ΔBcP12-S9	Tomato leaves, 3 dpi	14.70	2.98
T4	Bean leaves, 7 dpi	26.9	2.5
ΔCND5-1	Bean leaves, 7 dpi	3.5	0.4
SAS56	Bean leaves, 3 dpi	17.2	0.8
ΔBcP12-S9	Bean leaves, 3 dpi	17.3	0.7
ATCC 58025	Bean leaves, 7 dpi	23.1	1.3
ΔBcP12-G12	Bean leaves, 7 dpi	20.1	3.0

[a] Values are means of 12 to 20 lesions.

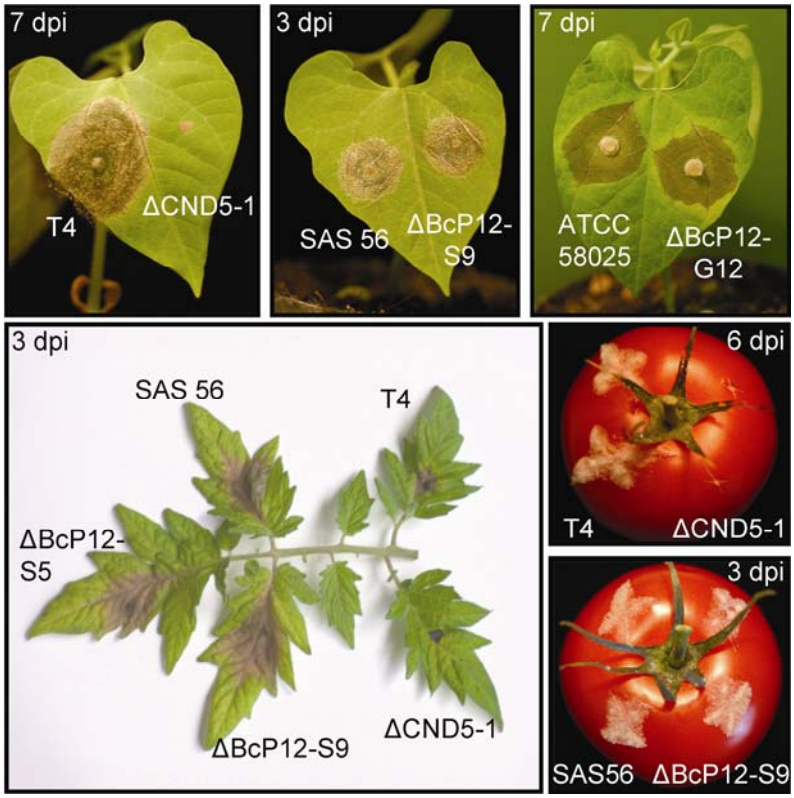

Fig. 8. Pathogenicity tests of recipient strains and mutants on young bean plants and detached tomato leaves and tomato fruits.

differs in the *B. cinerea* strains analyzed. In T4, botrydial is a virulence factor but, in strains SAS56 and ATCC 58025, is not. This was confirmed by tests using detached tomato leaves and tomato fruits (Table 2). Figure 8 demonstrates the difference in pathogenicity of SAS56 and T4 and their respective mutants in these systems.

DISCUSSION

The *B. cinerea* gene *bcbot1* encoding a P450 monooxygenase has been functionally characterized in different *B. cinerea* strains. Several observations suggested a possible role of *bcbot1* in botrydial production. First, expression of the gene in axenic culture coincides with botrydial production. Second, transcription in planta shows the highest level 44 hpi, i. e., at the beginning of soft-rot formation (Schulze Gronover et al. 2001), fitting to the report of Deighton and associates (2001), who detected botrydial in soft-rot lesions of B05.10 on *Capsicum annuum* fruits and leaves of *Phaseolus vulgaris*. Third, Δ*bcg1* deletion mutants (showing no expression of *bcbot1*) lack the capability to produce botrydial. And finally, mutants lacking a functional cytochrome P450 oxidoreductase (the essential partner for all P450 monooxygenases) also lack botrydial, strongly suggesting an involvement of P450 monooxygenases in the biosynthesis of the phytotoxin.

Deletion of *bcbot1* confirmed the participation of its gene product in botrydial biosynthesis. Mutants of three different *B. cinerea* strains lacking a functional copy of the gene did not show any botrydial production in chemical analyses. By contrast, the pathway intermediate 10-hydroxyprobotryane (Fig. 3, number 5) accumulated in the knock-out mutants ΔBcP12-G12 and ΔCND5-1, which suggests that BcBOT1 is involved in hydroxylation of the C-15 carbon atom of the botryane skeleton. This step, followed by the hydroxylation of the C-10 carbon atom, which leads to the trans-glycol (Fig. 3, number 6) and finally yields botrydial (Fig. 3, number 7) by oxidation of the C10-C15 bond, is crucial in the biosynthetic pathway of the botryane toxins and is thought to be important for their biological activity (Colmenares et al. 2002; Durán-Patrón et al. 1999). The observation that the SAS56-derived *Δbcbot1* mutants (ΔBcP12-S5, ΔBcP12-S9) did not accumulate any intermediate could be due to further metabolism of the intermediate 10-hydroxyprobotryane by an enzyme that is not active in the other strains.

The availability of defined botrydial nonproducing mutants for the first time allows an analysis of the role of the toxin in the development of disease. Therefore, all mutants were tested for their pathogenicity on different host plants. While ΔBcbot1 mutants of strains ATCC 58025 and SAS56 did not show any alteration in virulence, deletion of the gene in strain T4 caused a significant reduction in virulence on bean plants, detached tomato leaves, and tomato fruits (Fig. 8; Table 2), although no major influence on growth on agar plates could be detected (data not shown). Complementation of the ΔCND5-1 mutant with the wild-type allele fully restored pathogenicity. Therefore, botrydial must be regarded as a virulence factor, but limited only to one specific strain within the three tested strains. This shows the importance of including different field isolates in such investigations. To our knowledge, there is only one other case (also in *B. cinerea*) in which the strain dependency of a virulence factor has been reported. Deletion of the gene *bcpme1* (encoding a pectin methyl esterase) in strain Bd90 resulted in reduced virulence (Valette-Collete et al. 2003), whereas a deletion mutant of the same gene in strain B05.10 was fully pathogenic (I. Kars and J. van Kan, *personal communication*).

Some virulence factors might act in a host-dependent manner. This is true for host-specific toxins, such as the AF toxin in *Alternaria alternata* (Ito et al. 2004), but can also be the case for nonspecific toxins. In the genus *Fusarium*, it has been shown that unspecific phytotoxins can have a host plant–specific effect. Deletion of the trichodiene synthase encoding gene *tox5* responsible for trichothecene biosynthesis in *Gibberella pulicaris* (*Fusarium sambucinum*) resulted in reduced virulence of the fungus on parsnip root but did not affect virulence on potato tubers (Desjardins et al. 1992). On the other hand, trichothecene nonproducing mutants of *F. graminearum* caused less disease (than did the wild-type strain from which they were derived) on both wheat and maize (Proctor et al. 2002). A host-dependent effect was not observed for ΔBcbot1 mutants when tested on tomato leaves and fruits and bean plants.

A second class of phytotoxins that have been described in *B. cinerea* are the botcinolides (Fig. 1), highly substituted nonalactones that show biological activity such as induction of chlorosis and necrosis on bean, corn, and tobacco plants (Collado et al. 1996; Cutler et al. 1993, 1996). Interestingly, botcinolide has been detected in culture filtrates of strains SAS56, ATCC 58025, and the *bcbot1* deletion mutants of both wild types but not in strain T4 and the respective ΔBcbot1 mutant. This could be the explanation for the different impact of the knock-out of *bcbot1* in T4 and SAS56/ATCC 58025. The latter still have botcinolides and, hence, are still pathogenic. In order to prove this assumption, it would be necessary to identify genes involved in botcinolide biosynthesis for generation of single and double knock-out mutants together with *bcbot1*.

The strain-dependent impact of the knock-out of *bcbot1* may also suggest that *B. cinerea* could have developed strain-dependent infection strategies. Distinct populations of this species are known to have different ecological and molecular characteristics suggesting a possible host adaptation (Giraud et al. 1997; Fournier et al. 2003). SAS56 and T4 strains, isolated from grape and tomato, respectively, belong to populations that may have developed different infection strategies, in which toxins including botrydial and botcinolide play more or less important roles.

The mechanism of cytotoxicity of botrydial is not yet clear. An important feature is the light-dependency of its effect (Colmenares et al. 2002). It has been shown that, under light, botrydial decomposes to botryendial (less toxic) and H_2O_2 (I. G. Collado, *personal communication*). Since reactive oxygen species like H_2O_2 have been implicated in pathogenicity of *Botrytis cinerea* (Govrin and Levine 2000; Rolke et al. 2004; von Tiedemann 1997), this could be the major effect of botrydial. A detailed analysis of the reactive oxygen species generation of ΔBcbot1 mutants in planta will be necessary to clarify this aspect of the effect of botrydial.

Genes responsible for the generation of secondary metabolites are often clustered in fungi (Keller and Hohn 1997). It is thought that this arrangement could favor transmission of certain properties by horizontal gene transfer (Walton 2000). Analysis of neighboring genes of *bcbot1* revealed two further P450 monooxygenase encoding genes (*CND11*, *bcP450-13*) and one putative terpene cyclase (*CND15*) closely linked to each other. *CND11*, *CND15*, and *bcbot1* (*CND5*) had previously been shown to be coregulated, i. e., they are under control of calcineurin (Viaud et al. 2003). We could demonstrate a coregulation by BCG1 of *CND15*, *bcbot1*, and *bcP450-13*. Therefore, all three neighboring genes are promising candidates as further genes involved in the botrydial pathway, which—assuming that the oxygen atom of the tertiary hydroxyl group at C-9 originates from water (Bradshaw et al. 1982)—includes the cyclization of farnesyl diphosphate (possibly by *CND15*) and three additional hydroxylation steps (Durán-Patrón et al. 2001). Characterization of *CND15* by targeted gene deletion is currently in progress.

Bcbot1 (*CND5*) had previously been shown to be regulated by calcineurin (Viaud et al. 2003). We could demonstrate by Northern analysis that the transcription of *bcbot1* is also dependent on the G protein α subunit BCG1 in axenic culture as well as in planta. It is not yet known whether both signal chain components belong to the same or two different signaling cascades. In a recent SSH (suppression subtractive hybridization) analysis, Schulze Gronover and associates (2004) identified a large set of BCG1-controlled genes. Surprisingly, the expression of only some of these BCG1-target genes is also affected in the adenylate cyclase (BAC) mutant, suggesting that BCG1 is involved in at least one additional signaling cascade beside the cAMP pathway. The involvement of calcium and Gα proteins in one cascade has, so far, only been shown for animals and plants. We are currently investigating whether the calcineurin/calmodulin pathway is under control of a Gα protein also in *B. cinerea*. Interestingly, several of the BCG1-controlled genes encode enzymes that are probably involved in secondary metabolite pathways. Among them are three putative P450 monooxygenase genes (additional to those of the botrydial cluster) and a polyketide synthase. All these genes are not expressed in planta in the Δbcg1 mutant (Schulze Gronover et al. 2004). These data demonstrate that, beside botrydial, probably a whole set of (yet unidentified) secondary metabolites is under control of BCG1. So far, a relation between secondary metabolism and a Gα subunit has been shown only for *Aspergillus nidulans*. The biosynthesis of the carcinogenic mycotoxins sterigmatocystin and aflatoxin in *Aspergillus* species is negatively controlled by FadA, an alpha subunit of a heterotrimeric G protein. On the other hand, the dominant active allele, fadAG42R, stimulates the transcription of the penicillin biosynthetic genes (Tag et al. 2000). Furthermore, FadA links the sporulation in this fungus to the production of secondary metabolites through a shared G protein signaling pathway, which is partially mediated through the cAMP-dependent protein kinase, PkaA (Shimizu and Keller 2001). However, in *B. cinerea* botrydial biosynthesis is controlled by the Gα subunit BCG1 but not through the cAMP-pathway, since the Δbac mutant (lacking the adenylate cyclase [Klimpel et al. 2002]) is not affected in botrydial production (data not shown).

MATERIALS AND METHODS

Fungal strains.

Strain SAS56 of *Botrytis cinerea* Pers.: Fr. [*Botryotinia fuckeliana* (de Bary) Whetz] is an ascospore isolate from *Vitis*-derived field isolate (Faretra et al. 1988) B05.10, a strain used by most *Botrytis* molecular research groups for knock-out analyses, obtained after benomyl treatment of a *Vitis* isolate (Quidde et al. 1999), ATCC 58025, a nonsporulating overproducer of ABA (Marumo et al. 1982) and T4, an isolate from tomato plants grown in a glasshouse (Levis et al. 1997a). Strain Δ*bcg1* contains a deletion in the G protein α subunit *bcg1* and was described by Schulze Gronover and associates (2001).

Bacterial strains.

Escherichia coli TOP10F′ (Invitrogen, Groningen, The Netherlands) was used for propagation of plasmids. Propagation of lambda clones was performed in strain LE392 (Stratagene, La Jolla, CA, U.S.A.).

Media and culture conditions.

Standard growth medium for *B. cinerea* strains was 2% malt extract (Oxoid Ltd., Basingstoke, Hampshire, England) amended with 0.5% glucose, 0.1% casein peptone (Difco Laboratories, Sparks, MD, U.S.A.), 0.1% casamino acids (Difco), 0.1% yeast extract (Duchefa Biochemie BV, Haarlem, The Netherlands), and 0.02% ribonucleic acid sodium salt. For chemical analyses, mycelia were grown at 24 ± 2°C and 140 rpm in 500-ml Erlenmeyer flasks containing Czapek-Dox medium or in Fernbach flasks containing 200 ml of modified Czapek-Dox medium (5% glucose, 0.1% yeast extract, 0.05% KH_2PO_4, 0.2% $NaNO_3$, 0.05% $MgSO_4 \cdot 7\ H_2O$, and 0.0001% $FeSO_4$) at room temperature. In a typical experiment, 10 Erlenmeyer flasks, each containing 200 ml of culture medium, were inoculated with 2×10^7 fresh conidia per flask and were incubated for five days. For nonsporulating strains, the Erlenmeyer flasks were inoculated by addition of five, 1-cm diameter mycelial disks cut from an actively growing culture.

For DNA isolation, mycelium was grown for three to four days at 20°C on complex medium agar (Pontecorvo et al. 1953) with a cellophane overlay.

Chemical methods.

For TLC, 1 ml of culture filtrate was mixed with one droplet of 0.1 N HCl in a 2-ml Eppendorf cap and the solution was saturated with NaCl. Ethylacetate (1 ml) was added, and the solution was mixed and centrifuged. The upper phase was filtered through $NaSO_4$. After drying, the flow-through was diluted in 40 µl of acetone-mix (6 µl of Tween-20 in 60 ml of H_2O mixed with 40 ml of acetone). TLC was performed on Merck Kieselgel 60 F_{254}, 0.25 mm thick, using a mixture of hexan and ethylacetate (7:3). After spraying with oleum (40 ml of sulphuric acid, 8 ml of acetic acid, and 2 ml of water), the plate was incubated at 100°C for 10 min, and botrydial was visualized under UV light at 365 nm.

^1H and ^{13}C-NMR spectra were recorded on Varian Gemini 200 MHz (^1H at 199.975 MHz, ^{13}C at 50.289 MHz) and Varian Unity 400 MHz (^1H at 399.952 MHz, ^{13}C at 100.570 MHz) spectrometers. Chemical shifts are quoted relative to tretramethylsilane (Me_4Si) in $CDCl_3$. Mass spectra were recorded on a GC-MS Thermoquest spectrometer, model Voyager. HPLC was performed with a Hitachi/Merck L-6270 apparatus equipped with a UV-VIS detector (L 4250) and a differential refractometer detector (RI-71). Silica gel (Merck, Darmstadt, Germany) was used for column chromatography. Purification by means of HPLC was carried out with a Si gel column (LiChrospher Si-60, 10 µm, 1 cm wide, 25 cm long or 5 µm, 0.4 cm wide, 25 cm long). Culture fluid (2 liters) was acidified to pH 2.0 with HCl, was saturated with NaCl, and was extracted with EtOAc. The EtOAc extract was washed with $NaHCO_3$ and H_2O and then was dried over anhydrous Na_2SO_4. Evaporation of the solvent at reduced pressure gave a yellow oil that was separated by means of column chromatography on silica gel, with a mixture of ethyl acetate/petroleum ether (10, 20, 40, 60, 80, and 100% ethyl acetate) as solvent. The fractions were collected and analyzed in a MS-GC equipment, Thermoquest, model Voyager, by the addition of authentic samples. Samples of metabolites, previously isolated from strains of *B. cinerea* (Collado et al. 2000), were used as references in the analysis. On the other hand, compounds eluted in the fractions 10 to 100% were isolated by further purification using semi-preparative HPLC (hexane/ethyl acetate 90:10; 2.8 ml min^{-1}) to afford compounds 5 through 12, as indicated in Table 1 and Figure 1, from the wild isolates and mutants of *B. cinerea*.

Standard molecular methods.

DNA isolation. Fungal genomic DNA was isolated as described by Cenis (1992). Lambda DNA was isolated according to the standard method (Sambrook et al. 1989). Plasmid DNA was isolated using a plasmid DNA preparation kit (Genomed, Bad Oeynhausen, Germany).

Southern analysis. Genomic DNA was digested with restriction enzymes, was size-separated on a 1% agarose gel, and was blotted onto Hybond-N^+ membranes (Amersham Biosciences, Freiburg, Germany), according to Sambrook and associates (1989). Hybridization was carried out in 6× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 2 mM EDTA [pH 7.7]), 5× Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), and 0.01% salmon sperm DNA at 65°C for 16 to 20 h in the presence of a random-primed [α-^{32}P]dCTP-labeled probe. Membranes were washed in 2× SSPE, 0.1% SDS at 65°C before being exposed to an autoradiographic film.

Northern analysis. RNA was isolated from mycelial samples using the RNAgents Total RNA Isolation System (Promega, Mannheim, Germany). Samples of 10 to 15 μg of RNA were transferred to Hybond-N^+ filters after electrophoresis on a 1% agarose gel containing formaldehyde, according to Sambrook and associates (1989). Blot hybridizations were carried out in 0.6 M NaCl, 0.16 M Na_2HPO_4, 0.06 M EDTA, 1% N-lauroylsarcosine (Sigma, St. Louis), 10% dextran sulphate (Eppendorf AG, Hamburg, Germany), 0.01% salmon sperm DNA, pH 6.2, as described for Southern blots.

Sequencing. DNA sequencing of recombinant plasmid clones was performed with an automatic sequencer LI-COR 4200 (MWG Biotech, Munich, Germany) using the Thermo Sequenase fluorescent-labeled primer cycle sequencing kit (Amersham Biosciences). For sequence analysis, the program DNA Star (Madison, WI, U.S.A.) was used.

Cloning of the *bcP450-12* gene.

In the B05.10 strain, *bcP450-12* was cloned in a PCR approach by amplifying genomic DNA with primers according to the cDNA clone CNS01BNR (AL114463) out of a *B. cinerea* T4 strain cDNA library (Viaud et al. 2005). PCR reactions contained 25 ng of DNA, 10 pmol of each primer, 200 nM dNTPs, and 1 U *Taq* polymerase (Red Taq; Sigma-Aldrich, Deisenhofen, Germany) and were carried out at 94°C for 3 min followed by 30 cycles at 94°C for 1 min, 61°C for 1.5 min, and 72°C for 2 min. Primers are P12-F (5′-AAG CAG CTG GAG GCA TTC CCG-3′) and P12-R (5′-AAG CTT CTG GAG CTT GGC CGG-3′). The resulting 0.67-kb PCR fragment was used as a probe for screening a genomic λ-EMBL3 library of *B. cinerea* SAS56 (Quidde et al. 1999). Positive and purified phages were subcloned in pUC19 (Yanisch-Perron et al. 1985) and pBluescriptII SK(–) (Stratagene), respectively.

Amplification of cluster genes and intergenic regions by PCR.

Intergenic regions were amplified in a PCR approach by amplifying T4, B05.10, SAS56, and ATCC 58025 genomic DNA using the following primers (Fig. 2): c1 (5′-CAC TAA AAC GGC GCT GCT CAC G-3′), c2 (5′-CTG CCC AGG CGA GGC TAA GTG TT-3′), c3 (5′-TCA AGA TTG CGC GTG ACT ACA TCA-3′), c4 (5′-GCA GTG GCC CAG GGC AAT CTA-3′), c5 (5′-AGC GGA ATT GAC TTG CCT CCA GTA-3′), c6 (5′-ACG GCC ACC ATT CGT TCG TGT-3′), c7 (5′-AGC GGT GTT GTG GAG TTA GTG TCG-3′), c8 (5′-CGG CCC ATG CTA GGG GAG TTT-3′), c9 (5′-GCA AAG TAC GGA CTG GAC CGA CTG-3′), and c10 (5′-CAC ACG GCG ATG AAT CTA CAC AGG-3′). Reactions contained 25 ng of DNA, 5 pmol of each primer, 200 nM concentrations of deoxynucleosid triphosphates, and 1 U of *Taq* polymerase (BioTherm; GeneCraft, Lüdinghausen, Germany) and were carried out at 94°C for 4 min, followed by 35 cycles at 94°C for 1 min, 59°C for 62°C for 1 min, and 70°C for 2 min 40 s.

Vector constructions.

For construction of the gene replacement vector pP12Rep, the plasmid pOliHP (Rolke et al. 2004) carrying the *Escherichia coli* hygromycin phosphotransferase gene *hph* under control of the *Aspergillus nidulans oliC* promoter and *trpC* terminator was used as a basis vector. A 1-kb PCR fragment was amplified from the 5′-region of *bcP450-12* using the primers P12-VH (primer 1; 5′-GCT A**GG TAC C**CT TCT GAC GAT GGG-3′) and P12-VR (primer 2; 5′-CGC CGA G**CT CGA G**CT CAC TAG CGC-3′) (artificial *Kpn*I and *Xho*I sites, respectively, are indicated by bold print). From the 3′ end of *bcP450-12*, a 1.5-kb fragment was amplified using the primers P12-HH (primer 3; 5′-TGC CCT **GCA TGC** AGC TGC TAA CCG-3′) and P12-HR (primer 4; 5′-GGC TTA CCT TGT TTG TAT TTG CGG-3′) (an artificial *Sph*I site is indicated by bold print). Both fragments were cloned into pCR2.1-TOPO (Invitrogen), were cut with *Kpn*I/*Xho*I and *Sph*I/*Hin*dIII, respectively, and were cloned into the corresponding sites of pOliHP. By cutting with *Kpn*I and *Hin*dIII, the 5.2-kb replacement cassette was isolated from the vector prior to transformation (Fig. 5B).

The gene replacement vector p*CND5*Δ was generated by using the cDNA clone CNS01BNR (AL114463). The bialaphos resistance gene *BAR* from pCB1265 (Sweigard et al. 1997) was introduced into this plasmid at new *Eam1104I* restriction sites created using a Seamless cloning kit (Stratagene). The removal of the middle part of *CND5* from the cDNA clone, a first PCR amplification was realized using the primers 5′-AGT TA**C TCT TC**A CAC CGC CTG CAT CAA GGA GTC TCT-3′ (CND5A) and 5′-AGT TA**C TCT TC**A TGG AAT GAG GAT GGG GAA GTG CTT-3′ (CND5B) that both contain one *Eam1104I* recognition site (Fig. 4A). A second PCR with the primers 5′-TTA **CTC TTC** ACC ACC TGA ATG GCG AAT GGA AAT-3′ (bar-sean-up) and 5′-TTA **CTC TTC** AGT GCA CGG AAA TGT TGA ATA CTC-3′ (bar-sean-low) that both contain one *Eam1104I* site allowed amplification of the *BAR* gene. Both PCR products were digested by *Eam1104I*, and they were ligated together. In the resulting p*CND5Δ*, the *BAR* gene is located inside *CND5* cDNA in place of a 416-bp sequence (Fig. 5A).

For complementation analysis, a 2,500-bp *CND5* genomic fragment from T4 was PCR-amplified with DyNAzyme DNA polymerase (Finnzyme, Espoo, Finland), by using the upstream primer 5′-GCG **CTC GAG** ATT TTG GGT GCT GCA GAG TC-3′ (CND5F), containing a *Xho*I restriction site, and the lower primer 5′-AAA **CCG CGG** CTA ATT GAA ACA CCA GTC AAT AAG AG-3′(CND5R), containing a *Sac*II site (Fig. 5A). The complementation vector p*CND5* was constructed with pBS KS+ vector (Stratagene) in two steps. First, the *hph* gene coding for hygromycin resistance from pCB1003 (Sweigard et al. 1997) was introduced at the *Kpn*I restriction site. Then, the 2,500-bp PCR fragment containing the *CND5* locus was introduced between the *Xho*I and *Sac*II sites.

Transformation of *B. cinerea*.

Protoplasts from ATCC 58025 and SAS56 were generated as described before (Siewers et al. 2004). Protoplasts (10^7) were added to 10 μg of the replacement cassette and were transformed according to ten Have and associates (1998). Hygromycin B–resistant colonies were transferred to agar plates containing Gamborg's B5 medium (Duchefa Biochemie BV) and 2% glucose, complemented with 70 μg of hygromycin B per milliter. Homologous integration events and pure transformants were identified by PCR using the following primers (Fig. 5): P12a (primer 5; 5′-CTC CCC TAG CAT GGG CCG CTC-3′), P12b (primer 6; 5′-CCG ATG CAA GCA CGG GAG CCC-3′), P12c (primer 7; 5′-GTC CGG TGT GAT TCT CCG

ACA TTG-3′), and pLOF-oliP (primer 8; 5′-GGT ACT GCC CCA CTT AGT GGC AGC TCG CG-3′).

Protoplasts from T4 were prepared and transformed as described previously (Levis et al. 1997b). DNA-mediated transformation was carried out using 2 μg of linearized p*CND5Δ* or p*CND5*. Transformed protoplasts were plated in molten osmotically stabilized medium agar containing 100 μg of bialaphos per milliliter (glufosinate ammonium, GmbH, Augsburg, Germany) or hygromycin B (Invitrogen). Transformants were selected after 6 to 8 days at 23°C and were subcultured twice on selective media, and single-spore cultures were made to get genetically pure transformants. The screening for *CND5* gene inactivation event was done by PCR using the primers 5′-TCC TCA GAG CCA GCC CAG AT-3′ (CND5-1) and 5′-CAT GCGCAC GCT CGG GTC GTT-3′ (bar547) (Fig. 5A). *CND5* gene inactivation and complementation were both confirmed by Southern blot analysis.

Infection assays.

Conidia of strains SAS56 and T4 were collected from 10-day-old malt extract plates and were suspended in Gamborg's B5 medium (Duchefa Biochemie BV) supplemented with 2% glucose and 10 mM KH_2PO_4, pH 6.4, to a final concentration of 4×10^5 conidia per milliliter. Droplets (5 μl) were applied to primary leaves of *Phaseolus vulgaris* genotype N90598 (originated from J. D. Kelly, Michigan State University, East Lansing, MI, U.S.A.), detached leaves of *Lycopersicon esculentum*, and prewounded tomato fruits. Infection assays with strain ATCC 58025 were performed with agar plugs derived from 10-day-old malt extract plates on prewounded primary leaves of *Phaseolus vulgaris*. The infected plants and fruits were incubated in a plastic propagator box at 20°C with natural illumination conditions. Disease symptoms were scored two to seven days after inoculation.

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