

Characterisation of proteinaceous  
toxins isolated from *Pyrenophora*  
*teres* f. *teres*

by

Ismail Ahmed Ismail

A thesis submitted for the degree of Doctor of Philosophy

at

The University of Adelaide

Faculty of Science

School of Agriculture, Food and Wine

Waite Campus

Adelaide, South Australia

2013

## Table of contents

Table of contents.....	II
List of Tables.....	XIV
Abstract.....	XVI
Declaration .....	XX
Acknowledgment .....	XXI
Abbreviation .....	XXII
Chapter 1 General introduction .....	1
1.1 Fungal phytotoxins .....	1
1.2 Necrotrophic phytotoxins and disease development .....	3
1.3 Net blotch disease in barley .....	5
1.4 <i>Ptt</i> -barley interaction.....	7
1.4.1 Infection process.....	7
1.4.2 <i>P. teres</i> toxins .....	8
1.4.2.1 HNSTs .....	9
1.4.2.2 Proteinaceous HSTs.....	10
1.5 Virulence and proteinaceous HSTs.....	12
1.6 The mode of action of necrotrophic effectors (HSTs) .....	13
1.7 Identification of necrotrophic fungal toxins might contribute to the development of resistant barley .....	15
1.8 The objectives of this study.....	18
Chapter 2 Fungal growth and virulence of <i>Pyrenophora teres</i> f. <i>teres</i> on barley .....	20
2.1 Introduction .....	20
2.2 Materials and methods.....	22
2.2.1 Plant growth.....	22
2.2.2 Fungal isolation and culturing .....	22
2.2.3 Virulence testing of <i>Ptt</i> isolates.....	23
2.2.4 <i>In vitro</i> and <i>in planta</i> conidial germination assays.....	24
2.2.5 Determination of fungal growth <i>in planta</i> .....	27
2.2.6 Optimisation of proteinaceous toxin production and extraction ....	27

2.2.6.1	Effect of media type and incubation conditions on protein production.....	28
2.2.6.2	Protein extraction from <i>Ptt</i> culture filtrate .....	29
2.2.6.2.1	Ultrafiltration purification (UFP) .....	30
2.2.6.2.2	Ammonium sulfate precipitation (ASP).....	31
2.2.6.2.3	TCA protein extraction.....	31
2.2.6.3	PAGE.....	32
2.2.6.3.1	Coomassie Brilliant Blue staining (CBB R250).....	32
2.2.6.3.2	Silver staining.....	33
2.2.6.4	Evaluation of UFP and ASP extraction methods .....	34
2.2.6.5	Proteinaceous toxin bioassay for six isolates of <i>Ptt</i> .....	34
2.2.7	Statistical analysis .....	35
<b>2.3</b>	<b>Results.....</b>	<b>35</b>
2.3.1	Virulence of <i>Ptt</i> isolates .....	35
2.3.2	Germination and fungal growth are correlated with virulence.....	39
2.3.2.1	Germination <i>in vitro</i> and <i>in planta</i> .....	39
2.3.2.2	Fungal hyphal growth <i>in planta</i> .....	40
2.3.3	Effect of media on protein production.....	45
2.3.4	Optimisation of protein production conditions.....	46
2.3.5	Evaluation of UFP and ASP extraction methods .....	49
2.3.6	Bioassay of total protein extracted from culture filtrates of six <i>Ptt</i> isolates .....	49
<b>2.4</b>	<b>Discussion .....</b>	<b>52</b>
<b>Chapter 3 Identification of individual candidate proteinaceous toxins from <i>Pyrenophora teres f. teres</i>.....</b>		<b>58</b>
<b>3.1</b>	<b>Introduction .....</b>	<b>58</b>
<b>3.2</b>	<b>Material and methods .....</b>	<b>60</b>
3.2.1	Plant growth.....	60
3.2.2	Fungal isolation and toxin production.....	60
3.2.3	Proteinaceous toxin fractionation and bioassays .....	60
3.2.3.1	Bioassay of protein fractions from two isolates with different virulence .....	61
3.2.3.2	Bioassay of protein fractions and sub-fractions on barley cultivars of different susceptibility.....	62
3.2.4	Investigation of <i>Ptt</i> toxins in intercellular washing fluids (ICWFs) and cell components in infected plants.....	62
3.2.4.1	Plant inoculation.....	63

3.2.4.2	Protein extraction from ICWFs .....	63
3.2.4.3	Protein extraction from cell components.....	64
3.2.5	Visualisation of proteins and candidate toxin identification .....	66
3.2.5.1	PAGE.....	66
3.2.5.2	2DGE.....	66
3.2.5.3	Candidate proteinaceous toxin identification .....	67
<b>3.3</b>	<b>Results.....</b>	<b>69</b>
3.3.1	Identification of potential toxins .....	69
3.3.1.1	From fractions of culture filtrate proteins from two isolates with different virulence .....	69
3.3.1.2	From fractions and sub-fractions of culture filtrate proteins that induce symptoms differentially on barley cultivars of different susceptibility.....	75
3.3.1.3	Comparing active and less active protein fractions .....	86
3.3.2	Identification of virulence-related candidate proteins (VRCPs) ....	88
3.3.3	Characterisation of ICWFs and cell components from infected plants.....	91
<b>3.4</b>	<b>Discussion .....</b>	<b>99</b>
<b>Chapter 4</b>	<b>Isolation, characterisation and cloning of cDNA encoding for virulence-related candidate proteins (VRCPs) from <i>Pyrenophora teres f. teres (Ptt)</i> .....</b>	<b>108</b>
<b>4.1</b>	<b>Introduction .....</b>	<b>108</b>
<b>4.2</b>	<b>Materials and methods.....</b>	<b>111</b>
4.2.1	Isolation of the full length of cDNA for virulence-related candidate gene transcripts (VRCGs).....	111
4.2.1.1	Culturing the isolate .....	111
4.2.1.2	Primer design.....	111
4.2.1.3	RNA extraction, quantification and DNase treatment.....	112
4.2.1.4	Isolation of the full length of VRCGs .....	113
4.2.1.5	cDNA extraction from agarose gel and confirmation of sequences .....	114
4.2.2	Bioinformatics analysis .....	114
4.2.2.1	Conserved domains analyses for VRCPs .....	114
4.2.2.2	Alignment of individual VRCPs .....	115
4.2.2.3	Phylogenetic analysis .....	117
4.2.2.4	Secondary structure and three dimensional modelling of VRCPs	117
4.2.3	Semi-quantitative RT-PCR.....	118

4.2.3.1	<i>In vitro</i> .....	118
4.2.3.2	<i>In planta</i> .....	119
<b>4.3</b>	<b>Results</b> .....	<b>120</b>
4.3.1	Isolation of the full length of cDNA for virulence-related candidate gene transcripts (VRCGs).....	120
4.3.2	Conserved domains and phylogenetic analyses for VRCPs.....	120
4.3.2.1	PttXyn11A.....	120
4.3.2.2	PttCHFP1.....	130
4.3.2.3	PttGPI-CFEM .....	136
4.3.2.4	PttSP1 .....	140
4.3.3	Semi-quantitative RT-PCR.....	144
4.3.3.1	<i>In vitro</i> .....	144
4.3.3.2	<i>In planta</i> .....	145
<b>4.4</b>	<b>Discussion</b> .....	<b>146</b>
<b>Chapter 5</b>	<b>Heterologous expression and bioassays of virulence-related candidate proteins (VRCPs)</b> .....	<b>152</b>
<b>5.1</b>	<b>Introduction</b> .....	<b>152</b>
<b>5.2</b>	<b>Material and methods</b> .....	<b>153</b>
5.2.1	Cloning of cDNA of VRCGs .....	153
5.2.2	Heterologous expression of candidate proteins .....	155
5.2.2.1	Ligation of PCR amplicons in pDEST17 vector .....	155
5.2.2.2	Expression of recombinant protein.....	156
5.2.2.3	Purification of heterologously expressed proteins .....	156
5.2.2.3.1	Purification under denaturing conditions .....	157
5.2.2.3.2	Purification under native conditions.....	157
5.2.2.4	Visualisation and LC-eSI-IT MS identification of expressed recombinant proteins .....	158
5.2.2.5	The effect of recombinant protein on the viability of <i>E. coli</i> .....	158
5.2.3	Bioassay of heterologously expressed proteins.....	159
5.2.4	Activity of xylanase in six isolates of <i>Ptt in vitro</i> .....	160
5.2.5	Statistical analysis and photography .....	162
<b>5.3</b>	<b>Results</b> .....	<b>162</b>
5.3.1	Heterologous expression of VRCPs .....	162
5.3.1.1	PttGPI-CFEM .....	162
5.3.1.2	PttXyn11A.....	163
5.3.1.3	PttCHFP1.....	168
5.3.1.4	PttSP1 .....	172

5.3.2	Bioassay for candidate proteins.....	176
5.3.2.1	Biological activity of recombinant proteins on barley cultivars ..	176
5.3.2.2	Biological activity of recombinant proteins on cv. Sloop .....	183
5.3.3	Biological activity of commercial xylanase .....	186
5.3.4	Xylanase activity in isolates of <i>Ptt</i> with different virulence .....	187
<b>5.4</b>	<b>Discussion .....</b>	<b>188</b>
<b>Chapter 6</b>	<b>Expression of virulence-related candidate genes (VRCG) during the interaction between barley and isolates of <i>P. teres f. teres</i> with different virulence.....</b>	<b>194</b>
<b>6.1</b>	<b>Introduction .....</b>	<b>194</b>
<b>6.2</b>	<b>Material and methods .....</b>	<b>197</b>
6.2.1	Quantification of expression of VRCGs in six isolates of <i>Ptt</i> .....	197
6.2.1.1	Primer design for RT-qPCR .....	197
6.2.1.2	Plant inoculation and <i>in planta</i> cDNA synthesis.....	199
6.2.1.3	Optimisation of RT-qPCR for VRCGs gene expression.....	199
6.2.1.3.1	VRCGs expression using Comparative C <sub>T</sub> ( $\Delta\Delta C_T$ ).....	200
6.2.1.3.2	qPCR for genomic DNA for six isolates of <i>Ptt</i> at 192 hpi .....	201
6.2.1.3.3	VRCGs expression using absolute quantification .....	201
6.2.1.3.3.1	Primer efficiency .....	201
6.2.1.3.3.2	Standard curve optimisation .....	202
6.2.1.3.3.3	Optimisation of final cDNA and primer concentration for <i>in planta</i> analysis .....	203
6.2.1.3.4	Absolute quantification of VRCGs expression using the optimal cDNA and primer concentrations .....	203
6.2.2	Statistical analysis and photography .....	204
<b>6.3</b>	<b>Results.....</b>	<b>204</b>
6.3.1	Virulence of <i>Ptt</i> isolates .....	204
6.3.2	Optimisation of quantitative RT-PCR for VRCGs.....	206
6.3.2.1	VRCGs expression using Comparative C <sub>T</sub> ( $\Delta\Delta C_T$ ).....	206
6.3.2.2	qPCR for genomic DNA for six isolates at 192 hpi .....	207
6.3.2.3	VRCGs expression using absolute quantification .....	208
6.3.2.3.1	Primer efficiency and standard curves .....	208
6.3.2.3.2	Optimisation of cDNA concentration <i>in planta</i> .....	209
6.3.2.3.2.1	Absolute quantification of VRCGs during the interaction .....	210
<b>6.4</b>	<b>Discussion .....</b>	<b>216</b>
<b>Chapter 7</b>	<b>General discussion.....</b>	<b>222</b>

<b>7.1</b>	<b>Virulence needs both toxin production and fungal growth ....</b>	<b>223</b>
7.1.1	Xylanase contributes to <i>Ptt</i> virulence.....	224
7.1.2	PttCHFP1 might contribute to <i>Ptt</i> virulence .....	227
7.1.3	Contribution of other proteinaceous toxins to <i>Ptt</i> virulence .....	228
7.1.4	Proposed infection process of barley by <i>Ptt</i> .....	229
<b>7.2</b>	<b>Conclusion and future directions .....</b>	<b>232</b>
<b>References.. .....</b>		<b>234</b>
<b>Appendix 1 .....</b>		<b>257</b>
<b>Appendix 2 .....</b>		<b>259</b>
<b>Appendix 3 .....</b>		<b>266</b>
<b>Appendix 4 .....</b>		<b>269</b>
<b>Appendix 5 .....</b>		<b>278</b>

## List of Figures

Figure 1.1. Net blotch disease in barley, spot form net blotch (A) and net form net blotch (B).....	6
Figure 2.1. Specially designed Petri dish inoculation system used in this study to allow easy visualisation of <i>Ptt</i> growth on barley. ....	26
Figure 2.2. Representative images depicting differences in the virulence of the six <i>Ptt</i> isolates on barley cv. Sloop 6 days post inoculation.....	36
Figure 2.3. Representative images depicting differences in the virulence of the six <i>Ptt</i> isolates on barley cv. Sloop 17 days post inoculation.....	37
Figure 2.4. Representative images for fungal development of two isolates of <i>Ptt</i> after 11 days post inoculation <i>in planta</i> . ....	38
Figure 2.5. Percentage of conidia germinated for six isolates of <i>Ptt</i> after 24 h <i>in vitro</i> or <i>in planta</i> in barley cv. Sloop.....	40
Figure 2.6. Representative images for fungal development of six isolates of <i>Ptt</i> <i>in planta</i> .....	41
Figure 2.7. Differences in the hyphal length between six isolates of <i>Ptt</i> at 24 hours post inoculation (hpi) <i>in planta</i> in barley cv. Sloop. ....	43

Figure 2.8. Fungal development of six isolates of <i>Ptt</i> after 24 hours post inoculation (hpi) <i>in planta</i> . .....	44
Figure 2.9. Optimisation of toxin production in two isolates of <i>Ptt</i> (32/98, more virulent) and 08/08f (less virulent) using two types of media. ....	45
Figure 2.10. Optimisation of protein production in 32/98 and 152/09 (more virulent isolates) grown for 12 days (d) on two types of media. ....	47
Figure 2.11. Concentration of protein in the filtrates of two virulent isolates (32/98 and 152/09). .....	48
Figure 2.12. PAGE for total protein extracted from the filtrates of 32/98 and 152/09 grown for 23 days in two types of media. ....	48
Figure 2.13. Effect toxin extraction methods on the bioassay of toxins on barley cv. Sloop. ....	49
Figure 2.14. Bioassay test for proteinaceous toxins extracted from filtrates of the six isolates of <i>Ptt</i> . ....	51
Figure 3.1. Bioassays of 5 fractions (F1 to F5) from the culture filtrates of the more virulent isolate (32/98) and less virulent isolate (08/08f) after 5 days post injection. ....	70
Figure 3.2. Protein comparison on PAGE between protein fractions (F2 to F5) of the culture filtrates of the more (32/98) and less (08/08f) virulent isolates. ....	72
Figure 3.3. Bioassay of 10 protein fractions (F1 to F10) from filtrates of the more virulent isolate (32/98) on three barley cultivars 5 days post injection. ....	77
Figure 3.4. Bioassay of 6 protein sub-fractions (SF4/1 to SF4/6) of more virulent isolate (32/98) on three barley cultivars after 5 days post injection. ....	78
Figure 3.5. Separation of protein fractions (F3 to F10), protein sub-fractions (4/1-4/6) and crude toxin (32T) for more virulence isolate 32/98 on PAGE. ....	80

Figure 3.6. 2DGE comparison of protein profiles of fraction 4 (A) and fraction 6 (B) from 32/98 isolate. ....	87
Figure 3.7. Identification of proteins extracted from culture filtrates from the more virulent isolate 32/98 (A) and the least virulent isolate 08/08f (B) using 2-D gel electrophoresis.....	89
Figure 3.8. PAGE for proteins extracted after 6 and 9 day post inoculation (dpi) from uninfected leaves (C) and leaves infected with 32/98 isolate (32) of <i>Ptt</i> . ....	92
Figure 3.9. Separation of proteins extracted from 32/98 crude toxin (32T), fraction 4 (F4), sub-fraction 4/2 (SF4/2) and intercellular washing fluids (ICWFs) using PAGE.....	92
Figure 3.10. 2DGE comparison of protein profiles of ICWFs from infected and uninfected plants.....	97
Figure 4.1. The full length of <i>PttXyn11A</i> cDNA and PttXyn11A protein (JX900133) (A). ....	121
Figure 4.2. Multiple alignment and phylogenetic analysis of PttXyn11A and proteins containing Glycosyl hydrolases family 11 domain. ....	124
Figure 4.3. Alignment of PttXyn11A of <i>Ptt</i> with TrXyn11A, <i>Trichoderma reesei</i> and BcXyn11A, <i>B. cinerea</i> (Brito <i>et al.</i> , 2006). ....	126
Figure 4.4. Secondary and three dimensional structure of PttXyn11A of <i>Ptt</i> . .	129
Figure 4.5. The full length of <i>PttCHFPI</i> cDNA and PttCHFPI protein (JX900134).....	131
Figure 4.6. Phylogenetic tree of selected members of cysteine hydrolase superfamily. ....	132
Figure 4.7. Multiple alignment of PttCHFPI and selected members of isochorismatase family proteins. ....	134
Figure 4.8. The predicted three dimensional structure of PttCHFPI of <i>Ptt</i> (A) and <i>P. aeruginosa</i> 1NF8 protein (B). ....	135
Figure 4.9. The full length of <i>PttGPI-CFEM</i> cDNA and PttGPI-CFEM protein (A).....	137

Figure 4.10. Multiple alignment of PttGPI-CFEM and fungal proteins showing the eight cysteine-containing CFEM domain (A). .....	138
Figure 4.11. The predicted three dimensional structure of PttGPI-CFEM of <i>Ptt</i> . .....	139
Figure 4.12. The full length of <i>PttSP1</i> cDNA and PttSP1 protein (A). .....	141
Figure 4.13. Multiple alignment of PttSP1 and bacterial proteins showing lipid attachment site. ....	142
Figure 4.14. Secondary and three dimensional structure of PttSP1. ....	143
Figure 4.15. The predicted three dimensional structure of PttSP1 of <i>Ptt</i> . ....	144
Figure 4.16. Differences in the expression level of VRCGs for six isolates ( <i>in vitro</i> ) growing for 10 days in FCM. ....	145
Figure 4.17. Differences in the expression level of VRCGs for six isolates <i>in planta</i> at 192 hours post inoculation. ....	146
Figure 5.1. Denaturing purification of PttGPI-CFEM heterologously expressed in <i>E. coli</i> . ....	163
Figure 5.2. Optimisation of heterologous expression of PttXyn11A in <i>E. coli</i> with different temperatures. ....	165
Figure 5.3. Optimisation of heterologous expression of PttXyn11A in <i>E. coli</i> with different incubation time and L-arabinose concentration. ....	166
Figure 5.4. Heterologous expression of PttXyn11A in <i>E.coli</i> . ....	167
Figure 5.5. The effect of overexpression of PttXyn11A on the viability of <i>E. coli</i> . ....	168
Figure 5.6. Optimisation of heterologous expression of PttCHFP1 in <i>E. coli</i> with different incubation conditions and L-arabinose concentration. ....	169
Figure 5.7. Heterologous expression and denaturing purification of PttCHFP1 in <i>E.coli</i> . ....	170
Figure 5.8. Heterologous expression and native purification of PttCHFP1 in <i>E.coli</i> . ....	170

Figure 5.9. The effect of overexpression of PttCHFP1 on the viability of <i>E. coli</i> . .....	171
Figure 5.10. Optimisation of heterologous expression of PttSP1 in <i>E. coli</i> with different incubation conditions and L-arabinose concentration. ..	173
Figure 5.11. Heterologous expression and denaturing purification of PttSP1 in <i>E. coli</i> .....	174
Figure 5.12. Heterologous expression and native purification of PttSP1 in <i>E.</i> <i>coli</i> . .....	175
Figure 5.13. The effect of overexpression of PttSP1 on the viability of <i>E.coli</i> . .....	176
Figure 5.14. Bioassay of heterologously expressed PttXyn11A (X), PttCHFP1 (C) and PttSP1 (S) (expressed in <i>E. coli</i> ) unwashed protein. ....	179
Figure 5.15. Bioassay of heterologously expressed PttXyn11A (X), PttCHFP1 (C) and PttSP1 (S) (expressed in <i>E. coli</i> ) washed protein. ....	180
Figure 5.16. Bioassay of heterologously expressed PttXyn11A (X), PttCHFP1 (C) and PttSP1 (S) (expressed in <i>E. coli</i> ) on three cultivars. ....	181
Figure 5.17. Bioassay of heterologously expressed PttXyn11A (X), PttCHFP1 (C) and PttSP1 (S) (expressed in <i>E. coli</i> ), clear lysate on three cultivars. ....	182
Figure 5.18. Effect of protein concentration on the bioassay of heterologously expressed PttCHFP1 (expressed in <i>E. coli</i> ) on cv. Sloop.....	184
Figure 5.19. Bioassay of heterologously expressed PttSP1 (expressed in <i>E. coli</i> ) on cv. Sloop. ....	185
Figure 5.20. Bioassay of commercial xylanase on cv. Sloop. ....	186
Figure 5.21. Activity of xylanase (X) in the culture filtrate of six isolates of <i>Ptt</i> grown at FCM for 10 and 15 days. ....	188
Figure 6.1. Virulence of six isolates at different time on barley cv. Sloop, starting from 40 hours post inoculation (hpi) until 192 hpi.....	205
Figure 6.2. Virulence score of six isolates at different time on barley cv. Sloop, from 64 hours post inoculation (hpi) until 192 hpi.....	206

Figure 6.3. Fluorescent value of qPCR of <i>PttGAPDH</i> for six isolates at 192 hours post inoculation (hpi).....	208
Figure 6.4. The expression of VRCGs during the interaction of <i>Ptt</i> isolates and barley. ....	215
Figure 7.1. Simplified schematic showing the proposed scenario for <i>Ptt</i> infection process.....	231
Figure A1.1. Tekauz's scale (Tekauz, 1985); the scale describes the reaction of barley to <i>P. teres</i> f. <i>teres</i> and illustrates these reaction using numbers from 1 to 10. ....	257
Figure A2.1. RT-PCR cycle number optimisation for VRCGs <i>PttSP1</i> , <i>PttXyn11A</i> , <i>PttCHFP1</i> , <i>PttGPI-CFEM</i> and <i>PttGAPDH</i> . ....	259
Figure A2.2. Multiple alignment of PttCHFP1 and CSHase family proteins. .	260
Figure A2.3. The evolutionary history of PttCHFP1 and its homologues from CSHase family.....	261
Figure A2.4. Multiple alignment of PttCHFP1 and Nicotinamidase family proteins. ....	262
Figure A2.5. The evolutionary history of PttCHFP1 and its homologues from Nicotinamidase family.....	263
Figure A2.6. Secondary structure of PttGPI-CFEM and 4 selected members of CFEM family.....	264
Figure A2.7. Graphical summary of PttSP1 BLAST in UniProtKB databases. ....	265
Figure A3.1. Native extraction of heterologous expression of PttXyn11A from <i>E. coli</i> with different temperatures. ....	266
Figure A3.2. Denaturing extraction of heterologous expression of PttXyn11A in new transformant of <i>E. coli</i> . ....	267

Figure A3.3. Optimisation of heterologous expression of <i>PttCHF1</i> in <i>E. coli</i> with different incubation conditions and L-arabinose concentration. ....	267
Figure A3.4. Optimisation of heterologous expression of <i>PttSP1</i> in <i>E. coli</i> with different incubation conditions and L-arabinose concentration. ..	268
Figure A4.1. RT-PCR products (partial product) for four VRCGs isolated from 32/98 mycelium grown for 10 day on FCM. ....	269
Figure A4.2. Sequence confirmation and alignment of qRT-PCR primers products with the full length of corresponding genes. ....	273
Figure A4.3. Expression of VRCGs at 40 and 192 hours post inoculation (hpi) using comparative CT ( $\Delta\Delta C_T$ ) method. ....	274
Figure A4.4. Amplification efficiency for VRCGs at different primer concentrations. ....	274
Figure A4.5. Standard curves for VRCGs with low concentration of cDNA. ....	275
Figure A4.6. Optimising of standard curve for VRCGs at lower cDNA concentration. ....	275
Figure A4.7. Standard curve for VRCGs with high concentration of cDNA. ....	276
Figure A4.8. Optimising standard curve for VRCGs at higher cDNA concentration. ....	276
Figure A4.9. The expression of VRCGs ( <i>PttSP1</i> , <i>PttXyn11A</i> , <i>PttCHF1</i> , <i>PttGPI-CFEM</i> and <i>PttGAPDH</i> ) in (pg) at 40, 64, 120 and 144 hours post inoculation (hpi) in six isolates of <i>Ptt</i> in three experiments. ....	277

## List of Tables

Table 2.1. Origin and isolation year of <i>Pyrenophora teres</i> f. <i>teres</i> isolates used in this study and the host barley cultivar from which they were isolated.....	23
Table 2.2. Media and their components used for toxin production optimisation. ....	29
Table 3.1. Protein identification of the active fractions from more (32/98) and less (08/08f) virulent isolates.....	74
Table 3.2. Protein concentrations in fractions and sub-fractions of culture filtrates from the 32/98 isolate.....	75
Table 3.3. Protein identification of the active fractions and sub-fractions from the culture filtrate of a more virulent isolate (32/98). ....	82
Table 3.4. Protein identification of whole fraction 4 and 4/4 from filtrates of a more virulent isolate (32/98). ....	84
Table 3.5. Protein identification of differentially expressed two-dimensional gel electrophoresis (2DGE) protein spots (Figure 3.7). ....	90
Table 3.6. Protein identification of ICWFs from barley leaves infected with <i>Ptt</i> isolate 32/98 after 9 dpi. ....	93
Table 3.7. Protein identification of 2DGE comparison of ICWFs from infected and uninfected leaves after 9 dpi (Figure 3.10). ....	98
Table 3.8. The most dominant proteins as identified in the biologically active protein profiles and their accession number, source, family and proposed function. ....	104
Table 4.1. List of primers used in full length isolation and semi-quantitative RT-PCR to amplify the cDNA of the VRCGs <i>PttXyn11A</i> , <i>PttCHFPI</i> , <i>PttSP1</i> , <i>PttGPI-CFEM</i> and <i>PttGAPDH</i> . ....	112
Table 4.2. Selected proteins of GH11 family and their accession numbers and organisms which were used in multiple alignments with <i>PttXyn11A</i> (JX900133). ....	115

Table 4.3. Cysteine hydrolase families used in the alignments and phylogenetic analysis. ....	116
Table 6.1. List of primers used in RT-qPCR to amplify the partial length of cDNA of VRCGs <i>PttSPI</i> , <i>PttXyn11A</i> , <i>PttCHFPI</i> , <i>PttGPI-CFEM</i> and <i>PttGAPDH</i> . ....	198
Table A1.1. A numerical scale to assess development of <i>P. teres</i> during its growth upon barley using light microscopy (Lightfoot and Able, 2010). ....	258

## **Abstract**

*Pyrenophora teres f. teres* (*Ptt*) causes net form net blotch disease (NFNB), an important disease of barley in Australia and worldwide. This fungus uses proteinaceous toxins to cause necrosis and different isolates of *Ptt* differ in their ability to cause symptoms on different cultivars of barley. However, little is known about the roles of pathogen growth and individual toxins in symptom development. This project therefore aimed to determine whether there is a relationship between toxin production, fungal growth and virulence in NFNB.

Conidial germination, extent of fungal growth and culture filtrate toxicity were compared for six South Australian *Ptt* isolates with different virulence on the barley cultivar ‘Sloop’. In addition, *Ptt* toxin production was optimised before identification and selection of virulence-related candidate proteins (VRCPs) for further characterisation. The biological activity of recombinant VRCPs on susceptible and resistant cultivars and VRCPs gene expression during the interaction of Sloop with each isolate were also compared.

In general, the more virulent isolates had higher rates of conidial germination (both *in vitro* and *in planta*) and fungal development *in planta*, represented by longer hyphae and more appressoria, compared with less virulent isolates. Similarly, *PttGAPDH* and its transcript were more abundant during the interaction of barley with more virulent isolates.

A proteomics approach was used to identify proteins unique to the more virulent isolate, proteins from bioactive fractions on either susceptible (Sloop) or resistant cultivars (CI9214 and Beecher) and proteins from the intercellular washing fluids (ICWFs) of infected barley. These analyses revealed that *Ptt* produced proteins between 37 and 150 kDa that have biological activity.

Liquid Chromatography-Electrospray Ionisation Ion-Trap Mass Spectrometry (LC-eSI-IT MS), of individual biologically active proteins was used to identify peptides which matched to 17 proteins that belong to three groups of fungal proteins including virulence-related proteins; fungal growth and development proteins; and those with unknown function (hypothetical proteins). However, *Ptt* toxins were not detected in the ICWF protein profiles suggesting that *Ptt* toxins were either in trace amounts or might be internalised into the cell.

The four VRCPs selected, were identified as hypothetical proteins with unknown function in the *Ptt* database. Further bioinformatic analysis characterised these VRCPs as an isochorismatase (PttCHFP1), an endo-1, 4- $\beta$ -xylanase A (PttXyn11A), a glycoposphatidylinositol (GPI)-anchored common in fungal extracellular membrane (CFEM) domain-containing protein (PttGPI-CFEM) and an unknown proteinaceous secreted (but conserved) hypothetical protein (PttSP1). These VRCPs were heterologously expressed and characterised using gene expression studies.

PttXyn11A had strong homology with the well characterised endoxylanases, TrXyn11A from *Trichoderma reesei* and BcXyn11A from *Botrytis cinerea*, known to contribute to virulence. A necrosis-inducing region on the surface of the enzyme was also identified in PttXyn11A, suggesting a potential role in necrosis induction. The culture filtrates for more virulent isolates had significantly greater xylanase activity than those from less virulent isolates. Even though heterologously expressed *PttXyn11A* was toxic to *Escherichia coli*, xylanase activity was detectable at very low levels and was not enough to cause symptoms in the bioassay. In addition, semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) and RT-quantitative PCR (RT-qPCR) analysis demonstrated that *PttXyn11A* was expressed more abundantly by the more virulent isolates compared with the other isolates in culture and during the plant-pathogen interaction. Together, these results suggest that *PttXyn11A* plays a role in virulence, either through its ability to degrade the plant cell wall to assist fungal growth or through its necrosis-inducing ability.

PttCHF1 showed homology to an isochorismatase, an enzyme that has been proposed to have a role in plant defence via inhibition of salicylic acid production. PttSP1 showed homology to a membrane lipoprotein proposed to have a role in fungal development. Bioassay of recombinant *PttCHF1* and *PttSP1* induced chlorosis symptoms in the susceptible barley cultivar (Sloop). The cysteine-rich CFEM domain identified in PttGPI-CFEM has been

suggested to have an important role in hyphal attachment and fungal networking. However, *E. coli* was not able to express this gene probably due to its attachment to the plasma membrane and/or cell wall. Analysis of the gene expression profiles for *PttCHFPI*, *PttGPI-CFEM* and *PttSPI* showed no significant differences between isolates *in vitro* and *in planta* suggesting that all isolates regulated the expression of these genes to the essential level possibly required for pathogenesis.

This is the first study to identify the relationship between fungal growth and proteinaceous toxin production, characterise individual proteinaceous toxins in the mixture of *Ptt* culture filtrate and investigate the expression profiles of genes encoding VRCPs during the *Ptt*-barley interaction. This study therefore provides a better understanding of the *Ptt*-barley interaction by identifying the potential toxins which might lead to identify the toxin targets and ultimately support the breeding of resistant cultivars of barley.

## **Declaration**

I declare that the work presented in this thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Ismail Ahmed Ismail, and to the best of my knowledge and belief, this thesis does not contain any material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

The author acknowledges that copyright of the published works contained within this thesis (as listed in the Appendix 5) resides with the copyright holder (s) of those works. I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue, and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Ismail Ahmed Ismail

/ /

## **Acknowledgment**

I would like to thank the Ministry of Higher Education and Scientific Research of Iraq for providing me this PhD scholarship. My sincere thanks to my principal supervisor Associate Professor Amanda J. Able for the support, help, encouragement, patience, guidance and for believing in me. I am also grateful for my co-supervisor Dr. Dale Godfrey for her assistance, advices and support in my research.

Thanks to the Grains Research and Development Corporation for their support of this research and for providing a travel scholarship to attend the 4th Asian Conference on Plant Pathology and the 18th Biennial Australasian Plant Pathology Conference in Darwin. My thanks to Dr. Hugh Wallwork for providing *Pyrenophora teres* f. *teres* isolates and Mark Butt for his assistance in plant and fungal preparations.

I also would like to thank Dr William Bovill and Dr Kelvin Khoo for their help, Darren Wong, Wan Mohd Aizat Wan Kamaruddin, Duc Thong Le and all Able Lab members for their warm and beneficial discussion.

I am appreciative for the Iraqi Cultural Affair in Australia, the International Student Centre in Adelaide, especially Ms Jane Copeland, and the School of Agriculture, Food and Wine for their continuous support.

Finally, my special thanks to my beautiful wife Entesar for her incredible patience and love, my lovely three kids, Durar, Yousif and Ibrahim. Many thanks to my mother for her prayer, asking my lord to recuperate her, my brothers and sister for their sacrifices and support.

## Abbreviation

<u>Abbreviation</u>	<u>Full term</u>
×	Times
°C	Degree Celsius
2DGE	Two-Dimensional Gel Electrophoresis
3′	Three prime
5′	Five prime
ASP	Ammonium sulfate precipitation
BLAST	Basic Local Alignment Search Tool
BLM	Barley leaf medium
bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary DNA
CHAPS	3-[(3- cholamidopropyl) dimethylammonio]-1-propanesulfonate
cm	Centimeter
cv	Cultivar
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dpi	Day post inoculation
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
EST	Expressed sequence tag

FCM	Fries culture medium
FCM-noTE	FCM with no trace elements
<i>g</i>	<i>g</i> -force
<i>g</i>	Gram
<i>h</i>	Hour
<i>hpi</i>	Hour post inoculation
HNST	Host non-selective toxin
HST	Host specific toxin
ICWF	Intercellular washing fluid
IEF	Isoelectric focusing
IPTG	Isopropylthiogalactosidase
Kb	Kilobase
kDa	Kilodaltons
L	Liter
LB	Luria Bertani
LC-eSI-ITMS	Liquid Chromatography-Electrospray Ionisation Ion-Trap Mass Spectrometry
LMWC	Low molecular weight compound
LSD	Least significant difference
M	Molar
mA	Milliampere
MES	2-(N-morpholino) ethanesulfonic acid
mg	Milligram

min	Minute
mL	Millilitre
ML/min	Millilitre per minute
mM	Milimolar
mm	Millimeter
mRNA	Messenger ribonucleic acid
MS	Mass spectrophotometry
MW	Molecular weight
NCBI	National Center for Biotechnology Information
ng	Nanogram
Ni-NTA	Nikle-nitrilotriacetic acid
nm	Nanometers
OD	Optical density
ORF	Open reading frame
P	Probability
PAGE	Polyacrylamide Gel Electrophoresis
PCD	Programmed cell death
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PFCM	Phosphate buffered FCM
pH	Potential of Hydrogen
<i>pI</i>	Isoelectric point
PR-Protein	Pathogenesis related-protein

PS	Photosystem
QTL	Quantitative trait loci
r	Correlation coefficient
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
rpm	Revolutions per minute
RQ	Relative quantification
RT-PCR	Reverse transcriptase PCR
SDS	Sodium dodecyl sulphate
SDW	Sterile distilled water
sec	Second
SNW	Sterile nanopure water
TAE buffer	Tris-acetate EDTA buffer
TCA	Trichloroacetic acid
TE buffer	Tris EDTA buffer
Tm	Melting temperature
Tris	Tris (hydroxymethyl) amino methane
U	Unit
UFP	Ultrafiltration purification
UV	Ultra violet
V	Voltage
v/v	Volume for volume
Vhr	Volt hours
w/v	Weight for volume
µg	Micrograms
µg/mL	Micrograms per millilitre
µL	Micro litre
µM	Micromolar
µm	Micrometer