The molecular basis of RPS4/RRS1-mediated defense activation in Arabidopsis

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

July 2017

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ABSTRACT

Upon pathogen invasion, each plant cell has the ability to mount an innate immune response. Plants have evolved *R* genes, which typically encode nucleotide-binding domain and leucine-rich repeat-containing immune receptors (NLRs). The model plant species, Arabidopsis, harbors the paired NLRs, *RPS4* and *RRS1*, the products of which function cooperatively to confer recognition of the *Pseudomonas syringae* effector, AvrRps4, and the *Ralstonia solanacearum* effector, PopP2. The exact mechanism underlying RPS4/RRS1-mediated effector recognition remains unclear; therefore, the function of RPS4 and RRS1 was further elucidated.

Firstly, by investigating the avirulence activity of natural variants of PopP2 isolated from *R. solanacearum* strains from across the Republic of Korea, *popP2* was demonstrated to be well-conserved and RPS4/RRS1-mediated recognition of PopP2 could tolerate multiple natural polymorphisms in the *popP2* sequence. Moreover, a conserved PopP2 EAR motif was identified and characterized; the EAR motif was shown to be required for *in planta* PopP2 stability and recognition.

Secondly, utilizing *suppressor of slh1 immunity* (*sushi*) mutants generated in a forward genetic screen on *slh1* mutant seeds, insight was gained into the differential requirements for RRS1 auto-activity and effector perception. A leucine-rich repeat (LRR) mutation, L816F, was identified, which affected auto-activity but not effector recognition. Furthermore, a WRKY domain mutation, C1243Y, was identified, which conferred auto-activity with distinct features compared to other known auto-active RRS1 variants. Notably, a TIR mutant harboring a C15Y mutation was identified that impaired RPS4/RRS1 TIR/TIR heterodimer formation and full-length RRS1 function.

Finally, an analagous self-association interface (DE) identified in the crystal structure of the TNL, SNC1, was investigated for its role in RPS4 function. It was demonstrated that the DE interface mutations, R116A and M150R,

disabled RPS4 TIR domain effector-independent cell death induction and impaired full-length RPS4 signaling.

ACKNOWLEDGMENTS

Firstly, I would like to express my sincere gratitude to my supervisor, Kee Hoon Sohn, for his support and guidance throughout my PhD. His advice and enthusiasm significantly helped my progress and our frequent scientific discussions were always inspiring. I would also like to thank Cecile Segonzac who was an excellent lab mentor and project partner. Her guidance in the lab was invaluable and working alongside her was thoroughly enjoyable.

I would also like to extend my thanks to my co-supervisors, Rosie Bradshaw and Janet Reid, whose support was greatly appreciated. This PhD would not have been the same without the friendships from all lab members in NZ and Korea, especially other PhD students Jay, Maxim and Sera. We have experienced the challenges that a PhD brings together while also enjoying many entertaining conversations and fun times.

I would like to thank my family for their unconditional love, support and encouragement from overseas. Finally, I thank my partner, Janell, for her unwavering support, love and belief in me.

All research conducted by myself unless otherwise stated in figure legends.

LIST OF PUBLICATIONS

Sohn, K.H., Segonzac, C., Rallapalli, G., Sarris, P.F., Woo, J.Y., Williams, S.J., **Newman, T.E.**, Paek, K.H., Kobe, B. and Jones, J.D. (2014). The nuclear immune receptor RPS4 is required for $RRS1^{SLH1}$-dependent constitutive defense activation in *Arabidopsis thaliana*. *PLoS Genet*, *10*(10), p.e1004655.

Work in this thesis contributed to the publications below.

Zhang, X., Bernoux, M., Bentham, A.R., **Newman, T.E.**, Ve, T., Casey, L.W., Raaymakers, T.M., Hu, J., Croll, T.I., Schreiber, K.J., Staskawicz, B.J., Anderson, P.A., Sohn, K.H., Williams, S.J., Dodds, P.N., Kobe, B. (2017). Multiple functional self-association interfaces in plant TIR domains. *Proceedings of the National Academy of Sciences*, p.201621248.

Segonzac, C.†, **Newman, T.E.**†, Choi, S., Jayaraman, J., Choi, D.S., Jung, G., Cho, H., Lee, Y.K., Sohn, K.H. (In Revision). A conserved EAR motif is required for avirulence and stability of the *Ralstonia solanacearum* effector PopP2 *in planta*. *Frontiers in Plant Science*.

† These authors contributed equally

ABBREVIATIONS

aa: amino acids

Avr: avirulence

bp: base pair

CC: coiled-coil

cDNA: complementary deoxyribonucleic acid

cfu: colony forming unit

DNA: deoxyribonucleic acid

dpi: day post-inoculation

EDS1: enhanced disease susceptibility 1

ETI: effector-triggered immunity

HR: hypersensitive response

kb: kilobase

kDa: kilodaltons

LRR: leucine-rich repeat

ml: milliliter

mg: milligram

mM: millimolar

NLR: nucleotide-binding domain and leucine-rich repeat-containing protein

NLR-ID: NLR-integrated domain

OD: optical density

PAMP: pathogen-associated molecular pattern

Pf: *Pseudomonas fluorescens*

PCR: polymerase chain reaction

PR: pathogenesis-related

PRR: pattern recognition receptor

PTI: PAMP-triggered immunity

Pto: *Pseudomonas syringae pv. tomato*

R: resistance

RNA: ribonucleic acid

ROS: reactive oxygen species

RPS4: resistance to *Pseudomonas syringae* 4

RRS1: resistance to *Ralstonia solanacearum* 1

RT-PCR: reverse transcription polymerase chain reaction

SA: salicylic acid

slh1 mutant: *sensitive to low humidity 1* mutant (single leucine insertion in *RRS1* WRKY domain)

sushi mutant: *suppressor of slh1 immunity* mutant

TAE: tris acetate EDTA

Tris: tris(hydroxymethyl)aminomethane

TTSS: type-three secretion system

µl: microliter

µM: micromolar

WT: wild type

CHAPTER 1: GENERAL INTRODUCTION

1.1 Introduction

Plants are constantly under threat of infection by pathogenic bacteria, viruses, fungi and oomycetes. According to Oerke (2006), the total global potential loss of crop productivity due to pathogens is approximately 16%. Despite this, the majority of plants are resistant to the majority of pathogens they encounter.

Plants defend themselves against a broad range of pathogens with physical barriers, such as the leaf cuticle and plant cell wall, and the production of anti-microbial secondary metabolites (Martin, 1964; Hématy et al., 2009; Paiva et al., 2010). Furthermore, stomata close upon pathogen perception to further prevent pathogen entry (Sawinski et al., 2013). Some specialized pathogens are able to overcome these barriers and eventually penetrate plant cell walls. Each plant cell, however, is capable of detecting the pathogen (non-self) or the modification of the host's virulence target (modified self), and subsequently mounting an innate immune response to restrict pathogen growth.

Plant immunity, which is commonly described as multi-layered, can be separated into two distinct categories: PAMP (pathogen-associated molecular pattern)-triggered immunity (PTI), and effector-triggered immunity (ETI) (Figure 1.1). Pathogen effectors, which are typically secreted to suppress the first layer of plant defense, PTI, are recognized by resistance (R) proteins in plants; this was first described in flax by gene-for-gene interactions (Flor, 1942; 1955). The constant selective pressure for the pathogen to acquire new effectors to suppress immunity, and for the host to acquire novel *R* genes to recognize effectors and activate ETI, is commonly referred to as a co-evolutionary arms race (Jones and Dangl, 2006). It must be noted that the dichotomy between PAMPs and effectors and, similarly, PTI and ETI is not always clearly defined (Thomma et al., 2011). In light of this, an alternative

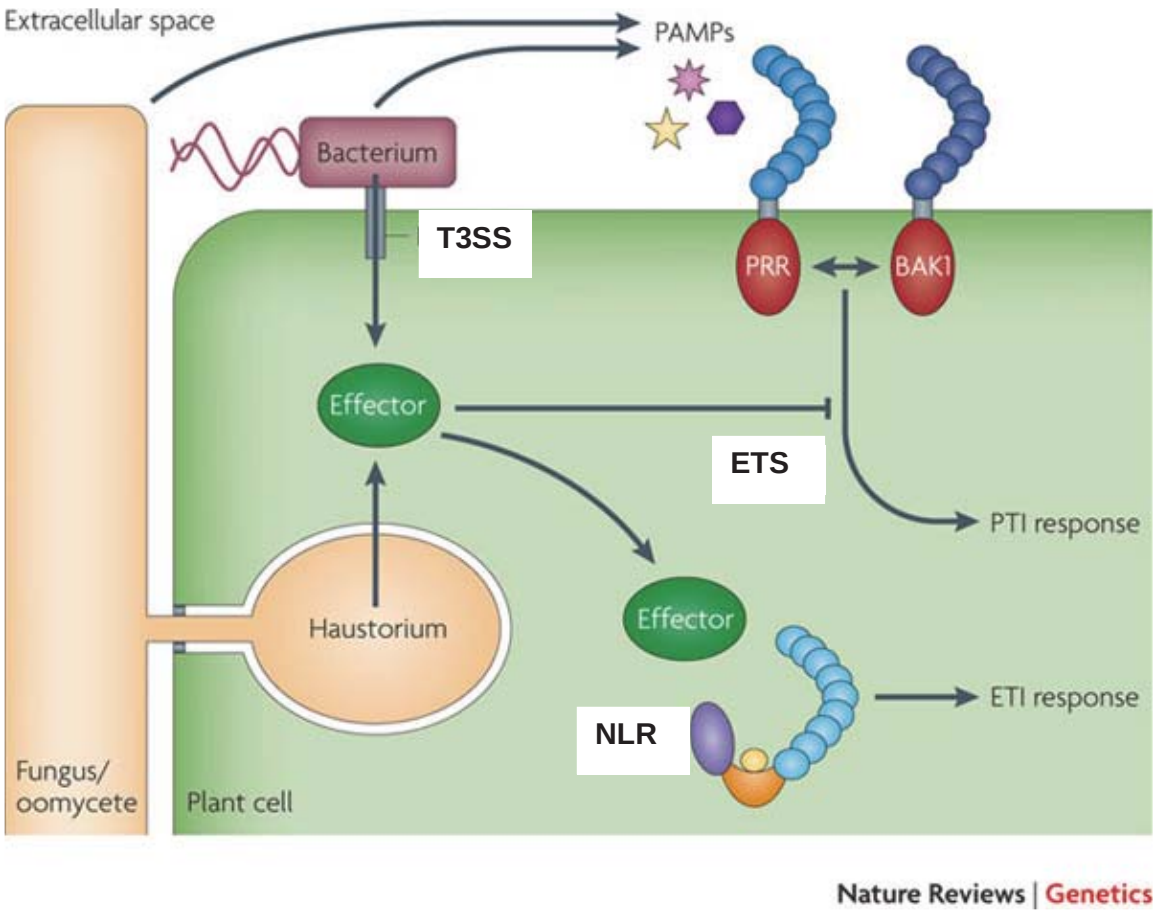


Figure 1.1. A schematic displaying a simplified model of plant innate immunity. Conserved microbial features (PAMPs) are recognized by cell surface receptors (PRRs), which elicit PAMP-triggered immunity (PTI), often by forming a complex with BAK1. Pathogens secrete proteinaceous effectors into the plant cell, which often function to inhibit PTI resulting in effector-triggered susceptibility (ETS). However, plants have evolved *R* genes that typically encode NB-LRRs (NLRs). These perceive the presence of effectors and activate a stronger defense response termed effector-triggered immunity (ETI). Adapted from Dodds & Rathjen, 2010.

model of the plant immune system has been proposed whereby the plant recognizes so-called invasion patterns (IPs) (Cook et al., 2015).

1.2 PAMP-triggered immunity (PTI)

The first layer of plant innate immunity, PTI, is activated by the detection of conserved microbial molecules (PAMPs) by plant membrane-associated receptors (pattern recognition receptors (PRRs)). Several bacterial PAMPs have been identified including lipopolysaccharide (LPS), the main component of the outer membranes of Gram-negative bacteria. Perception of LPS by *Arabidopsis thaliana* (hereafter; Arabidopsis) triggers induction of defense-associated genes via nitric oxide (NO) production (Zeidler et al., 2004). Flagellin, the primary component of bacterial flagella, is a well-characterized PAMP that elicits PTI in Arabidopsis. The epitope, flg22, that comprises 22 amino acids at the amino terminus of flagellin is sufficient to activate PTI, and is recognized by the leucine-rich repeat (LRR) receptor-like kinase (RLK), flagellin-sensing 2 (FLS2) (Felix et al., 1999; Gómez-Gómez and Boller, 2000). The abundant bacterial cytoplasmic protein elongation factor Tu (EF-Tu) has also been identified as a PAMP (Kunze et al., 2004). Similar to flg22 from flagellin, the EF-Tu derived epitope, elf18, is sufficient for recognition by the LRR-RLK EF-Tu receptor (EFR) (Zipfel et al., 2006). In rice (*Oryza sativa*), however, elf18 does not induce an immune response, but the EF-Tu central region comprising Lys176 to Gly225, termed EFa50, was demonstrated to be recognized in rice and induce PTI (Furukawa et al., 2014). Additionally, the necessary component of fungal cell walls, chitin, has been described as a PAMP recognized by CERK1 that functions cooperatively with other signaling components in Arabidopsis and rice (Shimizu et al., 2010; Wan et al., 2012; Cao et al., 2014).

All of the aforementioned PAMPs are widely conserved; however, some PAMPs have a much narrower distribution. For example, Pep-13 is a surface-exposed peptide within a cell wall calcium-dependent transglutaminase

(TGase) conserved only among *Phytophthora* species. Pep-13 activates PTI in parsley and potato (Brunner et al., 2002).

The Arabidopsis brassinosteroid insensitive 1 (BRI1)-associated receptor kinase 1 (BAK1) has roles in diverse signaling processes including plant immunity (Postel et al., 2010). BAK1 is proposed to interact with multiple PRRs to act as a positive regulator and signal amplifier of PTI (Chinchilla et al., 2007; Heese et al., 2007; Shan et al., 2008). In the case of flg22 perception, FLS2-mediated recognition of flg22 induces complex formation between FLS2 and BAK1 (Chinchilla et al., 2007; Heese et al., 2007). The plasma membrane-localized receptor-like cytoplasmic kinase (RLCK), *Botrytis*-induced kinase 1 (BIK1), is subsequently phosphorylated in an FLS2- and BAK1-dependent manner to transduce signaling (Zhang and Zhou, 2010; Lu et al., 2011). BR-signaling kinase 1 (BSK1) and stomatal cytokinesis-defective 1 (SCD1) have also been implicated in FLS2 downstream signaling (Korasick et al., 2010; Shi et al., 2013). Ultimately, activation of mitogen-activated protein kinases (MAPKs) and calcium-dependent protein kinases (CDPKs) leads to expression of flg22-responsive genes (Asai et al., 2002; Boudsocq et al., 2010).

The initiation of PTI involves many molecular responses within the plant cell in order to prevent infection, such as MAPK signaling, the production of reactive oxygen species (ROS), cytosolic Ca2+ influx, the accumulation of callose at the cell wall for increased strength, and transcriptional induction of pathogen-responsive genes (Nürnberger et al., 1994; Kitajima and Sato, 1999; Schwessinger and Zipfel, 2008; Luna et al., 2011; Daudi et al., 2012).

1.2.1 Suppression of PTI by effectors

PTI is, however, not sufficient to restrict the growth of some pathogens. Successful pathogens are able to overcome or suppress PTI via the action of secreted effectors. Effectors are proteins delivered into the plant cell by the pathogen, which are able to suppress PTI and/or ETI or otherwise enable

pathogen growth. Several plant pathogenic bacteria possess a type III secretion system (TTSS) for delivery of effectors (virulence factors) into the host cell (Alfano and Collmer, 1996). Some fungal and oomycete pathogens employ a different delivery method for the secretion of effectors, a specialized structure called a haustorium penetrates the cell wall and invaginates the host cell plasma membrane for delivery of effectors into the plant cell (Dodds et al., 2004). This suppression of PTI is the phase of effector-triggered susceptibility (ETS) (Figure 1.1) (Jones and Dangl, 2006).

A prime example of an effector interfering with PTI to enable pathogen growth is the *Pseudomonas syringae* secreted effector, AvrPto. Once secreted, the effector binds and inhibits receptor kinases including the aforementioned PRRs, FLS2 and EFR (Hauck et al., 2003; Xiang et al., 2008). Another *P. syringae* effector, HopF2, also suppresses PTI via BAK1 targeting (Zhou et al., 2014). Furthermore, the *P. syringae* effector, HopM1, targets the vesicle trafficking-related AtMIN7 for degradation, which abrogates PAMP-triggered oxidative burst and stomatal immunity (Lozano‐Durán et al., 2014). Further biochemical functions of pathogenic effectors are described below.

1.3 Biochemical functions of effectors

Virulence factors secreted by pathogens display a range of functions in the host cell, and the majority of effectors still remain uncharacterized. Identifying the biochemical activities of effector proteins is an important target to increase our understanding of bacterial pathogenesis (Staskawicz et al., 2001). Of the characterized effectors, the main groups divided based on their biochemical function are proteases, ubiquitin ligases, phosphatases, acetyltransferases and transcriptional activators. As described earlier, some plants have evolved *R* genes to monitor the activity of effectors *in planta* and induce a defense response. Under these circumstances, the effector is termed an avirulence (Avr) factor.

Cysteine proteases possess an invariant catalytic triad of C/H/D for proteolytic

activity on specific host virulence targets. The *P. syringae* effector AvrRpt2 is a cysteine protease, which targets and eliminates RIN4. RPS2 is the corresponding R protein, which detects the cleavage of RIN4 to elicit a defense response (Axtell and Staskawicz, 2003; Mackey et al., 2003). Similarly, another *P. syringae* effector AvrPphB targets its substrate, the serine/threonine kinase PBS1, for proteolytic cleavage. The Arabidopsis R protein RPS5 indirectly recognizes AvrPphB by detecting the activity of AvrPphB on PBS1 to consequently activate ETI (Shao et al., 2003). The crystal structure of AvrPphB has been determined and it has been shown to resemble a papain-like cysteine protease with a distinct binding site for PBS1 (Zhu et al., 2004). Notably, it was recently demonstrated that the AvrPphB cleavage site within PBS1 could be replaced with cleavage sites for other pathogen proteases. Thus, PBS1 can be engineered to act as a decoy for RPS5-mediated perception of PBS1 cleavage by other proteases (Kim et al., 2016). These examples of effector recognition both adhere to the guard/decoy hypothesis, as is discussed further in 1.4.3.2. Several other pathogen-derived effectors are biochemically defined as small ubiquitin-like modifier (SUMO) proteases. *Xanthomonas euvesicatoria* (formerly *Xanthomonas campestris* pv. *vesicatoria)* (*Xcv*), the causal agent of bacterial spot of tomato, harbors the effectors AvrXv4 and XopD both of which encode SUMO proteases, proteins which desumoylate their specific host targets *in planta* (Hotson et al., 2003; Roden et al., 2004). XopD was shown to target and desumoylate the tomato ethylene-responsive transcription factor, *Sl*ERF4, to suppress ethylene-induced transcription thereby increasing susceptibility to *Xcv* (Kim et al., 2013).

Host-encoded ubiquitin ligases, specifically E3 ubiquitin ligases, play important roles in the regulation of plant immunity signaling (Duplan and Rivas, 2014). For example, Arabidopsis E3 ubiquitin ligase plant U-box 13 (PUB13) is involved in turnover of the PRR, lysin motif receptor kinase 5 (LYK5), which is responsible for recognition of the fungal PAMPs long chain chitooligosaccharides (Liao et al., 2017). Moreover, some pathogen effectors possess E3 ubiquitin ligase activity, which aid the pathogen in abrogating host immunity of susceptible plants. The *P. syringae* pv. *tomato* (*Pto*) effector

AvrPtoB carries an E3 ubiquitin ligase domain and marks host kinase, Fen; the aforementioned PRR, FLS2; host kinase, BAK1; and LysM receptor-like kinase, Bti9, for degradation by the proteasome to increase disease susceptibility (Rosebrock et al., 2007; Göhre et al., 2008; Shan et al., 2008; Cheng et al., 2011; Zeng et al., 2012). AvrPtoB is recognized in Pto-carrying tomato and was identified by a yeast-two-hybrid (Y2H) screen of all *Pto* DC3000 effectors with *Pto* (Kim et al., 2002). This recognition was shown to be dependent on a decoy kinase and an R protein: the serine-threonine protein kinase, Pto, and the nucleotide-binding and leucine-rich repeat-containing (NLR) protein, Prf (Pedley and Martin, 2003). Tomato has, therefore, evolved to monitor the activity of AvrPtoB and mount an ETI response upon recognition of the effector. Despite Fen and Pto sharing 87% amino acid identity, AvrPtoB binding results in Fen ubiquitination and degradation whereas Pto is recalcitrant to degradation and triggers ETI. This difference was shown to be dependent on the ability of Pto to bind two distinct domains of AvrPtoB, one at the N-terminus (Pto-interacting domain (PID)) and another near the E3 ligase domain (Fen-interacting domain (FID)). Fen, however, can only bind at the FID. Both Fen and Pto bound to the FID become ubiquitinated and degraded. On the other hand, Pto bound to the PID evades degradation, as it is further away from the E3 ligase domain, and triggers ETI (Mathieu et al., 2014). It was previously reported that the greater kinase activity of Pto over Fen allowed it to inactivate the E3 ligase domain of AvrPtoB via threonine-450 (T450) phosphorylation, thus evading degradation; however, this was demonstrated not to be the case (Ntoukakis et al., 2009; Mathieu et al., 2014).

Protein tyrosine phosphatase activity is a further function of pathogen-derived virulence factors to interfere with host defense responses. The *P. syringae* effector, HopAO1 (formerly HopPtoD2), possesses tyrosine phosphatase activity. HopAO1 was proposed to inactivate MAPK pathways to suppress hypersensitive response (HR) as demonstrated in *Nicotiana benthamiana* (Espinosa et al., 2003). An HR is a form of localized programmed cell death, which often plays an important role in ETI by restricting the growth of biotrophic and hemibiotrophic pathogens. However, it was shown that

HopAO1 primarily targets PTI in Arabidopsis independent or downstream of MAPK activation (Underwood et al., 2007).

Additionally, some effector proteins function by transfer of an acetyl group onto the virulence target. These so-called acetyltransferases are able to perturb the activity of host immunity-related proteins. The *P. syringae* type III acetyltransferase effector HopZ1a has been demonstrated to target GmHID1 in soybean to suppress isoflavone biosynthesis, and tubulin in Arabidopsis to disrupt the cytoskeleton and secretion (Zhou et al., 2011; Lee et al., 2012). Similarly, the *Xcv* derived effector AvrBsT exhibits acetyltransferase activity on Arabidopsis ACIP1, perturbing PTI as well as triggering ETI (Cheong et al., 2014). Multiple host proteins that interact with AvrBsT have been identified in pepper (*Capsicum annuum*), namely CaADC1, CaALDH1, CaHSP70a, CaSGT1 and SnRK1 (Szczesny et al., 2010; Kim et al., 2013; Kim et al., 2014a; Kim and Hwang, 2015). Notably, all of the interacting proteins are involved in HR induction and immunity signaling (Han and Hwang, 2016). The mammalian pathogen *Yersinia* harbors the effector YopJ, which was originally thought to act as a cysteine protease due to sequence similarity, but has since been shown to possess acetyltransferase activity (Orth et al., 2000; Mukherjee et al., 2006). The *Ralstonia solanacearum* effector, PopP2, which belongs to the YopJ-like family of effectors, also requires acetyltransferase activity for function. Mutation of the PopP2 catalytic cysteine residue, C321, abolishes the avirulence function of PopP2. PopP2 has been shown to auto-acetylate a specific lysine residue required for protein activity, lysine residues in the WRKY domain of the cognate R protein, RESISTANT TO RALSTONIA SOLANACEARUM 1 (RRS1), and also other WRKY transcription factors involved in plant immunity (Tasset et al., 2010; Le Roux et al., 2015; Sarris et al., 2015).

The final group of effectors discussed here is transcriptional activators. Transcription activator-like effectors (TALEs) function to reprogram the transcription of specific host genes by mimicking host transcription factors. TALEs bind to host DNA and activate immunity-related genes (Moscou and Bogdanove, 2009). AvrBs3, and effectors of the AvrBs3 family, identified only

in *Xanthomonas spp.* and *R. solanacearum* are the largest class of type III TALEs (Kay and Bonas, 2009). The central repeat domain composed of a variable number of a tandemly arranged 34/35-amino acid motif is the most defining structural characteristic of TALEs (Schornack et al., 2006). AvrBs3 transcriptionally activates genes that contain the promoter element *UPA* (upregulated by AvrBs3) box (Kay et al., 2007). The pepper *R* gene *Bs3* mediates recognition of the *Xanthomonas campestris* pv *vesicatoria* effector, AvrBs3. *Bs3* has evolved to contain a *UPA* box, to which AvrBs3 usually binds to suppress immunity; however, the *Bs3 R* gene triggers ETI and disease resistance (Römer et al., 2009). This case is a clear indication of the constant molecular arms race between pathogen and host. Recently, a *Xanthomonas gardneri* TALE, AvrHah1, was shown to enhance water soaking in tomato, pepper and *N. benthamiana* by binding and upregulating two basic helix-loop-helix (bHLH) transcription factors. This resulted in upregulation of pectate lyase, hypothesized to increase cell wall hygroscopicity and, therefore, enable faster absorption of water into the apoplast, thus generating a beneficial environment for *X. gardneri* multiplication (Schwartz et al., 2017).

1.4 Effector-triggered immunity

1.4.1 Plant R proteins

Pathogen effectors are delivered into the host cell in order to suppress plant immunity and promote microbial growth. This has selected for the acquisition of plant *R* genes, the products of which are capable of recognizing effectors, either directly or indirectly, and triggering ETI (Figure 1.1).

The very first isolated *R* gene was uncharacteristic of the many *R* genes discovered since. *HM1*, which confers resistance to the fungus *Cochliobolus carbonum* race 1, encodes a HC toxin reductase (HCTR) (Johal and Briggs, 1992).

Another class of plant R proteins is the receptor-like proteins (RLPs) comprising extracellular LRRs, a transmembrane domain, and a small cytoplasmic domain, which lacks any obvious signaling motif (Martin et al., 2003). The RLP class of *R* genes includes tomato *Cf-2, Cf-4, Cf-5* and *Cf-9* that confer resistance to the fungal pathogen *Cladosporium fulvum* (Jones et al., 1994; Dixon et al., 1995; Thomas et al., 1997; Dixon et al., 1998).

By far the largest number of characterized plant R proteins belong to the nucleotide-binding (NB) and leucine-rich repeat (LRR) class; the NLRs. The Arabidopsis Col-0 genome harbors at least 149 NLRs, which structurally resemble mammalian NOD-like receptors (Meyers et al., 2003; Takken and Tameling, 2009; Jones et al., 2016).

1.4.2 NLR R proteins

The NLR family of R proteins can largely be divided into two subclasses based on their N-terminal domain, which is often a coiled-coil (CC) or Toll/Interleukin-1 receptor (TIR) domain (Figure 1.2). CC domain-containing NLRs (CNLs) and TIR domain-containing NLRs (TNLs) confer specific resistance to pathogens carrying the cognate avirulence protein (Dangl and Jones, 2001).

The CC domain of CNLs is predicted to form amphipathic alpha-helices, and act as a protein-protein interaction interface ensuring specificity of recognition with signaling partners and other protein interactants (Moffett et al., 2002; Meyers et al., 2003). The CNL class of R proteins includes: Arabidopsis RPM1, RPS2 and RPS5, which confer recognition of the *P. syringae* effectors AvrRpm1/AvrB, AvrRpt2 and AvrPphB, respectively; pepper Bs2 which confers recognition of the *Xanthomonas campestris* effector AvrBs2; the tomato Prf which confers recognition of the *P. syringae* effector AvrPto; and potato Rx which confers recognition of the potato virus X (PVX) coat protein (Bendahmane et al., 1995; Leister et al., 1996; Salmeron et al., 1996; Tai et al., 1999; Warren et al., 1999; Mackey et al., 2002). CNLs and TNLs differ not

only in their domain structure, but also in immune signaling components. Some NLRs with a CC N-terminus require the immune regulator NDR1 (non-race-specific disease resistance 1) for signal transduction, whereas nearly all characterized TNLs signal through the lipase-like protein EDS1 (ENHANCED DISEASE SUSCEPTIBILITY 1) (Feys and Parker, 2000). The Arabidopsis TNL, TTR1 (tolerance to tobacco ringspot virus), however, was uncharacteristically shown to function independently of EDS1 (Nam et al., 2011). The TIR domain of TNLs, which is homologous to the *Drosophila* Toll receptor and the cytoplasmic domain of the human interleukin-1 receptor, has been proposed and demonstrated to be involved in the cell death-signaling pathway (Martin et al., 2003; Swiderski et al., 2009). Examples of TNLs providing resistance to viral, fungal or oomycete pathogens are: the *Nicotiana tabacum* (tobacco) N protein which confers recognition of the tobacco mosaic virus (TMV) coat protein (CP); flax L6 which confers recognition of the effector AvrL567 from the flax rust pathogen *Melampsora lini*; and Arabidopsis RPP5 which confers recognition of the effector ATR5 from the downy mildew pathogen *Hyaloperonospora arabidopsidis* (Whitham et al., 1994; Lawrence et al., 1995; Bailey et al., 2011).

The central nucleotide-binding (NB) domain is present in all NLRs (Figure 1.2). This nucleotide-binding pocket is part of a larger domain called the NB-ARC domain, as it is a constituent of APAF-1 (apoptotic protease-activating factor-1), R proteins and CED-4 (*Caenorhabditis elegans* death-4 protein) (van der Biezen and Jones, 1998). Multiple conserved motifs within this NB-ARC domain of plant R proteins have been identified including the hhGRExE, Walker A or P-loop, Walker B, GxP, RNBS-A to D, and MHD motifs (Traut, 1994; Meyers et al., 1999; Lukasik and Takken, 2009). The requirement of these motifs has been demonstrated multiple times by the resulting loss-of-function or auto-activation of R proteins after motif mutation (Meyers et al., 1999; Van Ooijen et al., 2008). A random mutagenesis screen on NRC1 (NB-LRR required for HR-associated cell death-1), a tomato downstream signaling NLR, was carried out to identify mutant variants that induced an effector-independent HR in tobacco. 10 single amino acid substitution gain-of-function mutations were identified. Notably, all were in or in close proximity to highly

conserved motifs within the NB-ARC domain (Sueldo et al., 2015). The NB-ARC domain comprises 3 subdomains: NB, ARC1 and ARC2. The NB domain forms a pocket for binding and hydrolysis of ATP and this has been demonstrated for the tomato R proteins, I-2 and Mi-1 (Tameling et al., 2002). ATP hydrolysis is proposed to be required for the activation of NLRs. The ARC1 domain of potato Rx was shown to interact with several LRR domains, and it is therefore hypothesized that the ARC1 domain of NLRs acts as a molecular scaffold for binding of LRRs (Rairdan and Moffett, 2006). There is evidence to suggest that the ARC2 domain, on the other hand, is required for both auto-inhibition in the absence of an effector, and activation upon effector recognition (Van Ooijen et al., 2008). It has been demonstrated in multiple NLRs that substitution of aspartate to valine in the MHD motif within ARC2 results in a strong auto-activation of the R protein (Bendahmane et al., 2002; De La Fuente Bentem van et al., 2005; Howles et al., 2005). Furthermore, a domain-swap experiment on potato *Rx* with a homologous *R* gene, *Gpa2*, has demonstrated that the ARC2 domain is required for both maintaining the R protein in an auto-inhibited condition in the absence of effector and acting as a switch for the conformational change to an active state upon effector recognition (Rairdan and Moffett, 2006). One theory states that an inactive NLR remains in the ADP-bound state and NLR activation upon effector binding requires conformational changes and ADP/ATP exchange; however, this paradigm has recently been challenged (Tameling et al., 2006; Williams et al., 2011). It was proposed that the flax immune receptors, L6 and L7, exist in a dynamic equilibrium switching between a more favorable ADP-bound state (OFF) and a transient ATP-bound state (ON state). Binding of the cognate effector, AvrL567, results in stabilization of the ATP-bound ON state and defense induction (Bernoux et al., 2016). Whether this model can be regarded as a general mechanism for all or some NLRs remains to be seen. In fact, a further model of action has been hypothesized for NLRs such as RPS2 and RPM1 whereby they remain in the ATP-bound conformation but are held inactive by an interacting partner such as RIN4, a model put forward based on a crystal structure study of *Caenorhabditis elegans* CED-4 (Qi et al., 2010). This negative regulation is proposed to be perturbed by the respective effector protein.

The variable C-terminal LRR domain is a tandem array of 20-29 amino acid residues, which contains a conserved 11-residue segment with the consensus sequence LxxLxLxx$^{N}/_{C}$XL (Figure 1.2). LRRs are a constituent of many proteins involved in diverse plant processes (Kobe and Kajava, 2001). The LRR acts as a structural platform that is capable of allowing protein-protein interactions, and it appears to be involved in both negative regulation and activation (Takken and Tameling, 2009). Intramolecular interaction of the LRR domain with the ARC domain maintains the NLR in an auto-inhibited state and disruption of this can result in activation (Rairdan and Moffett, 2006). However, deletion of the LRR domain does not necessarily result in constitutive activity (Bendahmane et al., 2002; Hwang and Williamson, 2003). Additionally, the C-terminus of the LRR domain has been shown to confer recognition specificity of the appropriate effector in some cases (Parniske et al., 1997; Wang et al., 1998; Ellis et al., 1999). Many NLRs do not physically associate with their respective effectors via the LRR domain; however, the mechanism of perception of the *Hyaloperonospora arabidopsidis* effector, ATR1, by the RPP1 LRR domain has been well demonstrated (Takken and Tameling, 2009; Steinbrenner et al., 2012; Goritschnig et al., 2016). A further proposed function of the LRR domain is in downstream signal transduction. The *rps5-1* mutation within the LRR domain of *RPS5* resulted in compromised function of several NB-LRRs and it was, therefore, hypothesized that the LRR domain may interact with a downstream signaling component required for signal transduction for multiple R proteins (Warren et al., 1998).

In addition to the characteristic TIR/CC, NB-ARC and LRR domains, many R proteins possess an additional unorthodox domain. These domains are primarily thought to act as baits for pathogen-derived effector proteins, which mimic bona fide virulence targets, allowing effector recognition and ETI. These domains have been referred to as integrated decoys, integrated sensors or simply integrated domains (IDs) (hereafter, NLR-IDs) (Kroj et al., 2016; Sarris et al., 2016). NLR-IDs are emerging as a widespread feature of NLRs; Sarris et al. (2016) identified 720 NLR-IDs from 40 predicted

angiosperm proteomes and analysis of 31 plant genomes by Kroj et al. (2016) revealed that 3.5% of all NLR proteins encode an atypical domain.

Figure 1.2. A schematic displaying the modular structure of NLRs.
Plant NLRs possess a variable N-terminal domain, primarily either a coiled-coil (CC) or a Toll/Interleukin-1 receptor (TIR) domain, followed by the nucleotide-binding adaptor shared by APAF-1, R proteins, and CED-4 (NB-ARC) domain comprising three sub-domains: NB, ARC1 and ARC2. At the C-terminus, is a leucine-rich repeat (LRR) domain. Adapted from Cesari et al., 2014.

1.4.3 Mechanisms of pathogen recognition by R proteins

In order for plants to activate an effective ETI in response to pathogen infection, the initial step is recognition of pathogen effectors by R proteins. Perception of Avr proteins by the cognate R protein occurs via one of two mechanisms; either direct or indirect recognition.

1.4.3.1 Direct recognition

The first model to explain the molecular basis of effector recognition by R proteins was the gene-for-gene hypothesis proposed after work on rust in flax (Flor, 1942; 1955). According to the gene-for-gene hypothesis, protein products of single dominant *R* genes recognize avirulence proteins (effectors). This ligand-receptor model is able to explain the recognition of several pathogen-derived effectors by the corresponding R protein. The causal pathogen of rice blast fungus, *Magnaporthe oryzae*, delivers the AVR-Pita effector into rice. Recognition and defense elicitation occurs via the rice R protein Pi-ta, which has been reported to interact directly with AVR-Pita (Jia et al., 2000). Similarly, the flax R proteins L5, L6 and L7 confer recognition of the

flax rust fungus, *Melampsora lini*, effector AvrL567 by direct R-Avr protein interactions (Dodds et al., 2006). Recognition of the *Hyaloperonospora arabidopsidis* Avr protein, ATR1, by the cognate Arabidopsis TNL, RPP1, also occurs via direct recognition. The RPP1 LRR domain mediates interaction with ATR1 (Krasileva et al., 2010).

1.4.3.2 Indirect recognition: Guard/decoy hypothesis

Interestingly, only a few examples of direct effector recognition by the corresponding R protein have been demonstrated. The guard and decoy models have been proposed to explain the indirect recognition of effectors. The guard model states that a plant R protein monitors the host target of an effector and detects perturbation of the host target by the effector (Wu et al., 2014). The RIN4 protein of Arabidopsis is the host virulence target of three distinct *P. syringae* type III effectors: AvrRpt2, AvrRpm1 and AvrB. RIN4 is guarded by the R proteins RPS2 and RPM1, which recognize AvrRpt2 and AvrRpm1/AvrB, respectively. RPS2 detects AvrRpt2-induced posttranslational disappearance of RIN4, whereas RPM1 detects AvrRpm1/AvrB-mediated phosphorylation of RIN4 (Mackey et al., 2002; Mackey et al., 2003). Similarly, the aforementioned *P. syringae* effector AvrPphB cleaves the Arabidopsis host kinase, PBS1, which is detected by the NLR, RPS5 (Shao et al., 2003).

A similar yet biochemically distinct mechanism of effector recognition is the decoy model, in which the effector host target is not biologically functional. The host target (decoy) has evolved to be targeted by the effector and, as in the guard model, this perturbation of the protein is detected by an interacting R protein. The *P. syringae* effector HopZ1a is an acetyltransferase, which targets host kinases to promote pathogen virulence. However, Arabidopsis has evolved a non-functional pseudokinase decoy, ZED1, which is targeted by HopZ1a and guarded by the R protein, ZAR1. Modification of ZED1 by HopZ1a results in ZAR1-mediated immunity (Lewis et al., 2013). In fact, ZAR1 guards not only ZED1 but also PBL2 (a homolog and putative decoy of the PTI signaling kinase, BIK1) in complex with another pseudokinase, RKS2, for

recognition of the *Xanthomonas campestris* effector AvrAC; as well as the kinase, ZRK3, for recognition of the *P. syringae* effector HopF2a (Wang et al., 2015; Seto et al., 2017).

Interestingly, genes encoding a TIR domain but lacking NB-ARC and LRR domains (TIR-X (TX)) and genes encoding a TIR and NB-ARC domain but without an LRR domain (TR-NBS (TN)) have also been implicated in plant immunity; many examples are consistent with the guard model of effector recognition (Meyers et al., 2002). For example, an Arabidopsis TN, TN2 (TIR-NBS2) was demonstrated to guard EXO70B1, a subunit of the exocyst complex (Zhao et al., 2015). Interestingly, a TIR-only protein lacking the canonical NB-ARC and LRR domains mediates recognition of the *P. syringae* effector, HopBA1. The truncated NLR, response to HopBA1 (RBA1), interacts directly with HopBA1 and triggers a cell death response (Nishimura et al., 2017). This expands our understanding of the requirements of immune receptors. Although not yet known, it seems plausible that RBA1 may heterodimerize with a full length TNL in order to mediate its cell death response.

An additional recently proposed effector recognition model is the integrated decoy hypothesis, which describes the recognition of effectors by plant NLR pairs, such as RRS1/RPS4 and RGA4/RGA5. In this model, one R protein is required for effector recognition while the other is required for activation and downstream signaling (Cesari et al., 2014). This is discussed further in 1.4.4.

1.4.4 Paired R proteins

The vast majority of *R* genes function individually for effector recognition, activation and subsequent downstream signaling; however, it is an emerging theme that *R* genes can function as pairs. The first isolated pair of *NLR* genes that function as a pair were Arabidopsis *RPP2A* and *RPP2B*, identified based on the disease resistance they provide to the *Hyaloperonospora arabidopsidis* isolate Cala2 (Sinapidou et al., 2004). Similarly, the rice *R* gene pair *RGA4*

and *RGA5* are both required for recognition of the *M. oryzae* effectors AVR-Pia and AVR1-CO39 (Cesari et al., 2013). AVR-Pia associates with the RGA5 NLR-ID, related to ATX1 (RATX1), a heavy metal-associated (HMA) domain, which induces RGA4/RGA5 complex conformational changes and RGA4-mediated immunity (Cesari et al., 2013; Ortiz et al., 2017). As described in detail in section 1.5, the Arabidopsis TNLs RPS4 and RRS1 cooperate to recognize multiple effectors. Furthermore, it has recently been found that an *R* gene pair that resembles RPS4 and RRS1, namely RPS4B/RRS1B, function together for RPS4/RRS1-independent recognition of AvrRps4 (Saucet et al., 2015). Paired *R* genes generally conform to the integrated decoy model of action whereby one NLR acts as a sensor NLR and the other acts as a signaling NLR (Cesari et al., 2014).

1.5 The RPS4/RRS1 NLR complex

1.5.1 The *avrRps4/RPS4* gene-for-gene model

avrRps4 was first identified and cloned by constructing a genomic cosmid library of *P. syringae* pv. *pisi* strain 151, which was shown to trigger HR in the Arabidopsis accession Po-1 (Hinsch and Staskawicz, 1996). A 1.2kb fragment conferred avirulence to the normally virulent *P. syringae* pv. tomato (*Pto*) DC3000 in Po-1. The gene encoding the only significant open reading frame (ORF) within this fragment was designated *avrRps4*. The natural variation of AvrRps4 recognition in Arabidopsis accessions allowed identification of the cognate *R* gene. The resistant ecotype Ws-0 was crossed to the susceptible ecotype RLD and the *Pto* DC3000(*avrRps4*)-triggered HR in segregating F_2 lines were assayed. The single dominant locus conferring resistance which mapped to chromosome 5 was designated *RPS4* (Gassmann et al., 1999). Since the identification of *avrRps4/RPS4*, several studies have greatly advanced our understanding of the molecular mechanism underlying this *avr/R*-gene model.

RPS4 has been classified as belonging to the TNL class of *R* genes, and it resides in an extensive cluster of *R* genes called the *MRC-J* region (Holub et al., 1997; Gassmann et al., 1999). Nuclear localization of the RPS4 protein, mediated by the C-terminal nuclear localization signal (NLS), is required for AvrRps4 recognition and subsequent activation of immune responses (Figure 1.3) (Wirthmueller et al., 2007). This suggests that RPS4 has a nuclear function and, in fact, a negative regulator of RPS4 and another TNL SNC1 (suppressor of *npr1-1*, constitutive 1), named SRFR1, functions together with SGT1 (suppressor of G2 allele of *skp1*) to prevent nuclear R protein accumulation (the role of SGT1 is discussed in detail in section 1.6) (Li et al., 2010). As is the case for nearly all thus far characterized TNLs, RPS4-mediated resistance is EDS1-dependent (Wirthmueller et al., 2007).

The N-terminus (1-136aa) of AvrRps4 is sufficient for delivery into the plant cell and it can be fused to other effectors to signal the proteins through the TTSS (Sohn et al., 2007). Whereas the N-terminus is sufficient for TTSS delivery *in planta*, the C-terminal 88aa fragment is sufficient for avirulence function in turnip (Sohn et al., 2009). Once delivered into the plant cell, AvrRps4 is processed into a smaller fragment at G133-G134, and the 11-kD C-terminal fragment ($AvrRps4^{C}$) is necessary and sufficient for activation of RPS4-mediated immunity. This proteolytic processing is required for the virulence but not the avirulence function of AvrRps4. The conserved KRVY motif at the N-terminus of the processed protein is, however, required for both virulence and avirulence (Figure 1.3) (Sohn et al., 2009). AvrRps4 has a nucleo-cytoplasmic distribution *in planta* and it has been demonstrated that nuclear localization is necessary for AvrRps4-triggered immunity, which is consistent with the requirement of RPS4 nuclear localization for defense activation (Wirthmueller et al., 2007; Heidrich et al., 2011). Heidrich et al. (2011) also reported interaction of EDS1 with both RPS4 and $AvrRps4^{C}$, concomitant with previous findings (Bhattacharjee et al., 2011). It was hypothesized that EDS1 is guarded by RPS4 and this complex is disrupted by the interaction of $AvrRps4^{C}$ with EDS1. The direct interaction of AvrRps4 with EDS1 has since been disputed, as the interaction could not be replicated in *Nicotiana benthamiana* or yeast, suggesting that AvrRps4 may interact with

an immune signaling complex containing EDS1, but not directly with the EDS1 protein itself (Sohn et al., 2012). However, more recently, Huh et al. (2017) demonstrated AvrRps4-EDS1 direct association using co-immunoprecipitation (CoIP) and bimolecular fluorescence complementation (BiFC) dependent on specific epitope tags. This study also demonstrated the requirement of analyzing interactions in immune receptor complexes in the presence of all components.

In 2012, the crystal structure of AvrRps4^{C}, which forms an antiparallel alpha-helical coiled coil structure, was determined and residues required for function were identified. Glu175 and Glu187 are required for both RPS4/RRS1-dependent and -independent HR, but of these two residues, only Glu187 is required for RPS4/RRS1-dependent and -independent immunity (Figure 1.3). These residues both reside within a functionally important negatively charged surface patch and, additionally, this result displays that there can be a separation of HR and immunity signaling (Sohn et al., 2012). Li et al. (2014) demonstrated that the N-terminus of AvrRps4 (1-136aa), which harbors a chloroplast transit peptide, is localized to the chloroplasts wherein resides the proposed host virulence target (Figure 1.3). This choloroplast localization is in contrast to the nucleo-cytoplasmic distribution previously described for full-length AvrRps4 (Wirthmueller et al., 2007).

1.5.2 The *popP2/RRS1* gene-for-gene model

In contrast to the *avrRps4/RPS4* gene-for-gene model, for *popP2/RRS1* the *R* gene was identified prior to the *Avr* gene. F_9 recombinant inbred lines (RILs) were generated after crossing of Arabidopsis ecotypes Col-5 and Nd-1, which are susceptible and resistant to the soilborne bacterium *R. solanacearum* strain GMI1000, respectively. These RILs were used to map the resistance determinant *RRS1* to the same region as *RPS4*, the *MRC-J* cluster in chromosome 5 (Deslandes et al., 1998). *RRS1* is the only known genetically recessive NLR *R* gene, despite behaving as a dominant *R* gene in transgenic plants. *RRS1* is a TNL; however, it possesses an additional C-terminal WRKY

DNA-binding domain (DBD) and putative nuclear localization signal (NLS) (Figure 1.3) (Deslandes et al., 2002). WRKY DBDs are specific for transcription factors and are strongly implicated in plant defense. WRKY transcription factors regulate gene transcription by binding to the W-box in promoter elements of defense-associated genes (Eulgem et al., 2000).

The cognate Avr protein recognized by RRS1 was reported to be the YopJ-like type III effector PopP2. In yeast, PopP2 interacts directly with both RRS1-R (Nd-1) and RRS1-S (Col-5), and PopP2 and RRS1 co-localize to the nucleus in plant cells (Deslandes et al., 2003). RRS1-R (resistant) confers recognition of PopP2; however, RRS1-S (susceptible) does not. Their nucleotide sequences are 98% identical, but the largest difference is that *RRS1-S* encodes a premature stop codon resulting in a 90 amino acid (aa) truncation (Figure 1.3) (Deslandes et al., 2002). The evidence that both RRS1-R and RRS1-S co-localize to the nucleus with PopP2 suggests that interaction of RRS1 with PopP2 alone is not sufficient for recognition. It was demonstrated that PopP2 displays acetyltransferase activity, which is required for function. Mutation of the highly conserved catalytic cysteine residue (C321A) impairs RRS1-mediated immunity; however, it does not affect PopP2-RRS1 interaction. H260 and D279 make up the other two catalytic residues of the catalytic triad. PopP2 autoacetylates a specific internal lysine residue (K383), which is necessary for avirulence activity (Figure 1.3) (Tasset et al., 2010).

Noutoshi et al. (2005) isolated an interesting mutant of RRS1-R with a single leucine insertion in the WRKY domain after L1224 in the Arabidopsis accession No-0 (Figure 1.3). The *sensitive to low humidity* (*slh1*) mutant exhibits a severely stunted morphology and develops necrotic lesions when grown on soil. Salicylic acid (SA)-dependent upregulation of defense-associated genes and accumulation of callose and autofluorescent compounds indicate that the observed *slh1* phenotype is due to constitutive defense activation. Evidence indicates that the 3 bp insertion in the WRKY domain impairs binding of RRS1 to a synthetic W-box. This led to the hypothesis that the wild-type (WT) RRS1 allele negatively regulates immunity

by binding to the promoter elements of defense genes via the WRKY domain (Noutoshi et al., 2005). *RRS1* is an atypical *R* gene because of the presence of the C-terminal WRKY domain. It has characteristics of *R* genes, but also plant transcription factors, which could provide a direct link between effector recognition and transcriptional reprogramming (Rushton et al., 2010).

1.5.3 Recognition of multiple effectors

Remarkably, the aforementioned TNLs, RPS4 and RRS1, have been shown to function cooperatively for recognition of AvrRps4, PopP2, an unknown effector from the fungal pathogen *Colletotrichum higginsianum*, and more recently an unknown effector from the bacterial pathogen *Xanthomonas campestris* pv. *campestris* (Birker et al., 2009; Narusaka et al., 2009; Debieu et al., 2016). Growth of *Pto* DC3000(*avrRps4*) in the *rps4-21/rrs1-1* double mutant of the Arabidopsis accession Ws-0 was comparable to growth in the *rps4-21* and *rrs1-1* single mutants, suggesting that both TNLs are required for effector recognition. *RPS4* and *RRS1* are also genetically linked; they reside in a head-to-head orientation and share a 264bp 5'-regulatory sequence (Narusaka et al., 2009).

The biochemical function of RPS4/RRS1 was not well understood, but recent studies have helped to unravel the molecular mechanism by which this pair of TNLs recognize multiple effectors and trigger defense responses. Williams et al. (2014) demonstrated that RPS4 and RRS1 physically associate via TIR/TIR domain heterodimerization and that the TIR domains can also self-associate/homodimerize. Using tobacco agroinfiltration assays, it was shown that mutation of the SH motif, which mediates TIR/TIR homo- and hetero-dimerization of RPS4 and RRS1, impairs effector recognition. This disruption of the interaction interface and resulting impairment of effector recognition suggests that recognition of AvrRps4 and PopP2 is dependent on the formation of a heterodimeric RPS4/RRS1 complex (Williams et al., 2014). The RPS4 TIR domain plus a short stretch of the NB domain activates effector-independent cell death in tobacco; however, mutation of the SH motif residues

abolishes this cell death, suggesting that RPS4 TIR homodimerization is required for the cell death signaling pathway (Figure 1.3). The functional requirement of the P-loop motif for RPS4 but not RRS1 provides further evidence that RPS4 acts as the signaling partner in the complex (Swiderski et al., 2009; Williams et al., 2014). Heterodimerization of the RPS4 TIR domain with the RRS1 TIR domain likely inhibits signaling by RPS4 but forms a resting, inactive, signaling-competent complex, which can be activated by conformational changes upon recognition of AvrRps4 or PopP2. It is hypothesized that this conformational change releases the RPS4 TIR from the inhibitory effect of RRS1 TIR, allowing downstream signaling via RPS4 TIR/TIR homodimerization (Williams et al., 2014).

In another study, it was shown that RPS4 is required for $RRS1^{SLH1}$ dependent constitutive defense activation. Transcription profiling data indicate the significant overlap between $RRS1^{SLH1}$-, AvrRps4- and PopP2-mediated immune responses, suggesting that the *slh1* mutation confers effector-independent RPS4/RRS1 immune signaling. A forward genetic screen performed on the *slh1* mutant was employed to isolate *suppressor of slh1 immunity* (*sushi*) mutants based on the impairment of the *slh1* morphological phenotype. As expected, several *SUSHI* mutations were identified within *RRS1* but also, importantly, within *RPS4* (Sohn et al., 2014). In agreement with evidence provided by Williams et al. (2014), this indicates that RPS4 functions in complex with RRS1. PopP2 and AvrRps4 both interact with RRS1 and it is therefore hypothesized that RRS1 is required for direct effector sensing, while RPS4 activates defense after detection of effector-induced perturbation of the RRS1 protein (Sohn et al., 2014; Williams et al., 2014). This mechanism is in agreement with the integrated decoy model to explain the functioning of NLR protein pairs in plant immunity (Cesari et al., 2014).

PopP2 has been demonstrated to bind and acetylate the WRKY domain of RRS1 (specifically the critical lysine residue K1221); AvrRps4 was reported to bind the RRS1 WRKY domain with unknown enzymatic activity (Figure 1.3) (Le Roux et al., 2015; Sarris et al., 2015). It is likely that the single leucine insertion conferring the *slh1* mutant phenotype mimics the acetylated RRS1

WRKY domain, thereby constitutively activating immunity. As previously mentioned, WRKY transcription factors (TFs) are strongly implicated in plant immunity (Eulgem et al., 2000). Both PopP2 and AvrRps4 target other WRKY proteins, which are likely virulence targets in order to impair DNA binding and consequently suppress host immunity. In fact, PopP2 was shown to contribute to suppression of PTI and dissociation of WRKY TFs from their host DNA (Le Roux et al., 2015; Sarris et al., 2015). RRS1 has evolved to carry an integrated decoy (WRKY domain) that is targeted by AvrRps4/PopP2 and RPS4 has evolved to detect the effector-induced modification of the RRS1 WRKY domain so that, as a pair, RPS4/RRS1 can activate rapid, strong defense in response to *P. syringae* and *R. solanacearum* infection. Signaling of the RPS4/RRS1 complex is dependent on an RRS1 leucine zipper (LZ) motif, a motif primarily involved in protein-protein interaction (Narusaka et al., 2016). The function of the NLR RPS2 and, interestingly, the WRKY TFs WRKY18, WRKY40 and WRKY60 is also dependent on an LZ motif (Tao et al., 2000; Xu et al., 2006). The LZ motif in these WRKY TFs is required for self-association as well as association with each other (Xu et al., 2006). Narusaka et al. (2016) suggest that the RRS1 LZ motif is required for interaction of RRS1 with RPS4; however, future protein-protein interaction assays would be required to confirm this. The susceptibility of the *wrky18/wrky40* double mutant to *Pto* DC3000(*avrRps4*) suggests that the activated RPS4/RRS1 signaling complex may regulate gene transcription via the WRKY TFs WRKY18 and WRKY40 (Schön et al., 2013). Recently, further insight into the interactions involved in proper RPS4/RRS1 complex function was gained. It was demonstrated that the RRS1 protein stabilizes RPS4 as well as suppressing RPS4-mediated autoimmunity. Furthermore, in contrast to previous data, it was shown that AvrRps4 does not disrupt RPS4-EDS1 interaction (Huh et al., 2017). It was also proposed that RPS4 only forms TIR/TIR domain homodimers in the presence of RRS1; a result that is puzzling considering the RPS4 TIR SH motif is required for RPS4 TIR/TIR domain homodimerization and RPS4 TIR domain and full length HR induction in tobacco, which is suppressed not promoted by co-expression with RRS1 TIR domain (Williams et al., 2014; Huh et al., 2017).

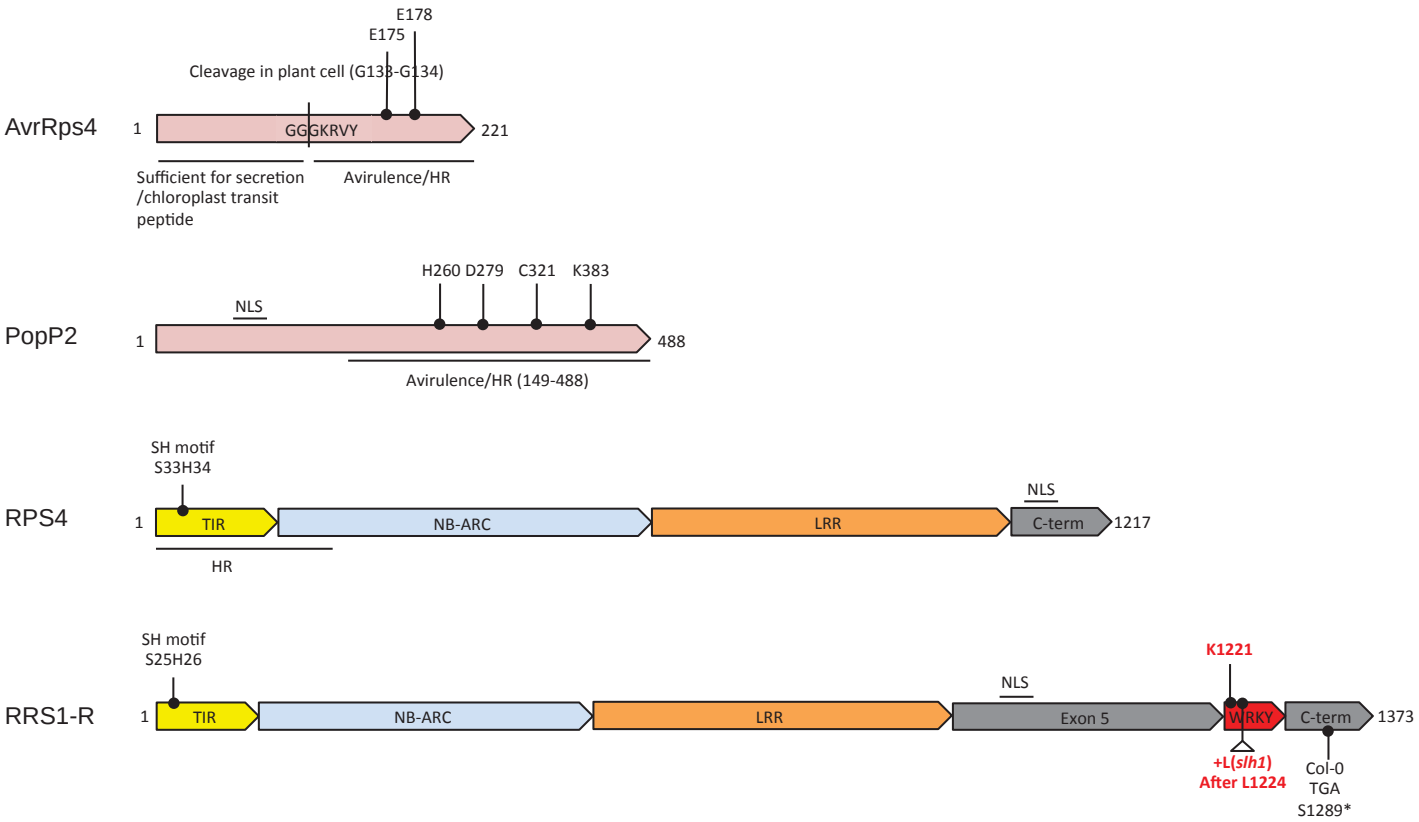


Figure 1.3. A schematic displaying the structure and important residues of effectors, AvrRps4 and PopP2, and NLRs, RPS4 and RRS1.
AvrRps4) N-terminus necessary and sufficient for secretion that also acts as a chloroplast transit peptide; *in planta* cleavage site; C-terminus required for avirulence/HR induction, KRVY motif required for virulence and avirulence; E175 required for RPS4/RRS1-dependent and –independent HR; and E178 required for RPS4/RRS1-dependent and –independent HR, and RPS4/RRS1-dependent immunity. PopP2) Nuclear localization signal (NLS); 149-488 aa sufficient for avirulence/HR induction; catalytic triad H260, D279 and C321; and autoacetylated residue K383. RPS4) SH motif required for TIR/TIR association with RRS1 and TIR/TIR self-association; TIR + short stretch of NB domain that triggers effector-independent cell death; C-terminal NLS. RRS1) SH motif required for TIR/TIR heterodimer formation with RPS4; critical lysine residue acetylated by PopP2 K1221; location of *slh1* leucine insertion; Col-0 premature stop codon S1289* (encodes RRS1-S).

1.5.4 RRS1- and RPS4-independent AvrRps4 recognition (RRIR)

The Arabidopsis accessions Ws-2 and Col-0 maintain recognition of AvrRps4 if *RPS4* or *RRS1* is mutated, as demonstrated by HR and bacterial growth respectively (Birker et al., 2009; Sohn et al., 2012). This suggested that Ws-2 and Col-0 carry a secondary locus, which confers recognition of AvrRps4 but not PopP2. Saucet et al. (2015) positionally cloned the *RRS1- and RPS4-independent AvrRps4 recognition* (*RRIR*) locus and discovered another pair of *R* genes with strong homology to *RPS4*/*RRS1*, which were termed *RPS4B*/*RRS1B*. Employing *Agrobacterium*-mediated transient expression in tobacco leaves, it was shown that RPS4B and RRS1B do, indeed, confer recognition of AvrRps4 but not PopP2. Moreover, RPS4B and RRS1B associate with each other. Inappropriate combinations (RPS4/RRS1B and RPS4B/RRS1) can occur but they are non-functional for AvrRps4 recognition (Saucet et al., 2015).

1.6 Genes required for ETI Signaling

Forward genetic screens, primarily in Arabidopsis, have identified multiple conserved plant immune regulators. These genes have been found to be necessary for ETI.

EDS1 was identified after mutational analysis of the *Arabidopsis* ecotype Ws-0. *EDS1* was initially reported to be required for the function of *RPP* (*RESISTANCE TO P. PARASITICA*) genes in providing resistance to *Peronospora parasitica* (Parker et al., 1996). The *EDS1* gene encodes a lipase-like protein; however, enzymatic activity is not required for its function (Falk et al., 1999; Wagner et al., 2013). EDS1 is required for downstream signaling of all thus far characterized TNLs, apart from TTR1 (Wiermer et al., 2005; Nam et al., 2011). Despite the greatly important role that EDS1 plays in ETI, the biochemical function of EDS1 remains elusive.

Studies have shown that EDS1 can homodimerize or heterodimerize with PAD4 or SAG101 (Feys et al., 2001; Feys et al., 2005). *PAD4* (*PHYTOALEXIN DEFICIENT 4*) was identified by mutagenesis of Arabidopsis and a subsequent assay for susceptibility after infection with *P. syringae* pv. *maculicola* and, like EDS1, has homology to lipases and is required for TNL-mediated immune response signaling (Glazebrook et al., 1996; Jirage et al., 1999). *SAG101* (*Senescence-Associated Gene 101*) was identified as a senescence-related gene and encodes an interacting partner of EDS1 (Feys et al., 2001; He et al., 2001). SAG101 is indispensable for TNL-mediated EDS1-dependent immunity (Feys et al., 2005).

EDS1, PAD4 and SAG101 are required for immune signaling mediated by nearly all TNLs; however, CNL-mediated signaling is regulated primarily by another protein, NDR1. *NDR1* was identified in a fast-neutron-mutagenesis screening identifying mutants of the Arabidopsis accession Col-0 that were susceptible to *Pto* DC3000(*avrB*) (Century et al., 1995). Intriguingly, the EDS1/PAD4 complex has also been shown to be involved in downstream signaling mediated by several CNLs such as RPS2 (Venugopal et al., 2009). For RPS2 as well as TNL and basal immunity, EDS1/PAD4 and the defense hormone salicylic acid (SA) function redundantly to regulate immunity-related genes. Although current models suggest that EDS1/PAD4 function upstream of SA, it appears that a further separate EDS1/PAD4 pathway also functions in parallel to SA-regulated immunity (Cui et al., 2017).

Studies have revealed that the molecular chaperone, HSP90 (heat shock protein 90), and its co-chaperones, SGT1 and RAR1 (required for *Mla12* resistance 1), are required for immunity mediated by multiple TNLs and CNLs (Takahashi et al., 2003; Liu et al., 2004). The HSP90 chaperone complex functions to maintain NLR proteins in the properly folded state and in the appropriate subcellular location (Shirasu, 2009). Moreover, the HSP90 chaperone complex also functions in PRR maturation and transport, as demonstrated in rice (Thao et al., 2007; Chen et al., 2010). In fact, HSP90s are conserved among the majority of living organisms including bacteria, yeast and mammals as well as plants (Corigliano and Clemente, 2016).

Further downstream, defense signaling converges on the central immune regulator, NPR1 (non-expressor of PR genes 1), which acts as a receptor for SA and controls the onset of systemic acquired resistance (Cao et al., 1997; Wu et al., 2012). NPR1 activity is regulated by several posttranslational modifications (PTMs) including phosphorylation and sumoylation (Withers and Dong, 2016).

1.7 Transcriptional repression in immunity

Transcriptional repression in plants is emerging as an important regulatory mechanism for many diverse plant processes. The Gro/Tup1-like proteins constitute a small family of 13 members in Arabidopsis (Liu and Karmarkar, 2008). They are named based on their structural similarity to the *Drosophila* and *Saccharomyces cerevisiae* co-repressors, Groucho and Tup1 (Keleher et al., 1992; Paroush et al., 1994).

The Gro/Tup1-like family can be divided into two subclasses based on their domain organization. The TPL/TPR/WSIP proteins possess central WD-repeats in addition to the C-terminal WD-repeats, and a CTLH (C-terminal to LisH) domain. The LUG/LUH subclass of proteins possess a LUFS domain at their N-termini and lack a CTLH domain (Liu and Karmarkar, 2008).

LEUNIG (LUG) was the very first Gro/Tup1-like co-repressor identified in plants in a genetic screen to identify enhancer mutations of the floral homeotic mutant *apetala2-1*. LUG is a negative regulator of the AGAMOUS (AG) gene (Liu and Meyerowitz, 1995). Gro/Tup1-like co-repressors function by recruiting histone deacetylases (HDACs) to epigenetically repress gene transcription via chromatin remodeling (Krogan and Long, 2009).

1.7.1 The EAR motif

Co-repressor proteins are recruited to genetic elements via proteins possessing repressor domains (RDs). RDs implicated in plant transcriptional regulation include the EAR (ERF-associated amphiphilic repression) motif and the Wuschel-box with the short amino acid sequences, L/FDLNL/F(x)P and TLxLFP, respectively (Ohta et al., 2001; Ikeda et al., 2009). It was reported that fusion of an EAR motif to transcription factors is sufficient for them to become dominant transcriptional repressors (Hiratsu et al., 2003).

Probably the best-known example of defense regulation by a transcriptional co-repressor is in the case of jasmonic acid (JA) signaling. The JAZ (jasmonate ZIM-domain) proteins in Arabidopsis recruit the Gro/Tup1-like corepressor TOPLESS (TPL) and TPL-related proteins (TPRs) primarily via the adaptor NINJA (novel interactor of JAZ), which recruits co-repressors via an EAR motif to negatively regulate JA signaling (Pauwels et al., 2010). Furthermore, the Arabidopsis TNL SNC1 requires a co-repressor for function; TPL and TPR1 were demonstrated to function redundantly in SNC1-mediated immune signaling. SNC1 represses negative regulators of immunity during pathogen infection via recruitment of a co-repressor, which itself recruits histone deacetylase 19 (HDAC19) to epigenetically silence negative regulators of defense (Zhu et al., 2010).

1.8 Aims of the study

Overall aim: to increase our understanding of the mechanism by which the two TNL R proteins, RPS4 and RRS1, mediate recognition of AvrRps4/PopP2 and immunity in the model plant species *Arabidopsis thaliana*.

Chapter 3 aim: to determine further functional PopP2 residues required for *in planta* RPS4/RRS1-mediated recognition

Chapter 4 aim: to gain further insight into the biochemical requirements of RPS4/RRS1 function in terms of auto-activity and effector recognition.

Chapter 5 aim: to ascertain if RPS4 employs a common mechanism of TIR domain self-association and signaling via two interfaces (Zhang et al., 2017).

CHAPTER 2: MATERIALS AND METHODS

2.1 Plant materials

Table 2.1. Arabidopsis genotypes used in these studies.

Genotype	WT/GM
Col-0	WT
No-0	WT
Ws-2	WT
slh1	GM
Ws-2 *rrs1-1*	GM
Several *sushi* mutants (see Table 3.1)	GM

2.2 Primers used

See next page.

Gene name	Primer name	Sequence	Purpose
Rs GMI1000/PopP2	PopP2 149aa forward (F)	GGTCTCCAATGGCTGGCCAGCAAG CAACC	Sequencing and Golden Gate cloning
Rs GMI1000/PopP2	PopP2 reverse (R)	GGTCTCAAAGCGTTGGTATCCAATA GGGAATCCTG	Sequencing and Golden Gate cloning
Rs GMI1000/PopP2	Pe_13 PopP2(149-241) R	GGTCTCACGAAGCGATATGTCGCG CCCC	Golden Gate cloning
Rs GMI1000/PopP2	C321A mut. F	CAGAAGTCCTTCTTCGATGCCCGG ATACTCTCCCTGTC	Site-directed mutagenesis
Rs GMI1000/PopP2	C321A mut. R	GACAGGGAGAGTATCCGGGCATCG AAGAAGGACTTCTG	Site-directed mutagenesis
Rs GMI1000/PopP2	PopP2_SRDX_R	GGTCTCTCGAACGCAAAGCCCAGG CGCAGTTCCAGATCCAGATCCAGG TTGGTATCCAATAGGGAATC	Golden Gate cloning (PopP2 + C-terminal SRDX)
Rs GMI1000/PopP2	PopP2_srdx_R	GGTCTCTCGAACGCAAAGCCCAGG CGAAATTCAAAATCAAAATCAAAGT TGGTATCCAATAGGGAATC	Golden Gate cloning (PopP2 + C-terminal srdx)
At Col-0/EF1alpha	EF1alpha_F	CAGGCTGATTGTGCTGTTCTTA	qPCR
At Col-0/EF1alpha	EF1alpha_R	GTTGTATCCGACCTTCTTCAGG	qPCR
At Col-0/PR1	PR1_F	ATACACTCTGGTGGGCCTTACG	qPCR
At Col-0/PR1	PR1_R	TACACCTCACTTTGGCACATCC	qPCR
At Col-0/PBS3	PBS3_F	TTCGCTGGCTTGTATAGGATGA	qPCR
At Col-0/PBS3	PBS3_R	CTGGAAATGTTGAGGTGTCAGC	qPCR
At Col-0/FMO1	FMO1_F	TGTGTTTGAAGATGGGACGACA	qPCR
At Col-0/FMO1	FMO1_R	GTTCGAGCTGCTTTGGACGTAT	qPCR
At Col-0/SARD1	SARD1_qPCR_F	CCGATATGCGAAGTTATGAAAGC	qPCR
At Col-0/SARD1	SARD1_qPCR_R	AGTGGCTCGCAGCATATTGTT	qPCR

At Ws-2/RRS1	RRS1 modA F	GGTCTCGAATGACCAATTGTGAAAAGGATG	Golden Gate cloning (gDNA)
At Ws-2/RRS1	RRS1 modA R	GGTCTCGATGATAAGGGTGAACGACGC	Golden Gate cloning (gDNA)
At Ws-2/RRS1	RRS1 modB F	GGTCTCGTCATTACTTGACAAATTTTGTTGTTATTC	Golden Gate cloning (gDNA)
At Ws-2/RRS1	RRS1 modB R	GGTCTCATGGAAACGTTGAGAAGTGGG	Golden Gate cloning (gDNA)
At Ws-2/RRS1	RRS1 modC F	GGTCTCGTCCATATCTATGTATCTTTTTACTTTCATG	Golden Gate cloning (gDNA)
At Ws-2/RRS1	RRS1 modC R	GGTCTCTCGAAATAATCGAAGAATGTT	Golden Gate cloning (gDNA)
At Col-0/HOS15	HOS15 CDS F1	GGTCTCCAATGTCTTCACTTACCTCCGTCG	Golden Gate cloning (cDNA)
At Col-0/HOS15	HOS15 CDS R1	GGTCTCAGTTGCAAGTAAAGTCCCTTCACC	Golden Gate cloning (cDNA)
At Col-0/HOS15	HOS15 CDS F2	GGTCTCACAACTGGTTCATGTGATGGC	Golden Gate cloning (cDNA)
At Col-0/HOS15	HOS15 CDS R2	GGTCTCACGAACATTCTGAAATCAAGAACGCAAACT	Golden Gate cloning (cDNA)
At Col-0/LUG	LUG CDS F1	GGTCTCCAATGTCTCAGACCAACTGGGAAGCTG	Golden Gate cloning (cDNA)
At Col-0/LUG	LUG CDS R1	GGTCTCAGTTGTCCAACATTGTCCCCAAATCG	Golden Gate cloning (cDNA)
At Col-0/LUG	LUG CDS F2	GGTCTCACAACTGTTGGACCCAAGTCATGCATC	Golden Gate cloning (cDNA)
At Col-0/LUG	LUG CDS R2	GGTCTCATGAGTTGAAGGTGTAGAAGG	Golden Gate cloning (cDNA)

At Col-0/LUG	LUG CDS F3	GGTCTCGCTCATACTCCTGGAGATGTGATC	Golden Gate cloning (cDNA)
At Col-0/LUG	LUG CDS R3	GGTCTCACGAACTTCCACAGTTTCACTAGCTTATC	Golden Gate cloning (cDNA)
At Col-0/LUH	LUH CDS F1	GGTCTCCAATGGCTCAGAGTAATTGGGAAGC	Golden Gate cloning (cDNA)
At Col-0/LUH	LUH CDS R1	GGTCTCAGTTGGCCATCTTTCGGATTAAGGTTTC	Golden Gate cloning (cDNA)
At Col-0/LUH	LUH CDS F2	GGTCTCACAACAAAATGCAAATGATGGATCTATAGG	Golden Gate cloning (cDNA)
At Col-0/LUH	LUH CDS R2	GGTCTCACGAACTTCCAAATCTTTACGGATTTGTC	Golden Gate cloning (cDNA)
At Col-0/SAP18	SAP18 cDNA F1	GGTCTCGAATGGCTGAAGCAGCGAG	Golden Gate cloning (cDNA)
At Col-0/SAP18	SAP18 cDNA R1	GGTCTCACGAAGTAAATTGCCACATCCAGATAATC	Golden Gate cloning (cDNA)
At Col-0/SEU	SEU CDS F1	GGTCTCCAATGGTACCATCAGAGCCGCCTAATC	Golden Gate cloning (cDNA)
At Col-0/SEU	SEU CDS R1	GGTCTCAGTTGGCCATCACGAACAACCCGAAG	Golden Gate cloning (cDNA)
At Col-0/SEU	SEU CDS F2	GGTCTCACAACTTCGAATAGTCTTCTCGCCAG	Golden Gate cloning (cDNA)
At Col-0/SEU	SEU CDS R2	GGTCTCACGAACGCGTTCCAATCAAAATTGTTGAAACC	Golden Gate cloning (cDNA)
At Col-0/SLK1	SLK1 cDNA F1	GGTCTCGAATGAACAGAACGGTCGTCTC	Golden Gate cloning (cDNA)
At Col-0/SLK1	SLK1 cDNA R1	GGTCTCAGAACTTTGCCAGTTGCCG	Golden Gate cloning (cDNA)

At Col-0/SLK1	SLK1 cDNA F2	GGTCTCGGTTCATGGAACTACAGTCGC	Golden Gate cloning (cDNA)
At Col-0/SLK1	SLK1 cDNA R2	GGTCTCACGAACAAGCCACCATAGATATCATTGT	Golden Gate cloning (cDNA)
At Col-0/SLK2	SLK2 cDNA F1	GGTCTCGAATGGCTTCTTCAACTTCTGG	Golden Gate cloning (cDNA)
At Col-0/SLK2	SLK2 cDNA R1	GGTCTCAGAGAAAAATACTCCGTCACAAATTTC	Golden Gate cloning (cDNA)
At Col-0/SLK2	SLK2 cDNA F2 (mutate SmaI site)	GGTCTCGTCTCCTCGGGCAAAGAAAAG	Golden Gate cloning (cDNA)
At Col-0/SLK2	SLK2 cDNA R2	GGTCTCACAACCTCAGAAATCTGCAGAC	Golden Gate cloning (cDNA)
At Col-0/SLK2	SLK2 cDNA F3	GGTCTCGGTTGTCAGCAGCATGAAAGA	Golden Gate cloning (cDNA)
At Col-0/SLK2	SLK2 cDNA R3	GGTCTCACGAATGACTTCCAAGAATATCCTCCC	Golden Gate cloning (cDNA)
At Col-0/TPL	TPL F1	GGTCTCGAATGTCTTCTCTTAGTAGAGAGCTCG	Golden Gate cloning (cDNA)
At Col-0/TPL	TPL R1	GGTCTCACGTCAGAGATTCCCATTGGTC	Golden Gate cloning (cDNA)
At Col-0/TPL	TPL F2	GGTCTCGGACGAGGTGAATCTAGGCG	Golden Gate cloning (cDNA)
At Col-0/TPL	TPL R2	GGTCTCAGAAACTACATTGGCTGATCGATCTG	Golden Gate cloning (cDNA)
At Col-0/TPL	TPL F3	GGTCTCGTTTCCATCCAAGGAATGAATGGAG	Golden Gate cloning (cDNA)
At Col-0/TPL	TPL R3	GGTCTCACGAATCTCTGAGGCTGATCAGATGC	Golden Gate cloning (cDNA)

At Col-0/TPR1	TPR1 cDNA F1	GGTCTCGAATGATGTCTTCTCTGAG CAGAGAGC	Golden Gate cloning (cDNA)
At Col-0/TPR1	TPR1 cDNA R1	GGTCTCACTACATTCGTACCAACTA GTAGCAGAG	Golden Gate cloning (cDNA)
At Col-0/TPR1	TPR1 cDNA F2	GGTCTCGGTAGGGGATATTGGGCT CTGGGAAGTCGGTTCTCGAGAACG TCTAGTC	Golden Gate cloning (cDNA)
At Col-0/TPR1	TPR1 cDNA R2 (mutate BsaI site)	GGTCTCAGAAACGACAGAGGCAGG CCTCTCTACCATCGGAATGCTGTTG ATTGC	Golden Gate cloning (cDNA)
At Col-0/TPR1	TPR1 cDNA F3	GGTCTCGTTTCCATCCCTGGAATGA ATGGAG	Golden Gate cloning (cDNA)
At Col-0/TPR1	TPR1 cDNA R3	GGTCTCACGAATCTCTGAGGCTGG TCAGAGG	Golden Gate cloning (cDNA)
At Col-0/TPR1	TPR1 cDNA mutate BsaI site F	GATGCTGATGGAGGACTCCAGGCA AGTCCA	Site-directed mutagenesis
At Col-0/TPR1	TPR1 cDNA mutate BsaI site R	TGGACTTGCCTGGAGTCCTCCATCA GCATC	Site-directed mutagenesis
At Col-0/TPR2	TPR2 cDNA F1	GGTCTCGAATGATGTCGTCTTTGAG CAGAGAGTTAG	Golden Gate cloning (cDNA)
At Col-0/TPR2	TPR2 cDNA R1	GGTCTCAGTTGACGTAGGTCTGAG CCT	Golden Gate cloning (cDNA)
At Col-0/TPR2	TPR2 cDNA F2	GGTCTCGCAACACCTCGAGATTGAT GCTCATGTAGGCTGTGTGAATGACT TGG	Golden Gate cloning (cDNA)
At Col-0/TPR2	TPR2 cDNA R2 (mutate BsaI site)	GGTCTCACATCATTTGCCATTAGGA GTCCGCTGTTTGGCTGCC	Golden Gate cloning (cDNA)
At Col-0/TPR2	TPR2 cDNA F3	GGTCTCGGATGTTCCCGAGAATCC TGAAGG	Golden Gate cloning (cDNA)

At Col-0/TPR2	TPR2 cDNA R3	GGTCTCACGAACCTTTGAATCTGATCCGAACTTGAGTTG	Golden Gate cloning (cDNA)
At Col-0/TPR2	TPR2 cDNA mutate BsaI F	GGATTCAAGATCCTTGCAAATACTGATGGACTCAGAACCTTAAGAGCATTTGAAGCGCGG	Site-directed mutagenesis
At Col-0/TPR2	TPR2 cDNA mutate BsaI site R	CCGCGCTTCAAATGCTCTTAAGGTTCTGAGACCTTCAGTATTTGCAAGGATCTTGAATCC	Site-directed mutagenesis
At Col-0/TPR3	TPR3 cDNA F1	GGTCTCGAATGATGTCGTCGTTGAGTCGAGAG	Golden Gate cloning (cDNA)
At Col-0/TPR3	TPR3 cDNA R1	GGTCTCACGTCGCAGATCCAACAAGAAGTA	Golden Gate cloning (cDNA)
At Col-0/TPR3	TPR3 cDNA F2	GGTCTCGGACGGGAGAAATCACATTGTGG	Golden Gate cloning (cDNA)
At Col-0/TPR3	TPR3 cDNA R2	GGTCTCAAGAGCCTCGTTCAACTTTACAATTG	Golden Gate cloning (cDNA)
At Col-0/TPR3	TPR3 cDNA F3	GGTCTCGCTCTCCTGTTAGACACTCACAAATG	Golden Gate cloning (cDNA)
At Col-0/TPR3	TPR3 cDNA R3	GGTCTCACGAATCTTTGTAACTGTTCTGGAGTTTGG	Golden Gate cloning (cDNA)
At Col-0/TPR3	TPR3 cDNA mutate x2 BsaI sites F	GGATTTTAATTGTATGTTGTCTATGGTCTTTAGTCTCAGTCTCTTATTGTGCAGGGGTTGCG	Site-directed mutagenesis
At Col-0/TPR3	TPR3 cDNA mutate x2 BsaI sites R	CGCAACCCCTGCACAATAAGAGACTGAGACTAAAGACCATAGACAACATACAATTAAAATCC	Site-directed mutagenesis
At Col-0/TPR4	TPR4 cDNA F1	GGTCTCGAATGATGTCGTCACTCAGCAGAGAAC	Golden Gate cloning (cDNA)

At Col-0/TPR4	TPR4 cDNA R1	GGTCTCAGAAATACCAAAAGGTCTG GGTCTCTT	Golden Gate cloning (cDNA)
At Col-0/TPR4	TPR4 cDNA F2	GGTCTCGTTTCAGATGGGGTTAATA ATCTTCCAGTCAAT	Golden Gate cloning (cDNA)
At Col-0/TPR4	TPR4 cDNA R2	GGTCTCAGTTGAGACGGCCAGCAG AG	Golden Gate cloning (cDNA)
At Col-0/TPR4	TPR4 cDNA F3	GGTCTCGCAACTACTGATAACGGAA TTAAGATTCTAGCG	Golden Gate cloning (cDNA)
At Col-0/TPR4	TPR4 cDNA R3	GGTCTCACGAACGAATCACTCGGTT GTTGATCTGAC	Golden Gate cloning (cDNA)
At Col-0/TPR4	TPR4 cDNA mutate BsaI site F	CGGCAGATTCTGAAAGTGTGTTGAA AAGACCCAGACCTTTTGGTATTTCA GATGGGG	Site-directed mutagenesis
At Col-0/TPR4	TPR4 cDNA mutate BsaI site R	CCCCATCTGAAATACCAAAAGGTCT GGGTCTTTTCAACACACTTTCAGAA TCTGCCG	Site-directed mutagenesis
At No-0/RRS1	RRS1_gSeq_F1	GAAAAAGCATCGTCGGCATAAACA	to sequence RRS1 CDS in sushis
At No-0/RRS1	RRS1_gSeq_R1	AAGTGCGTCTCGTACACATCTC	to sequence RRS1 CDS in sushis
At No-0/RRS1	RRS1_gSeq_F2	CCAGGTTTTCGCTTTATTCTACTC	to sequence RRS1 CDS in sushis
At No-0/RRS1	RRS1_gSeq_R2	CTCATTTAAACCCTGGACCTCATAT	to sequence RRS1 CDS in sushis
At No-0/RRS1	RRS1_gSeq_F3	TCTCGAGGGGTTTGACTGGCTAG	to sequence RRS1 CDS in sushis
At No-0/RRS1	RRS1_gSeq_R3	TTCTGCGCCTCTCGATCTGT	to sequence RRS1 CDS in sushis
At No-0/RRS1	RRS1_gSeq_F4	CCACATGTTGAAATTGATGTCCTTG	to sequence RRS1 CDS in sushis
At No-0/RRS1	RRS1_gSeq_R4	GATCGTCCTCAACATCTCCAGGTTC	to sequence RRS1 CDS in sushis
At No-0/RRS1	RRS1_gSeq_F5	GGTGGAACCAAGGTAAGCAA	to sequence RRS1 CDS in sushis
At No-0/RRS1	RRS1_gSeq_R5	GCCACCAAGATATAACTGTTTCAGA	to sequence RRS1 CDS in sushis
At No-0/RRS1	RRS1_gSeq_F6	CCAAACCATAGAGAGCTTGTGAATT	to sequence RRS1 CDS in sushis

At No-0/RRS1	RRS1_gSeq_R6	TGGAAAGAAAGAGAGCTAATGGAGAGGT	to sequence RRS1 CDS in sushis
At No-0/RRS1	RRS1_gSeq_F7	CGCTGACTTATGTAAAACACATACC	to sequence RRS1 CDS in sushis
At No-0/RRS1	RRS1_gSeq_R7	CTTTGTCTGCTGATTGATAGGAAAG	to sequence RRS1 CDS in sushis
At No-0/RRS1	RRS1_gSeq_F8	GTGATGCTACAGATGTTGGCA	to sequence RRS1 CDS in sushis
At No-0/RRS1	RRS1_gSeq_R8	TGTAAACTTTAGTTCAATCTAATAAATTGCACGT	to sequence RRS1 CDS in sushis
At No-0/RRS1	RRS1_gSeq_F10	GTGCGTAAGTGATCAAATTATGG	to sequence RRS1 CDS in sushis
At No-0/RRS1	RRS1_gSeq_R10	TTGTCGAGATCACCATATGTTGG	to sequence RRS1 CDS in sushis
At No-0/RRS1	CAPSslh1_L	GTTATATCGACGTTGGATGCAG	to genotype slh1 mutation - CAPS marker, digest with DdeI, uncut (352 bp) is slh1, cut is WT, from Noutoshi et al. (2005)
At No-0/RRS1	CAPSslh1_R	CCAGCAAGTTTAGGATGATTACG	to genotype slh1 mutation - CAPS marker, digest with DdeI, uncut (352 bp) is slh1, cut is WT, from Noutoshi et al. (2005)
At Ws-2/RRS1	RRS1 C15Y mutagenesis F	AGGAATTCGTGTGCATCAGCTACGTAGAAGAGGTACGGTACTCT	RRS1 sushi site-directed mutagenesis
At Ws-2/RRS1	RRS1 C15Y mutagenesis R	AGAGTACCGTACCTCTTCTACGTAGCTGATGCACACGAATTCCT	RRS1 sushi site-directed mutagenesis
At Ws-2/RRS1	RRS1 P68L mutagenesis F	GGGTTTCTGTGATGGTTTTACTCGGAAACTGTGATCCTTCCG	RRS1 sushi site-directed mutagenesis
At Ws-2/RRS1	RRS1 P68L mutagenesis R	CGGAAGGATCACAGTTTCCGAGTAAAACCATCACAGAAACCC	RRS1 sushi site-directed mutagenesis
At Ws-2/RRS1	RRS1 G176E mutagenesis F	CGATAGGCATCCGTTGTGTTGAAATTTGGGGTATGCCTGGCAT	RRS1 sushi site-directed mutagenesis
At Ws-2/RRS1	RRS1 G176E mutagenesis R	ATGCCAGGCATACCCCAAATTTCAACACAACGGATGCCTATCG	RRS1 sushi site-directed mutagenesis

At Ws-2/RRS1	RRS1 C607Y mutagenesis F	GGACGATCAGGCTTTACCATTCCCACCATCTAGTTGATATCG	RRS1 sushi site-directed mutagenesis
At Ws-2/RRS1	RRS1 C607Y mutagenesis R	CGATATCAACTAGATGGTGGGAATGGTAAAGCCTGATCGTCC	RRS1 sushi site-directed mutagenesis
At Ws-2/RRS1	RRS1 L816F mutagenesis F	GGCTAATTTAGAATTTCTCAAAGTTTTTGATCTCTCTGGTTGCTCAG	RRS1 sushi site-directed mutagenesis
At Ws-2/RRS1	RRS1 L816F mutagenesis R	CTGAGCAACCAGAGAGATCAAAAACTTTGAGAAATTCTAAATTAGCC	RRS1 sushi site-directed mutagenesis
At Ws-2/RRS1	RRS1 C1243Y mutagenesis F	CTTACAAGTTCACGCATGGTTATAAAGCTACAAAACAAGTCCAAC	RRS1 sushi site-directed mutagenesis
At Ws-2/RRS1	RRS1 C1243Y mutagenesis R	GTTGGACTTGTTTTGTAGCTTTATAACCATGCGTGAACTTGTAAG	RRS1 sushi site-directed mutagenesis
At Ws-2/RRS1	RRS1_K1221Q_F	GGCGAAAGTACGGTCAACAAGACATCTTAGGTTCTCG	Site-directed mutagenesis
At Ws-2/RRS1	RRS1_K1221Q_R	CGAGAACCTAAGATGTCTTGTTGACCGTACTTTCGCC	Site-directed mutagenesis
At Col-0/RRS1B	RRS1B CDS F1	GGTCTCGAATGACCGAGAGTGAGCAAATC	Golden Gate cloning (gDNA)
At Col-0/RRS1B	RRS1B CDS R1	GGTCTCACTGGAATGGTGTACTGATGAG	Golden Gate cloning (gDNA)
At Col-0/RRS1B	RRS1B CDS F2	GGTCTCGCCAGGAAAGAATGTAGTGACTC	Golden Gate cloning (gDNA)
At Col-0/RRS1B	RRS1B CDS R2	GGTCTCAAACTGTAAATCTTGAACAATCTAAGG	Golden Gate cloning (gDNA)
At Col-0/RRS1B	RRS1B CDS F3	GGTCTCGAGTTCCAATCCTGAAGTCCATC	Golden Gate cloning (gDNA)
At Col-0/RRS1B	RRS1B CDS R3	GGTCTCATGAAAGACCTGGGATTTCAGC	Golden Gate cloning (gDNA)

At Col-0/RRS1B	RRS1B CDS F4	GGTCTCGTTCAGGTGTCTCAAACCTTGA	Golden Gate cloning (gDNA)
At Col-0/RRS1B	RRS1B CDS R4	GGTCTCATAGGCTAAGACCTTGAGCC	Golden Gate cloning (gDNA)
At Col-0/RRS1B	RRS1B CDS F5	GGTCTCGCCTATAGGTCGCTCATACGTG	Golden Gate cloning (gDNA)
At Col-0/RRS1B	RRS1B CDS R5	GGTCTCACGAATCTATTCAGAATTTTACTAGGTAAATTG	Golden Gate cloning (gDNA)
At Col-0/RRS1B	RRS1B_mod1_C12Y_F	CGTCTACATCAGCTACATAGAGGAGGTACG	Site-directed mutagenesis
At Col-0/RRS1B	RRS1B_mod1_C12Y_R	CGTACCTCCTCTATGTAGCTGATGTAGACG	Site-directed mutagenesis
At Col-0/RRS1B	RRS1B_mod1_P63L_F	GGTTTCTGTTATGATTTTACTAGGAAACCGTACGG	Site-directed mutagenesis
At Col-0/RRS1B	RRS1B_mod1_P63L_R	CCGTACGGTTTCCTAGTAAAATCATAACAGAAACC	Site-directed mutagenesis
At Col-0/RRS1B	RRS1B_mod2_G167E_F	GGACATCCGTTGTGTTGAAATTTGGGGTATGCC	Site-directed mutagenesis
At Col-0/RRS1B	RRS1B_mod2_G167E_R	GGCATACCCCAAATTTCAACACAACGGATGTCC	Site-directed mutagenesis
At Col-0/RRS1B	RRS1B_mod3_C600Y_F	GTTGAAGACAATCAGGCTTTATCATTCCCAACAACTAG	Site-directed mutagenesis
At Col-0/RRS1B	RRS1B_mod3_C600Y_R	CTAGTTGTTGGGAATGATAAAGCCTGATTGTCTTCAAC	Site-directed mutagenesis
At Col-0/RRS1B	RRS1B_mod5_C1211Y_F	CCAGCTCGAAAGGTTATTTTGCTAGGAAACAAGTC	Site-directed mutagenesis
At Col-0/RRS1B	RRS1B_mod5_C1211Y_R	GACTTGTTTCCTAGCAAAATAACCTTTCGAGCTGG	Site-directed mutagenesis

At Col-0/RRS1B	gcR1B K1191Q mutagenesis F	GGCGAAAGTATGGTCAACAACCCATCAAAAGTTCTC	Site-directed mutagenesis
At Col-0/RRS1B	gcR1B K1191Q mutagenesis R	GAGAACTTTTGATGGGTTGTTGACCATACTTTCGCC	Site-directed mutagenesis
At Col-0/RRS1B	gcR1B SH-AA (SH 22/23) mutagenesis F	CGATACTCCTTCGTCGCCGCCCTCTCCAAAGCTCTCC	Site-directed mutagenesis
At Col-0/RRS1B	gcR1B SH-AA (SH 22/23) mutagenesis R	GGAGAGCTTTGGAGAGGGCGGCGACGAAGGAGTATCG	Site-directed mutagenesis
At Ws-2/RRS1	RRS1_modEx5-7_F	GGTCTCAATGGAACTCATCAACAAAGCTCC	Golden Gate cloning (gDNA)
At Col-0/RRS1B	RRS1B_modTIR_R (166aa)	GGTCTCTCGAAAACACAACGGATGTCCAACG	Golden Gate cloning (gDNA)
At Col-0/RPS4	RPS4_S33A/H34A mut. F	CGGAGATTCGTCGCCGCTCTCGTAACGGCC	Site-directed mutagenesis
At Col-0/RPS4	RPS4_S33A/H34A mut. R	GGCCGTTACGAGAGCGGCGACGAATCTCCG	Site-directed mutagenesis
At Col-0/RPS4	RPS4_R116A mut. F	GCCATCCACCGTTGCAGATTTGAAAGGAAA	Site-directed mutagenesis
At Col-0/RPS4	RPS4_R116A mut. R	TTTCCTTTCAAATCTGCAACGGTGGATGGC	Site-directed mutagenesis
At Col-0/RPS4	RPS4_M150R mut. F	CTTGATTCCTAACATTAGGGGCATCATCAT	Site-directed mutagenesis
At Col-0/RPS4	RPS4_M150R mut. R	ATGATGATGCCCCTAATGTTAGGAATCAAG	Site-directed mutagenesis

2.3 Bacterial strains

Strain	Plasmid	Selection
Agrobacterium tumefaciens AGL1	pICH86988 : PopP2 (149-488) : YFP	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : PopP2 / C321A (149-488) : YFP	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : PopP2 / LAAL (149-488) : YFP	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : PopP2 (149-488) / LAAL - SRDX : YFP	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : PopP2 (149-488) / LAAL - srdx : YFP	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : PopP2 (149-488) / Pe_1 : YFP	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : PopP2 (149-488) / Pe_2 : YFP	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : PopP2 (149-241) / Pe_13 : YFP	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : GFP	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RPS4 : 6xHA	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RPS4 / S33AH34A : 6xHA	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RPS4 / K242A : 6xHA	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : AvrRps4 : YFP	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1-R : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1-R / SLH1 : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1-R /C15Y : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1-R / P68L : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1-R / G176E : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1-R / C607Y : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1-R / L816F : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1-R / C1243Y : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1-R / S25AH26A : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1-R / SLH1 / C15Y : 3xFlag	Amp100/Km50

Agrobacterium tumefaciens AGL1	pICH86988 : RRS1-R / SLH1 / P68L : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1-R / SLH1 / G176E : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1-R / SLH1 / C607Y : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1-R / SLH1 / L816F : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1-R / K1221Q : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1-R / K1221Q / C15Y : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1-R / K1221Q / P68L : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1-R / K1221Q / G176E : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1-R / K1221Q / C607Y : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1-R / K1221Q / L816F : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1-R / C1243Y / C15Y : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1-R / C1243Y / P68L : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1-R / C1243Y / G176E : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1-R / C1243Y / C607Y : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1-R / C1243Y / L816F : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1-R / C1243Y / S25AH26A : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1-R / Ex7Col : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1-R / SLH1 / Ex7Col : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1-R / K1221Q / Ex7Col : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1-R / C1243Y / Ex7Col : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1-R / K185A : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RPS4 (1-236) : 6xHA	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RPS4 (1-236) : YFP	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RPS4 (1-250) : GFP	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1 (1-175) : YFP	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1 (1-175) / S25AH26A : YFP	Amp100/Km50

Agrobacterium tumefaciens AGL1	pICH86988 : RRS1 (1-175) / C15Y : YFP	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1 (1-175) / P68L : YFP	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1B : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1B / S22AH23A : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1B / C12Y : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1B / P63L : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1B / G167E : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1B / C600Y : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1B / C1211Y : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1B / C1211Y / S22AH23A : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1B / C1211Y / C12Y : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1B / C1211Y / P63L : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1B / C1211Y / G167E : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1B / C1211Y / C600Y : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1B / K1191Q : 3xFlag	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1-R / Ex5-7 : 6xHA	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1-R / Ex5-7 / K1221Q : 6xHA	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1-R / Ex5-7 / SLH1 : 6xHA	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1-R / Ex5-7 / C1243Y : 6xHA	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1-R / Ex5-7 : 6xHA	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1B (1-166) : YFP	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1B (1-166) / S22AH23A : YFP	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1B (1-166) / C12Y : YFP	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RRS1B (1-166) / P63L : YFP	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RPS4 (1-236) / S33AH34A : 6xHA	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RPS4 (1-236) / R116A : 6xHA	Amp100/Km50

Agrobacterium tumefaciens AGL1	pICH86988 : RPS4 (1-236) / M150R : 6xHA	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RPS4 (1-236) / S33AH34A / R116A : 6xHA	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RPS4 (1-236) / S33AH34A / M150R : 6xHA	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RPS4 (1-236) / S33AH34A : YFP	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RPS4 (1-236) / R116A : YFP	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RPS4 (1-236) / M150R : YFP	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RPS4 (1-236) / S33AH34A / R116A : YFP	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RPS4 (1-236) / S33AH34A / M150R : YFP	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RPS4 / R116A : 6xHA	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RPS4 / M150R : 6xHA	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RPS4 / S33AH34A / R116A : 6xHA	Amp100/Km50
Agrobacterium tumefaciens AGL1	pICH86988 : RPS4 / S33AH34A / M150R : 6xHA	Amp100/Km50
Escherichia coli DH5α		
Escherichia coli HB101 (pRK2013)		Km50
Pseudomonas syringae pv. *tomato* DC3000		Rif50
Pseudomonas syringae pv. *tomato* DC3000	pBBR1MCS-5	Rif50/Gm20
Pseudomonas syringae pv. *tomato* DC3000	pEDV6 : PopP2 (149-488) : HA	Rif50/Gm20
Pseudomonas syringae pv. *tomato* DC3000	pEDV6 : PopP2 / C321A (149-488) : HA	Rif50/Gm20
Pseudomonas syringae pv. *tomato* DC3000	pEDV6 : PopP2 / LAAL (149-488) : HA	Rif50/Gm20
Pseudomonas syringae pv. *tomato* DC3000	pEDV6 : PopP2 / LAAL (149-488) - SRDX : HA	Rif50/Gm20
Pseudomonas syringae pv. *tomato* DC3000	pBBR1MCS-5 : AvrRps4N : PopP2 / Pe_1 (149-488) : 6xHA	Rif50/Gm20
Pseudomonas syringae pv. *tomato* DC3000	pBBR1MCS-5 : AvrRps4N : PopP2 / Pe_2 (149-488) : 6xHA	Rif50/Gm20

Pseudomonas syringae pv. *tomato* DC3000	pBBR1MCS-5 : AvrRps4N : PopP2 / Pe_13 (149-241) : 6xHA	Rif50/Gm20
Pseudomonas fluorescens Pf0-1 (T3S)	pEDV6 : PopP2 (149-488) : HA	Chl20/Tet5/Gm20
Pseudomonas fluorescens Pf0-1 (T3S)	pEDV6 : PopP2 / C321A (149-488) : HA	Chl20/Tet5/Gm20
Pseudomonas fluorescens Pf0-1 (T3S)	pEDV6 : PopP2 / LAAL (149-488) : HA	Chl20/Tet5/Gm20
Pseudomonas fluorescens Pf0-1 (T3S)	pEDV6 : PopP2 / LAAL (149-488) - SRDX : HA	Chl20/Tet5/Gm20
Pseudomonas fluorescens Pf0-1 (T3S)	pBBR1MCS-5	Chl20/Tet5/Gm20
Pseudomonas fluorescens Pf0-1 (T3S)	pBBR1MCS-5 : AvrRps4N : PopP2 (149-488) : 6xHA	Chl20/Tet5/Gm20
Pseudomonas fluorescens Pf0-1 (T3S)	pBBR1MCS-5 : AvrRps4N : PopP2 / C321A (149-488) : 6xHA	Chl20/Tet5/Gm20
Pseudomonas fluorescens Pf0-1 (T3S)	pBBR1MCS-5 : AvrRps4N : PopP2 / C321A (149-488) - SRDX : 6xHA	Chl20/Tet5/Gm20
Pseudomonas fluorescens Pf0-1 (T3S)	pBBR1MCS-5 : AvrRps4N : PopP2 / C321A (149-488) - srdx : 6xHA	Chl20/Tet5/Gm20
Pseudomonas fluorescens Pf0-1 (T3S)	pBBR1MCS-5 : AvrRps4N : PopP2 / LAAL (149-488) : 6xHA	Chl20/Tet5/Gm20
Pseudomonas fluorescens Pf0-1 (T3S)	pBBR1MCS-5 : AvrRps4N : PopP2 / LAAL (149-488) - SRDX : 6xHA	Chl20/Tet5/Gm20
Pseudomonas fluorescens Pf0-1 (T3S)	pBBR1MCS-5 : AvrRps4N : PopP2 / LAAL (149-488) srdx : 6xHA	Chl20/Tet5/Gm20
Saccharomyces cerevisiae EGY48 (pSH18-34) (URA3) Mat (α)	pLexA-DBD	-His/-Ura
Saccharomyces cerevisiae EGY48 (pSH18-34) (URA3) Mat (α)	pLexA-DBD : PopP2 (149-488) : 3xFlag	-His/-Ura
Saccharomyces cerevisiae EGY48 (pSH18-34) (URA3) Mat (α)	pLexA-DBD : LUG : 3xFlag	-His/-Ura
Saccharomyces cerevisiae EGY48 (pSH18-34) (URA3) Mat (α)	pLexA-DBD : RPS4 (1-236) : 3xFlag	-His/-Ura

Saccharomyces cerevisiae RFY206 Mat (a)	pB42-AD	-Trp
Saccharomyces cerevisiae RFY206 Mat (a)	pB42-AD : HOS15 : 6xHA	-Trp
Saccharomyces cerevisiae RFY206 Mat (a)	pB42-AD : LUG : 6xHA	-Trp
Saccharomyces cerevisiae RFY206 Mat (a)	pB42-AD : LUH : 6xHA	-Trp
Saccharomyces cerevisiae RFY206 Mat (a)	pB42-AD : SAP18 : 6xHA	-Trp
Saccharomyces cerevisiae RFY206 Mat (a)	pB42-AD : SEU : 6xHA	-Trp
Saccharomyces cerevisiae RFY206 Mat (a)	pB42-AD : SLK1 : 6xHA	-Trp
Saccharomyces cerevisiae RFY206 Mat (a)	pB42-AD : SLK2 : 6xHA	-Trp
Saccharomyces cerevisiae RFY206 Mat (a)	pB42-AD : TPL : 6xHA	-Trp
Saccharomyces cerevisiae RFY206 Mat (a)	pB42-AD : TPR1 : 6xHA	-Trp
Saccharomyces cerevisiae RFY206 Mat (a)	pB42-AD : TPR2 : 6xHA	-Trp
Saccharomyces cerevisiae RFY206 Mat (a)	pB42-AD : TPR3 : 6xHA	-Trp
Saccharomyces cerevisiae RFY206 Mat (a)	pB42-AD : TPR4 : 6xHA	-Trp
Saccharomyces cerevisiae RFY206 Mat (a)	pB42-AD : RRS1 (1-175) : 6xHA	-Trp
Saccharomyces cerevisiae RFY206 Mat (a)	pB42-AD : RRS1 (1-175) / S25AH26A : 6xHA	-Trp
Saccharomyces cerevisiae RFY206 Mat (a)	pB42-AD : RRS1 (1-175) / C15Y : 6xHA	-Trp
Saccharomyces cerevisiae RFY206 Mat (a)	pB42-AD : RRS1 (1-175) / P68L : 6xHA	-Trp

Amp = ampicillin, Km = kanamycin, Rif = rifampicin, Gm = gentamicin, Chl = chloramphenicol, Tet = tetracycline, His = histidine, Ura = uracil, Trp = tryptophan.

Number after antibiotic indicates final concentration used in µg/ml.

2.4 Growth conditions

Arabidopsis plants were grown in short day conditions (11h light/13h dark) at 22°C. *Nicotiana benthamiana* and *Nicotiana tabacum* (tobacco) W38 were grown in long day conditions (14h light/10h dark). After sowing, seeds were covered with a plastic lid for 3-4 days to promote germination. After 2 weeks of growth, seedlings were transferred to individual pots, and 4-5 week old plants were used for infection assays.

2.5 Cross-fertilization of Arabidopsis

Mature siliques and open flowers were removed from the maternal plant, and unopened buds were opened and emasculated (anthers were removed). From the paternal plant, open, mature flowers were taken and tapped on the stigmata of the maternal plant. Siliques were left to develop and collected once dry.

2.6 Media

All recipes are for 1 liter of medium.

2.6.1 L

10g tryptone, 5g yeast extract, 5g NaCl, 1g glucose. For solid medium, 10g of agar was added.

2.6.2 King's B

20g Bacto-peptone, 1.5g K_2HPO_4, 10ml glycerol. For solid medium, 15g agar was added.

2.6.3 Murashige-Skoog (MS)

4.4g MS powder, 10g sucrose, pH adjusted to 5.8 with KOH. For solid medium, 8g of agar was added.

2.7 Antibiotics

The final concentrations of antibiotics used for bacterial cultures were: Ampicillin - 100µg/ml; Spectinomycin - 100µg/ml; Kanamycin - 50µg/ml; Gentamicin - 20µg/ml; Tetracycline - 5µg/ml; Chloramphenicol - 30µg/ml; Rifampicin - 50µg/ml.

2.8 Plant pathology

2.8.1 Arabidopsis infection

Pseudomonas strains were streaked on King's B medium and incubated for 48 hours at 28ºC. Bacteria were scraped from the plate and re-suspended in 2ml 10mM $MgCl_2$. The optical density was measured at 600nm, and adjusted to OD_{600} = 0.001 for bacterial growth assays, and OD_{600} = 0.2 for RNA, HR or ion leakage assays. Leaves of 4-5 week old Arabidopsis plants were hand-infiltrated on the abaxial surface of the leaf using a 1ml blunt-end syringe.

2.8.1.1 Hypersensitive response (HR) assay

Macroscopic cell death symptoms were observed and photographed at 20-24 hours post-infiltration (hpi).

2.8.1.2 Ion leakage assay

Following infection with Pf0-1(T3S), leaf discs were sampled at 0.5 hpi, washed in water for 30 minutes (with gentle shaking at room temperature) and

transferred to fresh water (0 hpi sample). Ion leakage measurements were taken at 6, 12, 24, 36 and 48 hpi using a conductivity meter (Horiba B-173).

2.8.1.3 *In planta* bacterial growth assay

Leaves were sampled at 4 days post-infiltration (dpi). Samples were ground in 10mM $MgCl_2$, serially diluted and spotted on KB agar containing appropriate antibiotics. These were incubated at 28^{o}C for 2 days prior to counting colonies in order to calculate the number of colony forming units (cfu)/cm^2 of infected leaf.

2.9 Molecular biology

2.9.1 DNA

2.9.1.1 Polymerase chain reaction (PCR)

Each PCR was carried out using 50-1000ng DNA as template. Each reaction contained: 1X PCR Taq or Phusion buffer, 0.2mM dNTPs, Taq DNA polymerase (NEB) or Phusion high-fidelity DNA polymerase (NEB), and 10µM of each primer. PCR cycles (denaturation, primer annealing and elongation) were optimized for different primers and length of product, and performed in a DNA thermal cycler.

2.9.1.2 Chelex plant genomic DNA extraction

One leaf disc (0.37cm^2) was ground in 150µl 5% chelex. This was boiled at 96ºC for 10 minutes, vortexed for 20 seconds and centrifuged at maximum speed for 1 minute. 100µl of the supernatant was transferred to a fresh tube, centrifuged at maximum speed for 1 minute and 2µl of the resulting supernatant was used for PCR (HwangBo et al., 2010).

2.9.1.3 Plasmid DNA preparation

Plasmid DNA was purified from bacterial cultures using one of two methods: the Axygen Plasmid Miniprep kit or manual miniprep.

2.9.1.3.1 Manual protocol

Manual miniprep involved centrifugation of 2ml liquid bacterial culture at 2500g for 5 minutes in 2ml microcentrifuge tubes. The supernatant was discarded and the pellet was re-suspended in 200µl of solution I. 200µl of solution II was added and the tubes were inverted 4-6 times. 200µl of solution III was added the tubes were inverted 6-8 times. The tubes were centrifuged at 10000g for 10 minutes, and 600µl of supernatant was transferred to new 1.5ml microcentrifuge tubes. 400µl of isopropanol was added and the tubes were mixed by gentle inversion. The tubes were centrifuged at maximum speed for 1 minute. The supernatant was discarded and the pellet was washed with 400µl 70% ethanol; this washing step was repeated and the pellet was air-dried. The pellet was re-suspended in 30µl sterile H_2O with 1µl RNase.

2.9.1.3.2 Axygen Plasmid Miniprep kit protocol

2ml of overnight liquid bacterial culture was centrifuged at 12000g for 1 minute. The supernatant was removed using a vacuum pump and the pellet was re-suspended in 250µl of Buffer S1 by vortexing. 250µl buffer S2 was added and the tube was inverted 4-6 times. 350µl buffer S3 was added and the tube was inverted 6-8 times. The tube was centrifuged at 12000g for 10 minutes. A miniprep column was placed into an uncapped 2ml microcentrifuge tube and the supernatant was transferred into the column. This was centrifuged at 12000g for 1 minute. The filtrate was discarded and 700µl of buffer W2 was added. The tube was centrifuged at 12000g for 1 minute. A

further 250µl of buffer W2 was added and the tube was centrifuged for 2 minutes. The miniprep column was transferred to a 1.5ml microcentrifuge tube and 50µl of eluent was added to the center of the membrane, left to stand for 1 minute and then centrifuged at 12000g for 1 minute.

2.9.1.4 Electrophoresis of DNA

For separation of DNA fragments, agarose gel electrophoresis was performed. The gels containing 0.8 - 2% agarose and ethidium bromide were prepared in 1x TAE (40 mM Tris, 20 mM NAOAc, 1 mM EDTA, pH7.9). The electrophoresis was carried out in tanks containing TAE buffer. DNA samples were loaded into wells after mixing with 10x loading buffer. 1kb or 100bp ladder was also added in wells alongside the samples for estimation of fragment length. Gels were run at 120V for 25-40 minutes until the bands were sufficiently separated. The DNA fragments were then visualized under UV light in a UV transilluminator.

2.9.1.5 Purification of DNA from agarose gel

The desired DNA band was visualised on a UV plate, excised with a clean razor blade and placed in a 1.5ml microcentrifuge tube. The fragment was purified using the Thermo Scientific Gene JET PCR Purification Kit.

2.9.1.6 Ligation

Blunt ligation of DNA fragments into the pICH41021 (pUC19b) vector was carried out with 10µl of insert fragment, 1µl of vector pICH41021, 1.5µl of T4 DNA ligase buffer, 0.5µl of SmaI, 0.5µl of T4 DNA ligase and 1.5µl sterile H_2O. Ligation was carried out at room temperature for 2 hours.

2.9.1.7 Preparation of electrocompetent *Escherichia coli* (DH5alpha) and *Agrobacterium tumefaciens* (AGL1) cells

A fresh plate was streaked from glycerol stock (DH5alpha on L, AGL1 on L+Amp) and seed cultures were incubated overnight (for DH5alpha enough

for 1/100 dilution, for AGL1 enough for 1/40 dilution). Seed cultures were grown in 500ml until OD_{600} reached 0.6-0.7 (~4h for DH5alpha, ~6h for AGL1). The cells were put on ice for at least 30 minutes and then the cells were pelleted in a megacentrifuge (15 minutes at 5000rpm). The supernatant was poured off and ~20ml of cold 10% glycerol was added. The pellet was resuspended and 10% glycerol was added up to 500ml. The cells were then pelleted again (15 min at 5000rpm) and the supernatant was poured off. ~20ml cold 10% glycerol was added and at this stage the bottles were swirled to resuspend the pellet. 10% glycerol was added up to half volume (250ml) and this 250ml wash was repeated. The final wash was performed in $1/10^{th}$ of the initial volume. The supernatant was poured off and cells were resuspended in 1ml 10% glycerol. 50µl aliquots were made using a pipette and these were flash frozen in liquid nitrogen immediately.

2.9.1.8 Transformation of competent *E. coli* and *A. tumefaciens* cells

Transformation was carried out via the electroporation method. 1µl of plasmid miniprep DNA or 15µl of ligation product was added to 50µl of electrocompetent cells and this was pipetted into a pre-chilled 1mm electroporation cuvette. The cuvette was placed in the electroporator and electroporation was performed. Immediately after electroporation, transformed cells were suspended in 0.5-1ml L medium and incubated on a shaker for 45 minutes at 37ºC for *E. coli* and at 28 ºC for *A. tumefaciens*. Transformed cells were selected on L agar containing the appropriate antibiotics.

2.9.1.9 Site-directed mutagenesis

A modified protocol of the Agilent site-directed mutagenesis kit was used. Using pre-designed specific mutagenic primers, Phusion PCR was carried out with 5µl plasmid miniprep template, 1.5µl + 1.5µl of primers at 10µM, 10µl of 5X HF buffer, 1µl of dNTPs, 1µl of Phusion and up to 51µl with H_2O. 1µl of DpnI was added to 15µl of PCR product and incubated at 37ºC for 2-3 hours. This was then sepharose-purified and transformed into *E. coli*.

2.9.1.10 Colony PCR

To screen recombinant plasmids during cloning, PCR of bacterial colonies was performed. A single colony was transferred to 50µl of sterile water using a pipette tip and agitated. 2µl of the colony suspension was used as the template for Taq PCR.

2.9.1.11 DNA sequencing

Sequencing reactions were carried out by Macrogen after preparation of tubes containing 5µl of template DNA (concentration 10-50ng/µl) and 5µl of primer.

2.9.1.12 Golden Gate cloning

Golden Gate technology was used for the assembly of DNA modules into a destination vector (primarily pICH86966 or pICH86988). This was carried out using the type II restriction endonuclease BsaI and ligation of the specific 4bp overhangs by T4 DNA ligase. Equimolar amounts of each entry vector and the destination vector were mixed in a PCR tube with 2µl BSA, 2µl T4 DNA ligase buffer, 1µl BsaI, 1µl T4 DNA ligase and made up to 20µl with sterile water. In a PCR thermocycler, the program was: 25 times (37ºC for 3 minutes, 16ºC for 4 minutes), 50ºC for 5 minutes and finally 80ºC for 5 minutes. The assembly was purified with a sepharose column, transformed into *E. coli* and plated on L medium.

2.9.1.13 Triparental mating

Recipient (*P. fluorescens* Pf0-1), donor (*E. coli* DH5α carrying a broad host range vector) and helper (*E. coli* HB101 carrying pRK2013) strains were streaked out on the appropriate medium with appropriate antibiotics and incubated at 28ºC (Pf0-1) or 37ºC (*E. coli*) for 24 hours. 3ml liquid culture of each strain was grown for 18 hours on a shaking incubator at the same respective temperatures. The strains were then mixed in a 1.5ml microcentrifuge tube as follows: recipient (0.6ml), donor (0.2ml) and helper (0.2ml). This was centrifuged at 4000rpm for 3 minutes and the supernatant

was discarded. 1ml of L broth was added and the cells were washed by pipetting. Once again, the cells were centrifuged at 4000rpm for 3 minutes and the supernatant was discarded. This time, cells were re-suspended in 0.2ml L broth. 10 individual 20µl spots were made on L agar and incubated at 28°C for 6-8 hours. Cells were collected with a 1ml pipette tip, re-streaked on King's B agar containing chloramphenicol, tetracycline and gentamycin, and incubated at 30°C for up to 48 hours until colonies appeared. Cells were cultured in L broth overnight and glycerol stocks were made.

2.9.2 RNA

2.9.2.1 Total RNA extraction

Frozen plant tissue was ground in liquid nitrogen using a pestle and mortar and transferred to a 2ml microcentrifuge tube. The tube was opened under the fume hood and 1ml Tri reagent was added. The mixture was vortexed and incubated for 5 minutes at room temperature. 100µl of BCP (1-bromo 3-chloropropane) was added, and the tube was shaken vigorously for 15 seconds. The tube was incubated for 10 minutes at room temperature and then centrifuged at 12000g at 4°C. The aqueous upper layer was transferred to a fresh 1.5ml microcentrifuge tube. 0.25ml of isopropanol and 0.25ml of high salt precipitation solution (0.8M sodium citrate and 1.2M NaCl, 0.45µM filtered) was added. This was mixed well by inversion and incubated for 5 minutes at room temperature. The tubes were centrifuged for 15 minutes at 12000g at 4°C, and the supernatant was discarded. The pellet was washed with 0.7ml 70% ethanol, and this was centrifuged for 5 minutes at 8000g at 4°C. The pellet was air dried for 10-15 minutes and resuspended in 50µl DEPC-treated water. The 50µl of RNA was treated with 5µl of DNase with 5µl of DNase 10X reaction buffer, and incubated at room temperature for 15 minutes. 5µl of DNase stop solution was added and this was incubated for 10 minutes at 70°C. To assess the integrity of RNA, 2µl of RNA was loaded and ran on a 2% agarose gel for visualization of discrete rRNA bands. To assess the concentration of RNA, 1.5µl of RNA was analyzed using the Nanodrop machine.

2.9.2.2 Reverse transcription PCR (RT-PCR)

Up to 5µg of RNA was mixed with DEPC-treated water up to 14µl. 6µl of RT mix (2µl of maxima enzyme mix and 4µl of maxima reaction mix) was added, so the final reaction volume was 20µl. Using a thermocycler, PCR was performed with the following conditions: 25°C for 10 minutes, 55°C for 30 minutes, 85°C for 5 minutes. The PCR product (cDNA) was topped up to 100µl with sterile water and 2µl was used for PCR reactions.

2.9.2.3 qRT-PCR

In wells of a 96-well qRT-PCR plate, 2µl of template cDNA along with 18µl of a qPCR mix (10µl 2X ThermoSYBR Master Mix, 2µl 10µM primers mix, 6µl H_2O) was added. The foil was placed over the plate and the plate was pulsed in a centrifuge. The qPCR conditions used were as follows: UDG pretreatment: 50ºC for 2 minutes; activation: 95ºC for 5 minutes; amplification: (95ºC for 15 seconds, 60ºC for 25 seconds, 72ºC for 25 seconds) 40 times; melting: 95ºC for 5 minutes, 65ºC for 1 minute, 98ºC continuous; cool: 40ºC for 15 seconds.

2.9.3 Protein

2.9.3.1 Protein expression *in planta*

2.9.3.1.1 *Agrobacterium*-mediated transient transformation

Agrobacterium strains were streaked on fresh L medium and incubated for 48 hours at 28ºC. Single colonies were taken to inoculate 3ml L broth and incubated overnight in a shaking incubator (200rpm) at 28ºC. 2ml of the cultures were centrifuged at 5000rpm for 3 minutes and bacteria were re-suspended in *Agrobacterium* infiltration medium (10mM MgCl2 and 10mM MES adjusted to pH5.6 with KOH). Optical density was measured and adjusted to OD_{600} = 0.1 - 0.5. Leaves of 4-5 week old *Nicotiana benthamiana*

or tobacco plants were infiltrated with the bacterial suspension on the abaxial surface of the leaf using a 1ml syringe. Cell death was generally observed at 2-3 dpi after infiltration into tobacco leaves. For protein expression, *N. benthamiana* leaves were used and *Agrobacterium* strains were co-infiltrated with P19 posttranscriptional gene silencing suppressor from the tomato bushy stunt virus (at OD_{600} = 0.1) to enhance expression (Shamloul et al., 2014). P19 could not be used in tobacco as it induces a hypersensitive response; therefore, immuno-detection was only carried out in *N. benthamiana* leaves (Angel et al., 2011).

2.9.3.1.2 Arabidopsis stable transformation

Arabidopsis stable transformants were generated using the floral dip method. *Agrobacterium* AGL1 carrying the desired vector was streaked on L agar and incubated at 28°C for 48 hours. 1ml of L broth was pipetted onto the plate and the bacteria was harvested and used to inoculate 250ml of L. This was incubated at 28°C for 36 hours and then chilled on ice for 30 minutes. The culture was centrifuged for 15 minutes at 5000rpm and resuspended in equal volume 5% sucrose. The optical density at 600nm was measured and adjusted to OD_{600} = 0.8-1 in 400ml 5% sucrose. 160µl Pulse (Silwet L-77) was added and mixed by inversion. Flowering Arabidopsis were dipped in solution and agitated for 2 minutes. Plants were covered with a plastic autoclave bag for 1 day before this was removed and then left to set seed.

2.9.3.2 Total protein extraction from plant tissue and Western blot

Plant protein samples were prepared from *N. benthamiana* 48 h after *Agrobacterium*-mediated transformation. Frozen plant tissue was ground with a pre-cooled pestle and mortar and mixed with an equal volume of GTEN (10% glycerol, 150 mM Tris-HCl pH 7.5, 1mM EDTA, 150 mM NaCl) extraction buffer supplemented with 5mM DTT, plant protease inhibitor tablet (1 tablet / 50 ml extraction solution) (Sigma) 0.2% (vol / vol) Nonpidet P-40 detergent. The mixture was vortexed and centrifuged for 15 minutes at 5000 rpm at 4°C. The supernatant was transferred to a new tube with SDS loading

buffer and DTT. Proteins were separated using SDS-PAGE, electroblotted onto a PVDF membrane and probed with appropriate antibodies. Proteins were detected with a mix of SuperSignal West Pico and SuperSignal West Femto chemiluminescent substrates (Pierce). Membranes were stained with Ponceau S to visualize protein loading.

2.9.3.3 Co-immunoprecipitation (CoIP) assay

Protein extracts were mixed with agarose antibody beads and GTEN buffer in 1.5ml microcentrifuge tubes and incubated on a rotator for 2 hours at 4°C. The CoIP tubes were centrifuged 3 times at 7000rpm for 5 seconds and the supernatant was discarded. The beads were washed with GTEN and centrifuged back down 3 times. 5X SDS loading buffer was added and the samples were boiled for 10 minutes at 96°C. The immunoprecipitated samples were centrifuged at maximum speed for 1 minute and then analyzed by SDS-PAGE and immunoblotting as in 2.7.3.2.

2.10 Yeast-two-hybrid (Y2H) assays

The *Saccharomyces cerevisiae* strains used for the Y2H assays were EGY48 Mat(α) and RFY206 Mat(a). RFY206 carries the pSH18-34 vector, which encodes the lacZ reporter gene under the control of 8 upstream LexA operators. Additionally, pSH18-34 encodes the URA3 selectable marker, allowing growth on media lacking uracil. EGY48 and RFY206(pSH18-34) were transformed with pB42-AD and pLexA-DBD constructs, respectively, using the 'Frozen-EZ Yeast Transformation II Kit' according to the manufacturer's recommendations (Zymo Research). pB42-AD encodes the TRP1 selectable marker, which allows yeast growth on media lacking tryptophan (Trp), pLexA encodes the HIS3 selectable marker, allowing growth on media lacking histidine (His). After transformation of yeast with the appropriate constructs, mating and interaction assays were performed as described in the Yeast Protocols Handbook (Clontech).

CHAPTER 3: A conserved EAR motif is required for avirulence and stability of the *Ralstonia solanacearum* effector PopP2 *in planta*

3.1 Introduction

The soil-borne pathogen *Ralstonia solanacearum* is the cause of devastating bacterial wilt in a wide range of host species including agronomically important *Solanaceae* species. Due to the wide genetic and host range diversity of strains, the concept of an *R. solanacearum* species complex (RSSC) is now generally accepted (Genin and Denny, 2012). The first RSSC sequenced strain, GMI1000, belongs to phylotype I isolated from tomato plants (Salanoubat et al., 2002). GMI1000 harbors around 70 predicted type 3 effectors (T3Es), also termed Rips (*Ralstonia* injected proteins), which are secreted into host cells to promote infection and enable bacterial growth (Mukaihara et al., 2010; Peeters et al., 2013). The *R. solanacearum* T3E repertoire is extensive when compared to other bacterial pathogens such as *Xanthomonas* spp. and *Pseudomonas syringae*, which both possess 30-40 T3Es (Alfano and Collmer, 2004; Büttner and Bonas, 2010). Moreover, further analysis of all sequenced *R. solanacearum* strains revealed that there is a large number of conserved core effectors (>30) (Genin and Denny, 2012). This suggests that the common ancestor already possessed a large arsenal of T3Es.

One of the primary roles for the T3Es is to dampen or suppress host defense responses. These responses are initially induced by the recognition of conserved microbial features termed pathogen/microbe-associated molecular patterns (PAMPs/MAMPs), such as flagellin, the building block of the bacterial flagellum or peptidoglycan from the bacterial envelope (Felix et al., 1999; Gust et al., 2007). Activation of the host pattern-recognition receptors (PRRs) by these molecules leads to pattern-triggered immunity (PTI), an efficient defense response impeding pathogen growth (Zipfel et al., 2004; Jones and Dangl, 2006). Consequently, numerous T3Es have been shown to target and inhibit components of the PTI signaling pathway, restoring susceptibility in the host plant (Macho and Zipfel, 2015). In turn, plants have evolved an

intracellular set of immune receptors belonging to the nucleotide-binding leucine-rich repeat resistance (NLR) protein family that can detect corresponding T3Es and activate effector-triggered immunity (ETI) (Jones et al., 2016). The T3Es that activate ETI are termed avirulence (Avr) proteins. ETI is often associated with a strong programmed cell death of the infected cells, the hypersensitive response (HR), which participates in pathogen growth restriction (Dodds and Rathjen, 2010).

At least 7 of the ~70 Rips trigger HR in *R. solanacearum* host species. The first identified Rip Avr protein, AvrA, triggers HR in *Nicotiana tabacum* (tobacco) (Carney and Denny, 1990; Robertson et al., 2004). PopP1, Awr2, Awr5 and RipTPS also trigger HR in tobacco leaf cells (Poueymiro et al., 2009; Solé et al., 2012; Poueymiro et al., 2014). Awr2 and Awr5 trigger HR in other *Nicotiana* species, while PopP1 also acts as an avirulence gene in a *Petunia* line (Lavie et al., 2002; Solé et al., 2012). In wild eggplant (*Solanum torvum*), the putative Zn-dependent protease, RipAX2 (formerly Rip36), induces a strong HR (Peeters et al., 2013; Nahar et al., 2014). Finally, the well-characterized acetyltransferase effector, PopP2, is one of two sequence-unrelated effectors that are recognized in the model plant species Arabidopsis by the paired NLRs RPS4 and RRS1-R (Deslandes et al., 2002; Deslandes et al., 2003; Narusaka et al., 2009). Besides PopP2 being the sole *R. solanacearum* effector recognized in Arabidopsis so far, the system allowing for its recognition is of particular interest due to the unusual structure of the RRS1-R receptor that harbors a WRKY-DNA binding domain (Deslandes et al., 2002). Multiple WRKY transcription factors (TFs) are involved notably in wound or defense response (Eulgem and Somssich, 2007). Of note, in the Arabidopsis ecotype Col-0, a shorter form of the RRS1 protein caused by a premature stop codon after the WRKY domain loses the ability to recognize PopP2 and is therefore denoted as RRS1-S (Deslandes et al., 2002). For the clarity of this report, *RRS1-R* (Ws-2), the allele conferring PopP2 recognition, is referred to as *RRS1*. Importantly, as a result of the conservation of immune signaling in plants, transgenic tomato expressing *RPS4* and *RRS1* from Arabidopsis are resistant to infection by *R. solanacearum* RS1002 strain that carries *popP2* (Narusaka et al., 2013). Considering the large plasticity of the

R. solanacearum effector repertoire across the species complex and geographic regions, investigation into PopP2 contribution to virulence and the structural requirement for its recognition by the RPS4/RRS1 complex is essential to envisage the deployment of these *R* genes in crop species (Dangl et al., 2013; Peeters et al., 2013).

PopP2 belongs to the YopJ-like family of cysteine proteases, which share conserved catalytic triad residues (histidine (H), aspartate (D)/glutamate (E), cysteine (C)). However, several YopJ-like effectors from mammals and plant pathogens can modify their host target by trans-acetylation rather than proteolytic activity (Ma and Ma, 2016). Indeed, PopP2 exhibits acetyltransferase activity, which is fully dependent on the catalytic cysteine residue, C321. Auto-acetylation of a lysine residue (K383) is required for the trans-acetylation activity of PopP2 and RPS4/RRS1-mediated recognition (Tasset et al., 2010). PopP2 co-localizes with RRS1 to the plant cell nucleus; however, the N-terminal 148 amino acids of PopP2 that include a putative nuclear localization signal (NLS) are dispensable for nuclear localization and avirulence (Deslandes et al., 2003; Sohn et al., 2014). Two recent studies brought evidence that PopP2 specifically targets the WRKY domain of RRS1. Acetylation of a key lysine residue in the RRS1 WRKY domain results in dissociation from the DNA and RPS4-dependent ETI. The RRS1 WRKY domain hence acts as a decoy to trap PopP2 activity, which may otherwise target WRKY transcription factors (TFs) to disable plant defense (Le Roux et al., 2015; Sarris et al., 2015).

PopP2, though not belonging *per se* to the core-effector repertoire, contributes significantly to *R. solanacearum* virulence when present (Macho et al., 2010). This contribution to virulence could now be attributed to the ability of PopP2 to acetylate multiple host WRKY TFs probably resulting in their dissociation from DNA (Le Roux et al., 2015). WRKY TFs are integral for the regulation of plant innate immunity and are implicated in PTI, ETI and systemic acquired resistance (SAR) responses (Eulgem and Somssich, 2007; Rushton et al., 2010). PopP2-mediated WRKY TF acetylation has been shown to abrogate PAMP-triggered immunity (PTI) and contribute to *R.*

solanacearum virulence (Le Roux et al., 2015). Thus, one can infer that the virulence function of the nuclear localized PopP2 is to manipulate host defense gene transcription via inhibition of WRKY TF DNA binding.

WRKY TFs play roles as both activators and repressors in plant immune signaling (Xu et al., 2006). Transcriptional repression is mainly achieved by chromatin modification at different levels (Berger, 2007). Transcriptional repressors can interact with co-repressors that recruit histone deacetylase for epigenetic silencing of gene expression (Long et al., 2006; Krogan et al., 2012). Direct interaction between repressor and co-repressor is mediated by the ethylene-responsive element binding factor-associated amphiphilic repression (EAR) motif (Song and Galbraith, 2006; Szemenyei et al., 2008). For instance, the co-repressor TOPLESS (TPL) is involved in the regulation of jasmonic acid (JA) signaling. Jasmonate ZIM-domain (JAZ) proteins function as transcriptional repressors of JA-regulated genes (Santner and Estelle, 2007). The majority of JAZ proteins directly bind to an adapter protein, Novel Interactor of JAZ (NINJA), which possesses an EAR motif and recruits TPL to epigenetically silence gene transcription via the histone deacetylase HDA19 (Pauwels et al., 2010; Pauwels and Goossens, 2011). TPL and TOPLESS-RELATED (TPRs) belong to the Groucho (Gro)/Tup1-like family of co-repressors encompassing 13 members in Arabidopsis (Liu and Karmarkar, 2008). Similar to TPL involvement in JA regulation of gene expression, TPRs play a role in the repression of negative regulators of defense during infection (Zhu et al., 2010). Although the recruitment of co-repressors for the repressor activity of some WRKY TFs has not been demonstrated yet, it is interesting to note that three members of the WRKY family contain an EAR motif (Kagale et al., 2010).

To gain further insights into the surveillance system that monitors virulence activity in the plant cell, the natural sequence variation of PopP2 in *R. solanacearum* strains isolated from diseased tomato and pepper fields across Republic of Korea was analyzed. It was found that the sequence is highly conserved and, among several, only one PopP2 allele lacked avirulence activity. This indicates that RPS4/RRS1-mediated recognition can tolerate

multiple natural polymorphisms in PopP2. Furthermore, a conserved EAR motif was identified in PopP2, which was required for *in planta* recognition, PTI suppression and protein accumulation. Besides providing valuable insight into the natural variation of PopP2 in virulent *R. solanacearum* strains, this study also unveils a novel mechanism by which a pathogenic effector could maintain its stability in the host cell.

3.2 Results

3.2.1 PopP2 is highly conserved among Korean *R. solanacearum* isolates and harbors a putative transcriptional repressor motif

In order to survey naturally occurring sequence variation in PopP2, 20 *R. solanacearum* strains isolated from commercially grown pepper or tomato plants showing wilting symptoms in the Republic of Korea were selected, on the basis of their geographic location, the host plant they were collected from (Pepper, strains 'Pe_' and Tomato, strains 'To_') and the year of collection (Table 3.1 & Figure 3.1). Using gene-specific primers, the presence of *popP2* was confirmed in 17 of the 20 *R. solanacearum* strains (Table 3.1). The genomic sequence encoding the C-terminal region of PopP2 that is necessary and sufficient for avirulence in Arabidopsis, amino acids 149-488, was analyzed in the 17 *popP2*-harboring strains and compared to the GMI1000 reference (Salanoubat et al., 2002; Sohn et al., 2014). 11 strains (Pe_2, Pe_3, Pe_18, Pe_24, Pe_27, Pe_42, Pe_45, Pe_56, To_1, To_7 and To_42) harbored 4 SNPs resulting in the following amino acid residue changes: G156D, S288N, G396E and V465M. These 11 strains also harbored 4 synonymous mutations at A169, A186, V291 and V406. 5 other strains (Pe_1, Pe_26, Pe_28, Pe_40 and To_63) harbored the 4 aforementioned non-synonymous SNPs as well as 2 additional SNPs resulting in S167C and Q179R. These 5 isolates all harbored the same aforementioned synonymous SNPs except for the mutation at A169. Finally, the Pe_13 strain harbored G156D, S167C and Q179R as well as the synonymous mutation at A186.

Table 3.1. Origin of the isolated *Ralstonia solanacearum* strains. List of the strains used in this study, including host and year of isolation. *popP2* presence as assessed by PCR amplification with specific primers is indicated by "+" and *popP2* absence is indicated by "-".

Name	Number	Host	Year of isolation	*popP2* presence
Pe_1	YKB3030	Pepper	2000	+
Pe_2	YKB3033	Pepper	2000	+
Pe_3	YKB3078	Pepper	2001	+
Pe_4	YKB4598	Pepper	2001	-
Pe_13	YKB5438	Pepper	2002	+
Pe_18	YKB5445	Pepper	2002	+
Pe_24	YKB5458	Pepper	2002	+
Pe_26	YKB5774	Pepper	2003	+
Pe_27	YKB5778	Pepper	2003	+
Pe_28	YKB5861	Pepper	1999	+
Pe_40	YKB6924	Pepper	2005	+
Pe_42	YKB6953	Pepper	2005	+
Pe_45	YKB7024	Pepper	2005	+
Pe_56	YKB7141	Pepper	2005	+
Pe_57	YKB7171	Pepper	2005	-
To_1	YKB9153	Tomato	2008	+
To_7	YKB9174	Tomato	2008	+
To_42	YKB9246	Tomato	2008	+
To_52	YKB9258	Tomato	2008	-
To_63	YKB9274	Tomato	2008	+

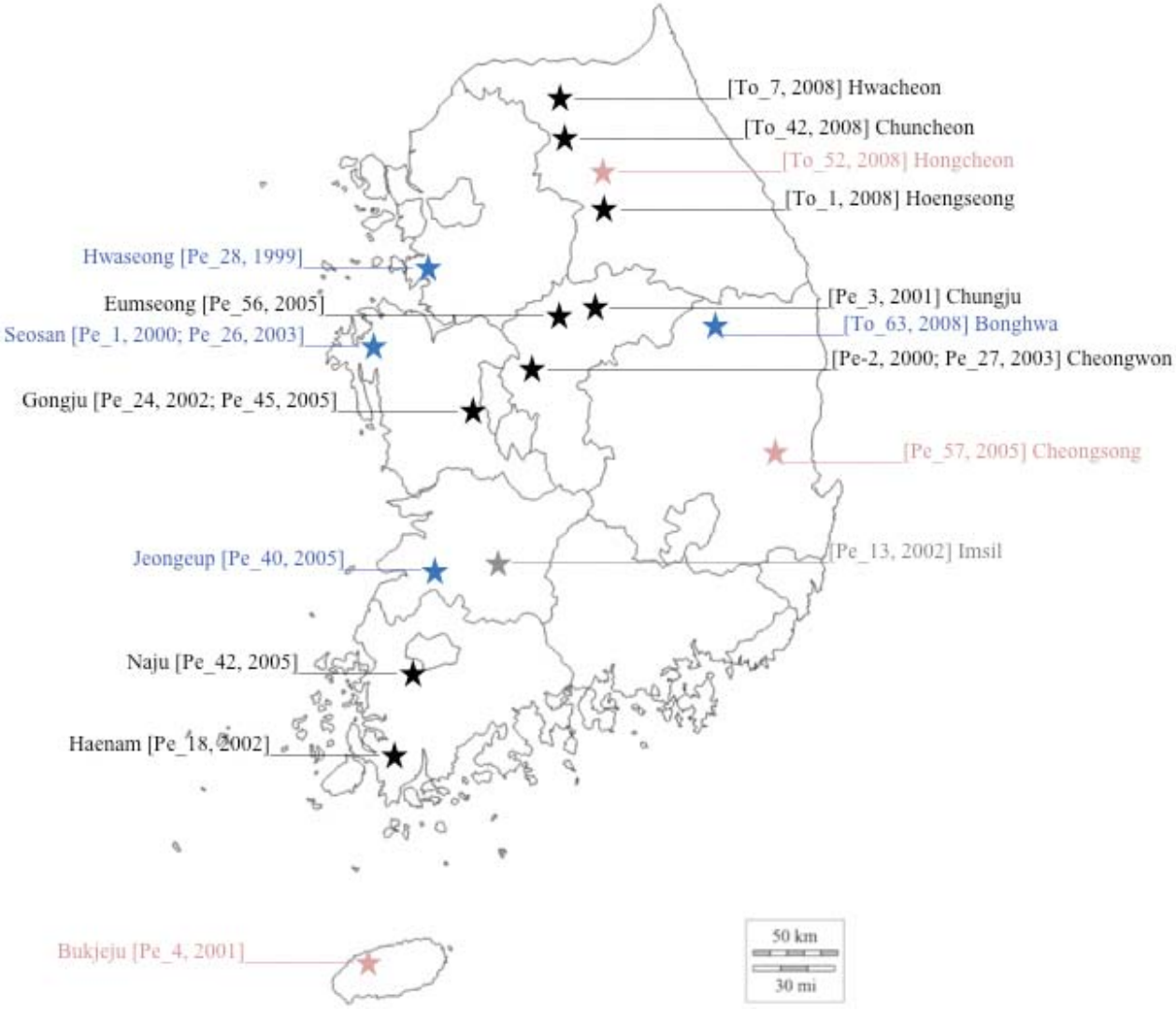


Figure 3.1. Regional map of the Republic of Korea with location of strain isolation. Strains labelled in pink did not harbor *popP2* (Pe_4, Pe_57, To_52). Strains labelled in blue harbored the “Pe_1” allele (Pe_1, Pe_26, Pe_28, Pe_40 and To_63). Strains labelled in black harbored the “Pe_2” allele (Pe_2, Pe_3, Pe_18, Pe_24, Pe_27, Pe_45, Pe_56, To_1, To_7 and To_42). Pe_13 is labelled in grey.

Cecile Segonzac produced this figure.

In addition to this, Pe_13 harbored a SNP resulting in A234G and a single nucleotide insertion, which resulted in a premature stop codon, E241* (Figure 3.2). Therefore, the survey identified three novel polymorphic PopP2 variant groups, which were termed PopP2^{Pe_2} (4 non-synonymous SNPs, present in 11 strains), PopP2^{Pe_1} (6 non-synonymous SNPs, present in 5 strains) and PopP2^{Pe_13} (4 non-synonymous SNPs and a frameshift insertion, present in only one of the selected strains) (Figure 3.2). The majority of strains analyzed here (11 of 17) harboring the "Pe_2" *popP2* allele were isolated from regions spanning the length of the Republic of Korea from Hwacheon in the north down to the southern coastal region, Haenam (Figure 3.1). Among the 5 strains harboring the "Pe_1" allele, 4 were isolated from pepper fields in western regions; the other was isolated from tomato in eastern Bongwha. The Pe_13 strain carrying the truncated PopP2 variant was isolated from Imsil in the south-west (Figure 3.1). The specific host cultivar genotypes are unknown. Thus, no obvious correlation between the host or the location of the isolated strains and the presence of a specific *popP2* allele could be revealed by this survey.

Nonetheless, closer analysis of the PopP2 coding sequence highlighted the presence of a putative LxLxL EAR motif. Characterized EAR motifs comprise three leucine residues with amino acid spacers; however, PopP2 possesses an additional fourth leucine residue (LSLSLAL) at amino acids 324-330, which is almost adjacent to C321, one of the three conserved catalytic residues, H260, D279 and C321 (Tasset et al., 2010). Similar to the catalytic residues, the putative EAR motif is fully conserved in all the sequenced PopP2 variants (Figure 3.2).

3.2.2 Only one of the newly identified PopP2 variants loses avirulence function *in planta.*

To investigate the effect of natural polymorphism on the avirulence activity of PopP2, the three newly identified PopP2 variants lacking N-terminal region were translationally fused with AvrRps4 N-terminal domain (AvrRps4N) and

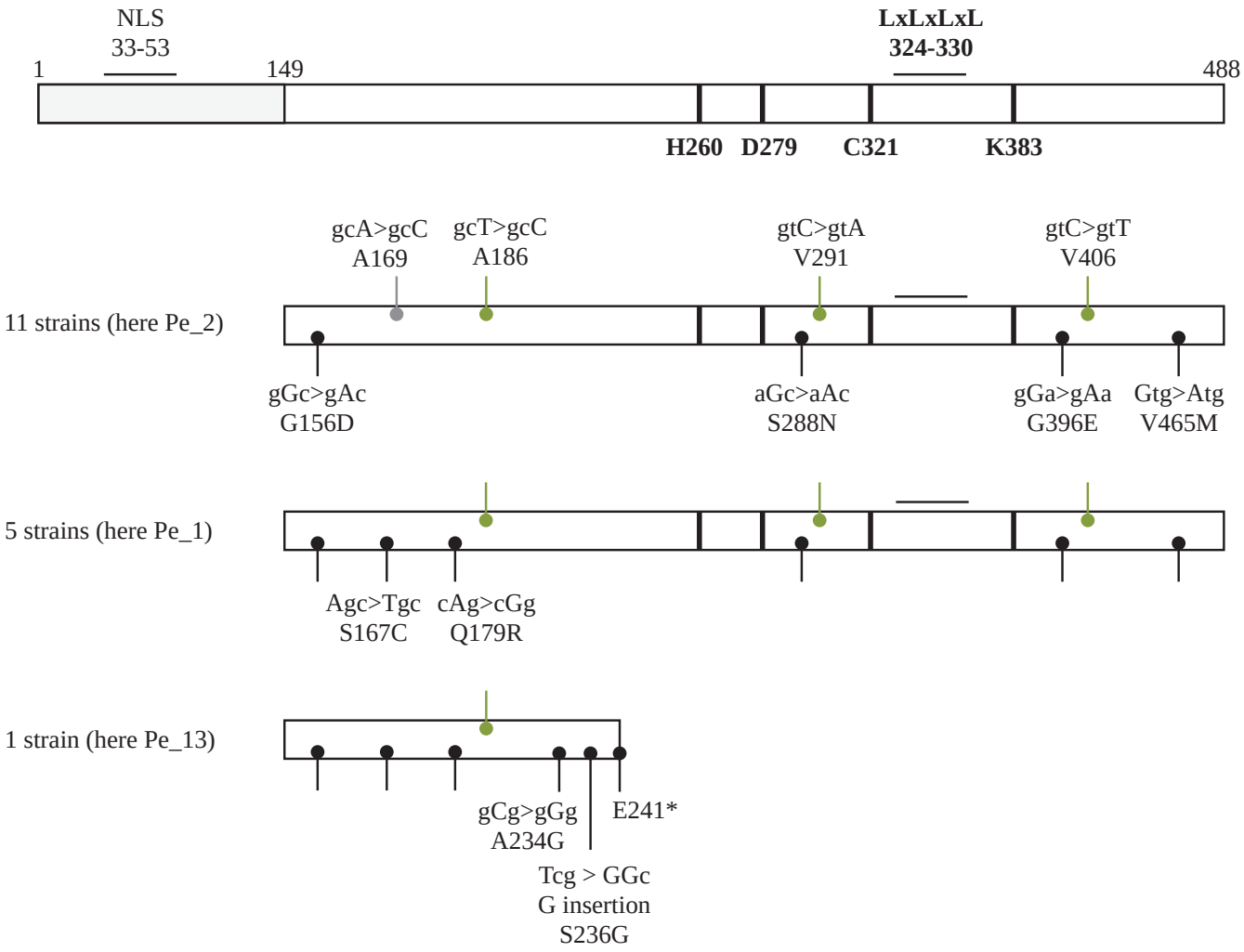


Figure 3.2. Natural variants and conserved residues of the PopP2 effector. A schematic of PopP2 sequence displaying natural variation and known or putative functional residues/motifs. GMI1000 PopP2 was used as the reference sequence. Labels in black show non-synonymous mutations and labels in green show synonymous mutations. See Table 3.1 for further details about the strains.

Gayoung Jung amplified *popP2* and sent samples for sequencing; Cecile Segonzac helped to produce the figure.

delivered by the *P. syringae* pv. *tomato* (*Pto*) DC3000 type 3 secretion system into the resistant Ws-2 Arabidopsis accession, carrying functional RPS4 and RRS1-R, to assay for HR (Sohn et al., 2014). At 1 day post-infiltration (dpi) *Pto* DC3000-delivered AvrRps4N:PopP2^{Pe_1} and AvrRps4N:PopP2^{Pe_2} variants triggered a strong HR (Figure 3.3A). Conversely, the truncated AvrRps4N:PopP2^{Pe_13} could not trigger HR in Arabidopsis. This lack of avirulence activity was expected as the catalytic residues required for acetyltransferase activity and RPS4/RRS1-mediated recognition are absent in this variant due to a premature stop codon (Figure 3.3A) (Tasset et al., 2010; Sohn et al., 2014). Similar recognition events were observed when PopP2 natural variants were co-expressed with RRS1 and RPS4 in tobacco leaf cells after *Agrobacterium*-mediated transient transformation (hereafter, agroinfiltration) (Figure 3.3B). Expression of all three protein variants was confirmed by immuno-detection in *Nicotiana benthamiana* leaf extracts (Figure 3.3C). Notably, none of the six identified SNPs present in the avirulent PopP2^{Pe_1} allele affected recognition by RPS4/RRS1 despite the close proximity of two polymorphisms to catalytic residues (S288N and G396E). This suggests that these polymorphisms do not impair PopP2 acetyltransferase activity and that RPS4/RRS1-mediated recognition can accommodate significant variation in the PopP2 sequence.

3.2.3 The conserved EAR motif is required for PopP2 avirulence activity in Arabidopsis.

To test if PopP2$^{149\text{-}488}$ (hereafter referred to as PopP2) requires an LxLxL motif (hereafter; EAR motif) for avirulence function, site-directed mutagenesis was performed on the PopP2 LxLxLxL sequence to generate the LxAxAxL variant (hereafter, PopP2^{LAAL}). Mutation of the two central leucine residues ensured that both LxLxL sequences were disrupted. Additionally, a synthetic EAR motif, SRDX (LDLDLELRLGFA, derived from the SUPERMAN repressor domain) or a mutated version of the artificial EAR motif, srdx (FDFDFEFRLGFA), was fused to the C-terminus of PopP2^{C321A} and PopP2^{LAAL} to test the specificity of EAR motif function (Hiratsu et al., 2003).

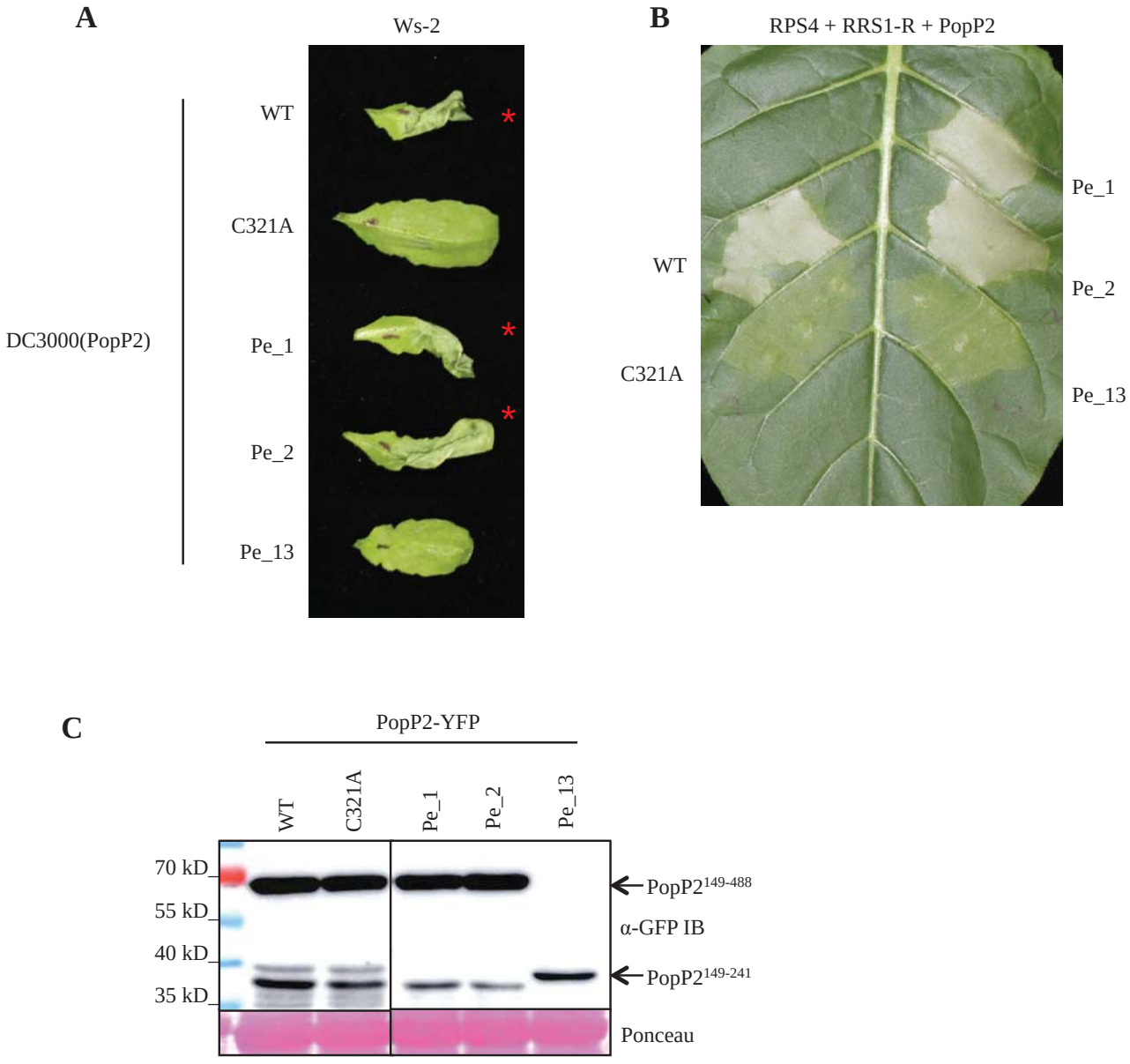


Figure 3.3. PopP2^{Pe_1} and PopP2^{Pe_2} are avirulent in Arabidopsis, PopP2^{Pe_13} is not. (A) PopP2 natural variants, PopP2^{Pe_1} and PopP2^{Pe_2}, elicit an HR in resistant Arabidopsis accession Ws-2. The truncated natural variant, PopP2^{Pe_13} is not recognized. PopP2 variants were delivered by *P. syringae* pv. *tomato (Pto)* DC3000 and photographs were taken 1 day post-infiltration (1dpi). Red asterisks indicate HR. This experiment was conducted three times with similar results. (B) *Agrobacterium*-mediated co-expression of PopP2 variants with RPS4 and RRS1-R in tobacco leaf cells. Red asterisks indicate strong programmed cell death (PCD) at 3 dpi. This experiment was conducted 3 times with similar results. (C) The PopP2 natural variant proteins accumulate to a similar amount in *N. benthamiana* after agro-infiltration. Immuno-detection of PopP2 variants C-terminally fused to YFP epitope tag was conducted using anti-GFP antibodies. Ponceau S staining of total protein demonstrates equal loading of the samples.

Sera Choi conducted the experiment for Figure 3.3B (I conducted another repeat).

To assay for HR in Arabidopsis, a modified *Pseudomonas fluorescens* strain, Pf0-1, which carries a functional type 3 secretion system (hereafter Pf0-1(T3S)) was used for delivery of the PopP2 variants (Thomas et al., 2009). As was previously reported, AvrRps4N:PopP2 triggered HR at 24 hours post-infection (hpi) in the resistant Ws-2 accession, but not in the susceptible Col-0 accession when delivered by Pf0-1(T3S) (Sohn et al., 2014). The catalytic cysteine mutant, PopP2^{C321A}, was unable to trigger HR in Ws-2 due to the loss of acetyltransferase activity required for RPS4/RRS1-mediated recognition, and this was unaffected by fusion of C-terminal SRDX or srdx (Figure 3.4) (Tasset et al., 2010; Sohn et al., 2014). Interestingly, the PopP2^{LAAL} variant with a disrupted EAR motif also failed to elicit HR in Ws-2. This suggests that PopP2 requires a functional EAR motif to trigger RPS4/RRS1-mediated HR. Indeed, fusion of the SRDX motif to the C-terminus of PopP2^{LAAL} partially restored the ability of PopP2 to trigger HR in Ws-2, suggesting that PopP2-triggered HR is dependent on a functional EAR motif (Figure 3.4, Figure 3.5A). Fusion of the mutated artificial EAR motif, srdx, to PopP2^{LAAL} had no effect as expected (Figure 3.4). PopP2 variants (WT, C321A and LAAL) were delivered *in planta* as demonstrated by a secretion assay in *N. benthamiana* leaves (Figure 3.6). In addition, ion leakage was measured to quantify the macroscopic HR symptoms and found that PopP2^{LAAL} induced ion leakage to the same extent as the negative control, PopP2^{C321A}. In agreement with the HR data, PopP2$^{LAAL\text{-}SRDX}$ induced more ion leakage than PopP2^{LAAL} to a level intermediate between PopP2^{LAAL} and PopP2^{WT} (Figure 3.5B).

It became apparent that the PopP2 EAR motif was required to trigger HR in Arabidopsis. However, HR does not always correlate with the defense response leading to immune transcriptional reprogramming (Yu et al., 1998; Cawly et al., 2005; Gassmann, 2005). Therefore, the requirement of this motif for the induction of defense-related genes and disease resistance was also investigated. To this end, expression of multiple defense marker genes known to be upregulated by bacterial T3S-delivered PopP2 were analyzed: *PR1*, *FMO1*, *PBS3* and *SARD1* (Sohn et al., 2014).

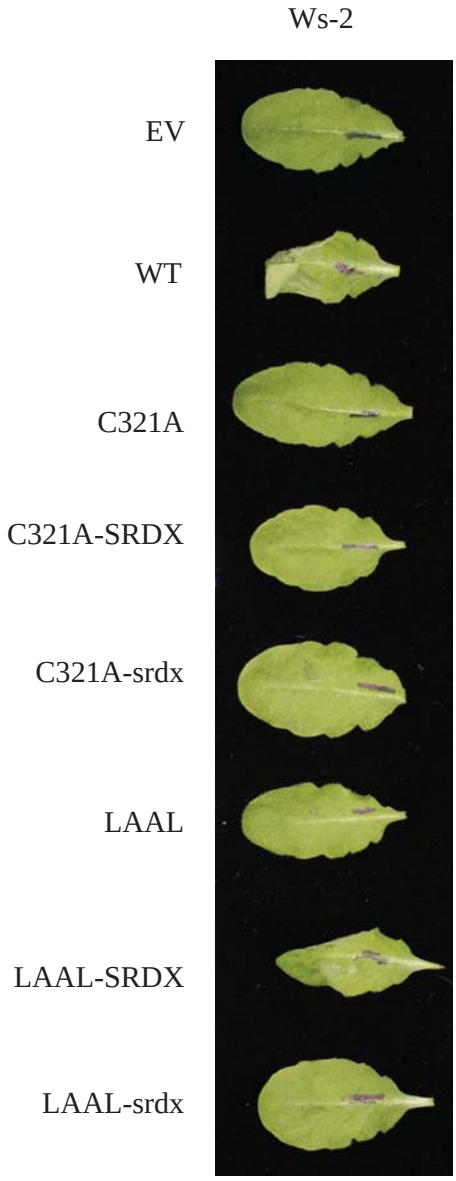


Figure 3.4. The SRDX motif partially restores PopP2^{LAAL} avirulence activity in Arabidopsis. PopP2 variants C-terminally tagged with 6xHA were delivered by *Pseudomonas fluorescens* Pf0-1(T3S) into Ws-2 leaves and photographs were taken 1 day after infiltration. A red asterisk indicates visible cell death. This experiment was conducted 3 times with similar results.

Sera Choi conducted this experiment.

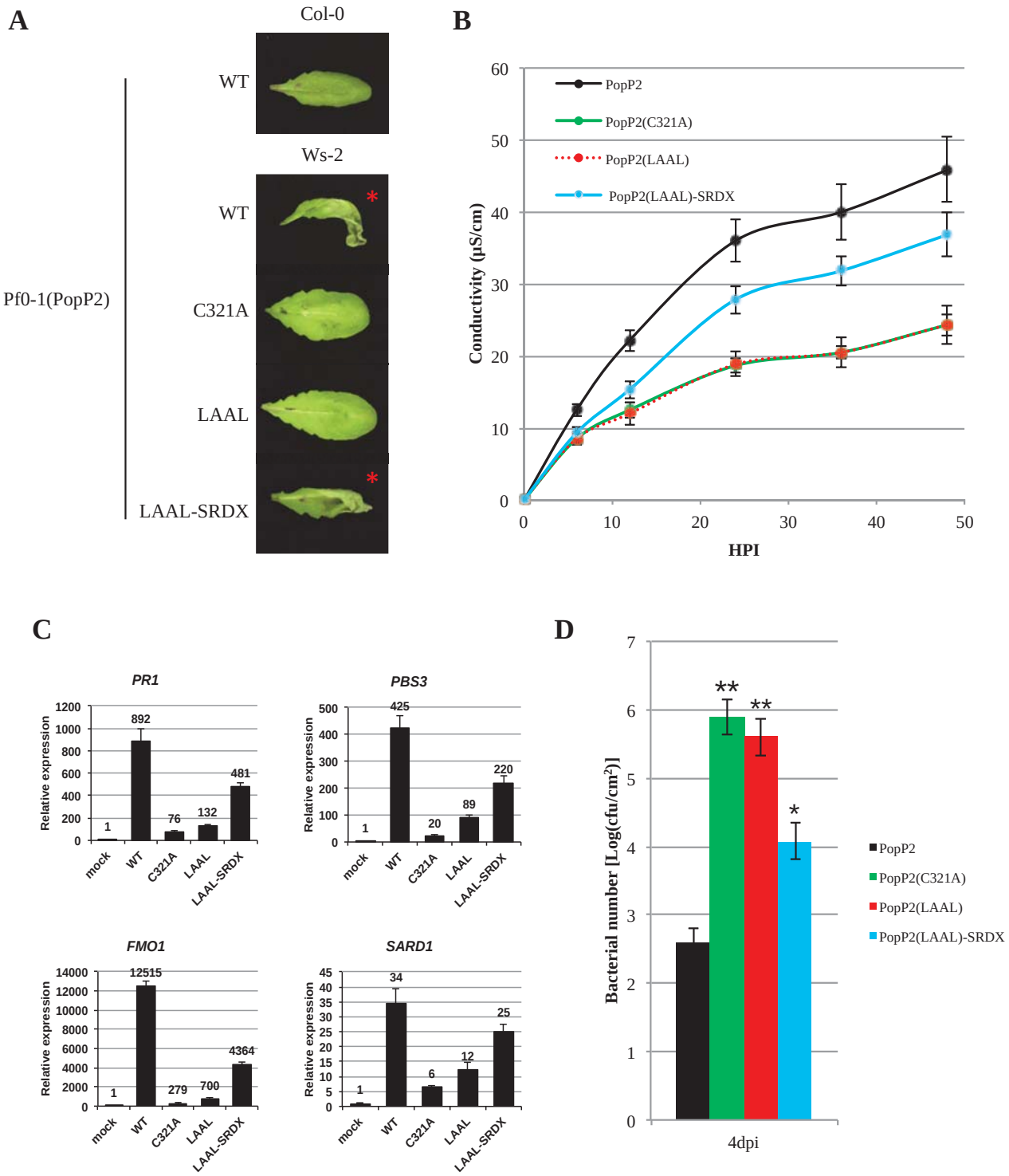


Figure 3.5. The EAR motif is required for PopP2 avirulence activity in Arabidopsis. (A) PopP2 EAR motif is required for HR elicitation in a resistant Arabidopsis accession (Ws-2). PopP2 variants were delivered by *P. fluorescens* Pf0-1(T3S) and photographs were taken 1 day after infiltration. This experiment was conducted 3 times with similar results. (B) PopP2 EAR motif is required for ion leakage in Ws-2. Data are means ± S.E. (*n* = 6) of one representative experiment. This experiment was conducted 3 times with similar results. PopP2 refers to PopP2(WT) (C) PopP2 EAR motif is required for the upregulation of defense marker genes *PR1*, *PBS3*, *FMO1* and *SARD1*. Values shown are the average of values obtained in 3 independent experiments ± S.E. (D) PopP2 EAR motif is required for *Pto* DC3000 growth restriction. Data are shown as mean colony-forming units (cfu).cm^{-2} ± S.E. (*n* = 5). Asterisks indicate statistically significant differences from *Pto* DC3000(PopP2) growth (* P<0.01, ** P<0.001). PopP2 refers to PopP2(WT). This experiment was conducted 3 times with similar results.

Cecile Segonzac conducted the experiment for Figure 3.5C

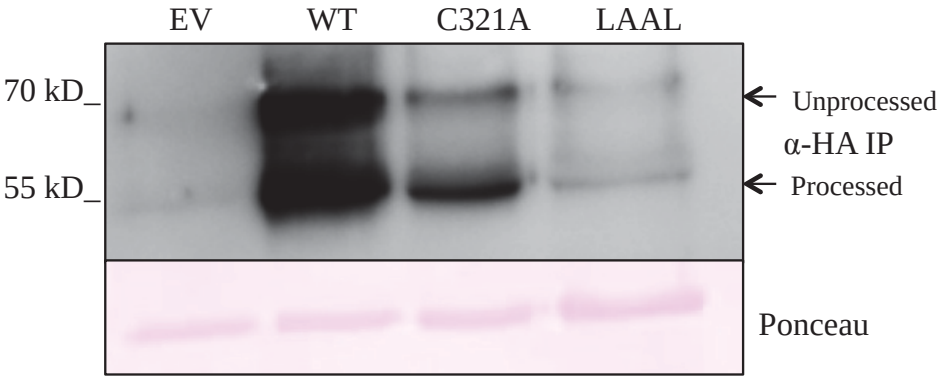


Figure 3.6. *In planta* processing of *Pto* DC3000-delivered AvrRps4N:PopP2:6xHA variants in *N. benthamiana*. The AvrRps4N:PopP2 variants C-terminally tagged with 6xHA were delivered by *Pto* DC3000 ($1x10^9$ cfu/ml) in *N. benthamiana*. *N. benthamiana* leaf samples were harvested at 10 hours post infiltration. Protein accumulation of PopP2 variants was confirmed by immunoprecipitation followed by immunoblot analysis using α-HA antibodies. Protein loading was confirmed with Ponceau staining. IP = immunoprecipitation.

Sera Choi conducted this experiment.

The Arabidopsis accession Ws-2 was infiltrated with Pf0-1(T3S) carrying PopP2 variants and samples were taken 8 hpi for RNA extraction and qRT-PCR analysis. Consistent with the loss of HR induction by $PopP2^{LAAL}$, the upregulation of all four defense genes was significantly impaired by mutation of the EAR motif. Additionally, fusion of the SRDX motif partially restored defense gene upregulation to a level more similar to $PopP2^{WT}$ (Figure 3.5C). To further test the requirement of the EAR motif for PopP2 avirulence function, bacterial growth of *Pto* DC3000 carrying PopP2 variants was assayed in Arabidopsis (Sohn et al., 2014). As expected from previous findings, *Pto* DC3000($PopP2^{LAAL}$) did not exhibit the growth restriction that $PopP2^{WT}$ did, and grew to a number comparable to *Pto* DC3000 ($PopP2^{C321A}$) (Figure 3.5D). Furthermore, *Pto* DC3000 ($PopP2^{LAAL-SRDX}$) growth was partially restricted, corroborating the other evidence that PopP2 recognition *in planta* is dependent on a functional EAR motif. Overall, the PopP2 EAR motif was demonstrated to be required for HR elicitation, upregulation of defense

marker genes and bacterial growth restriction. The specificity of these effects of PopP2 EAR motif was further confirmed by the gain of avirulence observed with PopP2^{LAAL} fused to a C-terminal synthetic EAR motif.

3.2.4 The conserved EAR motif is required for PopP2-mediated PTI suppression.

Delivery of PopP2 but not PopP2^{C321A} via *P. fluorescens* Pf0-1(T3S) has been demonstrated to inhibit PTI as indicated by cell death induction by subsequent *Pto* DC3000 infiltration (Crabill et al., 2010; Badel et al., 2013; Le Roux et al., 2015). This suggested that PopP2 acetyltransferase activity is required for virulence activity to suppress host PTI (Le Roux et al., 2015). Since discovering that the conserved EAR motif was required for avirulence, its requirement for PopP2 virulence activity was investigated, as measured by PTI suppression in *N. benthamiana* leaves. Infiltration of Pf0-1(T3S) carrying PopP2 variants alone (empty vector (EV), PopP2, PopP2^{C321A}, PopP2^{LAAL}, PopP2$^{LAAL\text{-}SRDX}$ and PopP2$^{LAAL\text{-}srdx}$) induced no cell death while infiltration of *Pto* DC3000 induced a cell death response. As previously shown, infiltration of Pf0-1(T3S)(PopP2) followed by infiltration of *Pto* DC3000 resulted in a cell death response due to PopP2 PTI suppression; however, infiltration of Pf0-1(T3S)(EV) or Pf0-1(T3S) (PopP2^{C321A}) followed by infiltration of *Pto* DC3000 resulted in no cell death induction due to *N. benthamiana* PTI-induced inhibition of *Pto* DC3000-induced cell death. Interestingly, it was discovered that Pf0-1(T3S)(PopP2^{LAAL}) infiltration into *N. benthamiana* leaves was also unable to suppress host PTI, as demonstrated by the lack of cell death induction by subsequent *Pto* DC3000 infiltration (Figure 3.7). This suggests that the EAR motif is not only required for avirulence activity but also for PopP2 virulence function. Fusion of the artificial EAR motif, SRDX, partially restored the PTI suppression ability of PopP2, whereas fusion of the mutated version, srdx, had no effect (Figure 3.7).

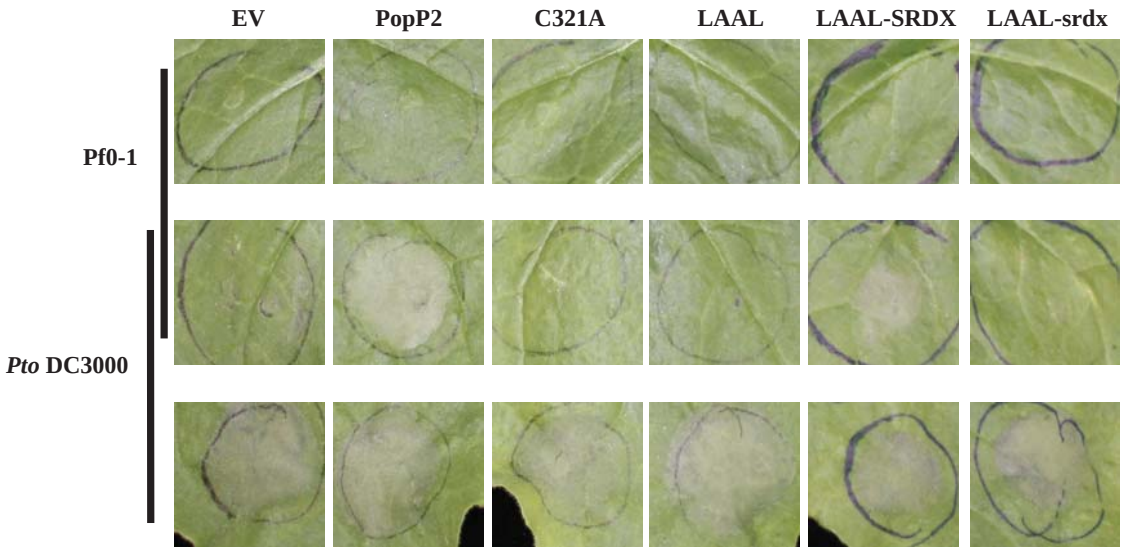


Figure 3.7. The EAR motif is required for PopP2-mediated inhibition of *P. fluorescens* Pf0-1-induced PTI in *N. benthamiana*. 3x10^8 cfu/ml of *P. fluorescens* Pf0-1 carrying empty vector or PopP2 variants (WT, C321A, LAAL, LAAL-SRDX or LAAL-srdx) were infiltrated in 5-week old *N. benthamiana* leaves (in indicated circles as 'Pf0-1'). After 8 hours, 2x10^7 cfu/ml of *Pto* DC3000 was infiltrated (in indicated circles as 'DC3000'). *Pto* DC3000-triggered tissue collapse was scored at 48hpi after *Pto* DC3000 infiltration. This experiment was conducted 4 times with similar results.

Sera Choi conducted this experiment.

3.2.5 PopP2 does not interact with known Arabidopsis transcriptional co-repressors in yeast.

The EAR motif is known to confer transcriptional repression activity via the recruitment of a co-repressor (Kagale and Rozwadowski, 2011). This led us to hypothesize that PopP2 recognition in Arabidopsis requires the recruitment of a transcriptional co-repressor. Therefore, a library of transcriptional co-repressors was screened for interaction with PopP2 using a LexA-based yeast-two-hybrid (Y2H) assay (Gyuris et al., 1993). The library comprised several members of the Groucho/Tup1-like family of co-repressors with known LisH domains and WD repeats (Liu and Karmarkar, 2008). LEUNIG (LUG) and the closely related LEUNIG_HOMOLOG (LUH) as well as TPL and its close homologs TPR1-4 are the best characterized of the Gro/Tup1-like co-repressors. SEUSS (SEU) and its close homologs, SEUSS-LIKE (SLK1-2),

can interact with LUG or LUH to form a functional repressor complex (Franks et al., 2002; Sitaraman et al., 2008; Grigorova et al., 2011; Shrestha et al., 2014). Finally, high expression of osmotically responsive genes 15 (HOS15) and SIN3-associated polypeptide of 18 kDa (SAP18) were included, as they are known to mediate transcriptional repression in Arabidopsis via chromatin modification (Song and Galbraith, 2006; Hill et al., 2008; Zhu et al., 2008).

Protein fusions were assayed for interaction in yeast with *LEU2* and *lacZ* reporter genes under the control of upstream LexA operators. Yeast cells expressing empty vector controls with fusion proteins were assayed for growth and the development of blue color on the induction medium, (-His(H)/-Trp(T)/-Ura(U)/-Leu(L)) + X-Gal, to test for auto-activity. TPL, TPR4 and SAP18 in the pB42-AD vector alone activated *LEU2* but not *lacZ* allowing growth on medium lacking leucine; all other yeast cells did not activate reporter genes (Figure 3.8). As expected, LUG interaction with SLK2, used as a positive control, showed strong interaction (Stahle et al., 2009). However, none of the yeast cells co-expressing PopP2-DBD and a transcriptional co-repressor-AD fusion protein showed clear protein-protein interaction in yeast cells (Figure 3.8).

3.2.6 The EAR motif is required for PopP2 stability *in N. benthamiana.*

The HR activation in response to PopP2 variants was assayed in the native Arabidopsis system. A heterologous tobacco overexpression system was also used to assay for RPS4/RRS1-mediated recognition of PopP2 variants (Figure 3.9A). Agroinfiltration of $PopP2^{WT}$, RPS4 and RRS1 resulted in a robust programmed cell death (PCD) response at 3 dpi in tobacco. Conversely, co-expression of the inactive $PopP2^{C321A}$ mutant with RPS4 and RRS1 resulted in significantly reduced PCD (Sohn et al., 2014). Intriguingly, the $PopP2^{LAAL}$, $PopP2^{LAAL}$-SRDX and $PopP2^{LAAL}$-srdx variants all elicited a strong PCD response when co-expressed with RPS4 and RRS1 (Figure 3.9A). Thus, loss of PopP2 avirulence activity as a result of EAR motif

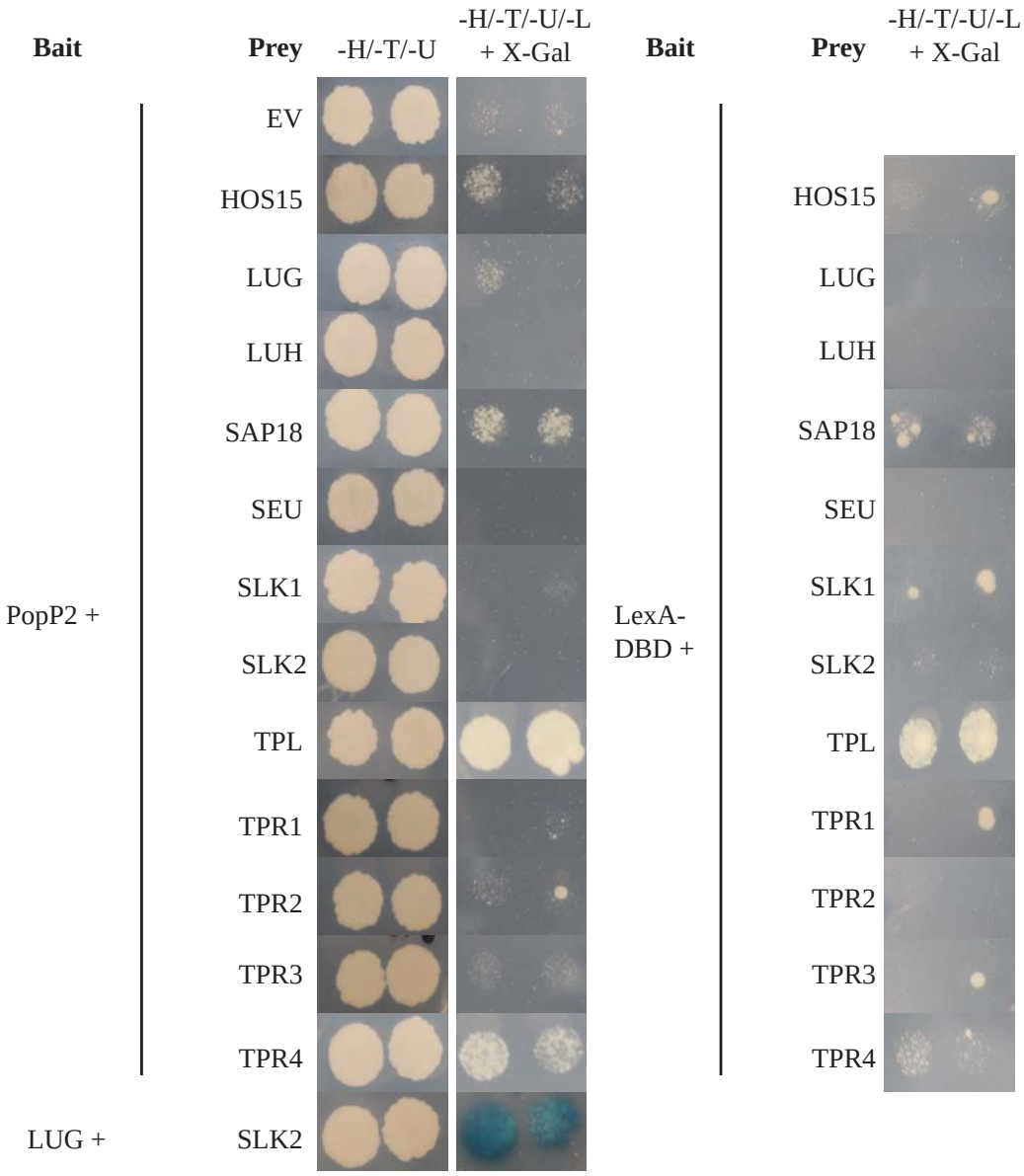


Figure 3.8. PopP2 does not interact with known transcriptional co-repressors in yeast. Growth of yeast cells expressing LexA-DNA-binding domain (DBD) fusion (bait) and B42-activation domain (AD) fusion (prey) as indicated. Yeast cells were grown on non-selective media lacking histidine, tryptophan and uracil (-H/-T/-U) or selective media also lacking leucine (-H/-T/-U/-L) and supplemented with X-Gal. TPL, TPR4 and SAP18 B42-AD fusions are weakly auto-active as indicated by the growth of yeast cells expressing LexA-DBD alone and B42-AD protein fusions. This experiment was conducted twice with similar results.

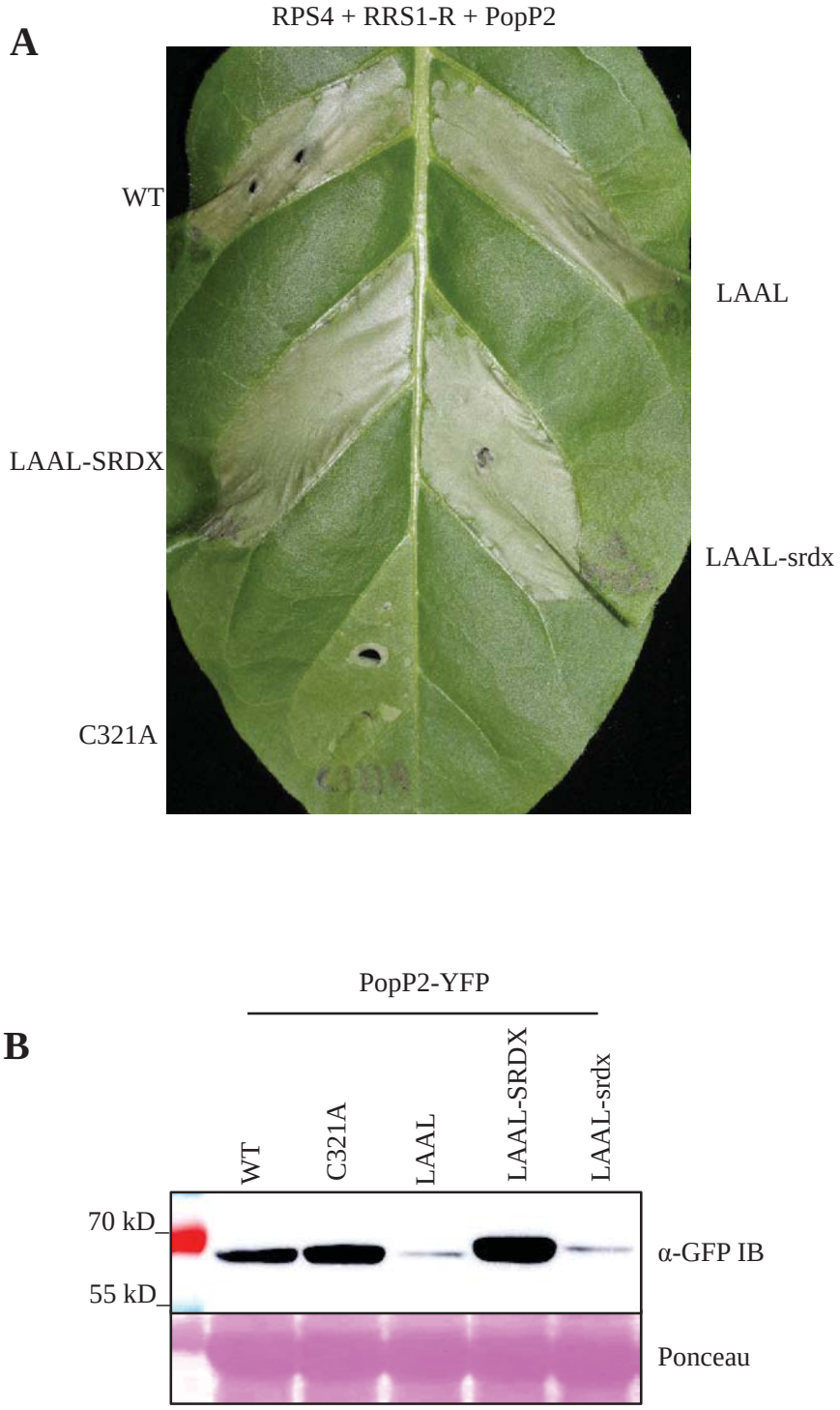


Figure 3.9. The EAR motif is required for PopP2 stability *in planta*. (A) Agro-mediated co-expression of the PopP2 variants with RPS4 and RRS1-R in tobacco. PopP2^{LAAL} triggers a cell death response when co-expressed with RPS4 and RRS1-R independent of SRDX fusion. This experiment was conducted 3 times with similar results. (B) Mutation of the PopP2 EAR motif affects PopP2 protein accumulation after transient expression following agro-infiltration in *N. benthamiana*. The wild-type level of protein accumulation could be restored by fusion of a functional synthetic EAR motif (SRDX), but not by the mutated version (srdx). Immuno-detection of PopP2 variants C-terminally fused to YFP tag was conducted using anti-GFP antibodies. Ponceau S staining of total protein demonstrates equal loading of the samples. IB = immunoblot.

Jay Jayaraman conducted the experiment for Figure 3.9A (I conducted another repeat).

disruption in Arabidopsis could not be reconstituted in the tobacco overexpression system.

To confirm the expression of PopP2 variants, sequences were fused with a C-terminal YFP epitope tag and transiently expressed in *N. benthamiana*. Total proteins were extracted and subjected to immuno-detection with anti-GFP antibodies (these antibodies also detect the YFP epitope tag). PopP2^{WT} and PopP2^{C321A} accumulated to similar amounts. Surprisingly, PopP2^{LAAL} protein accumulation was significantly lower as indicated by the low intensity band on the blot (Figure 3.9B). Furthermore, fusion of the synthetic EAR motif restored the protein level to the WT level while fusion of the non-functional srdx motif, had no effect on protein accumulation. This evidence suggests that the stability of PopP2 inside plant cells is dependent on an EAR motif (Figure 3.9B).

3.3 Discussion

The *R. solanacearum* acetyltransferase effector, PopP2, is known to activate RPS4/RRS1-mediated resistance in certain Arabidopsis accessions (Deslandes et al., 2003; Sohn et al., 2014). PopP2 acetyltransferase activity is required for auto-acetylation and trans-acetylation of key lysine residues in the RRS1 WRKY domain and activation of the RPS4/RRS1 immune complex (Tasset et al., 2010; Le Roux et al., 2015; Sarris et al., 2015). Here, the results indicate that while the RPS4/RRS1 immune complex can recognize the naturally occurring alleles of PopP2 in Korean *R. solanacearum* isolates, a conserved EAR motif is necessary for its avirulence activity by regulating the protein stability in the plant cell.

3.3.1 PopP2 natural variation in virulent *R. solanacearum* strains

The natural variation of PopP2 sequence was surveyed in *R. solanacearum* strains isolated from diseased tomato and pepper fields at different geographic locations across the Republic of Korea. Gene-specific sequencing

and subsequent analysis revealed that *popP2* is highly conserved across South Korean isolates, as only 3 polymorphic alleles were identified among the 17 *popP2*-harboring strains. In fact, all but two of the polymorphisms are conservative; they result in a change to an amino acid with similar properties. However, G156D and G396E result in a change from hydrogen to a negatively charged side chain. It was shown that, despite these polymorphisms, two of the natural variants, PopP2^{Pe_1} and PopP2^{Pe_2}, retained avirulence activity in Arabidopsis Ws-2. The significantly truncated variant, PopP2^{Pe_13}, did not trigger HR. This is consistent with the previous report showing that the catalytic triad is required for PopP2 acetyltransferase activity (Tasset et al., 2010).

It must be considered that the *R. solanacearum* strains from which the *popP2* alleles were isolated were highly virulent on pepper or tomato plants. Genome sequence analysis does not reveal any strong homology of *Solanaceae* disease resistance (*R*) genes with *RPS4* and *RRS1* (Consortium, 2012; Kim et al., 2014b). However, the existence of PopP2^{Pe_13} (a truncated variant that lacks avirulence) suggests that there might be a selective pressure in natural host plants of *R. solanacearum*. This hypothesis is further supported by the three *R. solanacearum* strains that were shown to lack *popP2* in this study. In this regard, it would be interesting to survey the presence/absence and sequence polymorphism of *popP2* in *R. solanacearum* strains from other host plants or geographic regions. Furthermore, in addition to RPS4/RRS1, it is plausible that PopP2 may be recognized by other R protein(s) and that natural variants found in this study may show altered avirulence activity.

3.3.2 EAR motif-dependent protein stability control

This study illustrates the first example of a plant pathogenic effector that is dependent on an EAR motif for avirulence activity. Of note, the *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) effector XopD possesses two EAR motifs (sequence (L/F)DLN(L/F)(X)P) that are both required for virulence. XopD represses defense gene transcription via the two EAR motifs to enable *Xcv*

growth in tomato (Kim et al., 2008). Likewise, it was demonstrated that the PopP2 EAR motif is required for PTI suppression, which may contribute to *R. solanacearum* virulence. In addition, it was showed that disruption of the LxLxLxL amino acid sequence rendered PopP2 unstable, but could be stabilized by addition of the synthetic EAR motif SRDX at the C-terminus. Therefore, PopP2 stability appears to depend specifically on the presence of the LxLxLxL sequence. In the tobacco heterologous system, despite being clearly reduced, accumulation of PopP2^{LAAL} might nonetheless reach a threshold required for detection by the over-expressed RPS4 and RRS1 receptors. Conversely, in the native Arabidopsis system, it can be inferred that PopP2 EAR motif could play an important role in protein stability, allowing its accumulation above the necessary amount to trigger RRS1 activation.

A novel mechanism of controlling protein stability via an EAR motif has recently been unveiled. Proteasomal and non-proteasomal degradation of the ZINC FINGER OF ARABIDOPSIS THALIANA12 (ZAT12) transcription factor is controlled by an LxLxL EAR motif (Le et al., 2016). Similarly to the reduced accumulation of PopP2^{LAAL}, the abundance of the ZAT12 variant carrying a mutation in the EAR motif was lower than the wild-type. Le et al. (2016) hypothesized that the ZAT12 EAR motif is involved in mediating interactions with different partners; at least one of which is H_2O_2 responsive and another that is a factor of proteasomal degradation targeting, such as an E3-ubiquitin ligase. Conversely, an earlier study investigating a poplar (*Populus* spp.) ortholog of ZAT12, *Pti* Cys2/His2 zinc-finger protein 1 (*Pti*ZFP1), reported that the *Pti*ZFP1 EAR motif promotes its degradation by the 26S proteasome through MAPK binding (Hamel et al., 2011). This is in contrast to the ZAT12 EAR motif-mediated protein stability model, but it provides additional clues to investigate the mechanism regulating PopP2 accumulation *in planta*.

PopP2 contributes to *R. solanacearum* virulence in tomato, eggplant, bean and Arabidopsis (Macho et al., 2010; Le Roux et al., 2015). It is conceivable that successful *R. solanacearum* strains have acquired PopP2 variants carrying an EAR motif to stabilize the secreted effector by circumventing *in planta* degradation. This mechanism could have been selected to enhance

the virulence of *R. solanacearum* on host plants. Indeed, it was shown that PopP2 stability is dependent on a functional EAR motif and that this is associated with both PopP2-mediated PTI suppression and host recognition ability.

3.3.3 Possible mechanisms of PopP2 EAR motif function

The requirement of PopP2 for an EAR motif to trigger an avirulence response coupled with the numerous reports of EAR motif-mediated recruitment of co-repressors led us to generate yeast two-hybrid constructs of known Arabidopsis transcriptional co-repressors to screen for interaction with PopP2 in yeast cells. The Groucho (Gro)/Tup1-like family of co-repressors make up the largest and best characterized family of co-repressors in Arabidopsis with at least 13 members, including LUG and LUH, TPL and TPRs and HOS15 (Liu and Karmarkar, 2008). LUG and LUH are partially redundant transcriptional co-repressors and together with SEU/SLKs are involved in embryo and floral development and abiotic stress responses (Sitaraman et al., 2008; Shrestha et al., 2014). Similarly, HOS15 and SAP18 have not been implicated in plant defense so far, but mediate gene repression in response to cold or salt stress (Song and Galbraith, 2006; Hill et al., 2008; Zhu et al., 2008). On the other hand, the recruitment of the functionally redundant TPL and TPR1-4 by EAR motif-containing proteins in defense signaling is well known. TPL is involved in the regulation of jasmonic acid (JA) signaling for disease resistance to necrotrophic pathogens and stomatal defense (Pauwels et al., 2010; Pauwels and Goossens, 2011). Similarly TPR1 and other TPRs associate with the *R* gene *SNC1* to participate in transcriptional repression of negative regulators of defense (Zhu et al., 2010).

The yeast-two-hybrid (Y2H) screen data indicate that PopP2 does not interact directly with any of the tested co-repressors in these experimental conditions. Further confirmation of this result could be obtained by testing *in planta* interaction between PopP2 and transcriptional co-repressors in the future. Nonetheless, based on these findings, it can be hypothesized that *in planta*

PopP2 stability may be dependent on EAR motif-mediated recruitment of an as yet untested co-repressor or another host component. Identification of the host factor(s) controlling PopP2 stability shall bring new insights into the systems used to monitor pathogen virulence in the plant cell.

Another scenario could be that mutation of the putative EAR motif renders PopP2 unstable by affecting its conformation. Fusion of the synthetic EAR motif, SRDX, might stabilize PopP2 via recruitment of a stable corepressor to the unstable PopP2, which would not otherwise associate with a transcriptional corepressor. On the other hand, it cannot be ruled out that PopP2^{LAAL}-SRDX may induce cell death independent of RPS4/RRS1. In order to assay this, cell death could simply be assayed in Arabidopsis ecotype Col-0, which does not recognize PopP2, or in an *rrs1* knockout (KO) Ws-2 background.

CHAPTER 4: Characterization of *SUSHI* mutations in the *RRS1* disease resistance gene

4.1 Introduction

Unlike animals that possess a circulatory immune system, the plant innate immune system is dependent on individual cells sensing pathogen presence and subsequently triggering a defense response. The initial form of defense is activated upon recognition of pathogen-associated molecular patterns (PAMPs) via membrane-localized receptors, namely pattern recognition receptors (PRRs). This results in pattern-triggered immunity (PTI), a defense-signaling pathway that induces a multitude of cellular changes to prevent pathogen proliferation (Nürnberger et al., 1994; Kitajima and Sato, 1999; Schwessinger and Zipfel, 2008; Luna et al., 2011; Daudi et al., 2012). This basal defense response can be overcome by successful pathogens through secretion of immunity-dampening proteinaceous effectors. Plants have, however, evolved resistance (*R*) genes, the products of which recognize pathogen effectors and activate an amplified form of defense termed ETI (effector-triggered immunity). This often culminates in an HR (hypersensitive response), a form of localized programmed cell death (Jones and Dangl, 2006).

R genes typically encode nucleotide-binding domain and leucine-rich repeat-containing (NLR) receptors. NLRs recognize intracellular pathogen effectors either directly through physical association or, more commonly, indirectly via the detection of perturbed host proteins. NLR activation results in a strong defense response that participates in the restriction of pathogen growth; however, defense activation is also negatively correlated with plant growth (Denancé et al., 2013). Therefore, it is important for plants to avoid inappropriate NLR activation. This is dependent partially on NLR auto-inhibition. In the absence of the appropriate stimulus (i.e. an effector), NLRs exist in an inactive, auto-inhibited state. Although other roles have been demonstrated, such as effector binding, it is thought that the main function for

the LRR domain is in auto-inhibition (Ade et al., 2007). Furthermore, the NB-ARC domain is responsible for maintaining an inhibited “OFF” state through ADP binding. ADP/ATP exchange by the NB-ARC domain results in a conformational switch to the active state (Takken and Tameling, 2009). Predominantly, the N-terminal TIR or CC domain is involved in downstream signaling and there are several examples of TIR/CC domains eliciting an HR-like cell death response when ectopically expressed *in planta* (Swiderski et al., 2009; Cesari et al., 2016). An emerging hypothesis is that plant and animal NLRs employ a similar mechanism of activation via signaling by cooperative assembly formation (SCAF) (Bentham et al., 2016).

The majority of NLRs function individually to recognize an effector and signal downstream; however, it is emerging that many NLRs function cooperatively in a dual NLR complex. Generally, one NLR acts as a sensor NLR by directly or indirectly perceiving the effector; the other NLR acts as a signaling NLR to activate a defence response. This has been termed the integrated decoy hypothesis (Cesari et al., 2014).

RPS4 and RRS1 were originally hypothesized to function independently for the recognition of AvrRps4 and PopP2, respectively; however, RPS4 and RRS1 have since been shown to function cooperatively for the recognition of AvrRps4 and PopP2 (Narusaka et al., 2009). More recently, a second NLR pair were shown to confer recognition of AvrRps4 but not PopP2. These were assigned the gene names *RPS4B* and *RRS1B* (Saucet et al., 2015).

An autoimmune mutant of *RRS1* was identified, which harbors a single leucine insertion in the RRS1 WRKY domain (Noutoshi et al., 2005). This mutant allele, *slh1* (*sensitive to low humidity 1*), confers constitutive defense activation resulting in a severely stunted morphology. A forward genetic screen identified *suppressor of slh1 immunity* (*sushi*) mutants, which display a recovered morphological phenotype. Intragenic *RPS4* mutations were previously characterized; here *SUSHI* mutations in *RRS1* were characterized (Sohn et al., 2014).

6 causal intragenic *RRS1 SUSHI* mutations were identified, which at least partially restored WT morphology. This was associated with suppression of defense gene upregulation. The *SUSHI* mutations differentially affected auto-activity and effector recognition as measured by tobacco PCD. Surprisingly, a further misregulated WRKY domain RRS1 variant, $RRS1^{C1243Y}$, was identified, which displays distinct characteristics from other auto-active RRS1 variants. It was demonstrated that the TIR C15Y mutation abolished RRS1 function by disrupting heterodimer formation with its signaling partner, RPS4. Moreover, RRS1B variants harboring corresponding *SUSHI* mutations were generated, which highlighted intriguing differences between RRS1 and RRS1B function, including the requirement of TIR/TIR heterodimer formation.

4.2 Results

4.2.1 Characterization of intragenic suppressors of *slh1*-mediated immunity

A single leucine residue insertion in the WRKY domain of RRS1 causes temperature-dependent auto-immunity in the *slh1* mutant. When grown at a high humidity or high temperature (up to 26°C described here), the autoimmunity is suppressed (Noutoshi et al., 2005). The severely stunted morphology of *slh1* plants resulting from constitutive defense activation was used as a readily observable defense marker for a forward genetic screen of ethyl methanesulfonate (EMS)-mutagenized *slh1* aiming at the identification of mutations suppressing auto-immunity (Noutoshi et al., 2005; Sohn et al., 2014). In short, *slh1* mutant seeds were treated with EMS, ~7000 M1 plants were grown at 28°C and M2 seeds were harvested. ~500,000 M2 plants were screened at 21°C for a wild-type (WT) morphology. 83 independent M2 families of *sushi* mutants were recovered and scored as exhibiting a fully or partially rescued WT phenotype, with 69 and 14 families respectively. In the M3 generation, the *sushi* mutants were genotyped for the presence of *slh1* alleles using a cleaved amplified polymorphic sequences (CAPS) marker and 72 out of 83 were confirmed to be homozygous for *slh1*. It was important that

plants were homozygous for *slh1* due to the recessive nature of *RRS1* (Deslandes et al., 1998). Sanger sequencing of *RPS4* and *RRS1* coding regions was carried out in the 72 *sushi* lines homozygous for the *slh1* mutation to identify intragenic mutations in either of these genes. Single nucleotide polymorphisms (SNPs) in *RPS4* were found in more than half (46) of the *sushi* lines. The critical role for defense activation of 12 RPS4 amino acid residues has previously been demonstrated in these *sushi* lines (Sohn et al., 2014). In contrast, only 19 *RRS1* mutations were identified, 9 of which encoded a premature stop codon and 2 of which harbored heterozygous mutations. To address the requirement of the 8 RRS1 residues affected by homozygous, non-stop codon, non-synonymous SNPs for auto-immunity, first the phenotype of the M3 plants was observed when grown at the permissive temperature of 22°C (Table 4.1, Figure 4.1, Figure 4.2) (Sohn et al., 2014). Control *slh1* plants were severely dwarfed whereas the 8 *rrs1-sushi* mutants displayed a gradient of rescued morphology. The stature of *sushi84*, *sushi87* and *sushi88* was similar to that of wild-type (WT) No-0 plants whereas growth of the other 5 *rrs1-sushi* mutants was intermediate (Figure 4.2A). Nonetheless, the 8 *rrs1-sushi* mutants showed at least partially rescued *slh1*-mediated growth retardation. It was previously reported that expression of the defense-associated genes *PR1*, *PBS3* and *FMO1* is induced by a temperature shift in the *slh1* mutant (Sohn et al., 2014). To correlate the rescued growth phenotype with suppression of defense responses in the *rrs1-sushi* mutants, marker gene expression was monitored in plants grown at 28°C and shifted to 19°C for 4 days before RNA extraction. Subsequent quantitative RT-PCR (qRT-PCR) revealed that transcript accumulation of *PR1*, *PBS3* and *FMO1* was dampened in the *rrs1-sushi* mutants to a level comparable to WT No-0 (Figure 4.2B). The rescued morphological phenotype was, therefore, associated with suppression of defense signaling.

To confirm if the *RRS1* SNPs identified were, indeed, the causal *sushi* mutations, the 8 *rrs1-sushi* lines and WT No-0 were crossed to the Ws-2 *rrs1-1* knockout mutant and the F1 growth phenotype was assayed at 22°C according to the previous finding indicating that in the homo- or hemi- but not

Table 4.1. *RRS1* intragenic mutations identified in *sushi* mutants.

sushi[a]	Genomic[b]	Exon[c]	Domain[d]	Protein[e]	RRS1B[f]
84	tGc>tAc	1	TIR	C15Y	C12
40	Cga>Tga	1	TIR	R33*	
45	cCc>cTc	1	TIR	P68L	P63
81	Cga>Tga	2	NB-ARC	R151*	
11	gGa>gAa	2	NB-ARC	G176E	G167
33	tGg>tAg	2	NB-ARC	W178*	
78	tGg>tAg	3		W441*	
26	tGc>tAc	4	LRR	C607Y	C600
23	cCa>cTa	4	LRR	P741L	P741
85	Caa>Taa	4	LRR	Q787*	
61	Cga>Tga	4	LRR	R800*	
88	Ctt>Ttt	4	LRR	L814F	
50	tCt>tTt	5		S981F	S945
87	tGt>tAt	7	WRKY	C1241Y	C1211

[a]Number of the sequenced *sushi* line

[b]Nucleotide mutation identified in RRS1 codon

[c]Localization of the mutation in RRS1 CDS

[d]Localization of the mutation in RRS1 conserved domain

[e]Resulting amino acid change in RRS1 protein (* indicates STOP codon)

[f]Conserved corresponding residue in *RRS1B* protein

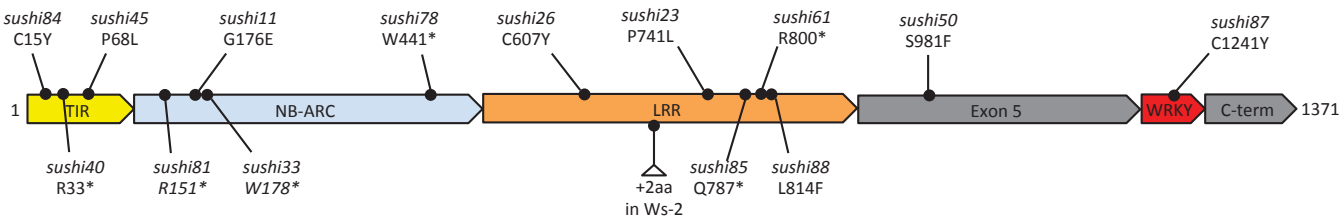


Figure 4.1. A schematic of RRS1 displaying *SUSHI* mutations.
Causal non-synonymous non-stop codon mutations *sushi84* (C15Y), *sushi45* (P68L), *sushi11* (G176E), *sushi26* (C607Y), *sushi88* (L814F) and *sushi87* (C1241Y); non-causal non-stop codon mutations *sushi23* (P741L) and *sushi50* (S981F); and premature stop codon mutations *sushi40* (R33*), *sushi81* (R151*), *sushi33* (W178*), *sushi78* (W441*), *sushi85* (Q787*) and *sushi61* (R800*). Also see Table 1.

heterozygous state, the *RRS1*^{SLH1} allele renders plants stunted due to elevated immunity (Noutoshi et al., 2005). Using an *slh1* genotyping CAPS marker, it was first confirmed that F1 hybrids were hemizygous *RRS1*$^{SLH1/sushi}$*/rrs1-1.* When grown at 22°C, 6 of the *sushi* x *rrs1-1* F1 hybrids grew to a similar size as the No-0 x *rrs1-1* plants; however, the F1 plants derived from the crosses of *sushi23* and *sushi50* with *rrs1-1* were significantly stunted (Figure 4.3A). This suggests that the *RRS1* mutations identified in *sushi84* (C15Y), *sushi45* (P68L), *sushi11* (G176E), *sushi26* (C607Y), *sushi88* (L814F) and *sushi87* (C1241Y) were responsible for *slh1* phenotype suppression whereas the *RRS1* mutations identified in *sushi23* (P741L) and *sushi50* (S981F) were not the causal *sushi* mutations (Figure 4.3). From this genetic analysis, it can be inferred that *sushi23* and *sushi50* harbor a secondary mutation at an unknown locus, which encodes a component of RRS1^{SLH1} signaling. To confirm that the morphological phenotype was associated with defense signaling, *PR1*, *PBS3* and *FMO1* transcript accumulation was measured in the same F1 hybrids. Consistent with the observed retardation in growth, expression of *PR1*, *PBS3* and *FMO1* was elevated in *sushi23* x *rrs1-1* and *sushi50* x *rrs1-1* F1 plants (Figure 4.3B). In summary, this analysis revealed 6 residues in RRS1 whose alteration leads to suppression of aberrant defense responses in the *slh1* mutant.

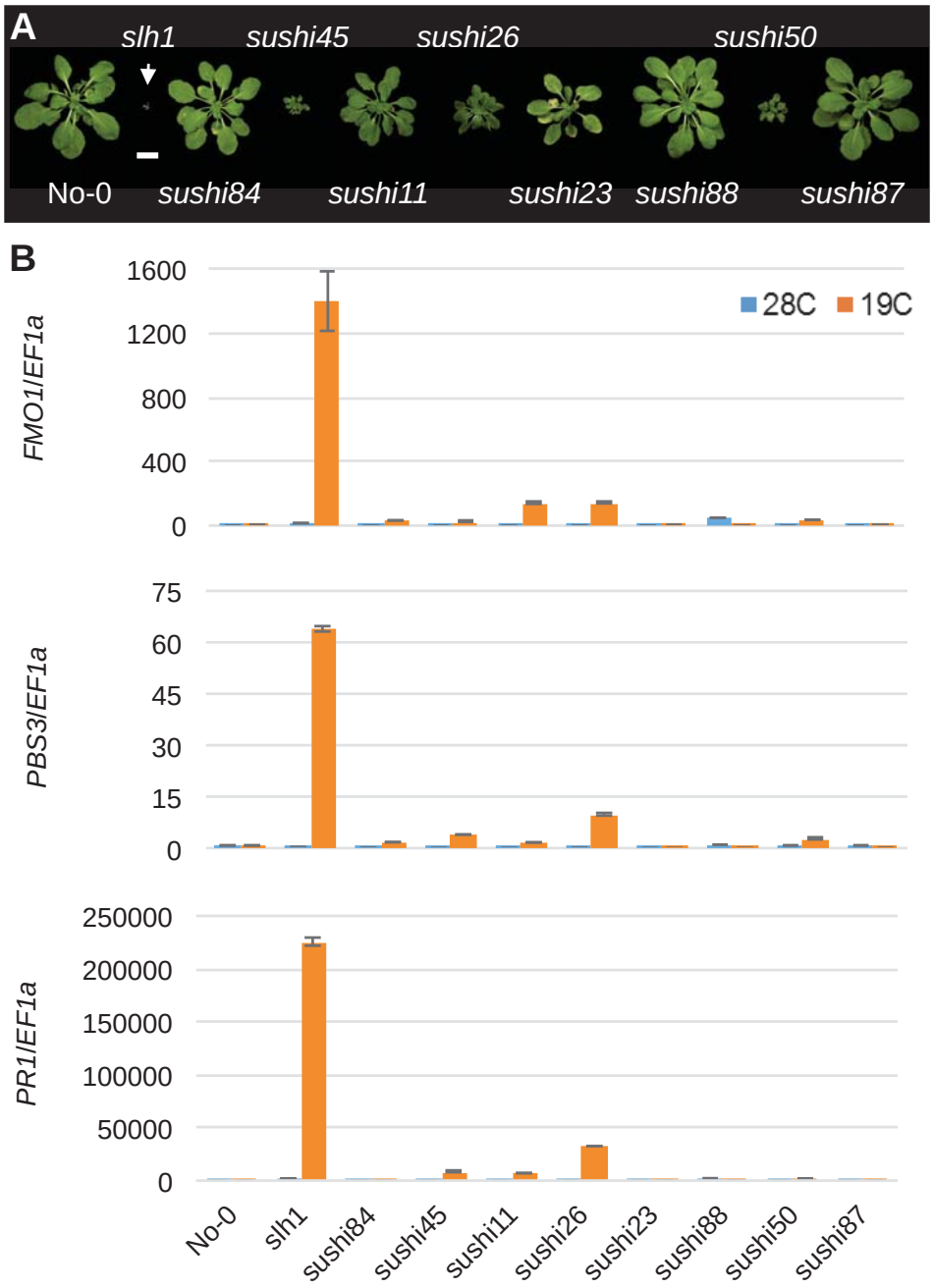


Figure 4.2. Phenotype of the *sushi* lines carrying non-stop non-synonymous mutations in *RRS1*. (A) Morphology of *sushi* mutants carrying mutations in *RRS1* (M3 generation), wild type No-0 and *slh1* plants grown at 22°C for five weeks. Scale bar represents 1 cm. (B) qRT-PCR analysis of selected $RRS1^{SLH1}$-regulated genes in wild type No-0, *slh1* and *sushi* carrying mutations in *RRS1*. Transcript accumulation is presented relative to No-0. Plants were grown at 28°C for 5 weeks then shifted to 19°C for 4 days prior to RNA isolation.

Cecile Segonzac conducted this experiment.

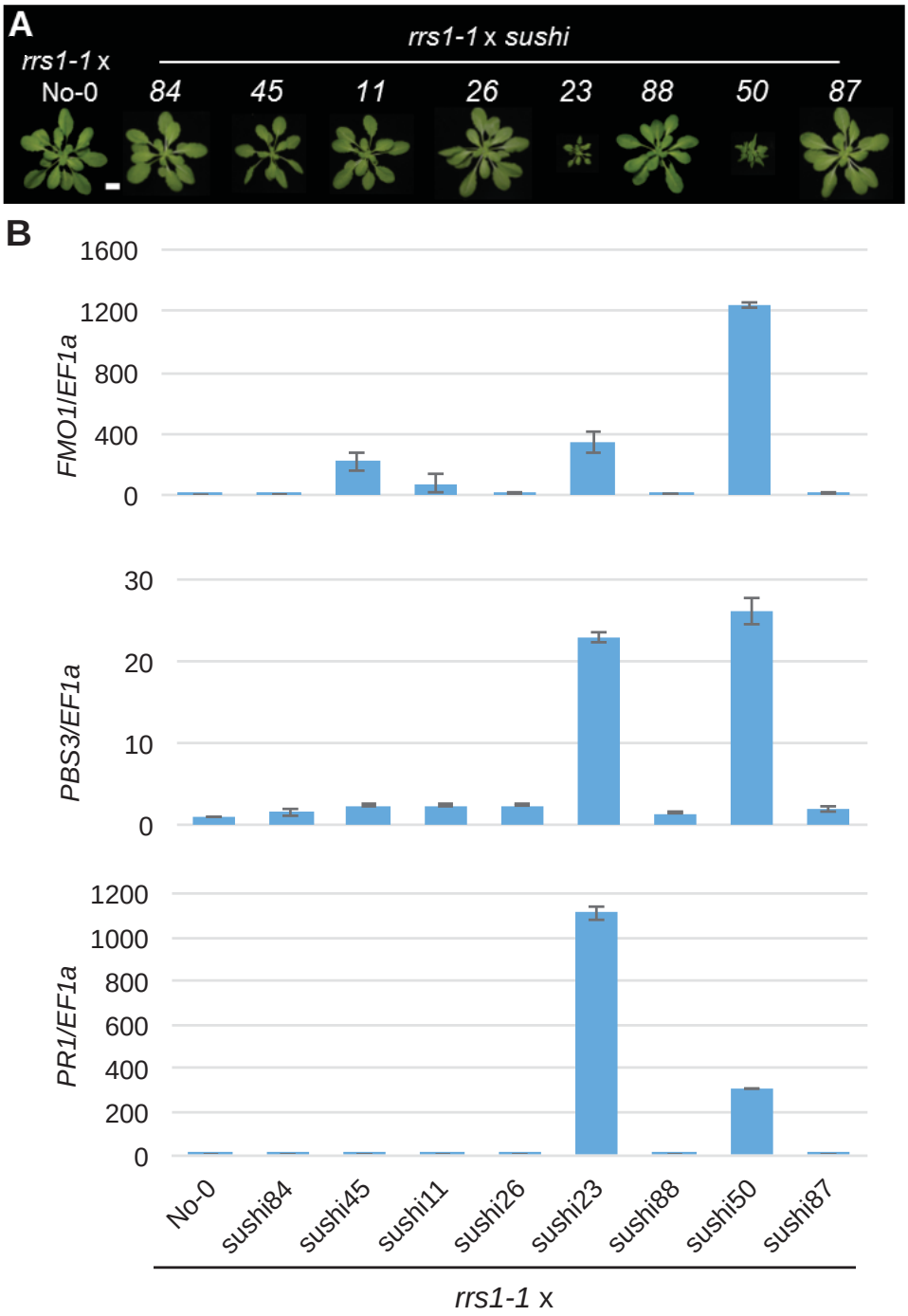


Figure 4.3. Identification of 6 non-synonymous mutations in *RRS1* that cause suppression of *slh1* auto-immunity. (A) F1 hybrids between *rrs1-1* and *sushi* were grown for five weeks at 22°C before the photograph was taken. Scale bar represents 1 cm. (B) Growth restriction of F1 hybrids (shown in (A)) correlates with *FMO1*, *PBS3* and *PR1* transcript accumulation as determined by qRT-PCR. Transcript accumulation is presented relative to the F1 No-0 x *rrs1-1* hybrid.

Cecile Segonzac conducted this experiment.

4.2.2 *RRS1 SUSHI* mutations differentially affect RPS4-dependent RRS1^{SLH1} auto-activity and effector recognition in tobacco

To characterize the effect of the *SUSHI* mutations on RRS1 function, a transient expression system was employed by *Agrobacterium* transformation of *Nicotiana tabacum* (tobacco) leaf cells (hereafter, agroinfiltration). Co-expression of RPS4, RRS1-R and AvrRps4 or PopP2 has been shown to induce a strong, programmed cell death (PCD) within 3 days post-infiltration (dpi). Additionally, co-expression of the truncated RRS1 variant, RRS1-S, which is encoded by Arabidopsis Col-0 accession, with RPS4 and AvrRps4 but not PopP2 induces a rapid PCD. Similarly, co-expression of RPS4 with auto-active RRS1^{SLH1} triggers a rapid PCD response in the absence of an effector (Sohn et al., 2014). To address the importance of the RRS1 residues identified in *sushi* mutants for RRS1 functions (auto-activity or effector recognition), the corresponding mutations were introduced in an RRS1-R construct obtained from Arabidopsis ecotype Ws-2 by site-directed mutagenesis. No-0 and Ws-2 RRS1-R alleles are almost identical apart from a 2 amino acid (aa) insertion in exon 4 of Ws-2 RRS1. Hence, in the following analysis No-0 RRS1^{L814F} corresponds to Ws-2 RRS1^{L816F} and No-0 RRS1^{C1241Y} to Ws-2 RRS1^{C1243Y} (Figure 4.1). Firstly, to test putative auto-activity of the RRS1^{SUSHI} variants, individual mutants were co-expressed with the green fluorescent protein (GFP). As expected, none of the RRS1^{SUSHI} variants induced PCD (Figure 4.4A). The RRS1^{SUSHI} variants were then agroinfiltrated with RPS4 and a cell death response was observed with the auto-active control RRS1^{SLH1}. Unexpectedly, RRS1^{C1243Y} also elicited PCD when co-infiltrated with RPS4 (Figure 4.4A). Intriguingly, mutation of this cysteine residue located in the RRS1 WRKY domain suppressed *slh1*-mediated defense signaling in the native Arabidopsis system yet led to auto-activation in the heterologous tobacco transient system. Next, it was tested if the *RRS1 SUSHI* mutations could suppress autoimmunity in tobacco. Specific *SUSHI* mutations were introduced into both RRS1^{SLH1} and RRS1^{K1221Q} auto-active alleles and individual variants were co-expressed with RPS4. K1221 is the critical lysine residue of RRS1 that is acetylated by PopP2 resulting in activation of the RRS1/RPS4 complex. Changing K1221 to a glutamine (Q)

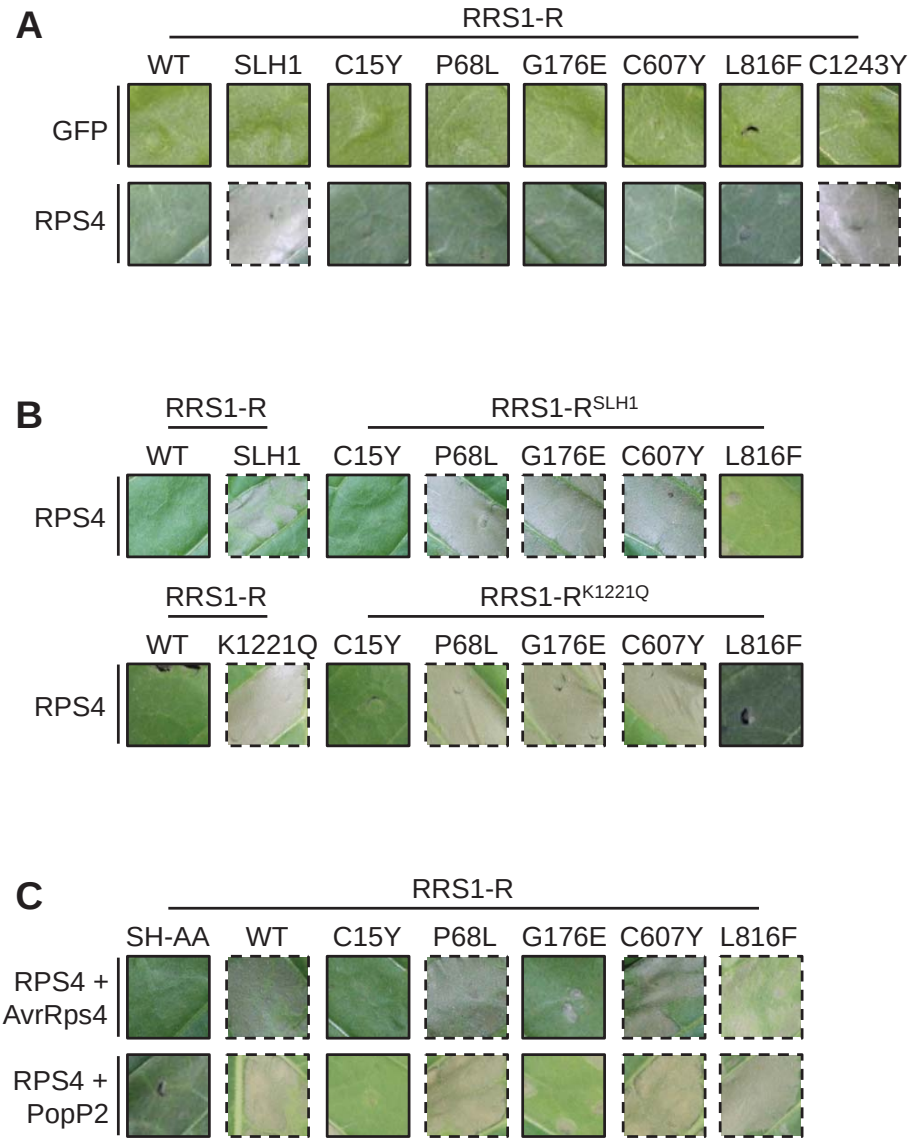


Figure 4.4. RRS1^{SUSHI} variants differentially affect auto-activity and effector recognition by the RRS1/RPS4 complex when transiently expressed in tobacco leaf cells. Photographs were taken 3 days after agro-infiltration. (A) RRS1^{C1243Y} protein variant is auto-active in tobacco leaf cells. RRS1-R wild type or carrying SUSHI mutation was co-expressed with GFP or RPS4. Like RRS1^{SLH1}, RRS1^{C1243Y} triggers cell death when co-expressed with RPS4. (B) C15 and L816 residues are required for auto-activity of RRS1^{SLH1} or RRS1^{K1221Q} protein variants in the presence of RPS4. (C) C15 and G176 residues are required for AvrRps4 and PopP2 recognition by the RRS1-R/RPS4 complex.

Figure produced from joint work with Cecile Segonzac. Jun Zhou helped with cloning.

residue mimics acetylation thus rendering RRS1 auto-active (Le Roux et al., 2015; Sarris et al., 2015). $RRS1^{SLH1}$ and $RRS1^{K1221Q}$, but not $RRS1^{WT}$, induced PCD when co-expressed with RPS4 (Figure 4.4B). P68L (located in the TIR domain), G176E (NB-ARC) and C607Y (LRR) mutations had no effect on the auto-activation of $RRS1^{SLH1}$ and $RRS1^{K1221Q}$. Conversely, C15Y (TIR) and L816F (LRR) suppressed PCD mediated by both auto-active alleles, indicating that C15 and L816 residues are required for auto-activity (Figure 4.4B). In addition, the effect of *RRS1 SUSHI* mutations on effector recognition in tobacco was tested. To this end, $RRS1^{SUSHI}$ variants were co-infiltrated with RPS4 and AvrRps4 or PopP2 and PCD was assessed. Agroinfiltration of RRS1, but not $RRS1^{S25AH26A}$ (hereafter, $RRS1^{SH\text{-}AA}$), with RPS4 and AvrRps4 or PopP2 induced a strong PCD response (Figure 4.4C). S25 and H26 constitute the so-called "SH motif" of RRS1, which is critical for heterodimer formation with RPS4 and, thus, effector recognition (Williams et al., 2014). PCD was also induced when $RRS1^{P68L}$, $RRS1^{C607Y}$ and $RRS1^{L816F}$ variants were agroinfiltrated with RPS4 and either effector. $RRS1^{C15Y}$ failed to induce a cell death response when co-expressed with RPS4 and effectors whereas $RRS1^{G176E}$ only induced a weak PCD response. Therefore, C15 and G176 residues appear to be required for effector recognition in tobacco. Notably, the C15 residue was required for both auto-activity and effector recognition, G176 was required exclusively for effector recognition and L816 was required exclusively for auto-activity. All RRS1 variants were expressed *in planta* as demonstrated by Western blots (Figure 4.5). In summary, a further mis-activated WRKY domain mutant of RRS1 was identified as well as specific residues important for the proper functioning of RRS1. Moreover, residues that are able to uncouple the auto-active and effector recognition functions of the immune receptor were identified, hinting at a differential mechanism of activation between wild-type and autoimmune RRS1 variants.

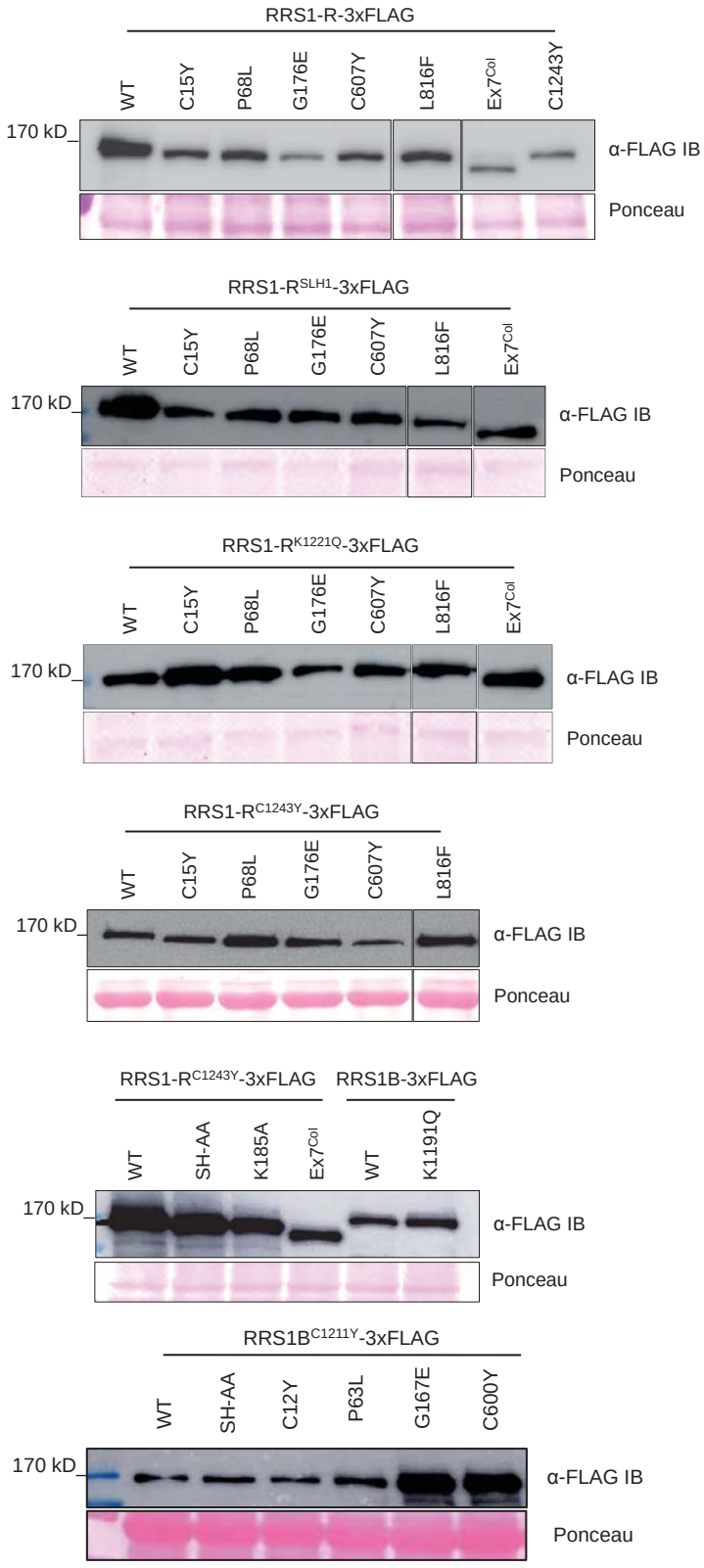


Figure 4.5. RRS1 and RRS1B variants are expressed *in planta*. Expression of protein variants in *N. benthamiana* leaves after *Agrobacterium* delivery demonstrated by Western blots. Equal protein loading shown using Ponceau S solution.

4.2.3 RRS1^{C1243Y} auto-activity in tobacco displays distinct features from other auto-active RRS1 variants

In apparent contradiction, the RRS1 (Ws-2) C1243Y mutation conferred auto-activity in tobacco, but the RRS1^{SLH1} (No-0) C1241Y mutation suppressed auto-activity in Arabidopsis. Of note, a double mutant variant RRS1$^{SLH1/C1243Y}$ (Ws-2) was also tested for auto-activity in tobacco and it did, indeed, induce RPS4-dependent cell death (Figure 4.6A). To further characterize this auto-activation mechanism, RRS1 variants carrying the C1243Y mutation as well as secondary *SUSHI* mutations were generated and co-expressed with RPS4 (Figure 4.6A). C15, G176 and L816 residues were required for RRS1^{C1243Y} auto-activity whereas mutation of P68 and C607 had no effect on the induction of RRS1^{C1243Y}-mediated PCD. The C15Y mutation completely abolished PCD whereas G176E and L816F mutations significantly weakened the PCD (Figure 4.6A). The invariant lysine residue in the NB domain P-loop motif of plant immune receptors has been shown to be required for ATP binding, which results in a conformational change and a switch to the active state (Tameling et al., 2002; Ueda et al., 2006; Williams et al., 2011). To further characterize RRS1^{C1243Y} auto-activity, RRS1^{C1243Y} and RPS4 SH and P-loop motifs were mutated. As was found for RRS^{SLH1} auto-activity, the SH motif of both RRS1 and RPS4 was required for PCD triggered by RRS1^{C1243Y}; however, only RPS4, not RRS1, P-loop was required (Figure 4.6B). RRS1^{C1243Y}-induced PCD was, therefore, dependent on TIR/TIR heterodimerization with RPS4, and RPS4 but not RRS1 ATP binding. Arabidopsis Col-0 accession harbors an RRS1 allele, RRS1-S, which confers recognition of AvrRps4 but not PopP2 (Deslandes et al., 2002). *RRS1-S* encodes a premature stop codon, which results in a truncated exon 7 (Ex7^{Col}). It was shown here that this C-terminal truncation also disabled RRS1^{SLH1} and RRS1^{K1221Q} RPS4-dependent auto-activity in tobacco (Figure 4.6C). The RRS1-R variant present in Arabidopsis ecotypes Ws-2 and No-0, which possesses an extended exon 7 (Ex7^{Ws}), confers recognition of both AvrRps4 and PopP2. Intriguingly, the RRS1^{C1243Y} variant induced PCD independent of the C-terminal extension when co-expressed with RPS4 (Figure 4.6C). This

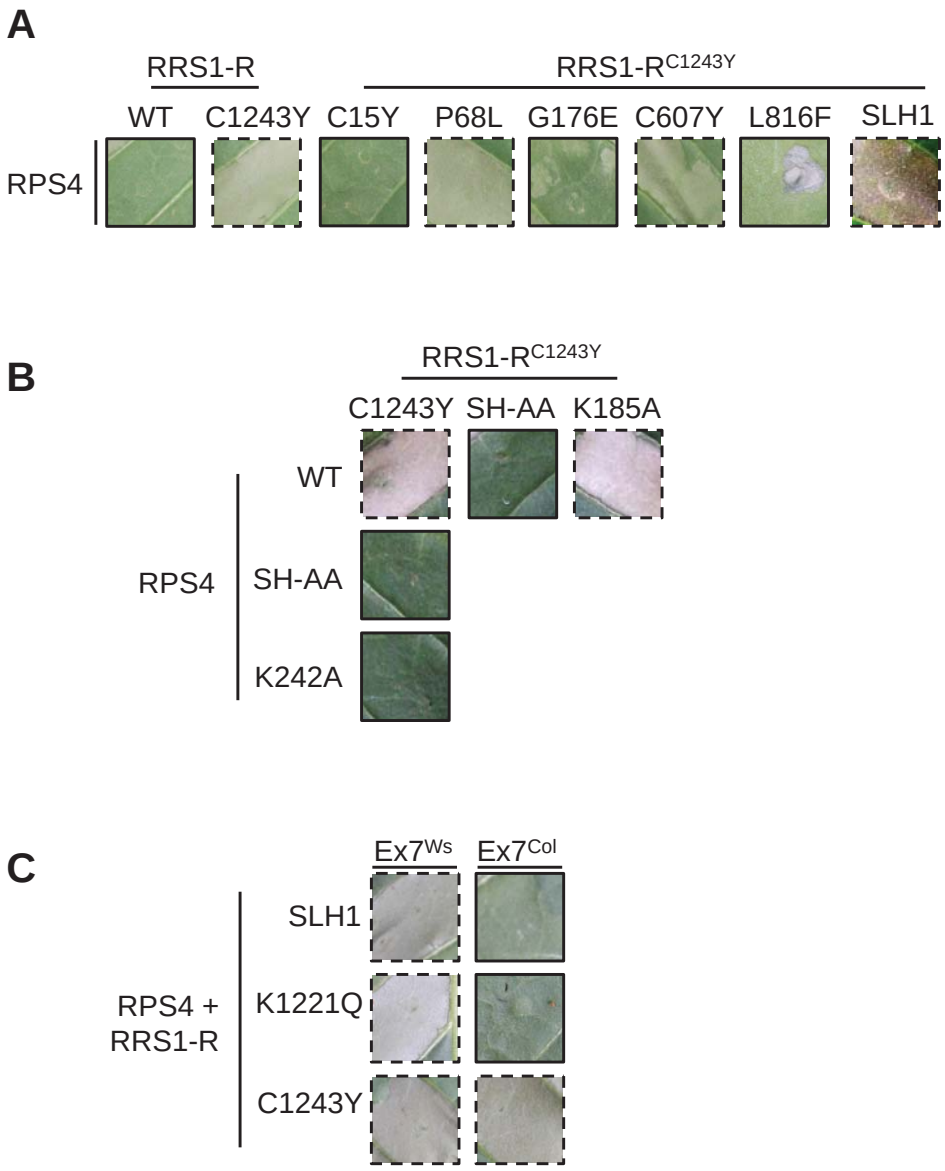


Figure 4.6. Characterization of RRS1^{C1243Y}-induced cell death in tobacco leaf cells. Photographs were taken 3 days after agro-infiltration. (A) C15, G176 and L816 residues are required for RRS1^{C1243Y}-induced cell death in the presence of RPS4. (B) RRS1^{C1234Y}-dependent programmed cell death requires RPS4 P-loop (K242A) and TIR/TIR domain hetero-dimerization (SH-AA) but not RRS1 P-loop (K185A). (C) Unlike SLH1 and K1221Q mutations, C1243Y confers auto-activity of the RRS1-S protein variant in the presence of RPS4.

suggests a mechanistic difference of auto-activation by the RRS1^{C1243Y} variant.

4.2.4 The NB-ARC and LRR domains are required for RRS1-R *in trans* interference with auto-activity in tobacco

The RRS1^{SLH1} allele is genetically recessive (Noutoshi et al., 2005). This recessive nature could be recapitulated in the heterologous tobacco system, as RRS1 but not GFP interfered with RRS1^{SLH1}-mediated RPS4-dependent PCD. It was suggested that the recessive nature of RRS1 is a result of an RRS1 allele acting as a "poison subunit" via *in trans* interference, such as RRS1-S interfering with RRS1-R PopP2 responsiveness or, as discussed here, RRS1 interfering with RRS1^{SLH1} auto-activity (Sohn et al., 2014). The requirements of RRS1 *in trans* interference were investigated.

In terms of RRS1 defense signaling, the SH motif but not the P-loop (K185) is required for RRS1 function. Conversely, for RRS1 *in trans* interference function, RRS1 P-loop but not the SH motif is required (Sohn et al., 2014). It was tested if RRS1^{WT} could also interfere with RRS1^{K1221Q} and RRS1^{C1243Y} auto-activity in tobacco. When co-expressed with RPS4 and RRS1^{SLH1}, RRS1^{K1221Q} or RRS1^{C1243Y}, RRS1^{WT} suppressed PCD, whereas RRS1^{K185A} had no effect (Figure 4.7A). The requirement of the SH motif for RRS1^{WT} interference with RRS1^{K1221Q} and RRS1^{C1243Y} auto-activity was then investigated, as well as the requirement of RRS1 SUSHI residues and the C-terminal extension for RRS1 interference with RRS1^{SLH1}, RRS1^{K1221Q} and RRS1^{C1243Y} auto-activity. Accordingly, RRS1 "suppressor" variants were co-infiltrated with an auto-active RRS1 variant and RPS4. The SH motif, known to be dispensable for the *in trans* interference of RRS1 with RRS1^{SLH1} auto-activity, was also not required for the *in trans* interference of RRS1 with RRS1^{K1221Q} and RRS1^{C1243Y} auto-activity (Figure 4.7B). Similarly, C15 (TIR) was not required for the *in trans* interference of RRS1 with RRS1^{SLH1} and RRS1^{K1221Q} auto-activity, and P68 (TIR) was not required for RRS1 interference with any of the auto-active RRS1 variants. Intriguingly, C15 was,

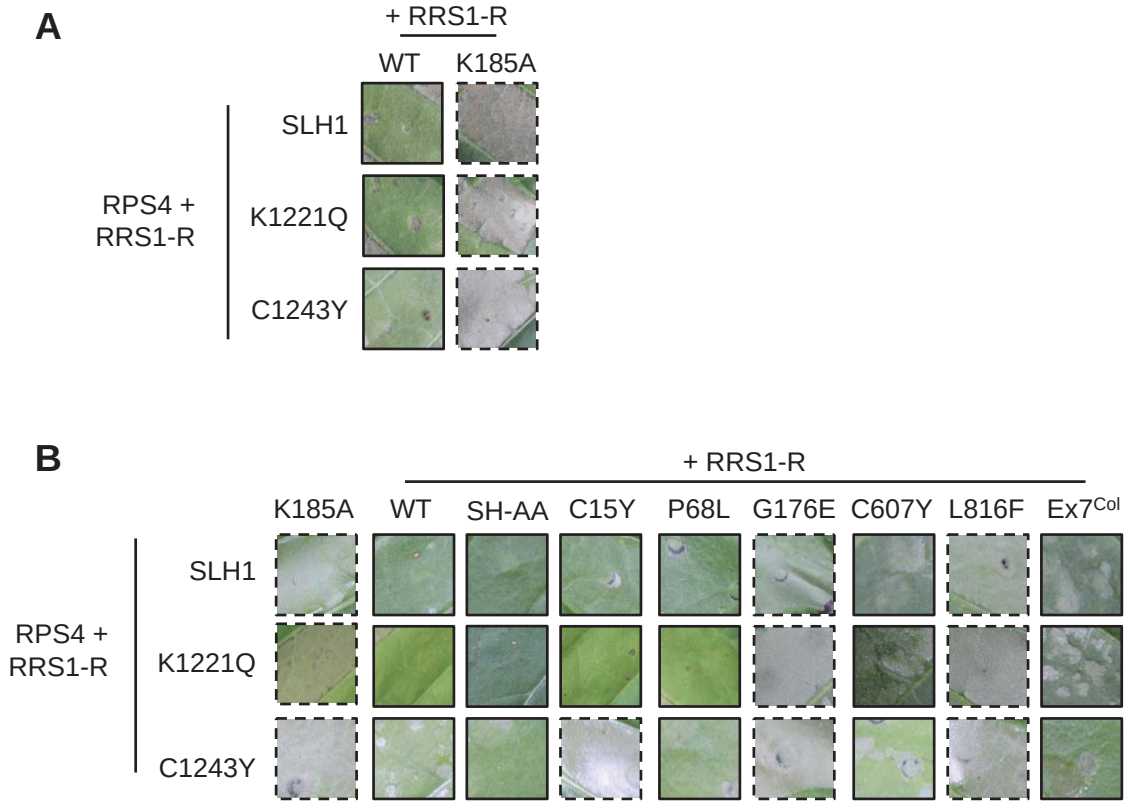


Figure 4.7. Characterization of the interference of RRS1-R with auto-active RRS1 variants in tobacco leaf cells. Photographs were taken 3 days after agro-infiltration. (A) The interference of $RRS1^{C1243Y}$-induced programmed cell death by RRS1 requires the P-loop motif (K185). (B) G176 and L816 are required for interference with $RRS1^{SLH1}$- and $RRS1^{K1221Q}$-induced programed cell death. C15, G176 and L816 are required for interference with $RRS1^{C1243Y}$-induced programmed cell death.

however, required for RRS1 interference with RRS1^{C1243Y}-mediated PCD. Apart from this exception, TIR mutations did not affect the *in trans* interference function of RRS1, suggesting that RPS4/RRS1 TIR/TIR heterodimerization is generally inessential for this function. G176 (NB-ARC) and L816 (LRR) residues were fully required for *in trans* interference of all three auto-active alleles, indicating a role of ATP-binding and the LRR domain in this unresolved mechanism. C607 and the C-terminal extension of RRS1-R may be partially required, indicated by the partial suppression of cell death induction. The patchy cell death could, however, be an artefact of the heterologous overexpression system used to assay for *in trans* interference. The residues shown to be required for cell death signaling via WT or auto-active RRS1 variants in tobacco, C15 (TIR), G176 (NB-ARC) and L816 (LRR) were also involved in the *in trans* interference function of the RRS1 NLR. Notably, the NB-ARC and LRR SUSHI residues, G176 and L816, were fully required for the *in trans* interference of RRS1 with all known RRS1 auto-active variants. This gives some insight into the molecular basis of RRS1 recessivity.

4.2.5 RRS1 TIR domain SUSHI residues are required for TIR domain function and heterodimer formation with RPS4

Two *sushi* mutants, *sushi84* (C15Y) and *sushi45* (P68L), were found to harbor mutations in the *RRS1* TIR domain. Functional characterization of RRS1^{SUSHI} variants demonstrated that C15, but not P68, is critical for RRS1 defense signaling in tobacco. The TIR mutants were further characterized in both TIR domain and full-length contexts. The N-terminal region of RPS4 (1-236), which comprises the TIR domain and a short stretch of the NB domain, induces effector-independent cell death in tobacco (Zhang et al., 2004; Swiderski et al., 2009). Co-expression of the RRS1 TIR domain (1-175) results in SH motif-dependent suppression of RPS4 TIR domain-mediated cell death (Williams et al., 2014). RRS1 TIR domain variants carrying the C15Y and P68L *SUSHI* mutations were investigated to test if they retained this suppressive activity. Firstly, expression of RRS1(1-175)^{C15Y} and RRS1(1-175)^{P68L} TIR variants alone did not induce cell death, similar to wild-type

RRS1(1-175) and RRS1(1-175)$^{SH-AA}$ (Figure 4.8A) (Williams et al., 2014). As expected, expression of RPS4(1-236) induced a cell death response and this could be suppressed by co-expression with RRS1(1-175) but not RRS1(1-175)$^{SH-AA}$. Like the RRS1(1-175)$^{SH-AA}$ variant that is impaired in heterodimer formation, RRS1(1-175)^{C15Y} and RRS1(1-175)^{P68L} also failed to suppress RPS4(1-236)-induced cell death (Figure 4.8A). This indicates that C15 and P68 might be required for RPS4/RRS1 TIR/TIR interaction or for the suppression activity of RRS1.

Thus, the effect of the *RRS1* TIR domain *SUSHI* mutations on RPS4/RRS1 TIR/TIR domain interaction was assayed in yeast cells and *in planta*. RPS4 and RRS1 TIR domains interact in yeast (Williams et al., 2014). Using a LexA-based yeast-two-hybrid (Y2H) assay, heterodimer formation of RRS1^{SUSHI} TIR domain variants as prey with RPS4 TIR domain as bait was tested. RPS4(1-236) was cloned into the LexA DNA-binding domain (DBD) vector, pLexA, to generate an RPS4 TIR-DBD fusion. RRS1(1-175) variants (WT, SH-AA, C15Y and P68L) were cloned into the B42 activation domain (AD) vector, pB42-AD, to generate RRS1 TIR-AD fusions. RPS4 TIR-DBD was assayed for interaction with RRS1 TIR-AD variants in yeast using *LEU2* and *lacZ* reporter genes regulated by upstream LexA operators. LEU2 allows growth in the absence of leucine and β-galactosidase, encoded by *lacZ*, hydrolyzes its substrate, X-Gal, to produce a blue-colored product. All mated yeast cells carried the three plasmids required for the assay (pLexA, pB42-AD and pSH18-34 *lacZ* reporter plasmid) as indicated by their growth on –His(H)/-Trp(T)/-Ura(U) medium (Figure 4.8B). In order to test for auto-activity, yeast cells co-expressing RPS4(1-236)-DBD and the AD alone (pB42-AD empty vector (EV)) were assayed for growth and the development of blue color on the induction medium, (-His(H)/-Trp(T)/-Ura(U) /-Leu(L)) + X-Gal. The reporter genes were not induced, as the yeast did not grow. As expected, yeast cells co-expressing RPS4(1-236)-DBD and RRS1(1-175)-AD fusion proteins grew well and developed a deep blue color due to the strong physical interaction between RPS4 and RRS1 TIR domains and subsequent induction of reporter genes (Figure 4.8B). Co-expression of RPS4(1-236)-DBD and RRS1(1-175)$^{SH-AA}$-AD fusion proteins, however, did not induce reporter genes and the

yeast cells did not grow as mutation of the SH motif impaired interaction. Likewise, yeast cells co-expressing RPS4(1-236)-DBD and RRS1(1-175)^{C15Y}-AD or RRS1(1-175)^{P68L}-AD variants did not grow, suggesting that the residues, C15 and P68, are involved in RPS4/RRS1 TIR/TIR heterodimerization. Yeast protein extraction and immuno-blotting showed that all RRS1(1-175) variants were expressed (Figure 4.8B).

The interaction of RRS1 TIR domain variants with RPS4 TIR domain was the assayed *in planta*. RRS1 TIR domain variants were fused to a C-terminal YFP tag and RPS4 TIR domain was fused to a C-terminal 6xHA tag. These constructs were co-expressed in *Nicotiana benthamiana* leaves by *Agrobacterium* delivery and the resulting proteins were immuno-purified. Interestingly, RPS4 TIR domain was pulled down with all RRS1 TIR domain variants (WT, SH-AA, C15Y and P68L), which demonstrates that these mutations do not significantly impair TIR/TIR heterodimer formation in this assay (Figure 4.8C). Perhaps this *in planta* overexpression assay is less sensitive to minor modifications of interaction. Following on from this, the interaction of full-length RRS1 carrying *SUSHI* mutations with RPS4 was assayed using a similar *in planta* CoIP assay. Full-length RRS1 was fused to a C-terminal 3xFLAG tag and full-length RPS4 was fused to a C-terminal 6xHA tag. In agreement with previous data, RRS1 physically associated with RPS4 and the interaction of RRS1^{SH-AA} with RPS4 was impaired (Figure 4.9) (Williams et al., 2014). All RRS1^{SUSHI} variants interacted with RPS4, but the interaction of RRS1^{C15Y} with RPS4 was significantly impaired and comparable to the interaction of RRS1^{SH-AA} with RPS4 (Figure 4.9). This suggests that the four non-TIR domain *SUSHI* mutations do not suppress RRS1^{SLH1} signaling by disrupting heterodimer formation in *N. benthamiana*. Despite impairing TIR/TIR domain interaction in yeast, the P68L mutation did not affect *in planta* interaction, but the effect of this mutation in the native Arabidopsis system cannot yet be deduced. It appeared that the C15 residue was responsible for RPS4/RRS1 heterodimer formation via the TIR domains.

To sum up, two *RRS1 SUSHI* mutations have been identified in the TIR domain, C15Y and P68L, which fully and partially restore the WT Arabidopsis

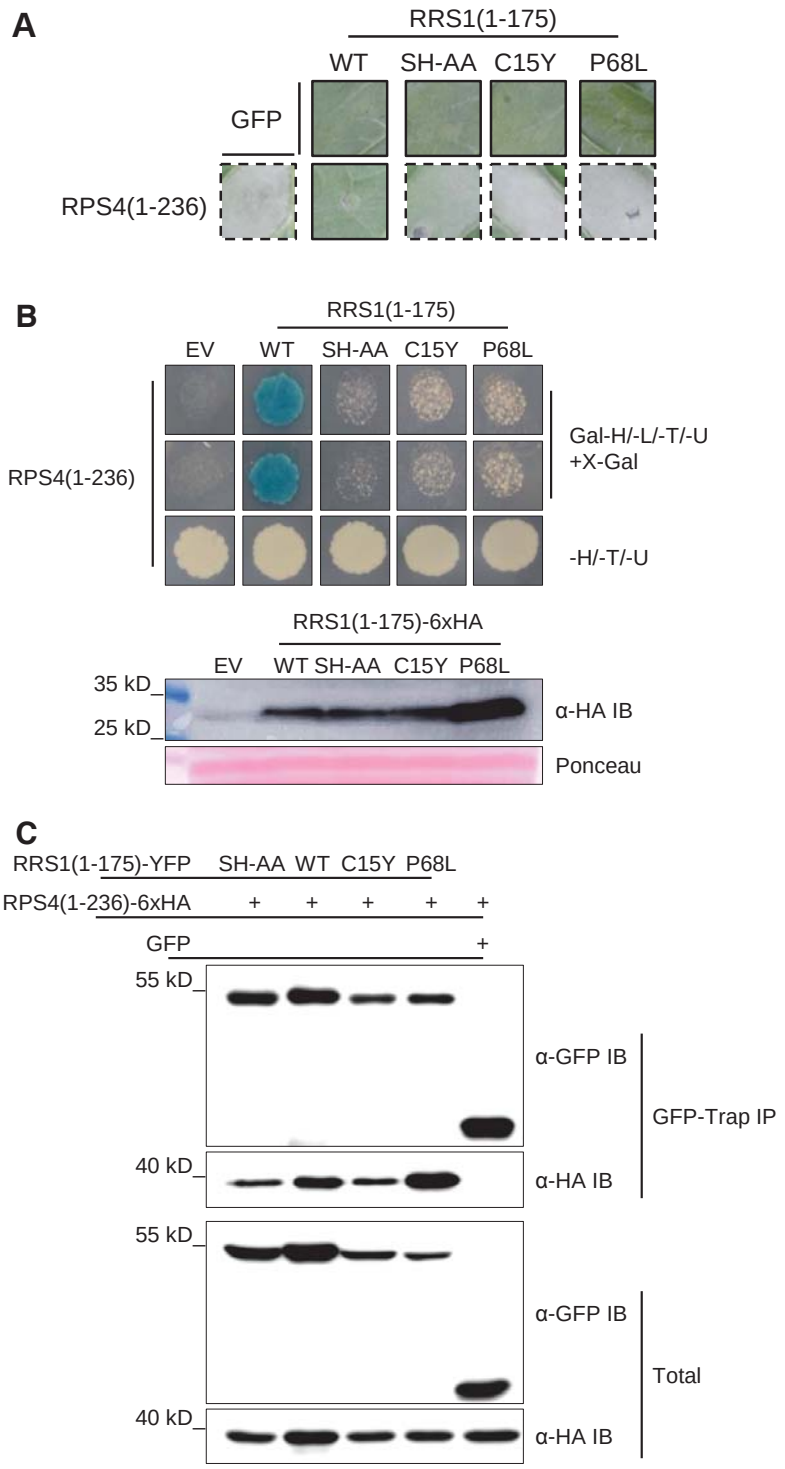


Figure 4.8. RRS1 TIR domain carrying SUSHI mutations can no longer suppress RPS4 TIR domain-induced cell death signaling in tobacco leaf cells. (A) RRS1(1-175) TIR domain wild type or carrying SH-AA, C15Y or P68L amino acid changes was co-expressed with GFP or with RPS4(1-236) TIR domain. Photographs were taken 3 days after agro-infiltration. (B) RRS1(1-175)^{C15Y} and RRS1(1-175)^{P68L} cannot interact with RPS4(1-236) when co-expressed in yeast cells. The lower panel shows the expression of RRS1(1-175) protein variants in yeast cells. (C) *In planta* interaction between RRS1(1-175) and RPS4(1-236) TIR domains after co-expression in tobacco leaf cells. The lower bands in the GFP blots are free GFP (27 kD). IB = immunoblot, IP = immunoprecipitation.

Cecile Segonzac helped with cloning and conducted CoIP for Figure 4.8C.

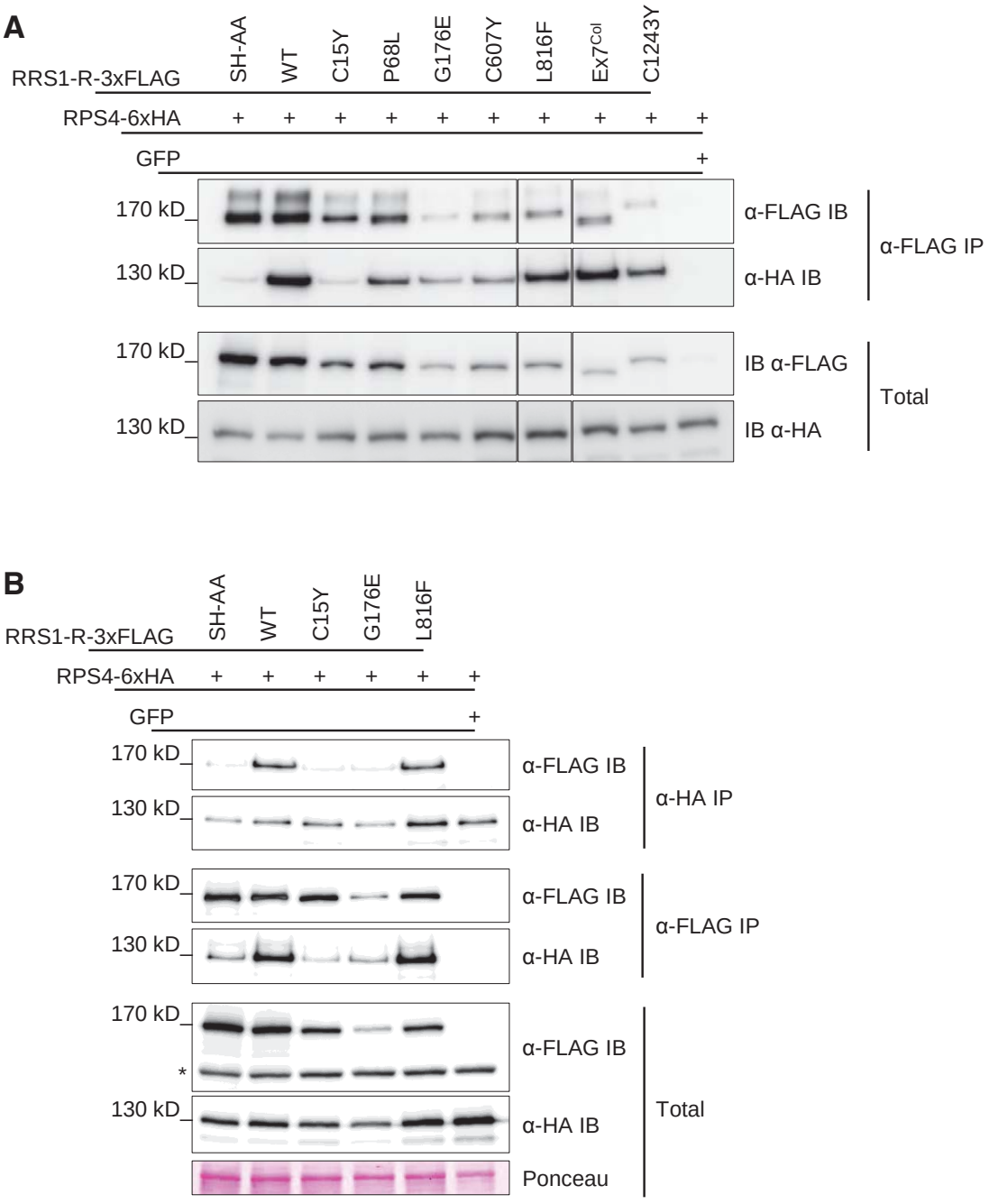


Figure 4.9. The C15 residue in RRS1 TIR domain is crucial for the interaction between RRS1-R and RPS4 *in planta*. Full length RRS1 protein variants fused with 3xFLAG tag were co-expressed with full length RPS4 fused with 6xHA tag in *N. benthamiana*. Total protein extracts were subjected to anti-FLAG or anti-HA immunoprecipitation. (A) All of the strong causal SUSHI variants and RRS1-S. (B) C15Y, G176E and L816F only.

Cecile Segonzac helped with cloning and conducted CoIPs for this figure.

phenotype, respectively. Both C15Y and P68L mutations abolish RRS1 TIR suppression of RPS4 TIR-mediated HR in tobacco and TIR/TIR heterodimerization in yeast. Furthermore, C15Y impairs full length RPS4/RRS1 heterodimer formation *in planta* and TIR/TIR heterodimer formation *in vitro*. The critical requirement of RPS4/RRS1 TIR/TIR interaction has been demonstrated previously (Sohn et al., 2014; Williams et al., 2014). Here, further evidence has been provided to support this and additional residues have been uncovered that are involved in heterodimerization and, thus, RRS1 function.

4.2.6 The NB-ARC and LRR domain SUSHI residues that are conserved in RRS1B are required for function in tobacco

A closely homologous NLR pair to RPS4 and RRS1, RPS4B and RRS1B, function cooperatively to confer recognition of AvrRps4 but not PopP2 (Saucet et al., 2015). Sequence analysis revealed that some of the residues mutated in the *sushi* mutants are conserved in RRS1B. Out of the six residues that were significantly required for $RRS1^{SLH1}$-induced autoimmunity, five of them are conserved in RRS1B (C12, P63, G167, C600 and C1211) (Figure 4.10, Table 4.1). The requirement of these SUSHI residues for RRS1B function was investigated. Initially, the auto-activity of RRS1B variants was tested by co-expressing with GFP and RPS4B. No cell death was induced by co-expression of RRS1B variants with GFP; however, $RRS1B^{C1211Y}$ induced PCD when co-expressed with RPS4B (Figure 4.11A). The cysteine to tyrosine WRKY mutation that rendered RRS1 auto-active in tobacco had the same effect on the closely related RRS1B. All other RRS1B variants were not auto-active. To assay for the effector recognition function of the mimic RRS1B SUSHI variants, RRS1B variants were co-expressed with RPS4B and AvrRps4. As expected, WT RRS1B induced a strong cell death response. $RRS1B^{C12Y}$ and $RRS1B^{P63L}$ TIR variants also induced PCD whereas $RRS1B^{G167E}$ and $RRS1B^{C600Y}$ did not. Additionally, it was discovered that RRS1B-mediated AvrRps4 recognition is dependent on its SH motif, as the $RRS1B^{SH\text{-}AA}$ variant failed to induce a PCD response when co-expressed with

RPS4B and AvrRps4 (Figure 4.11A). AvrRps4 recognition by RRS1B^{C12Y} was rather intriguing given the RRS1^{C15Y} phenotype in tobacco (Figure 4.4). After finding that RRS1B^{C1211Y} was auto-active, the effect of other mimic *SUSHI* mutations on this auto-activity function was also investigated. The same residues that were required for AvrRps4 recognition were also required for RRS1B^{C1211Y} auto-activity (SH motif (TIR domain), G167 (NB-ARC domain) and C600 (LRR domain)). C12Y and P63L weakened but did not fully abolish the cell death response (Figure 4.11A). All RRS1B variants were expressed in *N. benthamiana* as demonstrated by immunodetection (Figure 4.11C)

It was recently demonstrated that RRS1 and RRS1B require their appropriate co-receptor to confer AvrRps4 recognition (Saucet et al., 2015). In other words, RRS1 does not function with RPS4B and RRS1B does not function with RPS4. Whether this specificity also applies to RRS1 or RRS1B autoimmune alleles remained unknown. This was investigated by co-expressing known autoimmune RRS1 (RRS1^{SLH1}, RRS1^{K1221Q}, RRS1^{C1243Y}) or RRS1B (RRS1B^{C1211Y}) variants with RPS4 or RPS4B. Additionally, an acetyl lysine mimic RRS1B variant was generated by mutation of the corresponding critical WRKY lysine residue, lysine 1911, to glutamine (K1191Q). RRS1B^{K1191Q} was assayed for auto-activity with both RPS4 and RPS4B. Consistent with the effector recognition function, all RRS1/RRS1B auto-active variants required their appropriate signaling partner for cell death activation in tobacco. RRS1B^{C1211Y} induced PCD when co-expressed with RPS4B but not RPS4 and, conversely, RRS1^{SLH1}, RRS1^{K1221Q} and RRS1^{C1243Y} induced PCD when co-expressed with RPS4 but not RPS4B. Moreover, the acetyl lysine mimic RRS1B variant, RRS1B^{K1191Q}, did not induce PCD with RPS4 or RPS4B (Figure 4.11B). The RPS4B/RRS1B NLR pair do not recognize the *R. solanacearum* acetyltransferase effector, PopP2, and this result indicates that the B pair lacks PopP2-induced signaling capacity regardless of lysine acetylation.

Of the four SUSHI residues significantly required for RRS1^{SLH1}-induced autoimmunity in Arabidopsis and conserved in RRS1B, two were shown to be involved in the RRS1 signaling function in tobacco, C15 and G176. In a full

length RPS4/RRS1 CoIP, it was shown that C15Y but not G176E impairs heterodimer formation. This was supported by Y2H and *in vitro* data. Therefore, whether the corresponding mutations in RRS1B, C12Y and G167E, affected heterodimer formation with RPS4B was investigated using an *in planta* CoIP assay. RRS1B interacted with RPS4B; however, RRS1B$^{SH\text{-}AA}$ interaction with RPS4B was significantly impaired suggesting that the SH motif also plays a role in TIR/TIR domain heterodimerization in the B pair. Furthermore, this assay revealed that RRS1B^{C12Y} but not RRS1B^{G167E} was impaired in RPS4B interaction (Figure 4.11D). The TIR cysteine residue is, therefore, required for heterodimer formation between signaling and sensor NLRs in both RRS1 and RRS1B. Intriguingly, RRS1B^{C12Y} was functional for AvrRps4 recognition and RRS1B$^{C12Y/C1211Y}$ retained auto-activity whereas C15Y fully abolished effector recognition and auto-active functions of RRS1.

Figure 4.10. A schematic of RRS1B displaying mimic causal *SUSHI* mutations.
Mimic causal non-synonymous non-stop codon mutations in RRS1B: TIR domain C12Y and P63L, NB-ARC domain G167E, LRR domain C600Y and WRKY domain C1211Y. See Table 4.1.

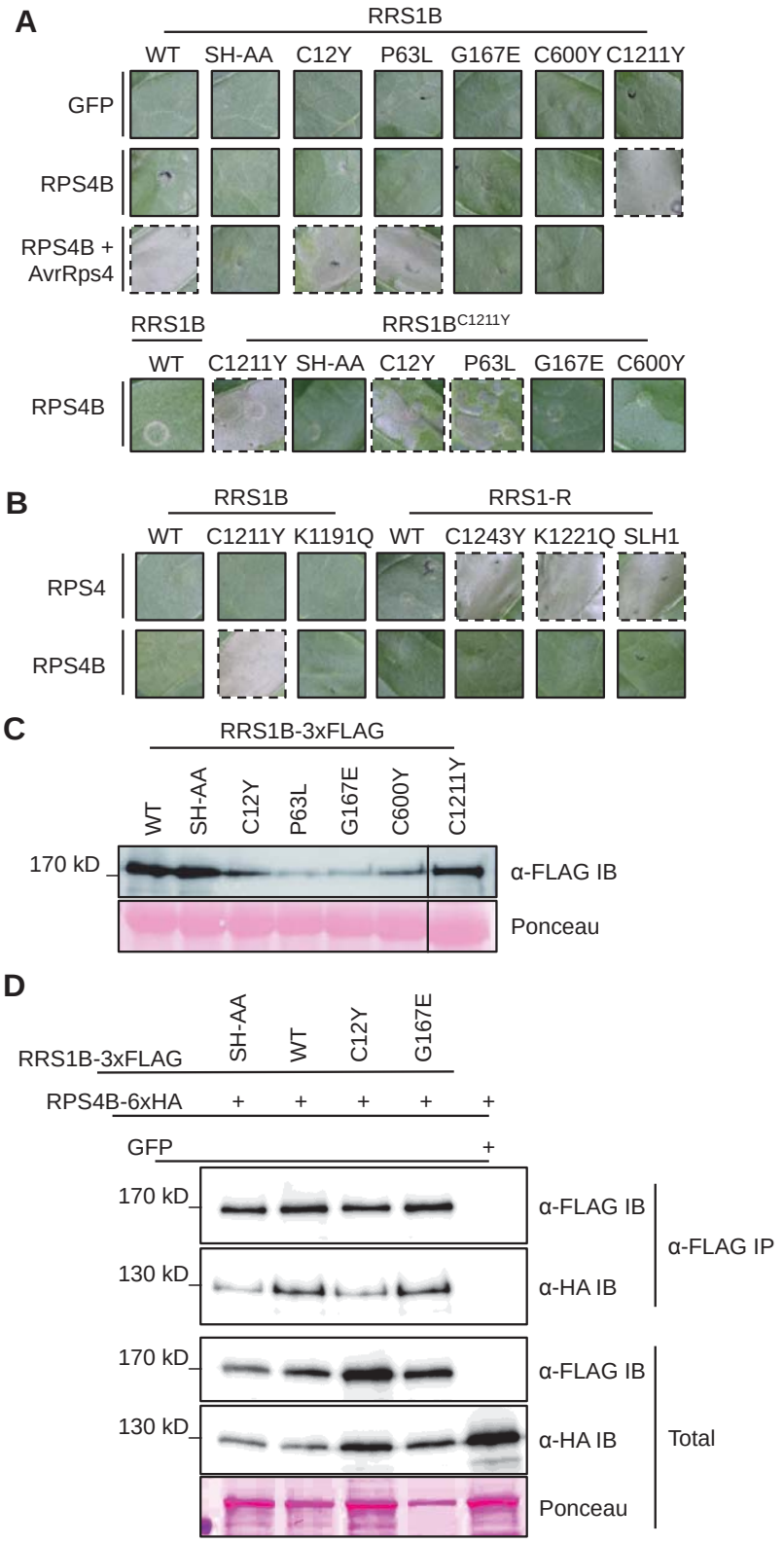


Figure 4.11. Corresponding *SUSHI* mutations in *RRS1B* affect its function. (A) Co-expression of RRS1B variants with GFP, RPS4B, or RPS4B and AvrRps4. (B) Auto-activity requires cognate co-receptor. Known or putative auto-active RRS1 and RRS1B variants were co-expressed with RPS4 or RPS4B. (C) A Western blot to demonstrate that all RRS1B protein variants were expressed. (D) Co-immunoprecipitation (CoIP) assay showing that RRS1B^{C12Y} is impaired in RPS4B interaction.

Cecile Segonzac helped with cloning and conducted CoIP for Figure 4.11D.

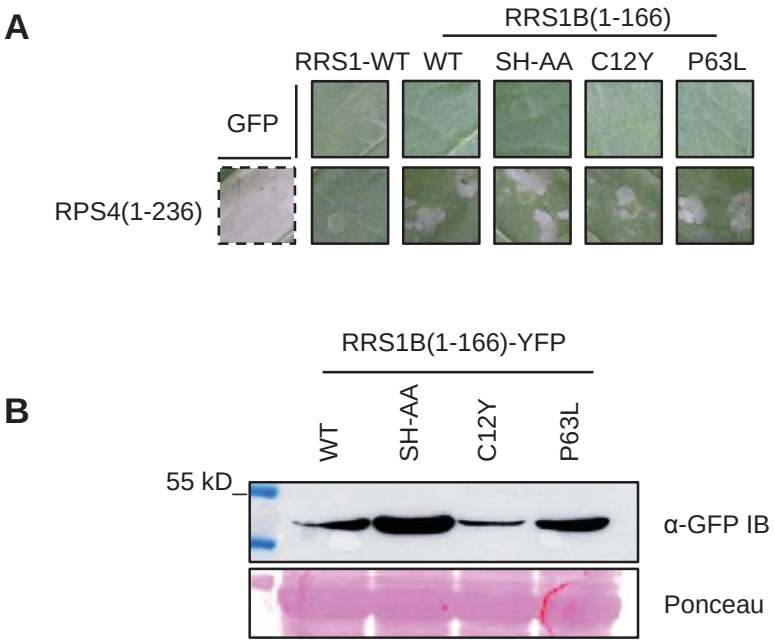


Figure 4.12. RRS1B TIR mutations do not significantly impair the partial suppression of RPS4 TIR-induced HR. (A) The TIR domains of RRS1, RRS1B and RRS1B variants were co-expressed with GFP or RPS4(1-236) in tobacco. (B) Expression of all RRS1B(1-166)-YFP variants in *N. benthamiana* leaves as demonstrated by a Western blot with anti-GFP antibodies.

This suggests a functional difference between RRS1 and RRS1B TIR domains as RPS4B/RRS1B can still function despite the RRS1B C12Y-induced TIR/TIR domain dissociation. It was demonstrated that, unlike the RPS4 TIR domain, the RPS4B TIR domain does not induce effector-independent PCD (Saucet et al., 2015). The RRS1B TIR domain might, therefore, play a different role in terms of regulation than its homologous counterpart. The RRS1B TIR domain can, however, suppress RPS4 TIR domain-induced cell death (Saucet et al., 2015). It was shown here that this suppression is not dependent on the SH motif, C12 or P63 unlike RRS1 TIR domain suppression of RPS4 TIR domain-induced PCD, which is fully dependent on the SH motif, C15 and P68 (Figure 4.12). Overall, these results suggest important mechanistic similarities and differences between RPS4/RRS1 and RPS4B/RRS1B immune complex activation.

4.2.7 RRS1 SUSHI mutations do not affect TIR domain-C-terminus intramolecular interaction

The TIR domain mutations, C15Y and possibly P68L, affected RPS4/RRS1 heterodimer formation. A CoIP assay revealed that the four other *SUSHI* mutations did not impair intermolecular interactions with RPS4. Whether RRS1 also formed intramolecular interactions was unknown. If RRS1 does, indeed, form intramolecular interactions that are required for function, it is plausible that one or more of the SUSHI residues are responsible for these associations. Firstly, the RRS1 TIR domain (1-175 aa) was assayed for interaction with the RRS1 C-terminal region including the WRKY domain (coded for by exons 5, 6 and 7; hereafter, Ex5-7) using an *in planta* CoIP assay. Interestingly, a strong *in trans* interaction was observed between these RRS1 domains (Figure 4.13).

The effect of *SUSHI* mutations on this interaction was then investigated by introducing TIR domain (C15Y and P68L) and Ex5-7 (C1243Y) mutations, and assaying the variants for interaction. To further characterize the RRS1 TIR domain:Ex5-7 intramolecular interaction, $TIR^{SH\text{-}AA}$, $Ex5\text{-}7^{SLH1}$ and $Ex5\text{-}7^{K1221Q}$ were also assayed for interaction. It was found that the RRS1 TIR domain and Ex5-7 could physically associate irrespective of any mutations (Figure 4.13). C15, P68 and C1243 are, therefore, not required for TIR domain:Ex5-7 interaction. As the RRS1 TIR domain physically associated with the RRS1 C-terminal region, Ex5-7, it was tested if the RPS4 TIR domain (1-250 aa) could also form an interaction with RRS1 Ex5-7. Using a CoIP assay, it was found that the RPS4 TIR domain also physically associated with Ex5-7. As with RRS1 TIR domain:Ex5-7 interaction, the Ex5-7 mutations S983F, K1221Q, SLH1 and C1243Y did not affect interaction with the RPS4 TIR domain (Figure 4.14A).

Furthermore, RPS4 TIR domain *SUSHI* mutations that were previously characterized were assayed for their effect on this intermolecular interaction (Sohn et al., 2014). It was shown that R28H, A38V, P105L and G120R did not impair RPS4 TIR domain:RRS1 Ex5-7 interaction; however, L101F appeared

to attenuate the association (Figure 4.14B). The RPS4 TIR domain E88K variant fused to a C-terminal GFP tag was not well expressed in the total plant extract and, therefore, the effect of this mutation on RPS4 TIR domain:RRS1 Ex5-7 interaction could not be accurately determined. The E88K mutation may result in destabilization of the RPS4 TIR domain protein.

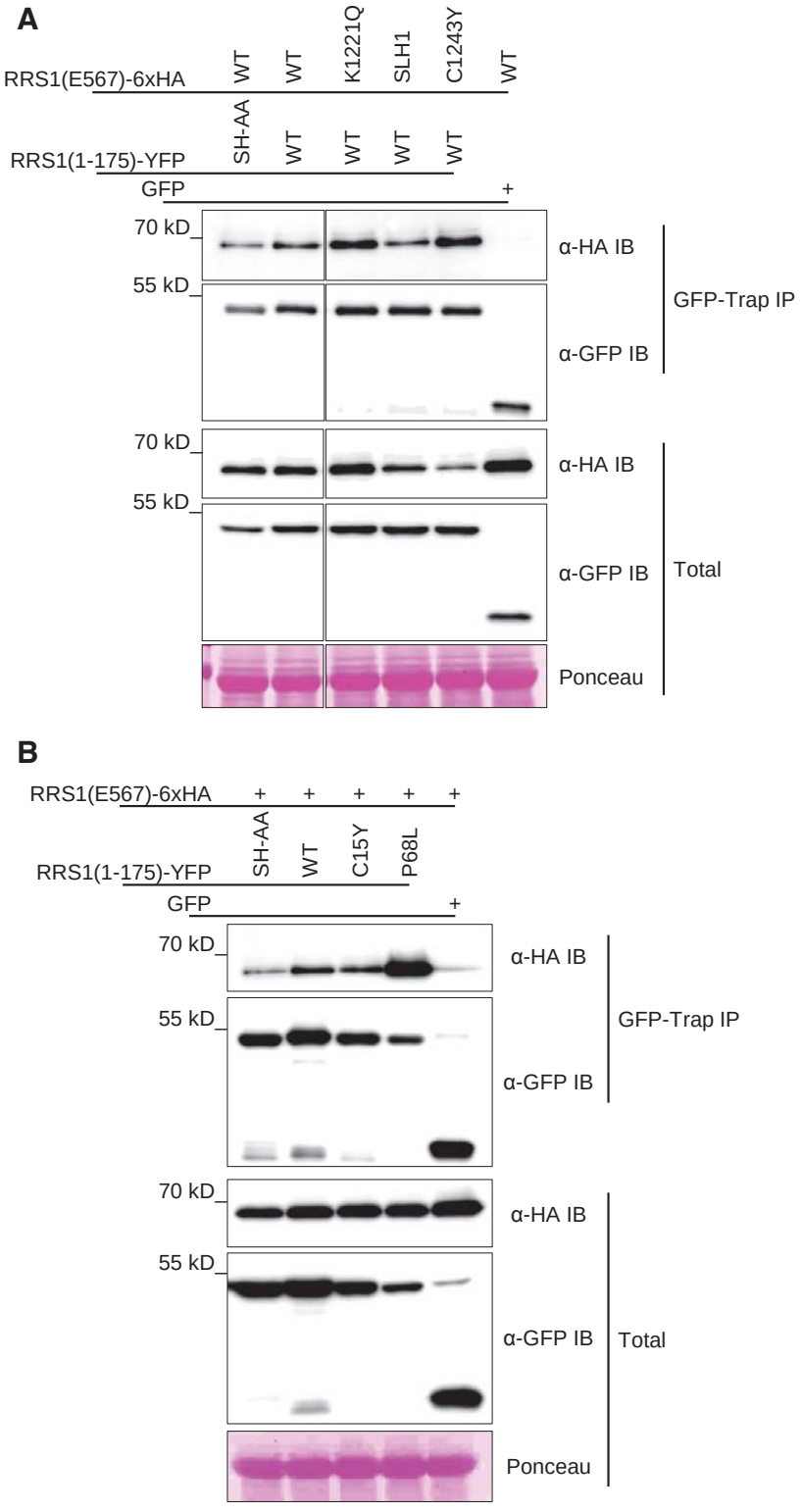


Figure 4.13. Intramolecular interaction between RRS1(1-175) TIR domain and RRS1 C-terminus (Ex5-7). (A) RRS1 TIR WT CoIP interaction assay with Ex5-7 SUSHI variants. (B) RRS1 TIR SUSHI variants CoIP interaction assay with RRS1 Ex5-7 WT. The lower bands in the GFP blots are free GFP (27 kD). Both experiments were performed twice.

Cecile Segonzac helped with cloning and conducted CoIPs for this figure.

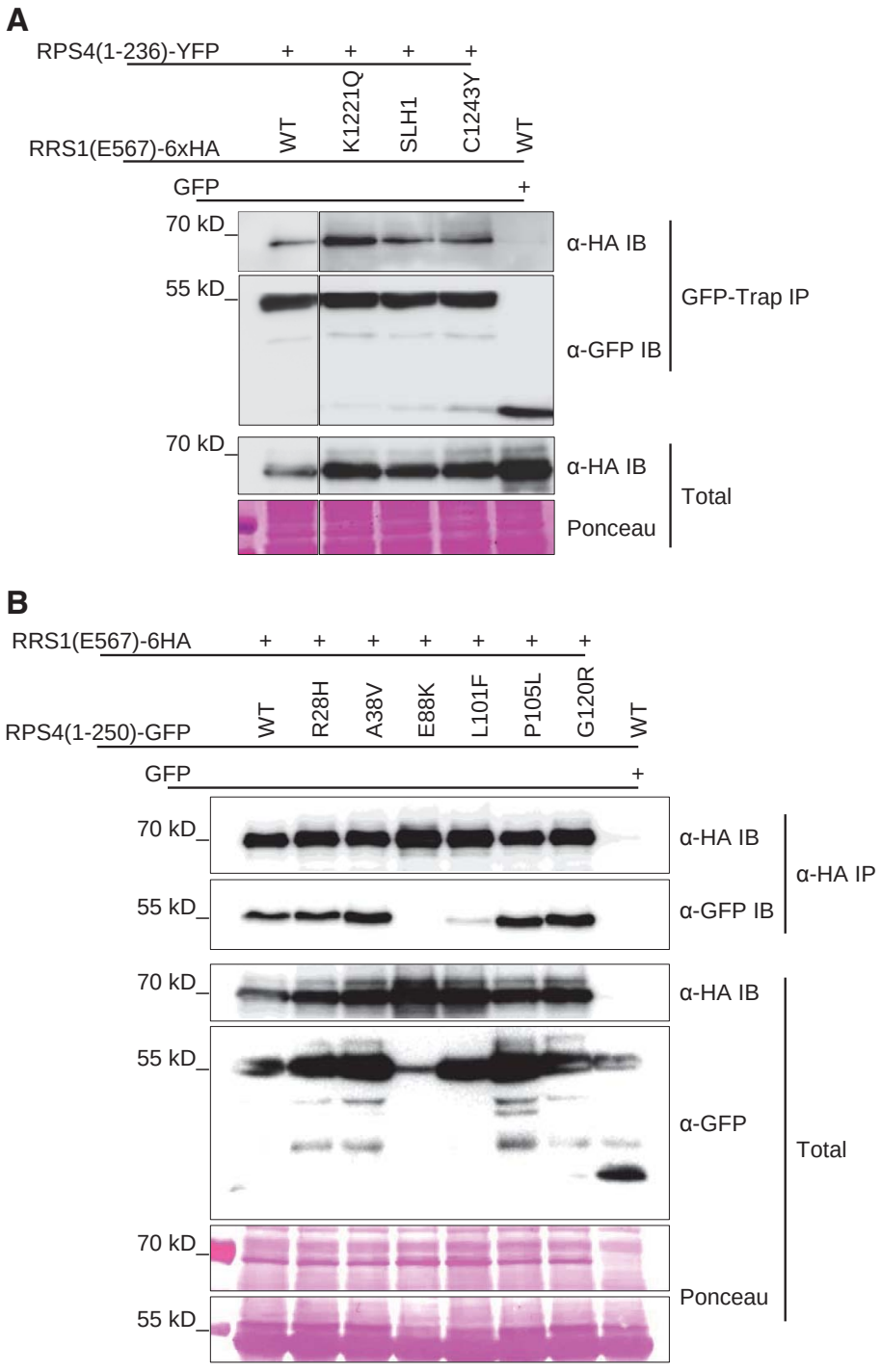


Figure 4.14. Intermolecular interaction between RPS4 TIR domain and RRS1 Ex5-7. (A) RPS4(1-236) TIR WT CoIP interaction assay with RRS1 Ex5-7 SUSHI variants. (B) RPS4 TIR(1-250) SUSHI variants CoIP interaction assay with RRS1 Ex5-7 WT. The lower bands in the GFP blots are free GFP (27 kD). Both experiments were only performed once.

Cecile Segonzac helped with cloning and conducted CoIPs for this figure.

4.3 Discussion

4.3.1 Identification of RRS1 *sushi* mutants and tobacco HR characterization.

Since the initial characterization of *RPS4* and *RRS1* nearly 20 years ago, much has been learned about the function of these NLRs; however, there are mechanistic details that still remain unsolved (Deslandes et al., 1998; Gassmann et al., 1999). A forward genetic screen has enabled detailed insights into the molecular mechanism of RPS4/RRS1 regulation and activation (Sohn et al., 2014). Overall, 46 intragenic *RPS4 sushi* mutants were identified whereas only 19 intragenic *RRS1 sushi* mutants were identified, despite *RRS1* being approximately 2kb longer than *RPS4*. From this, it can be inferred that *RPS4* is more sensitive to SNPs than *RRS1*. In other words, *RRS1* can accommodate many SNPs and retain function, whereas *RPS4* cannot, suggesting that RRS1 is more flexible to structural modifications than RPS4.

Of the 16 *RRS1 sushi* mutants, 8 carried homozygous, non-synonymous, non-stop codon mutations. The mutant plants were morphologically identifiable when compared to *slh1* plants, as the wild-type (WT) phenotype was at least partially restored. This was associated with a suppression of defense gene upregulation. Genetic crosses of the *sushi* mutants to *rrs1-1* knockout (KO) plants indicated that 6 of the 8 *sushi* mutations caused a significant suppression of $RRS1^{SLH1}$-mediated immunity and, therefore, functional characterization focused on these RRS1 variants. The transient Agro-infiltration assays in tobacco revealed some fascinating results, the first of which was the auto-activity of the $RRS1^{C1243Y}$ variant when co-expressed with RPS4. The mutant, *sushi87,* which harbored the $RRS1^{SLH1/C1241Y}$ variant, grew similarly to WT No-0. Therefore, the WRKY domain cysteine to tyrosine mutation suppressed the *slh1* phenotype in Arabidopsis; however, co-expression of $RRS1^{C1243Y}$ and RPS4 in tobacco elicited an effector-independent programmed cell death (PCD) response. This result demonstrates a differential response in the two systems. All WRKY domains

contain a zinc-finger motif and C1241/C1243 is, in fact, the first conserved cysteine residue of the RRS1 WRKY domain zinc-finger motif (C-X_5-H-X-C-X_{23}-H-X-H). Zinc-finger motifs have been shown to be involved in DNA binding and protein-protein interaction (Takatsuji, 1998).

Another interesting finding from the tobacco transient assay was that the majority of sushi mutations did not disable $RRS1^{SLH1}$ signaling. Only C15Y (TIR) and L816F (LRR) suppressed $RRS1^{SLH1}$ auto-activity in tobacco, suggesting that while important insights can be gained using this system, it must be considered that the native Arabidopsis phenotype is not always recapitulated. Likewise, only C15Y and L816F suppressed $RRS1^{K1221Q}$ auto-activity. Intriguingly, an uncoupling of auto-active and effector recognition functions of RRS1 was discovered. Assaying the effect of *RRS1 SUSHI* mutations on RPS4/RRS1-mediated effector recognition (both AvrRps4 and PopP2) revealed that C15Y and G176E (NB-ARC), but not L816F, impaired effector recognition in tobacco. This is the first example of single nucleotide polymorphisms (SNPs) that discriminate between auto-activity and effector recognition in a plant NLR.

4.3.2 Characterization of the auto-active $RRS1^{C1243Y}$ variant.

As discussed previously, the $RRS1^{C1243Y}$ variant was auto-active in tobacco. Investigation of the molecular basis of this unexpected auto-activity revealed some interesting findings. It was demonstrated that C15 was fully required for $RRS1^{C1243Y}$-induced PCD, whereas G176 and L816 were partially required. Interestingly, C15 and L816 were involved in signaling mediated by the other auto-active alleles; however, G176 was not (Figure 4.3B). This is not the only distinct feature of $RRS1^{C1243Y}$ auto-activity that was discovered. $RRS1^{C1243Y}$ also induced a significantly stronger cell death response than $RRS1^{SLH1}$ and $RRS1^{K1221Q}$ (Figure 4.15) and was not dependent on the RRS1-R C-terminal extension, unlike $RRS1^{SLH1}$ and $RRS1^{K1221Q}$ (Figure 4.6C).

As mentioned in the introduction, the No-0 and Ws-2 RRS1 alleles differ only by a 2 amino acid insertion in exon 4 of the Ws-2 allele. Although this insertion does not affect the ability to recognize PopP2 or AvrRps4, there remains a possibility that it affects regulation of RRS1 in such a way that RRS1^{C1243Y} (Ws-2) is auto-active yet RRS1^{C1241Y} (No-0) is not. Therefore, it would be informative to assay cell death induction of the No-0 RRS1 allele carrying the C1241Y mutation when co-expressed with RPS4 in tobacco.

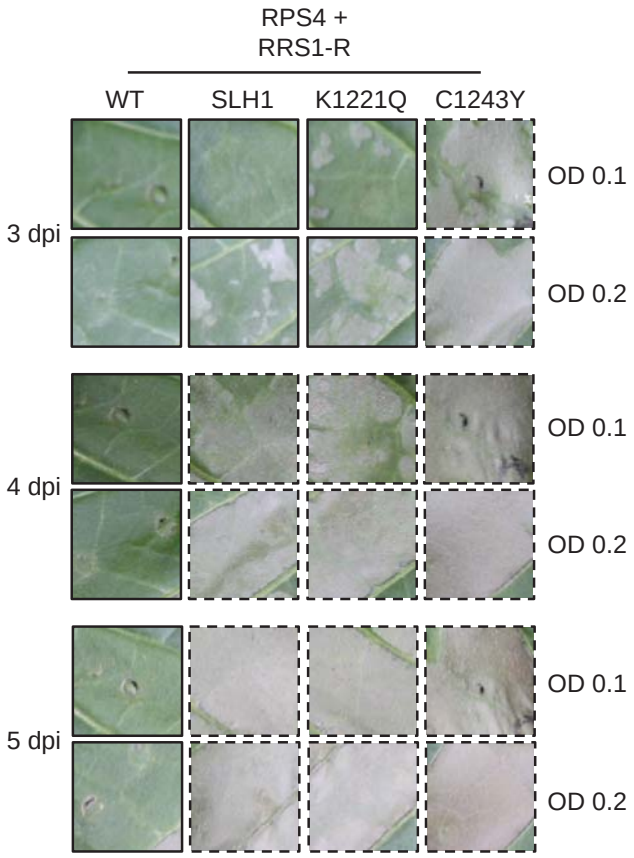


Figure 4.15. RRS1^{C1243Y} induces a stronger cell death response than other known auto-active RRS1 alleles. RPS4 was co-expressed with RRS1 WT, RRS1^{SLH1}, RRS1^{K1221Q} and RRS1^{C1243Y} by *Agrobacterium* delivery at OD_{600} = 0.1 and 0.2. Photographs were taken at 3 dpi, 4 dpi and 5 dpi.

4.3.3 The effect of *SUSHI* mutations on *in trans* interference and inter/intramolecular RRS1/RPS4 interactions

The previously demonstrated RRS1 *in trans* interference activity was further characterized (Sohn et al., 2014). Most notably, the particular domains required for this mechanism were defined as RRS1 NB-ARC and LRR domains. The exact mechanism by which this occurs remains elusive; however, this study has advanced our understanding of the RRS1 requirements for *in trans* interference function.

The RRS1^{C15Y} phenotype corroborates previous evidence of the requirement of RPS4/RRS1 TIR/TIR domain interaction (Williams et al., 2014). It is plausible that the cysteine (C) to tyrosine (Y) mutation affects the positioning or angle of the αA helix; the tyrosine aromatic side chain would likely clash with arginine (R) 20 positioned at the start of the αA helix, thus disrupting the RPS4/RRS1 heterodimer (Williams et al., 2014). Interestingly, analysis of the close *RRS1* orthologues and progenitors as discussed in Saucet et al. (2015) revealed that this TIR cysteine residue is conserved in all, suggesting that it may have been maintained due to its critical requirement.

4.3.4 *RRS1B* corresponding *SUSHI* mutations

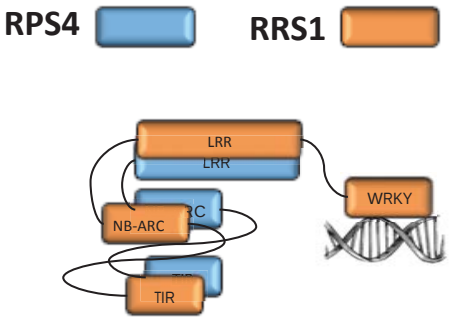
As a result of investigating the effect of *SUSHI* mutations on RRS1B function, some interesting comparisons between RRS1 and RRS1B can be drawn. Perhaps most intriguingly, a difference in the effect of the TIR domain cysteine to tyrosine mutation, C15Y and C12Y in RRS1 and RRS1B, respectively, was observed. In both cases, the TIR domain cysteine is required for TIR/TIR domain heterodimer formation with the appropriate signaling partner; however, only RRS1 is dependent on the TIR domain cysteine for signaling function.

4.3.5 Models of the effect of *RRS1 SUSHI* mutations

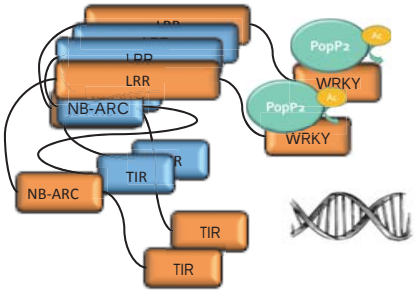
Overall, 4 *SUSHI* mutations with particularly interesting effects on RRS1 function have been identified: C15Y (TIR), G176E (NB-ARC), L816F (LRR) and C1243Y (WRKY). Unequivocal evidence has been accumulated that shows that the TIR domain C15Y mutation impairs heterodimer formation with the RPS4 TIR domain. Therefore, it can be hypothesized that the effect on RRS1 function is a result of the loss of sensor NLR (RRS1) communication with the activator/transducer NLR (RPS4), which is required for RPS4/RRS1 complex activation (Figure 4.16, Model 3).

The NB-ARC domain G176 residue may be required for conformational changes within the RRS1 protein to the active state (Figure 4.16, Model 4). The LRR domain of the potato NLR Rx has been demonstrated to interact with the ARC domain to maintain an auto-inhibited state (Rairdan and Moffett, 2006). The L816F mutation results in an amino acid change from leucine, with an aliphatic hydrophobic side chain, to phenylalanine, with an aromatic hydrophobic side chain. This mutation may strengthen LRR/ARC domain auto-inhibition and this can be overcome by AvrRps4/PopP2 interaction but RRS1 carrying an auto-active mutation (SLH1 and K1221Q) remains in a clamped-like inactive state (Figure 4.16, Model 5).

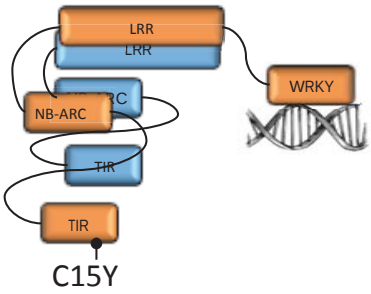
Finally, it can be hypothesized that the WRKY domain C1243Y mutation that resides within a zinc finger motif may disrupt DNA binding and structure of the WRKY domain, thus altering the conformation of the RPS4/RRS1 complex thereby inducing auto-activity in tobacco (Figure 4.16, Model 6). Why the Arabidopsis *sushi87* mutant harboring the $RRS1^{SLH1/C1243Y}$ allele was not autoimmune remains puzzling, although it is likely that the presence of C1243Y neutralizes the SLH1 mutation (a single leucine insertion after L1224) and vice versa in Arabidopsis.



1) Inactive signaling-competent complex.

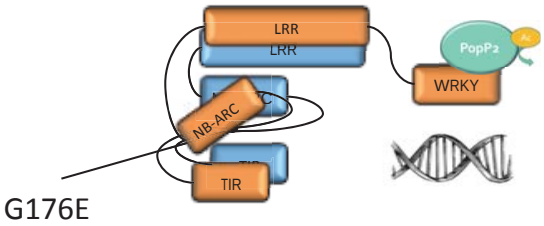


2) Active complex.



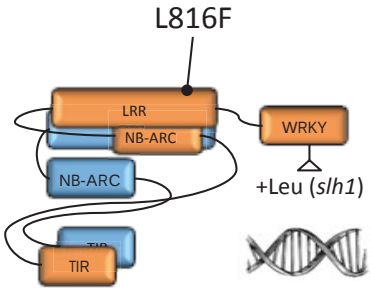
3) C15Y impairs heterodimer formation.

- Loss of sensor-activator communication



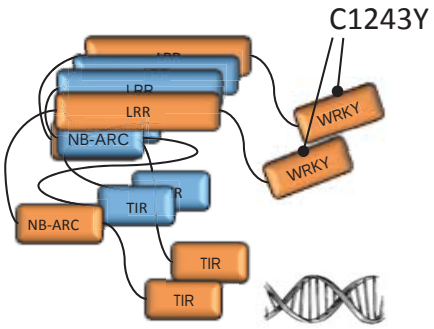
4) G176E impairs conformational change.

- Disabled molecular switch



5) L816F retains RRS1 in auto-inhibited state.

- RRS1 'clamped'



6) C1243Y dissociates WRKY domain from DNA & disrupts WRKY domain structure.

Figure 4.16. Models of the effect of *RRS1 SUSHI* mutations.
Schematics depicting inactive and active RPS4/RRS1 complexes as well as the proposed effect of 4 interesting amino acid changes resulting from *SUSHI* mutations: C15Y, G176E, L816F and C1243Y. Model 1) RPS4/RRS1 form a complex bound to the DNA via the RRS1 WRKY domain. The complex is inactive but signaling-competent. Model 2) After acetylation by the PopP2 effector, the RRS1 WRKY domain dissociates from DNA and structural reconfiguration occurs, allowing RPS4 TIR/TIR domain homodimer formation and downstream signaling. Model 3) The RRS1 TIR C15Y mutation impairs RRS1 TIR domain heterodimer formation with the RPS4 TIR domain. This results in a loss of sensor (RRS1) – activator (RPS4) communication. Model 4) The G176E NB-ARC mutation may impair the ability of RRS1 to switch from the inactive to active state upon effector recognition. Model 5) The LRR domain L816F mutation may enhance auto-inhibition so much that auto-active mutations cannot activate the complex as normal. Model 6) The WRKY domain C1243Y mutation that disrupts the zinc finger motif likely affects DNA binding and conformation of the WRKY domain. These models are applicable to both Arabidopsis and tobacco.

4.3.6 Is RRS1 a functional transcription factor?

Since the initial characterization of RRS1, its modular structure and function thereof has been both intriguing and puzzling. RRS1 comprises typical NLR domains (TIR, NB-ARC and LRR) yet with an additional WRKY domain characteristic of transcription factors. In fact, RRS1 is the first R protein containing a group III conserved WRKY domain (Deslandes et al., 2002). Logically, the structure of RRS1 suggests a fascinating “shortcut” in immune signaling components – an immune receptor that directly reprograms transcription upon effector recognition.

Deslandes et al. (2002) suggested that activation of the C-terminal WRKY domain upon effector perception might induce a signaling cascade or direct activation of immunity-related genes. The same group later demonstrated that nuclear localization of RRS1 is dependent on PopP2, suggesting that the default state of RRS1 is in the cytoplasm, thus preventing WRKY domain DNA binding. Upon *R. solanacearum* infection, the NLS-harboring effector PopP2 interacts with RRS1, which is then localized to the nucleus via a “piggyback mechanism” enabling transcriptional activity of the WRKY domain to activate expression of defense genes (Deslandes et al., 2003). This was a compelling hypothesis; however, it has since been shown that RRS1 and RPS4 localize to the nucleus independent of PopP2 (or AvrRps4) and that RRS1 associates with DNA in the absence of PopP2 (Le Roux et al., 2015; Sarris et al., 2015; Huh et al., 2017). The idea that RRS1 may act as a transcriptional activator was initially suggested following characterization; however, Noutoshi et al. (2005) first put forward the suggestion that RRS1 may in fact function as a transcriptional repressor. This model was inspired by their study on the Arabidopsis *slh1* mutant, which carries a single leucine insertion in the RRS1 WRKY domain resulting in impaired RRS1-DNA binding and constitutive defense activation. It was proposed that RRS1 might bind to the promoter of defense-associated genes and repress their expression. Upon effector recognition or in the case of the *slh1* allele, de-repression of defense genes occurs and, therefore, a defense response is elicited. Noutoshi et al. (2005) also proposed a model whereby the WRKY domain functions as a

guardee, which is modified by PopP2 causing activation of the RRS1 NLR. This guard model was also supported by a study demonstrating that PopP2 physical interaction with RRS1 is not sufficient for recognition; acetyltransferase activity is additionally required (Tasset et al., 2010). Tasset et al. (2010) also suggested an alternative whereby RRS1 functions as an "enabler" of PopP2 activity that facilitates in the targeting of host components, which ultimately results in defense activation following perception of PopP2 acetyltransferase activity by another immune receptor. Still, there was no consensus on the model of action and function of RRS1 – is it a transcriptional regulator, an NLR fused with a guardee (WRKY domain), or an enabler of effector enzymatic activity? Support was given to the transcriptional regulator model in two other studies but without any conclusive evidence (Sohn et al., 2012; Sohn et al., 2014). To further complicate things, a theory was proposed that AvrRps4 targets the immune regulator EDS1, which is guarded by RPS4 (Bhattacharjee et al., 2011; Heidrich et al., 2011).

In 2015, two breakthrough studies shed some light on the enigmatic RRS1 disease resistance protein. Sarris et al. (2015) and Le Roux et al. (2015) demonstrated that RPS4/RRS1-mediated effector recognition is dependent on RRS1 WRKY domain acetylation by PopP2 or binding by AvrRps4. They show that the RRS1 WRKY domain acts as an integrated decoy and that PopP2 and AvrRps4 target bona fide WRKY transcription factors in order to suppress PTI. RRS1 has, therefore, integrated an effector bait into an NLR, which is able to intercept the virulence activity of at least two effectors inducing activation of the RPS4/RRS1 NLR complex and, ultimately, a rapid defense response. Interestingly, DNA dissociation is not sufficient or even required for RPS4/RRS1 activation. Recognition of AvrRps4 does not reduce RRS1 affinity for W-box DNA (the sequence to which the WRKY domain binds); therefore, loss of DNA binding is not required for RPS4/RRS1-mediated AvrRps4 recognition. A critical lysine residue acetylated by PopP2 resulting in immunity activation is lysine 1221 (K1221). Mutation of the lysine residue to glutamine (K1221Q) mimics acetylated RRS1 and results in dissociation from DNA and auto-activity as demonstrated in tobacco and Arabidopsis. Interestingly, mutation of this lysine residue to arginine (K1221R)

results in dissociation from DNA but no auto-activity, as demonstrated in Arabidopsis and tobacco, showing that loss of DNA binding is not always associated with defense activation (Le Roux et al., 2015; Sarris et al., 2015). This counters the theory that loss of RRS1 DNA binding results in de-repression of defense gene expression.

Furthermore, RRS1 requires the typical TNL RPS4 for signaling. RPS4 likely induces cell death via its TIR domain upon detection of specific RRS1 perturbation and conformational changes (Swiderski et al., 2009; Sohn et al., 2014). In the default state, RRS1 may negatively regulate RPS4 activation (Sohn et al., 2014; Huh et al., 2017). Direct association of the lipase-like immune regulator EDS1 with RPS4 would enable rapid signal transduction to induce transcriptional reprogramming as other TNLs do (Huh et al., 2017). With all studies in mind, it appears that RRS1 does not act as a functional transcriptional regulator despite harboring a WRKY domain. Instead, RRS1 is poised at the chromatin with an integrated decoy to monitor the presence of effectors that would otherwise disable defense by targeting bona fide WRKY TFs. The knowledge that RRS1 harbors a WRKY domain and associates with DNA may have been a red herring in the quest to decipher its function.

CHAPTER 5: Investigating the RPS4 TIR domain interfaces required for defense signaling

5.1 Introduction

In collaboration with other laboratories researching the functional self-association interfaces in plant TIR domains, a project focusing on the RPS4 TIR domain was conducted. Zhang et al. (2017) solved the crystal structure of the TIR domain of the Arabidopsis NLR suppressor of npr1-1, constitutive 1 (SNC1). Analysis revealed the presence of an L6-like interface involving helices αD and αE (DE interface) and an RPS4-like interface involving helices αA and αE (AE interface). The RPS4 AE interface has previously been reported to be involved in RPS4 TIR domain auto-activity and RPS4 full-length signaling activity (Williams et al., 2014). The RPS4 DE interface, however, was not detected in the crystal structure of the RPS4 TIR domain or its heterodimer with the RRS1 TIR domain. A model of the RPS4 TIR domain DE interface was generated by superposition of the RPS4 TIR domain onto the L6 TIR domain DE interface dimer. The stability of the L6 TIR domain DE interface structure and, therefore, homodimerization and auto-activity was shown to be dependent on R164 and K200. Mutation of the corresponding RPS4 TIR residues, R116A and M150R, impaired RPS4 TIR self-association but not RPS4 TIR interaction with RRS1 TIR in a yeast-two-hybrid (Y2H) assay. Furthermore, R116A was shown to reduce RPS4 TIR self-association using size-exclusion chromatography (SEC) coupled to multi-angle light scattering (MALS) (Zhang et al., 2017). Here, the requirement of the RPS4 TIR domain DE interface was further investigated by assaying TIR domain auto-activity, RPS4 TIR homodimerization, RPS4/RRS1 TIR heterodimerization and full-length effector-dependent and effector-independent cell death in tobacco leaf cells. It was found that the DE interface is required for RPS4 TIR domain-mediated cell death induction and RPS4 full length signaling. In CoIP experiments, mutation of the DE interface did not impair RPS4 TIR/TIR homodimerization or RPS4/RRS1 TIR/TIR heterodimerization. Characterization of TIR domain signaling will allow us to elucidate a general mechanism by which TNL immune receptors become

activated. With this knowledge, there is the potential to engineer resistance genes with greater efficiency.

5.2 Results

5.2.1 R116A and M150R mutations disable RPS4 TIR domain auto-activity

It was previously demonstrated that the RPS4 TIR domain triggers effector-independent programmed cell death (PCD) in tobacco and that this is dependent on homodimer formation via the SH motif at the AE interface (Zhang et al., 2004; Swiderski et al., 2009; Williams et al., 2014). Since the DE interface mutations disrupted RPS4 TIR domain self-association, it was proposed that they may also impair RPS4 TIR domain-induced PCD in tobacco. To this end, RPS4(1-236) variants carrying the two DE interface mutations, R116A and M150R, were generated and an *Agrobacterium*-mediated overexpression assay was used in tobacco leaves to assay for a cell death response. RPS4 TIR variants (WT, SH-AA, R116A and M150R) were tagged with either C-terminal 6xHA or C-terminal YFP tags. As expected, the $RPS4^{WT}$ TIR domain induced strong PCD at 3 dpi and the AE interface mutant, $RPS4^{SH\text{-}AA}$ TIR domain, did not induce PCD. Interestingly, it was found that the R116A and M150R mutations abolished RPS4(1-236)-6xHA-triggered cell death signaling; however, only R116A fully abolished RPS4(1-236)-YFP triggered PCD. AE and DE interface double mutant TIR domains, $RPS4(1\text{-}236)^{SH\text{-}AA/R116A}$ and $RPS4(1\text{-}236)^{SH\text{-}AA/M150R}$ did not trigger PCD (Figure 5.1). Expression of all constructs was confirmed by immunoblot analysis with anti-GFP or anti-HA antibodies (Figure 5.2).

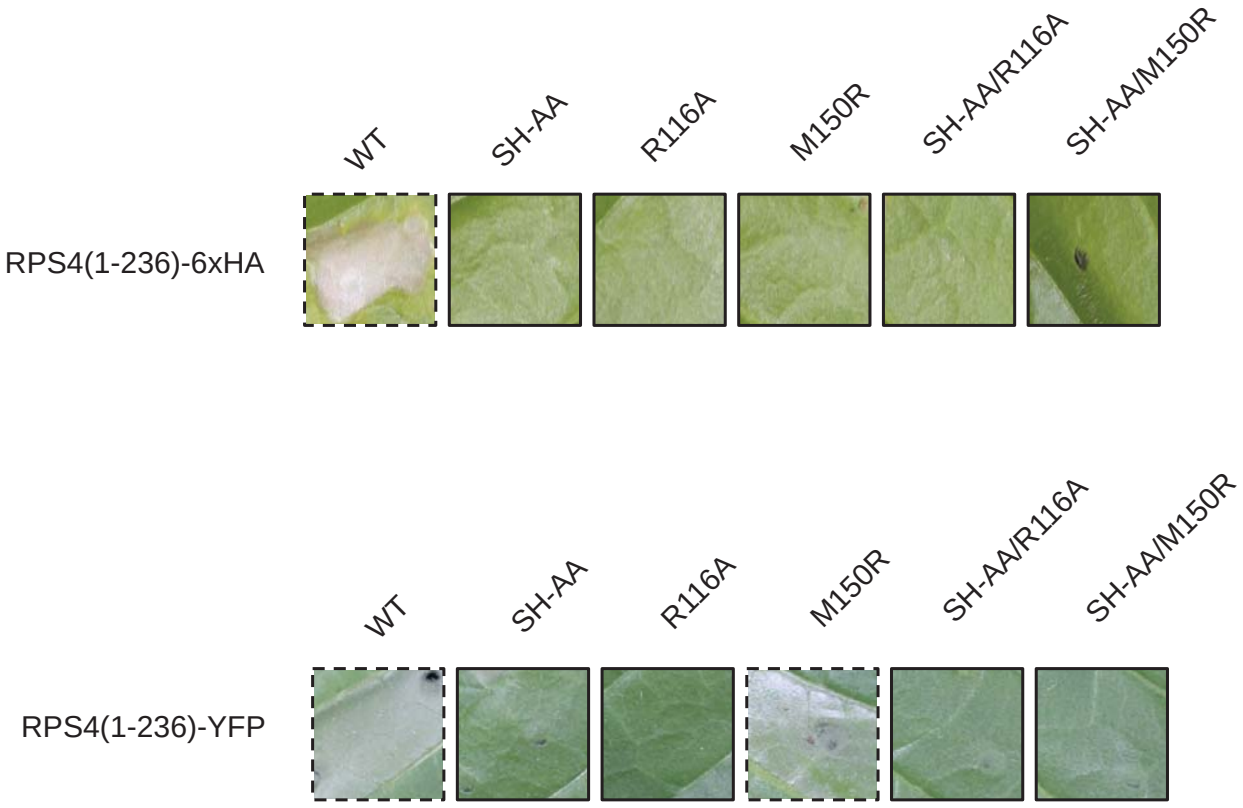


Figure 5.1. RPS4 TIR domain DE interface mutations R116A and M150R disable RPS4 TIR-induced dell death in tobacco. Cell death signaling activity of *Agrobacterium*-delivered RPS4 TIR variants fused to 6xHA or YFP C-terminal epitope tags in tobacco. Photos were taken 3 dpi.

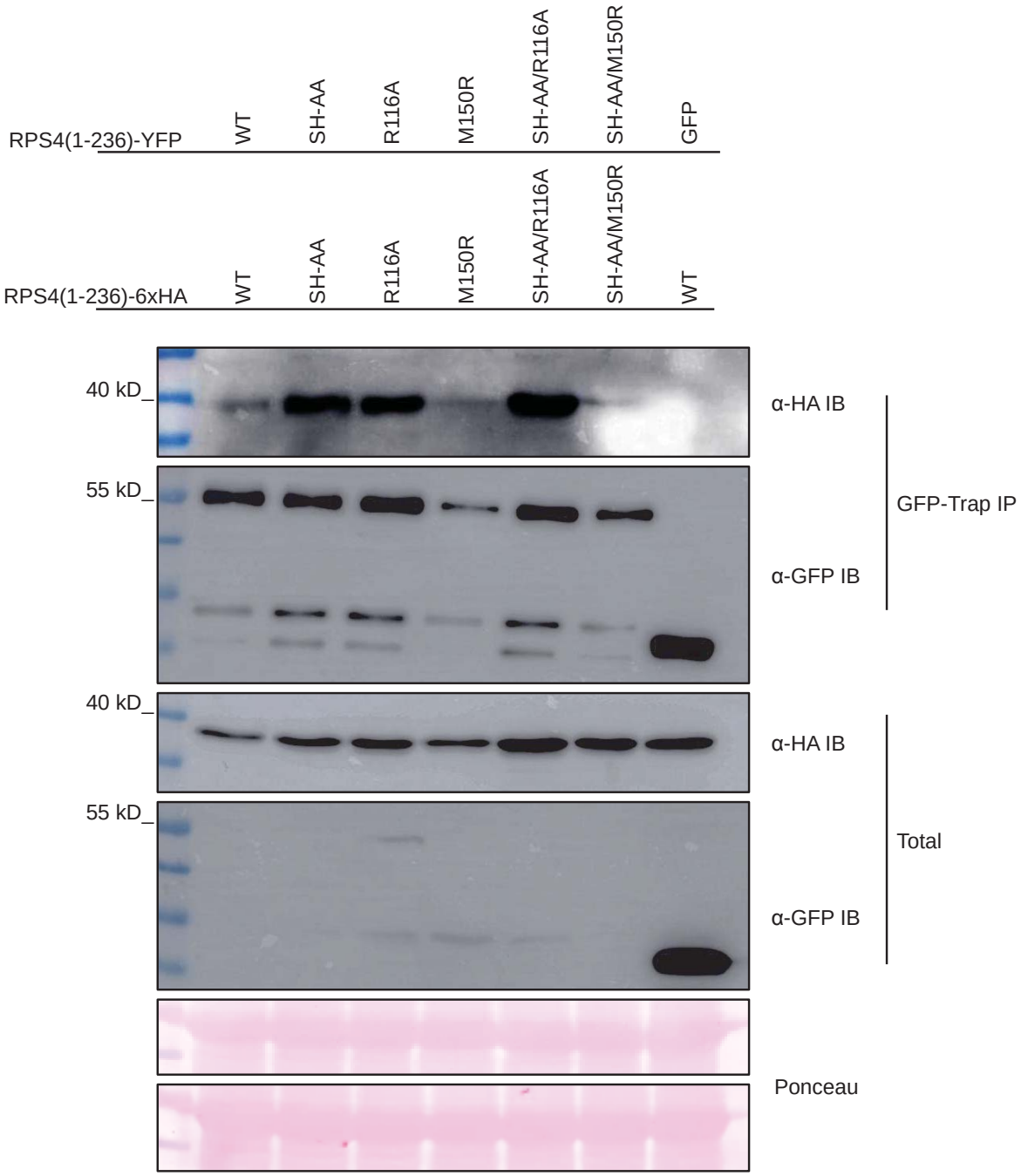


Figure 5.2. RPS4 TIR DE interface mutants maintain self-association *in planta*. Self-association CoIP assay of RPS4(1-236)-YFP and RPS4(1-236)-6xHA AE and DE interface mutants in *N. benthamiana* leaves. RPS4(1-236)-YFP variants were pulled down with anti-GFP beads and probed with anti-HA antibodies. The lower bands in the GFP blots are free GFP (27 kD). IB = immunoblot, IP = immunoprecipitation. This experiment was conducted 3 times with similar results.

5.2.2 R116A and M150R mutations do not affect RPS4 homodimerization or RPS4/RRS1 heterodimerization in a CoIP assay

As previously mentioned, a Y2H assay revealed that the RPS4 TIR DE interface mutations, R116A and M150R, impaired self-association but not association with RRS1 TIR domain, RRS1(1-175) (Figure 5.3) (Zhang et al., 2017). This interaction was assayed using an *in planta* coimmunoprecipitation (CoIP) assay. Using the RPS4 TIR constructs described in 5.2.1 and RRS1 TIR variants fused to a C-terminal YFP tag, CoIP assays were performed using *Agrobacterium*-mediated delivery in *Nicotiana benthamiana* leaves. In order to test RPS4 TIR domain self-association, RPS4(1-236)-YFP and RPS4(1-236)-6xHA variants were co-expressed. Using anti-GFP beads, RPS4(1-236)-YFP was pulled down and subsequently probed with anti-HA antibodies. Surprisingly, it was found that RPS4 variants harboring a DE interface mutation, R116A or M150R, were not impaired in homodimerization in this assay (Figure 5.3).

In order to test RPS4 TIR domain heterodimer formation with RRS1 TIR domain, RPS4(1-236)-6xHA and RRS1(1-175)-YFP variants were co-expressed in *N. benthamiana* leaves by *Agrobacterium* delivery. RRS1(1-175)-YFP was pulled down and subsequently probed with anti-HA antibodies. Consistent with the Y2H data, neither R116A nor M150R impaired RPS4/RRS1 TIR heterodimer formation (Figure 5.4). Overall, the DE interface mutations had no effect on RPS4 TIR domain self-association or interaction with RRS1 TIR domain in this CoIP assay, despite impairing self-association in a Y2H assay.

5.2.3 R116A and M150R mutations impair RPS4 full-length signaling

Interaction of RPS4 with RRS1 via their TIR domains is thought to maintain the RPS4/RRS1 complex in an active, signaling-competent state. Upon effector recognition, the complex undergoes a conformational change resulting in the activated state in which the RPS4 TIR domain self-associates

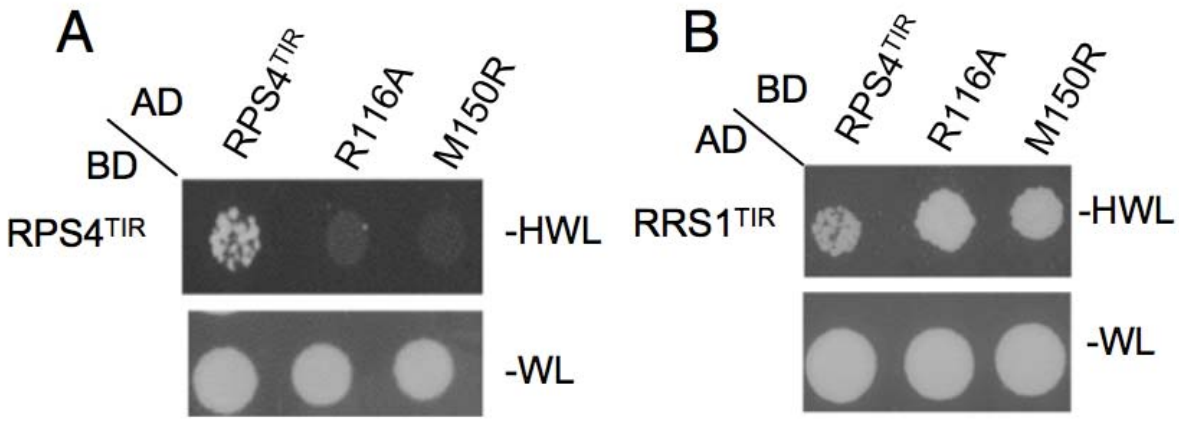


Figure 5.3. RPS4 TIR domain DE interface mutations affect RPS4 TIR self-association but not RPS4/RRS1 TIR/TIR heterodimer formation in yeast. (A) Growth of yeast cells expressing GAL4-BD fusion of RPS4 TIR (1-183) and GAL4-AD fusion of RPS4 TIR (1-183 or RPS4 TIR mutants on non-selective media lacking tryptophan and leucine (-WL) or selective media additionally lacking histidine (-HWL). (B) Growth of yeast cells co-expressing GAL4-BD fusion of RPS4 TIR or RPS4 TIR mutants and GAL4-AD fusion of RRS1 TIR (1-185) on –WL and –HWL media. Taken from Zhang et al. (2017).

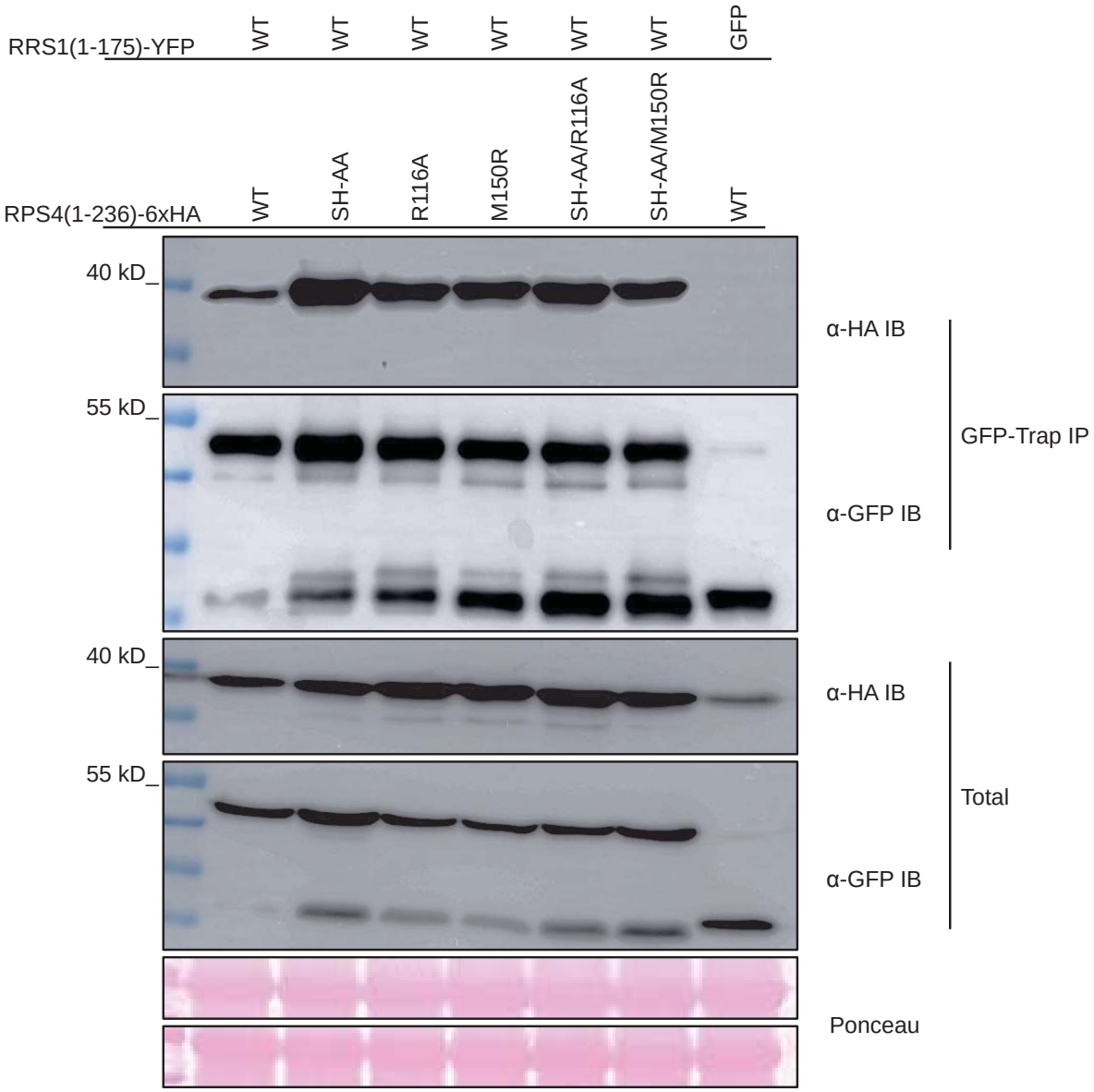


Figure 5.4. RPS4 TIR DE interface mutants maintain interaction with RRS1 TIR domain *in planta*. RPS4/RRS1 TIR/TIR heterodimer CoIP assay of RPS4(1-236)-6xHA AE and DE interface mutants with RRS1(1-175)-YFP. RRS1(1-175)-YFP variants were pulled down with anti-GFP beads and this was probed with anti-HA antibodies. The lower bands in the GFP blots are free GFP (27 kD). This experiment was conducted 3 times with similar results.

to signal downstream (Williams et al., 2014). Therefore, RPS4 TIR domain self-association is a critical requirement for RPS4 full-length function. As the DE interface residues, R116 and M150, were shown to be required for self-association in yeast and for RPS4 TIR-induced programmed cell death in tobacco, it was investigated whether they were, indeed, required for RPS4 full length function (Zhang et al., 2017). Accordingly, an *Agrobacterium*-mediated overexpression assay was employed in tobacco to assay RPS4 function in effector recognition and auto-active RRS1 signaling. To assay for effector recognition, RPS4 variants (WT, SH-AA, R116A, M150R, SH-AA/R116A and SH-AA/M150R) were co-expressed with RRS1 and either AvrRps4 or PopP2. As expected, RPS4 induced strong PCD when co-expressed with RRS1 and AvrRps4 or PopP2, whereas $RPS4^{SH\text{-}AA}$ did not (Williams et al., 2014). It was demonstrated that the DE interface mutations, R116A and M150R, impaired signaling by full-length RPS4. The RPS4 R116A mutation did not fully abolish PopP2 recognition, but PCD was significantly impaired. Additionally, the double AE and DE interface RPS4 variants, $RPS4^{SH\text{-}AA/R116A}$ and $RPS4^{SH\text{-}AA/M150R}$, were unable to induce cell death in response to either effector (Figure 5.5A).

Next, it was investigated if the DE interface residues, R116 and M150, were required for signaling by an auto-active RRS1 variant or if they were exclusively required for effector recognition. Therefore, the six aforementioned RPS4 variants were co-infiltrated with $RRS1^{SLH1}$ in the absence of an effector. Interestingly, it was found that M150R but not R116A affected $RRS1^{SLH1}$ signaling (Figure 5.5A). Expression of all RPS4-6xHA constructs was confirmed by immunoblotting with anti-HA antibodies (Figure 5.5B).

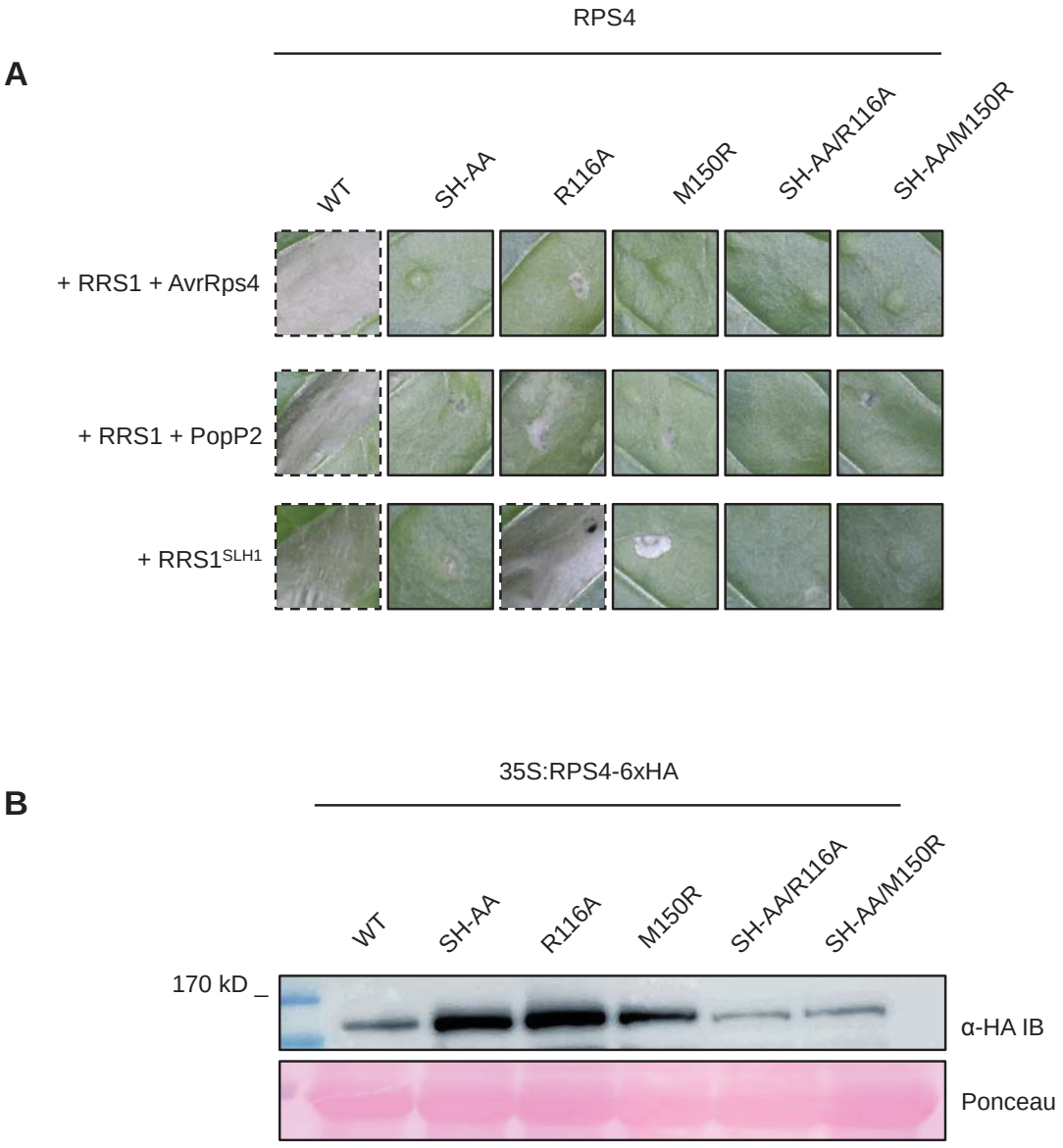


Figure 5.5. RPS4 DE interface mutations impair effector-dependent and –independent cell-death signaling. Cell death activity of RPS4 variants fused to a C-terminal 6xHA tag upon *Agrobacterium*-mediated co-expression with RRS1 and AvrRps4 or PopP2; or with auto-active RRS1^{SLH1}. *Agrobacterium* cultures were adjusted to OD 0.1 and photos were taken 5 dpi.

5.3 Discussion

NLRs with an N-terminal TIR domain comprise the largest class of NLRs. Despite this, little is known about the specific interactions involved in TIR domain-mediated defense signaling. Here, data was provided to support Zhang et al. (2017) in elucidating a general mechanism by which TNLs may self-associate via their TIR domains in order to signal downstream.

The effector-independent PCD that is induced by the RPS4 TIR domain was suppressed by the two DE interface mutations, R116A and M150R, when tagged with C-terminal 6xHA. This result corroborates Y2H data, which revealed that R116A and M150R DE interface mutations result in dissociation of RPS4 TIR domain homodimers. Surprisingly, when C-terminally tagged with YFP, RPS4(1-236)^{M150R} induced a cell death response. This initially puzzling result is likely a consequence of the native ability of *Aequorea*-derived fluorescent proteins to form weak dimers, thus maintaining the RPS4 TIR domains in close enough proximity to induce PCD in spite of the M150R mutation (Shaner et al., 2005). Of note, Krasileva et al. (2010) reported that the RPP1-WsB TIR domain (1-266) elicited PCD in tobacco, although this response was dependent on a dimeric GFP tag. When fused to monomeric GFP carrying an A206K mutation, the RPP1-WsB TIR domain no longer induced a cell death response (Krasileva et al., 2010).

The tobacco PCD results indicated that R116 and M150 residues are involved in cell death signaling likely via the proper formation of TIR/TIR homodimers. In a coimmunoprecipitation (CoIP) assay, however, R116A and M150R mutant TIR domain variants maintained self-association (Figure 5.2). It is possible that this contrasting result to the Y2H assay is a consequence of expressing a small fragment of the RPS4 NB-ARC domain as well as the TIR domain. In the Y2H assay, an RPS4 TIR domain construct was used (residues 1-183); however, in the *N. benthamiana* CoIP assay a construct with an extra 53 residues of the NB-ARC domain (residues 1-236) was used, as this is required for cell death induction in tobacco. R116A and M150R mutations disabled cell death signaling via disruption of DE interface self-

association. Perhaps in the CoIP assay, the RPS4 DE interface mutant variants could not interact via the DE interface, but maintained interaction via the NB-ARC fragment, thus making it appear as if R116A and M150R had no effect on interaction. Therefore, the $RPS4^{(1-236)}$ DE interface mutant variants are likely lost in critical association at the DE interface and, as a result, could no longer induce cell death. Notably, in CoIP experiments, the $RPS4^{SH-AA}$ mutant and double mutants (SH-AA/R116A and SH-AA/M150R) also retained self-association. It is probable that the CoIP experiment could not demonstrate this loss of interaction as the $RPS4^{(1-236)}$ variants could self-associate independent of the AE or DE interfaces.

Furthermore, the DE interface mutant RPS4(1-236) variants maintained interaction with the RRS1 TIR domain (residues 1-175). As with the homodimer CoIP assay, it would be interesting to confirm these results with an RPS4(1-183) variant. It would be expected that the RPS4/RRS1 TIR heterodimer would be unaffected by the DE interface mutations, as this was demonstrated in yeast.

Finally, it was demonstrated that R116 and M150 residues were required in the full-length context. Mutation of both residues impaired effector recognition (both AvrRps4 and PopP2), whereas mutation of M150 but not R116 affected $RRS1^{SLH1}$ auto-activity. It appears, therefore, that both AE and DE interface RPS4 self-association is required for RPS4/RRS1-mediated signaling.

CHAPTER 6: GENERAL DISCUSSION AND OUTLOOK

6.1 Summary of findings

Overall, this research has advanced our understanding of plant immune receptor signaling, specifically in the exciting area of integrated decoys. Chapter 3 focused on the impact of variation in the effector PopP2 on avirulence, whereas Chapters 4 and 5 both focused on the impact of variation in NLRs (RRS1 and RPS4) on their signaling function.

In Chapter 3, *popP2* was shown to be well conserved in *Ralstonia solanacearum* strains isolated from diseased tomato and pepper fields from across the Republic of Korea and RPS4/RRS1-mediated recognition was tolerant of multiple polymorphisms in the *popP2* sequence. Moreover, a conserved EAR motif was identified and demonstrated to be required for *in planta* PopP2 stability and recognition.

In Chapter 4, single nucleotide polymorphisms were identified that discriminated between auto-activity and effector recognition functions. Furthermore, a mutation in the integrated decoy WRKY domain was shown to confer auto-activity with distinct features compared to other known auto-active RRS1 variants. Notably, TIR domain mutant was also identified that impaired RPS4/RRS1 TIR/TIR heterodimer formation and full-length RRS1 function.

Finally, in Chapter 5, it was shown that two DE interface mutations disabled RPS4 TIR domain effector-independent cell death induction and impaired full-length RPS4 signaling. This supports the theory that plant NLR TIR domains signal using a common mechanism via two distinct interfaces.

The requirements of host recognition of effectors by this specific RPS4/RRS1 NLR pair have been further characterized. Perhaps more importantly, this research has added important findings to a growing body of evidence on the mechanism of NLR perception of pathogen-derived effectors.

6.2 Comparison to other systems

As described in Chapter 3, it was unexpectedly revealed that PopP2 is dependent on its EAR motif for stability; if the EAR motif is mutated the effector is degraded *in planta*. It was hypothesized that PopP2 EAR motif-mediated stability is conferred through interaction with an as yet unknown host protein. The causal agent of potato late blight, oomycete pathogen *Phytophthora infestans*, secretes the effector Avr3A that, like PopP2, was shown to be dependent on a specific sequence for accumulation inside host cells. The so-called “effector domain”, a positively charged surface patch, was demonstrated to mediate binding to phosphatidylinositol monophosphates (PIPs), which was required for stability and virulence activity (Yaeno et al., 2011). It was proposed that PIP binding renders AvrA3 unable to be detected and degraded by the host proteasome or otherwise. Indeed, the same could hold true for PopP2 whereby interaction with a host component via the EAR motif protects the effector from host degradation.

The bacterial pathogen, *Salmonella enterica*, delivers type III effectors into mammalian cells. It was found that many membrane-localized effectors encode a coiled-coil (CC) domain and that disruption of the CC domain in several of these effectors affects host membrane association and effector stability both in *Salmonella* and in the host cells (Knodler et al., 2011). A mis-targeted effector may become susceptible to host proteasomal degradation. Evasion of host degradation, then, may apply to not only plant pathogen effectors but also effectors secreted into mammalian cells. It is likely that in order to fulfill their respective virulence activities in host cells, regardless of kingdom, effectors must evade the host degradation machinery, which may require interaction with a host component.

Plant cells have the ability to recognize non-self/foreign proteins and subject them to degradation (Vierstra, 1993). Bacterial, fungal, oomycetal and viral effectors may be sensed by the host cell as foreign and become degraded; however, the fact that effectors are commonly demonstrated to accumulate *in*

planta and function properly indicates that many effectors do evade degradation. It was discussed that interaction with an appropriate cofactor or co-subunit is necessary for many host proteins to avoid selective degradation in plant cells (Callis, 1995). For example, when the amount of the chloroplast-synthesized large subunit of ribulose 1,5-bisphosphate (RuBP) is low, the imported RuBP small subunit becomes selectively and rapidly degraded by a protease (Schmidt and Mishkind, 1983). It is plausible that the same applies for microbial-derived proteins in plant cells; without appropriate complex formation, the effector may not accumulate.

Integrated decoys are emerging as a frequent and widespread form of immune receptor in plants. In all characterized NLR integrated decoy pairs, there is one partner that harbors the unorthodox decoy domain, which acts as the sensor, whereas the interacting partner functions as the signal transducer. This is consistent with the RPS4/RRS1 data. The rice NLR pair RGA4/RGA5 that mediates resistance to the fungal pathogen *Magnaporthe oryzae* is strikingly similar to RPS4/RRS1. RGA4 induces effector-independent cell death, much like the RPS4 N-terminus; RGA5 heterodimer formation with RGA4 suppresses this cell death, much like the RRS1 TIR domain repression of RPS4 TIR domain-mediated cell death; effector perception occurs via physical binding of effector to the RGA5 integrated domain, as is the case for RRS1; and RGA4/RGA5 also recognize multiple sequence unrelated effectors. Furthermore, RGA4 and RGA5 form heterodimers via their N-terminal domains; however, in this case they happen to be CC domains and not TIR domains (Cesari et al., 2014).

Intriguingly, many mammalian NLRs have also been demonstrated to form heteromeric complexes (von Moltke et al., 2013). In mice macrophages, it was shown that the NLRs NAIP5 (NLR family, apoptosis inhibitory proteins 5) and NLRC4 (NLR family CARD domain-containing protein 4) heterodimerize and function together for resistance to *Legionella pneumophila* via flagellin recognition (Kofoed and Vance, 2011; Zhao et al., 2011). In this system, NAIP5 acts as the direct flagellin sensor whereas NLRC4 is required for downstream signaling responses. This is reminiscent of the RPS4/RRS1

immune complex; however, there are also some notable differences between these NLR pairs. For example, NAIP5 and NLRC4 are not co-located in the mouse genome and they are monomeric in the absence of the ligand (Halff et al., 2012). In spite of the differences, it appears that there are several interesting parallels between the way in which plants and animals fight disease.

6.3 Outlook

Following on from this thesis, there are several experiments that could be carried out to build on the research carried out here. The next logical step of Chapter 3 would be to determine if PopP2 does, indeed, interact with a host protein via its EAR motif and, if so, what the identity of this protein is. An interaction screen, such as a yeast-two-hybrid (Y2H) screen, could be employed with further transcriptional corepressor candidates or even the Arabidopsis proteome. In the scenario whereby PopP2 does not interact with a host protein via its EAR motif, but the PopP2 LAAL mutation disrupts stability, solving the crystal structure of the PopP2^{LAAL} mutant could provide insight into how the polymorphisms affect PopP2 conformation and stability.

It would be interesting and highly useful to enhance Chapter 4 by generating transgenic Arabidopsis lines harboring the RRS1 SUSHI variants. By transforming an *rrs1rrs1b* knockout (KO) Arabidopsis line with RRS1 SUSHI variants, the resulting transgenics could be assayed for PopP2 and AvrRps4 recognition upon *Pseudomonas* delivery in the native Arabidopsis system. This would hopefully corroborate the results observed in tobacco. Bacterial growth assays could also be conducted with *Pto* DC3000 carrying either AvrRps4 or PopP2. Further dissection of the requirements of RRS1 for its function could be carried out through random mutagenesis of one or more of its domains and subsequent functional assays in tobacco, as carried out in Chapter 4. This would help to uncover additional residues involved in effector recognition and/or signaling.

The research in Chapter 5 could be continued through investigation of further TNLs to determine if the two interfaces (AE and DE) required for RPS4, SNC1 and L6 signaling are involved in signaling via other TNLs. It would be interesting to solve the crystal structure of other TIR domains and then use Y2H interaction, CoIPs and cell death assays upon agroinfiltration to determine the requirement of these interfaces in a broad range of TNL immune receptors. Furthermore, whether modification of these interfaces could bring about an enhancement of the defense response could be investigated through site-directed mutagenesis and subsequent cell death and interaction assays of RPS4, SNC1 and L6.

These studies have collectively expanded our knowledge of RPS4/RRS1 function. As an already extensively studied system, much is known about the mechanism of RPS4/RRS1-mediated defense signaling. However, several aspects of function by this NLR pair remain to be elucidated. Throughout this thesis, I have aimed to further our knowledge of the molecular basis of RPS4/RRS1-mediated defense activation. Why study this and what is the potential agricultural benefit of characterizing the mechanistic details of this NLR pair? Importantly, RPS4 and RRS1 confer resistance to four different pathogens and have been shown to function in multiple plant families, highlighting their potential for deployment in crop species not limited to *Brassicaceae* (Narusaka et al., 2013). RPS4 and RRS1 are the best-characterized NLR pair thus far, perhaps owing to the ease of studying the Arabidopsis-*Pseudomonas* pathosystem.

Collectively, these data can be utilized to develop a general model by which many plant NLRs may function. This will allow us to understand more readily the function and mechanism of action of newly discovered paired NLRs. Additionally, an advanced understanding of NLR function will ultimately aid in the engineering of novel NLRs. With greater knowledge of the mechanism of NLR function, it will become increasingly easier to design and generate novel NLRs with efficient *in planta* functioning and without inappropriate activation for deployment in crop species. The integration of other or additional NLR-IDs into *RRS1* is an exciting prospect in order to alter or expand the recognition

specificity of RPS4/RRS1 for any desired pathogen effector. A breakthrough study by Kim et al. (2016) demonstrated the potential of using decoys to improve disease resistance. The *P. syringae* AvrPphB (effector) cleavage site within the targeted host kinase PBS1 (guardee) was substituted with other bacterial or viral protease cleavage sites to successfully expand the recognition specificity of RPS5 (guard/NLR). A similar approach to RPS4/RRS1 holds great potential.

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