FURTHER CHARACTERISATION OF THE DOTHISTROMIN GENE CLUSTER OF *DOTHISTROMA PINI*

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ABSTRACT

The polyketide dothistromin is a toxin produced by the filamentous fungus *Dothistroma pini* that is thought to play a role in causing *Dothistroma* needle blight in *Pinus radiata.* Dothistromin is structurally similar to aflatoxin B1 (AF), a highly carcinogenic toxin with no known function that is produced by the fungus *Aspergillus parasiticus* and also to versicolorin, an intermediate of the well characterised biosynthetic pathways of AF and sterigmatocystin (ST). The structural similarities between AF/ST and dothistromin suggest that genes homologous to AF biosynthetic genes will be involved in dothistromin biosynthesis. AF/ST biosynthetic genes of *A. parasiticus* and *A. nidulans* are clustered and hence it is likely that the dothistromin biosynthetic genes are also clustered in a similar manner. Two λ clones, λKSA and λCGV1 containing portions of the putative dothistromin cluster have been isolated in previous studies. Another λ clone λCGV2 was also identified using an aflatoxin gene probe but it is unknown whether it is part of the dothistromin biosynthetic cluster.

The λKSA clone contains part of a putative polyketide synthase pks^{dot} (64% identical to *A. parasiticus* AF biosynthetic gene *pksA).* Two crucial domains required for functioning are contained within λKSA, the β-keto acyl synthase (KS) and acyl transferase (AT) domains. The putative pks^{dot} is thought be involved in the beginning of the dothistromin biosynthetic pathway, working in a complex with a fatty acid synthase (FAS) to produce the intermediate noranthrone. A gene replacement construct was made using Multisite Gateway ™Recombination, replacing the AT and KS domains with an *hph* cassette. Disruption of the pks^{dot} gene will confirm it's involvement in dothistromin biosynthesis and could also confirm the role of dothistromin in pathogenicity as if the putative polyketide synthase (pks^{dot}) is involved in the first step of the dothistromin pathway thus a knockout would form a mutant devoid of any intermediates. Confirming the involvement of pks^{dot} would also provide evidence that like λCGV1, λKSA contains a portion of the dothistromin biosynthetic gene cluster.

As the positioning of the three lambda clones λKSA, λCGV1 and λCGV2 relative to one another in the *D. pini* genome was unknown Southern blot analysis was implemented to identify any relationship between the three lambda clones. No evidence was found to suggest the close linkage of the three lambda clones however this does not discount any linkage at all. Southern blot analysis did provide evidence that *ver-2* (77%

identity to melanin biosynthetic gene *phn1* of *Cochliobolus heterostrophus)* of λCGV2 is within close proximity to a putative *aflR* gene (regulatory gene for activating gene transcription in AF/ST biosynthesis) suggesting a regulatory role of this putative *aflR* gene in melanin biosynthesis and not dothistromin biosynthesis.

Further nucleotide sequencing of the λKSA clone revealed three putative dothistromin genes. Mox^{dot} and ord^{dot} have high amino acid identity to genes involved in the AF/ST pathways (70% identity to *moxY* and 51% identity to *avfA* of *A. parasiticus* respectively), suggesting similar roles in dothistromin biosynthesis. $Epox^{dot}$ showed high amino acid identity to an epoxide hydrolase of *A. niger (hyll)* suggesting it has a unique role in dothistromin biosynthesis as no homologs are seen in the AF/ST clusters. Southern blotting was also used to confirm the arrangements of genes from the λKSA clone within the *D. pini* genome.

Further characterisation of genes involved in dothistromin biosynthesis will firstly enable understanding of the role of dothistromin in needle blight and secondly will enable further comparative studies between AF/ST and dothistromin.

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'It is in hoping that we dream, In dreaming that we seek, In seeking that we find our life's desire'

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ACP	Acyl-carrier protein
Amp	Ampicillin
AF or AFB1	Aflatoxin B1
AT	Acyl transferase
AVF	Averufin
Bp	base-pairs (DNA)
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
cDNA	Complementary DNA
CSPD	Disodium 3-(4-methoxyspiro (1, 2- dioxetane-3, 2' – (5' chloro) tricyclo [3, 3.1.3.1] decan}-4-yl) phenyl phosphate
CTAB	Hexadecyltrimethylammonium bromide
CU	Cerato ulmin
DB	*D. pini* broth
DIG	digoxygenin
DH	Dehydratase
DM	*D. pini* media
DNA	Deoxyribonuclic acid
dNTP	deoxy-nucleotide tri phosphate
dot	Putative dothistromin gene
D. pini	*Dothistroma pini*
DSM	*D. pini* sporulation media
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
EH	Epoxide hydrolase
ER	Enoyl reductase
Etbr	Ethidium bromide
FAS	Fatty acid synthase
Hph	Hygromycin B phosphate transferase gene
HPLC	High pressure liquid chromatography

IPTG	Isopropyl-β-D-galactoside
Kb	kilobase (DNA)
KDa	Kilodalton
KS	β-keto acyl synthase
Kv	Kilovolts
LB	Luira-Bertani
NA	Norsolorinic acid
PCR	Polymerase chain reaction
PEG	poly-ethyl glycol
PKS	Polyketide synthase
RACE	Rapid amplification of cDNA ends
RE	Restriction endonuclease
REMI	Restriction enzyme-mediated integration
Rpm	Revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulphate
ST	Sterigmatocystin
TAGKO	Transposition-arrayed gene knockouts
Taq	Thermus aquaticus
TE	Thioesterase
Tm	Melting temperature
UV	Ultra violet
VHA	Versiconal hemiacetal acetate
Xgal	5-bromo-4-chloro-3-idoly-β-D-galactopyranoside
Ω	Ohms
λ	Lambda
μf	Microfarad