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The botrydial biosynthetic gene cluster of *Botrytis cinerea* displays a bipartite genomic structure and is positively regulated by the putative $Zn(II)_2Cys_6$ transcription factor BcBot6

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ABSTRACT

Botrydial (BOT) is a non-host specific phytotoxin produced by the polyphagous phytopathogenic fungus *Botrytis cinerea*. The genomic region of the BOT biosynthetic gene cluster was investigated and revealed two additional genes named *Bcbot6* and *Bcbot7*. Analysis revealed that the G + C/A + T-equilibrated regions that contain the *Bcbot* genes alternate with A + T-rich regions made of relics of transposable elements that have undergone repeat-induced point mutations (RIP). Furthermore, BcBot6, a $Zn(II)_2Cys_6$ putative transcription factor was identified as a nuclear protein and the major positive regulator of BOT biosynthesis. In addition, the phenotype of the *ΔBcbot6* mutant indicated that BcBot6 and therefore BOT are dispensable for the development, pathogenicity and response to abiotic stresses in the *B. cinerea* strain B05.10. Finally, our data revealed that *B. pseudocinerea*, that is also polyphagous and lives in sympatry with *B. cinerea*, lacks the ability to produce BOT. Identification of BcBot6 as the major regulator of BOT synthesis is the first step towards a comprehensive understanding of the complete regulation network of BOT synthesis and of its ecological role in the *B. cinerea* life cycle.

1. Introduction

Filamentous fungi are well-known producers of so-called secondary metabolites (SMs) or natural products, many of which confer either beneficial or detrimental properties. For example, the polyketide lovastatin is widely used as an anti-cholesterol drug (Manzoni and Rollini, 2002) whereas aflatoxin is carcinogenic and is a threat for both humans and animals (Hoffmeister and Keller, 2007). These molecules, generally of low molecular weight, can be considered as conditional for fungal growth and their production is often linked to developmental programs (Calvo et al., 2002). In most cases, their role is unknown but some studies demonstrate their importance for protecting fungi *e.g.* against predation (Rohlfs and Churchill, 2011) or to survive in hostile environment (Eisenman and Casadevall, 2012). Unique features of the genes involved in SM biosynthesis is their genomic co-localization (Smith et al., 1990; Keller and Hohn, 1997; Hoffmeister and Keller, 2007) as well as their co-regulation (Yin and Keller, 2011).

To be able to finely respond to different stimuli, fungi have evolved sophisticated gene regulation mechanisms. In particular, the ability of fungi to produce SMs during orchestrated time and in different environments implies complex hierarchical regulatory mechanisms. Around 60% of known fungal secondary metabolism gene clusters possess a pathway specific transcription factor (TF; Brakhage, 2012). In general, those proteins belong to the $Zn(II)_2Cys_6$ TF family and specifically regulate the genes of their corresponding cluster (Knox and Keller, 2015).

In addition to a specific type of regulators, broad domain TFs are involved in the regulation of fungal genes involved in SMs produced in response to environmental stimuli (*e.g.* pH, carbon and

Abbreviations: SM, secondary metabolite; BOT, botrydial; BOA, botcinic acid; PKS, polyketide synthase; TF, transcription factor; TE, transposable element; RIP, repeat-induced point mutation; GFP, green fluorescent protein; HPLC, High-pressure liquid chromatography, NMR, Nuclear Magnetic Resonance.

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nitrogen source or redox status) (Brakhage, 2012). One example is the GATA TF AreA that regulates different clusters in diverse fungal species according to the available nitrogen source (Tudzynski et al., 1999; Kim and Woloshuk, 2008; Tudzynski, 2014).

Even though the action of TFs is necessary to regulate fungal SM genes, it is not always sufficient. Indeed, chromatin is a dynamic structure that can switch from the transcriptionally permissive euchromatin (opened state) to the repressive heterochromatin (condensed state) and *vice versa* (Palmer and Keller, 2010). This process is mediated by post-translational modifications of histone residues such as the trimethylation of the lysine 9 residue of histone 3 (H3K9me3) deposited by DIM-5 in *Neurospora crassa* (Tamaru and Selker, 2001). The presence of H3K9me3 mark is often associated with A + T-rich sequences that result through a process known as RIP for Repeat-Induced Point mutations (Hane et al., 2015). This pre-meiotic defense system aims to protect the genome from invasive elements such as transposable elements (TEs) or viruses by mutating C to T bases within duplicated sequences.

The putative methyltransferase LaeA is another important global regulator of SM in fungi is the putative methyltransferase LaeA. This nuclear protein interacts with VeA and VelB to form the fungal specific Velvet complex that mediates development and SM genes expression in response to light (Bayram et al., 2008). The mode of action of LaeA is currently unknown but a study in *Aspergillus nidulans* by Reyes-Dominguez et al. (2010) suggests that it might counteract the establishment of the repressive H3K9me3 mark and thereby have an impact on chromatin structure.

Botrytis cinerea is an Ascomycetous necrotrophic fungus that induces gray mold disease on more than 1400 plant species (Elad et al., 2016). This fungus is considered as one of the most important fungal plant pathogens and causes important economic losses on grapevine and many other fruits and flowers (Dean et al., 2012). Numerous SMs have been isolated from *B. cinerea* fermentation cultures (Collado et al., 2007; Collado and Viaud, 2016) including two non-host specific phytotoxins: the sesquiterpene botrydial (BOT) and its derivatives (botryanes) as well as the polyketide botcinic acid (BOA) and the structurally related botcinins. The genes involved in the production of both BOT and BOA are organized in clusters within the *B. cinerea* genome. The BOT gene cluster contains five genes encoding biosynthesis enzymes. *Bcbot2* encodes a sesquiterpene cyclase which is the first fungal key enzyme demonstrated to be able to convert farnesyl pyrophosphate into presilphiperfolan-8-beta-ol (Pinedo et al., 2008). *Bcbot1*, *Bcbot3* and *Bcbot4* encode cytochrome P450 proteins. Finally, *Bcbot5* encodes a putative acetyl transferase probably also involved in the biosynthesis of the toxin (Siewers et al., 2005; Collado and Viaud, 2016). The BOA cluster is composed of sixteen predicted genes with two encoding the polyketide synthase (PKS) key enzymes (namely *Bcboa6* and *Bcboa9*), ten encoding enzymes putatively involved in the further steps of the biosynthesis and two encoding putative regulators: a putative NmrA-like regulator (*Bcboa1*) and a putative specific regulator encoding a $Zn(II)_2Cys_6$ TF (*Bcboa13*) (Dalmais et al., 2011).

Both BOT and BOA are phytotoxic. BOT is produced during infection *in planta* (Deighton et al., 2001) and has been shown to induce chlorosis symptoms typical of the gray mold when applied exogenously (Rebordinos et al., 1996; Colmenares et al., 2002). Dihydrobotrydial and other derivatives also display phytotoxic activity but to a lesser extent than BOT (Durán-Patrón et al., 1999; Colmenares et al., 2002; Collado and Viaud, 2016). In the same manner, exogenous application of BOA and derivatives leads to chlorosis and necrosis on different plant hosts underlying their phytotoxic activity (Cutler et al., 1993, 1996). Inactivation of both *Bcbot2* and *Bcboa6* and the subsequent absence of BOT and BOA production showed that these toxins are involved in the colonization of plant tissues (Dalmais et al., 2011). Nevertheless, their mode of action remains enigmatic and only scarce information is available regarding their regulation.

To date it has been demonstrated that both BOA and BOT gene clusters are under the control of two signal transduction pathways. The first one cascade involves BCG1 α subunit of a heterotrimeric G protein, phospholipase C (BcPlc1), calcineurin phosphatase and the downstream C_2H_2 TF BcCrz1 (Viaud et al., 2003; Pinedo et al., 2008; Schumacher, 2008a, 2008b; Dalmais et al., 2011). The second is the stress-induced MAP kinase cascade (Heller et al., 2012) with the downstream bZIP TF BcAtf1 (Temme et al., 2012) and the BcReg1 transcriptional regulator (Michielse et al., 2011). In addition, it was demonstrated that *B. cinerea* secondary metabolism is greatly influenced by light-dependent development (Viaud et al., 2016). In *B. cinerea*, the perception of light triggers the production of macroconidia and represses the formation of sclerotia. This developmental program involves the members of the Velvet complex BcVel1, BcVel2 and BcLae1 (the orthologs of VeA, VelB and LaeA, respectively) (Schumacher et al., 2012, 2015; Yang et al., 2013). The deletion of the Velvet partners leads to abnormal constant production of conidia even in sclerotia-inducing conditions *i.e.* darkness, increase in conidial melanin (Schumacher, 2016), loss of oxalic acid formation and reduced virulence. In addition, sixteen SM gene clusters are affected by either deletion of *Bcvel1* or *Bclae1* or by both. In particular, the BOT and BOA gene clusters were shown to be positively regulated by BcLae1.

The gene deletion of all the regulators presented above results in pleiotropic phenotypes and does not point to specific regulation of the biosynthesis of the BOT and BOA phytotoxins. As introduced above, the gene *Bcboa13* located in the BOA gene cluster is predicted to encode a $Zn(II)_2Cys_6$ TF and is likely a pathway specific regulator (Dalmais et al., 2011). In contrast, no gene encoding a putative regulator has been identified to date in the BOT gene cluster. To find a BOT specific regulator, Simon et al. (2013) constructed a Yeast One Hybrid library containing nearly all *B. cinerea* TFs (396 out of the 406 predicted TFs). The screening of the library using the bidirectional promoter of *Bcbot1-2* as bait led to the identification of the C_2H_2 TF BcYoh1. A global transcriptomic analysis of the *ΔBcyoh1* mutant revealed this TF to be a global transcriptional regulator not only acting on the BOT and other SM gene clusters but also on carbohydrate metabolism, transport, virulence and detoxification mechanisms.

Until this report, the existence of a BOT-specific TF was still hypothetical. The publication of the complete *de novo* assembly of the WT strain B05.10 allowed us to identify a putative $Zn(II)_2Cys_6$ TF-encoding gene located adjacent to known *Bcbot* genes. In this study, we aimed to functionally characterize this gene and to investigate its presumed regulatory role on the BOT gene cluster. Through gene inactivation, the function of BcBot6 as a positive regulator of BOT production was examined. Moreover, we explored the genomic region containing *Bcbot* genes for A + T-rich regions/islands and the presence of RIPed TEs. Finally, we questioned the role of BOT in fungal development, virulence and response to abiotic stresses through a combination of genetic and molecular approaches.

2. Material and methods

2.1. Cultivation of B. cinerea strains

The *Botrytis cinerea* Pers.: Fr. model strain B05.10 was used as a recipient strain for genetic modifications (Quidde et al., 1998). Routine cultivation of the strains was carried out on rich medium (RM; 20 g/L malt extract, 5 g/L yeast extract and 15 g/L agar) supplemented, for mutant strains, with hygromycin B (Sigma-Aldrich; 100 μg/ml) or nourseothricin (Werner Bioagents; 80 μg/