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Molecular Quest for Avirulence Factors in *Venturia inaequalis*

Joe Win

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biological Sciences, The University of Auckland, 2003
Abstract

The molecular basis for the gene-for-gene relationship of $V_m$-resistance in apple to *Venturia inaequalis* was investigated. Incompatible reactions involved a hypersensitive response (HR), which was accompanied by the accumulation of dark brown pigments and autofluorescent materials in epidermal and mesophyll cells at the site of invasion. Cell-free culture filtrates of the avirulent isolate elicited an HR in the $V_m$ host (h$_5$) leaves, but not in the susceptible host (h$_1$). The elicitor activity was resistant to boiling but was abolished by proteinase K digestion. Elicitation of HR was used to monitor purification of the avirulence factor, AVRVm, from liquid cultures of the avirulent isolate following ultrafiltration, acetone precipitation and ion-exchange chromatography. The purest fraction contained three major proteins all with low isoelectric points (pI 3.0-4.5). The fraction also elicited HR on the differential host h$_4$, but not on other resistant hosts (h$_2$, h$_3$ and h$_6$) tested. Three candidate AVRVm proteins were identified and amino acid sequences were obtained using Edman degradation and mass spectrometry. Nucleotide sequences corresponding to these proteins were found in databases of *V. inaequalis* expressed sequence tags. There were no polymorphisms evident between avirulent and virulent isolates (representing races 1 and 5 respectively) either at genomic DNA or cDNA level of the full open reading frames. RT-PCR revealed that all genes were expressed in both avirulent and virulent isolates during *in vitro* and *in planta* growth. All three genes showed similar levels of expression between avirulent and virulent isolates during their *in vitro* growth. However, preliminary RT-PCR experiments showed that two of these genes were likely to be expressed at lower levels in the virulent compared with the avirulent isolate during compatible infection. Implications of this difference in expression and the future experiments to identify the genuine *AvrVm* gene were discussed.
Dedication

To my mother, Mi Nyunt Kyi, who unconditionally supported (and she still does) me with love and everything else I asked of her throughout this thesis and my life.
Acknowledgments

Foremost, I thank my Supervisor Dr. Kim Plummer and Co-supervisor Dr. David Greenwood. Kim is the chief architect of this project. I appreciate her visionary guidance on the project and all the hours she put into this thesis editing and commenting. Dave acquired nano-spray ES-MS data from HPLC fractions and helped me a lot with protein biochemistry as well as my writing.

I acknowledge the involvement of Dr. Christiane Stehmann in acquiring MS data from the protein spots and her advice on protein purification. I also acknowledge the contributions from Dr Wei Cui who sequenced and aligned the genomic DNA from the fungal races, and Sarah Hollingworth who constructed the in vitro cDNA library.

Special thanks to Dr. Jan van Kan, Wageningen University, for his critical and insightful comments and discussions on the contents of this thesis and our paper, and his fresh ideas brought to the lab. Thanks also to Vincent Bus who supplied me with apple hosts that were essential part of the project. I owe Anna Fitzgerald a million thanks for her generosity with apple seedlings used in in planta experiments and for providing phage plates for library sequencing.

I value the moral and social support from all PHDie/GeneTechies who also are great source of expert information and techniques. Database searches would not have been possible without the help of Marcus Davy who not only compiled all the in vitro EST sequences into a database but also turned my PC into a BLAST work station.

All experiments in this thesis were performed at HortResearch, Mt Albert Research Centre, Auckland. This research was supported by The Royal Society of New Zealand through the Marsden Fund (Contract Number: HOR602) and by the Foundation for Research, Science and Technology, New Zealand (Contract Number: C06X0207).
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Abbreviations

SI units (Système International d'unités) and their derived units were used throughout this thesis. One-letter symbols for amino acids and nucleotides were according to IUPAC-IUB (The International Union of Pure and Applied Chemistry -International Union of Biochemistry) guidelines (International Union of Biochemistry, 1978).

<table>
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<th>Description</th>
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<tr>
<td>°C</td>
<td>degree Centigrade</td>
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<tr>
<td>2D</td>
<td>two-dimensional</td>
</tr>
<tr>
<td>APAF</td>
<td>Australian Proteome Analysis Facility</td>
</tr>
<tr>
<td>Avr</td>
<td>avirulence</td>
</tr>
<tr>
<td>AvrVx</td>
<td>avirulence gene from <em>V. inaequalis</em> corresponding to resistance gene <em>V.x</em></td>
</tr>
<tr>
<td>AVRVx</td>
<td>avirulence protein from <em>V. inaequalis</em> corresponding to resistance Protein <em>V.x</em></td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>CC</td>
<td>coiled-coil domain</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CF</td>
<td>cell-free culture filtrate</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate</td>
</tr>
<tr>
<td>cM</td>
<td>centi-Morgan</td>
</tr>
<tr>
<td>d</td>
<td>day</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>E-value</td>
<td>Expect value</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionisation</td>
</tr>
<tr>
<td>EST</td>
<td>expressed sequence tag</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HR</td>
<td>hypersensitive response</td>
</tr>
<tr>
<td>h_x</td>
<td><em>Malus</em> differential host, <em>x</em> = number</td>
</tr>
<tr>
<td>IEF</td>
<td>isoelectric focusing</td>
</tr>
<tr>
<td>IPG</td>
<td>immobilised pH gradient</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>LC</td>
<td>liquid chromatography</td>
</tr>
<tr>
<td>LRR</td>
<td>leucine rich repeat</td>
</tr>
<tr>
<td>LZ</td>
<td>leucine zipper</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>matrix-assisted laser desorption/ionisation-time of flight</td>
</tr>
<tr>
<td>MES</td>
<td>2-[N-morpholino]ethanesulphonic acid</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>MWCO</td>
<td>molecular weight cut off</td>
</tr>
<tr>
<td>N-terminal</td>
<td>amino terminal</td>
</tr>
<tr>
<td>NBS</td>
<td>nucleotide binding sites</td>
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<td>number</td>
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<td>ORF</td>
<td>open reading frame</td>
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<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PDA</td>
<td>potato dextrose agar</td>
</tr>
<tr>
<td>PDB</td>
<td>potato dextrose broth</td>
</tr>
<tr>
<td>pI</td>
<td>isoelectric point</td>
</tr>
<tr>
<td>PK</td>
<td>protein kinase</td>
</tr>
<tr>
<td>PR</td>
<td>pathogenesis-related</td>
</tr>
<tr>
<td>PTH</td>
<td>3-phenyl-2-thiohydantoin</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>Q-TOF</td>
<td>quadrupole-time of flight</td>
</tr>
<tr>
<td>R</td>
<td>resistance</td>
</tr>
<tr>
<td>RGH</td>
<td>resistance gene homologs</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcriptase-primed polymerase chain reaction</td>
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<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>TAE</td>
<td>tris-acetate EDTA running buffer</td>
</tr>
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<td>TBP</td>
<td>Tributylphosphine</td>
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<td>trifluoroacetic acid</td>
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<td>TIR</td>
<td>Toll/Interleukin 1 receptor</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane region</td>
</tr>
<tr>
<td>Tm</td>
<td>melting temperature</td>
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<tr>
<td>U</td>
<td>unit</td>
</tr>
<tr>
<td>UV</td>
<td>ultra-violet</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
</tr>
<tr>
<td>Vh</td>
<td>Volt hours</td>
</tr>
<tr>
<td>V_x</td>
<td>Resistance gene against <em>V. inaequalis</em> from <em>Malus</em> spp., x = initial of the species of <em>Malus</em> (e.g., <em>V_m</em> = <em>V. inaequalis</em> resistance gene from <em>M. micromalus</em>)</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per volume</td>
</tr>
</tbody>
</table>