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

Jay Jayaraman

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Contents

Abstract 6
Acknowledgements 8
Contributions to thesis 9
Abbreviations 10

Chapter 1: General Introduction
1.1 The kiwifruit industry & *Pseudomonas syringae pv. actinidiae* 14
1.2 *Arabidopsis thaliana* as a model for plant defence 19
1.2.1 *Pseudomonas syringae* infection of Arabidopsis 19
1.2.2 *Hyaloperonospora arabidopsidis* infection of Arabidopsis 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 *R* 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 *Psa* 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 *Arabidopsis thaliana* accessions 78
2.3.2 *Nicotiana tabacum* 79
2.3.3 *Nicotiana benthamiana* 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 *In planta* 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 *Agrobacterium tumefaciens* transient infiltration 82
2.5.5.1 Confocal laser scanning microscopy for subcellular localization 82
2.5.6 *N. benthamiana* 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$_{PsaV13}$ 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 *Nicotiana* spp. and requires *SGT1* 110
3.2.5 HopZ5 triggered immunity in Col-0 requires *SID2*, *EDS1* & *NDR1* 113
3.2.6 HopZ5 is a *bona fide* plasma membrane-localized acetyltransferase that autoacetylates *in planta* 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 *in planta* 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 *Psa* LV5 triggers HR and avirulence in Arabidopsis conferred by the *RPS5*/*SUMM2* locus 130
4.2.2 A homolog of AvrPphB in *Psa* LV5 triggers RPS5-mediated HR in Col-0 when delivered under its native promoter 135
4.2.3 *Psa* LV5 triggers AvrPtoB homolog-dependent HCD in *Nicotiana benthamiana* 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, *Sl*PtoB 150
4.3 Discussion 156
4.3.1 *Psa* 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 *Pseudomonas* and *Hyaloperonospora arabidopsidis* interact with Arabidopsis WRKYs 165
5.2.2 Overexpressing AtWRKYs in Nicotiana spp. triggers a conditional HCD dependent on their putative DNA-binding ability and *SGT1* 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 *Nicotiana* 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 – *Escherichia coli*)

Appendix 3 (*Psa* T3E delivery library in pBBR5-A4p/pVSP61 - *Pseudomonas*)

Appendix 4 (*Psa* T3E library in pICH86988/pBTEX *Agrobacterium tumefaciens*)

Appendix 5 (Nucleus-localized effector bait library in pLexA-GG - *Saccharomyces cerevisiae* EGY48)

Appendix 6 (AtWRKY prey library in pB42AD-GG - *Saccharomyces cerevisiae* 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$_{Psa\text{V13}}$, 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 (*Sl*PtoB) 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.

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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 matierials 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	BOTRYITIS 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 *Escherichia coli*
ET	Ethylene
ETI	Effector-triggered immunity
EV	Empty vector
FLS2	Flagellin-sensitive 2 (a sensor PRR/RLK)
flg22	Flagellin-derived epitope from *Pseudomonas aeruginosa*
g	gram
GA	Gibberellic acid

h	hours
HCD	Hypersensitive response-like cell death
His	Histidine
Hpa	*Hyaloperonospora arabidopsidis*
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	Nucelotide-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	*Pseudomonas phaseolicola* genomic island (a type of ICE)
PR	Pathogenesis-related

PRR	Pattern recognition receptor (cell plasma membrane)
PTI	PAMP-triggered immunity
Pf	*Pseudomonas fluorescens*
Pgy	*Pseudomonas syringae* pv. *glycinea*
Pma	*Pseudomonas syringae* pv. *maculicola*
Pph	*Pseudomonas syringae* pv. *phaseolicola*
Psa	*Pseudomonas syringae* pv. *actinidiae*
Psy	*Pseudomonas syringae* pv. *syringae*
Pto	*Pseudomonas syringae* pv. *tomato*
qPCR	Quantitative polymerase chain reaction
RAC	Resistance to *Albugo candida* (NLRs for *A. candida* 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 *Peronospora parasitica* (NLRs for *Hyaloperonospora arabidopsidis*)
RPM1	RESISTANCE TO *PSEUDOMONAS SYRINGAE* PV. *MACULICOLA* 1 – detects AvrRpm1
RPS2	RESISTANCE TO *PSEUDOMONAS SYRINGAE* 2 – NLR detects AvrRpt2
RPS4	RESISTANCE TO *PSEUDOMONAS SYRINGAE* 4 – NLR detects AvrRps4 (and PopP2)
RPS5	RESISTANCE TO *PSEUDOMONAS SYRINGAE* 5 – NLR detects AvrPphB (HopAR1)
RPS6	RESISTANCE TO *PSEUDOMONAS SYRINGAE* 6 – NLR detects HopPsyA (HopA1)
RRS1	RESISTANCE TO *RALSTONIA SOLANACEARUM* 1 – NLR detects PopP2 (and AvrRps4)
Rso	*Ralstonia solanacearum*

s	seconds
SA	Salicylic acid
SAG101	Senescence associated gene 101
SAR	Systemic acquired resistance
SDS	Sodium dodecyl sulphate
SGT1	Suppressor of G2 allele of *skp1* (required for most NLRs)
SID2	Salicylic acid induction deficient 2
SOBIR1	Suppressor of *bir1-1* (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)
Xcv	*Xanthomonas campestris* pv. *vesicatoria*
ZAR1	HopZ activated resistance 1 – NLR detects HopZ1a
µg	microgram
µL	microlitre
µM	micromolar

Chapter 1: General introduction

1.1 The kiwifruit industry & *Pseudomonas syringae pv. actinidiae*

Kiwifruit is New Zealand's largest horticultural export with the industry contributing in excess of $1.4 billion to GDP (Skallerud and Olsen, 2011). With the detection of *Pseudomonas syringae* pv. *actinidiae* (hereafter, *Psa*), a virulent and highly damaging pathogen causing bacterial canker in all currently commercially important cultivars of kiwifruit, in November 2010, the industry faces one of its greatest challenges to date. The implementation of biosecurity measures has not been successful at limiting the spread of the disease and most orchards in the Bay of Plenty region where the disease was first detected have now shown some level of infection, including a strain that results in significant loss of not only fruit yield but damages vine growth and thus future yield potential as well (Serizawa et al., 1989). As a result, as with other commercial horticultural projects in the past, the immediate aim was to develop a kiwifruit cultivar that was resistant to bacterial infection – in particular, understanding the immune defence prospect of the kiwifruit-*Psa* system (Scortichini et al., 2012). Recently, the identification of a *Psa*-tolerant cultivar of gold-fleshed kiwifruit (*Actinidia chinensis* var sungold) has allowed for the switch to this new cultivar, albeit without an understanding of the mechanisms behind said tolerance.

Psa was first identified as a disease on kiwifruit (*Actinidia deliciosa*) in Japan in 1984 (Takikawa et al., 1989) manifesting as brown leaf spots with chlorotic haloes, brown discoloration of buds, cankers with exudates on woody tissues, collapsed leaders and eventual plant death with a similar disease reported in China (Wang et al., 1989). Subsequently reports had identified it in Korea (Koh and Lee, 1992) and Italy (Scortichini, 1994). A more recent and considerably more severe outbreak in Italy in 2008 (Ferrante and Scortichini, 2010) was followed by reports in Portugal (Balestra et al., 2010), Chile (Marcelletti et al., 2011), and New Zealand (Everett et al., 2011). The severe outbreak in Italy demonstrated devastatingly rapid spread in just 2 years from central Italy to

orchards in the northern reaches and was genetically different to the original strain from Italy (Ferrante and Scortichini, 2010).

Considering the large family of Pseudomonads constituted by pathogenic *Pseudomonas syringae* strains, these have been divided into four broad monophyletic groups (Figure 1.1) based on genome similarity (Sarkar and Guttman, 2004). Based on host range and disease symptoms, the *P. syringae* strains that are plant pathogens can be assigned to different pathovars, such as that represented by *Psa*, which in turn falls into Group 1 of pathogenic *P. syringae* strains. Interestingly, although a group of strains from the same host may be assigned to the same pathovar type, the pathovar designation may not correspond to a natural phylogenetic group owing to evolutionary dynamics. This model was built on by recent work identifying several clades making up the *Psa* pathovar (Figure 1.2), namely *Psa* LVs (also called NZLV) a new set of strains from New Zealand, *Psa* Vs (also called NZV from the so called global outbreak clade) related to the 2008 Italian strain as well as the virulent Chinese, Chilean and New Zealand strains, *Psa* Js related to the original 1984 Japanese *Psa* type strain, and *Psa* Ks related to the 1992 Korean strain (McCann et al., 2013). Interestingly, the original Italian strain with less severe disease symptoms (like the original Japanese strains) appeared to be from the J clade and the subsequent Italian V strains appear to be evolutionarily diverged. Furthermore, close examination of the V clade strains has led to the identification of each of these strains as independently originated from China rather than a gradual spread between countries (Butler et al., 2013). This was determined by examination of integrative conjugated elements (ICEs) or genomic islands that were gained idependently from different *Pseudomonas syringae* strains, probably from China, into *Psa* (Figure 1.3).

These findings suggest that strain origin may indeed be associated with disease severity on kiwifruit and future work on developing resistance to these strains should factor in these considerations. The presence of a wide spectrum of genetic determinants in each strain would then determine the success of a particular resistance strategy.

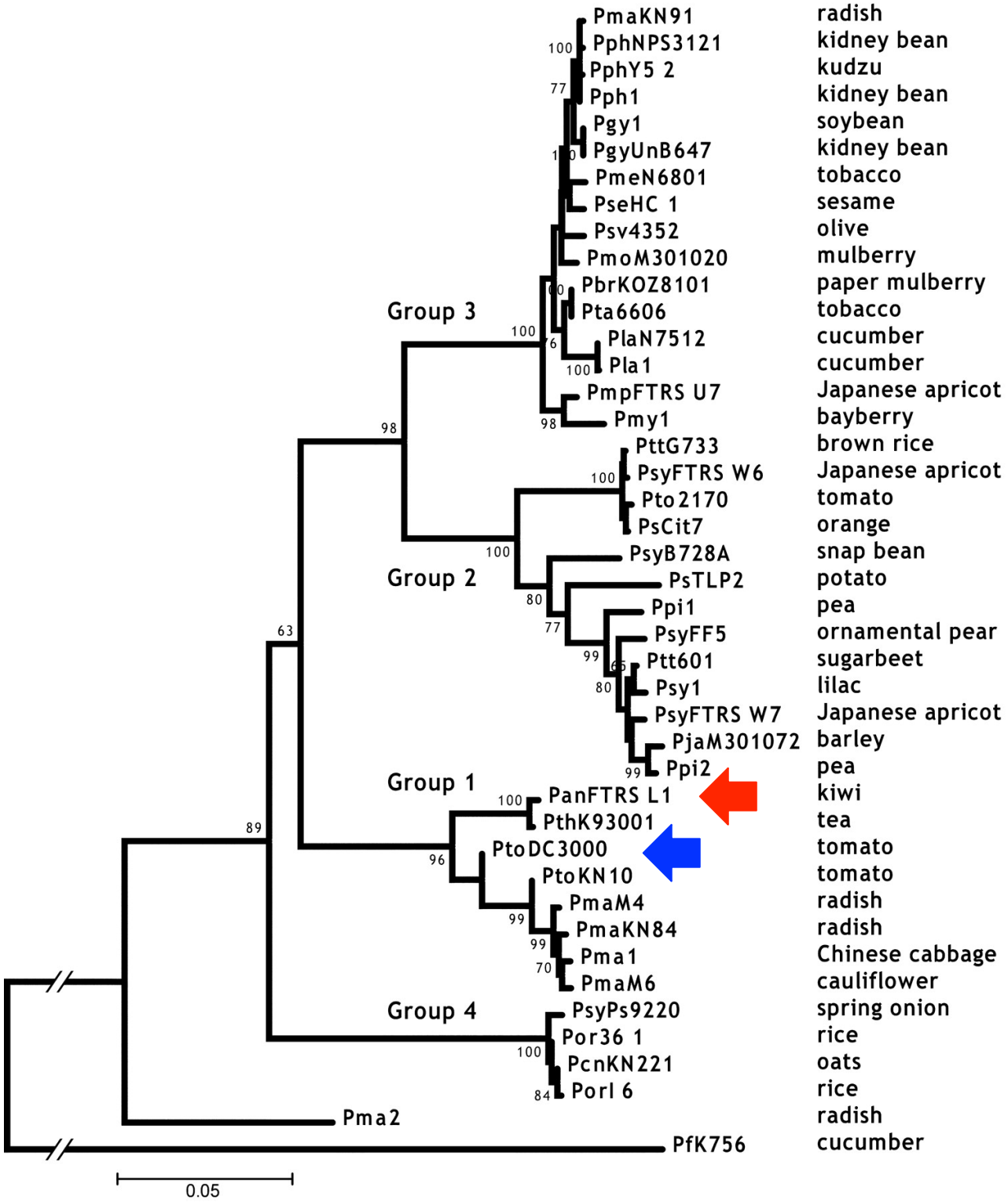


Figure 1.1: *Pseudomonas syringae* pathovars. Neighbour-joining tree of *Pseudomonas syringae* pathovars of plants. The four major groups *P. syringae* is divided into are indicated and host of isolation is given next to each strain. *Psa* (type strain isolated in Japan indicated with red arrow) falls into group 1 with the well-characterized tomato pathogen *Pseudomonas syringae* pv *tomato* DC3000 (blue arrow). The non-pathogenic *P. fluorescens* PfK756 strain is used as the outgroup (reproduced from Sarkar & Guttman, 2004).

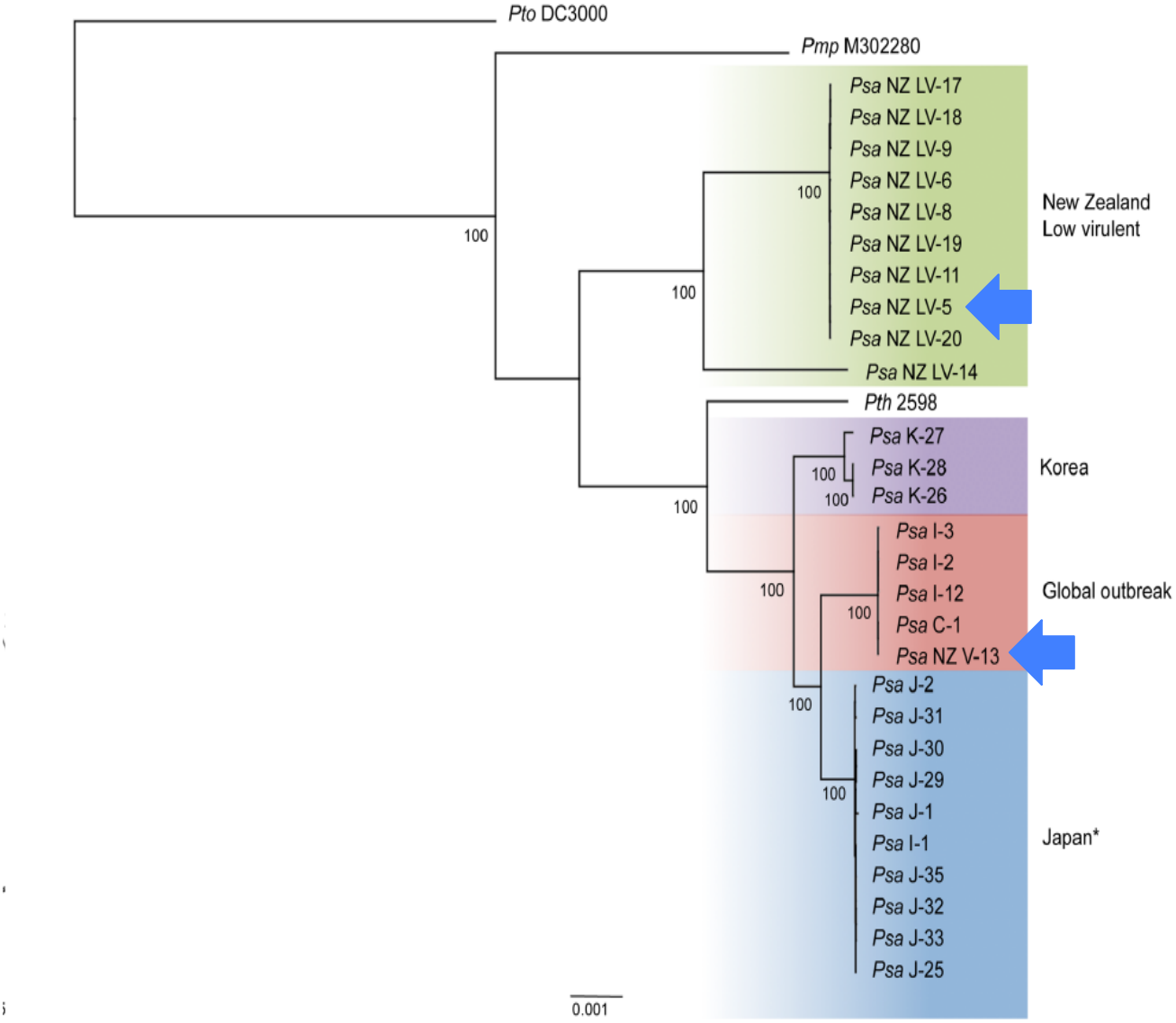


Figure 1.2: *Pseudomonas syringae* pv. *actinidiae* strain phylogeny. Maximum likelihood phylogenetic tree of genomes of *Psa* strains identify three moderately related clades of strains: V (virulent or global outbreak), J (Japan) and K (Korea). A more distantly related LV (low virulence) clade is also identified. Strains are identified by geographic origin (as for clade designation). I, C and NZ strains were isolated in Italy, China and New Zealand, respectively. The type *Psa* strain from Figure1.1 (PanFTRS_L1) is *Psa* J-1 here and the J clade (*) is considered the type clade for *Psa*. Pto DC3000 was used as outgroup and strains closely related to *Psa*, *P. syringae* pv. *morsprunorum* (stone-fruit pathogen) and *P. syringae* pv. *theae* (tea pathogen) are included for reference. The key strains discussed in this thesis *Psa* V13 and *Psa* LV5 are indicated here with blue arrows (reproduced from McCann et al., 2013).

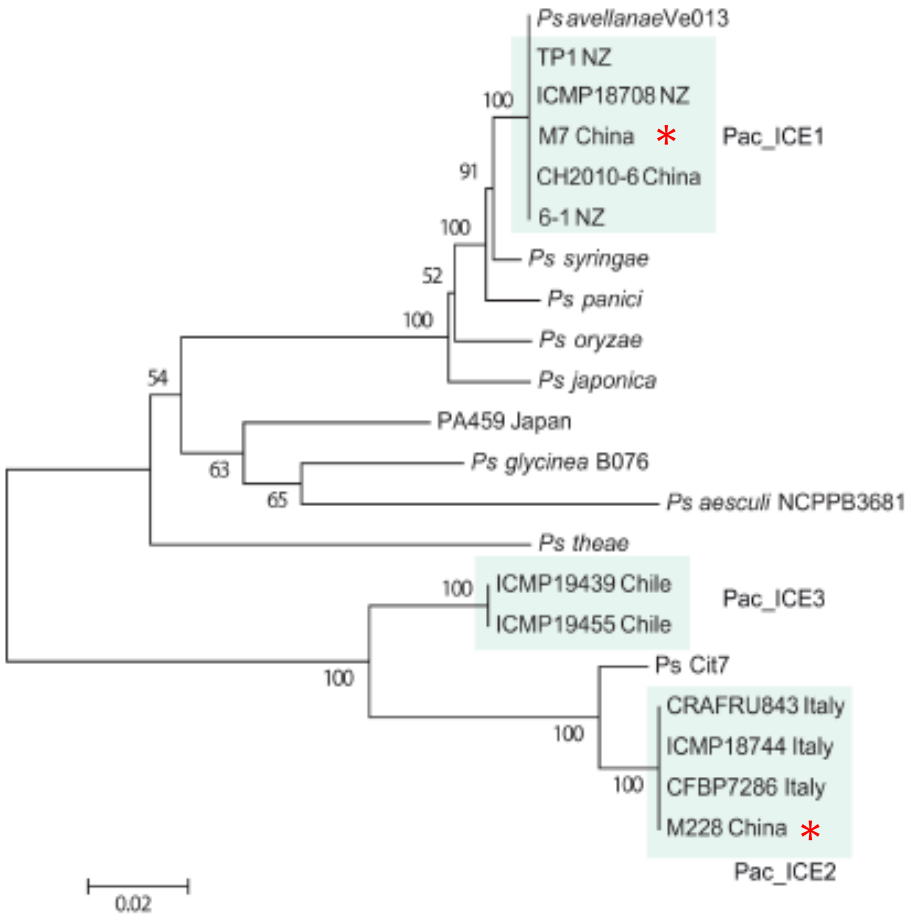


Figure 1.3: *Pseudomonas syringae* pv. *actinidiae* V strains are independently introduced. Neighbour-joining phylogenetic tree of *Psa* V strains based on seven highly conserved ICE protein sequences for each strain. Phylogeny indicated three unique ICEs present in *Psa* V strains (green squares). Grouping indicates relatedness to be closer to the Chinese isolates (red asterisks) rather than to each other implicating independent origins from China for each strain ICE group. *Psa* V13 is likely to be part of ICE1 group since all NZ isolates fall into this group (reproduced from Butler et al., 2013).

1.2 *Arabidopsis thaliana* as a model for plant defence

The fact that most pathogens do not infect most plants indicates that plants possess a certain level of defence innately that must be specifically overcome by successful pathogens. Such defences should thus presumably be effective against a broad variety pathogens and recognize conserved molecular signals, effectively constituting a nonhost-type resistance, rendering all strains of a pathogen unable to cause disease on the plant (hence the plant is a nonhost) (Katagiri et al., 2002). The evolutionary battle between pathogens and hosts would then drive pathogens to develop mechanisms of resistance evasion that would then need to be countered by plants in a never-ending battle, a veritable 'arms-race' (Jones and Dangl, 2006). Early studies in this secondary form of resistance in flax has suggested a gene-for-gene relationship (Flor, 1971) that could be transferable between plant cultivars using breeding techniques – a system already well developed for a wide variety of plants over centuries.

Arabidopsis thaliana (hereafter Arabidopsis) is a small dicotyledonous plant of the *Brassicaceae* native to Eurasia but long since naturalized around the world. It often grows as a winter annual with a very short life cycle of around 6 weeks and possesses the considerable virtue of being the first plant to have fully sequenced genome (Kaul et al., 2000; Mitchell-Olds, 2001). Being a typical self-pollinator, evolution has led to a wide variety of plant accessions (also called ecotypes, akin to races or cultivars), which have genetically distinct phenotypes including varied disease resistances. In fact, the use of Arabidopsis has led to many early breakthroughs in understanding how plants manifest plant disease resistance (Bent et al., 1993; Dangl et al., 1992; Dong et al., 1991; Holub, 1994; Holub et al., 1995; Whalen et al., 1991).

1.2.1 *Pseudomonas syringae* infection of Arabidopsis

Pseudomonas syringae is a Gram-negative, rod-shaped γ-proteobacterium with polar flagella, strains of which possess the ability to infect a wide variety of plants, as mentioned earlier (Sarkar and Guttman, 2004). Different strains of *P. syringae*, however, are remarkable for their host-specific interactions with plants

(Hirano and Upper, 2000). A specific strain may be assigned to one of approximately 50 pathovars based on its host-range (Gardan et al., 1999; Sarkar and Guttman, 2004) and then additionally to a race (which can be grouped into clades) based on differential interactions between cultivars of the host plant. Using *P. syringae* as a model for the study of host-pathogen interactions has been pivotal to elucidating the molecular basis of such host specificity.

In the late 1980s, several *P. syringae* pathovars (a designation of their ability to infect a plant species) including *tomato*, *maculicola* (cauliflower and other *Brassicaceae*), and *pisi* (pea), were discovered to infect Arabidopsis, identifying two very virulent type pathogens *P. syringae* pv. *tomato* DC3000 (*Pto* DC3000) and *P. syringae* pv. *maculicola* ES4326 (*Pma* ES4326) and several resistance genes (*R* genes) (Dangl et al., 1992; Dong et al., 1991; Whalen et al., 1991). These studies principally identified assay mechanisms for rapidly determining resistance and susceptibility in Arabidopsis. Namely, leaves infected with *P. syringae* showed water-soaked patches, which eventually become chlorotic (Figure 1.4) and then necrotic, surrounded by diffuse chlorosis if the plants were susceptible. In resistant plants, conversely, *P. syringae* triggered a macroscopic hypersensitive response (HR) – a rapid, defence-associated programmed cell death of large sections of plant leaf tissue in contact with the pathogen when the pathogen is delivered at a high titer (Dong et al., 1991; Katagiri et al., 2002). Apart from *Pto* DC3000, however, few *P. syringae* strains isolated from tomato were found to be virulent on Arabidopsis, indicating that Arabidopsis is largely a nonhost for tomato-infecting strains of *P. syringae* (Sohn et al., 2012b).

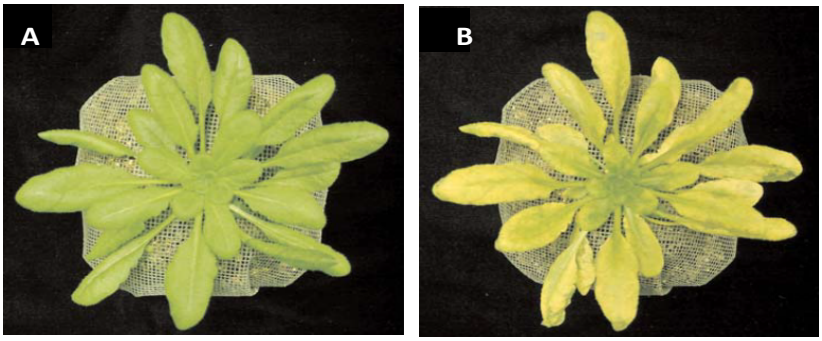


Figure 1.4: *Arabidopsis thaliana* Col-0 infected with *Pto* DC3000. (A) A five week old plant of Arabidopsis accession Col-0. (B) Five week old Col-0, 3 days after being dipped in a concentrated solution containing virulent *Pseudomonas syringae* pv. *tomato* DC3000 demonstrates typical chlorosis due to infection (reproduced from Katagiri et al., 2002).

Interestingly, a screen of *Pseudomonas* strains that naturally infect Arabidopsis in the American wild identified several virulent and avirulent strains that helped highlight the role played by effectors in this dynamic (Jakob et al., 2002; Karasov et al., 2014). Several *P. viridiflava* strains and a few *P. syringae* strains isolated from select Arabidopsis wild accessions were found to trigger accession-specific HR indicating the occurrence of a type-III system effector-triggered responses in some accessions of Arabidopsis (Jakob et al., 2002). Many investigations of host and nonhost resistance in *Pseudomonas*-Arabidopsis interactions are currently underway and are expected to reveal as yet unknown mechanisms about pathogen defence in Arabidopsis.

1.2.2 *Hyaloperonospora arabidopsidis* infection of Arabidopsis

Downy mildew infection of Arabidopsis, first identified over a century ago, is caused by the obligate biotrophic oomycete, *Hyaloperonospora arabidopsidis* (hereafter, *Hpa*, formerly *Hyaloperonospora parasitica*/*Peronospora parasitica*) (Holub, 2008). Oomycetes are closely related to brown algae and diatoms but are often misclassified as true fungi. Oomycetes may appear similar to some fungal pathogens through their production of similar infection structures, such as appressoria, infection hyphae, and haustoria (Slusarenko and Schlaich, 2003). However, several features separate fungi and oomycetes: oomycetal cell walls are primarily cellulose-based rather than fungal chitin; oomycetal hyphae are coenocytic (not separated by septa as they are in fungi); oomycetes have diploid vegetative nuclei, compared to the haploid or dikaryotic nuclei of fungi; several differences exist between the two groups' biochemistry; and many oomycetes produce motile biflagellate zoospores (Coates and Beynon, 2010).

Much in the way that *P. syringae* has pathovars determined by their ability to infect plant hosts, *Hpa* demonstrates extensive levels of phenotypic and genotypic variation, reflecting the natural coevolution that has taken place between it and Arabidopsis (Figure 1.5) (Holub, 1994). *Hpa* isolates collected are named after the location where they were isolated, abbreviated to two letters, and a susceptible Arabidopsis accession, again abbreviated to two letters. For

instance, the isolate Emoy was isolated in East Malling, Kent, UK = Em and results in a compatible interaction with the Arabidopsis accession Oystese = Oy.

The Arabidopsis-*Hpa* relationship serves as a case study for understanding the numerous oomycetal pathogens of a large number of economically important crops: *Phytophthora* spp., *Pythium* spp., and *Bremia* spp. Another model oomycete pathogen of Arabidopsis, *Albugo candida* – the causal agent of Arabidopsis white rust, is also being investigated for its ability to reveal details about immunity in Arabidopsis (Holub et al., 1995). In fact, several resistance loci against *Hpa* have been identified as conferring resistance to *Albugo candida* as well, implying the transferability of research into the two biotrophic pathogens (Borhan et al., 2001).

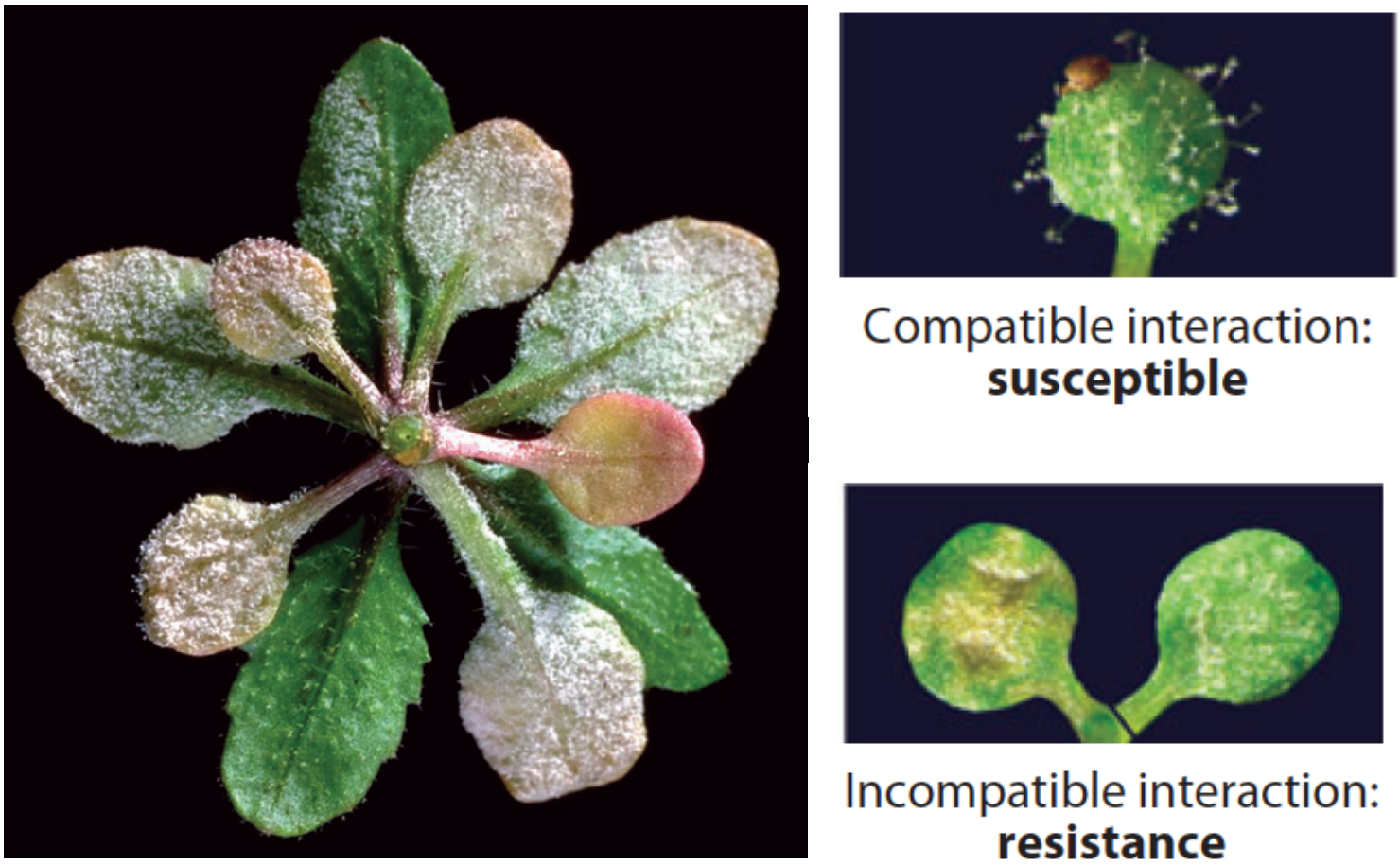


Figure 1.5: *Arabidopsis thaliana* infected with *Hpa.* Downy mildew infection by the virulent biotrophic pathogen *Hyaloperonospora arabidopsidis* on the underside of a susceptible Arabidopsis plant (left). The spectrum of interactions between Arabidopsis and *Hpa* determines whether conidiophores are produced after 7 days post infection in susceptible plants (top right) or pitting/flecking resistance restricts growth of *Hpa* with the development of HR in resistant plants giving the appearance of flecks or pits in the leaf tissues (bottom right) (reproduced from Coates & Beynon, 2010).

Further study using Arabidopsis-*P. syringae* and Arabidopsis-*Hpa* plant disease models allowed comprehensive study of the plant disease resistance systems described since the early work of H.H. Flor. Such work has identified two broad systems of defence that are intricately linked but essentially resolvable: a primary cell periphery-associated response that is broad and robust – pathogen associated molecular pattern (PAMP)-triggered immunity (PTI) – and a highly specific but devastatingly potent secondary response – effector triggered immunity (ETI) (summarized in Figure 1.6). The plant defence models that have been developed over the past few decades have been referred to as the central dogma of plant defence (Bent and Mackey, 2007) or the zig-zag model of the pathogen-plant evolutionary arms-race (Jones and Dangl, 2006) tying together the dynamics of PTI and ETI through actions of and evolutionary drivers acting on pathogen effectors. More recently, however, accumulated evidence of nonhost resistance and HR triggered by recognition of extracellular effectors at the cell membrane as well as lineage-specificity of certain PAMPs has led to the development of a more universal invasion model that blurs the delineation between PTI and ETI (Cook et al., 2015). This new model retains the strengths of the zig-zag model, vis-à-vis the parameters of pathogen-host interactions, but allows for PAMPs and effectors to be associated together as a spectrum of invasion-associated signals to trigger similar (but selectively varied) defence responses as well as accommodates for symbiotic relationships in the invasion model.

Notably, studies of *P. syringae* have led to identification of numerous bacterial avirulence effectors and their corresponding *R* genes, examples of classical gene-for-gene resistance: AvrRpm1/AvrB recognized by RPM1 (also called RPS1 or RPS3), AvrRpt2 recognized by RPS2, AvrRps4 recognized by RPS4, AvrPphB recognized by RPS5, HopA1 recognized by RPS6, and HopZ1a/HopF2a recognized by ZAR1 (and unfortunately not called RPS7) (Bisgrove et al., 1994; Debener et al., 1991; Gassmann et al., 1999; S. H. Kim et al., 2009; Kunkel et al., 1993; Lewis et al., 2010; Seto et al., 2017; Simonich and Innes, 1995).

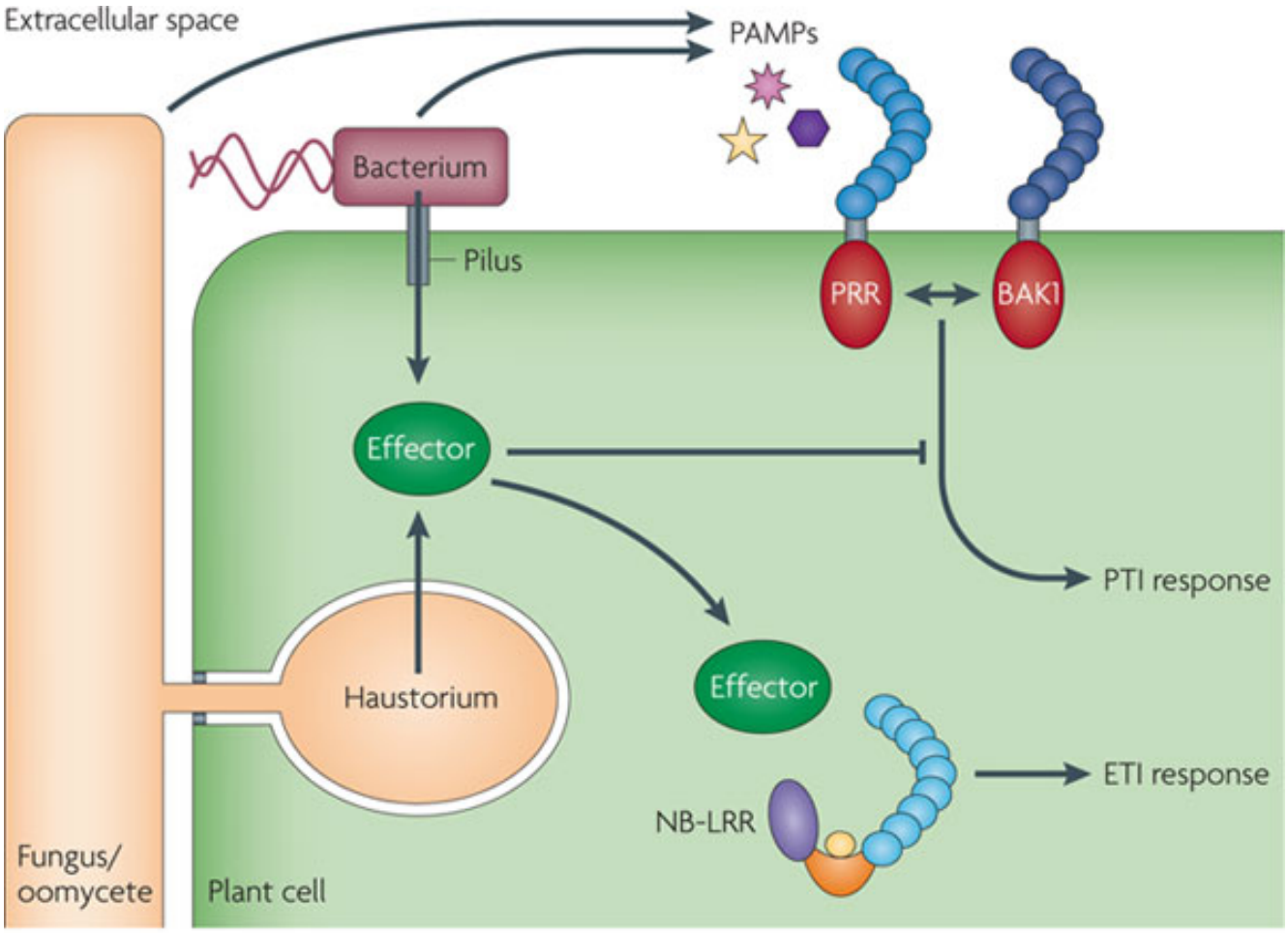


Figure 1.6: Plant primary and secondary defence systems. Proteins from the pathogens within the extracellular spaces, such as lipopolysaccharides, flagellin and chitin (PAMPs) are detected by membrane-bound pattern recognition receptors (PRRs) and elicit PAMP-triggered immunity (PTI). PRRs may interact with Brassinosteroid insensitive 1-associated kinase 1 (*BAK1*) to initiate PTI signalling. Bacterial pathogens deliver effector proteins into the host cell by a type III secretion injectisome, while fungi and oomycetes deliver effectors from haustoria or other intracellular structures by an unknown mechanism. These intracellular effectors often act to suppress PTI. However, many are recognized by intracellular nucleotide-binding (NB)-LRR receptors, which induces effector-triggered immunity (ETI) (reproduced from Dodds & Rathjen, 2010 – Lic#4179571493826).

The *Hpa*-Arabidopsis relationship demonstrates significant complexities for identification of both the effector in question (due to difficulties in mapping by crosses between *Hpa* isolates) as well as the *R* gene involved (due to the presence of multiple recognized loci in each isolate and allelism in the Arabidopsis accessions) (Bailey et al., 2011; Coates and Beynon, 2010; Rehmany et al., 2005). So far around 40 loci in *Hpa* isolates have been associated with resistance in Arabidopsis (Goritschnig et al., 2012; Holub, 1994), but only four have been linked to an actual effector, termed *Arabidopsis thaliana* recognized (ATR): ATR1 from eight isolates, including Emoy2, differentially recognized by RPP1 from Arabidopsis accessions Niederzenz or Wassilewskija (Botella et al., 1998; Rehmany et al., 2005), ATR13 from isolate Maks9 recognized by RPP13 from Arabidopsis accession Niederzenz (also called RPP11 in accession RLD) (Allen et al., 2004), ATR5 (a non-canonical effector lacking the RxLR motif) from isolate Emoy2 recognized by RPP5 from Arabidopsis accession Landsberg *erecta* (Bailey et al., 2011), and ATR39-1 from isolate Emoy2 recognized by Arabidopsis accession Weiningen (Goritschnig et al., 2012). Several R genes/proteins have been characterized in Arabidopsis but their corresponding ATRs have not yet been identified: RPP2A and RPP2B in Col-0 that work together to trigger resistance to isolate Cala2 (Sinapidou et al., 2004), RPP4 (a homolog of *RPP5* in Landsberg *erecta*) in Col-0 that triggers resistance against Emoy2 but not against $ATR5^{Emoy2}$ (Bailey et al., 2011, p. 5; van der Biezen et al., 2002), and RPP8 in Landsberg *erecta* that recognizes Emoy2 (McDowell et al., 1998). In addition to this, *RPP7* in Col-0 that recognizes isolate Hiks1 has been mapped to a region about 100kb in size, carrying the largest group of *RPP* genes yet (~15 homologs) within a region of poor sequence quality and thus still remains to be identified (Holub, 2001; Tor, 1994). *RPP9* from Arabidopsis accession Weiningen is another *R* gene of note with an unknown corresponding ATR from isolate Hiks1, and was also found to confer resistance to *Albugo candida* (called *RESISTANCE TO ALBUGO CANDIDA1*, *RAC1*, in accession Keswick-1) (Borhan et al., 2001; Holub, 1994). Interestingly, two other *R* genes conferring resistance to *Albugo candida* were identified in Arabidopsis accession Keswick, *RAC2* and *RAC3* that are allelic to a gene in the *RPP1/RPP13* locus (Niederzenz) and *RPP8* (Landsberg *erecta*), respectively (Borhan et al., 2001), indicating the interwoven

mechanisms of virulence utilized by the two biotrophic pathogens, *Albugo candida* and *Hpa*.

Apart from revealing gene-for-gene relationships in ETI between Arabidopsis and *P. syringae* or *Hpa*, characterizing effector function has led to findings in PTI as well as signaling mechanisms important for both PTI and ETI (Azevedo et al., 2002; Büttner, 2016; Century et al., 1995; Muskett et al., 2002; Parker et al., 1996; Takahashi et al., 2003). It seems clear that study of these two key pathogens of Arabidopsis have contributed greatly to the understanding of immunity in Arabidopsis and, by application, to commercially important plants as well.

1.3 PAMP-triggered immunity

1.3.1 Pathogens, PAMPs & perception

When a potential pathogen can overcome primary barriers to entry into the plant, including waxy cell layers and robust cell wall structures, they are subject to a primary and innate form of defence whereby the plant detects molecules that are typically conserved between pathogens and therefore termed pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs). They achieve this through cell-membrane localized pattern-recognition receptors (PRRs; Figure 1.7) that bind to a specifically recognized PAMP, thereby triggering a cascade of intracellular responses associated with defence termed PAMP-triggered immunity (PTI) (Chisholm et al., 2006; Zipfel and Robatzek, 2010). By definition, PAMPs would be extracellular and highly conserved. Bacterial PAMPs such as flagellin from the motility and adhesion structure flagellum (Felix et al., 1999), lipopolysaccharide (LPS) from cell walls of Gram-negative bacteria (Newman et al., 2007), peptidoglycan (PGN) from the cell wall (Gust, 2015), cytoplasmic cold-shock protein (CSP) (Felix and Boller, 2003), and elongation factor-Tu (EF-Tu) (Kunze et al., 2004) have all been reported to trigger PTI.

Perhaps the best characterized bacterial PAMP is flagellin, used in the construction of bacterial flagella. Most plant species, with rice being the only

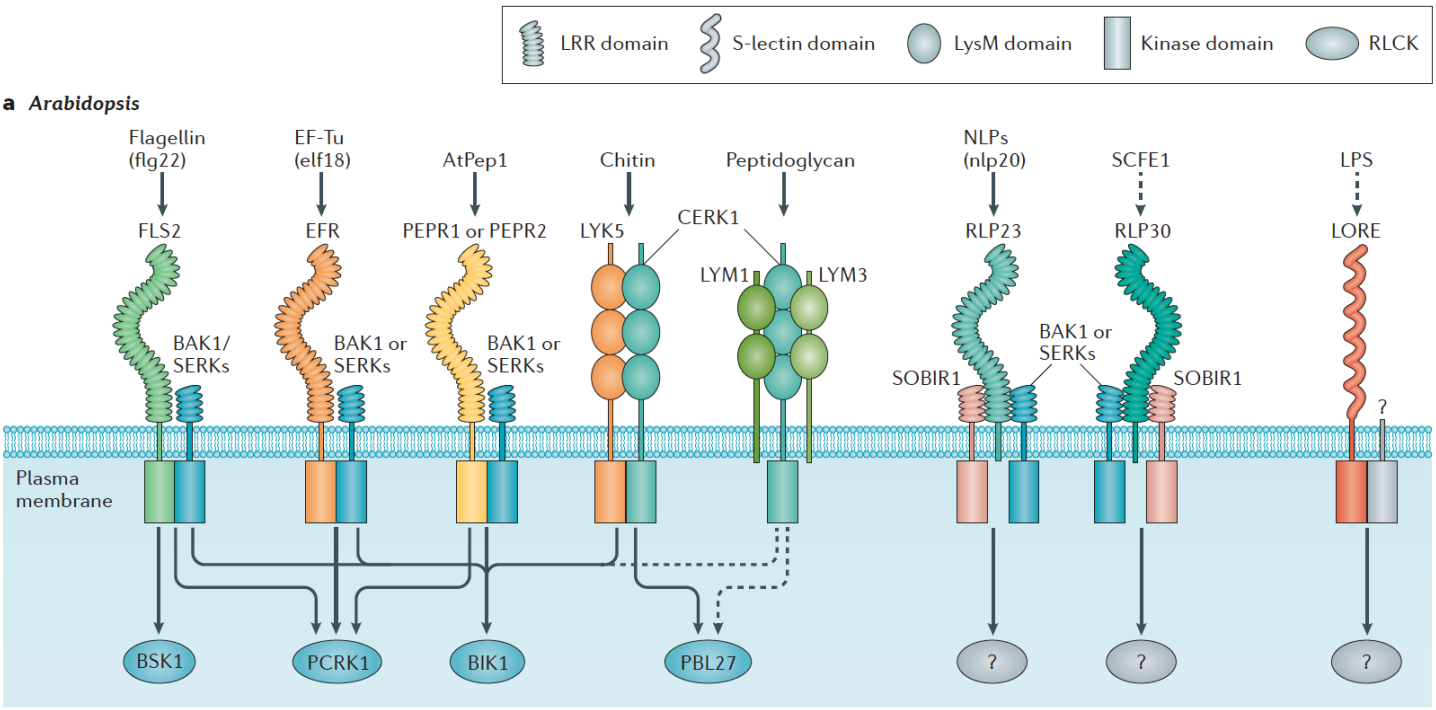


Figure 1.7: Plant PRRs. Pattern recognition receptors (PRRs) recognize multiple PAMPs from bacteria (flagellin, EF-Tu, peptidoglycan, LPS), fungi (chitin, SCFE1), necrosis-inducing peptides (NLPs), or damage-associated molecules (AtPep1). PRRs work in conjunction with several different regulatory receptor kinases according to their ectodomain. In addition, RLCKs are specifically recruited to different PRR complexes. In Arabidopsis, BAK1/SERK3, related SERKs and CERK1 are recruited upon ligand perception by LRR-receptor kinases and LysM-receptor kinases or RLPs, respectively. Constitutive bimolecular LRR–RLP–SOBIR1 complexes recruit BAK1 and SERKs upon ligand binding. No regulatory receptor kinases interacting with the LPS-detecting LORE S-lectin-receptor kinase have yet been identified. BIK1 and similarly other cytoplasmic kinases are a convergent point for multiple PRR pathways (reproduced from Couto & Zipfel, 2016 – Lic#4179580174160).

known exception (Fujiwara et al., 2004), recognize a specific epitope of the flagellin peptide sequence 22 amino acids in length (flg22) at the N-terminus (Felix et al., 1999), binding and triggering PTI via the plant leucine rich repeat receptor-like kinase (LRR-RLK) PRR, FLAGELLIN SENSING 2 (FLS2) (Chinchilla, 2006). The corresponding PRR for EF-Tu has also been identified as a *Brassicaceae* specific LRR-RLK, ELONGATION FACTOR-TU RECEPTOR (EFR) (Kunze et al., 2004; Zipfel et al., 2006). PGN is detected in Arabidopsis by a pair of LysM-domain carrying receptor-like proteins (RLPs) LYM1 and LYM3, in conjunction with the LysM-domain carrying RLK, CERK1/LYK1 (Willmann et al., 2011), a scenario mirrored by detection of PGN in rice through orthologous LysM-RLPs, OsLYP4 and OsLYP6 (Liu et al., 2012). Recently, the PRRs for LPS (a bulb-type lectin S-domain-1 receptor-like kinase, LORE) and cold-shock proteins (the RLK-type COLD SHOCK PROTEIN RECEPTOR, CORE, which also recognizes cold-shock protein epitopes, csp15 and csp22) have been found in Arabidopsis and *Nicotiana benthamiana*, respectively (Ranf et al., 2015; Wang et al., 2016). Unlike flagellin, PGN and LPS, EF-Tu and cold-shock protein are intracellular and are proposed to have a extracellular moonlighting function (in bacteria) and thus are detectable *in planta* (Granado et al., 1995; reveiwed in Kainulainen & Korhonen, 2014). Plants lacking in these PRRs are more amenable to infection as in the case of Arabidopsis *fls2*, *efr, lore* or *lym1/lym3* plants in their response to various pathogens possessing the corresponding PAMP (Ranf et al., 2015; Willmann et al., 2011; Zipfel, 2014; Zipfel et al., 2006, 2004).

Fungal PAMPs include ethylene-inducing xylanases (EIX), which are recognized by the tomato receptor-like protein PRRs LeEIX1 and LeEIX2 (Ron and Avni, 2004), and chitin, which is recognized by LysM-domain carrying chitin oligosaccharide elicitor binding protein (CEBiP) in rice and Arabidopsis (Kaku et al., 2006) as well as the Arabidopsis LysM-RLK, CERK1/LYK1 (Miya et al., 2007) and RLPs, LYM1 and LYM3 previously associated with detection of bacterial PGN, but involving other Lys-M-RLKs, LYK4 and LYK5 as well (Wan et al., 2012; Willmann et al., 2011).

Oomycete heptaglucans have also been reported as a PAMP (Sharp et al., 1984) in leguminous crops such as soybean, and are bound by soluble β–glucan-binding protein (GBP) (Fliegmann et al., 2004). Transglutaminase-derived epitope Pep-13 and cellulose binding elicitor lectin (CBEL) from oomycetes have also been implicated as PAMPs but knowledge of signaling mechanisms are still lacking (Brunner et al., 2002; Gaulin et al., 2006). It is unfortunate that as yet no PRRs of the RLK or RLP types have been identified to recognize any of the aforementioned oomycetal PAMPs (GBP, Pep-13 or CBEL). Future work in this area will certainly be illuminating for perspectives on PTI and its amenability for application against oomycete pathogens.

1.3.2 PTI signaling & defence

Much concerted effort has been focused on elucidating the mechanisms behind PTI signaling with little reward. The best understood yet is the flagellin-FLS2 signaling model (Chinchilla, 2006). FLS2 associates with BAK1 (BRI1-ASSOCIATED KINASE 1) upon flg22 treatment, triggering endocytosis and recycling of FLS2 during PTI in Arabidopsis (Chinchilla et al., 2007). The fact that *BAK1*-silenced plants are lacking in a diverse range of PAMP signaling indicates an evolutionarily conserved or shared mechanism of signaling that is currently being elucidated (Heese et al., 2007; Liebrand et al., 2014; Zipfel, 2008).

What is apparent from the burgeoning body of research on PRRs in Arabidopsis is that the perception of PAMPs is mediated by a large family of RLKs and RLPs that physically interact with the PAMP to trigger signaling (Couto and Zipfel, 2016). With the identification of BAK1 as a co-receptor of sorts for the perception of flg22, several subsequent experiments led to the finding that BAK1 is required for signaling in many RLP and RLK-mediated signaling events. This includes EFR and PEPR1/PEPR2 that senses endogenous damage-signaling peptide Pep1 (Postel et al., 2010; Yamaguchi et al., 2010, 2006), RLP23 that recognizes necrosis and ethylene-inducing peptide 1-like proteins (NLPs) from multiple bacterial, fungal and oomycetal pathogens (Albert et al., 2015), RLP30 that recognizes *Sclerotinia sclerotiorum* PAMP SsE1 (Zhang et al., 2013), and

CORE that recognizes bacterial cold-shock protein (Wang et al., 2016). Notably, BAK1 was not required for perception of several notable PAMPs including chitin (via CERK1) or LPS (via LOREs) indicating that it is not a universal PAMP signaling regulator and further work is required to address whether other regulators are involved.

The issue of signal transduction for RLPs, in particular, remained shrouded in mystery due to their lack of a signaling kinase function (Wang and Fiers, 2010). Recently, investigations of tomato RLPs Cf-4 (considered a component of ETI against fungal pathogen *Cladosporium fulvum* extracellular effector Avr4) and Ve1 (another ETI component RLP against fungal pathogen *Verticillium dahliae* effector Ave1) found that they required a homolog of Arabidopsis RLK protein SUPPRESSOR OF BIR1,1/EVERSHED (SOBIR1/EVR) (Liebrand et al., 2013). Subsequent research found that SOBIR1 was required for immunity triggered by many RLPs including aforementioned RLP30 and RLP23 also dependent on BAK1 (Albert et al., 2015; Zhang et al., 2013), and RESPONSIVENESS TO BOTRYTIS POLYGALACTURONASE-1 (RBPG1) that specifically recognizes fungal endopolygalacturonases (Zhang et al., 2014). Collectively, these studies led to SOBIR1 identification as a regulatory RLK specifically involved in RLP complexes. The *SOBIR1* gene was identified as a suppressor mutation of the mutant RLK *bir1-1* (for BAK1-INTERACTING RECEPTOR1) that triggered autoimmunity when lost (Gao et al., 2009). This led to work revealing a family of BIR1 homologs that are currently thought to act as signaling suppressors of BAK1 and SOBIR1 that, in the event of activation or loss of BIR1-mediated suppression, associate with each other and act together to trigger immunity (Domínguez-Ferreras et al., 2015; Liu et al., 2016).

In addition to the dynamics with BAK1/SOBIR1, FLS2, EFR and CERK1 interact with receptor like cytoplasmic kinase BOTRYTIS-INDUCED KINASE1 (BIK1) that has been found to be phosphorylated by BAK1 and is involved in PTI signaling (Lu et al., 2010). This phosphorylation step is followed by rapid calcium influx and calcium-dependent kinase activation (Boudsocq et al., 2010), reactive oxygen species accumulation (ROS burst) through NADPH oxidases like RBOHD

(Smith and Heese, 2014), and mitogen-activated protein kinase (MAPK) cascades (Pitzschke et al., 2009). In fact, it been found that BIK1 directly phosphorylates and activates RBOHD to trigger the ROS burst believed to be critical to PTI defence in a BAK1-dependent manner (L. Li et al., 2014).

PTI involves a myriad of chemical and biological responses, which include cross-membrane ion fluxes, MAPK activation, ROS production, rapid defence gene expression and cell wall reinforcement (including callose deposition) (Bigeard et al., 2015; Zhang and Klessig, 2001; Zipfel, 2008). Following activation of the PRR (e.g. FLS2 and BAK1 by flg22, or CERK1 and LYK4/5 by chitin), the subsequent steps leading to activation of MAPK cascades that activate transcription factors, such as those from the WRKY, TGA and NAC families, is currently of great interest (Couto and Zipfel, 2016). The current consensus dictates that following PRR activation, the direct transphosphorylation of receptor like cytoplasmic kinases (RLCKs), like BIK1 and other PBS1-like (PBL*s*) RLCKs, leads to phosphorylation of MAPKKKs/MEKKs (like MEKK1 or MAPKKK5) that subsequently phosphorylate MAPKKs (like MKK4/MKK5 or MKK1/MKK2) that then phosphorylate and activate MAPKs (MPK3/MPK6 or MPK4/MPK11) to then similarly activate transcription factors (Bigeard et al., 2015; Couto and Zipfel, 2016; Yamada et al., 2016). The Arabidopsis genome encodes around 60 MAPKKKs, 10 MAPKKs and 20 MAPKs involved in stress responses (biotic and abiotic), hormone responses, cell division, and development (MAPK Group, 2002). The Arabidopsis immunity-associated MAPK cascade is arranged as a pair of independent pathways: an unknown-MAPKKKK/MEKK-MKK4/MKK5-MPK3/MPK6 pathway and a MEKK1-MKK1/MKK2-MPK4/MPK11 pathway (Asai et al., 2002; Bethke et al., 2012; Huang et al., 2000; Nühse et al., 2000). Recently, the discovery of CERK1-associated activation of PBL27 was found to activate MAPKKK5 upon chitin perception that led to activation of both pathways via MKK4/5 and MKK2, validating this model (Yamada et al., 2016). Yamada and colleagues also found that *mapkkk5* mutants allowed increased flg22-mediated activation of MAPKs suggesting an inhibitory response of MAPKKK5 on the MAPK pathways in response to flg22. This finding is interesting as it suggests a more complex pattern of activation beyond MAPK activation is responsible for

the differential defence outputs seen for responses to bacterial (flg22-triggered) versus fungal (chitin-triggered) pathogens.

The calcium burst, seen as one of the earliest plant responses to pathogen recognition, also plays a role in PTI signaling. The calcium burst cooperates with PRR-mediated phosphorylation to activate calcium dependent protein kinases (CDPKs) that participate in activation of RBOHs (thus leading to the ROS burst) and are key regulators of transcriptional reprogramming during PTI (Ranf et al., 2011). Multiple Arabidopsis CDPKs including CPK4, CPK5, CPK6 and CPK11 are involved in coregulating defence gene expression through activation of immunity-related transcription factors through both MAPK-dependent (cooperatively) and independent activity (Boudsocq et al., 2010). Intriguingly, Gao and colleagues found CDPK activation of several WRKY transcription factors during ETI (Gao et al., 2013). This overlap of activities between CDPKs and MAPKs coupled to their activation of differential responses to differing PAMPs and NLR-mediated recognition events suggests a possible point of convergence between PTI and ETI signaling (Couto and Zipfel, 2016).

The nuanced activation of a network of immunity-related transcription factors is believed to be a major function of the CDPKs and MAPK cascades in response to pathogen perception (Bigeard et al., 2015). For example MPK3, MPK6 and MPK4 coordinately phosphorylate Arabidopsis transcription factor WRKY33 (AtWRKY33) to trigger camalexin biosynthesis upon detection of multiple pathogens, both biotrophic and necrotrophic (Mao et al., 2011). The WRKY family of transcription factors, in particular, has been studied extensively for their MAPK-dependent activation by phosphorylation on selected sites (so-called proline-directed serine residues, or SPs) that facilitate their binding to their DNA targets (Adachi et al., 2015; Ishihama et al., 2011). MPK3 also phosphorylates bZIP transcription factor VIP1, allowing it to specifically localize to the nucleus and trigger transcription of the immunity-related *PR1* gene (Djamei et al., 2007). PR1, long used as a marker of defence activation in plants, was recently found to encode a sterol-binding protein particularly potent as an antimicrobial agent against biotrophic sterol-auxotrophic oomycete pathogens like *Phytophthora* and

Hyaloperonospora spp. (Gamir et al., 2017). MAPK cascades/CDPKs play other roles not related to transcription activation that include activation of proteins involved in hormone synthesis and regulation of the plant proteasome, all of which additively contribute to the complex dynamics behind robust PTI signaling (Bigeard et al., 2015; Couto and Zipfel, 2016).

1.4 Effectors of plant pathogenic microbes

Many fungal, oomycete and bacterial pathogens secrete proteins called effectors into plant cells to be able to overcome PTI and trigger disease. To initiate entry into their plant hosts, fungi and oomycetes are either able to utilize the stomata of leaf and stem tissues to passively invade or more often develop special structures called appresoria that forcefully penetrate waxy cuticle layers on plants to allow access to apoplastic spaces beneath (Stergiopoulos and de Wit, 2009). Fungal and oomycete effectors are largely delivered either through *in planta* cellular fungal invaginations called haustoria that serve as feeding structures and plant/fungal interface for protein secretion (e.g. *Melampsora lini* and *Phytophthora infestans*) or through development of many filamentous hyphae within the plant structure that directly act as secretory structures themselves (eg. *Cladosporium fulvum*) (Dou and Zhou, 2012; Stergiopoulos and de Wit, 2009). While it has long been observed that these structures are formed within plant cells/tissues, it is still unclear how effector proteins are directed to and secreted from such structures and is under active investigation. Oomycete effectors may have an RXLR-dEER motif or FLAK motif, predicted to be involved in effector translocation into the host cell, while fungal effectors in plant pathogens *Magnporthe oryzae* and *Colletotrichum higginsianum* accumulate in haustoria biotrophic interfacial complexes (BICs) for delivery (Khang et al., 2010; reviewed in Dou and Zhou, 2012).

Bacterial diseases include speck, spot and blotch diseases of various plant tissues and are primarily successful due to the ability of bacteria to inject effectors into healthy plant cells. Bacterial plant pathogens often use a type III secretion system (T3SS) to inject effector proteins directly from the bacterial cytoplasm

into plant cells (Feng and Zhou, 2012). Such structures are of ancient origin and while the T3SS remains perhaps the best studied, as the name implies, there remain other secretion systems developed by bacteria that could be of interest as well.

1.4.1 Bacterial effector delivery systems

Bacterial pathogens do not have a particularly specialized mechanism for entering plant tissues themselves but rather utilize stomatal openings of leaves, wounds and well as feeding tissues of insect carriers (Jin et al., 2003; Katagiri et al., 2002). Pseudomonads, however, are notorious for expressing ice nucleating proteins that facilitate ice crystal formation thereby utilizing this process to indirectly wound the plant and gain entry (Hirano and Upper, 2000).

Regardless, once within the apoplastic space of the plant, conditions within trigger drastic changes, including bacterial transcriptional reprogramming, which allows for the construction of specialized structures that facilitate the delivery of protein effectors to establish disease (Tang et al., 2006). Although many bacterial protein delivery systems have been identified to date, the type III and type IV delivery systems remain the best studied (reviewed in Fronzes et al., 2009). However, other systems may function in concert with or additional to these and are of interest in studying novel systems of effector delivery.

1.4.1.1 Type I, II and V secretion systems

Bacterial type I secretion systems (T1SS), typified by the haemolysin secretion system in *Escherichia coli* (Figure 1.8) (Koronakis et al., 1991), are basic, tripartite systems facilitating the passage of proteins of various sizes across the cell membranes of largely Gram-negative mammalian pathogens. The T1SS is made up of an ATP-binding cassette (ABC) transporter or a proton-antiporter, an adaptor protein that bridges the inner membrane (IM) and outer membrane (OM), and an outer membrane pore. The T1SS secretes substrates in a single step without a stable periplasmic intermediate. T1SSs are involved in the secretion of cytotoxins belonging to the RTX (repeats-in-toxin) protein family, cell surface

layer proteins, proteases, lipases, bacteriocins and haem-acquisition proteins (Fronzes et al., 2009; Omori and Idei, 2003). For plants, the rice pathogen *Xanthomonas oryzae* pv. *oryzae* avirulent effector called AvrXa21/RaxX is the sole characterized plant effector that appears to be secreted by a T1SS during infection (Lee et al., 2006; Pruitt et al., 2015) along with the cell adhesion protein LapA from *P. fluorescens* (Boyd et al., 2014).

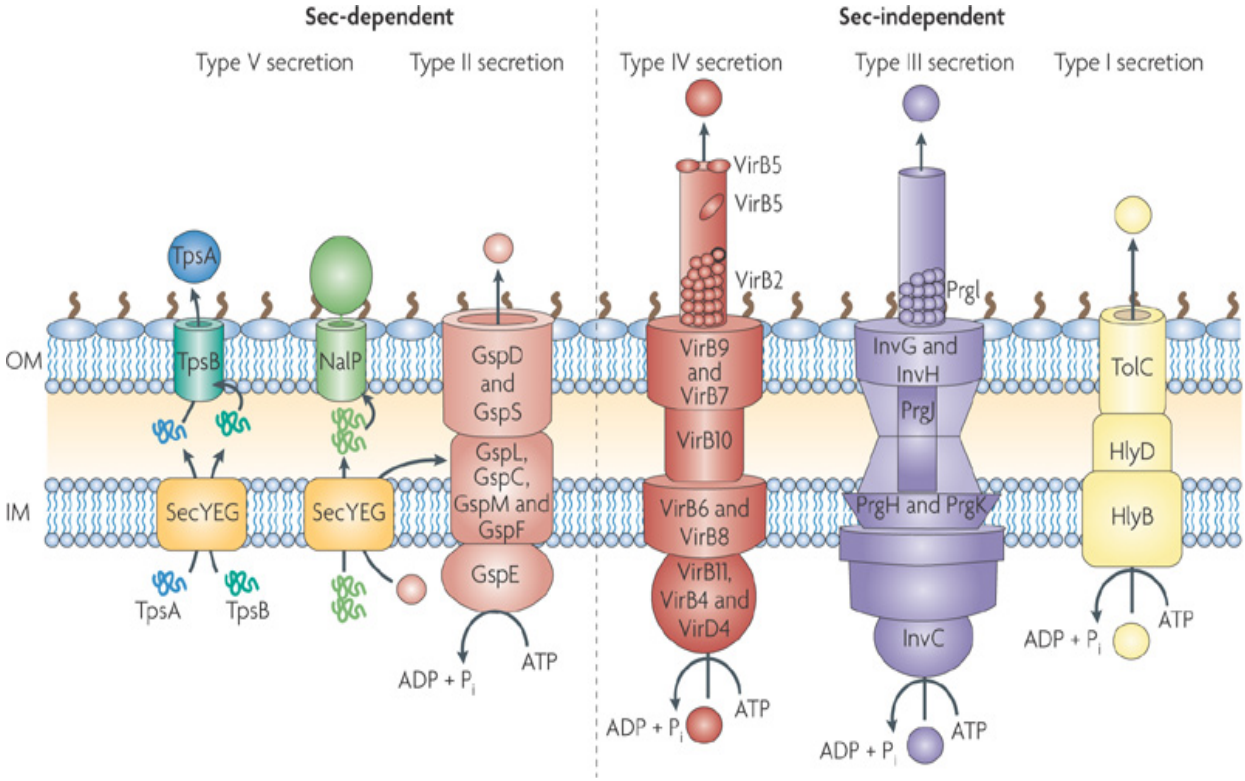


Figure 1.8: Bacterial secretion systems. Sec translocon-dependent and Sec-independent machinery involved in bacterial secretion systems involves a large number of components and continues to be studied today. Of particular note to this thesis are the type III (*Pseudomonas*) and type IV (*Agrobacterium*) secretion systems of effector proteins and T-DNA, respectively (Reproduced from Fronzes et al. 2009– Lic#4179580649540)

Type II secretion systems (T2SS) are built with many components and use a two-step mechanism for protein translocation (Figure 1.8). First, the unprocessed pre-effector protein is delivered through the inner membrane by the Sec translocon (Sec-dependent) or the twin-arginine translocation (TAT) pathway. Then from the periplasm, the effector protein is delivered by the T2SS through the outer membrane. The T2SS translocon is built with 12–14 protein components called secretins (Filloux, 2004) that are found in both bacterial membranes, the cytoplasm and the periplasm that function in conjunction with 10-15 other poorly characterized protein components to achieve protein delivery (Tosi et al., 2014). A typical example of a T2SS, the general secretion pathway (Gsp) system, is shown (Figure 1.8). The T2SS shares an evolutionary link with the type IV pilus delivery mechanism, which is Sec-independent as well as some evolutionarily conserved similarity to archaeal flagella construction (Peabody et al., 2003). To date, there no known effectors of plant pathogens that are secreted using T2SS, but its evolutionary link to type IV pili make it noteworthy nonetheless.

Finally, the bacterial two-step type V secretion system (T5SS) was discovered in recent studies to consist of either autotransporters or two-partner systems that facilitate protein delivery in two resolvable steps (Henderson et al., 2004). Autotransporter systems such as the NalP delivery system from *Neisseria meningitides,* uses a Sec-dependent delivery of proteins into the periplasmic space followed by a self-contained translocator domain-mediated delivery across the OM. Conversely, the second step of secretion could be facilitated by a second protein such as in the case of TspA delivery system whereby TspB facilitates its delivery across the OM (Mazar and Cotter, 2007). So far, this system has not been described for plant pathogens.

1.4.1.2 Type III secretion

Arguably the best characterized protein delivery system in bacteria, the bacterial type III secretion system (T3SS), forms a complex injectisome that delivers proteins from Gram-negative pathogens in a single step directly into host cells, both plants (particularly *Pseudomonas* spp., *Ralstonia* spp. and *Xanthomonas*

spp.) and mammals (including *Salmonella*, *Shigella*, *Yersinia, Vibrio* and pathogenic *E. coli*) (Cornelis and Van Gijsegem, 2000; Galan and Wolf-Watz, 2006; Hueck, 1998). While being termed Sec-independent due to a lack of Sec involvement during secretion, assembly of the T3SS does require Sec involvement (Cambronne and Roy, 2006).

To date, T3SS-delivered effectors remain the chief pathogenicity determinant in bacterial-plant interactions with a loss of the T3SS often resulting in complete failure of the pathogen to infect the host (He et al., 2004). The T3SS shares evolutionary links with bacterial flagella assembly systems and T3SS injectisome construction involves ordered secretion and assembly much like flagella assembly (Kubori et al., 2000; van Gijsegem et al., 1995) suggesting a shared evolutionary history for the two systems.

The T3SS of plant pathogenic bacteria is encoded by *hrp* (*hypersensitive response and pathogenicity*) and *hrc* (*hr and conserved*) genes carried in the *hrp/hrc* pathogenicity island (Buell et al., 2003; Diepold and Wagner, 2014). *Pto* DC3000 genome sequence availability facilitated the identification of a conserved *hrp/hrc* locus with at least 27 open reading frames (ORFs) that could be divided into 3 groups: ~3-5 transcriptional regulator genes, ~15 structural components, and a suite of putative secreted effectors, the last of which are the most varied in sequence (Büttner, 2012; Galan and Wolf-Watz, 2006; Jin et al., 2003).

The T3SS mechanism is underpinned by the needle complex formed from its numerous parts collectively called the 'injectisome', first identified in *Salmonella typhimurium* (Kubori et al., 1998). This needle complex is built by proteins encoded by *hrp/hrc* genes under control of a *cis*-acting *hrp*-box promoter element that specifically triggers expression under controlled conditions of low nutrition, low osmotic strength and low pH proposed to mimic plant apoplastic conditions (Jin et al., 2003; Tang et al., 2006). HrpR, HrpS and HrpL are key proteins involved in regulation in *P. syringae* (Innes et al., 1993; Xiao et al., 1994). HrpL, an alternative RNA polymerase sigma factor regulated through HrpR and HrpS, binds to the *hrp*-box (with consensus: GGAACC–n15-17-

C/ACACNCA), present upstream of injectisome genes as well as effectors, that acts as a promoter that gene transcription is initiated from (Ferreira et al., 2006; He et al., 2004). While structural and transcriptional regulator genes within the *hrp/hrc* complex are largely conserved, there appears to be considerable variation between the putative secreted protein effectors, strongly suggesting that this underlies the mechanism behind host diversity between very similar strains (Alfano and Collmer, 2004). Studies on assembly components of the needle complex itself have identified intracellular (ATPase HrcN, and export proteins HrcR, HrcS, HrcT, HrcU, and HrcV) and periplasmic (Secretin HrcC and structural ring protein HrcJ) components to be highly conserved and critical to function between both mammalian and plant pathogens (Büttner, 2012; Diepold and Wagner, 2014).

While expression control has been well studied for the *hrp/hrc* system, the exact mechanism of assembly has only been revealed recently (reviewed in Diepold and Wagner, 2014) with limited knowledge on regulation of effector specificity and recognition for delivery. Investigation of the T3SS in mammalian pathogens suggest a substrate specificity switch from secretion of the injectisome proteins to secretion of translocators and effector proteins, efficiently delivering proteins only when the injectisome is fully assembled with the needle having a fixed length (Ferris and Minamino, 2006; Galan and Wolf-Watz, 2006). There is also more recent evidence to suggest that calcium ion concentrations further assist in gating mechanisms to facilitate timed delivery of effectors *in planta* (Tang et al., 2006).

While most, if not all, T3SS proteins possess non-cleavable 'secretion signals' identifiable from location at the N-terminus and their requirement for T3SS delivery, no clear peptide consensus can be found as yet (Samudrala et al., 2009). Furthermore, certain T3SS effectors require chaperone proteins often encoded in the same operon as the effector protein itself and are required not just for delivery (due to size constraints within the injectisome needle) but also possibly for correct folding for biochemical function and also targeting to the T3SS itself (Lohou et al., 2013).

1.4.1.3 Type IV secretion

While not nearly as well characterized as the T3SS, the type IV secretion system (T4SS) of pathogenic bacteria was identified to be involved in plant disease through study of the crown gall pathogen *Agrobacterium tumefaciens*. The T4SS is considered to be linked to bacterial twitching motility and ancient bacterial conjugation processes much like the relationship between the T3SS and flagella (Christie et al., 2005; Mattick, 2002). The T4SS is orthologous to the conjugation machinery of bacteria, being capable of translocating both DNA and proteins. In *A. tumefaciens*, which uses T4SS to deliver its single-stranded T-DNA coated in Vir proteins into the plant host, the translocated DNA then integrates into the host genome and its subsequent expression causes the affected area to develop into a crown gall (tumor). Most T4SSs identified so far are closely related to the *A. tumefaciens virB/virD4* system (Figure 1.8) comprising around 12 proteins that deliver a wide variety of substrate proteins for many functions (Fronzes et al., 2009). In *Agrobacterium,* these components are often encoded by a so-called Ti (tumor-inducing) plasmid possessing *Vir* genes that encode both structural proteins to form the delivery apparatus as well as DNA-binding proteins (Vergunst et al., 2000; reviewed in Fronzes et al., 2009).

1.4.2 Effector delivery by filamentous pathogens

To initiate entry into their plant hosts, fungi and oomycetes are either able to utilize the stomata of leaf and stem tissues to passively invade or more often develop special structures called appressoria that forcefully penetrate waxy cuticle layers on plants to allow access to apoplastic spaces beneath (Stergiopoulos and de Wit, 2009). Once within the plant tissues, fungal and oomycete effectors are largely either delivered directly into plant cells through cellular invaginations called haustoria or invasive hyphae that serve as feeding structures and plant/pathogen interface for protein secretion (e.g. *Hyaloperonospora*, *Albugo*, *Phytophthora*, *Colletotrichum*, *Melampsora*, *Blumeria*, and *Magnaporthe*) or simply secreted into the plant apoplast by the many filamentous hyphae that act as secretory structures themselves (eg.

Cladosporium, *Botrytis*, *Sclerotinia*, *Leptosphaeria*, and *Fusarium*) (reviewed in Dou and Zhou, 2012; Petre and Kamoun, 2014; Selin et al., 2016; Stergiopoulos and de Wit, 2009). While it has long been observed that these structures are formed within plant cells/tissues, how effector proteins are directed to and secreted from such structures is under active investigation.

Stemming from studies on effectors from *Hpa* and *Phytophthora* spp., oomycete effectors often have one of three possible amino acid stretches that direct secretion into plant cells: an RxLR (sometimes with a downstream dEER) motif, LxLFLAK (or crinkler/CRN) motif, or CHxC motif at their N-terminus following the signal peptide (Jiang et al., 2008; Petre and Kamoun, 2014; Rehmany et al., 2005). The RxLR-dEER dual motifs are perhaps the best-studied with the dEER motif being present in only some examples (Jiang et al., 2008; Tyler, 2006). Examination of the role of these RxLR motifs has led to the finding that these motifs are involved in binding the plant-derived phospholipid PI3P that is abundant at the haustoria/plant interface and such binding facilitates transfer into the plant cell via lipid raft-mediated endocytosis (Kale et al., 2010). However, due to several experiments disagreeing with this paradigm, there currently no clear consesnsus on how RxLR-dEER effectors are transmitted into the plant host cytoplasm with a possible role played by both the plant and the pathogen itself (Petre and Kamoun, 2014; Yaeno and Shirasu, 2013). In addition, a bioinformatics analysis of *Phytophthora* RxLR effectors led to the identification of a secondary C-terminal three-α-helix bundle fold termed the WY motif that has recently been implicated in PI3P binding as well, but is restricted to *Phytophthora* and downy mildew lineages (R. H. Y. Jiang et al., 2013; Win et al., 2012; Yaeno and Shirasu, 2013).

Meanwhile, fungal effectors in plant pathogens *Magnporthe oryzae* and *Colletotrichum higginsianum* that form invasive hyphae accumulate in biotrophic interfacial complexes (BICs) formed for delivery (reviewed in Dou and Zhou, 2012; Khang et al., 2010; Kleemann et al., 2012). Recently, several studies have identified RxLR-like motifs (without an analogous dEER motif) in some fungal secreted effectors from these BICs with the implication that such presumably

convergently evolved motifs allow for uptake into the plant cell through binding of PI3P (Kale et al., 2010; Rafiqi et al., 2010; Tyler et al., 2013).

1.4.3 Effectors & avirulence proteins

Pathogen-delivered effectors were originally understood in the context of the detection of resistance against these effectors (phenotypically presenting as a hypersensitive response, details in ETI section), making them, in effect, avirulence proteins (encoded by *avr* genes) (Bent et al., 1993; Dong et al., 1991; Staskawicz et al., 1984). Particularly in bacterial systems, this ability to render pathogens avirulent was of interest as they provided an easy and rapid method of screening for plant genes against an otherwise virulent pathogen and capitalizing on bacterial-delivery systems allowed for the same for eukaryotic pathogen effectors (Fabro et al., 2011; Guttman et al., 2002; Roden et al., 2004; Sohn et al., 2007; Upadhyaya et al., 2014). The detection of these proteins has long been ascribed to a 'secondary' defence system in plants (Flor, 1971; Jones and Dangl, 2006; van der Planck, 1968).

The first plant pathogen *avr* gene cloned was *Pseudomonas syringae* effector AvrA, which triggered avirulence in soybean plants carrying the *R* gene *RPG2* (Staskawicz et al., 1984). This and similar early studies utilized screening of a large cosmid library (consisting of fragments of the pathogen's genome) on resistant and susceptible plant lines to determine the bacterial genomic component(s) responsible for avirulence, thus laboriously locating and identifying the *avr* gene. Since then, bacterial genome sequencing tools coupled to transposon-mediated reporter fusions to previously characterized Avr proteins, AvrRpt2 from *P. syringae* or AvrBs2 from *Xanthomonas campestris*, have allowed for rapid identification of avirulence effectors (Guttman et al., 2002; Roden et al., 2004). Furthermore, genome sequences of plant pathogens, particularly of *P. syringae* coupled to bioinformatics analysis such as *hrp*-box prediction, ORF-prediction, and use of the basic local alignment search tool (BLAST) to rapidly predict effectors (by identifying local alignments to peptide

stretches from proteins of known function), allow sequential cloning and screening of large numbers of effectors (Altschul et al., 1990; Vinatzer et al., 2005). To date, well in excess of 40 avirulence effectors have been identified in a wide variety of plant hosts including soybean, tomato, flax, wheat, and Arabidopsis (Rouxel and Balesdent, 2010).

The tomato pathogenic *P. syringae* pv. *tomato* strains carry several avirulent effectors that were identified during initial forays into understanding plant resistance. Due to the availability of genome sequence information for these pathogens coupled to well-developed methods of screening plant lines as well as the pathogens' versatility for use on Arabidopsis, identification of *avr-R* relationships could be accelerated (Katagiri et al., 2002). These *avr* genes include *avrRpt2*, encoding a cysteine protease, which triggers defence in soybean and Arabidopsis dependent on their allele of the *R* gene *RPS2* (Mindrinos et al., 1994), as well as *avrPto* that triggers resistance dependent on a specific allele of the *R* gene kinase *Pto* in tomato (Ronald et al., 1992). The discovery of *avrRpt2* avirulence added to earlier studies on bean pathogen *P. syringae* pv. *maculicola* and its avirulence gene *avrRpm1* in both bean and Arabidopsis due to the *RPM1* gene (Dangl et al., 1992) and *P. syringae* pv. *glycinea* race 4 avirulence gene *avrB* on soybean possessing the *RPG1* gene (a homolog of Arabidopsis *RPM1* which also detects *avrB*) (Innes et al., 1993). These three bacterial *avr* genes and their associated plant *R* genes allowed for the development of perhaps the best-studied model of indirect *R* gene recognition (see below), namely the RPM1/RPS2/RIN4 system in Arabidopsis (Mackey et al., 2003). Conversely, the *avrPto* discovery coupled with another *avr* gene *avrPtoB* from a different *P. syringae* pv. *tomato* strain (*Pto* DC3000) (Kim et al., 2002) was subsequently developed into the best characterized direct interaction *R* gene model (see below), namely the Pto/Fen/Prf system (Balmuth and Rathjen, 2007). Both models above are far from resolved, however, and research into the mechanisms involved in triggering defence is still revelatory (Hou et al., 2011; Mathieu et al., 2014).

Interestingly, of known bacterial *avr* genes, none appear to be evolutionarily closely related apart from the *avrBs3* family from *X. campestris* and the pan-species *yopJ/avrRxv/hopZ* superfamily (Bonas and Lahaye, 2002; Lewis et al., 2008; Ma et al., 2006). AvrBs3 from *X. campestris* pv. *vesicatoria* is a transcriptional activator-like (TAL) effector, recognized by the pepper executor *R* gene *Bs3* (Roemer et al., 2009). AvrBs3 and its homolog TALs activate transcription of susceptibility targets in the plant, but through its promoter specifically designed to exploit this function of AvrBs3, the *Bs3* gene instead triggers defence (Roemer et al., 2009). From the same family of TALs is the *X. campestris* pv. *vesicatoria avr* gene *avrBs4,* which triggers defence on tomato plants carrying the *Bs4 R* gene. *Bs4* also detects the TALs *Hax3* and *Hax4* from *X. campestris* pv. *armoraciae*, as well as several TALs of *X. oryzae* pv. *oryzae*: *avrXa3*, *avrXa4* and *avrXa5,* which each trigger defence in rice dependent on *R* genes *Xa3*, *Xa4* and *Xa5*, respectively (Chisholm et al., 2006). The identification and characterization of *avrBs3* due to its avirulence status in pepper plants facilitated swift functional characterization of other family members.

Members of the *yopJ* family includes cysteine proteases (XopJ/AvrRxv from *X. campestris* pv. *vesicatoria* and AvrXv4 from *X. campestris* pv. *euvesicatoria*) and acetyltransferases (PopP2 from *Ralstonia solanacearum*, HopZ1/HopZ2/ HopZ3/HopZ4 from *P. syringae,* and AvrBsT from *X. campestris* pv. *vesicatoria*) each demonstrating a unique preference for plant targets and are recognized by different *R* genes (Bonshtien et al., 2005; Cheong et al., 2014; Lewis et al., 2008; Üstün et al., 2014, 2013). An example of this diversity is the HopZ1 cluster, made up by HopZ1a, HopZ1b, and HopZ1c. The Arabidopsis R protein ZAR1, in conjunction with putative pseudokinase decoy ZED1, recognizes HopZ1a alone out of the three due to retention of catalytic core residues and enzymatic activity (Lewis et al., 2013; Morgan et al., 2010; Zhou et al., 2009). Interestingly, HopZ2 (triggers immunity in bean plants; targets Arabidopsis negative regulator of resistance to oomycete pathogens MLO2), HopZ3 (triggers immunity in *N. benthamiana* and bean plants; targets Arabidopsis immune regulator MPK4 and immune-related protein RIN4) and HopZ4 (not known to trigger immunity; targets Arabidopsis proteasomal component RPT6) of the same family appear to

have varied *in planta* targets and therefore likely different cognate *R* genes (Lee et al., 2015; Lewis et al., 2012, 2011; Üstün et al., 2014).

Despite the large number of fungal diseases currently being battled, avirulent effectors from ascomycetes barley and corn powdery mildew pathogen *Blumeria graminis*, barley and rye scald pathogen *Rhynchosporium secalis*, tomato leaf mold causing *Cladosporium fulvum*, Fusarium wilt pathogen *Fusarium oxysporum*, rice blast pathogen *Magnaporthe grisea* and Brassicaceae blackleg disease causal agent *Leptosphaeria maculans* represent the largest share of the total fungal effectors cloned (Stergiopoulos and de Wit, 2009). The clear majority of these cloned avirulence effectors represent small cysteine-rich secreted proteins with little to no homology shared between them. Perhaps the best studied of these are the small secreted effectors of *C. fulvum* that were identified by laboriously mapping the dominant *avr* locus in segregating *C. fulvum* populations or through extensive investigations of pathogen 'secreted' peptides (possessing characterized signal peptides for secretion) identified from RNA sequencing data: Avr2 – an inhibitor of plant cysteine protease Rcr3 activating Cladosporium fulvum 2 (Cf-2) (Dixon et al., 1996), Avr4 – a chitin binding protein protecting fungal cell walls from plant chitinases and triggering tomato Cf-4-dependent resistance (Thomas et al., 1997), Avr4E – a small cysteine-rich avirulence protein that is independently recognized by a Cf-4 homolog, Cf-4E (Takken et al., 1999; Westerink et al., 2004), Avr5 - a small cysteine-rich protein of unknown virulence function recognized by Cf-5 (Dixon et al., 1998; Mesarich et al., 2014), and Avr9 – a carboxypeptidase inhibitor recognized by Cf-9 (Jones et al., 1994). The identification of these avirulent effectors in *C. fulvum* and consequently their matching R proteins in wild resistant tomato relatives (Boukema, 1981; de Wit and Spikman, 1982; de Wit et al., 1985) allowed introgression of the leucine-rich repeat (LRR)-type *Cf-2*, *Cf-4*, *Cf-5* and *Cf-9 R* genes into commercially cultivated tomato lines, generating a superior *C. fulvum* resistant line but also to understand the mechanism behind said resistances.

Of particular interest, due to the economic impact of rice blast disease, are the various recently identified rice blast avirulent effectors, the metalloprotease

effector AVR-Pita with the rice *R* gene *Pi-ta* conferring resistance, AvrPiz-t and its corresponding rice *R* gene *Piz-t*, as well as AvrPia recognized by rice *Pia*; these three effectors are cysteine-rich putatively secreted effectors unlike the sole non-small avirulent protein, Ace1, recognized by the R protein Pi33 (reviewed in Rouxel and Balesdent, 2010). Fungal avirulent effectors are significantly more difficult to identify compared to their bacterial counterparts as their manipulation and delivery is considerably more complex. Additionally, due to limited sequence information availability for these fungal pathogens until recently, and the issue of multiple homologous effectors being present, the issue of resolving the avirulent allele may be complicated.

Like fungal effectors, oomycete avirulence effectors from potato and tomato late-blight pathogen *Phytophthora infestans*, soybean stem and root rot causal agent *Phytophthora sojae* and cruciferous downy mildew pathogen *Hyaloperonospora arabidopsidis*, are similarly difficult to identify (Kamoun, 2006). The identification of RXLR domains in putative effectors coupled to new methods of screening using bacterial delivery systems are making identification of avirulent effectors from fungal and oomycete pathogens easier (Fabro et al., 2011; Roden et al., 2004; Sohn et al., 2007). Recently, using a bacterial promoter and an *in planta* cleaved N-terminus fragment of bacterial *avrRps4* (an effector from *P. syringae* pv. *pisi*), *H. arabidopsidis* effectors ATR1 and ATR13 were examined for their ability to promote growth of bacterial pathogens in susceptible plants while restricting growth in resistant ones (Fabro et al., 2011; Sohn et al., 2007). Coupling such systems to a resistant versus susceptible plant screening can allow processing of large numbers of fungal and oomycete effectors to rapidly identify avirulent effectors.

1.4.4 Biochemical functions of effectors as virulence agents

Due to their ability to trigger a secondary defence system (ETI) in plants, thereby rendering pathogens avirulent, it may seem counterintuitive for pathogens to carry effectors. However, as mentioned earlier, animal and plant pathogens need to manipulate host processes to overcome innate primary defence systems for

successful disease establishment (Cambronne and Roy, 2006; Jones and Dangl, 2006). With this regard, effectors from plant pathogens act as 'weapons' that, when delivered into host cells, abrogate PTI in plants thereby allowing for growth and spread of the pathogen. With focus on bacterial T3SS delivered effectors (T3Es), there have been a large number of studies in the past few decades that have demonstrated the effectiveness of T3Es in facilitating pathogen spread in otherwise non-hosts (Alfano and Collmer, 2004; Dou and Zhou, 2012). This delicate balance of risk and reward typifies why effectors are so diverse and widespread among pathogens despite the chance of reduced viability if detected in the occasional resistant host.

1.4.4.1 Extracellular effectors

Many pathogens, particularly fungal and oomycetal, secrete plant cell wall degrading enzymes, toxins and cysteine-rich peptides into the plant apoplastic space. Cell wall enzymes break down plant walls and facilitate nutrient acquisition by the pathogen while toxins allow for cellular pore formation or cell death to release nutrients into the apoplast – a mechanism more important for necrotrophic pathogens that kill their host for nutrients (Cantu et al., 2008). Small cysteine-rich proteins have been postulated to be inhibitors of a wide variety of plant defence enzymes in the apoplast including hydrolases and chitinases and other proteins, with LysM motifs, act complementarily to suppress chitinase-assisted PTI triggering (CERK and CEBiP-mediated) by binding to chitin and blocking chitinases or mopping up chitin fragments in the apoplast (de Jonge et al., 2011; Stergiopoulos and de Wit, 2009). While these cysteine-rich effectors can be detected by plants to trigger defence responses (see ETI below), certain necrotrophic pathogens are believed to utilize this very process to facilitate their growth, such as those that utilize the necrosis and ethylene inducing peptide (NEP) family of proteins. Another example is the ToxA effector from wheat pathogens *Stagonospora nodorum* and *Pyrenophora tritici-repentis* that co-opts defence-related cell death in 'susceptible' plants ironically carrying the classical *R* gene *Tsn1* (Faris et al., 2010; Lu et al., 2014).

1.4.4.2 Suppression of PTI signaling

Screens to identify effectors that directly affect PTI signaling have found that many successful pathogens effectively target PTI. Several different effectors from varied pathogens target the same conserved FLS2/BAK1/BIK1 or EFR/BAK1/BIK1 complexes involved in detecting PAMPs and triggering PTI. AvrPto from *P. syringae* pv. *tomato* JL1065 is a kinase inhibitor that directly targets FLS2 and EFR (but not BAK1) to suppress signaling and abrogate PTI (Xiang et al., 2011, 2008). AvrPtoB from *Pto* DC3000 also targets the same complex by inhibiting kinase activity of FLS2 as well as BAK1 and CERK1 (Cheng et al., 2011; Goehre et al., 2008; Zeng et al., 2012). These two effectors play such an important role in *P. syringae* infections that knocking them out simultaneously results in a radically decreased virulence suggesting that they facilitate the activity of other effectors by targeting a vital component of PTI (Cunnac et al., 2011). HopF2 from *Pto* DC3000, an ADP-ribosyltransferase, also targets BAK1 (Zhou et al., 2014) and a key suppressor of PTI, RIN4, that is phosphorylated during early stages of PTI activation to derepress PTI (Chung et al., 2014; Wilton et al., 2010), as well as interfering with MAP kinase cascades triggered during PTI activation (Wu et al., 2011), all suggesting a kinase inhibitor role. BIK1 is targeted by two known effectors, AvrPphB a cysteine protease from *P. syringae* pv. *phaseolicola* that targets and cleaves both BIK1 and other RLCK family members, including PBS1, in Arabidopsis plants (J. Zhang et al., 2010). AvrAC a uridylyltransferase from *Xanthomonas campestris* that putatively blocks BIK1 kinase activity along with that of RIPK – another BIK1 RLCK family member that acts as a signal transducer for defence (Feng et al., 2012). Interestingly, AvrB from *P. syringae* pv. *glycinea* also targets RIPK but rather than inhibiting it, co-opts its function to hyperphosphorylate RIN4 and as a result suppresses basal defence (Chung et al., 2014; Liu et al., 2011). HopAO1 is another effector from *Pto* DC3000 that was recently implicated in targeting EFR-mediated PTI signaling through its tyrosine phosphatase activity to dampen the immune response (Macho et al., 2014). Much like HopF2, HopAI1 also targets MAP kinases, albeit through a phosphothreonine lyase activity that targets the MAPK, MPK4 (Zhang et al., 2007).

Finally, a trio of fungal effectors ECP6 from tomato pathogen *Cladosporium fulvum* (de Jonge et al., 2010; Sánchez-Vallet et al., 2015), Mg3LysM from wheat pathogen *Mycosphaerella graminicola* (Marshall et al., 2011) and Slp1 from rice pathogen *Magnaporthe oryzae* (Mentlak et al., 2012) are LysM containing proteins that are predicted to bind chitin to competitively inhibit plant receptor-mediated fungal detection. Knocking out these effectors from their respective fungal strains severely restricts the pathogens' ability to avoid detection and reduces disease symptoms and, in the case of Mg3LysM, only wheat CERK1 and CEBiP knockout plants were infected by *M. graminicola* lacking this effector (Lee et al., 2014; Mentlak et al., 2012).

1.4.4.3 Phytohormone manipulation

Plant hormones play a critical role in plant-pathogen interactions (Kazan and Lyons, 2014). The classical defence hormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) take center stage in these interactions often acting antagonistically or synergistically (Figure 1.9). SA is generally required for biotrophic pathogens (that acquire nutrients from a living host) and JA/ET against necrotrophic pathogens (that acquire nutrients from non-living host tissues). Important crosstalk between these three primary defence hormones and other classically developmental hormones are pivotal in the process of successfully defending against pathogens. An example of this network is the modulation of PTI signaling by brassinosteroid (BR) treatments that act to upregulate (BAK1-dependent) and simultaneously repress (BAK1-independent) defence that is mediated by SA (Albrecht et al., 2012; Belkhadir et al., 2012).

Pathogens have sought to exploit either mechanisms of defence suppression or defence hyperactivity depending on their mode of nutrition (biotrophy versus necrotrophy). The classic example of a pathogen utilizing a plant hormone system to bypass defence is the use of coronatine, a degradation-resistant mimic of jasmonyl-L-isoleucine (JA-Ile), carried by many *Pseudomonas syringae* strains to activate the JA pathway signaling through the SCF-box mediated ubiquitination and degradation of ZIM-domain-containing JAZ repressors (Demianski et al., 2012). This allows JA pathway activation to antagonize the SA

pathway critical for defence against the hemi-biotroph *P. syringae*. Additionally, crosstalk between JA and abscisic acid (ABA) pathways allows these pathogens to keep stomata open through dampening of PAMP-induced ROS burst (Melotto et al., 2006).

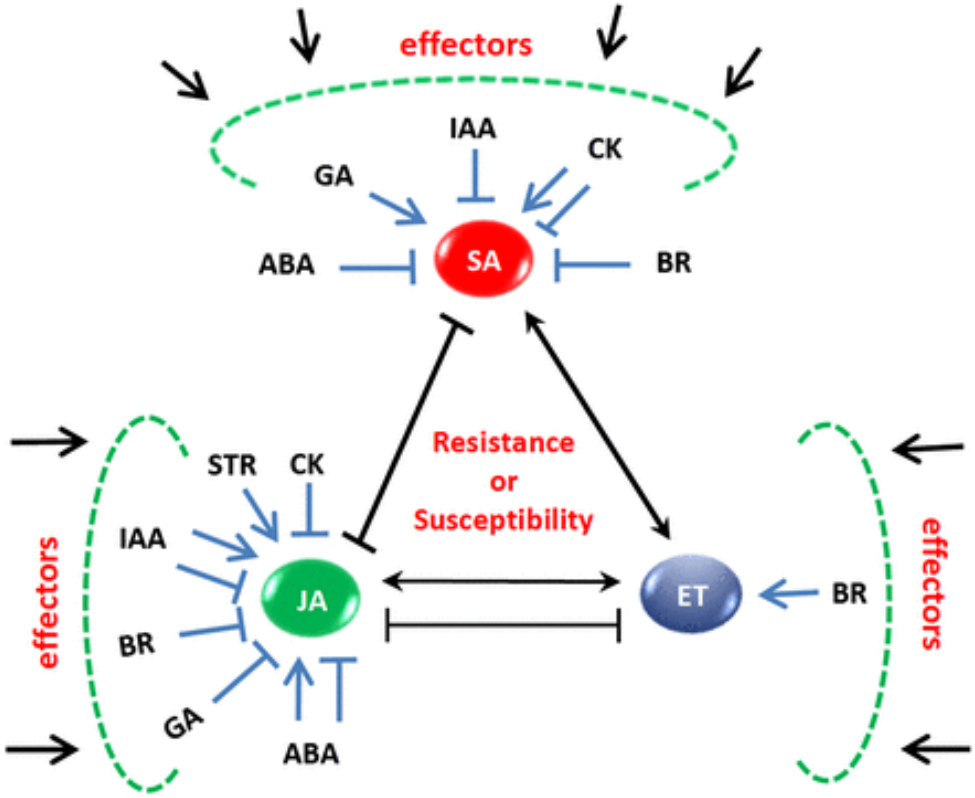


Figure 1.9: Phytohormone networks in plants the participate in defence against pathogens. The plant defence hormones JA, SA, and ET are centrally involved in plant defence, with abscisic acid ABA, auxin IAA, cytokinins CK, brassinosteroids BR, gibberellins GA, and strigolactones STR assisting, often in conjunction with the primary defence hormones to trigger non-self resistance. Pathogens have developed strategies to either interfere with or hijack phytohormone pathways to induce susceptibility. Forward and blunt arrows indicate positive and negative interactions, respectively (reproduced from Kazan & Lyons 2014).

AvrPto and AvrPtoB from *P. syringae* pv. *tomato* strains induce ET synthesis to trigger cell death and suppress defence (Cohn and Martin, 2005) and AvrPtoB also carries the ability to hijack the Arabidopsis ABA pathway to suppress defence (de Torres-Zabala et al., 2007). Both *P. syringae* pv. *tomato* AvrRpt2 and *X. campestris* pv. *vesicatoria* effector AvrBs3 activate auxin-mediated signaling by

degradation of the auxin pathway repressor, Aux/IAA, or transcriptional activation of the auxin pathway, respectively, to similarly suppress plant defences (Cui et al., 2013; Kay et al., 2007). The conserved effector locus (CEL) of *P. syringae*, *Erwinia amylovora* and *Pantoea stewartii* all act to suppress SA-dependent callose deposition and cell-wall based defence responses (DebRoy et al., 2004). Furthermore, *X. oryzae* pv. *oryzae* effectors XopN, XopQ, XopX, and XopZ all appear to suppress rice PTI induced by cell wall-degrading enzymes, albeit with undiscovered mechanisms (Sinha et al., 2013).

Mechanistically, the exact dynamics of how pathogens hijack these hormone systems remains limited. AvrB from *P. syringae* pv. *glycinea* can co-opt JA-mediated suppression of defence by binding and altering MPK4 activity supported by plant chaperone HSP90 (part of the HSP90/SGT1 chaperone complex) in a RIN4-dependent manner, possibly with the assistance of RIPK (Cui et al., 2010). This ability of AvrB at least partially phenocopies coronatine-mediated mechanisms. Cysteine protease HopX1 from *P. syringae* pv. *tabaci* 11528 similarly suppresses defence including callose deposition via the JA pathway through targeted degradation of JAZ repressors (Gimenez-Ibanez et al., 2014), a mechanism shared by the acetyltransferase HopZ1a from *P. syringae* pv. *syringae* A2 (Jiang et al., 2013). HopI1 from *P. syringae* pv. *maculicola* ES4326 utilizes its J-domain to bind the plant HSP70 ubiquitin ligase from the HSP90/SGT1 chaperone complex, and targets it to chloroplasts where HopI1 is purported to hijack the degradation activity of HSP70 to target components important for SA accumulation (Jelenska et al., 2010). The putative nucleoside hydrolase HopQ1 from *Pto* DC3000 can bind 14-3-3 proteins *in vivo* and activate cytokinin (CK) signaling, which in turn downregulates FLS2 levels and thus defence (Hann et al., 2014; Li et al., 2013). Interestingly, previous studies in Arabidopsis have shown that yeast cdc25, a potent mediator of cell cycle progression, binds 14-3-3 proteins. Furthermore, yeast cdc25 expression *in planta* was found to upregulate CK and downregulate ET pathways, thus possibly resulting in low FLS2 due to FLS2 expression reliance on ET signaling (Mersmann et al., 2010; Spadafora et al., 2012). XopN, a HEAT-repeat carrying effector also interacts with two different tomato 14-3-3 proteins including TFT1

and an LRR-receptor like kinase R protein TARK1 to functionally interfere with PTI signaling (J. G. Kim et al., 2009; Mudgett, 2006).

The fungal pathogen *Ustilago maydis* effector Cmu1 is a chorismate mutase that is able to inhibit SA biosynthesis through redirecting chorismate away from the SA biosynthetic pathway in plant chloroplasts (Djamei et al., 2011). The relative abundance of putative chorismate mutases in the various hemi-biotrophic and biotrophic plant pathogens suggests an important mechanism herein that may be conserved throughout. Another conserved virulence mechanism includes fungal, oomycetal, or bacterial targeting of JAZ repressors in their plant host. This was determined by protein interactome studies looking at oomycete downy mildew *H. arabidopsidis*, powdery mildew fungus *Golovinomyces orontii* and *P. syringae* effectors against Arabidopsis that suggests that manipulation of the JA pathway may be conserved between kingdoms (Mukhtar et al., 2011; Wessling et al., 2014).

1.4.4.4 Targeting pathogen entry barriers and secretion

Once the pathogen is detected, mobilization of barriers-to-entry are often implemented during PTI to prevent disease establishment. Entry restriction of pathogens has been studied in terms of resistance to penetration through several genetic studies identifying *PEN1*, *PEN2* and *PEN3* genes, important in maintaining resistance at the cell margin in non-adapted barley fungal pathogen *Blumeria graminis* fsp. *hordei* infection of Arabidopsis (Collins et al., 2003). *PEN1* encodes a secretory pathway associated syntaxin that interacts with VAMP721/722 and SNAP33 (making up the SNARE complex), to mediate secretion during the formation of penetration-resistant structures called papillae (Collins et al., 2003). *PEN2* encodes a peroxisome-localized myrosinase involved in hydrolyzing indole glucosinolates, important antimicrobial compounds (Lipka et al., 2005). *PEN3* encodes a plasma membrane-localized ABC transporter for putative antimicrobial compounds (Stein et al., 2006). While having other functions as well, losses of these three critical genes result in maximal penetration by pathogens into the plant despite carrying detectable PAMPs. Knowledge of the functions of these critical plant processes lends clues to

mechanisms that could be exploited by pathogens to overcome PTI-instigated barriers. For example, the previously mentioned tyrosine phosphatase HopAO1 from *Pto* DC3000 either targets secretory pathways downstream of MAPK signaling cascades or MAPK-independent pathways to effectively restrict callose deposition (critical for papillae development) thereby limiting the effectiveness of PTI (Underwood et al., 2007).

HopZ1a from *P. syringae* pv. *syringae* A2, from the *yopJ/avrRxv/hopZ* family of serine/threonine acetyltransferases, targets soybean HID1 that is involved in the production of a critical antimicrobial compound, diazedin, secreted during PTI (H. Zhou et al., 2011). Another function ascribed to HopZ1a is its ability to target and disrupt the plant microtubule network that participates in secretory pathways during PTI (A. H.-Y. Lee et al., 2012). *P. syringae* pv. *pisi* 895A HopZ2, a family member of the same superfamily, also putatively targets a pathway mediated by Arabidopsis MLO2 (Lewis et al., 2012). This homolog of pea *MLO*, the gene identified during studies of resistance to downy mildew in mutant peas, has been shown to facilitate defence, when mutated, in a *PEN1*-, *PEN2*- and *PEN3*-dependent manner (Consonni et al., 2010). AvrBsT from *Xanthomonas campestris* pv. *vesicatoria* is another member of the YopJ family that also targets callose deposition as well as repressing defence gene expression (Kim et al., 2010) and was recently found to acetylate microtubule-associated protein ACIP1, a member of a family of proteins critical for immunity (Cheong et al., 2014).

HopM1 from *Pto* DC3000 was possibly one of the first effectors found to target secretion of callose deposition and vesicle trafficking of antimicrobial compounds through an as yet unknown mechanism of targeting Arabidopsis MIN7 via proteasomal degradation (Nomura et al., 2006), a process prevented by ETI if present (Nomura et al., 2011). Additionally, HopM1 has recently been found to further target MIN10, a 14-3-3 protein associated with proteasomal degradation thereby suppressing PAMP triggered ROS burst thus compromising further stomatal immunity (Lozano-Duran et al., 2014). While HopM1 mechanism of action is unknown, it has been shown to act redundantly with

AvrE1 in the CEL along with HopAA1 that, when deleted, severely restricts pathogenicity (Badel et al., 2006; Kvitko et al., 2009). HopM1 and AvrE1 along with HopR1 act in a redundant effector group (REG) to facilitate restriction of antimicrobial delivery following AvrPto or AvrPtoB action implying a highly important role in allowing disease establishment that is conserved in many studied virulent Pseudomonads (Cunnac et al., 2011). AvrE was recently localized to the plant cell plasma membrane where it decreased expression of NHL13, a membrane associated protein required for Arabidopsis antibacterial immunity (Xin et al., 2015). However, the most striking finding was the role played by members of the CEL, HopM1 and AvrE1, in providing an aqueous apoplast following infection that in PTI-compromised plants was sufficient to allow for full disease establishment (Xin et al., 2016). Taken together, these pivotal findings explained the series of events necessary for successful infection of Arabidopsis plants – a high humidity situation to facilitate infection through leaf stomata, suppression of PTI by early-acting T3Es (including critical activity of AvrPto/AvrPtoB), followed by maintenance of an aqueous apoplast independent of humidity (particularly by HopM1/AvrE) to enable full disease progression.

In the case of non-bacterial effectors, the oomycete *Phytophthora infestans* RxLR effector AVRblb2 that triggers Rpi-blb2 mediated defence has been shown to restrict export of papain-like cysteine protease C14 (Bozkurt et al., 2011; Oh et al., 2014). Although still unknown, this may function in a manner similar to *R. solanacearum* effector PopP2-mediated restriction of Arabidopsis apoplastic cysteine protease RD19 (Bernoux et al., 2008). Additionally, several effectors from fungi and oomycetes have been shown to have cysteine protease inhibitor-like roles including *C. fulvum* Avr2 and *P. infestans* EPIC1 and EPIC2B against tomato apoplastic proteases RCR3 and PIP1 that participate in enabling pathogen detection and resistance (Song et al., 2009; Tian et al., 2007).

Perhaps the most unusual effector mode of action proposed yet is the bacterial cysteine protease HopN1 from *Pto* DC3000 which targets tomato PsbQ, a member of the oxygen evolving complex of photosystem II (PSII), effectively

restricting callose deposition and ROS burst but also inhibiting effector-triggered immunity (discussed later, herein) (Rodriguez-Herva et al., 2012). In fact, this underlines the difficulty in separating PTI-abrogation from ETI-abrogation mediated by effectors as they often appear to share pathways, signaling mechanisms and, indeed, purposes.

1.4.4.5 Modulation of effector-triggered immunity and susceptibility

Effector triggered immunity, ETI (discussed in detail later, herein) often results in a sacrificial programmed cell death of infected cells (a macroscopic phenotype termed hypersensitive response, HR, develops when a large dose of bacterial cells are delivered) upon detection of pathogen effectors *in planta*, thereby restricting pathogen growth to a limited area (Bent and Mackey, 2007). If the pathogen is present in small quantities (as is common in natural infections), this programmed cell death is microscopic and limited to a few leaf cells and the majority of the plant's leaves remain healthy. Therefore, biotrophic pathogens would best be served by effectors that actively restrict HR while hemi-biotrophic pathogens would benefit from a similar process at least early during infection. Necrotrophic pathogens, however, would not be affected as cell death is a requirement for their success and, as in the case of ToxA action in wheat, pathogens may be best served by promoting innate plant HR-type defence (Coll et al., 2011; Faris et al., 2010).

Pathogen effectors can target plant proteins required during ETI signaling. While *P. syringae* effectors AvrB and AvrRpm1 can facilitate hyperphosphorylation of RIN4 to suppress basal defence, this triggers RPM1-mediated ETI (Dangl et al., 1992; Innes et al., 1993). AvrRpt2, a cysteine protease cleaves RIN4, allowing *P. syringae* strains carrying *avrRpt2* to escape *RPM1*-mediated ETI (Kim et al., 2005). Certain plant lines carry *RPS2* to detect the AvrRpt2 effect on RIN4, restoring ETI, but the effect of *RPM1*-mediated resistance is lost and is a mechanism of virulence contribution by AvrRpt2 in non-*RPS2* plants. Interestingly, HopF2 from certain *P. syringae* strains appears to partially repress the cleavage of RIN4 by AvrRpt2 allowing escape from RPS2-mediated surveillance (Wilton et al., 2010). While as yet there are no identified

Arabidopsis *R* genes that recognize HopF2, tobacco appears to carry such a gene that makes *P. syringae* strains carrying HopF2 avirulent (Robert-Seilaniantz et al., 2006). This model clearly suggests a struggle between plant and pathogen that has been driven by the pathogen successfully evading ETI using effectors targeting components of ETI *in planta*.

P. syringae pv. *syringae* 61 effector HopA1 and *P. syringae* pv. *pisi* 895A effector AvrRps4 are believed to target a conserved ETI-signaling component EDS1 if the plants lack the putative EDS1-guarding R proteins RPS6 and RPS4, respectively (Bhattacharjee et al., 2011). HopA1 triggers HR in Arabidopsis due to the *RPS6* gene (S. H. Kim et al., 2009) but using this as a tool, several ETI-suppressing effectors have been found within the commonly used *Pto* DC3000 strain alone that were also able to suppress *RPM1*-mediated ETI (Guo et al., 2009). A novel, as yet uncharacterized, effector HopS2 was found in this study to strongly suppress ETI and, interestingly, PTI suppressors AvrPtoB, HopAO1 and HopF2 were also identified as ETI suppressors.

HopD1, from *P. syringae* pv. *phaseolicola* 1302A, can interact with defence-promoting transcription factor NTL9 at the endoplasmic reticulum and suppress ETI-specific immunity (Block et al., 2014). Interestingly, while direct effects of transcription factor like (TAL) effectors from *Xanthomonas* spp. and *Pantoea agglomerans* is unknown, these appear to target host systems to increase susceptibility. An example of this includes *X. oryzae* pv. *oryzae* TALs PthXo6 and PthXo1 that induce rice genes TFX1 and 8N3 that have been linked to decreased or increased pathogen virulence when overexpressed or knocked down, respectively (Sugio et al., 2007; Yang et al., 2006).

The selection between necrotrophic versus biotrophic defence is best typified by the study of RxLR effectors from biotrophic *H. arabidopsidis*, hemi-biotrophic *Phytophthora* spp. and necrotrophic *Pythium ultimum*. *H. arabidopsidis* and *P. sojae* RxLR effectors (such as Avr1b from *P. sojae*) carry a conserved WY domain that acts to suppress cell death by ETI while cell death preferring *P. ultimum* RxLR effectors lack this WY domain (Bozkurt et al., 2012). *P. infestans* effector

Avr3a stabilizes host E3 ubiquitin ligase CMPG1 that has been linked to HR related activity and appears to be otherwise degraded by the 26S proteasome (Bos et al., 2010). Interestingly, bacterial effectors HopZ4 from *P. syringae* pv. *lachrymans* and XopJ from *X. campestris* pv. *vesicatoria* has been shown to interact with proteasomal subunit RPT6 and thus could alleviate SA-dependent immunity (ETI) in a similar manner in their respective hosts (Üstün et al., 2014, 2013). Meanwhile, necrotrophic pathogens have been known to carry NEP-like proteins (NLPs that include bacterial toxin, ToxA) that facilitate host cell death such as NLPs from *Pectobacterium carotovorum* that are critical for aggressive growth (Ottmann et al., 2009). Much like in the ToxA-Tsn1 system, *Cochliobolus victoriae* toxin Victorin requires Arabidopsis *R* gene *LOV1* that it subverts into triggering ETI-like cell death allowing for necrotrophic growth (Gilbert and Wolpert, 2013).

Finally, several studies of *P. syringae* effectors such as HopM1, AvrPto and AvrPtoB, as well as *X. campestris* effector XopN are involved in early establishment of the disease while in the late stages contributing heavily to host tissue necrosis (Cohn and Martin, 2005; Taylor et al., 2012). This implies that a single effector may carry myriad roles in the process of disease establishment and proliferation.

1.5 Effector-triggered immunity

The fact that pathogens can and do secrete effectors to suppress primary plant defence using some myriad mechanisms implies that plants had to have coevolved a secondary defence system to combat such effectiveness, or perish. This takes the form of a substantial suite of *R* genes that encode intra- and extra-cellular proteins to screen for the presence of interfering pathogen effectors *in planta* to trigger a powerful, sacrificial form of defence termed effector-triggered immunity (ETI) (DeYoung and Innes, 2006). These *R* genes come in a variety of forms and are subdivided into classes based on their structure and mechanism of function and arguably only differ from PRRs involved in PTI (extracellular) by their recognition of proteins within the cell itself (Bent and Mackey, 2007; Spoel

and Dong, 2012). The four broad classes of non-PTI *R* genes thus are: Nucleotide-binding Leucine-rich repeat (NB-LRRs) representing by far the largest group, serine/threonine kinases that have been found to act in conjunction with NB-LRRs, receptor-like proteins (RLPs), and receptor-like kinases (RLKs) both arginine-aspartate (RD) and non-RD.

Detection of an effector presence *in planta*, achieved through either direct interaction with the effector in some cases or through effector targets or decoys in others, triggers a cascade of *R* gene-dependent downstream events that can culminate in a localized hypersensitive response (HR), eventually leading a long-term resistance against a broad swathe of pathogens in a phenomenon termed systemic acquired resistance (SAR) (Jones and Dangl, 2006; Ross, 1961). The HR is primarily understood to be a programmed cell death response of infected cells that rapidly acts to restrict the spread of the pathogen beyond the infection site and that can be extremely taxing on the plant but beneficial in terms of the plant's longevity and success (Burdon and Thrall, 2003; Dong et al., 1991; Johansson et al., 2015).

1.5.1 NB-LRRs

Nucleotide-binding leucine-rich repeat (NB-LRR) proteins represent the largest group of plant *R* genes, with greater than 150 in *A. thaliana* identified to date (Meyers et al., 2003). NB-LRRs typically consist of a variable amino terminus (N-terminus), a nucleotide-binding site and two ARC subdomains (due to resemblance to Apoptotic protease activating factor-1, R protein and *Caenorhabiditis elegans* death-4 protein domains) forming an NB-ARC (NB domain) in the middle and an LRR domain at the carboxyl terminus (Van Ooijen et al., 2008). Remarkably, NB-LRR proteins show similarity to NLR proteins (nucleotide-binding oligomerization domain (NOD)- and LRR-containing proteins), which serve, perhaps analogously, as intracellular immune receptors in animals. Work on the well-studied potato NB-LRR protein Rx has led to a mechanistic model for R protein activation (Moffett et al., 2002; Takken et al., 2006). In the absence of effectors, intramolecular interactions often occur

between the N-terminus, NB and LRR domains of NB-LRRs. Such interaction restricts nucleotide hydrolysis of the NB domain, effectively inhibiting the activity of the receptor (Qi and Innes, 2013; Takken et al., 2006). Upon detection of either the effector or effector-activated ligand, intramolecular inhibition is released, resulting in receptor activation, activating nucleotide turnover and downstream signaling. Furthermore, it is likely that NB-LRR activation leads to protein conformational changes, potentially facilitated by the plant chaperone complex consisting of HEAT SHOCK PROTEIN 90 (HSP90) and SUPPRESSOR OF G2 ALLELE OF SKP1 (SGT1) as well as REQUIRED FOR *MLA12* RESISTANCE (RAR1) (Shirasu, 2009).

Building on seminal work by H. H. Flor (Flor, 1971) on the flax rust fungal pathogen *Melampsora lini*, researchers could isolate the first set of identified *R* genes in plants at the flax *L* locus, a collection of 11 alleles (*L1-11*) conferring resistance to a variety of *M. lini* effectors (Ellis et al., 1999). These *R* genes encode NB-LRR type resistance proteins with a Drosophila Toll-like and mammalian interleukin1 receptor-like domain (TIR) at the N-terminal end making it a TIR-NB-LRR (TNL) subclass of the NB-LRR family (Radons et al., 2003). Arabidopsis *R* genes *RPP5* and *RPP2A/B* conferring resistance against downy mildew pathogen *H. arabidopsidis* Emoy2 and Cala2, respectively, TAO1 involved in *Pseudomonas syringae* pv. *glycinea* race 4 AvrB detection and signaling, as well as *RRS1* and *RPS4* conferring resistance against both *P. syringae* pv. *pisi* 151 effector AvrRps4 and *Ralstonia solanacearum* GMI1000 effector PopP2 are also well studied members of this TNL subclass (Deslandes et al., 2003; Eitas et al., 2008; Holub et al., 1995; Narusaka et al., 2009; Sinapidou et al., 2004).

Research in Arabidopsis working with *P. syringae* pv. *tomato* JL1065 and *P. syringae* pv. *maculicola* M2 identified a pair of NB-LRR resistance proteins: RPS2 recognizing AvrRpt2 and RPM1 recognizing AvrRpm1, from respective pathogens (Dangl et al., 1992; Mindrinos et al., 1994; Whalen et al., 1991). RPS2 and RPM1 belong to a different subclass of NB-LRRs, with a coiled-coil domain (CC) at their N-terminal end making them CC-NB-LRRs (CNLs) (Pan et al., 2000).

The well-characterized potato Rx protein is another example of a CNL (Moffett et al., 2002). Included in this *R* gene subclass are the pepper *Bs2* gene for resistance against AvrBs2 from *Xanthomonas campestris* pv. *euvesicatoria*, tomato *Prf* gene conferring resistance against AvrPto and AvrPtoB from *P. syringae* pv. *Tomato* strains, Arabidopsis *RPS5* conferring resistance to *P. syringae* pv. *phaseolicola* race 3 AvrPphB, as well as rice paired *R* genes *RGA4* and *RGA5* conferring resistance to both *Magnaporthe oryzae* effectors Avr1-CO39 and Avr-Pia (Belkhadir et al., 2004; Cesari et al., 2013; Ronald et al., 1992; Warren et al., 1998).

The presence of either an N-terminus CC or TIR domain in the triggered NB-LRR typically determines whether the resistance response requires either NON-RACE-SPECIFIC DISEASE RESISTANCE 1 (NDR1) or the ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1)/ PHYTOALEXIN DEFICIENT 4 (PAD4)/ SENESCENCE ASSOCIATED GENE 101 (SAG101) complex, respectively, for signaling during defence activation (see Mechanisms of signaling section).

1.5.2 Cytoplasmic serine/threonine kinases

Serine/threonine kinases, specifically cytoplasmic kinases and pseudokinases, represent a small group of important *R* genes with several currently known and characterized (including *Pto* and *Fen* in tomato, *ZED1*, *PBS1, CRCK3* and *PBL2* in Arabidopsis, and a novel *Rpg5* gene in barley) that recognize and participate in triggering defence against pathogens, particularly *Pseudomonas syringae* (Brueggeman et al., 2008; Chang et al., 2002; Lewis et al., 2013; Swiderski and Innes, 2001; G. Wang et al., 2015; Z. Zhang et al., 2017). Cytoplasmic serine/threonine kinases are not believed to function independently as *R* genes *per se* but through association with NB-LRR proteins that act as a sort of guard, screening for the presence of an effector without directly interacting with it (see Guardee/Decoy hypothesis below) (Spoel and Dong, 2012). The barley Rpg5 protein, notably, has NB and LRR domains, but also carries a C-terminal serine/threonine kinase domain reminiscent of an integrated decoy NB-LRR (see

Integrated decoy section below) that has the serine/threonine kinase fused to the NB-LRR gene itself (Brueggeman et al., 2008).

Pto and *Fen* were initially identified in wild tomato species (*Solanum pimpinellifolium*) with the former conferring resistance to strains of *P. syringae* pv. *tomato*, the causal agent of bacterial speck disease, and subsequently bred into commercial tomato cultivars (Pitblado and MacNeill, 1983). Later work discovered that this resistance was specific to *avrPto*- and *avrPtoB*-carrying strains (Kim et al., 2002; Ronald et al., 1992) and genetic studies into the *Pto* locus identified a multi-gene cluster of serine/threonine kinase homologs (including *Fen*) that resulted in this resistance, dependent on the NB-LRR *Prf* from the same genetic locus (Chang et al., 2002; Martin et al., 1993). Interestingly, while cultivated and wild tomatoes carried *PtoA/B/C/D/F*, wild tomato varieties carried 'pseudogene' versions of cultivated tomato *PtoA* and *PtoF* as well as a novel functional allele *Pto* (*PtoE*), which conferred resistance against effectors AvrPto and AvrPtoB (Chang et al., 2002). Interestingly, AvrPtoB, while not being evolutionarily related to AvrPto, is still recognized by Pto and possesses an E3-ligase-like domain that, when removed or deactivated, allowed for detection by Fen (so-called because plants carrying *Fen* demonstrate a sensitivity to the fungicide fenthion). *Fen* plants, however, did not mount resistance against AvrPto (Chang et al., 2002; Rosebrock et al., 2007). Both *Pto* and *Fen* required the NB-LRR gene *Prf* to be able to confer this specificity (Salmeron et al., 1994). Later studies revealed Pto/Prf and Fen/Prf associate to form multimeric transphosphorylated (P+1) molecular traps facilitated by Prf for kinase inhibitor effectors like AvrPto/AvrPtoB (Ntoukakis et al., 2013). Interestingly, maladapted forms of AvrPtoB such as that from bean pathogen *P. syringae* pv. *syringae* B728a (also called VirPphA) are still able to trigger defence in tomato lines that lack *Pto* due to their inability to degrade Fen or its close homologs (Chien et al., 2013). While Pto and Fen have been demonstrated to be able to trigger defence, tomato *PtoF* remains a mystery as previous studies have shown its ability to facilitate *Prf*-dependent cell death against AvrPto in heterologous plant systems but does not appear to confer significant resistance in cultivated tomato (Chang et al., 2002).

AVRPPHB SUSCEPTIBLE1 (PBS1), a cytoplasmic serine/threonine kinase from Arabidopsis, was identified during studies of loss of resistance against *P. syringae* pv. *phaseolicola* race 3 avirulent effector AvrPphB (Shao et al., 2003; Swiderski and Innes, 2001). AvrPphB is a cysteine protease that cleaves PBS1, leading to phosphorylation and activation of the NB-LRR, RPS5, triggering defence. The PBS1-unique sequence that RPS5 binds as part of a pre-activation complex is a SEMPH loop that is located opposite the AvrPphB cleavage site and therefore, while AvrPphB can cleave the kinase, it simultaneously allows for conformational change of phosphorylated RPS5 at the other end of PBS1 (Qi et al., 2014). AvrPphB has been shown to target and cleave other cytoplasmic kinases important for PTI including BIK1, and as such PBS1 is therefore believed to serve as a decoy for this activity (J. Zhang et al., 2010). Similar to the AvrPphB-PBS1-RPS5 relationship, Arabidopsis serine/threonine kinases CRCK3 and PBL2 act to trigger NB-LRRs SUMM2- and ZAR1-mediated immunities for activities of their corresponding effectors HopAI1 (a phosphothreonine lyase targeting MPK4) and AvrAC (a uridylyltransferase targeting multiple cytoplasmic kinases), respectively (G. Wang et al., 2015; Z. Zhang et al., 2017). Recently, Seto and colleagues found that ZAR1 is able to recognize and confer resistance against an allele of HopF2 from *P. syrinage* pv. *aceris* M302273PT through its action on another serine/threonine kinase, ZRK3 (Seto et al., 2017). Another notable example, a nonfunctional serine/threonine kinase (pseudokinase), is ZED1/ZRK1 that triggers ZAR1-mediated immunity against HopZ1a (Lewis et al., 2013), making ZAR1 a guard NB-LRR for multiple cytoplasmic kinases and pseudokinases via different effector-triggered modifications, reliant on several serine/threonine kinases.

1.5.3 Receptor-like proteins

Fungal PRRs such as CEBiP (see PTI section) are classic examples of receptor like proteins (RLPs) with LRR or Lysine motif (LysM) domains fused to a transmembrane domain that allows the protein to bind to and interact with an extracellular target and other host proteins to facilitate signaling. The earliest

RLPs were identified in seminal work on tomato, the transmembrane LRR (TM-LRR) type RLP alleles *Cf-4/Cf-9*, *Cf-2* and *Cf-5* genes that confer resistance against *Cladosporium fulvum* effectors, discussed earlier (Thomas et al., 1997). Typical of extracellular RLPs, they contain LxxLxxLxxLx(N/C/T)x(x)LxxIPxx type motifs that allow for interaction with their small cysteine-rich effector targets (Jones and Jones, 1997). Interestingly, while these *R* genes retain a distinct ability to interact with effector targets directly, they lack a direct signaling component. Recently, BAK1 and SOBIR1 were implicated in defence triggered by Avr4 and Avr9, two effectors that require the RLPs Cf-4 and Cf-9, respectively (Postma et al., 2016). However, investigations of other genetic requirements of Cf-4-mediated immunity for example, identified the CNL NRC1 (and other similar NB-LRR-type family members NRC2 or NRC3) that could also interact with a wide variety of RLP-type proteins upstream including Cf-9, as well as being required for immunity mediated by Pto/Prf, and the NB-LRRs Rx and Mi (Gabriels et al., 2007; Wu et al., 2016).

1.5.4 Receptor-like kinases

Like RLPs, receptor-like kinases (RLKs) also contain LRR or LysM type domains as a transmembrane domain to facilitate membrane localization, but additionally have a signaling component as well in the form of a kinase – typically a serine/threonine kinase (Afzal et al., 2008). While RLKs with a conserved arginine (R) and a highly conserved catalytic aspartate (D) in the activation loop of sub-domain VI represent a very large group of genes in plants, it has been noted that a subgroup lacking these two residues (non-RD RLKs) are particularly associated in a pathogen defence role.

FLS2, *EFR* and *GBP* (see PTI section) are good examples of PTI-associated non-RD RLKs (Spoel and Dong, 2012). This class of R proteins include the first identified PRR gene from rice, *Xa21*, that confers resistance against rice bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae* carrying the tyrosine sulfated protein RaxX (Pruitt et al., 2015; Song et al., 1995). A second non-RD RLK identified more recently, *Xa3/Xa26*, also functions against *X. oryzae* pv. *oryzae*, albeit possessing

a different avirulent effector (Li et al., 2012). While structurally similar to *FLS2*, one of the few known interactors and a putative signaling component of *Xa21*- and *Xa3*-mediated resistance, is rice kinase SERK2 but the overall mode of action for the two RLKs remains largely elusive and under study (Chen et al., 2014).

1.5.5 Mechanism of signaling during ETI

While NB-LRRs are critical mediators of defence, they are not the sole components of the defence response during ETI. Signaling cascades that stem from *R* gene triggers being activated are critical to the development and maintenance of conditions that constitute a defence response including the HR and eventually SAR (Dodds and Rathjen, 2010). One of the largest gaps in our knowledge of ETI signaling lies in processes immediately following triggering of the NB-LRR protein. However, as mentioned earlier, based on the subclass of NB-LRR triggered, several different key ETI signaling components have been identified (Aarts et al., 1998).

Most genes identified through ETI suppressor screens are either genes specific to the *R* gene signaling pathway itself or members of the chaperone complex (SGT1-HSP90 complex) required by many NB-LRRs. One of the first identified shared component was ENHANCED DISEASE SUSCEPTIBILITY 1 (*EDS1*) that is required for signaling for most known TIR-NB-LRRs (TNLs), first identified for *RPP* genes conferring resistance to *H. arabidopsidis* (Parker et al., 1996). Despite the indication that the TIR domain of TNLs are involved, the direct connection to *EDS1* remains elusive (Buscaill and Rivas, 2014). Interestingly, *EDS1* homolog, *PHYTOALEXIN DEFICIENT* 4 (*PAD4*) is involved in phytoalexin production and *PATHOGENESIS RELATED* (*PR*) gene expression, markers of ETI (Feys et al., 2001; Jirage et al., 1999). EDS1 directly binds either PAD4 or SENESCENCE ASSOCIATED GENE 101 (SAG101) in a complex *in planta*, the former of which is required for salicylic acid and TNL-dependent defence signaling (Feys et al., 2005; Rietz et al., 2011). Not unlike EDS1 and TNLs, plasma membrane-bound NON-RACE-SPECIFIC DISEASE RESISTANCE 1 (NDR1) is required for signaling

from some CC-NB-LRRs (CNLs) all of which are similarly membrane-bound (Aarts et al., 1998; Day et al., 2006).

While the exact mechanism of activation from NB-LRR to EDS1/NDR1 to defence responses including ROS bursts and *PR* gene expression is unclear, what is known is that kinase signaling cascades are inherently tied to ETI. Of particular interest are the MAPK pathways (see PTI signaling section, above) that are involved in eukaryotic signal transduction cascades (Dodds and Rathjen, 2010). Critically, both PTI and ETI involve MAPK cascades, albeit to different extents (Pitzschke et al., 2009). As mentioned earlier, CALCIUM-DEPENDENT PROTEIN KINASE (CPK) pathways involved in PTI have also been implicated in ETI downstream signaling (Boudsocq et al., 2010).

1.5.5.1 Direct Avr-R interaction

With very limited exceptions, most R proteins interact only indirectly with their Avr targets (Figure 1.10) (Spoel and Dong, 2012). Perhaps the best-characterized direct interaction is the rice blast disease *Magnapothe oryzae* effector AVR-Pita interaction with rice R protein Pi-ta (Jia et al., 2000). Pi-ta is an NB-LRR protein with a novel leucine-rich domain (LRD) at the C-terminus that binds the metalloprotease AVR-Pita via this domain. The flax rust *M. lini* effector AvrL5/6/7 and the products of their cognate *R* genes in flax, *L5*, *L6* and *L7* also bind each other through R protein polymorphic LRR domains (Dodds et al., 2006). The sole bacterial avirulent effector to have known direct interaction with its R protein is PopP2 from *Ralstonia solanacearum*, which interacts with its corresponding NB-LRR protein RRS1-R in Arabidopsis, albeit through the WRKY domain that is proposed to be an integrated decoy (Deslandes et al., 2003).

1.5.5.2 Guard-guardee/decoy hypothesis

The lack of evidence of direct interactions between R proteins and their avirulent effectors led to the proposal of the guard hypothesis, whereby NB-LRR type R proteins monitor for the presence of an effector through the latter's interaction/action on a secondary plant protein: the guardee (Figure 1.10)

(DeYoung and Innes, 2006). The classical example described earlier is the AvrPphB-PBS1-RPS5 interaction in Arabidopsis plants infected with *Pseudomonas syringae* pv. *phaseolicola* race 3 (Qi et al., 2014). In this example, it has been shown that AvrPphB (the effector) is a cysteine protease that enzymatically cleaves PBS1 (the guardee), upon which RPS5 (the guard) triggers ETI. Heterologous co-expression of *avrPphB*, *PBS1* and *RPS5* genes in *Nicotiana benthamiana* results in the formation of a protein complex, with the PBS1-RPS5 interaction occurring via the RPS5 CC-NB domains and its LRR domain serving as a repressor of ectopic activation (Ade et al., 2007).

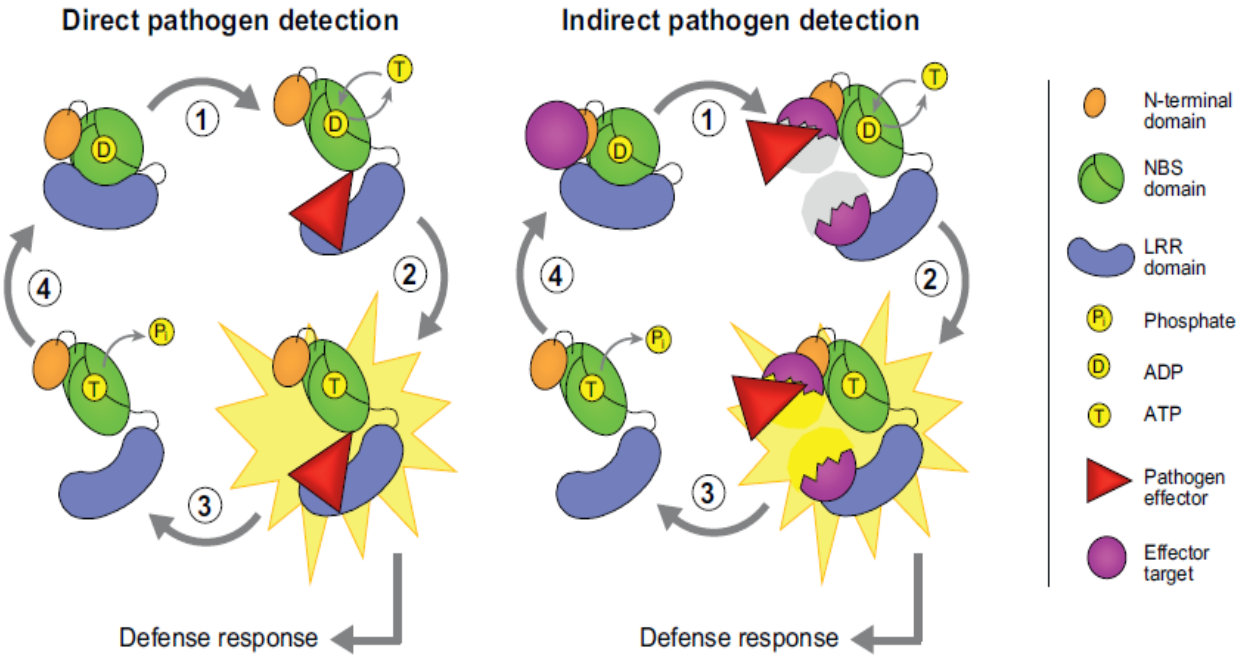


Figure 1.10: Direct and indirect modes of effector detection via NB-LRRs. Signalling is activated in a similar way for both direct (*left*) and indirect (*right*) modes of pathogen detection. Presence of the pathogen effector (1) alters the structure of the NBS-LRR protein through direct binding (*left*) or modification of additional plant proteins (*right*), allowing exchange of ADP for ATP. Note that the eliciting protein may initially interact both with the N-terminal domain and the LRR. Binding of ATP to the NBS domain (2) results in activation of signal transduction through the creation of binding sites for downstream signaling molecules and/or the formation of NBS-LRR protein multimers. Dissociation of the pathogen effector and modified effector targets (if present) (3), along with hydrolysis of ATP (4), returns the NBS-LRR protein to its inactive state. (Reproduced from Bent & Mackey, 2007)

The interaction between AvrPto/AvrPtoB and Pto is also deemed an indirect interaction due to the NB-LRR (Prf) only signaling through Pto mediated detection of these effectors (Ntoukakis et al., 2014). AvrPto and AvrPtoB along with *P. syringae* pv. *tomato* DC3000 effector HopF2 target BAK1 kinase function to repress PTI (Zhou et al., 2014). Therefore, the Prf/Pto complex is ideal for such kinase inhibitors albeit only effective against mechanisms utilized by AvrPto and AvrPtoB (Ntoukakis et al., 2013). Recent findings indicate that AvrPtoB interacts with both Fen and Pto from the same multimeric complex but through a unique interface for Pto suggesting that Fen possibly signals in a manner unlike the P+1 loop disruption trap used by Pto (Mathieu et al., 2014). Due to no known contribution to immunity apart from serving as a trap for their effectors, Pto homologs as well as PBS1 are also classified as decoys (Hou et al., 2011). Pto is predicted to serve as a decoy for immunity-related kinases like Pti1 while AvrPphB targets multiple immunity-related RLCKs like BIK1 that PBS1 serves as a decoy for (Hou et al., 2011; J. Zhang et al., 2010).

Perhaps the best characterized guard-guardee model stems from work with Arabidopsis and *P. syringae* pv. *maculicola* M2 avirulent effector AvrRpm1 which phosphorylates RPM1 INTERACTING 4 (RIN4), a negative regulator of PTI (Mackey et al., 2002). Arabidopsis CNL RPM1 can recognize this modification of RIN4 by AvrRpm1 as well as that by AvrB from *P. syringae* pv. *glycinea* race 4 that is normally recognized by orthologous proteins to RPM1 and RIN4 in soybean (Selote and Kachroo, 2010). Interestingly, the cysteine protease AvrRpt2 from *Pseudomonas syringae* pv. *tomato* JL1065 targeted the same RIN4 protein but through cleavage, a modification detected by Arabidopsis RPS2 (Mackey et al., 2003). Since phosphorylated RIN4 was able to suppress PTI, it was not clear how AvrB and AvrRpm1 were able to phosphorylate and derepress PTI in a way detectable as foreign by RPM1 until recently. It was recently shown that RIN4 acts as a phosphoswitch for PTI derepression at residue S141 upon flg22 perception while AvrRpm1/AvrB-mediated phosphorylation of a different residue T166 in RIN4, that is epistatic to S141 phosphorylation, is detectable by RPM1 (Chung et al., 2014). Downstream signaling of RPM1 and RPS2 is still

unclear but NDR1 association with RIN4 suggests a potential link to downstream defence activation (Day et al., 2006).

1.5.5.3 *R* gene pairs

Several recent findings suggest that a single NB-LRR alone may not be able to trigger ETI. Building on the finding that many NB-LRRs are clustered and could thus potentially be coexpressed, several NB-LRR pairs that work together mechanistically to trigger immunity have been discovered (Cesari et al., 2014; Eitas and Dangl, 2010; Jones and Banfield, 2017). The earliest discovered pair of NB-LRRs were the RPP2A and RPP2B proteins which confer resistance to oomycete *H. arabidopsidis* Cala2 that could potentially perceive multiple Avrs (Sinapidou et al., 2004). The requirement of TNL *RPM1* and its partner CNL *TAO1* for ETI against AvrB and putatively other Avrs as well hinted at a dynamic between CC and TIR domain NB-LRRs that had previously not been seen (Eitas et al., 2008).

The detection of *Ralstonia solanacearum* effector PopP2, evolutionarily unrelated *P. syringae* pv. *pisi* 151 effector AvrRps4, and an as yet unidentified effector from fungal pathogen *Colletotrichum higginsianum* share dual TNLs in Arabidopsis: the WRKY domain containing *RRS1-R* and *RPS4* that act in concert to trigger ETI (Narusaka et al., 2009; Sohn et al., 2014). Recent studies into the mechanics behind such pairing has revealed that the two RPS4 and RRS1-R proteins pair through their TIR domain interactions at two unique interfaces (Williams et al., 2014; X. Zhang et al., 2017). A similar interface of pairing TNLs was also observed for flax TNL L6 as well as Arabidopsis SNC1 suggesting that TNL pairing, both heteromeric and homomeric is possibly a structural norm (Cesari et al., 2014; X. Zhang et al., 2017). Pairing of CNLs are also notable, with wheat Lr10 and RGA2 CNLs mediating resistance to wheat leaf rust caused by *Puccinia triticina* (Loutre et al., 2009), barley Rpg5 and RGA1 mediating resistance to *Puccinia graminis* (Wang et al., 2013) and melon Prv and Fom-1 mediating resistance against *Fusarium oxysporum* and Papaya ring-spot virus (Brotman et al., 2013). Paired NB-LRRs are also required for the *Magnporthe grisea* avirulence effector PO6-6 where both NB-LRRs *Pi5-1* and *Pi5-2* are CNLs (Lee et

al., 2009). Other paired CNLs include RGA4/RGA5 and Pik-1/Pik-2 that confer resistance triggered by *M. grisea* effectors Avr1-CO39/Avr-Pia and Avr-Pik, respectively (Ashikawa et al., 2008; Okuyama et al., 2011; Yuan et al., 2011; Zhai et al., 2014). While most of the paired NB-LRRs seen to date are colocalized (and therefore putatively coexpressed), the single deviance was the *RPM1-TAO1* system (Eitas et al., 2008).

1.6 Aims of this study

While *Psa* has been studied for just over a decade now, the most recent global outbreaks and the reason for their resounding success as pathogens on kiwifruit remains a burning question. It seems obvious that *Psa* will have evolved an adaptation that allowed it to bypass the usual basal resistance offered by PTI mechanisms in kiwifruit. *Psa* effectors, particularly those secreted using a type III secretion system may have played a role. Indeed, the knowledge that *Psa* secretes effectors into kiwifruit and potentially the nonhost plants *Arabidopsis thaliana* and *Nicotiana benthamiana* could thus be used to garner knowledge about which, if any, of these effectors are capable of triggering avirulence in the large collective repertoire of *A. thaliana* and *N. benthamiana R* genes now globally available.

With the advent of next generation sequencing technologies allowing access to the full genome sequence of a representative of each of the unique *Psa* clades, coupled to new technology used for eukaryotic effector screening using a bacterial host, we could potentially harness a thousand different accessions of *A. thaliana* in the search for avirulence and therefore resistance to *Psa*. Acquiring this knowledge will allow us to better understand the mechanisms of virulence that have made *Psa* such a successful pathogen and facilitate the study of how *A. thaliana* and *Nicotiana* plants mount effective resistance in the form of ETI against *Psa*, with the hopes of transitioning this knowledge through research efforts downstream into kiwifruit. This PhD thesis, thus, aimed to investigate the effector biology of *Psa* specifically on aforementioned non-host plants (*Arabidopsis thaliana* and *Nicotiana* species).

The aims of this research project, thus, were to characterize effectors of interest from *Psa* with the following objectives:

1. **Identify avirulence effectors against *Psa* V13 in Arabidopsis**
   I sought to identify sources of avirulence using delivery of individual *Psa* V13 effectors by *Pseudomonas fluorescens* Pf0-1(T3S) (an empty strain that artificially possesses the ability to deliver type 3 secreted effectors).

This chapter also aimed to understand the biochemical basis of avirulent effector work with the hope that such studies would lead to identification of an NB-LRR-type R-gene that could confer recognition of *Psa* V13 in transgenic kiwfruit.

2. **Identify avirulence effectors against *Psa* LV5 in Arabidopsis and *Nicotiana* spp**

   The rationale for this was that any avirulence in these non-hosts may be potential candidates for host resistance on kiwifruit. The primary aim was to narrow down interesting targets for studies in kiwifruit via reporter eclipse assays (developed in collaboration with Dr. David Greenwood at PFR, Palmerston North) could not be deployed as the early trials failed. The reporter eclipse assay is currently still being optimized.

   Instead, I used Pf0-1(T3S) delivery of *Psa* LV5 effectors and mapping with *Psa* LV5 was used to identify an *R* gene in Arabidopsis associated with HR against *Psa* LV5. I also used *Agrobacterium* transient expression to study *Psa* LV5 homologs of previously well-characterized effectors from other pathogenic strains to seek out novel phenotypes of effector recognition *in planta.*

3. **Identify nucleus-localized effectors from *Psa* that can target Arabidopsis WRKY transcription factors**

   This aim arose from a side-project looking at effectors PopP2 from *Ralstonia solanacearum* and AvrRps4 from *Pseudomonas syringae* pv. *pisi* for interactions with a Y2H library of WRKY TFs from Arabidopsis. Interestingly we found that these two effectors appeared to specifically target a subset of closely related WRKYs that are guarded by the decoy NB-LRR (RRS1). We reasoned that since there is such specificity perhaps plant nucleus-localized effectors from other pathogens, particularly *Psa*, targeted WRKYs as well to achieve virulence but due to different specificities were able to avoid detection by RRS1. The aim of this chapter was to seek out *Psa* effectors that could interact with Arabidopsis WRKYs in Y2H assays. Effectors from *Hyaloperonospora arabidopsidis* were used

as controls for this screen since it is a virulent pathogen of Arabidopsis while *Psa* is not. A second aim of this project was to develop and test multiple screening systems (HR suppression and LUC assay) for studying these effector-WRKY interactions that will potentially be useful in future work to engineer RRS1 to detect currently undetected effectors that target WRKYs.

Overall, this multi-pronged approach has identified several interesting routes to developing resistance against *Psa*. They provide a solid basis for future work and contribute towards general understanding of effector biology, not just against *Psa* but also other bacterial pathogens.

Chapter 2: Materials & methods

Materials

2.1 Plasmid constructs*, **

Vector	Tag	Host	Description
pICH41021 (Engler et al., 2008)	n/a	*E. coli* DH5α (Appendix 2)	Shuttle vector for blunt-end *Sma*I restriction enzyme cloning of each ~1kb fragment/module; Amp^R
pBBR1MCS-5B::avrRps4$_{pro}$ (#2.1.1)	C-terminal 6xHA	Pf0-1(T3S), or *Pto* DC3000 (Appendix 3)	Golden gate cloning compatible broad host-range vector with avrRps4 promoter driven expression; Gm^R
pVSP61 (Debener et al., 1991) (Kunkel et al., 1993)	n/a	Pf0-1(T3S), or *Pto* DC3000 (Appendix 3)	Broad host-range vector for *avrRpm1*$_{PmaM2}$, *avrRpt2*$_{PtoJL1065}$ or *avrPphB*$_{PphR3}$; Km^R
pICH86988 (35SCaMV$_{pro}$) (Weber et al., 2011)	C-terminal YFP or 3xFLAG	*A. tumefaciens* AGL1 (Appendix 4)	Golden gate cloning compatible binary vector for *Agrobacterium* transient/stable infiltration or floral dips; Km^R

pBTEX (Lin et al., 2006)	n/a	*A. tumefaciens* AGL1 (Appendix 4)	Binary vector for *Agrobacterium* transient infiltration of AvrPtoB$_{PsyB728a}$; Km^R
pLexA-GG (#2.1.2) or pNLexA (Sohn et al., 2012)	N-terminal 3xFLAG HIS3	*Saccharomyces cerevisiae* EGY48 + pSH18-34 (URA3) Mat (a) (Appendix 5)	Golden gate cloning compatible vector carrying C-terminal activation domain for GAL promoter; Amp^R
pB42AD-GG (#2.1.2)	N-terminal 6xHA TRP1	*Saccharomyces cerevisiae* RFY206 Mat (α) (Appendix 6)	Golden gate cloning compatible vector carrying C-terminal LexA DNA-binding domain for GAL promoter; Amp^R
pWboxA (W6 W-box) pWboxB (W43 W-box) (#2.1.3)	n/a	*A. tumefaciens* AGL1 (Appendix 4)	Dual luciferase vector with dual W-boxes and 35S minimal promoter for measuring activity of AtWRKY transcription factor

* Appendices list all biological materials produced using the vectors listed in this table

**Antibiotic resistance*:* Km^R = kanamycin; Gm^R = gentamicin ; Amp^R = ampicillin

2.1.1 Construction of Golden Gate-compatible broad host-range plasmid

The multiple cloning site (MCS) of the broad host range vector, pBBR1MCS-5 (Kovach et al., 1995), was modified by inserting a fusion construct containing 128 bp upstream sequence from the start codon of *avrRps4* (*avrRps4* promoter) and 596 bp of the golden gate cloning system compatible *lacZ* gene containing flanking *Bsa*I restriction enzyme sites from binary vector pICH86988 (Weber et al., 2011). This fusion was generated by standard PCR using primers 5′-CGCAGATCTTTCCCCGAAGATTAGGAACT-3′ and 5′-GCCAGCTGCGGTCTCCCATTGGGAAGCCTCTTTGTCAAAG-3′ for the *avrRps4* promoter and 5′-CTTTGACAAAGAGGCTTCCCAATGGGAGACCGCAGCTG-3′ and 5′-TATCGATAAGCTGAGACCGTCACAG-3′ for *lacZ* followed by overlap PCR to create a DNA fragment containing 5′-*Bgl*II-*avrRps4* $_{pro}$ -*lacZ*-*Cla*I-3′. The amplified DNA fragment was digested with *Bgl*II and *Cla*I, purified from a 1.0% agarose gel, and ligated into *Bgl*II- and *Cla*I-treated pBBR1MCS-5. The sequence of the final pBBR1MCS-5:*avrRps4* $_{pro}$ construct was confirmed by sequencing with M13F and M13R primers.

2.1.2 Construction of Golden Gate-compatible yeast 2-hybrid plasmids

The yeast 2-hybrid vector, pLexA (Gyuris et al., 1993), carrying the LexA DNA binding domain (BD), was modified by first utilizing site-directed mutagenesis to remove all internal *Bsa*I restriction enzyme sites (#2.6.2.10; generating pLexA-B) and then inserting a 596 bp fragment of the golden gate cloning system compatible *lacZ* gene containing flanking *Bsa*I restriction enzyme sites from binary vector pICH86988 (Weber et al., 2011) into the pLexA-B multiple cloning site (MCS) using *BamH*I and *EcoR*I sites. This *lacZ* fragment was generated by standard PCR using primers 5′-TAGAGGATCCGGAGGGAGACCGCAGCTGG-3′ and 5′-TTACGAATTCAGCGTGAGACCGTCACAGCTTGT-3′ to create a DNA fragment containing 5′-*BamH*I-*lacZ*-*EcoR*I-3′. The amplified DNA fragment was digested with *BamH*I and *EcoR*I, purified from a 1.0% agarose gel, and ligated into *BamH*I- and *EcoR*I-treated pLexA-B (generating pLexA-GG). The sequence of the final pLexA-GG construct was confirmed by sequencing with M13F and M13R primers.

The yeast 2-hybrid vector, pB42AD (Gyuris et al., 1993), carrying the GAL4 activation domain (AD), was modified by first utilizing site-directed mutagenesis to remove all internal *Bsa*I restriction enzyme sites (#2.6.2.10; generating pB42AD-B) and then inserting a 596 bp fragment of the golden gate cloning system compatible *lacZ* gene containing flanking *Bsa*I restriction enzyme sites from binary vector pICH86988 (Weber et al., 2011) into the pB42AD-B multiple cloning site (MCS) using *Xho*I and *EcoR*I sites. This *lacZ* fragment was generated by standard PCR using primers 5′-GAATTCACGGAGGGAGACCGCAGCTGGCACGAC-3′ and 5′-CTCGAGAAGCGTGAGACCGTCACAGCTT-3′ to create a DNA fragment containing 5′- *Xho*I-*lacZ*-*EcoR*I-3′. The amplified DNA fragment was digested with *Xho*I and *EcoR*I, purified from a 1.0% agarose gel, and ligated into *Xho*I- and *EcoR*I-treated pB42AD-B (generating pB42AD-GG). The sequence of the final pB42AD-GG construct was confirmed by sequencing with M13F and M13R primers.

2.1.3 Construction of dual luciferase reporter vector

Two W-box sequences: WboxA (binds WRKY6-like AtWRKYs), or WboxB (binds WRKY43-like AtWRKYs), were derived from variants in a previously published study (Ciolkowski et al., 2008). Both W-boxes were were synthesized as dual tandem constructs in partially overlapping forward and reverse primers (Appendix 1) flanked with 5' *Xho*I and 3' *EcoR*I restriction sites and used in a short 'filling' PCR (to fill ends of the overlapping primers) resulting in a 48 bp PCR fragment. The PCR product was then run in an agarose gel, correct band extracted from gel, and then subjected restriction digestion with *Xho*I and *EcoR*I before cloning into the MCS of *Xho*I- and *EcoR*I-digested vector sites upstream of a 35S minimal promoter for the firefly luciferase gene (orange) in the dual luciferase vector pNWA62 (courtesy of Dr. Nick Albert, PFR, NZ) between the T-DNA left and right borders (LB/RB) to create pWboxA (2xWboxA promoters) and pWboxB (2 x WboxB promoters) constructs. The 35S minimal promoter for the firefly luciferase allows determination of transcription factor repression activity (reduction of basal level of transcription from the 35S minimal

promoter) as well as activation activity (increase of basal level of transcription) when coexpressed AtWRKYs bind either of the dual W-box constructs upstream of this minimal promoter. pNWA62 also posseses a 35S CaMV promoter driving a renilla luciferase gene as a internal control that is used to compare relative luciferase activities to assess activation/repression of firefly luciferase gene transcription.

2.2 Bacterial strains**

Bacterial strain	Genotype	Description
Escherichia coli DH5α	*fhuA2 lacΔU169 phoA glnV44 Φ80' lacZΔM15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Used for cloning plasmids,: effector modules, binary, broad host-range, and yeast vectors
E. coli HB101 (pRK2013)	*hsd20(r_{B^-}, m_{B^-}) recA13, rpsL20, leu, proA2* Km^R	Used as helper for triparental mating
Pseudomonas fluorescens Pf0-1 (pLN18; hereafter Pf0-1(T3S))	Cm^R and Tet^R; Has stably integrated pLN18 type III secretion system of *P. syringae* pv. *syringae* 61 with HopA1 deleted (Thomas et al., 2009)	Used for infiltration of isolated type III secreted effectors from *Psa* expressed in a broad-host range vector (Km^R or Gm^R)
Pseudomonas syringae pv. *tomato* DC3000 (NCPPB 4369; hereafter, *Pto* DC3000)	A spontaneously generated Rif^R mutant of the causal agent of bacterial speck in tomato DC52 (Cuppels, 1986)	Used for bacterial growth curve studies following conjugation with broad host-range vector (Km^R or Gm^R) expressing selected

		effectors
Agrobacterium tumefaciens AGL1	AGL0 (C58 pTiBo542) Amp^R recA::bla, T-region deleted Mop(+) Cb^R] (Lazo et al., 1991)	Used for transient infiltrations into *Nicotiana benthamiana* and *N. tabacum* and stable transformation of *Arabidopsis thaliana* by floral dip method. Carrying binary construct (Km^R).

**Antibiotic resistance*:* Km^R = kanamycin; Cm^R = chloramphenicol; Tet^R = tetracycline; Rif^R = rifampicin; Gm^R = gentamicin ; Amp^R = ampicillin

2.2.1 Wildtype *Psa* strains

Pseudomonas syringae pv. *actinidiae* (*Psa*) strains used in this study:

***Psa* strain**	**ICMP #**	**Isolated**	**Source**
Psa V13	ICMP18884	Bay of Plenty, NZ	PFR, Mt Albert, NZ
Psa LV5	ICMP18803	Bay of Plenty, NZ	PFR, Mt Albert, NZ

2.2.2 Triparental mating of broad host-range plasmids

Recipient Pf0-1(T3S) (or *Pto* DC3000), donor *E. coli* DH5α carrying the broad host-range plasmid to be conjugated into the *Pseudomonas* strain and helper *E. coli* HB101 with pRK2013 were streaked on King's B or L-agar medium (see #2.2/2.4/2.5.1) with appropriate antibiotics (and grown overnight before inoculation in liquid L medium with antibiotics on shaker at 28°C (Pf0-1(T3S) or *Pto* DC3000). Overnight cultures were mixed as: Recipient (0.6mL) + Donor (0.2mL) + Helper (0.2mL) at OD600=~2 in a 1.5mL eppendorf tube and pelleted at 4000g for 3 min, resuspended/washed with 1mL L medium and repelleted. After resuspending the pellet in 0.2mL fresh liquid L medium, approximately ten 20µL spots of cells were made on solid L medium plates without antibiotics,

dried and incubated for 6-8 hours at 28°C. Spots were then scraped off from the plate and restreaked on King's B plates with either 30mg/L chloramphenicol + 5mg/L tetracycline (Pf0-1(T3S)) or 50mg/L rifampicin (*Pto* DC3000) selection and additional broad-host-range plasmid conferred antibiotic selection. Plates were incubated for 48 h at 28°C and then single colonies were restreaked before making glycerol stocks.

2.2.3 Competent cell preparation & transformation

DH5α, GV3101::pMP90, or AGL1 were made electrocompetent by growth to OD 0.7 and multiple centrifugation and cold 10% glycerol washes followed snap freezing aliquots in liquid nitrogen and storage at -80°C. Competent cells were transformed by electroporation in a MicroPulser Electroporator [BioRad, NZ] at recommended settings and immediately added to 10 volumes antibiotic-free liquid L medium and culture at appropriate temperature for 0.5-1 hour before plating on antibiotic selection plates.

2.2.4 Glycerol stocks

Glycerol stocks were made from overnight cultures of bacteria grown in liquid L-medium containing appropriate antibiotics. 2 volumes of overnight-grown culture was mixed with 1 volume sterile 60% glycerol, vortexed briefly, and frozen at -80°C for long-term storage.

2.3 Plant material

2.3.1 *Arabidopsis thaliana* accessions

Arabidopsis accessions used here were obtained from the Arabidopsis stock centre (NASC), The Sainsbury Laboratory (Norwich, UK), or Plant and Food Research (Mt Albert, NZ). Seeds were sown on Dalton's Premium Seed raising mix [Fruitfed, NZ] mixed with coarse grain vermiculite and transferred to

individual cell trays two weeks after germination and grown to 4-5 weeks old before use. Plants were grown in short day conditions with 11 hours light at 22°C with humidity regulated at approximately 60%.

2.3.2 Nicotiana tabacum

Nicotiana tabacum Wisconsin 38 (a.k.a. Havana 38) seeds were sown on Dalton's Premium Seed raising mix [Fruitfed, NZ] and transferred to individual pots one week after germination and grown for 4-5 weeks before use. Plants were grown in short day conditions with 12 hours light at 24°C with humidity regulated at approximately 60%.

2.3.3 Nicotiana benthamiana

Nicotiana benthamiana seeds were sown on Dalton's Premium Seed raising mix [Fruitfed, NZ] and transferred to individual pots one week after germination and grown for 4-6 weeks before use. Plants were grown in short day conditions with 12 hours light at 24°C with humidity not regulated at approximately 60%.

2.4 Bacterial strains for cloning

E. coli DH5α and HB101 strains were grown on L-medium (Tryptone 10g/L [Sigma Aldrich, NZ], yeast extract 5g/L [Sigma Aldrich, NZ], sodium chloride 5g/L [Merck, NZ] and D-glucose 1g/L [Merck, NZ]; solidified with bacteriological agar 15g/L [Sigma Aldrich, NZ]) with antibiotics appropriate for plasmid vector carried.

Methods

2.5 Pathology methods

2.5.1 Bacterial infiltrations for HR assays in Arabidopsis

Psa strains were grown on King's B medium (peptone 20g/L [Sigma Aldrich, NZ], K_2HPO_4 1.5g/L [Merck, NZ], glycerol 10ml/L [Merck, NZ]; solidified with bacteriological agar 8g/L [Sigma Aldrich, NZ]) without antibiotics. Pf0-1(T3S)

strains were grown on King's B medium with chloramphenicol 10mg/L [Sigma Aldrich, NZ], tetracycline 5mg/L [Sigma Aldrich, NZ] and either gentamicin 20mg/L [Sigma Aldrich, NZ], or kanamycin 50mg/L [Sigma Aldrich, NZ], for plasmid pBBR1MCS5-derivatives or pVSP61-derivatives, respectively. *Pto* DC3000 strains were grown on King's B medium with rifampicin 50mg/L [Duchefa Biochimie, Belgium] and either gentamicin 20mg/L [Sigma Aldrich, NZ], or kanamycin 50mg/L [Sigma Aldrich, NZ], for plasmid pBBR1MCS5-derivatives or pVSP61-derivatives, respectively. *Psa*, Pf0-1(T3S) or *Pto* DC3000 strains were streaked from glycerol stocks onto antibiotic selection L-medium or King's B plates and grown for two days at 28^{o}C and then subsequently harvested and resuspended in 10mM $MgCl_2$ and diluted to OD 0.2 for HR. Infiltrations were carried out on 4-5 week old fully expanded leaves of Arabidopsis with a blunt 1mL syringe with as little damage as possible. HR was assayed visually at ~20-24 hours post infiltration (hpi).

2.5.2 Type-III secreted effector electrolyte leakage assays

Psa, Pf0-1(T3S) or *Pto* DC3000 strains were streaked and resuspended as before for HR assays (#2.5.1) and diluted to OD 0.2 (Pf0-1(T3S)) or 0.01 (*Psa* or *Pto* DC3000) before blunt syringe infiltrations into four fully expanded leaves of four 4-5 week old Arabidopsis plants. One 0.38 cm^2 disc was taken with a #3 cork borer from each infiltrated leaf ~0.5 hpi and washed in deionized water for 30 minutes before transfer of four discs into each of four wells with 3mL deionized water (n=4). Electrolyte leakage was measured by loading 60µL from each well on a Horiba B-173 Twin Cond conductivity meter. Measurements were taken at 2, 6, 12, 24, 36, 48 and 72 hours post infiltration (hpi). Significant electrolyte leakage associated with HR was determined over the full life of the experiment for Pf0-1(T3S) infiltrations while only early (6-12 h) increases in *Psa*/*Pto* experiments were indicative of the same.

2.5.3 *In planta* bacterial growth assays

Pto DC3000 strains were streaked and resuspended as before for HR assays (#2.5.1) and diluted to OD 0.0002 (1 x 10^5 CFU/mL) or 0.001 (5 x 10^5 CFU/mL), depending on experimental requirements, before blunt syringe infiltrations into

three fully expanded leaves of two 4-5-week-old Arabidopsis plants for each bacterial strain. Two 0.38 cm^2 discs were taken with a #3 cork borer from two different plants at 4 dpi and ground in 250µL sterile 10mM $MgCl_2$ (n=6). Each sample was then serially diluted in sterile 10mM $MgCl_2$ and 20µL spots of each sample and dilution were plated on King's B plates with appropriate antibiotics, incubated for 2 days at 28°C and then colony forming units (CFU) counted for least dilute sample with clear discrete CFU counts visible.

2.5.3.1 Bacterial spray inoculation assays for growth in Arabidopsis

Psa or *Pto* DC3000 were grown on King's B medium with or without antibiotics, resuspended in 10mM $MgCl_2$ as before (#2.5.1) and diluted to OD 1 (5 x 10^8 CFU/mL), sufficient to make up 50mL of bacterial spray suspension. Mild surfactant Vac-in-Stuff™ Silwet L-77 [Thermo Fischer, Korea] was added to a final concentration of 0.02% (v/v). The bacterial suspension was sprayed onto eight 4-5-week-old Arabidopsis plants for each bacterial strain and observed for disease symptom development for 1-2 weeks.

2.5.4 Floral dip transformation of Arabidopsis

Transgenic Arabidopsis stable lines were developed by floral dip method (Clough and Bent, 1998). Flowering Arabidopsis plants were dipped in a 5% sucrose solution containing the *A. tumefaciens* AGL1 strain at OD 1, carrying pICH86988 vector construct with the transgene (*hopZ5* or variants tagged with YFP) under the CaMV 35S promoter. Seeds were harvested when matured and bleach-sterilized (1mL 1% bleach treated for 1 min; followed by four sterile water washes, 1 volume each) before screening. Transgenic *Arabidopsis thaliana* seeds were germinated on Murashige & Skoog medium (MS salts + vitamins) 4.4g/L [Sigma Aldrich, NZ], 0.5g/L sucrose [Sigma Aldrich, NZ]; solidified with bacteriological agar 15g/L [Sigma Aldrich, NZ]) with kanamycin 50mg/L [Sigma Aldrich, NZ]. Seedlings positive for selection were subsequently transferred to soil and transformants confirmed by qPCR for *hopZ5* transgene expression. T2 seeds harvested from each confirmed T1 parent line were used for experiments after selection on agar-solidified MS medium supplemented with kanamycin.

2.5.5 *Agrobacterium tumefaciens* transient infiltration

Agrobacterium tumefaciens AGL1 were grown on L-medium with ampicillin 100mg/L [Sigma Aldrich, NZ] and kanamycin 50mg/mL [Sigma Aldrich, NZ]. *A. tumefaciens* AGL1 was streaked onto agar-solidified plates from glycerol stocks and grown for two days at 28^{o}C. These were then used to make fresh inoculations in liquid L-medium with antibiotics and grown overnight before centrifugation at 2500g and resuspension in Agroinfiltration buffer (10mM $MgCl_2$, 10mM MES) and dilution to required OD for blunt syringe infiltrations into *N. benthamiana* or *N. tabacum*. HR was assayed at between 2-7 days post infiltration (dpi), depending on the experimental requirements. For protein expression, where cell death would preclude observation of protein accumulation, 3 µM $LaCl_3$ [Sigma Aldrich, NZ] was coinfiltrated into the leaf patches at 0 and 1 dpi to suppress cell death (Sasabe et al., 2000). For measurement of proteins subject to degredation by the plant proteasomal pathway, leaf patches were pretreated with 100µM solution of MG132 [Sigma Aldrich, NZ] 3 hours before harvest.

2.5.5.1 Confocal laser scanning microscopy for subcellular localization

SGT1-silenced *N. benthamiana* plants were transiently infiltrated with *A. tumefaciens* AGL1 carrying YFP-tagged HopZ5 variants (#2.5.5). Leaf discs were taken at 2 dpi from randomly selected infiltrated areas, avoiding leaf veins, and mounted in water for confocal laser scanning microscopy. A Carl Zeiss LSM700 with excitation at 488 nm with a 20 mW Argon laser with emission filters set between 500 and 530 nm was used for imaging.

2.5.6 *N. benthamiana* electrolyte leakage assay

Electrolyte leakage measurements were carried out as described previously (Oh et al., 2010). Briefly, Agroinfiltration was carried out as described above for transient expression and two leaf discs taken per sample (n = 6) at 0 and 2 dpi, floated on 2 mL deionized water with shaking at 160 rpm for 2 hours before conductivity measurements were taken on a Horiba B-173 Twin Cond conductivity meter.

2.5.7 Virus-induced gene silencing

Two-week-old *N. benthamiana* seedlings were infiltrated with pTRV1 and pTRV2 (carrying the target gene sequence) at total OD_{600}= 1 (Mixture of OD_{600}= 0.5 of each pTRV1 and pTRV2), into 2 cotyledons. Infiltrated plants were grown 4-5 weeks before infiltration with *A. tumefaciens* AGL1 carrying YFP-, 6xHA- or 3xFLAG-tagged genes of interest. Samples were also taken from infiltrated plants to confirm silencing by RNA extraction (#2.6.3.1) and qPCR (#2.6.3.2 AND #2.6.3.3).

2.5.8 Genotyping T-DNA knockout Arabidopsis lines for homozygous mutants

Genotyping was performed on individual plants of Arabidopsis T-DNA knockout mutant plants (SALK lines). Gene-specific primers were designed using SALK online T-DNA primer design tool (http://signal.salk.edu/tdnaprimers.2.html). Plant gDNA was extracted as described below (#2.6.2.2). Three different mastermixes were prepared: One with the forward gene-specific primer and T-DNA primer (LBb1.3), the second with reverse gene-specific primer and T-DNA primer, and finally both gene-specific primers for standard PCR (#2.6.2.3). Finding bands in either of the first two meant T-DNA was present in at least one copy of gene. A band in the third setup meant the plant possessed a wild type copy of the gene. Finding a band only either the first two setups meant a homozygous T-DNA knockout plant line. A band in only the third setup meant a homozygous wild type plant line. Bands in one of the first two AND the third meant the plant was heterozygous for the knockout. Only homozygous plants found were used for bulking seeds and subsequent experiments.

2.5.9 WRKY reporter activation/repression assay

Protocol adapted from (Hellens et al., 2005). *A. tumefaciens* AGL1 was streaked and prepared as for transient expression assays (#2.5.5). AGL1 carrying the pWbox reporter plasmid was mixed with AGL1 carrying WRKY activator/repressor to be tested and AGL1 carrying the effector to be tested (or empty vector with YFP tag alone) at OD_{600} ratios of 1:6:9 to a final OD_{600} of up to 2 (OD_{600} was normalized each time with empty vector strain). Infiltrations of *N. benthamiana* leaves were carried out on three different leaves per plant per

sample. Two leaf discs per leaf were taken at 3 dpi with a #1 cork borer (n=6) and ground up in 500µL of 1x PBS buffer (137mM sodium chloride, 10mM phosphate, 2.7mM potassium chloride; pH 7.4) and 0.1 volume of this sample was mixed 2:1 with reagents from Promega Dual-Glo™ luciferase kit [Promega, Korea] to assay firefly and renilla luciferase activities sequentially on a luminometer as per kit instructions in an opaque 96-well microtitre plate. Reads were taken for 1 second per well with six wells set up for each sample (technical repeats, n=6).

2.6 Molecular biology methods

2.6.1 Enzymes

Restriction enzymes were purchased from New England Biolabs [NEB, Thermo Fischer, NZ] and used with 10X buffer supplied with the enzymes under recommended conditions.

Phusion Hifi polymerase and buffer [Thermo Fischer, NZ] was used for PCR amplification for cloning and Agilent Quickchange kit for site directed mutagenesis. T4 DNA ligase was from NEB and used with supplied buffer under recommended conditions.

Golden gate and Blunt-end cloning was done with restriction enzymes from NEB and with T4 ligase and buffer as above.

2.6.2 DNA

2.6.2.1 Bacterial gDNA extraction methods

Alkaline lysis-type method of genomic DNA extraction from bacteria was achieved through use of iNtRON Genomic DNA extraction kit [Ngaio Diagnostics, NZ] according to recommended conditions. DNA concentration was quantified by Nanodrop 1000 Specrophotometer [Thermo Fischer, NZ] and agarose gel electrophoresis.

2.6.2.2 Plant gDNA extraction methods

Genomic DNA from Arabidopsis or *Lycopersicon esculentum* was extracted from tissue frozen in liquid nitrogen using the GeneJet Plant Genomic DNA Kit [Thermo Fischer, NZ] according to instructions. DNA concentration was quantified by Nanodrop and agarose gel electrophoresis.

2.6.2.3 Polymerase chain reaction (standard)

Polymerase chain reactions (PCR) were carried out using Phusion High-Fidelity Polymerase [NEB, Thermo Fischer, NZ] using recommended conditions in an Eppendorf Mastercycler Nexus machine [MediRay, NZ]. The following PCR conditions were used: one cycle at 95^{o}C for 5min; 35 cycles at 95^{o}C for 30s, 52-60^{o}C for 30s, and 72^{o}C for 30s per kb; one cycle at 72^{o}C for 5min.

2.6.2.4 Agarose gel electrophoresis

DNA fragments and PCR product purification was achieved through agarose gel electrophoresis. Gels made were 1-2.5% (w/v) Low EEO Agarose [Sigma Aldrich, NZ] in TAE buffer, melted by microwave heating. Gels were cooled and solidified with combs in racks and then transferred to gel tanks [BioRad, NZ] containing TAE buffer stained with 20µL ethidium bromide for nucleic acid visualization. Samples were loaded into wells in the agarose gel after mixing with 10X gel loading buffer (to 2X) along with 1kb and/or 100bp DNA ladder [NEB, Thermo Fischer, NZ] and run under constant voltage. Nucleic acid was visualized on a BioRad GelDoc 312nm UV transilluminator [BioRad, NZ] and photographed.

2.6.2.5 Agarose gel purification of DNA

DNA fragments separated on agarose gels were excised using clean razor blades and then extracted using AxyPrep DNA gel extraction kit [MediRay, NZ] according to kit instructions before agarose gel electrophoresis and Nanodrop quantification.

2.6.2.6 Blunt-end cloning Golden Gate modules in pICH41021 (shuttle vector)

Agarose gel purified PCR products (10 volume) were blunt ligated into pICH41021 (1 volume) using single-pot cloning with SmaI (1 volume) and T4 DNA ligase (1 volume) under conditions recommended for ligation reactions

with T4 Buffer. pICH41021 is a shuttle vector possessing a multiple cloning site carrying SmaI within a *lacZ* gene for blue/white selection and ampicillin resistance gene. Reactions were conducted for 2-3 hours at room temperature before Sepharose 4B [Sigma Aldrich, NZ] desalting and transformation into DH5α competent cells for selection on agar-solidifed L-medium containing ampicillin (#2.4). Positive transformants were confirmed by plasmid extraction (#2.6.2.8), BsaI restriction enzyme digestion, and agarose gel electrophoresis (#2.6.2.4) of digested products to visualize the Golden Gate-compatible module. Module sequence fidelity was confirmed by sequencing using the M13 forward and reverse primers (M13-F/M13-R; Appendix 1) using sequencing services provided by Macrogen (Korea).

2.6.2.7 Golden gate cloning

Golden gate cloning was undertaken as recommended by Engler et al (2008). Briefly, assembly was undertaken in single-pot method with recepient vector (pICH86988, pBBR1MCS-5B::avrRps4$_{pro}$, pLexA-GG, or pB42AD-GG) and equimolar amounts of each module to be assembled (in shuttle vector pICH41021) as well as the epitope tag shuttle vector, restriction enzyme BsaI-HF [NEB, Thermo Fischer, NZ] and T4 DNA ligase [NEB, Thermo Fischer, NZ] in the presence of 1X BSA [Sigma Aldrich, NZ] and 1X T4 DNA Ligase buffer in 20µL reaction volume. Incubation was undertaken in a PCR thermocycler as for normal PCR, with the following conditions: 25 cycles at 95^{o}C for 5min; 35 cycles at 37^{o}C for 3min, and 16^{o}C for 4min; one cycle at 50^{o}C for 5min; and one cycle at 80^{o}C for 5min.. Typically 10-15µL of this reaction mix was then desalted by running through a pierced tube containing 50µL Sepharose 4B [Sigma Aldrich, NZ] and then transformed by electroporation on a Bio-Rad GenePulser electroporator (2.1 kV, 100 Ω, and 25 µF) into DH5α electrocompetent cells for selection.

2.6.2.8 Plasmid DNA isolation

Alkaline lysis method AxyPrep Miniprep kit [MediRay, NZ] was used according to instructions to extract plasmid DNA for cloning and transformation purposes. DNA concentration was quantified by Nanodrop and agarose gel electrophoresis.

2.6.2.9 Alkaline Lysis Miniprep

Bulk isolation of plasmid for restriction digest confirmation was carried out using manual alkaline lysis miniprep (Birnboim and Doly, 1979). Briefly, 2mL overnight cultures were centrifuged at 5000g for 5min and supernatant discarded. Supernatant was then resuspended in 0.125 volume cold Solution I (50 mM glucose; 25 mM Tris-Cl, pH 8.0; 10 mM EDTA, pH 8.0) and then 0.125 volume of Solution II (0.2 N NaOH; 1% (w/v) SDS) added, gently mixed by inversion 4-5 times and incubated at room temperature for 2 min. 0.175 volume Solution III (3 M potassium; 5 M acetate solution, pH 4.8) was then added and mixed by inversion incubated again for 2 min before centrifugation at 17000g for 10 min. Supernatant was transferred to a fresh 1.5mL tube and 0.2 volume isopropanol added, mixed by inversion and centrifuged at 17000g for 2 min to pellet plasmid DNA. Supernatant was then discarded, pellet washed with 70% ethanol and centrifuged at 17000g for 1 min. Ethanol was removed and pellet air dried before resuspension in 50µL dH_2O for use in restriction digests. Concentration was confirmed via Nanodrop quantification and via agarose gel electrophoresis.

2.6.2.10 Site-directed mutagenesis

Site directed mutagenesis of RE sites and amino acid changes were undertaken using the Agilent Quikchange Site-directed mutagenesis kit [Agilent, NZ] using DpnI-dependent methylated DNA degradation *in vitro* according to instructions.

2.6.2.11 DNA sequencing

DNA sequencing was done through Macrogen Inc, Korea or Bioneer, Korea according to supplier instructions. 5µL of DNA template was sent with 5µL of 5µM sequencing primer using the EZ-seq barcoded label system (Macrogen) or with proprietary labeling (Bioneer).

2.6.3 RNA

2.6.3.1 Total RNA extraction

Total RNA was extracted from leaf discs from 4-6 week old *A. thaliana* plants or *N. benthamiana*. Samples were harvested and snap frozen in liquid nitrogen. 3-4 leaf discs from *A. thaliana* (or 2 leaf discs from *N. benthamiana*) were then removed from -80^{o}C storage and ground in a sterile mortar and pestle and RNA extracted with 1mL Tri Reagent [Sigma Aldrich, NZ] and 0.1mL BCP [Sigma Aldrich, NZ]. Samples were vortexed vigorously followed by centrifugation at 12000g at 4^{o}C. Upper aqueous phase was taken and 0.5 volume isopropanol added with 0.5 volume High Salt buffer (0.8 M sodium citrate and 1.2 M NaCl) and vortexed briefly. Samples were then centrifuged at 12000g at 4^{o}C for 15 min, supernatant discarded and pellet washed with 70% ethanol and re-centrifuged at 8000g at 4^{o}C for 5 min. Pellet was then resuspended in DEPC water and DNAse I [Sigma Aldrich, NZ] treated according to kit instructions. RNA was assessed by agarose gel electrophoresis to visualize rRNA bands as well as quantified by Nanodrop.

2.6.3.2 cDNA synthesis (reverse transcription)

cDNA was synthesized from RNA using the Maxima First strand synthesis kit [Thermo Fischer, NZ] according to kit instructions. Using a PCR thermocycler, first strand synthesis was achieved using conditions: 25^{o}C for 10 min, 55^{o}C for 30 min, and 85^{o}C for 5 min.

2.6.3.3 Quantitative reverse transcription PCR (RT-qPCR)

Using a 96-well qPCR plate, 2µl of template cDNA along with 18µl of a qPCR mix according to the Maxima qPCR kit instructions [Thermo Fischer, NZ], (10µl 2X Thermo Maxima SYBR Green Mastermix, 2µl 10µM primers mix, 6µl H_2O) were added. The plate was protected from light and pulse-centrifuged to collect samples. qPCR was conducted under the following conditions: one cycle of UDG pretreatment at 50^{o}C for 2min; one cycle at 95^{o}C for 5min; 40 cycles at 95^{o}C for 15s, 60^{o}C for 25s, and 72^{o}C for 25s; and melting at 95^{o}C for 5 min then 65^{o}C-98^{o}C ramped for 10min. RT-qPCR primers for reporter genes were described and used previously and thus were not checked for primer efficiency (Sohn et al., 2014). Primers for transgenes are listed in Appendix 1 and were checked by melt curve for presence of multiple products and by running agaorose gels for visualizing

products produced, but were not assessed for primer efficiency, which was assumed to be 100%. Quantification was analyzed out by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen et al., 2001) with EF1α as the internal reference gene and comparisons to wildtype (no treatment), mock (water)-treatment, or empty vector (EV)-treatment (indicated for each figure in Results) determining gene expression changes.

2.6.4 Protein

2.6.4.1 Total protein extraction

N. benthamiana leaves were infiltrated with *A. tumefaciens AGL1* carrying binary vectors for tagged proteins (#2.5.5). Plant tissue was frozen in liquid nitrogen after 2-3 dpi. Frozen tissue was ground in a liquid nitrogen chilled mortar and pestle and transferred to an equal volume of protein extraction buffer: GTEN (10% glycerol, 25 mM Tris pH 7.5, 1 mM EDTA, 150 mM NaCl) , 2% w/v PVPP [Sigma Aldrich, NZ], 10 mM DTT [Sigma Alrich, NZ], 1X Complete™-mini protease inhibitor tablet [Sigma Aldrich, NZ], 0.1% NP40 [Sigma Alrich, NZ]. For protein degradation experiments (Chapter 4; blocking the plant proteasomal pathway), MG132 [Sigma Aldrich, NZ] was added to a final concentration of 150µM in the protein extraction buffer. Ground protein samples with extraction buffer were thawed on ice before vortexing. 2mL of this sample was centrifuged at 5000rpm at 4^{o}C for 15min, samples made up with SDS Loading buffer (to final concentration: 100 mM Tris-HCl (pH 6.8) [Sigma Aldrich, NZ], 4% (w/v) SDS [Sigma Aldrich, NZ], 0.2% (w/v) bromophenol blue [Sigma Aldrich, NZ], 200 mM DTT [Sigma Aldrich, NZ]) and then heated to 96^{o}C for 10min. The sample was then vortexed before centrifugation at maximum speed before loading on a 10% Tris-glycine SDS-polyacrylamide gel. PageRuler™ prestained protein ladder [Thermo Fischer, NZ] was run alongside for determination of protein band sizes.

2.6.4.1.1 Immunoprecipitation

N. benthamiana leaves were infiltrated with *A. tumefaciens AGL1* carrying binary vectors for tagged proteins (#2.5.5). Leaf tissue was harvested at 2 dpi and snap frozen before grinding and total protein extraction. Samples were immunoprecipitated (IP) using anti-FLAG affinity gel [Sigma Aldrich, NZ] and

then treated the same as total protein at the stage of adding SDS Loading buffer (#2.6.4.1).

2.6.4.2 SDS-PAGE & Western blot

Protein extracts in SDS Loading buffer were separated by SDS–PAGE, according to the method of Laemmli (Laemmli 1970). SDS-polyacrylamide gel electrophoresis was performed at 130V for 1.5-2.5h in Running buffer (25 mM Tris-HCl, 192 mM glycine, 0.1% SDS) to separate proteins by mass. Subsequently gels were blotted onto PVDF membranes [Sigma Aldrich, NZ] in Transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% (v/v) methanol) overnight at 30V (4^{o}C). Membranes were then blocked with with 5% non-fat milk in TBST buffer (20 mM Tris-HCl, pH7.5, 150 mM NaCl, 0.1% Tween-20) for 30 min at room temperature and subsequently incubated with horseradish peroxidase-conjugated antibody against the epitope tag (anti-HA [Sigma Aldrich, NZ], anti-FLAG [Sigma Aldrich, NZ], anti-GFP [Santa Cruz Biotechnology, USA], or against acetylated lysine residues (anti-AcK) [Cell Signaling, USA]). After washing with TBST buffer (3 x 10 min) visualization was achieved using Pierce Pico and Femto reagents [Thermo Fischer, NZ] carrying chemiluminescent substrate for the HRP enzyme. Reagents were applied to the blot at , and light emitted was captured by photographic film [Kodak, NZ]. Equal sample loading per lane was demonstrated by Ponceau red staining [Sigma Aldrich, NZ] for 10min and visualizing the Rubisco band following destain with de-ionized water for 15min.

2.6.5 Yeast 2-hybrid

All protocols for yeast experiments were obtained from Clontech (www.clontech.com). Yeast medium used was either rich YPD medium (20 g/L Difco peptone, 10 g/L Yeast extract with 20 g/L Difco agar for plates) or SD medium (6.7 g/L Yeast nitrogen base without amino acids and 20 g/L Difco agar for plates supplemented with appropriate Dropout amino acid solution as per manufacturer instructions).

2.6.5.1 Yeast transformation

Yeast strains EGY48 and RFY206 were transformed with pLexA-GG or pB42AD-GG constructs, respectively, using the Frozen-EZ Yeast Transformation II Kit™ [Zymo Research, Korea] as per kit instructions. Materials produced are listed in Appendices 5 & 6).

2.6.5.2 Yeast mating and interaction

Inoculums of *Saccharomyces cerevisiae* EGY48 carrying pLexA-GG constructs (Appendix 5) and RFY206 carrying pB42AD-GG constructs (Appendix 6) were mixed and mated overnight in liquid YPD medium and plated on SD media lacking uracil, tryptophan, and histidine. Single colonies that formed after 2-3 days were selected as diploid mated yeast and screened for protein-protein interactions. Protein interaction was verified by streaking and then spotting (at OD_{600} = 0.5) the diploid yeast cells on SD media lacking uracil, tryptophan, histidine and leucine (quadruple knockout) and containing BU salts and X-β-gal at 40μg/mL [Sigma, Korea]. Diploid yeast that grew on this media and turned blue were selected as positive interactants. Strong interactions were dark-medium blue with strong confluent growth for streaked as well as spotted colonies.

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

3.1 Introduction

Pseudomonas syringae pv. *actinidiae* (*Psa*) causes a bacterial canker disease in kiwifruit and has been a global threat to the kiwifruit industry since the 1980s and consists of several distinct clades known as biovars. The source of a recent global outbreak of bacterial canker disease in kiwifruit orchards in major exporters like Italy (2008), Chile (2010) and New Zealand (2010) has been identified as biovar 3 strains of *Psa* which includes the V13 strain (ICMP 18884, CP011972-3) from New Zealand (Chapman et al., 2012). The *Psa* V13 genome was recently sequenced in order to understand its origin and virulence mechanisms (McCann et al., 2013; Templeton et al., 2015). Like other *P. syringae* strains, *Psa* V13 carries the genes encoding the T3SS and a repertoire of 34 predicted T3Es. Putative biochemical activities of *Psa* V13 T3Es include ribosyltransferase, glutaminase, phospholyase, cysteine protease, and acetyltransferase. Interestingly, HopZ5 (AKT29515.1), a putative acetyltransferase, and HopH1 (AKT29516.1) are uniquely present among the global outbreak strains of biovar 3 including *Psa* V13 (McCann et al., 2013).

The YopJ (Yersinia outer protein J) effector protein from the human gastroenteritis pathogen *Yersinia pseudotuberculosis* was shown to have an acetyltransferase activity. YopJ acetylates multiple MITOGEN ACTIVATED PROTEIN KINASE KINASE (MAPKK) proteins, key components of host defence, which results in inhibition of their phosphorylation and suppression of NFκB mammalian defence signaling pathways (Mukherjee et al., 2006; Orth et al., 1999). Interestingly, plant pathogenic bacteria carry several YopJ-class T3Es including *Ralstonia* PopP2 and the *Pseudomonas* HopZ family of effectors (Lewis et al., 2011; Tasset et al., 2010). HopZ1a from *P. syringae* pv. *syringae* A2 autoacetylates a specific threonine residue (T346) with the involvement of two serine residues (S349 and S351) following *in planta* activation of HopZ1a by the plant cofactor IP6 (Ma et al., 2015; Zhang et al., 2016). In addition, HopZ1a

acetylates jasmonate ZIM-domain (JAZ) repressors of jasmonic acid (JA) signaling leading to their proteasomal degradation, thereby activating the JA pathway and antagonizing salicylic acid-mediated disease resistance (Jiang et al., 2013). Additionally, HopZ1a targets tubulin to block defence at the cell wall while simultaneously interfering with the host's secretory pathway that has been linked to systemic immunity (A. H.-Y. Lee et al., 2012; Macho et al., 2010; Wang et al., 2005). However, in the presence of a functional CNL gene, *ZAR1*, HopZ1a activates ETI in Arabidopsis (Lewis et al., 2010). HopZ1a-triggered immunity requires Arabidopsis pseudokinase ZED1 that is monitored by ZAR1 (Lewis et al., 2013). The downstream genetic components required for HopZ1a-triggered immunity remains elusive (Lewis et al., 2013, 2010). The *Ralstonia solanacearum* GMI1000 effector PopP2 is a nucleus-localized acetyltransferase that autoacetylates a lysine residue (K383) required for its *trans*-acetylation activity (Tasset et al., 2010) and targets WRKY transcription factors involved in defence activation (Le Roux et al., 2015; Sarris et al., 2015). The TNL protein pair RRS1 and RPS4 confers the recognition of PopP2 in Arabidopsis. PopP2 acetylates the WRKY DNA-binding domain of RRS1 resulting in destabilized RRS1 binding to its target DNA (Deslandes et al., 2002; Le Roux et al., 2015; Sarris et al., 2015). It is hypothesized that PopP2 targets other WRKY transcription factors to enhance bacterial virulence yet a detailed mechanism is unknown. Based on these findings, despite their conserved enzymatic activity, different members of the YopJ acetyltransferase family may have been specialized for targeting distinct plant defence components.

In this study, we sought to examine the T3E involvement of the *Psa* V13-triggered HR in Arabidopsis. By using T3SS delivery of *Psa* effectors from *P. fluorescens* Pf0-1 (T3S), a non-pathogenic strain engineered to carry a functional T3SS (Thomas et al., 2009), we sought to identify T3Es that trigger ETI in Arabidopsis. We also sought to characterize interesting putative avirulence effectors from this study to understand their biochemical activity in both Arabidopsis and *Nicotiana benthamiana* using effector delivery and transient expression studies, respectively. Furthermore, we exploited both these systems to understand the genetic requirements for avirulence in both Arabidopsis and

N. benthamiana and to uncover cryptic differences in HR and immunity triggered by the avirulence effector, HopZ5.

3.2 Results

3.2.1 HopZ5$_{Psa\text{V13}}$ triggers accession-specific immunity in Arabidopsis

Several *P. syringae* strains have been shown to trigger *NB-LRR*-dependent immunity in Arabidopsis associated with a hypersensitive response (HR), thought to be indicative of a strong immune response (Bisgrove et al., 1994; Hinsch and Staskawicz, 1996; Johansson et al., 2015; Kunkel et al., 1993; Lewis et al., 2010). To examine if *P. syringae* pv. *actinidiae* (*Psa*) V13 can also trigger immunity in Arabidopsis, we screened several accessions that were used to found a Multiparent Advanced Generation Inter-Cross (MAGIC) recombinant inbred line set (Kover et al., 2009) for *Psa* V13-triggered HR as an indicator for effector-triggered immunity (ETI). Of the 19 MAGIC parents (accessions used to generate the MAGIC RIL library set), we checked 11 accessions that were available to us (Burren-0 (Bur-0), Canary Islands-0 (Can-0), Columbia-0 (Col-0), Catania-1 (Ct-1), Edinburgh-0 (Edi-0), Hilversum-0 (Hi-0), Landsberg *erecta*-0 (L*er*-0), Nossen-0 (No-0), Oystese-0 (Oy-0), San Feliu-2 (Sf-2), and Wassilewskija (Ws-2)). *Psa* V13 triggered a strong HR in the Ct-1 and L*er*-0 accessions but not in other accessions tested including Col-0 and Ws-2 (Figure 3.1).

In order to identify the *Psa* V13 type-III secreted effectors (T3Es) that trigger HR in Ct-1, we cloned all 32 predicted full length T3Es from *Psa* V13 (Table 3.1; Figure 3.2) by the golden gate cloning method (#2.6.2.6-7) (Engler et al., 2008) into the broad host-range vector pBBR 1MCS-5 (Kovach et al., 1995). The previously characterized *avrRps4* promoter was used to drive T3E expression (Hinsch and Staskawicz, 1996; Sohn et al., 2007). Several T3E genes identified in the *Psa* V13 genome (McCann et al., 2013) are excluded from Table 1 for which homologs were cloned instead (*hopAM1-2* or *hopBB1-1*) or that are not likely expressed due to truncations/early stop codons indicating that they were pseudogenes (*avrRpm2*, *hopA1*, *hopW1*, *hopAA1-1*, *hopAA1-2*, and *hopAG1*).

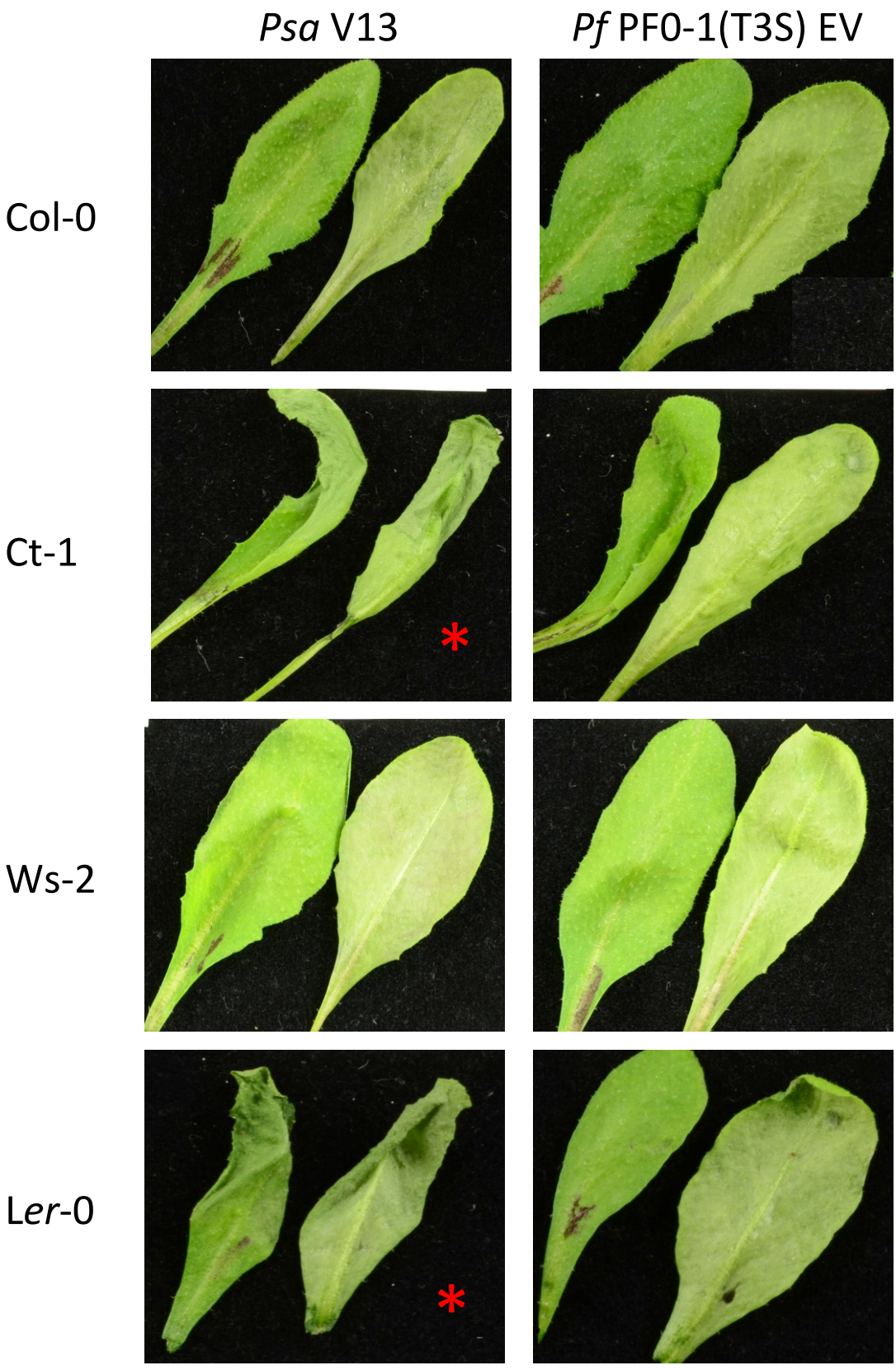


Figure 3.1. *Psa* V13 triggers HR in Arabidopsis in an accession-specific manner. Leaves of *Arabidopsis* plants (Col-0, Ct-1, Ws-2, or L*er*-0) were infiltrated with *P. syringae pv. actinidiae V13 or P. fluorescens* Pf0-1 (T3S) carrying the empty vector pBBR1MCS-5 (EV). Bacterial suspensions (1×10^8 CFU/mL) were blunt-syringe infiltrated into the leaves (#2.5.1) and photographs were taken 20 hours after infiltration (1 dpi). Development of the HR is indicated by a red asterisk

Table 1. Full predicted type-III system secreted effector (T3E) repertoire of *Psa* V13

Psa V13 T3E	Locus tag	Hrp box	Chaperone	Predicted protein size (kDa)
AvrB4-1	IYO_03480	✔		36.0
AvrD1	IYO_03455	✔		34.6
AvrE1	IYO_06540	✔		195.3
AvrPto5	IYO_11535	✔		17.2
AvrRpm1	IYO_07800	✔		24.9
HopD1	IYO_03415	✔		34.6
HopF2	plasmid	✔	ShcF	22.0
HopH1	IYO_23150	✔		24.3
HopI1	IYO_05035	✔		49.3
HopM1	IYO_06530	✔	ShcM	75.9
HopN1	IYO_06505	✔	ShcN	38.7
HopQ1	IYO_03410	✔		48.8
HopR1	IYO_07915	✔		210.3
HopS2	IYO_03945	✔	ShcS2	18.7
HopX3 (HopF3)	IYO_03510	none	ShcF	40.9
HopY1	IYO_00845	✔		30.9
HopZ3	IYO_27805	✔		45.1
HopZ5	IYO_23155	✔		39.0
HopAE1	IYO_19340	✔		126.6
HopAF1	IYO_03560	✔		23.4
HopAH1	IYO_08085	none		42.8
HopAI1	IYO_08090	none		29.7
HopAM1-1	IYO_23055	✔		31.3
HopAO2	IYO_03600	✔		38.1
HopAS1	IYO_26235	✔		149.0
HopAU1	IYO_29455	✔		88.2
HopAV1	IYO_29370	none		123.5
HopAW1	IYO_03535	✔		24.9
HopAY1	IYO_03495	none		27.1
HopAZ1	IYO_13315	✔		24.7
HopBB1-2	IYO_03610	✔	ShcF	30.7
HopBN1	IYO_013150	✔	ShcF	56.8

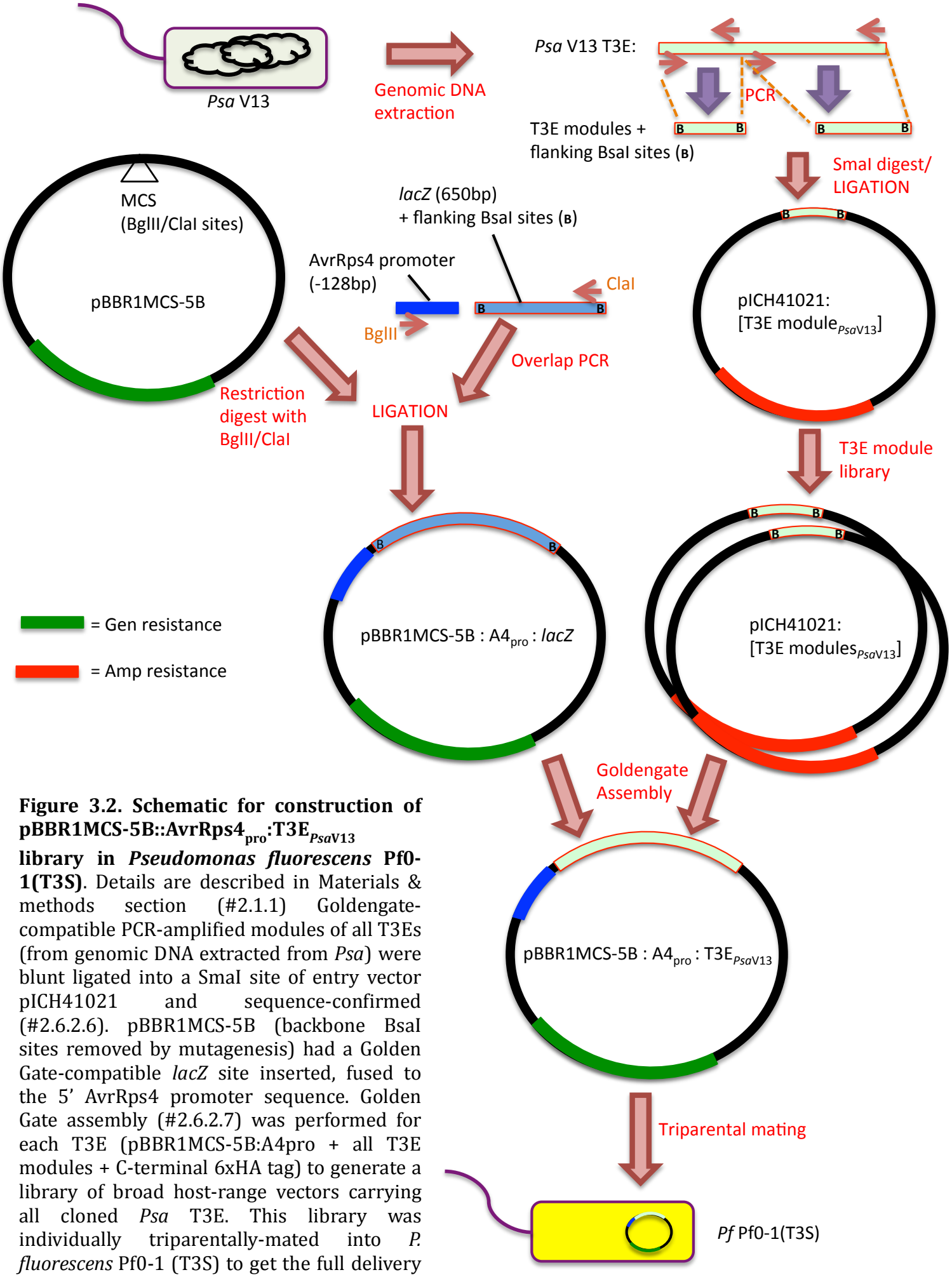


Figure 3.2. Schematic for construction of pBBR1MCS-5B::AvrRps4$_{pro}$:T3E$_{PsaV13}$ library in *Pseudomonas fluorescens* Pf0-1(T3S). Details are described in Materials & methods section (#2.1.1) Goldengate-compatible PCR-amplified modules of all T3Es (from genomic DNA extracted from *Psa*) were blunt ligated into a SmaI site of entry vector pICH41021 and sequence-confirmed (#2.6.2.6). pBBR1MCS-5B (backbone BsaI sites removed by mutagenesis) had a Golden Gate-compatible *lacZ* site inserted, fused to the 5' AvrRps4 promoter sequence. Golden Gate assembly (#2.6.2.7) was performed for each T3E (pBBR1MCS-5B:A4pro + all T3E modules + C-terminal 6xHA tag) to generate a library of broad host-range vectors carrying all cloned *Psa* T3E. This library was individually triparentally-mated into *P. fluorescens* Pf0-1 (T3S) to get the full delivery effector library for *Psa* for screening Arabidopsis plants.

Individual T3E delivery by *Pseudomonas fluorescens* (*Pf*) Pf0-1(T3S) that carries a stably integrated T3SS from *P. syringae* pv. *syringae* 61 (hereafter, Pf0-1(T3S)) identified HopZ5 as a cause of the HR in Ct-1 (Figure 3.3A). Col-0 and Ws-2 did not develop HR in response to Pf0-1(T3S)-delivered HopZ5 or empty vector (EV). However, as expected, Pf0-1(T3S)-delivered AvrRpm1 (from *P. syringae* pv. *maculicola* M6) triggered HR in all three Arabidopsis accessions tested, indicating that our experimental conditions were optimal (Debener et al., 1991). To quantify HopZ5-triggered HR, we measured the leakage of ions caused by HR development in the infected leaves. As expected, Pf0-1(T3S)-delivered AvrRpm1 induced significant increase in electrolyte leakage levels in all three Arabidopsis accessions tested whereas EV did not cause a notable change. Consistent with HR data, Pf0-1(T3S)-delivered HopZ5-triggered significantly increased electrolyte leakage level as compared to EV in Ct-1 but not in Ws-2 nor Col-0 (Figure 3.3B).

The development of HR is indicative but not evidence of an immune response as several examples of HR without immunity has been described (Century et al., 1995; Cooley et al., 2000). To examine the HopZ5-triggered immunity in Arabidopsis, we assessed HopZ5-triggered defence gene expression for four previously characterized early defence marker genes *WRKY51*, *SARD1*, *PBS3,* and *FMO1* by Pf0-1(T3S)-delivery (Sohn et al., 2014). As expected, Pf0-1(T3S)-delivered HopZ5 upregulated transcript accumulation of these defence genes in Ct-1 but not in Col-0 and Ws-2 (Figure 3.4).

3.2.2 HopZ5 triggers immunity independent of HR in Col-0

A commonly used assay for the vigor of an immune response is measuring an avirulence effector's effect on *in planta* growth of otherwise virulent *P. syringae* pv. *tomato* (*Pto*) DC3000 in Arabidopsis (Century et al., 1995; Hinsch and Staskawicz, 1996; S. H. Kim et al., 2009; Simonich and Innes, 1995). We assessed the effect of HopZ5 on *in planta* growth of *Pto* DC3000 in Ct-1, Col-0 and Ws-2. *Pto* DC3000 strains carrying *hopZ5* or *avrRpm1* grew significantly less (a reduction of more than one log) than EV in Ct-1 (Figure 3.5). *Pto* DC3000 strains carrying *avrRpm1* grew significantly less (at least a reduction of one log) than EV in both Ws-2 and Col-0. Interestingly, although neither Col-0 nor Ws-2

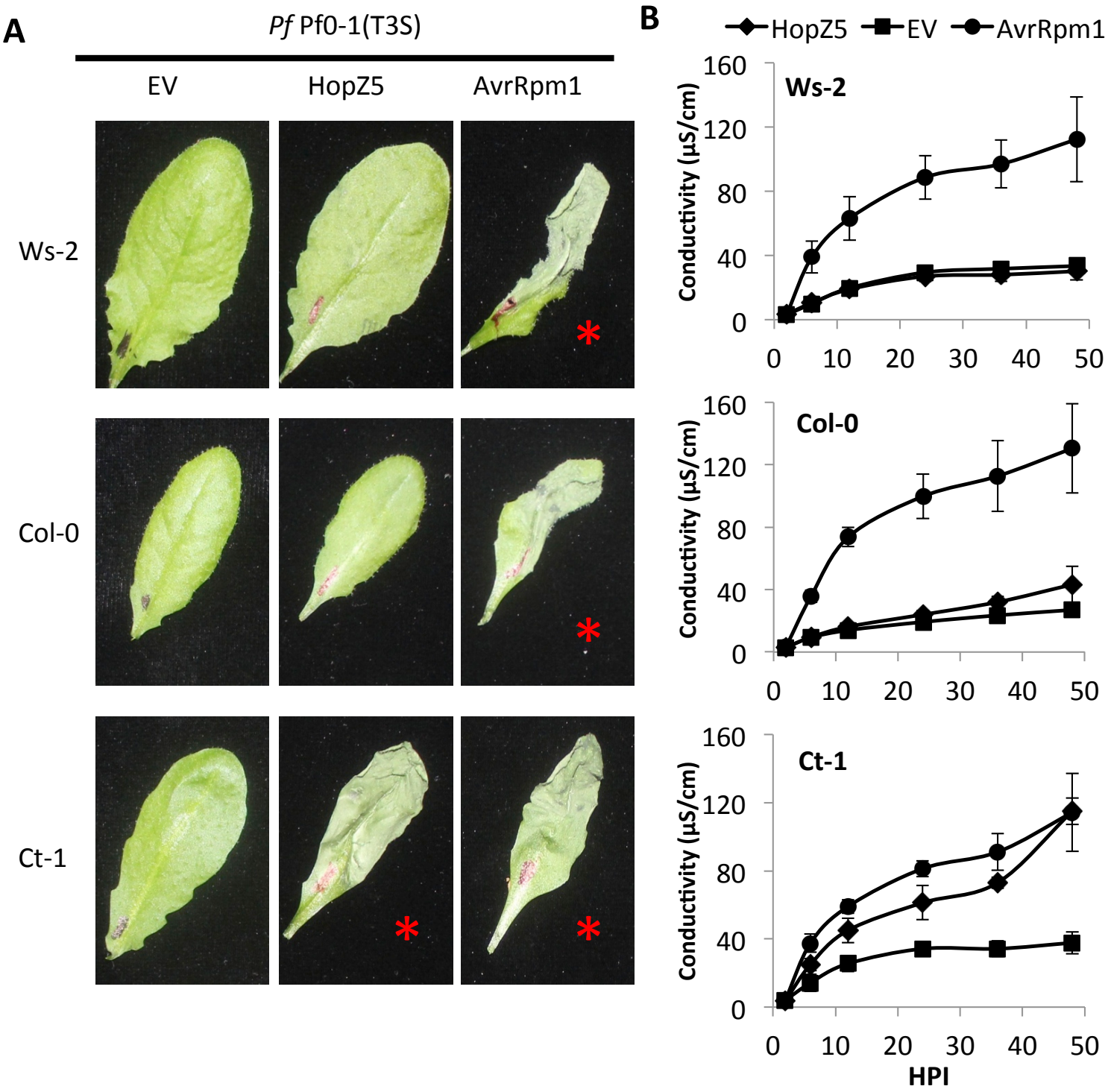


Figure 3.3. HopZ5 triggers HR and ion leakage against Ct-1 but not Col-0 or Ws-2. (A) Leaves of *Arabidopsis* plants were infiltrated with *P. fluorescens* Pf0-1 (T3S) carrying an EV, *hopZ5* with a C-terminal 6xHA tag (*hopZ5-HA*) under the control of the *avrRps4* promoter in pBBR1MCS-5 (Appendix 3), or untagged *avrRpm1* under its native promoter in pVSP61 (Debener et al., 1991). Bacterial suspensions (1×10^8 CFU/mL) were blunt-syringe infiltrated into the leaves and photographs were taken 20 hours after infiltration (1 dpi) (#2.5.1). Development of the HR is indicated by a red asterisk. **(B)** Electrolyte leakage from *Arabidopsis* leaf discs after infiltration with *P. fluorescens* Pf0-1 (T3S) carrying the indicated constructs, as in (A) (#2.5.2). Bacteria (1×10^8 CFU/mL) were blunt syringe-infiltrated into the leaves. The error bars indicate the standard error from four technical replicates. The experiment was conducted three times with similar results.

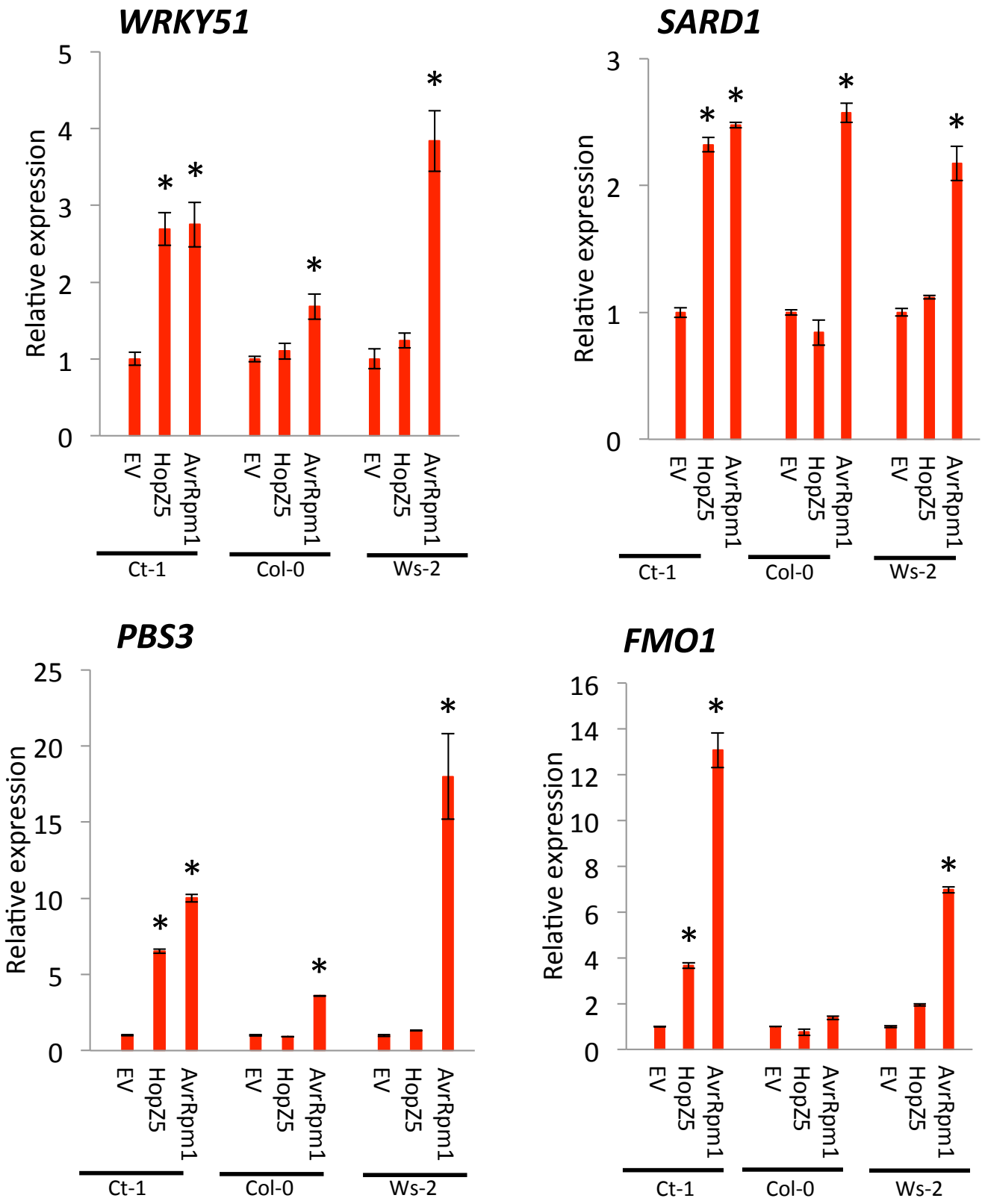


Figure 3.4. HopZ5 delivery by *Pf* Pf0-1 (T3S) triggers defence gene expression in Ct-1 but not Col-0 or Ws-2. Defence gene expression in Arabidopsis in response to EV, HopZ5-HA or AvrRpm1. *P. fluorescens* Pf0-1 (T3S) (1×10^8 CFU/mL) carrying EV, *hopZ5-HA* or *avrRpm1* (as in Figure 3.3; #2.5.1) was blunt syringe-infiltrated into leaves of Ct-1, Col-0 or Ws-2, and samples taken at 8 hours post infiltration. Defence gene (*WRKY51/SARD1/PBS3/FMO1*) expression was determined from cDNA by reverse transcription quantitative polymerase chain reaction (RT-qPCR; #2.6.3.1-3). Expression for each defence gene is relative to internal *EF1α* expression and defence gene expression for EV samples indicated. Error bars indicate standard error from three technical replicates. Asterisks indicate results of Student's t-test between selected sample and EV for that accession; * ($P < 0.01$). The experiment was conducted three times with similar results.

accessions show HR in response to Pf0-1(T3S)-delivered HopZ5, we consistently observed a significant growth restriction in Col-0 (a reduction of almost one log) but not in Ws-2.

Since HopZ5 restricts *in planta Pto* DC3000 growth in both Ct-1 and Col-0, and due to the poor defence gene expression from Pf0-1(T3S)-delivered AvrRpm1 (Figure 3.4), we tested whether *Pto* DC3000 delivery of HopZ5 induces defence marker gene expression in Col-0. As expected, Col-0 showed upregulated *PBS3* and *FMO1* transcript accumulation in response to *Pto* DC3000-delivered HopZ5 and AvrRpm1 compared to EV (Figure 3.6). These data suggest that HopZ5 triggers defence response in Ct-1 and Col-0, despite the lack of HR development in the latter.

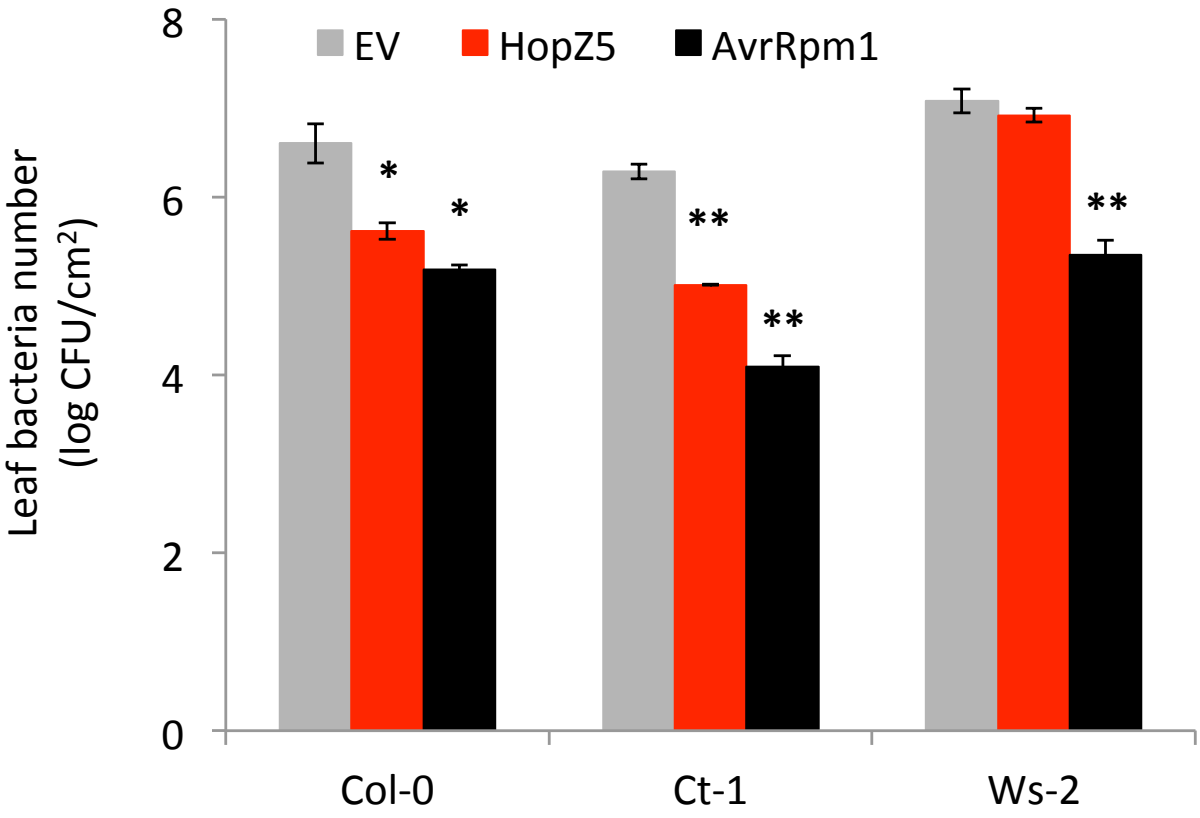


Figure 3.5. HopZ5-triggered *Pto* DC3000 growth restriction in Ct-1 and Col-0 but not Ws-2. *P. syringae* pv. *tomato* DC3000 (*Pto* DC3000) carrying EV, *hopZ5-HA* (Appendix 3) or *avrRpm1* (Debener et al., 1991) was blunt syringe-infiltrated at 5×10^5 CFU/mL into *Arabidopsis* (Ct-1, Col-0 or Ws-2) leaves, and bacterial growth was determined 4 days post-infection (4 dpi) (#2.5.3). Error bars represent standard error from six technical replicates. Asterisks indicate results of Student's *t*-test between selected sample and EV for that accession; * ($P < 0.05$), ** ($P < 0.01$). The experiment was conducted six times with similar results.

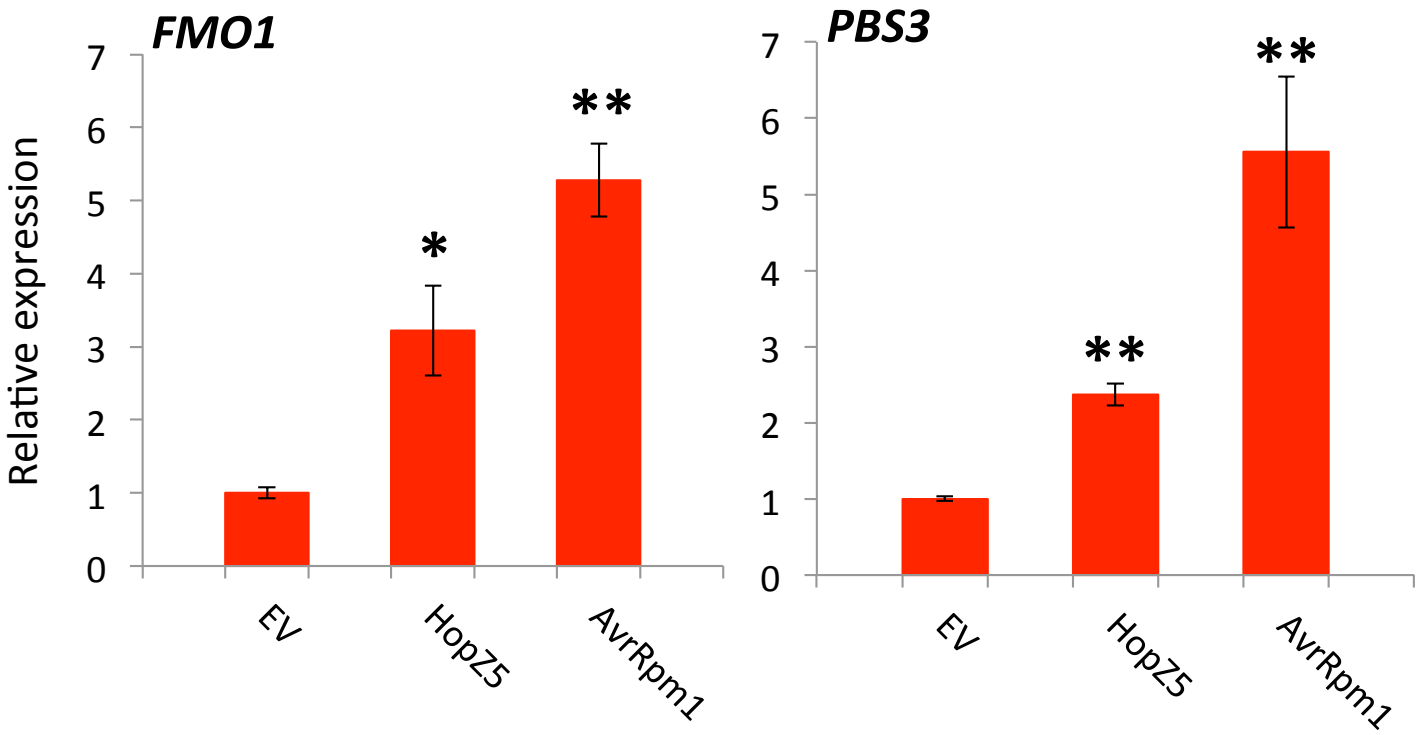
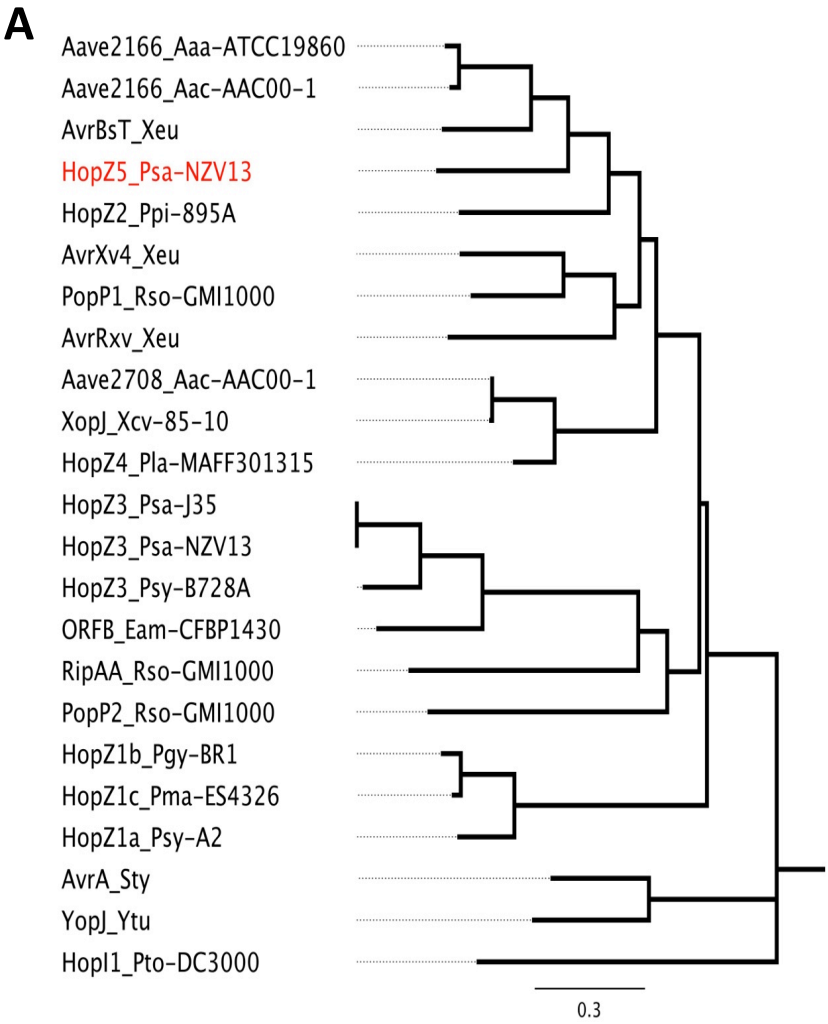


Figure 3.6. HopZ5 delivery by *Pto* DC3000 triggers defence gene expression in Col-0. *Pto* DC3000 (2×10^7 CFU/mL) carrying EV, *hopZ5-HA* or *avrRpm1* (as in Figure 3.5; #2.5.3) was blunt syringe-infiltrated into leaves, samples taken at 8 hours post infiltration and defence gene (*PBS3/FMO1*) expression determined from cDNA by RT-qPCR (#2.6.3.1-3). Expression for each defence gene is relative to internal *EF1α* expression and defence gene expression for EV samples indicated. Error bars indicate standard error from three technical replicates. Asterisks indicate results of Student's t-test between selected sample and EV; * ($P < 0.05$), ** ($P < 0.01$). The experiment was conducted twice with similar results.

3.2.3 HopZ5 is a putative acetyltransferase with key residues required for triggering both HR and immunity, independently in Arabidopsis

In silico analysis of HopZ5 suggests that it belongs to the YopJ superfamily of acetyltransferases and cysteine proteases (Figure 3.7A). Several members of this family including *Pseudomonas syringae* HopZ1, *Xanthomonas campestris* AvrBsT and *R. solanacearum* PopP2 have been characterized previously (Cheong et al., 2014; Ma et al., 2015; Tasset et al., 2010). HopZ5, like other YopJ family members, possesses several conserved key residues including a catalytic triad consisting of histidine (H150), glutamate (E169) and cysteine (C218) and a lysine residue putatively required for auto-/trans-acetylation (K278) (Figure 3.7B). Additionally, HopZ5 possesses a glycine (G2) that is conserved in most members of the HopZ family of T3Es and required for myristoylation-dependent plasma membrane localization (Lewis et al., 2008). The phylogenetic



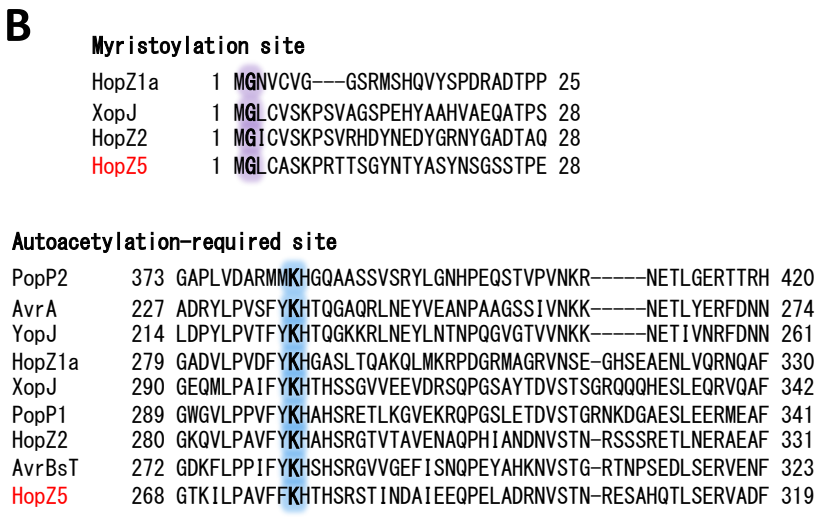


Figure 3.7. HopZ5 is a member of the YopJ superfamily with conserved catalytic residues. **(A)** HopZ5 (in red) from *Pseudomonas syringae* pv. *actinidiae* V13 is a putative acetyltransferase from the YopJ family with *Xanthomonas* type-III secreted effector AvrBsT as its closest homolog. Jukes Cantor neighbour-joining tree using HopI1 from *Pto* DC3000 as outgroup and root, generated in Geneious software with various YopJ-family members: Aave2166_Aaa-ATCC19860 (*Acidovorax avenae* pv. *avenae* ATCC19860), Aave2166_Aac-AAC00-1 (*Acidovorax citrulli* AAC00-1), AvrBsT_Xeu (*Xanthomonas campestris* pv *euvesicatoria* Bv5-4a), HopZ2_Ppi-895A (*P. syringae* pv. *pisi* 895A), AvrXv4_Xeu (*X. campestris* pv *euvesicatoria* race T3), PopP1_Rso-GMI1000 (*Ralstonia solanacearum* GMI1000), AvrRxv_Xcv (*X. campestris* pv *euvesicatoria* 85-10), Aave2708_Aac-AAC00-1 (*Acidovorax citrulli* AAC00-1), XopJ_Xcv-85-10 (*X. campestris* pv. *vesicatoria* 85-10), HopZ4_Pla-MAFF301315 (*P. syringae* pv. *lachrymans* MAFF301315), HopZ3_Psa-J35 (*P. syringae* pv. *actinidiae* J35), HopZ3_Psa-V13 (*P. syringae* pv. *actinidiae* V13), HopZ3_Psy-B728A (*P. syringae* pv. *syringae* B728A), ORFB_Eam-CFBP1430 (*Erwinia amylovora* str. CFBP1430), RipAA_Rso-GMI1000 (*R. solanacearum* GMI1000), PopP2_Rso-GMI1000 (*R. solanacearum* GMI1000), HopZ1b_Pgy-BR1 (*P. syringae* pv. *glycinea* BR1), HopZ1c_Pma-ES4326 (*P. syringae* pv. *maculicola* ES4326), HopZ1a_Psy-A2 (*P. syringae* pv. *syringae* A2), AvrA_Sty (*Salmonella typhimurium* str. *14028s), YopJ_Ytu (Yersinia pseudotuberculosis str.* IP32953). Node length is indicated. **(B)** Critically conserved residues in selected YopJ family members are shown: Putatively myristoylated glycine highlighted in purple, acetyltransferase catalytic core histidine, glutamate and cysteine residues highlighted in orange, and autoacetylation-required lysine residue highlighted in blue.

relationships and predicted conserved residues suggest that HopZ5 may be a plasma membrane-localized acetyltransferase.

We therefore sought to test whether these key residues in HopZ5, vis-à-vis their biochemical activities, were important for avirulence (Figure 3.8A). To determine this, we utilized site-directed mutagenesis to generate HopZ5 variants carrying a G2A (glycine to alanine), C218A (cysteine to alanine) or K278R (lysine to arginine) mutations that would eliminate the predicted plasma membrane localization, acetyltransferase activity or auto-/trans-acetylation activity, respectively. These variants were then examined for the development of defence responses when delivered by a T3SS in the resistant Arabidopsis accession Ct-1. Pf0-1(T3S)-delivered HopZ5 C218A or K278R variants did not trigger HR in Ct-1 leaves (Figure 3.8B). Interestingly, the G2A variant only showed a partial loss of HR as observed through a qualitative assessment of leaf appearance (Figure 3.8B). This may be due to the presence of a cysteine residue (C4) at the N-terminus of HopZ5 that could be palmitoylated. Electrolyte leakage quantification showed a significant but not complete attenuation of ion leakage for the G2A variant while both C218A and K278R variants induced a much-reduced level, similar to EV (Figure 3.8C).

These HopZ5 variants were then assessed for their ability to trigger early defence responses, by determining the expression of three defence marker genes *FMO1*, *PBS3*, and *WRKY51* at 8 hours post delivery by Pf0-1(T3S). Notably, wild-type HopZ5 but not G2A, C218A or K278A variants induced significant transcript accumulation of defence marker gene expression as compared to EV (Figure 3.9A). To further elucidate defence responses triggered by HopZ5 variants late in the infection process, restriction of growth for *Pto* DC3000 in Ct-1 was determined. Although the G2A variant induced significantly reduced ion leakage as compared to wild-type HopZ5 (Figure 3.8C), *in planta* growth of *Pto* DC3000 carrying the G2A variant was comparable to *Pto* DC3000 carrying wild-type *hopZ5* (Figure 3.9B). In addition, both C218A and K278A variants did not significantly restrict *Pto* DC3000 growth compared to EV. Taken together, these

data suggest that all three predicted functional residues are at least partially required for a full induction of ETI.

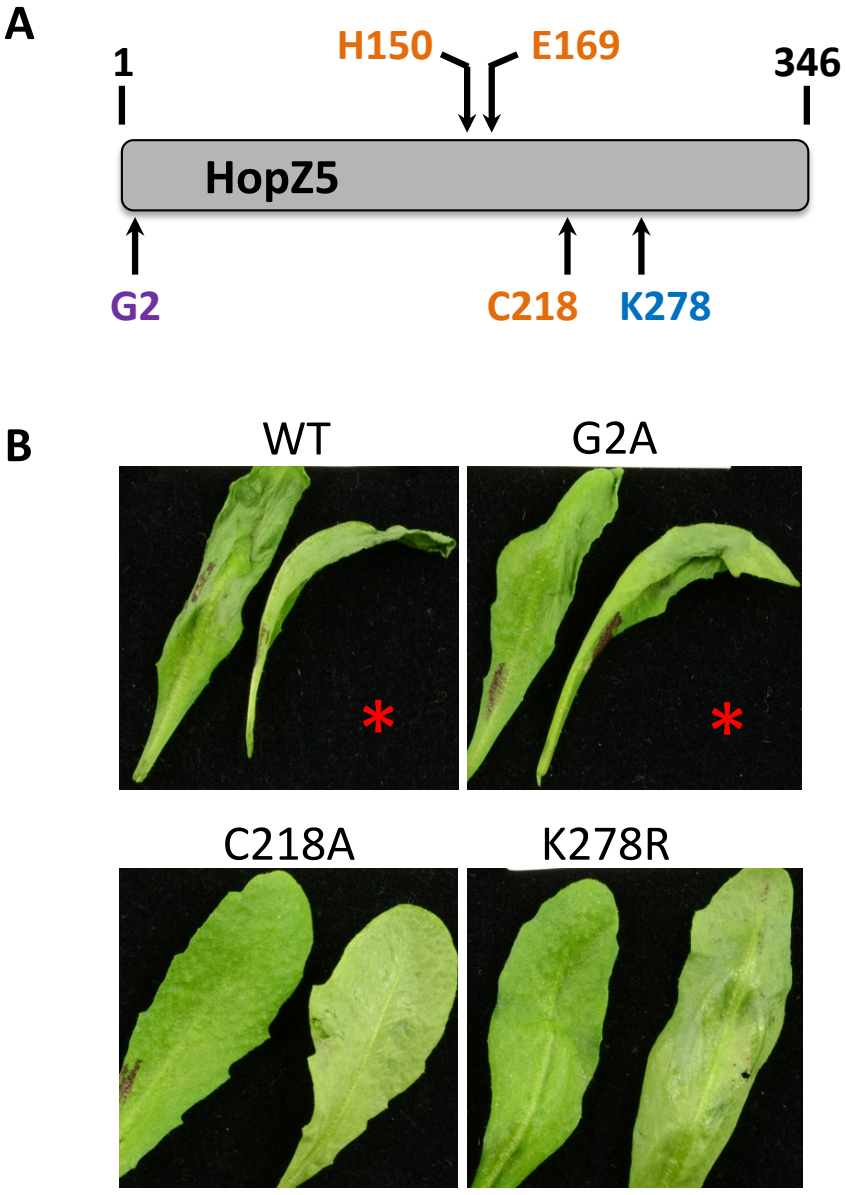


Figure 3.8. HopZ5 variants are affected in their ability to trigger HR and ion leakage in Ct-1. (A) Key residues for HopZ5 function predicted through conservation between YopJ family members. Line model for HopZ5 peptide with putative myristoylation residue (Glycine, G2), acetyltransferase catalytic core residues (Histidine, H150; Glutamate E169; Cysteine, C218) and autoacetylation required site (Lysine, K278) is shown. Mutants generated (#2.6.2.10) in this study include a glycine to alanine mutation (G2A), cysteine to alanine (C218A) and a lysine to arginine (K278R) (Appendix 3). **(B)** Development of the hypersensitive response in the Arabidopsis accession Ct-1 against HopZ5 variants. *P. fluorescens* Pf0-1 (T3S) (1×10^8 CFU/mL) carrying *hopZ5-HA* (WT), *hopZ5*(G2A)-*HA*, *hopZ5*(C218A)-*HA*, or *hopZ5*(K278R)-*HA* was blunt syringe infiltrated into leaves and photographs taken 20 hours after infiltration (#2.5.1). The red asterisk indicates development of HR. **(C)** Electrolyte leakage from *Arabidopsis* accession Ct-1 leaf discs after infiltration with *P. fluorescens* Pf0-1 (T3S) expressing HopZ5 variants, as in (B) (#2.5.2). Bacteria (1×10^8 CFU/mL) were blunt syringe infiltrated into the leaves. The error bars indicate the standard error from four technical replicates. The experiment was conducted five times with similar results.

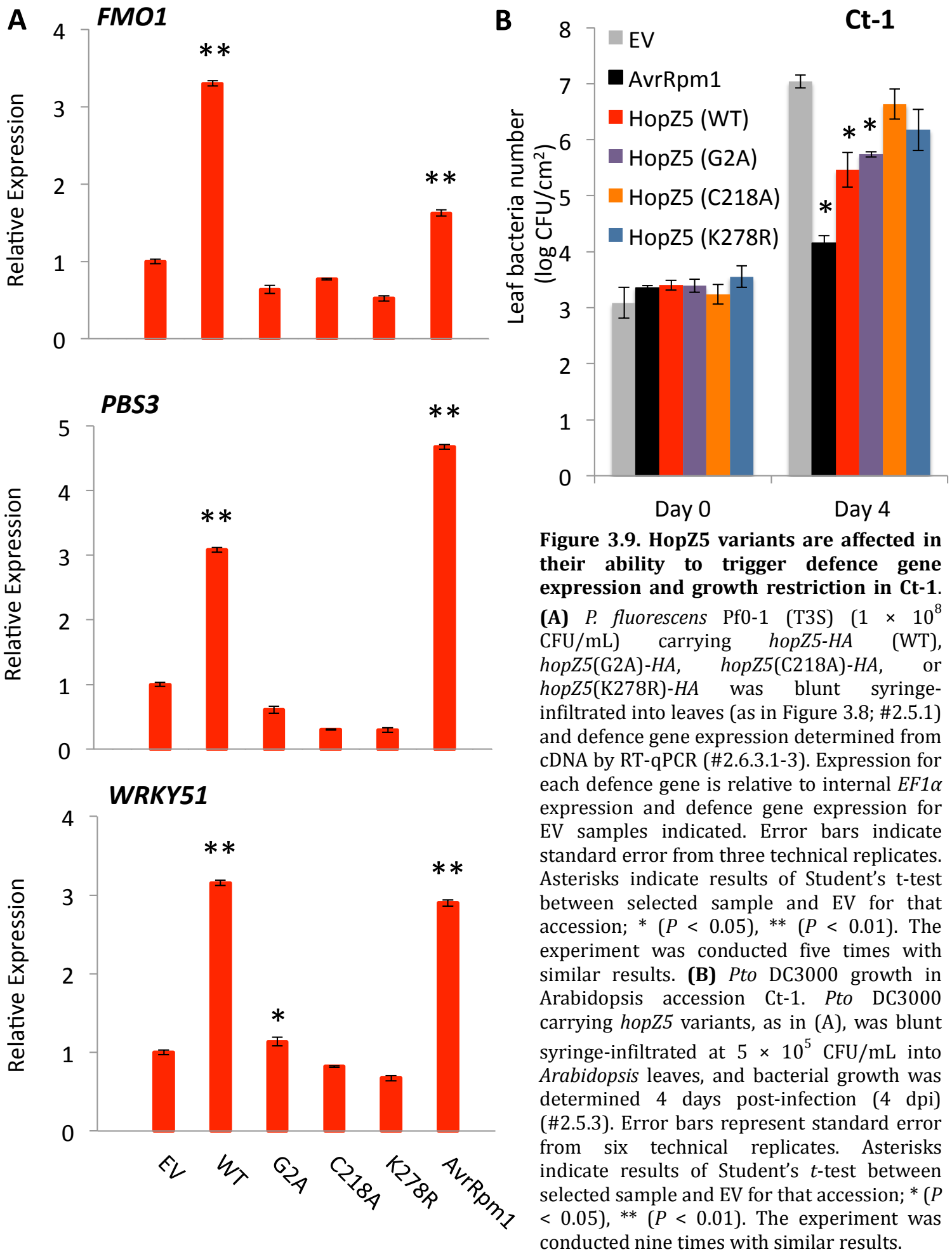


Figure 3.9. HopZ5 variants are affected in their ability to trigger defence gene expression and growth restriction in Ct-1. **(A)** *P. fluorescens* Pf0-1 (T3S) (1 × 10^8 CFU/mL) carrying *hopZ5-HA* (WT), *hopZ5*(G2A)-*HA*, *hopZ5*(C218A)-*HA*, or *hopZ5*(K278R)-*HA* was blunt syringe-infiltrated into leaves (as in Figure 3.8; #2.5.1) and defence gene expression determined from cDNA by RT-qPCR (#2.6.3.1-3). Expression for each defence gene is relative to internal *EF1α* expression and defence gene expression for EV samples indicated. Error bars indicate standard error from three technical replicates. Asterisks indicate results of Student's t-test between selected sample and EV for that accession; * ($P < 0.05$), ** ($P < 0.01$). The experiment was conducted five times with similar results. **(B)** *Pto* DC3000 growth in Arabidopsis accession Ct-1. *Pto* DC3000 carrying *hopZ5* variants, as in (A), was blunt syringe-infiltrated at 5 × 10^5 CFU/mL into *Arabidopsis* leaves, and bacterial growth was determined 4 days post-infection (4 dpi) (#2.5.3). Error bars represent standard error from six technical replicates. Asterisks indicate results of Student's *t*-test between selected sample and EV for that accession; * ($P < 0.05$), ** ($P < 0.01$). The experiment was conducted nine times with similar results.

To clarify the HopZ5-triggered bacterial immunity without HR in Col-0 plants, we generated stable transgenic Col-0 lines expressing HopZ5-YFP variants under the control of the 35S promoter. Using multiple independent lines for wild-type HopZ5 (Z-1, Z-4, Z-5), C218A (C-1, C-4), K278R (K-2, K-6) and G2A (G-2, G-3), we first tested for expression of the transgene in T2 plants. Notably, we were only able to identify low-expression lines for wild-type HopZ5 (Z) and G2A (G) variants while C218A (C) and K278R (K) variant lines had significantly higher transgene expression levels (Figure 3.10A). Additionally, T2 plants for the Z lines alone showed some stunted growth and early flowering phenotypes compared to wild-type Col-0 and other mutant variants (Figure 3.10B), despite the lack of any lesioning symptoms often associated with autoimmune phenotypes (Bomblies et al., 2007; Noutoshi et al., 2005; Zhang et al., 2003). Importantly, we found that *PR1* expression was significantly upregulated in the Z lines despite the low HopZ5 transgene expression level (Figure 3.10A). G plant lines showed *PR1* transcript accumulation levels intermediate between Z lines and wild-type Col-0, while in C plant lines, *PR1* expression was indistinguishable from Col-0. The K plant lines showed only slightly elevated *PR1* transcript accumulation compared to Col-0 (approximately 2-fold), indicating a minor upregulation of defence responses. Next, we assessed whether the transgenic expression of HopZ5 variants could restrict *Pto* DC3000 growth because of upregulated defence responses. Consistent with *PR1* expression analysis, Z plant lines showed significant reduction in *Pto* DC3000 growth compared to wild-type Col-0 (Figure 3.10C). Unexpectedly, the G plant lines did not show significant reduction of *Pto* DC3000 growth compared to Col-0 which could be due to the low expression levels of the G2A variant.

Taken together, the elevated *PR1* expression and reduced *Pto* DC3000 growth relative to wild-type Col-0 in HopZ5 Z lines (Figure 3.10A-C), coupled to upregulation of ETI marker genes in response to HopZ5 delivery by *Pto* DC3000 (Figure 3.6) and virulent pathogen growth restriction (Figure 3.5) suggest that HopZ5 triggers defence that is not associated with HR in Col-0.

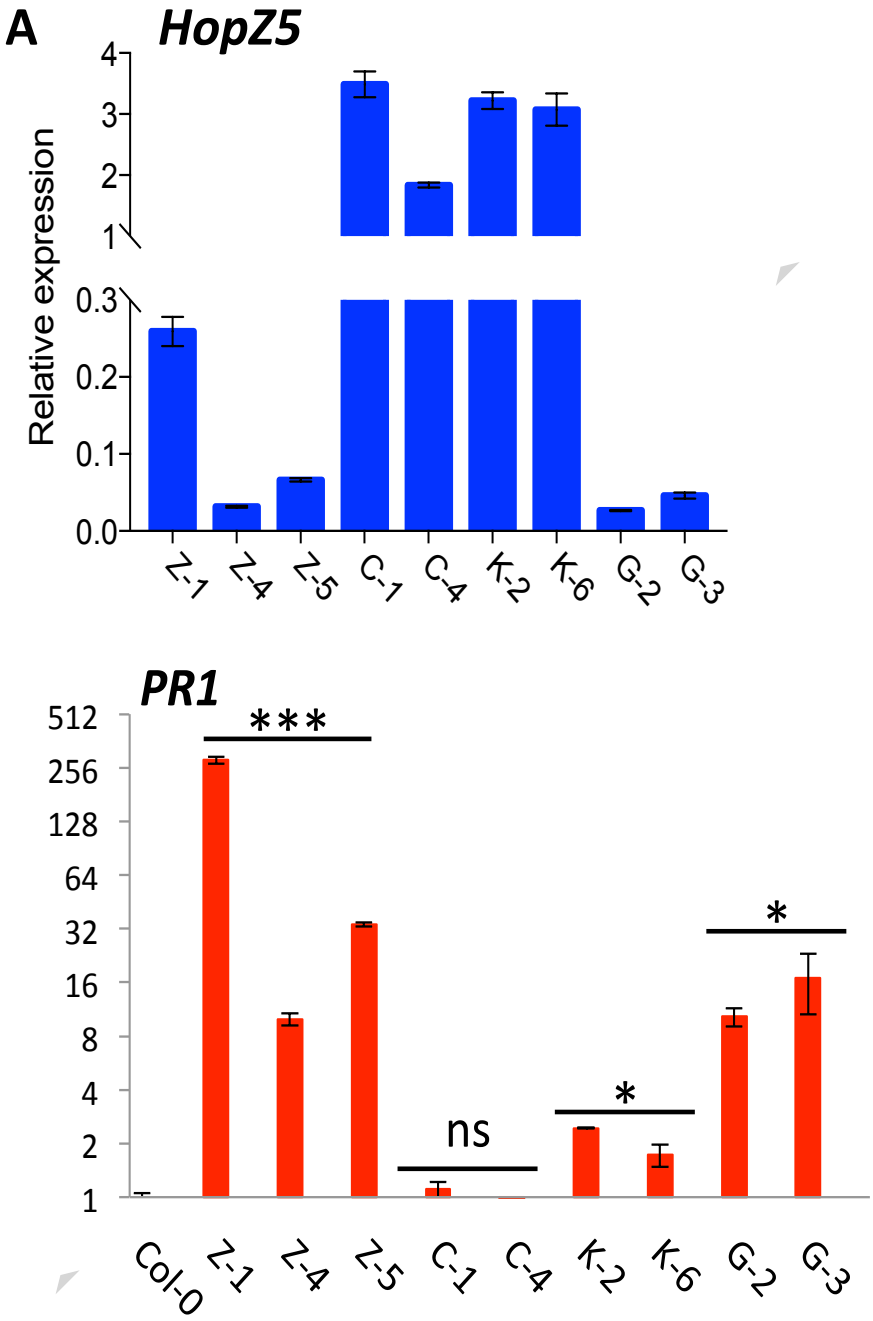


Figure 3.10. HopZ5 but not its variants triggers immunity when stably expressed in Col-0. **(A)** *hopZ5*-transgene and *PR1* marker gene expression was determined in *hopZ5* stable expression lines in the Col-0 background. Floral dips (#2.5.4) of wildtype Col-0 plants with YFP-tagged hopZ5 wildtype or variants (Appendix 4) were conducted and seeds collected for screening for T1 transformants. Seeds from T1 transformants were harvested after selection for three weeks on solid media containing kanamycin and then these seeds were resown on solid medium containing kanamycin to obtain 15-20 T2 seedlings from two or three independent T1 lines per *hopZ5* variant (*hopZ5*-wild-type: Z-1, Z-4, Z-5; *C218A*: C-1, C-4; *K278R*: K-2, K-6; *G2A*: G-2, G-3) were pooled for RT-qPCR. RT-qPCR was conducted for the *hopZ5-YFP* transgene or *PR1* expression (#2.6.3.1-3). Expression is relative to internal *EF1α* expression only (*hopZ5*) or *EF1α* and *PR1* expression for wildtype (*PR1*). Error bars indicate standard error from three technical replicates. Asterisks indicate results of Student's t-test between selected sample and Col-0 wild-type also grown on plates but without kanamycin; *** (P < 0.0001). This experiment was conducted three times with similar results.

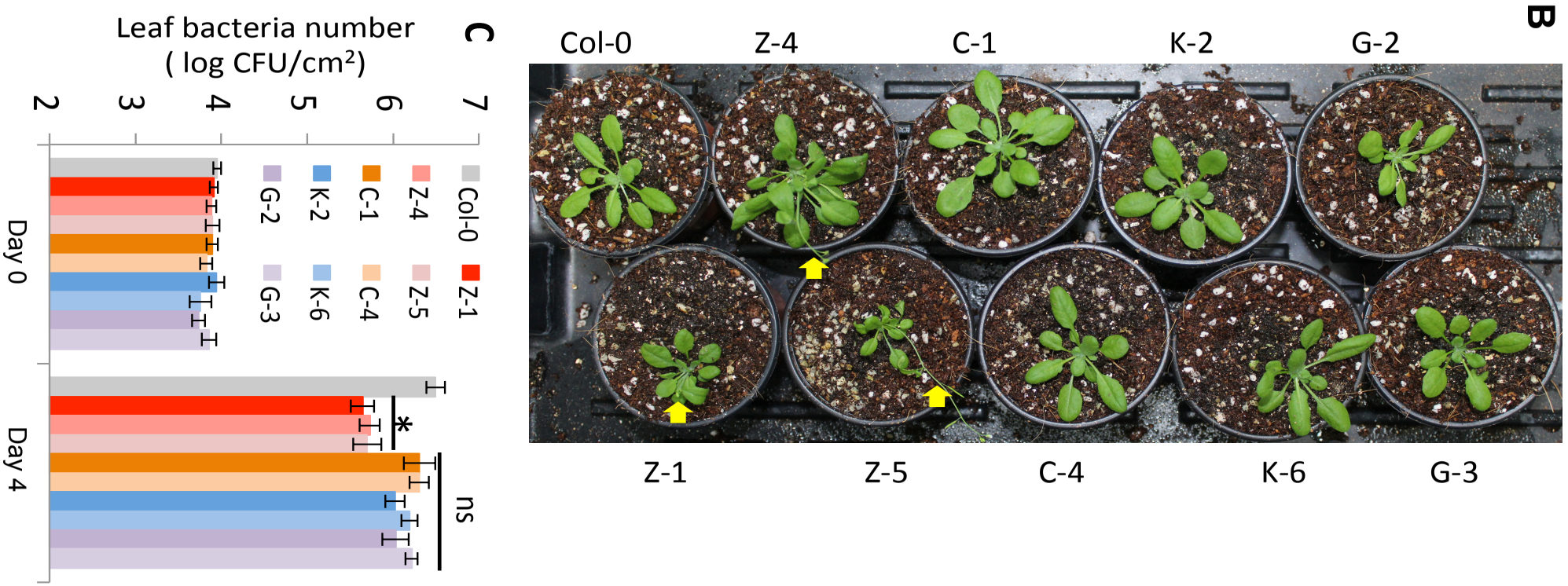
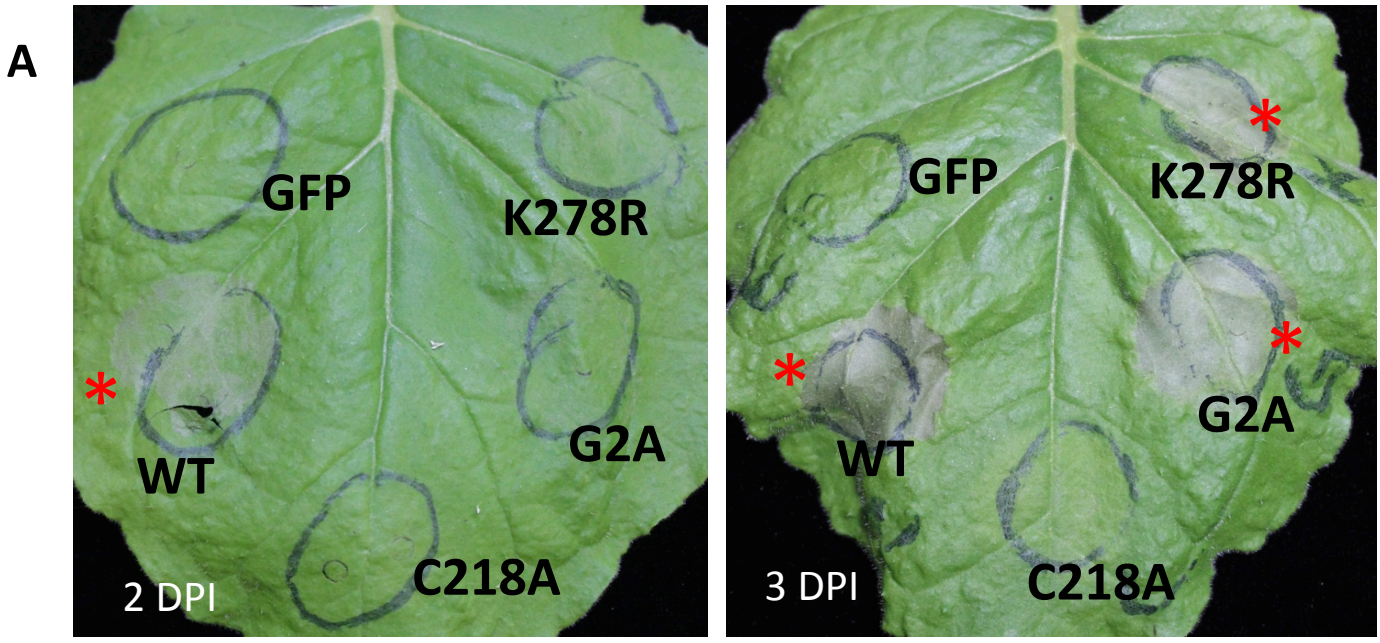


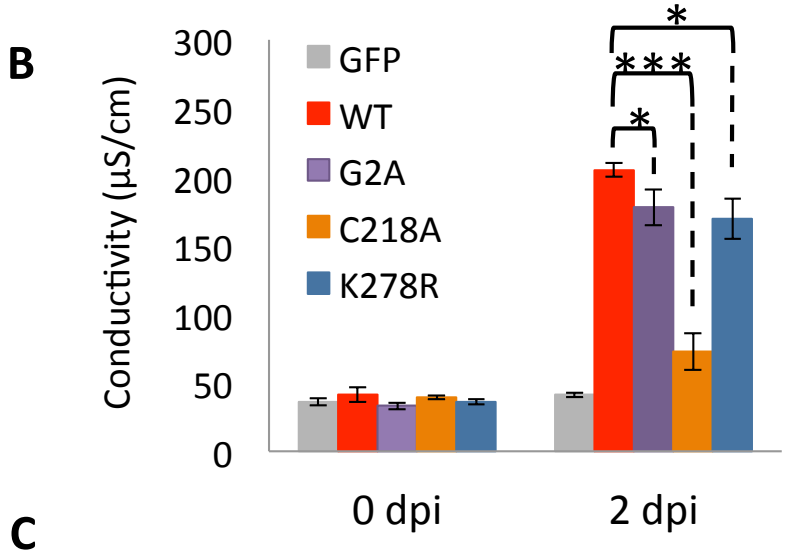
Figure 3.10. HopZ5 but not its variants triggers immunity when stably expressed in Col-0. **(B)** 3-week-old T2 plants expressing C-terminally YFP-tagged *hopZ5*-variants, as in (A), were transferred to soil after selection on kanamycin. Photographs are of 5-week-old representative plants that showed a differential phenotype only. Typically between 1-in-3 (Z-1) to 1-in-4 plants (Z-4 and Z-5) showed a stunted/early flowering phenotype, putatively representative of Mendelian segregation and individuals homozygous for the transgene. Yellow arrows indicate the inflorescence in the early-flowering plants. **(C)** *Pto* DC3000 growth in *hopZ5* stable expression lines in Col-0. *Pto* DC3000 was blunt syringe-infiltrated at 5×10^5 CFU/mL into leaves of each stable expression line from (A) and bacterial growth was determined 4 days post-infection (4 dpi) (#2.5.3). Error bars represent standard error from six technical replicates. Asterisks indicate results of Student's *t*-test between selected sample and EV for that accession; ns ($P > 0.05$), * ($P < 0.05$), ** ($P < 0.01$). The experiment was conducted three times with similar results.

3.2.4 HopZ5 triggers HCD in *Nicotiana* spp. and requires *SGT1*

Agrobacterium-mediated transient expression (hereafter, agroinfiltration) of pathogen effectors in *Nicotiana* spp. often induces HR-like cell death (HCD) (Clarke et al., 2015; Wei et al., 2007; Wroblewski et al., 2009). To test if HopZ5 triggers HCD in *Nicotiana* spp., we transiently expressed C-terminally YFP-tagged HopZ5 under the control of constitutive cauliflower mosaic virus 35S promoter (35S promoter) in *Nicotiana benthamiana* via agroinfiltration. Interestingly, HopZ5 triggered a robust HCD within 2 dpi (Figure 3.11A). In order to test if this HopZ5-triggered defence responses required similar properties of HopZ5 as for Arabidopsis, HopZ5-YFP variants were transiently expressed in *N. benthamiana* and *N. tabacum*. Agroinfiltration of wild-type HopZ5 and its G2A and K278R variants but not GFP control induced rapid development of HCD in both *N. benthamiana* (Figure 3.11A) and *N. tabacum* (Figure 3.11C). Consistent with *Pseudomonas*-delivered HopZ5 results in Arabidopsis, the C218A variant did not induce any visible cell death. Interestingly, both the G2A and K278R variants induced delayed cell death development (symptom development for G2A and K278R variants were delayed by a day), indicating that avirulence activity of these variants was significantly reduced in both *Nicotiana* spp. To quantify the effect of the mutations on HopZ5-triggered HCD, we carried out an electrolyte leakage assay after agroinfiltration of G2A, C218A and K278R variants in *N. benthamiana* (Figure 3.11B). Electrolyte leakage measurement at 2 dpi, when HCD symptoms started to appear prior to leaf collapse, demonstrated a statistically significant reduction for both the G2A and K278R variants and a complete loss of HCD-associated leakage for the C218A variant. These results demonstrate that the partial loss of avirulence function for HopZ5 G2A and K278A variants applies in more than one plant host.

We used a well-established virus-induced gene silencing (VIGS) system to examine the genetic components required for HopZ5-triggered HCD in *N. benthamiana*. We generated VIGS constructs for *NbEDS1*, *NbNDR1* and *NbSGT1* as they have been previously identified as critical components in rendering HCD (Cai et al., 2006; Liu et al., 2002). While silencing enabled significant reduction in transcriptional expression of *NbEDS1*, *NbNDR1* and *NbSGT1*, only *NbSGT1*-





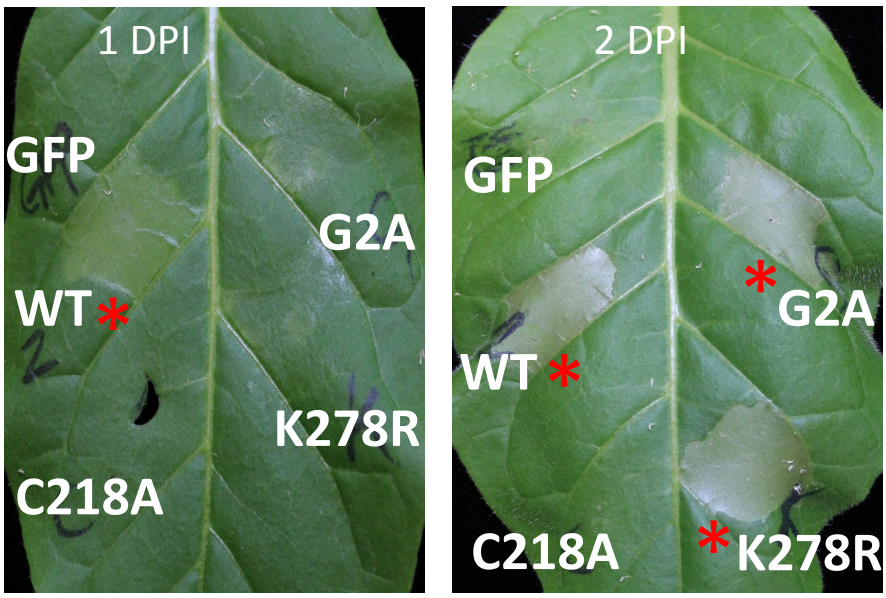


Figure 3.11. HopZ5 triggers HCD and ion leakage in *Nicotiana* spp. (A) HopZ5 (WT) or mutated myristoylation (G2A) catalytic core (C218A) and autoacetylation-required (K278R) variants (Appendix 4) tagged with 3xFLAG were transiently expressed under the CaMV 35S promoter in *N. benthamiana* (#2.5.5). Photographs were taken at 2-3 days post infiltration (dpi). Red asterisks indicate development of HR-like cell death symptoms. This experiment was conducted six times with identical results. **(B)** Electrolyte leakage from *N. benthamiana* leaf discs triggered by *Agrobacterium*-mediated transient expression of HopZ5 or variants, as in (A), was determined at 0 and 2 days post infiltration (dpi) (#2.5.6). Infiltrations were carried out by blunt syringe at OD_{600}= 0.4 for all samples. Error bars represent standard error from four technical replicates. Asterisks indicate results of Student's *t*-test between selected samples; * ($P < 0.05$), *** ($P < 0.001$). This experiment was conducted three times with identical results. **(C)** HopZ5 or variants, as in (A), were transiently expressed under the constitutive CaMV 35S promoter in *Nicotiana tabacum* (#2.5.5). Photographs were taken at 1-2 days post infiltration (dpi). Red asterisks indicate development of HR-like cell death symptoms. This experiment was repeated five times with identical results.

silenced plants lost the ability to mount HCD in response to HopZ5 expression (Figure 3.12A-B). This is in contrast to the control RPS4-TIR domain expression which showed loss of HCD in *SGT1*- and *EDS1*-silenced plants as seen previously (Swiderski et al., 2009). This result suggests that the HopZ5-triggered activation of defence signaling, at least in *N. benthamiana*, requires *SGT1*.

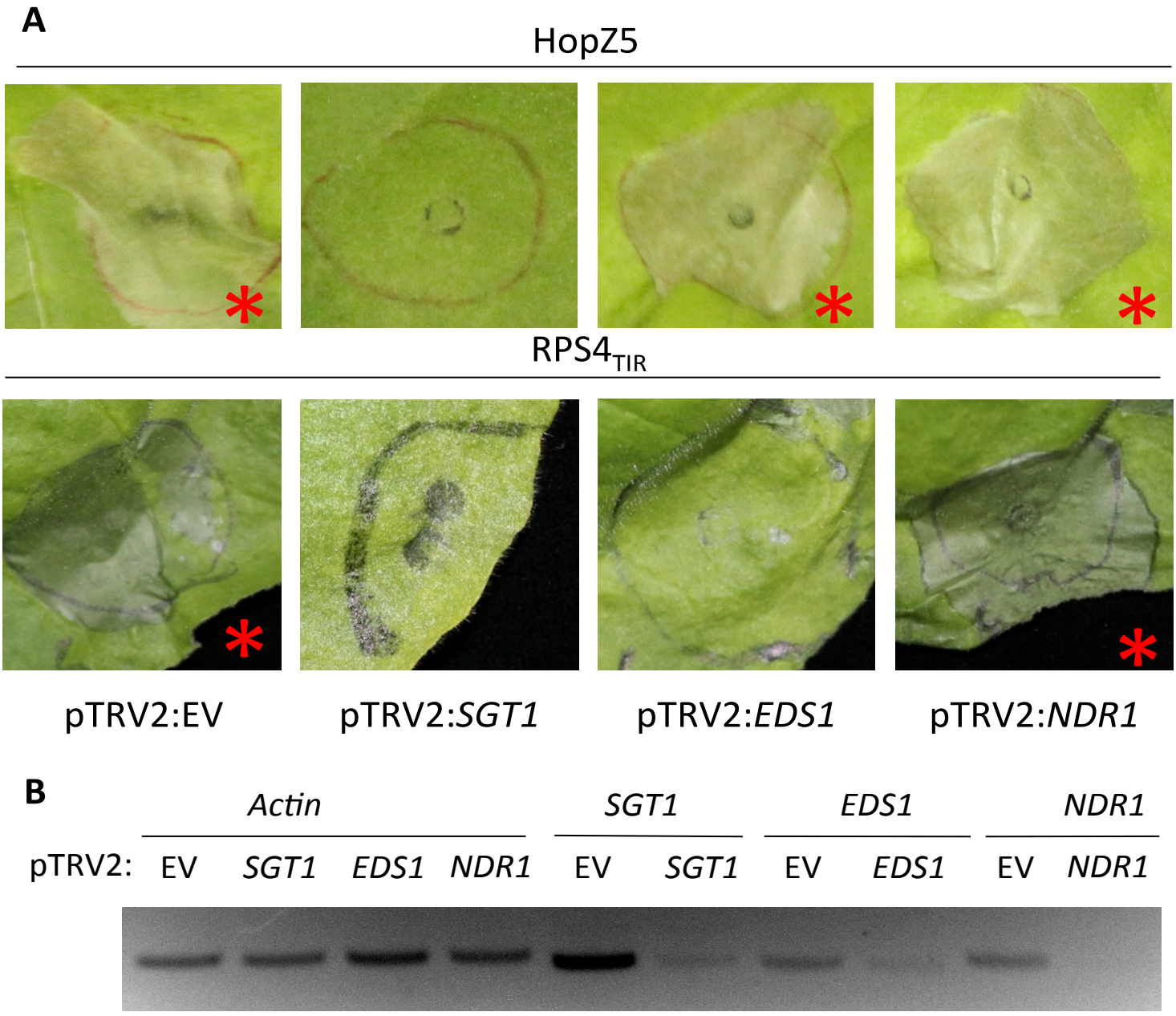


Figure 3.12. *SGT1* but not *EDS1* or *NDR1* is required for HopZ5-triggered HCD in *N. benthamiana*. **(A)** *SGT1*-silenced *Nicotiana benthamiana* loses HopZ5-triggered HR-like cell death. Two-week-old *N. benthamiana* seedlings were infiltrated with *Agrobacterium tumefaciens* AGL1 carrying pTRV2 EV or targeting *SGT1*, *EDS1* or *NDR1* for silencing (#2.5.7). 4-5 weeks post VIGS treatment, HopZ5 or RPS4-TIR domain was transiently expressed and photographs taken at 3 days post infiltration. Red asterisks indicate development of cell death symptoms. The experiment was conducted three times with similar results. **(B)** Silencing of target genes in VIGS treated plants was confirmed by RNA extraction from multiple leaves followed by semi-quantitative polymerase chain reaction following 28 cycles (qPCR #2.6.3.1-3 and agarose gel electrophoresis #2.6.2.4). Actin was used as control for RNA.

3.2.5 HopZ5 triggered immunity in Arabidopsis Col-0 requires *SID2*, *EDS1* and *NDR1*

To test if the lack of requirement for *NDR1* and *EDS1* for HopZ5-triggered responses is reproduced in Arabidopsis, we tested if Col-0 mutants, *eds1-2*, *ndr1-1* and *sid2-1*, were able to restrict growth of *Pto* DC3000 delivering HopZ5 or two different avirulence effectors, AvrRps4 or AvrRpt2 (Aarts et al., 1998; Nawrath and Métraux, 1999). *SID2* (*SALICYLIC ACID INDUCTION-DEFICIENT2*), also called *ISOCHORISMATE SYNTHASE1* (*ICS1*), is a biosynthetic gene involved in production of SA and has been reported to be required for full resistance to avirulent strains of *Pto* DC3000 carrying effectors AvrRpt2 and AvrRps4 but does not affect HR development in response to these strains (Nawrath and Métraux, 1999; Wildermuth et al., 2001).

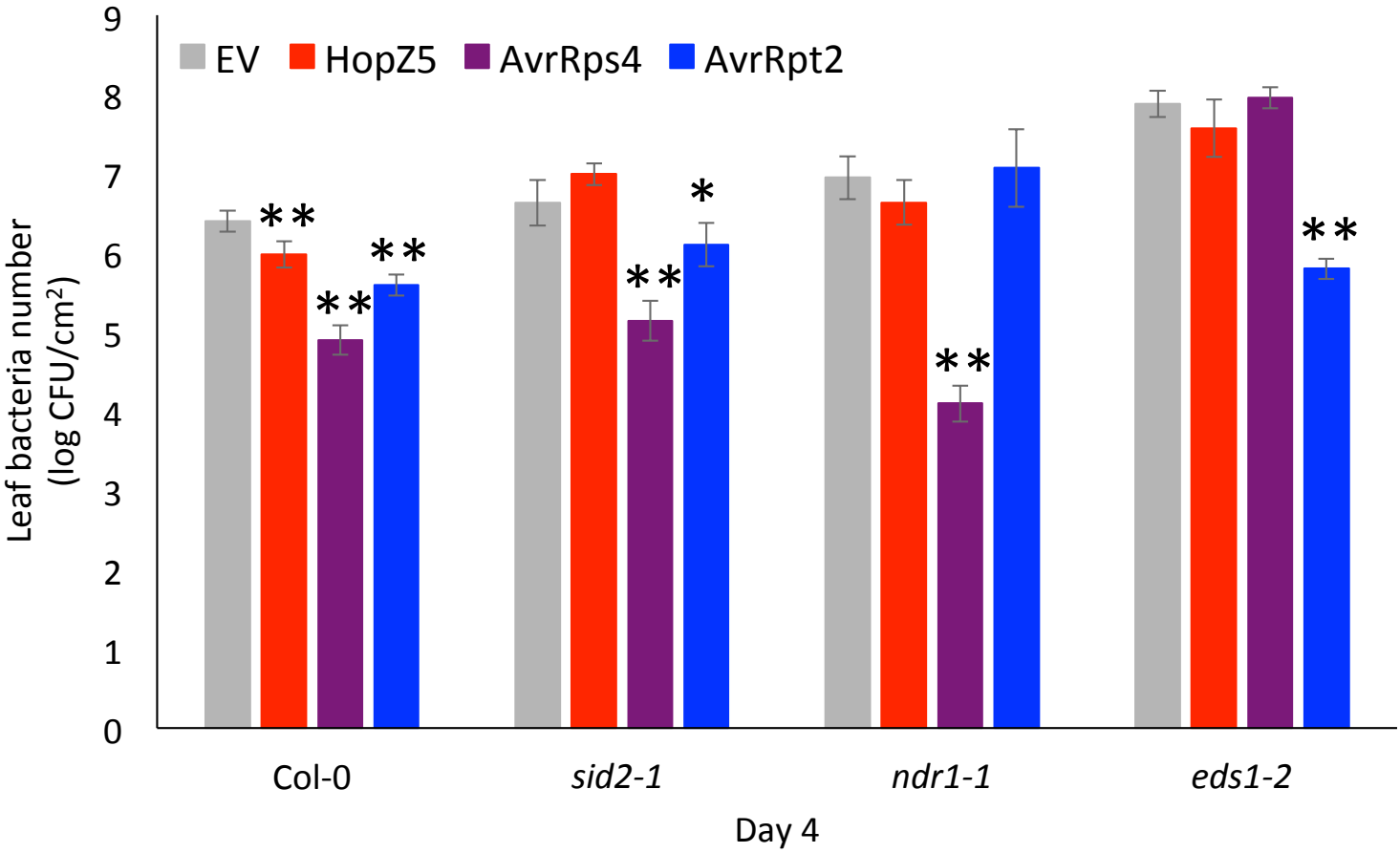


Figure 3.13. *EDS1*, *NDR1* and *SID2* are required for HopZ5-triggered immunity in Arabidopsis. *Pto* DC3000 carrying EV, *hopZ5*-HA, *avrRps4* or *avrRpt2* (Appendix 3) was blunt syringe-infiltrated at 5×10^5 CFU/mL into leaves of Arabidopsis wildtype Col-0, *sid2-1, ndr1-1,* or *eds1-2* mutant lines (#2.5.3). Bacterial growth was determined 4 days post-infection (4 dpi). Error bars represent standard error from six technical replicates. Asterisks indicate results of Student's *t*-test between selected sample and EV for that accession; * ($P < 0.05$), ** ($P < 0.01$). The experiment was conducted three times with similar results.

Surprisingly, in contrast to wild-type Col-0, all three mutants (*sid2-1*, *ndr1-1* and *eds1-2*) were affected in their ability to restrict growth of *Pto* DC3000 delivering HopZ5 (Figure 3.13). As expected, resistance to *Pto* DC3000 expressing AvrRpt2 or AvrRps4 was abolished in *ndr1-1* or *eds1-2*, respectively (Aarts et al., 1998; Nawrath and Métraux, 1999). When comparing this to the lack of an *NDR1* or *EDS1* requirement for HCD in *N. benthamiana*, this further indicated a clear separation of HR/HCD and immunity in response to HopZ5 in Arabidopsis.

3.2.6 HopZ5 is a *bona fide* plasma membrane-localized acetyltransferase that autoacetylates *in planta*

Similar to the work carried out for PopP2 (Tasset et al., 2010), AvrBsT (Cheong et al., 2014), and HopZ1a (A. H.-Y. Lee et al., 2012; Lewis et al., 2008) for which critical amino acid residues were mutagenized to analyze biochemical functions, we utilized site-directed mutagenesis (#2.6.2.10) to generate HopZ5 variants carrying a C218A (cysteine to alanine) or K278R (lysine to arginine) mutation that would eliminate the predicted acetyltransferase activity or auto-/trans-acetylation, respectively. We also generated a G2A (glycine to alanine) HopZ5 variant to alter the predicted subcellular localization. To confirm the biochemical requirement for these key residues, we then used *NbSGT1*-silenced *N. benthamiana* since HopZ5-triggered HCD was reduced in these plants, promoting HopZ5 protein accumulation and detection. Agroinfiltration of C-terminally YFP-tagged HopZ5 (HopZ5-YFP) variants allowed detection of subcellular localization of HopZ5 by confocal microscopy. We found that while wild-type HopZ5 and both C218A and K278R variants localized to the cell periphery, the G2A variant localized in the cytoplasm and nuclei of *Agrobacteria*-infected cells (Figure 3.14). Because wildtype HopZ5 normally localizes at the cell periphery and was absent from cytoplasmic strands, the presence of YFP signal in the cytoplasmic strands in HopZ5-G2A infiltrated plants (as well as in nuclei) suggests that the wildtype HopZ5 protein localized to the plant plasma membrane at the cell periphery.

R. solanacearum effector PopP2 was shown to auto-/trans-acetylate lysine residues (Tasset et al., 2010). Furthermore, the predicted 'autoacetylation

residue' analog in AvrBsT and HopZ1a were shown to be required for trans- or

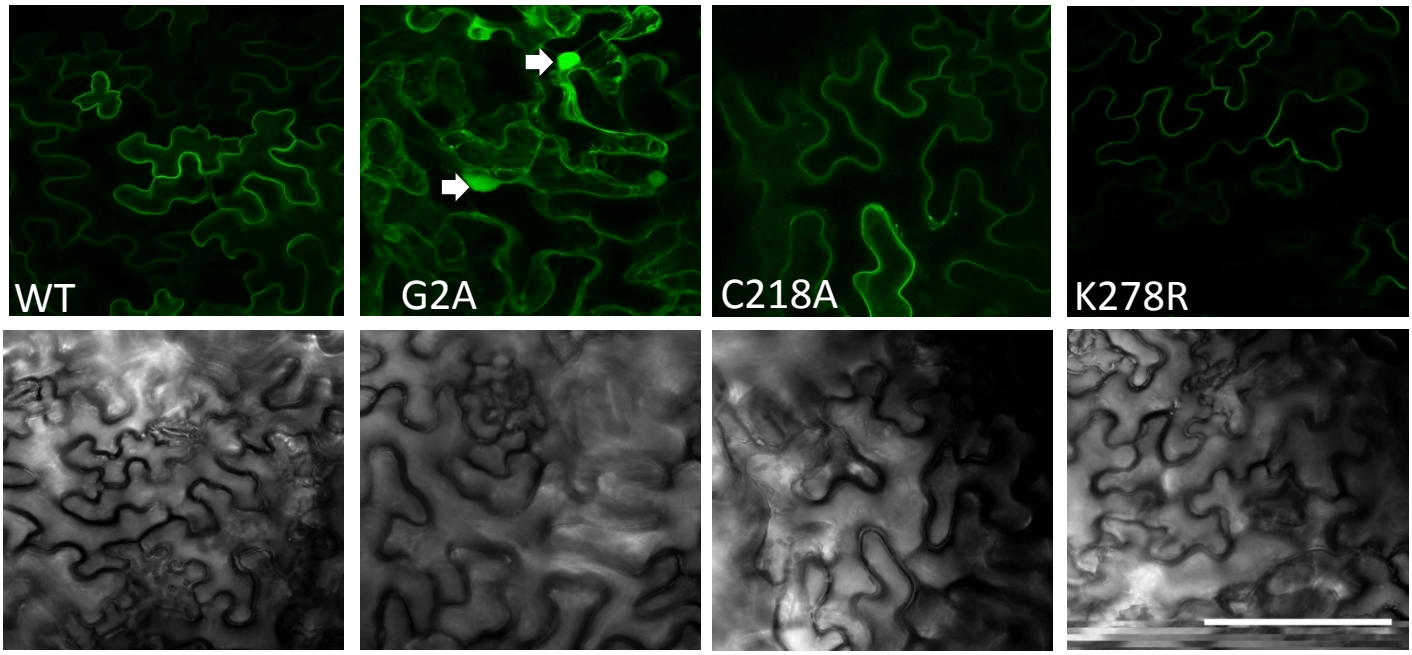


Figure 3.14. HopZ5 localizes to the plasma membrane through putative myristoylation of its G2 residue. *In planta* subcellular localization of HopZ5 variants was determined in *SGT1*-silenced *N. benthamiana*. Leaves were infiltrated with *A. tumefaciens* AGL1 carrying YFP-tagged *hopZ5* (WT), *hopZ5* (G2A), *hopZ5* (C218A) or *hopZ5* (K278R) under a constitutive CaMV 35S promoter (#2.5.5). Leaf discs were taken at 2 days post infiltration and visualized under confocal laser-scanning microscopy for localization of the YFP tag (#2.5.5.1). Top and bottom panels are confocal images and corresponding bright-field images, respectively. White arrows in G2A sample indicate plant cell nuclei. Scale bar represents 50 µm.

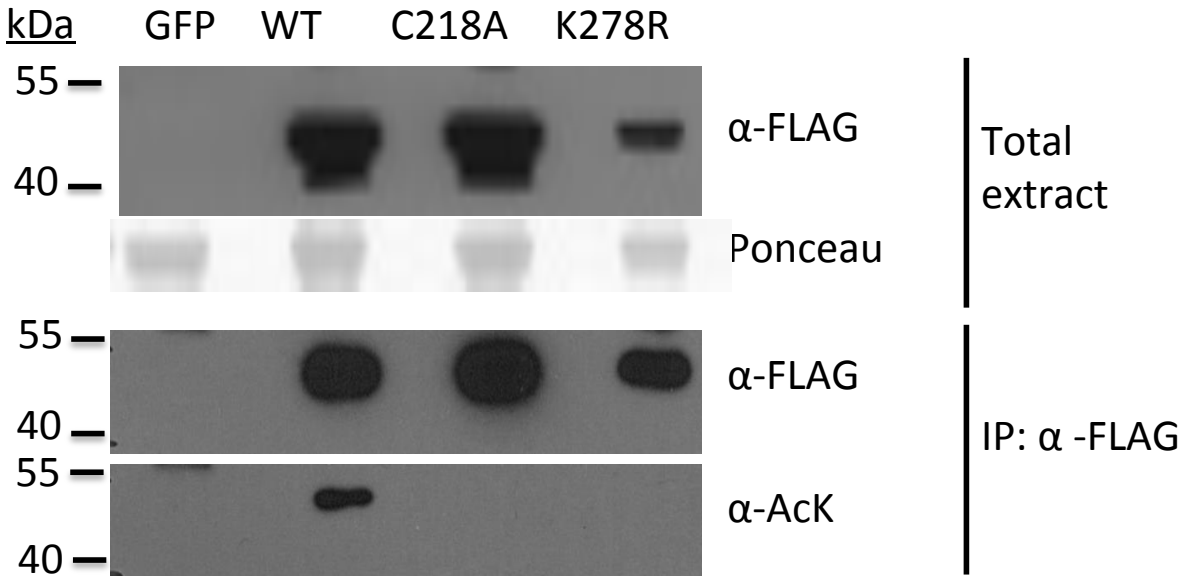


Figure 3.15. Catalytic site cysteine residue and downstream lysine residue are required for *in planta* autoacetylation of HopZ5. Detection of acetylated wild-type HopZ5 (WT), but not HopZ5-C218A and HopZ5-K278R from transient expression in *N. benthamiana SGT1*-silenced plants. *SGT1*-silenced *N. benthamiana* leaves were infiltrated with *A. tumefaciens* AGL1 carrying YFP-tagged *hopZ5* (WT), *hopZ5* (C218A) or *hopZ5* (K278R) under a constitutive CaMV 35S promoter (#2.5.5). Protein was extracted from leaf samples at 2 dpi. Protein presence in total extract was tested by α-FLAG antibody and acetylation status tested by immunoblot with an α-AcK antibody after α-FLAG immunoprecipitation (#2.6.4). Ponceau Red staining of total protein extract showing the Rubisco protein band indicates even sample loading. Numbers to the left of the panels indicate band sizes for the PageRuler™ protein ladder in kDa.

HopZ5 C218A and K278R variants were thus further examined for their ability to autoacetylate lysine residues. Transiently expressed FLAG-tagged HopZ5 variants (#2.5.5) were immunoprecipitated via the FLAG tag (#2.6.4.1.1), and probed with an antibody that detects acetylated lysine residues, used as a marker for autoacetylation ability (#2.6.4.2). We found that wild-type HopZ5 but not the C218A variant was lysine-acetylated, suggesting that C218A has lost its acetyltransferase activity and that importantly that HopZ5 lysine residues were autoacetylated (Figure 3.15). In addition, although catalytic residues (H150, E169 and C218) are present, lysine-acetylation of the K278R variant was not detectable, indicating that this residue may be the sole acetylated lysine residue and is autoacetylated in HopZ5, or that it is required for autoacetylation in general.

3.2.7 HopZ5 does not suppress ETI

T3Es have been shown to have a role in suppression of ETI (Guo et al., 2009; Jamir et al., 2004; Macho et al., 2010). For example, co-delivery of AvrRpm1 with ETI-suppressor HopD1 suppressed AvrRpm1-trigger HR and alleviated ETI (Block et al., 2014). To assess whether HopZ5 had a role in suppression of ETI we co-delivered AvrRpm1 and HopZ5, AvrPtoB$_{Pto\text{DC3000}}$ (a known suppressor of ETI), HopAM1-1$_{Psa\text{V13}}$ or HopF2$_{Psa\text{V13}}$ (two T3Es from *Psa* V13 that are homologous to known suppressors of ETI; (Guo et al., 2009)) by Pf0-1(T3S). We found that HopZ5 co-delivery with HopZ5 was unable to suppress AvrRpm1-triggered HR, while the control AvrPtoB could do so, as expected (Figure 3.16A). Interestingly, we found that *Psa* V13 HopAM1-1, a homolog of HopAM1 from *Pto* DC3000, could suppress AvrRpm1-triggered HR. This suggested a lack of ETI-suppression ability for HopZ5, at least in the Arabidopsis accession used, Ws-2.

Recently, HopZ1a was shown to have a role in the suppression of systemic acquired resistance (SAR) triggered by preinoculated with an avirulent pathogen (Macho et al., 2010). Preinoculation with virulent or avirulent bacteria is known to trigger SAR through the upregulation of plant immunity prior to a secondary infection that is subsequently examined for restricted growth attributable to SAR (Cameron et al., 1994). This test is particularly interesting as it allows us to

assess HopZ5 for a role in virulence, independent of its avirulence effect, in a plant that has a demonstrable response to HopZ5, Arabidopsis accession Ct-1.

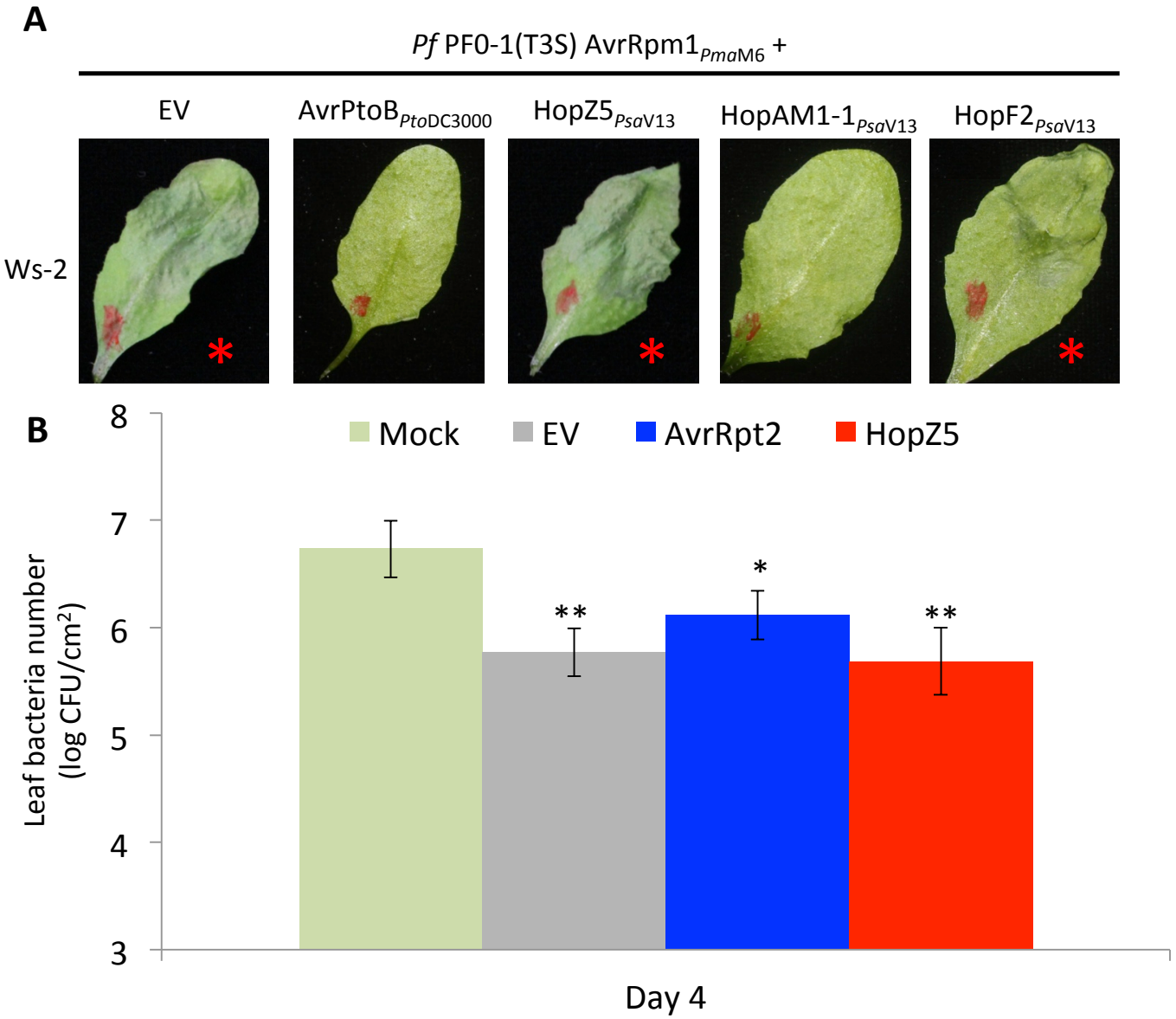


Figure 3.16 – HopZ5 does not suppress ETI in Arabidopsis. **(A)** Leaves of Arabidopsis Ws-2 plants were infiltrated with *P. fluorescens* Pf0-1 (T3S) carrying *avrRpm1* under its native promoter in pVSP61, and either EV (pBBR1MCS-5B), $avrPtoB_{PtoDC3000}$, $hopZ5_{PsaV13}$, $hopAM1\text{-}1_{PsaV13}$, or $hopF2_{PsaV13}$ (Appendix 3) to assess their co-delivery suppression of HR triggered by AvrRpm1. Bacterial suspensions (1×10^8 CFU/mL) were blunt-syringe infiltrated into the leaves and photographs were taken 20 hours after infiltration (1 dpi) (#2.5.1). Development of the hypersensitive response is indicated by a red asterisk. **(B)** Growth of *Pto* DC3000 in secondary leaves after preinoculation of primary leaves to assess SAR-mediated growth restriction. Primary leaves of Arabidopsis Col-0 plants were infiltrated with 10 mM $MgCl_2$ (mock), or bacterial suspensions (5×10^5 CFU/mL) of *Pto* DC3000 carrying EV, *hopZ5*, or *avrRpt2*. After 2 days, secondary leaves were infiltrated with wild-type *Pto* DC3000 and growth was determined at 4 dpi (#2.5.3). Error bars represent standard error of the mean for six technical replicates. Asterisks indicate results from Student's *t*-test for indicated samples compared to plants preinoculated with mock; * ($P < 0.05$), ** ($P < 0.01$).

Notably, preinoculation with *Pto* DC3000 carrying EV, *avrRpt2* or *hopZ5* restricted growth of the secondary *Pto* DC3000 infection (Figure 3.16B), unlike the SAR-suppression seen previously for HopZ1a (Macho et al., 2010). This suggests that HopZ5 does not appear to have a role in SAR-suppression as seen for its homolog HopZ1a.

3.3 Discussion

We have identified HopZ5 from *Psa* V13 as an avirulence effector in Arabidopsis accessions Ct-1 and Col-0. HopZ5 delivery by *Pseudomonas* T3S resulted in HR and bacterial growth restriction in Ct-1. Interestingly, in Col-0, neither HR nor elevated electrolyte leakage was observed but *Pto* DC3000 growth was significantly restricted in the presence of HopZ5. Enhanced defence marker gene expression when HopZ5 was delivered from *Pseudomonas* or transgenically expressed in Col-0 indicates the presence of a functional recognition system in Col-0. The difference between Pf0-1(T3S) and *Pto* DC3000 delivery of HopZ5 is unknown but likely to be an issue of delivery efficiency since in our Pf0-1(T3S) delivery experiments defence marker gene upregulation in Col-0 was poor even for the positive control AvrRpm1 (Figure 3.4). In addition, acetyltransferase activity and plasma membrane localization are required for HopZ5-triggered immunity in both Arabidopsis and *Nicotiana benthamiana*. These findings in addition to the requirement of *SGT1* alone for HCD (in *N. benthamiana*) and requirement of *EDS1*, *NDR1* and SID2 for immunity (in Arabidopsis) further supports a separation of HR and immunity in plant responses to HopZ5.

Unfortunately, determination of HopZ5-associated virulence function was complicated due to HopZ5-triggered resistance and HR in a wide variety of plant species tested (S. Choi, J. Jayaraman, K. H. Sohn, unpublished). Elucidation of the genetic components required for HopZ5-triggered immunity in Arabidopsis will be critical to future work, as this will facilitate further investigations of HopZ5-associated virulence. This of interest in the interaction between *Psa* and kiwifruit given that HopZ5 is unique to the global outbreak strains of *Psa*. The gain of effectors HopZ5 and HopH1 was proposed as a transposon-mediated lateral

transfer event (McCann et al., 2013). HopZ5 is unique to *Psa*, while HopH1 is present in multiple pathogenic *Pseudomonas* strains and is believed to be a helper protein (Mohr et al., 2008). We do not yet know whether this lateral transfer event played a significant role in the rapid global spread of this disease, but if it did this research may help us develop potent options for application of resistance to *Psa*.

3.3.1 Resistance without HR in Arabidopsis

Separation of HR from pathogen growth restriction was demonstrated in *ndr1* mutant plants that showed HR in response to *Pto* DC3000 delivering AvrRpm1, AvrB or AvrPphB but lost the ability to restrict growth of these strains (Century et al., 1995). Similarly, Turnip crinkle virus (TCV)-triggered HR is mediated by the CNL gene *HRT* while the full disease resistance is mediated in conjunction with a second recessive determinant, *rrt* (Cooley et al., 2000). Moreover, TCV resistance has a non-canonical requirement for *EDS1* as well as *RAR1* and *SGT1* but these genes are not required for HR (Chandra-Shekara et al., 2004). The converse, HR-independent resistance, was recently shown in Arabidopsis metacaspase mutants, *mc1* and *mc2*. *mc1* and *mc2* mutants show reduced and increased cell death responses to *Pto* DC3000 delivering AvrRpm1, respectively, but have no effect on bacterial growth (Coll et al., 2010). Similarly, immunity triggered by unrelated bacterial type III effectors HopA1 (previously HopPsyA) and AvrRps4 specifically in the Col-0 accession are HR-independent (Gassmann, 2005; Gassmann et al., 1999). The locus that confers suppression of AvrRps4- or HopA1-triggered HR in Col-0, *HR REGULATOR IN EDS1 PATHWAY* (*HED1*), was previously mapped to the bottom arm of chromosome 5 in Arabidopsis but the exact genetic determinant of this phenotype remains to be elucidated (Gassmann, 2005). Furthermore, Heidrich and colleagues have elegantly demonstrated that the cell compartment-specific localization of EDS1 is linked to pathogen growth restriction (nucleus alone) or HR (nucleo-cytoplasmic) in response to *Pto* DC3000 carrying *avrRps4* (Heidrich et al., 2011). Two other effectors have shown immunity independent of HR in Col-0: HopAM1-1, which has a minor recognition represented by multiple quantitative trait loci (Iakovidis et al., 2016), and the recently studied HopBA1, which triggers HR and immunity

in accession Ag-0 dependent on the TIR-domain-only protein RBA1 but only triggers immunity in Col-0 (Nishimura et al., 2017). The latter is particularly interesting as, firstly, it is a TIR-domain protein, thereby adding to the list of TNL or TNL-like NB-LRRs that trigger HR-independent defences in Col-0, and secondly, HR in Col-0 can be introduced by the transgenic expression of the Ag-0 allele of RBA1.

Yet another example of HR uncoupled from defence is the *defence, no death1* (*dnd1*) mutant that shows constitutively elevated defence responses but is unable to mount HR in response to avirulent bacterial strains (Yu et al., 1998). *dnd1* carries a null mutation in a cyclic nucleotide-gated ion channel *AtCNGC2* predicted to be involved in Ca^{2+} signaling during defence responses (Clough et al., 2000). A similar gain of constitutive defence phenotype was observed in *dnd2*/*AtCNGC4* and *cpr22*/*AtCNGC11-AtCNGC12* mutants although the latter can show HR (Balagué et al., 2003; Clough et al., 2000; Yoshioka et al., 2006). Intriguingly, the *cpr22* phenotype, much like the HR-independence of HopZ5-triggered immunity, demonstrates genetic requirement of EDS1 and NDR1 for elevated defences but not autoimmune-related stunting or SA accumulation, products of lesioning/HR response (Yoshioka et al., 2006). The involvement of different *AtCNGCs* in HR-independent defence suggests that they are negative regulators of defence. Notably, it is possible due to its membrane-localization that HopZ5 targets an ion channel in Col-0 triggering HR-independent immunity.

The closest homolog of HopZ5 is AvrBsT from *X. campestris* pv. *vesicatoria* (*Xcv*; also called *X. euvesicatoria*), sharing 58% amino acid identity. AvrBsT triggers HR and immunity (as measured by virulent bacterial growth) in Arabidopsis accession Pitzal-0 (Pi-0) but not in Col-0 and Landsberg *erecta*-0 (L*er*-0) (Cunnac et al., 2007). Notably, Pi-0 was the only accession out of 71 total tested that showed AvrBsT-triggered HR. Suppression of AvrBsT-triggered immunity/HR is conferred by the *SUPPRESSOR OF AVRBST ELICITED RESISTANCE1* (*SOBER1*) gene encoding a carboxylesterase. In contrast to AvrBsT, Col-0 showed disease resistance to virulent bacterial pathogen carrying *hopZ5* (Figure 3.5-3.6 & Figure 3.10). Thus, it is likely that the genetic basis of suppression of HopZ5-triggered

HR is different from AvrBsT. However, it remains to be seen if *SOBER1* plays a role in HopZ5-mediated immunity in Col-0.

The separation of HR incidence from immunity was originally ascribed to a matter of amplitude, particularly because ETI was perceived as an amplified PTI response (Coll et al., 2011). However, as in the aforementioned examples, the resolution of HR from immunity can be complex. In the case of HopZ5, it is conceivable that a dominant suppressor or negative regulator of HR may exist in Col-0. This suppressor would specifically interfere with HR signaling triggered by HopZ5 or reduce the general immunity level below the threshold that is required for HR development but not immunity. Further investigation into the dynamics behind HR-associated and HR-independent HopZ5-triggered immunity could serve to illustrate a larger role of HR in relation to immunity.

3.3.2 The YopJ family is diverse with little conservation of *in planta* targets

The YopJ family members are present in various plant pathogenic bacteria including *Pseudomonas*, *Xanthomonas*, *Acidovorax* and *Ralstonia* (Figure 3.7A) (Lewis et al., 2011). *Xcv*, the causal agent of tomato bacterial spot disease, carries four different members of the YopJ family, namely *avrXv4*, *avrRxv*, *avrBsT* and *xopJ* (Astua-Monge et al., 2000; Ciesiolka et al., 1999; Noël et al., 2003). AvrXv4 is a cytoplasmic effector with small ubiquitin modifier (SUMO)-protease activity that modifies a large number of proteins *in planta* and triggers defence responses in wild tomato relative *Solanum pennellii* and *N. benthamiana* (Roden et al., 2004). AvrRxv recruits a plant 14-3-3 protein for its virulence and avirulence activities (Whalen et al., 2008). AvrBsT, originally shown to trigger disease resistance in pepper plants (Minsavage, 1990), is a cytoplasm-localized acetyltransferase that binds to and suppresses cytoplasmic SNF1-related kinase (SnRK1) that is required for AvrBs1-triggered HR (Szczesny et al., 2010). Furthermore, AvrBsT acetylates microtubule-associated proteins to disrupt the microtubule network that is required for plant defence (Cheong et al., 2014). XopJ, much like its closest homolog, HopZ4, binds and proteolytically degrades proteasomal subunit RPT6 to suppress plant immunity at the plant cell

membrane (Üstün and Börnke, 2015). *R. solanacearum* GMI1000 carries three YopJ family members *avrA*, *popP1* and *popP2*. AvrA and PopP1 trigger acetyltransferase activity-dependent HR in *N. tabacum* and *N. glutinosa*, respectively, that is involved in restriction of host range (Le Roux et al., 2015; Poueymiro et al., 2009). The HopZ family of effectors is found in many *Pseudomonas syringae* strains. HopZ1a was shown to target both, JAZ repressors affecting salicylic acid-mediated resistance via activation of jasmonate signaling, and tubulin that results in disruption of the microtubule network and associated protein secretion and defence at the cell wall (Jiang et al., 2013; A. H.-Y. Lee et al., 2012). HopZ2 from pea pathogen *P. syringae* pv. *pisi* 895A physically associates with an Arabidopsis transmembrane domain containing protein MILDEW RESISTANCE LOCUS2 (MLO2) that is required for resistance against necrotrophic pathogens *Botrytis cinerea* and *Magnaporthe oryzae* (Lewis et al., 2012). Cytoplasmic HopZ3 from bean pathogen *P. syringae* pv. *syringae* B728a has recently been shown to target Arabidopsis RIN4, various receptor-like cytoplasmic kinases (RLCKs) and the MAP kinase MPK4, all involved in defence signaling *in planta* (Lee et al., 2015). HopZ4 from cucumber pathogen *P. syringae* pv. *lachrymans* MAFF301315 binds to proteasomal subunit RPT6 and inhibits proteasome activity that is required for disease resistance (Üstün et al., 2014). Most HopZ effectors have been shown to be localized at the plant cell membrane, likely via myristoylation, and their subcellular localization is required for function (Lewis et al., 2008; Üstün et al., 2014). Based on these findings and our results (Figure 3.13), it is expected that HopZ5 targets a plasma membrane-localized defence component(s).

Plant resistance against YopJ family members has been genetically characterized so far for HopZ1a, AvrBsT and PopP2. HopZ1a targets pseudokinase ZED1 and this event is recognized by ZAR1 (Lewis et al., 2013, 2010). PopP2 directly acetylates WRKY DNA-binding domain of RRS1 and activates RPS4-dependent immunity (Deslandes et al., 1998; Le Roux et al., 2015; Sarris et al., 2015). AvrBsT-mediated immunity in pepper involves both CaSGT1 and CaPIK1 (N. H. Kim et al., 2014). CaPIK1 is a pepper receptor-like cytoplasmic kinase that forms a complex with CaSGT1 and triggers phosphorylation of CaSGT1, subsequent

monomerization and nuclear localization. AvrBsT appears to interact with this complex and interferes with this process, leading to a cell death response in an AvrBsT catalytic activity-independent manner but dependent on CaPIK1 phosphorylation of CaSGT1 through specific accumulation of AvrBsT-CaSGT1 in the cytoplasm (N. H. Kim et al., 2014). Furthermore, two other interactors have been found to contribute to the cell death response triggered by AvrBsT in pepper, CaHSP70a and CaALDH1 (Kim and Hwang, 2015a, 2015b). As yet it remains unclear how these multiple targets integrate to trigger a cell death response to AvrBsT.

As mentioned previously, few of the characterized homologs of HopZ5 share an *in planta* target while some have a large number of targets that could all additively contribute to its virulence function (Jiang et al., 2013; A. H.-Y. Lee et al., 2012; Lee et al., 2015; Lewis et al., 2011). Additionally, distinct NB-LRRs recognize corresponding YopJ family T3Es to activate plant immunity. However, certain cues can be taken from previous studies on YopJ family T3Es to predict circumstances for HopZ5. Based on these findings, we hypothesize that HopZ5 could target a host protein(s) that has not been implicated in other HopZ effector-triggered immunity or susceptibility studies. Furthermore, we predict that the *in planta* target and the corresponding NB-LRR of HopZ5 are likely to be plasma membrane localized.

3.3.3 Quantitative involvement of autoacetylation in bacterial acetyltransferase activity

A lysine residue that was shown to be autoacetylated in PopP2 is conserved among the majority of the YopJ family of effectors. In the case of PopP2, the autoacetylated lysine is required for its avirulence activity (Tasset et al., 2010). However, it was shown that HopZ1a does not require this conserved lysine for its function in a native promoter context (A. H.-Y. Lee et al., 2012; Ma et al., 2015; Rufián et al., 2015). Some members of the YopJ family of effectors were shown to acetylate serine and threonine residues, including YopJ (Mukherjee et al., 2006) and HopZ1a (Ma et al., 2015). We have shown here that the conserved lysine residue (K278) is required for HopZ5 autoacetylation of lysine residues *in planta*

(Figure 3.14). Lysine 278 of HopZ5 seems to either be the only lysine residue that is autoacetylated since the mutation of lysine 278 to arginine (K278R) abolished HopZ5 lysine autoacetylation, or is required for autoacetylation activity. Since the antibody (α-AcK) we used for testing HopZ5 autoacetylation did not allow us to detect non-lysine acetylation, additional autoacetylation of other residues in HopZ5 may still occur. Importantly, it is plausible that K278 is the only lysine residue critical for HopZ5 avirulence (Figures 3.8-3.11). However, agroinfiltration of K278R variant of HopZ5 in *Nicotiana* spp. could still induce delayed HCD. This partial loss of avirulence activity of the K278R variant of HopZ5 in *Nicotiana* spp. suggests that the requirement of K278 in HopZ5-triggered immunity might be dose-dependent.

We also observed a similar partial loss of function of the G2A variant, which lost plasma membrane localization, presumably through lack of myristoylation (Figure 3.13). This partial loss of function could be due to the cytoplasm-localized HopZ5 G2A variant that could still interact with its target protein and induce HCD. Identification of the host *in planta* target(s) of HopZ5 will be essential in better understanding the requirement for subcellular localization in initiation of HopZ5-triggered immunity in the future.

3.3.4 Genetic requirement for HopZ5-triggered immunity suggests recognition by a novel NB-LRR

We showed here that *SGT1* is required for HopZ5-mediated HCD in *N. benthamiana*. Analysis of the genetic requirement for AvrBsT-triggered immunity in Arabidopsis indicated that *NDR1* (fully), *EDS1* (partially), and *SID2* (partially) were required in Pi-0 plants, with no recognition in Col-0 (Cunnac et al., 2007). A similar study of ZAR1-mediated immunity against HopZ1a in Arabidopsis Col-0 could not identify a requirement for *NDR1*, *EDS1, SID2,* or *SGT1* (Lewis et al., 2010). In comparison, our results (recognition in Col-0 with full requirement of *NDR1*, *EDS1*, and *SID2*; Figure 3.13) thus strongly suggests a novel recognition of HopZ5. It must be noted, however, that due to the weak immunity triggered by HopZ5 in Arabidopsis Col-0, the full requirements for *SID2* and *EDS1* seen in our experiments may mirror the partial requirements for

these two genes for AvrBsT-triggered immunity. Despite this, our results strongly suggest that the recognition of HopZ5 (and possibly AvrBsT) is likely mediated by an as yet uncharacterized NB-LRR. The identification that both *NDR1* and *EDS1* are required for immunity against HopZ5 in Arabidopsis was initially surprising and appeared to contradict our expectations from observations of HCD development in *N. benthamiana*. However, due to the uncoupling of HR (or HCD) from immunity in Col-0 plants, we were able to explain the requirement for both *NDR1* and *EDS1* in immunity mounted in response to HopZ5 irrespective of their requirement for HCD/HR.

Regardless, we found that HopZ5 does not require *NDR1* nor *EDS1* to activate HCD in *N. benthamiana*. This can be explained through a redundancy between these two canonical pathways in *N. benthamiana*, or that the EDS1 or NDR1 levels that do accumulate in the silenced plants are not rate-limiting. The latter possibility is unlikely since the control $RS4_{TIR}$ domain-triggered HCD was suppressed by *SGT1*- and *EDS1*-silencing, indicating that insufficient levels of SGT1 and EDS1 were accumulating in these plants to allow for $RPS4_{TIR}$-triggered HCD (Figure 3.12). Redundancy between *EDS1* and *NDR1* occurs in Arabidopsis *RPP7*- and *RPP8*-mediated immunity against the oomycete pathogen *Hpa* that was not abolished in the absence of *EDS1* or *NDR1* alone but was attenuated in the *eds1 ndr1* double mutant (McDowell et al., 2000). Therefore, it is plausible that EDS1 and NDR1 play a functionally redundant role in HopZ5-triggered immunity. Alternatively, neither NDR1 nor EDS1 are required for HopZ5-triggered immunity as it may require an as yet undiscovered ETI signaling component, a scenario seen for a number of NB-LRR-mediated immunities including that of RPP13-Nd (Bittner-Eddy and Beynon, 2001).

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

4.1 Introduction

Kiwifruit plants display a wide spectrum of responses to *Pseudomonas syringae pv. actinidiae* (*Psa*) with some strains (V clade; biovar 3) displaying extreme virulence on certain cultivars (particularly Hort16A), other strains (J and K clades; biovars 1 and 2, respectively) displaying moderate virulence (on multiple cultivars including Hort16A and Hayward), and yet others displaying little to no virulence on several tested cultivars (LV clade; biovar 4) (McCann et al., 2013). This intricate relationship between *Psa* and their host plant, kiwifruit, or nonhost plants (the model plants Arabidopsis and *Nicotiana* spp.) offered an important opportunity to study the difference between host versus nonhost resistance (NHR) against the various *Psa* strains, with *Psa* LV5 as a suitable strain for studying host resistance in kiwifruit.

Host resistance is an example of a gene-for-gene response, typically mediated by recognition of a single effector triggering ETI (akin to observation for HopZ5 in Chapter 3), while NHR is considered an amalgamation of different modes of resistance conferred by multiple genes against a maladapted pathogen (Gill et al., 2015; Mysore and Ryu, 2004). Furthermore, NHR is divided into two categories based on the plant response to a high load of pathogen delivery: type-I where there is resistance without any visible development of symptoms, or type-II where there is development of an HR (Mysore and Ryu, 2004). Determination of effectors from *Psa* LV5 that were good targets for host resistance was problematic due to the large number of effectors shared by the various *Psa* strains that were virulent on kiwifruit (McCann et al., 2013), and particularly because we could not determine from genome sequence information alone if these effectors were all functional. This was further complicated by the fact that several attempts at delivering individual effectors into kiwifruit proved unsuccessful or laborious. Therefore, because the NHR triggered by multiple *Psa* strains, including *Psa* LV5, on Arabidopsis Col-0 and *Nicotiana benthamiana* was associated with an HR in both plants (type-II NHR), we reasoned that the ETI

triggered by effectors unique to *Psa* LV5 (using well developed tools for these model plants) could be good sources of host resistance in kiwifruit as they would thus be proven functional. The reasoning behind this is due to the observation that sometimes *R* genes with specificity for the same effectors are found in distantly related plant species either through conservation of an ancestral specificity in multiple plant lineages or convergent evolution. An example of this is the recognition of *P. syringae* avirulence genes *avrB* and *avrRpm1* by Arabidopsis and soybean. The Arabidopsis RPM1 gene has a dual specificity for AvrB or AvrRpm1 (Bisgrove et al., 1994) while soybean expresses *R* genes able to distinguish between these two avirulence genes with alleles (Rpg1-b specific for AvrB and Rpg1-r for AvrRpm1) of the *R* gene tightly linked at the *Rpg1* locus (Ashfield et al., 2003, 1995). Yet another strong example of this is the recognition of pepper pathogen *Xanthomonas campestris* pv. *vesicatoria* effector AvrRxV in bean, corn, or cotton, three long-diverged plant lineages (Whalen et al., 1988). AvrPphB (also called HopAR1) from bean *pathogen P. syringae* pv. *phaseolicola* race 3 is another effector that is recognized in both Arabidopsis and bean (*Phaseolus*) (Jenner et al., 1991; Simonich and Innes, 1995). In fact, some *P. syringae* strains naturally infecting Arabidopsis in Europe have been found to carry the homologous effector AvrPphB2, that are recognized by the same *R* gene, *RPS5,* first identified for its recognition of AvrPphB (Karasov et al., 2014). Thus, convergent evolution may be driven by shared effectors from different pathogens acquired through horizontal gene transfer events, a mechanism proposed for *avrPphB* (Lovell et al., 2009).

AvrPto (from *P. syringae* pv. *tomato* JL1065) and AvrPtoB (from *Pto* DC3000) are two independently evolved effectors that target PTI and are recognized by the wild tomato *Solanum pimpinellifolium* R protein kinase Pto in conjunction with NB-LRR Prf (Kim et al., 2002; Ronald et al., 1992). AvrPto is an 18kDa protein with a central α-helix bundle structure but has no overall sequence homology to any known proteins (Wulf et al., 2004). A large Ω-loop, called the GINP loop, the central core was required for interaction with Pto in a yeast 2-hybrid system and mutations in this GINP loop abolishes interactions with Pto as well as Pto-mediated resistance in tomato (Wulf et al., 2004). A homolog of AvrPto from *Pto*

DC3000 was also shown to trigger Pto/Prf-mediated immunity in tomato lines with *S. pimpinellifolium* Pto/Prf locus introgressed (Lin and Martin, 2005; Salmeron et al., 1994). AvrPtoB, on the other hand, is a larger 59kDa protein that possess two distinct domains at either end, a N-terminal domain required for virulence activity in tomato plants, and a C-terminus carrying an E3 ubiquitin ligase domain required for virulence in Arabidopsis and for programmed cell death suppressing ability (Abramovitch et al., 2006, 2003; Goehre et al., 2008; Xiao et al., 2007). Work on the dynamics of Pto and Prf function led to the discovery that Prf and Pto form a heterodimeric complex with the two Pto molecules in the complex serving two functions, one a sensor (Pto_{sensor}) and the other an activator ($Pto_{activator}$), with the AvrPto-Pto_{sensor} or AvrPtoB-Pto_{sensor} interaction disrupting the P+1 loop setup by the $Pto_{activator}$ molecule within the complex and thereby triggering Prf-mediated signaling to immunity (Mucyn et al., 2006; Ntoukakis et al., 2013).

The finding that Pto could trigger resistance against both AvrPto and AvrPtoB then led to structural work that identified unique interaction surfaces for AvrPto and AvrPtoB with Pto (Dong et al., 2009; Xing et al., 2007), implying the possibility that versions of Pto could exist in nature that recognized one but not the other. In fact, two examples of such recognition exist. The first was the existence of a so-called Rsb (resistance suppressed by AvrPtoB C-terminus) phenotype for recognition of an artificial E3 ligase mutant version of AvrPtoB (Abramovitch et al., 2003; Janjusevic et al., 2006; Tang et al., 1996). The Rsb phenotype was found to be conferred by a Pto homolog, Fen, that bound to E3 ligase-deactivated AvrPtoB but not AvrPto, an interaction not detectable for fully functional AvrPtoB (Rosebrock et al., 2007). This lack of Fen binding and immunity to non-mutated AvrPtoB was found to be a result of the ability of AvrPtoB to bind Pto or Fen at a site within AvrPtoB that then allowed it to use its E3 ligase activity to degrade Pto/Fen (Mathieu et al., 2014). However, due to a secondary Pto-specific binding site in AvrPtoB distal to the E3 ligase domain, Pto could avoid degradation and was therefore able to trigger resistance (Mathieu et al., 2014). In fact, a natural variant of AvrPtoB, the allele from *P. syringae* pv *syringae* B728a, carried a mildly defective E3 ligase domain that was seen to

trigger Pto-independent resistance in tomato cultivars that carried a Fen homolog (Chien et al., 2013). Thus this was considered a Pto-homolog recognition of AvrPtoB but not AvrPto due to lack of E3 ligase activity. The second example of differential AvrPto/AvrPtoB recognition was found in another tomato wild accession, *S. chmielewskii*, carrying a homolog of *Pto* that facilitated recognition of AvrPtoB but not AvrPto due to a single amino acid change in its sequence (Kraus et al., 2016).

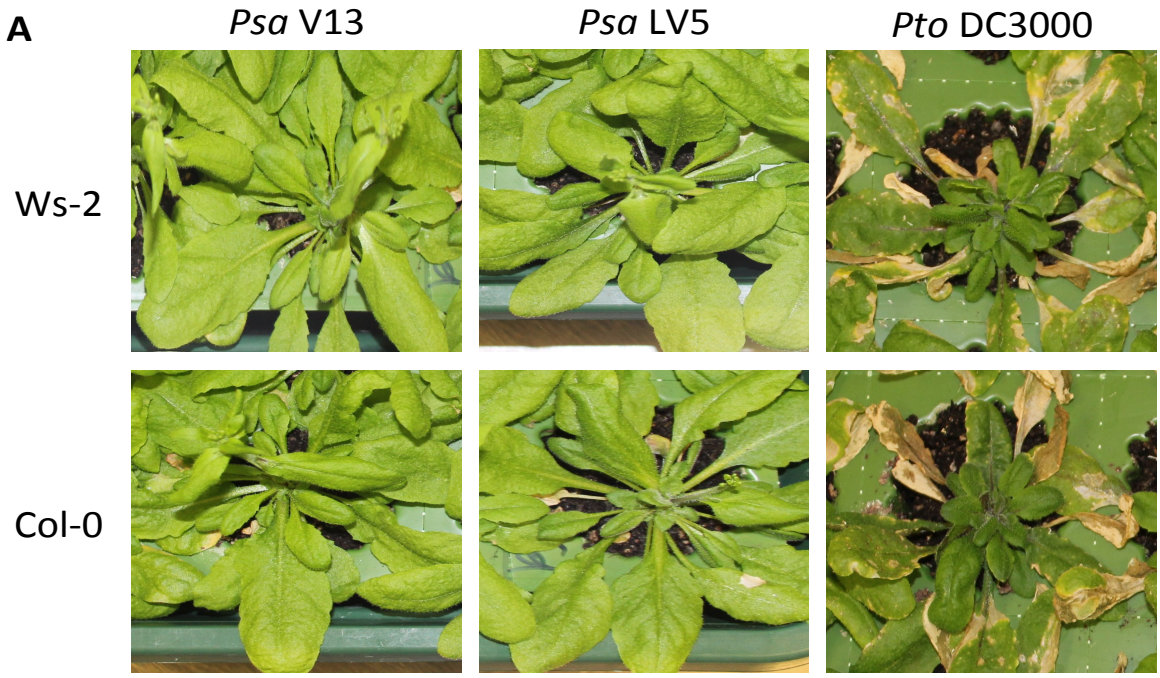
Recently, AvrPto from *Pto* DC3000 was found to trigger an HR and associated growth restriction in Arabidopsis accessions Bu-22 and Bu-25 (Velásquez et al., 2017). This finding suggests a conserved/convergent effector recognition event (AvrPto recognition in Arabidopsis and tomato) across at least two different plant families. This is thus a good example of host recognition and, since AvrPto recognition in these plants was associated with an HR response, makes consideration of AvrPtoB homologs interesting for type-II NHR as well.

In this chapter, we sought ot examine effector candidates from *Psa* LV5 for host resistance in kiwifruit. With the aim of ultimately examining effectors for for Avr status in kiwifruit using reporter-gene eclipse assays (a time-consuming and laborious process), we sought to prioritize effector Avr candidates by examining NHR triggered in tractable model plants Arabidopsis and *Nicotiana benthamiana*. The genome sequence of *Psa* LV5 identified several good candidates for avirulence related to previously characterized effectors from other pathogens, namely *hopA1*, *hopAB3*, *hopAI1*, and *hopAR1* (McCann et al., 2013); we investigated whether these effectors were indeed classical *avr* genes contributing to host resistance in kiwifruit. Our particular focus was to identify the Avr determinant in *Psa* LV5 in Arabidopsis (via *Pf* Pf0-1(T3S) delivery and mapping the HR locus by bulk segregant analysis) or *N. benthamiana* (by characterizing HR-like cell death produced by transient expression), thus indicating that it was functional before testing it. We furthermore investigated the novel HR-like cell death for HopAB3 (a homolog of AvrPtoB) discovered in this screen with the aim of understanding the molecular determinants of this novel phenotype.

4.2 Results

4.2.1 *Psa* LV5 triggers HR and avirulence in Arabidopsis conferred by the *RPS5/SUMM2* locus

High-dose (10^8 CFU/mL) spray inoculations of *Psa* LV5 and *Psa* V13 did not cause disease on Arabidopsis accessions, exemplified by accessions Columbia-0 (Col-0) and Wassilewskija (Ws-2), unlike the virulent pathogen *Pto* DC3000 at 7 dpi (Figure 4.1A). Several other strains (*Psa* J35, K28, and I4 from biovar 1 through 3, respectively) also did not cause disease in multiple Arabidopsis



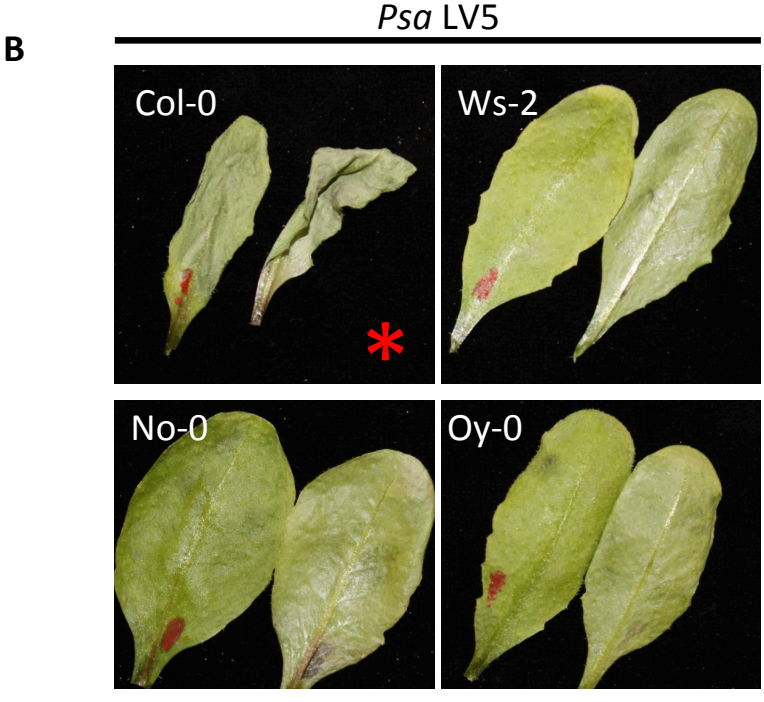


Figure 4.1. *Psa* LV5 triggers type-II NHR in Arabidopsis Col-0. (A) Growth and disease symptom development assay on leaves of *Arabidopsis* Col-0 or Ws-2 plants were spray-inoculated (5×10^8 CFU/mL) with *Pto* DC3000 (virulent), *P. syringae pv. actinidiae* (*Psa*) V13 (avirulent), or *Psa* LV5 (avirulent) (#2.5.3.1). Photographs of representative plants displaying disease symptoms, if any, were take at 7 dpi. **(B)** HR assay on leaves of *Arabidopsis* plants (Col-0, Ws-2, No-0, or Oy-0) were infiltrated with *Psa* LV5. Bacterial suspensions (1×10^8 CFU/mL) were blunt-syringe infiltrated into the leaves and photographs were taken 20 hpi (#2.5.1). Development of HR is indicated by a red asterisk.

accessions, indicating that Arabidopsis displayed NHR to *Psa* (data not shown). To identify the nature of the NHR against *Psa* LV5 in Arabidopsis, we inoculated high doses of *Psa* LV5 through blunt-syringe infiltration into Arabidopsis leaves and identified several accessions in which *Psa* LV5 triggered HR. Notably, Col-0 triggered a strong HR while MAGIC line parents Nossen-0 (No-0), Oystese-0 (Oy-0), and Ws-2 did not (Figure 4.1B). This indicated to us that a type-II NHR was present in Col-0 against *Psa* LV5 probably conferred by recognition of an effector from *Psa* LV5. Similar to our strategy for *Psa* V13 in Chapter 3, we identified 26 T3Es present in the *Psa* LV5 genome. Only ten of these T3Es (indicated in red, Table 4.1) were found to have homologs in *Psa* V13 (>90% homology to *Psa* V13; with <20 amino acids difference in size), which did not trigger HR in Col-0. We therefore cloned all 16 unique full length T3Es in *Psa* LV5 T3Es by the golden gate cloning method into the broad host-range vector pBBR 1MCS-5 under the *avrRps4* promoter as in Chapter 4 (Figure 3.2).

Individual T3E delivery by Pf0-1(T3S) did not identify the cause of the HR in Col-0 as none of the effectors alone triggered an HR (data not shown). To examine this quantitatively, all *Psa* LV5 unique T3Es were measured for leakage of ions caused by HR development in Col-0 leaves with AvrRpm1 delivery by Pf0-1(T3S) and *Psa* LV5 itself as controls (Figure 4.2A). As expected, both *Psa* LV5 and Pf0-1(T3S)-delivered AvrRpm1 induced significant increase in electrolyte leakage levels in Col-0 whereas neither any of the T3Es nor EV caused a notable change. As mentioned in Chapter 3, effectors may be affected in their ability to trigger ETI/HR through the effects of other effectors in the repertoire of a pathogen (Guo et al., 2009). We therefore reasoned that the lack of an HR in Col-0 for *Psa* V13 (triggered by an effector shared with *Psa* LV5) could be a result of another *Psa* V13 effector that can suppress HR. An example of this ability for a *Psa* V13 effector was seen for HopAM1-1$_{PsaV13}$ in Chapter 3. We therefore individually checked each *Psa* V13 homolog of *Psa* LV5 effectors for HR upon Pf0-1(T3S) delivery. Unfortunately, we were still unable to identify a T3E that triggered HR in Col-0 (Figure 4.2B).

Table 4.1 Full predicted type-III system secreted effector (T3E) repertoire of *Psa* LV5

Psa LV5 T3E	Locus tag	Hrp box	Chaperone	Predicted protein size (kDa)
AvrE1	A237_25990	✔		195.3
HopA1*	A237_05249	✔	ShcA	42.2
HopE1	A237_11125	✔		24.1
HopF1	A237_04013	✔	ShcF	21.9
HopN1	A237_25950	✔		38.7
HopO1	A237_01140	*hopS1* operon		32.6
HopR1	A237_16231	none		210.3
HopS1	A237_01150	✔	ShcS1	19.9
HopS2	A237_01125	✔	ShcS2	18.7
HopT1	A237_01135	*hopS1* operon		41.5
HopW1	A237_16224	none		82.9
HopX1	A237_26145	*hrpK1* operon		40.6
HopX2	A237_14679	✔		38.0
HopY1	A237_03183	✔		30.9
HopAA1	A237_25960	✔		50.5
HopAB3*	A237_23148	✔		62.6
HopAE1	A237_12751	✔		126.6
HopAF1-2	A237_28116	✔		30.9
HopAG1	A237_16469	✔		77.6
HopAH1	A237_16474	none		42.8
HopAI1*	A237_16479	none		29.7
HopAR1*	A237_04016	✔		28.5
HopAS1	A237_06036	✔		149.0
HopAY1	A237_04058	✔		35.4
HopAZ1	A237_21936	✔		24.7

* Cloned *Psa* LV5 effectors that were screened for avirulence in *Nicotiana benthamiana* by transient expression due to published homologs possessing avirulence activities

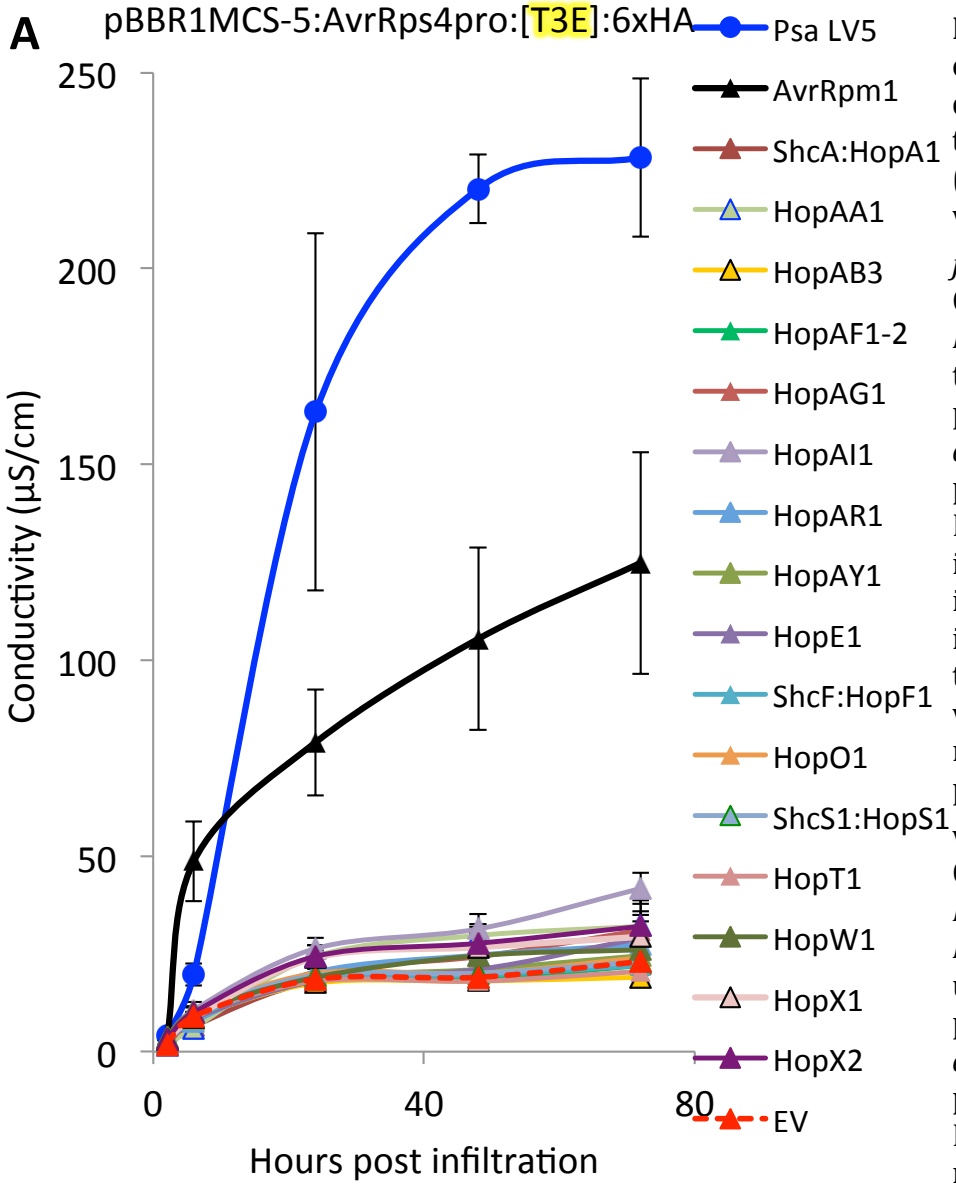
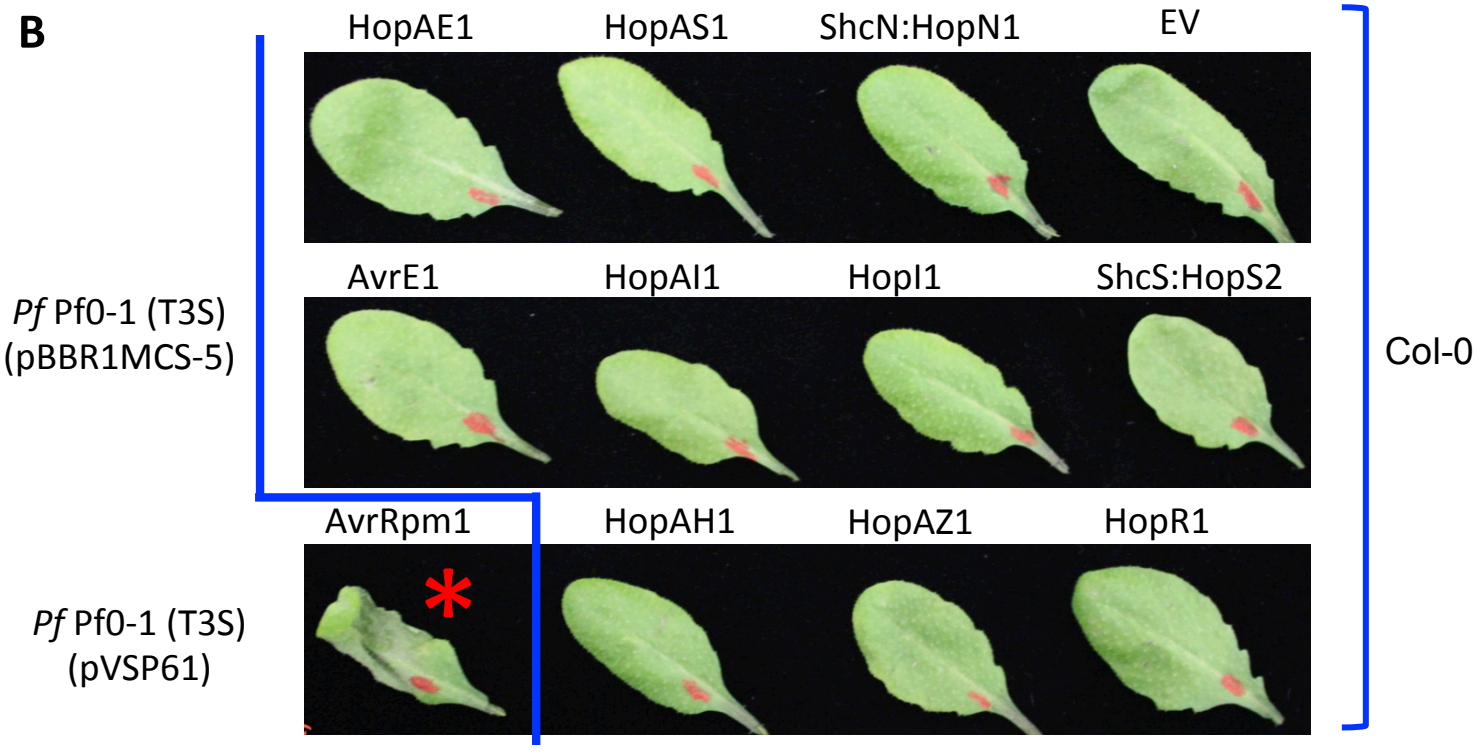


Figure 4.2. Individual *Psa* LV5 effector or its *Psa* V13 homolog delivery by *Pf* PF0-1(T3S) does not trigger ion leakage or HR in Col-0. **(A)** Leaves of *Arabidopsis* Col-0 plants were blunt syringe-infiltrated with *P. fluorescens* Pf0-1 (T3S) (1×10^8 CFU/mL) carrying an EV, the indicated *Psa* LV5 T3E with a C-terminal 6xHA tag under the control of the *avrRps4* promoter in pBBR1MCS-5, or untagged *avrRpm1* under its native promoter in pVSP61 (Appendix 3; #2.5.1). Electrolyte leakage from leaf discs after infiltration was determined at indicated timepoints. The error bars indicate the standard error from four technical replicates. The experiment was conducted twice with similar results. **(B)** Leaves of *Arabidopsis* Col-0 plants were blunt syringe-infiltrated with *P. fluorescens* Pf0-1 (T3S) (1×10^8 CFU/mL) carrying an EV, the indicated *Psa* V13 T3E (homologous to that in *Psa* LV5) with a C-terminal 6xHA tag under the control of the *avrRps4* promoter in pBBR1MCS-5, or untagged *avrRpm1* under its native promoter in pVSP61. (Appendix 3; #2.5.1). Development of HR is indicated by a red asterisk.



Due to the inability to identify the T3E responsible for *Psa* LV5-triggered HR in Col-0, we instead decided to use a Col-0 (HR) x Ws-2 (no-HR) crossed F_2 population to map the gene conferring the HR response to *Psa* LV5 itself. We screened around 400 F_2 individuals to collect 80 clearly no-HR individual F_2 plants from which we extracted gDNA for conducting bulk segregant analysis (pooled gDNA) and mapping (individual gDNA) (Figure 4.3). Bulk segregant analysis to identify the no-HR phenotype-linked locus (the only clearly defined homozygous phenotype) in the F_2 mapping population involved screening the pool of 80 no-HR individuals for enrichment of PCR-based marker(s) for Ws-2 (no-HR) allele(s). The markers used were identified in previous studies (Bell and Ecker, 1994; Pacurar et al., 2012). Using these indel (insertion-deletion/ microsatellite) markers CH1A-CH5E (Appendix 1), with at least 5 markers per chromosome, we found enrichment of the Ws-2 (no-HR) allele at the locus containing marker CH1A. We thus ascertained that the corresponding region on the upper arm of chromosome 1 in Col-0 (where marker CH1A is located, in both Col-0 and Ws-2) was conferring the *Psa* LV5-triggered HR. Using further markers CH1A-2 (Pacurar et al., 2012), INDEL-8A, and INDEL-12A (these two markers were designed in Geneious software [Biomatters, NZ] using genome alignments for Ws-0 and Col-0 in the relevant region; Appendix 1) we fine mapped the HR locus. This led to the identification of a broad 4 Mbp region housing the *NB-LRR* genes *RPS5* and *SUMM2* within the HR-conferring locus (Figure 4.4A).

RPS5 is a CNL that recognizes AvrPphB (also known as AvrPph3) from *P. syringae* pv. *phaseolicola* through its cysteine protease cleavage of the Arabidopsis RLK PBS1 (Shao et al., 2003; Warren et al., 1999). Interestingly, *Psa* LV5 carries an *avrPphB* homolog, *hopAR1* with 96% amino acid identity to AvrPphB (Figure 4.4B). SUMM2 is a CNL that guards the Arabidopsis MPK4 substrate kinase CRCK3 in the MEKK1-MKK1/MKK2-MPK4 signaling pathway targeted by the transgenically expressed phosphothreonine lyase effector HopAI1 (Zhang et al., 2012; Z. Zhang et al., 2017). *Psa* LV5 carries a HopAI1 homolog with 91% amino acid identity (Figure 4.4B), but is like HopAI1 from *Pto* DC3000 in that it lacks a T3S promoter (HrpL binding site) signal and is therefore likely not expressed. Taken together, this is highly suggestive of a

problem in the screening of T3Es undertaken earlier to identify the T3E responsible for *Psa* LV5-triggered HR in Col-0.

4.2.2 A homolog of AvrPphB in *Psa* LV5 triggers *RPS5*-mediated HR in Col-0 when delivered under its native promoter

To examine more closely if the delivery system was in fact responsible for the lack of an HR upon delivery of *Psa* LV5 T3Es, we cloned the *avrPphB* gene (from *P. syringae* pv. *phaseolicola* R3) under the *avrRps4* promoter as we did for

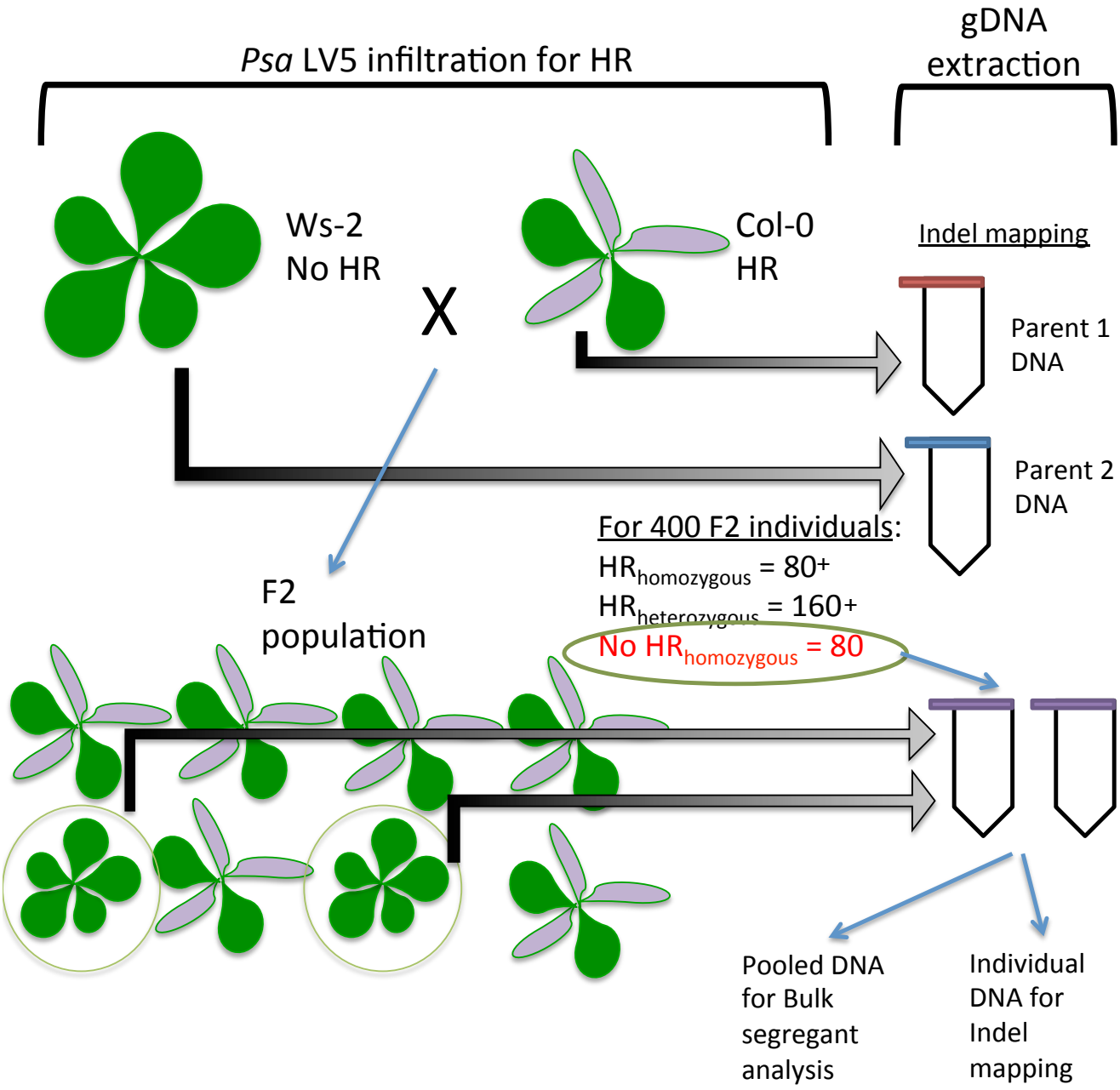


Figure 4.3. Schematic for mapping *R*-gene locus in Col-0 x Ws-2 F2 population. Ws-2 and Col-0 parents were crossed to generate F1 plants from which an F2 population was generated for mapping HR to *Psa* LV5. Roughly 400 individual F2 plants were blunt-syringe infiltrated with *Psa* LV5 (1×10^8 CFU/mL) and the no-HR F2 individuals (~25%) were harvested and genomic DNA extracted, along with Ws-2 and Col-0 parents as controls, for bulk segregant analysis and mapping.

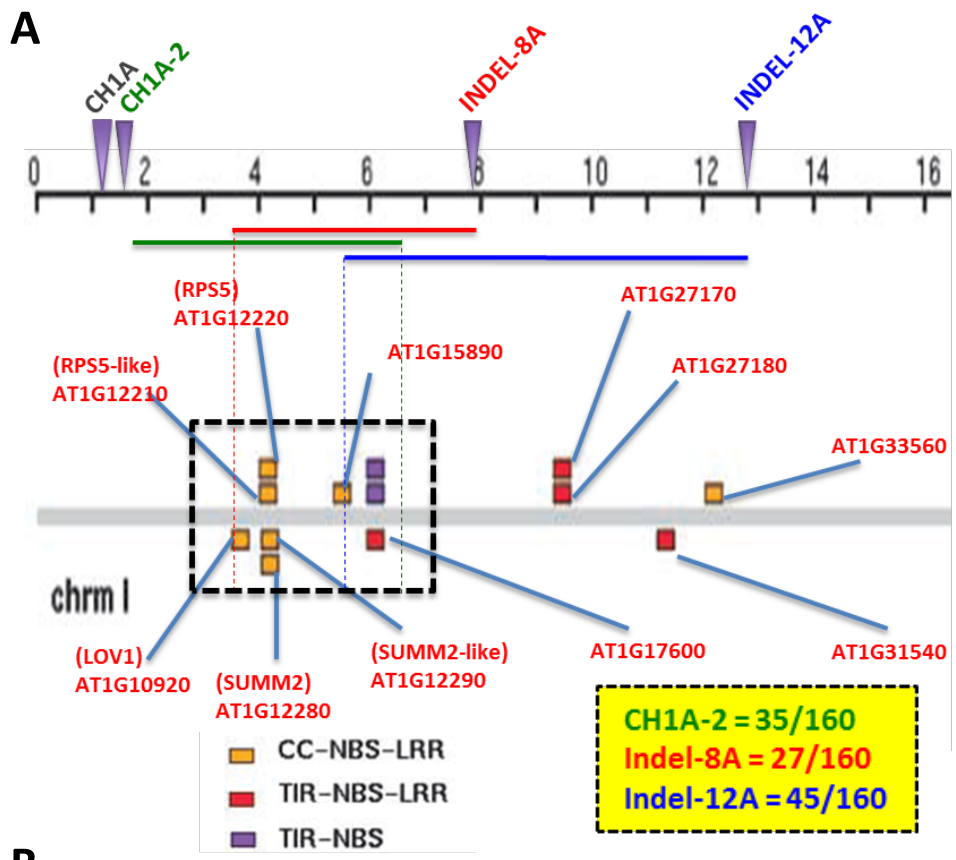
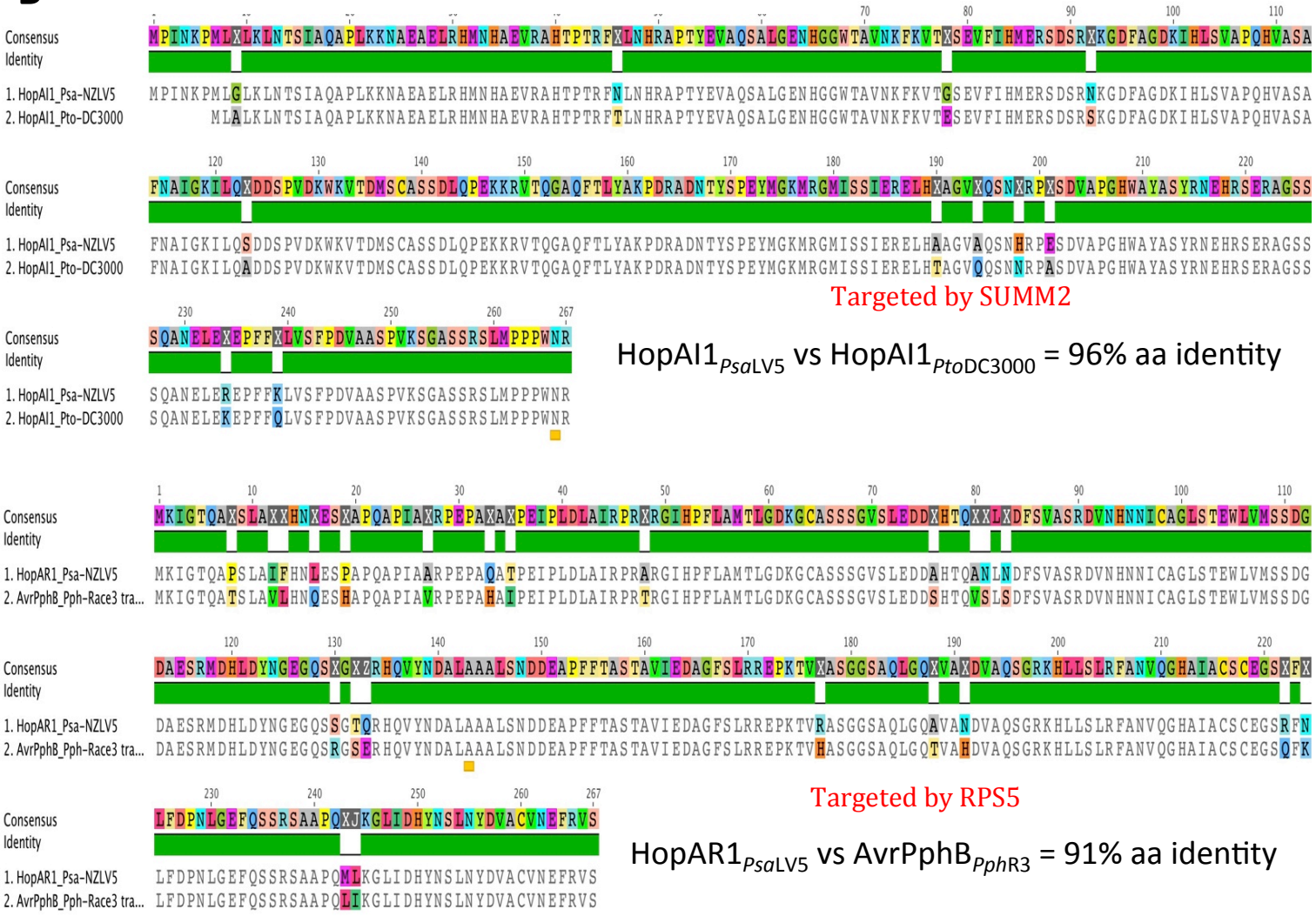


Figure 4.4. Bulk segregant analysis and genotyping identified *RPS5*/*SUMM2* locus conferring HR to *Psa* LV5. (A) Bulk segregant analysis of pooled gDNA from 80 F_2 individuals using indel markers identified the upper arm of chromosome 1 in Arabidopsis Col-0 as the locus conferring HR to *Psa* LV5. Using original screening marker CH1A and three independent indel markers (CH1A-2, INDEL8A, and INDEL12A; refer to Appendix 1) the HR-locus was mapped to a roughly 4Mbp region of chromosome 1 in Col-0 carrying the *RPS5* and *SUMM2* NLR genes. **(B)** ClustalW alignments from Geneious software for *Psa* LV5 homologs of effectors previously shown to be recognized by both SUMM2 and RPS5: HopAI1 (96% amino acid identity) and HopAR1 (91% amino acid identity; also called AvrPphB), respectively.

B



hopAR1 for delivery by Pf0-1(T3S). Similarly, we cloned *hopAR1* under its native promoter into the original pBBR 1MCS-5 vector (blunt ligated into a SmaI site in the MCS). Unfortunately, due to the lack of a clear upstream HrpL site and thus unlikeliness of expression and delivery by the T3SS, this was not done for HopAI1. Pf0-1(T3S) delivery of HopAR1 under a native promoter resulted in a strong HR while AvrPphB delivered from Pf0-1(T3S) under the *avrRps4* promoter did not give an HR (Figure 4.5A). To examine if this poor response to AvrPphB when under the AvrRps4 promoter was a product of inherently poor T3E delivery by Pf0-1(T3S) or was due to the promoter alone, we utilized *Pto* DC3000 to deliver our constructs and tested for electrolyte leakage associated with the development of HR. Notably, delivery of AvrPphB, HopAR1, $HopAI1_{PtoDC3000}$, or $HopAI1_{PsaLV5}$ when expressed from the AvrRps4 promoter failed to trigger ion leakage significantly different from EV (Figure 4.5B). AvrPphB expressed from its native promoter, however, triggered significant ion leakage associated with the development of a strong HR response. Taken together, this strongly suggested that *Psa* LV5-triggered HR in Col-0 may be due to delivery of HopAR1.

To confirm recognition of *Psa* LV5 by RPS5, we tested EMS/T-DNA knockout mutants for every NB-LRR in the *RPS5*/*SUMM2* locus for HR in response to *Psa* LV5, and Pf0-1(T3S) delivering AvrPphB and HopAR1 under their native promoters. T-DNA mutants were checked by genotyping PCR for presence of homozygous T-DNA insertion (#2.5.8): *rps5-3* (SALK_127201), *rps5-4* (SALK_015294), *lov1-1* (SALK_152273), *summ2-8* (SALK_060102), *summ2-like-1* (SALK_012475), *rps5-like-1* (SALK_098988), *at1g15890-1* (SALK_055219), and *soc3-7* (SALK_069931). *rps5-2* (an EMS mutant of *RPS5*), *rps5-3,* and *rps5-4* resulted in significant loss of HR in response to *Psa* LV5 and did not confer HR in response to Pf0-1(T3S) delivery of HopAR1 or AvrPphB, as expected (Figure 4.6A). Quantification of electrolyte leakage from leaf discs of Col-0, *rps5-2* and *rps5-3* plants infiltrated with *Psa* LV5, *Pto* DC3000 wild-type or carrying *avrRpt2* or *avrPphB* confirmed the lack of electrolyte leakage associated with HR in response to *Psa* LV5 and AvrPphB delivery (Figure 4.6B). This result confirms

that the source of *Psa* LV5 avirulence in Arabidopsis Col-0 is most likely to be RPS5 recognition of HopAR1$_{PsaLV5}$.

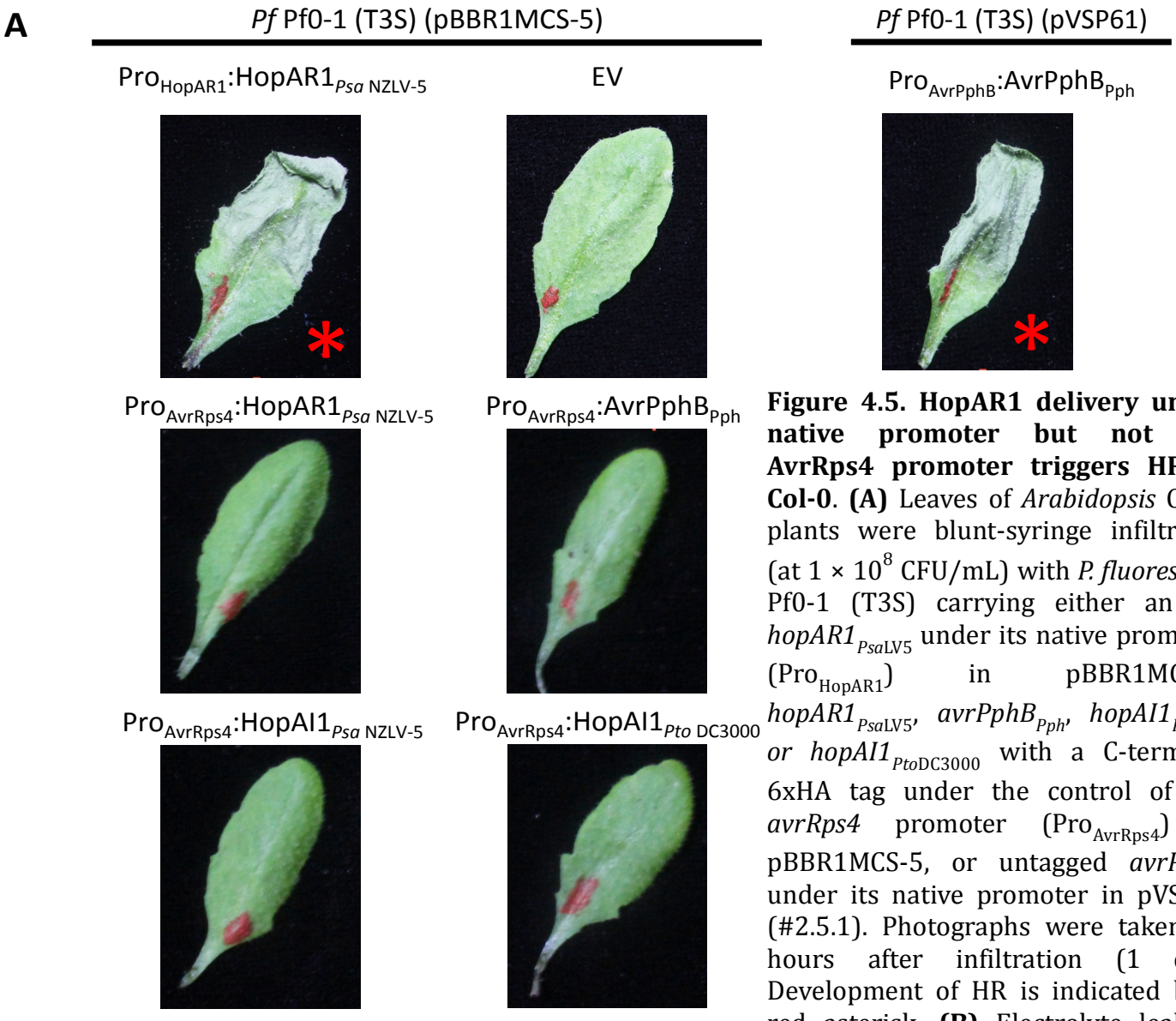
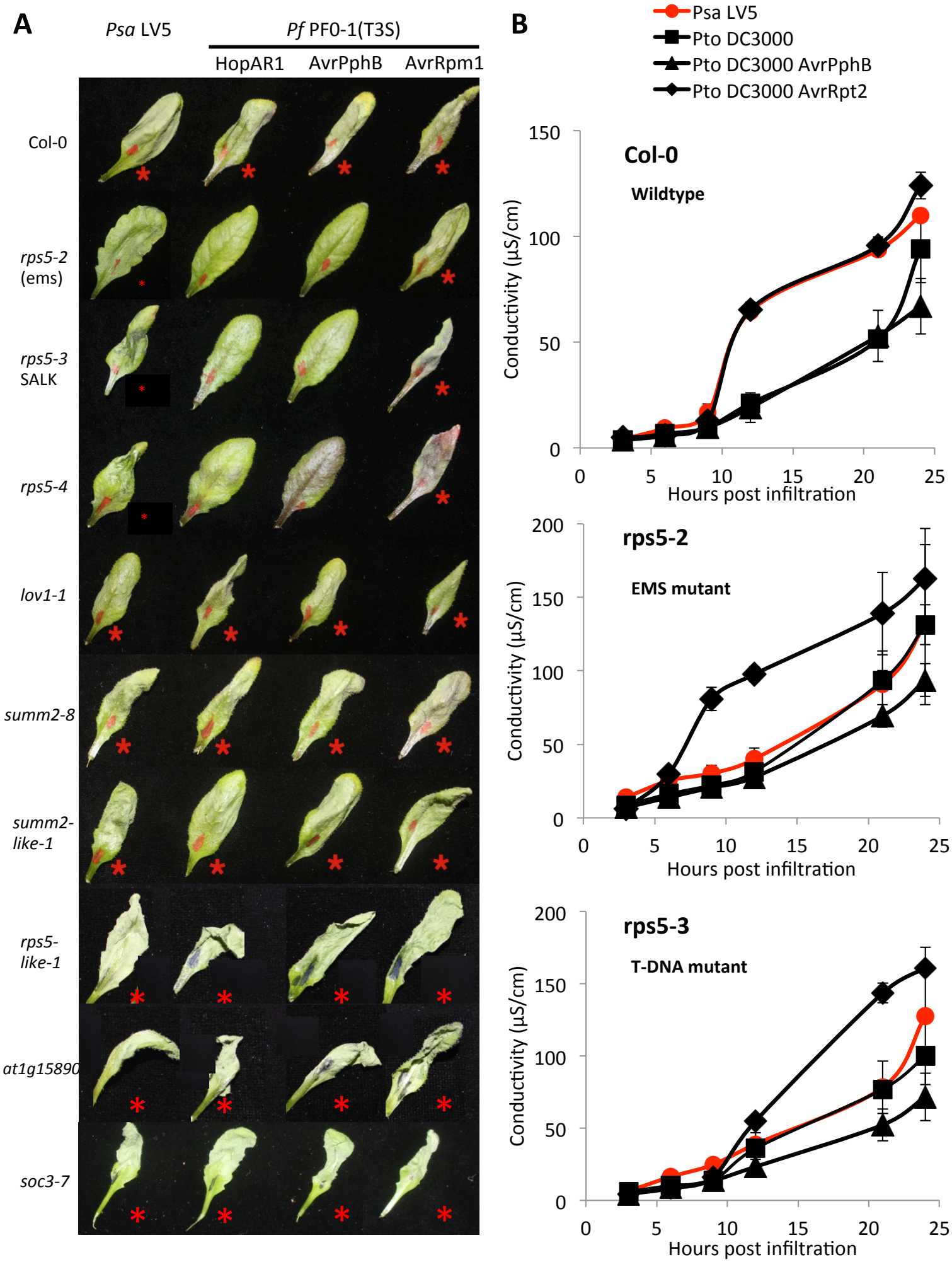


Figure 4.5. HopAR1 delivery under native promoter but not the AvrRps4 promoter triggers HR in Col-0. (A) Leaves of *Arabidopsis* Col-0 plants were blunt-syringe infiltrated (at 1 × 10^8 CFU/mL) with *P. fluorescens* Pf0-1 (T3S) carrying either an EV, *hopAR1*$_{PsaLV5}$ under its native promoter (Pro$_{HopAR1}$) in pBBR1MCS-5, *hopAR1*$_{PsaLV5}$, *avrPphB*$_{Pph}$, *hopAI1*$_{PsaLV5}$ or *hopAI1*$_{PtoDC3000}$ with a C-terminal 6xHA tag under the control of the *avrRps4* promoter (Pro$_{AvrRps4}$) in pBBR1MCS-5, or untagged *avrPphB* under its native promoter in pVSP61 (#2.5.1). Photographs were taken 22 hours after infiltration (1 dpi). Development of HR is indicated by a red asterisk. **(B)** Electrolyte leakage from leaves of Col-0 plants blunt syringe infiltrated (at 1 × 10^8 CFU/mL) with *Pto* DC3000 carrying EV (pBBR1MCS-5), or *avrPphB* under its native promoter in pVSP61, or *avrPphB*, *hopAR1*, *hopAI1*$_{PsaLV5}$ or *hopAI1*$_{PtoDC3000}$ in pBBR1MCS-5 under the *avrRps4* promoter. Electrolyte leakage from leaf discs after infiltration was determined at indicated timepoints (#2.5.2) – note difference to Pf0-1(T3S). The error bars indicate the standard error from four technical replicates. The experiment was conducted twice with similar results.


A
Psa LV5
Pf PF0-1(T3S)
HopAR1
AvrPphB
AvrRpm1
Col-0
rps5-2 (ems)
rps5-3 SALK
rps5-4
lov1-1
summ2-8
summ2-like-1
rps5-like-1
at1g15890
soc3-7
B
Psa LV5
Pto DC3000
Pto DC3000 AvrPphB
Pto DC3000 AvrRpt2
Col-0
Wildtype
rps5-2
EMS mutant
rps5-3
T-DNA mutant
Conductivity (µS/cm)
Hours post infiltration
0
50
100
150
200
0
5
10
15
20
25

4.2.3 *Psa* LV5 triggers AvrPtoB homolog-dependent HCD in *Nicotiana benthamiana*

To test if another model plant species, *Nicotiana benthamiana*, is capable of mounting resistance to *Psa* LV5, we infiltrated *N. benthamiana* leaves with low dose inoculations of *Psa* LV5 (10^5 CFU/mL) with the control avirulent *Pto* DC3000 strain as performed in previous research (Wei et al., 2007). Apart from some minor chlorosis, *Psa* LV5 was unable to grow and trigger necrotic symptoms after 7 days indicating that, like *Pto* DC3000, *Psa* LV5 is avirulent in *N. benthamiana* (Figure 4.7A). A high dose inoculum of *Psa* LV5 (10^8 CFU/mL), however, triggered development of an HR like that seen for *Pto* DC3000 (Figure 4.7B). Therefore, we reasoned that there was a possibility that one or more T3Es from *Psa* LV5 could trigger resistance in *N. benthamiana* in the same way that HopQ1-1 or HopAD1 rendered *Pto* DC3000 avirulent in *N. benthamiana* (Wei et al., 2007, 2015). Interestingly, homologs of neither avirulence effectors from *Pto* DC3000, HopQ1-1 or HopAD1, are present in *Psa* LV5.

We used *Agrobacterium tumefaciens*-mediated transient expression to isogenically observe responses to individual effectors in *N. benthamiana* to avoid the confounding effects of multiple effectors secreted by *Psa* LV5, as achieved previously for other pathogens (Du et al., 2014; Wroblewski et al., 2009). Expression of four homologs of known avirulence effectors from *Psa* LV5 in

(See previous page)

Figure 4.6. *Psa* LV5 triggered HR and ion leakage in *rps5* plants is compromised. **(A)** T-DNA knockout lines for all NLR genes in the *RPS5/SUMM2* locus identified RPS5 as conferring the *Psa* LV5-triggered HR in Col-0. Leaves of *confirmed homozygous* mutant or knockout plants (EMS-mutagenized: *rps5-2*; SALK T-DNA insertion, confimed by method in #2.5.8: *rps5-3/at1g12220*, *rps5-4/at1g12220*, *lov1-1/at1g10920*, *summ2-8/at1g12280*, *summ2-like-1/at1g12290*, *rps5-like-1/at1g12210*, *at1g15890*, and *soc3-7/at1g17600*) were blunt-syringe infiltrated (at 1×10^8 CFU/mL) with *Psa LV5 or P. fluorescens* Pf0-1 (T3S) carrying $hopAR1_{PsaLV5}$, $avrPphB_{Pph}$, or $avrRpm1_{PmaM2}$ under their respective native promoters (#2.5.1). Photographs were taken 20 hours after infiltration (1 dpi). Development of HR is indicated by a red asterisk with the size of the asterisks denoting the strength of response. (rps2-2, rps5-3 and rps5-4 showed some weak cell death/HR at 1 dpi) **(B)** Electrolyte leakage from leaves of Col-0, rps5-2 or rps5-3 plants blunt-syringe infiltrated (at 1×10^8 CFU/mL) with *Psa* LV5 or *Pto* DC3000 carrying EV, or delivering AvrPphB or AvrRpt2 (#2.5.2) – note difference to Pf0-1(T3S). Electrolyte leakage from leaf discs after infiltration was determined at indicated timepoints. The error bars indicate the standard error from four technical replicates. The experiment was conducted twice with similar results.

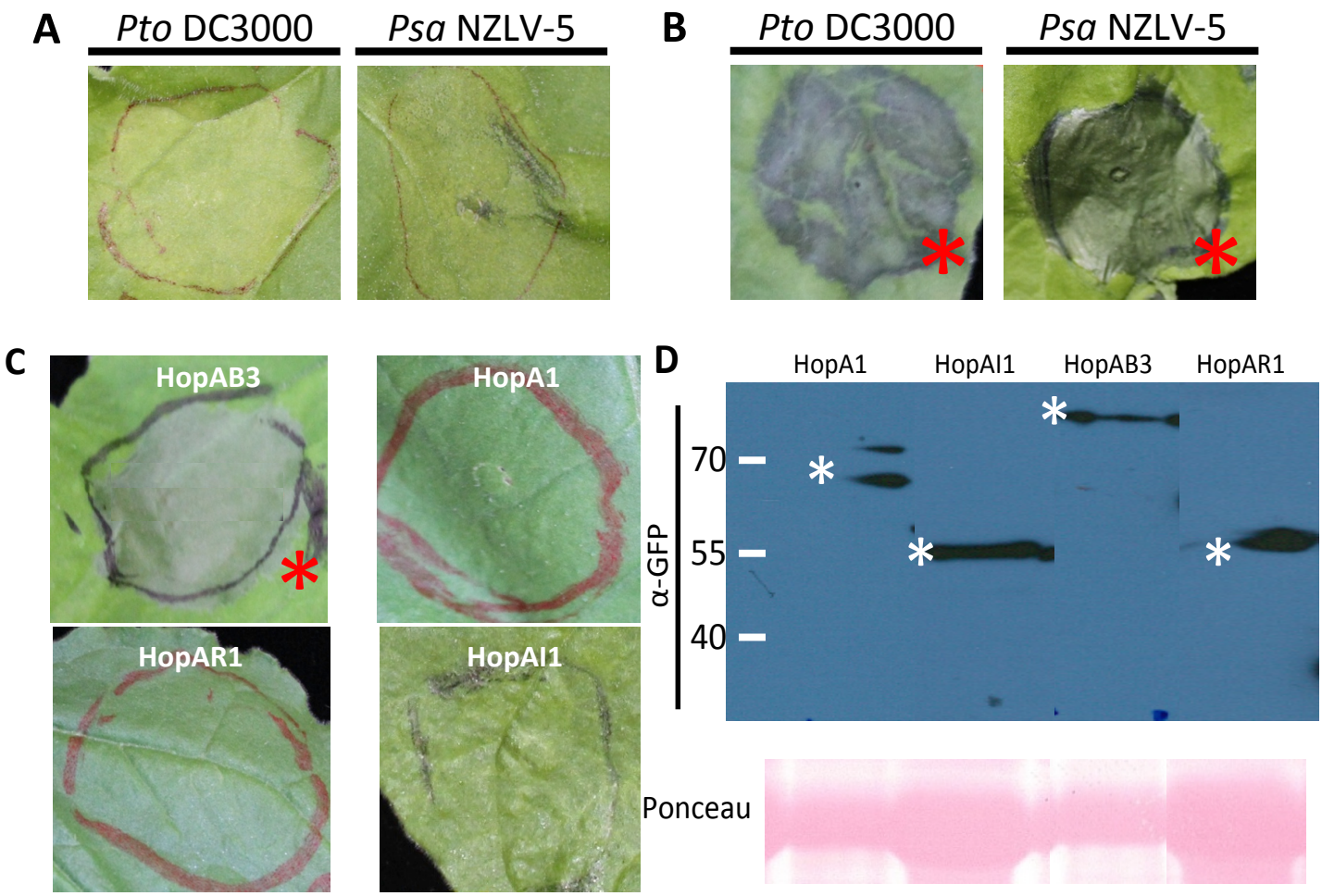


Figure 4.7. *Psa* LV5 triggers HR in *Nicotiana benthamiana* contributed to by HopAB3. (A) Leaves of *Nicotiana benthamiana* were infiltrated with *Psa* LV5 or *Pto* DC3000. Bacterial suspension (1×10^5 CFU/mL) was blunt-syringe infiltrated into the leaves and photographs were taken 7 days after infiltration (identical to method in #2.5.1). **(B)** Leaves of *Nicotiana benthamiana* were infiltrated with *Psa* LV5 or *Pto* DC3000. Bacterial suspension (1×10^8 CFU/mL) was blunt-syringe infiltrated into the leaves and photographs were taken 24 hours after infiltration (1 dpi) (identical to method in #2.5.3). Development of HR is indicated by a red asterisk. **(C)** Known Avr homologs for HopA1, HopAB3, HopAR1 or HopAI1 from *Psa* LV5 were cloned, and were transiently expressed by *Agrobacterium tumefaciens* AGL1 infiltration (OD_{600} = 0.4) under the CaMV 35S promoter with C-terminal YFP tags in *N. benthamiana.* Photographs were taken at 3 days post infiltration (dpi) (#2.5.5). Red asterisks indicate development of HR-like cell death symptoms. This experiment was conducted four times with identical results. **(D)** Samples at 2dpi for transiently expressed effectors from (C) were assayed by western blot using α-GFP antibody (#2.6.4). White asterisks indicate expected size for the T3E+YFP band. Ponceau Red staining of total protein extract showing the Rubisco protein band indicates even sample loading. Numbers to the left of the panel indicate band sizes for the PageRuler™ protein ladder in kDa.

multiple plant species (HopAB3, HopA1, HopAI1 and HopAR1 homologs from *Pto* DC3000 or *P. syringae* pv. *glycinea* in tomato, Arabidopsis or tobacco) under the control of a 35S CaMV promoter and tagged with YFP identified an HR-like cell death response (HCD) triggered by HopAB3 only (Figure 4.7C) (Gassmann, 2005; Kim et al., 2002; Simonich and Innes, 1995; Zhang et al., 2007). All four T3Es were expressed well at 2 dpi as determined by an immunoblot against the YFP tag by α–GFP antibodies (Figure 4.7D).

HopAB3 from *Psa* LV5 is a homolog of AvrPtoB from *Pto* DC3000 (64% amino acid identity; hereafter AvrPtoB), HopAB3 from *Pto* T1, and VirPphA from *Psy* B728a (45% amino acid identity; hereafter VirPphA), forming a large family of E3 ubiquitin ligase domain-carrying effectors predominantly from tomato and bean pathogens (Figure 4.8A-B; (Lin et al., 2006)). AvrPtoB has been studied extensively and is a virulence factor targeting PTI through suppression of RLKs involved in signaling (Gimenez-Ibanez et al., 2009; Goehre et al., 2008). AvrPtoB and its homologs are detected in resistant wild tomato *Solanum pimpinellifolium* by the combined action of kinase decoy Pto and NB-LRR Prf; genes for which

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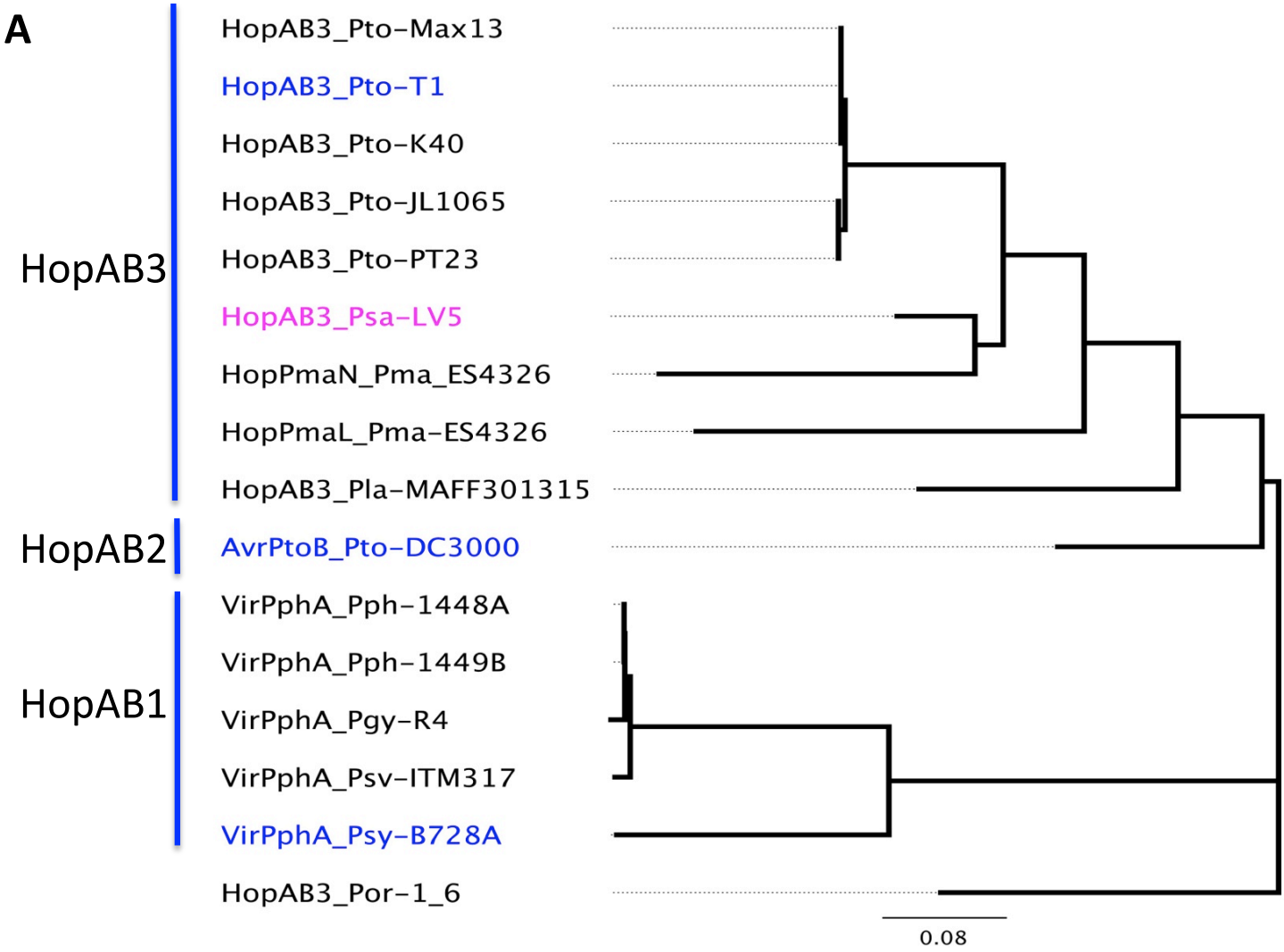
Figure 4.8. HopAB3 is a homolog of AvrPtoB and VirPphA. **(A)** HopAB3 (in pink) from *Psa* LV5 is a homolog of E3 ligase carrying effector AvrPtoB/HopABs from multiple *Pto* and *Pma* strains. This Jukes Cantor neighbour-joining tree was generated using Geneious software with various HopAB family members: HopAB3_Pto-Max13 (*P. syringae* pv. *tomato* Max13), HopAB3_Pto-T1 (*P. syringae* pv. *tomato* T1), HopAB3_Pto-K40 (*P. syringae* pv. *tomato* K40), HopAB3_Pto-Jl1065 (*P. syringae* pv. *tomato* JL1065), HopAB3_Pto-T23 (*P. syringae* pv. *tomato* T23), HopPmaN_Pma-ES4326 (*P. syringae* pv. *maculicola* ES4326), HopPmaL_Pma-ES4326 (*P. syringae* pv. *maculicola* ES4326), HopAB3_Pla-MAFF301315 (*P. syringae* pv. *lachrymans* MAFF301315), AvrPtoB_Pto-DC3000 (*P. syringae* pv. *tomato* DC3000), VirPphA_Pph-1448A (*P. syringae* pv. *phaseolicola* 1448A), VirPphA_Pph-1449B (*P. syringae* pv. *phaseolicola* 1449B), VirPphA_Pgy-R4 (*P. syringae* pv. *glycinea* R4), VirPphA_Psv-ITM317 (*P. savastanoi* pv. *savastanoi* ITM317), VirPphA_Psy-B728a (*P. syringae* pv. *syringae* B728a), HopAB3_Por-1_6 (*P. syringae* pv. *oryzae* 1_6). Node length for the tree is indicated as are the sub-clades and their type strains in blue (AvrPtoB/HopAB3$_{Pto\text{T1}}$, AvrPtoB/HopAB2$_{Pto\text{DC3000}}$, and VirPphA/HopAB1$_{Psy\text{B728a}}$). **(B)** ClustalW alignment between HopAB3$_{Psa\text{LV5}}$ and type strains AvrPtoB$_{Pto\text{DC3000}}$ and VirPphA$_{Psy\text{B728a}}$ was generated in Geneious software. The percentage amino acid identity between each of the three comparisons is indicated above the alignment. The presence of all previously published functional peptide fragments and required functional residues in AvrPtoB$_{Pto\text{DC3000}}$ are indicated, including Pto-recognized 1-307aa fragment, Pto/Fen-recognized 1-387aa fragment, Pto-interacting (PID) F173, Pto/Fen-interacting (FID) G325, putative phosphorylation site T450, E3 ligase catalytic residues (F479, F525, F533), and putative auto-ubiquitinated lysines (K512, K520, K521, K529). Note that the F525 residue in the catalytic site is substituted for a histidine in VirPphA$_{Psy\text{B728a}}$.


A
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HopAB3_Pto-T1
HopAB3_Pto-K40
HopAB3_Pto-JL1065
HopAB3_Pto-PT23
HopAB3_Psa-LV5
HopPmaN_Pma_ES4326
HopPmaL_Pma-ES4326
HopAB3_Pla-MAFF301315
AvrPtoB_Pto-DC3000
VirPphA_Pph-1448A
VirPphA_Pph-1449B
VirPphA_Pgy-R4
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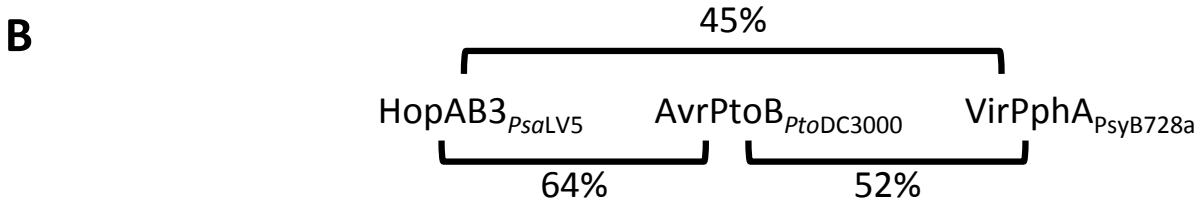
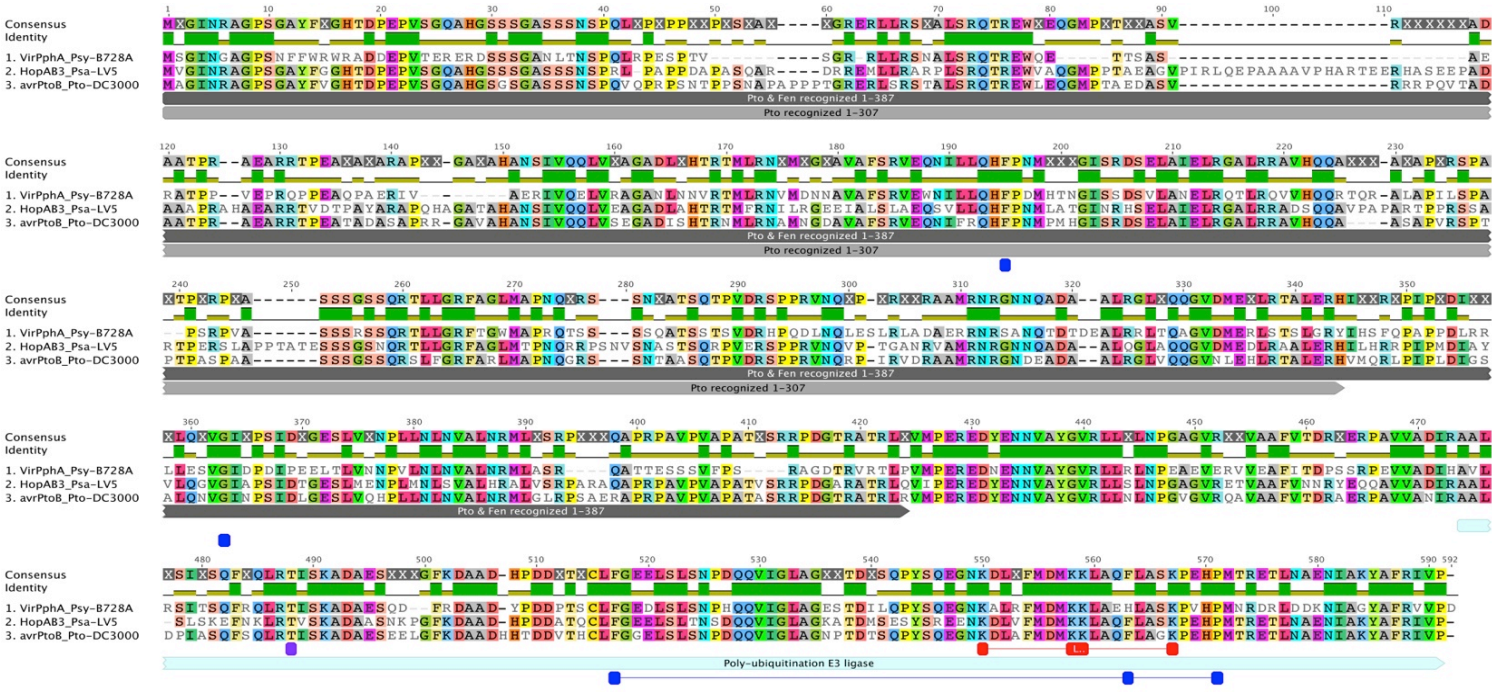
VirPphA_Psy-B728A
HopAB3_Por-1_6
HopAB3
HopAB2
HopAB1
0.08


B
45%
HopAB3PsaLV5
AvrPtoBPtoDC3000
VirPphAPsyB728a
64%
52%


Consensus
Identity
1. VirPphA_Psy-B728A
2. HopAB3_Psa-LV5
3. avrPtoB_Pto-DC3000
Pto & Fen recognized 1-387
Pto recognized 1-307
Poly-ubiquitination E3 ligase

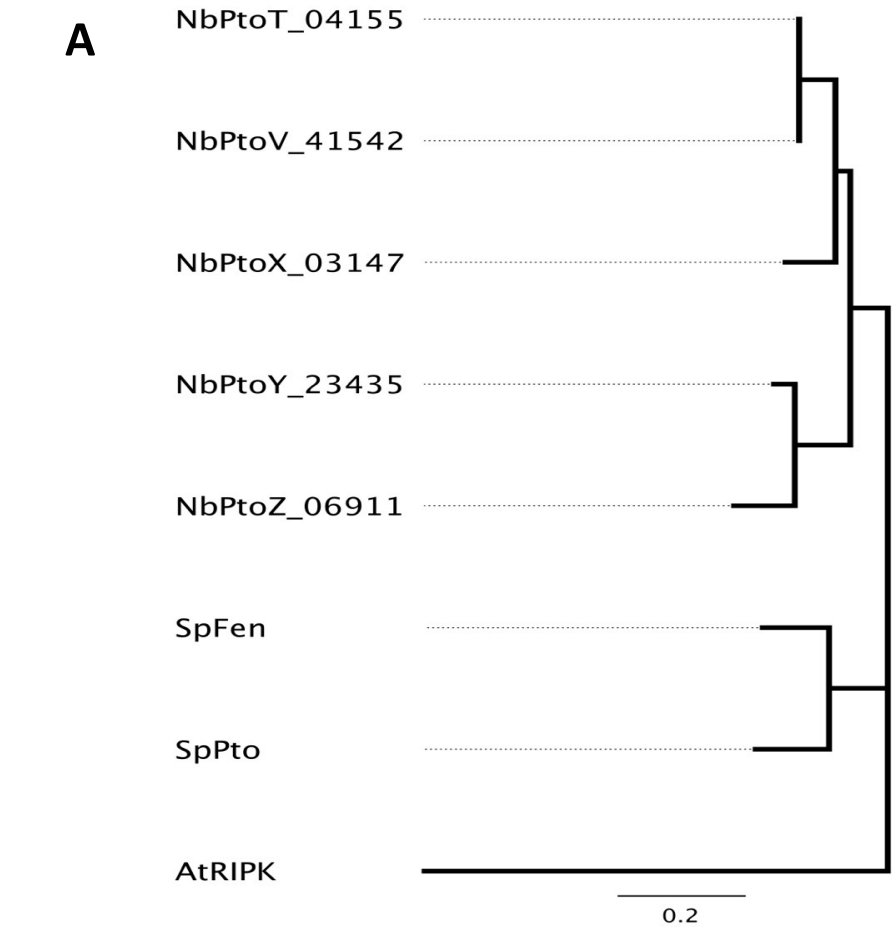
have been introgressed into cultivated tomato *Solanum lycopersicum* (Lin and Martin, 2007; Mathieu et al., 2014; Ntoukakis et al., 2013; Salmeron et al., 1994). AvrPtoB has also been found to require its E3 ligase activity to avoid detection by another kinase decoy Fen (in combination with Prf), which it degrades through the host proteasomal pathway. Known key amino acid residues required for this activity, as well as Fen and Pto recognition, are conserved in HopAB3 (Figure 4.8B; (Abramovitch et al., 2006; Mathieu et al., 2014; Rosebrock et al., 2007)).

4.2.4 HopAB3 is recognized by Pto but not Fen

Interestingly, no homolog of AvrPtoB is known to trigger HCD in *N. benthamiana* when expressed alone (Lin et al., 2006). However, sequencing of the *N. benthamiana* genome identified of homologs of Pto (Figure 4.9A) but more importantly Prf (Figure 4.9B) (Bombarely et al., 2012). NbPrf could serve as a native recognition determinant in *N. benthamiana* and explains previously reported recognition of AvrPtoB in *N. benthamiana* only expressing Pto (Mucyn et al., 2006). However, HopAB3 is recognized when expressed alone in *N. benthamiana.* To examine this in detail, HopAB3 and AvrPtoB were transiently expressed at OD_{600} = 0.4 where only HopAB3 triggered HCD or OD_{600} = 0.1 where neither triggered visible HCD at 2-3 dpi (Figure 4.10A).

At this lower OD, when Pto (from *S. pimpinellifolium*; *Sp*Pto) is expressed, only HopAB3 can trigger strong HCD, possibly through recruitment of native *N. benthamiana* Prf homologs, while *Sp*Pto alone does not trigger a response and AvrPtoB only triggers a weak HCD as reported previously (Mucyn et al., 2006). Interestingly, when *Sp*Pto and *Sp*Prf are coexpressed with either HopAB3 or AvrPtoB, the HCD triggered appears equally strong, suggesting a difference between AvrPtoB and HopAB3 for requirement of *Sp*Prf (HopAB3 does not require *Sp*Prf). However, due to the ability of HopAB3 alone to trigger HCD in *N. benthamiana* no clear conclusions about Pto/Prf requirement can be drawn. Notably, we did not see a ligand-independent HCD as reported previously for *Sp*Pto + *Sp*Prf under our conditions (Mucyn et al., 2006). Nevertheless, these results suggest that *Sp*Pto (with *Sp*Prf) recognizes HopAB3 in *N. benthamiana* in

a manner similar to the recognition seen for AvrPtoB, contributed to by a further recognition event native to *N. benthamiana*, possibly through Pto/Prf homologs.



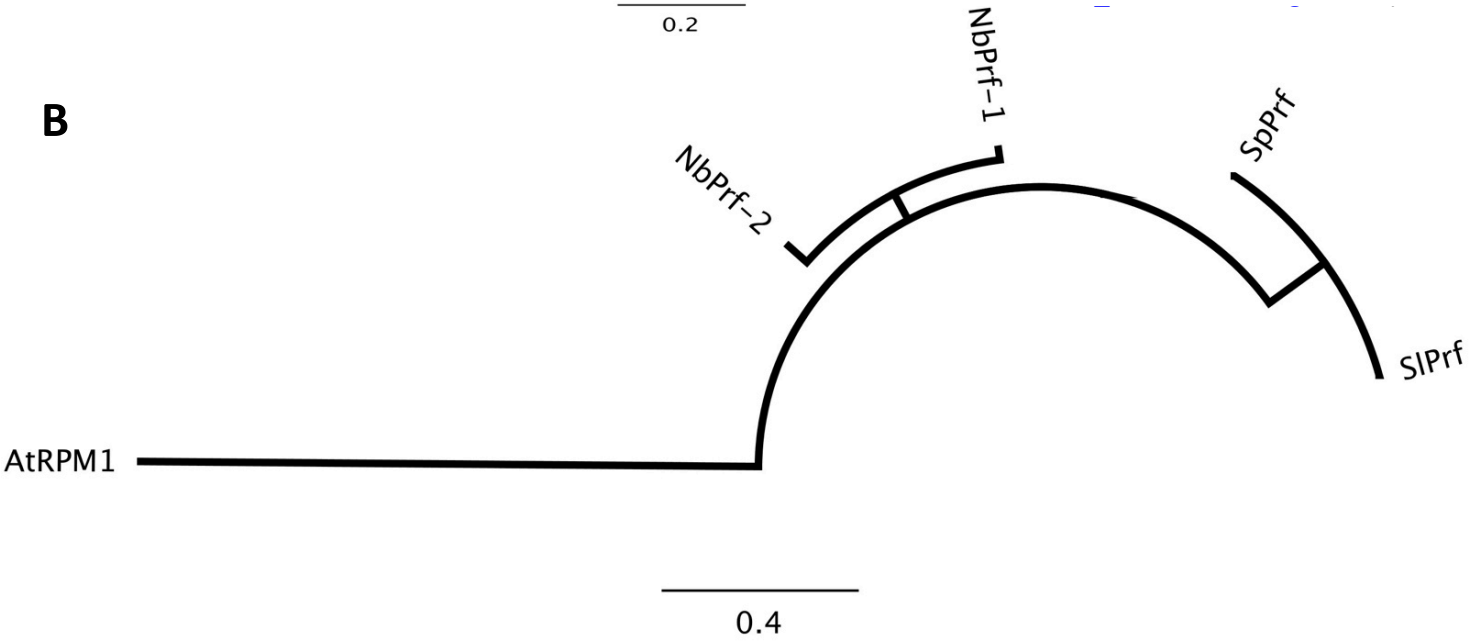


Figure 4.9. Pto and Prf homologs are present in *N. benthamiana*. **(A)** *N. benthamiana* has at least five full-length homologs of Fen/Pto receptor-like kinases (RLKs), *Sp*Fen and *Sp*Pto, from BLAST sequences of the Solgenomics database. Jukes Cantor neighbour-joining tree was generated using Geneious software with Arabidopsis RLK, AtRIPK, set as outgroup. The node length is indicated. **(B)** *N. benthamiana* has two homologs of the NB-LRR Prf from cultivated (*Sl*Prf) and wild tomato (*Sp*Prf), denoted *Nb*Prf-1 and *Nb*Prf-2, from BLAST sequences of the Solgenomics database. Jukes Cantor neighbour-joining tree was generated using Geneious software with Arabidopsis NB-LRR, *At*RPM1, set as outgroup. The node length is indicated.
(Source for *N. benthamiana* sequences:https://solgenomics.net/organism/Nicotiana_benthamiana/genome)

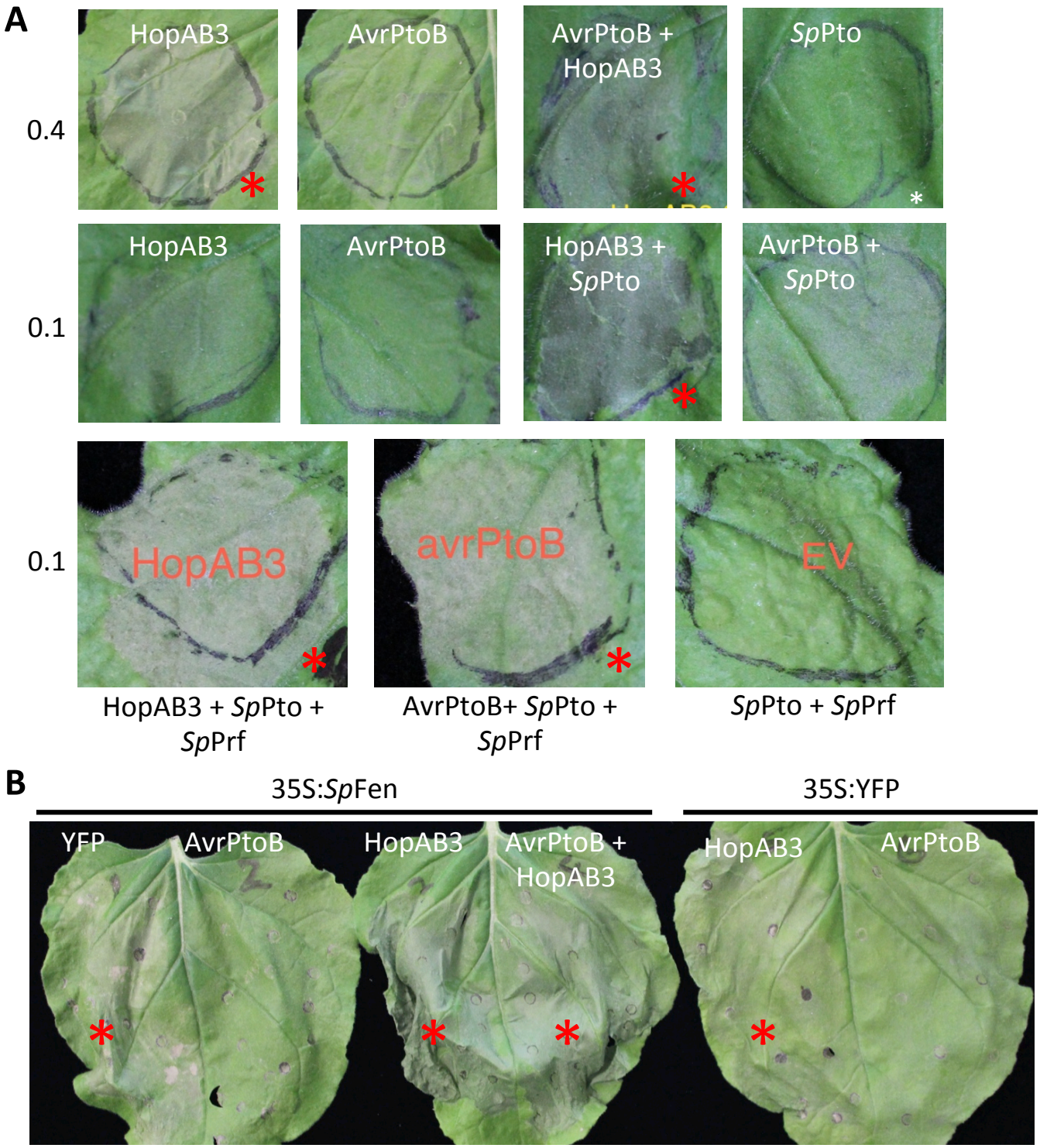


Figure 4.10. HopAB3 triggers Pto-mediated but not Fen-mediated HR in *N. benthamiana*. (A) AvrPtoB HopAB3 with a C-terminal YFP tag was transiently expressed by *Agrobacterium tumefaciens* AGL1 infiltratic alone (OD_{600} = 0.4 or 0.1), together (OD_{600} = 0.4), with *Sp*Pto (AvrPtoB/HopAB3 at OD_{600} = 0.1; *Sp*Pto at OD_{60} 0.4), or with *Sp*Pto and *Sp*Prf (AvrPtoB/HopAB3 at OD_{600} = 0.1; *Sp*Pto/*Sp*Prf at OD_{600} = 0.4). All constructs we expressed under the CaMV 35S promoter except for *Sp*Prf, which was expressed under its native promoter in *benthamiana.* OD was adjusted for each infiltration using expression of just YFP-tag. Numbers indicated on t left are OD for HopAB3, AvrPtoB or YFP tag (EV) used in that panel (#2.5.5). Photographs were taken at 3 da post infiltration (dpi). Red asterisks indicate development of cell death symptoms. The smaller asterisk f AvrPtoB + *Sp*Pto indicates a weaker cell death response. This experiment was conducted three times with simil results. **(B)** AvrPtoB or HopAB3 with a C-terminal YFP tag was transiently expressed by *Agrobacteriu* infiltration (OD_{600} = 0.2) alone, or together with SpFen (OD_{600} = 0.2) under the CaMV 35S promoter in *benthamiana.* OD was adjusted for each infiltration using expression of just YFP-tag (#2.5.5). Photographs we taken at 3 days post infiltration (dpi). Red asterisks indicate development of cell death symptoms. Th experiment was conducted three times with similar results.

Interestingly, *N. benthamiana* native recognition of HopAB3 was not suppressed by coexpression of HopAB3 and AvrPtoB implying that Fen homologs in *N. benthamiana* are not responsible for HopAB3-induced HCD, as AvrPtoB should facilitate their degradation (Figure 4.10A). To test this further, we expressed *Sp*Fen in *N. benthamiana*, which has been shown to trigger an ectopic HCD-type response (Rosebrock et al., 2007). Coexpression of AvrPtoB could suppress the *Sp*Fen-triggered HCD, but neither HopAB3 expression alone nor HopAB3 + AvrPtoB dual expression appeared to suppress *Sp*Fen-triggered HCD, although it is possible that an unidentified *N. benthamiana* R protein-mediated recognition of HopAB3 is responsible for the HCD seen in these latter two samples (Figure 4.10B). Nevertheless, this suggests that a homolog of *Sp*Fen is unlikely to be responsible for HopAB3-triggered recognition since the coexpression of AvrPtoB did not block recognition of HopAB3 in *N. benthamiana* (Figure 4.10A-B). However, the possibility remains that if HopAB3 lacked E3 ligase activity, *Sp*Fen could trigger HCD before degradation by AvrPtoB, especially since the ubiquitination/degradation would be acting in *trans* to the recognition event.

4.2.5 HopAB3-triggered HCD is dependent on its fully functional E3 ligase domain

To explore a possible role for a weakened E3 ligase activity in HopAB3, we utilized a domain-swap approach. To determine which domains in HopAB3 conferred native *N. benthamiana* HCD compared to known domains in AvrPtoB, we generated N-terminus/C-terminus swaps between AvrPtoB and HopAB3 (Figure 4.11A). The domain swaps were carried out between AvrPtoB and HopAB3 in a conserved region at the 395th and 425th amino acid residue, respectively, effectively swapping the N-terminus Pto/Fen-binding regions (carrying the demonstrated AvrPtoB-mediated PTI suppression activity) and E3-ligase regions (Abramovitch et al., 2006). We opted for a region slightly upstream of the predicted E3 ligase regions (at 436aa and 466aa, respectively, in AvrPtoB and HopAB3) due to the clearer delineation of the Pto- and Fen-binding sites in the N-terminus.

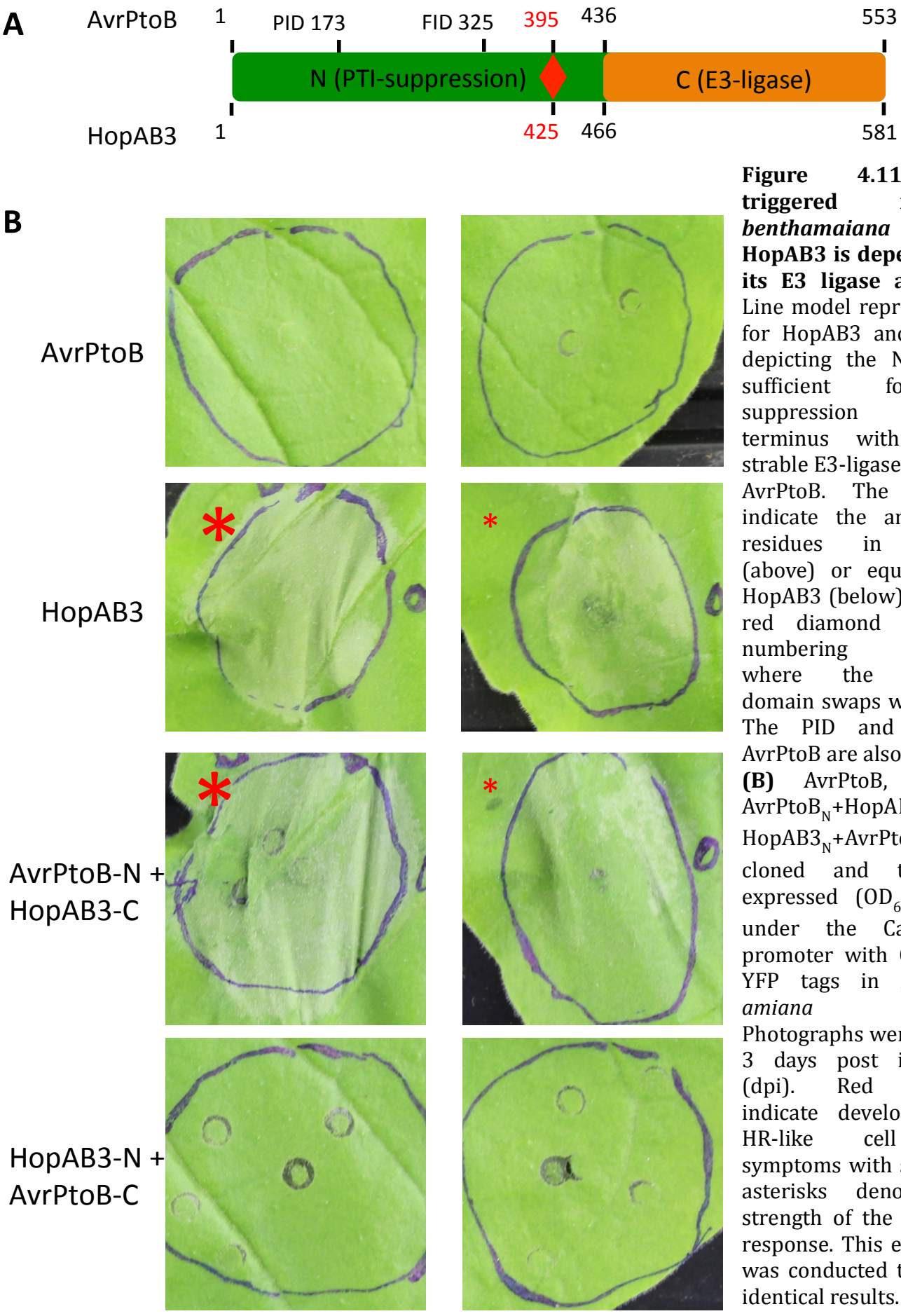


Figure 4.11. HCD triggered in *N. benthamaiana* by HopAB3 is dependent on its E3 ligase allele. (A) Line model representation for HopAB3 and AvrPtoB depicting the N-terminus sufficient for PTI suppression and C-terminus with demonstrable E3-ligase activity in AvrPtoB. The numbers indicate the amino acid residues in AvrPtoB (above) or equivalent in HopAB3 (below) while the red diamond with red numbering indicates where the E3-ligase domain swaps were made. The PID and FID for AvrPtoB are also indicated. **(B)** AvrPtoB, HopAB3, $AvrPtoB_N$+$HopAB3_C$ or $HopAB3_N$+$AvrPtoB_C$ were cloned and transiently expressed (OD_{600} = 0.5) under the CaMV 35S promoter with C-terminal YFP tags in *N. benthamiana* (#2.5.5). Photographs were taken at 3 days post infiltration (dpi). Red asterisks indicate development of HR-like cell death symptoms with size of the asterisks denoting the strength of the cell death response. This experiment was conducted twice with identical results.

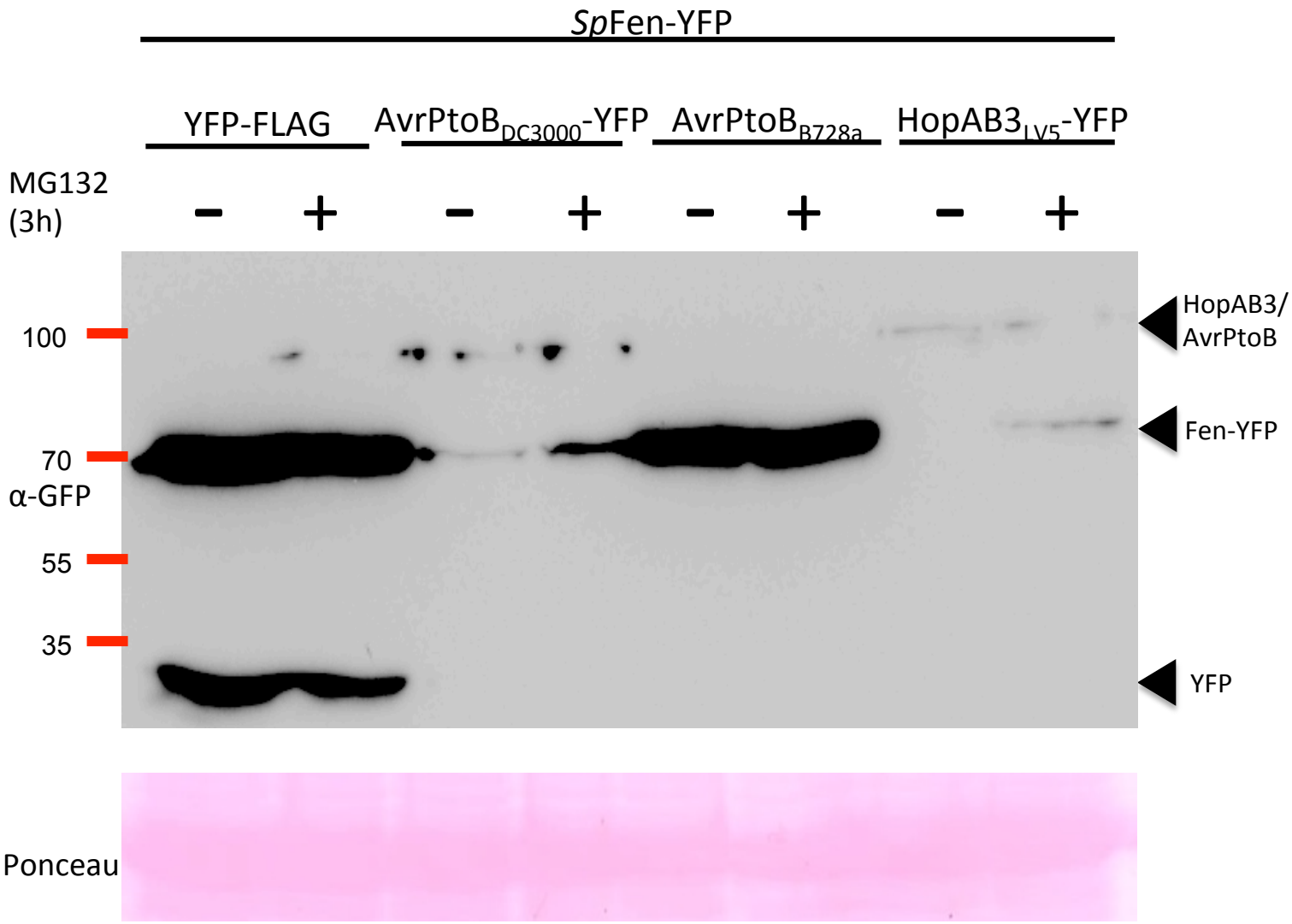


Figure 4.12. HopAB3 and AvrPtoB$_{PtoDC3000}$ both degrade *Sp*Fen in *N. benthamiana*. Detection of YFP-tagged *Sp*Fen (*Agrobacterium tumefaciens* AGL1, OD_{600} = 0.6) from transient expression coinfiltrated with YFP-tag alone, YFP-tagged AvrPtoB$_{PtoDC3000}$, YFP-tagged HopAB3 or untagged VirPphA/AvrPtoB$_{PsyB728a}$ (OD_{600} = 0.1) in *N. benthamiana* and infiltrated with 3 µM $LaCl_3$ at 0 and 1 dpi to suppress cell death (Sasabe et al., 2000). Protein was extracted from leaf samples at 2 dpi with or without pretreatment with 100µM solution of MG132 3 hours before harvest. Protein presence in total extract was tested by immunoblot using α-GFP antibody (#2.6.4). Ponceau Red staining of total protein extract showing the Rubisco protein band indicates even sample loading. Numbers to the left of the panel and associated red bars indicate band sizes for the PageRuler™ protein ladder in kDa.This experiment was conducted twice with identical results.

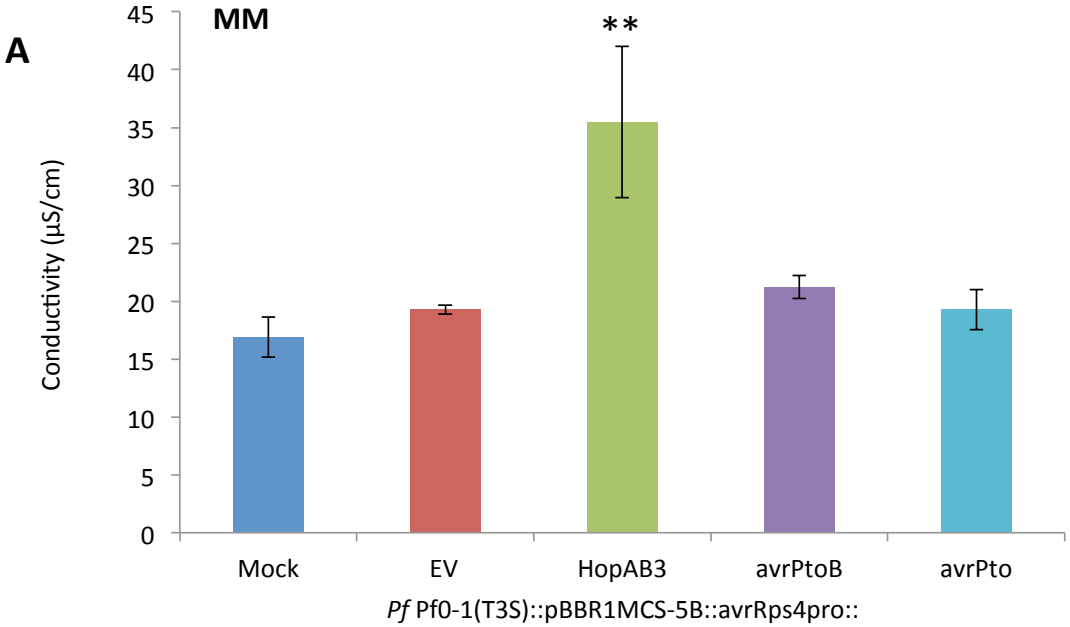
Interestingly, we found that the *N. benthamiana* HCD is determined by the presence of the C-terminus of HopAB3 at both OD 0.4 and 0.1 (Figure 4.11B). Conversely, neither AvrPtoB nor its E3 ligase domain could trigger HCD when coupled to the N-terminus of HopAB3. This clearly suggests that, since the known Pto- and Fen-binding sites are present in the N-terminus of AvrPtoB, the differential binding of a Pto/Fen homolog(s) could not be the determinant of HCD. It is more likely that a non-functional E3 ligase domain in HopAB3 is responsible for a Fen-like *N. benthamiana* homolog triggering HCD.

To demonstrate this lack of E3 ligase activity, we checked whether HopAB3 can degrade YFP-tagged *Sp*Fen *in planta*. When coinfiltrated with AvrPtoB (with a functional E3 ligase activity), *Sp*Fen was degraded and could not be detected in an immunoblot with the α–GFP antibody (Figure 4.12). When the known E3 ligase defective VirPphA from *P. syringae* pv. *syringae* B728a is coinfiltrated, Fen accumulation is indistinguishable from empty vector (YFP-tag alone) coinfiltration. Surprisingly, HopAB3 coinfiltration lacked *Sp*Fen accumulation, suggesting a functional E3 ligase activity (Figure 4.12). MG132 treatment to block proteasomal degradation could at least partially rescue some *Sp*Fen accumulation in both HopAB3 and AvrPtoB samples, demonstrating that the lack of *Sp*Fen accumulation in the HopAB3/AvrPtoB samples is likely to be due to proteasomal degradation of Fen through E3 ligase activity of HopAB3/AvrPtoB. This result contradicted our prediction of E3 ligase domain functionality for HopAB3. Therefore, HopAB3 appears to be an AvrPtoB homolog with a functional E3 ligase that is detected in *N. benthamiana* independent of Pto, possibly through a Pto-like homolog native to *N. benthamiana*.

4.2.6 HopAB3 triggers ion leakage in tomato putatively dependent on a Fen homolog, *Sl*PtoB

Due to our inability to easily distinguish between the different Pto homologs in *N. benthamiana* (Figure 4.9A), we decided to investigate other recognition events of HopAB3 that could be introduced into *N. benthamiana*. AvrPtoB can trigger resistance measurable through quantification of HR and ion leakage in a Pto-

dependent manner in tomato plants (Lin and Martin, 2005). Conversely, since HopAB3 can trigger Pto-independent HCD in *N. benthamiana*, we investigated if HopAB3 is able to trigger HR and ion leakage when delivered by Pf0-1(T3S) in tomato lines that lack Pto. Using *S. lycopersicum* cv. Moneymaker (MM), Pf0-1(T3S)-delivery of AvrPtoB or AvrPto (from *Pto* JL1065) was unable to trigger any significant ion leakage associated with HR, as was expected due to the lack of



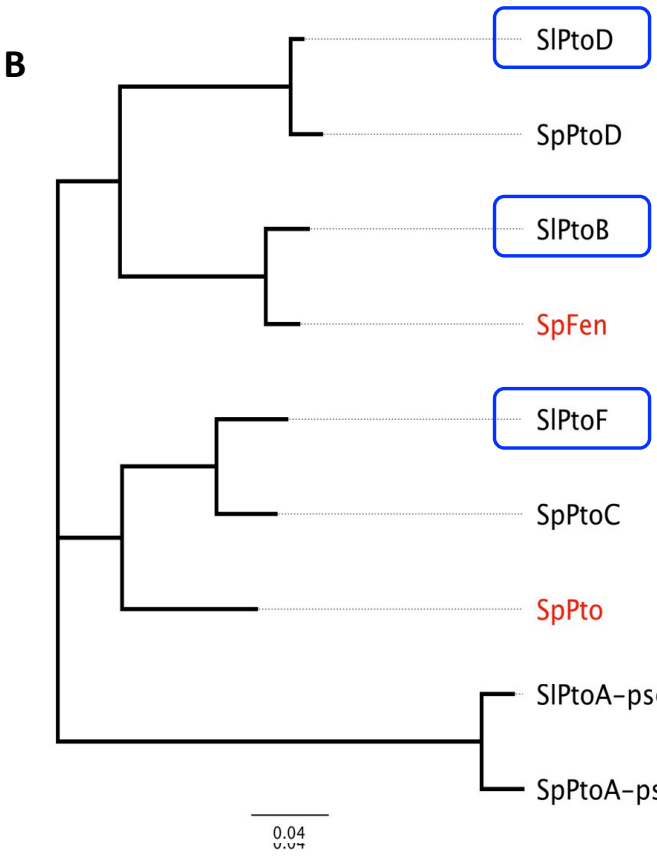


Figure 4.13. HopAB3 triggers ion leakage in tomato putatively dependent on a Pto homolog. (A) Electrolyte leakage from *Solanum lycopersicum* cv. Moneymaker leaf discs mock treated or infiltrated with *P. fluorescens* Pf0-1(T3S) carrying either EV, HopAB3, AvrPtoB$_{PtoDC3000}$ or AvrPto$_{PtoJL1065}$ by blunt syringe at OD_{600}= 0.2 (infiltrations identical to method in #2.5.1 but with a single timepoint for ion leakage determination at 6 hpi, similar to method in #2.5.6). Error bars represent standard deviation from six technical replicates. Asterisks indicate results of Student's *t*-test between selected samples; ** (P < 0.01). This experiment was conducted twice with identical results. **(B)** *S. lycopersicum* has at least three full-length homologs (*Sl*PtoB, *Sl*PtoD, and *Sl*PtoF indicated by blue squares) of *Sp*Fen and *Sp*Pto, indicated in red, from BLAST sequences of the Solgenomics database. Jukes Cantor neighbour-joining tree was generated using Geneious software. The node length is indicated. (Source of *Solanum lycopersicum* sequences: https://solgenomics.net/organism/Nicotiana_benthamiana/genome)

Pto-mediated recognition in this plant (Figure 4.13A). Interestingly, we saw a small but clear ion leakage due to HopAB3-triggered HR in MM leaves.

The tomato line *S. lycopersicum* cv. VFNT Cherry is known to carry several Pto/Fen-like homologs (Chang et al., 2002). We generated a tree for these homologs and predicted that the recognition of HopAB3 in MM plants was likely due to one or more of these homologs: *Sl*PtoB (most Fen-like), *Sl*PtoC, *Sl*PtoD, or *Sl*PtoF (Figure 4.13B). We therefore cloned and individually expressed each of these homologs from tomato (MM) in *N. benthamiana* with a YFP-tag. *Sl*PtoC could not be cloned due to a lack of amplification of its corresponding gene. We found that transiently expressing *Sl*PtoB triggered a HCD like that triggered by *Sp*Fen, at 3 dpi (Figure 4.14A; compare Figure 4.10B – *Sp*Fen + YFP). Interestingly, when coexpressed with AvrPtoB, HopAB3, or YFP alone, even at the lower OD_{600} = 0.3 and at 2dpi when symptoms of *Sp*Fen/*Sl*PtoB (with YFP alone) did not develop, HopAB3 triggered HCD when coexpressed with *Sl*PtoB while AvrPtoB did not (Figure 4.14B).

Coexpression of domain swapped variants of HopAB3 (with E3 ligase domain of AvrPtoB) or AvrPtoB (with E3 ligase domain of HopAB3) demonstrated that the *Sl*PtoB-mediated HCD phenotype was dependent on the presence of the E3 ligase domain of HopAB3 (Figure 4.14C). To check if there is indeed differential degradation of *Sp*Fen/*Sl*PtoB, we coexpressed them with HopAB3/AvrPtoB alleles. Supporting our previous findings, HopAB3 is unable to completely degrade *Sl*PtoB despite possessing full E3 ligase activity against *Sp*Fen (Figure 4.15). Notably, AvrPtoB was unable to degrade *Sl*PtoB and this may be due to an inability to interact with it since it still possesses E3 ligase activity against *Sp*Fen. The E3 ligase mutant, VirPphA/AvrPtoB$_{Psy\text{B728a}}$ was equally unable to degrade *Sp*Fen/*Sl*PtoB. This suggests that the HR triggered by HopAB3 in tomato is at least partially contributed to by *Sl*PtoB, the first known recognition by a cultivated tomato homolog of Pto/Fen to recognize an AvrPtoB family member that carries a fully functional E3 ligase domain capable of *Sp*Fen degradation.

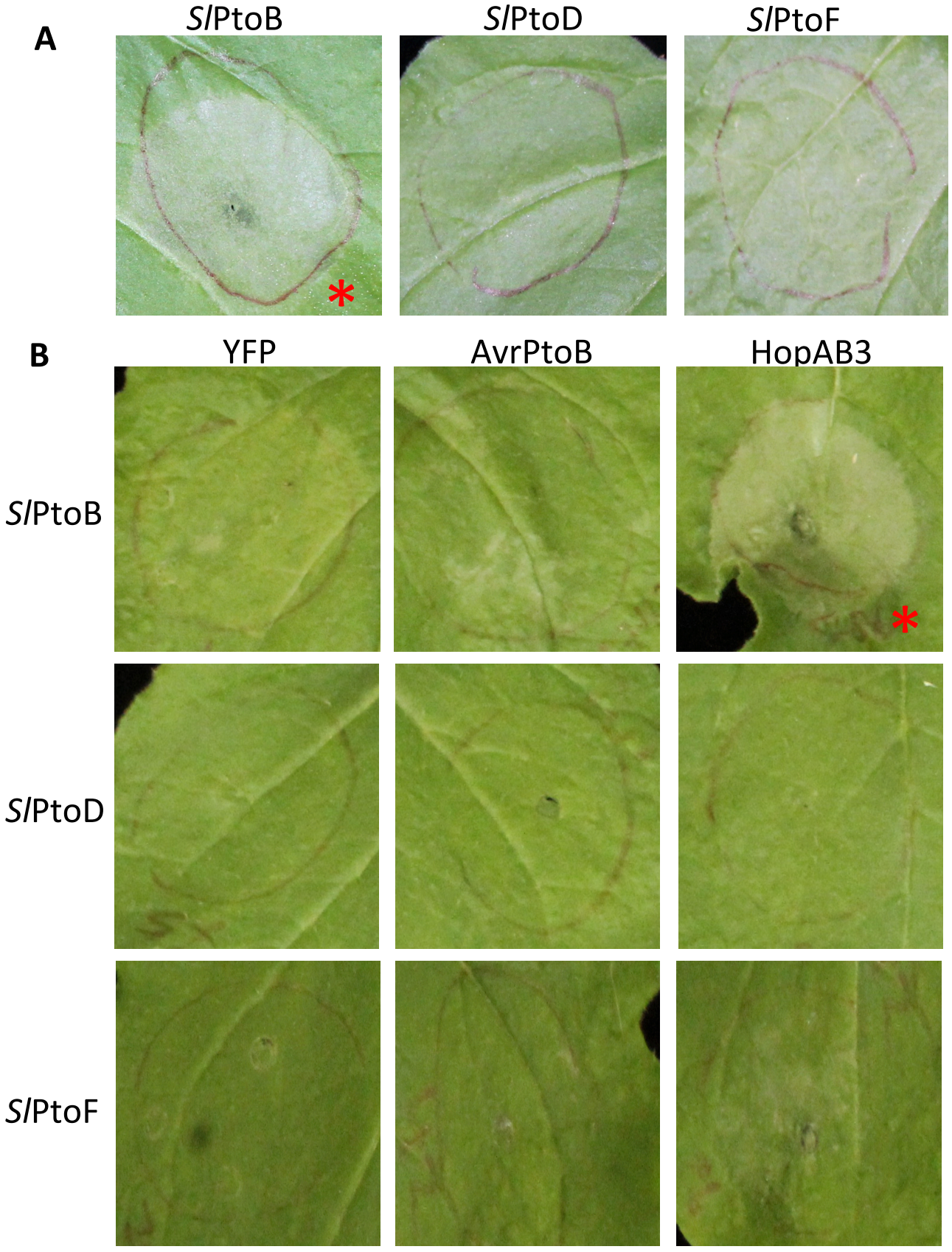


Figure 4.14. HopAB3 triggers Fen homolog *Sl*PtoB-dependent HCD in *N. benthamiana*. (A) *Sl*PtoB, *Sl*PtoD, or *Sl*PtoF with a C-terminal YFP tag was transiently expressed by *Agrobacterium tumefaciens* AGL1 infiltration (OD_{600}=0.5) under the CaMV 35S promoter in *N. benthamiana* (#2.5.5). Photographs were taken at 3 dpi. This experiment was conducted three times with identical results. **(B)** AvrPtoB or HopAB3 with a C-terminal YFP tag or YFP alone was transiently expressed by *Agrobacterium tumefaciens* AGL1 infiltration (OD_{600}=0.1), with *Sl*PtoB, *Sl*PtoD, or *Sl*PtoF (OD_{600}=0.3) under the CaMV 35S promoter in *N. benthamiana* (#2.5.5). Photographs were taken at 2 dpi. This experiment was conducted four times with identical results. Red asterisks in (A) or (B) indicate development of cell death symptoms.

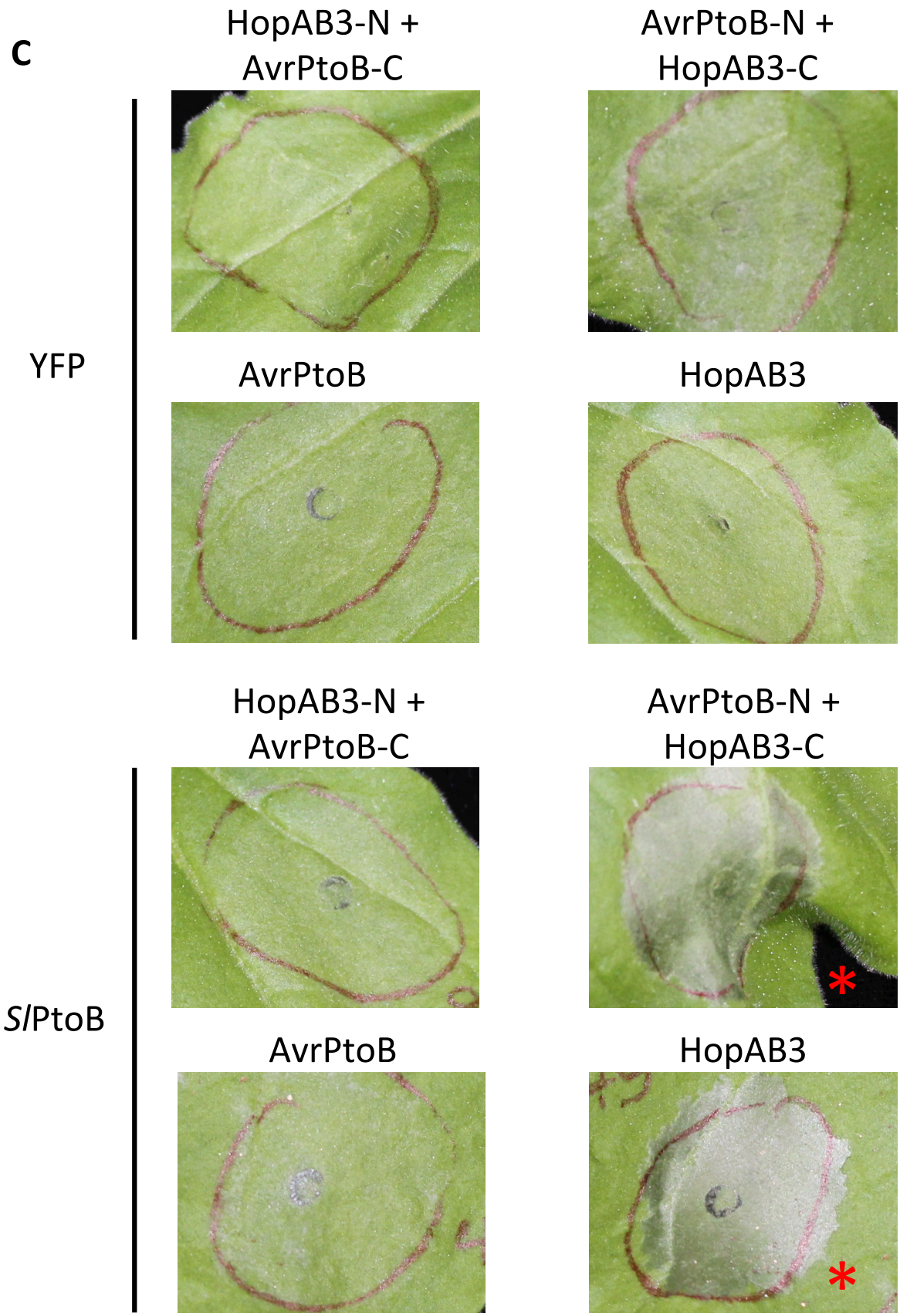


Figure 4.14. HopAB3 triggers Fen homolog *Sl*PtoB-dependent HCD in *N. benthamiana*. (C) AvrPtoB, HopAB3, $AvrPtoB_N$+$HopAB3_C$ or $HopAB3_N$+$AvrPtoB_C$ with a C-terminal YFP tag was transiently expressed by *Agrobacterium tumefaciens* AGL1 infiltration (OD_{600}=0.1), with *Sl*PtoB (OD_{600}=0.3) under the CaMV 35S promoter in *N. benthamiana* (#2.5.5). Photographs were taken at 2 dpi. This experiment was conducted twice with identical results. Red asterisks indicate development of cell death symptoms.

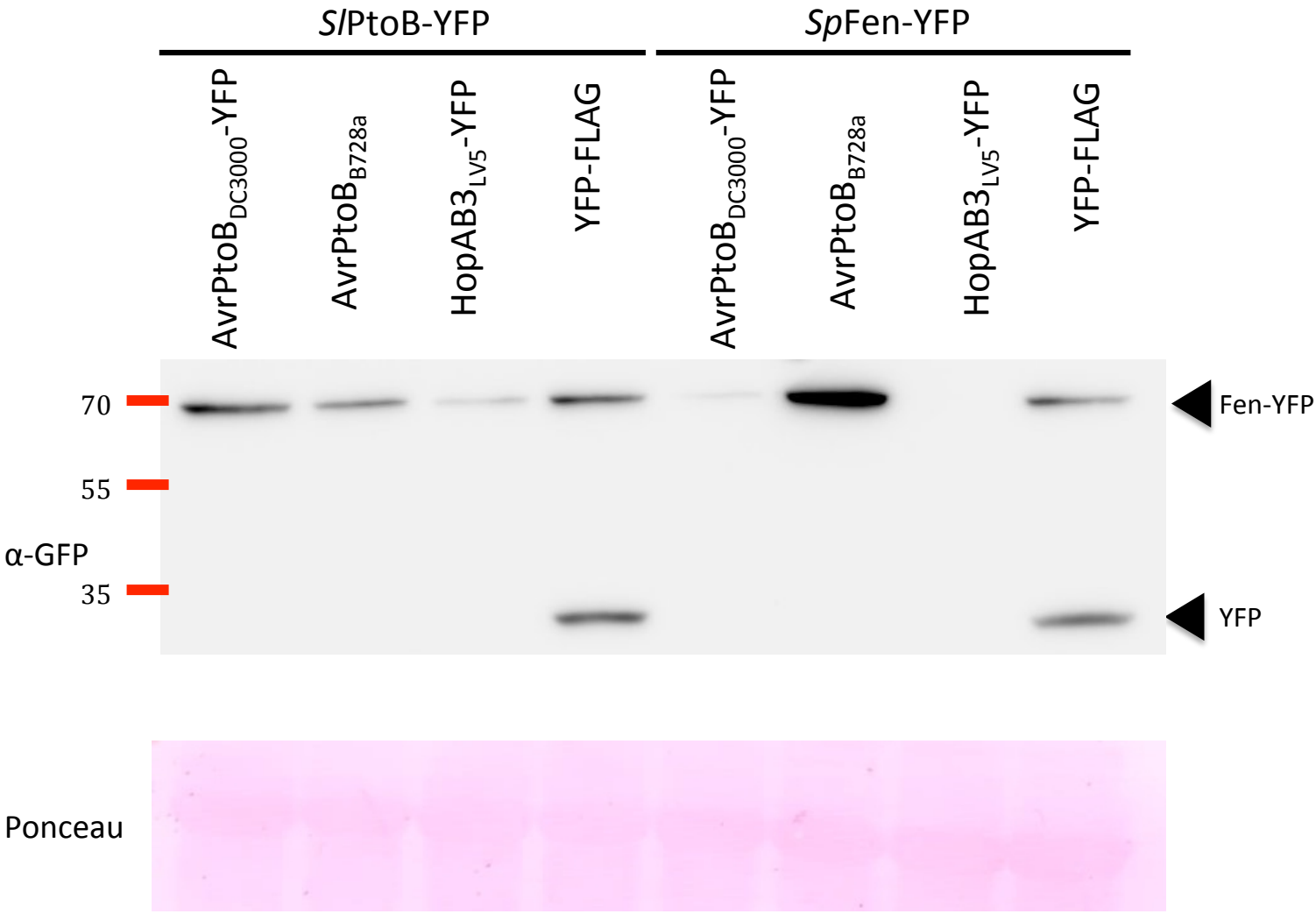


Figure 4.15. HopAB3 degrades *Sl*PtoB incompletely in *N. benthamiana*. Detection of YFP-tagged *Sp*Fen or *Sl*PtoB (*Agrobacterium tumefaciens* AGL1, OD_{600} = 0.6) from transient expression coinfiltrated with YFP-tag alone, YFP-tagged AvrPtoB$_{PtoDC3000}$, YFP-tagged HopAB3 or untagged VirPphA/AvrPtoB$_{PsyB728a}$ (OD_{600} = 0.1) in *N. benthamiana* treated with 3 µM $LaCl_3$ at 1 dpi to suppress cell death. Protein was extracted from leaf samples at 2 dpi. Protein presence in total extract was tested by immunoblot using α-GFP antibody. Ponceau Red staining of total protein extract showing the Rubisco protein band indicates even sample loading. Numbers to the left of the panel and associated red bars indicate band sizes for the PageRuler™ protein ladder in kDa.

4.3 Discussion

We identified both model plant species Arabidopsis (Col-0) and *N. benthamiana* to have nonhost resistance to *Psa*, including against *Psa* LV5. Using these two nonhost species, we identified two T3Es that serve as avirulence effectors, namely, HopAR1 and HopAB3, unique to *Psa* LV5. Both are homologs of AvrPphB and AvrPtoB, respectively, which are well-characterized examples of avirulence effectors. We showed that Arabidopsis nonhost resistance to *Psa* LV5 is, unsurprisingly, at least partially due to the T3E HopAR1, which triggers an RPS5-dependent HR. We additionally showed that *N. benthamiana* plants are able to mount an HCD in response to HopAB3, contributing to the nonhost resistance seen against *Psa* LV5. Interestingly, this recognition of HopAB3 is unique within the AvrPtoB family and is reproduced in cultivated tomato as well. It is likely that this response is due to a homolog of *S. pimpinellifolium* cytoplasmic kinase *Sp*Fen, present in cultivated tomato – *S. lycopersicum* PtoB (*Sl*PtoB). Both HopAR1 and HopAB3 are now high priority candiates for study of nonhost resistance in kiwifruit for their potential ability to render *Psa* LV5 non-pathogenic on kiwifruit, particularly the Hort16a cultivars they were isolated from.

4.3.1 *Psa* LV5 is a pathogen of sour cherry that only grows epiphytically on kiwifruit

Recently, during our investigation of nonhost resistance mechanisms against *Psa* LV5, it and its close relatives in clade/biovar 4 of *Psa* strains were suggested for reclassification as *Pseudomonas syringae* pv. *actinidifoliorum* since several different tests have shown that it is, in fact, not a pathogen of kiwifruit (Cunty et al., 2015). This 'resistance' by kiwifruit was not determined conclusively to be host or nonhost resistance since only a single kiwifruit cultivar (*Actinidia deliciosa* cv. 'Hayward') was tested. However, multi-locus sequence typing, biochemical analyses and pathogenicity tests have demonstrated it to be a close relative of *P. avellenae*, the causal agent of hazelnut canker disease, and found *Psa* LV strains to cause disease only on sour cherry, *Prunus cerasus* (Ferrante and Scortichini, 2015). This raised the question of why *Psa* LV5 and its T3Es needed

to be studied as a pathogen of kiwifruit if all it can achieve is epiphytic growth, perhaps due to what now appears to be nonhost resistance.

Previous studies have demonstrated the ability of an avirulent pathogen to grow epiphytically on a nonhost (Daub, 1981; J. Lee et al., 2012). Perhaps most notably, several studies have shown a gain in virulence can be achieved through simple mutations/deletions in the pathogen's effector repertoire (Lim and Kunkel, 2004; Schwartz et al., 2015; Wei et al., 2007, 2015). This is particularly true for host resistance, which can be easily overcome (Gill et al., 2015). These gain-of-virulence type occurrences are thought to reflect evolutionary adaptations in nature whereby a pathogen evades recognition and thus plant immunity through loss of an effector. Therefore, to identify additional sources of resistance against currently avirulent pathogens existent as epiphytic populations on a host plant could prove critical to crop biosecurity. In fact, the current strategy in the kiwifruit industry has been to substitute the susceptible kiwifruit cultivar (Hort16a) with a more resistant one (Gold3). Previously, a similar substitution to cherry crops that were resistant to bacterial canker disease was subverted and resulted in the emergence of three newly virulent pathogenic strains that were believed to be epiphytic prior to the introduction of the resistant cherry crop (Vivian and Gibbon, 1997).

In addition to this, due to the co-occurrence of *Psa* LV strains with other more virulent strains of *Psa* on kiwifruit plants, the possibility of mixed infections introduces an unknown factor when analyzing the effect of disease in kiwifruit orchards. In fact, a recent examination of mixed infections of virulent and avirulent pathogenic *Pseudomonas* strains found that a diverse number of interactions could occur between the different pathogens, including the event where the avirulent strains could proliferate when coinoculated with virulent strains (Rufián et al., 2017). Therefore, choosing to study the effector repertoire of the avirulent *Psa* LV5 strain becomes significantly more interesting from the standpoint of plant disease as a multipartite community of pathogens interacting with the kiwifruit host through their combined repertoire of effectors.

Due to the ease of utilizing model plants to examine nonhost resistance, we opted for characterizing the genetic sources of resistance to *Psa* LV5 in these model plants. In particular, due to the close homology of HopAR1 to AvrPphB, we sought to investigate whether it was responsible for *Psa* LV5-triggered HR in Col-0 plants. HopAR1 (another homolog of AvrPphB and HopAR1$_{Psa\text{LV5}}$) from *P. syringae* pv. *phaseolicola* 1302A resides on the excisable genomic island PPHGI-1 that can be lost through even limited selective pressure on the pathogen (Pitman et al., 2005). A recent genome sequence analysis identified a PPHGI-1-like genomic island termed the Pacific island, in several *Psa* strains that carry HopAR1 or HopAR1-like truncated sequences (McCann et al., 2013). This was particularly of concern since, if HopAR1 from *Psa* LV5 is also responsible for the nonhost resistance seen in kiwifruit, it could serve as a veritable Achilles heel due to its ease of loss. The first step to check was the ability of HopAR1$_{Psa\text{LV5}}$ to trigger an HR in Col-0, the original Arabidopsis accession to show avirulence to AvrPphB (Simonich and Innes, 1995). When delivered under its native promoter HopAR1 did indeed trigger an RPS5-dependent HR that demonstrated a similarity to HopAR1$_{Pph1302A}$. Unfortunately, checking delivery of HopAR1 in kiwifruit plants, however, could not be achieved at this time due to restrictions in plant availability and time. Future assessments of immunity triggered by *Psa* LV5 in kiwifruit should focus on the ability of HopAR1 to trigger such and whether a lack of HopAR1 would be sufficient to render *Psa* LV5 virulent on currently resistant kiwifruit cultivars.

4.3.2 Col-0 responds without an HR to some avirulent effectors when delivered under the AvrRps4 promoter

The causal effector for HR triggered by *Psa* LV5, HopAR1, proved difficult to identify using T3SS delivery by Pf0-1(T3S). In fact, we needed to map the *R*-gene using *Psa* LV5 in a Col-0 x Ws-2 crossed F_2 population to identify the *RPS5* locus as responsible for the HR triggered by *Psa* LV5. Subsequently, we found that the AvrRps4 promoter we used to identify and characterize HopZ5 (Chapter 3) was unsuccessful for delivering HopAR1. Indeed, we showed that HopAR1 homolog AvrPphB was also unable to trigger an HR when delivered under this AvrRps4 promoter (Figure 4.5).

This raises an interesting issue regarding the choice of promoter for delivery by the T3SS. Herein we utilized the promoter of AvrRps4 previously described for delivery of AvrRps4 as well as fungal and oomycetal effectors fused to the AvrRps4 N-terminus (Fabro et al., 2011; Kemen et al., 2011; Sharma et al., 2013; Sohn et al., 2007). However, these studies have largely relied on the bacterial T3SS recognition of the N-terminal AvrRps4 'secretion signal'. In our work, we eliminated the use of this N-terminal peptide and instead opted for the AvrRps4 promoter alone and relied on the T3Es from *Psa* LV5 for their inherent 'secretion signal'. However, we found that the AvrRps4 promoter was unable to effectively serve as a promoter for neither AvrPphB nor HopAR1. This is confusing as native promoters of both HopAR1 and AvrPphB were sufficient for Pf0-1(T3S)-mediated delivery into Arabidopsis leaves to trigger RPS5-dependent HR, indicating that the effectors themselves are functional. Conversely, the AvrRps4 promoter allows for both AvrRps4 (Hinsch and Staskawicz, 1996) and HopZ5 (Chapter 3) delivery sufficient to trigger an HR in Arabidopsis accessions Ws-2 and Ct-1, respectively. Therefore, the only plausible explanation would be that the AvrRps4 promoter allows for either differential (poorer) accumulation of these effectors thereby resulting in a different response when delivered by a T3SS. Explicitly, the AvrRps4 promoter resulted in sufficient levels of AvrRps4 and HopZ5 to be expressed to trigger an HR when delivered by Pf0-1(T3S) but not for AvrPphB or HopAR1. This could also be affected by the Arabidopsis accession the effectors were being delivered into. AvrRps4 does not trigger an HR in Col-0 plants but does in Ws-2 plants and HopZ5 again does not trigger an HR in Col-0 but does so in Ct-1, despite both effectors being able to trigger immunity in Col-0. Additionally, in our conditions in the lab, we noted a decided reduction in the immune response of Col-0 in response to not only HopZ5 when delivered by Pf0-1(T3S) but also AvrRpm1. Therefore, it is possible that a combination of poor delivery by Pf0-1(T3S) and a poor response by Col-0 to effectors expressed at levels achieved when under the AvrRps4 promoter could result in the observations seen herein for HopAR1. The latter is likely the more significant contributor due to our observations that even *Pto* DC3000 delivery of

HopAR1 or AvrPphB under the AvrRps4 promoter failed to trigger any HR-associated electrolyte leakage (Figure 4.5B).

AvrRps4, in Col-0 plants, is still able to trigger immunity sufficient to restrict pathogen growth, albeit without the HR (Gassmann et al., 1999). We did not examine pathogen growth for HopAR1 or AvrPphB when delivered under the AvrRps4 promoter. Thus, while no HR was seen for HopAR1 or AvrPphB when expressed under the AvrRps4 promoter, they may still have triggered an immune response in Col-0. As mentioned in Chapter 3, it is possible that the reported shuttling of immune hub, EDS1, between nucleus and cytoplasm may influence HR versus immunity for certain TNL-mediated responses (Heidrich et al., 2011). If a similar mechanism is true for delivery of HopAR1/AvrPphB, both of which trigger CNL RPS5-mediated immunity, it will be interesting to see if this is a dose-dependent response. Indeed, EDS1 has been reported as a partial requirement for immunity triggered by AvrPphB before (Aarts et al., 1998) and another CNL, RPS2, has recently been shown to require *EDS1* or *SID2/ICS1* redundantly for triggering immunity against its corresponding effector, AvrRpt2 (Cui et al., 2017).

Naturally, the best way to test whether this difference in HR phenotype is due to differential delivery dependent on the promoter used for HopAR1/AvrPphB would be to test for secretion of HopAR1/AvrPphB when delivered by the T3SS and expressed under different promoters. Several secretion assays exist including immunoblots of tagged proteins expressed in pathogens grown in minimal media (Huynh et al., 1989) or calmodulin-dependent adenlyate cyclase fusion to N-terminal fragments of T3Es delivered directly into plants (Schechter et al., 2004). Unfortunately, due to a lack of time, we were unable to carry out such work for this project. Future work on examining promoter-dependent secretion by such assays will be critical to understanding this interesting phenotype.

4.3.3 AvrPtoB/HopAB3 recognition is multipartite and variable

In our research, herein, we found that HopAB3, much like AvrPtoB from *Pto* DC3000, triggers a Pto-dependent HCD in *N. benthamiana* plants transiently expressing the NB-LRR *Sp*Prf as well. Additionally, HopAB3 triggers an *Sl*PtoB-dependent recognition in cultivated tomato unique to members of the AvrPtoB family of E3 ligase-carrying effectors. We predict this recognition event is reminiscent of previously described minor recognition of AvrPto by *Sl*PtoF (Chang et al., 2002), albeit to a stronger extent as HopAB3 was able to trigger significant ion leakage in tomato leaves when delivered by Pf0-1(T3S) whilst AvrPto did not, despite the presence of *Sl*PtoF in these tomato plants (Moneymaker). Additionally, we predict that the sequence variation in HopAB3 compared to other AvrPtoB family members that trigger recognition in tomato allows for a similar recognition by an *N. benthamiana* homolog of *Sl*PtoB.

The crystal structure of AvrPto-Pto and AvrPtoB-Pto identified shared and unique interfaces for each interaction (Dong et al., 2009; Xing et al., 2007). This prompted a recent investigation of natural variants of *Pto* in wild tomato relatives, which identified a *Pto* homolog from *S. chmielewskii* that facilitated recognition of AvrPtoB (from *Pto* DC3000) but not AvrPto (from *Pto* JL1065 or *Pto* DC3000) (Kraus et al., 2016). In fact, several accessions of *S. chmielewskii* could support full growth of *Pto* DC3000, which carries both *avrPtoB* and *avrPto*, despite having a functionally expressed Pto homolog. Interestingly, while both *Sp*Pto and *Sc*Pto require the NB-LRR Prf to confer recognition, sequence variation (carrying His193 instead of Asp193) outside the characterized interaction interface in *Sc*Pto allows for recognition of AvrPtoB but not AvrPto (Kraus et al., 2016). The finding suggested an evolutionary pressure on the plant to adapt to evolving pathogens that lacked AvrPto but carried an allele of AvrPtoB. This paradigm then naturally proposes another possibility: that an AvrPtoB allele could exist that is recognized by a Pto/Fen homolog that does not recognize either AvrPto or AvrPtoB.

At first glance, the cause of HopAB3-triggered HCD in *N. benthamiana* and ion leakage in tomato could be attributed to a non-functional E3 ligase activity

allowing for *Sp*Fen- or Fen-homolog-dependent immunity similar to E3 ligase-defective AvrPtoB mutant variants (Abramovitch et al., 2006; Rosebrock et al., 2007). However, analysis of the HCD triggered by HopAB3 coexpressed with *Sp*Fen in *N. benthamiana* and immunoblots of *Sp*Fen coexpressed with HopAB3 indicated that HopAB3 has a fully functional E3 ligase ability capable of degrading *Sp*Fen (Figure 4.12) and that a Fen-homolog is not likely to be responsible for HopAB3 triggered HCD in *N. benthamiana* (Figure 4.10). This result appeared to contradict the observation that terminal swaps between HopAB3 and AvrPtoB identified the C-terminus (carrying the E3 ligase domain) as associated with recognition in *N. benthamiana*, a fragment not known to house interfaces previously identified for interaction with Pto/Fen (Figure 4.11 & 4.14B). The domain-swap experiments intriguingly suggested that the reason for detection of HopAB3 by *Sl*PtoB is either due to a lack of *Sl*PtoB degradation, despite a functional E3 ligase, or a novel interaction between *Sl*PtoB and HopAB3 within the E3 ligase domain that allows for this recognition. The immunoblot of *Sp*Fen or *Sl*PtoB coexpressed with AvrPtoB or HopAB3 indicated that the E3 ligase of HopAB3 is specific for *Sp*Fen degradation but is weakened against *Sl*PtoB, a novel finding with no such specificity reported to date. Moreover, this adds to the currently held theory that AvrPtoB has evolved to bind *Sp*Fen and degrade it, thereby evading recognition. It appears that unlike *Sp*Fen, *Sl*PtoB has lost ability to interact with AvrPtoB, but retained that ability to bind HopAB3-type alleles despite strong E3 ligase activity which works well against *Sp*Fen, thus triggering immunity against select alleles.

Future work on this matter would also ideally focus on the nature of the *Sl*PtoB-HopAB3/AvrPtoB interaction to examine residues/domains necessary for this interaction. Examination of residues in HopAB3 that are different from AvrPtoB in the E3 ligase domain would also serve to identify regions in HopAB3 that have been exclusively targeted by *Sl*PtoB that conversely cannot recognize AvrPto or AvrPtoB (possibly due to a lack of interaction with the latter). *Psa* LV5 is not known to be a pathogen on tomato plants and while we have not demonstrated this, we can reasonably predict that tomato plants would show some nonhost resistance to *Psa* LV5, at least partially dependent on HopAB3. From an

evolutionary perspective, the finding that HopAB3 triggers immune responses in cultivated tomato that otherwise cannot recognize AvrPto or AvrPtoB is an interesting one that suggests an evolutionary balancing act between pathogens carrying alleles of AvrPto/AvrPtoB and tomato alleles of Pto/Fen.

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

5.1 Introduction

The WRKY transcription factor (TF) family represents one of the largest families of TFs in higher plants with around 72 genes in Arabidopsis (Eulgem and Somssich, 2007). While WRKY TFs are also found in the unicellular eukaryote *Giardia lamblia* and the slime mold *Dictyostelium discoideum* (Ulker and Somssich, 2004), there is no evidence that they exist in animals. However, WRKY TFs along with another plant defence-related NAC TF family, do belong to a WRKY-GCM1 (GLIAL CELL MISSING1) superfamily including the animal-specific GCM TFs, widespread in eukaryotes (Babu et al., 2006; Yamasaki et al., 2008).

WRKYs are so called due to their possession of a WRKY domain, a conserved N-terminal sequence of WRKYGQK along with a Zn finger-like motif, which can be either $Cx_{4-5}Cx_{22-23}HxH$ (C2H2-type) or $Cx_{7}Cx_{23}HxC$ (C2HC-type) (Eulgem et al., 1999; Rushton et al., 1995). WRKYs are divided into three broad groups based on the the number of WRKY DNA-binding motifs and the Zn finger motif they carry (Eulgem et al., 2000). Group I and II WRKYs possess C2H2-type Zn finger motifs while group III WRKYs have the C2HC-type, both of which require Zn-binding to carry out their DNA-binding activity (de Pater et al., 1996). While group II and III WRKYs have a single WRKYGQK motif, group I WRKYs possess two such motifs with several experiments showing that the C-terminal motif is responsible for the DNA binding activity (de Pater et al., 1996; Eulgem et al., 1999). These WRKYs have been shown to bind a specific DNA sequence termed a W-box with the consensus (T)TTGACC/T with the core 4-base TGAC element being critical for DNA binding (Eulgem et al., 1999; Rushton et al., 1996). Solution and crystal structures of WRKYs bound to DNA targets has demonstrated that a typical WRKY consists of a 4/5-stranded β-sheet, with a zinc binding pocket formed by the conserved C/H residues located at the end of the β-sheets and the WRKYGQK residues of the most N-terminal β-strand, kinked in the middle of the sequence by the G residue enabling hydrophobic interactions involving the Y residue and electrostatic interactions involving the R and K

residues within the major groove of the DNA strand (Duan et al., 2007; Yamasaki et al., 2005). Recently further analysis using extensive examination of the interface between a WRKY and its DNA target has revealed that the WRKYGQK motif interacts with DNA bases within the groove mainly through extensive apolar contacts with thymine methyl groups within the conserved (T)TTGACC/T sequence in the W-boxes (Yamasaki et al., 2012). Investigation into the DNA-binding selectivity of the WRKYs revealed that DNA sequence variation in the region flanking the core TTGACC/T sequence along with peptide sequence variation flanking the WRKYGQK motif in the WRKYs contributes to high specificity and selectivity of DNA binding for each WRKY (Ciolkowski et al., 2008). Apart from their individual DNA binding activities, several WRKYs have demonstrated interactions with other WRKYs to collectively activate or repress their targets in a complex network (Besseau et al., 2012; Chi et al., 2013; Xu et al., 2006). WRKYs are also able to regulate their own expression and expression of other WRKYs through W-boxes in their promoters, generating a complex cascade of reactions with built in feedback loops (Chen et al., 2010; Hu et al., 2012; Wang et al., 2006).

Over two decades since their discovery (Rushton et al., 1995), many functions have been ascribed to WRKY TFs. WRKYs participate in a variety of plant processes including the responses to pathogens (Birkenbihl et al., 2017; Hu et al., 2012; Pandey and Somssich, 2009), abiotic stresses (Rushton et al., 2010; Scarpeci et al., 2013; Yan et al., 2013), and senescence (Besseau et al., 2012; X. Zhou et al., 2011). In Arabidopsis, almost 70% of WRKY genes were differentially expressed in response to avirulent *Pseudomonas syringae* or treatment with SA (Dong et al., 2003). Many studies to date have identified roles for WRKYs in both PTI and ETI as both positive and negative regulators (Eulgem, 2006; Pandey and Somssich, 2009; Sohn et al., 2014; Zipfel et al., 2004). Promoters of multiple defence-related marker genes are enriched for W-box elements (Navarro et al., 2004; Zipfel et al., 2004). One of the earliest studies on the Arabidopsis response to flg22 identified upregulation of WRKY22 and WRKY29 that are also upregulated in chitin-triggered immunity (Asai et al., 2002; Wan et al., 2004). The contribution of these WRKYs can be difficult to determine due to functional

redundancy within the WRKY network and the aforementioned complexities involving WRKY-WRKY interactions as well as the network of auto- and *trans*-regulation by these WRKYs. However, several landmark studies have utilized a combination of overexpression and deletion plant lines in Arabidopsis to determine the relationship between the various WRKYs and pathogen defence responses.

Arabidopsis group I WRKY, WRKY33, activation has been linked to increased resistance to necrotrophic pathogens and overexpression of WRKY33 led to increased susceptibility to the hemi-biotrophic pathogen *P. syringae* (Zheng et al., 2006). Similarly, group I WRKYs from Arabidopsis WRKY3 and WRKY4 show a positive regulation of resistance to necrotrophic pathogens and associated susceptibility to *P. syringae*. Moreover, *wrky4* mutant plants show increased resistance to *P. syringae* (Lai et al., 2008). Yet another group I WRKY, Arabidopsis WRKY58, has been linked to repression of SA-mediated immunity with mutant *wrky58* lines displaying enhanced resistance to virulent *P. syringae* strains with weak immune induction (Wang et al., 2006). Finally, overexpression of Arabidopsis WRKY25, another group I WRKY, conferred enhanced susceptibility to *P. syringae* (Zheng et al., 2007). Taken together, the activity of group I WRKYs largely appears to be supportive of defence responses against necrotrophic pathogens through antagonism of SA-mediated defence.

Group II WRKYs from Arabidopsis have been divided into multiple subgroups based on sequence diversity within the WRKY motif. Group II-a WRKYs from Arabidopsis constitute just the three WRKYs: WRKY18, WRKY40 and WRKY60 that have been studied intensively due to their partially redundant negative regulation of immunity in response to several biotrophic pathogens including *P. syringae* and powdery mildew pathogen *Golovinomyces orontii* (Shen et al., 2007; Xu et al., 2006). WRKY18 alone appears to have a positive role in mediating SA-dependent defences and along with WRKY40 is an activator-type transcription factor in group II-a while WRKY60 appears to be a potent repressor-type WRKY (Chen et al., 2010; Wang et al., 2006). Group II-b and II-c WRKYs from Arabidopsis have not been extensively studied for roles in defence. A notable

exception is WRKY6 (group II-b) that acts as a positive regulator of senescence and overexpression of WRKY6 led to upregulation of the defence marker gene *PR1* suggesting a positive regulation function in immunity (Robatzek and Somssich, 2002). Other WRKYs closely related to WRKY6 have recently been tenuously linked to roles in immunity including WRKY72 and WRKY61 (Bhattarai et al., 2010; Gao et al., 2016) but the dynamics of this complex subgroup within group II-b remains to be explored in detail. Another notable exception is WRKY28 from group II-c that has been identified as an activator of SA-mediated immunity including binding of the ICS1 promoter to activate transcription (van Verk et al., 2011) but also has been reported to primarily activate, along with another group II-c member WRKY75, the JA/ET pathway traditionally considered antagonistic to the SA-mediated pathway (Chen et al., 2013). This latter activity of WRKY28 and WRKY75 is reminiscent of group I WRKYs in their ability to promote resistance to necrotrophic pathogens. WRKYs from groups II-d and II-e have been found to be repressors and activators of immunity, respectively, including WRKY7/WRKY11/WRKY17 and possibly WRKY15 (group II-d) that redundantly work to suppress basal immunity (Journot-Catalino et al., 2006; Kim et al., 2006) and the previously mentioned basal immunity activators WRKY22 and WRKY29 (group II-e) (Asai et al., 2002).

Group III WRKYs from Arabidopsis are divided into two subgroups, III-a and III-b, based on evidence of evolutionary diversification within this group (Figure 5.1; (Wu et al., 2005)). Like work done for previous WRKY groups, findings for group III-a indicate that they are largely activators of SA-mediated immunity. A pivotal immunity-related member of group III-a is Arabidopsis WRKY70 that plays a critical role in ETI, and the balance between SA- and JA/ET-dependent defence responses, with WRKY70 acting to both activate and suppress these pathways (Knoth et al., 2007; Li et al., 2006; Ulker et al., 2007). Furthermore, WRKY70 and another group III-a member WRKY53, positively regulate SAR with the double *wrky53/wrky70* mutant displaying an enhanced susceptibility phenotype to *P. syringae* (Wang et al., 2006). Additionally, WRKY54 (another group III-a member) and WRKY70 were found to promote SA signaling but suppress SA accumulation, although the latter may be a function of a feedback

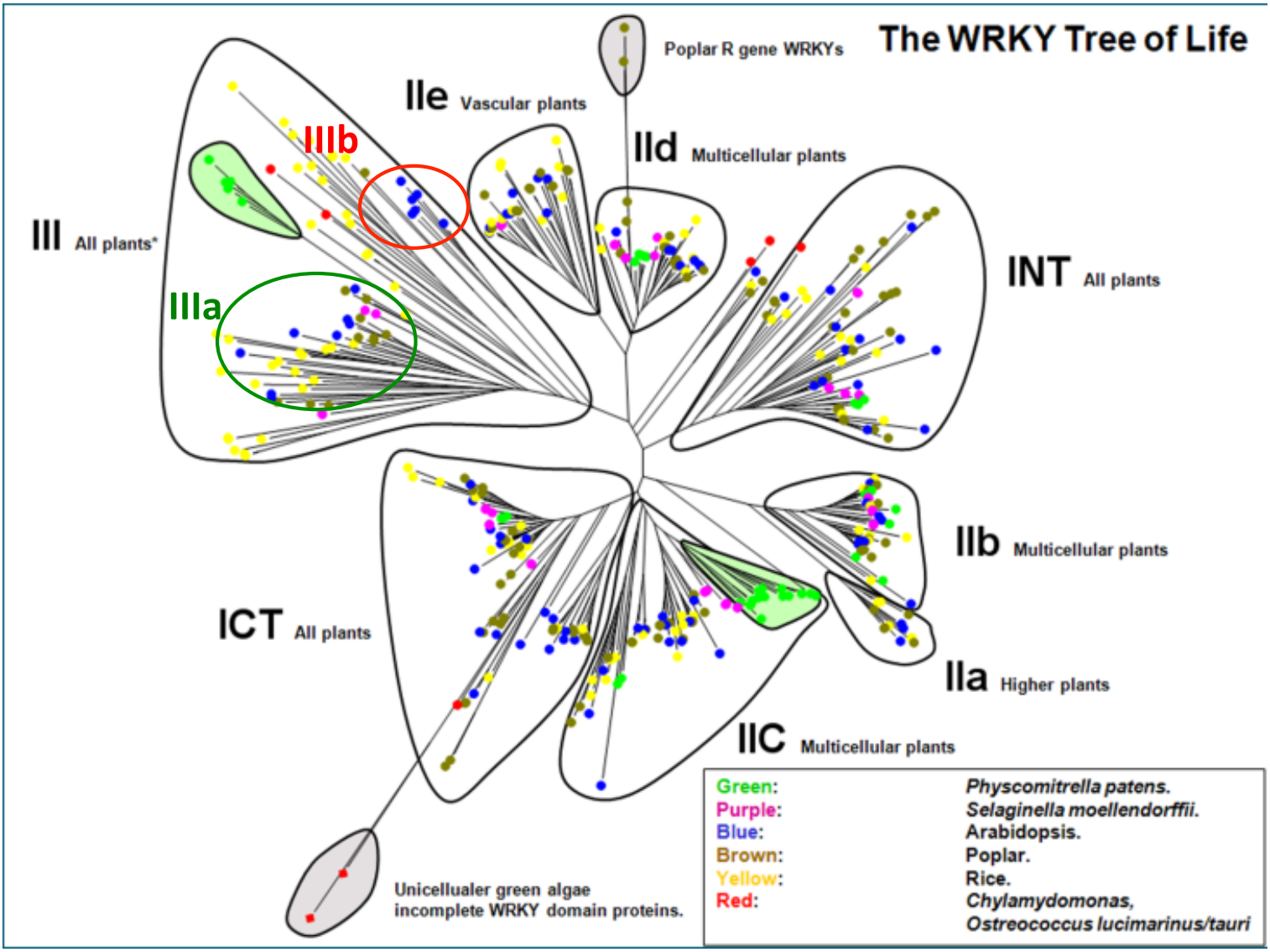


Figure 5.1 The WRKY tree of life. Neighbor joining phylogenetic tree derived from a MUSCLE alignment of WRKY domains from the complete WRKY gene families from the following species: *Arabidopsis thaliana* and *A. lyrata* (Blue), *Selaginella moellendorffii* (Purple), *Physcomitrella patens* (Green), *Populus trichocarpa* (Brown), *Oryza sativa* (Yellow), and *Chlamydomonas reinhardtii, Ostreococcus lucimarinus, and O. tauri* (Red). The higher plant WRKY groups are marked I-III (with subgroups a, b, d, or e indicated). I NTD and I CTD denote the N-terminal and C-terminal domains from Group I proteins. Group III subdivision into group IIIa and IIIb for Arabidopsis are indicated in green and red, respectively within group III. (Reproduced from Rinerson et al 2015)

response due to a deficiency in SA sensing in the *wrky54/wrky70* double mutant (Wang et al., 2006). A role for other Arabidopsis group III-a WRKYs in SA-mediated immunity include findings for WRKY41, which was found to promote resistance to hemi-biotrophic *P. syringae* but suppress resistance against necrotrophic bacterium *Pectobacterium carotovorum* (Higashi et al., 2008), and WRKY46, which coordinates activity with WRKY70 and WRKY53 to promote resistance to *P. syringae* (Hu et al., 2012). Indeed, recent studies on WRKY46, understood to be representative of other group III-a WRKYs, further indicated that, upon perception of PAMPs, MAPK pathways activate WRKY46 and that it then acts as a transcriptional activator to upregulate PAMP-responsive defence genes (Sheikh et al., 2016). Group III-b WRKYs from Arabidopsis, however, display a converse relationship with SA-mediated immunity, with WRKY38 and WRKY62 both contributing negatively to basal defence (Kim et al., 2008).

Several previous studies looking at *P. syringae* T3E AvrRps4 and *R. solanacearum* T3E PopP2 have identified their interaction with several WRKYs as an occurrence during their promotion of bacterial virulence (Le Roux et al., 2015; Sarris et al., 2015). Both AvrRps4 and PopP2 have been shown to localize to the plant cell nucleus when transiently expressed (Sohn et al., 2012a; Tasset et al., 2010). Additionally, their detection by the WRKY-domain carrying nuclear-localized NB-LRR, RRS1, in conjunction with another TNL, RPS4, is mediated by an interaction with this WRKY-domain that mimics their proposed virulence targets (Le Roux et al., 2015; Sarris et al., 2015). In this pair of studies, PopP2 and AvrRps4 could selectively bind to several WRKYs, from multiple WRKY groups, and in the case of PopP2, acetylate them. These findings then raised several questions:

1) How are these WRKYs exclusively selected for by PopP2/AvrRps4?

2) Are other WRKYs not examined in these studies also selected for by PopP2/AvrRps4?

3) Do other pathogen-derived effectors also target WRKYs but through a measure of selectiveness avoid RRS1/RPS4-triggered immunity?

This chapter sought to answer these questions by looking at nucleus-localized effectors from multiple pathogens as well as AvrRps4 and PopP2. Using a yeast

2-hybrid screen we sought to examine if PopP2 and AvrRps4 interacted with a large number of AtWRKYs or whether their targeting of AtWRKYs was specific. We also sought to identify other effectors, notably from *Psa*, that interacted with AtWRKYs and whether the AtWRKYs they interacted with were distinguishable from those interacting with AvrRps4/PopP2.

5.2 Results

5.2.1 Multiple nuclear-localized effectors from *Pseudomonas* and *Hyaloperonospora arabidopsidis* interact with Arabidopsis WRKYs

To answer the questions discussed above for WRKY targeting by effectors, we sought to employ a classical yeast two-hybrid (Y2H) approach (Figure 5.2A). We focused on WRKYs from *Arabidopsis thaliana* Col-0 (hereafter, AtWRKYs) for interactions with AvrRps4 and PopP2 in addition to effectors from four different pathogens: *Psa* V13 and LV5 (both avirulent on Col-0), *R. solanacearum* (*Rso*) GMI1000 (virulent on Col-0), and *Hyaloperonospora arabidopsidis* (*Hpa*) Emoy2 (avirulent on Col-0 due to *RPP4*). Using the LexA system, we individually mated all non-autoactive AtWRKYs fused to an activation domain against each of the non-autoactive effectors fused to DNA binding domain of LexA to screen for activation of the *LEU2* and *β–galactosidase* reporter genes (#2.6.5; Figure 5.2A-B). This interaction was confirmed via a secondary system using either bimolecular fluorescence complementation (BiFC) or coimmunoprecipitation studies (coIP) followed by characterization of the effector's effect on WRKY activity by a dual luciferase reporter system.

We previously identified a large number T3Es from *Psa* V13 and *Psa* LV5 that localized to the plant cell nucleus when expressed transiently in *N. benthamiana* leaves (Table 5.1; S. Choi, J. Jayaraman, K.H. Sohn, *manuscript in preparation*). Additionally, we cloned several nuclear localization-predicted *Rso* T3Es estimated using the nuclear localization signal (NLS) prediction algorithm, NLStradamus (Table 5.2; (Nguyen Ba et al., 2009)) and *Hpa* Emoy2 effectors localized to the nucleus previously (Table 5.3; (Baxter et al., 2010; Caillaud et al., 2012a, 2012b)). Effectors indicated in bold in the aforementioned tables indicate

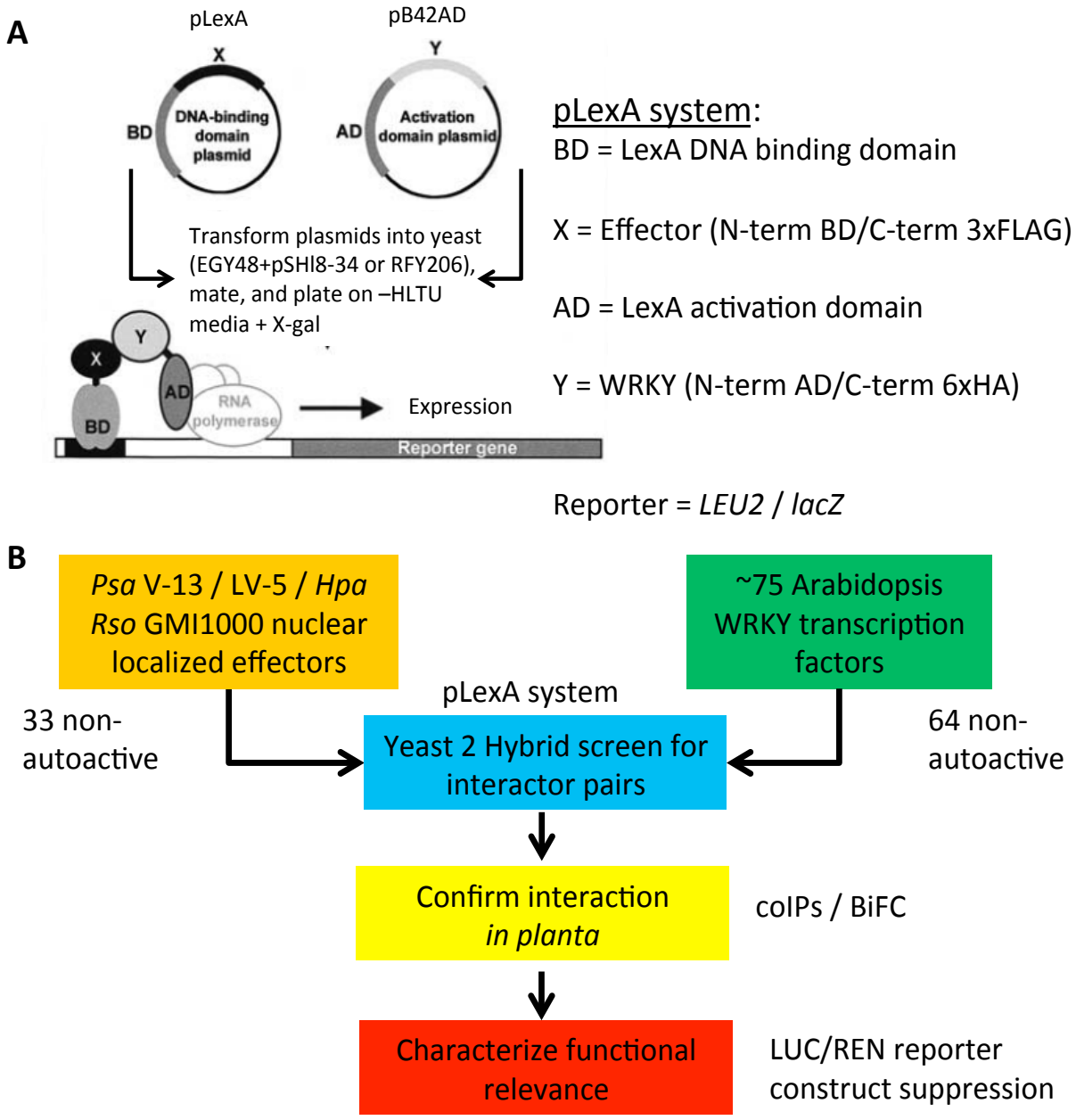


Figure 5.2. The mechanism of the yeast two hybrid screen and schematic for screening of effectors against AtWRKY library. (A) Effectors were cloned into pLexA vector tagged with N-terminal LexA DNA-binding domain (DBD) and a C-terminal 3xFLAG tag. Arabidopsis WRKYs (AtWRKY) were cloned into the pB42AD vector tagged with N-terminal B42 activation domain (AD) and a C-terminal 6xHA tag. Reporter genes *LEU* and *lacZ* activation by their GAL promoter is detected upon interaction between effector and AtWRKY, bringing the AD and DBD of LexA together to reconstitute the transcription factor. **(B)** Nuclear-localized *Psa* V13, *Psa* LV5 and *Hyaloperonospora arabidopsidis* Emoy2 effectors along with nuclear-predicted effectors from *Ralstonia solanacearum* GMI1000 (33 out of 44 non-autoactive in pLexA) and AtWRKY transcription factors (64 out of 65 nonautoactive in pB42AD) were cloned. These were then transformed into appropriate yeast strains and checked for autoactivation of reporter genes (#2.6.5). All non-autoactive effectors were individually mated against all non-autoactive AtWRKY TFs to identify reporter activation. This was subsequently confirmed by coIPs and/or BiFC and further tested for AtWRKY suppression by reporter assays.

Table 5.1. *Psa* V13/*Psa* LV5 effectors that localize to the nucleus

Strain	T3E	size (aa)	WRKY interaction
***Psa* V13**	**AvrD1**	**312**	**Various strong + weak**
Psa V13	HopD1	706	No interactors
Psa V13	HopF2	206	Weak only
Psa V13	HopH1	219	No interactors
Psa V13	HopI1	457	No interactors
Psa V13	HopQ1	448	Autoactive
Psa V13	HopX3	375	Autoactive
***Psa* V13**	**HopY1**	**287**	**Various strong + weak**
Psa V13	HopZ3	408	Weak only
Psa V13	HopAF1	214	Weak only
Psa V13	HopAI1	268	No interactors
Psa V13	HopAO2	340	Weak only
Psa V13	HopAY1	247	No interactors
Psa V13	HopBN1	298	Autoactive
Psa LV5	HopA1	381	Weak only
Psa LV5	HopE1	212	Weak only
Psa LV5	HopF1	205	Autoactive
Psa LV5	HopX1	381	Weak only
Psa LV5	HopX2	353	Autoactive
Psa LV5	HopAI1	268	No interactors
Psa LV5	HopAY1	324	No interactors

*Nucleus-localized effectors from Psa V13 and LV5 (Choi, S. Jayaraman, J., & Sohn K.H. *manuscript in preparation*)

effectors that were found to have significant interactions with AtWRKYs in the Y2H screen. As seen in these tables, several effectors were found to be autoactive and thus were excluded from the screen. Interestingly, while we found a few AtWRKYs could interact with *Rso* effector RipAF1 (data not shown), investigation of its subcellular localization led us to exclude it from further analysis due to its lack of a nuclear localization (Figure 5.3). RipAB, another T3E from *Rso*, was validated as nuclear localized as predicted using NLStradamus.

Table 5.2. *Ralstonia solanacearum* GMI1000 predicted nuclear-localized effectors

Strain	T3E	size (aa)	WRKY interaction
Rso GMI1000	RipA1	1063	No interactors
Rso GMI1000	RipA2	1127	No interactors
Rso GMI1000	RipD	643	No interactors
Rso GMI1000	RipE1	425	No interactors
Rso GMI1000	RipL	1336	Autoactive
Rso GMI1000	RipAB	174	Weak only
Rso GMI1000	RipAD	310	Weak only
***Rso* GMI1000**	**RipAF1**	**350**	**Various strong + weak**
Rso GMI1000	RipAO	498	No interactors
Rso GMI1000	RipAY	416	Autoactive

Table 5.3. *Hyaloperonospora arabidopsidis* Emoy2 nuclear-localized effectors

Strain	Hpa RxL	size (aa)	WRKY interaction
Hpa Emoy2	RxL2	183	No interactors
Hpa Emoy2	RxLL3a	391	Autoactive
Hpa Emoy2	RxL10	221	Autoactive
Hpa Emoy2	RxL16	213	Autoactive
Hpa Emoy2	RxL18	123	No interactors
***Hpa* Emoy2**	**RxL24**	**376**	**Various strong + weak**
Hpa Emoy2	RxL36	126	No interactors
Hpa Emoy2	RxL44	105	No interactors
Hpa Emoy2	RxL45a	92	Weak only
Hpa Emoy2	RxL45b	87	Weak only
***Hpa* Emoy2**	**RxLL60**	**188**	**Various strong + weak**
***Hpa* Emoy2**	**RxL73**	**449**	**Various strong + weak**

ripAB *ripAF1*

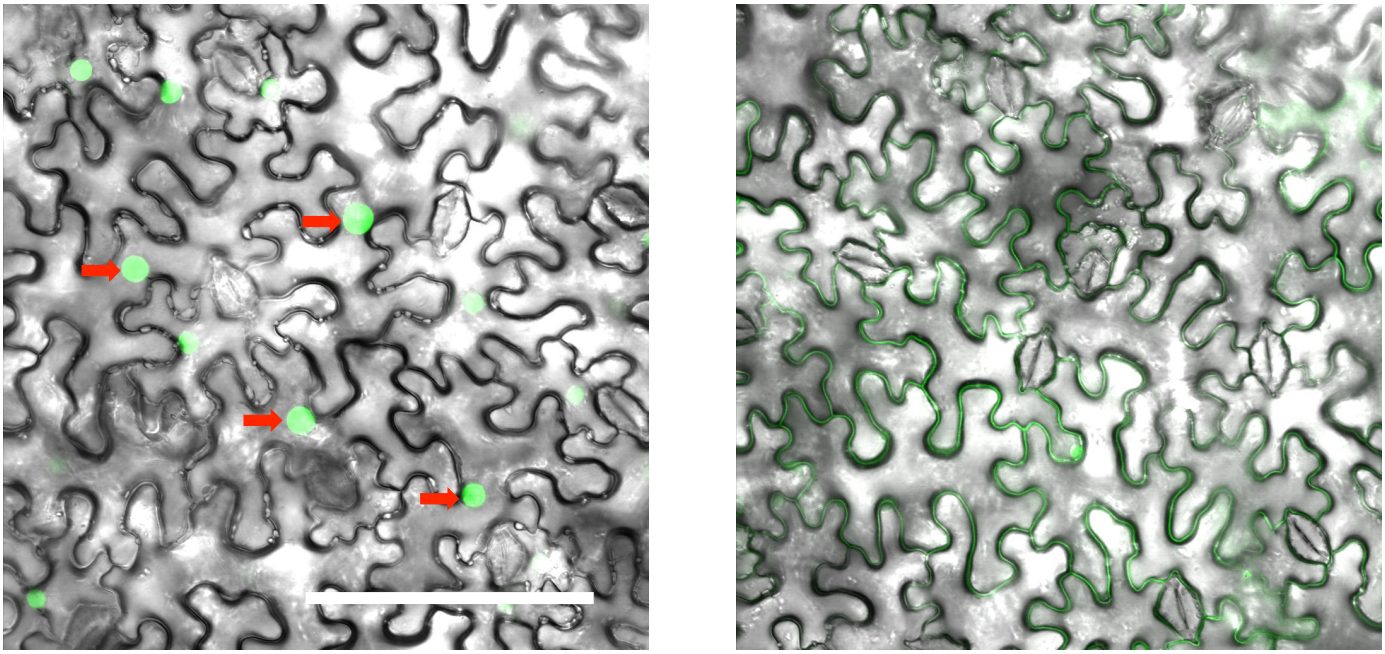
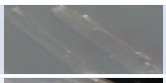
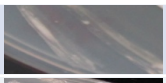
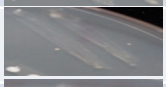
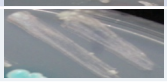
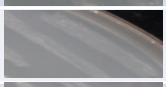
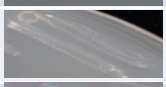
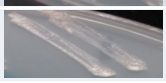
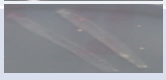
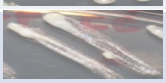
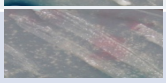
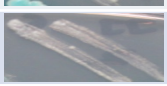
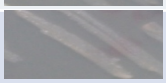
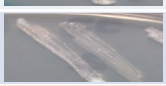
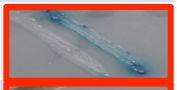
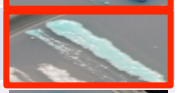
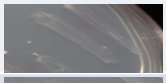
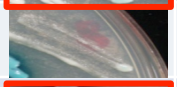
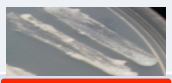
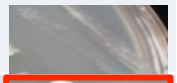


Figure 5.3. RipAF1 is not nuclear localized. *In planta* subcellular localization of RipAB and RipAF1 was determined in *N. benthamiana*. Leaves were infiltrated with *Agrobacterium tumefaciens* AGL1 at OD_{600} = 0.4 carrying YFP-tagged *ripAB* or *ripAF1* under a constitutive CaMV 35S promoter (#2.5.5 & #2.5.5.1). Leaf discs were taken at 2 days post infiltration and visualized under confocal laser-scanning microscopy for localization of the YFP tag. Panels are confocal images merged with bright-field images. White scale bar = 50 µm. Red arrows in RipAB sample indicate plant cell nuclei.

Using the Y2H screen (#2.6.5) we identified several AtWRKYs that interacted with several effectors from *Psa* and *Hpa*, both strongly and weakly – indicated with red borders for each panel (Figure 5.4). We validated these interactions by independently re-mating these selected AtWRKYs with their interacting effectors and carrying out dilutions to OD_{600} = 0.5 and spotting them on quadruple dropout selection medium containing X-gal (#2.6.5.2). AtWRKY1 did not interact with any effectors and thus was used as a negative control along with EV (empty pB42AD-GG vector) mated to each of the interacting effectors (Figure 5.5).

(See following 3 pages)

Figure 5.4. AtWRKY interacting effectors in Y2H (streaks). (A-C) Yeast expressing DBD-Effector and AD-AtWRKY (or AD-EV), were mated and then two independent colonies were restreaked and grown on SD/-His/-Leu/-Trp/-Ura/X-β-Gal medium for 5 days and then photographed (#2.6.5). The AvrRps4-AtWRKY41 interaction (green border) was used as positive control while Effector-EV (EV is pB42AD-GG) 'interactions' were used as negative controls. Putative positive interactions selected for further analysis are highlighted with a red border. Only AvrRps4, PopP2, AvrD, HopY1, RxL24, RxL73 and RxLL60 interactions are shown as only these effectors showed any strong interactions with the AtWRKYs. RipAF1 showed some strong interactions (possibly through an artifact of the screen) but was excluded because it was not nuclear-localized (Figure 5.3).

WRKY	AvrRps4	PopP2	AvrD1	HopY1	RxL24	RxL73	RxLL60
EV							
WRKY1							
WRKY2							
WRKY4							
WRKY6							
WRKY8							
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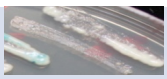
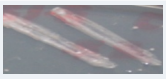
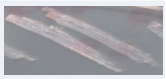
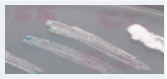
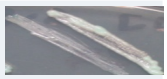
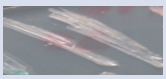
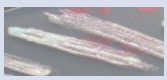
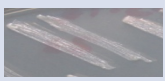
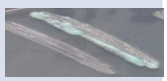
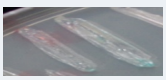
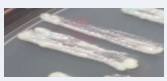
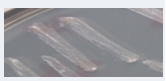
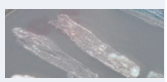
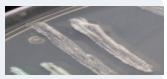
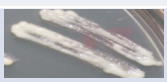
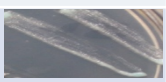
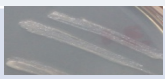
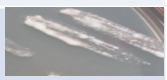
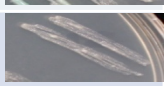
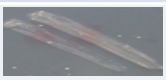
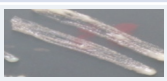
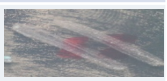
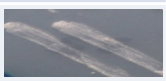
WRKY20							
WRKY21							
WRKY22							
WRKY23							
WRKY24							
WRKY25							

WRKY	AvrRps4	PopP2	AvrD1	HopY1	RxL24	RxL73	RxLL60
WRKY26							
WRKY27							
WRKY28							
WRKY30							
WRKY31							
WRKY33							
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WRKY46							
WRKY47							
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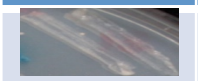
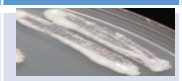
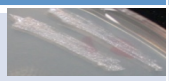
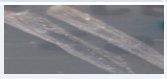
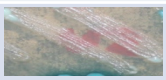
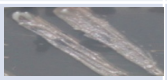
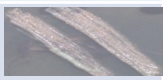
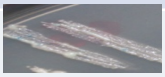
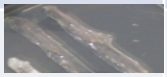
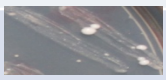
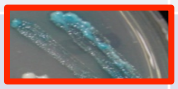
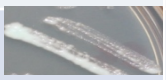
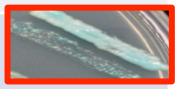
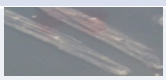
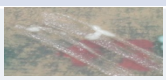
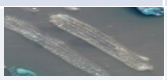
WRKY50							

WRKY	AvrRps4	PopP2	AvrD1	HopY1	RxL24	RxL73	RxLL60
WRKY51							
WRKY53							
WRKY54							
WRKY55							
WRKY56							
WRKY57							
WRKY58							
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WRKY60							
WRKY61							
WRKY62							
WRKY63							
WRKY64							
WRKY65							
WRKY66							
WRKY67							
WRKY69							
WRKY70							
WRKY72							
WRKY74							
WRKY75							

WRKY1
AvrRps4
AvrD1
HopY1
RxL24
RxL73
RxLL60
PopP2
WRKY22
WRKY1
PopP2
AvrRps4
WRKY41
WRKY53
WRKY54
WRKY70
RxL24
WRKY9
WRKY12
WRKY13
WRKY44
WRKY46
WRKY72
RxLL60
WRKY4
WRKY6
WRKY9
WRKY12
WRKY13
WRKY18
AvrD1
WRKY6
WRKY9
WRKY12
WRKY13
WRKY24
WRKY26
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WRKY44
WRKY46
WRKY51
WRKY56
WRKY58
AvrD1
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WRKY60
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WRKY61
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WRKY45
WRKY51
WRKY56
WRKY60
RxL73
WRKY4
WRKY6
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WRKY18
WRKY24
WRKY40
WRKY42
WRKY43
WRKY44
WRKY56
WRKY57
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WRKY66
WRKY72

(See previous page)
Figure 5.5. AtWRKY interacting effectors in Y2H (spots). Yeast expressing DBD-Effector and AD-AtWRKY, were mated and then two independent colonies were resuspended in SD /-His/-Leu/-Trp/-Ura liquid medium, diluted to OD_{600} = 0.5, and then spotted on SD/-His/-Leu/-Trp/-Ura/X-β-Gal medium (#2.6.5.2). The AvrRps4-AtWRKY41 interaction (green border) was used as positive control while Effector-AtWRKY1 interactions were used as negative controls (outlined in yellow). Putative positive interactions selected for further analysis are highlighted with a red border.

Notably, we found that PopP2 was slightly autoactive in our screen (Figure 5.4 compare interaction with EV) and as such it was difficult to identify any weak interactors using this Y2H approach. We were only able to confirm an interaction with the group II-e WRKY, AtWRKY22, using our conditions for PopP2 while previous studies have shown several other interactors (Table 5.4; (Le Roux et al., 2015)). Intriguingly, we found that AvrRps4 interacted strongly with several AtWRKYs from group III-a, including previously characterized AtWRKY41 and AtWRKY70 (Table 5.4; (Sarris et al., 2015)). We could not identify an interaction between AvrRps4 and either AtWRKY60 or AtWRKY33 as shown in this previous study, but identified novel interactors AtWRKY53 and AtWRKY54 that have previously not been described (Table 5.4).

Table 5.4. Summary of AtWRKY interacting effectors and their AtWRKY targets*

Grp	WRKY	popP2	avrRps4	hopY1	avrD1	RxL24	RxLL60	RxL73
III-a	WRKY41		■					
	WRKY53		■					
	WRKY46				■	■		
	WRKY54		■					
	WRKY70		■					
II-e	WRKY22	■						
II-b	WRKY72			■	■	■		■
	WRKY9			■		■	■	
II-c	WRKY12					■		
	WRKY13				■	■	■	
	WRKY43			■	■		■	■
I	WRKY44				■	■		■
	WRKY58				■			

*Blue filled cells indicate a putative strong interaction

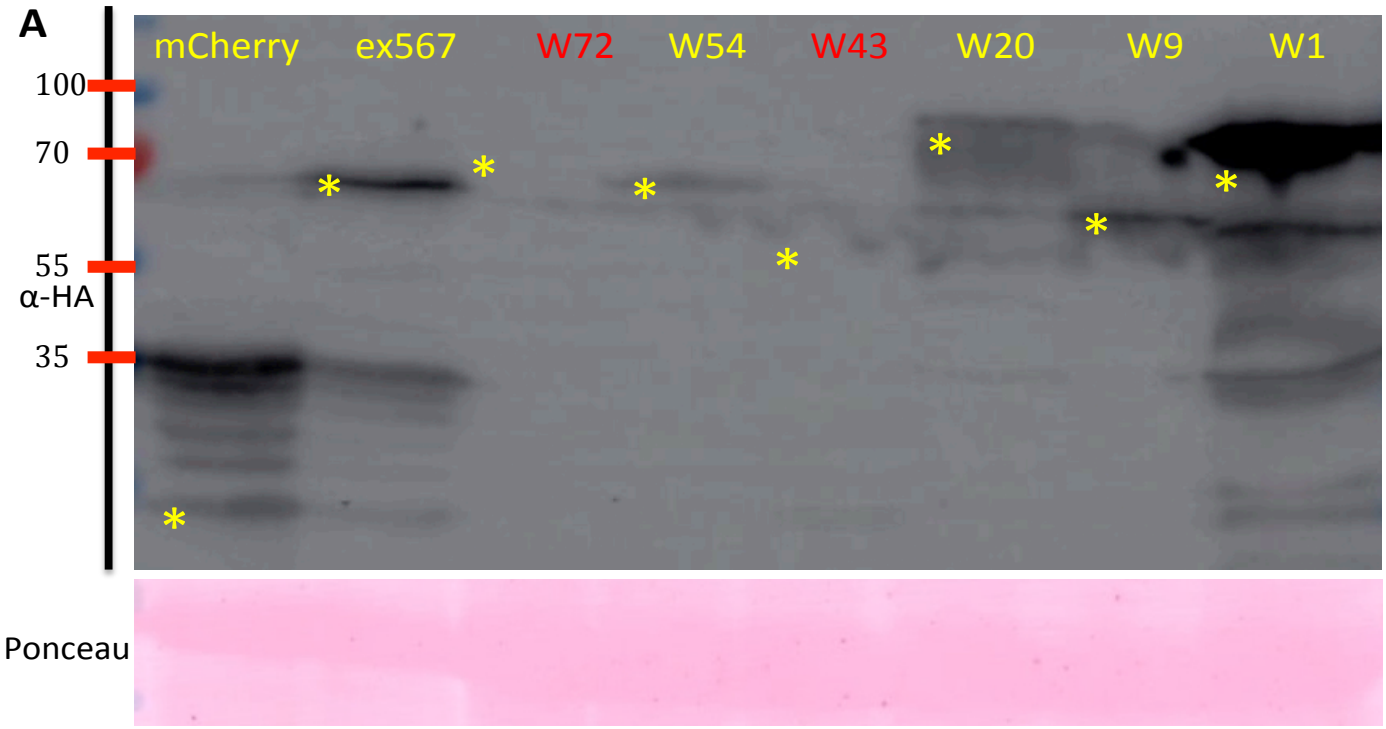
We identified T3Es AvrD and HopY1 from *Psa* V13 and RxLR effectors RxL24, RxL73, and RxLL60 from *Hpa* Emoy2 as interactors of AtWRKYs (Figure 5.5; Table 5.4). These effectors appear to interact with a subset of AtWRKYs (largely group I, II-b and II-c) unique to the subset interacting with AvrRps4 or PopP2 (group III-a or II-e). Notably, AtWRKY72 (W72) and AtWRKY43 (W43), highlighted in yellow (Table 5.4) are of particular interest, as they appear to interact with at least 4 different effectors from both pathogens. These two WRKYs, amongst others identified in this study, could serve as hubs for triggering immune responses and thus serve as attractive targets to be targeted by pathogens.

To validate the interactions seen in the Y2H screen, we used *Agrobacterium*-mediated transient expression in *N. benthamiana* leaves for either W72 or W43 fused to an N-terminal fragment of YFP under the CaMV 35S promoter and coexpressed it with a C-terminal fragment of YFP fused to HopY1 (BiFC). Using confocal laser scanning microscopy, we were unfortunately unable to see an interaction *in planta* (data not shown). Alternative constructs, swapping C- and N-terminal fragments of YFP to tag W72 and HopY1 at their N- or C-termini, did not give a detectable signal either. An immunoblot for selected HA-tagged WRKYs transiently expressed in *N. benthamiana* showed that W72 and W43 were undetectable at 2 dpi under the conditions used (Figure 5.6A). Controls mCherry-HA, HA-tagged exons5-7 of RRS1 (containing the WRKY domain), and HA-tagged AtWRKY1 were expressed well but other HA-tagged WRKYs, AtWRKY20 and AtWRKY54 and AtWRKY9 were poorly expressed.

This result indicated that certain AtWRKYs could be under tight regulation (post-transcriptionally or post-translationally) when expressed *in planta* and therefore may not accumulate to levels that are easily detectable. To confirm if the interactor and control AtWRKYs are detectable at all as well as to identify their subcellular localization, we conducted confocal fluorescence microscopy of full-length mCherry-tagged AtWRKY1, AtWRKY9, AtWRKY12, AtWRKY13, AtWRKY41, AtWRKY43, AtWRKY44, AtWRKY46, AtWRKY53, AtWRKY54, AtWRKY58, AtWRKY70, and AtWRKY72. Unfortunately, we were unable to

visualize their *in planta* localization even when coexpressed with the viral p19 silencing suppressor (data not shown) (Silhavy et al., 2002). Due to our suspicion that the mCherry tag may affect accumulation of the tagged AtWRKYs for visualization, we repeated the confocal microscopy test for YFP-tagged W72 and AtWRKY44, a known transcriptionally regulated WRKY from Arabidopsis (Han et al., 2013). We found that while AtWRKY44 accumulated to easily detectable levels (gain set at 450V) like the YFP tag expressed alone, W72 did not accumulate to high levels even with the YFP tag (gain set at 2260V) like mCherry tag alone (Figure 5.6B). We therefore theorized that any AtWRKYs that accumulated poorly *in planta* would not be detectable with an mCherry tag due to the combined effect of low accumulation of the WRKY and mCherry tag.

Furthermore, since we could detect W72 when tagged with YFP, albeit at low levels, we could confirm *in planta* expression of at least this one interactor AtWRKY. Unfortunately, the low accumulation of W72 suggests that, at least for the conditions used here, detection of an interaction between W72 and HopY1 or other effectors by BiFC will not be feasible.



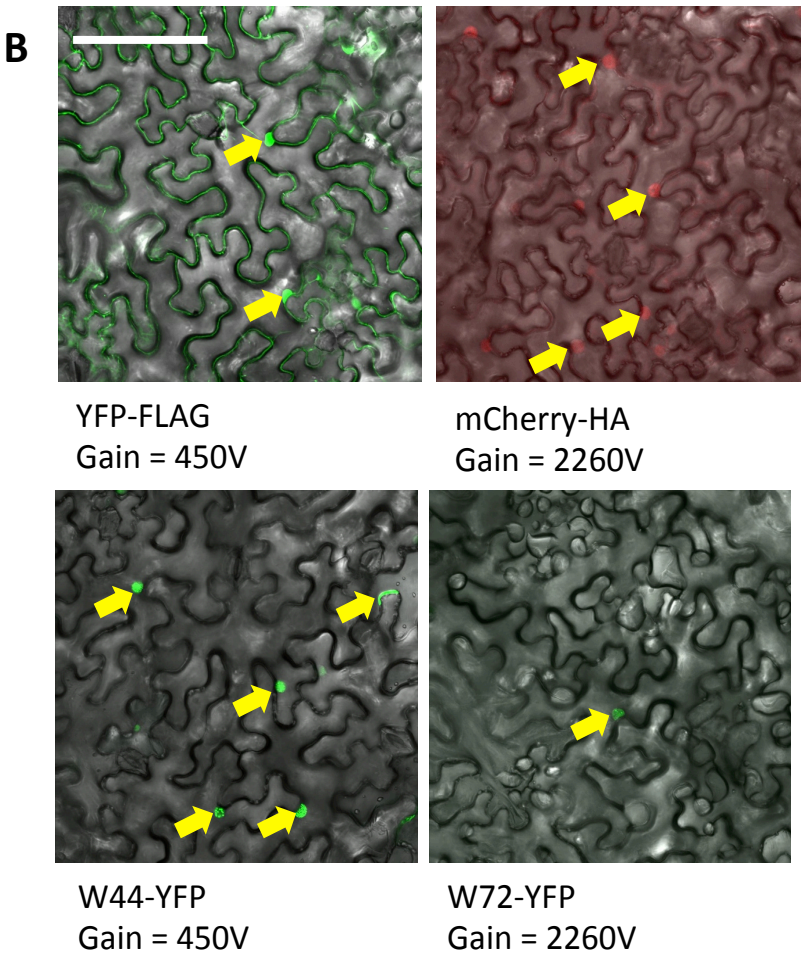


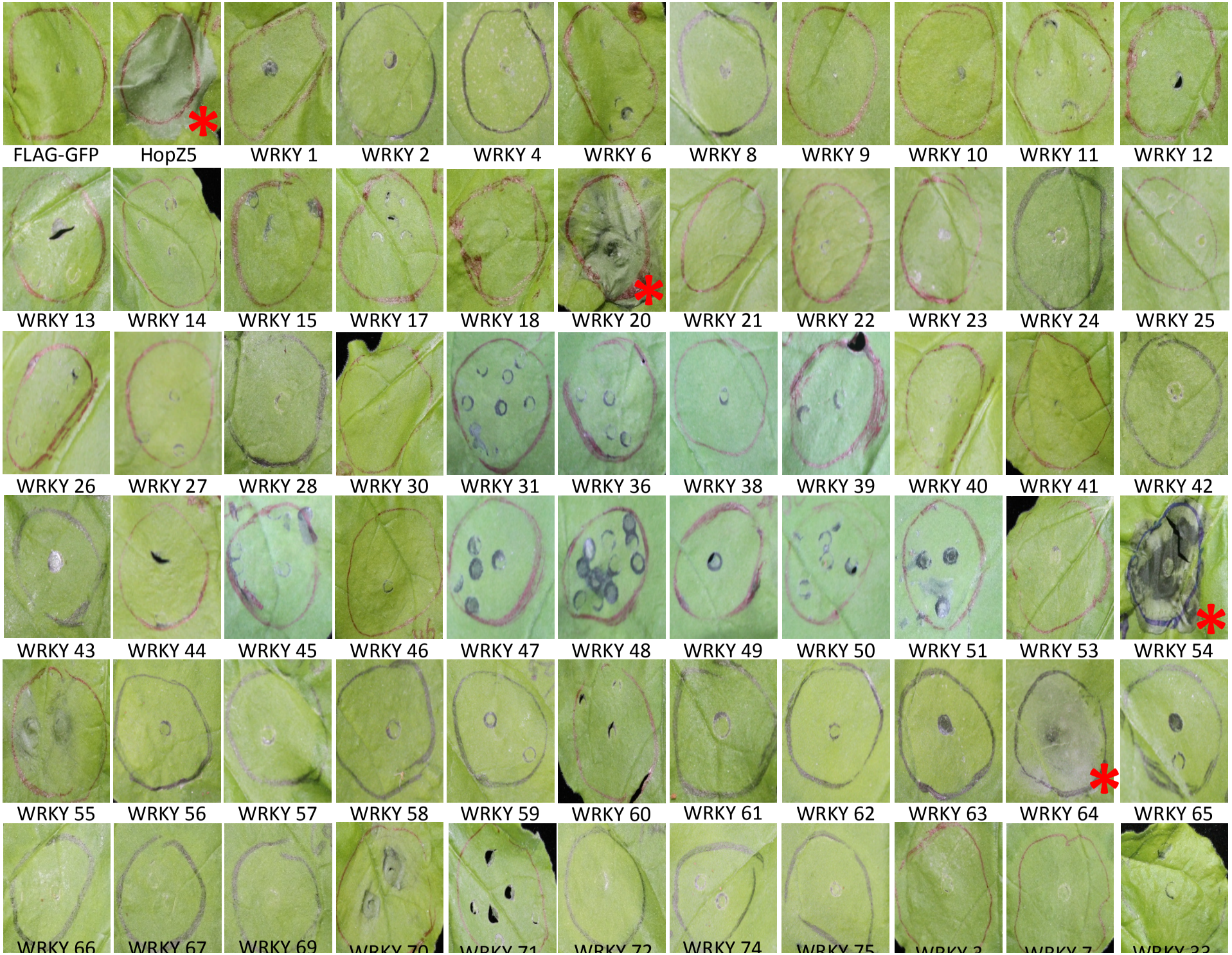
Figure 5.6. Interactor AtWRKYs are poorly expressed *in planta*. (A) Detection of selected HA-tagged WRKY transcription factors, mCherry alone tagged with HA, or HA-tagged exons 5-6-7 (C-term carrying WRKY domain) for RRS1 using α–HA antibody. Protein was extracted from *N. benthamiana* leaf discs at 2 days post infiltration with *Agrobacterium tumefaciens* AGL1 at OD_{600} = 0.5. Yellow asterisks mark bands of correct size for each AtWRKY. AtWRKY72 and AtWRKY43 showed no bands in the immunoblot. Ponceau Red staining of total protein extract showing the Rubisco protein band for sample loading control. Numbers to the left of the panel and associated red bars indicate band sizes for the PageRuler™ protein ladder in kDa. This experiment was conducted twice with similar results. **(B)** *In planta* subcellular localization of AtWRKY72, AtWRKY44, YFP-FLAG tag and mCherry-HA tag was determined in *N. benthamiana*. Leaves were infiltrated with *Agrobacterium tumefaciens* AGL1 at OD_{600} = 0.4 carrying genes under a constitutive CaMV 35S promoter. Leaf discs were taken at 2 days post infiltration and visualized under confocal laser-scanning microscopy for localization of the YFP/mCherry tag. Panels are confocal images merged with bright-field images. White scale bar = 50 µm. Yellow arrows indicate plant cell nuclei.

5.2.2 Overexpressing AtWRKYs in *Nicotiana* spp. triggers a conditional HCD dependent on their putative DNA-binding ability and *SGT1*

Interestingly, we found that when transiently overexpressed in *N. benthamiana* leaves, several AtWRKYs could trigger an HR-like cell death response (HCD) at 4 dpi specifically under low-temperature, low-humidity, low-light conditions. These included AtWRKY20, AtWRKY54 and AtWRKY64 (Figure 5.7A). Lack of an HCD response for these three AtWRKYs when the full AtWRKY library was overexpressed in related species, *N. tabacum*, demonstrated that this HCD development was specific to *N. benthamiana*. Additionally, we identified AtWRKY15 and AtWRKY55 as triggering an *N. tabacum*-specific HCD at 4 dpi (Figure 5.7B).

Previous studies of PopP2 activity on RRS1 and AtWRKY41 showed that PopP2-mediated acetylation of key lysine residues in the WRKYGQK motif blocked the DNA-binding ability of RRS1 and AtWRKY41 and that a mutant of this lysine residue in RRS1 was not able to bind DNA either (Le Roux et al., 2015; Sarris et al., 2015). To observe if the HCD-triggered by any of the AtWRKYs in *N. benthamiana* required their DNA-binding ability, we generated double mutants of AtWRKY20 (C-terminal WRKY motif), AtWRKY54, and AtWRKY64 so that the WRKYGQK motif was changed to a WRQYGQQ instead. We then observed if the HCD-triggering ability was compromised in *N. benthamiana* when transiently expressed. Notably, all three mutated AtWRKYs (AtWRKY20-QQ, AtWRKY54-QQ, and AtWRKY64-QQ) lost their ability to trigger HCD at 4dpi when they putatively lost their DNA-binding ability (Figure 5.8A). To examine if this loss of HCD was attributable to a loss in protein accumulation of the relevant mutated AtWRKY, we optimized conditions for expression of these AtWRKYs at 3dpi and coexpressed it with viral p19 silencing suppressor. Immunoblotting for the HA-

A


FLAG-GFP
HopZ5
WRKY 1
WRKY 2
WRKY 4
WRKY 6
WRKY 8
WRKY 9
WRKY 10
WRKY 11
WRKY 12
WRKY 13
WRKY 14
WRKY 15
WRKY 17
WRKY 18
WRKY 20
WRKY 21
WRKY 22
WRKY 23
WRKY 24
WRKY 25
WRKY 26
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WRKY 62
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WRKY 70
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WRKY 75

B

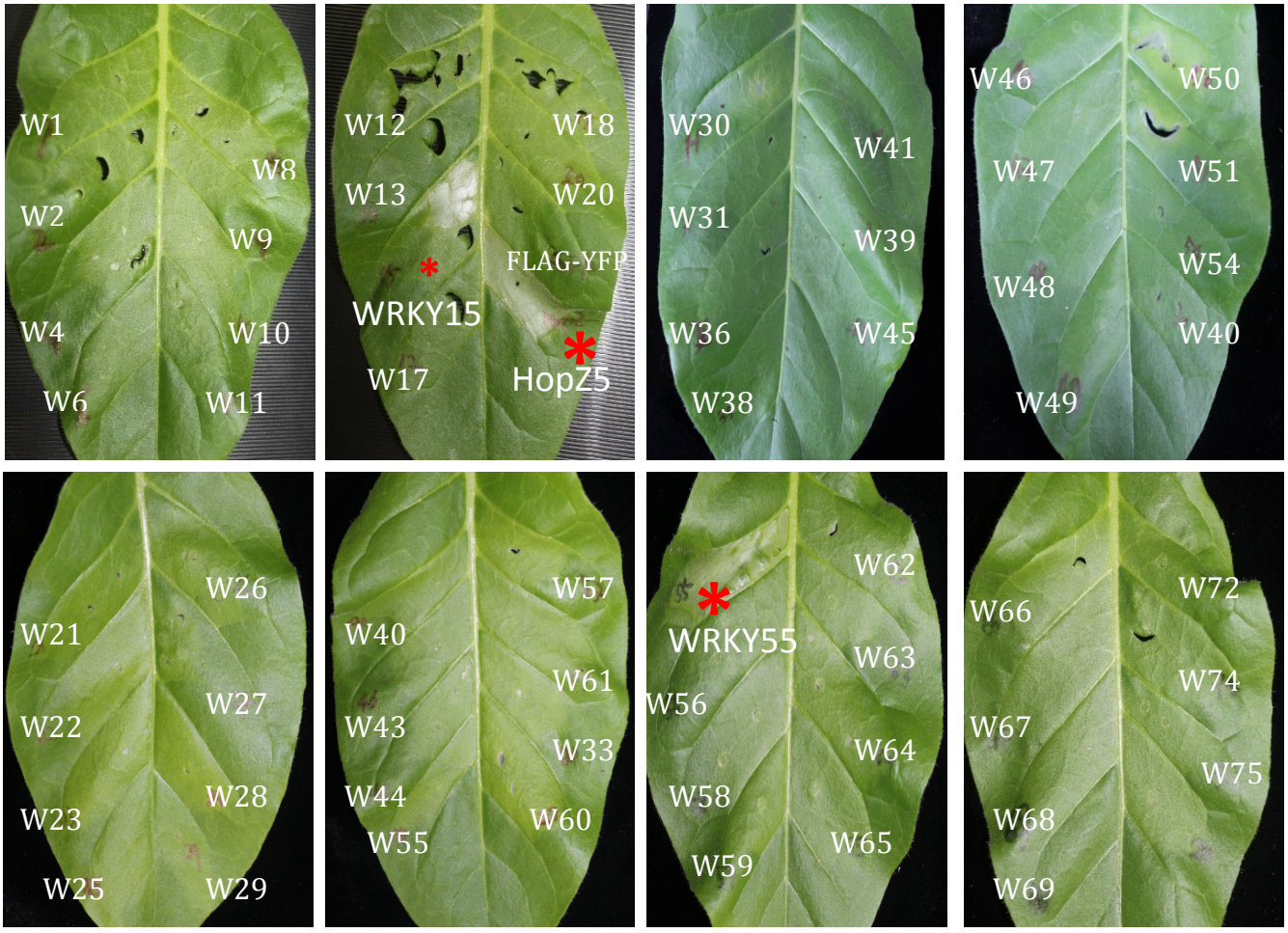
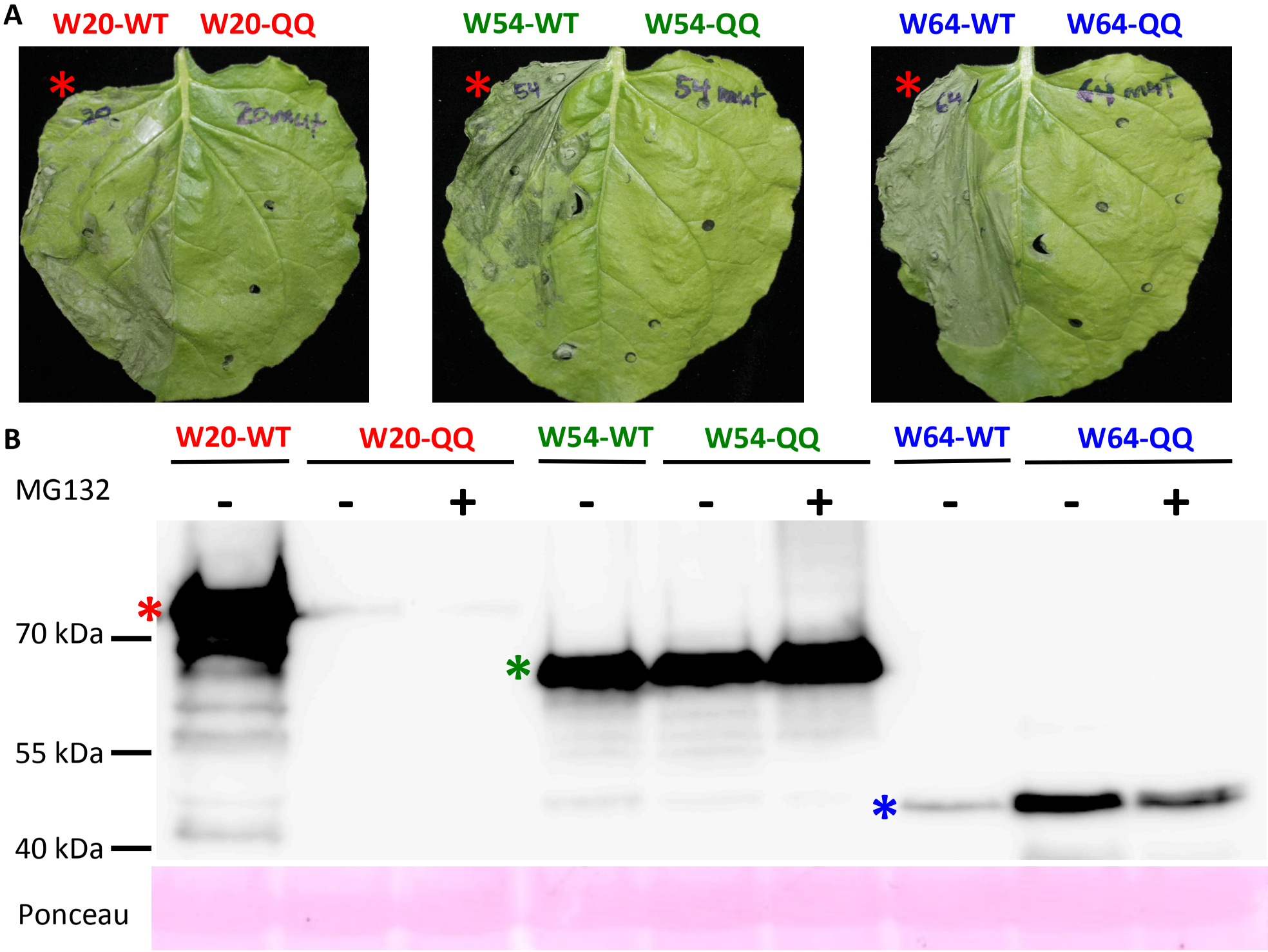


Figure 5.7. AtWRKY-triggered HR-like cell death in *Nicotiana* spp. (A) All cloned AtWRKY transcription factors used in the Y2H library screen were transiently expressed by *Agrobacterium tumefaciens* AGL1 infiltration (OD_{600}=0.5) under the CaMV 35S promoter with a C-terminal 6xHA tag in *N. benthamiana* (#2.5.5; Appendix 4). Photographs were taken at 4 days post infiltration (dpi). FLAG-YFP and HopZ5-HA were used as negative and positive controls for HR-like cell death (HCD), respectively. **(B)** Infiltrations carried out as in (A) for *N. tabacum* (#2.5.5). Photographs were taken at 4 days post infiltration (dpi). FLAG-YFP and HopZ5-HA were used as negative and positive controls for HCD, respectively. Red asterisks (in both panels) indicate strong development of HCD symptoms only. The experiment (for both panels) was conducted three times with identical results.

(See following page)

Figure 5.8. AtWRKY20/AtWRKY54/AtWRKY64-triggered HCD depends on their WRKY motif. (A) The WRKYGQK residues of the WRKY motif for AtWRKY20 (C-term), AtWRKY54, and AtWRKY64 were mutagenized to predicted non-DNA binding variants, WRQYGQQ. Mutant variants (QQ) along with wildtype (WT) were transiently expressed by *Agrobacterium tumefaciens* AGL1 infiltration (OD_{600}=0.5) under the CaMV 35S promoter with a C-terminal 6xHA tag in *N. benthamiana* half-leaves (#2.5.5). Photographs were taken at 4 days post infiltration (dpi). Red asterisks indicate development of HCD symptoms. This experiment was conducted twice with identical results. **(B)** Detection of HA-tagged AtWRKYs and their variants using α–HA antibody. Protein was extracted from *N. benthamiana* leaf discs at 3 days post infiltration with *Agrobacterium tumefaciens* AGL1 at OD_{600}=0.5, with (+) or without (-) a 100μM MG132 treatment 3 hours prior to harvest (#2.6.4). Asterisks mark expected size for each AtWRKY. Ponceau Red staining of total protein extract showing the Rubisco protein band for sample loading control. Numbers to the left of the panel and associated bars indicate band sizes for the PageRuler™ protein ladder in kDa.


A
W20-WT
W20-QQ
W54-WT
W54-QQ
W64-WT
W64-QQ
B
W20-WT
W20-QQ
W54-WT
W54-QQ
W64-WT
W64-QQ
MG132
- - + - - + - - +
70 kDa
55 kDa
40 kDa
Ponceau

tag of these AtWRKYs showed normal accumulation of AtWRKY54-QQ compared to wildtype AtWRKY54(-WT) and slightly better accumulation of AtWRKY64-QQ compared to AtWRKY64-WT (Figure 5.8B). The latter is probably due to the strong HCD response triggered by AtWRKY64-WT even at 3dpi leading to poor protein extraction. Surprisingly, AtWRKY20-QQ failed to accumulate to the same level as AtWRKY20-WT, suggesting that the loss of DNA-binding affected protein accumulation. This further suggested that a possible reason for the loss of HCD for AtWRKY20-QQ expression could simply be a result of poor protein accumulation (indirectly a result of loss of DNA-binding) but for both AtWRKY54-QQ and AtWRKY64-QQ, the lack of DNA-binding ability was likely a proximate cause for the loss of HCD.

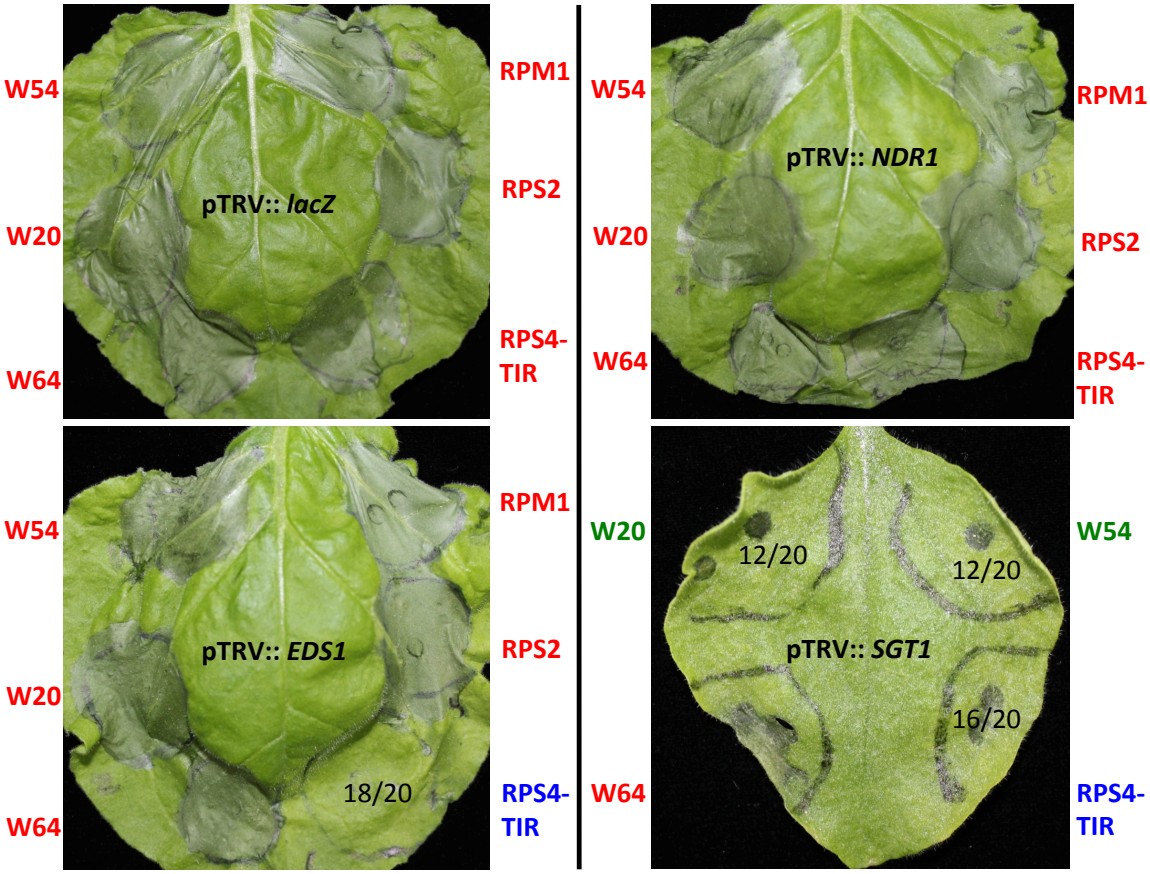


Figure 5.9. AtWRKY20/AtWRKY54-triggered HCD partially requires *SGT1* but not *EDS1* or *NDR1*. Two-week-old *N. benthamiana* seedlings were infiltrated with *Agrobacterium tumefaciens* AGL1 carrying pTRV:EV or pTRV2 targeting *SGT1*, *EDS1* or *NDR1* for silencing (#2.5.7). 4-5 weeks post VIGS treatment, AtWRKY20, AtWRKY54, and AtWRKY64 were transiently expressed in silenced plants along with RPM1, RPS2, and RPS4-TIR domain all under the control of 35S CaMV promoter (#2.5.5; Appendix 4). Photographs were taken at 4 dpi. Red colored labels indicate development of cell death symptoms, blue colored labels indicate successful control HCD suppression by silencing, and green colored labels indicate partial suppression. The experiment was conducted twice with similar results. Numbers indicated on infiltrated patches without HCD demonstrate HCD-suppressed/total patches infiltrated for both experimental runs.

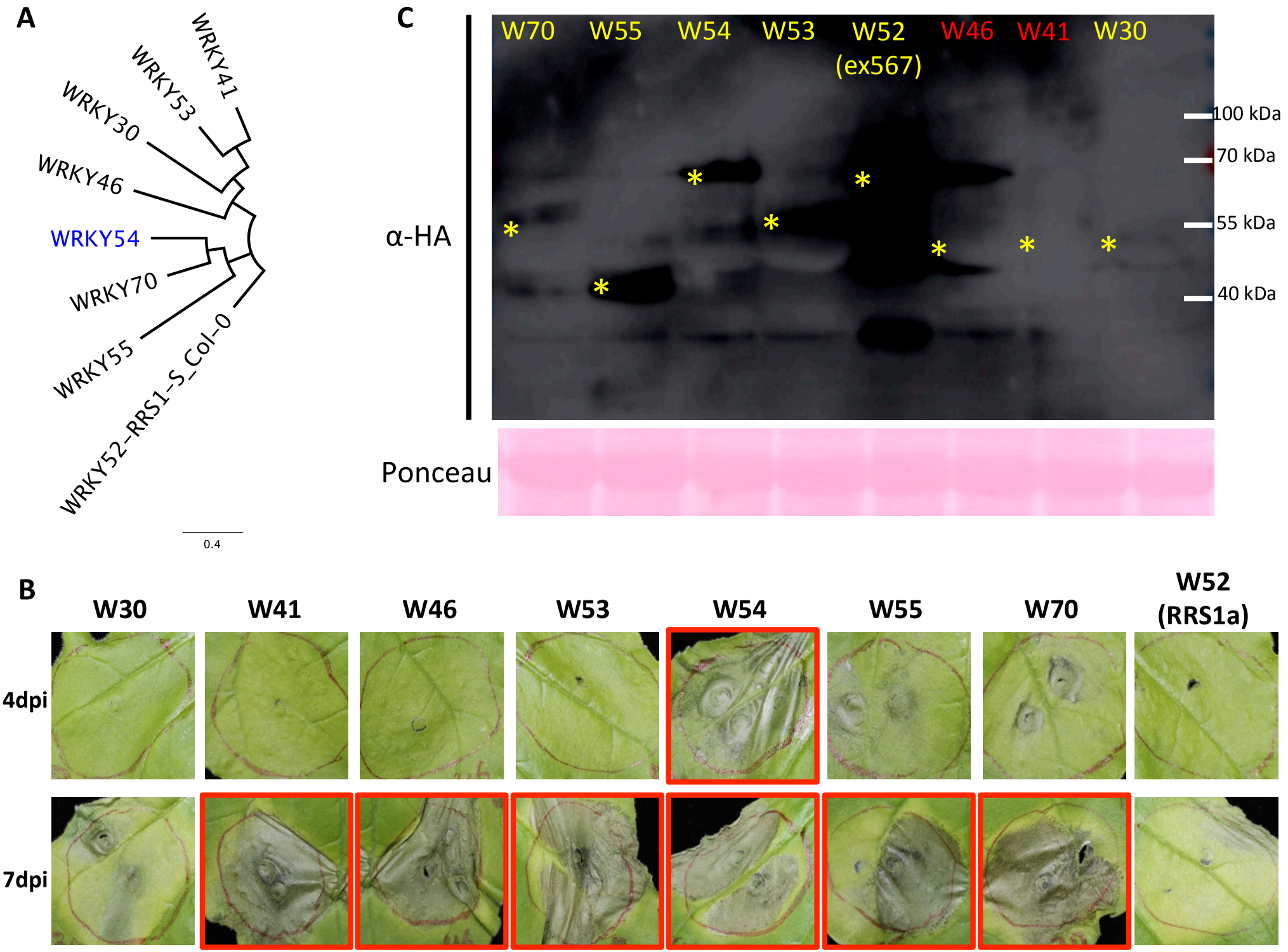
To examine the genetic requirements for the HCD triggered by AtWRKY20, AtWRKY54, and AtWRKY64, we utilized virus-induced gene silencing (VIGS) of important immune regulators *NDR1*, *EDS1* and *SGT1*. Following silencing, infiltration of *N. benthamiana* leaves with AtWRKY20, AtWRKY54, AtWRKY64, the full length CNLs RPS2, or RPM1, or the RPS4-TIR domain generated HCD symptoms at 4 dpi in EV-silenced plants (Figure 5.9). *EDS1*-silenced plants lost RPS4-TIR-triggered HCD as expected but was unaffected for any of the AtWRKYs, while *NDR1*-silenced plants were unaffected for any of the infiltrations including RPS2 and RPM1, surprisingly. Most notably, *SGT1*-silenced plants largely lost the HCD in response to AtWRKY20 (40% of infiltrated patches triggered HCD) and AtWRKY54 (40% of infiltrated patches triggered HCD), similar to loss for RPS4-TIR (20% of infiltrated patches triggered HCD). Interestingly, for AtWRKY64, only 15% of infiltrated patches showed a reduction of HCD symptoms. This result suggested that *SGT1* was required for AtWRKY-triggered HCD for AtWRKY20 and AtWRKY54 but not for AtWRKY64.

5.2.3 AtWRKY54-triggered HCD is representative for immune-triggering ability of group III-a AtWRKYs

From the transient expression screen of our AtWRKY library in *N. benthamiana*, the HCD triggered by AtWRKY54 was particularly interesting since it interacts with AvrRps4 in our Y2H screen. Even more curious is the interaction of many effectors from this AtWRKY group III-a family with AvrRps4 (Table 5.4). This suggested to us that AvrRps4 targets the whole family of AtWRKY54 homologs.

(See following page)

Figure 5.10. AtWRKY54 homologs are expressed *in planta* but only trigger weak HCD. **(A)** Jukes-Cantor neighbor-joining tree for group III-a homologs of AtWRKY54 from Aabidopsis Col-0 was generated using Geneious software. Node length is indicated. **(B)** AtWRKY54-homologous AtWRKYs were transiently expressed by *Agrobacterium tumefaciens* AGL1 infiltration (OD_{600}=0.5) under the CaMV 35S promoter with a C-terminal 6xHA tag in *N. benthamiana* (#2.5.5; Appendix 4). Photographs were taken at 4 and 7 days post infiltration (dpi). Full length RRS1 was used as negative control for HCD. Red borders on each panel indicate development of HCD symptoms. This experiment was conducted twice with identical results. **(C)** Detection of AtWRKY54-homologous HA-tagged AtWRKYs using α–HA antibody. Protein was extracted from *N. benthamiana* leaf discs at 3 days post infiltration with *Agrobacterium tumefaciens* AGL1 at OD_{600} = 0.5. Yellow asterisks mark bands of correct size for each AtWRKY. AtWRKY46 and AtWRKY41 showed no bands in the immunoblot and are indicated in red. AtWRKY52 (exons 5-7 of RRS1) was used as a positive control. Ponceau Red staining of total protein extract showing the Rubisco protein band for sample loading control. Numbers to the right of the panel and associated bars indicate band sizes for the PageRuler™ protein ladder in kDa.


A
WRKY41
WRKY53
WRKY30
WRKY46
WRKY54
WRKY70
WRKY55
WRKY52-RRS1-S_Col-0
0.4
C
W70
W55
W54
W53
W52
(ex567)
W46
W41
W30
100 kDa
70 kDa
55 kDa
40 kDa
α-HA
Ponceau
B
W30
W41
W46
W53
W54
W55
W70
W52
(RRS1a)
4dpi
7dpi

The AtWRKY54 family also includes AtWRKY52 (RRS1-S) which is known to be involved in recognition of AvrRps4 (Narusaka et al., 2016, 2009) as well as several AtWRKYs involved in plant immunity (Figure 5.10A).

During the screen of the AtWRKY library in *N. benthamiana*, we noticed that several AtWRKYs occasionally triggered a weak HCD at 4 dpi but were not noted as strong HCD triggers (data not shown). These included AtWRKY55 and AtWRKY70. We therefore, examined all AtWRKY54 homologs by transient expression for their ability to trigger HCD at a later time (7 dpi) as an indicator of a later development of HCD (weak HCD) in *N. benthamiana*. Interestingly, we found that all AtWRKY54 homologs, except AtWRKY30 and RRS1-S could trigger HCD at this later time (Figure 5.10B). Immunoblots to examine expression of these AtWRKYs allowed us to detect expression of all AtWRKY54 homologs at 3 dpi except for AtWRKY46 and AtWRKY41 (despite their ability to trigger weak HCD) and poor expression of AtWRKY70 and AtWRKY30 (Figure 5.10C). The latter could thus explain the lack of HCD triggered by AtWRKY30. Taken together, our findings indicate that most if not all group III-a AtWRKYs can trigger an ectopic HCD response when overexpressed in *N. benthamiana* and that AtWRKY54 could serve as a marker for this ability. Furthermore, the targeting of this family by AvrRps4 is of interest.

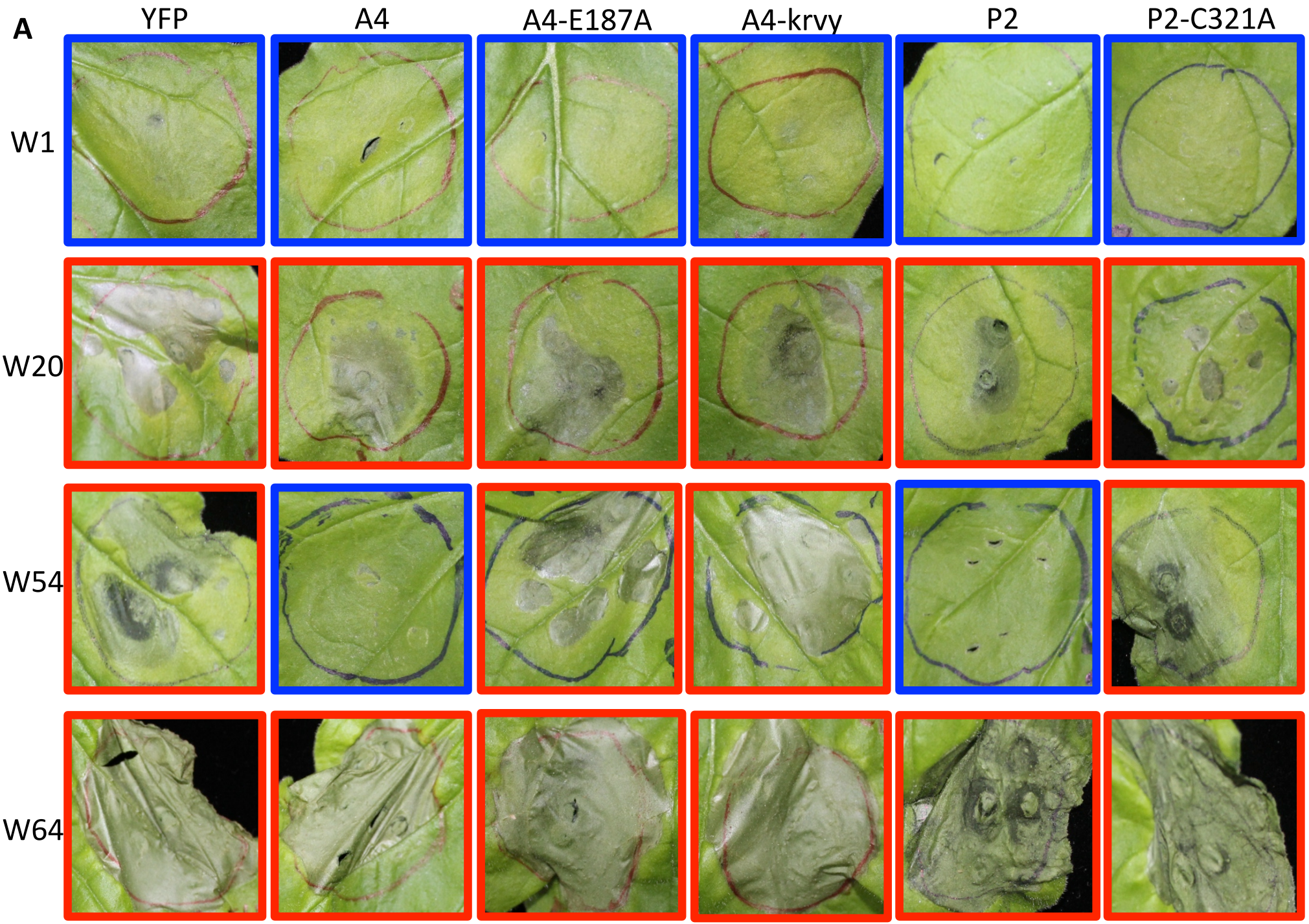
5.2.4 AvrRps4 and PopP2 specifically suppress the AtWRKY54 overexpression HCD phenotype

As mentioned earlier, due to the interaction of AvrRps4 (A4) with AtWRKY54 in the Y2H screen, we were interested in whether A4 could suppress AtWRKY54-triggered HCD. To this end, we transiently coexpressed AtWRKY1 (W1; does not trigger HCD/does not interact with A4), AtWRKY20 (W20; triggers HCD/does not interact with A4), AtWRKY54 (W54; triggers HCD/interacts with A4), or AtWRKY64 (W64; triggers HCD/does not interact with A4) with A4 or a putative non-functional mutant variant (A4-E187A/A4-krvy) that is known to not activate ETI in Arabidopsis (Sohn et al., 2012a). The A4-E187A variant has a glutamic acid to alanine substitution while the A4-krvy variant has all four residues (lysine, arginine, valine and tyrosine) following the A4 self-processing site

substituted for four alanines. While A4 did not interfere with W20- and W64-triggered HCD, W54-triggered HCD was completely suppressed by A4 (Figure 5.11A). Critically, this W54-triggered HCD suppression by A4 was dependent on both its E187 residue and its KRVY motif.

Due to the ability of PopP2 (P2) to also interact with RRS1-S (Sarris et al., 2015), we checked if P2 was also able to suppress the W54-triggered HCD. Surprisingly, even though P2 failed to interact with W54 in our Y2H screen, we found that P2 could suppress W54-triggered HCD, but did not affect W20- and W64-triggered HCD (Figure 5.11A). Additionally, this W54-triggered HCD suppression by P2 was dependent on its catalytic activity as an acetyltransferase since the catalytic site inactive mutant C321A (Tasset et al., 2010) was unable to suppress the HCD.

While the acetyltransferase activity of P2 on its target WRKY transcription factors are known, the activity of A4 is not. To investigate further the ability of A4 to suppress W54-triggered HCD, we coexpressed several different mutant variants of A4 with W54. In addition to the E187A and krvy mutant variants, we coexpressed another mutant in the *in planta* processed C-terminal fragment, E175A (a glutamic acid to alanine substitution) that failed to initiate RRS1/RPS4-mediated HR (Sohn et al., 2012a), with W54. We also coexpressed an A4 mutant defective in *in planta* processing, R112L (an arginine to leucine substitution), and both the C-term and N-term processed fragments of A4 (Sohn et al., 2009) with W54. We found that along with the E187 residue and the KRVY motif in the C-terminus fragment, E175A is also required for suppression of W54-triggered HCD (Figure 5.11B). Interestingly, like wildtype A4, the R112L variant of A4 was also able to largely suppress W54-triggered HCD, while neither the C-terminus nor N-terminus fragments could do so (Figure 5.11B). Additionally, this suppression of W54-triggered HCD was specific as the A4-R112L variant was unable to suppress W20-triggered HCD, in a manner similar to wildtype A4 that was compromised in the A4-E175A variant (Figure 5.11C). Quantification of electrolyte leakage for these variants of AvrRps4 as well as P2 and its C321A variant further supported that while E187A, E175A and krvy mutant variants of A4 were compromised in their ability to specifically suppress W54-triggered


A
YFP
A4
A4-E187A
A4-krvy
P2
P2-C321A
W1
W20
W54
W64

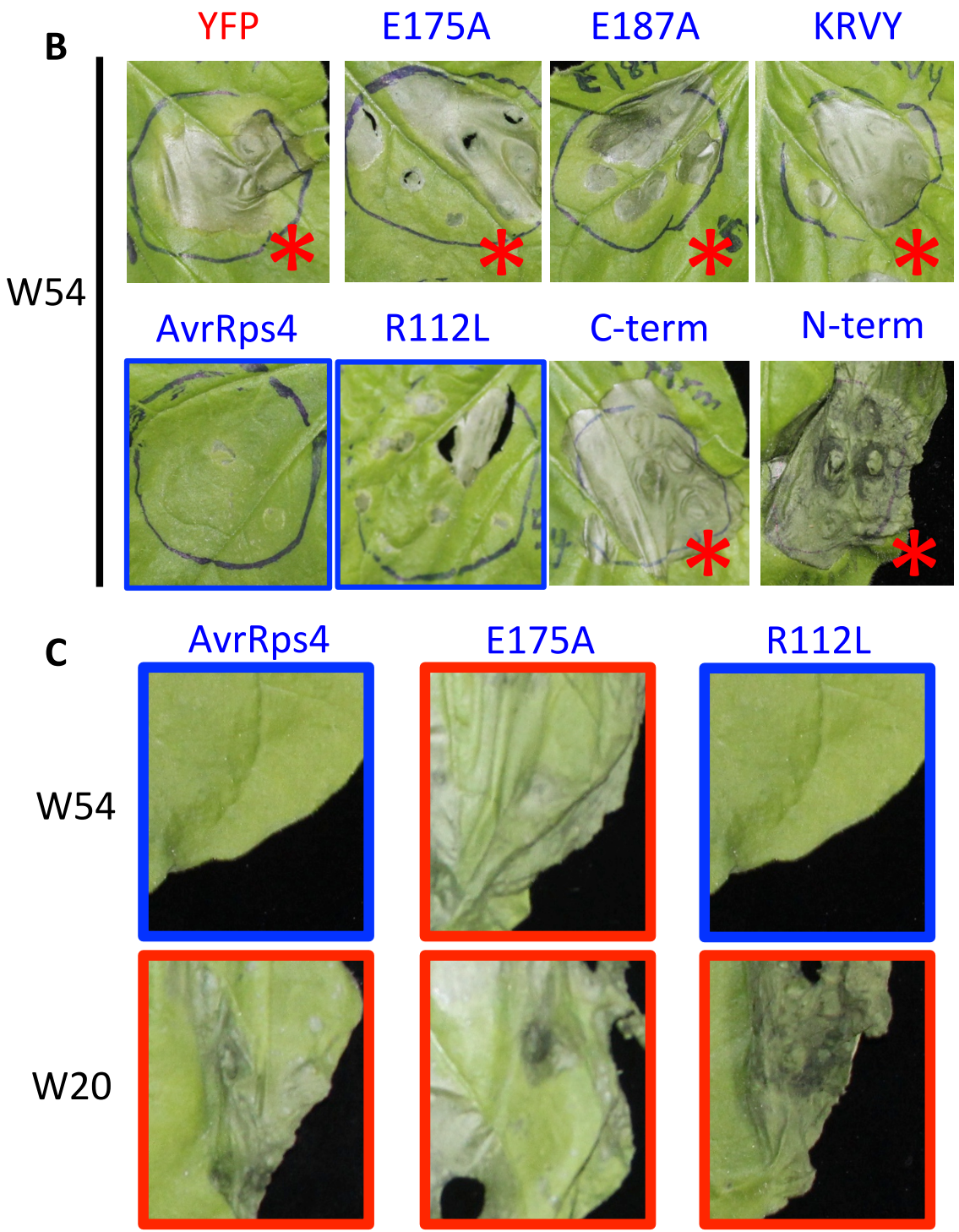


Figure 5.11. AvrRps4/PopP2 specifically suppress AtWRKY54-triggered HCD. **(A)** HA-tagged AtWRKY1 (negative HCD control), AtWRKY20 (negative interactor control), AtWRKY54 or AtWRKY64 (negative interactor control) were transiently coexpressed with FLAG-tagged AvrRps4 (A4), A4-E187A mutant, A4-krvy mutant, PopP2 (P2), or P2-C321A mutant by *Agrobacterium tumefaciens* AGL1 infiltration (AtWRKYs at OD_{600}=0.4, effectors at OD_{600}=0.6) under the CaMV 35S promoter in *N. benthamiana* (#2.5.5; Appendix 4). Photographs were taken at 4 days post infiltration (dpi). Red and blue borders on each panel indicate development of HCD symptoms and lack thereof, respectively. This experiment was conducted five times with identical results. **(B)** HA-tagged AtWRKY54 was transiently coexpressed with FLAG-tagged AvrRps4, or its E175A, E187A, KRVY, or R112L mutants, C- or N-terminal fragment by *Agrobacterium tumefaciens* AGL1 infiltration (AtWRKYs at OD_{600} = 0.4, effectors at OD_{600}=0.6) under the CaMV 35S promoter in *N. benthamiana* (#2.5.5; Appendix 4). Photographs were taken at 4 days post infiltration (dpi). Blue borders on each panel indicate suppression of HCD symptoms, red asterisks indicate successful development of HCD symptoms. This experiment was conducted four times with identical results. **(C)** HA-tagged AtWRKY20 (negative interactor control), or AtWRKY54 were transiently coexpressed with FLAG-tagged AvrRps4, or its E187A or R112L mutants by *Agrobacterium tumefaciens* AGL1 infiltration (AtWRKYs at OD_{600}=0.4, effectors at OD_{600}=0.6) under the CaMV 35S promoter in *N. benthamiana* (#2.5.5; Appendix 4). Photographs were taken at 4 days post infiltration (dpi). Red and blue borders on each panel indicate development of HCD symptoms and lack thereof, respectively. This experiment was conducted twice with identical results.

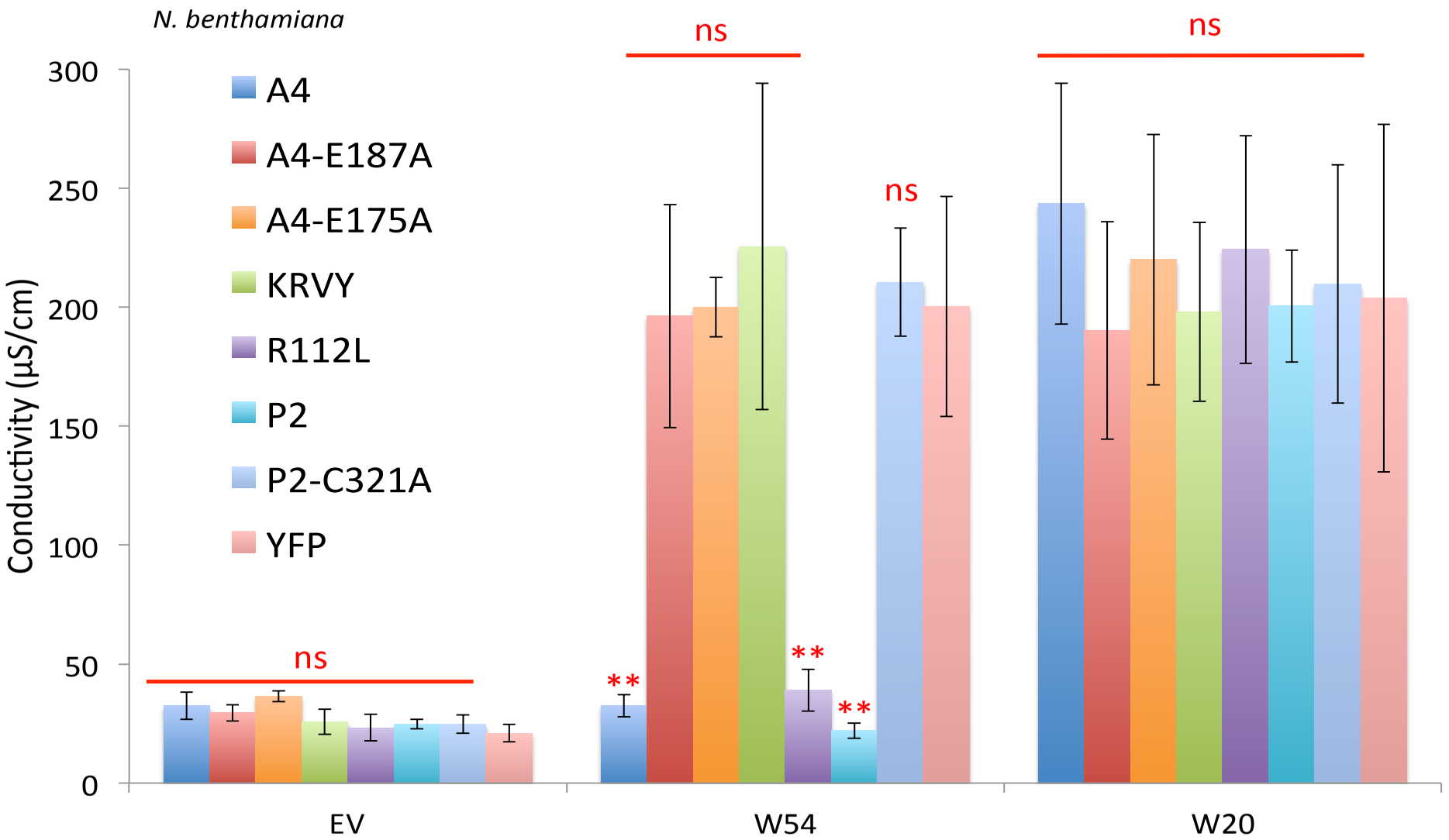
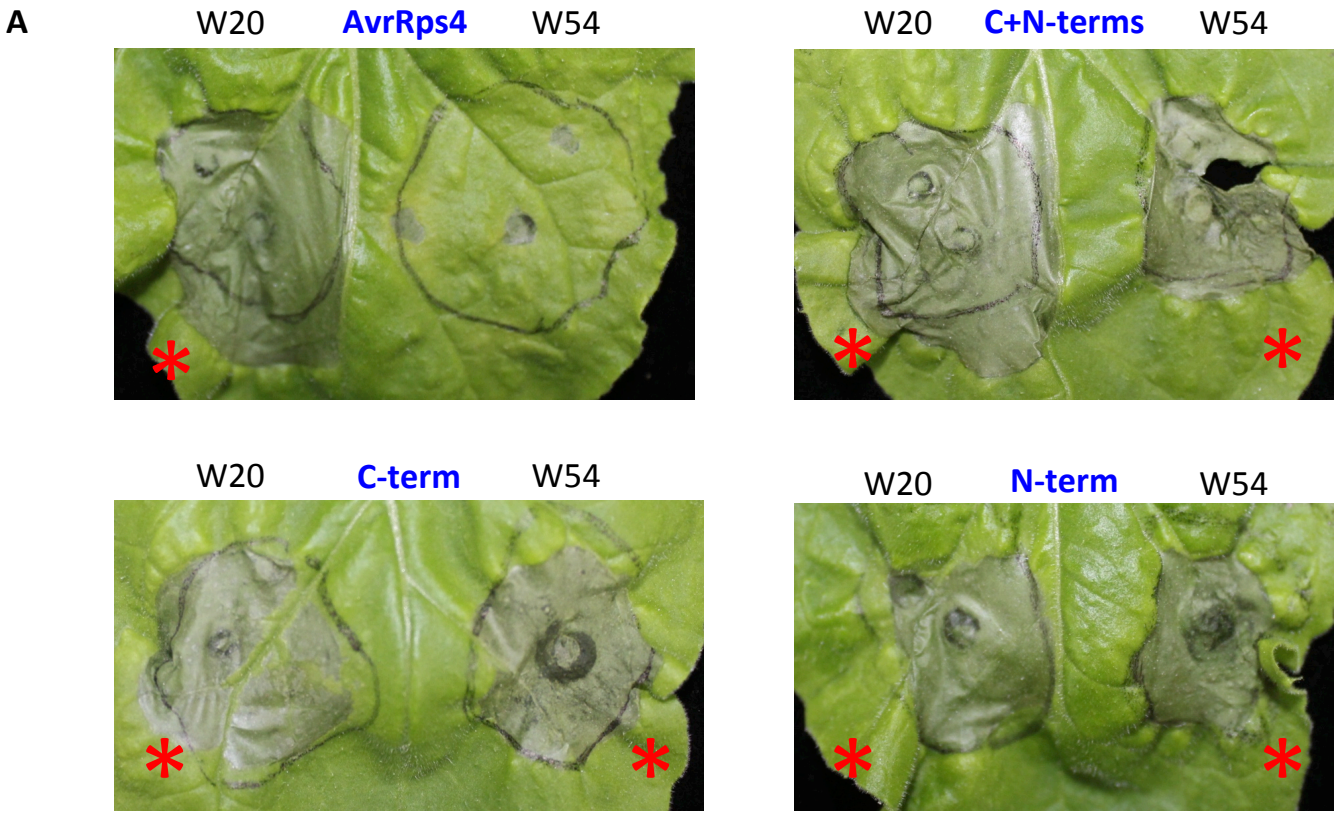


Figure 5.12. AvrRps4/PopP2 suppress ion leakage from AtWRKY54-triggered HCD. Electrolyte leakage from *N. benthamiana* leaf discs taken at 3 dpi triggered by *Agrobacterium*-mediated transient expression of EV, AtWRKY54 or AtWRKY20 (Appendix 4) coexpressed with YFP, A4 (AvrRps4), A4-E187A mutant, A4-E175A mutant, (A4-)KRVY mutant, (A4-)R112L mutant, P2 (PopP2), or P2-C321A mutant (Sohn et al., 2007; Tasset et al 2010; AtWRKYs at OD_{600}=0.4, effectors at OD_{600}=0.6) under the CaMV 35S promoter in *N. benthamiana* (#2.5.5). Error bars represent standard deviation from four technical replicates. Asterisks indicate results of Student's *t*-test between selected samples and the YFP expressing sample for that AtWRKY; ** ($p < 0.01$), "ns" indicates non-significant difference. This experiment was conducted four times with identical results.

HCD, the A4-R112L variant was still able to suppress this HCD (Figure 5.12). Additionally, this assay confirmed that P2 can measurably suppress W54-triggered HCD specifically, possibly through an interaction with W54 not visualized in the Y2H screen, and depends on a functional catalytic activity to do so.

5.2.5 Unprocessed AvrRps4 alone can suppress AtWRKY54-triggered HCD

Due to the unexpected result that the R112L variant of A4 could suppress W54-triggered HCD, while the N- and C-terminus fragments were not, we wanted to investigate whether this suppression was a result of a lack of all components of A4 being present. We therefore coexpressed both N- and C-terminus fragments together with W54 as well as each fragment alone, or full length A4 with W54 in *N. benthamiana*. As seen earlier, neither N- nor C-terminal fragments of A4 could suppress W54-triggered HCD. Notably, when coexpressing both N- and C-terminal fragments with W54, the HCD still developed but did not do so for full length A4 (Figure 5.13A).



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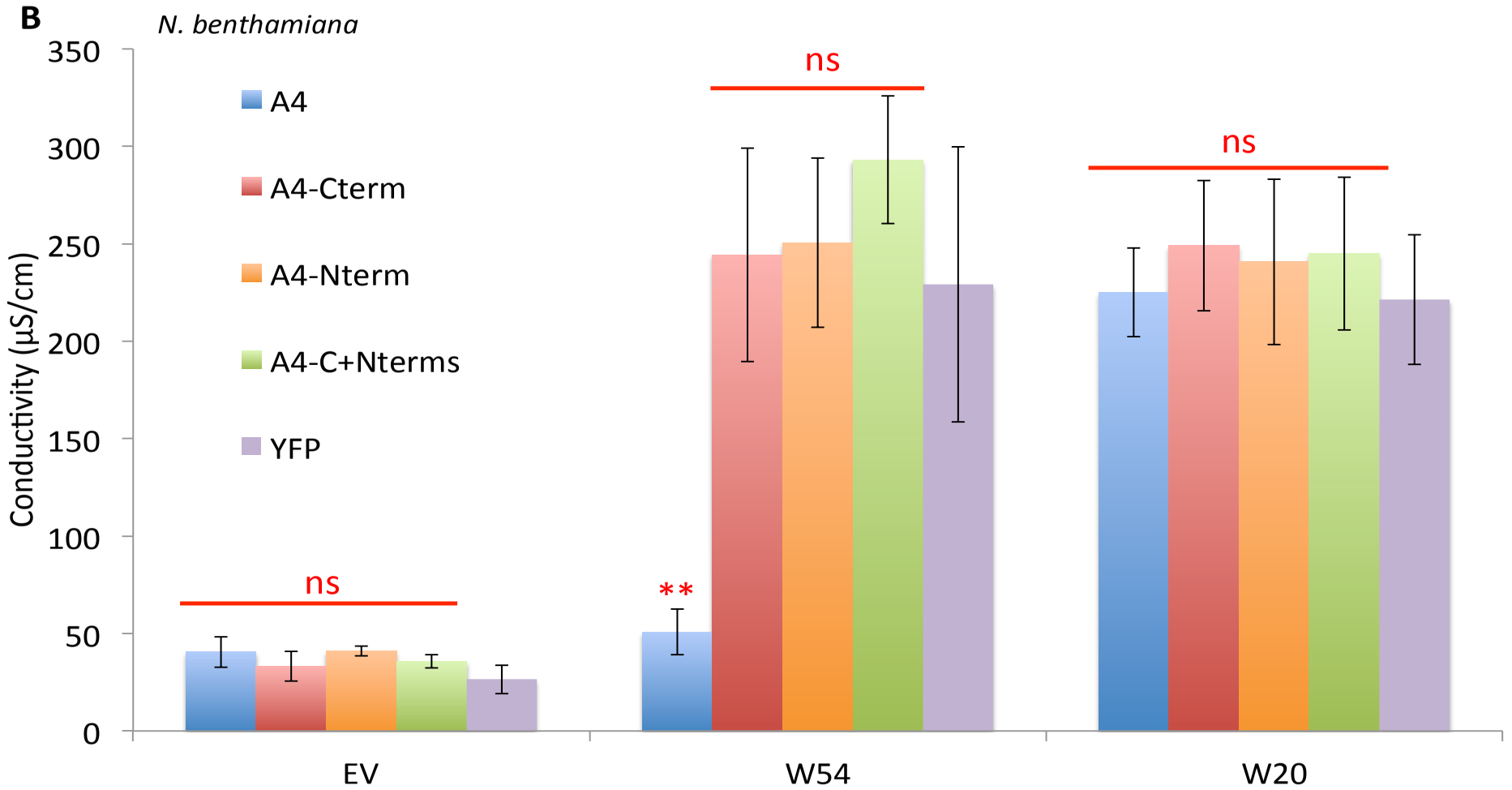
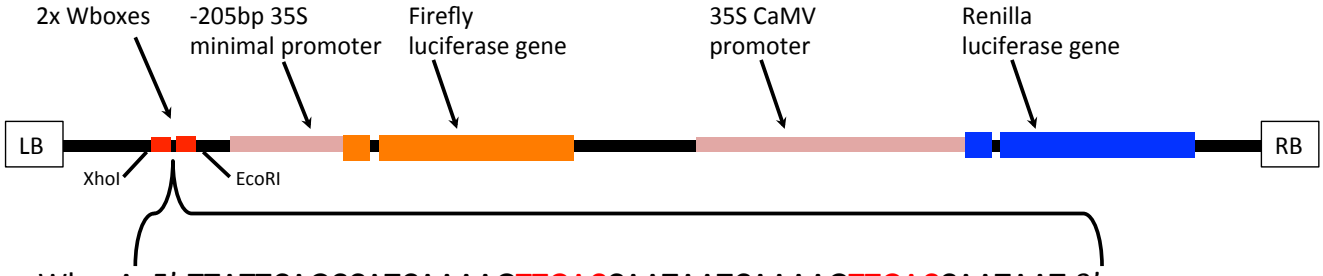


Figure 5.13. AvrRps4 fragments do not suppress AtWRKY54-triggered HCD and ion leakage. (A) HA-tagged AtWRKY20 (negative interactor control), or AtWRKY54 were transiently coexpressed with FLAG-tagged AvrRps4 (full length), its C-terminal fragment, N-terminal fragment or both C- and N-terminal fragments by *Agrobacterium tumefaciens* AGL1 infiltration (AtWRKYs at OD_{600}=0.4, effectors at OD_{600}=0.6) under the CaMV 35S promoter in *N. benthamiana* (#2.5.5; Appendix 4). Photographs were taken at 4 days post infiltration (dpi). Red asterisks indicate development of HCD symptoms. This experiment was conducted twice with identical results. **(B)** Electrolyte leakage from *N. benthamiana* leaf discs taken at 3 days post infiltration (dpi) triggered by *Agrobacterium*-mediated transient expression of EV, AtWRKY54 or AtWRKY20 coexpressed with YFP, AvrRps4 (A4), A4-Cterm, A4-Nterm or A4-C+Nterms (AtWRKYs at OD_{600}=0.4, effectors at OD_{600}=0.6) under the CaMV 35S promoter in *N. benthamiana*. Error bars represent standard deviation from four technical replicates. Asterisks indicate results of Student's *t*-test between selected samples and the YFP expressing sample for that AtWRKY; ** ($p < 0.01$), "ns" indicates non-significant difference. This experiment was conducted twice with identical results.

Quantification of electrolyte leakage revealed the same, with N-terminal fragment alone, C-terminal fragment alone and N-terminal and C-terminal fragments together being unable to suppress electrolyte leakage associated with W54-triggered HCD (Figure 5.13B). Taken together, this revealed that only full length A4 has a novel ability to suppress W54-triggered HCD with the detection by RRS1/RPS4 being unaffected by the R112L mutation due to retention of its ability to target AtWRKYs (represented by W54). This further reveals an interesting virulence function of A4 not just independent of its *in planta* processing but rather dependent on it not being processed.

5.2.6 AtWRKY54 is a transcriptional activator suppressed by functional AvrRps4 or PopP2

WRKY transcription factors exert control over transcriptional programs by binding to *cis*-regulatory elements, termed W-boxes, upstream of their target genes, with a core minimal consensus sequence of 5'-TTGAC-C/T-3' (de Pater et al., 1996; Rushton et al., 1996). An investigation of binding specificity of five different AtWRKYs determined sequences adjacent to the W-box element as determining binding specificity for AtWRKYs (Ciolkowski et al., 2008). Using the findings from this study, we developed two different tandem W-box promoter element sequences that showed different binding affinities for different AtWRKYs, pWboxA (showing greater affinity for AtWRKY6 and AtWRKY11) or pWboxB (showing greater affinity for AtWRKY43).



pWboxA: 5'-TTATTCAGCCATCAAAAGTTGACCAATAATCAAAAGTTGACCAATAAT-3'
pWboxB: 5'-TTATTCAGCCATCAAAATTTGACCAATAATCAAAATTTGACCAATAAT-3'

Figure 5.14. Schematic for dual luciferase inducible-promoter vector construct. Two W-box sequences (WboxA – binds WRKY6-like or WboxB – binds WRKY43-like) derived from variants in a previously published study (Ciolkowski et al 2008) were duplicated tandemly into the MCS (XhoI/EcoRI cloned) upstream of a 35S minimal promoter for the firefly luciferase gene (orange) in dual luciferase vector pNWA62 between the T-DNA left and right borders (LB/RB) to create pWboxA (2xWboxA promoter) and pWboxB (2 x WboxB promoter) constructs (#2.1.3). The activation of this artificial promoter by a coexpressed AtWRKY is measured relative to the constitutively expressed (CaMV 35S promoter) renilla luciferase (blue) in the same construct. Presence of the minimal promoter allows assessment of repression as well as activation.

To investigate the functional relevance of effectors targeting AtWRKYs, we developed a dual luciferase reporter construct featuring either of these paired W-box *cis*-elements (Figure 5.14) whereby the sensor luciferase (firefly luciferase; *LUC*) was under the control of a W-box element (pWboxA or pWboxB) fused 5' to a 35S minimal promoter (-205bp upstream sequence of the CaMV 35S promoter) as well as a full length 35S promoter fused to an internal control luciferase (renilla luciferase; *REN*) in a binary vector (Hellens et al., 2005). This construct allowed us to assess the activation of the *LUC* reporter by an activator-AtWRKY binding to the W-boxes thereby promoting transcription. It also allowed the detection of repression by a repressor-AtWRKY binding to the W-boxes through any competitive negative activity on the 35S minimal promoter. Furthermore, the use of W-box *cis*-elements in our reporter constructs allowed us to assess not only the native activator/repressor activity but also allowed us to assess the native DNA-binding ability of the AtWRKY, and its selectivity, at the same time.

Using coexpression of the dual luciferase reporter constructs with an EV (mCherry-HA tag alone), HA-tagged AtWRKY40 (W40; repressor-AtWRKY control), HA-tagged AtWRKY33 (W33; activator-AtWRKY control), or HA-tagged W54, we sought to determine the effect of W54 on transcription via the two different W-box elements. Following coexpression, at 3 dpi, leaf tissue transiently expressing infiltrated constructs were sampled and assayed for LUC and REN activities with relative LUC/REN readings used as a readout of LUC activation (Hellens et al., 2005). A reporter construct with no promoter (lacking W-box elements and no 35S minimal promoter) showed little to no LUC expression (Figure 5.15). Meanwhile, constructs with no W-box elements but with the 35S minimal promoter alone showed some expression of LUC with coexpression of either W40 or W54 not affecting this level significantly. Using the pWboxA construct, we observed a small but significant reduction or elevation in LUC levels relative to REN for W40 or W33, respectively, as expected (Figure 5.15). Most notably, using pWboxA, we could see a large upregulation of LUC levels relative to REN (nearly two-fold) indicating that W54 is an authentic transcriptional activator. Results using the alternative reporter construct

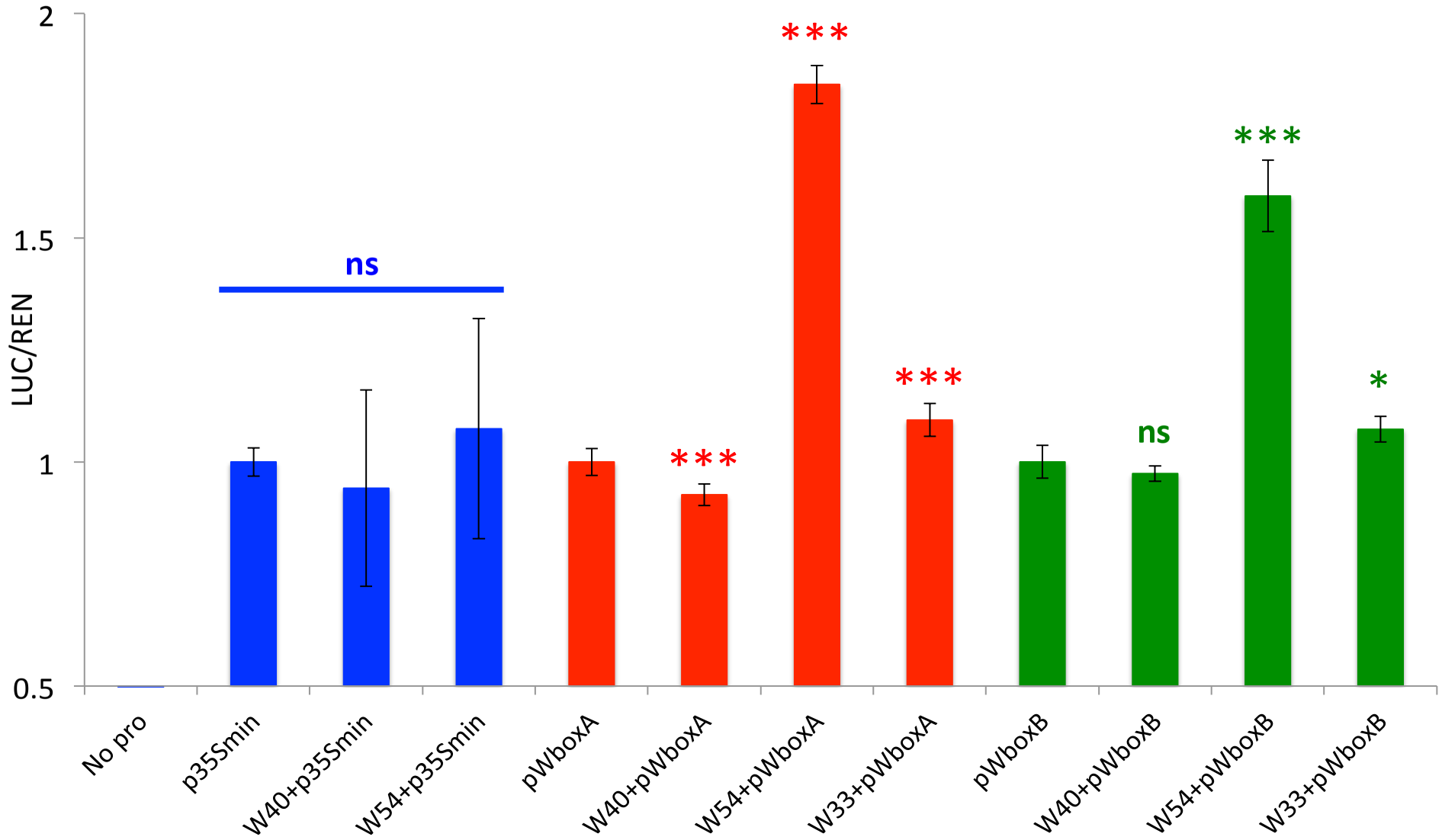


Figure 5.15. AtWRKY54 is an activator of transcription. Relative firefly (LUC) to renilla (REN) luciferase activity in six leaf discs from three independent *N. benthamiana* leaves for a dual luciferase reporter vector with no firefly luciferase promoter (No pro), a dual luciferase reporter vector with only the 35S min promoter (p35Smin), pWboxA (red bars), or pWboxB (green bars) coexpressed by *Agrobacterium*-mediated transient infiltration with AtWRKY54, AtWRKY40 (repressor control) or AtWRKY33 (activator control) was determined by dual luciferase assay (#2.5.9). (pWboxA/B at OD_{600}=0.1 and AtWRKYs at OD_{600}=0.9). Error bars represent standard deviation from six technical replicates. Asterisks indicate results of Student's *t*-test between selected samples and the pWboxA/B-expressing sample alone; *** ($p < 0.001$), * ($p < 0.05$), "ns" indicates non-significant difference. This experiment was conducted twice with similar results.

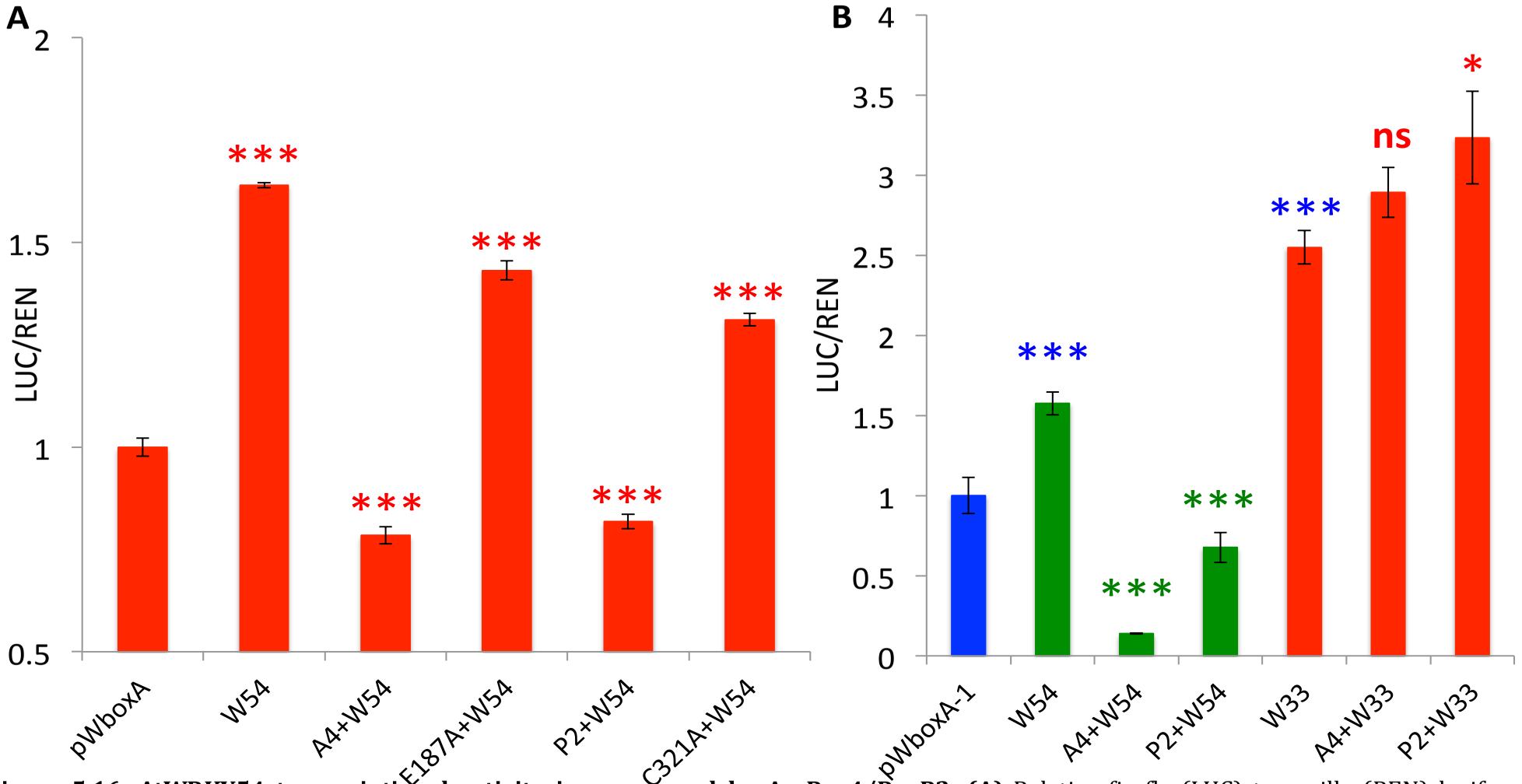


Figure 5.16. AtWRKY54 transcriptional activity is suppressed by AvrRps4/PopP2. (A) Relative firefly (LUC) to renilla (REN) luciferase activity in six leaf discs from three independent *N. benthamiana* leaves for pWboxA coexpressed by *Agrobacterium*-mediated transient infiltration with AtWRKY54 alone, or AtWRKY54 with AvrRps4 (A4), (A4-)E187A mutant, PopP2 (P2), or (P2-)C321A mutant was determined by dual luciferase assay (#2.5.9). (pWboxA at OD_{600} = 0.1, AtWRKY54 and effectors each at OD_{600} = 0.9) Error bars represent standard deviation from six technical replicates. Asterisks indicate results of Student's *t*-test between selected samples and the pWboxA-expressing sample alone; *** ($p < 0.001$). (B) Relative firefly (LUC) to renilla (REN) luciferase activity as in (A) with AtWRKY54 (green bars) or AtWRKY33 (red bars) alone, or respective AtWRKY with AvrRps4 (A4) or PopP2 (P2) was determined by dual luciferase assay (#2.5.9). (pWboxA at OD_{600} = 0.1, AtWRKYs and effectors each at OD_{600} = 0.9) Error bars represent standard deviation from six technical replicates. Asterisks indicate results of Student's *t*-test between selected samples and the pWboxA-expressing sample alone (blue) or selected sample and its AtWRKY-alone (green or red); *** ($p < 0.001$), * ($p < 0.05$), "ns" indicates non-significant difference.

pWboxB gave similar results with a small (bot non-significant) reduction in LUC levels for W40 and significantly elevated levels of LUC for both W33 and W54, relative to REN (Figure 5.15).

We next investigated whether this transcriptional activation by W54 could be suppressed by W54. Coexpression of A4 or P2 (or their mutant variants) with W54 and pWboxA showed that both A4 and P2 are able to suppress W54-mediated transcriptional activation via pWboxA (Figure 5.16A). Furthermore, this effector-mediated suppression was dependent on A4-E187 and P2-C321 residues as was seen earlier for suppression of W54-triggered HCD. Additonally, this suppression was specific for W54-mediated transcriptional activation of LUC using pWboxA since a similar activation mediated by W33 was unaffected by coexpression with either A4 or P2 (Figure 5.16B). There was, however, an as yet inexplicable, small but significant upregulation of LUC levels when P2 was coexpressed with W33 and pWboxA.

5.3 Discussion

From observation of the WRKY tree of life (Figure 5.1) and the observation of roles of the different groups of WRKYs in either promoting or suppressing SA-mediated responses (in Arabidopsis), it seems clear that evolutionarily linked sub-groups have opposing roles. Group $I_{C\text{-}term}$ (suppressor) + group II-c (putative activator), group II-a (suppressor) + group II-b (activator), group II-d (suppressor) + group II-e (activator), and group III-a (activator) + group III-b (suppressor) pairs all appear to have evolved together (Figure 5.1). This suggests an evolutionary balancing act by these WRKYs to retain an antagonism between activation and suppression that is reflected in their genomic sequences. This would then make it difficult for effectors to target SA-promoting WRKYs without also targeting SA-suppressing WRKYs simultaneously. However, effectors like AvrRps4 and PopP2 have done just that. This begs the question of how they can achieve such specificity and whether such specificity allows for other effectors to target WRKYs as well but not get detected by RPS4/RRS1.

5.3.1 AtWRKYs targeted by multiple effectors could serve as immunity hubs

Through investigation of multiple nuclear-localized effectors for interaction with AtWRKY transcription factors using a yeast 2-hybrid approach, we discovered several effectors from two different pathogens targeting multiple AtWRKYs that have not been described previously. We validated the targeting of several AtWRKYs by AvrRps4 but found PopP2 to interact poorly in our screen (Sarris et al., 2015). Nevertheless, we identified interesting AtWRKY interactions with HopY1 and AvrD from our *Psa* V13 pathogen and RxL24, RxL73 and RxLL60 from *Hpa* Emoy2, relatives of two biotrophic pathogens of Arabidopsis. Validating these interactions *in planta* proved difficult as several interesting target AtWRKYs (AtWRKY72 and AtWRKY43) were expressed poorly under our conditions. Future work will seek to build on this discovery, with particular focus on the *in planta* confirmation of these interactions as well as the effect of these interactions on plant immunity.

HopY1 and AvrD homologs from *Pto* DC3000 and *P. syringae* pv. *phaseolicola* 1448A, respectively, have been confirmed for secretion by the T3SS via assays for HR triggered by Δ79aa AvrRpt2 fusions (Chang et al., 2005; Vencato et al., 2006). While a biochemical function for HopY1 is still not ascribed, AvrD1 (and other homologs from various *P. syringae* pv. *glycinea* strains) have been shown to trigger bacterial biosynthesis of syringolides that trigger Rpg4-dependent HR in soybean plants (Keith et al., 1997). This function of AvrD1 does not require transmission of AvrD1 into plant cells as a normal effector would since its role in production of syringolide is primarily within the bacteria. Recently, a novel *in planta* role for AvrD1 was described whereby it is able to bind to soybean NDR1 and if *NDR1* is silenced in these plants AvrD1 is able to promote bacterial virulence (Selote et al., 2014). The interaction described here for the AvrD1 homolog from *Psa* V13 has not been described previously. Inducible expression lines for these effectors in Col-0 will be ideal to study any contributions to increased virulence of pathogens (*Pto* DC3000, both wild-type and weakened, as well as *Hpa*).

The RxLR effectors RxL24, RxL73 and RxLL60 have been described previously (Caillaud et al., 2012b; Fabro et al., 2011). RxL24 is the only of the three that carries a WY motif but possesses a variant RxLR motif (it has a KxLR motif instead; (Jiang et al., 2008)). Assessment of contribution to bacterial virulence was previously undertaken for these three RxLRs with RxL73 and RxLL60 found to significantly promote virulence in 9 and 5 Arabidopsis accessions, respectively, out of 12 accessions tested (Fabro et al., 2011). Unfortunately, only fully virulent *Pto* DC3000 was checked for increased virulence in these accessions. Due to the possession of multiple effectors, wild-type *Pto* DC3000 may not be the most ideal test strain and a weakened *Pto* DC3000 strain (such as the *ΔavrPto/ΔavrptoB* strain) is perhaps better suited for future tests. Additionally, only RxLL60 was assessed for its ability to suppress PTI, and significant suppression of ROS production was detected for this effector, albeit with no effect on callose production (Caillaud et al., 2012b). Stable expression of the three RxLRs in Col-0 found varied growth phenotypes with RxL24 producing double apical meristems, RxL73 inducing chlorosis in leaves, and RxLL60 resulting in bushy compact growth (Caillaud et al., 2012b). Again, inducible expression lines for these three RxLR effectors in Col-0 will be developed in the future to study contributions to increased virulence weakened *Pto* DC3000 strains as well as investigating any effects on PTI.

As mentioned earlier, several AtWRKYs were targeted by multiple effectors from both pathogens that have previously been implicated as having some role in immunity but not characterized in depth. AtWRKY72 was targeted by HopY1, AvrD, RxL24 and RxL73 and has been shown to be required for basal resistance to *Hpa* Noco2 but not for the virulent hemi-biotroph, *Pto* DC3000 (Bhattarai et al., 2010). The same paper demonstrated that tomato ortholog SlWRKY72 was also required for basal resistance to *P. syringae* pv. *tomato* T1 strain in tomato plants. Interestingly, this paper also demonstrated that AtWRKY72 is not required for avirulent *Pto* DC3000 strains nor for *Hpa* Emoy2 which is avirulent in Col-0 due to it carrying the NB-LRR *RPP4* (van der Biezen et al., 2002). Conversely, another recent paper analyzing the response to avirulent pathogens carrying *avrRps4* in Col-0, Ws-2 and Nd-1 accessions of Arabidopsis found that

AtWRKY72 was one of the upregulated genes during PTI (Sohn et al., 2014). This is backed up by similar findings for flg22-triggered upregulation of AtWRKY72 in the L*er*-0 background from another study that first identified many AtWRKYs upregulated during PTI (Zipfel et al., 2004). Taken together, the involvement of AtWRKY72 in basal resistance to virulent pathogens and the existence of several close homologs including AtWRKY61 (showed a weak interaction in yeast with HopY1) and AtWRKY9 (showed strong interactions with HopY1, RxL24, and RxLL60 and weak interactions with AvrD and RxL73) suggests some involvement of these AtWRKYs in immunity (Figure 5.5; Table 5.4). Recently, AtWRKY61 was also implicated in resistance to turnip crinkle virus, further adding to the suggestion that this group of AtWRKYs (part of AtWRKY group II-b) is a hub for immunity (Gao et al., 2016). We would be interested in exploring the role of all three of these AtWRKYs individually as well as in a triple knockout line to explore their contribution to basal resistance during PTI.

Another AtWRKY of interest is AtWRKY43, which was targeted by HopY1, AvrD, RxLL60 and RxL73. Recently, an analysis of Arabidopsis infection by *Pseudomonas syringae* demonstrated that several transcription factors including AtWRKY43 were differentially coexpressed with other immunity related genes (differentially coexpressed genes, DCGs) during the infection process (Jiang et al., 2016). Analogous to differential gene expression analysis, differential coexpression analysis (also called differential network analysis) involves pairwise subtraction of interactions that have been mapped in differential experimental conditions (Hsu et al., 2015). This form of analyzing traditional transcriptomic data thus has a higher sensitivity to smaller changes in transcript levels as it selectively focuses on multiple genes that are believed to be co-regulated under a very specific set of conditions. The same work that identified AtWRKY43 as a DCG for *Pseudomonas* infections also identified AtWRKY38, RAP2.2, ANAC019, ANAC072, MYB51 as a DCGs (Jiang et al., 2016), validating this identification since these transcription factors are all known immune regulators in Arabidopsis basal defence (Gigolashvili et al., 2007; Kim et al., 2008; Zhao et al., 2012; Zheng et al., 2012). Moreover, AtWRKY43 was also identified as one of several transcription factors that physically associate with

calmodulin (CaM) and calmodulin-like (CML) proteins that act as calcium ion sensors particularly important for responding to calcium bursts during PTI (Popescu et al., 2007). This is particularly intriguing since Popescu and colleagues also found AtWRKY53 to be a CaM/CML-binding transcription factor, possibly a mechanism utilized by AtWRKY53 to contribute to basal resistance against *P. syringae* (Hu et al., 2012). Therefore, future research on AtWRKY43 should look at revealing further details on how the effectors from *Psa* and *Hpa* can target AtWRKY43 function to facilitate virulence.

Several AtWRKYs have not been implicated for a role in plant immunity as yet but are targeted by multiple effectors. These include AtWRKY12, AtWRKY13, and AtWRKY44 (Table 5.4). AtWRKY12 and AtWRKY13, two closely related AtWRKYs from group II-c, have recently been reported to participate in flowering time control under short day conditions in conjunction with the plant hormone gibberellin (Li et al., 2016). These two transcription factors work antagonistically, AtWRKY12 promotes early flowering while AtWRKY13 represses it in short day conditions. No direct role in pathogen response has been observed for either AtWRKY12 or AtWRKY13, but an investigation of the necrotroph *Pectobacterium carotovorum* infection in Arabidopsis and cabbage found that a homolog of AtWRKY12 in cabbage, BrWRKY12, contributes to resistance to the bacterial soft rot disease caused by this pathogen in cabbage as well as when transgenically expressed in Arabidopsis (H. S. Kim et al., 2014). Due to the antagonistic nature of AtWRKY12 and AtWRKY13 in flowering, we can speculate that a similar antagonism exists for pathogen responses with AtWRKY13 promoting resistance to biotrophic pathogens. This would then suggest that AtWRKY13 is a more likely target for effectors from a biotroph (such as *Pseudomonas* and *Hpa*). Coincidently, this is what we see in the interaction of *Psa* and *Hpa* effectors with AtWRKY12 or AtWRKY13, with the latter having a larger share of strong interactions (Table 5.4).

AtWRKY44, also known as TRANSPARENT TESTA GLABRA 2 (TTG2), has previously been identified as having tissue-specific roles in controlling lethality in interploidy crosses (Dilkes et al., 2008) as well trichome formation,

anthocyanin accumulation, seed color pigmentation, and differentiation of root hairless cells (Ishida et al., 2007). More recently, investigations of the phenomenon whereby plants accelerate flowering during drought conditions, termed drought escape, identified regulation of AtWRKY44 and its interactor TOE1 by flowering regulator GIGANTEA (GI) and miRNA172 during drought stress during long day conditions (Han et al., 2013). Han and colleagues discovered that AtWRKY44 and TOE1 work together through effects on sugar signaling to affect plant osmotic responses to drought. This is critical particularly since observations have found a link between GI-controlled early flowering responses and a susceptibility to the hemi-biotrophic pathogen *Fusarium oxysporum* in Arabidopsis (Lyons et al., 2015). Interestingly, a similar association of flowering time genes and resistance to hemi-biotrophic *Verticillium* spp. that cause vascular wilt disease in Arabidopsis has also been reported (Haffner et al., 2010). It will be fascinating to see if this link between flowering time and disease resistance is mediated by AtWRKY12/AtWRKY13 and/or AtWRKY44, and whether the targeting of these AtWRKYs by effectors contributes to pathogen virulence.

5.3.2 AtWRKYs can trigger HCD in *Nicotiana* reminiscent of an immune response

MAP kinase (MAPK) cascades play significant roles in activation of defences and several examples, particularly the evolutionarily conserved MEKK2-SIPK/WIPK cascade, have been shown to phosphorylate WRKY transcription factors to facilitate their W-box binding and transcriptional activity necessary to trigger defence responses that may include cell death (Ishihama et al., 2011; Mao et al., 2011; Menke et al., 2005). Notably, several studies have found that transient overexpression of NbWRKYs in *Nicotiana benthamiana* triggered only weak cell death responses and required some activation by a MAPK in their SP clusters to trigger a stronger cell death response (Adachi et al., 2015; Menke et al., 2005). We reasoned that, since basal activity of some WRKYs without prior phosphorylation was sufficient to trigger observable cell death, transient overexpression of AtWRKYs in a heterologous host (*Nicotiana* spp.) may trigger an ectopic immune response as well. Indeed this is exactly what we observed for

AtWRKY20, AtWRKY54 and AtWRKY64 that triggered a strong cell death response when the *Nicotiana* plants were kept in low-temperature, low-humidity, low-light conditions. AtWRKY20 is closely related to AtWRKY33, an AtWRKY known to require MPK3/MPK6-mediated activation (Mao et al., 2011). Meanwhile, AtWRKY54 and AtWRKY64 are members of group III AtWRKYs that are pivotal to multiple immune responses (Higashi et al., 2008; Hu et al., 2012; Wang et al., 2006).

That *SGT1* was required for the HCD triggered by AtWRKY20 and AtWRKY54 suggests that the HCD was indeed a *bona fide* immune response and could putatively involve NB-LRR signaling. The HCD triggered by these two AtWRKYs were significantly weaker than that triggered by AtWRKY64. Therefore, due to the susceptibility of the HCD phenotype to the plant conditions used, we cannot discount that *SGT1*-silencing affected the HCD development (or lack thereof) in some other way. In fact it seems strange that *SGT1* was required for AtWRKY20- and ATWRKY54-triggered HCD but not for the same triggered by AtWRKY64. Additionally, the requirement of *SGT1* is non-canonical. The traditional understanding of SGT1 involvement in immunity lies in its complex formation with HSP90 and RAR1 to facilitate NB-LRR stability and homeostasis (M. Zhang et al., 2010), a process understood to be upstream of the MAPK cascade and WRKY transcription factor activation (Bigeard et al., 2015). Nevertheless, further investigation will be required to elucidate any true involvement of SGT1 and its co-chaperones in the response of *N. benthamiana* to overexpression of these AtWRKYs.

Perhaps most convincingly, the HCD response in *N. benthamiana* required a putative DNA-binding ability that was disrupted in mutant variants of AtWRKY20, AtWRKY54 and AtWRKY64. The mutants generated were double lysine to glutamine substitutions in the key WRKYGQK motif (making it WRQYGQQ) with the new glutamines mimicking acetylated lysine residues that are products of the acetyltransferase activity of PopP2 (Le Roux et al., 2015). Such acetylation modifications, and mimicry of the same through use of glutamines, have previously been shown to facilitate loss of DNA-binding ability

for RRS1 and AtWRKY41 (Le Roux et al., 2015; Sarris et al., 2015). These modifications to AtWRKY20, AtWRKY54 and AtWRKY64 did not significantly affect protein accumulation but did affect the ability of these AtWRKYs to trigger the HCD (Figure 5.8). We therefore predict that DNA-binding ability is critical for these AtWRKY-triggered HCD responses in *N. benthamiana* and the HCD development likely represents an overactive immune signal when these AtWRKYs are overexpressed. For future work, it will be vital to see if DNA-binding ability is indeed lost in these mutant variants through use of electromobility assays (EMSA) using DNA probes carrying W-box elements as used in earlier studies (Ciolkowski et al., 2008; Le Roux et al., 2015).

An intriguing observation from the AtWRKY transient overexpression screen was that several effectors could weakly trigger HCD at later timepoints (up to 7 dpi) than that used for AtWRKY20/AtWRKY54/AtWRKY64-triggered HCD (4 dpi). This included many AtWRKYs closely related to AtWRKY54 that are known to be involved in immunity. In fact, apart from AtWRKY30 that accumulated poorly *in planta*, all other AtWRKYs related to AtWRKY54 could trigger an HCD at 7 dpi (Figure 5.10B). This suggested that this family of AtWRKY54-related AtWRKYs represent a pro-HCD clade (group III-a) that together collectively work to activate immunity. Indeed, several studies have shown a similar relationship for pathogen resistance as well as senescence (Besseau et al., 2012; Higashi et al., 2008; Hu et al., 2012; Wang et al., 2006).

5.3.3 Full length AvrRps4 has evolved to specifically target group III WRKYs

The discovery that two evolutionarily distant T3Es, AvrRps4 and PopP2, bind to and activate resistance mediated by WRKY-domain carrying NB-LRR, RRS1, was suggested to be an example of an integrated decoy NB-LRR, i.e. a target or target-mimic for the effector located within an NB-LRR protein (Le Roux et al., 2015). Indeed, several lines of evidence were presented that these two effectors in fact selectively target AtWRKYs to contribute to virulence, lending credibility to the claim that RRS1-R served as a decoy for these AtWRKYs (Le Roux et al., 2015; Sarris et al., 2015). The primary focus of these two papers, however, was the

acetyltransferase activity of PopP2 that acetylated several immune-related AtWRKY transcription factors and demonstrably led to reduced DNA binding by these targeted AtWRKYs. The functional activity of AvrRps4, however, remains a mystery despite over two decades of research since its discovery (Hinsch and Staskawicz, 1996; G. Li et al., 2014; Sohn et al., 2012a, 2009). Additionally, how these effectors selectively targeted AtWRKYs that activate immunity against biotrophic pathogens versus AtWRKYs that suppressed this activity in favor of defence against necrotrophic pathogens remains unresolved.

Our yeast 2-hybrid work led to the interesting observation that AvrRps4 bound to several members of group III AtWRKYs specifically. AvrRps4 was originally found to bind to AtWRKY41 from yeast 2-hybrid screens and our reproduction of this interaction is thus used in our experiments as the positive control (Mukhtar et al., 2011). As mentioned earlier, many these AtWRKYs, including AtWRKY54, triggers an immune response (HCD) when overexpressed in *N. benthamiana*. Notably, when coexpressed with AvrRps4, AtWRKY54-triggered HCD was suppressed. This led us to the conclusion that due to selective binding of these group III-a AtWRKYs by AvrRps4 in the yeast 2-hybrid screen and given their propensity to trigger HCD, it appears that AvrRps4 targets these pro-HCD AtWRKYs specifically to mediate virulence. Furthermore, this AtWRKY54-specific HCD-suppression ability of AvrRps4 required several key residues noted for their requirement in triggering RPS4-dependent avirulence in Arabidopsis (Sohn et al., 2012a, 2009). Interestingly, another residue, required for *in planta* processing, R112L, was not required for suppression of AtWRKY54-triggered HCD. Even more curiously, this residue was also required for virulence contribution in *N. benthamiana* but not required for avirulence mediated by RPS4 (Sohn et al., 2009). This finding is best reconciled by work carried out by Li and colleagues that described a chloroplast-localized function for AvrRps4 that required *in planta* processing (G. Li et al., 2014). In this work, they demonstrated that only full length AvrRps4 was localized within the nucleus, presumably the site of AtWRKY activity, and processed forms existed outside the nucleus, particularly within the chloroplasts. This then suggests two distinct virulence roles for AvrRps4. The first is a virulence function that requires *in planta*

processing and is localized within chloroplasts and the second is a full length AvrRps4 virulence function yet to be demonstrated that targets AtWRKYs and, in the presence of RPS4 and RRS1, can trigger defence (G. Li et al., 2014; Sohn et al., 2012a). Indeed, our findings that AvrRps4 N-terminus fragment alone, C-terminus fragment alone, or both C- and N-termini fragments coexpressed with AtWRKY54 was unable to suppress the HCD, indicating that the targeting of AtWRKY54 by AvrRps4 is dependent on intact full length AvrRps4, possibly due to a localization requirement. To test whether this is indeed true for a virulence function of AvrRps4 within the nucleus alone, we would need to examine if processable (WT) and unprocessable (R112L) forms of AvrRps4, when transgenically expressed in Arabidopsis *rrs1a/rrs1b* mutants, can confer a virulence benefit to *Pto* DC3000 strains that have been weakened in their virulence (such as a *ΔavrPto/ΔavrptoB* or a *ΔhrcC* mutant variant). Li and colleagues demonstrated a clear PTI-suppression (reduction of callose deposition and ROS burst suppression) ability for full length AvrRps4 when expressed transgenically in *RPS4*-lacking plants that could not be reproduced for the C-terminal fragment of AvrRps4 alone, but still could develop a previously rare HR in Col-0 plants for both AvrRps4 variants (G. Li et al., 2014). However, it is possible that both full length and the C-terminal fragment could trigger HR symptoms in Col-0 plants due to their forced estradiol-induced expression (with far larger quantities accumulating in the chloroplast/cytoplasm than when expressed by bacteria and delivered into the plant (G. Li et al., 2014), a scenario seen for forced cytoplasm localization of AvrRps4 allowing for AvrRps4-triggered HR in Col-0 plants (Heidrich et al., 2011). There is also no mention in this study by Li and colleagues of *RPS4b/RRS1b*-mediated resistance to AvrRps4 in their Col-0 *rps4-2* plants (relevance discussed further, below) (G. Li et al., 2014; Saucet et al., 2015). Nevertheless, the exact mechanisms behind the disparate phenotypes described here remains to be explored and future work will seek to unravel these convoluted mechanisms.

The observation that AvrRps4 can bind to other non-group III-a AtWRKYs (including AtWRKY60 and AtWRKY33) in coimmunoprecipitation studies is interesting as we have not been able to reproduce this interaction in our yeast 2-

hybrid screens (Sarris et al., 2015). Another interaction that we did not see in our yeast 2-hybrid screen was that between AvrRps4 and any AtWRKYs from group II-d, including AtWRKY22 and AtWRKY29. This group is particularly interesting because a secondary pair of *RPS4/RRS1* homologs, called *RPS4b/RRS1b*, act in Arabidopsis to trigger ETI specifically against AvrRps4 (Saucet et al., 2015). The WRKY domain of *RRS1b* most closely resembles group II-d AtWRKYs, suggesting that like *RRS1*, which appears to fall within group III-a AtWRKYs and putatively guards them specifically, the same could be true for *RRS1b* and group II-d AtWRKYs. Future work would explore this further to identify if these interactions influence the activity of these non-group III AtWRKYs similar to its effect on AtWRKY54.

Despite our inability to detect more than a single AtWRKY interaction with PopP2 in our yeast 2-hybrid screen, we found that PopP2 could suppress AtWRKY54-triggered HCD as well. Other members of group III-a AtWRKYs are known to interact with and be acetylated by PopP2 including AtWRKY41, AtWRKY30, AtWRKY53 and AtWRKY70 (Le Roux et al., 2015; Sarris et al., 2015). Moreover, this suppression required acetyltransferase ability of PopP2. This result served as a validation mechanism for our HCD suppression screens particularly because PopP2-mediated HCD suppression was specific to AtWRKY54, much like AvrRps4. Despite the wide variety of AtWRKYs targeted by PopP2 for acetylation (Le Roux et al., 2015), it is interesting that the ability of PopP2 to suppress AtWRKY54-triggered HCD is so selective. This is especially notable due to the close phylogenetic relationship of AtWRKY20 C-terminal WRKY motif to that of AtWRKY33, an AtWRKY targeted by PopP2 (Sarris et al., 2015; Wu et al., 2005). AtWRKYs from group I carry two WRKY motifs, an N-terminus motif that is believed to facilitate protein-protein interactions between WRKYs and a C-terminus domain that has been shown to bind to DNA targets directly (Ciolkowski et al., 2008; de Pater et al., 1996; Eulgem et al., 1999). The fact that, despite the very close sequence identity of AtWRKY33 C-terminal WRKY motif and that of AtWRKY20, PopP2 was unable to suppress AtWRKY20-triggered HCD is evidence of the specificity exerted by these AtWRKY-targeting effectors. Further investigation will be required to examine how PopP2 exploits

the subtle differences between these AtWRKYs to achieve virulence contributions to *Rso* GMI1000.

The possibility remained that AvrRps4 was indirectly affecting the HCD triggered by AtWRKY54 through some other mechanism as multiple *Hpa* and *Pseudomonas* effectors have been shown to have the ability to suppress cell death responses when delivered into plants, a critical ability for biotrophic/hemi-biotrophic pathogens (Dou et al., 2008; Guo et al., 2009). Therefore, to elucidate whether the ability of AvrRps4 to suppress AtWRKY54-triggered HCD specifically was a product of a direct effect on AtWRKY54 function or a general HCD suppression mechanism we implemented a transcriptional reporter assay. The use of the reporter allowed us to, firstly, determine that AtWRKY54 was an activating transcription factor (not a repressor) via the W-box *cis*-elements in the reporter. Secondly, we could determine that AvrRps4 was able to suppress this activation of the reporter when coexpressed with AtWRKY54 and the reporter. We were also able to reproduce this suppression of AtWRKY54-mediated reporter activation with PopP2. This indicated that the suppression of AtWRKY54-triggered HCD was likely a product of AvrRps4/PopP2 suppression of AtWRKY54 transcriptional activity specifically since AtWRKY33 transcriptional activation of the reporter was unaffected. Even more conclusively, this transcriptional suppression was dependent on a 'functional' allele of AvrRps4 or PopP2 since respective E187A or C321A variants of these effectors were unable to suppress the reporter. We envision that future work would investigate whether the reporter suppression holds true for other AtWRKYs that interact with AvrRps4 (and PopP2) as well as expanding this tool to examine activation/repression activity for other interesting AtWRKYs identified in the yeast 2-hybrid screen in conjunction with their interactor effectors.

One of our concerns when using this assay was the poor activation of the reporter above the level induced by the minimal 35S promoter fused to the W-box *cis*-elements. In fact we found that replicated experiments, while holding true to the relationship in our figures herein, often varied greatly in the level of induction from coexpression with the AtWRKY. We believe that this is a product

of the weak transcriptional activity of the AtWRKYs used without phosphorylation-type activation (by a MAPK) as well a complication with HCD symptoms triggered in the *N. benthamiana* plants used. We therefore predict that the reporter assay can be improved through use of either mutationally 'activated' forms of the AtWRKYs vis-a-vis their phospho-mimic variants at any SP sites (Adachi et al., 2015; Mao et al., 2011) or using pre-treatments of a PAMP to trigger activation (by phosphorylation) of these AtWRKYs prior to assessing reporter activation. The latter would also serve to identify AtWRKYs that are predisposed to 'activation' in response to PAMP-perception and thus would clarify their involvement in immunity.

To summarize, our main findings herein demonstrate a virulence function of AvrRps4 because of its ability to exclusively suppress transcriptional activation by AtWRKY54. We suggest that AtWRKY54, in our experiments, serves as a proxy for group III-a AtWRKYs. This is made particularly evident due to the significant number of interactions between AvrRps4 and specifically group III-a AtWRKYs in the yeast 2-hybrid screen coupled to the ability of most of these AtWRKYs to trigger an ectopic cell death response in *N. benthamiana* much like AtWRKY54, albeit to a lesser extent. Additionally, due to the inability of processed fragments of AvrRps4 to carry out a similar role in suppressing AtWRKY54, we suggest the virulence function of full length AvrRps4 is resolvable from that mediated by its processed form. Finally, the virulence function of full length AvrRps4, namely that of targeting group III-a AtWRKYs, is guarded against by the presence of the paired NB-LRR genes *RPS4*/*RRS1* that trigger ETI instead. While it is fair to suggest that the evidence supplied here is far from conclusive, it does give a tantalizing glance into a controversial topic regarding the authentic virulence function of AvrRps4, how this enigmatic effector carries out its role, and how its function is subverted by multiple NB-LRRs to trigger ETI.

Chapter 6: General Discussion

6.1 HopZ5 virulence function by association

During our investigations of HopZ5, we identified that HopZ5 was unable to block AvrRpm1-triggered HR in Arabidopsis. We also showed that HopZ5 was ineffective at blocking AvrRpt2-triggered SAR, a mechanism of virulence proposed for HopZ1a, a homolog of HopZ5 (Macho et al., 2010). Other proposed virulence targets of HopZ1A include tubulin from the host cell cytoskeleton (A. H.-Y. Lee et al., 2012) and JAZ repressors that are targeted by HopZ1a to trigger JA/ET pathway antagonism of SA-mediated resistance (Jiang et al., 2013). Despite its targets being typically localized within the cytoplasm/nucleus, HopZ1a is targeted to the plasma membrane with membrane localization being demonstrably required for virulence/avirulence (Lewis et al., 2014, 2008). This raises the question of what the authentic *in planta* targets are to promote virulence and whether these different proposed targets are additively required for this function. HopZ3 is another effector that has been shown to have multiple *in planta* targets that include, surprisingly, other co-delivered effectors (Lee et al., 2015).

Unfortunately, we have not yet managed to demonstrate a clear virulence function for HopZ5, particularly because most plants tested demonstrated resistance to HopZ5 (sometimes with an associated HR). This made it difficult to identify plants that would be suitable for investigating HopZ5 virulence function. This is particularly true for Arabidopsis as we found that a suppressor of HR (but not resistance) likely exists in Col-0 and possibly other accessions, including Ws-2, and until identification of this repressor is undertaken (S. Choi, thesis) we will not be able to distinguish which plants are authentically susceptible to HopZ5 while lacking the repressor. This is because the lack of growth restriction in Ws-2 may be associated with a true susceptibility or a stronger expression of the suppressor present in Col-0 that allows it to evade resistance as well as HR.

However, many clues to HopZ5-associated virulence/aviruence exist, particularly in the relationship between *Psa* and kiwifruit. McCann and colleagues have noted that HopZ5 and helper HopH1 were limited to, but conserved in, the global 'outbreak clade' of *Psa* strains causing particularly damaging disease on susceptible kiwifruit cultivars such as Hort16A (McCann et al., 2013). Both these effectors appeared to be acquired together as inferred by their presence on a transposable element. Association of HopZ5 and HopH1 with such increased disease severity could imply that either of these effectors are required for boosted virulence or disease symptom production in susceptible kiwifruit varieties. Additionally, resistant kiwifruit cultivars, such as the Hayward variety, showed increased resistance against outbreak strain *Psa* V13 compared to non-outbreak strains that cause disease (McCann et al., 2013). While the mechanism of this resistance remains to be validated in kiwifruit, it is tempting to speculate that HopZ5- or HopH1-triggered recognition could at least be partly contributory.

A similar linkage was recently made for homologs from the YopJ superfamily of effectors from *Acidovorax citrulli*, a pathogen of watermelon (*Citrullus* spp.) and melon (*Cucumis* spp.). Eckshtain-Levi and colleagues found that *A. citrulli* strains from group I that were virulent on and isolated from melon and other non-watermelon cucurbits prevalently either had a truncation in Aave_2166 (closest homolog to AvrBsT and HopZ5) or completely lacked Aave_2708 (closest homolog XopJ and HopZ4) (Eckshtain-Levi et al., 2014). Interestingly, group II strains that are virulent on watermelon possess functional versions of both these effectors and are significantly reduced in virulence on non-watermelon hosts (Eckshtain-Levi et al., 2014). Another similar observation was recently made for *R. solanacearum* effector *ripAA* (also called *avrA*) from the YopJ superfamily. *ripAA* is also coincidentally lost in a monophyletic clade of strains that have lost the ability to infect banana plants but have simultaneously gained the ability to infect melon plants (Ailloud et al., 2015). This suggests that these effectors contribute to disease intensity in susceptible hosts and thus is an important consideration for disease control.

How does HopZ5 mediate virulence in kiwifruit? Why are the *Psa* global outbreak strains that trigger significant disease in Hort16A able to do so? How is the Gold3 variety able to resist *Psa* V strains that possess HopZ5 – is this a lack of a virulence target or possession of an *R* gene? Answers to these questions will be important for determining the future resilience of resistance to *Psa* and could lead to key discoveries in the field regarding the balance of virulence versus avirulence in plant-pathogen interactions.

6.2 Nonhost resistance versus host resistance: a distinction without a difference?

NHR remains the gold standard for resistance breeding in horticulture. Several studies have suggested the involvement of PTI during NHR (Ham et al., 2007; Jie Zhang et al., 2010). The role of ETI in NHR remains complex to discern as ETI is considered a hallmark of host resistance but can also be involved in host-range restriction (a hallmark of NHR) (Ferrante et al., 2009; Mysore and Ryu, 2004; Jie Zhang et al., 2010). The claim that PTI is the primary form of NHR was proposed by Ham and colleagues due to the observation that NHR to *P. syringae* pv. *phaseolicola* NPS3121 (*Pph*) is compromised in the presence of AvrRpm1 and HopM1 (Ham et al., 2007), two effectors known to target PTI (Belkhadir et al., 2004; Nomura et al., 2006). However, more recent studies on AvrRpm1 and HopM1 indicate that they also carry the ability to suppress ETI (Guo et al., 2009). Additionally, Arabidopsis Col-0 resistance to *Pph* and another non-adapted strain, *P. syringae* pv. *glycinea* race 4 (*Pgy*), that both display type-I NHR (without HR) found that this NHR requires *EDS1* (Mishina and Zeier, 2007), a genetic component required for TNL signaling (Aarts et al., 1998). As mentioned earlier in Chapter 3, several well-characterized effectors fail to trigger an HR in Arabidopsis Col-0, and most of these trigger HR-independent immunity depedent on a TNL and *EDS1* (Aarts et al., 1998; Gassmann, 2005; S. H. Kim et al., 2009; Nishimura et al., 2017).

Moreover, several studies have identified involvement of T3E recognition and ETI contributing significantly during NHR (Ferrante et al., 2009; Poueymiro et al., 2009; Wei et al., 2007, 2015; Jie Zhang et al., 2010). Recently several *P. syringae*

strains (pathogens of tomato, bean, snapdragon, and hemp) were found to trigger immunity in Col-0 plants due to their possession of an avirulence effector, HopAS1 (Sohn et al., 2012b). This immunity was not associated with a prototypical HR because delivery by a T3SS only triggered a weak HR response that is difficult to recognize as type-II NHR. Of the strains that possessed this full length HopAS1, *Pph* and several strains of *P. syringae* pv. *glycinea* were amongst them. This then raises the question of whether NHR against the aforementioned *Pph* and *Pgy* strains are indeed examples of obscure pathways of basal immunity (PTI) or could just simply be a lack of characterization of an NB-LRR-mediated recognition of an avirulence effector.

For HopAS1, all strains of *P. syringae* pathogenic on Col-0 possessed a truncated form of HopAS1 (Sohn et al., 2012b). Because *Psa* LV5 possesses full length HopAS1, we predict that it is likely to be avirulent, but we excluded examination of HopAS1 in our research since our aim was to identify *Psa* LV5-unique T3Es; putative candidates for NHR in kiwifruit as well. *Psa* V13, fully virulent on kiwifruit, also carries full length HopAS1 (with over 98% identity to that in *Psa* LV5) and thus HopAS1 is not likely to be responsible for NHR in kiwifruit.

Our aim, in the future, will be to identify if the two T3Es that trigger immunity in Arabidopsis and *N. benthamiana*, HopAR1 and HopAB3, are also avirulence effectors in kiwifruit. This process involves coexpressing the individual effectors along with a reporter gene in kiwifruit leaves to observe if the T3Es are able to trigger HR in the leaves sufficient to suppress expression of the reporter. This so-called 'reporter eclipse assay' is being undertaken in conjunction with Plant & Food Research, PN. The process will necessitate use of biolistics (gene gun) since traditional delivery methods (pathogen-mediated T3SS delivery or *Agrobacterium*-mediated transient expression) are not developed as yet for kiwifruit. Finding an avirulence effector in *Psa* LV5 would precede knockout of that effector to see if a gain of virulence is possible for *Psa* LV5 on kiwifruit. We predict that comprehension of the ETI contribution to NHR will serve to elucidate the difference, if any, between NHR and host resistance by ETI.

6.3 Plant transcription factors as immunity hubs

Induction of plant immunity requires the expression of *PATHOGENESIS-RELATED* (*PR*) genes that encode proteins with antimicrobial activities (Gamir et al., 2017; Van Loon and Van Strien, 1999). SA accumulation is critical for both branches of immunity, PTI and ETI, and dependent on NPR1 detection of SA to trigger expression of *PR* genes through TGA transcription factors (TFs), a subfamily of bZIP TFs (Cao et al., 1997; Després et al., 2000). The identification of TFs that can act upon pathogen perception to trigger the expression of defence related genes now spans many families, including the TGA-bZIP, MYB, NAC, and WRKY families (Tsuda and Somssich, 2015).

In a series of recent large-scale studies of the Arabidopsis defence network, several transcription factors including some from the TCP family of TFs, TCP13, TCP14, TCP15, and TCP19 were identified as interactants with a number of effectors from multiple biotrophic pathogens: *Hpa*, *P. syringae* and *Golovinomyces orontii*, the causal agent of powdery mildew of Arabidopsis (Mukhtar et al., 2011; Wessling et al., 2014). These studies found that multiple effectors from the same species target these conserved hubs of immunity (intra-specific convergence), a phenomenon also seen for multiple effectors from different pathogens (inter-specific convergence), thus lending credibility to the idea that TFs could be targeted by effectors as a conserved strategy for promoting virulence. TCP14 and TCP15 have been implicated in *SUPPRESSOR OF RPS4-RLD,1* (*SRFR1*)-associated immunity and along with TCP8 are required for ETI mediated by the resistance genes *RPS2*, *RPS4*, *RPS6* and *RPM1* (S. H. Kim et al., 2014). Other TCP transcription factors involved in immunity include the TCP8/TCP9/TCP20-coordination with TFs WRKY28 and NAC019 to regulate *SID2* expression and associated SA biosynthesis (X. Wang et al., 2015). It seems clear that pathogen targeting of this family of TFs would be crucial for enhanced virulence in Arabidopsis.

One of the identified TFs from the aforementioned large-scale studies on the Arabidopsis immune network, NTL9 (a NAC family TF), has been shown recently to be targeted by HopD1, a T3E from *P. syringae*, to suppress ETI activation (Block et al., 2014). Follow-on work from the large-scale studies looked at

coexpressed defence-related genes in Arabidopsis and identified many TFs from several defence-related TF familes (Tully et al., 2014). Many of the genes found to be coexpressed differentially in response to pathogens were from the WRKY family of TFs, including WRKY15, WRKY33, WRKY75, WRKY3, WRKY30, WRKY27, WRKY22, and WRKY7. In fact, WRKY TFs are considered one of the major defence-related TF families with notable examples identified in Arabidopsis, barley and rice (Pandey and Somssich, 2009).

Due to the presence of a guard/integrated decoy NB-LRR gene resembling WRKYs in Arabidopsis (*RRS1*), several studies have proposed and found several WRKYs targeted by the effectors AvrRps4 and PopP2 (Le Roux et al., 2015; Sarris et al., 2015). As discussed earlier, WRKYs are distributed in multiple groups with closely related groups possessing a role in either promoting or repressing SA-mediated immunity. Due to their selectivity for effectors in a particular group of WRKYs that promote SA-mediated immunity (Le Roux et al., 2015), we propose that AvrRps4 and PopP2 avoid off-target effects that could otherwise affect WRKYs that repress SA-mediated immunity. Studying this selectivity allows us to address issues regarding how effectors that target immunity via WRKYs would achieve this and can also reveal how RRS1 and RPS4 can trap these effectors into triggering ETI instead.

Selectivity for WRKYs futher implies that other effectors not detected by RRS1/RPS4, but nevertheless target WRKYs, may exist. Our work here has identified HopY1 and AvrD from *Psa* V13, two as yet uncharacterized effectors that target multiple WRKYs. We have also identified uncharacterized effectors from *Hpa* Emoy2, RxL24, RxL73 and RxLL60 that bind to WRKYs. We foresee that further work on these effectors could reveal how they select their targeted WRKYs and how this leads to enhanced virulence for the pathogen. We are developing transgenic Arabidopsis lines (both inducible and stable expression) that carry these effectors that will allow us to investigate whether expression of these effectors confers enhanced virulence. We are also developing multiple knockout lines for their targeted WRKYs, particularly ones that appear to be inter-specific hubs, to observe if these WRKYs are indeed required for basal immunity. We predict that findings from this research will add to the growing

body of work that suggests a major virulence function of several effectors from biotrophic pathogens is to suppress defence-related TFs like members of the WRKY family.

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