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LaeA negatively regulates dothistromin production in the pine needle pathogen *Dothistroma septosporum*

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ABSTRACT

In filamentous fungi both pathway-specific and global regulators regulate genes involved in the biosynthesis of secondary metabolites. LaeA is a global regulator that was named for its mutant phenotype, loss of *aflR* expression, due to its effect on the aflatoxin-pathway regulator AflR in *Aspergillus* spp. The pine needle pathogen *Dothistroma septosporum* produces a polyketide virulence factor, dothistromin, that is chemically related to aflatoxin and whose pathway genes are also regulated by an ortholog of AflR. However, dothistromin biosynthesis is distinctive because it is switched on during early (rather than late) exponential growth phase and the genes are dispersed in six loci across one chromosome instead of being clustered. It was therefore of interest to determine whether the function of the global regulator LaeA is conserved in *D. septosporum*. To address this question, a LaeA ortholog (*DsLaeA*) was identified and its function analyzed in *D. septosporum*. In contrast to aflatoxin production in *Aspergillus* spp., deletion of *DsLaeA* resulted in enhanced dothistromin production and increased expression of the pathway regulatory gene *DsAflR*. Although expression of other putative secondary metabolite genes in *D. septosporum* showed a range of different responses to loss of DsLaeA function, thin layer chromatography revealed increased levels of a previously unknown metabolite in *DsLaeA* mutants. In addition, these mutants exhibited reduced asexual sporulation, germination and hydrophobicity. Our data suggest that although the developmental regulatory role of DsLaeA is conserved, its role in the regulation of secondary metabolism differs from that of LaeA in *A. nidulans* and appears to be species specific. This study provides a step towards understanding fundamental differences in regulation of clustered and fragmented groups of secondary metabolite genes that may shed light on understanding functional adaptation in secondary metabolism.

1. Introduction

Filamentous fungi produce a diverse array of secondary metabolites that do not normally have a role in growth and development but can contribute towards fitness factors such as defense, communication, niche adaptation and UV protection (Trienens et al., 2010; Amare and Keller, 2014). Fungal secondary metabolite genes are usually clustered and transcriptionally co-regulated by a combination of pathway-specific and global regulator proteins. For example, the pathway-specific transcription factor AflR regulates expression of genes in aflatoxin clusters in *Aspergillus flavus* and *A. parasiticus* and the sterigmatocystin cluster of *A. nidulans* (Chang et al., 1993; Fernandes et al., 1998). Some global regulators orchestrate regulation of both secondary metabolism and fungal development in response to environmental cues (Butchko et al., 2012; Calvo and Cary, 2015) by means of chromatin modification (Strauss and Reyes-Dominguez, 2011; Brakhage, 2013).

The global regulator gene *laeA* (named for loss of *aflR* expression) is predicted to encode a methyltransferase (Bok and Keller, 2004; Sarikaya Bayram et al., 2010). Identification and characterisation of *laeA* in *A. nidulans* was an important milestone to better understand global secondary metabolite gene regulation in filamentous fungi (Bok and Keller, 2004) and its crucial function in secondary metabolism has now been documented for many fungi (reviewed by Jain and Keller, 2013). Most of the initial studies were done in *Aspergillus* spp., in which *laeA* deletion mutants showed marked reductions in production of secondary metabolites such as sterigmatocystin, aflatoxin, gliotoxin and endocrocin, (Bok and Keller, 2004; Amaike and Keller, 2009; Bok et al., 2005; Lim et al., 2012). LaeA was also shown to control both asexual and sexual development in *A. nidulans* (Sarikaya Bayram et al., 2010).

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A role of LaeA in development was also demonstrated in *Fusarium fujikuroi* (Wiemann et al., 2010), *Cochliobolus heterostrophus* (Wu et al., 2012), *A. flavus* (Amaike and Keller, 2009), *Trichoderma reesei* (Seiboth et al., 2012), *Monascus ruber* (Liu et al., 2016) and *Alternaria alternata* (Takao et al., 2016).

LaeA occurs as part of a protein complex with the light-regulated developmental regulatory proteins VeA and VelB in *A. nidulans* (Bayram et al., 2008). In the presence of light, LaeA is required for proper asexual development whereas VeA and VelB are also required for sexual fruiting body development in dark conditions (Bayram and Braus, 2012). A similar type of protein complex interaction involving LaeA, VeA and VelB was shown in other fungi such as *Fusarium* spp., *Penicillium chrysogenum*, *Botrytis cinerea* (Wiemann et al., 2010; López-Berges et al., 2013; Hoff et al., 2010; Schumacher et al., 2015).

LaeA has also been shown to have a role in virulence of pathogenic fungi. For example an *A. flavus laeA* mutant was significantly impaired in the ability to degrade host cell lipid reserves in peanut and also showed reduced host cell colonization compared to wild type strains (Amaike and Keller, 2009). Similarly, *laeA* mutants of *F. fujikuroi* showed reduced virulence on rice (Wiemann et al., 2010), and those of the Dothideomycete pathogens *C. heterostrophus* and *A. alternata* had reduced pathogenicity in maize and tomato, respectively, compared to the wild type (Wu et al., 2012; Takao et al., 2016).

In this study we characterised the LaeA ortholog of another Dothideomycete, the forest pathogen *Dothistroma septosporum*. This fungus is the main causal agent of Dothistroma needle blight, one of the most devastating diseases of pine trees worldwide (Watt et al., 2009; Drenkhan et al., 2016). The impact of *D. septosporum* on forest health has increased substantially in recent years, particularly in the Northern hemisphere (Drenkhan and Hanso, 2009; Woods et al., 2005). Climate change has been implicated in the increased severity of the disease (Woods, 2011), leading to efforts to understand the pathogen and its interaction with its host at the molecular level (Bradshaw et al., 2016). Identification of key molecules such as secondary metabolites that are involved in pathogenesis, and understanding how the production of these molecules is regulated may lead to new methods of disease management.

D. septosporum produces dothistromin, a polyketide that is chemically similar to a precursor of aflatoxin (AF) and sterigmatocystin (ST) (Gallagher and Hodges, 1972). Dothistromin is a virulence factor required for expansion of necrotic disease lesions (Kabir et al., 2015a) and certain aspects of the genetics of dothistromin biosynthesis are unusual. Whilst fungal secondary metabolite genes are generally clustered (Keller and Hohn, 1997), dothistromin biosynthetic genes are instead arranged at six separate loci spread across a 1.3-Mb chromosome (Chettri et al., 2013; Bradshaw et al., 2013). Furthermore, dothistromin is mainly produced during early exponential phase in culture (Schwelm et al., 2008), instead of during late exponential and stationary phases as seen for other fungal secondary metabolites, in keeping with high levels of dothistromin production during periods of rapid fungal biomass accumulation *in planta* (Kabir et al., 2015b). Due to its unique gene arrangement and timing of biosynthesis we were interested to determine whether LaeA has a role in regulation of the fragmented dothistromin gene cluster.

To date the importance of LaeA proteins in regulation and coordination of secondary metabolism and development has not been examined in a forest foliar pathogen. As a first step towards achieving our goal we genetically characterised the LaeA ortholog in *D. septosporum* and showed its role in governing morphology and development. We provide evidence that LaeA could act as a negative regulator of secondary metabolism in this fungus.

2. Materials and methods

2.1. Fungal strain and culture conditions

Wild-type *D. septosporum* NZE10 was used throughout this work. Cultures were grown at 22 °C on Dothistroma medium (DM), Dothistroma sporulation medium (DSM) or pine needle extract minimal medium (PMMG) (Chettri et al., 2012). For growth in liquid media the cultures were inoculated with 1×10^5 spores per ml in 125 ml flasks containing 25 ml of media and shaken at 180 rpm for seven days. To study the effect of light and dark the growth chamber had three Sylvania GRO-LUX (F30w/GRO-TB) and two Philips-lifemax (TLD-30W/840 cool white) lights, used with continuous illumination (light conditions) or with culture flasks double wrapped in foil (dark conditions).

2.2. Identification of Dothistroma septosporum LaeA (DsLaeA)

To confirm the identity of the previously reported *D. septosporum* LaeA ortholog (de Wit et al., 2012), BLASTP and reciprocal BLASTP were performed using the LaeA protein sequence from *A. nidulans* (accession number AAQ95166). To further confirm its authenticity phylogenetic analysis was done using additional putative or confirmed LaeA orthologs from other species. Phylogenetic analysis was done using MEGA 5.0 with default parameters (Tamura et al., 2011).

2.3. Manipulation of the DsLaeA gene in Dothistroma septosporum

Genomic DNA was isolated from *D. septosporum* using a CTAB method (Moller et al., 1992). A *DsLaeA* gene knockout construct, pOscLaeA, was made via One Step Construction of Agrobacterium-Recombination-ready-plasmids (OSCAR) using PCR-based methods described previously (Paz et al., 2011). Vectors pA-HYG OSCAR and pOSCAR were purchased from the Fungal Genetics Stock Center (http://www.fgsc.net). The pOscLaeA knockout construct was made such that 1035 bp of *DsLaeA* coding region (i.e. nucleotides 1803893 – 1805158 of *D. septosporum* Scaffold 3; http://genome.jgi.doe.gov/Dotse1/Dotse1.home.html) was replaced by a hygromycin resistance gene, flanked with 1250 bp and 1850 bp of 5′ and 3′ *DsLaeA* flanking regions to guide targeted integration. All primers used for PCR are listed in Supplementary Table 1. *D. septosporum* NZE10 was transformed with pOscLaeA using methods described previously (Bradshaw et al., 2006) and transformants were single-spore purified.

To complement the phenotype of the *D. septosporum* LaeA replacement strain KO1 with *DsLaeA*, co-transformation was done with a 1:1 ratio of *DsLaeA* coding sequence flanked by 1 kb upstream and downstream sequence (i.e. nucleotides 1802651 to 1806194 of *D. septosporum* scaffold 3), and the linearized vector pBCphleo (pR224) that confers phleomycin resistance. Transformants were selected on 10 μg/ml phleomycin and were single-spore purified. Confirmation of *DsLaeA* gene replacement was determined by PCR and Southern hybridisation of *AvaI*-digested DNA with a digoxigenin (DIG)-labeled probe encompassing DsLaeA 3′ flank and hph gene regions, following the protocol described earlier (Bradshaw et al., 2006). The copy number of *DsLaeA* gene in the complemented strains was determined as previously described (Chettri et al., 2015).

To determine if CfLaeA, the LaeA ortholog of *Cladosporium fulvum*, a close relative of *D. septosporum*, is functionally orthologous to DsLaeA, the *D. septosporum DsLaeA* KO1 mutant was transformed with a plasmid (pR224) containing the phleomycin resistance gene and a 3.1 kb genomic DNA fragment containing the *CfLaeA* gene (nucleotides 11511–14650 of *C. fulvum* scaffold