

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.

**Effector-triggered immunity
against *Pseudomonas syringae* pv.
actinidiae in nonhost plants**

Jay Jayaraman

Thesis submitted to the Massey University
for the degree of Doctor of Philosophy

April 2017

© This copy of the thesis has been supplied on the condition that anyone who consults it is understood to recognize that its copyright rests with the author and that no quotation from the thesis, nor any information derived therefrom, may be published without the author's prior written consent.

Contents

Abstract	6
Acknowledgements	8
Contributions to thesis	9
Abbreviations	10

Chapter 1: General Introduction

1.1 The kiwifruit industry & <i>Pseudomonas syringae</i> pv. <i>actinidiae</i>	14
1.2 <i>Arabidopsis thaliana</i> as a model for plant defence	19
1.2.1 <i>Pseudomonas syringae</i> infection of <i>Arabidopsis</i>	19
1.2.2 <i>Hyaloperonospora arabidopsidis</i> infection of <i>Arabidopsis</i>	21
1.3 PAMP-triggered immunity.....	26
1.3.1 Pathogens, PAMPs & perception.....	26
1.3.2 PTI signalling & defence.....	29
1.4 Effectors of plant pathogenic microbes.....	33
1.4.1 Bacterial effector delivery system.....	34
1.4.1.1 Type I, II and V secretion.....	34
1.4.1.2 Type III secretion.....	36
1.4.1.3 Type IV secretion.....	39
1.4.2 Effector delivery by filamentous pathogens	39
1.4.3 Effectors & avirulence proteins.....	41
1.4.4 Biochemical functions of effectors as virulence agents	45
1.4.4.1 Extracellular effectors	46
1.4.4.2 Suppression of PTI signaling	46
1.4.4.3 Phytohormone manipulation	48
1.4.4.4 Targeting pathogen entry barriers and secretion	51
1.4.4.5 Modulation of effector-triggered immunity and susceptibility	54
1.5 Effector triggered immunity	56
1.5.1 NB-LRRs	57
1.5.2 Cytoplasmic serine/threonine Kinases	59
1.5.3 Receptor-like proteins	61
1.5.4 Receptor-like kinases	62
1.5.5 Mechanism of signalling during ETI	63
1.5.5.1 Direct Avr-R interaction	64
1.5.5.2 Guard-guardee/decoy hypothesis	64
1.5.5.3 <i>R</i> gene pairs	67
1.6 Aims of this study	69

Chapter 2: Materials & Methods

Materials

2.1 Plasmid constructs	72
2.1.1 Construction of Golden Gate-compatible broad host-range plasmid	74
2.1.2 Construction of Golden Gate-compatible yeast 2-hybrid plasmids	74
2.1.3 Construction of dual luciferase reporter vector	75
2.2 Bacterial strains	76
2.2.1 Wildtype <i>Psa</i> strains	77
2.2.2 Triparental mating	77
2.2.3 Competent cell preparation & transformation	78
2.2.4 Glycerol stocks	78
2.3 Plant material	78
2.3.1 <i>Arabidopsis thaliana</i> accessions	78
2.3.2 <i>Nicotiana tabacum</i>	79
2.3.3 <i>Nicotiana benthamiana</i>	79

2.4 Bacterial strains for cloning	79
Methods	
2.5 Pathology methods	79
2.5.1 Bacterial infiltrations for HR assays in Arabidopsis	79
2.5.2 Type-III secreted effector electrolyte leakage assay	80
2.5.3 <i>In planta</i> bacterial growth assays	80
2.5.3.1 Bacterial spray inoculation assays for growth in Arabidopsis	81
2.5.4 Floral dip transformation of Arabidopsis	81
2.5.5 <i>Agrobacterium tumefaciens</i> transient infiltration	82
2.5.5.1 Confocal laser scanning microscopy for subcellular localization	82
2.5.6 <i>N. benthamiana</i> electrolyte leakage assay	82
2.5.7 Virus-induced gene silencing	83
2.5.8 Genotyping T-DNA knockout Arabidopsis lines for homozygous mutants	83
2.5.9 WRKY reporter activation/repression assay	83
2.6 Molecular biology methods	84
2.6.1 Enzymes	84
2.6.2 DNA	84
2.6.2.1 Bacterial gDNA extraction methods	84
2.6.2.2 Plant gDNA extraction methods	84
2.6.2.3 Polymerase chain reaction	85
2.6.2.4 Agarose gel electrophoresis	85
2.6.2.5 Agarose gel purification of DNA	85
2.6.2.6 Blunt-end cloning	85
2.6.2.7 Golden gate cloning	86
2.6.2.8 Plasmid isolation	86
2.6.2.9 Alkaline lysis miniprep	87
2.6.2.10 Site-directed mutagenesis	87
2.6.2.11 DNA sequencing	87
2.6.3 RNA	87
2.6.3.1 Total RNA extraction	87
2.6.3.2 cDNA synthesis	88
2.6.3.3 Quantitative reverse transcription PCR (RT-qPCR)	88
2.6.4 Protein	89
2.6.4.1 Total protein extraction	89
2.6.4.1.1 Immunoprecipitation	89
2.6.4.2 SDS-PAGE & Western blot	90
2.6.5 Yeast 2-hybrid	90
2.6.5.1 Yeast transformation	90
2.6.5.2 Yeast mating and interaction	91

Chapter 3: HopZ5, a plasma membrane-localized acetyltransferase effector from *Psa* V13, dissects hypersensitive response from immunity in Arabidopsis

3.1 Introduction	92
3.2 Results	94
3.2.1 HopZ5 _{<i>Psa</i>V13} triggers accession-specific immunity in Arabidopsis	94
3.2.2 HopZ5 triggers immunity independent of HR in Col-0	98
3.2.3 HopZ5 is a putative acetyltransferase with key residues required for triggering both HR and immunity, independently in Arabidopsis	102
3.2.4 HopZ5 triggers HR in <i>Nicotiana</i> spp. and requires <i>SGT1</i>	110
3.2.5 HopZ5 triggered immunity in Col-0 requires <i>SID2</i> , <i>EDS1</i> & <i>NDR1</i>	113
3.2.6 HopZ5 is a <i>bona fide</i> plasma membrane-localized acetyltransferase that autoacetylates <i>in planta</i>	114
3.2.7 HopZ5 does not suppress ETI	116
3.3 Discussion	118
3.3.1 Resistance without HR in Arabidopsis	119

3.3.2	The YopJ family is diverse with little conservation of <i>in planta</i> targets	121
3.3.3	Quantitative involvement of autoacetylation in bacterial acetyltransferase activity	123
3.3.4	Genetic requirement for HopZ5-triggered immunity suggests recognition by a novel NLR	124

Chapter 4: Multiple sources of avirulence present in the *Psa* LV5 effectome mediate nonhost resistance

4.1	Introduction	126
4.2	Results	130
4.2.1	<i>Psa</i> LV5 triggers HR and avirulence in Arabidopsis conferred by the <i>RPS5/SUMM2</i> locus	130
4.2.2	A homolog of AvrPphB in <i>Psa</i> LV5 triggers RPS5-mediated HR in Col-0 when delivered under its native promoter	135
4.2.3	<i>Psa</i> LV5 triggers AvrPtoB homolog-dependent HCD in <i>Nicotiana benthamiana</i>	140
4.2.4	HopAB3 is recognized by Pto but not Fen	144
4.2.5	HopAB3-triggered HCD is dependent on its E3 ligase allele despite functional E3 ligase activity	147
4.2.6	HopAB3 triggers ion leakage in tomato putatively dependent on a Fen homolog, <i>SI</i> PtoB	150
4.3	Discussion	156
4.3.1	<i>Psa</i> LV5 is a pathogen of sour cherry that only grows epiphytically on kiwifruit	156
4.3.2	Col-0 responds without an HR to some avirulent effectors when delivered under the AvrRps4 promoter	158
4.3.3	The AvrPto/AvrPtoB recognition is multipartite and variegated	161

Chapter 5: Multiple effectors from *Pseudomonas* and *Hyaloperonospora arabidopsidis* target WRKY transcription factors in plant hosts

5.1	Introduction	164
5.2	Results	165
5.2.1	Multiple nuclear-localized effectors from <i>Pseudomonas</i> and <i>Hyaloperonospora arabidopsidis</i> interact with Arabidopsis WRKYs	165
5.2.2	Overexpressing AtWRKYs in <i>Nicotiana</i> spp. triggers a conditional HCD dependent on their putative DNA-binding ability and <i>SGT1</i>	183
5.2.3	AtWRKY54-triggered HCD is representative for immune-triggering ability of group III-a AtWRKYs	188
5.2.4	AvrRps4 and PopP2 specifically suppress the AtWRKY54 overexpression HCD phenotype	190
5.2.5	Unprocessed AvrRps4 alone can suppress AtWRKY54	195
5.2.6	AtWRKY54 is a transcriptional activator suppressed by functional AvrRps4 or PopP2	197
5.3	Discussion	201
5.3.1	AtWRKYs targeted by multiple effectors could serve as immunity hubs	202
5.3.2	AtWRKYs can trigger HCD in <i>Nicotiana</i> reminiscent of an immune response	206
5.3.3	Full length AvrRps4 has evolved to specifically target group III WRKYs	208

Chapter 6: General discussion

6.1	HopZ5 virulence function by association	214
6.2	Nonhost resistance versus host resistance: a distinction without a difference?	216

6.3 Plant transcription factors as immunity hubs	218
References	221
Appendices	(included in CD-ROM attached)
Appendix 1 (Primers)	
Appendix 2 (Cloning modules in pICH41021 – <i>Escherichia coli</i>)	
Appendix 3 (<i>Psa</i> T3E delivery library in pBBR5-A4p/pVSP61 - <i>Pseudomonas</i>)	
Appendix 4 (<i>Psa</i> T3E library in pICH86988/pBTEX <i>Agrobacterium tumefaciens</i>)	
Appendix 5 (Nucleus-localized effector bait library in pLexA-GG - <i>Saccharomyces cerevisiae</i> EGY48)	
Appendix 6 (AtWRKY prey library in pB42AD-GG - <i>Saccharomyces cerevisiae</i> RFY206)	

Abstract

Pseudomonas syringae pv. *actinidiae* (*Psa*) is a virulent and highly damaging pathogen causing bacterial canker in all currently commercially important cultivars of kiwifruit (*Actinidia* spp.). *Arabidopsis* and *Nicotiana* spp. plants, however, are nonhosts to *Psa*. In our course of investigating the various nonhost resistance mechanisms in play against *Psa*, we identified several sources of resistance against several *Psa* strains as well as a possible novel virulence mechanism used by *Psa* and *Hyaloperonospora arabidopsidis* (*Hpa*), a biotrophic pathogen of *Arabidopsis*.

Firstly, we discovered that the highly virulent strain, *Psa* V13, triggers hypersensitive response (HR) in *Arabidopsis* in an accession-specific manner and that HopZ5_{*Psa*V13}, a member of the YopJ family of putative acetyltransferases, confers this bacterial avirulence. We also show that the immunity triggered by HopZ5 is independent from HR in the *Arabidopsis* accession Col-0. Through mutagenesis, we show that key amino acid residues predicted for acetyltransferase activity are vital to HopZ5-triggered immunity and HR, phenotypes reproduced in *Nicotiana* spp.

Secondly, we identified multiple sources of avirulence for the kiwifruit low-virulence strain, *Psa* LV5, in *Arabidopsis* and *Nicotiana benthamiana*, namely homologs of previously characterized effectors, HopAR1 and HopAB3, respectively. We additionally show that HopAB3 can trigger resistance in cultivated tomato putatively due to a novel recognition by a cultivated tomato homolog (*SIPtoB*) of the resistance gene *Fen*.

Finally, we identified several nuclear-localized effectors from *Psa* and *Hpa* that interact with *Arabidopsis* WRKY transcription factors, different to WRKYs targeted by previously identified AvrRps4 and PopP2. We show that some WRKYs can trigger a cell death response in *N. benthamiana* when overexpressed and that coexpression of AvrRps4 or PopP2 is able to suppress this cell death response for the WRKYs they interact with. We show that this suppression is associated with suppression of transcriptional activation ability of the WRKY and

propose that this mechanism of transcription suppression may be utilized by other *Psa* and *Hpa* effectors identified in this study.

Acknowledgements

I would like to thank my supervisor, Prof. Kee Hoon Sohn, for his support, guidance and friendship during my PhD. His keen mind has often served as a benchmark during the many struggles whilst undertaking my PhD and I hope that some of his enthusiasm for science has rubbed off on me. I would also like to thank Prof. Cecile Segonzac who was irreplaceable as a lab mentor and coffee companion; her guidance in the lab was second only to her compassion during the tough times.

I would also like to thank Dr. Janet Reid, Dr. Rosie Bradshaw, Dr. Matt Templeton and Dr. Erik Rikkerink, who collectively supported me during my undertaking in a myriad unseen, but not unappreciated, ways. I have thoroughly enjoyed the many conversations with KSL lab members both in NZ and Korea: Toby, Maxim, Gayoung, Jeongmin, Hayoung, Haseong, Wanhui, and last but certainly not least, Sera! Sera and I undertook this project as partners-in-crime and I am eternally grateful to her for the many hours of toil she has put in by my side to make this project a successful one.

I thank my parents, and my sister, Jeya, for their loving support and comfort, during good times and bad. Finally, I would like to thank my partner, Nathalie, who stood by me during the ordeal of failed results, writing dilemmas, and the many challenges of moving to a foreign country to finish my research. Without you I am without a purpose

Contributions to this thesis

Several individuals have contributed towards the work described in this thesis.

These include:

Amandine Spiandore – for assistance with cloning of *Psa* V13 effector modules described in Chapter 3 (Appendix 2).

Sera Choi – for cloning of *Psa* V13 effector modules described in Chapter 3 (Appendix 2), assembly of GoldenGate constructs of HopZ5 and its variants (Appendix 4), and production of results used for Figure 3.12 (VIGS) and Figure 3.15 (immunoblots).

Jun Zhou – for cloning of WRKY transcription factor modules from Arabidopsis in Chapter 5 (resulting in materials in Appendix 6).

Without these significant contributions, this thesis would not have been able to achieve the level of results produced herein. All other work described in this thesis is mine alone conducted under the guidance of Prof. Kee Hoon Sohn or Prof. Cecile Segonzac.

Abbreviations

_aa	Amino acids
ABA	Abscisic acid
Avr	Avirulence
bp	Base pair
BAK1	BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 (a helper RLK)
BIC	Biotrophic interfacial complex
BIK1	BOTRYTIS INDUCED KINASE 1 (a cytoplasmic kinase)
BR	Brassinosteroid
CC	Coiled-coil (a domain in NB-LRRs)
cDNA	Complementary deoxyribonucleic acid
CDPK	Calcium-dependent protein kinase (Also abbreviated to CPK)
CEL	Conserved effector locus
CFU	Colony forming unit
CK	Cytokinin
CNL	Coiled-coil nucleotide-binding leucine-rich-repeat receptor (a class of NLR)
DNA	Deoxyribonucleic acid
dpi	Days post inoculation
DTT	dithiothreitol
EDS1	Enhanced disease susceptibility 1 (required for most TNLs)
EDTA	ethylenediamine tetraacetic acid
EFR	EF-Tu receptor (a sensor PRR/RLK)
EF-Tu	Elongation factor thermo unstable
elf18	EF Tu-derived epitope from <i>Escherichia coli</i>
ET	Ethylene
ETI	Effector-triggered immunity
EV	Empty vector
FLS2	Flagellin-sensitive 2 (a sensor PRR/RLK)
flg22	Flagellin-derived epitope from <i>Pseudomonas aeruginosa</i>
g	gram
GA	Gibberellic acid

h	hours
HCD	Hypersensitive response-like cell death
His	Histidine
Hpa	<i>Hyaloperonospora arabidopsidis</i>
hpi	Hours post infiltration
HR	Hypersensitive response
HSP90	Heat shock protein 90
ICE	Integrated conjugative element
JA	Jasmonic acid
kb	kilobase
kDa	kilodaltons
LPS	Lipopolysaccharide
LRR	Leucine rich repeat (domain common in PRRs and NB-LRRs)
Leu	Leucine
M	molar
MAPK	Mitogen-activated protein kinase
mg	milligram
min	minutes
mL	millilitre
mM	millimolar
NAC	NAM, ATAF, and CUC (stress-related plant transcription factor family)
NB-LRR	Nucleotide-binding leucine-rich-repeat receptor (intracellular)
NBS	Nucleotide binding site (domain of NB-LRR)
NLR	Nod-like receptors
NLS	Nuclear localization signal
NDR1	Nonrace-specific disease resistance 1 (required for many CNLs)
OD	Optical density of bacterial suspension with 600nm wavelength light
PAD4	Phytoalexin deficient 4
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PGN	Peptidoglycan
PPHGI	<i>Pseudomonas phaseolicola</i> genomic island (a type of ICE)
PR	Pathogenesis-related

PRR	Pattern recognition receptor (cell plasma membrane)
PTI	PAMP-triggered immunity
<i>Pf</i>	<i>Pseudomonas fluorescens</i>
<i>Pgy</i>	<i>Pseudomonas syringae</i> pv. <i>glycinea</i>
<i>Pma</i>	<i>Pseudomonas syringae</i> pv. <i>maculicola</i>
<i>Pph</i>	<i>Pseudomonas syringae</i> pv. <i>phaseolicola</i>
<i>Psa</i>	<i>Pseudomonas syringae</i> pv. <i>actinidiae</i>
<i>Psy</i>	<i>Pseudomonas syringae</i> pv. <i>syringae</i>
<i>Pto</i>	<i>Pseudomonas syringae</i> pv. <i>tomato</i>
qPCR	Quantitative polymerase chain reaction
RAC	Resistance to <i>Albugo candida</i> (NLRs for <i>A. candida</i> effectors)
RAR1	Required for Mla12 resistance
RBOHD	RESPIRATORY BURST OXIDASE HOMOLOG PROTEIN D
RIN4	RPM1-interacting 4
RLCK	Receptor-like cytoplasmic kinase (intracellular)
RLK	Receptor-like kinase (a class of PRR)
RLP	Receptor-like protein (a class of PRR)
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPP	Resistance to <i>Peronospora parasitica</i> (NLRs for <i>Hyaloperonospora arabidopsidis</i>)
RPM1	RESISTANCE TO <i>PSEUDOMONAS SYRINGAE</i> PV. <i>MACULICOLA</i> 1 – detects AvrRpm1
RPS2	RESISTANCE TO <i>PSEUDOMONAS SYRINGAE</i> 2 – NLR detects AvrRpt2
RPS4	RESISTANCE TO <i>PSEUDOMONAS SYRINGAE</i> 4 – NLR detects AvrRps4 (and PopP2)
RPS5	RESISTANCE TO <i>PSEUDOMONAS SYRINGAE</i> 5 – NLR detects AvrPphB (HopAR1)
RPS6	RESISTANCE TO <i>PSEUDOMONAS SYRINGAE</i> 6 – NLR detects HopPsyA (HopA1)
RRS1	RESISTANCE TO <i>RALSTONIA SOLANACEARUM</i> 1 – NLR detects PopP2 (and AvrRps4)
<i>Rso</i>	<i>Ralstonia solanacearum</i>

s	seconds
SA	Salicylic acid
SAG101	Senescence associated gene 101
SAR	Systemic acquired resistance
SDS	Sodium dodecyl sulphate
SGT1	Suppressor of G2 allele of <i>skp1</i> (required for most NLRs)
SID2	Salicylic acid induction deficient 2
SOBIR1	Suppressor of <i>bir1-1</i> (a helper RLK)
STR	Strigolactone
SUMM2	SUPPRESSOR OF MKK1 MKK2 2- NLR detects HopAI1
TAE	tris acetate EDTA
TAL	Transcriptional activator-like (effector)
TEMED	N,N,N',N'-teramethyl-ethylenediamine
TIR	Toll-interleukin-1 receptor (a domain in NB-LRRs)
TNL	Toll-interleukin-1 receptor nucleotide-binding leucine-rich-repeat receptor (a class of NLR)
Tris	tris(hydroxymethyl)aminomethane
Trp	Tryptophan
T3SS	Type-three secretion system
T3E	Type-three secreted effector (bacterial)
<i>Xcv</i>	<i>Xanthomonas campestris</i> pv. <i>vesicatoria</i>
ZAR1	HopZ activated resistance 1 - NLR detects HopZ1a
µg	microgram
µL	microlitre
µM	micromolar