Regulation of Secondary metabolism in the gray mold fungus Botrytis cinerea

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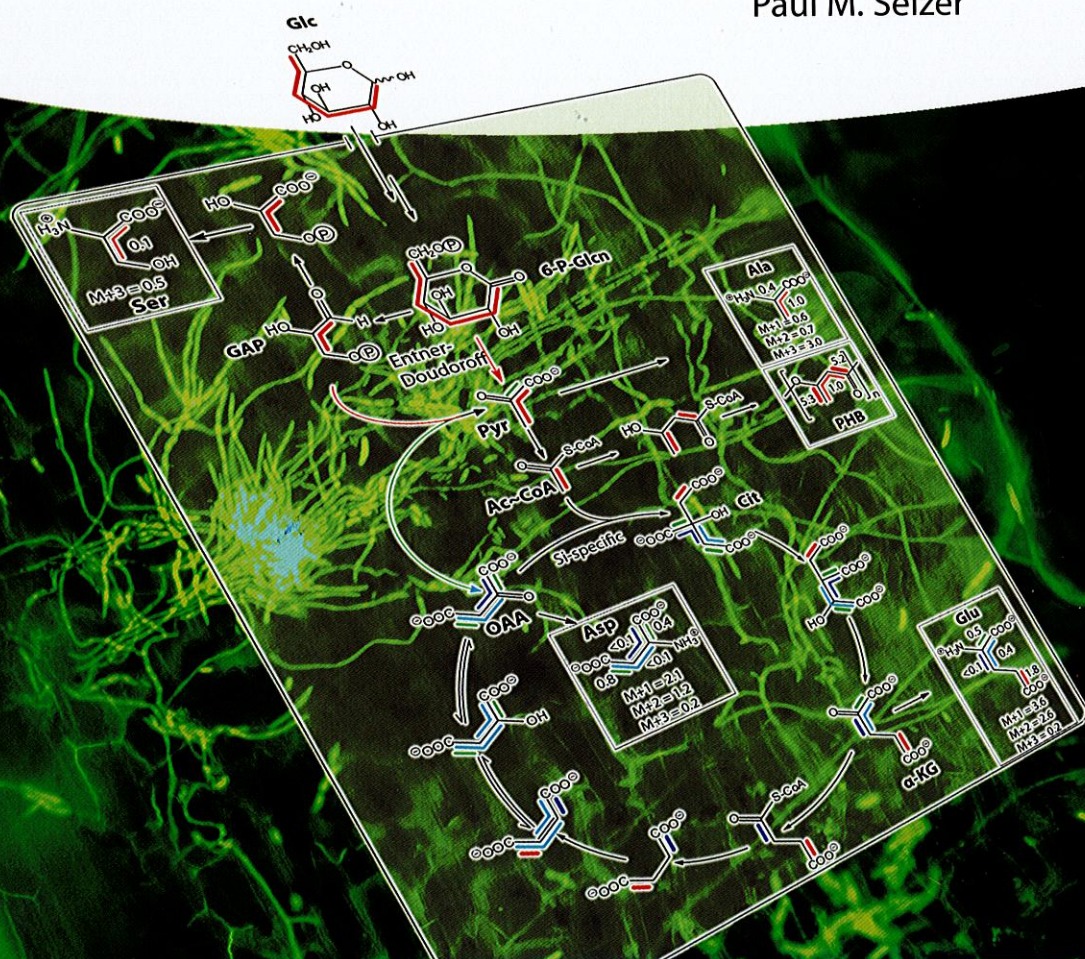
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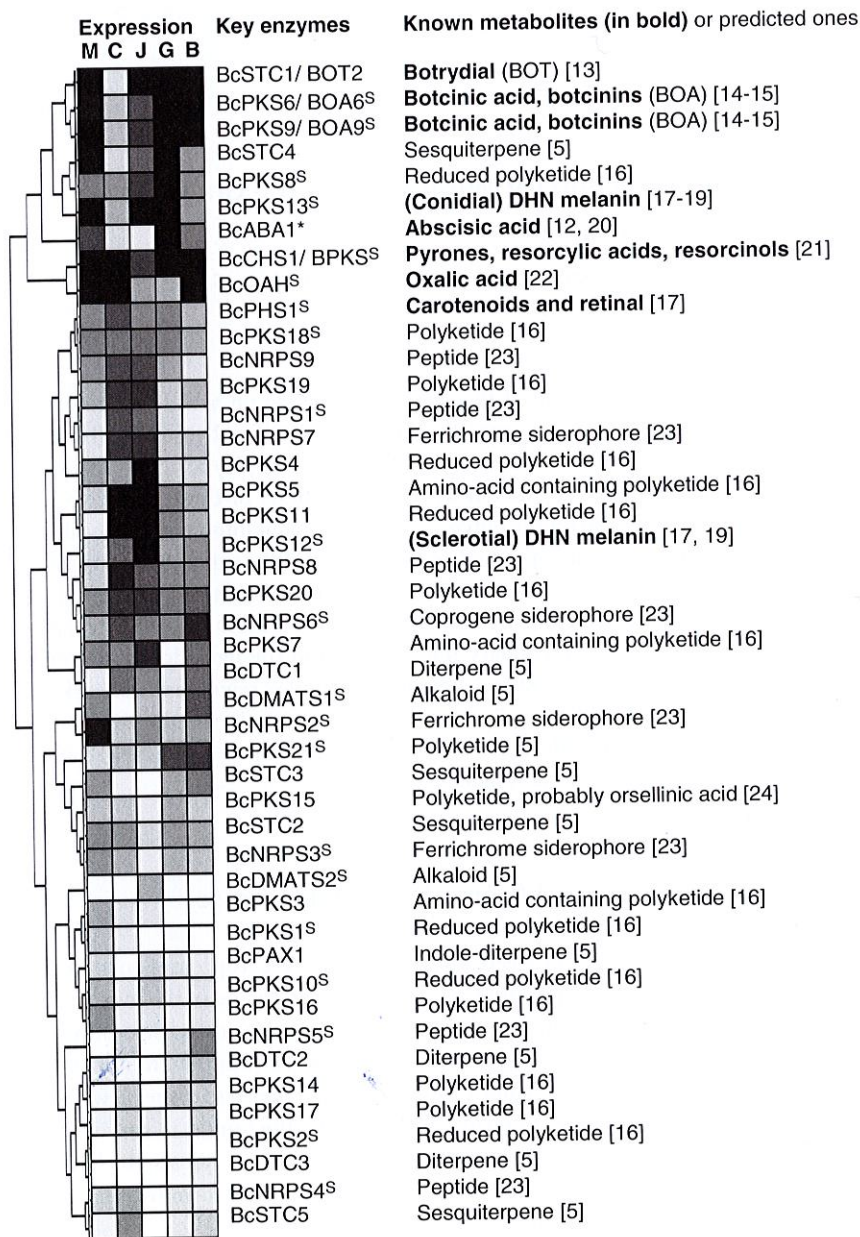Muriel Viaud*, Julia Schumacher, Antoine Porquier, and Adeline Simon

Abstract

Botrytis cinerea is responsible of the gray mold disease on a wide range of host plants. During the infection process, it produces unspecific phytotoxins (botrydial and botcinic acid) and other secondary metabolites that play a significant role in plant tissue colonization. Regulation of the biosynthesis of these bioactive compounds is therefore crucial for the outcome of fungus/plant interactions. Combining genetics and transcriptomic approaches, the role of different signaling cascades, transcription factors, and other regulators such as those belonging to the VELVET complex have recently been investigated. Overall, these studies suggest a strong link between the regulation of secondary metabolism and the light-dependent development in *B. cinerea*. Further knowledge of the complex regulatory network controlling secondary metabolism in *B. cinerea* is crucial to understand the interaction between *B. cinerea* and its different host plants and may help to define new strategies to control this crop-devastating fungus.

Botrytis cinerea, the Causal Agent of Gray Mold Disease

Botrytis cinerea Pers. Fr. causes severe pre- and postharvest crop losses on more than 200 host plants including important crops and is therefore one of the most studied phytopathogenic fungi [1]. This Ascomycete (Leotiomycetes class, Sclerotiniaceae family) is also named the gray mold agent because of the production of masses of melanized conidia on infected plants. Conidia allow *B. cinerea* to disperse widely on further plants while the ability to form sclerotia allows the fungus to survive in the soil for extended periods. These melanized survival structures can also be fertilized by microconidia from a strain of the opposing mating type to form fruiting bodies (apothecia) with sexual recombinants (ascospores) [2, 3].

   B. cinerea is considered a typical necrotroph, inducing host cell death and feeding on dead tissues. Thanks to an arsenal of plant cell wall – degrading enzymes, to

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Expression M C J G B	Key enzymes	Known metabolites (in bold) or predicted ones
	BcSTC1/ BOT2	**Botrydial (BOT)** [13]
	BcPKS6/ BOA6[S]	**Botcinic acid, botcinins (BOA)** [14-15]
	BcPKS9/ BOA9[S]	**Botcinic acid, botcinins (BOA)** [14-15]
	BcSTC4	Sesquiterpene [5]
	BcPKS8[S]	Reduced polyketide [16]
	BcPKS13[S]	**(Conidial) DHN melanin** [17-19]
	BcABA1*	**Abscisic acid** [12, 20]
	BcCHS1/ BPKS[S]	**Pyrones, resorcylic acids, resorcinols** [21]
	BcOAH[S]	**Oxalic acid** [22]
	BcPHS1[S]	**Carotenoids and retinal** [17]
	BcPKS18[S]	Polyketide [16]
	BcNRPS9	Peptide [23]
	BcPKS19	Polyketide [16]
	BcNRPS1[S]	Peptide [23]
	BcNRPS7	Ferrichrome siderophore [23]
	BcPKS4	Reduced polyketide [16]
	BcPKS5	Amino-acid containing polyketide [16]
	BcPKS11	Reduced polyketide [16]
	BcPKS12[S]	**(Sclerotial) DHN melanin** [17, 19]
	BcNRPS8	Peptide [23]
	BcPKS20	Polyketide [16]
	BcNRPS6[S]	Coprogene siderophore [23]
	BcPKS7	Amino-acid containing polyketide [16]
	BcDTC1	Diterpene [5]
	BcDMATS1[S]	Alkaloid [5]
	BcNRPS2[S]	Ferrichrome siderophore [23]
	BcPKS21[S]	Polyketide [5]
	BcSTC3	Sesquiterpene [5]
	BcPKS15	Polyketide, probably orsellinic acid [24]
	BcSTC2	Sesquiterpene [5]
	BcNRPS3[S]	Ferrichrome siderophore [23]
	BcDMATS2[S]	Alkaloid [5]
	BcPKS3	Amino-acid containing polyketide [16]
	BcPKS1[S]	Reduced polyketide [16]
	BcPAX1	Indole-diterpene [5]
	BcPKS10[S]	Reduced polyketide [16]
	BcPKS16	Polyketide [16]
	BcNRPS5[S]	Peptide [23]
	BcDTC2	Diterpene [5]
	BcPKS14	Polyketide [16]
	BcPKS17	Polyketide [16]
	BcPKS2[S]	Reduced polyketide [16]
	BcDTC3	Diterpene [5]
	BcNRPS4[S]	Peptide [23]
	BcSTC5	Sesquiterpene [5]

Figure 11.1 Repertoire of the predicted secondary metabolism key enzymes (KE) genes in *Botrytis cinerea* and their expression in different growth conditions. Conidial suspensions of the wild type strain B05.10 were incubated for 48 h on minimal medium (M), complete medium (C), grape Juice medium (J), grape berries (G; *Vitis vinifera*), or on bean leaves (B; *Phaseolus vulgaris*). Expressions (i.e., log2-normalized intensities) from NimbleGen array data were clustered and depicted by a color scale, where light gray represent weakly expressed genes and dark gray represent highly expressed genes. [S]Indicates that the KE is also present in the close species *Sclerotinia sclerotiorum* [5]. [*]The KE responsible for ABA synthesis remains unknown, so the gene encoding the BcABA1 P450 monooxygenase was included in the analysis [12].

phytotoxic small proteins and metabolites, and to the ability to encounter strong oxidative stress, the fungus can develop on many different host tissues under a wide range of environmental conditions [3–7]. *B. cinerea* is more destructive on mature or senescent tissues, but it can gain entry to these tissues at a much earlier stage and remain quiescent until the host physiology becomes favorable for its proliferation [3]. In some cases, the endophytic behavior of *B. cinerea* was clearly demonstrated [8, 9]. Finally, under specific environmental conditions in vineyards, *B. cinerea* may not cause gray mold but noble mold, which allows the production of sweet wines like Sauternes. In this case, the development of the fungus is limited to the outermost layers of grape epidermis [10]. In conclusion, *B. cinerea* encounters many different biotic and abiotic interactions and consequently modifies its metabolism and physiology to adapt to the altering environmental conditions.

Repertoire of Secondary Metabolites Produced by *B. cinerea*

Secondary metabolites (SMs) are expected to play an essential role in the versatile life of *B. cinerea* as described in the previous section. In the pregenomic era of this fungus, about eight families of SMs have been isolated from *in vitro* mycelium, but sequencing of the genome revealed a repertoire of gene clusters dedicated to the synthesis of approximately 40 different SMs [5, 11]. As in other fungi, these clusters include one (or two) gene(s) encoding the key enzyme (KE) responsible for synthesis of the raw product, genes encoding enzymes for further modifications and in some cases, genes encoding transporters and/or regulators. Among the 44 KEs (Figure 11.1) are many polyketide synthases (21 PKSs) [16, 21, 24], nonribosomal peptide synthetases (9 NRPSs) [23], and sesquiterpene cyclases (5 STCs) and diterpene cyclases (3 DTCs). Notably, only 21 of these KE-encoding genes are shared with the closest related genus *Sclerotinia* [5]. To date, only a small number of SMs have been identified and connected with the genetic make-up of *B. cinerea*. Among them are the predominant nonspecific toxins botrydial (BOT) and botcinic acid (BOA) (Figure 11.2) and other SMs that may be involved in plant/fungus interactions:

- *Botrydial (BOT)* and related compounds are sesquiterpenoids [26]. The discovery of the genetic basis for BOT biosynthesis included the identification of a cluster of coregulated genes [13, 27]. Deletion analyses of *bcbot1, bcbot3,*

bcbot4 encoding cytochrome P450 monooxygenases and *bcbot2/bcstc1* encoding a STC proved their requirements for BOT biosynthesis [13, 25] (I.G. Collado *et al.*, unpublished). BOT would trigger plant cell death via induction of the hypersensitive response [28].

- *Botcinic acid (BOA)* and its derivatives (botcinins) are combined polyketides [29, 30]. The cluster of coregulated genes (*bcboa1-17*; Figure 11.2) includes two PKS-encoding genes essential for BOA biosynthesis: BcBOA6 mediates the formation of the tetraketide core, whereas BcBOA9 is responsible for the synthesis of the side chain [14, 15]. Analysis of mutants lacking the ability to produce BOT, BOA, or both toxins suggested a redundant role in plant tissue colonization [14]. Additionally, several botcinins would have antifungal activities [29, 30].

- *Abscisic acid (ABA)* is a plant hormone and belongs to the group of sesquiterpenoids [31]. While in higher plants, its biosynthesis is mediated via the carotenoid (CAR) pathway, *B. cinerea* synthesizes ABA directly from farnesyl diphosphate via different oxidative steps. The *B. cinerea* KE gene responsible for the first step of ABA biosynthesis remains unknown but a cluster of four genes (*bcaba1-4*) involved in the later steps was characterized [12, 20]. To date, no evidence exists that fungus-derived ABA influences fungus–host interactions (V. Siewers and P. Tudzynski, unpublished).

- *Oxalic acid (OA)* is a compound that is produced via the oxaloacetate acetylhydrolase (OAH) in *B. cinerea* and *Sclerotinia sclerotiorum*. Loss of OA formation affects virulence in closely related fungi in different ways: while it results in avirulent mutants in *S. sclerotiorum*, *bcoahA* mutants are still able to colonize the host tissue [22, 32, 33].

B. cinerea also produces several pigments that can act as protectants against UV and other exogenous stresses (oxidative stresses, desiccation):

- *1,8-Dihydroxynaphthalene (DHN)-melanin* is a polyketide derivative accumulating in the conidiophores, conidia, and sclerotia of *B. cinerea*, giving them their characteristic color [34, 35]. In contrast to other DHN-melanin forming fungi, *B. cinerea* possesses two developmentally regulated PKS (KE)-encoding genes (*bcpks12, bcpks13*), while the other melanogenic genes are present in single copies [17]. Recently, it was shown that BcPKS12 and BcPKS13 provide the precursors for sclerotial and conidial melanogenesis, respectively [18, 19].

- *Carotenoids (CARs)* are tetraterpenoids produced by plants and fungi to protect cells from free radicals and singlet oxygen [36]. A cluster of four light-induced genes (*bop2-bcphd1-bcphs1-bccao1*) was recently identified in *B. cinerea* [17] that shares similarity with the CAR gene cluster in *Fusarium fujikuroi* suggesting that *B. cinerea* produces CAR via a similar biosynthetic pathway than *F. fujikuroi* [37].

- *Bikaverin (BIK)*, a red pigment, is a well-known polyketide in some *Fusarium* species [38] but that is also produced by rare strains of *B. cinerea* due to a probable horizontal gene transfer [39, 40].

The repertoire of KEs (Figure 11.1) suggests that *B. cinerea* can also produce several siderophores [23] and many other SMs that remain to be chemically identified.

(a)   Botrydial (sesquiterpene)

Botcinic acid (polyketide)

(b)   French bean, 3 dpi   Grape, 10 dpi
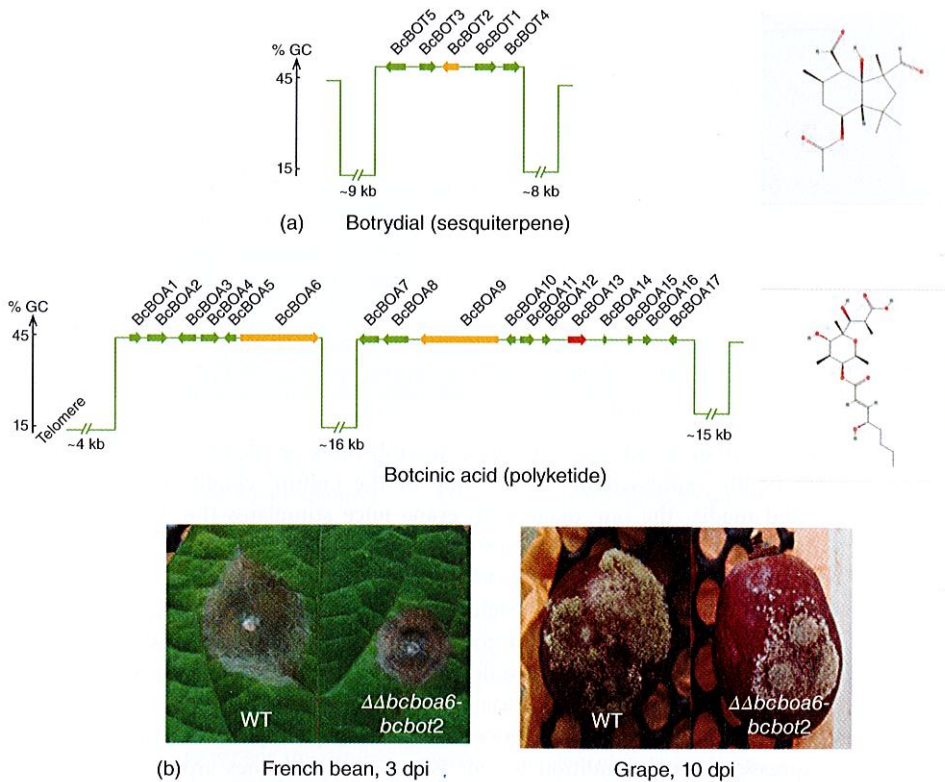
Figure 11.2 The two toxins botcinic acid (BOA) and botrydial (BOT) are produced from clusters of co-regulated genes and have a redundant role in virulence. (a) BOT and BOA clusters predicted in the wild strain B05.10 [13, 14] and surrounding AT-rich regions (J. van Kan *et al.*, unpublished); chemical structures of BOT and BOA (PubChem). The KE for BOT synthesis is the Sesquiterpene cyclase (STC) encoded by *bcbot2/stc1*. Other co-regulated genes encode for P450 monooxygenases (*bcbot1, 3,* and *4*) also involved in BOT synthesis [12] (I. G. Collado *et al.*, unpublished) and a putative acetyl transferase (*bcbot5*). Two KEs are required for BOA synthesis: the polyketide synthases (PKSs) *bcbot6/pks6* and *bcboa9/pks9* [14, 15]. Other co-regulated genes encode for putative monooxygenases (*bcboa2, 3, 4,* and *7*), dehydrogenases (*bcboa5* and *17*), a FAD-binding protein (*bcboa8*), a dehydratase (*bcboa16*), a thioesterase (*bcboa10*), a transferase (*bcboa11*), and unknown proteins (*bcboa12, 14,* and *16*). A pathway-specific Zn(II)$_2$Cys$_6$ transcription factor (TF) is encoded by *bcboa13* and a Nmr-A like regulator is putatively encoded by *bcboa1*. (b) While both single mutants Δ*bcboa6* and Δ*bcbot2*, impaired in BOA and BOT production, respectively, are not altered in virulence compared to the wild type (WT) strain (not shown), the double ΔΔ*bcboa6-bcbot2* mutant, unable to produce any toxin, causes significant smaller necrotic lesions on several host plants. Here, conidial suspensions were inoculated on leaves of *Phaseolus vulgaris* (French bean) and *Vitis vinifera* (grape) berries.

Importantly, the property to produce the mentioned SMs varies among *B. cinerea* wild strains. ABA and BIK are only formed by few strains [12, 40], and not all wild strains produce OA, BOT, and BOA [41, 42].
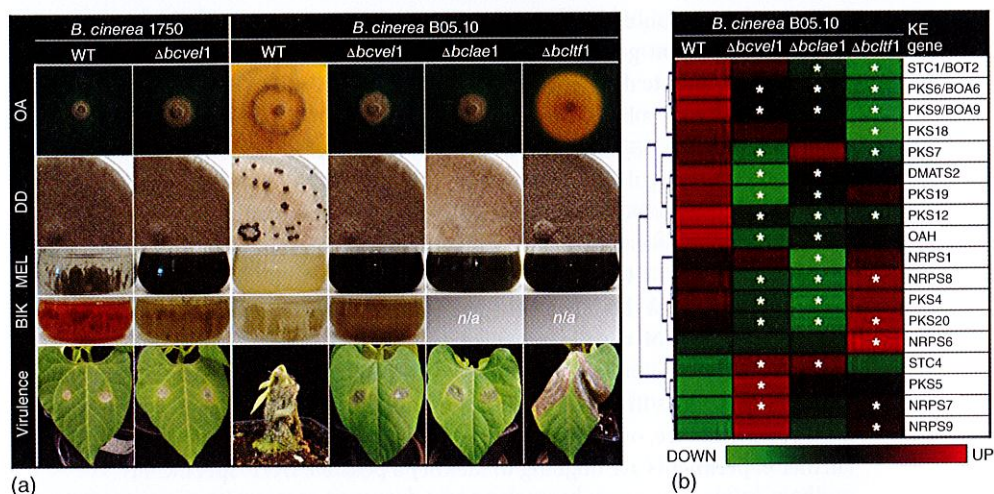
Expression of SM Genes during *In Vitro* and *In Planta* Conditions

In the past years, several genome-wide expression studies were designed to investigate the transcriptome of *B. cinerea* either during saprophytic growth on various culture media or during the infection process on different host plants. Figure 11.1 presents the expression patterns of the SM KE-encoding genes of the model strain B05.10 after 2 days of growth in three *in vitro* conditions *versus* two *in planta* conditions. The media used were minimal medium (**M**) [43], complete medium (**C**) [17], and grape juice medium (**J**) [44]. The *in planta* conditions are grape berries (**G**) [43] and bean leaves (**B**) [41] both at 48- h postinfection. Figure 11.1 highlights several features about the regulation of SM in *B. cinerea*:

- About half of the KE-encoding genes (mainly those on the top of the Figure 11.1) are highly expressed in at least one of the culture conditions. Among the tested media, the one made with grape juice stimulates the highest number of KE-encoding genes (16 genes highly expressed). This medium with a high sugar content (about $200 \, g \, l^{-1}$), a low pH (around 4), and the presence of plant compounds was originally designed to mimic the conditions that the fungus encounters during infection of grape berries [44] and then chosen to compare the expression of SM genes in different regulation mutants (see Section 11.5 and Figure 11.3).
- In opposite, five of the KE-encoding genes (e.g., *bcpks2* or *bcdtc3*) are not expressed in any condition tested. Whether these genes are functional in *B. cinerea* remains to be elucidated.
- Finally, the clustering shows that BOT and BOA KE-encoding genes have similar expression patterns. This coregulation includes also the other *bcbot* and *bcboa* genes presented in Figure 11.2. These data indicate that the biosynthesis of both toxins is activated during plant tissue colonization and in grape juice medium but also in synthetic minimal medium. The moderate expression under *in vitro* conditions has allowed for the identification of the underlying biosynthetic pathways [45].

Overall, the transcriptomic data indicate that, on one hand, the SM genes belonging to one cluster are tightly coregulated [13, 14, 20] and, on the other hand, almost each cluster has its own expression pattern when we consider the different growth conditions. It is known from other fungi that the regulation of SM gene clusters occurs at several levels, that is, by pathway-specific transcription factors (TFs) that are usually encoded by a cluster gene, by broad domain TFs that integrate environmental signals such as the availability of carbon and nitrogen sources and the ambient pH, and by other global regulators that could affect gene expression via chromatin remodeling [46–48]. Recent advances regarding *B. cinerea* are presented in the following sections.

Figure 11.3 panel (a): phenotypes of deletion mutants (rows OA, DD, MEL, BIK, Virulence) for *B. cinerea* 1750 (WT, Δbcvel1) and *B. cinerea* B05.10 (WT, Δbcvel1, Δbclae1, Δbcltf1). The BIK row for Δbclae1 and Δbcltf1 is marked n/a.

Panel (b): *B. cinerea* B05.10 comparative gene expression heat map.

WT	Δbcvel1	Δbclae1	Δbcltf1	KE gene
		*	*	STC1/BOT2
	*	*	*	PKS6/BOA6
	*	*	*	PKS9/BOA9
			*	PKS18
	*		*	PKS7
	*	*		DMATS2
	*	*		PKS19
	*	*	*	PKS12
	*	*		OAH
		*		NRPS1
	*		*	NRPS8
	*	*		PKS4
	*		*	PKS20
			*	NRPS6
	*	*		STC4
	*			PKS5
	*		*	NRPS7
			*	NRPS9

DOWN ▭ UP

(a)   (b)

Figure 11.3 Members of the VELVET complex (BcVEL1, BcLAE1) and the light-responsive transcription factor BcLTF1 control light-dependent differentiation, secondary metabolism, and virulence. (a) Phenotypes of the deletion mutants in wild strain 1750 (BIK producer) and the standard recipient strain B05.10. OA – detection of oxalic formation by acidification of the culture medium 6 dpi (bluish green: pH >7, yellow pH <6). DD – differentiation phenotype 12 dpi in constant darkness; the WT:B05.10 produces sclerotia while the other strains produce conidia. MEL – accumulation of DHN melanin in liquid cultures 7 dpi (minimal medium with $NaNO_3$ as nitrogen source). BIK – accumulation of bikaverin in liquid cultures 7 dpi (minimal medium with NH_4NO_3 as nitrogen source). Virulence – lesion formation on primary leaves of *Phaseolus vulgaris* (French bean) 6 dpi. (b) Comparative gene expression studies of the mutants. Conidial suspensions of the wild type B05.10 and the three deletion mutants were incubated for 48 h on solid grape juice medium with cellophane overlays. Material from four biological replicates were used for hybridization of NimbleGen arrays. Statistical analyses revealed 18 KE-encoding genes that are differentially expressed in at least one mutant (*: fold change >2; p <0.05). Relative expression values of these genes (i.e., log2-normalized intensities scaled by gene) were clustered and depicted by color scale, in which shades of green and red represent under- and overexpressed genes, respectively. GEO accession GSE63021 and (A. Simon et al., unpublished).

Pathway-Specific Regulation by Transcription Factors

Many fungal SM gene clusters contain a gene that encodes a Zn(II)2Cys6 zinc finger TF that specifically regulates the adjacent biosynthetic genes [48]. In *B. cinerea*, 40% of the SM clusters, including the BOA one, contain a gene encoding such a candidate TF [5, 14]. The function of the candidate *bcboa13* (Figure 11.2) was recently investigated by gene deletion and expression analysis. Results indicated that BcBOA13 regulates genes that are part of the BOA cluster but not other SM genes such as those of the BOT cluster, suggesting that BcBOA13 is indeed a BOA-specific TF (A. Porquier et al., unpublished). Another pathway-specific TF identified in *B. cinerea* is the Zn(II)2Cys6 protein BcRUM1 (regulator of unknown metabolite).

Bcrum1 inactivation demonstrated that this TF is required for the co-expression of the four adjacent genes that are all possibly involved in the biosynthesis of an unknown metabolite during the early stages of the infection process [49].

As opposed to the BOA cluster, the predicted BOT cluster lacks a TF-encoding gene (Figure 11.2) [13]. Therefore, a yeast one-hybrid (YOH) approach was developed to identify regulators of the BOT genes [44]. The bidirectional promoter of *bcbot1/bcbot2* was used as bait for screening a library containing 396 out of 406 *B. cinerea* TFs, revealing a physical interaction of the BOT promoter with a Cys2His2 TF (BcYOH1). A global transcriptomic analysis of the Δ*bcyoh1* mutant further revealed that this TF is not specific to the BOT cluster but is rather involved in the regulation of a wide range of genes. The expression of 22 SM clusters including the BOT and BOA ones appeared BcYOH1 dependent. Moreover, the expression of genes involved in other processes such as carbohydrate metabolism, transport, virulence, or detoxification was also affected in the Δ*bcyoh1* mutant [44]. Further experiments are ongoing to identify a possible BOT-specific TF.

Concerted Regulation of Secondary Metabolism and Light-Dependent Development

A prominent global regulator of fungal SM is the putative methyltransferase LaeA (loss of aflR expression) that was first identified in *Aspergillus nidulans* as a regulator of SM gene clusters [50, 51]. Later, LaeA was shown to form a protein (VELVET) complex with the fungal regulatory proteins VeA and VelB that coordinates development and secondary metabolism in response to light in *A. nidulans* and other filamentous ascomycetes [52–54]. Light is also an important environmental cue for *B. cinerea* as it triggers the formation of macroconidia and represses the formation of sclerotia. Like *A. nidulans*, *B. cinerea* contains a VELVET complex composed of the orthologs BcVEL1 (VeA), BcVEL2 (VelB), and BcLAE1 (LaeA) whereby BcVEL1 functions as the bridging partner by interacting with both BcVEL2 and BcLAE1 [55]. General features of Δ*bcvel1* and Δ*bclae1* mutants are the "always conidia" phenotype (conidiation in light and darkness accompanied by the loss of sclerotia formation), increased production of conidial/BcPKS13-derived melanin, loss of OA formation, deregulation of several SM-related genes, and reduced virulence on bean and tomato (Figure 11.3) [41, 55]. The deletion of the VelB ortholog BcVEL2 results in a similar phenotype to that of Δ*bcvel1* regarding differentiation and virulence [56], which is in accordance with the interaction found between these two proteins. The outstanding role of BcVEL1 as the central part of the VELVET complex that links development and virulence is furthermore supported by the finding that mutations of this gene (single nucleotide polymorphisms causing stop codons) were identified in two wild strains (T4, 1750) showing the "always conidia" phenotype and reduced aggressiveness on the host [40, 41]. The latter strain (1750) belongs to the group of the BIK producers; and by targeted deletion of the whole *bcvel1* in this genetic background it was shown that the formation of the red pigment depends on the VELVET complex (Figure 11.3) [40].

Recently, the light-responsive TF1 (BcLTF1) was identified by a random mutagenesis approach. As deletion mutants of the VELVET complex, the Δ*bcltf1* mutant exhibits hyper-conidiation accompanied by increased production of conidial/BcPKS13-derived melanin and loss of sclerotia formation (Figure 11.3). However, in contrast to the other mutants, Δ*bcltf1* mutants massively suffer from oxidative stress and are therefore severely impaired in growth in the light. Microarray analyses were performed to compare the transcriptional responses to 60 min of light treatment between undifferentiated mycelia of the wild type and the mutant demonstrating that BcLTF1 is required for proper regulation of the majority of light-responsive genes and additionally for the regulation of SM-related genes in a light-independent manner [17]. However, the question remains open whether BcLTF1 directly affects SM by its function as a TF or whether the effect is indirect and possibly due to changes in primary metabolism and/or the cellular redox status.

For the direct comparison of the impacts of BcVEL1, BcLAE1, and BcLTF1 on gene expression, a microarray approach was performed using grape juice medium and the three deletion mutants (Figure 11.3). Even under this condition, the deletion of *bcltf1* affected the expression profile of 11 KE-encoding genes; six genes are underexpressed and five genes are overexpressed compared to the wild type. *Bcpks13* is also overexpressed in the mutant, but to a lesser extent than on complete medium [17]. This is because *bcpks13* is already strongly expressed in the wild type on grape juice medium compared to the complete medium (Figure 11.1). The deletions of both VELVET complex members affect the expression levels of several KE-encoding genes; the profiles were similar but not identical. Thus, *bcpks7* is underexpressed in Δ*bcvel1* and Δ*bcltf1* but not in Δ*bclae1*, and *bcnrps1* is underexpressed in Δ*bclae1* only [55] (J. Schumacher *et al.*, unpublished).

BcWCL1 ("white collar"-like 1) is an example for a broad-domain TF that allows for the integration of light signals due to a blue light-sensing LOV (light-oxygen-voltage) domain. Deletion mutants exhibit hyper-conidiation as well but are not affected in OA formation and virulence in standard illumination conditions. Moreover, the mutants are partially "blind": expression of certain light-responsive genes is no longer induced by light [57]. Recent genome-wide expression analyses revealed that a couple of KE-encoding genes are differentially expressed in the mutant, for example, *bcpks13* (conidial melanin) is overexpressed while *bcphs1* (CARs and retinal) is not induced in response to light treatment in the mutant (J. Schumacher *et al.*, unpublished).

The strong interdependence of SM and light-dependent development in *B. cinerea* is furthermore supported by identification of other mutants that are affected in both processes. For instance, the deletion of the bZIP-TF BcATF1 results in increased accumulation of aerial hyphae, reduced conidiation, loss of sclerotia formation, and increased production of BOA and BOT, which is accompanied by slightly increased colonization efficiencies on different host plants. Though BOA- and BOT-encoding genes are underexpressed in the mutant, no physical interactions between their promoters and BcATF1 could be established by yeast one-hybrid experiments, which suggests an indirect effect on SM-related gene expression [58].

Regulation of Secondary Metabolism by Conserved Signal Transduction Pathways

Another level of regulation of SM is mediated by conserved signal transduction pathways that comprise cAMP (cyclic adenosine monophosphate) and Ca^{2+} as second messengers and further include Ras superfamily proteins and mitogen-activated protein (MAP) kinases. These pathways may regulate broad domain and pathway-specific TFs. The key components of the pathways including the three MAP kinases BMP1 (FUS3/KSS1 ortholog), BMP3 (SLT2 ortholog) and BcSAK1 (HOG (high osmolarity glycerol) ortholog), the adenylate cyclase BAC and the protein kinase A (PKA) that are part of the cAMP cascade, and the calcineurin phosphatase (CN) as one of the main actors of the Ca^{2+} signaling pathway. Heterotrimeric G proteins that act as upstream elements have also been functionally studied in *B. cinerea* with regard to development, responses to different kinds of stresses as well as virulence [59, 60]. However, not much is known how these signaling pathways influence SM. The deletions of the three MAP kinases affect the production of melanin, which is accompanied by defects in proper differentiation of conidia and/or sclerotia [61]. A recent study revealed impact of the stress-activated MAP kinase BcSAK1 on the formation of the known phytotoxins: BOT, BOA, and its derivatives are produced in much lower quantities in axenic cultures of $\Delta bcsak1$ mutants compared to the wild type [62]. The same effect was found in deletion mutants of the Gα subunit BCG1. As mutants of the cAMP pathway, that is, Δbac and $\Delta bcpka1$ mutants are not impaired in the formation of toxins [63], a second downstream pathway of BCG1 has been hypothesized. This turned out to be the Ca^{2+}/CN pathway that is linked with the heterotrimeric G protein *via* a phospholipase C (BcPLC1) [64]. The inhibition of CN by cyclosporine A and the deletion of components of the latter pathway such as the CN regulator BcRCN1 and the CN-responsive TF BcCRZ1 resulted in decreased expression levels of BOT- and BOA-biosynthetic genes [27, 66]. More recently, the contribution of Ca^{2+}-dependent signal transduction to the regulation of the carotenogenic genes has been revealed; their expression levels are induced by Ca^{2+} addition, suggesting a link between Ca^{2+}- and light-/BcWCL1-dependent signaling (J. Schumacher *et al.*, unpublished).

Role of the Chromatin Landscape in the Regulation of Secondary Metabolism?

Active TFs are not always sufficient to express SM genes as clusters can be embedded within transcriptionally silent heterochromatin. Chromatin-based regulation through histone acetylation and methylation has recently been shown to play a significant role in the regulation of SM clusters in *A. nidulans* and other model fungi [47, 48, 67]. In *B. cinerea*, the occurrence of such mechanisms has not yet been shown, but several relevant clues are already available.

Firstly, some of the clusters are in particular genomic environments. The whole genome sequence of *B. cinerea* was recently assembled into 16 core chromosomes

(J. van Kan *et al.*, unpublished) and this revealed that six SM gene clusters including the BOA one are located in subtelomeric regions. *bcboa1* is even the closest gene to one of the telomeres of chromosome 1. Studies in other fungi have shown that these regions are more often subjected to chromatin-mediated regulation [67]. In addition, both BOT and BOA clusters are surrounded by several kb of AT-rich regions (>80%; Figure 11.2) [14] (J. van Kan *et al.*, unpublished). In the case of the BOA cluster, a 16 kb-stretch of AT-rich sequence is even present inside the cluster between *bcboa6* and *bcboa7*. Again, such AT-rich regions may correspond to heterochromatin structure and may be under the control of histone modifiers as demonstrated for effector genes that are specifically expressed during infection in *Leptosphaeria maculans* [68]. The fact that the clusters encoding the two main phytotoxins of *B. cinerea* share similar AT-rich surrounding regions raises questions about the importance of the role of these genomic features in chromatin structure and gene expression.

Secondly, the characterization of several regulators in *B. cinerea* revealed a possible involvement of chromatin-mediated regulation. The mode of action of the global regulator LaeA mentioned above remains enigmatic, but they are some evidences *in A. nidulans* that it counteracts some silencing heterochromatic marks [69].

Another example is the transcriptional regulator BcREG1 that is required for conidiogenesis, production of BOA and BOT, and colonization of the host tissues [70]. The mode of action of BcREG1 remains obscure, but a recent study on the ortholog SGE1 in *Fusarium fujikuroi* suggests that these regulators may act on the chromatin level [71].

Finally, the mutation of *bcspt3*, encoding a component of the multisubunit SAGA (Spt-Ada-Gcn5-acetyltransferase) complex, resulted in an altered brownish/yellowish pigmentation suggesting that the disruption of the SAGA complex may affect the repertoire of expressed SM genes in *B. cinerea* [72]. The SAGA complex is required to activate transcription of a subset of RNA polymerase II-dependent genes by modifying the histones, especially in response to environmental stresses [73, 74].

Genetic studies on histone modifiers are currently in progress to investigate the role of chromatin modifications (methylation and acetylation) in the regulation of secondary metabolism in *B. cinerea* (J. Schumacher *et al.*, unpublished; A. Porquier *et al*, unpublished).

Concluding Remarks

Although many actors of the regulation of the secondary metabolism in *B. cinerea* have been identified in the past years, important studies remain to be conducted to understand the complex regulatory network from the upstream environmental signals to the downstream regulators that directly interact with SM biosynthetic gene clusters to allow the production of bioactive compounds. This knowledge is crucial to understand the development and virulence of *B. cinerea* and may provide useful start points for the identification of new biological targets to control

this crop-devastating fungus. This knowledge could also provide approaches to "wake up" silent SM clusters during *in vitro* growth and discover new bioactive compounds. Possible approaches include genetic engineering (e.g., overexpression of pathway-specific TFs, modification of the chromatin landscape) and co-culturing with other microorganisms [47, 74]. In addition to their activities toward plants, new SMs isolated from *B. cinerea* could have interesting toxic activities toward other organisms (fungi, oomycetes, insects, bacteria, nematodes, etc.) that can be exploited in biotechnology. Indeed, as more fungal genome sequences are becoming available and more SM biosynthetic genes are being identified, fungi re-emerge as important resources for new therapeutic and agrochemical agents [75, 76].

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