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The Sesquiterpene Synthase from the Botrydial Biosynthetic Gene Cluster of the Phytopathogen *Botrytis cinerea*

Cristina Pinedo^{1,†}, Chieh-Mei Wang^{2,†}, Jean-Marc Pradier³, Bérengère Dalmais³, Mathias Choquer³, Pascal Le Pêcheur³, Guillaume Morgant³, Isidro G. Collado^{1,*}, David E. Cane^{2,*}, and Muriel Viaud^{3,*}

¹Departamento de Química Orgánica, Facultad de Ciencias, Universidad de Cádiz, 11510 Puerto Real, Spain.

²Department of Chemistry, Box H, Brown University, Providence, Rhode Island 02912–9108 USA.

³UMR BIOGER, INRA, Route de Saint-Cyr, 78086 Versailles, France

Abstract

The fungus *Botrytis cinerea* is the causal agent of the economically important gray mold disease that affects more than 200 ornamental and agriculturally important plant species. *B. cinerea* is a necrotrophic plant pathogen that secretes nonspecific phytotoxins, including the sesquiterpene botrydial and the polyketide botcinic acid. The region surrounding the previously characterized *BcBOT1* gene has now been identified as the botrydial biosynthetic gene cluster. Five genes including *BcBOT1* and *BcBOT2* were shown by quantitative Reverse Transcription-PCR to be co-regulated through the calcineurin signaling pathway. Inactivation of the *BcBOT2* gene, encoding a putative sesquiterpene cyclase, abolished botrydial biosynthesis, which could be restored by *in trans* complementation. Inactivation of *BcBOT2* also resulted in over-production of botcinic acid that was observed to be strain-dependent. Recombinant BcBOT2 protein converted farnesyl diphosphate to the parent sesquiterpene of the botrydial biosynthetic pathway, the tricyclic alcohol presilphiperfolan-8 β -ol.

Filamentous fungi produce a wide variety of medically and agriculturally important secondary metabolites. Although, the native biological role of many of these naturally occurring compounds may often remain unclear, relationships with fungal development, pathogenicity, chemical defense, and fitness have been established (1). For example, the well-known mycotoxic trichothecenes are fungal sesquiterpenes that have important consequences for both human and animal health, while the fungal sesquiterpene abscisic acid and the diterpene gibberellins can act as plant hormones. The key enzymes for the biosynthetic pathways leading to each of these metabolites are the sesquiterpene and diterpene synthases that catalyze the carbocationic cyclization of the universal precursors farnesyl diphosphate and geranylgeranyl diphosphate, respectively. The genes encoding microbial terpene cyclases are usually part of a physical cluster of genes whose encoded protein products together catalyze all or most of the steps in the biosynthetic pathway leading to the final terpene metabolite (2,3).

Botrytis cinerea is the causal agent of the economically important gray mold disease that affects more than 200 ornamental and agricultural plant host species (4,5). In contrast to its usual

*To whom correspondence should be addressed. E-mail: isidro.gonzalez@uca.es; E-mail: David_Cane@brown.edu; E-mail: viaud@versailles.inra.fr.

†These authors contributed equally to this work.

Supporting Information Available: Tables of PCR primers, Supplementary Figures 1–6 (SDS Page gel, NMR and mass spectra.) This material is free of charge via the Internet.

deleterious role, infection of Sémillon grapes with *B. cinerea* under specific climatic conditions also plays a vital role in the production of Sauternes and other botrytised dessert wines, leading to the designation “la pourriture noble” or “noble rot” (6). As a necrotrophic and polyphageous pathogen, the gray mold fungus secretes nonspecific phytotoxins that kill cells from a large spectrum of plants. Among the numerous metabolites isolated from fermentation broths (7), the most well known are the bicyclic sesquiterpene botrydial (**1**) and several related compounds with lower phytotoxic potential (Figure 1) (8). Botrydial, which is produced during plant infection, induces chlorosis and cell collapse (9). In addition to this toxic sesquiterpene, aggressive strains of *B. cinerea* also produce polyketides such as botcinic acid and related botcinins that display phytotoxic and antifungal activity (Figure 1) (10). We have recently shown that the *BcBOT1* (for *Botrytis cinerea* **BO**Trydial **bio**synthesis) gene, which encodes a P450 monooxygenase, is responsible for one of the final steps of botrydial biosynthesis (Figure 2a) (11). Indeed, *bcbot1Δ* deletion mutants were unable to produce either 10β,15α-dihydroxybotryane (**8**), botrydial (**1**), or dihydrobotrydial (**2**) (Figures 1 and 2a). Inactivation of the *BcBOT1* gene in three different strains demonstrated that botrydial is a strain-dependent virulence factor, with some strains also producing botcinic acids as virulence agents.

The sequencing of the genomes of the B05–10 and T4 strains of *B. cinerea* at the Broad Institute and the Genoscope, respectively (12), has provided the opportunity to further characterize the *BcBOT1* gene locus. Our previous study indicated that this gene is surrounded by two other putative P450 monooxygenase-encoding genes (*BcBOT3* and *BcBOT4*) as well as the *BcBOT2* gene, encoding a probable sesquiterpene synthase (Figure 3a) (11). The dataset from the *B. cinerea* T4 strain (<http://www.genoscope.cns.fr/>) provided a 16 kb DNA contig containing this region. Using data from Expressed Sequence Tags (13), we have confirmed the cDNA sequences of each of the four previously identified genes along with that of a fifth expressed gene, *BcBOT5*, located at the 5'-end of the cluster (Figure 3a). The deduced amino-acid sequence for this fifth gene showed high homology to known acetyl transferases, suggesting that it is likely responsible for the introduction of the characteristic acetyl group of botrydial (**1**) and related late-stage biosynthetic intermediates (Figure 1).

RESULTS AND DISCUSSION

The *BcBOT2* Gene is Predicted to Encode a Terpene Synthase

The predicted 399 amino-acid sequence of the BcBOT2 protein shows significant levels of similarity to microbial terpene synthases. Most importantly, the BcBOT2 protein has both of the two strictly conserved active-site motifs that are essential to binding of the three Mg²⁺ cations that constitute the required cofactor for all terpene synthase reactions: an aspartate-rich sequence, **DDQFD**, that is found at amino-acid 141 and an “N(S/T)(E/D)” triad, **NDVLSYRKD**, located 144 amino acids downstream of the **DDXXD** motif (14). Thus the *BcBOT2* gene product would be expected to correspond to the key sesquiterpene synthase responsible for catalyzing the cyclization of farnesyl diphosphate.

B. cinerea *bcbot2Δ* Deletion Mutants are Blocked in the First Step of Botrydial Biosynthesis

In order to test this hypothesis, we first used a gene inactivation approach based on homologous recombination. The knock-out plasmid p*BcBOT2Δ*, harboring the *BAR* gene that confers resistance to bialaphos in place of the *BcBOT2* gene, was initially used to transform protoplasts of the *B. cinerea* B05–10 and T4 strains, but no *bcbot2Δ* deletion mutant could be isolated from a total of 30 transformants. Gene targeting in fungi was recently facilitated by the construction of *ku* recipient strains that are altered in the Non-Homologous-end-Joining DNA repair system and therefore show a high level of homologous recombination (15). The recipient strains B05–10 *ku70Δ* and T4 *ku80Δ*, recently developed in *B. cinerea* (16), were therefore

transformed with the plasmid *pBcBOT2Δ*, thereby yielding two *bcbot2Δ* deletion mutants within the B05–10 background (#6 and #12) and one such mutant in the T4 background (#3) out of a total of ten analyzed transformants. The metabolites produced by the wild type *B. cinerea* strains and by the corresponding *BcBOT2Δ* mutants were each identified by high-pressure liquid chromatography (HPLC) comparison with authentic samples as well as ¹H and ¹³C NMR (Figure 1 and Table 1). The wild-type B05–10 strain and the derived *kuΔ* recipient strain produced both botrydial (**1**) and its derivatives (**2** and **3**) as well as small amounts of botcinin A (**7**). As expected (11), both the wild-type T4 strain and the derived *kuΔ* recipient strain produced botrydial but neither botcinin A nor any other botcinic acid derivative. Significantly, neither of the *bcbot2Δ* null mutants generated from either the parent B05–10 or the T4 backgrounds was able to produce either botrydial or any of its derivatives and biosynthetic intermediates. These data established that the protein encoded by *BcBOT2* is required for the first step of botrydial biosynthesis, the presumptive cyclization of FPP to the key probotryane alcohol intermediate, presilphiperfolan-8β-ol (**10**) (Figure 2a). Interestingly, inactivation of the *BcBOT2* gene in the B05–10 background also led to an apparent redirection of metabolism, resulting in a significant overproduction of both botcinic acid (**6**) (57 mg/l) and botcinin A (**7**) (3 mg/l), compared to the minimal level of <0.2 mg/l of **7** in the wild-type *B. cinerea* B05–10 parent strain, and the absence of **6** altogether. (Table 1). By contrast, neither of these latter botcinic acid derivatives was detected in the corresponding *B. cinerea* T4 *ku80Δ bcbot2Δ* mutant.

In order to validate the inferred function of the BcBOT2 protein, we reintroduced a copy of the native *BcBOT2* gene into the B05–10 *ku70Δbcbot2Δ-6* null mutant. Southern-blot analysis showed that the integration occurred at the targeted locus resulting in a tandem pattern with the knock-out copy of the gene. The metabolite profile of the complemented transformant indicated that the biosynthesis of botrydial and its derivatives had been restored (Table 1), while interestingly the production of botcinic acid and its derivatives reverted to the original low levels characteristic of the parent B05–10 strain. These data demonstrate that the *BcBOT2* gene encodes the enzyme responsible for the committed step of botrydial biosynthesis, proposed to correspond to the cyclization of FPP.

The Five Contiguous Genes of the Botrydial Biosynthetic Cluster are Co-regulated by a Common Pathway

The *BcBOT2* gene is adjacent to the *BcBOT1* gene that has previously been shown to encode a P450 monooxygenase required for botrydial biosynthesis (11) (Figure 3a). The combined evidence that two neighboring genes, *BcBOT1* and *BcBOT2*, are involved in different steps of the toxin synthesis thereby validates the designation of the genomic locus as the botrydial biosynthetic gene cluster. Surrounding the *BcBOT1* and *BcBOT2* loci are three additional co-regulated genes that code for enzymes with putative functions in the biosynthesis of botrydial (Figure 3a). Macro-array analyses have previously shown that the three central genes *BcBOT3*, *BcBOT2* and *BcBOT1* (previously known as *CND11*, *CND15* and *CND5* for *CalciNeurin-Dependent*) are co-regulated by a transduction pathway that depends on both the *Gα* protein BCG1 and calcineurin (17,18). In order to evaluate whether the two flanking genes *BcBOT4* and *BcBOT5* are also co-regulated through this same pathway, we used quantitative Reverse Transcription - PCR (qRT-PCR) to analyze expression of all five genes of the cluster in both wild-type *B. cinerea* B05–10 and a derived *bcg1Δ* mutant from which the gene for the BCG1 protein had been deleted (19). The qRT-PCR results (Figure 3b) indicated that all five clustered genes are significantly down-regulated both by the calcineurin inhibitor cyclosporin A and by inactivation of the *BCG1* gene, thus clearly demonstrating that these five contiguous genes are co-regulated by the same BCG1-calcineurin transduction pathway (18). In this regulatory pathway, the external signal is known to be relayed successively through the *Gα* protein BCG1, phospholipase C, calcineurin phosphatase and CRZ1, a Cys₂His₂ Zinc finger transcription

factor (20). The gene promoters of the botrydial biosynthetic cluster are therefore presumed to be activated directly by the global transcription factor CRZ1, or indirectly by a CRZ1-dependent transcription factor. This putative specific transcription factor might then be of the Zn(II)₂Cys₆ class as commonly observed in genes clusters for secondary metabolism (3). Both the 5'- and 3'-ends of the 16-kb botrydial cluster are TA-rich (>80%) which has made difficult the cloning and sequencing of the surrounding genomic sequences and the identification of potential additional *BcBOT* genes. Although, the minimal set of enzymes required for the biosynthesis of botrydial is apparently present in this five-gene biosynthetic cluster, additional enzymes, a transporter, or a specific transcription factor may also be necessary for production of botrydial by *B. cinerea*.

Botrydial is a Strain-Dependent Virulence Factor

Neither saprophytic growth nor conidiation of the *bcbot2Δ* mutants raised on either standard culture (NY) or vegetable rich (V8) media were affected compared to the corresponding wild-type strains of *B. cinerea*. Our previous studies with the botrydial non-producing mutant, *bcbot1Δ*, had suggested, however, that the sesquiterpene toxin must be regarded as a strain-dependent virulence factor. Indeed inactivation of the *BcBOT1* gene had led to reduced virulence by the T4 recipient strain but not in the more virulent strain SAS56 (11).

The availability of the *bcbot2Δ* mutants allowed investigation of the role of botrydial in the highly virulent model *B. cinerea* B05–10 strain. Standard virulence assays performed by inoculating young mycelium (Figure 4 and Table 2) or conidia onto bean and tomato leaves gave similar results. Lesions caused by *B. cinerea* B05–10 *ku70Δbcbot2Δ* mutants were similar to those due to wild-type, suggesting that botrydial is not essential for infection in this genetic background. In contrast, the virulence of the *B. cinerea* T4 *ku80Δ bcbot2Δ* mutant was reduced by an amount similar to that of the previously described T4 *bcbot1Δ* mutant (11) (Figure 4 and Table 2). The botrydial-defective T4 mutants were still able to penetrate the plant tissues but the infection was arrested in the early stages of colonization. This *in planta* growth defect of the *B. cinerea* T4 *ku80Δ bcbot2Δ* mutant was also observed on grapevine berries inoculated with conidial suspensions (Figure 4c and Table 2). Taken together, these data indicated that botrydial is necessary for colonization of plant tissue by the *B. cinerea* T4 strain but not by the more virulent B05–10 strain.

As a necrotrophic pathogen, *B. cinerea* induces host cell death as it colonizes plant tissue (5). Among the probable effectors are phytotoxic secondary metabolites such as botrydial and botcinic acid derivatives whose specific modes of action are still unclear. The properties of the botrydial non-producing *bcbot2Δ* mutant have confirmed that the sesquiterpene toxin is a strain-dependent virulence factor, consistent with the results previously obtained with the *bcbot1Δ* mutant (11). Thus despite the inability of the *bcbot1Δ* and *bcbot2Δ* mutants of highly aggressive strains such as *B. cinerea* SAS56 and B05–10 to produce botrydial, they still retain substantial virulence on all of the tested hosts. Botcinic acid derivatives are overproduced in the fully virulent *B. cinerea* B05–10 *bcbot2Δ* mutants, while botcinates are completely absent in the T4 *bcbot2Δ* mutant which exhibits a defect in colonization of plant tissues. Botrydial and botcinic acid derivatives may therefore have a redundant function in highly virulent strains whereby botcinic acids are able to compensate for the absence of botrydial by an unknown regulatory mechanism. One hypothesis would be feed-back control by botrydial on the signal transduction pathways regulating synthesis of both toxins, but such mechanisms have not been described so far in fungi. Expression analysis of the *B. cinerea* genes encoding polyketide synthases are in progress to identify botcinic acid biosynthetic genes and to address these questions.

The BcBOT2 protein is a sesquiterpene synthase

We have also directly established the biochemical function of the protein encoded by the *BcBOT2* protein. Following heterologous bacterial expression in *Escherichia coli* of a 1.2-kb synthetic gene corresponding to *BcBOT2* with codons optimized for *E. coli*, the soluble BcBOT2 protein, carrying an N-terminal His₆-tag, was purified to >95% homogeneity by metal affinity, Ni-NTA chromatography. The recombinant BcBOT2 protein was shown to have the expected molecular mass by MALDI-TOF mass spectrometry. GC-MS analysis of the products resulting from the incubation of farnesyl diphosphate (FPP) with recombinant BcBOT2 protein revealed the formation of the predicted sesquiterpene alcohol, presilphiperfolan-8 β -ol (**10**, C₁₅H₂₆O, *m/z* 222), as the major (>95%) product, which was shown to be identical with an authentic sample of **10** by direct comparison of GC retention times on both achiral and chiral capillary columns as well as mass spectra (Supplementary Figure 2) (21). Preparative-scale incubation with FPP yielded a sample of **10** whose ¹H NMR spectrum was identical with that of authentic **10** (Supplementary Figure 3). Similar incubation of [1,1-²H₂]FPP with recombinant BcBOT2 protein gave [5,5-²H₂]-**10** (C₁₅H₂₂D₂, *m/z* 224) (Figure 2b), whose ¹H NMR spectrum was identical to that of unlabeled **10** except for the absence of the characteristic 1 H doublets (*J* = 12 Hz) at δ 2.25 and 1.30 corresponding to each of the geminal H-5 methylene protons (Supplementary Figures 4 and 5). Using [1-³H]FPP as substrate, the reaction exhibited typical Michaelis-Menten kinetics (Supplementary Figure 6), with a turnover number *k*_{cat} 3.23 \pm 0.15 s⁻¹ and Michaelis constant *K*_m for FPP of 6.04 \pm 0.98 μ M, comparable to typical steady-state kinetic parameters for other microbial terpene synthases (14, 22).

Cyclization Mechanism

Incorporation experiments with isotopically labeled precursors using intact cultures of *B. cinerea* have previously established the origin of the carbon skeleton of botrydial as well as the operation of a 1,3-hydride shift during the cyclization of FPP, leading to a proposal for the biosynthesis of botrydial (7,23) that is now reinforced by the demonstrated enzymatic conversion of FPP to presilphiperfolan-8 β -ol (**10**) (21) (Figure 2b). The finding that recombinant BcBOT2 protein catalyzes the cyclization of FPP to the sesquiterpene alcohol presilphiperfolan-8 β -ol (**10**), combined with the observation that the corresponding *BcBOT2* gene is essential to the formation of botryane and probotryane sesquiterpenes, firmly establishes the biochemical function of this gene which is thus shown to be responsible for the first committed step in the biosynthesis of botrydial.

Although several probotryane metabolites such as **8**, presumptive intermediates of botrydial biosynthesis, have previously been isolated along with the phytotoxin botrydial (**1**) and its derivatives **2–5** from the fungal plant pathogen *B. cinerea* (7), the parent sesquiterpene alcohol presilphiperfolan-8 β -ol (**10**) has never been isolated from *B. cinerea* itself. The tricyclic alcohol **10** and its presilphiperfolan-9-ol isomer are, however, known compounds, having previously been isolated from the plants *Eriophyllum staechadifolium* and *Fluorensia heterolepsis* (21). Interestingly, both the enzymatically-generated fungal sesquiterpene alcohol and the plant-derived presilphiperfolan-8 β -ol (**10**) have the same absolute configuration, as confirmed by direct chiral GC-MS comparison.

The Presilphiperfolan-8-yl Cation **11** Plays a Central in the Biosynthesis of Numerous Polycyclic Sesquiterpenes

This report describes the expression and biochemical characterization of the first presilphiperfolan-8 β -ol synthase obtained from any fungal or plant source. Enhancing the significance of our findings, the presilphiperfolan-8-yl cation **11** is in fact thought to play a central role in the formation of a wide range of polycyclic sesquiterpenes of both plant and fungal origin (Figure 5). For example, it has been shown that acid-catalyzed solvolysis of presilphiperfolan-8 β -ol (**10**) results in rearrangement to the tricyclic sesquiterpene

terrecyclene, the precursor of the *Aspergillus terreus* antibiotic quadrone (24,25). There is also strong experimental evidence supporting the conversion of **11** to a variety of plant-derived triquinane sesquiterpenes, including silphinenes, modhephene, and isocomene as well as several other tricyclic sesquiterpenes (24-29) (Figure 5). It is therefore likely that close protein homologs of the fungal presilphiperfolan-8 β -ol synthase will be found in numerous plant and fungal species that are responsible for the biosynthesis of a wide range of triquinane and related sesquiterpene metabolites.

MATERIALS AND METHODS

Fungal Strains and Culture Conditions

Strain B05-10 of *Botrytis cinerea* Pers.: Fr. [*Botryotinia fuckeliana* (de Bary) Whetz] is derived from a *Vitis* field isolate (30). The B05-10 *bcg1A* mutant obtained by inactivation of the gene encoding the Ga protein BCG1 (19) was provided by B. Tudzynski (University of Münster, Germany). The T4 wild-type strain was isolated from infected tomatoes (*Lycopersicon esculentum*) grown in a greenhouse in Avignon, France (31). The T4 *bcbot1A* mutant is described in our previous study (11). The recipient strains B05-10 *ku70A* and T4 *ku80A*, recently developed to increase gene deletion efficiency (16). Standard procedures for culture and maintenance of *B. cinerea* wild-type strain and mutants were carried out on NY medium (2 g malt extract, 2 g yeast extract, 15 g agar per l water) at 21 °C with 16 h daylight per 24 h. Growth and conidiation rates were estimated on cultures on NY and on Vegetables-8 (32) solid media. Conidia were counted using microscopy and a Malassez chamber.

Standard Molecular Methods

Genomic DNA was extracted from fungal mycelium using a Sarcosyl-based protocol (33). Gel electrophoresis, restriction enzyme digestion, and Southern experiments were performed using standard protocols (34). DNA probes were labeled by the random primer method using the Q-Biogen Nonaprimer Kit and 20 μ Ci α -³²P-dCTP as previously described (31).

Quantitative RT-PCR analysis

Conidia from the wild-type *B. cinerea* B05-10 WT and from the *bcg1A* mutant were germinated for 62 h at 21 °C in 100 ml liquid medium (2 g yeast extract, 10 g glucose, 2 g KH₂PO₄, 1.5 g K₂HPO₄, 1 g (NH₄)₂SO₄, 0.5 g MgSO₄•7 H₂O per l water) rotated at 120 rpm. Then mycelia were quickly filtered and transferred in 100 ml of the same medium minus the nitrogen sources. After 4 h at 120 rpm, 100 μ l of cyclosporin A (CsA; Calbiochem) suspended in 99% ethanol / 1% DMSO was added to obtain final concentrations of 0 or 10 μ g CsA/ml. After 3 h at 120 rpm, samples were filtered, washed with sterile water and frozen at -80°C. RNA was extracted from fungal tissues with Extract-All reagent (Eurobio).

Primer pairs were designed to amplify the individual *BcBOT1* to *BcBOT5* genes (Accession number AY277723; Figure 3a) and the constitutively expressed elongation factor gene *EF1b* (35) (Supplementary Table 1). These specific primers were defined in the 3' region of the each gene with the Primer Express 3.0 program according to the supplier's recommendations (ABI) and purchased from Eurogentec (40 nmol, SePOP purification). Primers were designed to span intron splice sites (BD1 from the *BcBOT4* primer pair, BD17 from the *BcBOT2* primer pair) or flanking intron (*BcBOT1*, *BcBOT3*, and *EF1b* primer pairs) in order to prevent or identify amplification of contaminating genomic DNA. This strategy was not optimal for the design of the primer pair for the *BcBOT5* gene. Efficiency tests were carried out with a range of four cDNA dilutions (1/10) of wild-type *B. cinerea* B05-10 cultivated under standard conditions. All gene-specific primer pairs achieved an efficiency between 90 and 100% and no primer dimers were observed for any pairs.

Real-Time PCR assays were carried out using a qPCR ABI Prism® SDS 9700 thermocycler including the associated software program SDS 2.2.2 from Applied Biosystems. All reactions were set up in 96-well reaction plates. Each 25- μ l reaction was performed in the MastermixPlus kit (RT-SN2X-03) from Eurogentec containing the SYBRGreen I dye as a fluorophor. Forward and reverse primer pair concentrations of 200 nM each were chosen for all genes. Negative control reactions without cDNA template were included. Each reaction was carried out in duplicate for efficiency experiments and in triplicate for quantification experiments. PCR cycling parameters were 95 °C for 10 min, followed by 40 cycles to 95 °C for 15 s, 60 °C for 1 min with the 9600 emulation protocol. For each experiment, melting curves were acquired by cooling to 60 °C for 30 s, followed by ramping up the temperature to 95 °C for 1 min with a ramp of 2%. Fluorescence data were continuously collected during the ramping from 60 to 95 °C.

Relative quantification of gene expression was achieved using the comparative Ct method with the *EF1b* gene as a control for constitutive expression (35). The expression levels of each of the five BCL genes from the B05–10 WT strain grown without CsA were used for calibration and defined value of 1. The Ct value for each gene was measured and the expression level of the genes in the different samples (B05–10, B05–10 + CsA and *bcb1A*) was calculated by the formula $2^{-\Delta\Delta Ct}$, representing the x-fold difference from the calibration reference. The relative expression software tool included in the SDS 2.2.2 software was used to test whether the expression differences were significant with an interval confidence of 95%.

Gene Inactivation and Complementation by Protoplast Transformation

The T4 genomic fragment containing the *BcBOT2* gene and promoter was PCR-amplified with DyNAzyme DNA polymerase (Finnzymes) by using the upstream primer Cyc1 (Supplementary Table 2) and the downstream primer Cyc2. The resulting 2648-bp PCR fragment was cloned into the pGEMt vector (Promega) yielding pBcBOT2 plasmid. The bialaphos resistance gene *BAR* from pCB1265 (36) was introduced into pBcBOT2 at an *Eam1104I* restriction site created using the Seamless cloning kit (Stratagene). In order to remove the central portion of the *BcBOT2* gene, an initial round of PCR amplification was carried out using the primers CycA-seam and CycB-seam that both contain a unique *Eam1104I* recognition site. A second PCR with the primers bar-seam-up and bar-seam-low, each containing an *Eam1104I* site allowed amplification of the *BAR* gene. Both PCR products were digested by *Eam1104I* and then ligated together. In the resulting pBcBOT2 Δ , the *BAR* gene is located between the codons for amino acid 65 and 248 of the BcBOT2 protein.

The *BcBOT2* complementation vector was constructed by introducing the *Nat1* nourseothricin resistance gene into pBcBOT2. The *Nat1* gene and OliC promoter were first PCR-amplified from pNR1 vector (kindly provided by B. Tudzynski, U. of Munster, Germany) using the primers Nat1-F-NN and Nat1-R-SS that contain *Nco* I and *Sac* II sites, respectively (Supplementary Table 2). The PCR product and pBcBOT2 were each digested by these two enzymes and ligated together to obtain the pBcBOT2-*Nat1* complementation vector.

Protoplasts from *B. cinerea* wild-type strains, *ku* recipient strains and B05–10 *ku70A bcbot2 Δ -6* mutant were prepared and transformed as described previously (31). DNA-mediated transformation was carried out using 2 μ g of linearized pBcBOT2 Δ or circular pBcBOT2-*Nat1*. Transformed protoplasts were plated in molten osmotically stabilized medium agar containing 100 μ g/ml bialaphos (glufosinate ammonium, Dr. Ehrenstorfer GmbH, Augsburg, Germany) or 70 μ g/ml nourseothricin (Werner). Transformants were selected after 6 to 8 days at 23°C, sub-cultured twice on selective media and single spore cultures were prepared to obtain genetically pure transformants. The screening for inactivation of the *BcBOT2* gene was carried out by PCR using the Cyc5 primer located upstream of Cyc1 and Bar547 primer located inside

the *bar* gene. Gene inactivation and complementation were verified by Southern-blot hybridization.

Metabolite Analysis

For analysis of metabolite production, strains were grown on malt agar medium (20 g D-glucose, 10 g malt extract, 20 g agar, pH 6.5–7 per l water) at 25 °C and used to inoculate Roux bottles or Erlenmeyer flasks. All studied strains were fermented in both surface and shaken cultures. For surface cultures, mycelia were grown in 1–l Roux bottles containing 150 mL of modified Czapek-Dox medium (50 mg D-glucose, 1 g yeast extract, 5 g KH₂PO₄, 2 g NaNO₃, 0.5 g MgSO₄•7H₂O and 0.01 g FeSO₄•7H₂O, pH 6.5–7.0 per l water) at room temperature. For shaken cultures, mycelia were grown in Erlenmeyer flasks containing 200 ml of the same medium agitated on an orbital shaker at 140 rpm at 25 °C. Each Roux bottle or Erlenmeyer flask was inoculated with mycelium on six small slices of agar (1 cm). After 7 days incubation under fluorescent light, the culture medium was filtered, saturated with NaCl, extracted with ethyl acetate (3 × 0.5 vol.) and washed with water (3 × 0.25 vol.). The organic extracts were dried over Na₂SO₄ and concentrated to dryness.

¹H and ¹³C NMR measurements on metabolites isolated from culture extracts were obtained on Varian Unity 400 and Varian Innova 600 NMR spectrometers with SiMe₄ as the internal reference. Mass spectra were recorded on a GC-MS Thermoquest Voyager spectrometer, and a VG Autospec-Q spectrometer. HPLC was performed with a Hitachi/Merck L-6270 apparatus equipped with a UV-VIS detector (L 6200) and a differential refractometer detector (RI-71). TLC was performed on Merck Kiesegel 60 F₂₅₄, 0.2 mm thick. Silica gel (Merck) was used for column chromatography. HPLC purification was accomplished with a silica gel column (Hibar 60, 7 m, 1 cm wide, 25 cm long). Chemicals were products of Fluka or Aldrich. All solvents were freshly distilled.

For metabolite isolation, the culture filtrate (3 liter) was saturated with NaCl and the aqueous phase was extracted with ethyl acetate. This extract was washed with H₂O and then dried over anhydrous Na₂SO₄. Evaporation of the solvent at reduced pressure gave 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), and 20% methanol in ethyl acetate as solvent. Extensive spectroscopic analysis by ¹H-NMR and ¹³C-NMR was used to detect the presence of the various toxins in each fraction. Candidate fractions were further purified by HPLC with an increasing gradient of ethyl acetate to petroleum ether. The toxin structures were analyzed by spectroscopic methods and direct comparison with authentic samples, previously isolated from strains of *B. cinerea* (37). Semi-preparative HPLC afforded compounds **1–7**, from both wild-type strains and mutants of *B. cinerea* (Table 1 and Figure 1).

Infection Assays

The infection of bean (*Vicia fabae*), tomato (*Lycopersicon esculentum*) and grape berries (*Vitis vinifera*) by *B. cinerea* was studied by inoculating detached leaves or fruit with young unsporulated mycelium or conidial suspensions from cultures on NY medium. Bean plants (Caruso cultivar) and tomato plants (Moneymaker cultivar) were grown under greenhouse conditions. Leaves were harvested from 3–4-week plants and placed in a transparent plastic box lined with tissue moistened with sterile water. Leaves were inoculated with 5-mm diameter-plugs of 3-day-old mycelium. Alternatively, conidia were collected from 10-day-old plates and suspended in sucrose phosphate buffer (10 mM sucrose, 10 mM KH₂PO₄) to a final concentration of 10⁵ conidia per ml. Droplets of 10 µl were applied to the leaves and at the top of the grape berries. Storage boxes containing inoculated leaves and berries were incubated in a growth cabinet at 21 °C with 16 h daylight. Disease development on leaves was recorded

daily as radial spread from the inoculation point to the lesion margin. Pathogenicity assays on leaves were repeated 3 times using at least 5 leaves per assay.

Expression and Purification of Recombinant BcBOT2 Protein

Plasmid pJ201:10027 harboring a synthetic *BcBOT2* gene, constructed using optimized *E. coli* codons (DNA2.0 Inc.), was extracted from *E. coli* DH10B and treated with *NdeI* and *XhoI*. The resulting *BcBOT2* DNA was then ligated into expression vector pET28a that had been digested with the same enzymes. The resulting plasmid pET28a-*BcBOT2* was transformed into *E. coli* XL-1 Blue competent cells by electroporation, and the insert sequence was verified by resequencing. The pET28a-*BcBOT2* construct was then introduced into *E. coli* BL21(DE3) cells by heat shock transformation. The *E. coli* BL21(DE3) cells harboring the pET28a-*BcBOT2* construct were grown in Luria-Bertani broth containing 50 mg/l kanamycin. Cultures were incubated at 37 °C with shaking. At an OD₆₀₀ of 0.7, IPTG was added to a final concentration of 0.2 mM, and the culture was incubated overnight at 18 °C with shaking. The cells were harvested by centrifugation and suspended in 30 ml lysis buffer (50 mM Tris, 300 mM NaCl, 10 mM imidazole, 5 mM β-mercaptoethanol, pH 7.0). The suspension was lysed by sonication and centrifuged. The supernatant was bound to Ni-NTA resin (5 ml) that had been equilibrated with lysis buffer by shaking at 4 °C for 1 h. The resin slurry was applied to a column and prewashed with buffer containing 10 mM to 30 mM imidazole. The protein was eluted with 70 mM imidazole buffer (50 mM Tris, 300 mM NaCl, 70 mM imidazole, 5 mM β-mercaptoethanol, pH 7.0) at a flow rate of 2 ml/min. The fractions containing BcBOT2 protein (>95% pure by SDS PAGE, Supplementary Figure 1) were pooled, concentrated by Amicon Ultracel PL-10 centriprep, and exchanged into storage buffer (50 mM PIPES, 20% (vol/vol) glycerol, 10 mM MgCl₂, 100 mM NaCl, 5 mM β-mercaptoethanol, pH 7.0) with a PD-10 desalting column. The typical yield was ~8.5 mg protein/l culture. The protein was flash-frozen in liquid nitrogen and stored at -80 °C. MALDI-TOF analysis of recombinant His₆-tag-BcBOT2 protein was carried out on an Applied Biosystems Voyager-DE Pro spectrometer with bovine serum albumin (M_D 66431) added as an internal standard: M_D of 47221 ± 50 (calcd for His₆-tag-BcBOT2 minus *N*-terminal Met-Gly M_D 47246.)

Incubation of BcBOT Protein with FPP and GC-MS Analysis

Purified recombinant BcBOT2 (1 μM) was incubated with 60 μM FPP in 4 ml of assay buffer (50 mM PIPES, 10 mM MgCl₂, 100 mM NaCl, pH 7.0), overlaid with 4 ml of HPLC-grade pentane, for 1 h at 30 °C. The reaction mixture was extracted with the 4 ml pentane overlay followed by 3 × 4 ml of HPLC-grade dichloromethane. The combined organic extract was dried over Na₂SO₄ and concentrated to 200 μl under reduced pressure at 0 °C. A 1-μl portion of the concentrated extract was analyzed by capillary GC-MS (Hewlett-Packard Series II Gas Chromatograph, 5971 Series Mass Selective Detector, 70 eV, EI, positive ion mode; 30 m × 0.25 mm HP5MS capillary column, using a temperature program of 60–280 °C, a temperature gradient of 20 °C/min and a solvent delay of 4.0 min). The incubation gave as major product (*m/z* 222) (–)-presilphiperfolan-8β-ol (**10**), that was identical in retention time and mass spectrum (Supplementary Figure 2) by direct GC-MS comparison with authentic **10** that had been isolated from *E. staecheifolium* (gift of Drs. Robert M. Coates and Juan A. Faraldos of the University of Illinois, Urbana, Illinois, USA). Both the GC-MS of enzymatically-generated **10** and the reference sample of **10** also exhibited <5% of the dehydration product presilphiperfol-7-ene (*m/z* 204), identical to the reference spectrum in the MassFinder database. This dehydration product was possibly generated during the GC-MS analysis. Chiral GC-MS analysis (Hydrodex-β-6-TBDM, 25 m × 0.25 mm, using a temperature program of 50–200 °C, a temperature gradient of 10 °C/min and a solvent delay of 4.0 min) confirmed that the enzymatic reaction product **10** was a single enantiomer identical to authentic (–)-**10**.

The same procedure was used for the incubation of BcBOT2 protein with [1,1-²H₂]-FPP and GC-MS analysis of the resulting [5,5-²H₂]-presilphiperfolan-8-ol (**10**) (*m/z* 224).

Incubation of BcBOT2 Protein with FPP and with [1,1-²H₂]-FPP and NMR Analysis

Purified BcBOT2 protein (1 μM) was incubated with FPP (60 μM) in 500 μl of assay buffer, overlaid with 100 μl of HPLC-grade pentane at 37 °C for 2 h. The reaction mixture was extracted with an equal volume of HPLC-grade dichloromethane. The combined organic extract was dried over Na₂SO₄ and concentrated. The product was purified through a mini-SiO₂ gel column (0.5 × 7 cm, 2:1 pentane/dichloromethane) and concentrated. The ¹H NMR spectrum of the purified sample of **10** was recorded in C₆D₆ (Supplementary Figure 3). The same procedure was also carried out using [1,1-²H₂]-FPP (60 μM) to give [5,5-²H₂]-presilphiperfolan-8-ol (**10**) (Supplementary Figures 4 and 5). The original ¹H NMR assignments (21) of the H-5 protons of **10** to the doublets at δ 2.25 and 1.30 were revised and reassigned by ¹H-¹H COSY NMR, as well as NOESY, HSQC, and HMBC analysis.

Kinetic Assay of Recombinant BcBOT2

Variable substrate [1-³H]-FPP (0.5 to 35 μM; 77 Ci/mol) was incubated with 0.09 nM purified BcBOT2 protein in 1 ml of assay buffer. The assay mixtures, overlaid with 1.2 ml of hexanes, were incubated at 30 °C for 20 min. The reactions were terminated by the addition of 75 μl of 500 mM EDTA (pH 8.0) followed by immediate vortexing for 30 s. The organic layer was loaded onto a mini SiO₂ gel column (2 cm) in a Pasteur pipette and expelled with a stream of nitrogen into a scintillation vial containing 7 ml of Opti-Fluor. The samples were extracted further with two 1-ml portions of diethyl ether, and the extracts were applied to the same mini column, which was further washed with 750 μl of diethyl ether. The tritium content of the combined extracts was determined by liquid scintillation counting. Kinetic constants were determined by using Kaleidagraph software and fitting the data to the Michaelis-Menten equation by non-linear least-squares regression, which gave the values of $K_m = 6.04 \pm 0.98$ μM, $k_{cat} = 3.23 \pm 0.15$ s⁻¹ (Supplementary Figure 6). Reported standard deviations in the steady-state kinetic parameters represent the calculated statistical errors in the non-linear, least squares regression analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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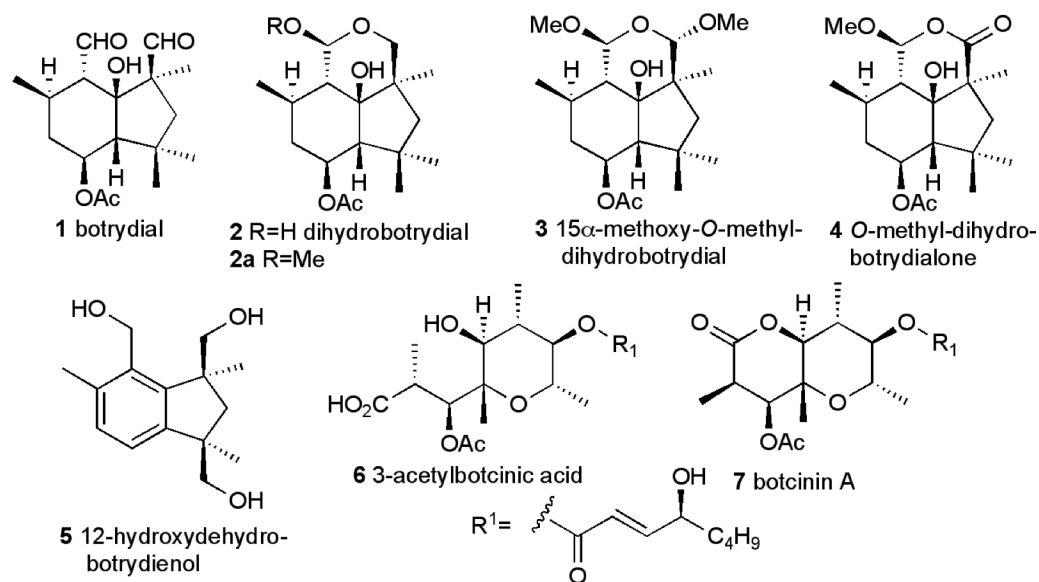


Figure 1.
Metabolites isolated from *B. cinerea* strains and mutants.

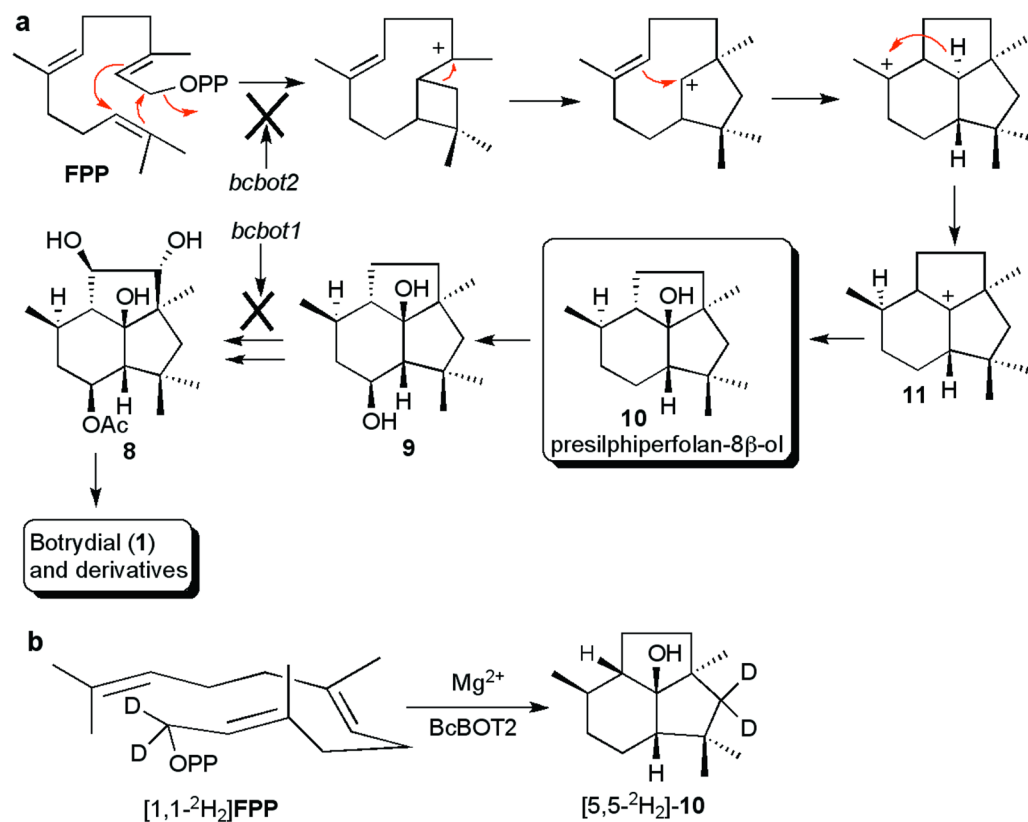


Figure 2. Biosynthesis of botrydial by *Botrytis cinerea*. **a.** Cyclization of farnesyl diphosphate (FPP) to presilphiperfolan-8 β -ol (**10**) catalyzed by the BcBOT2 protein and biosynthesis of botrydial from **10**. *B. cinerea* mutant *bcbot1* does not form botrydial and other late stage intermediates but does accumulate **9** and other probotryanes, while mutant *bcbot2* is blocked in the entire pathway. **b.** Cyclization of [1,1-²H₂]farnesyl diphosphate to [5,5-²H₂]presilphiperfolan-8 β -ol.

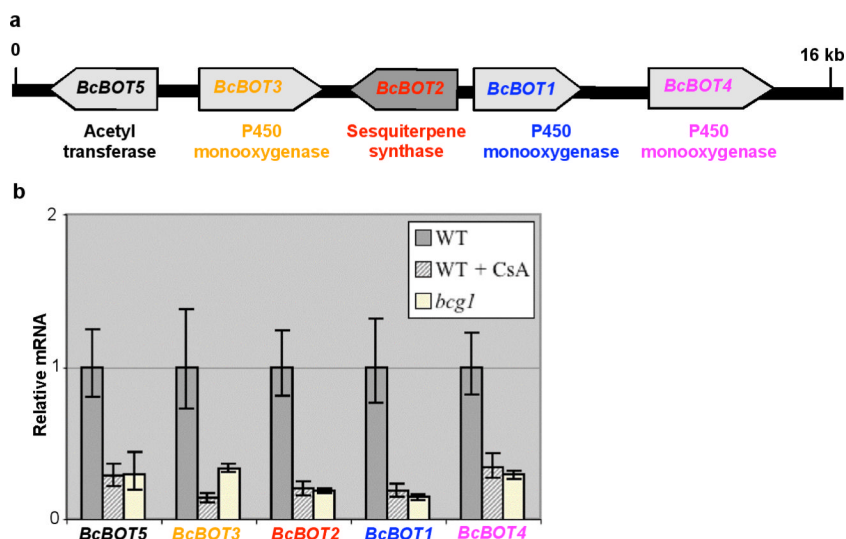


Figure 3. Physical cluster of co-regulated genes of *Botrytis cinerea* flanking the botrydial biosynthesis gene *BcBOT1*. **a.** Five open reading frames of the botrydial biosynthetic gene cluster (accession number at NCBI: AY277723). Putative functions predicted by protein sequence similarity are indicated under each gene. **b.** Down-regulation of the clustered genes in the presence of the calcineurin inhibitor cyclosporin A (10 μ g/ml) and in the *bcg1* Δ mutant (18). The expression level of each of the five clustered genes was analysed by qRT-PCR with the constitutively expressed elongation factor gene *EF1b* serving as a control (35).

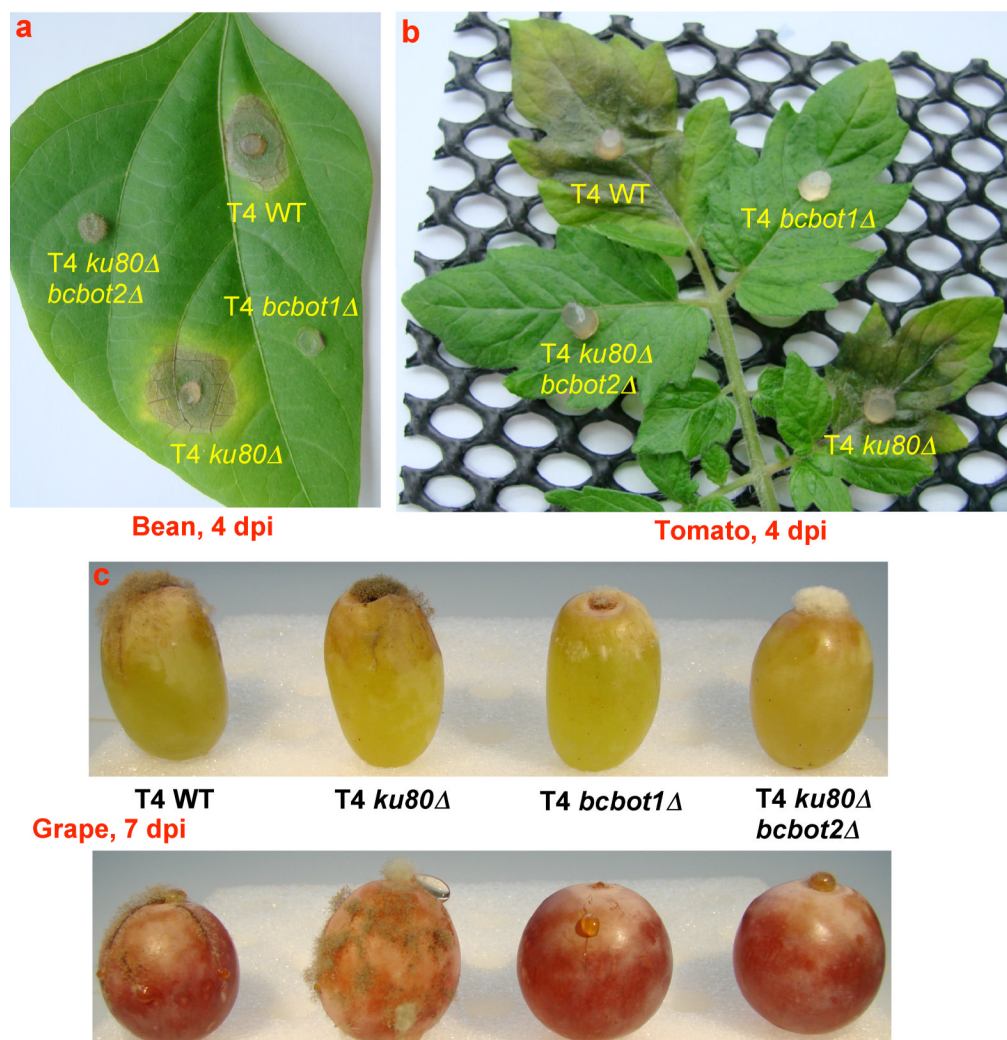


Figure 4. Pathogenicity assays of *Botrytis cinerea*. Wild-type T4 strains, recipient strains (*ku* mutants) and botrydial mutants on (a) bean leaves, (b) tomato leaves, and (c) grape berries. Leaves were inoculated with plugs of mycelium while the tops of the berries were inoculated with 10^3 conidia.

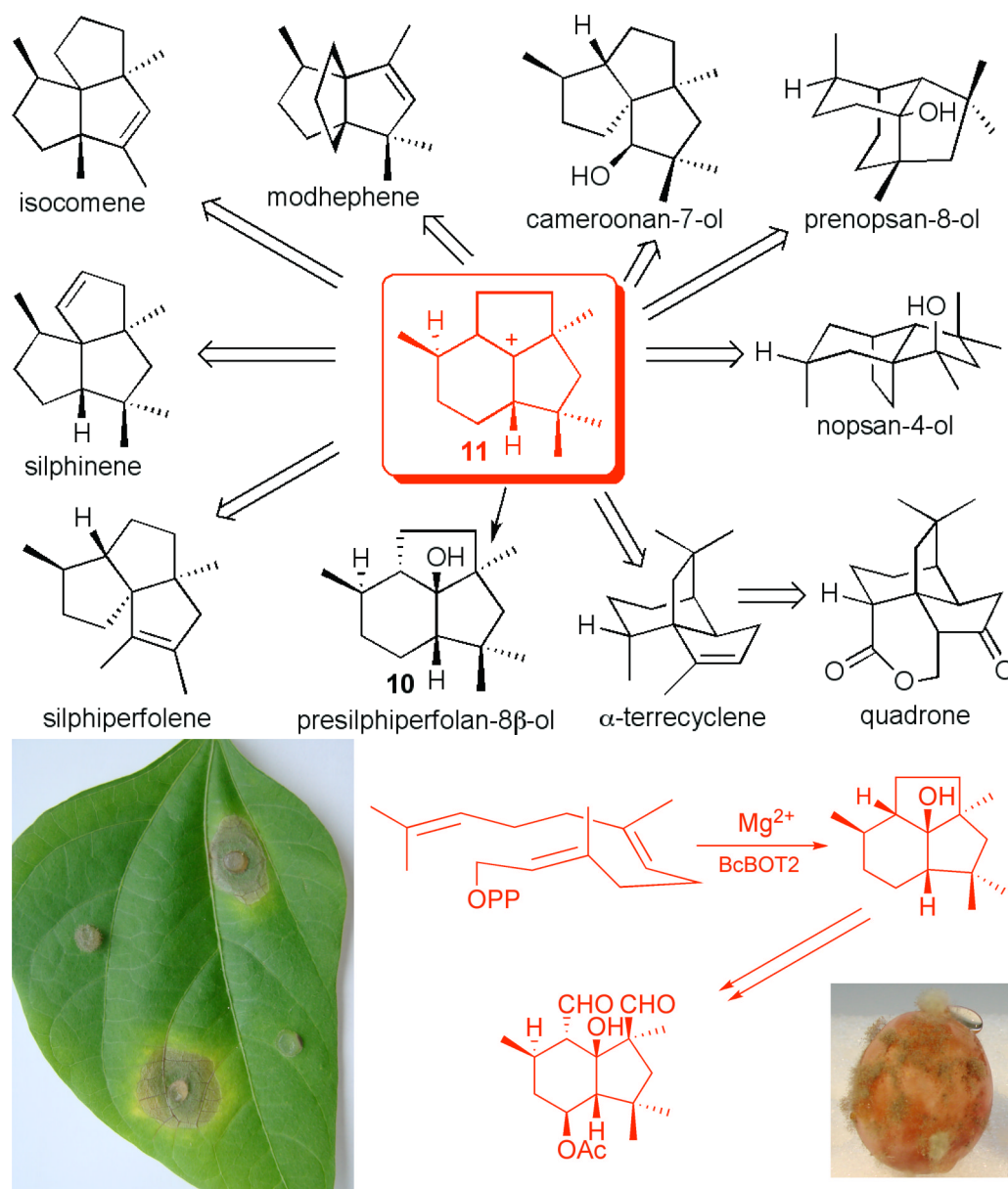


Figure 5. The presilphiperfolan-8-yl cation **11** is the likely precursor of a wide variety of triquinane and related sesquiterpenes from fungi and higher plants.

Metabolites identified in *Botrytis cinerea* wild-type strains, recipient strains (*ku* mutants) and *bcbot2* mutants (numbers correspond to compounds in Figure 1).

Table 1

Strain or mutant	Botryanes(mg/l)				Botcinic acid & derivatives (mg/l)		
	1	2	2a	3			
B05-10 wild-type	2	1	1.1	1.7	7	0.17	
B05-10 <i>ku70Δ</i>	1	2	2a	3	7		
	2	0.7	1	1.3	0.2		
B05-10 <i>ku70Δ bcbot2Δ-6</i>			none		6		7
B05-10 <i>ku70Δ bcbot2Δ BcBOT2</i>	1	2	2a	3	7		3
	1.7	0.7	1	2	0.2		
T4 wild-type	2		4	5	none		
	1.7		33	1			
T4 <i>ku80Δ</i>	2		4	5	none		
	2.3		30	1.7			
T4 <i>ku80Δ bcbot2Δ</i>			none		none		

Table 2

Virulence of *B. cinerea* strains on bean and tomato leaves illustrated in Figure 4. Values are means and standard deviation of at least 15 lesions resulting from 3 independent assays with 5 to 7 independent leaves per strain or mutant.

<i>B. cinerea</i> strain	Bean leaves, 5 dpi ^a : Lesion size (mm)	Tomato leaves, 4dpi: Lesion size (mm)
B05-10 WT	30.9±4.5	35.1±3.8
B05-10 <i>ku70Δ</i>	29.5±5.1	36.1±5.2
B05-10 <i>ku70Δ bcbot2Δ-6</i>	29.5±5.3	35.8±3.4
B05-10 <i>ku70Δ bcbot2Δ-12</i>	28.6±4.6	35.8±3.6
B05-10 <i>ku70Δ bcbot2Δ BcBOT2</i>	31.7±3.1	33.0±3.6
T4 WT	22.7±5.0	28.5±4.9
T4 <i>ku80Δ</i>	21.6±3.7	25.0±5.3
T4 <i>ku80Δ bcbot2Δ</i>	2.8±4.1	3.9±7.2
T4 <i>bcbot1Δ</i>	6.1±4.8	7.5±7.9

^a dpi: days post inoculation.