

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

**Further Studies of Dothistromin  
Toxin Genes in the Fungal Forest Pathogen  
*Dothistroma septosporum***

**A thesis presented in partial fulfillment of the requirements  
for the degree of Master of Science in Biochemistry  
at Massey University, Palmerston North, New Zealand**

**Zhilun Feng**

**2007**

## ABSTRACT

The fungal pathogen *Dothistroma septosporum* is the main causal agent of *Dothistroma* (red-band) needle blight, which is a devastating foliar disease of a wide range of pine species. Dothistromin is a difuranoanthraquinone toxin produced by *D. septosporum* and is considered as a possible virulence factor for the disease. Based on the similarity of chemical structure between dothistromin and aflatoxin (AF) /sterigmatocystin (ST) precursors, nine putative dothistromin biosynthetic genes have been identified, which are homologous to their corresponding genes in the AF/ST gene clusters. However, in contrast to all 25 AF biosynthetic genes tightly clustered in one region (70-Kb) of the genome, the dothistromin gene clusters are located on a 1.3-Mb chromosome and separated into three mini-clusters along with non-dothistromin genes.

The *dotC* gene, located in the mini-cluster 1, is predicted to encode a major facilitator superfamily (MFS) membrane transporter involved in secretion of dothistromin. In this work, by constructing DotC-eGFP fusion protein containing mutants, the subcellular localization of the DotC protein was determined to be mainly targeted to the plasma membrane. The biological function of the *dotC* gene was characterized by targeted gene disruption. The *dotC* gene disrupted mutants showed a significant reduction of dothistromin production in both the medium and mycelium. In addition, the exponential growth of *dotC* null mutants was inhibited when exogenous dothistromin was presented and these mutants also displayed more sensitivity than the wild type strain to exogenous dothistromin. The results indicated that the DotC protein is a membrane associated protein and might have a role in dothistromin production and be involved in secretion of exogenously supplied dothistromin toxin.

Two novel dothistromin biosynthetic genes, *norA/B* and *verB* (partial sequence), were identified by using degenerate PCR and *D. septosporum* genomic library screening. The putative NorA/B and VerB are postulated to encode a dehydrogenase and a desaturase, respectively and are similar to AF/ST genes. These findings further confirmed that the dothistromin shares biosynthetic pathway steps with AF/ST.

## **ACKNOWLEDGEMENTS**

First and foremost, I would like to gratefully acknowledge my supervision Dr Rosie Bradshaw for her constant encouragement and guidance during this research. Thanks for your kindness and patience to make the last two and half years be the wonderful and inspiring time in my life.

It is a pleasure to thank my lab colleagues. Thanks for Shuguang always ready with helpful technical support and advice. Thanks for Ping sharing her knowledge to give me an easy beginning in the lab. Thanks for Arne kindly providing GFP and microscopy technical support. Thanks for Naydene for her ELISA support and English help. Thanks for Yanan and Justine, it was so happy to work together in the “fungaljungle”.

I would also like to thank Dr Kathryn Stowell for giving me very useful advice when I first stepped into the IMBS. Thanks for Kathy Hamilton arranging my study course. Many thanks for other staff working in the IMBS, who gave me help over the duration of my studies.

Finally I would like to thank my parents and my wife. With your understanding and encouraging, I could easily stay by myself in Palmy, which is a quiet and small town far away from Auckland and China.

## ABBREVIATIONS

ABC transporter	ATP-binding cassette transporter
AF	aflatoxin
amp <sup>r</sup>	ampicillin resistance
bp	base pair
cm	centimeter
°C	degree celsius
dATP	deoxyadenosine triphosphate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
Doth	dothistromin
Fig.	figure
g	gram
GFP	green fluorescent protein
eGFP	enhanced green fluorescence protein
IPTG	isopropyl- $\beta$ -d-thiogalactoside
Kb	kilobase pair
L	litre
M	molar
Mb	megabase
MFS transporter	major facilitator superfamily transporter
ml	milliliter
mM	millimolar
OD <sub>600</sub>	optical density at 600 nm
ORF	open reading frame
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
ST	sterigmatocysin
TMD	transmembrane domain
$\mu$ l	microlitre
$\mu$ M	micromolar
$\mu$ g	microgram
UV	ultraviolet
v/v	volume per volume
WT	wildtype
w/v	weight per volume
X-Gal	5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside
~	approximate

# TABLE OF CONTENTS

ABSTRACT.....	i
ACKNOWLEDGEMENTS .....	ii
ABBREVIATIONS .....	iii
TABLE OF CONTENTS .....	iv
LIST OF FIGURES .....	ix
LIST OF TABLES .....	x
<b>CHAPTER ONE: INTRODUCTION .....</b>	<b>1</b>
<b>1.1 <i>Dothistroma</i> needle blight and <i>Dothistroma septosporum</i> .....</b>	<b>1</b>
1.1.1 <i>Dothistroma</i> needle blight: the red band blight .....	1
1.1.2 <i>Dothistroma septosporum</i> : the causal agent of blight .....	3
1.1.3 The infection process.....	3
1.1.4 Current methods of disease control .....	4
<b>1.2 Dothistromin toxin.....</b>	<b>5</b>
1.2.1 General features of dothistromin .....	5
1.2.2 Biochemical aspects of dothistromin.....	6
1.2.3 Dothistromin biosynthetic gene clusters .....	7
1.2.3.1 Fungal gene clusters of AF/ST .....	7
1.2.3.2 Dothistromin gene clusters .....	8
<b>1.3 Membrane transporters in filamentous fungi .....</b>	<b>13</b>
1.3.1 ABC transporters .....	13
1.3.2 MFS transporters .....	14
1.3.3 DotC, a putative MFS transporter.....	16
<b>1.4 Green fluorescent protein .....</b>	<b>17</b>
1.4.1 Applications of GFP .....	18
1.4.2 GFP improvements .....	19
<b>1.5 Targeted gene disruption.....</b>	<b>20</b>
<b>1.6 Aims and objectives .....</b>	<b>23</b>

<b>CHAPTER TWO: MATERIALS AND METHODS.....</b>	<b>24</b>
<b>2.1 FUNGAL, BACTERIAL STRAINS AND PLASMIDS.....</b>	<b>24</b>
<b>2.2 GROWTH AND MAINTENANCE OF CULTURES.....</b>	<b>25</b>
2.2.1 Growth and maintenance of <i>E. coli</i> cultures .....	25
2.2.2 Growth and maintenance of <i>D. septosporum</i> cultures.....	26
2.2.2.1 General growth and maintenance of <i>D. septosporum</i> cultures.....	26
2.2.2.2 Single spore purification.....	26
2.2.2.3 Growth and harvest of <i>D. septosporum</i> mycelia.....	26
<b>2.3 DNA MANIPULATION .....</b>	<b>28</b>
2.3.1 DNA isolation from <i>D. septosporum</i> cultures: CTAB Method .....	28
2.3.2 Isolation of plasmid DNA from <i>E. coli</i> .....	29
2.3.3 Agarose gel electrophoresis of DNA.....	29
2.3.4 Agarose gel purification of DNA fragments.....	29
2.3.5 Determination of DNA concentration by fluorometric assay .....	29
<b>2.4 DNA LIGATION AND CLONING.....</b>	<b>30</b>
2.4.1 Restriction endonuclease digestion of DNA.....	30
2.4.2 Standard ligation reactions.....	31
2.4.3 Gateway recombination reactions.....	31
2.4.3.1 BP recombination.....	31
2.4.3.2 LR recombination.....	32
<b>2.5 TRANSFORMATION PROTOCOL .....</b>	<b>33</b>
2.5.1 Transformation of <i>E. coli</i> competent cells.....	33
2.5.1.1 Preparation of competent cells and transformation by electroporation.....	33
2.5.1.2 Transformation by CaCl <sub>2</sub> competent cells .....	34
2.5.2 Transformation of <i>D. septosporum</i> .....	34
2.5.2.1 Preparation of competent <i>D. septosporum</i> protoplasts .....	34
2.5.2.2 Transformation of <i>D. septosporum</i> protoplasts.....	35
<b>2.6 AMPLIFICATION OF DNA BY THE POLYMERASE CHAIN REACTION .....</b>	<b>36</b>
2.6.1 Normal oligonucleotide primers and degenerate primers .....	36
2.6.2 Reagents and cycling conditions for basic PCR .....	38
2.6.3 Optimization of PCR conditions.....	39
2.6.4 Touchdown PCR and inverse PCR.....	39
2.6.4.1 Touchdown PCR.....	39
2.6.4.2 Inverse PCR.....	41
2.6.5 <i>E. coli</i> colony PCR .....	41
2.6.6 Purification of PCR products.....	42
<b>2.7 DNA SEQUENCING.....</b>	<b>42</b>

<b>2.8 HYBRIDISATION OF DIG-LABELED PROBES TO BLOTS.....</b>	<b>42</b>
2.8.1 DIG labeling of DNA probe .....	42
2.8.2 Southern blotting (Capillary) .....	43
2.8.3 Preparing colony lifts .....	43
2.8.4 Probe concentration determination.....	44
2.8.5 Hybridization of DIG labeled probe.....	45
2.8.6 Immunological detection.....	45
2.8.7 Stripping blots.....	46
<b>2.9 DOTHISTROMIN ISOLATION FROM MYCELIUM OF     <i>D.SEPTOSPORUM</i> .....</b>	<b>46</b>
<b>2.10 QUANTIFICATION OF DOTHISTROMIN (ELISA METHOD) .....</b>	<b>47</b>
<b>2.11 OBSERVATION OF GFP EXPRESSION IN <i>D.SEPTOSPORUM</i>     CULTURES.....</b>	<b>48</b>
2.11.1 Fluorescent microscopy .....	48
2.11.2 Confocal microscopy.....	49
<b>2.12 GROWTH RATE ANALYSIS .....</b>	<b>49</b>
<b>2.13 OBTAINING <i>D. SEPTOSPORUM</i> CONIDIA AND     QUANTIFICATION .....</b>	<b>49</b>
<b>2.14 DOTHISTROMIN RESISTANCE ASSAY.....</b>	<b>50</b>
<b>CHAPTER THREE: DOTC-EGFP MUTANTS.....</b>	<b>51</b>
<b>3.1 INTRODUCTION .....</b>	<b>51</b>
<b>3.2 CONSTRUCT THE DOTC-EGFP CONTAINING VECTOR.....</b>	<b>51</b>
3.2.1 Obtaining the <i>dotC-egfp</i> fusion gene.....	51
3.2.2 Cloning the <i>dotC-egfp</i> fusion gene into the pGEM <sup>®</sup> -T Easy vector .....	54
3.2.3 Cloning the <i>dotC-gfp</i> fusion gene and the <i>hph</i> gene into the pBluescript II KS plus vector .....	57
3.2.3.1 Cloning the <i>dotC-egfp</i> fusion gene into the pBluescript vector.....	57
3.2.3.2 Cloning the <i>hph</i> gene into the pBlue-DGrev (pR264).....	59
<b>3.3 TRANSFORMATION OF THE pR265 VECTOR     INTO <i>D. SEPTOSPORUM</i> .....</b>	<b>61</b>
3.3.1 <i>D. septosporum</i> protoplasts mediated fungal transformation .....	61
3.3.2 Screening of <i>dotC-egfp</i> mutants by PCR .....	61
3.3.3 Confirmation of the integration of <i>egfp</i> by Southern blotting analysis.....	63



<b>3.4 OBSERVATION OF THE DOTC-EGFP MUTANTS.....</b>	<b>66</b>
3.4.1 Growth rate of the <i>dotC-egfp</i> integrated mutants .....	66
3.4.2 Observation of the colonies and spores of the <i>dotC-egfp</i> integrated mutants.....	67
3.4.3 Observation of the mycelia of the <i>dotC-egfp</i> integrated mutants by confocal microscopy .....	68
<b>3.5 DISCUSSION .....</b>	<b>71</b>
3.5.1 Obtaining the <i>dotC-egfp</i> gene containing vector (pR265) .....	71
3.5.2 Fungal transformation .....	71
3.5.3 GFP observation in <i>dotC-egfp</i> integrated mutants .....	72
<b>CHAPTER FOUR: DOTC GENE DISRUPTION .....</b>	<b>75</b>
<b>4.1 INTRODUCTION .....</b>	<b>75</b>
<b>4.2 CONSTRUCT THE DOTC GENE DISRUPTION VECTOR.....</b>	<b>75</b>
4.2.1 Step 1: Producing <i>attB</i> attached PCR products .....	76
4.2.2 Step 2: Cloning the 5'and 3'elements into the pGEM <sup>®</sup> -T Easy vector.....	78
4.2.3 Step 3: BP ( <i>attB</i> : <i>attP</i> ) recombination to generate entry clones.....	78
4.2.4 Step 4: LR recombination to obtain the final disruption vector .....	79
<b>4.3 TARGETED DELETION OF THE DOTC GENE     IN D. SEPTOSPORUM .....</b>	<b>81</b>
4.3.1 Screening of <i>dotC</i> deletion mutants by PCR .....	81
4.3.2 Confirmation of <i>dotC</i> disrupted mutants by Southern blotting analysis.....	85
<b>4.4 CHARACTERIZATION OF THE DOTC DISRUPTED MUTANTS .....</b>	<b>89</b>
4.4.1 Growth rate of the <i>dotC</i> mutants .....	89
4.4.2 Dothistromin production of <i>dotC</i> mutants .....	90
4.4.3 Dothistromin resistance of <i>dotC</i> mutants.....	94
4.4.4 Sporulation of <i>dotC</i> mutants .....	97
<b>4.5 DISCUSSION.....</b>	<b>98</b>
4.5.1 Construction of the <i>dotC</i> mutants .....	98
4.5.2 Characterization of the <i>dotC</i> mutants.....	99
4.5.2.1 Dothistromin production.....	99
4.5.2.2 Dothistromin resistance .....	100
4.5.2.3 Sporulation .....	101
4.5.3 The role of the <i>dotC</i> in <i>D. septosporum</i> .....	102

<b>CHAPTER FIVE: NOVEL DOTHISTROMIN GENES.....</b>	<b>104</b>
<b>5.1 INTRODUCTION .....</b>	<b>104</b>
<b>5.2 IDENTIFICATION OF NOVEL DOTHISTROMIN</b>	
<b>SYNTHETIC GENES .....</b>	<b>104</b>
5.2.1 Design of degenerate PCR primers .....	104
5.2.2 Touchdown PCR using degenerate primers.....	107
5.2.3 Inverse PCR to determine the whole sequences of the <i>norA/B</i> and <i>verB</i> ..	108
5.2.4 Screening the <i>D. septosporum</i> genomic library with a <i>norA/B</i> probe.....	111
5.2.5 Sequencing the isolated clone pR268.....	112
<b>5.3 DISCUSSION .....</b>	<b>117</b>
5.3.1 Obtaining the novel dothistromin biosynthetic genes .....	117
5.3.2 The possible roles of the <i>norA/B</i> and <i>verB</i> in dothistromin biosynthesis ..	118
5.3.3 The dothistromin biosynthetic clusters .....	119
<b>CHAPTER SIX: CONCLUSIONS AND FUTURE WORK .....</b>	<b>121</b>
<b>6.1 LOCALIZATION OF THE DOTC PROTEIN .....</b>	<b>121</b>
<b>6.2 TARGETED DISRUPTION OF THE DOTC GENE.....</b>	<b>122</b>
<b>6.3 THE NOVEL DOTHISTROMIN SYNTHETIC GENES .....</b>	<b>124</b>
<b>REFERENCES .....</b>	<b>126</b>
<b>APPENDIX I: MEDIA .....</b>	<b>135</b>
<b>APPENDIX II: BUFFERS AND SOLUTIONS.....</b>	<b>137</b>
<b>APPENDIX III: The DOTC-EGFP SEQUENCE.....</b>	<b>140</b>
<b>APPENDIX IV: SOLVING THE PROBLEMS IN <i>D. SEPTOSPORUM</i></b>	
<b>TRANSFORMATION.....</b>	<b>144</b>
<b>APPENDIX V: PLASMID MAPS .....</b>	<b>149</b>

## List of Figures

Page	Figure	
2	Fig. 1.1	Infected pine needles
7	Fig. 1.2	Chemical structures of aflatoxin B <sub>1</sub> , versicolorin A, versicolorin B, and dothistromin
9	Fig. 1.3	Syntenly comparison of gene clusters
17	Fig. 1.4	Schematic representation of ABC and MFS transporters
22	Fig. 1.5	MultiSite Gateway
45	Fig. 2.1	A sample result of probe concentration determination
52	Fig. 3.1	Schematic diagram shows the procedure obtaining a <i>dotC-egfp</i> fusion gene
53	Fig. 3.2	The overall process for cloning the <i>dotC-egfp</i> vector
56	Fig. 3.3	Checking the plasmid pR263 with PCR and enzyme digestion
58	Fig. 3.4	Checking the plasmid pBlue-DGrev (pR264) with PCR and enzyme digestion
60	Fig. 3.5	Checking the plasmid pR265 with PCR and enzyme digestion
62	Fig. 3.6	Screening of <i>dotC-egfp</i> mutants by PCR
65	Fig. 3.7	Southern blotting results
67	Fig. 3.8	The green fluorescence of the FJT73 and FJT75 colonies
68	Fig. 3.9	WT and <i>egfp</i> containing mutants under normal microscopy
70	Fig. 3.10	Mycelia of the FJT73 and FJT75 under confocal microscopy
76	Fig. 4.1	PCR products of 5' and 3' elements of the <i>dotC</i> gene
77	Fig. 4.2	Experimental procedure for constructing the disruption vector
80	Fig. 4.3	LR recombination
83	Fig. 4.4	The plasmid map of the <i>dotC</i> disruption vector pR260
84	Fig. 4.5	PCR screening of the purified <i>dotC</i> disrupted transformants
87	Fig. 4.6	Schematic outline of expected Southern blot hybridization patterns
88	Fig. 4.7	Southern blot results to confirm the identified <i>dotC</i> mutants
91	Fig. 4.8	The <i>dotC</i> mutants' phenotype
94	Fig. 4.9	Resistance of the <i>dotC</i> mutants (FJT15 and 16), ectopic strain (FJT79) and WT (NZE10) to a contaminating fungus
106	Fig. 5.1	The multiple amino acid alignments of the NorA and VerB proteins
107	Fig. 5.2	Touchdown PCR results using degenerate primers
110	Fig. 5.3	Inverse PCR results
112	Fig. 5.4	PCR results of screening the <i>D. septosporum</i> genomic library
113	Fig. 5.5	The sequencing result of pR268
114	Fig. 5.6	Nucleotide and amino acid sequences of the putative <i>norA/B</i>
116	Fig. 5.7	The amino acid alignments of the putative NorA/B and partial VerB proteins
119	Fig. 5.8	The schematic AF biosynthetic pathway

## List of Tables

Page	Table	
11	Table 1.1	Characterization of genes identified in the three dothistromin gene mini-clusters
24	Table 2.1	Fungal, bacterial strains and plasmids
36	Table 2.2	Sequencing and PCR primers
40	Table 2.3	The procedure of touchdown PCR
66	Table 3.1	Radial growth of <i>dotC-egfp</i> integrated mutants
79	Table 4.1	Construction of the <i>dotC</i> 5' and 3' elements containing entry clones
90	Table 4.2	Radial growth of <i>dotC</i> mutants
93	Table 4.3	The dothistromin production of the <i>dotC</i> mutants
96	Table 4.4	The radial growth of <i>dotC</i> mutants in dothistromin containing media
97	Table 4.5	Sporulation of the <i>dotC</i> mutants
100	Table 4.6	Ratios of dothistromin levels WT: mutant
103	Table 4.7	Examples of the MFS transporters' function
105	Table 5.1	Degenerate primers for amplifying the <i>norA</i> and <i>verB</i> genes
110	Table 5.2	BLASTX results of inverse PCR products
114	Table 5.3	BLASTX results of pR268
117	Table 5.4	Amino acid identity of NorA/B between <i>D. septosporum</i> and <i>Aspergillus</i> species