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# Early expression of aflatoxin-like dothistromin genes in the forest pathogen *Dothistroma septosporum*

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ABSTRACT

The forest pathogen *Dothistroma septosporum* produces the polyketide dothistromin, a mycotoxin very similar in structure to versicolorin B, a precursor of aflatoxin (AF). Dothistromin is a broad-range toxin and possibly involved in red-band needle blight disease. As the role of dothistromin in the disease is unknown the expression of dothistromin genes was studied to reveal clues to its function. Although the genes of AF and dothistromin biosynthesis are very similar, this study revealed remarkable differences in the timing of their expression. Secondary metabolites, like AF, are usually produced during late exponential phase. Previously identified dothistromin genes, as well as a newly reported versicolorin B synthase gene, *vbsA*, showed high levels of expression during the onset of exponential growth. This unusual early expression was also seen in transformants containing a green fluorescent protein (GFP) gene regulated by a dothistromin gene promoter, where the highest GFP expression occurred in young mycelium. Two hypotheses for the biological role of dothistromin are proposed based on these results. The study of dothistromin genes will improve current knowledge about secondary metabolite genes, their putative biological roles, and their regulation.



## Introduction

The dothideomycete *Dothistroma septosporum* causes red-band needle blight of pines. The disease leads to premature defoliation, reduced growth, and sometimes death of the infected tree. Red-band disease has been a problem in commercial pine plantations in the Southern Hemisphere for decades, but the occurrence of the disease has recently increased in the Northern Hemisphere where it affects trees in their native ranges in addition to those planted as exotics (reviewed in Bradshaw 2004). A recent epidemic in British Columbia was shown to be related to global climate change (Woods *et al.* 2005) suggesting the disease might become more severe in the future.

*D. septosporum* synthesizes and secretes the polyketide dothistromin in culture as well as in pine needles. The toxin has been isolated from diseased needles and is thought to be responsible for the characteristic red–brown colouration commonly associated with the disease (Shain & Franich 1981). Dothistromin is similar in structure to versicolorin B, a precursor of the aflatoxin (AF) and sterigmatocystin (ST) mycotoxins produced predominantly by *Aspergillus* species. There is

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evidence that the biosynthesis of dothistromin, AF, and ST is the same up to the versicolorins (Bradshaw *et al.* 2002; Shaw *et al.* 1978).

Genes required for dothistromin synthesis have been identified in two regions of the *D. septosporum* genome, which contains genes similar to genes required for AF and ST biosynthesis (Bradshaw & Zhang 2006b). One region contains the ketoreductase gene *dot*A, an orthologue of the AF pathway gene *aflM* (*ver-1*), which is adjacent to three other putative dothistromin genes. The other region contains the polyketide synthase gene *pks*A, an orthologue of the AF pathway gene *aflC* (*pks*A), which is clustered with four other putative dothistromin genes (Bradshaw & Zhang 2006b). Both *dot*A and *pks*A have been confirmed to be involved in dothistromin biosynthesis by gene replacement (Bradshaw *et al.* 2002; Bradshaw *et al.* 2006). A further putative dothistromin gene *vbs*A has been identified by degenerate PCR (Bradshaw & Zhang 2006b, this paper). It was expected that the identified groups of dothistromin genes form part of a bigger cluster. Secondary metabolite genes, such as the AF and ST genes, are usually clustered in fungal genomes, in contrast to genes of primary metabolism (Zhang *et al.* 2005). However, recent results indicate that this is not the case for dothistromin (Bradshaw & Zhang 2006a). Cary & Ehrlich (2006) speculated that some of the dothistromin genes could represent an ancestral version of the AF gene cluster.

The expression of secondary metabolite gene clusters is regulated by pathway-specific and global regulators (reviewed by Yu & Keller 2005). The production of AF and ST occurs towards the end of the exponential growth phase (Skory *et al.* 1993) and is coupled with asexual sporulation and morphological development in *Aspergillus* spp. (Calvo *et al.* 2002; Yu & Keller 2005). A G-protein-mediated signalling pathway negatively regulates both AF (and ST) biosynthesis and sporulation such that, in general, de-repression of both systems occurs when growth signals are low (Yu & Keller 2005; Adams & Yu 1998). Whether similar patterns of regulation are seen in the biosynthesis of dothistromin by *D. septosporum* is yet to be determined.

Although the genes involved in AF and ST biosynthesis have been intensively studied (Yu *et al.* 2004; Yabe & Nakajima 2004; Bhatnagar *et al.* 2003) no definitive biological role for those compounds has yet been identified. Similarly, no specific role for dothistromin is known. The generation of disease symptoms by injecting purified dothistromin into needles (Shain & Franich 1981), and the abundance of the toxin in the red bands of diseased needles implies an important role of dothistromin in the disease process. However, it has not been shown that dothistromin has any influence on pathogenicity or virulence, and indeed, red bands are not always seen in *Dothistroma*-infected needles (A. Woods & M. Dick, pers. comm.).

As part of a research programme to elucidate the biological role of dothistromin, we studied the expression of dothistromin biosynthesis genes. Real-time PCR studies supported by green fluorescent protein (GFP) reporter gene studies revealed remarkable differences in dothistromin gene expression compared with AF and ST genes. This is also the first report of expression of GFP in *D. septosporum* and description of the isolation of the putative dothistromin gene, *vbs*A.

## Materials and methods

### *Fungal and bacterial strains*

*Dothistroma septosporum* isolates NZE7 (Bradshaw *et al.* 2006) and NZE10 were obtained from *Pinus radiata* needles near Rotorua, New Zealand. Identity was confirmed by the production of dothistromin and by sequencing the rRNA ITS region (Bradshaw *et al.* 2000; Barnes *et al.* 2004). New Zealand isolates of *D. septosporum* tested so far appear to be clonal (Bradshaw *et al.* 2000) and an earlier isolate (NZE1) is available from ATCC (MYA-605). Strains were routinely grown on dothistroma medium (DM) (Bradshaw *et al.* 2000) or potato dextrose agar (PDA; Difco, Sparks, MD) at 22 °C as previously described (Bradshaw *et al.* 2000). *Escherichia coli* strains XL1-blue (Bullock *et al.* 1987) or Top 10 (Invitrogen, Carlsbad, CA) were used for propagating plasmids.

### *Degenerate PCR for isolation of the* vbsA *gene*

PCR was performed in a volume of 25 μl containing 0.5 U *Taq* DNA polymerase (Invitrogen), 1 × PCR buffer provided by the manufacturer, 1.5 mM $MgCl_2$, 50 μM of each deoxynucleotide triphosphate (dNTP), 0.4 μM of each primer vbs-1aF and vbs-4R (Table 1) and 10 ng genomic NZE7 DNA. Amplification was carried out in a gradient Mastercycler (Eppendorf, Hamburg) with an initial step of 94 °C for 2 min, followed by three cycles of 94 °C for 30 s, 66 °C for 60 s, and 72 °C for 80 s. In subsequent cycles the annealing temperature was reduced by 3 °C every three cycles down to 42 °C, followed by 72 °C for 5 min. Degenerate primers were designed by alignment of Vbs polypeptide sequences from *Aspergillus flavus* (AAS90042, AAS90088, AAS90106, AAS90019), *A. parasiticus* (AAC49319), and *A. nomius* (AAS90066). Resulting PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Hilden), cloned into pGEM-T Easy vector (Promega, Madison, WI) and sequenced using an ABI Prism BigDye™ Terminator cycle sequencing ready reaction kit and an ABI3730 genetic analyser (Applied Biosystems, Foster City, CA). The GenBank accession number for the *vbs*A gene is EF177826.

### *Growth experiments*

Conidia were collected from 10–12 d cultures of *Dothistroma septosporum* grown on dothistroma sporulation media (DSM) (Bradshaw *et al.* 2000). Approximately $10^6$ conidia $ml^{-1}$ were inoculated into 25 ml Dothistroma Broth (DB) medium [2.5 % (w/v) malt extract (Oxoid, Basingstoke), 2 % (w/v) nutrient broth (Oxoid)] or potato dextrose broth (PDB; Difco) in 125 ml conical flasks and incubated at 22 °C on an orbital shaker at 180 rev $min^{-1}$. The mycelium was harvested by vacuum filtration and weighed. The mycelium from replicate flasks was then mixed and divided into two approximately even parts. One part was weighed, freeze-dried, and re-weighed to calculate total D.W., while the other was frozen in liquid nitrogen and used for RNA extraction. A competitive enzyme-linked immunosorbant assay (ELISA) was used to determine the