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Evolution of polyketide synthesis in a Dothideomycete forest pathogen

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ABSTRACT

Fungal secondary metabolites have many important biological roles and some, like the toxic polyketide aflatoxin, have been intensively studied at the genetic level. Complete sets of polyketide synthase (PKS) genes can now be identified in fungal pathogens by whole genome sequencing and studied in order to predict the biosynthetic potential of those fungi. The pine needle pathogen *Dothistroma septosporum* is predicted to have only three functional PKS genes, a small number for a hemibiotrophic fungus. One of these genes is required for production of dothistromin, a polyketide virulence factor related to aflatoxin, whose biosynthetic genes are dispersed across one chromosome rather than being clustered. Here we evaluated the evolution of the other two genes, and their predicted gene clusters, using phylogenetic and population analyses. *DsPks1* and its gene cluster are quite conserved amongst related fungi, whilst *DsPks2* appears to be novel. The DsPks1 protein was predicted to be required for dihydroxynaphthalene (DHN) melanin biosynthesis but functional analysis of DsPks1 mutants showed that *D. septosporum* produced mainly dihydroxyphenylalanine (DOPA) melanin, which is produced by a PKS-independent pathway. Although the secondary metabolites made by these two PKS genes are not known, comparisons between strains of *D. septosporum* from different regions of the world revealed that both PKS core genes are under negative selection and we suggest they may have important cryptic roles *in planta*.

1. Introduction

Many well-characterized fungal secondary metabolites (SMs) are polyketides, such as aflatoxin, fumonisin, and T-toxin, which have polyketide synthases as a core enzyme (Minto and Townsend, 1997; Proctor et al., 1999; Yang et al., 1996). In efforts to discern the capacity of fungi to produce additional SMs, genome sequences have been mined for the presence of key genes such as non-ribosomal peptide synthases and polyketide synthases (PKS). Such screens have shown a high level of diversity in the types of PKS genes and, consequently, in the polyketide products predicted to be made by each species (Li et al., 2016). They have also revealed variation in PKS gene number between species. For example between one and 37 PKS genes per genome were identified amongst 75 Dothideomycetes (Noar and Daub, 2016).

In general, pathogens with a necrotrophic or hemibiotrophic lifestyle have larger numbers of secondary metabolite core genes then biotrophs (de Wit et al., 2012; Spanu et al., 2010). However the hemibiotrophic pine needle pathogen *Dothistroma septosporum* has only four PKS genes, one of which is a pseudogene. In contrast its close relative *Cladosporium fulvum*, a biotrophic pathogen of tomato, has eight intact PKS genes (Collemare et al., 2014; de Wit et al., 2012). However all of these are only expressed at very low levels *in planta*, except *PKS6* that encodes the cytotoxic anthraquinone cladofulvin (Griffiths et al., 2016, 2015). This finding lead to the suggestion that down-regulation of secondary metabolite biosynthetic pathways might be a mechanism for adaptation to a biotrophic lifestyle (Collemare et al., 2014).

Amongst the three intact *D. septosporum* PKS core genes (*DsPksA, DsPks1, DsPks2*), only *DsPksA* has been characterized so far. The DsPksA protein is required for biosynthesis of the mycotoxin dothistromin (Bradshaw et al., 2006), a virulence factor required for expansion of necrotic disease lesions in Dothistroma needle blight disease (Kabir et al., 2015). Its structure is similar to that of versicolorin B, a precursor of aflatoxin, and its synthesis involves 19 other genes in addition to *DsPksA*, many of which are orthologs of aflatoxin biosynthetic genes (Chettri et al., 2013). Dothistromin genes are not clustered as those for aflatoxin biosynthesis, but instead are dispersed between six loci spread

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across chromosome 12 (Chettri et al., 2013). Despite their dispersed arrangement, dothistromin genes are co-regulated by an ortholog of the aflatoxin pathway regulator, AflR (Chettri et al., 2013). Phylogenomic analyses of genes in the aflatoxin/sterigmatocystin/dothistromin family suggested the presence of a large ancestral gene cluster that preceded the diversification seen in Eurotiomycete and Dothideomycete fungi today (Bradshaw et al., 2013). A full set of dothistromin genes is present in the *C. fulvum* genome but two of the key biosynthetic genes (*HexA* and *Nor1*) are pseudogenised so this species does not make dothistromin, in keeping with its biotrophic lifestyle (Chettri et al., 2013; de Wit et al., 2012; van der Burgt et al., 2014).

Besides the dothistromin PKS gene, *D. septosporum* has only two other functional PKS genes. *DsPks1* was predicted to be involved in synthesis of a melanin-related product, whilst no functional predictions were made for *DsPks2* (de Wit et al., 2012). Both are expressed at significantly higher levels during the necrotrophic than biotrophic stage of growth *in planta* (Bradshaw et al., 2016). To define the complete set of PKS genes in *D. septosporum,* we analysed *DsPks1* and *DsPks2* using phylogenetics and comparative genomics. We also made predictions about whether these genes are associated with complete or fragmented gene clusters and performed preliminary functional analysis by generation of *DsPks1* mutants. This work is expected to help define a minimal set of PKS genes required for a hemibiotrophic fungal pathogen and to shed light on their evolutionary history.

2. Materials and methods

2.1. PKS gene and protein sequence analyses

Nucleotide and predicted amino acid sequences for PKS genes from *D. septosporum* strain NZE10 were obtained from the Joint Genome Institute (JGI) (http://genome.jgi.doe.gov/Dotse1). Domain predictions were made using PKS/NRPS Analysis (Bachmann and Ravel, 2009) and confirmed using Structure Based Sequence Analysis of Polyketide Synthases (SBSPKS) (Anand et al., 2010) and NCBI domain finder (Marchler-Bauer et al., 2015). For inter-specific phylogenetic analyses of DsPks1 and DsPks2, best FASTA hits (Pearson and Lipman, 1988) were obtained from each of the species belonging to all fungi from the classes Dothideomycetes, Eurotiomycetes, and Sordariomycetes for which genome sequences were available in the JGI MycoCosm database (Grigoriev et al., 2012). Sequences confirmed by a best reciprocal FASTA hit back to the *D. septosporum* NZE10 protein models were selected as putative orthologs. Phylogenetic trees of DsPks1 and DsPks2 were built by aligning amino acid sequences using the Fast Fourier Transform (MAFFT) multiple alignment program with the E-INS-I set of parameters (Katoh et al., 2005), then trees built using the maximum likelihood method in PhyML software with default parameters (Guindon et al., 2010). The phylogenetic trees were visualized and shaded with the ETE3 toolkit (Huerta-Cepas et al., 2016).

2.2. Intraspecific sequence analyses

Intraspecific analyses of *DsPks1* and *DsPks2* genes were performed using genome sequences from 18 *D. septosporum* strains from different regions of the world (Supplementary Table 1). Illumina sequences for these strains are available from NCBI (accession numbers SAMN06689740 to SAMN06689757). To identify *DsPks1* and *DsPks2* sequences in those genomes, paired-end reads were mapped to the *D. septosporum* NZE10 reference genome, which was accessed through the JGI website (NZE10; http://genome.jgi.doe.gov/Dotse1/Dotse1.home.html) (de Wit et al., 2012) using Bowtie 2 (version 2.2.6) (Langmead and Salzberg, 2012) at between 36-80x coverage. The corresponding *DsPks1* and *DsPks2* sequences were imported into Geneious (version 8.0.3) (Kearse et al., 2012) for alignments, SNP identification and amino acid predictions.

For each PKS gene, all SNPs from each sample were concatenated and aligned. This alignment was then used to rebuild a phylogeny with PhyML. The obtained tree was used as a reference for computing dN/dS ratio and P-values for each site using SLR (Massingham and Goldman, 2005). The sites showing selection with a corrected P-value < 0.05 are reported.

For coalescent analysis, DNA sequences for each gene were imported into SNAP Workbench (Price and Carbone, 2005) and collapsed into haplotypes using SNAP Map (Aylor et al., 2006), excluding indels (insertions/deletions) and infinite sites violations. Genealogical inference (coalescent analysis) for each locus was obtained using Genetree (Bahlo and Griffiths, 2000) in the SNAP Workbench. This type of analysis only considers non-recombining partitions within each locus; therefore, any incompatible sites that related to recombination were manually removed. Convergence to the best tree was achieved by multiple independent runs of between 1000 and 10 million simulations, all assuming panmixia and constant population size.

2.3. Gene cluster analyses

To predict genes clustered near to the PKS core genes with potential roles in secondary metabolite production, 20 genes upstream and downstream of each PKS core gene were submitted for Antibiotics & Secondary Metabolite Analysis SHell (antiSMASH) analysis (Weber et al., 2015). Amongst the genes predicted to be part of a putative gene cluster, functions were predicted by a combination of gene ontology (GO) terms analysis of *D. septosporum* NZE10 gene sequences (Bradshaw et al., 2016) and manual InterProScan analysis (Jones et al., 2014). Finally, each of these candidate genes were added to the putative gene cluster if genes with similar predicted functions were found in other fungal PKS gene clusters.

2.4. Functional analysis of the DsPks1 gene by targeted gene knockout

The *DsPks1* gene was deleted by replacing it with a selectable marker in *D. septosporum* NZE10. A *DsPks1* gene knockout construct (pR414) was made via One Step Construction of Agrobacterium-Recombination-ready-plasmids (OSCAR) using PCR-based methods described previously (Paz et al., 2011) with genomic DNA template isolated from *D. septosporum* using a CTAB method (Moller et al., 1992). For pR414, 7283 bp (nucleotides 127,162–134,445 of *D. septosporum* NZE10 scaffold 10, containing the complete coding region of *DsPks1,* was replaced by a hygromycin resistance cassette, flanked with 1058 bp (5′) and 1021 bp (3′) of *DsPks1* flanking regions to guide targeted integration. *D. septosporum* NZE10 was transformed with pR414 using protoplast-mediated transformation methods described previously (Chettri et al., 2013) and transformants were single-spore purified prior to analysis. Confirmation of *DsPks1* gene replacement was determined by PCR (see Supplementary Fig. 6 for primers) and Southern hybridisation of *Pst1* digested DNA with a digoxigenin (DIG)-labeled probe encompassing *DsPks1* 3′flank and hph gene regions, following the protocols described earlier (Chettri et al., 2012).

2.5. Melanin extraction and characterisation

Melanin was extracted from both mycelia and culture filtrates from *D. septosporum* grown for 9 days in DM media (Bradshaw et al., 2000) at 22 °C with shaking at 200 rpm. Mycelia were collected by filtering through miracloth and ground with a mortar and pestle prior to extraction of melanin as described previously (Selvakumar et al., 2008; Zhang et al., 2015) except that non-hydrolysable residues obtained after precipitation with concentrated hydrochloric acid were successively treated with ethanol and chloroform to remove lipids. To detect melanin, the purified precipitates were dissolved in 1 ml of 0.1 N NaOH, alongside control melanin M8631 (Sigma-Aldrich, St. Louis, Missouri, USA) that was dissolved at a final concentration of 0.5 mg/ml. Ultraviolet–visible absorption spectra were determined using a NanoDrop