Determining the molecular mechanism of plant disease resistance following pathogen effector perception by the resistance gene pair *RPS4/RRS1*

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Abstract

Plant and animal cells have an assortment of receptors employed for pathogen surveillance. The Nucleotide-binding domain and Leucine-rich Repeat-containing (NLR) family of proteins are important receptors involved in cell autonomous surveillance. In animals, upon perception of virulence molecules or conserved molecular patterns from pathogens several NLRs initiate immune signalling by the induced proximity of signalling domains. In order to do this, these NLRs form oligomeric wheel-shaped complexes, called inflammasomes, that bring the N-terminal signalling domains of the component NLRs into close proximity and initiates downstream signalling. In plants, much less is known about signal initiation and transduction during NLR-mediated immunity. Plant NLRs recognise virulence factors that are often race specific, called effectors. I used the model NLR pair RRS1/RPS4 to investigate effector recognition by NLRs.

RRS1 and RPS4 have the archetypal TIR-NB-LRR architecture of a large subset of plant NLRs, but also contain non-canonical domains. Of particular note, RRS1 contains a C-terminal WRKY DNA-binding domain. RRS1 and RPS4 act in concert to recognise at least three effectors: AvrRps4 secreted by *Pseudomonas syringae*; PopP2, an acetyltransferase secreted by *Ralstonia solanacearum*; and an unidentified effector from *Colletotrichum higginsianum*.

In this thesis, I present evidence that supports a new conceptual framework for effector recognition. In this model, the virulence target of PopP2 and AvrRps4 has fused to an NLR (in this case the WRKY domain in RRS1) and acts as a decoy, baiting the effector to attack the NLR and trigger immunity, instead of enhancing susceptibility by interfering with the function of its intended target. This mechanism of effector perception may be widespread in plants and I discuss other examples of NLRs with integrated atypical domains.

NLRs exert intramolecular inhibition of immune signalling in the absence of effectors in order to prevent autoimmunity. The domains of RRS1 C-terminal to the WRKY exert negative regulation of activation. Upon establishing the

method of effector recognition by RRS1 and RPS4, I leverage this knowledge of the mechanism of RRS1 autoinhibition to engineer recognition of viral proteases.

In the final chapter of this thesis I engineer a mammalian inflammasome-forming NLR system, fused to the signalling domain of RPS4, into plants to 1) introduce into plants an intracellular receptor that recognises conserved pathogen associated molecular patterns and 2) test if inducing the proximity of plant NLR signalling domains is sufficient for activation of NLR-mediated immunity.

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Publications arising from this thesis

Some of the material offered in this thesis has been published in the following journal articles, of which I am co-first author. At the start of each chapter that contains published material, I have indicated the article in which the material was published. Where published or unpublished material that is essential to the narrative of this thesis, but was not performed by me, is included, I have indicated who performed the work.

Duxbury, Z.*, Ma, Y.*, Furzer, O.J.*, Huh, S.U., Cevik, V., Jones, J.D.G. and Sarris, P.F., 2016. Pathogen perception by NLRs in plants and animals: Parallel worlds. BioEssays, 38(8), pp.769–781.

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* indicates co-first authors.

Chapter 1. General Introduction

Multicellular eukaryotes are continually exposed to microbial pathogens. In spite of this, most of the time eukaryotes maintain good health. The innate immune system allows each cell to maintain a disease-free state by mounting an effective immune response to attempted pathogen infection.

In this thesis, I present research into the model plant receptors RRS1 and RPS4. I provide strong evidence that supports a new paradigm in recognition of pathogen virulence factors, called the "integrated decoy hypothesis". In the latter chapters of my thesis, I present three strategies to engineer synthetic disease resistance against different classes of pathogens. To provide context to my research, here I summarise the relevant literature on both plant and animal immune receptors.

1.1. Innate immunity

Cells rely on a membrane-bound and intracellular immune-receptor surveillance system to detect the molecular patterns of pathogen infection. The innate immune system utilises pattern recognition receptors (PRRs) to detect conserved pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), and other receptors to detect less conserved, pathogen race-specific molecules.

In mammals, Toll-like receptors (TLRs) carry extracellular leucine-rich repeat (LRR) domains to detect external PAMPs, for example, bacterial peptidoglycans or flagellin (Eitas and Dangl, 2010; Kofoed and Vance, 2011; Maekawa et al., 2011a; von Moltke et al., 2013). Upon PAMP perception, TLRs dimerise and transduce the perception signal via cytosolic TIR (Toll/Interleukin-1 receptor/Resistance protein) domains that recruit downstream signalling partners.

Plants perceive PAMPs via receptor-like kinases (RLKs), or receptor-like proteins (RLPs) without kinase domains, that span the plasma membrane. Perception of PAMPs triggers a response called PAMP-triggered immunity (PTI). The LRR ectodomain perceives PAMPs by direct interaction, which

induces homo- or hetero-dimerisation and the recruitment of cytoplasmic downstream signalling molecules (Macho and Zipfel, 2014; Zipfel, 2014). For example, the Arabidopsis RLK FLS2 senses a 22 amino-acid epitope (flg22) from bacterial flagellin (Chinchilla et al., 2006). Upon ligand binding, the RLK co-receptor BAK1 is recruited to the complex and binds both flg22 and FLS2, and phosphorylates FLS2 and a pre-associated receptor-like cytoplasmic kinase (RLCK) called BIK1 (Chinchilla et al., 2006; Lu et al., 2010; Schulze et al., 2010; Sun et al., 2013; Zhang et al., 2010). BIK1 transphosphorylates BAK1 and FLS2 and dissociates from the complex, going on to phosphorylate RbohD, an NADPH oxidase that generates apoplastic reactive oxygen species (ROS) required for PTI (Kadota et al., 2014; Li et al., 2014b). Another early PTI response is the influx of Ca^{2+} into the cell, which activates Ca^{2+}-dependent protein kinases (CDPKs) (Seybold et al., 2014). CDPKs further regulate RbohD function and transduce PTI signalling to the nucleus (Dubiella et al., 2013; Seybold et al., 2014). PAMP perception also triggers MAPK signalling cascades, a Ca^{2+} burst, and reprogramming of more than 1000 genes by WRKY transcription factors (Asai et al., 2002; Boudsocq et al., 2010; Dubiella et al., 2013).

Pathogens deliver effector molecules to host cells to promote virulence. Effectors often disrupt the initial immune response by compromising the function of PRRs or proteins required for PRR signalling (Boller and He, 2009; Dodds and Rathjen, 2010; Hogenhout et al., 2009). Bacterial pathogens deliver effectors to eukaryotic hosts via needle-like type III or type IV secretion systems (TTSS and TFSS). Some filamentous pathogens, including rusts, mildew and oomycetes, deliver apoplastic effectors via hyphae and intracellular effectors through haustoria, penetrating structures that invaginate the plant membrane (Petre and Kamoun, 2014). Other fungi, for example, the blast fungus *Magnaporthe* and smut *Ustilago*, grow intracellularly upon appresorial penetration, and effectors are delivered by intracellular invasive hyphae, not haustoria (Djamei and Kahmann, 2012; Zhang and Xu, 2014). The mechanism of delivery of effectors from these filamentous pathogens is not yet known.

The first effectors of plant pathogens were called avirulence (Avr) factors. This is because the presence of an *Avr* gene in a pathogen was a determinant for a failed infection (an incompatible interaction) if a corresponding *Resistance* (*R*) gene allele was present in the plant. The determination of resistance by the presence of a plant *R* gene allele and a pathogen *Avr* gene allele in an incompatible plant-pathogen interaction is defined by the "gene-for-gene" hypothesis (Flor, 1951, 1971). *R* genes have been the major means that plant breeders have employed to protect crops against disease. Many *R* genes have been cloned and the majority encode members of the Nucleotide-binding domain and Leucine-rich Repeat-containing (NLR) family of proteins (Ting et al., 2008).

In plants, NLRs are the major receptors for intracellular race-specific pathogen perception. NLRs mediate effector-triggered immunity (ETI), which shares many of the same features as PTI, such as CDPK and MAPK signalling, and the production of ROS (Gao et al., 2013; Jones and Dangl, 2006; Thomma et al., 2011). However, ETI is usually stronger than PTI and often results in a cell-death output termed the hypersensitive response (HR) (Jones and Dangl, 2006). HR may restrict the growth of biotrophic pathogens, but it is possible to block HR by genetic mutation without detriment to pathogen growth suppression (Coll et al., 2011). For example, Arabidopsis mutants for the gene encoding metacaspase 1 (*AtMC1*) have greatly reduced CNL- and TNL-mediated HR but maintain suppression of pathogen growth equivalent to WT (Coll et al., 2010). Uncoupling of resistance and HR can be performed by manipulating Avrs that would normally trigger HR. For example, delivering to Arabidopsis a nuclear-excluded form of AvrRps4, which is normally nucleocytoplasmic, abrogates the HR that is normally observed but maintains pathogen growth suppression associated with Avr perception (Heidrich et al., 2011).

NLRs are present in animals, too, and are important cytosolic receptors of PAMPs, DAMPs and effectors. However, while Arabidopsis has ~120 genes encoding full length NLRs, most mammals have ~20 NLRs (Jacob et al., 2013). Animal NLR-triggered immunity often triggers inflammation through the production of Interleukin (IL)-1β and through a cell-death response

termed pyroptosis. Several mammalian NLRs form higher-order complexes that act as scaffolds for downstream signalling components. These complexes are termed inflammasomes. Three inflammasomes are well-characterised: NLRP1, NLRP3 and the NAIP/NLRC4 inflammasomes.

1.2. NLRs are intracellular receptors that have analogous roles across kingdoms

1.2.1. NLR structure

Plant and animal NLRs have similar modular structures that are characteristic of the STAND (signal transduction ATPases with numerous domains) AAA+ ATPases (Leipe et al., 2004). Core to this structure is the nucleotide binding domain (NBD). Plant NLRs are in the AP subclass of the STAND superfamily and have an NBD of the NB-ARC (nucleotide-binding, Apaf-1, R-protein and CED-4) subtype (Leipe et al., 2004). The plant NB-ARC has three subdomains: the NB fold, and two helical domains (HDs), ARC1 (or HD1 in animals) and ARC2 (winged HD; WHD, in animals) (van der Biezen and Jones, 1998). Animal NLRs have NBDs that fall within the NACHT subclass and contain an additional helical bundle termed ARC3 or HD2 (Albrecht and Takken, 2006; Lechtenberg et al., 2014; Takken et al., 2006). NB-ARC and NACHT domains bind ATP or ADP, and the identity of the bound nucleotide may determine the activation state of the whole protein (Duncan et al., 2007; Maekawa et al., 2011b; Takken et al., 2006; Tameling et al., 2002; Williams et al., 2011).

The STAND superfamily NBDs have two conserved motifs, the Walker A (P-loop) and Walker B motifs, which are involved in nucleotide and Mg^{2+} binding, and nucleotide hydrolysis (Hu et al., 2013; Riedl et al., 2005; Walker et al., 1982). Plant NLRs have additional motifs, including the MHD motif, which contains a histidine that may be conserved across plant and animal NLRs (Bonardi et al., 2012; Lechtenberg et al., 2014; Riedl et al., 2005). ATP/ADP-binding is important for regulating the activation state of NLRs because mutating these motifs can suppress (e.g. P-loop mutations) or constitutively activate (e.g. MHD mutations) NLR-mediated immune signalling (Roberts et al., 2013; Sohn et al., 2014; Takken et al., 2006;

Tameling et al., 2002; Wang et al., 2015b; Williams et al., 2011; Xu et al., 2014a; Zhao et al., 2015).

NLR proteins canonically contain an LRR domain C-terminal to the NBD. The LRRs of TLRs and plant PRRs are often the site of ligand interaction (Jin and Lee, 2008; Zipfel, 2014), but this is less common in NLRs. In NLRs, LRRs inhibit NBD activation, which suppresses activity in the absence of ligands; indeed, deletion of LRR domains in both plant and animal NLRs often results in constitutive activation of immunity (Ade et al., 2007; Bendahmane et al., 2002; Inohara et al., 1999; Kofoed and Vance, 2011; Swiderski et al., 2009). The crystal structure of the inflammasome-forming NLRC4 reveals that the interactions between the NB and the LRR, as well as between the NB and ARC3, sterically prevent inflammasome oligomerisation, providing a mechanism for the LRR-mediated auto-inhibition of NLR activation (Hu et al., 2013).

The N-terminal domains of NLRs are typically involved in signal transduction. There are two canonical N-terminal signalling domains in animal NLRs: the caspase recruitment domain (CARD) and the Pyrin domain (PYD). Elicitor perception by NLRs often triggers oligomerisation that brings the N-terminal signalling domains of many NLRs into close proximity (described in detail in section 1.2.4). Most plant NLRs have N-terminal domains in two classes: TIR and coiled-coil (CC). Oligomerisation is important for the function of most TIR-NLRs (TNLs) and CC-NLRs (CNLs). For example, the TNL RPS4 has a dimerization interface discovered by obtaining the crystal structure of homodimerised RPS4 (Williams et al., 2014). Another TNL called L6 has a distinct and separate homodimerisation interface similarly characterised by determining the crystal structure of the TIR homodimer (Bernoux et al., 2011). Both interfaces are in regions of both proteins that are conserved across TIRs. Surprisingly, mutating either region disrupts dimer formation and immune signalling in both proteins (Zhang et al., 2017). This suggests that plant TIR domains can form open ended oligomers through end-to-end interactions at these two conserved dimerisation motifs.

Not all plant NLRs involved in immunity contain an N-terminal CC or TIR. The wheat NLR Tsn1, which is a determinant for sensitivity to the effector ToxA

(secreted by the fungal pathogens *Stagonospora nodorum* and *Pyrenophora tritici-repentis*), contains a serine/threonine protein kinase domain N-terminal to a canonical NB-ARC domain (Faris et al, 2010). Another example is the BED-NLRs, which contain a BED (BEAF and DREF) zinc-finger DNA-binding domain at their N-terminus, and that are found fused to the N-terminus of 32 poplar NLRs and in a few monocots (Germain and Séguin, 2011).

Other than the comparative architecture with animal NLRs, the most compelling evidence that the N-terminal domain of plant NLRs is involved in signalling is that many TIR- or CC-domains from NLRs trigger HR when overexpressed without the rest of the protein (Bernoux et al., 2011, 2016; Collier et al., 2011; Frost et al., 2004; Krasileva et al., 2010; Maekawa et al., 2011a, 2011b; Michael Weaver et al., 2006; Swiderski et al., 2009). Similarly to CARDs, N-terminal domains of plant NLRs facilitate the assembly of dimers or higher-order complexes. For example, activation of the CNL MLA10 requires homo-dimerisation via homophilic CC interactions (Bai et al., 2012).

Although there are similarities between plant and animal NLRs, it is likely that the analogous assembly of comparable modules between plant and animal NLRs arose through convergent evolution (Ausubel, 2005). Key evidence that suggests convergent evolution is the independent fusion of component modules. NBD and LRR domains exist independently in prokaryotes and eukaryotes, but NACHT-LRR fusions exist only in animals and NB-ARC-LRR fusions exist only in plants (Yue et al., 2012; Yuen et al., 2014). Urbach and Ausubel (2017) reconstructed the phylogeny of NACHT- and NB-ARC-containing proteins from a diverse set of organisms, including plants, animals and fungi. This study concluded that plant and animal NLRs most likely arose from independent fusion events. Taken together with the expansion of NLRs in plants and some animals, this demonstrates the propensity for proteins with an NLR-like architecture to function as receptors.

1.2.2. Mechanisms of pathogen recognition by NLRs

As the gene-for-gene hypothesis describes, many incompatible plant-pathogen interaction outcomes are determined by the recognition of secreted effectors by NLRs. This recognition can be via direct or indirect

molecular interactions and instead of a single NLR, can require NLR cooperativity (Dangl and Jones, 2001; Dodds and Rathjen, 2010).

1.2.2.1. Direct (receptor-ligand)

I stated earlier that TLRs and plant PRRs sense pathogens via direct LRR-PAMP interactions. This ligand-receptor style interaction is sometimes true of NLRs. For example, the LRR of human NLRX1 directly binds RNA ligands (Hong et al., 2012). There is some evidence that NOD1 directly binds the ligand L-Ala-γ-D-Glu-meso-diaminopimelic acid via its LRR (Chamaillard et al., 2003; Girardin et al., 2003; Laroui et al., 2011). NAIP2 binds the rod protein of the bacterial TTSS, and NAIP5 and NAIP6 bind flagellin (Kofoed and Vance, 2011; Zhao et al., 2011); however, these interactions occur at the NACHT, not the LRR (Tenthorey et al., 2014).

Some plant NLRs recognise effectors by direct interaction. For example, the direct interaction of the *Melampsora lini* (flax rust fungus; an important pathogen of flax, an oil and linen crop) effector AvrL567 with the flax TNL L6 results in pathogen recognition and resistance (Dodds et al., 2006). In addition, the recognition in rice of Avr-PikD and Avr-Pita, secreted by *Magnaporthe oryzae,* is mediated by direct interaction with the CNLs Pik-1 and Pi-ta, respectively (Jia et al., 2000; Maqbool et al., 2015).

Other NLRs utilise indirect methods of detection, or have yet undemonstrated mechanisms of recognition but are unlikely to interact directly with their ligand. For example, NLRP3-mediated immunity is stimulated by a dizzying plethora of molecules and it is almost inconceivable that it has the utility to directly bind each elicitor (Monie, 2013).

1.2.2.2. Indirect (Guard/Guardee and Decoy hypotheses)

Indirect detection of effectors may explain how hosts can cope with the enormous number of effectors they potentially face. Many plant NLRs monitor the integrity of host proteins that are targeted by pathogens, and perturbation of the function of the "guarded" host protein by an effector triggers an NLR-dependent immune response. The "guard hypothesis" describes this model and explains the evolutionary advantage to the host of monitoring effector targets instead of directly detecting effectors (Van Der

Biezen and Jones, 1998; Dangl and Jones, 2001; Jones and Dangl, 2006). Effectors of several model pathogens converge disproportionately to target a relatively small number of *Arabidopsis thaliana* (henceforth Arabidopsis) proteins, providing strong evidence for the hypothesis (Mukhtar et al., 2011; Weßling et al., 2014).

The guard hypothesis was initially formulated to rationalise the role of the tomato NLR Prf in the resistance-promoting interaction between the *Pseudomonas syringae* effector AvrPto and the tomato Ser/Thr kinase Pto (Van Der Biezen and Jones, 1998). In this system, AvrPto binds Pto to inhibit kinase activity, and this perturbation is recognised by Prf, which triggers an immune response (Van Der Biezen and Jones, 1998; Tang et al., 1996; Xiang et al., 2008; Xing et al., 2007).

Many examples of the systems that behave according to the guard hypothesis have been described. One archetypal example is the Arabidopsis RPM1 INTERACTING PROTEIN 4 (RIN4), which is targeted by multiple *P. syringae* effectors and is guarded by two NLRs (Grant et al., 1995; Mackey et al., 2002). The effectors AvrB and AvrRpm1 direct the host kinase RIPK to hyperphosphorylate RIN4 (Chung et al., 2011; Liu et al., 2011). The CNL RPM1 directly interacts with RIN4, but not with either effector, and upon effector-mediated modification of RIN4, an RPM1-dependent immune response is triggered (Mackey et al., 2002). In the absence of RPM1, RIN4-suppression of PTI is enhanced by AvrB and AvrRpm1 (Chung et al., 2014; Kim et al., 2005b; Lee et al., 2015; Ritter and Dangl, 1995). RIN4 is also cleaved by the effector AvrRpt2, which triggers an immune response mediated by RPS2, another CNL that monitors RIN4-integrity (Axtell and Staskawicz, 2003; Axtell et al., 2003; Mackey et al., 2003). AvrRpt2-mediated cleavage of RIN4 suppresses PTI in the absence of RPS2 (Afzal et al., 2011).

In some cases, guarded proteins that are targeted by effectors can be experimentally mutated without detriment to immunity. The guarded protein resembles the true virulence target of an effector and acts as “bait” to trap pathogens that secrete these effectors. This model is described by the “decoy hypothesis” (van der Hoorn and Kamoun, 2009). The Arabidopsis protein kinase PBS1, which is targeted by the protease effector AvrPphB, is an

archetypal decoy (Ade et al., 2007; Shao et al., 2003). PBS1 is monitored by the CNL RPS5, and upon cleavage of PBS1 by AvrPphB, an RPS5-mediated immune response is triggered. However, in the absence of RPS5, mutating PBS1 does not alter the susceptibility of Arabidopsis.

Another example of a decoy is the Arabidopsis ZED1 pseudokinase, which does not have a catalytically active kinase motif (Lewis et al., 2013). ZED1 is directly bound and acetylated by the acetyltransferase effector HopZ1a, but is also bound and monitored by the CNL ZAR1 (Lewis et al., 2010). ZAR1 probably detects the acetylation of ZED1 by HopZ1a and upon detection activates an immune response (Lewis et al., 2013). ZAR1 additionally indirectly monitors the kinase PBL2, a homologue of BIK1 (Wang et al., 2015a; Wei et al., 2010). It does this through constitutive interaction with another pseudokinase that is related to ZED1, called RKS1 (Huard-Chauveau et al., 2013).

The *Xanthomonas campestris* effector AvrAC is also recognised by ZAR1. AvrAC uridylates BIK1, blocking BIK1 phosphorylation to attenuate PTI (Feng et al., 2012). AvrAC uridylates RKS1 also, and this modification recruits the ZAR1/RKS1 complex, initiating an immune response (Wang et al., 2015a). The remarkable versatility of ZAR1 to recognise diverse effectors by binding related pseudokinases demonstrates how indirect recognition of effectors increases the utility of NLRs.

There is less evidence for mammalian NLRs than for plant NLRs utilising indirect recognition. I stated earlier that there is some evidence that the mammalian NLR NOD1 directly binds a ligand (Laroui et al., 2011), but NOD1 has more convincingly been shown to indirectly detect a bacterial effector. SopE, an effector delivered by *Salmonella*, activates the Rho GTPases Rac1 and Cdc42, which has the downstream consequence of altering the actin cytoskeleton to promote pathogen growth (Keestra et al., 2013). NOD1 monitors Rac1 and Cdc42 and triggers an immune response upon perturbation by SopE. Pyrin, a non-NLR protein with an N-terminal PYD, also monitors Rho GTPases for deactivation by a variety of toxins from several pathogens, demonstrating the versatility of indirect recognition (Xu et al., 2014c).

NOD1 also cooperates with NOD2 to detect ER stress induced by the *Brucella abortus* effector VceC or by chemical ER stress inducers, indicating that VceC recognition is through indirect mechanisms (Keestra-Gounder et al., 2016). NLRP3 is another NLR that detects cellular stress, and as described earlier, is triggered by a vast number of stimuli (Monie, 2013). Although the mechanism of detection of NLRP3-inflammasome elicitors is not known, it has been recently discovered that NLRP3 requires LRR binding to the kinase NEK7 for inflammasome function in response to several elicitors (He et al., 2016; Shi et al., 2015a). This suggests that NLRP3 may monitor NEK7 as a proxy for cellular integrity.

1.2.2.3. Engineering the specificity of effector perception of NLRs

NLR loci can be highly polymorphic, with multiple alleles of each individual NLR gene. This diversity in recognition capacity allows plant populations to maintain broad resistance to many pathogens. The diversity in recognition capacity between alleles, and between paralogues, provides a good resource to study the mechanisms that underpin differences in pathogen recognition specificity. Key example systems are: *Pik-1* alleles in rice which demonstrate differential recognition to *Magnaporthe oryzae Avr-Pik* alleles (Kanzaki et al., 2012); thirteen alleles of the flax *R* gene *L* which confers gene-for-gene recognition of flax rust strains (Ellis et al., 1999; Flor, 1965); Arabidopsis RPP13, which is highly polymorphic across Arabidopsis ecotypes and confers ecotype-specific differential resistance to three strains of *Hyaloperonospora arabidopsidis* (*Hpa*) (Bittner-Eddy et al., 2000; Rose et al., 2004); the NAIP NLRs, which have differential recognition of bacterial PAMPs (Vance, 2015); and RRS1 and RPS4, which I will describe in detail in following sections. As our mechanistic understanding of recognition has increased, the potential for engineering resistance has increased.

Several NLR allelic series exemplify the potential for rational engineering of NLRs to expand specificity. For example, the wheat NLR gene *Pm3* is in an allelic series with differential recognition specificities for powdery mildew isolates (Brunner et al., 2010). One allelic variant, PM3D, recognises the effector AvrPm3d from an isolate called DB Asosan, while PM3E recognises AvrPm3e from the 97019 isolate. These NLRs differ by three amino acids.

Brunner et al (2010) captured the genetic variation of both alleles in a single allele, *Pm3d+e*, creating a chimaera with dual recognition capacity that conferred recognition to both effectors. Similarly, the *Glycine max R* genes *Rpg1b* and *Rpg1r* share the roles of Arabidopsis *RPM1* by conferring recognition to the effectors *AvrB* and *AvrRpm1*, respectively (Ashfield et al., 1995). Ashfield et al (2014) modulated recognition of both effectors into a single allele by creating chimaeras of *Rpg1b* and *Rpg1r*. Although this approach helps inform us of NLR function and allows us to predict the structural requirements for broad recognition specificity, it has not yielded expanded, novel recognition of effectors.

Random mutagenesis of NLRs to generate expanded recognition is an alternative approach that has yielded some variants with novel recognition specificities. Directed evolution of the LRR domain of the NLR Rx was the first successful demonstration of an NLR with engineered recognition specificity (Farnham and Baulcombe, 2006). Rx confers resistance to Potato Virus X (PVX) strains with the TK-variant of the coat protein (CP), but not to variants with the KR variant; Farnham and Baulcombe (2006) stacked three point mutations to create RxM123, which gained recognition of CP-KR and the related Poplar Mosaic Virus (PopMV), and retained recognition of CP-TK. A stepwise mutation that led to RxM123, resulting in RxM1, conferred recognition of CP-KR but conferred only weak resistance to PopMV, resulting in a trailing necrosis phenotype. In a subsequent study, the researchers found that mutations in the NB or ARC1 domains strengthen the immune response to PopMV, resulting in full immunity and loss of the trailing necrosis phenotype (Harris et al., 2013). This demonstrated that RxM123 conferred strong PopMV resistance because it activated defence more rapidly than the stepwise mutants RxM1, RxM2 and RxM3.

The other notable engineering attempt of an NLR was random mutagenesis of the NLR R3a to extend its recognition specificity. R3a confers resistance to strains of *Phytophthora infestans* that carry AVR3a^{KI}, but confers only weak recognition to *P. infestans* strains harbouring the AVR3a^{EM} variant. Segretin et al (2014) generated several R3a alleles that were able to trigger an HR when transiently co-expressed with AVR3a^{EM} in *Nicotiana benthamiana*, and one

that was even able to recognise a related effector from *P. capsici*, PcAVR3a4. Curiously, none of the R3a variants conferred resistance to *P. infestans* strains carrying AVR3a^{EM}. Giannakopoulou et al (2015) introduced some of the gain-of-function mutations into the related NLR I-2, which confers resistance to the fungal pathogen *Fusarium oxysporum* f. sp. *lycopersici*, and found that one mutation greatly increased the weak recognition of I-2 for AVR3a^{EM}. Importantly, this mutant conferred quantitative resistance to *P. infestans* when transiently expressed in *N. benthamiana*. Although these innovative approaches generated expanded recognition, they still rely on chance and do not allow an NLR to be engineered to confer recognition to a specific effector from a list of characterised effectors from a selected pathogen. Engineering NLRs to perceive novel effectors is an under-utilised approach to providing genetic resistance to pathogens.

1.2.3. NLR activation

1.2.3.1. Relief of negative regulation

Intramolecular interactions within NLR molecules are important to activate immunity in the presence of pathogens, but also to suppress immunity in the absence of pathogens to limit the damage caused by ectopic activation. Exchange of ADP for ATP at the NB is associated with NLR activation, and the associated conformational change probably releases the negative regulation imposed by interacting domains within the protein (Takken et al., 2006; Lukasik and Takken, 2009; Takken and Tameling, 2009). Consistent with this, rearrangement or deletion of domains releases autoinhibition and results in a constitutively active NLR (Inohara et al., 1999; Bendahmane et al., 2002; Ade et al., 2007; Swiderski et al., 2009; Kofoed and Vance, 2011; see Section 1.2.1) The tight intramolecular regulation of immunity is subtle, and domains have roles in both suppression and activation. For example, although the deletion of the LRR in many NLRs renders them autoactive, the LRR of the maize CNL Rp1 is required for the constitutive activity of autoactive variants (Wang et al., 2015b). Another example that highlights the subtlety in intramolecular interactions that regulate NLR function is the interaction between the domains of the potato CNL Rx. Various mutations in the NB-ARC and LRR of Rx render it autoactive, but expressing the CC and the mutated NB-ARC-LRR

domains separately *in trans* suppresses autoactivity while maintaining perception of the cognate Avr of Rx, the PVX coat protein (Rairdan et al., 2008).

1.2.3.2. NLR cooperativity

Activated NLRs sometimes require other NLRs to transduce a signal. For example, the tobacco TNL requires the C_RNL NRG1 to confer resistance to TMV (Peart et al., 2005). The *N. benthamiana* homologue of the previously described Prf requires the CNLs NRC2a/b and NRC3 for recognition of AvrPto (Wu et al., 2016). Several unrelated Arabidopsis NLRs require a group of paralogous C_RNLs termed ADR1 (ACTIVATED DISEASE RESISTANCE 1), ADR1-LIKE1 (ADR1-L1) and ADR1-L2 (Bonardi et al., 2011; Dong et al., 2016). NLRs that cooperate downstream of other NLRs are often referred to as helpers. Some cooperating plant NLRs are genetically linked as pairs in converging or diverging orientations. These include the Arabidopsis gene pairs *RPS4*/*RRS1* and *CSA1*/*CHS3*, and the rice gene pairs *RGA4*/*RGA5* and *Pik-1*/*Pik-2* (Cesari et al., 2013; Maqbool et al., 2015; Narusaka et al., 2009; Xu et al., 2015). Some similarities between paired NLRs can be observed. One similarity is that paired NLRs have a division of labour. In cases where the mechanism of effector recognition as been characterised, the effector interacts with one NLR partner, termed the sensor. The other partner interacts with the sensor to transduce immune signalling, and is termed the executor (Griebel et al., 2014). For example, the rice CNL RGA5 is a sensor that directly interacts with the two effectors AVR-Pia and AVR1-CO39 from *Magnaporthe oryzae* to confer via interaction with the executor RGA4 (Cesari et al., 2013, 2014b).

Parallels can be drawn between plant paired NLRs and the murine NLRC4/NAIP inflammasome. In this system, the NAIPs are sensors that bind PAMPs and NLRC4 acts as the executor by transducing the immune signal via oligomerisation of CARDs (Kofoed and Vance, 2012). In humans, there is one NAIP homologue (hNAIP), but alternate splicing produces at least two splice variants which determine specificity through protein isoforms that confer recognition to either flagellin or the TTSS needle protein (Kortmann et al., 2015). Another example of NLR cooperativity in animals includes the

cooperation of NOD2 and NLRP1 for recognition of the PAMP muramyl dipeptide (Hsu et al., 2008).

1.2.3.3. Activation models

Several models have been proposed to unify our understanding of the mechanisms of plant-pathogen interactions (Chisholm et al., 2006; Jones and Dangl, 2006). A key concept that captures this interplay is the zigzag model of plant immunity (Jones and Dangl, 2006). This schema describes the selection pressures on both plant and pathogen as the pathogen "attempts" to subvert both PTI and ETI to establish a compatible disease interaction. The Arabidopsis-*Pseudomonas syringae* pathosystem provides an excellent illustration of the arms race described by this model. The susceptibility of Arabidopsis to *P. syringae* is enhanced by the effectors AvrB and AvrRpt2, which target RIN4. However, the plant contains the R proteins RPM1 and RPS2, which monitor RIN4 integrity for assault by these effectors. *P. syringae* that can colonise plans with RPM1 and RPS2 contains one or both of the effectors AvrPphB and HopF2, which suppress R-protein-mediated detection of AvrB and AvrRpt2, respectively (Russell et al., 2015; Wilton et al., 2010). AvrRpt2 itself can suppress AvrB recognition (Kim et al., 2005a). In an arms race model, host R proteins evolved to maintain recognition of effectors, and pathogen effectors evolved to enable colonisation by subverting the host immune system.

Recently, the invasion model has been put forward in an attempt to unify observations that are seemingly exceptional to the zigzag model (Cook et al., 2014). This model avoids describing observations in terms of PTI and ETI, or pathogen-derived molecules as PAMPs or effectors, and instead describes plant responses to pathogens as either lineage-specific or conserved, and elicitors of immunity as either structural or functional invasion patterns.

The zigzag model describes a landscape of resistance activation, at the extreme height of which the plant initiates HR. A systematic description unifying the observations of NLR activation, specifically focussing on the mechanisms of activation of the flax TNLs L6 and L7, was outlined in the recently proposed equilibrium-based switch activation model (Bernoux et al., 2016). The model proposes that NLRs exist in the plant cell in equilibrium

between “on” and “off” states. Recognised effectors stabilise the on state and move the position of the equilibrium toward more “on” molecules, promoting immunity.

1.2.4. Downstream of NLR activation

1.2.4.1. Induced proximity

In the case of inflammasome-forming NLRs, induced-proximity of CARDs promotes the recruitment of Caspase-1 proteins via homophilic CARD-CARD interaction between the NLR and Caspase-1 (Salvesen and Dixit, 1999). The recruited Caspase-1 or ASC may form filaments that are nucleated at the inflammasome and that are important for signal transduction (Cai et al., 2014a; Lu et al., 2014a; Zhang et al., 2015b). This forces a high local concentration of Caspase-1, which activates by proteolytically processing itself (Martinon and Tschopp, 2004).

Mature Caspase-1 activates immunity by cleaving pro-IL-1β and gasdermin D (Martinon and Tschopp, 2004; Shi et al., 2015b). Processed IL-1β is secreted from the cell and promotes inflammation, while the N-terminal fragment of gasdermin D oligomerises in the cell membrane, producing pores which mediate the pyroptotic cell-death response (Kayagaki et al., 2015; Liu et al., 2016; Shi et al., 2015b). PYD-containing NLRs instead recruit ASC, an adaptor protein that contains both CARD and PYD (Martinon et al., 2001; Masumoto et al., 1999, 2001). The PYD interacts with the NLR and the CARD recruits Caspase-1.

Two well studied NLRs that do not form inflammasomes, NOD1 and NOD2, recruit the kinase RICK1/RIP2 through homophilic interactions between the CARDs of NOD1/2 and RIPK1 (Akira et al., 2006; Inohara and Nuñez, 2003). RIPK1 is the first step in a signalling cascade that leads to the activation of NF-κB and the transcriptional upregulation of proinflammatory cytokines and antimicrobial peptides (Akira et al., 2006; Caruso et al., 2014).

1.2.4.2. Plant immune signalling adaptors

The mechanism of plant NLR-signalling is not as well understood as animal NLR-signalling. TNLs require the downstream signalling lipase-like protein

EDS1 (ENHANCED DISEASE SUSCEPTIBILITY 1) to confer resistance, but CNLs can function without it (Aarts et al., 1998). EDS1 interacts with the TNLs RPS4, RPS6 and SNC1 (Bhattacharjee et al., 2011; Heidrich et al., 2011) and may be required for signal transduction. Both nuclear and cytoplasmic pools of EDS1 are required for immune signalling for the TNLs RPS4 and RPS6 (García et al., 2010; Heidrich et al., 2011). However, restricting EDS1 to the nucleus or cytoplasm does not block immune signalling mediated by another TNL, called VICTR (Kunz et al., 2016).

EDS1 does not function alone but forms functional heterodimers with either PAD4 or SAG101, which are structurally related proteins (Wagner et al., 2013). These interactions contribute to signal transduction in a subset of TNLs. Recognition of the *P. syringae* effector AvrRps4 by the TNLs RRS1 and RPS4 requires a heterodimer of EDS1 and PAD4, but SAG101 is partially redundant with PAD4 in this role (Feys et al., 2005; Rietz et al., 2011; Wagner et al., 2013). However, SAG101 cannot compensate for mutated PAD4 during recognition of *Hyaloperonospora arabidopsidis* (*Hpa*) (Rietz et al., 2011). The mechanistic role of the EDS1/SAG101/PAD4 complex in signalling is an area of ongoing research.

A subset of CNLs which localise to the plasma membrane require *NDR1* (*NON-RACE SPECIFIC DISEASE RESISTANCE 1*), a glycosylphosphatidylinositol-anchored protein involved in maintaining the cell wall-plasma membrane junction (Aarts et al., 1998; Century et al., 1997; Coppinger et al., 2004; Elmore et al., 2011; Knepper et al., 2011). There are two phylogenetically distinct classes of CNLs (Andolfo et al., 2014; Jupe et al., 2012): those with a conserved EDVID motif (Rairdan et al., 2008), and those with CC domains that resemble the CC of the R protein RPW8, termed CC_R-NLRs or C_RNLs (Bonardi et al., 2011). Several C_RNLs have a demonstrated "helper" role and are required for the function of unrelated NLRs. Helper C_RNLs will be discussed later, and include the tobacco NRG1 and Arabidopsis ADR1 and ADR1-like proteins (Bonardi et al., 2011; Peart et al., 2005).

A big question in the mechanism of plant NLR function is, "Do plant NLRs activate defence by induced proximity of signalling domains or downstream executor proteins?". Overexpression of TIR or CC domains with intact

oligomerisation domains often triggers cell death, suggesting that oligomerisation induced by both high concentration of protein and a lack of intramolecular suppression usually enforced by the LRR is sufficient to trigger cell death. However, most plant NLRs exist in an oligomeric state prior to activation – the TNLs N and RPP1 are the exceptions that are monomeric prior to activation and oligomerise upon effector perception (Mestre and Baulcombe, 2006; Schreiber et al., 2016) – suggesting that if induced proximity of N-terminal signalling domains is the trigger for activating immunity that effector perception induces conformational changes that allow TIR or CC domains to come into close proximity.

1.2.4.3. Transcriptional regulation mediated by NLRs

Transcriptional regulation and salicylic acid (SA) accumulation are important downstream responses of NLR-mediated immunity. Other than TNLs and the lipase-like proteins PAD4 and SAG101, EDS1 interacts with a negative regulator of immunity, SRFR1 (Bhattacharjee et al., 2011; Kim et al., 2009; Kwon et al., 2009). Some effectors disrupt this interaction, but it is not known how this affects immune regulation (Bhattacharjee et al., 2011). SRFR1 interacts with TEOSINTE BRANCHED1/CYCLOIDEA/PCF (TCP) transcription factors TCP8, TCP14 and TCP15, and the triple mutant for these transcription factors are more susceptible to *P. syringae* carrying the effector AvrRps4 (Kim et al., 2014). TNL resistance was compromised in the triple mutant, but so too was resistance conferred by the CNLs RPS2 and RPM1. This establishes TCPs as a convergence point for all plant NLRs, not just the TNLs. Effectors from the bacterium *P. syringae*, the oomycetes *Hpa* and *Phytophthora capsici,* the fungus *Golovinomyces orontii* and the Aster Yellows phytoplasma strain Witches' Broom target TCPs, highlighting their importance for plant immunity (Mukhtar et al., 2011; Stam et al., 2013; Sugio et al., 2011, 2014; Weßling et al., 2014).

Some NLRs appear to regulate transcription more directly. Mammalian NLRs CIITA (MHC class 2 transcription activator) and NLRC5, which do not form inflammasomes, are each recruited to the nucleus and regulate gene expression (Devaiah and Singer, 2013; Kobayashi and van den Elsen, 2012; Ludigs et al., 2015). NLRC5 and CIITA regulate the expression of Major

Histocompatibility Complex I and II genes (MHCI and MHCII) genes, respectively. A recent study shows that NLRP3 is also recruited to the nucleus of Type 2 helper T cells, where it can act as a transcriptional regulator of cell differentiation, independent of its role in the inflammasome (Bruchard et al., 2015).

The plant TNLs SNC1 and RPS4 interact with the transcriptional activator bHLH84 (Xu et al., 2014b), and N interacts with the transcription factor SPL6 (Padmanabhan et al., 2013). SNC1 also associates with the transcriptional co-repressor TPR1, which in turn associates with the histone deacetylase HDA19 and binds the promoters of the negative immune regulators DND1 and DND2 (Zhu et al., 2010).

The barley CNL MLA1 interacts with the antagonistically acting transcription factors WRKY1 and MYB6, and the rice CNL PB1 interacts with WRKY45 to mediate immunity (Chang et al., 2013; Inoue et al., 2013). It was recently shown that other rice NLRs can interact with WRKYs, but no functional link was demonstrated in the conditions of the study (Liu et al., 2015b). Although there are examples of nuclear-localised NLRs, there is little evidence that NLRs directly regulate transcription.

Some NLRs may be able to bind DNA directly, without the need for an intermediary transcription factor. It was recently reported that, specifically after pathogen perception, the potato CNL Rx1 can bind and distort DNA via its NB-ARC domain (Fenyk et al., 2015). It remains to be seen if a binding partner of Rx1 confers this DNA-binding specificity. The tomato CNL I-2, which confers resistance to *Fusarium oxysporum* f.sp. *lycopersici*, also directly binds and melts DNA (Fenyk et al., 2016). ATP-bound I-2 has a higher affinity for DNA, and DNA-bound I-2 has a higher ATPase activity, establishing equilibrium between ATP/DNA-bound and ADP-bound/DNA-unbound I-2.

1.3. RRS1 and RPS4: The Rosetta Stone of plant immune signalling?

1.3.1. A model to study NLR-mediated plant immunity

The conservation of protein architecture in plant and animal NLRs suggests that there may be conservation in the mechanisms of NLR function. The genetic requirement for EDS1 in the TNLs, in particular, indicates that there may be commonalities in immune signalling in this branch of plant NLRs. In order to better understand molecular mechanisms of NLR activation and signalling, a model system is required. The RRS1 and RPS4 R protein pair have a number of characteristics for such a model system, including: two functionally different alleles that enable dissection of the stepwise or potentially unrelated requirements for recognition of each allele (Birker et al., 2009; Narusaka et al., 2009); at least three effectors, two of which are known, and which are unrelated in their amino acid sequence, structure and biochemical activity (PopP2 is an acetyltransferase and AvrRps4 has no known enzymatic activity); contribution to qualitative and quantitative disease resistance, allowing us to interrogate the reason that one system can give such different resistance responses (Debieu et al., 2015); a crystal structure of the homo- and hetero-dimerised TIR domains (Williams et al., 2014); a series of loss-of-function mutations in both NLRs (Sohn et al., 2014); and functionality of the system in distantly related plant species (Narusaka et al., 2013). It has frequently been proposed that RRS1 is a protein that has the capability to recognise, signal and activate defence through transcriptional reprogramming. It had been considered a missing link or "Rosetta stone", because gene fusion events are good predictors for functional association of proteins (Enright and Ouzounis, 2001). When I began my thesis work, it was still unknown how the RPS4/RRS1 complex perceived effectors.

1.3.2. Genetic context

RPS4 (*Resistance to Pseudomonas syringae 4*) and *RRS1* (*Resistance to Ralstonia solanacearum 1*) are tightly linked Arabidopsis TNLs that are divergently transcribed from a locus on chromosome five (Gassmann et al., 1999; Narusaka et al., 2009). Their start codons are separated by only 264 base pairs that may be a bidirectional promoter. *RPS4* (At5g45250) was cloned as a gene that conferred race-specific resistance to *P. syringae* pv. *tomato* (*Pst*) carrying the effector AvrRps4 from *P. syringae* pv. *pisi* (Gassmann et al., 1999; Hinsch and Staskawicz, 1996). Similarly, *RRS1*

(At5g45260) was mapped to the same locus from the ability of the Arabidopsis Nd-1 allele to confer resistance to *R. solanacearum* GMI1000, which carries the effector PopP2 (Deslandes et al., 1998, 2002). Resistance to the fungal pathogen *Colletotrichum higginsianum* was later mapped to the *RRS1*/*RPS4* locus, and it was found that mutations in either *RRS1-R* or *RPS4* compromised not only *C. higginsianum* resistance, but also resistance to *R. solanacearum* GMI1000 and *Pst-AvrRps4* (Birker et al., 2009; Narusaka et al., 2009). Later studies demonstrated that RRS1 and RPS4 physically interact via their TIR and other domains, and that the TIR interactions are required for resistance (Williams et al., 2014).

There are two functionally different alleles of RRS1: the *RRS1-R* allele, characteristic of the ecotypes Ws-2 and Nd-1, which confers recognition to PopP2, AvrRps4 and *C. higginsianum*; and the "susceptible" allele *RRS1-S* characteristic of Col-0, which confers recognition to AvrRps4 only (Deslandes et al., 1998, 2002; Narusaka et al., 2009). Plants with mutated RRS1 and RPS4 retain some level of resistance against *Pst-AvrRps4* compared to *Pst* that does not carry *AvrRps4* and maintain HR against AvrRps4 delivered by the non-pathogenic *Pseudomonas fluorescens* (*Pf0-1*) that carries a TTSS (Kim et al., 2010; Narusaka et al., 2009; Sohn et al., 2012), indicating that an RRS1/RPS4-independent recognition of AvrRps4 exists. This resistance mapped to two nearby homologues of *RRS1* and *RPS4*, which were termed *RRS1B* (*At5g45050*) and *RPS4B* (*At5g45060*) and were demonstrated to confer recognition to AvrRps4, but not PopP2 (Saucet et al., 2015).

1.3.3. Protein architecture and structure

The architecture of the RPS4 protein comprises the typical TIR-NB-ARC-LRR, but also contains a domain C-terminal to the LRR that I will refer to as the CTD (C-terminal domain), but has also been referred to as the PL (Post-LRR) domain or CNL (C-terminal non-LRR) (Figure 1.1) (Claverie et al., 2011; Dodds et al., 2001; Ghelder and Esmenjaud, 2016; Sohn et al., 2014). Domains with homology to the RPS4 CTD have been identified C-terminal of the LRR in TNLs across several plant species, including Arabidopsis (e.g. *RPS4*, *RPP1*), peach (e.g. *Ma*), flax (e.g. *P2*) and tobacco (e.g. *N*) (Claverie et al., 2011;

Dodds et al., 2001; Sohn et al., 2014). The function of this domain is not known, but it has been hypothesised to function as a DNA binding or transcriptional regulation domain, as a decoy, in ligand binding or in signalling. Its role in signalling is supported by the observation that four mutations in this domain abolish either AvrRps4 recognition or RRS1-mediated autoimmunity (Gassmann et al., 1999; Sohn et al., 2014).

RRS1 also contains the canonical TIR-NB-ARC-LRR domains, but additionally contains three C-terminal domains: an uncharacterised region of approximately 320 amino acids between the LRR domain and the WRKY domain, which I will term domain four (DOM4) for this thesis, the WRKY domain itself, and a C-terminal domain that varies in sequence and length between alleles, which I will term DOM6 (Deslandes et al., 2002) (Figure 1.1). RRS1 is one of three Arabidopsis TNLs that carry WRKY domains (Narusaka et al., 2009). WRKY proteins are transcription factors harbouring a conserved WRKY domain, which contains the WRKYGQK sequence followed by a $Cx_{4-5}Cx_{22-23}HxH$ or $Cx_7Cx_{23}HxC$ zinc-finger motif (Chi et al., 2013). The role of the WRKY domain in RRS1 has been subject to scrutiny and speculation since it was discovered. Other NLRs interact with WRKY proteins, which suggests that RRS1 could be a "Rosetta stone", exemplifying the functional relationship between NLRs and WRKYs through gene fusion.

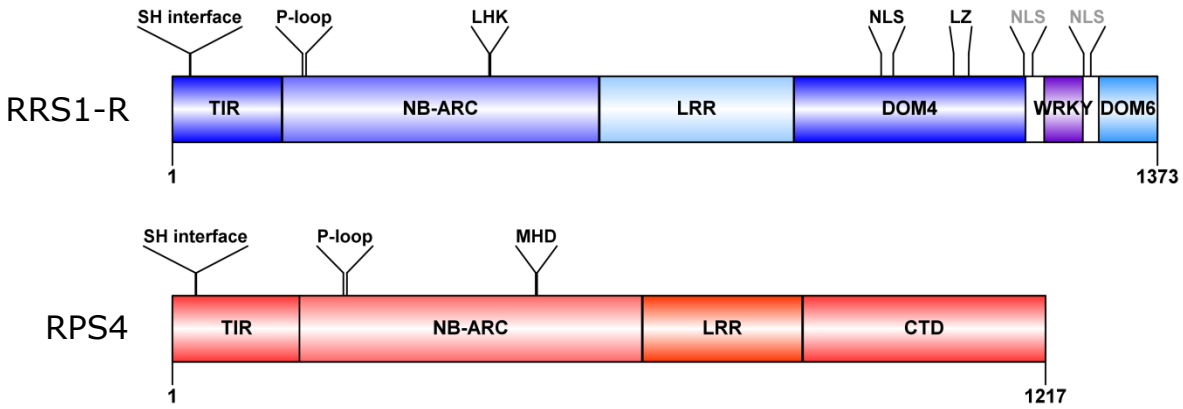

Figure 1.1. Domain architecture of RRS1-R and RPS4. RRS1-R has six domains: TIR; NB-ARC; LRR; DOM4; WRKY; DOM6. RPS4 has four domains: TIR; NB-ARC; LRR; CTD. Some common features of NLRs are indicated. SH interface, homo- and hetero-dimer interface for RRS1 and RPS4 [Williams et al 2014); P-loop, motif involved in nucleotide and Mg^{2+} binding, and nucleotide hydrolysis; MHD, conserved methionine-histidine-

aspartate motif at the C-terminus of ARC2 predicted to fulfil the role of the sensor II motif of AAA+ proteins, mutations in which often trigger autoimmunity; LHK, the amino acid residues at the MHD of RRS1; NLS, nuclear localisation signal (black = predicted in Deslandes et al. (2002); grey = predicted by NLS prediction software cNLS Mapper and NucPred (Brameier et al., 2007; Kosugi et al., 2009)); LZ, putative leucine zipper (Narusaka et al., 2016). The main difference between RRS1-S and RRS1-R is DOM6, which is 83 amino acids shorter on RRS1-R. Relative domain lengths are to scale.

In Chapter 3, I outline a model that assigns an "integrated decoy" role to the WRKY domain of RRS1 that has evolved to detect and protect against pathogenic threats to transcription. Although RRS1 binds DNA *in vitro* and *in planta*, and loss of DNA-binding is often (but not always) correlated with an autoimmune phenotype, it remains to be seen if RRS1 behaves as a bona fide transcription factor or cofactor (Noutoshi et al., 2005; Le Roux et al., 2015; Sarris et al., 2015; Sohn et al., 2014).

A mutant allele of *RRS1* termed *sensitive to low humidity 1* (*RRS1*^{slh1}) contains a leucine insertion in the WRKY domain that disrupts DNA-binding and results in an *RPS4*-dependent constitutive autoimmune phenotype (Noutoshi et al., 2005; Sohn et al., 2014). A suppressor screen of this autoimmunity identified many intragenic mutations that abolish *RRS1*^{SLH1} autoimmunity, including several mutations in DOM4 (Sohn et al., 2014). Additionally, there is a leucine zipper motif in DOM4, which also triggers constitutive RPS4-dependent autoimmunity when mutated (Narusaka et al., 2016).

The co-crystal structure of the RPS4 and RRS1 TIR domains revealed an interaction interface with key adjacent Ser and His residues (SH motif) (Williams et al., 2014). Mutation of these residues abolishes TIR interactions and immune signalling, but the full-length proteins retain association through interactions at other domains. Overexpression of the RPS4 TIR or the full-length protein triggers HR in tobacco, *N. benthamiana* and Arabidopsis, but overexpression of the RRS1 TIR does not (Heidrich et al., 2013; Swiderski et al., 2009; Williams et al., 2014; Zhang et al., 2004). This establishes a "division of labour" in the R protein pair, in which RPS4 is the executor, and RRS1 is the sensor. Mutating the P-loop of RPS4, but not RRS1, abolishes recognition of effectors, supporting this annotation further (Sohn

et al., 2014). Evidence for RRS1 as a sensor is provided in Chapter 3: both AvrRps4 and PopP2 are sensed by direct interaction with the WRKY domain of RRS1.

Deslandes et al (2002) predicted that RRS1 contains a nuclear localisation signal (NLS) in DOM4 and a simple search identifies another potential NLS at the N-terminus of the WRKY domain (Brameier et al., 2007; Kosugi et al., 2009). RPS4 contains an NLS in its CTD (Wirthmueller et al., 2007). Different studies have detected RRS1 in RPS4 in different compartments using fluorescence microscopy and organelle fractionation, and the consensus would suggest that both proteins are present in the nucleus, cytoplasm, and microsomes (Bhattacharjee et al., 2011; Deslandes et al., 2003; Heidrich et al., 2011; Narusaka et al., 2016; Wirthmueller et al., 2007). However, most studies place RRS1 in the nucleus, only, and the observations of the proteins in other compartments may be an artefact of overexpression or of a failure to co-express RPS4 and RRS1 together. Mutation of the putative leucine zipper of RRS1 not only triggers constitutive HR, but also reduces accumulation of heterologously expressed RRS1 and RPS4, and abolishes the nuclear accumulation of both proteins (Narusaka et al., 2016). Nuclear accumulation is required for RPS4 activity (Wirthmueller et al., 2007). Heidrich et al (2011) confined AvrRps4 to the nucleus, which suppressed cell death but not immunity. However, Sohn et al (2014) observed HR when AvrRps4 was restricted to either the nucleus or cytoplasm. Although the experiments have conflicting results, they demonstrate that RPS4 and RRS1 are active in both the nucleus and cytoplasm.

1.3.4. Effectors: PopP2 and AvrRps4

PopP2 is an acetyltransferase that enhances *R. solanacearum* virulence on susceptible tomato, bean and eggplant hosts (Macho et al., 2010). It is in the YopJ family of effectors and contains conserved amino acids that resemble the catalytic triad of CE family proteases (Mukherjee et al., 2006; Orth et al., 2000; Tasset et al., 2010). The crystal structure of HopZ1a, a related YopJ acetyltransferase from *P. syringae*, revealed that YopJ family acetyltransferases have lost the canonical SUMO binding pocket of CE

proteases, and instead have adapted the pocket to form an acetyl-enzyme intermediate during acetyltransferase catalysis (Zhang et al., 2016).

PopP2 interacts with both RRS1-R and RRS1-S, demonstrating that RRS1-S interaction with PopP2 per se is insufficient for defence activation (Deslandes et al., 2003; Williams et al., 2014). The region of RRS1 that interacts with PopP2 was not identified. Furthermore, PopP2 enzymatic activity is essential for immune signalling activation via RRS1-R, because mutating the catalytic core Cys321 abolishes recognition (Tasset et al., 2010). A continuing puzzle is the role of the host cysteine protease RD19, which is required for PopP2-triggered immunity (Bernoux et al., 2008). PopP2 re-localises RD19 to the nucleus, but no physical or enzymatic interaction has been established (Bernoux et al., 2008).

When secreted into the plant cell, AvrRps4 is proteolytically cleaved by an unknown host protease (Sohn et al., 2009). The C-terminal fragment is recognised by RRS1/RPS4 and co-immunoprecipitation of RRS1 and AvrRps4 suggests that association between the two proteins is required for AvrRps4 detection (Williams et al., 2014). AvrRps4 interaction with RRS1 probably occurs at the WRKY domain because a yeast two-hybrid (Y2H) screen identified an interaction between AvrRps4 and a WRKY closely related to RRS1, WRKY41 (Mukhtar et al., 2011), and because mutations in the RRS1-WRKY domain disrupt the association between AvrRps4 and the C-terminus of RRS1 (Sarris et al., 2015). The C-terminal fragment of cleaved AvrRps4 contains two features that are required for detection by RRS1/RPS4: Lys135-Arg-Val-Tyr138, termed the KRVY motif, and two glutamates comprising a surface-exposed negative patch (Sohn et al., 2012). AvrRps4 cleavage and the KRVY motif are required for virulence when expressed in Arabidopsis, although AvrRps4 does not confer virulence when delivered by *Pst DC3000* (Sohn et al., 2009). After cleavage, the N-terminal fragment translocates to the chloroplast, probably due to a putative chloroplast transit peptide (Li et al., 2014a). AvrRps4 has been detected in complexes containing EDS1, and may disrupt these complexes (Bhattacharjee et al., 2011; Heidrich et al., 2011). AvrRps4 virulence may require interactions between either or both WRKY proteins (suggested by the interaction between AvrRps4 and WRKY41)

and EDS1. In Chapter 3, I discuss evidence that the former interaction is required.

Interaction between RRS1 and both effectors is essential for recognition and occurs in the nucleus (Deslandes et al., 2003; Sarris et al., 2015). Indeed, exclusion of PopP2 from the nucleus abolishes recognition (Sohn et al., 2014). Heidrich et al (2011) restricted the activation of RPS4/RRS1 to the nucleus by expressing AvrRps4 fused to an NLS (AvrRps4^{NLS}); this suppressed HR but retained pathogen resistance. However, Sohn et al (2014) observed HR and elevated ion leakage when AvrRps4^{NLS} or AvrRps4 fused to a nuclear export signal (NES) was delivered to Arabidopsis.

1.4. Aims

In this thesis, I am to understand the mechanism of effector recognition by the NLR pair RRS1/RPS4, and to use that knowledge to engineer synthetic resistance. Chapter 3 outlines my research that contributed to our understanding of how RRS1 recognises both PopP2 and AvrRps4. This work provides some of the fundamental observations for the integrated decoy model of effector recognition. I hypothesise that an ancestor NLR to RRS1 monitored the integrity of a WRKY protein involved in immunity, the function of which was abrogated by effectors. This guard/guardee relationship had positive selection and co-inheritance of the NLR and WRKY protein was favoured. The WRKY protein may have exerted negative regulation on the NLR, suppressing autoactivity in the absence of effectors and further favouring tight linkage between the NLR and WRKY. In this scenario, a genome rearrangement occurred that fused the tightly linked NLR and WRKY to resemble RRS1. This close genetic association was highly favoured because it ensured the NLR and WRKY were inherited as a unit. The WRKY domain of the RRS1 progenitor may have lost its original function and evolved to maintain recognition of effectors.

In Chapter 4, I leverage knowledge of the mechanism of RRS1 autoinhibition to engineer recognition of viral proteases. I hypothesise that NLRs maintain a steady-state autoinhibition of immune signalling through intramolecular

interactions. Effector recognition disrupts this autoinhibition and activates immune signalling. The strategies described in Chapter 4 aim to generate new alleles of RRS1 that are relieved of autoinhibition by novel effectors.

Chapter 5 details my progress to-date engineering a plant intracellular PRR system based on the mammalian NLRC4/NAIP inflammasome. In this chapter I have two aims: first, to transfer a mechanism of intracellular PAMP perception from mammals into plants; and second, to use this system to determine if NLR signalling is initiated similarly in plants and mammals. I hypothesise that plant NLR immune signalling is activated by the induced proximity of N-terminal signalling domains, similarly to mammalian NLRs.

The overarching hypothesis of my thesis is that plant and animal NLRs employ the following common mechanisms for response to elicitors that can be engineered to expand NLR specificity: autoinhibition of signalling domains, release of autoinhibition by elicitor perception and conformational changes which bring the signalling domains into close proximity.

Chapter 2. Materials and Methods

2.1. Molecular and biochemical methods

2.1.1. Plasmid purification

5 mL lysogeny broth (LB) was inoculated with a single bacterial colony and supplemented with the appropriate antibiotic(s). Cultures were incubated at 37 °C with shaking overnight. Overnight cultures were centrifuged for 5 min at 5000 x *g*. Plasmids were extracted from the bacterial pellet using a QIAprep Spin Miniprep Kit (Qiagen), eluted in 20-50 µL of water and stored at -20 °C. Plasmids were sequenced by the GATC Biotech company (http://www.gatc-biotech.com/en/index.html).

2.1.2. Polymerase Chain Reaction (PCR)

DNA was amplified for cloning using Phusion High Fidelity DNA polymerase (NEB). Reaction mixes contained 1X reaction buffer, 0.2 mM dNTPs, 0.5 µM each of forward and reverse primer, 0.4-4 ng/µL DNA and 20 U/mL Phusion DNA polymerase. For mapping and colony PCR I used Taq DNA polymerase (NEB) at a concentration of 10 U/mL. For colony PCR, instead of purified DNA I used a single picked colony diluted in 25 µL water. To amplify DNA for USER cloning, I used 2X HiFi Uracil+ DNA Polymerase Readymix (KAPA).

Reactions were cycled between denaturing, annealing and extension temperatures appropriate to the primers and template in a thermocycler. PCR amplicons were either purified by a QIAquick PCR Purification Kit or QIAquick Gel Extraction Kit (QIAGEN).

2.1.3. DNA Electrophoresis

PCR amplicons and restriction digested plasmids were mixed with 1/10th volume 10X DNA loading dye (40% (v/v) glycerol, 0.5% (w/v) SDS, 10 mM EDTA, Orange G) and loaded into a 1-1.5% (w/v) agarose TAE (40 mM Tris, pH 7.6, 20 mM acetic acid, 1 mM EDTA, 0.5 µg/mL ethidium bromide) gel with an appropriate DNA ladder. 100-150 V was applied until the dye migrated approximately halfway down the gel, then the gel was imaged under UV light in a Geldoc (Bio-Rad).

2.1.4. Golden Gate Cloning

Most constructs were created using Golden Gate cloning. Golden Gate cloning is a method that simplifies the construction of multi-fragment plasmids in a single reaction containing the component DNA parts, including a recipient plasmid, a type IIS restriction enzyme (BpiI or BsaI) and DNA ligase (Engler et al., 2008; Weber et al., 2011). One requirement of this method is that the donor DNA must not contain BpiI or BsaI recognition sites. To remove sites from donor DNA, I performed Site Directed Mutagenesis (SDM) or ordered the DNA synthesised. During the single reaction, 4 bp overhangs are generated in each donor DNA plasmid or segment during a three min 37 °C stage. Type IIS restriction enzymes cut outside their recognition site, so repeated digestion stages will not cleave ligations that do not restore recognition sites and overhangs are unique to the destination of each DNA fragment. Digestion is followed by a four min 16 °C ligation stage. Stages are cycled 25 times in a thermocycler. Digestion/ligation reactions contained: 0.1 mg/mL BSA, 1X T4 DNA ligase buffer (NEB), 20 U/μL T4 DNA ligase (NEB), 1 U/μL BsaI-HF or BpiI (NEB), 0.67 fmol/μL recipient plasmid, 1.33 fmol/μL each donor DNA.

2.1.5. USER Cloning

In this cloning technique, PCR amplicons were generated with deoxyuridine-containing primers and HiFi Uracil+ DNA Polymerase (KAPA), a proofreading DNA polymerase that is compatible with deoxyuridine templates. Deoxyuridines were excised from PCR products with a mixture of uracil DNA glycosidase and DNA glycosylase-lyase endo VIII enzymes in the USER (NEB) mixture. After overhangs were generated by deoxyuridine excision, PCR products were ligated to recipient plasmid with complementary overhangs generated by digestion with the restriction enzyme PacI and the nicking endonuclease Nt.BbvCI. Reactions were performed in a single tube. Reactions were incubated for 15 min at 37 °C and 15 min at 25 °C. After this, T4 DNA ligase (NEB) and DNA ligase buffer were added to final concentrations recommended by the manufacturer and incubated for two hours at room temperature.

2.1.6. Colony PCR

To expedite cloning, white transformant colonies were picked with a pipette tip and resuspended in 25 µL Taq PCR reactions. After analysis by DNA electrophoresis, three colonies containing plasmids that recovered amplicons of the correct size were inoculated into 5 mL of LB for plasmid purification. After sequencing and restriction digestion to confirm the correct plasmid is present, the culture was stored in 20% (v/v) glycerol for storage at -80 °C.

2.1.7. Bacterial transformation

Plasmids and ligation reactions were electroporated in 0.1 cm cuvettes using a Micropulser (Biorad) into electrocompetent *Escherichia coli* DH10B cells with a competency of ~10^8 cfu/µg DNA using the manufacturer's preprogrammed *Ec1* setting. Plasmids reactions were electroporated in 0.1 cm cuvettes using a Micropulser (Biorad) into electrocompetent *Agrobacterium tumefaciens* GV3101 or Agl-1 cells with a competency of ~10^6 cfu/µg DNA using the manufacturer's preprogrammed *Agr* setting. Transformed *E. coli* or *A. tumefaciens* were recovered in 2X yeast extract tryptone (YT) medium for 1 hour with shaking at 37 °C or 18 °C, respectively. Recovered transformation reactions were spread on LB plates supplemented with the appropriate antibiotic, and with X-Gal and IPTG for blue/white screening, if appropriate.

2.1.8. Site Directed Mutagenesis (SDM)

Mutations were introduced into plasmids using the QuikChange II Site Directed Mutagenesis kit (Agilent) or by using back-to-back internal primers with mutations introduced at the 5' end.

2.1.9. Plant protein extraction

Leaves were harvested and ground in liquid nitrogen. Total proteins were extracted in GTEN buffer (10% (v/v) glycerol, 100 mM Tris-HCl pH 7.5, 1 mM EDTA, 150 mM NaCl) supplemented extemporaneously with 5 mM DTT, 1% (v/v) plant protease inhibitor cocktail (Sigma-Aldrich) and 0.2% (v/v) Nonidet P-40 Substitute (Sigma-Aldrich).

2.1.10. Immunoprecipitation

Protein samples were prepared from *N. benthamiana* 48hs after *Agrobacterium*-mediated transformation and the immunoprecipitation protocol was performed as was previously described (Sohn et al., 2014). Lysates were centrifuged for 20 min at 5,000 g at 4 °C and passed through a 0.2 μm syringe filter. 1.5 mL of filtered extract was incubated with 30 μL anti-FLAG M2 or EZview anti-HA affinity gel (Sigma-Aldrich), or GFP-Trap anti-GFP agarose beads (ChromoTek) for 2 hat 4 °C with gentle mixing. Antibody-coupled agarose beads were collected and washed three times in GTEN buffer, resuspended in SDS-loading buffer (63 mM Tris-HCl, pH 6.8, 1% (w/v) SDS, 10% (v/v) glycerol, 5 mM DTT, 0.003% (w/v) bromophenol blue) and denatured for 10 min at 96 °C. Proteins were separated by SDS-PAGE and analysed by immunoblotting using anti-FLAG M2, anti-HA-HRP, or anti-GFP conjugated antibodies.

2.1.11. SDS-PAGE

Protein samples in loading buffer were loaded onto acrylamide gels. Gels were cast in two phases: a lower resolving phase (6-12% (w/v) acrylamide, pH 8.8) and an upper stacking phase (5% (w/v) acrylamide, pH 6.8). Gels were submerged in running buffer (25 mM Tris-HCl, 192 mM glycine, 0.1 % (w/v) SDS) and 150 V was applied across the gels. PageRuler (Thermofisher) was used as a marker for protein size.

2.1.12. Blue Native PAGE

Blue Native PAGE was performed with the NativePAGE Bis-Tris Gel System (ThermoFisher). Protein extracted from plants was mixed with the NativePAGE Sample Buffer (4×) and NativePAGE 5% G-250 Sample Additive according to the manufacturer's instructions and run on a 3-12% NativePAGE Bis-Tris gel at 150V. The cathode chamber was filled with NativePAGE Dark Blue Cathode buffer and the anode buffer was filled with NativePAGE Anode buffer. When the dye-front had travelled ~1/3 down the gel, the NativePAGE Dark Blue Cathode buffer was replaced with the NativePAGE Light Blue Cathode buffer.

2.1.13. Immunoblotting

Proteins were transferred from the acrylamide gel to an Immobilon-P PVDF membrane (Merck Millipore) using a TransBlot Turbo Transfer System (Bio-Rad) according to the manufacturer's instructions. The membrane was blocked for 2 h at room temperature with 5 % (w/v) skim milk powder (DBC Foodservice) in Tris-buffered saline with 0.1% (v/v) Tween-20 (TBST). Membranes were incubated with the appropriate horseradish peroxidase (HRP)-conjugated antibody dissolved in TBST supplemented with 5% skim milk overnight at 4 °C with shaking. To probe for Myc-epitope-tagged proteins, membranes were incubated with anti-Myc antibody produced in rabbit (Sigma-Aldrich). Probed membranes were washed three times for 10 min with TBST, followed by a 10-min wash with TBS (Tris-buffered saline). Membranes probed with anti-Myc antibody were incubated with goat anti-rabbit IgG conjugated to HRP (Sigma-Aldrich) dissolved in TBST supplemented with 5% skim milk for 2 hours at room temperature with gentle shanking. Proteins of interest were detected by luminescence of HRP using the chemiluminescent substrate SuperSignal West Pico and Femto (ThermoFisher). Chemiluminescence was imaged with an ImageQuant LAS 4000 (Life Sciences).

2.1.14. Mass spectrometry

Samples for LC-MS analysis were excised from SDS-PAGE gels, destained, reduced and alkylated, and digested with trypsin. Peptides were analysed on a hybrid mass spectrometer LTQ-Orbitrap XL (ThermoFisher Scientific) and a nanoflow-UHPLC system (nanoAcquity, Waters Corp.) and masses were searched on Mascot server v.2.4.1 (Matrix Science) against the TAIR (version 10) database to identify acetylated peptides. Selected reaction monitoring (SRM) of acetylated peptides and non-modified control peptides by triple quadrupole MS was performed as described in Kadota et al. (2014) using nano-spray LC ESI and a TQ-S MS (Waters Corp., MA, USA). At least one replicate injection was performed per experiment and each experiment was repeated at least three times. The resultant TQ-S files were analysed in Skyline (see Supplementary methods).

2.1.15. Electrophoretic Mobility Shift Assay (EMSA)

Proteins to be analysed were expressed and extracted from *N. benthamiana* using *Agrobacterium*-mediated transient expression. Extracted proteins were purified by FLAG-IP and released from anti-FLAG antibodies by incubation with 3 X FLAG peptide (SIGMA). Purified proteins were concentrated by Amicon Ultra filter (Millipore Amicon). Synthetic W-box DNA oligonucleotide (W-box: 5′ -CGTTGACCGTTGACCGAGTTGACTTTTTA-3′), or a mutant form (mW-box: 5′ -CGTaGACgGTaGACgGAGTaGACgTTTTA-3′), were 5′-labelled with ^{32}P using T4 polynucleotide kinase (Invitrogen). FLAG-immunopurified protein was then co-incubated with DNA probes (100 fmol) for 60 min on ice, in binding buffer (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 2.5 mM DTT, 2.5 mM EDTA, 5 mM $MgCl_2$, 20% glycerol, 1 g dIdC). Samples were separated on a 4% native gel in 0.5 × TBE buffer at 100 V for 45 min. Following electrophoresis, the gel was dried on 3MM chromatography paper and exposed to an IP plate. Signal was detected by a FLA-5000 image analyser (Fujifilm).

2.2. Plant material and methods

2.2.1. Arabidopsis growth

Arabidopsis seeds were sown directly onto compost, stratified at 4 °C for four days. They were then transferred to a controlled environment room and were grown in short days (10 hr light/14 hr dark) at 21°C and 75% humidity.

2.2.2. Nicotiana spp. growth

Nicotiana benthamiana and *N. tabacum* "Petit Gerard" plants were grown in long days (16 hr light/8 hr dark) at 24 °C.

2.2.3. Transient protein expression by agroinfiltration

Agrobacterium strains carrying various constructs were grown in liquid LB-medium supplemented with the appropriate antibiotic for 24 hr at 28 °C with shaking. Cells were harvested by centrifugation, washed in 5 ml of 10 mM MgCl2, and re-suspended at OD600 0.5 in infiltration medium (10 mM MgCl2, 10 mM MES, pH 5.6). *N. tabacum* was infiltrated for HR assays and *N. benthamiana* for protein expression at 4–5 weeks old.

2.2.4. Seed selection

Arabidopsis seeds transformed with plasmids containing the FAST(fluorescence-accumulating seed technology)-Red selectable marker (Shimada et al., 2010) were screened for the presence of the fluorescent red selectable marker using a MZ16 fluorescence stereomicroscope (Leica). FAST-Red consists of the promoter of the *OLE1* oleosin gene of Arabidopsis driving a fusion of *OLE1* C-terminally tagged with the red fluorescent protein (RFP) TagRFP, which was derived by semi-random mutagenesis of the RFP eqFP578, from the sea anemone *Entacmaea quadricolor* (Merzlyak et al., 2007). OLE1-TagRFP is expressed only in dry seeds, and allows rapid, non-destructive screening of T1 seed.

2.2.5. Pseudomonas fluorescens Pf0-1 infiltration

Pf0-1 strains carrying PopP2, PopP2-C321A or empty vector were streaked on King's B agar plates and incubated at 28 °C for 24 hours. The bacterial lawn was resuspended in 10 mM $MgCl_2$ to an OD_{600} of 0.2. The bacterial suspension was syringe-infiltrated into the leaves of 5-week-old Arabidopsis plants. Leaves were assessed for HR 16 hpi.

2.2.6. Confocal microscopy

Agrobacterium strains carrying plasmids encoding NLRC4 fused to GFP were agroinfiltrated into *N. benthamiana*. Leaf discs were mounted in water 2 dpi and observed with a Leica SP5 confocal microscope (Leica Microsystems). Images were captured under a 63× objective after excitation at 488 nm or XX, for GFP- and auto-fluorescence, respectively.

2.3. Appendices

Appendix 2.1. List of primers used in this study.

Name	Nucleotide sequence	Purpose
ZD_W70-1F	AGGAAGACAAAATGGATACTAATAAAGCAA	Cloning WRKY70
ZD_W70-1R	AGGAAGACAACCTCTTCCTTCATTGAGGTAGATAA	Cloning WRKY70
ZD_W70-2F	AGGAAGACAAGAGGACAATCCTCATCGTCATCATGG	Cloning WRKY70
ZD_W70-2R	AGGAAGACAACGAACCAGATAGATTCGAACATGAACTG	Cloning WRKY70
ZD53_W33-1F	AGGAAGACAAAATGGCTGCTTCTTTTCTTACAATGG	Cloning WRKY33
ZD_W33-1R	AGGAAGACAAAGATGAAGAAGATCTTCTAGTAGACTG	Cloning WRKY33
ZD_W33-2F	AGGAAGACAAATCTTCTTCGACTTTTCATTCAGCTGTG	Cloning WRKY33
ZD_W33-2R	AGGAAGACAAGAGGACGAATCCTGTGGTGCTCTGTTTG	Cloning WRKY33
ZD_W33-3F	AGGAAGACAACCTCAGTCCCGATTAGACCAGCTG	Cloning WRKY33
ZD_W33-3R	AGGAAGACAACGAACCGGGCATAAACGAATCGAAAAATGAGGTTTCCTC	Cloning WRKY33
ZD_W41F-1	AGGAAGACAAAATGGAAATGATGAATTGGGAGCGGAGG	Cloning WRKY41
ZD_W41R-1	AGGAAGACAAAGGACGAACCCTGAAGCTGCTTGG	Cloning WRKY41
ZD_W41F-2	AGGAAGACAATCCTCTCCATCGTTGTCAGCTTCTTCTT	Cloning WRKY41
ZD_W41R-2	AGGAAGACAACGAACCAATCGAATTGTGGAAAAAAGTGGGG	Cloning WRKY41
ZD131_W60F	AGGAAGACAAAATGGACTATGATCCCAACAC	Cloning WRKY60
ZD132_W60R	AGGAAGACAACGAACCTGTTCTTGAATGCTCTATCAATCTC	Cloning WRKY60
ZD77_RRS1-K1217R-Fw	GGACTTGGCGAAGGTACGGTCAAAAAGACATCTTAGGTTCTCG	SDM primer to generate RRS1 K1217R
ZD78_RRS1-K1217R-Rv	CGAGAACCTAAGATGTCTTTTTGACCGTACCTTCGCCAAGTCC	SDM primer to generate RRS1 K1217R
ZD79_RRS1-K1221R -Fw	GGACTTGGCGAAAGTACGGTCAAAGAGACATCTTAGGTTCTCG	SDM primer to generate RRS1 K1221R
ZD80_RRS1- K1221R -Rv	CGAGAACCTAAGATGTCTCTTTGACCGTACTTTCGCCAAGTCC	SDM primer to generate RRS1 K1221R
ZD90_RRS1-KK/RR-Fw	GGACTTGGCGAAGGTACGGTCAAAGAGACATCTTAGGTTCTCG	SDM primer to generate RRS1 K1217R/K1221R
ZD91_RRS1-KK/RR-Rv	CGAGAACCTAAGATGTCTCTTTGACCGTACCTTCGCCAAGTCC	SDM primer to generate RRS1 K1217R/K1221R
ZD98_RRS1-K1217Q-Fw	GGACTTGGCGACAGTACGGTCAAAAAGACATCTTAGGTTCTCG	SDM primer to generate RRS1 K1217Q
ZD99_RRS1-K1217Q-Rv	CGAGAACCTAAGATGTCTTTTTGACCGTACTGTCGCCAAGTCC	SDM primer to generate RRS1 K1217Q
ZD100_RRS1-K1221Q -Fw	GGACTTGGCGAAAGTACGGTCAACAAGACATCTTAGGTTCTCG	SDM primer to generate RRS1 K1221Q
ZD101_RRS1- K1221Q -Rv	CGAGAACCTAAGATGTCTTGTTGACCGTACTTTCGCCAAGTCC	SDM primer to generate RRS1 K1221Q
ZD102_RRS1-KK/QQ-Fw	GGACTTGGCGACAGTACGGTCAACAAGACATCTTAGGTTCTCG	SDM primer to generate RRS1 K1217Q/K1221Q
ZD103_RRS1-KK/QQ-Rv	CGAGAACCTAAGATGTCTTGTTGACCGTACTGTCGCCAAGTCC	SDM primer to generate RRS1 K1217Q/K1221Q
ZD242_SOC1_KboxCT_Fw	AGGAAGACAATTCGAAGGATCGAGTCAGCACC	Cloning SOC1 K-box for RRS1 C-term fusion
ZD243_SOC1_KboxCT_Rv	AGGAAGACAAAAGCCTAAGATCCCCACTTTTCAGAG	Cloning SOC1 K-box for RRS1 C-term fusion
ZD270_Med19a_CT_Fw	AGGAAGACAATTCGATGGAGCCTGAACGTTTAAAATTTGG	Cloning MED19A for RRS1 C-term fusion
ZD271_Med19a _CT_Rv	AGGAAGACAAAAGCTTAGCCAGCAACCCTTATTGC	Cloning MED19A for RRS1 C-term fusion
ZD356_D4/5_R2	AGGAAGACAACTTCTCCCTGCATCCAACG	To generate RRS1-S-protease trap
ZD357_D4/5_F3	AGGAAGACAAAAACATCGCGAAAGTAAGG	To generate RRS1-S-protease trap
ZD378_D4/5 TuMV insFv2	AGGAAGACAAGAAGGGTTCTCTCGTTAGACATCAATCAGGGTCGAAACTTGTCTTCCT	To generate RRS1-S-TuMV

ZD379_D4/5 TuMV insRv2	AGGAAGACAAGTTTCGACCCTGATTGATGTCTAACGAGAGAACCCTTCTTGTCTTCCT	To generate RRS1-S-TuMV
ZD395D4/5_1xHA-GS5insF	AGGAAGACAAGAAGGGTTCTTATCCATACGATGTCCCAGATTATGCGGGTAGCGGTTCAGGCTCTGGAAGTGGAAGTAAACTTGTCTTCCT	To generate RRS1-S-[GS]5
ZD396D4/5_1xHA-GS5insR	AGGAAGACAAGTTTACTTCCACTTCCAGAGCCTGAACCGCTACCCGCATAATCTGGGACATCGTATGGATAAGAACCCTTCTTGTCTTCCT	To generate RRS1-S-[GS]5
ZD397D4/5_1xHA-TuMVinsF	AGGAAGACAAGAAGGGTTCTTATCCATACGATGTCCCAGATTATGCGGGTTCTCTCGTTAGACATCAATCAGGGTCGAAACTTGTCTTCCT	To generate RRS1-S-HA-TuMV
ZD398D4/5_1xHA-TuMVinsR	AGGAAGACAAGTTTCGACCCTGATTGATGTCTAACGAGAGAACCCGCATAATCTGGGACATCGTATGGATAAGAACCCTTCTTGTCTTCCT	To generate RRS1-S-HA-TuMV
ZD354_TuMV_NIa_F	AGGAAGACAAAATGAGTAACTCCATGTTCAGAGG	To clone TuMV NIa-pro
ZD355_TuMV_NIa_R	AGGAAGACAACGAACCTTGTGCGTAGACTGCCGTGC	To clone TuMV NIa-pro
ZD435_TuMV_NIaC/A_Fv2	AGGAAGACAAAGCCGGAAGTCCAATGGTGAGC	To mutate TuMV NIa-pro to catalytic dead variant
ZD436_TuMV_NIaC/A_Rv2	AGGAAGACAAGGCTTGGCCGTCTTTAGTGC	To mutate TuMV NIa-pro to catalytic dead variant
ZD565_PVYNTN_NIa_F	AGGAAGACAAAATGGCCAAATCACTCATGAGAGG	To clone PVY NIa-pro
ZD566_PVYNTN_NIa_R	AGGAAGACAACGAACCTTGCTCTACAACAACATCATGATC	To clone PVY NIa-pro
ZD560_CBSV_NIa_F	AGGAAGACAAAATGGTTGCAAAACCAGAAACAAC	To clone CBSV NIa-pro
ZD561_CBSV_NIa_R	AGGAAGACAACGAACCTTGCACTGTTATTGTGTTGATTGC	To clone CBSV NIa-pro
ZD421_D4/5_ZIMinsF:	AGGAAGACAAGAAGGGTTCTTCACAGCCAGGAAGTTCGC	To generate RRS1-S-HA-ZIM
ZD422_D4/5_ZIMinsR:	AGGAAGACAAGTTTCGACCCTTGTTTTGCTACTTCCATAATCTC	To generate RRS1-S-HA-ZIM
ZD478_D4/5_1xHA-CBSVinsF	AGGAAGACAAGAAGGGTTCTTATCCATACGATGTCCCAGATTATGCGGGTTCTACAATTGATGTTCAAGCTGGGTCGAAACTTGTCTTCCT	To generate RRS1-S-HA-CBSV
ZD479_D4/5_1xHA-CBSVinsR	AGGAAGACAAGTTTCGACCCAGCTTGAACATCAATTGTAGAACCCGCATAATCTGGGACATCGTATGGATAAGAACCCTTCTTGTCTTCCT	To generate RRS1-S-HA-CBSV
ZD415_Us_4xMyc_F	GGCTTAAUGGAACAAAAGTTGATCTCTG	USER cloning N-term 4xMyc tag
ZD416_Us_4xMyc-FlaA_R	ACTTGAGCAAGGUCCTCTTCAGAAATAAG	USER cloning 4xMyc-FlaA
ZD417_Us_FlaA_F	ACCTTGCTCAAGUAATCAACACTAATGTGG	USER cloning 4xMyc-FlaA
ZD418_Us_FlaA-R	GGTTTAAUAAGCTCACTATCGACCTAACAAAG	USER cloning 4xMyc-FlaA
ZD546_Us_4xMyc-PrgJ_R	ATGCGGCAAGGUCCTCTTCAGAAATAAG	USER cloning 4xMyc-PrgJ
ZD547_Us_PrgJ_F	ACCTTGCCGCAUCGATTGCAACTATTGTC	USER cloning 4xMyc-PrgJ
ZD548_Us_PrgJ-R	GGTTTAAUAAGCTCATGAGCGTAATAGCGTTTCAACAGC	USER cloning 4xMyc-PrgJ
ZD608_Us_YscF_F	ACCTTAGCAAUCCCCCGACTCCATTGCTG	USER cloning 4xMyc-YscF
ZD609_Us_YscF_R	GGTTTAAUAAGCTCAGCGGAAGTTCGAGACGATCG	USER cloning 4xMyc-YscF
ZD610_Us_4xMyc-YscF_R	ATTGCTAAGGUCCTCTTCAGAAATAAG	USER cloning 4xMyc-YscF
ZD611_Us_4xMyc-RsHrpB2-R	ATCATAAGGUCCTCTTCAGAAATAAG	USER cloning 4xMyc-RsHrpB2
ZD612_Us_RsHrpB2-F	ACCTTATGAUCCAGGGTCCGACTGC	USER cloning 4xMyc-RsHrpB2
ZD613_Us_RsHrpB2-R	GGTTTAAUAAGCTCATTGGTTCTTCATCAAGG	USER cloning 4xMyc-RsHrpB2
ZD614_Us_4xMyc-DC3000HrpB-R	ATGGTCATAAGGUCCTCTTCAGAAATAAG	USER cloning 4xMyc-PsHrpB
ZD615_Us_DC3000HrpB-F	ACCTTATGACCAUTTCCCAACTCAGC	USER cloning 4xMyc-PsHrpB
ZD616_Us_DC3000HrpB-R	GGTTTAAUAAGCTCAACTGCAGGTTGGTCAACTTG	USER cloning 4xMyc-PsHrpB
ZD617_Us_4xMyc-DC3000FliC-R	AGCCATAAGGUCCTCTTCAGAAATAAG	USER cloning 4xMyc-PsFliC
ZD618_Us_DC3000FliC-F	ACCTTATGGCUTTAACAGTAAACACCAACG	USER cloning 4xMyc-PsFliC
ZD619_Us_DC3000FliC-R	GGTTTAAUAAGCTCACTGAAGCAGTTTCAGTACAG	USER cloning 4xMyc-PsFliC
ZD542_Us_NAIP5_F	GGCTTAAUGGCTGAGCATGGGGAGTCC	USER cloning NAIP1/5-6xHA

ZD563_Us_NAIP5-R	AACCCGAUCCCTCCAGGATAACAGGAGAGAATGG	USER cloning NAIP5-6xHA
ZD544_Us_NAIP2_F	GGCTTAAUGGCAGCCCAGGGAGAAG	USER cloning NAIP2-6xHA
ZD564_Us_NAIP2-R	AACCCGAUCCCTTCTGAATGACAGGAGAGAATGG	USER cloning NAIP2-6xHA
ZD414_Us_NAIP1-R	AACCCGAUCCCTCCAGGACAACAGGAGAGAACG	USER cloning NAIP1-6xHA
ZD556_Us_6xHA_F	ATCGGGTUCGGGGTCTCATTCGTACCCA	USER cloning NAIP-6xHA
ZD557_Us_6xHA_R	GGTTTAAUAAGCTCAAAGCTCAGGCGTAATCT	USER cloning NAIP-6xHA
ZD558_RPS4TIR+80fusFgg	TGGTCTCTAATGGAGACATCATCTATTTCCACTGTG	Clone RPS4-TIR-NLRC4-HF
ZD559_RPS4TIR+80fusRgg	TGGTCTCTAAGACCCAACAACTCCAATGATACG	Clone RPS4-TIR-NLRC4-HF
ZD540_RRS1TIR+80fusFgg	TGGTCTCTAATGACCAATTGTGAAAAGGATGAGG	Clone RRS1-TIR-NLRC4-HF
ZD562_RRS1TIR+80fusRgg	TGGTCTCTAAGAACCCCAAATTCCAACACAACGG	Clone RRS1-TIR-NLRC4-HF
ZD482_MLA15CCfusFwgg	TGGTCTCTAATGGATATTGTCACCGGTGC	Clone MLA-CC-NLRC4-HF
ZD483_MLA15CCfusRvgg	TGGTCTCTAAGACAAAGCTCGAAGGCAAGGG	Clone MLA-CC-NLRC4-HF
ZD576_NLRC4_Fwgg	TGGTCTCTAATGAACTTTATAAGGAACAACAGACGAG	Clone and domesticate CC/TIR-NLRC4-HF
ZD571_NLRC4_CCRgg	TGGTCTCTCGAACCAGCAGTCACTAGTTTAAAGG	Clone and domesticate NLRC4
ZD530_NLRC4_CCRvgg1	TGGTCTCTGTCACCACAGTAGTCTAGGCTC	Clone and domesticate NLRC4
ZD531_NLRC4_CCFwgg2	TGGTCTCTTGACCTGGCCCTAGAAGG	Clone and domesticate NLRC4
ZD591_NLRC4_fusFwgg	TGGTCTCTTCTTCTAACTTTATAAGGAACAACAGACG	Clone and domesticate NLRC4-HF
ZD574_NLRC4ΔLRR_Rvgg	TGGTCTCTCGAACCCTTCCAGTTGAAGAACAAAGACAC	Clone and domesticate NLRC4△LRR-HF
ZD592_NLRC4ΔCARD_Fw	TGGTCTCTAATGTCTTATCAGGTCACAGAAGAAG	Clone and domesticate NLRC4△CARD-HF

Appendix 2.2. List of backbone plasmids used in this study.

Plasmid	Purpose	Selection
pICSL01002	GG N-term tag	Spectinomycin
pICSL01003	GG C-term tag	Spectinomycin
pICSL01005	GG CDS	Spectinomycin
pICSL86977	GG 35S binary vector	Kanamycin
pAGM4723	GG binary vector for gene stacking	Kanamycin
pICSLUs0004	USER 35S binary vector	Kanamycin

Chapter 3. RRS1 and RPS4 form an immune complex that confers recognition of effectors that target WRKY transcription factors

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3.1. Introduction

In this chapter, I define the RRS1-WRKY domain as the site of interaction between RRS1 and the effector PopP2. In particular, I focus on characterising the PopP2 recognition mechanism. PopP2 acetylates both RRS1-R and RRS1-S WRKY domains at both lysines (Ks) of the canonical WRKYGQK sequence. Acetyl-lysine (Ac-K) mimetic alleles of RRS1-R, but not RRS1-S, trigger effector-independent RPS4-dependent HR. PopP2 disrupts W-box DNA-binding of RRS1, but this is not sufficient for immune signalling activation. AvrRps4 and PopP2 interact with other Arabidopsis WRKY proteins, and several other WRKY proteins are acetylated by PopP2, suggesting that the RPS4/RRS1 complex enables plants to detect effectors which interfere with WRKY protein function in plant immunity. The discovery of multiple effectors that target WRKY proteins, and the evolution of immune receptors that detect such effectors, emphasises the significance of WRKY proteins for plant immunity. These data imply a general mode of evolution of new immune recognition capacities, by which NLR receptor pairs are selected, in which one member carries protein domains that enable perception of the action of pathogen effectors that target that domain, while the other activates defence upon such perception.

3.2. Results

3.2.1. RRS1-S and RRS1-R WRKY domains interact with, and are acetylated by, PopP2

PopP2 enzymatic activity is essential for defence activation via RRS1-R (Tasset et al., 2010). This suggests that RPS4/RRS1-R-mediated resistance to

PopP2 may involve acetylation of RRS1-R protein by PopP2. Cecile Segonzac in the lab tested this by co-expressing 35S:PopP2-GFP or 35S:PopP2-C321A-GFP, with 35S:RPS4-HA and 35S:RRS1-R or 35S:RRS1-S fused to a tandem 6xHis and 3xFLAG epitope (HF) tag in *Nicotiana benthamiana* leaves by agroinfiltration. After protein extraction and immunoprecipitation (IP) with FLAG beads, immunoblot analysis with anti-acetyl-lysine (α-Ac-K; Tasset et al., 2010) antibody revealed acetylation of RRS1-R and RRS1-S in the presence of PopP2, but not with GFP, AvrRps4-GFP or PopP2-C321A-GFP (Figures 2a and S2a of Sarris et al 2015). The signal of acetylated RRS1 was stronger in the absence of RPS4 or with TIR domain hetero-dimerization mutants RRS1-$R^{SH\text{-}AA}$ co-expressed with RPS4 SH-AA (Williams et al., 2014; Figure 2a of Sarris et al 2015). This suggests proper assembly of RRS1 protein into an RPS4/RRS1 complex might result in fewer available sites of RRS1 for acetylation by PopP2.

In order to identify residues on RRS1 protein that were acetylated, I purified RRS1-HF by IP and subjected it to mass spectrometry. In collaboration with the Sainsbury Laboratory Proteomics team (Frank Menke, Jan Sklenar and Paul Derbyshire), I identified acetylated RRS1 peptides in the presence of PopP2 (Figure 3.1; Appendix 3.1). Several acetylated peptides were detected, including mono- and di-acetylated peptides that showed a ~42 kDa mass shift on K residues in the WRKY domain (Figure 3.1). I transiently expressed HF-tagged RRS1-R and RRS1-S in *Nicotiana benthamiana*, digested affinity-purified protein, and used selected reaction monitoring (SRM), in collaboration with TSL proteomics, to quantify the relative level of acetylation of these peptides in the presence of PopP2, PopP2-C321A or GFP (Figure 3.1). This method allowed relative quantification of acetylated peptides between samples by normalising to protein levels using several non-modified control peptides from the protein of interest (Appendix 3.2). I identified four PopP2-dependent Ac-Ks, in and nearby the WRKY domains of both RRS1-R and RRS1-S, corresponding to acetylation of K1217, K1221, K1247 and K1276 in RRS1-R (Figure 3.1; Appendix 3.3).

PopP2 acetylates the WRKY domain of RRS1, but also requires the WRKY domain to interact with RRS1. Previously, only full length RRS1 protein has

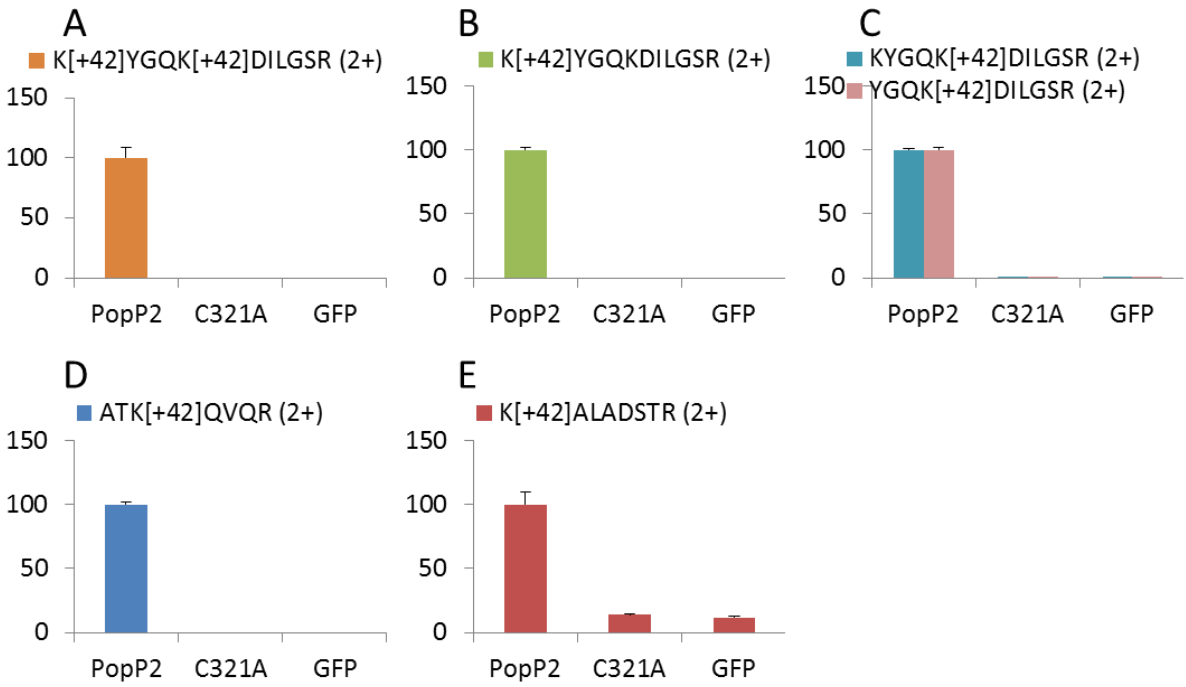
been reported to interact with PopP2 (Deslandes et al., 2003). Panos Sarris replaced the WRKY domain of both RRS1-R and RRS1-S with the bacterial LexA DNA-binding domain (Figure S2N, Sarris et al. 2015). LexA contains a DNA binding domain that has no known target in plant DNA (Fogh et al., 1994). PopP2 failed to co-IP with RRS1-R WRKY/LexA or RRS1-S WRKY/LexA (Figure S2o from Sarris et al 2015), suggesting that the WRKY domain is necessary for the interaction of RRS1 with PopP2.

3.2.2.Acetylation of the WRKY domain of RRS1-R, but not RRS1-S, is sufficient and required to trigger an immune response

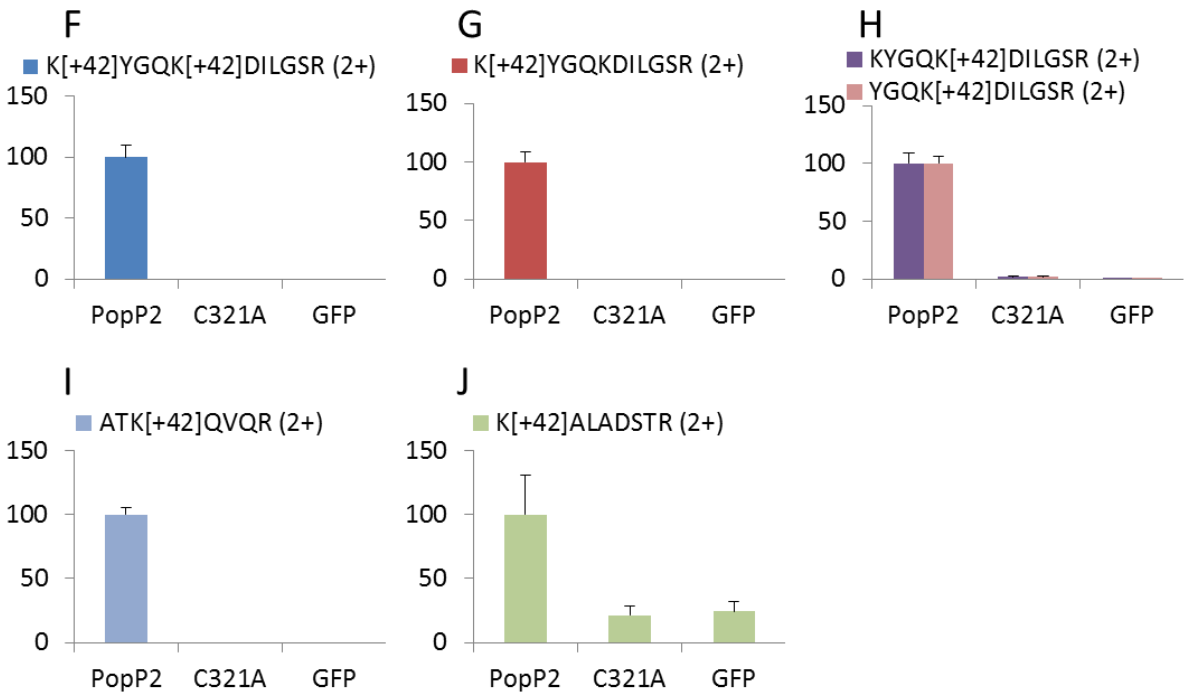
After demonstrating that RRS1 was acetylated by PopP2, I sought to discover if this acetylation was the cause of recognition and response. In order to do this, I utilised mimetic mutations of K and Ac-K. Glutamine (Q) has similarity in charge and structure to Ac-K, while arginine (R) is charge-conserving but is not able to be acetylated; both Q and R are easily substitutable for K by mutagenesis. Using these amino acid substitutions, I was able to infer the effect of acetylation on RRS1 in the absence of effector. The RRS1-R Ac-K mimetic mutants WRQ^{1217}YGQK^{1221} (RRS1-R^{K1217Q}) or WRK^{1217}YGQQ^{1221} (RRS1-R^{K1221Q}), and the double mutant (WRQ^{1217}YGQQ^{1221}), all trigger HR in the absence of an effector when expressed in *Nicotiana tabacum* in combination with RPS4 (Figure 3.2a). This indicates that acetylation of the WRKY domain is sufficient to trigger a response to PopP2 , and establishes a causal relationship between PopP2 acetylation of lysines in the WRKY domain and RRS1/RPS4-mediated HR.

I also tested the activity of K-to-R mutations in RRS1 in *N. tabacum* transient assays in the absence of effectors. When WRRYGQK, WRKYGQR or WRRYGQR mutations were introduced in RRS1-R or RRS1-S, no constitutive HR was observed when co-expressed with RPS4 in *N. tabacum* (Figure 3.2b). I also tested the K-to-R mutants for PopP2 recognition and response. RRS1-R^{K1217R} retained response to PopP2, but RRS1-R^{K1221R} did not respond to PopP2 (Figure 3.2b). This indicates that specific acetylation of K1221 is sufficient and necessary for response toPopP2 by RRS1-R. It is interesting that the K1221R mutation was sufficient to suppress HR in the presence of PopP2; in this case it is presumed that K1217 is still acetylated.

RRS1-R



RRS1-S



K

RRS1-R (Ws-2)	1215	W R K Y G Q K D I L G S R F P R G Y Y R C A Y K F T H G C K A T K Q V	1249
RRS1-S (Col-0)	1213	W R K Y G Q K D I L G S R F P R G Y Y R C A Y K F T H G C K A T K Q V	1247

RRS1-R (Ws-2)	1250	Q R S E T D S N M L A I T Y L S E H N H P R P T K R K A L A D S T R	1283
RRS1-S (Col-0)	1248	Q R S E T D S N M L A I T Y L S E H N H P R P T K R K A L A D S T R	1281

Figure 3.1. PopP2 acetylates the WRKY domain of RRS1-R and RRS1-S proteins. A-E) Quantitative acetylation of RRS1-R peptides doubly acetylated at K1217 and K1221 (A), or singly acetylated at K1217 (B), K1221 (C), K1247 (D) and K1276 (E). F-J) Quantitative acetylation of RRS1-S peptides doubly acetylated at K1215 and K1219 (F), or singly acetylated at K1215 (G), K1219 (H), K1245 (I) and K1274 (J). Relative levels of acetylated peptides from RRS1-R and RRS1-S, determined by SRM, are shown as an average (n=4) with error bars representing standard error (SE). Peptide sequence and charge are shown in each histogram. Relative abundance of RRS1 peptides derived from tryptic-digestion of RRS1 co-expressed with PopP2 were set to 100%, and peptides derived from RRS1 co-expressed with PopP2-C321A or GFP were normalized accordingly. Samples in which peptides were not detected were annotated *n.d.* K) Acetylated lysines in and around the RRS1 WRKY domain (underlined) are indicated by asterisks.

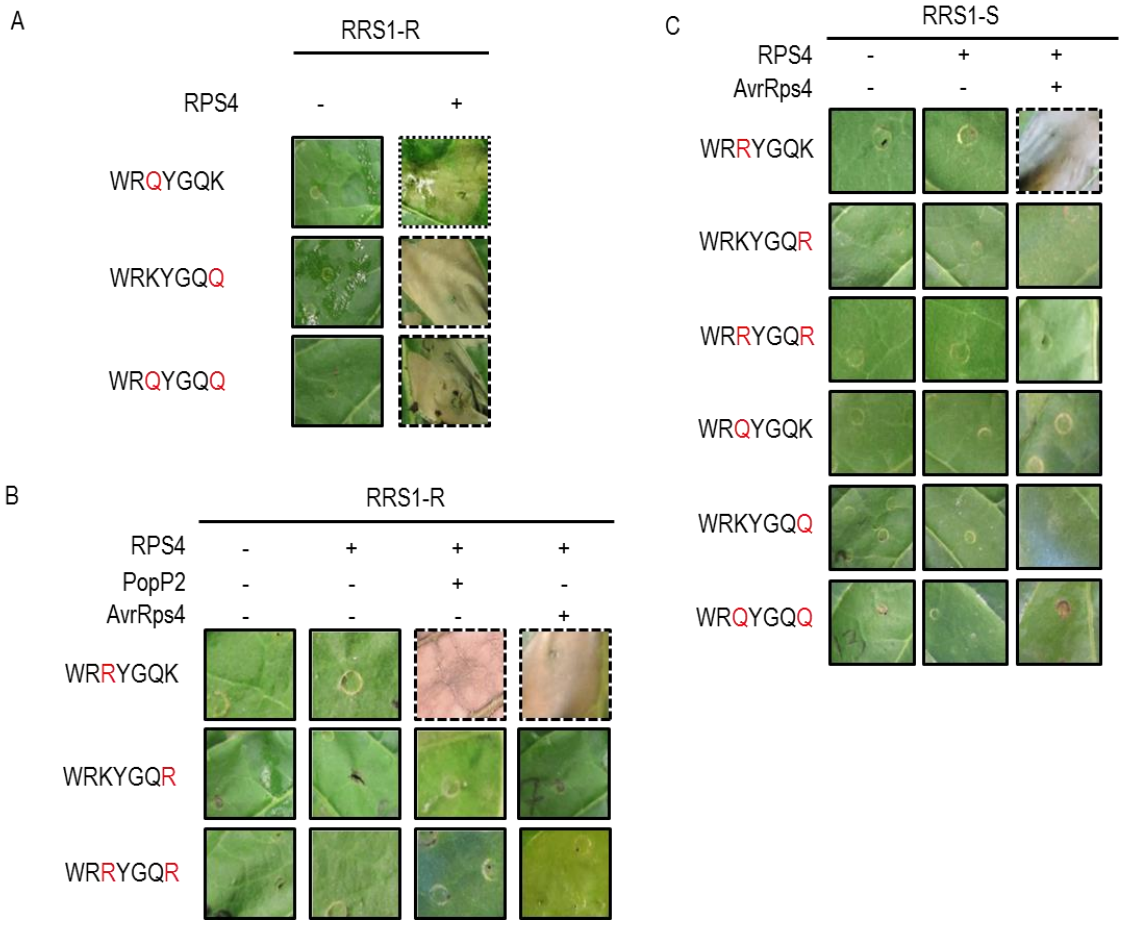


Figure 3.2. K1221 of RRS1-R is necessary for response to PopP2 and AvrRps4. A) Agroinfiltration assays in *Nicotiana tabacum* leaves show Ac-K mimetic alleles of RRS1-R exhibit constitutive effector-independent RPS4-dependent HR (K1217Q = WRQYGQK, K1221Q = WRKYGQQ and K1217Q/K1221Q = WRQYGQQ). B) Agroinfiltration assays in *N. tabacum* leaves show response to PopP2 and AvrRps4 requires K1221 of RRS1-R. RRS1-R mutants (K1217R = WRRYGQK, K1221R = WRKYGQR and K1217R/K1221R = WRRYGQR) expressed alone and co-expressed with RPS4 and PopP2 or AvrRps4. C) Agroinfiltration assay shows Ac-K mimetic alleles (K-to-Q) of RRS1-S lose responsiveness to AvrRps4 when co-expressed with RPS4. Each *N. tabacum* leaf section was infiltrated to co-express RRS1-S mutants (K1215R = WRRYGQK, K1219R = WRKYGQR, K1215R/K1219R = WRRYGQR, K1215Q = WRQYGQK, K1219Q = WRKYGQQ and K1215Q/K1219Q = WRQYGQQ) with or without RPS4 and AvrRps4. Each strain show was infiltrated at an OD_{600} of 0.5. In experiments with a variable number of co-infiltrated strains, 35S:GFP-carrying strains were included in cell mixtures with fewer component strains to ensure that the combined OD_{600} of each mixture was equal. HR pictures were taken 4 dpi. This was repeated at least three times with similar results.

However, the K1217Q mutation triggered constitutive HR. This can be explained in one of two ways: PopP2 acetylates K1217 to a lesser degree than K1221, more RRS1 molecules with Ac-K1217 are required to pass the threshold to HR, and the K1217Q mutation would be present in all RRS1 molecules in the experiments. The other explanation is that Q is a poor substitute for Ac-K and is inducing the change of RRS1 from an inactive form to an active form in a distinct way to that of Ac-K. To test these separate hypotheses, it would be useful to compare the levels of Ac-K1217 and Ac-K1221 in a sample; however, this comparison is not a reliable metric, because Ac-K blocks tryptic digestion and therefore peptides containing these moieties could fly vastly differently in the mass spectrometer. Because K1221 mimetic mutations reconstituted response and recognition phenotypes as expected, and because the K-to-R mutant of the nearby K1217 could confer HR when co-expressed with PopP2, I concluded the K1221 residue is the key target for PopP2 and did not further investigate the other two acetylated Ks (K1247 and K1276 of RRS1-R; Figure 3.1).

In addition to determining the relative importance of K1217 and K1221 for PopP2 response, I co-expressed the equivalent mimetic mutants of RRS1-S with RPS4 and AvrRps4 in order to determine their effect on AvrRps4 response. The K-to-Q mutations in RRS1-S do not trigger HR (Figure 3.2c), which reconstitutes the effect of PopP2 acetylation (i.e. RRS1-S does not trigger HR when acetylated by PopP2) and provides us with a tool to test the effect of acetylation on AvrRps4 recognition and response; we could not use RRS1-R K-to-Q mutants because they confer constitutive HR. The mutations of RRS1-S to WRQ^{1215}YGQK^{1219}, WRK^{1215}YGQQ^{1219} and WRK^{1215}YGQR^{1219}, but not to WRR^{1215}YGQK, lose PopP2 and AvrRps4 responsiveness (Figure 3.2C). These experiments point to the RRS1 WRKY domain as the target of two separate effectors, PopP2 and AvrRps4.

3.2.3.RRS1-S and RRS1-R lose affinity for W-box DNA sequences when acetylated by PopP2

In the previous section, I determined that acetylation of K1221 was required and sufficient for RRS1-R-mediated HR in response to PopP2. I next wanted to characterise the mechanistic reason for activation of immunity after

PopP2 acetylation of the WRKY domain. In other WRKY proteins, the equivalent residues that correspond to RRS1-R K1221 and K1217 are required to bind W-box DNA sequences (Duan et al., 2005; Maeo et al., 2001). Furthermore, the RRS1-R WRKY mutant allele *slh1* has reduced W-box DNA binding ability and confers constitutive activation of immunity (Noutoshi et al., 2005). I hypothesised that acetylation of K1221, which would neutralise the positively charged K, would disrupt WRKY-domain interaction with W-box DNA.

To test this, Sung Un Huh and I performed electrophoretic mobility shift assays (EMSA), checking the ability of both RRS1-R and RRS1-S to bind radiolabelled W-box DNA in the presence of PopP2 or the PopP2-C321A mutant (Figure 3.3a,b). For the EMSA, we transiently co-expressed RRS1-R or RRS1-S and RPS4 in the presence of PopP2 or PopP2-C321A mutant in *N. benthamiana*. The target DNA was a 29 bp double-stranded oligonucleotide containing three W-boxes. Furthermore, a mutant form of the 29 bp double-stranded oligonucleotide was used as a negative control (Figure 3.3). Both RRS1-R and RRS1-S WRKY domains bound radiolabelled W-box DNA; this binding can be competed with unlabelled W-box DNA (Figure 3.3a,b). The interaction of both RRS1 alleles with W-box DNA was inhibited in the presence of PopP2 but not in the presence of PopP2-C321A or GFP (Figures 3A,B). These results indicate that the acetyl-transferase activity of PopP2 is required to inhibit RRS1 DNA-binding. Furthermore, EMSA revealed that the autoimmune K1221Q mutation inhibits RRS1 binding to W-box DNA (Figure 3.3c). These data further indicate that the RRS1 WRKY-domain is the target of PopP2, and thus I infer that acetylation of lysines in the WRKYGQK domain can reduce the affinity of the RPS4/RRS1 complex for W-box DNA sequences.

The RRS1-R^{SLH1} and RRS1-R^{K1221Q} alleles cause effector-independent, RPS4-dependent HR in *N. tabacum* transient assays but RRS1-S versions of these alleles do not (Figure 3.2a,b; Sarris et al 2015). However, the DNA-binding capacity of both RRS1-R and RRS1-S is similarly disrupted by the *slh1* mutation and by PopP2-dependent acetylation (Figure 3.3a,b; Sarris et al 2015). Furthermore, AvrRps4 does not affect the abundance of RRS1/W-box

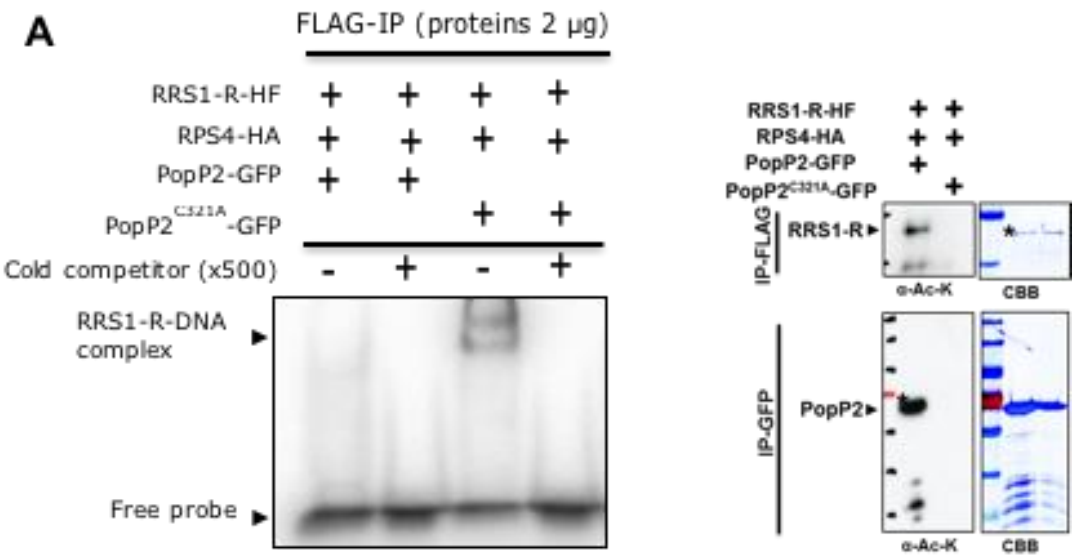
DNA complex that migrates on an EMSA, indicating it does not affect the affinity of RRS1 for DNA (Figure S3c,d of Sarris et al 2015). Although WRKY-domain acetylation is required for HR, and loss of DNA-protein interaction is correlated with HR, there must be another factor determining RRS1-R response to PopP2. To investigate which protein domains confer PopP2 responsiveness, Panos Sarris constructed protein chimaeras between RRS1-R and RRS1-S by swapping the seventh exon of these two genes, which contains DOM6 (Sarris et al., 2015). It was already known that when RRS1-R is truncated to resemble RRS1-S it does not confer recognition of PopP2 (Deslandes et al., 2002). P. Sarris found that the extra amino acids of DOM6 of RRS1-R (DOM6R) fused to DOM6 of RRS1-S (DOM6S) are sufficient to provide RPS4-dependent PopP2 recognition and response, and HR in tobacco (Sarris et al., 2015). The mechanism by which the C-terminal extension of RRS1-R confers RPS4-dependent responsiveness to acetylation of the WRKY domain, and to the leucine insertion in $RRS1^{SLH1}$, remains to be investigated.

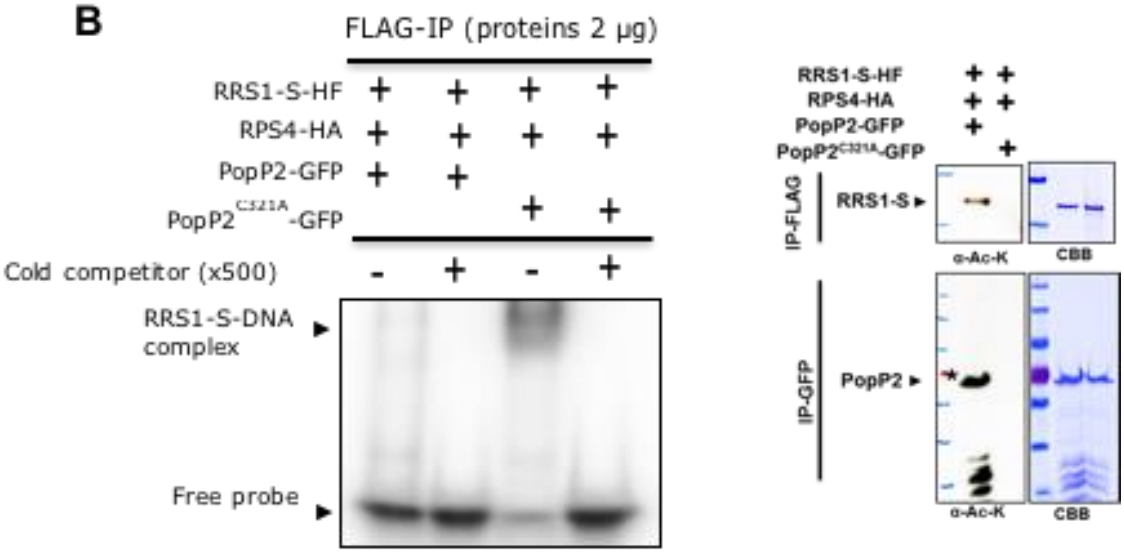
3.2.4.Other WRKY-domain proteins associate with AvrRps4 and PopP2, and are acetylated by PopP2

Both PopP2 and AvrRps4 interact with the WRKY domain of RRS1 and this interaction is required for activation of immunity. From the perspective of the pathogen, targeted attack of an R protein that results in an immune response is undesirable, and any effector that utilises such a strategy would have a strong selection pressure against it, unless it had another, vital function. In Arabidopsis, more than 70% of WRKY genes are implicated in defence (Chi et al., 2013; Dong et al., 2003). I hypothesised that the RRS1-WRKY domain was a decoy that mimicked the true target of PopP2 and AvrRps4, and that RRS1 detects effectors that interact with WRKY-domain proteins and impair their contribution to plant immunity.

To determine whether PopP2 associates with other WRKY-domain proteins, I co-expressed C-terminally GFP-tagged PopP2-C321A with several C-terminally HF-tagged WRKY proteins, selected because of their demonstrated involvement in plant defence. PopP2-C321A was chosen because it interacts more strongly with RRS1 than does PopP2 (Williams et

al., 2014). WRKY41, WRKY70, WRKY60 and WRKY33 co-IP with PopP2-C321A (Figure 3.4a). I analysed IP-purified WRKY proteins by discovery mass spectrometry and detected acetylation of WRKY41, WRKY70 and WRKY33, but not WRKY60, by PopP2 at the conserved WRKYGQK motif K that corresponds to K1221 in RRS1-R (Appendix 3.4). I was able to purify much greater amounts of plant-expressed WRKY41 than the other WRKYs, so S. U. Huh performed EMSAs and determined that PopP2 disrupted WRKY41 interaction with W-box DNA (Figure 3.4). In addition, AvrRps4 co-IPs with WRKY41, WRKY70, WRKY33 and WRKY60 (Sarris et al., 2015). These results confirm that both PopP2 and AvrRps4 associate with some, but not all, WRKY-domain proteins. Acetylation of the WRKY-domain of any WRKY protein is likely to interfere with its capacity to bind W-box DNA, and AvrRps4 binding may interfere with WRKY protein function by other means. Therefore, I propose that the RPS4/RRS1 immune complex has evolved to detect effectors that interfere with the function of WRKY-domain proteins (Figure 3.5).


A
FLAG-IP (proteins 2 μg)
RRS1-R-HF + + + +
RPS4-HA + + + +
PopP2-GFP + +
PopP2C321A-GFP + +
Cold competitor (x500) - + - +
RRS1-R-DNA complex
Free probe
RRS1-R-HF + +
RPS4-HA + +
PopP2-GFP +
PopP2C321A-GFP +
IP-FLAG
RRS1-R
α-Ac-K
CBB
IP-GFP
PopP2
α-Ac-K
CBB


B
FLAG-IP (proteins 2 μg)
RRS1-S-HF + + + +
RPS4-HA + + + +
PopP2-GFP + +
PopP2C321A-GFP + +
Cold competitor (x500) - + - +
RRS1-S-DNA complex
Free probe
RRS1-S-HF + +
RPS4-HA + +
PopP2-GFP +
PopP2C321A-GFP +
IP-FLAG
RRS1-S
α-Ac-K
CBB
IP-GFP
PopP2
α-Ac-K
CBB

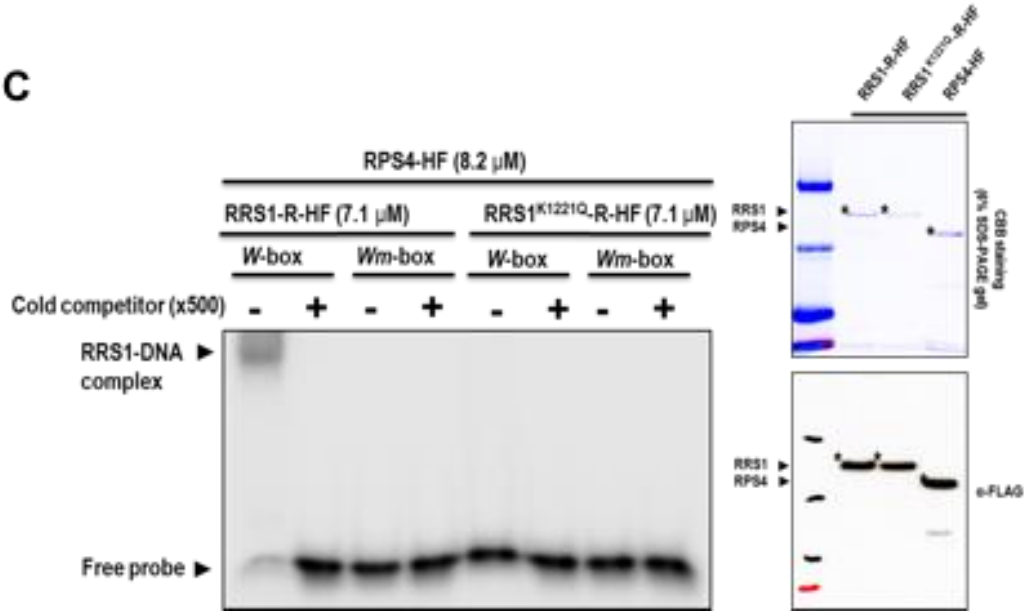

C
RPS4-HF (8.2 μM)
RRS1-R-HF (7.1 μM)
RRS1K1221Q-R-HF (7.1 μM)
W-box
Wm-box
W-box
Wm-box
Cold competitor (x500) - + - + - + - +
RRS1-DNA complex
Free probe
RRS1-R-HF
RRS1K1221Q-R-HF
RPS4-HF
RRS1
RPS4
CBB staining (4% SDS-PAGE gel)
RRS1
RPS4
α-FLAG

Figure 3.3. PopP2 acetylation reduces RRS1 affinity for a synthetic W-box. The electrophoretic mobility shift assays (EMSA) were performed with IP-purified RRS1-R (A) or RRS1-S (B) expressed with RPS4 and PopP2 or the control PopP2-C321A. 2µg of proteins was incubated with ^{32}P labelled synthetic 3x*W*-box double stranded DNA (dsDNA) oligonucleotide for 1 hr. The complexes were electrophoresed in 4% native acrylamide gel. The signals were detected by FLA-5000 (Fujifilm). For the competition assay of RRS1-S and RRS1-R proteins, RRS1-S (2 µg) and RRS1-R (2 µg) proteins were co-incubated with ^{32}P labelled synthetic *W*-box dsDNA mixed with 500-fold cold *W*-box dsDNA competitor. Control Western-blots were performed using the same co-IP samples for RRS1-R (A) and RRS1-S (B). These experiments were repeated three times with identical results. The relative expression levels and purity of co-purified protein used in each EMSA is shown to the right. Acetylation of PopP2 and RRS1 was confirmed by immunoblot with α-Ac-K antibody and total protein was stained with Coomassie (CBB). C) EMSA showing RRS1-R^{K1221Q}-HF loses affinity for 3xW-box DNA. RRS1-R-HF, RRS1-R^{K1221Q}-HF, and RPS4-HF were separately expressed and FLAG-purified. RPS4-HF (8.2 µM) was then mixed with either RRS1-R-HF (7.1 µM) or RRS1-R^{K1221Q}-HF (7.1 µM) before incubation with ^{32}P labelled synthetic 3x*W*-box dsDNA, or mutant 3x*W*-box dsDNA. EMSA was then performed as above. The purified protein was assessed by SDS-PAGE stained with Coomassie and probed with FLAG antibody. Sung Un Huh and I performed replicates; the experiment represented was performed by S. U. Huh.

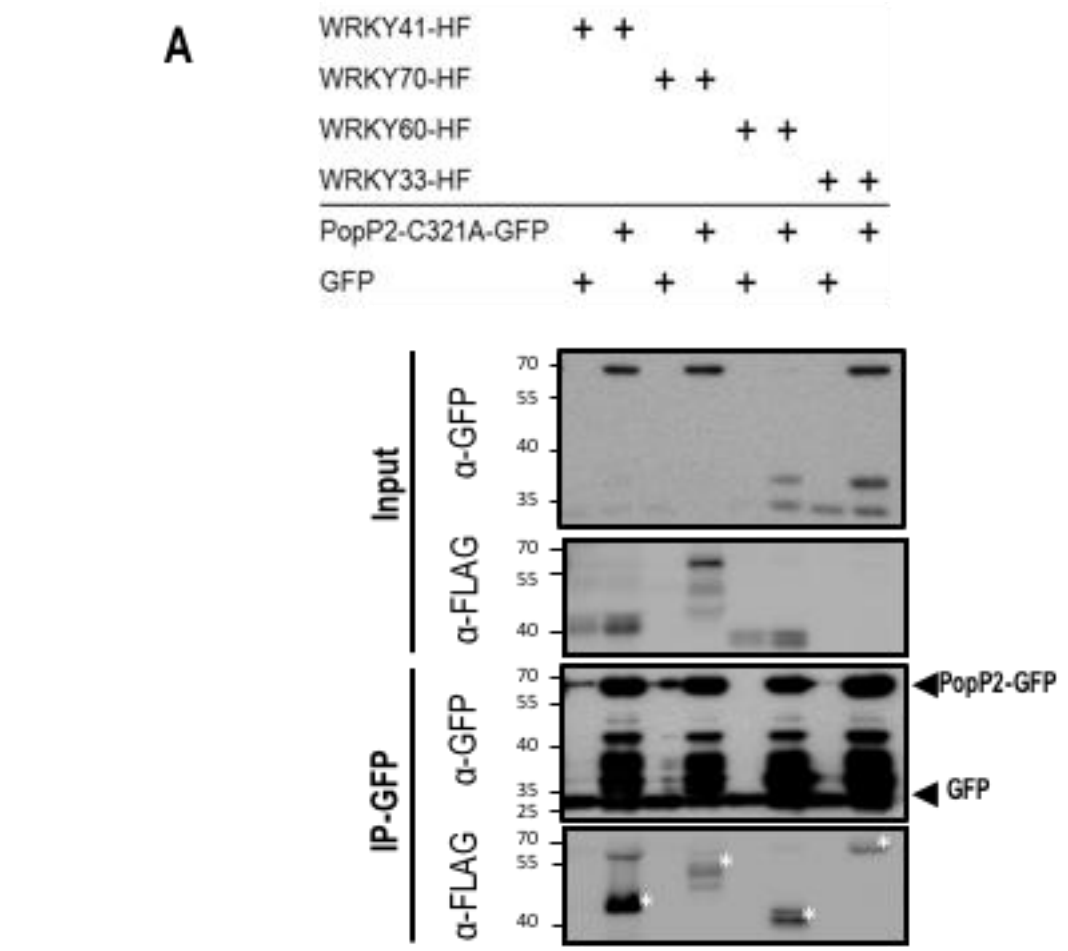
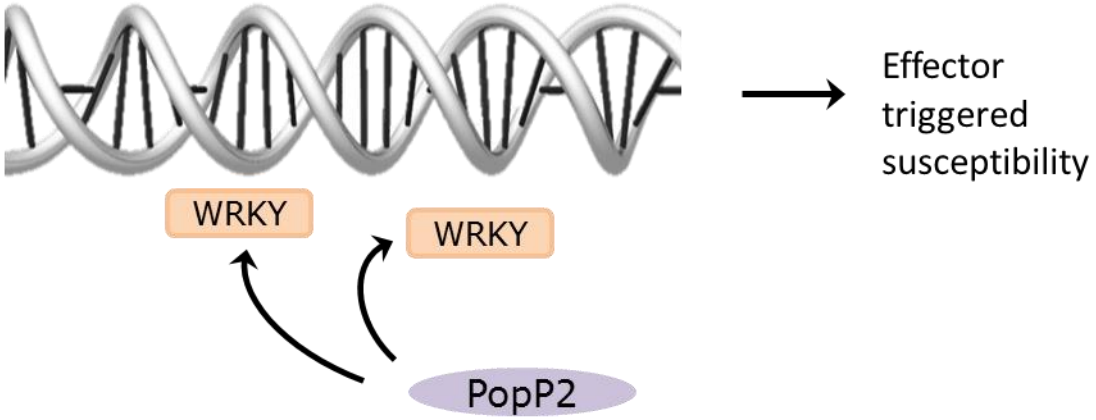

A
WRKY41-HF
WRKY70-HF
WRKY60-HF
WRKY33-HF
PopP2-C321A-GFP
GFP
Input
IP-GFP
α-GFP
α-FLAG
PopP2-GFP
GFP
B
FLAG-IP (proteins 2 µg)
AtWRKY41-HF
PopP2-GFP
PopP2C321A-GFP
Cold competitor (x500)
Protein-DNA complex
Free probe
C
AtWRKY41-HF
PopP2-GFP
PopP2C321A-GFP
IP-FLAG
AtWRKY41
PopP2
α-FLAG
α-GFP

Figure 3.4. Other WRKY proteins associate with PopP2 and AvrRps4, and are acetylated by PopP2. (A) Co-IP assays assessing association between PopP2-C321A-GFP and WRKY33-HF, WRKY41-HF, WRKY70-HF and WRKY60-HF. Immunoblots show the presence of proteins in total extracts (input) and after IP-GFP. GFP was used as a negative control. Asterisks indicate expected protein bands. Each interaction was repeated at least twice. (B) The electrophoretic mobility shift assays (EMSA) were performed with co-expression of proteins WRKY41 and PopP2 or control PopP2-C321A. The WRKY41 protein co-IPed with FLAG and 2μg were used for co-incubation with ^{32}P labelled synthetic 3x*W*-box double stranded DNA (dsDNA) oligonucleotide for 1 hr. The complexes were electrophoresed in 4% native acrylamide gel. The signals were detected by FLA-5000 (Fujifilm). For the competition assay of WRKY41 protein, WRKY41 (2 μg) protein were co-incubated with ^{32}P labelled synthetic *W*-box dsDNA mixed with 500-fold cold *W*-box dsDNA competitor. A total of three replicates were performed. (C) IP of WRKY41-HF co-expressed with PopP2-GFP or PopP2-C321A-GFP showing equivalent expression levels. I cloned WRKY41 and helped Sung Un Huh purify protein; S. U. Huh performed the EMSA. A total of three replicates were performed.

A


WRKY
WRKY
Transcriptional regulation mediating immunity

B


WRKY
WRKY
PopP2
Effector triggered susceptibility

C

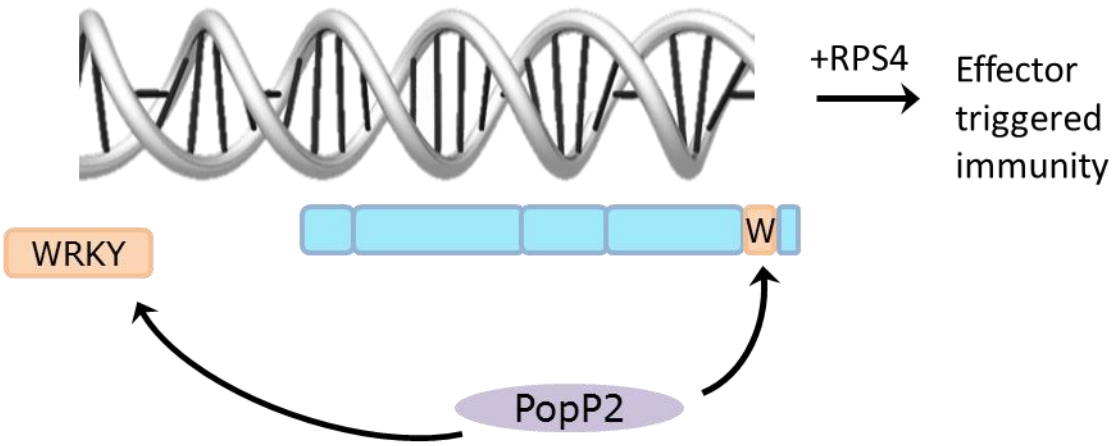

+RPS4
Effector triggered immunity
W
WRKY
PopP2

Figure 3.5. PopP2 acetylates the WRKY domain of RRS1 to initiate effector triggered immunity (ETI). The model depicts two scenarios. A) A pathogen challenges a non-host plant and infection is successfully resisted by PAMP-triggered immunity (PTI). WRKY transcription factors upregulate immunity-related genes to contribute to the immune response. B) PopP2 is injected into the plant cell and acetylates WRKY proteins involved in immunity, disrupting their ability to bind DNA and thereby attenuating PTI. C) In plant cells containing RRS1-R, PopP2 binds and acetylates WRKY domain-containing proteins. This includes the WRKY domain of RRS1-R, which triggers RPS4-dependent immunity. It should be noted that the WRKY domain of RRS1-R does not titrate out PopP2, but is instead mediating an immune response when acetylated.

3.3. Discussion

3.3.1. The WRKY domain of RRS1 is an integrated decoy

The presence of atypical domains fused to NLRs has led to much speculation on their role and several hypotheses on each individual fusion domain's function. The discovery of Arabidopsis NLRs that carry WRKY domains initially led to the proposal that such proteins reveal a direct link between NLR detection of effectors (directly or indirectly), and activation of defence via WRKY transcription factors (Jacob et al., 2013). Both PopP2 and AvrRps4 interact with the WRKY domain of RRS1. Although AvrRps4 interacts with other domains within RRS1, PopP2 interacts specifically with the WRKY domain, and acetylates lysines within the canonical WRKYGQK motif. Acetylation of these lysines in the RRS1-S allele, which does not activate immunity in response to PopP2, abolishes its capacity to support AvrRps4 recognition and response (Sarris et al., 2015). Mutating either K residue in the WRK^{1215}YGQK^{1219} motif to Q, and mutating K^{1219} to R, abolishes AvrRps4 recognition and response in RRS1-S, and also binding of AvrRps4 to the WRKY domain (Sarris et al., 2015). For the RRS1-R allele, an Ac-K mimetic Q residue at the K^{1221} position in WRK^{1217}YGQK^{1221} confers constitutive RPS4-dependent immunity activation, similar to that conferred by the RRS1-R^{SLH1} allele. Importantly, I show that the K^{1221} in the WRK^{1217}YGQK^{1221} motif plays the most important role in recognition and response of both PopP2 and AvrRps4. Considered together, these data show unambiguously that the RPS4/RRS1-R complex recognizes both AvrRps4 and PopP2 via their interactions with the RRS1-R WRKY domain.

In a companion paper to Sarris et al (2015), Le Roux et al (2015) provided strongly corroborating evidence on the mechanism of PopP2 recognition and response by RRS1-R, and for the virulence function of PopP2 by acetylation of WRKY proteins. The observations that two unrelated effectors target the atypical WRKY domain fused to the C-terminus of RRS1 provides strong support for the unifying hypothesis that atypical domain fusions are "integrated decoys".

The term decoy specifically refers to an NLR-monitored host protein that exists solely to be targeted by an effector in order to initiate an immune

response; in order to be a decoy a protein cannot have a role in basal immunity. Wu et al (2015) warned against the inaccurate blanket use of the term "integrated decoy" without first testing if the integrated domain has a role in basal immunity; the authors favour the term "sensor domain", which is agnostic about a role in basal immunity. The most obvious way to test if RRS1 has a role in basal immunity is to compare pathogen virulence on genotypes with and without *RRS1*, specifically to determine if the genotype that lacked a functional *RRS1* was more susceptible, compared to a genotype with functional *RRS1*, to a bacterial pathogen that did not carry either AvrRps4 or PopP2. Unfortunately, a lack of observable difference between phenotypes would not be sufficient to determine that RRS1 has no role in basal defence because WRKY proteins often function redundantly (Eulgem and Somssich, 2007). WRKY proteins often confer increased basal resistance when overexpressed; more conclusive evidence toward the potential role of RRS1 in basal immunity would be provided by including a transgenic line overexpressing RRS1 with the previous experiment, and determining whether susceptibility to a pathogen (which lacks either recognised effector) is altered. One remaining puzzle is whether or not RRS1 is solely a decoy or if it participates as a transcription factor in the transcriptional reprogramming that occurs subsequent to activation. $RRS1^{SLH1}$ does not bind DNA but plants expressing this protein have transcriptional profiles that indicate activated immunity (Sohn et al., 2014). In this case, RRS1 may behave as a cofactor that positively regulates immunity without directly binding DNA or it may have no role in transcriptional regulation.

The integrated decoy hypothesis is parsimonious because it suggests a single role for all atypical domain fusions. However, it is unwise to assume that all integrated domains are decoys or sensors and each domain must be evaluated on a case-by-case basis. In the article that first postulated the integrated decoy hypothesis (Cesari et al., 2014a), two cases were evaluated. RRS1/RPS4 was one. The other was RGA4/RGA5, a gene pair in rice that has several similarities to RRS1 and RPS4; most notably, the signalling active partner that confers constitutive activity when overexpressed without its partner (RPS4 and RGA4) is suppressed by its partner (RRS1 and RGA5), which contains an integrated domain. RGA5 contains a heavy metal-associated

(HMA) domain, termed a RATX1 domain (for Related to yeast ATX1), fused to its C-terminus. This integrated RATX1 domain binds directly to cognate effectors from the fungal pathogen *Magnaporthe oryzae* (causal agent of rice blast) to confer resistance to avirulent strains (Cesari et al., 2013, 2014b).

Another rice R protein, Pik-1, has an HMA domain integrated between its CC and NB-ARC domains (Cesari et al., 2013; Zhai et al., 2011). Pik-1, along with its signalling partner Pik-2, forms an allelic series with varying recognition specificity (Zhai et al., 2011). The HMA domain of the Pik-1 allele Pikp-1 was recently co-crystallised with its cognate effector AVR-PikD, demonstrating direct interaction (Maqbool et al., 2015). In order for these HMA domains to be considered integrated decoys, the cognate effectors that target them must also target other HMA-containing proteins to confer virulence, and this remains to be demonstrated. However, the first evidence that suggests that these integrated HMAs are decoys lies with the rice protein Pi21. Pi21 is a non-NLR rice blast susceptibility factor that contains an HMA domain (Fukuoka et al., 2009). This is an example of one HMA-domain-containing protein that is manipulated by one or more pathogen effectors to enhance pathogen virulence.

Are integrated decoys common amongst plants? The first published work that hypothesised that an integrated domain may function as a decoy proposed that the BED zinc-finger DNA-binding domain at the N-terminus of BED-NLRs functioned as a decoy for other host proteins containing BED-domains that had a role in basal defence and were targeted by effectors (Germain and Séguin, 2011). The mechanism or function of poplar BED-NLRs is still unknown, but rice contains several NLRs with integrated BED domains. One of these, Xa1, confers strain specific resistance to the bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae* (Yoshimura et al., 1998). No effectors are known to target Xa1 or any other rice BED-containing protein, but ZBED, a rice protein containing three BED domains homologous to Xa1-BED, contributes to basal defence and is a good candidate to test for effector targeting (Kroj et al., 2016). Other examples of functional NLRs with integrated atypical domains include CHS3 (LIM domain), RLM3 (brevis radix domains), Rpg5 (protein kinase domain), Xa1 (BED domain) and RPP2A

(DUF640, and additional TIR and NBS domains) from Arabidopsis (Brueggeman et al., 2008; Sinapidou et al., 2004; Staal et al., 2008; Xu et al., 2015; Yoshimura et al., 1998). Two recent studies performed computational searches of predicted gene models from a range of plant genomes and revealed a widespread tendency for integrated domains in NLR proteins, with kinase, WRKY and BED domains among the most common (Kroj et al., 2016; Sarris et al., 2016). In general, the presence of protein domain fusions in various NLR immune receptors, in almost all sequenced plant genomes, suggests *R* gene pairs with integrated decoy domains may be widespread in angiosperm plants. I predict that such protein domains in other NLR proteins are effector targets, and that such NLR domain fusions are likely to require a partner NLR protein for defence activation.

Atypical integrated domains are widespread in plant NLRs and they also appear to be present in animal NLRs. A good example of this is the human NAIP protein, an NLR that has three N-terminal BIR (baculovirus inhibitor of apoptosis repeats) domains of unknown function. Another example is the mouse NLR NLRP1b, which may have a motif that behaves like an integrated decoy. NLRP1b acts as a sensor for the protease activity of the lethal factor (LF) metalloprotease from *Bacillus anthracis* (Anthrax). LF cleaves multiple mitogen activated protein kinases to suppress defense activation (Turk, 2007), but NLRP1b has integrated a cleavage site for LF into its structure. Upon cleavage of NLRP1b by LF, inflammasome formation and caspase activation occurs (Chavarría-Smith and Vance, 2013; Levinsohn et al., 2012).

The "Rosetta Stone principle", which posits that evolution has favoured the fusion of proteins that are functionally linked, has been a guiding hypothesis for RRS1 characterisation (Lahaye, 2002). Specifically, the existence of an NLR-WRKY fusion has led to speculation that other archetypal NLRs may initiate immune responses by modulating transcriptional regulation through the modulation of WRKY proteins. Indeed, a good example is the CNL MLA1 from barley, which interacts with WRKY proteins via its CC domain (Shen et al., 2007). Another example of a Rosetta Stone situation is with the CNL AetRGA2a from *Aegilops tauschii*. AetRGA2a has an exocyst component domain, the exo70 domain, integrated at its C-terminus, and thus may be an

NLR with an integrated sensor domain (Periyannan et al., 2013). Evidence supporting this includes the observation that rice exo70 is targeted by *M. oryzae* effectors, and a loss-of-function mutant of exo70 is autoimmune due to a TIR-NB-ARC-only protein in Arabidopsis (Fujisaki et al., 2015; Zhao et al., 2015). Taken together, the Rosetta Stone principle probably still applies to integrated domains, but instead of domain integration being indicative of a close functional association between an NLR and its downstream signalling partner, the functional association is more likely between a guard and its decoy.

3.3.2. Differential recognition of PopP2 is conferred by a C-terminal motif of RRS1-R

Response to PopP2 is often correlated with DNA-binding, but two key experiments demonstrate that loss of DNA-binding ability is not sufficient to trigger an immune response. First, EMSA of RRS1-S co-expressed with PopP2 demonstrated that loss of DNA-binding was not sufficient for HR. Second, Le Roux et al (2015) demonstrated by FRET-FLIM (fluorescence resonance energy transfer-fluorescence lifetime imaging microscopy) of GFP-tagged RRS1-R^{K1221R} with a DNA-binding fluorescent dye in stable transgenic lines does not interact with DNA (but WT RRS1-R does), and the stable lines do not have an autoimmune phenotype. This latter observation matches my transient expression HR assay results. Additionally, overexpression of RRS1, with or without RPS4, does not trigger HR; in this scenario, it is likely that the majority of RRS1 molecules are not bound to DNA. An interesting correlation to this is that both alleles of RRS1 confer responsiveness to AvrRps4, even though this effector does not disrupt DNA-binding of RRS1 (Sarris et al 2015). Activation of immunity upon WRKYGQK acetylation must instead provoke intra- and inter-molecular reconfiguration within the RPS4/RRS1-R complex that also involves the C-terminal extension present in RRS1-R alleles, but not in RRS1-S alleles. Acetylation of the WRKY domain and the presence of the C-terminal extension are not required for AvrRps4 to activate the complex. Although loss of DNA-binding is not sufficient to trigger an immune response, it remains to be seen if dissociation of RRS1 from DNA is a requirement for a fully competent immune response. Understanding how DOM6R confers specific responsiveness to PopP2 is key to understanding

how an NLR can evolve to recognise multiple, unrelated pathogens that target the same host protein.

3.3.3.Plant WRKY proteins involved in immune regulation are good targets of effectors

Plant WRKY domain proteins are strongly implicated in plant defence (Chi et al., 2013; Dong et al., 2003). Mutations in several Arabidopsis WRKY genes, notably WRKY33, are associated with reduced pathogen resistance (Birkenbihl et al., 2012). I propose that both AvrRps4 and PopP2 evolved to modulate or block WRKY protein function, either by direct binding to, or acetylation of, WRKY proteins. In turn, effector interference with WRKY proteins led to selection for a resistance complex, RPS4/RRS1, which detects effectors that target WRKY domains. I hypothesise that the not yet identified *Colletotrichum higginsianum* fungal effector recognised by RPS4/RRS1-R also binds WRKY domains.

PopP2 appears to be able to preferentially acetylate WRKYs with a positive effect on immune responses to biotrophic pathogens, such as WRKY41, WRKY70 and WRKY33 (Birkenbihl et al., 2012; Higashi et al., 2008; Knoth et al., 2007; Li et al., 2006), and avoid acetylating the negative regulator WRKY60 (Xu et al., 2006). Le Roux et al (2015) additionally demonstrated that PopP2 does not acetylate the negative regulator WRKY40. A widespread yeast two-hybrid screen detected an interaction between WRKY41 and AvrRps4, but no other interactions were detected between WRKY proteins and effectors from a bacterium (*Pseudomonas syringae*), an oomycete (*Hyaloperonospora arabidopsidis*) or an ascomycete fungus (*Golovinomyces orontii*) (Mukhtar et al., 2011; Weßling et al., 2014). Nishimura et al (2015) proposed that integrated domains were decoys of host proteins that were convergent targets of multiple effectors; this does not appear to be the case for WRKY proteins.

The guard hypothesis outlines a mechanism by which a plant can compete with a microbe in an evolutionary arms race: *R* genes are under selection pressure to recognise perturbations to the function of host proteins, while pathogen effectors are under selection pressure to perturb the function of host proteins involved in basal defence in plants that lack a guard *R* gene.

However, PopP2 has a diverse range of WRKY proteins it can choose to target in order to abrogate immunity, and it has the ability to at least discriminate between WRKYs that positively or negatively regulate immunity. Since acetylation of RRS1 is detrimental to infection success, why has PopP2 not evolved to discriminate between the RRS1 WRKY domain, and the WRKY domains of positive regulators? The most likely answer to this question is that the WRKY domains of negative regulators (WRKY18, WRKY40 and WRKY60) have diverged from RRS1 more than most of the other Arabidopsis WRKYs (Eulgem et al., 2000). Another explanation is that PopP2 exists in *Ralstonia solanacearum*, which has not been recorded as a pathogen of Arabidopsis outside of the laboratory (brassicas are considered "distant" hosts of *R. solanacearum* (Guidot et al., 2014)), and there is no evidence that an NLR-WRKY fusion in a solanaceous host confers responsiveness to PopP2, which would lead to subsequent diversifying selection to evade RRS1 recognition.

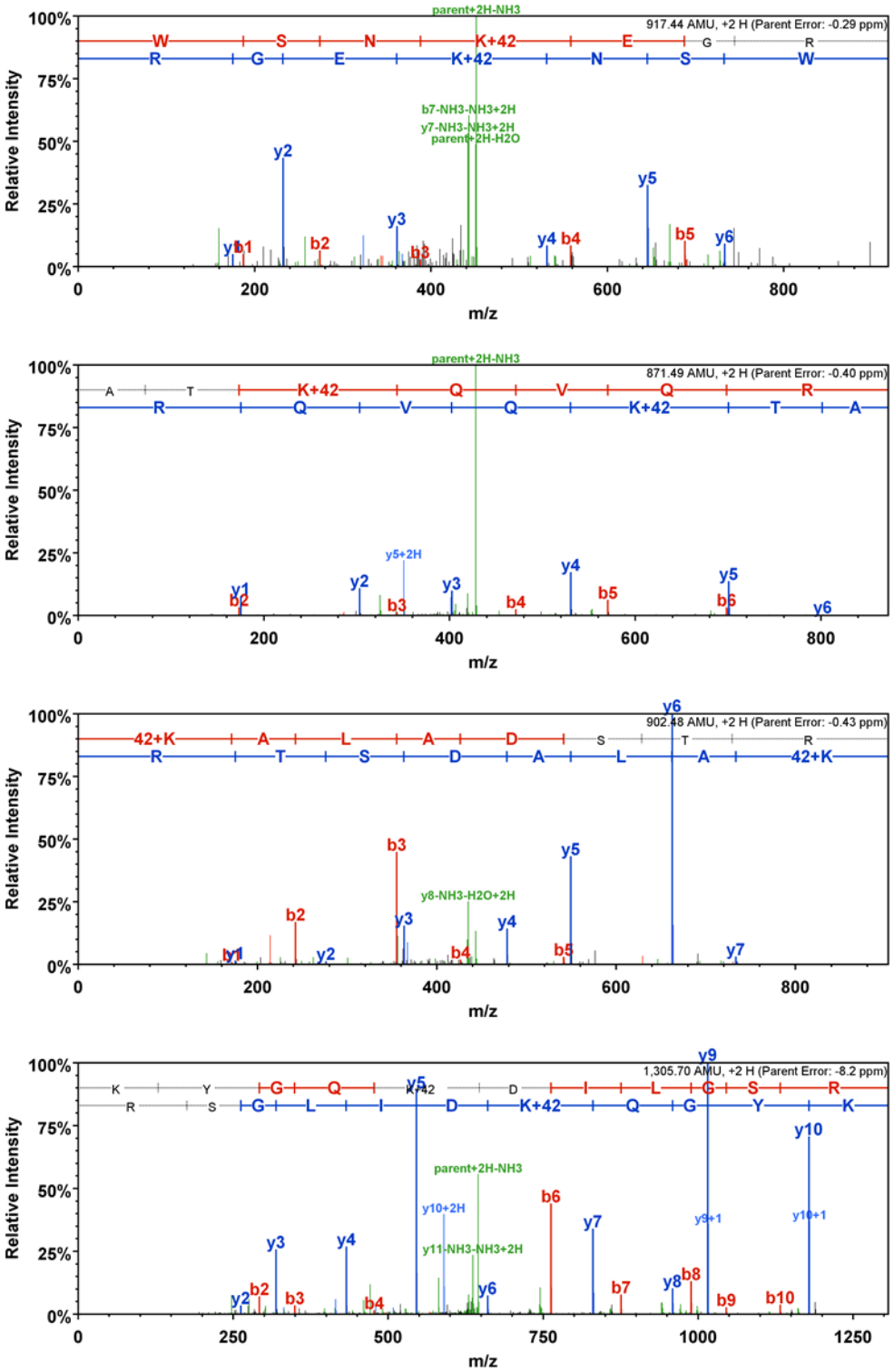
3.3.4.Does this greater understanding of RRS1/RPS4 recognition aid in our efforts to understand ETI and ultimately engineer it?

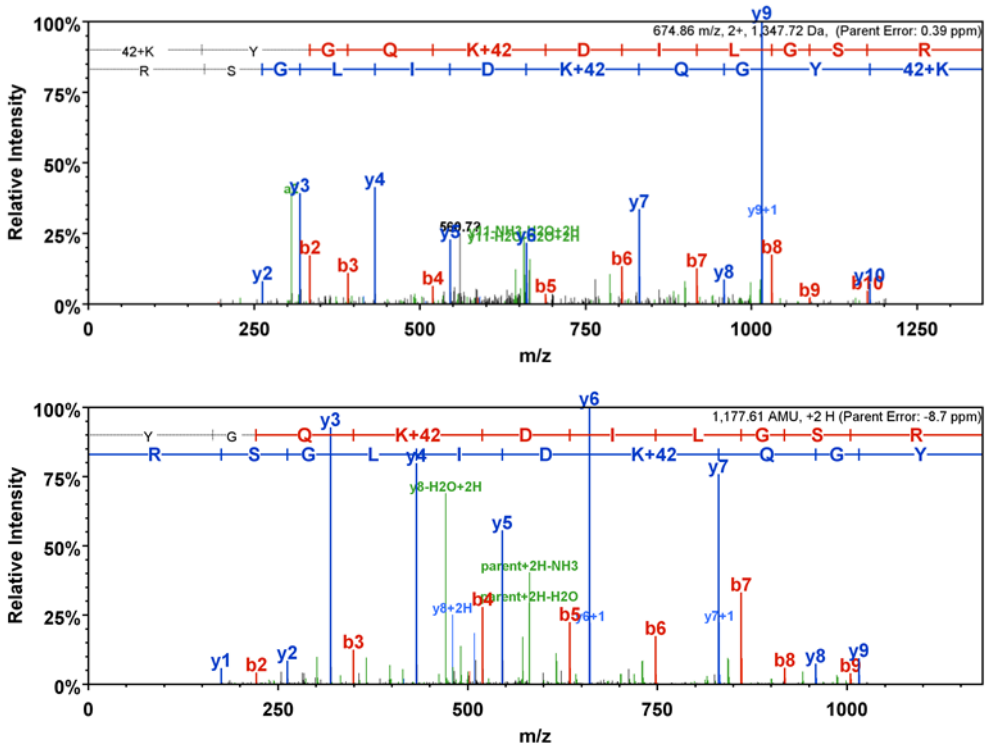
A corollary to the RRS1 integrated domain being a decoy is that if one switches the WRKY domain of RRS1 with another domain that is known to be targeted by another effector, one can change recognition specificity of RRS1 from WRKY-targeting effectors to effectors that target the new integrated domain. The simplest way to test this concept is to swap the RRS1 WRKY-domain with the closely related WRKY41 WRKY-domain, which is also known to interact with AvrRps4 (Mukhtar et al., 2011). The prediction is that this NLR will confer responsiveness to AvrRps4. Yan Ma generated this clone; she demonstrated that it conferred constitutive HR when co-expressed in tobacco with RPS4, but including AvrRps4 increased the onset of HR (data not shown). Clearly, the WRKY domain is required both for bait for effectors and for autoinhibition of RRS1.

Elucidating the molecular mechanism of effector recognition and response by RPS4 and RRS1 positions this system as a useful tool to investigate downstream NLR signalling. It also creates the opportunity to engineer novel resistance, using the presumptions described in this chapter, to generate

technology to better protect crops and to demonstrate a deep understanding of NLR-mediated resistance.

3.3.5. Appendices





Appendix 3.1. Peptide mass fingerprints of acetylated peptides identified by mass spectrometry. Tryptic peptides were analysed by LC-MS/MS using a hybrid mass spectrometer LTQ-Orbitrap XL and a nanoflow-UHPLC system.

Appendix 3.2. List of peptides and precursor/products used for SRM of RRS1 protein.

Acetylated peptides

Peptide Modified Sequence	Precursor *z*	Precursor	Fragmentation	Product *m/z*	Product *z*
K[+42]YGQK[+42]ILGSR	2	674.8646	y9	1015.5531	1
			b7	917.4727	1
			y7	830.4730	1
			b6	804.3886	1
			b4	519.2562	1
			y4	432.2565	1
			b2	334.1761	1
			y3	319.1724	1
YGQK[+42]DILGSR	2	589.8118	y7	830.4730	1
			y6	660.3675	1
			y5	545.3406	1
			b4	519.2562	1
			y4	432.2565	1
			y3	319.1724	1
KYGQK[+42]DILGSR	2	653.8649	y7	830.4842	1
			y8	958.5428	1
			y9	1015.5643	1
K[+42]YGQKDILGSR	2	653.8593	b4	519.2562	1
			b3	391.1976	1
			b2	334.1761	1

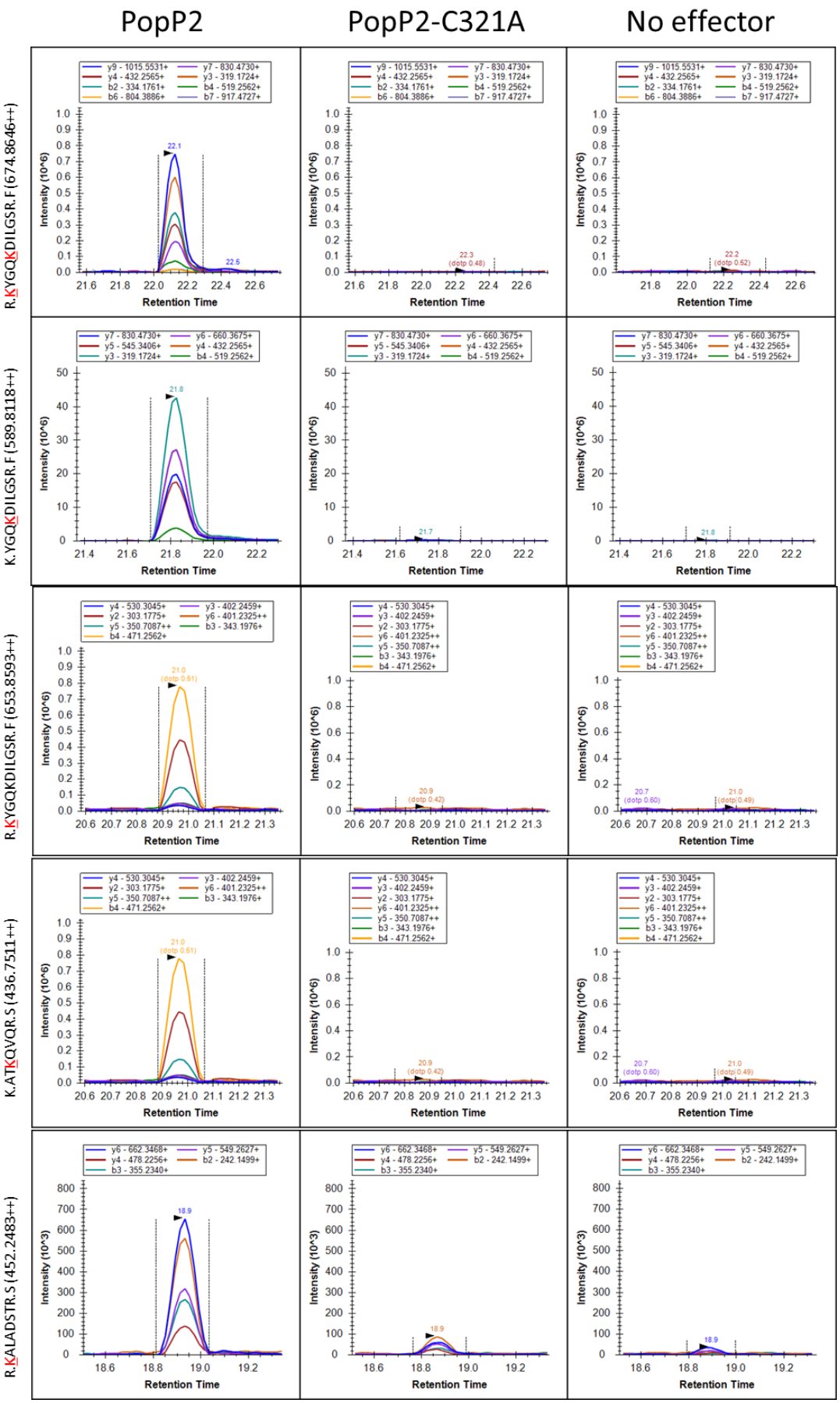
			b1	171.1128	1
ATK[+42]QVQR	2	436.7511	y4	530.3045	1
			b4	471.2562	1
			y3	402.2459	1
			y6	401.2325	2
			y5	350.7087	2
			b3	343.1976	1
			y2	303.1775	1
K[+42]ALADSTR	2	452.2483	y6	662.3468	1
			y5	549.2627	1
			y4	478.2256	1
			b3	355.2340	1
			b2	242.1499	1

Control peptides

Peptide Sequence	**Precursor *z***	**Precursor**	**Fragmentation**	**Product *m/z***	**Product *z***
AQGSEEIEGLFLDTSNLR	2	989.9895	y11	1264.6532	1
			y10	1135.6106	1
			y8	965.5051	1
			y7	818.4367	1
			y6	705.3526	1
			y5	590.3257	1
C[+57]VGIWGM[+16]PGIGK	2	645.8203	y8	861.4287	1

			y7	675.3494	1
			y5	471.2926	1
			y2	204.1343	1
GYTQELINK	2	533.2824	y7	845.4727	1
			b7	805.4090	1
			b6	692.3250	1
			y7	423.2400	2
			y3	374.2398	1
			y2	261.1557	1
LNHSWR	2	406.7117	y4	585.2892	1
			y3	448.2303	1
			b3	365.1932	1
			y5	350.1697	2
			y4	293.1482	2
			b2	228.1343	1
QLYLGGTAIR	2	546.3140	y8	850.4781	1
			y7	687.4148	1
			y6	574.3307	1
			y5	517.3093	1
RLWEPWSIK	2	607.8377	b8	1068.5625	1
			b7	955.4785	1
			y5	630.3610	1
			b4	585.3144	1
SVLEIPPNIEK	2	619.8532	y8	939.5146	1

			y7	810.4720	1
			y6	697.3879	1
			b5	542.3184	1
			y9	526.8030	2
			b4	429.2344	1
YSFVSHLSEALR	3	470.2455	y11	623.3329	2
			y10	579.8169	2
			y9	506.2827	2
			y8	456.7485	2


PopP2
PopP2-C321A
No effector
R.KYGQKDILGSR.F (674.8646++)
K.YGQKDILGSR.F (589.8118++)
R.KYGQKDILGSR.F (653.8593++)
K.ATKQVQR.S (436.7511++)
R.KALADSTR.S (452.2483++)
Intensity (10^6)
Intensity (10^3)
Retention Time

Appendix 3.3. Chromatograms showing transition peak areas of RRS1-R peptides with acetylated Ks. Transition peaks were obtained by analyzing peptides using nanospray ESI and a TQ-S mass spectrometer (Xevo). The y and b ions measured are indicated in the key superimposed over each chromatogram. Peptide information, including sequence, charge and mass, is to the left of each set of chromatograms, and acetylated lysines are highlighted in red and underlined.

Appendix 3.4. Acetylated WRKY peptides from several WRKY-domain proteins in the presence of PopP2 or PopP2-C321A. All WRKY proteins were detected by MS/MS. Acetylated and unacetylated peptides containing the WRKYGQK motif are presented.

Protein name	Acetyl-peptide sequence	Total WRKY-acetylpeptide count		Total unacetylated WRKY peptide count	
		PopP2	PopP2-C321A	PopP2	PopP2-C321A
WRKY33[a]	YGQK[+42]QVK[+42]GSENPR	1	0	0	0
	YGQK[+42]VVK[+42]GNPNPR	1	0		
WRKY41	(K)YGQK[+42]DILGAK(FPR)	10	0	0	6
	(K)YGQK[+42]DILGAK[+42]FPR	1	0		
WRKY60	nd	0	0	0	0
WRKY70	(K)YGQK[+42]EILNAK(FPR)	3	0	0	1
	(K)YGQK[+42]EILNAK[+42]FPR	1	0		

[a]WRKY33 contains two WRKY domains; acetyl-peptides derived from both these domains are shown. These are representative results from a single run. Acetylation of each WRKY protein was measured at least twice.

Chapter 4. Rational design of synthetic resistance against specific pathogens using the RRS1/RPS4 gene pair

4.1. Introduction

The potential for engineering novel resistance specificities by designing modified NLRs was discussed in Chapter 2. I believe that RRS1 and RPS4 are good candidates for engineering novel effector perception specificities because we have a good understanding of the mechanism of effector perception in this system: we know that RPS4 is involved in signalling and that RRS1 is involved in effector perception. In addition, RRS1/RPS4 has broad utility because they are functional in several plant families (Narusaka et al., 2013). In this chapter, I introduce two strategies to engineer NLRs to confer resistance to novel, selected effectors. The first strategy leverages our understanding of inter- and intramolecular negative regulation of RRS1. This system relies on an NLR that negatively regulates a second NLR, and the negative regulation is released upon effector-directed degradation of the first NLR. The second approach engineers a protease cleavage site into an NLR, which is recognised by a pathogen-secreted protease, such that upon proteolytic cleavage at this site the NLR activates immunity. The two strategies are informed by the depth of knowledge available on the mechanism of RRS1/RPS4 function.

4.2. Results

4.2.1. Synthetic resistance strategy one: Dominant negative suppression of an autoimmune allele may be engineered to specifically activate immunity in response to effectors that degrade host proteins

The recessive resistance gene *RRS1-R* confers recognition to PopP2 when homozygous, for example in the ecotype Nd-1, but not when heterozygous with *RRS1-S*, for example in the F1 cross between Nd-1 and the *RRS1-S*-carrying ecotype Col-5 (Deslandes et al., 1998, 2002). However, *RRS1-R* behaves as a dominant *R*-gene in transgenic plants: Nd-1 lines transformed

with *RRS1-S* and Col-5 lines transformed with *RRS1-R* are both resistant to *Ralstonia solanacearum* carrying PopP2 (Deslandes et al., 2002). The autoimmune *RRS1*^{SLH1} mutant is similarly recessive and is suppressed by an allelic copy of *RRS1-R* in heterozygous plants (Noutoshi et al., 2005). In contrast to *RRS1-R* suppression by *RRS1-S*, the autoimmune phenotype of *RRS1*^{SLH1} is suppressed by a single, hemizygous transgenic copy of *RRS1-R* (Noutoshi et al., 2005). Similarly, *RRS1-R* and *RRS1-S* are able to suppress *RRS1*^{SLH1} autoimmunity when overexpressed in tobacco with *RPS4* (Sohn et al., 2014) (and data not shown). My goal in the first part of this chapter is to construct a resistance gene system that recognises previously unrecognised effectors by taking advantage of the recessive nature of *RRS1*^{SLH1} to create a novel method of detecting effectors that degrade host protein targets.

I hypothesise that a plant expressing two forms of a resistance gene, where one form encodes an autoactive protein and the other form exerts dominant negative suppression on the autoactive protein, but is fused to an effector-dependent degron; that is, a protein known to be degraded in the presence of an effector, will be resistant to pathogens that secrete that effector (Figure 4.1). In the proof of concept for this system, the autoactive allele will be *RRS1*^{SLH1}, the dominant negative allele will be *RRS1* fused to any one of a number of plant proteins (that localise to the nucleus) which are known to be targeted by effectors. Co-expressing *RRS1-S* in the presence of *RPS4* and *RRS1*^{SLH1} results in a WT phenotype (Sohn et al., 2014). Effector-dependent destruction of RRS1-S protein in the presence of RPS4/RRS1^{SLH1} should thus activate defence. Many effectors provoke destruction of host targets, via the proteasome, such as JAZ proteins (by HopZ1a or by coronatine) (Jiang et al., 2013), TCPs (by Phytoplasma effector SAP11) or MADS-box transcription factors (by SAP54) (MacLean et al., 2014; Sugio et al., 2011, 2014). If RRS1-S is fused to a known effector target X (making RRS1-S-X), I expect the whole protein to be degraded in the presence of the effector. If RRS1-S-X is transformed into *slh1*, I would expect the effector-directed degradation of RRS1-S-X to relieve negative regulation of RRS1^{SLH1} autoimmunity and, as a result, trigger an immune response.

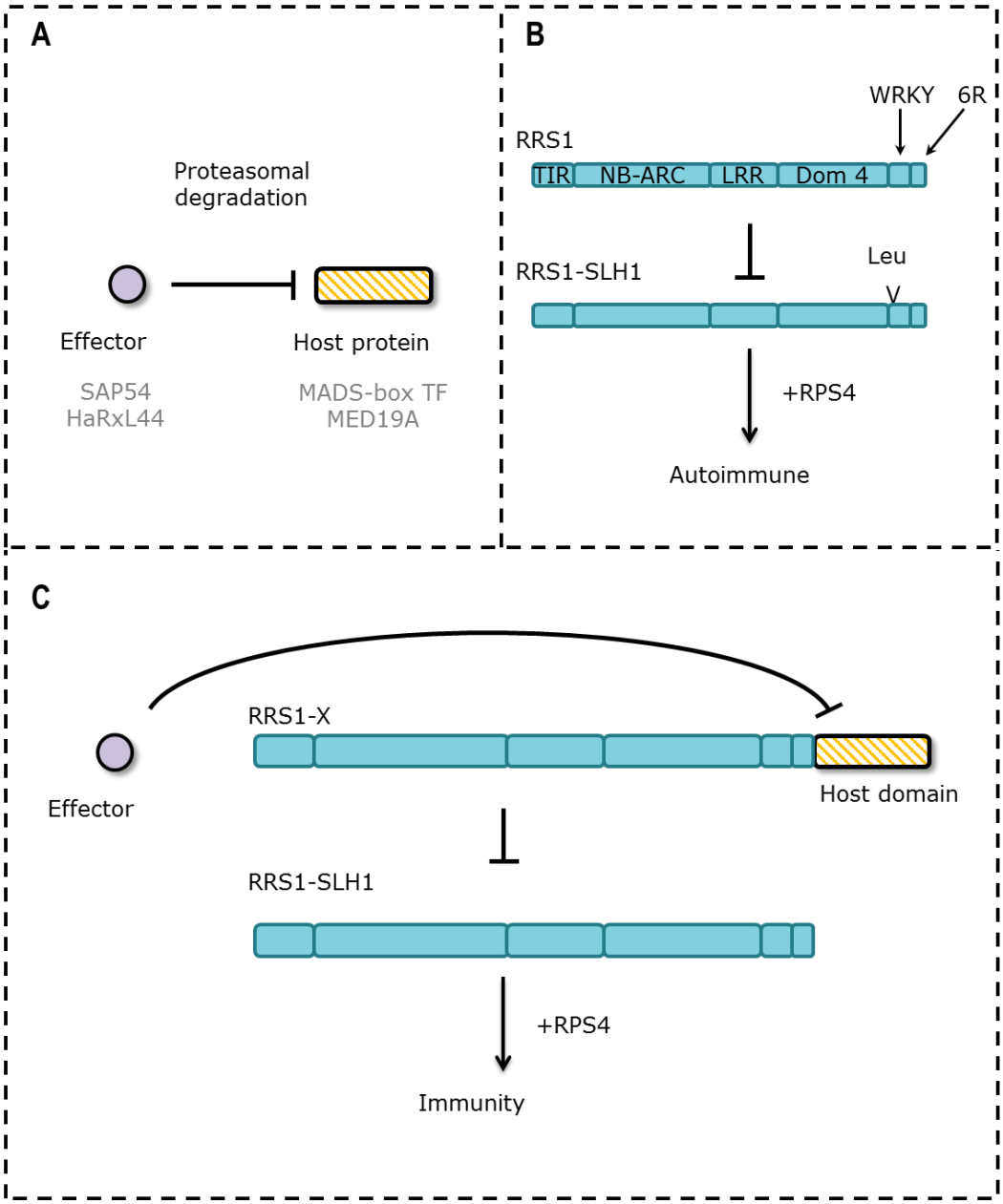


Figure 4.1. Outline of the concept for synthetic resistance strategy one. (A) Some effectors target host proteins and ultimately destine them for proteasomal degradation. Examples are indicated below their respective category of protein. (B) The recessive RRS1^{SLH1} allele is autoactive and triggers constitutive HR when expressed with RPS4 in tobacco, and confers an autoimmune phenotype in Arabidopsis. WT RRS1 alleles can suppress RRS1^{SLH1} autoimmunity. (C) The dominant suppression of RRS1^{SLH1} by WT RRS1 could be released by an effector that designates host proteins for proteasomal degradation if it could be made to target WT RRS1. Effectors could be made to determine the degradation of RRS1 by fusing a host target domain, corresponding to the domain in 4.1A, to RRS1 (creating RRS1-X). Thereby, during infection and secretion of the effector, RRS1-X is degraded and its suppression of RRS1^{SLH1} is released, triggering RPS4- and pathogen-dependent HR.

4.2.2.Most C-terminal fusions on RRS1 confer constitutive RPS4-dependent autoimmunity

The first step in establishing this strategy was to create the RRS1-S-X chimaeras. I chose to fuse effector-targeted domains to the C-terminus of RRS1-S, because N-terminal fusions of RRS1-S often result in a constitutive immune response (unpublished data). I generated constructs of RRS1-S-X with either the MED19A subunit of the transcriptional co-activating Mediator complex or the K-box domain of SOC1 ($SOC1^{K\text{-}box}$)(Figure 4.2), which gained this name because it structurally resembles the coiled-coiled domain of Keratin (Immink et al., 2010). MED19A is targeted for proteasomal degradation by the downy mildew effector HaRxL44 (Caillaud et al., 2013) and the phytoplasma effector SAP54 binds to the K-box domain of the host protein SOC1, a MADS-domain transcription factor, and proteasomally degrades the whole protein (MacLean et al., 2014).

When co-expressed in tobacco with RPS4, both chimaeras triggered HR in the absence of any effector; this occurred with and without an N-terminal influenza haemagglutinin (HA) epitope tag (Figure 4.2). I generated RRS1-R-X chimaeras with the same two degrons and these were also autoactive (Figure 4.2). I surmised two possible explanations for autoactivity: 1) a C-terminal fusion that surpasses a threshold in amino acid length destabilises RRS1 intramolecular inhibition, or 2) both MED19A and $SOC1^{K\text{-}box}$ were diverting RRS1 to other nuclear complexes, preventing *trans*-inhibition of immunity by RRS1. To test these hypotheses, I simply expressed RRS1-R C-terminally fused to GFP; this construct conferred RPS4-dependent autoimmunity (Figure 4.2). GFP (239 amino acids), MED19A (221 amino acids) and the SOC1 K-box (100 amino acids) confer autoimmunity when fused to the C-terminus of RRS1, while HF (52 amino acids) does not, suggesting that a C-terminal autoimmune threshold is between 52-to-100 amino acids. However, I have subsequently tagged RRS1-R with a 183 amino acid tag consisting of streptavidin binding protein (SBP), a human rhinovirus 3C protease cleavage site and two tandem copies of Protein G, and this construct was not autoimmune (data not shown). Effector relievable auto-inhibitory regulation of RRS1 can be disrupted by large C-terminal tags, but whether a tag disrupts

such autoinhibition cannot be predicted purely on the basis of length of the tag.

Mutating the TIR SH dimer-interface of RRS1 disrupts the formation of homo- and hetero-dimers of RRS1 and RPS4, respectively (Sohn et al., 2014). Substituting alanines at the SH interface of $RRS1^{SLH1}$ ($RRS1^{SLH1(SH\text{-}AA)}$) abolishes RPS4-dependent autoimmunity and, intriguingly, SH-to-AA mutants of RRS1 ($RRS1^{SH\text{-}AA}$) maintain suppression of $RRS1^{SLH1}$-autoimmunity (Sohn et al., 2014). Therefore, in an attempt to suppress the constitutive immunity conferred by the chimaeras, I decided to introduce the SH-to-AA mutation into my HA-RRS1-S-X constructs. I did not observe constitutive autoimmunity when SH-to-AA mutants were co-expressed in tobacco with RPS4 (Figure 4.3). However, although HA-$RRS1^{SH\text{-}AA}$ -S-HF suppressed $RRS1^{SLH1}$-autoimmunity, HA-$RRS1^{SH\text{-}AA}$ -S-MED19A and $RRS1^{SH\text{-}AA}$ -S-$SOC1^{K\text{-}box}$did not (Figure 4.3). This suggests that RRS1 alleles with derepressed autoinhibition are unable to suppress $RRS1^{SLH1}$ autoimmunity, even when their own autoimmune signalling is blocked by a TIR mutation.

This synthetic resistance approach holds promise but requires further development. In order for this method to be viable, I must find a way of fusing degrons to RRS1 without disrupting autoinhibition of immune signalling. Laurence Tomlinson has inserted circularly permuted GFP into the domain junctions of RRS1, and some of these chimaeras were still functional and were not autoactive (data not shown). I will insert degrons into these domain junctions and determine if this will disrupt autoinhibition of immunity and suppression of $RRS1^{SLH1}$ autoimmunity. Crucially, I must also determine that with any stable chimaera, my target effectors degrade the protein.

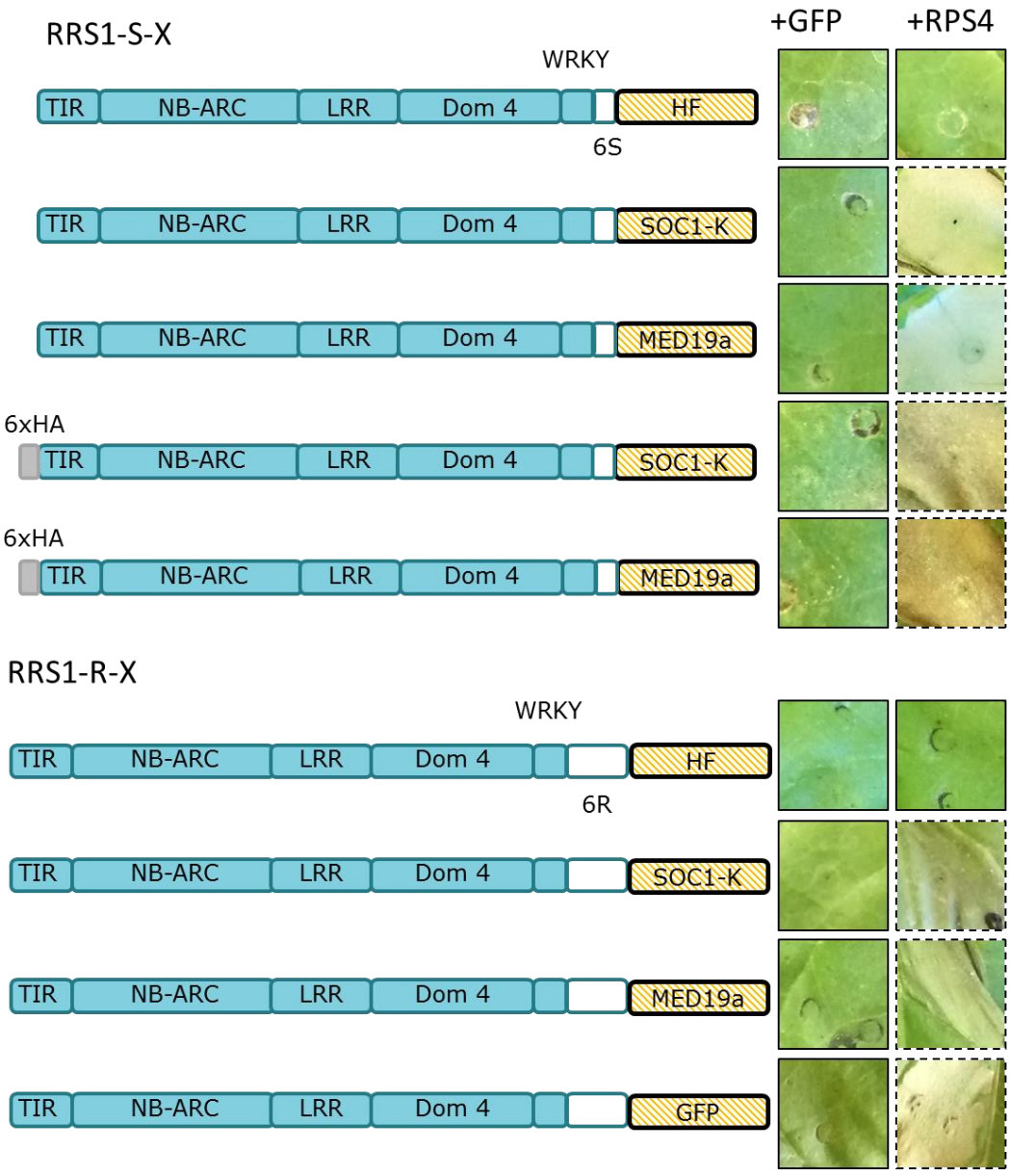


Figure 4.2. RRS1 with a C-terminal fusion is often autoactive. The RRS1-S- and RRS1-R-X-fusion constructs (X is an epitope tag or domain from another host protein targeted by an effector) shown were agro-infiltrated into *N. tabacum* with either GFP or RPS4. Leaf panels show phenotype. A solid outline indicates no HR, and a dashed outline indicates HR. HF, 6xHis.3xFLAG; SOC1-K, SOC1 keratin-like domain. All Agrobacterium strains were infiltrated at an OD_{600} of 0.5. This experiment was repeated three times and leaf panels from a representative experiment are shown.

4.2.3.Synthetic resistance strategy two: Introducing a protease effector recognition sequence between the WRKY and DOM6 domains of RRS1 may confer recognition to secreted protease effectors

In exploring the mechanisms of intracellular regulation of RRS1, Yan Ma and Panos Sarris discovered that a C-terminally truncated form RRS1 (RRS1△), that consists of domains one-to-four (TIR-NB-LRR-DOM4), activates autoimmunity when co-expressed with RPS4, and that this autoimmunity can be suppressed by co-expression with RRS1-R, but not RRS1-S (Figure 4.4a). Panagiotis Sarris and I leveraged this knowledge to design a synthetic resistance gene approach that could enable gene combinations that confer recognition of secreted protease effectors. The concept requires that a plant expresses a resistance gene that is known to be autoactive when C-terminally truncated, in this case RRS1-S with a protease recognition site introduced between DOM4 and the WRKY domain (termed RRS1-S$^{protease\ trap}$ hereon), will provide resistance to a pathogen that secretes protease effectors that cleave RRS1-S$^{protease\ trap}$. The resulting cleavage product is the autoactive, C-terminally truncated RRS1△ (Figure 4.4b). Specific effectors can be targeted by different variants of RRS1-S which contain the known protease cleavage sites of known effectors.

Although an observation of autoimmunity with a truncated form of RRS1 was the inspiration for this protease trap design, other NLRs could conceivably be used. For example, a cleavage site between the NB-ARC and LRR of Rx, or the TIR and NB-ARC of RPS4 would conceivably generate constructs that would trigger autoimmunity once cleaved. Using an alternative NLR may reduce the requirement of two genes (RRS1$^{protease\ trap}$ and RPS4) to one gene, and may expand the site of recognition from the nucleus to the nucleus and cytoplasm. I decided to use RRS1 for the proof of concept for a number of reasons: 1) RRS1△ triggers a consistent HR that is stronger than RPS4^{TIR} overexpressed in tobacco (data not shown). I hypothesised that this was because RPS4 was being fully activated by RRS1△, while RPS4^{TIR} cell death was triggered by a subset of activated RPS4^{TIR} present in overexpressed cells. 2) Well-studied pathogen-secreted proteases from potyviruses would be

ideal targets for a proof-of-concept study. 3) RRS1 and RPS4 function in species of plants from divergent families (Narusaka et al., 2013).

To test the concept, I first designed a cloning strategy that allowed rapid creation of RRS1-S$^{protease\ trap}$ alleles. I first considered Gibson assembly for constructing this gene, but because many intended protease recognition sites are short, this method was not appropriate. I instead settled on Golden Gate cloning because the cloned version of RRS1-S that I was using lacked the restriction nuclease recognition sites required to shuttle genes cloned by the Golden Gate method between plasmids and these recognition sites could be easily introduced with appropriate overhangs by PCR (Figure 4.4c). I compiled a list of pathogen secreted proteases and their host targets (Table 4.1). In principle, this system should be most effective against proteases that are targeted to the plant nucleus, where RRS1 is localised (Deslandes et al., 2003).

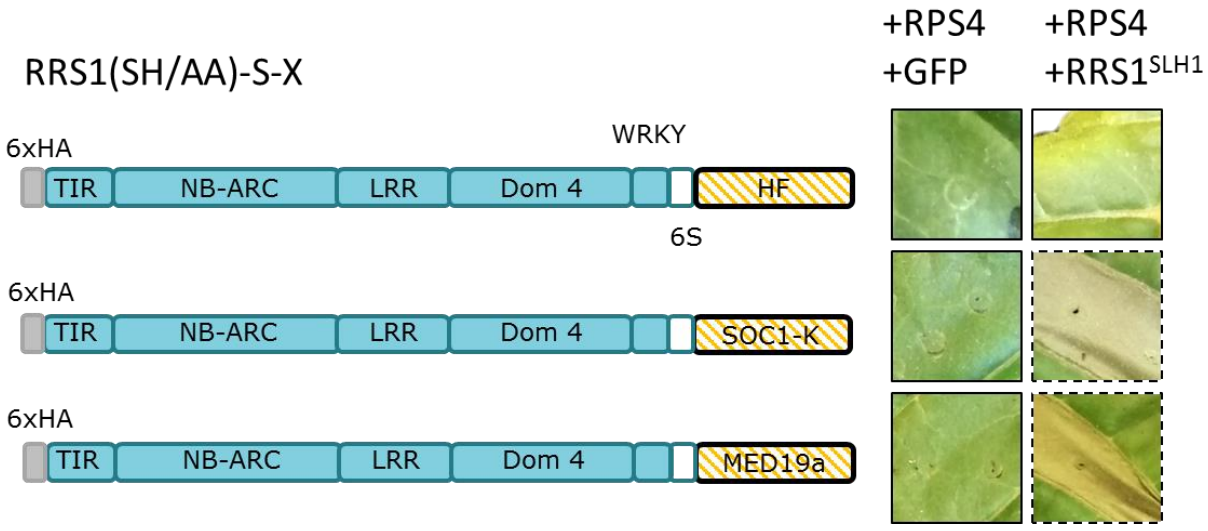
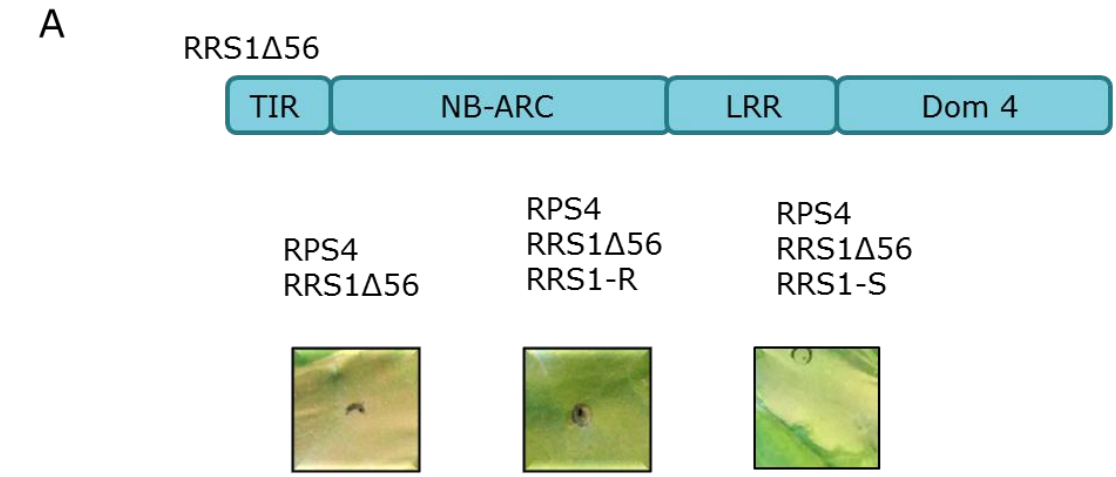


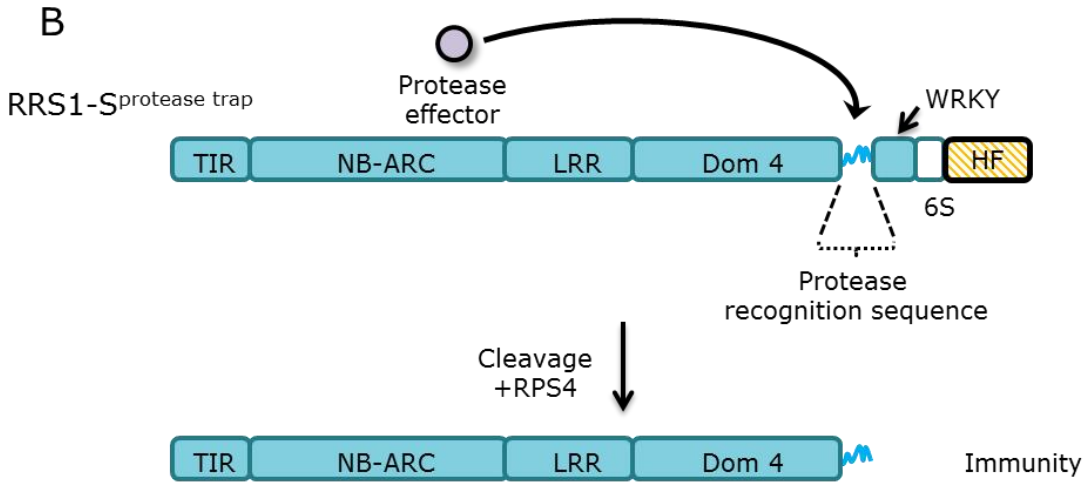
Figure 4.3. RRS1-S with a C-terminal fusion and a mutated SH dimer interface does not trigger constitutive HR but cannot suppress RRS1^{SLH1} HR. The RRS1$^{(SH/AA)}$-S-X fusion constructs (X is an epitope tag or domain from another host protein targeted by an effector) shown were agro-infiltrated into *N. tabacum* with either RPS4 or both RPS4 and RRS1^{SLH1}. Leaf panels show phenotype. A solid outline indicates no HR, and a dashed outline indicates HR. HF, 6xHis.3xFLAG; SOC1-K, SOC1 keratin-like domain. All Agrobacterium strains were infiltrated at an OD600 of 0.5. This experiment was repeated three times and leaf panels from a representative experiment are shown.

Table 4.1. Pathogen secreted proteases and their recognition sequences.

Protease	Recognition site*	Local.**	Notes
AvrRpt2	RIN4 RCS1: RSNVPKFG↓NWEA RCS2: VTVVPKFG↓DWDE	PM	Cleavage monitored by RPS2. From *P. syringae* (Kim et al., 2005a)
XopD	SUMO AFLFDGRRLRAEQTPDELEMEEGDEIDAMLH QTGG↓SCCTCFSNF	N	Conserved underlined. From *Xcv* and *Xcc* (Hotson et al., 2003)
AvrPphB	PBS1 RDFKSSNILLDEGFHPKLSDFGLAKLGPTGDK↓ SHVSTRV	PM	Guarded by RPS5 (Shao et al., 2003)
HopPtoN (HopN1)	PsbQ	C	(Rodríguez-Herva et al., 2012)
HopX1 (AvrPphE)	ZIM domain of JAZ proteins	N	From *P. syringae* pv. *tabaci* (*Pta*) 11528 (Gimenez-Ibanez et al., 2014)
XopJ	RPT6 (proteasomal subunit)	PM	(Üstün and Börnke, 2015)

***When known, amino acid recognition sequences are shown. RCS, RIN4 cleavage site. Underlined amino acids indicate amino acids indispensable for recognition. Arrows indicate cleavage site. **Localisation. PM, plasma membrane; N, nucleus; C, chloroplast.


A
RRS1Δ56
TIR
NB-ARC
LRR
Dom 4
RPS4
RRS1Δ56
RPS4
RRS1Δ56
RRS1-R
RPS4
RRS1Δ56
RRS1-S


B
RRS1-Sprotease trap
Protease effector
WRKY
TIR
NB-ARC
LRR
Dom 4
HF
6S
Protease recognition sequence
Cleavage +RPS4
TIR
NB-ARC
LRR
Dom 4
Immunity

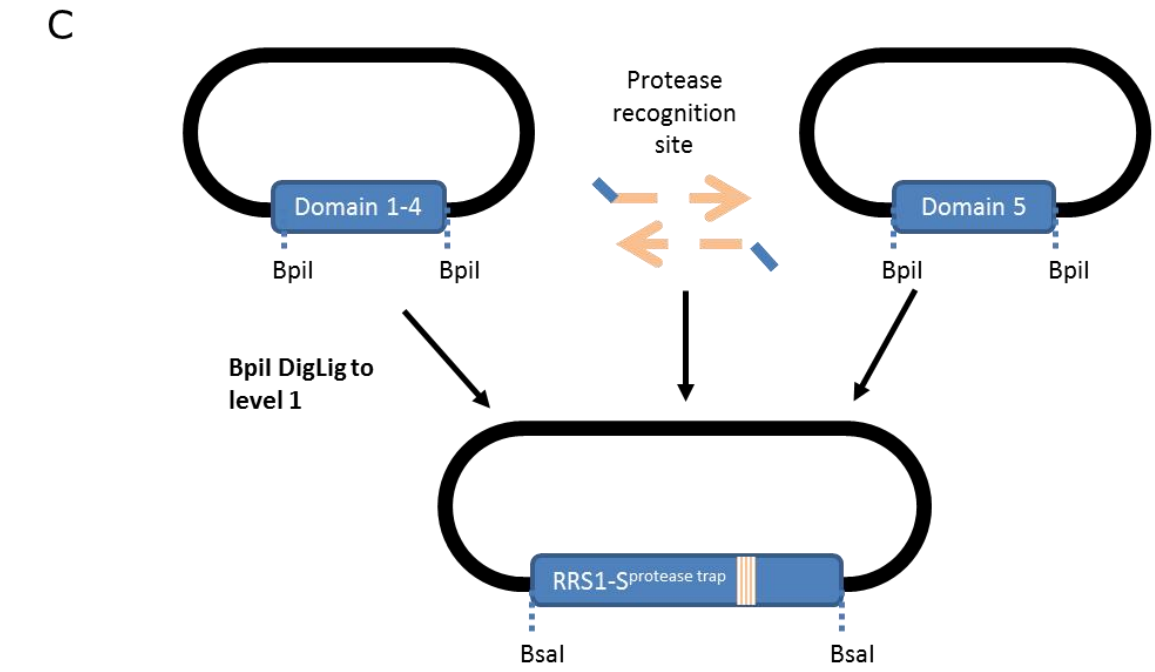

C
Protease recognition site
Domain 1-4
BpiI
BpiI
Domain 5
BpiI
BpiI
BpiI DigLig to level 1
RRS1-Sprotease trap
BsaI
BsaI

Figure 4.4. Outline of the concept for synthetic resistance strategy one. (A) Domains 1-4 of RRS1 (RRS1△56) trigger HR when co-expressed with RPS4. This HR can be suppressed by RRS1-R but not RRS1-S. *Agrobacterium* strains containing the constructs displayed above each panel were agro-infiltrated into *N. tabacum.* Leaf panels show phenotype. A solid outline indicates no HR, and a dashed outline indicates HR. All *Agrobacterium* strains were infiltrated at an OD_{600} of 0.5. Experiment performed by Yan Ma and Panagiotis Sarris. (B) A truncated form of RRS1 lacking the WRKY domain and domain 6 is autoactive; this observation may allow RRS1 to be engineered to recognise secreted protease effectors. (C) Cloning strategy for generating RRS1-$S^{protease\ trap}$ rapidly. Two Level 0 golden gate constructs containing Domains 1-4 and Domain 5-6 of RRS1-S were generated with distinct BpiI-generated overlaps. Overlapping oligonucleotides that encode the selected protease recognition sequence are synthesised with 5' BpiI sites that generate complementary overhangs upon BpiI digestion. Upon digestion and ligation (DigLig), a level 1 construct containing RRS1-$S^{protease\ trap}$ is generated.

4.2.4. The recognition site for a protease encoded by the turnip mosaic potyvirus (TuMV) inserted in the junction between DOM4 and the WRKY domain of RRS1-S (RRS1-S^{TuMV}) is sufficient to confer RPS4 and protease-dependent HR in tobacco

The first protease I chose to target was the Nuclear Inclusion a protease (NIa-pro) from Turnip Mosaic Virus (TuMV). TuMV is a single stranded RNA virus that is responsible for economically important diseases of brassicas and can infect Arabidopsis. During infection, TuMV replicates by utilising host translational machinery to synthesise a long polyprotein that is subsequently processed by two proteases contained within the polyprotein: HC-pro (helper component protease) and NIa-pro. NIa-pro has several features which make it an attractive target of our system: 1) NIa-pros are essential factors for potyviral replication and virulence. 2) Conceivably, it would be difficult for TuMV to evolve to evade recognition of an R gene that recognises NIa-pro activity, because the protease cleaves the potyviral polyprotein at seven sites; several cumulative mutations would be required to evade recognition, and would probably first generate a non-viable virus. Indeed, *Ry*, a resistance gene in potato against the potyvirus Potato Virus Y (PVY), recognises PVY NIa-pro protease activity and this *R* gene has not been overcome in more than 35 years of deployment (García-Arenal and McDonald, 2003; Mestre et al., 2000; Tomczyńska et al., 2014). 3) TuMV

infects brassicas so testing the RRS1-S^{TuMV} in Arabidopsis would be straightforward.

In order to determine if RRS1-S^{TuMV}, which contained a single GlySer linker on either side of the TuMV NIa-pro recognition site between DOM4 and the WRKY domain of RRS1-S (Table 4.2), could recognise TuMV NIa-pro, I co-expressed both proteins with RPS4 in tobacco (Figure 4.5). I observed HR in each replicate leaf, but cell death was never as rapid as with the positive control of RRS1-S^{TuMV} (which retained recognition of AvrRps4) and AvrRps4, and never affected the entire infiltrated area. RRS1-S^{TuMV} was C-terminally tagged with an HF epitope in these assays, so I was able to perform a Western blot to determine if TuMV NIa-pro cleaved RRS1-S^{TuMV}. The cleaved C-terminal fragment that contained the tag was ~18 kDa and difficult to detect with an anti-FLAG immunoblot, and there was no clear difference between the abundance of full-length RRS1-S^{TuMV} protein in the presence or absence of TuMV NIa-pro (data not shown). To make it easier to detect a cleavage event, I generated an RRS1-S^{TuMV} construct with a single HA-tag N-terminal to the TuMV NIa-pro cleavage site between DOM4 and the WRKY domain, and called it RRS1-S$^{HA\text{-}TuMV}$. Remarkably, this construct conferred stronger HR than RRS1-S^{TuMV} when co-expressed with TuMV NIa-pro and RPS4 (Figure 4.5a). I was able to observe TuMV NIa-pro-dependent cleavage of RRS1-S$^{HA\text{-}TuMV}$, measured by anti-FLAG immunoblotting as the disappearance of a band corresponding to full-length RRS1-S$^{HA\text{-}TuMV}$ and appearance of a smaller fragment corresponding to the size of the C-terminal cleavage product carrying the HF-tag, , and no cleavage in the presence of a catalytically dead mutant of TuMV NIa-pro (Figure 4.6). Anti-HA immunoblotting revealed the appearance of a band corresponding to the N-terminal cleavage product of RRS1-S$^{HA\text{-}TuMV}$, presumably present only in the presence of TuMV NIa-pro because the accessibility of the internal epitope tag to the antibody is increased after cleavage. Furthermore, I co-expressed a version of RRS1-S that had an HA-tag followed by five each of alternating Gly and Ser in the place of a protease recognition site (RRS1-S$^{HA\text{-}[GS]5}$) as a negative control (Table 4.2), with RPS4 and TuMV NIa-pro, and saw no HR or cleavage (Figure 4.5a and 4.6).

The TuMV NIa-pro recognition sequence I used in RRS1-S$^{HA\text{-}TuMV}$ overlaps with the consensus recognition sequence for PVY NIa-pro (Table 4.2). Therefore, I cloned NIa-pro from PVY (generously provided by Jacek Henning) and co-expressed it with RRS1-S$^{HA\text{-}TuMV}$ and RPS4. Unfortunately, I did not see any HR (Figure 4.5a). I also wanted to see if the approach worked for another potyvirus, Cassava Brown Streak Virus (CBSV), so I created RRS1-S$^{HA\text{-}CBSV}$ with a recognition sequence of CBSV NIa-pro (Table 4.2). When I co-expressed this construct with RPS4 and CBSV NIa-pro (cloned from a genomic fragment of CBSV generously provided by James Carrington), I did not observe any HR (Figure 4.5b). This may be due to several reasons. CBSV NIa-pro may have been poorly expressed or unstable, or may not have co-localised with RRS1-S$^{HA\text{-}CBSV}$, preventing it from cleaving its recognition sequence and triggering HR. I will determine the protein abundance of mCherry-CBSV NIa-pro by immunoblot and determine its localisation relative to RRS1 by fluorescence microscopy. I constructed another RRS1-S$^{protease\ trap}$ allele that contained the ZIM domain of JAZ5; the bacterial effector HopX1, from coronatine-deficient *Pseudomonas syringae* pv. *tabaci* (*Pta*) 11528, binds the ZIM domain of JAZ5 and results in the proteolytic cleavage of JAZ5 (Gimenez-Ibanez et al., 2014). This 40 amino acid insert resulted in an allele that conferred constitutive RPS4-dependent, effector-independent HR (Figure 4.5c). I am troubleshooting these preliminary negative results.

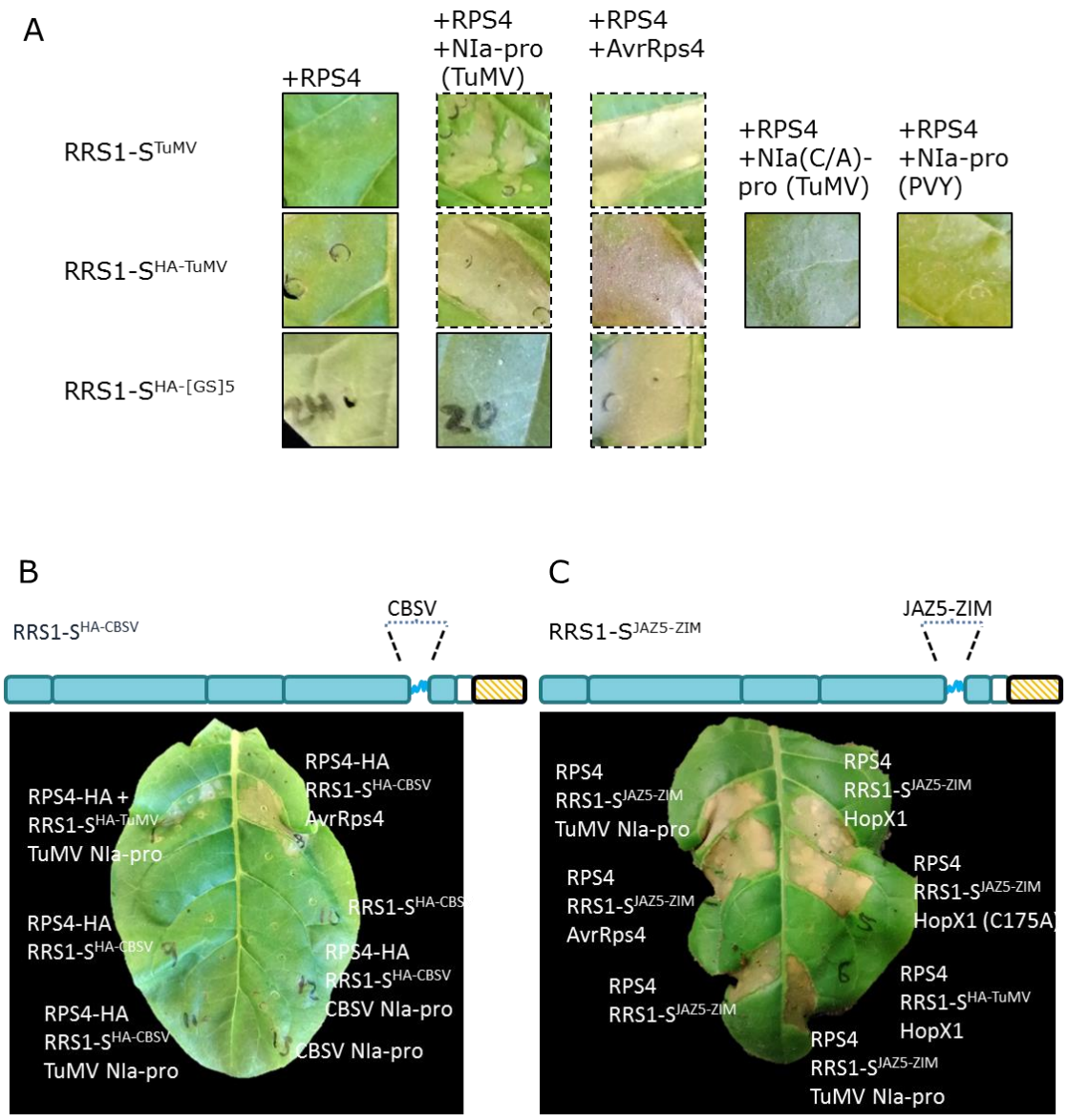


Figure 4.5. The recognition site for a protease encoded by the turnip mosaic potyvirus (TuMV) inserted in the junction between domain four and five of RRS1-S (RRS1-S^{TuMV}) is sufficient to confer RPS4 and protease-dependent HR in tobacco. (A) Three RRS1$^{protease\ trap}$ constructs were expressed in tobacco with RPS4 and either AvrRps4 or potyviral NIa-pros. RRS1-S^{TuMV} conferred weak HR when co-expressed with WT NIa-pro from TuMV. Enhanced HR was exhibited when a single influenza haemagglutinin (HA) epitope tag between domain 4 and TuMV NIa-pro recognition site was included. A catalytically dead mutant of NIa-pro (TuMV), with a C151 mutation (NIa(C/A)-pro (TuMV)), or NIa-pro from PVY , did not trigger HR. Agrobacterium strains containing the constructs displayed above each panel were agro-infiltrated into tobacco. Leaf panels show phenotype. A solid outline indicates no HR, and a dashed outline indicates HR. All Agrobacterium strains were infiltrated at an OD600 of 0.5. In experiments with a variable number of co-infiltrated strains, 35S:GFP-carrying strains were included in cell mixtures with fewer

component strains to ensure that the combined OD_{600} of each mixture was equal. (B) RRS1-$S^{HA\text{-}CBSV}$ does not trigger HR when co-expressed with NIa-pro CBSV. (C) RRS1-$S^{JAZ5\text{-}ZIM}$ is autoactive. This experiment was repeated three times.

RRS1-S$^{HA\text{-}TuMV}$-HF	+	+	+	-
RRS1-S$^{HA\text{-}GS5}$-HF	-	-	-	+
RPS4^{K242A}-HA	+	+	+	+
mCherry-NIa (TuMV)	-	+	-	+
mCherry-NIa C/A	-	-	+	-

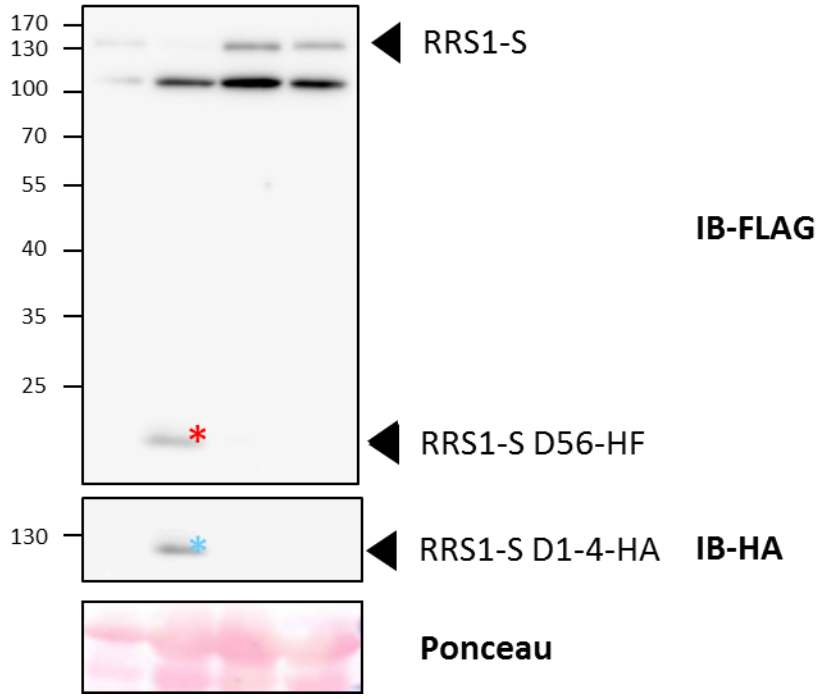


Figure 4.6. RRS1-S$^{HA\text{-}TuMV}$-HF is cleaved by NIa-pro (TuMV). Immunoblot showing cleavage of RRS1$^{HA\text{-}TuMV}$-HF by mCherry-NIa (TuMV). Appearance of a much shorter FLAG-tagged product indicated cleavage and generation of a Domain 5-6-HF fragment (red asterisk: HF-tagged cleavage product). Increased accessibility of the internal HA tag after cleavage (blue asterisk) also indicates cleavage. RRS1$^{HA\text{-}TuMV}$-HF was not cleaved by a catalytically-dead version of NIa-pro (mCherry-NIa (C/A)) and RRS1$^{HA\text{-}[GS]5}$-HF was not cleaved.

Table 4.2. Possible amino acids at NIa cleavage sites.

	Amino acid position*						
Potyvirus**	P5	P4	P3	P2	P1	↓	P1'
TuMV	X	V	R	H	Q	↓	[SIY]
used	L	V	R	H	Q	↓	S
PVY	X	V	X	H	Q	↓	[RSAG]
used	L	V	R	H	Q	↓	S
CBSV	X	[IV]	[DTE]	X	Q	↓	[VASGC]
used	T	I	D	V	Q	↓	A

*Arrows indicate cleavage site. Brackets indicate conserved amino acids that can all be found at the indicated position. **The name of a virus indicates the consensus sequence of its NIa, and "used" indicates the sequence that I used in the DOM4-WRKY junction to confer recognition to the Nia of that virus. References: (Kang et al., 2001; Kim et al., 1996; Mbanzibwa et al., 2009a, 2009b; Mestre et al., 2000; Robaglia et al., 1989).

4.2.5.RRS1-S$^{HA\text{-}TuMV}$ does not confer resistance to TuMV in the T1 generation when stacked with RPS4 in Arabidopsis

Transient co-expression of RRS1$^{HA\text{-}TuMV}$, RPS4 and TuMV NIa-pro elicited HR in tobacco. I next wanted to determine whether RRS1$^{HA\text{-}TuMV}$ and RPS4, when expressed stably in Arabidopsis, conferred resistance to TuMV. I stacked RRS1$^{HA\text{-}TuMV}$-HF or RRS1$^{HA\text{-}[GS]5}$-HF with RPS4-HA in a single T-DNA in the binary plasmid pAGM4723 under control of the promoters of the genes At4G34620 and At3G61440, respectively. These promoters were chosen because they exhibited much lower constitutive expression levels than the CaMV35S promoter, but still much higher expression than the lowly expressed native promoters of RPS4 and RRS1 (Sohn et al., 2014). However, it is not known in which cells these promoters are expressed. I included a FAST (fluorescence-accumulating seed technology)-Red selectable marker (Shimada et al., 2010). I transformed the triple mutant *rrs1-1*/*rps4-21*/*rps4b-1* (Ws-2) with either of these T-DNAs. I germinated 24 T1 seeds from each transformation and screened the resultant T1 plants for the presence of functional RRS1$^{HA\text{-}TuMV}$ and RPS4 by infiltrating with a modified *Pseudomonas fluorescens*, which carries a functional T3SS (Pf0-1), and the effector AvrRps4, or the unrecognised KRVY-AAAA mutant version of AvrRps4 (Sohn et al., 2009). Plants carrying functional transgenes were indicated by a visible HR when infiltrated with AvrRps4 but not AvrRps4-KRVY-AAAA (Appendix 4.1). Six plants carrying RRS1$^{HA\text{-}TuMV}$-HF and RPS4-HA displayed HR when challenged with AvrRps4 via Pf0-1, while none of the RRS1$^{HA\text{-}[GS]5}$-HF plants showed any tissue collapse. Pf0-1 delivery of AvrRps4 often gives ambiguous results, so I collected tissue from each plant for Western blot analysis, kept half the plants for T2 seed production, and gave the other half to collaborators Prof. John Walsh and Lawrence Bramham (University of Warwick) for TuMV resistance tests.

Lawrence Bramham challenged 8-week-old transgenic lines carrying RPS4-HA and either RRS1$^{HA\text{-}TuMV}$-HF or RRS1$^{HA\text{-}[GS]5}$-HF (*rrs1-1*/*rps4-21*/*rps4b-1* Ws-2) with TuMV isolate UK1. Infected plants were visually inspected for signs of TuMV infection and compared to uninfected and mock-infected plants (Appendix 4.2). At 14 dpi, all infected plants had yellowing around the edges of older leaves and distorted new leaves, but the plants carrying RRS1$^{HA\text{-}TuMV}$-

HF had slightly reduced visible symptoms (Figure 7A). At 19 dpi, infected plants were flowering but infected plants were not. To determine if there was a quantitative difference in disease resistance, L. Bramham performed an ELISA. Line #10 carrying RRS1$^{HA\text{-}TuMV}$-HF may have had a slight increase in resistance compared to the RRS1$^{HA\text{-}[GS]5}$-HF controls, but there was no appreciable difference between the other transformants (Appendix 4.2b). Only one plant infected with TuMV, RRS1$^{HA\text{-}TuMV}$-HF #2, showed AvrRps4 sensitivity (Appendix A). These first experiments are preliminary, and more decisive tests will be performed on homozygous T2 plants. At 35 dpi, all infected plants died (Appendix 4.2a). I am regenerating transformants with different promoters; the promoters used in this study have not been tested before, and although they confer recognition of AvrRps4 sometimes, a phloem-specific or ubiquitous promoter may be more appropriate for TuMV resistance.

4.3. Discussion

4.3.1. Release of negative regulation may be an effective method for recognising classes of pathogen effectors

Giannakopoulou et al (2016) summarises three approaches to engineer NLRs to gain novel recognition specificity: substituting amino acids in specific domains to expand recognition, swapping or integrating a host target domain into an NLR to confer recognition to the effector targeting that domain, or modifying the host protein guarded by an NLR so that it is targeted by a new effector. The first approach is exemplified by the engineering of R3a to retain recognition of its cognate effector and gain recognition of the effector AVR3a^{EM} (Giannakopoulou et al., 2015). At the time I initiated this project, no NLR or host protein had been rationally engineered to recognise completely novel effectors or pathogens. Substituting an alternative domain to the RRS1-WRKY, as a decoy, destabilised autoinhibition of immunity and triggered constitutive autoactivity (performed by Yan Ma, described at the end of chapter 3).

My first approach to the rational design of synthetic *R* genes targeted pathogens that secrete effectors that degrade host proteins via the proteasome. The fusion of effector targets to the C-terminus of RRS1

resulted in an autoimmune chimaera. To overcome autoactivity, I will try to generate RRS1 alleles with degrons inserted into interdomain junctions. This may work in some junctions; I have already shown that at the Domain 4-WRKY junction inserts that contain about 20 amino acids, like HA-TuMV, do not disrupt autoinhibition, but the 40 amino-acid ZIM domain from JAZ5 results in autoactivity when inserted in the same location.

For this approach to work, I must generate RRS1-S-degron chimaeras that are not autoactive, that suppress RRS1-R^{SLH1}, and that are degraded by the effectors for which they are designed to confer recognition. Assuming that this chimaera can be generated, will this concept work? An important factor that will affect this is protein abundance. RRS1-S-X must be at low enough levels to be sufficiently degraded by effectors to de-repress RRS1-R^{SLH1} autoimmunity. RRS1-R^{SLH1} must be expressed at high enough levels to trigger autoimmunity when de-repressed, but must be at low enough levels to be repressed by RRS1-S-X. This strategy of regulation has precedent in natural systems. The most famous is the control of gene expression by the hormone jasmonate. MYC transcription factors mediate jasmonate signalling, but in the absence of jasmonate they are repressed in a complex with jasmonate ZIM-domain (JAZ) transcriptional repressors. In the presence of jasmonate, JAZ proteins form a complex with COI1 (Coronatine Insensitive 1), the receptor for jasmonate. The formation of a JAZ-COI1 complex triggers the ubiquitination and subsequent proteasomal degradation of the JAZ protein, releasing repression of MYC-mediated transcriptional regulation (Chini et al., 2007; Thines et al., 2007; Zhang et al., 2015a).

If this synthetic resistance concept is functional, there are a number of effectors towards which it could confer recognition. These effectors must be nuclear localised and target a host protein for degradation via the proteasome. Aster Yellows phytoplasma strain Witches' Broom (AY-WB; *Candidatus* Phytoplasma asteris) contains at least two effectors, mentioned above, toward which RRS1-S-X could confer effective recognition: SAP11, which targets TCP transcription factors for proteasomal degradation (Sugio et al., 2011, 2014), and SAP54, which targets MADS-box transcription factors (MacLean et al., 2014). Phytoplasmas are phloem-restricted, so engineered

recognition must occur in the phloem. The companion cell-specific SUC2 promoter from Arabidopsis has been used to drive the expression of the salicylic acid receptor NPR1 in transgenic sweet orange to enhance resistance to the phloem-restricted bacterial pathogen *Candidatus* Liberibacter asiaticus (Dutt et al. 2015). The effectors that AY-WB secretes manipulate non-phloem tissue, so it is conceivable that engineered NLRs in the surrounding tissue will perceive these effectors. It remains to be seen if ETI triggered by the effector perception of companion cell-expressed NLRs will be sufficient to restrict the growth of phytoplasmas. Another effector may be a good target. HopZ1a, from *P. syringae,* both promote the proteasomal degradation of JAZ repressors (Jiang et al., 2013). However, in this case the plant hormone jasmonate also degrades JAZ proteins, which may lead to effector-independent activation of the system during jasmonate signalling events.

Hyaloperonospora arabidopsidis secretes a number of effectors that could be selected for: HaRxL44, which targets MED19A (Caillaud et al., 2013), and HaRxL45, which directly interacts with TCP14 but requires further characterisation to establish any virulence function, if any (Weßling et al., 2014).

There are well-characterised cytoplasmic and endoplasmic effectors that fit the criteria for targets of RRS1-S-X. For example, the *P. syringae* effector HopM1 targets the Arabidopsis proteins AtMIN7/BEN1 and AtMIN10/GRF8 in the trans-Golgi network and early endosome (Lozano-Durán et al., 2014; Nomura et al., 2006). AvrPtoB is an E3 ubiquitin ligase secreted by *Pst* DC3000 that targets membrane-spanning and cytosolic kinases, including Fen (Rosebrock et al., 2007), BAK1(Shan et al., 2008), FLS2 (Göhre et al., 2008), CERK1 (Gimenez-Ibanez et al., 2009) and Pto (Kim et al., 2002). In order to target these effectors, I anticipate I will need to set up a similar system with an autoactive R protein that is under dominant negative suppression in *trans*. HR triggered by the recognition of the *Cladosporium fulvum* avirulence gene *Avr9* by the tomato *R* gene *Cf-9* can be suppressed by a truncated form of *Cf-9* (*Cf-9*^{tr}) (Barker et al., 2006). A chimaera gene called *Hcr9-M205*, comprising the C-terminus of *Cf-9* and the N-terminus of

a *Cf-9* paralogue called *Hcr9-9A*, is weakly autoimmune and can be suppressed by (*Cf-9*^{tr}). The Cf-9 protein is localised to the plasma membrane (Piedras et al., 2000). Co-expression of *Cf-9*^{tr} and *Hcr9-M205* fits the conceptual requirements for a detector of a cytoplasmic or plasma-membrane-localised effector that targets host proteins for proteasomal degradation, if the target protein is fused to *Cf-9*^{tr}.

Overexpression of the signalling partner of some *R* gene pairs can trigger effector-independent cell-death that is suppressed by co-expression of the "sensor" partner. For example, overexpression of RPS4 and RGA4 triggers cell-death that is suppressed by RRS1 and RGA5, respectively (Cesari et al., 2014b; Williams et al., 2014). Fusion of proteasomally-degraded effector targets to the sensor partner may be a simpler system for this synthetic resistance approach.

4.3.2.Effector stabilisation of an integrated domain may be exploited for synthetic resistance

An alternative approach to integrating a degron into a dominant negative allele is to integrate a domain that normally determines high protein turnover and that is targeted and stabilised by an effector into an autoimmune protein like RRS1^{SLH1}. An example of this concept is the interaction between the *P. infestans* effector Avr3a and its host target, the E3 ligase CMPG1. In steady state, CMPG1 is degraded by the 26S proteasome (Bos et al., 2010). Avr3a directly interacts with and stabilises CMPG1 (Bos et al., 2010). A chimaera of RRS1^{SLH1} and CMPG1 may be continually degraded by the 26S proteasome. If this is the case, it would not be autoactive if protein abundance is maintained at a low level. Upon delivery of Avr3a, I hypothesise that RRS1^{SLH1}-CMPG1 might be stabilised, and the subsequent autoimmune response would suppress growth of *P. infestans*.

4.3.3.HR may be uncoupled from resistance with RRS1-S^{TuMV} because of the subcellular localisation of RRS1△

RRS1$^{HA\text{-}TuMV}$ confers a strong HR when transiently over-expressed with RPS4 and TuMV NIa-pro. However, Arabidopsis lines carrying RRS1$^{HA\text{-}TuMV}$ did not display resistance to TuMV. This may be due to expression levels of the transgene (I am testing expression levels and generating new constructs

under the control of different promoters), but one of the TuMV-challenged plants demonstrated AvrRps4 recognition. Mutations in R3a that triggered HR in the presence of an expanded repertoire of AVR3a alleles failed to confer resistance to *P. infestans* (Segretin et al., 2014), but when one of these mutations was introduced into I-2, the resultant mutant gained both recognition of $AVR3a^{EM}$ and partial resistance to *P. infestans* (Giannakopoulou et al., 2015). It is not yet understood why mutant I-2, but not mutant R3a, confers resistance to *P. infestans* carrying $AVR3a^{EM}$.

HR was a good indicator for viral resistance when Farnham and Baulcombe (2006) expanded the recognition specificity of Rx, but HR can be uncoupled from pathogen resistance (Bai et al., 2012; Hao et al., 2013; Heidrich et al., 2012; Segretin et al., 2014). Hao et al (2013) rationalised that HR could occur in a transient overexpression system, even if in the native system HR would not occur, because overexpression in a heterologous system could amplify a slow, weak cell death.

The CNL MLA10 triggers HR when overexpressed in *N. benthamiana* (Bai et al., 2012). Restricting MLA10 to the nucleus suppressed cell-death associated with HR, but did not affect resistance to barley powdery mildew when transiently expressed in barley (Bai et al., 2012). Cell death was enhanced when MLA10 was restricted to the cytoplasm by fusion to an NES or the steroid binding domain of the mammalian glucocorticoid receptor (Bai et al., 2012). Therefore, it is possible that nuclear localisation of RRS1 is required for resistance but not for HR.

RPS4-mediated resistance against *Pst* DC3000 carrying AvrRps4 in transgenic Arabidopsis is abrogated by mutation of the RPS4 nuclear localisation signal (NLS), but so too is HR in tobacco (Wirthmueller et al., 2007). Deslandes et al (2002) predicted an NLS in DOM4 of RRS1, which would still be present in RRS1△. However, two NLS prediction software, cNLS Mapper and NucPred (Brameier et al., 2007; Kosugi et al., 2009), predict that there is an NLS just C-terminal to DOM4, with much higher probability than a Domain 4 NLS (Figure 1.1). This WRKY-domain NLS would be removed from RRS1△ and may be the reason $RRS1^{HA\text{-}TuMV}$ triggers HR but not resistance. If this is the case, then $RRS1^{protease\ trap}$ may be a useful tool to understand the

requirement of cytoplasmic and nuclear signalling for resistance and HR. Adding an NLS N-terminal to the protease cleavage site of RRS1$^{protease\ trap}$ may confer resistance to pathogens carrying protease effectors.

4.3.4. A repertoire of modified NLRs and guarded host proteins with different subcellular locations can be used to maximise the number of protease effectors that can be targeted

When we conceived and initiated the RRS1$^{protease\ trap}$ project, there were no rationally designed R genes that could confer novel resistance to completely unrelated pathogens of choice. Giannakopoulou et al (2015) engineered I-2 to confer resistance to *P. infestans* carrying AVR3a, but this enhanced weak recognition of AVR3a that was already intrinsic to I-2. Since beginning this project, Kim et al (2016) have enhanced the RPS5/PBS1 guard/decoy system to recognise additional, unrelated effectors to the original effectors. Their method was simpler than my method; by substituting the protease cleavage site of AvrPphB in PBS1 with that of the AvrRpt2-cleavage site in RIN4 (RIN4 Cleavage Site 2; RCS2), or other protease cleavage sites, they allowed RPS5 to recognise AvrRpt2, instead of AvrPphB, and conferred resistance to *Pst* DC3000 that was almost as effective as that conferred by RPS2, the cognate *R* gene to AvrRpt2. Arabidopsis carrying RPS5 and both PBS1^{WT} and PBS1^{RCS2} recognised both effectors. A modified PBS1 that contains the TuMV NIa-pro cleavage site conferred HR in the presence of TuMV NIa-pro, and conferred a weak resistance to TuMV. The abundance of PBS1^{TuMV} in transgenic Arabidopsis was negatively correlated with TuMV proliferation. However, the weak immune response triggered a slow HR that did not completely inhibit viral spread. This resulted in a trailing necrosis phenotype, and ultimately led to the death of TuMV infected plants at a faster rate than WT plants. This contrasted with the problem I faced, which was that RRS1-S$^{HA\text{-}TuMV}$ triggered HR in transient assays but does not confer resistance to TuMV in Arabidopsis.

Although RPS5 confers an effective immune response to *P. syringae*, perhaps it could be modified to initiate a stronger response to rapidly spreading viruses. In order to remove the trailing necrosis phenotype, a more rapid and effective immune response needs to be initiated. RxM1, the engineered allele of Rx that has expanded recognition specificity to include PopMV, also confers trailing necrosis to PopMV infection (Farnham and Baulcombe,

2006). By mutating the NB or ARC1 domains of RxM1, Harris et al (2013) heightened the sensitivity and rapidity of the RxM1 immune response to PopMV and overcame the trailing necrosis phenotype. Another alternative is to relocate RPS5/PBS1^{TuMV} within the cell. RPS5/PBS1^{TuMV} is at the plasma membrane, while TuMV NIa-pro is mainly nuclear, with a small amount localising to the cytoplasm (Restrepo et al., 1990; Wei et al., 2010). It is likely that TuMV has progressed to a late stage in the cell before NIa-pro accumulates to significant levels in the cytoplasm where it is accessible for RPS5/PBS1^{TuMV} recognition (Kim et al., 2016). Relocating the complex to the nucleus may enhance the rate of recognition and alleviate trailing necrosis, if RPS5 immune-signalling is still possible in the nucleus. RPS5 function requires that the N-terminus of PBS1 is S-acylated, which mediates its localisation to the plasma membrane (Qi et al 2013). If I can optimise RRS1$^{HA\text{-}TuMV}$ to confer resistance, then it may be better suited to TuMV NIa-pro recognition because it localises to the nucleus.

My approach differs from the RPS5/PBS1$^{protease\ trap}$ approach because it modifies an NLR, not a decoy. Currently, it requires both RPS4 and RRS1, and would probably work best on nuclear-localised effector proteases. However, the concept could work with other NLRs. I could introduce a protease cleavage site between the TIR or CC domain and NB-ARC domain of RPS4 or MLA, respectively. The N-terminal domains of both these proteins trigger HR when overexpressed in transient assays (Bai et al., 2012; Swiderski et al., 2009). Mutating the p-loop of these proteins abrogates the autoimmunity conferred by overexpression of the full-length protein (Bai et al., 2012; Zhang et al., 2004). If the C-terminal domains do not suppress the TIR/CC domain autoimmunity, then these protease traps could simplify the system to just one gene, and they could be more useful for cytoplasmic or membrane-associated effectors.

If RRS1-S$^{protease\ trap}$ could be made to function, against what pathogens could it be deployed? Potyviruses are a good target, because NIa-mediated *in cis* processing of the polyprotein is a conserved method of replication within the *Potyviridae* family. Table 4.1 contains other potential effectors, including the bacterial effectors XopD and HopX1, which both localise to the plant

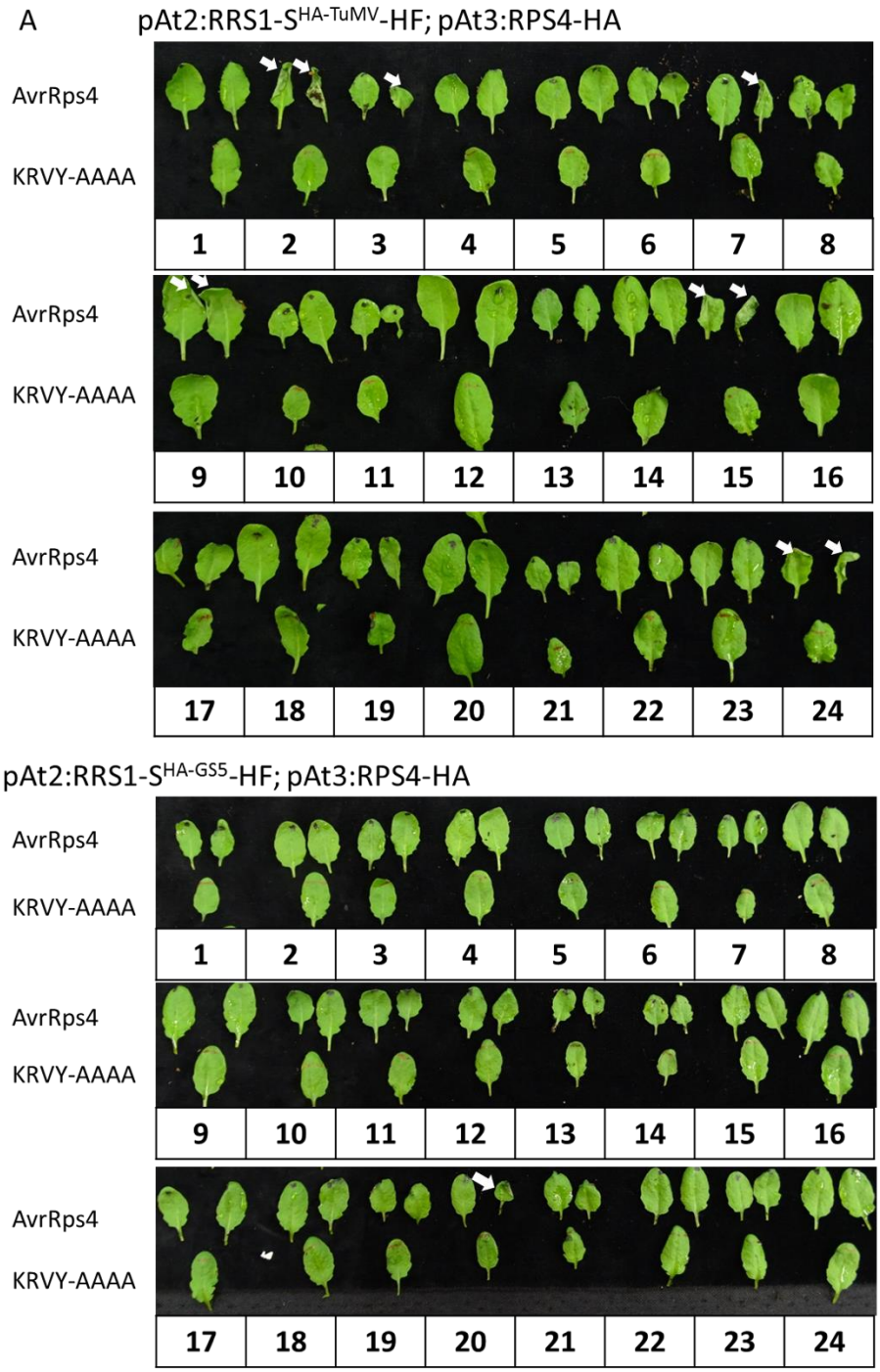
nucleus. However, other bacterial protease effectors have targets that are localised to the plasma membrane. For example, XopJ localises to the plasma membrane and targets the proteasomal subunit RPT6 (Üstün and Börnke, 2015). MLA$^{protease\ trap}$, which would contain the protease recognition site between the CC and NB-ARC, could be designed to target this effector. One fungal effector protease, BEC1019 from *Blumeria graminis* f. sp. *Hordei*, is translocated into the host cell during infection (Whigham et al., 2015), and could be targeted to confer recognition of the fungus. Pathogens secrete apoplastic proteases, the activities of which are recognised by host factors to elicit resistance (Lu et al., 2015; Nissinen et al., 2009). Identification of the host factor that is cleaved to initiate an immune response could allow engineering of recognition of apoplastic effector proteases.

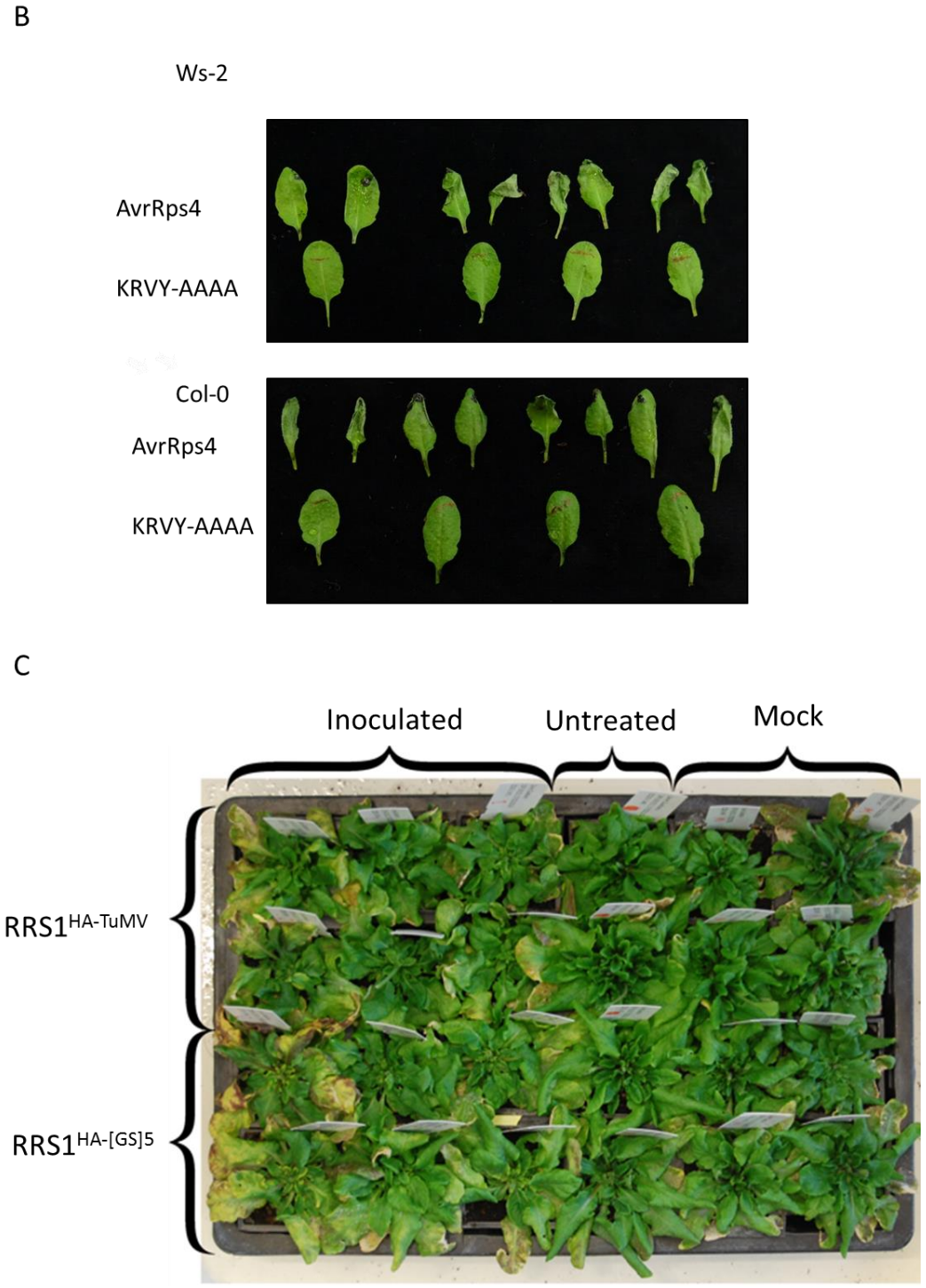
This approach could be adapted to extend recognition of pathogens in mammals. NLRP1 in rat, and its homologue in mice NLRP1B, are cleaved by the protease lethal factor (LF) from anthrax (*Bacillus anthracis*), inducing inflammasome formation and immune signalling (Chavarría-Smith and Vance, 2013; Levinsohn et al., 2012). It is conceivable that this recognition site could be substituted to the recognition site of another protease secreted by a pathogen. For example, *Escherichia coli* secretes the protease effector NleC that cleaves the p65 subunit of host NF-κB (Yen et al., 2010); substitution of the NLRP1 LF-cleavage site with the p65 NleC-cleavage site may confer recognition to *E. coli* in transgenic rats. This hypothesis is supported by Chavarría-Smith et al. (2016), which demonstrated that NLRP1B could be engineered to initiate an immune response in the presence of NIa-pro from Tobacco Etch Virus (TEV) by substituting the LF cleavage site in NLRP1B with an amino acid sequence cleaved by TEV NIa-pro.

There are four cloned *R* genes that confer resistance to TuMV; three of these confer extreme resistance (Moffett and Klessig, 2008). Furthermore, RNAi-mediated resistance against potyviruses has been effectively deployed (e.g. Jan et al., 2000; Patil et al., 2011; Yadav et al., 2011). There is no specific requirement for novel transgenic sources of TuMV resistance. However, demonstrating that novel recognition of TuMV as a proof-of-concept for either RRS1-S$^{protease\ trap}$ or PBS1^{TuMV} is an important first step in designing

NLRs to confer effective resistance to any pathogen of choice. As the field of "effectoromics" continues to catalogue effector repertoires of crop pathogens with increasingly more sophisticated sequencing and genomics techniques, and host targets of effectors are identified, the ability to design NLRs against these effectors will usher in a new era of disease management of crops. Devising both strategies described in this chapter relied on a deep understanding on the inter- and intra-molecular interactions of RRS1/RPS4 and, if functional, will target two different classes of effectors. This highlights the importance of continuing to characterise the mechanisms of NLR function and the mechanisms of the repertoires of effectors utilised by pathogens.

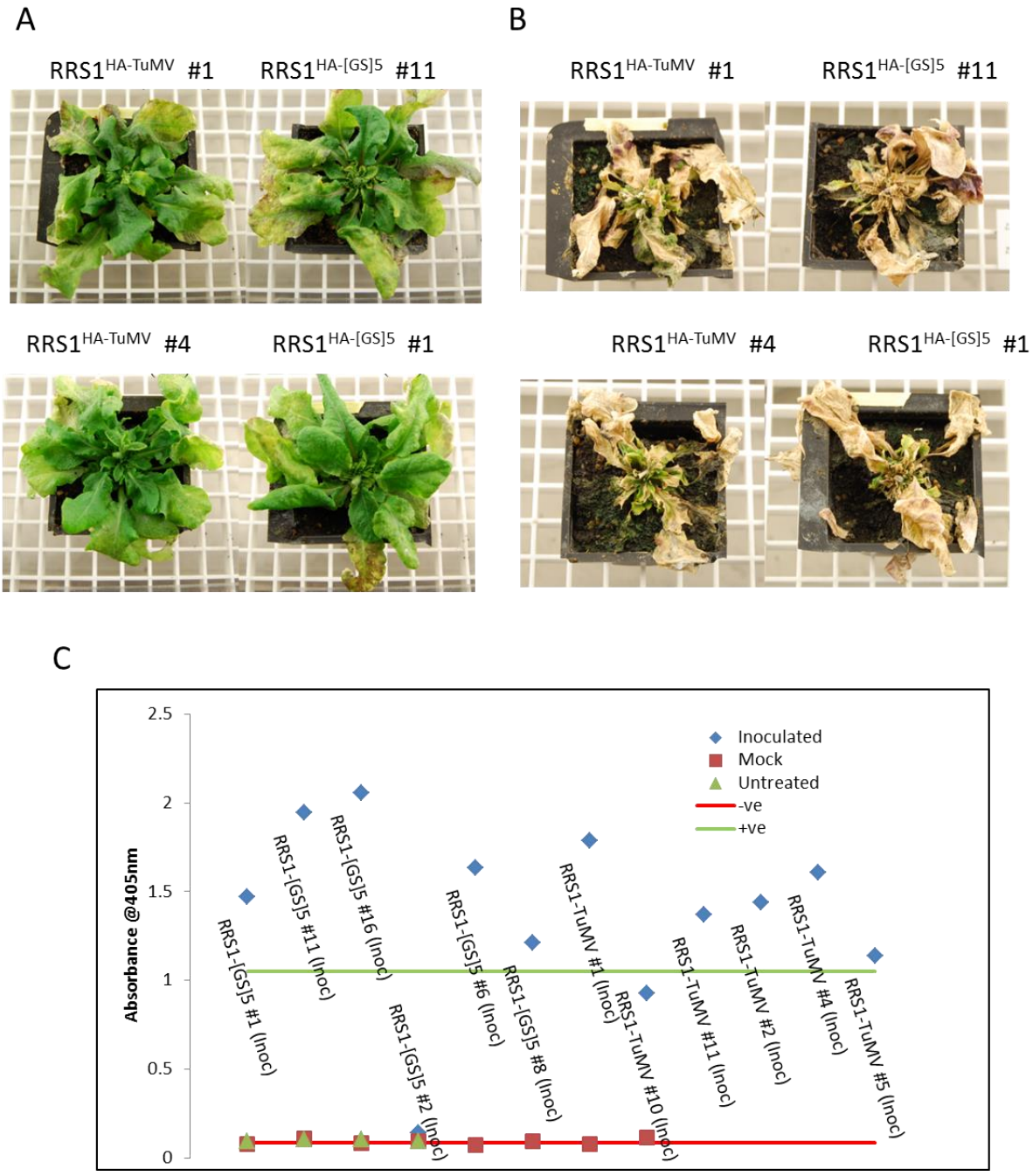
4.4. Appendices





Appendix 4.1. Phenotype of T1 transformants carrying RRS1-S$^{HA\text{-}TuMV}$-HF and RPS4-HA in the *rrs1-1/rps4-21/rps4b-1* (Ws-2) triple mutant background. (A) Complementation of AvrRps4-recognition by T1 transformants carrying RRS1-S$^{HA\text{-}TuMV}$-HF and RPS4-HA in the *rrs1-1*/*rps4-21*/*rps4b-1* (Ws-2) triple mutant background. Twenty-four of each were screened. Leaves were infiltrated with *Pf0-1* strains carrying either AvrRps4 or the unrecognised mutant AvrRps4$^{KRVY\text{-}AAAA}$ at an OD_{600} = 0.2 in infiltration medium (10 mM $MgCl_2$, 10 mM MES, pH 5.6) and assessed for complementation by leaf collapse (white arrows). Two leaves per plant were infiltrated with *Pf0-1*-AvrRps4 and one with *Pf0-1*-

KRVY-AAAA. (B) Col-0 and Ws-2 lines were screened as positive controls. (C) Twelve T1 plants of each transformant were given to Lawrence Bramham and John Walsh for TuMV infection. Layout of infection experiment is shown at 14 dpi (photo taken by Lawrence Bramham).



Appendix 4.2. RRS1$^{HA\text{-}TuMV}$ lines do not confer resistance to TuMV UK isolate 1. (A, B) TuMV was inoculated onto Arabidopsis leaves by macerating systemically infected leaves of infected plants in inoculation buffer (K_2HPO_4, 10g/L; Na_2SO_3,1g/L) and abrading the resulting mixture using sterile muslin cloth against target plants dusted with fine carborundum 300 grit (36µm). Plants were visually inspected at 14 dpi (A) and 35 dpi (B). (C) ELISA showing relative viral load of infected, mock-inoculated (buffer only) and un-inoculated of leaves. Fresh leaf samples of test plants were macerated using electric rollers and diluted 1:1 with 0.05M sodium carbonate buffer (Na_2CO_3, 1.6g/L; $NaHCO_3$,

3g/L) in duplicate wells of a 96-well microtiter plate. Positive and negative control plant samples were tested on each ELISA plate. After incubation with buffer overnight and binding of antigens to plate wells, plates were washed using phosphate buffered saline with tween (PBS-T) (Na_2HPO_4, 2.9g/L; KH_2PO_4, 0.2g/L; NaCl, 8g/L; KCl, 0.2g/L; Tween20, 0.5ml/L; pH 7.3) a minimum of three times, soaking for three minutes between washes. The first antibody used (EMA 67) was produced against TuMV isolate CZE 1, and has been shown to be capable of recognizing all TuMV isolates by targeting a conserved region of TuMV's coat protein (Jenner *et al.*, 1999). Plates were initially probed with an antibody raised in mouse against the TuMV coat protein (EMA 67) at a concentration of 1/500 in bovine serum albumin (BSA)/PBS-T solution (0.2g/100ml) and incubated for 2 hours at room temperature, then washed with PBS-T. Plates were then probed with goat anti-mouse IgG conjugated to alkaline phosphatase (Sigma-Aldrich) (diluted 1/3000 within BSA/PBS-T solution; 0.2g/100ml) and incubated for 2 hours, then washed with PBS-T. Substrate solution (1 alkaline phosphatase substrate tablet per 5ml 10% diethanolamine solution, pH 9.8) was subsequently added to each well and incubated at room temperature until the reaction progressed sufficiently to allow visualisation of TuMV antigens and then absorbance at 405nm was measured. Positive controls and uninfected negative controls were derived from TuMV-infected *Brassica rapa* ssp. *perviridis* cv. Tendergreen (Tendergreen Mustard; TGM) a highly susceptible host of TuMV. There absorbances were averaged and are indicated on the graph as lines. Inoculation, inspection and ELISAs were performed by Lawrence Bramham, University of Warwick.

Chapter 5. Engineering an intracellular PRR based on the murine NLRC4/NAIP inflammasome into plant cells

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5.1. Introduction

NLR-mediated immunity in plants is activated in response to effectors and often culminates in HR; this has been the resistance strategy I have attempted to understand and engineer in the previous chapters. However, in animals, NLRs typically initiate signalling in response to cognate PAMPs or in response to cellular stress, although some NLRs are activated (usually indirectly) in response to intracellular secreted virulence factors, like effectors (Duxbury et al., 2016). Plants recognize apoplastic and intracellular PAMPs via the extracellular domains of cell-surface receptors (Monaghan and Zipfel, 2012; Wei et al., 2013; Zipfel, 2014). Plant and animal NLRs share a similar modular architecture and have overlapping principles of activation. In this chapter, I tested whether the intracellular PAMP recognition mechanism of animal NLRs could be transferred into plants.

In animals, the NLRC4/NAIP inflammasome provides the best understood example of the mechanism by which NLRs confer bacterial PAMP recognition, activation, and specificity. In mice, different PAMPs are bound by different NAIP paralogues; in this way, NAIPs confer specificity for PAMP perception. NAIP1 binds TTSS needle proteins (for example, YscF from *Yersinia pestis*) (Rayamajhi et al., 2013; Yang et al., 2013), NAIP2 binds TTSS rod proteins (for example, PrgJ from *Salmonella enterica*) (Kofoed and Vance, 2011; Zhao et al., 2011), and NAIP5 and NAIP6 bind flagellin (for

example, FlaA from *Legionella pneumophila*) (Kofoed and Vance, 2011; Zhao et al., 2011) (Table 5.1). This interaction activates NAIPs and allows them to activate and recruit NLRC4, which interacts with the activated NAIP at a specific NBD interface, termed the receptor surface (Hu et al., 2015; Zhang et al., 2015b). NLRC4 recruitment to the NAIP/PAMP complex induces a conformational change which alleviates LRR steric obstruction of the "catalytic" surface of NLRC4, which recruits and activates other NLRC4 molecules. This continues until a wheel-like complex composed of the NAIP, PAMP and 9-11 NLRC4 proteins is formed (Figure 5.1). The CARDs of the NLRC4s are clustered at the centre of this wheel, and it is hypothesised that the induced proximity of the CARDs triggers the recruitment of caspases and ASC adaptor-proteins, initiating immune signalling. The elegant self-propagating formation of the inflammasome can be induced *in vitro*, suggesting it could form in plants.

Here, I demonstrate that the NLRC4/NAIP2/PrgJ and NLRC4/NAIP5/FlaA inflammasomes assemble *in planta* but that signalling via the CARD does not occur. I also demonstrate that N-terminal fusion of a plant NLR signalling-domain to NLRC4, specifically RPS4^{TIR}, triggers HR when the appropriate inflammasome components are expressed *in planta*. *Pseudomonas syringae* strains contain variation in flagellin alleles that modulate evasion of recognition without affecting motility, and no known flagellin allele from *Ralstonia solanacearum* is recognised by FLS2 in Arabidopsis (Clarke et al., 2013; Pfund et al., 2004). Installing the NLRC4/NAIP inflammasome in plants could create another layer of PAMP perception that bacterial pathogens have not co-evolved with, and could be a useful tool for studying plant NLR signalling initiation.

Table 5.1. Murine NAIPs and the cognate bacterial PAMPs to which they bind used in this study.

NAIP	PAMP	Notes
NAIP1	YscF	T3SS needle protein from *Yersinia pestis*
NAIP2	PrgJ	T3SS rod protein from *Salmonella enterica* serovar Typhimurium
NAIP5	FlaA	Flagellin monomer from *Legionella pneumophila*

5.2. Results

5.2.1. Inflammasome-like interactions between NLRC4, NAIPs and PAMPs are reconstituted in the appropriate specificities in planta

In mice, the NLRC4 inflammasome forms from a NAIP2-rod protein or NAIP5-flagellin heterodimer, which acts as a nucleus for sequential additions of NLRC4 molecules, until 9-11 NLRC4s are bound to make the full wheel-like structure (Figure 5.1). To test if the NLRC4-inflammasome forms in plants, I co-expressed murine NLRC4, murine NAIP2 and PrgJ from *Salmonella enterica*, or murine NAIP5 and FlaA from *Legionella pneumophila*, in *Nicotiana benthamiana*. After extracting total protein, I immunoprecipitated NLRC4 and the NAIPs by their respective epitope tags and probed with antibodies to determine if inflammasome components co-precipitated in a ligand-dependent manner. The complex associations were reconstituted *in planta*; NLRC4 precipitated with NAIP2 only in the presence of PrgJ, which was also in the complex, and vice versa (Figure 5.2a). However, PrgJ was only faintly visible when detected by Myc immunoblot after IP of NAIP2. NLRC4 precipitated with NAIP5 only in the presence of FlaA, and vice versa; the ligand co-IPed with NLRC4 and NAIP5 only when both were present (Figure 5.2a). NLRC4 did not precipitate with NAIP2 or NAIP5 in the presence of an inappropriate ligand, FlaA or PrgJ, respectively (Figure 5.2a).

Recently published structures of the NLRC4/NAIP2 inflammasomes utilised an N-terminally truncated NLRC4 that lacked a CARD (NLRC4△CARD) (Hu et al., 2015; Zhang et al., 2015b). I performed co-IP *in planta* with NLRC4△CARD and the other inflammasome components and observed similar immunoprecipitation specificities as full length NLRC4 (Figure 5.2b). Specifically, NLRC4△CARD co-IPed with both NAIP2 and PrgJ, and with NAIP5 and FlaA, in those combinations. Again, PrgJ was difficult to detect, particularly when probed in the NAIP IP. NLRC4△CARD did not co-IP with a NAIP or ligand when co-expressed with the inappropriate combinations of NAIP2 and FlaA, or NAIP5 and PrgJ (Fig 5.2b).

It has been reported that phosphorylation of NLRC4 at Ser533 is required to induce conformational changes required for inflammasome activity and immunity (Matusiak et al., 2015; Qu et al., 2012), but it is not clear if this modification is required for the assembly of NLRC4/NAIP5 into an inflammasome. I co-expressed NAIP2 and NAIP5 with PrgJ and FlaA, and with either WT NLRC4 or with a phosphomimetic mutant of NLRC4, NLRC4-S533D, or the phospho-dead mutant, NLRC4-S533A. All versions of NLRC4 could form the appropriate inflammasome associations in *N. benthamiana* (Figure 5.2a). That is, the mimetic forms of NLRC4 co-IPed with both NAIP2 and PrgJ, and with NAIP5 and FlaA, in those combinations (Fig 5.2a). To simplify further experiments, I continued using the WT NLRC4 only.

5.2.2. Inducible oligomerisation of NLR signalling domains fused to NLRC4 is sufficient to trigger cell death in planta

Because the NLRC4/NAIP/PAMP inflammasome can form in plants, I next wanted to engineer it to test if it can initiate an immune response to bacterial PAMPs. In order to do this, I fused the TIR-domain of RPS4 to the N-terminus of NLRC4 ($RPS4^{TIR}$-NLRC4). The TIR-domains of several TNLs, including RPS4, trigger HR when overexpressed *in planta* as a separate domain. Induced dimerization of TIR domains can enhance HR. For example, fusing the TIR domain of RPP1 to dimer-forming GFP triggered a strong HR, but $RPP1^{TIR}$ fused to monomeric GFP did not trigger HR (Krasileva et al., 2010).

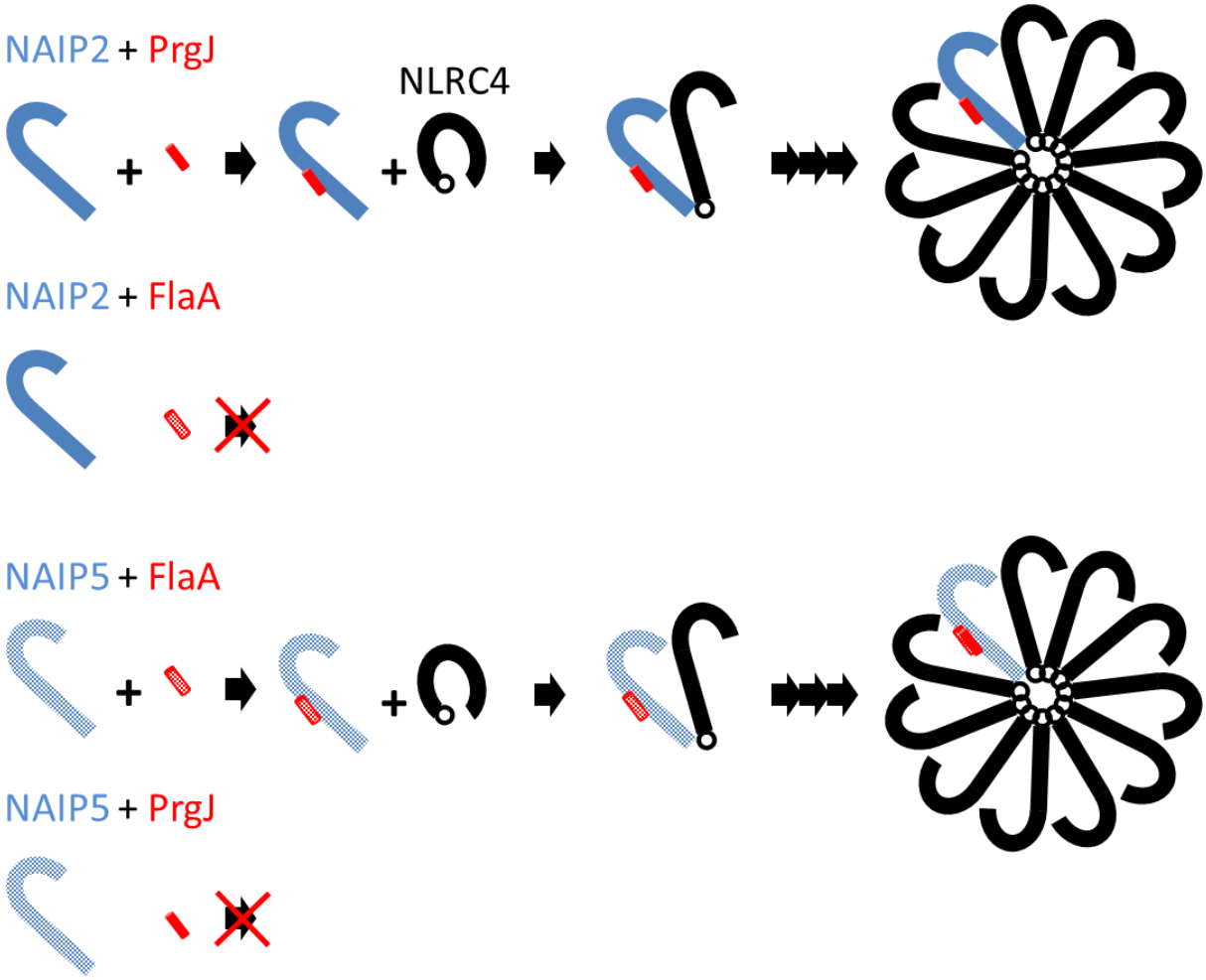


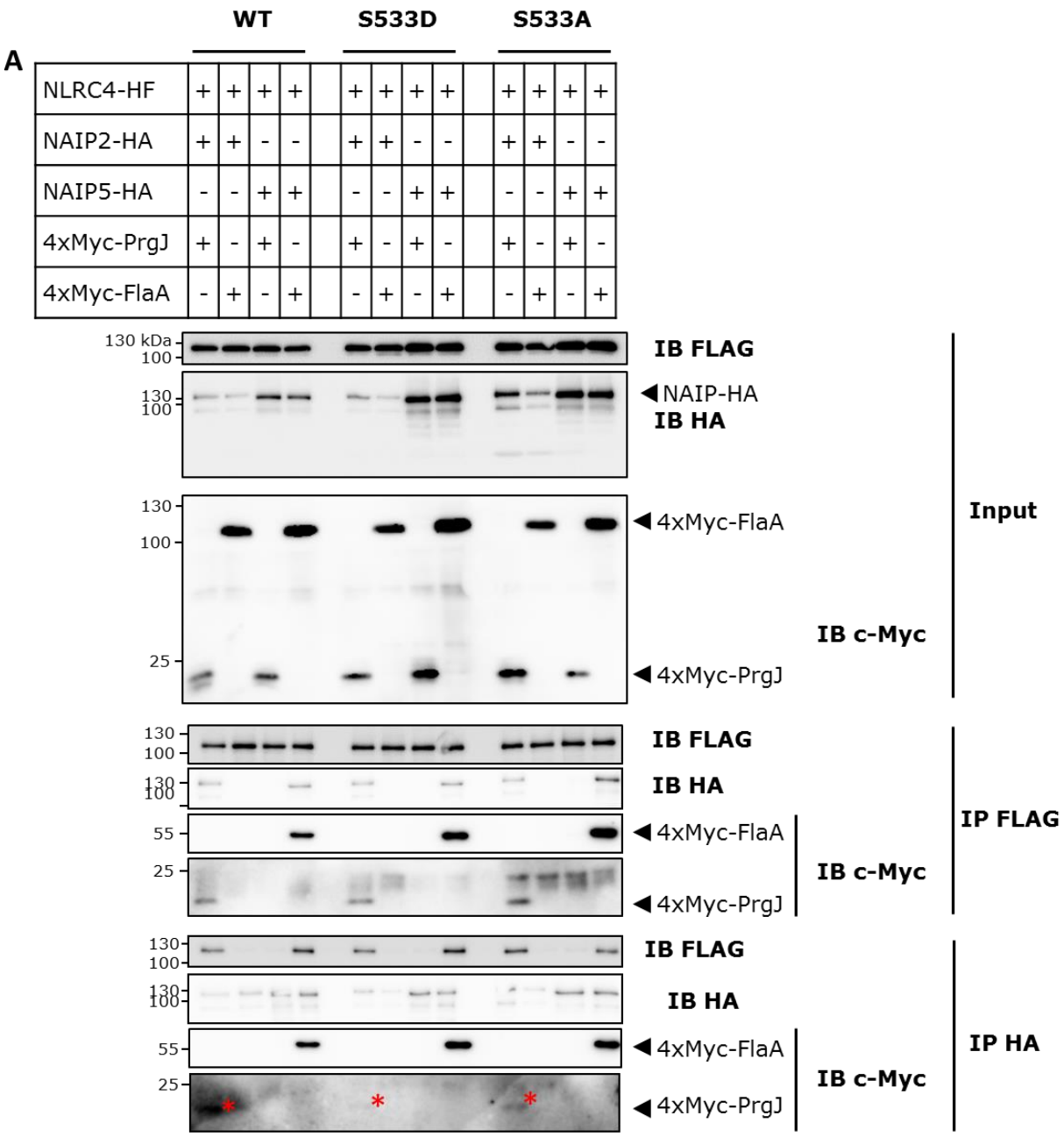
Figure 5.1. Pectoral diagram of NLRC4/NAIP inflammasome activation. Inflammasomes are metazoan cytosolic pattern recognition receptor complexes that regulate caspase-1 activity and IL-1β production. These complexes are formed of NLR superfamily proteins which sense pathogen-derived molecules and initiate an immune signal by forming a platform for the recruitment of signalling adaptors. The NAIP NLRs directly bind elicitors (e.g. PrgJ, solid red line; FlaA, chequered red line) in order to initiate formation of an inflammasome. The best structural data is for the PrgJ/NAIP2/NLRC4 inflammasome. The NAIP molecule (blue stick) binds PrgJ at its NB, then forms a heterodimer with an inactivated NLRC4 molecule, and in doing so induces conformational changes that "activates" NLRC4 by exposing the "catalytic surface" of NLRC4. This surface recruits and activates another NLRC4, which in turn recruits another NLRC4, and this continues until 9-10 NLRC4 molecules and one NAIP2 molecule have formed a wheel-like structure. Similarly, there appears to be 10-11 NLRC4 molecules to each NAIP5-flagellin in the flagellin/NAIP5/NLRC4 inflammasome. The CARDs (circle on end of NLRC4) of the NLRC4/NAIP inflammasome then recruit and activate caspase-1. Activation of caspase-1 is popularly considered to be activated by induced proximity upon recruitment to the centre of the inflammasome. The NLRC4/NAIP inflammasome only form in the appropriate combination of NAIP and PAMP.

I anticipated that RPS4^{TIR}-NLRC4 would not trigger HR when overexpressed because of the repression of self-propagating oligomerisation conferred by the LRR domain. Upon NAIP-mediated PAMP recognition, LRR repression would be relieved and I would expect an oligomerisation-dependent HR. Indeed, this is what I observed (Figure 5.3a). RPS4^{TIR}-NLRC4 triggered HR in the presence of the inflammasome-forming combination of NAIPs and PAMPs – that is, when coexpressed with NAIP1, NAIP2 or NAIP5, and YscF, PrgJ or FlaA, respectively (Figure 5.3a). RPS4^{TIR}-NLRC4 did not trigger HR when co-expressed with NAIP1 and PrgJ or FlaA, or with NAIP2 and YscF or FlaA, or with NAIP1 and PrgJ or FlaA (Figure 5.3a). I confirmed by co-IP that associations between all components occurred in the appropriate combinations: RPS4^{TIR}-NLRC4 with either NAIP1 and YscF, NAIP2 and PrgJ, or NAIP5 and FlaA, only (Figure 5.3b). This further demonstrated that the specificity of perception observed in mice could be reconstituted in plants.

Sung Un Huh in the Jones lab performed Blue Native PAGE with RPS4^{TIR}-NLRC4, NAIP1, NAIP2 or NAIP5, and YscF, PrgJ or FlaA. Blue Native PAGE separates proteins in their native conformation, without denaturation, based on the migration rate of native protein complexes through a polyacrylamide gel. He observed a ligand-dependent higher-order complex when RPS4^{TIR}-NLRC4 was co-expressed with a NAIP and its appropriate ligand, which is characteristic of inflammasome formation (Figure 5.4). In addition, expression of the RPS4^{TIR}-NLRC4 construct that lacks the oligomerisation-inhibitory LRR domain (RPS4^{TIR}-NLRC4$\triangle$LRR), which forms a higher order complex in the absence of NAIPs or PAMPs, triggered an HR (Figure 5.5a). There is no requirement for a CARD for RPS4^{TIR}-NLRC4-mediated HR (Figure 5.5b).

Several CC domains from CNLs trigger HR when overexpressed *in planta*. The CC from MLA7 (MLA^{CC}), which consists of the first 160 amino acids of MLA7, has only one amino acid difference when compared to the first 160 amino acids of MLA10, which is sufficient to trigger constitutive HR when expressed in *N. benthamiana* (Bai et al., 2012). I fused MLA^{CC} (cloned from a plasmid generously provided by Inmaculada Hernandez-Pinzon and Matthew Moscou) to the N- terminus of NLRC4 and overexpressed it with the other

components of the NLRC inflammasome. In this case, yellowing of the leaf was consistently observed, but not HR (Figure 5.5b). However, MLA^{CC}-NLR4ΔLRR triggered HR (Figure 5.5a). This observation was puzzling because the predicted signalling domain, the MLA^{CC} domain, was identical in both constructs. This result may be explained by some suppressive interaction between the MLA^{CC} and LRR domains. If this is the case, then the PAMP-induced attenuation of LRR-mediated suppression of NACHT activation and recruitment of NLRC4 NACHTs is not sufficient to allow HR as a result of MLA^{CC} induced proximity.


A
WT
S533D
S533A
NLRC4-HF
NAIP2-HA
NAIP5-HA
4xMyc-PrgJ
4xMyc-FlaA
130 kDa
100
IB FLAG
130
100
NAIP-HA
IB HA
Input
4xMyc-FlaA
IB c-Myc
25
4xMyc-PrgJ
55
IP FLAG
IP HA
*

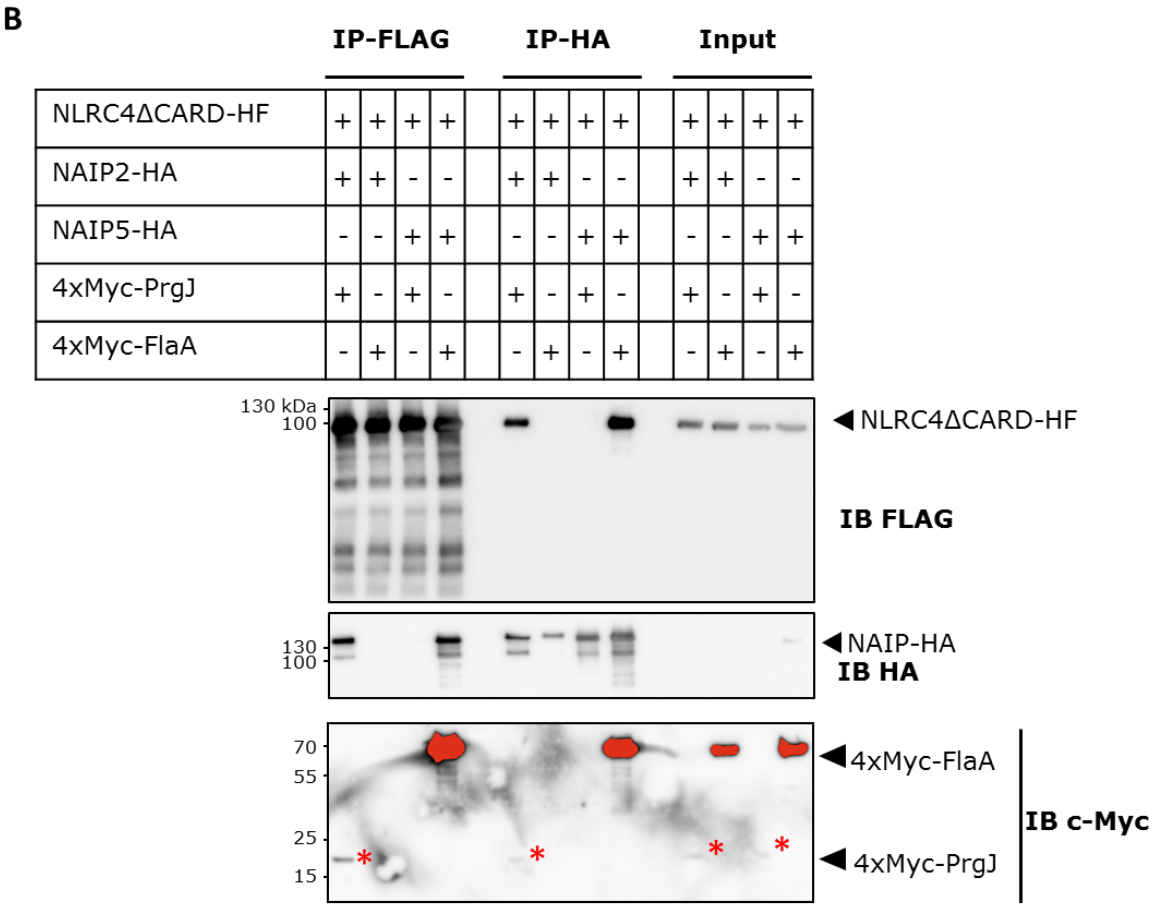


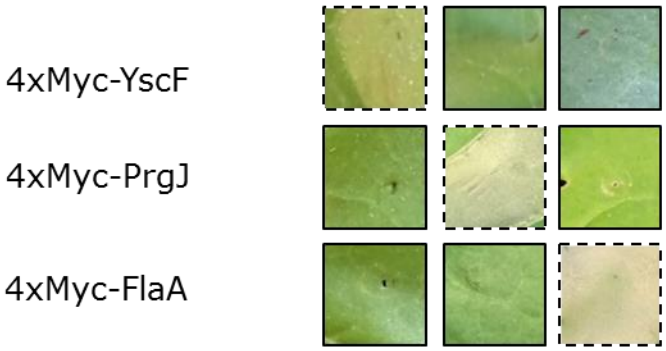
Figure 5.2. NLRC4, NAIP2/5 and FlaA/PrgJ assemble in plants in inflammasome-appropriate combinations. A) Full-length epitope-tagged components were co-agroinfiltrated in *Nicotiana benthamiana.* At 3 dpi, protein was extracted and tagged protein was immunoprecipitated on anti-FLAG or anti-HA antibodies bound to agarose beads. Beads were boiled in Laemmli buffer and equal volumes were loaded on SDS-PAGE (8% polyacrylamide) gels and electrophoresed. After separation, proteins were transferred to a nitrocellulose membrane. Membranes were blocked with 5% skim milk powder and probed with either epitope-specific primary antibodies followed by animal-specific, HRP-conjugated antibody, or an HRP-conjugated primary antibody. After washing, the membrane was provided with chemiluminescent substrate and luminescence was imaged with a luminomineter (ImageQuant LAS 4000). Asterisks indicate bands corresponding to predicted 4xMyc-PrgJ. A complete Myc immunoblot is shown for the input because both 4xMyc-FlaA and 4xPrgJ could be seen with the same exposure. However, two separate exposures were required for the Myc IB of both the HA and FLAG IPs because a stronger exposure was required to observe 4xMyc-PrgJ in both cases. All the bands revealed by immunoblot are shown. Additional bands not indicated by a triangle are most likely degradation products, because they are smaller than the predicted size. B) NLRC4 lacking a CARD also assembles correctly in plants. These experiments were repeated three times with similar results.

5.2.3.NAIP5, but not NAIP2, binds a PAMP from a plant pathogenic bacterium

Demonstrating that an inflammasome-like complex can form *in planta* and trigger HR when an NLR signalling domain is fused to its N- terminus are the first steps in installing a PRR in the plant cytoplasm. In order for the system to be useful, it must confer recognition to PAMPs from plant pathogenic bacteria, for example, *Pseudomonas syringae*, *Ralstonia solanacearum* and *Xanthomonas euvesicatoria*. Our collaborators, Jeannette Tenthorey and Russell Vance at the University of California, Berkeley, ordered the synthesis of plasmids encoding flagellin and TTSS rod proteins from these bacteria. FliC, the FlaA homologue in the plant pathogenic bacterial genomes examined, or HrpB(2), the PrgJ homologue, were co-transfected into HEK293T cells with NLRC4, Caspase-1, pro-IL-1β and either NAIP2 or NAIP5. The generation of mature IL-1β (p17) by activated Caspase-1-mediated conversion of pro-IL-1β was a readout for inflammasome formation. All PAMPs induced p17 production, but *P. syringae* HrpB (*Ps*HrpB) and *Ps*FliC triggered the strongest production in response to a plant pathogenic bacterial PAMP, although not as strong as PrgJ and FlaA (Figure 5.6a).

A

RPS4^{TIR}-NLRC4-HF	+	+	+
NAIP1-HA	+	-	-
NAIP2-HA	-	+	-
NAIP5-HA	-	-	+



B

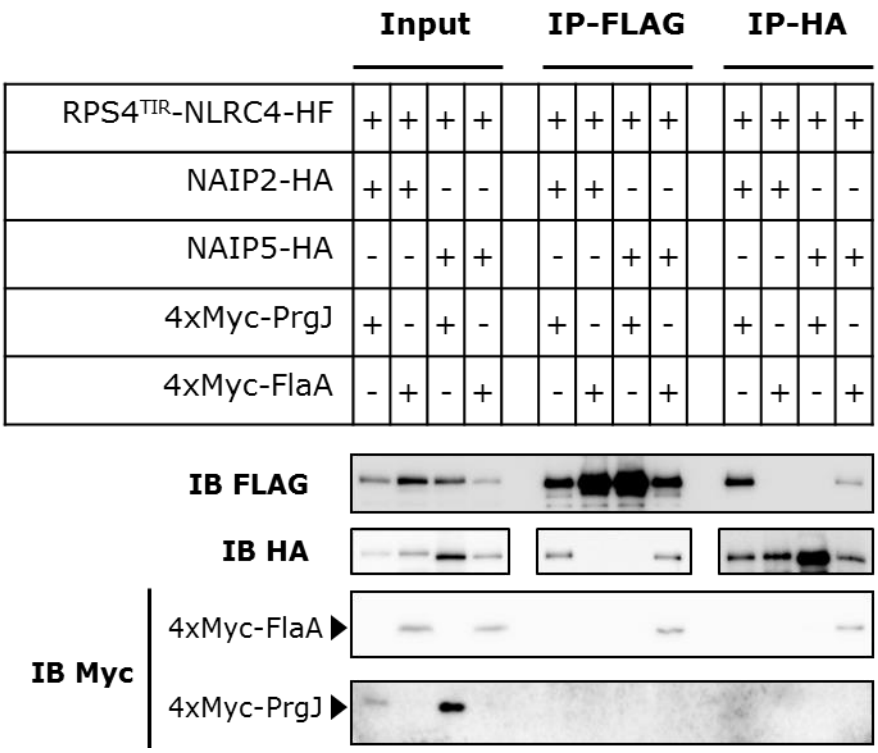


Figure 5.3. RPS4^{TIR}-NLRC4-HF assembles into an inflammasome-like complex that triggers a cell-death response when the appropriate NAIP and PAMP are present. A) *Nicotiana tabacum* was agroinfiltrated with a mix of strains carrying RPS4^{TIR}-NLRC4-HF, a NAIP (combinations indicated in the table above the leaf panels) and a PAMP (rows correspond to a PAMP with an NLRC4/NAIP combination). Each strain was infiltrated at an OD600 of 0.5. Leaves were assessed for cell-death at 3 dpi. Panels indicate representative leaves from at least three replicate infiltrations. A solid outline indicates green tissue and a dashed outline indicates HR. HF, 6xHis.3xFLAG; HA, 6xHA. B) The RPS4^{TIR}-NLRC4, NAIP2/5 and FlaA/PrgJ combinations were agroinfiltrated into *N. benthamiana* and co-immunoprecipitated under the same conditions as Figure 5.2. 4xMyc-PrgJ was consistently difficult to detect by immunoblot after IP and was not observed in these co-IPs. This experiment was repeated three times.

RPS4^{TIR}-NLRC4-HF	+	+	+	+
NAIP-HA	+	+	+	+
4xMyc-FlaA	-	+	-	-
4xMyc-PrgJ	-	-	+	-
4xMyc-YscF	-	-	-	+

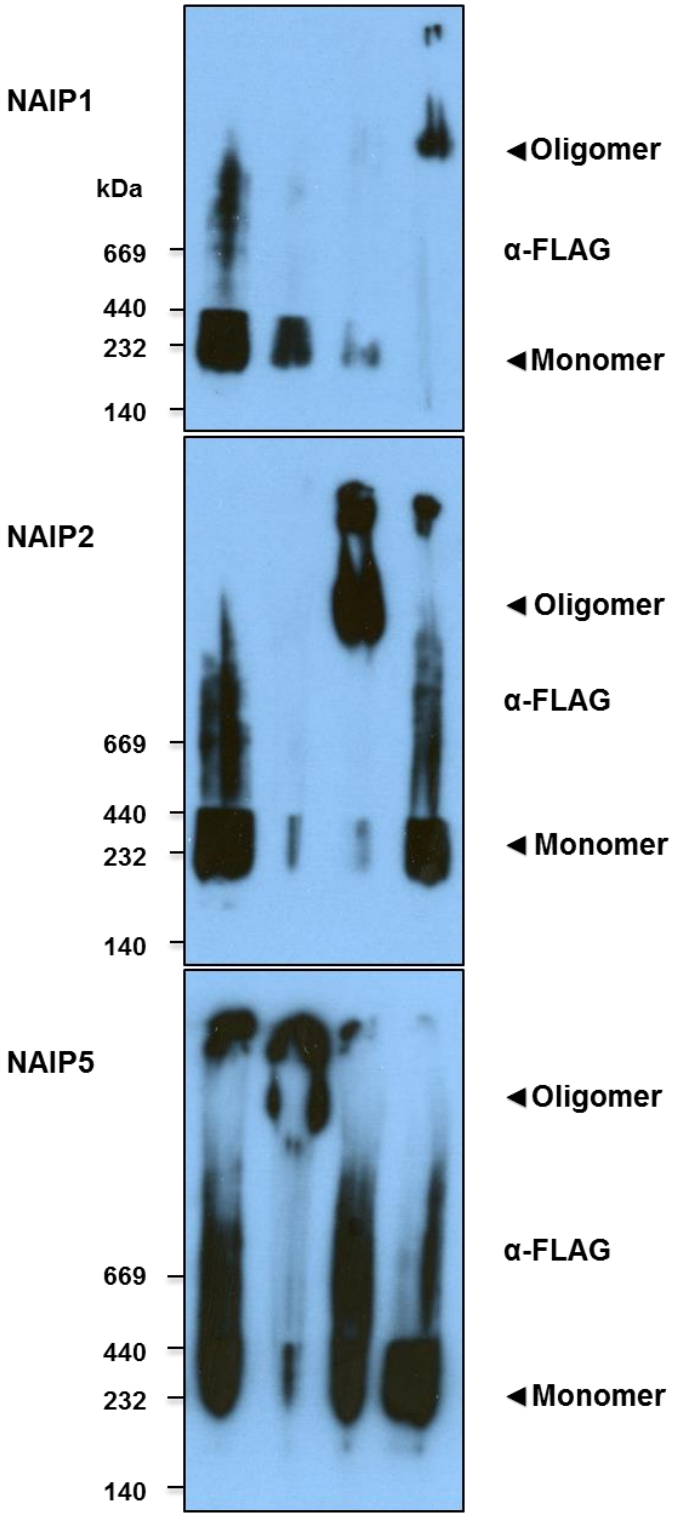
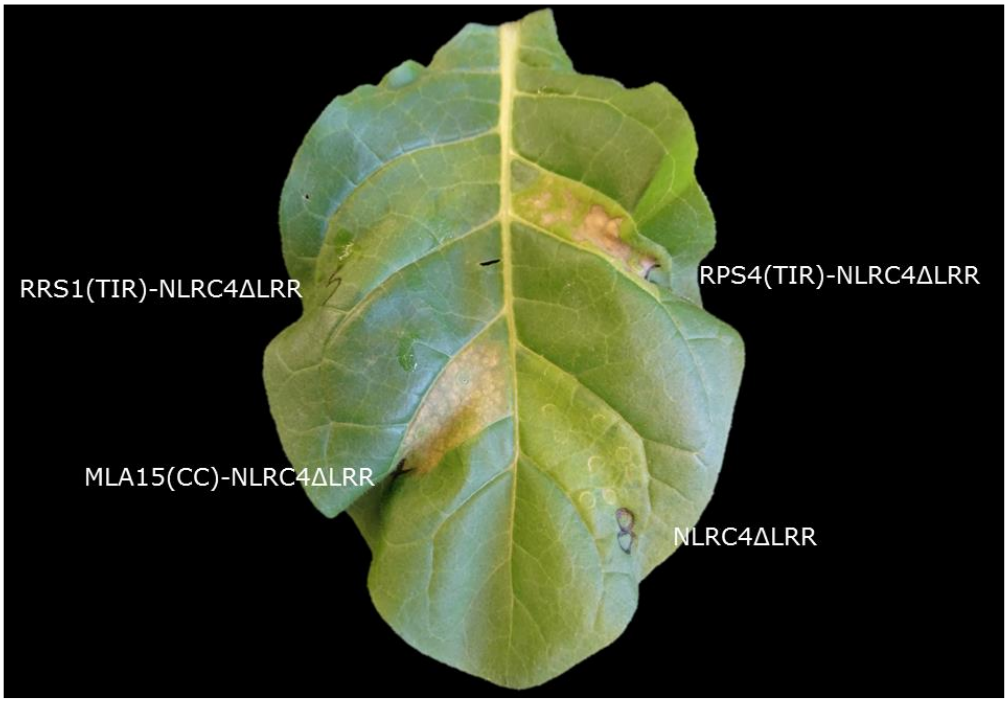


Figure 5.4. RPS4^{TIR}-NLRC4-HF assembles into a higher-order complex when the inflammasome-appropriate NAIP and PAMP are present. Sung Un Huh performed this experiment. Blue Native PAGE showing assembly of higher-order complexes (indicated by arrows) of co-expressed RPS4^{TIR}-NLRC4-HF, NAIP and PAMP. Oligomers were observed in the following appropriate combinations with RPS4^{TIR}-NLRC4-HF: NAIP1-6×HA and 4×Myc-YscF (top panel); NAIP2-6×HA and 4×Myc-PrgJ (middle panel); NAIP5-6×HA and 4×Myc-FlaA (bottom panel). Predicted oligomers (corresponding to inflammasome-like complexes) and RPS4^{TIR}-NLRC4-HF monomers are indicated. The smear in between the predicted oligomer and monomer bands may indicate an overexpression artefact of a series of partially formed NLRC4-HF oligomers. Protein was produced and purified with anti-FLAG beads in a similar method as 5.3b, but were eluted from the beads by competitive binding with 3xFLAG peptide. Eluted protein was mixed with NativePAGE sample buffer (4×)(Life Technologies) loaded on a 3-12% NativePAGE Bis-Tris gel and electrophoresed, then transferred to nitrocellulose membrane and probed with an HRP-conjugated anti-FLAG antibody. Appendix 5.1 shows expression of proteins. This experiment was repeated three times.

A



B

RPS4^{TIR}-NLRC4ΔCARD-HF	+	+	-	-
MLA^{CC}-NLRC4ΔCARD-HF	-	-	+	+
NAIP2-HA	+	-	+	-
NAIP5-HA	-	+	-	+

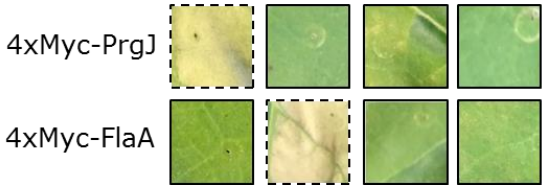


Figure 5.5. Oligomerisation of RPS4^{TIR} during assembly of the RPS4^{TIR}-NLRC4ΔCARD-HF/NAIP/PAMP inflammasome-like complex or constitutive assembly of RPS4^{TIR}-NLRC4ΔLRR oligomers triggers HR. A) The RPS4^{TIR} and MLA^{CC} N-terminal fusions of the auto-assembling NLRC4ΔLRR truncated protein trigger HR. *Nicotiana tabacum* was agroinfiltrated with strains carrying either RPS4^{TIR}-, RRS1^{TIR}-, MLA^{CC} -NLRC4ΔLRR or NLRC4ΔLRR at an OD_{600} of 0.5. Leaves were assessed for cell-death at 3 dpi. The leaf shown is a representative of three infiltrated leaves. B) RPS4^{TIR}- or MLA^{CC}-NLRC4ΔCARD-HF was co-agroinfiltrated with a NAIP (combinations indicated in the table above the leaf panels) and a PAMP (rows correspond to a PAMP with an NLRC4/NAIP combination).

Each strain was infiltrated at an OD_{600}=0.5. Leaves were assessed for cell-death at 3 dpi. Panels indicate representative leaves from at least three replicate infiltrations. A solid outline indicates green tissue and a dashed outline indicates HR. HF, 6xHis.3xFLAG; HA, 6xHA.

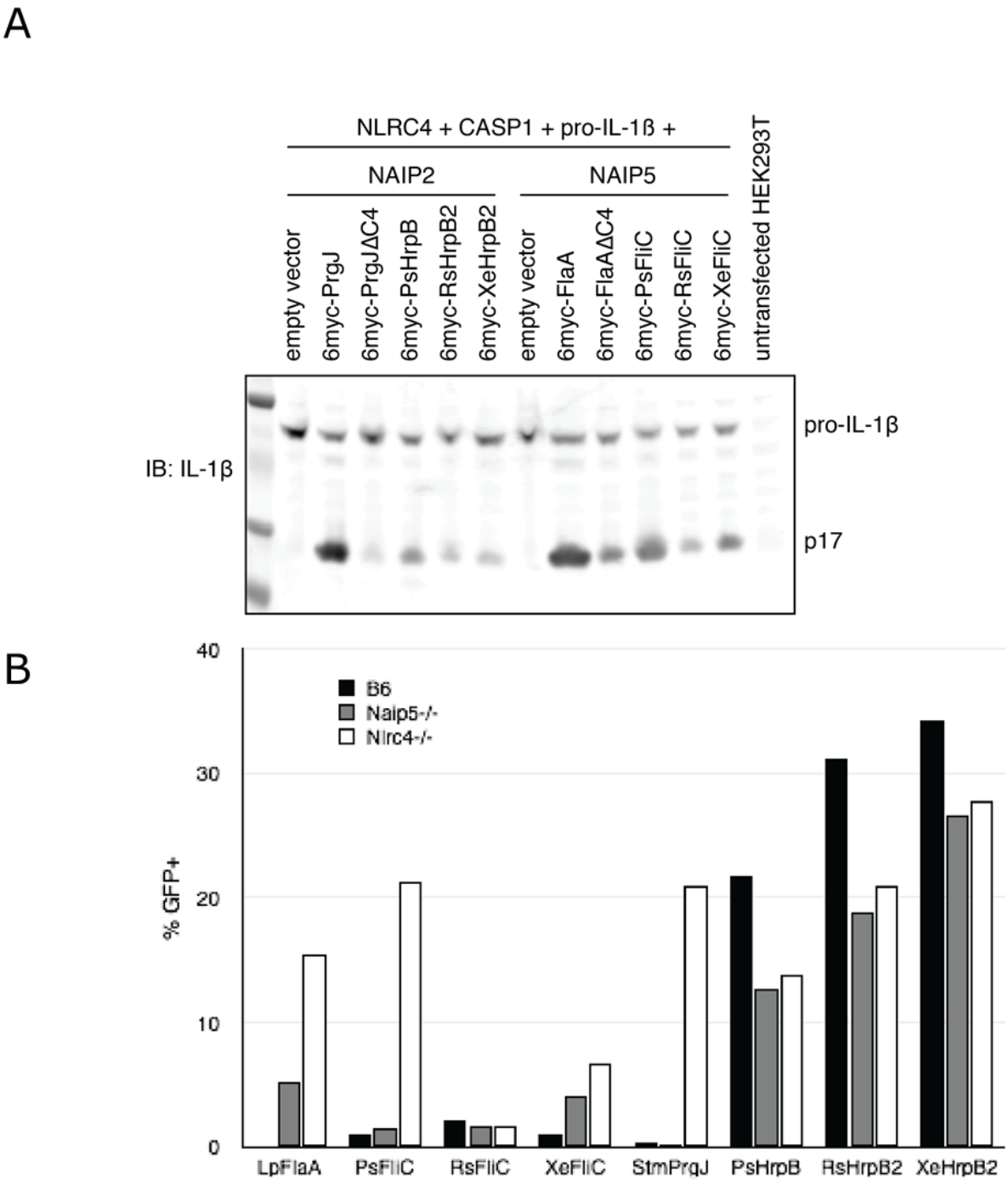


Figure 5.6. Flagellin, but probably not TTSS rod protein, from plant pathogenic bacteria is recognised by the murine NLRC4/NAIP inflammasome. These experiments were performed by Jeannette Tenthorey at the University of California, Berkeley. A) FliC, the

FlaA homologue in the plant pathogenic bacterial genomes examined, or HrpB(2), the PrgJ homologue, were co-transfected into HEK293T cells with NLRC4, CASP1, pro-IL-1β and either NAIP2 or NAIP5. Conversion of pro-IL-1β into mature IL-1β (p17) by activated CASP1 was a readout for inflammasome formation. B) A GFP-eclipse assay, whereby murine blastocyst B6 cells, or isogenic *Naip5*$^{-/-}$ or *Nlrc4*$^{-/-}$ mutants, were retrovirally transduced with PAMP-GFP fusions from plant pathogenic bacteria. The percentage of cells expressing GFP is a measure of recognition efficiency; the lower the percentage, the lower the survival rate of transformed cells and hence the higher the recognition efficiency of the NLRC4/NAIP complex for the PAMPs. The percentage of GFP-transduced cells in the negative control for *Rs*FliC-GFP transduction was very low, but this was probably because less DNA was transfected due to lower availability of the DNA of this construct. GFP-expressing cells were counted by flow cytometry 3-4 days after transfection. *Ps*, *Pseudomonas syringae*; *Rs*, *Ralstonia solanacearum*; *Xe*, *Xanthomonas euvesicatoria*.

J. Tenthorey also performed a GFP-eclipse assay, whereby she transfected murine blastocyst B6 cells, or *Naip5*$^{-/-}$ or *Nlrc4*$^{-/-}$mutants, with PAMP-GFP fusions from plant pathogenic bacteria. The percentage of cells expressing GFP is a measure of recognition efficiency: the higher the recognition efficiency of the NLRC4/NAIP complex for the PAMPs, the lower the survival rate of transformed cells and the lower the percentage of GFP-expressing cells. *Ps*FliC and *X. euvesicatoria* (*Xs*)FliC decreased the percentage of cells producing GFP, indicating that they were recognised by NAIP5/NLRC4 (Figure 5.6b). However, none of the plant pathogenic rod proteins were recognised (Figure 5.6b).

J. Tenthorey performed a co-IP by immunoprecipitating 6xMyc-tagged ligand and immunoblotting to determine if NAIPs or NLRC4 co-precipitated. NAIP5 co-IPed with *Ps*FliC, but it was not detected in any interaction with the other plant pathogenic PAMPs (Figure 5.7a). NLRC4 co-IPed with *Ps*FliC, but also with *Ps*HrpB and *Rs*HrpB2, although no interaction with NAIP2 was detected (Figure 5.7a).

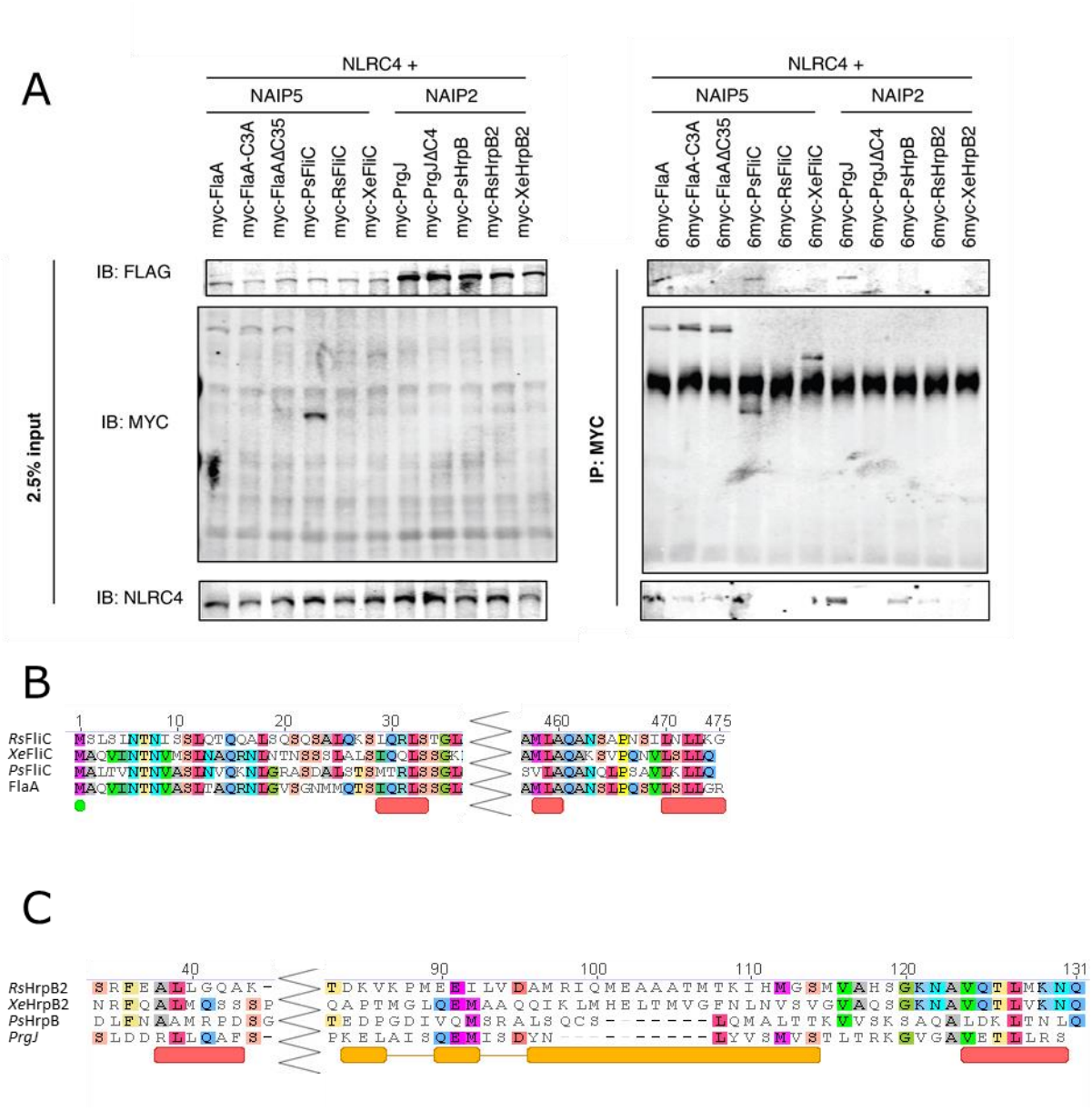


Figure 5.7. Recognition sequences from flagellin and TTSS rod proteins of plant pathogenic bacteria may be conserved enough for NLRC4/NAIP inflammasome recognition. A) *Ps*FliC and *Ps*HrpB can initiate the formation of a hetero-oligomeric complex with NLRC4/NAIP. HEK293T cells were co-transfected with wild-type NLRC4 and NAIP2 or NAIP5 in the indicated combinations with 6x-Myc-FlaA, 6x-Myc-PrgJ or unrecognised mutants of these PAMPs or with homologues from plant pathogenic bacteria, followed by anti-Myc immunoprecipitation, analysis by SDS-PAGE and western blotting. *Ps*, *Pseudomonas syringae*; *Rs*, *Ralstonia solanacearum*; *Xe*, *Xanthomonas euvesicatoria*; C35, C-terminal 35 amino acids of FlaA; FlaA-C3A, acids 470-472 of FlaA are substituted with Ala, as in LeuSerLeu-AlaAlaAla; C4, C-terminal four amino acids of PrgJ. ΔC constructs lack the indicated number of amino acids from the C terminus. FlaA-C3A, FlaA-ΔC35 and PrgJ-ΔC4 are unrecognised by NAIP5 and NAIP2 (Lightfield et al

2008; J. Tenthorey and R. Vance, unpublished data). B,C) Alignment of the amino acid sequences of FlaA (B) and PrgJ (C) with homologues from plant pathogenic bacteria. Regions that contribute to NLRC4/NAIP recognition are indicated by red labels. The yellow label indicates a region that contributes weakly to recognition of PrgJ. Jagged lines indicate non-conserved amino acids that were removed to condense the image.

The different assays used to assess PAMP recognition have their strengths and weaknesses and it is useful to use each test to get an overall picture of recognition. In summary, J. Tenthorey's experiments that examined the recognition by the NAIP/NLRC4-inflammasome of PAMPs from plant pathogenic bacteria observed strong recognition of *Ps*FliC by NAIP5/NLRC4, weaker recognition of *Xe*FliC, and weak, if any, recognition of rod proteins by NAIP2/NLRC4. Ultimately, the proof will be in the pudding: i.e. Do NLRC4 and a NAIP confer recognition to plant pathogenic bacteria when expressed in a plant?

I sought to verify the observations from mammalian cells *in planta*. I co-expressed RPS4^{TIR}-NLRC4 with NAIP2 or NAIP5 and either *Ps*HrpB, *Ralstonia solanacearum* (*Rs*)HrpB or *Ps*FliC. *Ps*FliC was triggered cell death when co-expressed with RPS4^{TIR}-NLRC4 and NLRC4 (Figure 5.8). However, neither rod protein was triggered any cell death when co-expressed with RPS4^{TIR}-NLRC4 and NAIP2 (Figure 5.8). The C-terminus of FlaA contains a region that is required for detection by NAIP5/NLRC4, and if amino acids 29-33 are mutated to alanines there is a week loss of detection (Lightfield et al., 2008; J. Tenthorey unpublished data). These regions are well-conserved between FlaA and FliC from plant pathogenic bacteria (Figure 5.7b). PrgJ has an N- and a C-terminal region that is recognised by NAIP2 (J. Tenthorey personal communication; Figure 5.7b); the C-terminal site is conserved between PrgJ and both *Rs*HrpB2 and *Xe*HrpB2, while *Ps*HrpB has a PrgJ-like N-terminus (Figure 5.7c). The differences at these sites probably explain the lack of recognition of the rod proteins from plant pathogenic bacteria by NAIP2. It is important to acknowledge that HR is just one readout of immunity. The tests in mammalian cells indicate that different PAMPs produce different magnitudes of output, dependent on the measure of activation of the NLRC4/NAIP inflammasome. The HR observed *in planta* may be due to a

threshold of RPS4^{TIR}-NLRC4/NAIP oligomers forming and triggering a strong immune response. A lack of HR may still correlate with reduced recognition of a PAMP, which may confer resistance when a stably transformed plant is challenged with a pathogen presenting the PAMP.

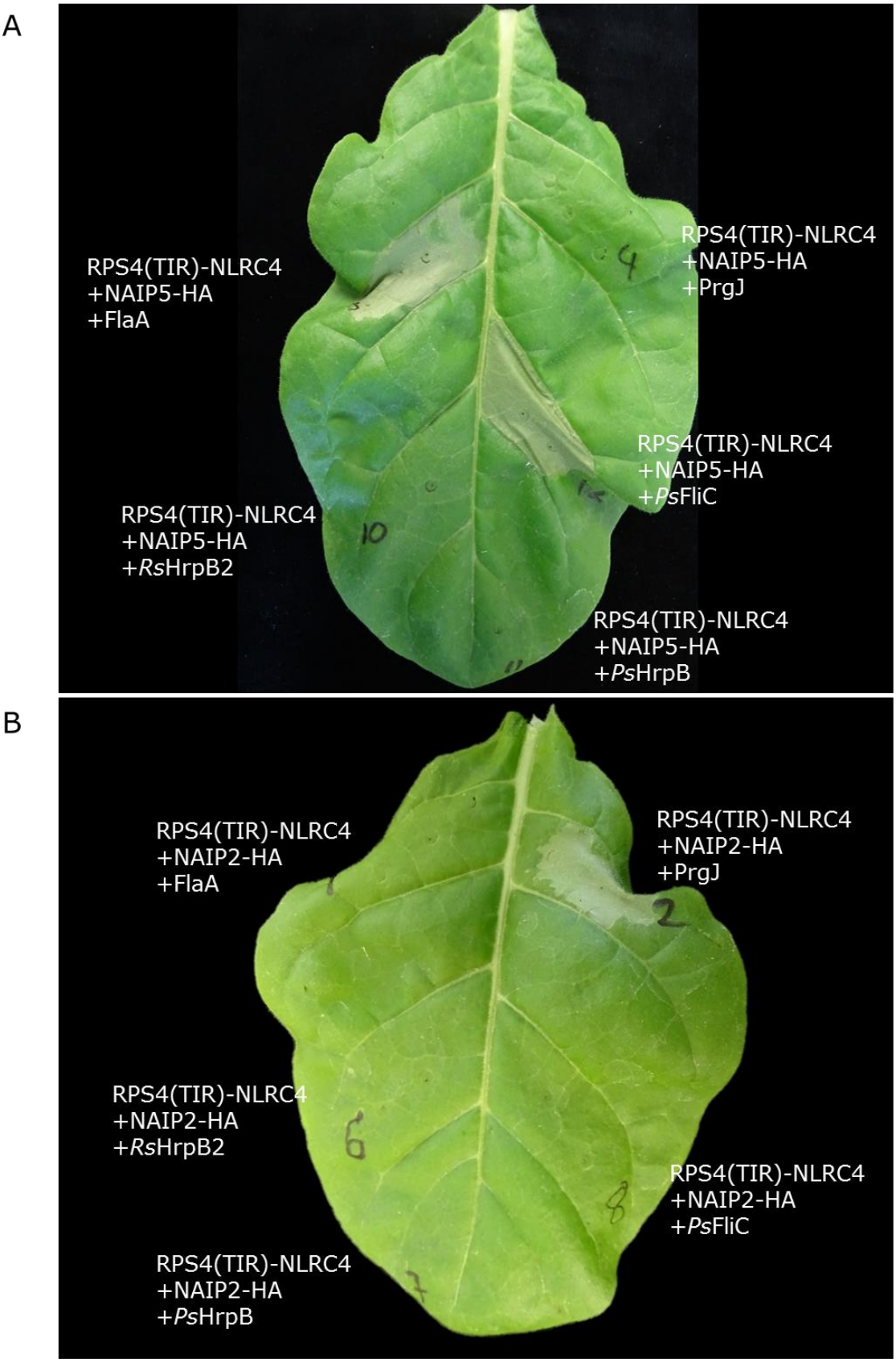


Figure 5.8. PAMPs from plant pathogenic trigger RPS4^{TIR}-NLRC4-dependent HR when expressed in plants. *Nicotiana tabacum* was agroinfiltrated with a mix of strains carrying RPS4^{TIR}-NLRC4-HF, NAIP5-HA (A) or NAIP2-HA (B) and a PAMP with an N-terminal Myc tag. Each strain was infiltrated at an OD_{600} of 0.5. Leaves were assessed for cell-death at 3 dpi. The leaves shown are representative of least three replicate infiltrations.

These tests have provided support to the hypothesis that the NLRC4/NAIP inflammasome could perform as an intracellular PRR *in planta*. In order to determine if it could confer resistance to bacterial pathogens, I agro-infiltrated RPS4^{TIR}-NLRC4 and either NAIP2 or NAIP5 into *N. benthamiana* leaves and, 48 hours later, infiltrated with *Pseudomonas syringae* CUCPB5460, a *hopQ1* mutant capable of compatible growth on *N. benthamiana* (Wei et al., 2007). The results of these experiments are still inconclusive; I needed to suspend them to write the thesis. I will generate Arabidopsis lines stably expressing RPS4^{TIR}-NLRC4/NAIP and challenge these plants with *Pst*DC3000. In addition, I will include two constructs as negative controls. RPS4$^{TIR(SH/AA)}$-NLRC4 will not trigger HR in the presence of a ligand and RPS4^{TIR}-NLRC4-R288A (which will be tested in transient assays when generated), a catalytic surface mutant which can form dimers with NAIPs but cannot oligomerise with other NLRC4 molecules. This would be a control for increased resistance from overexpression that you can see with some TIR-NLRs; I would predict that the catalytic surface mutant would prevent TIR-dimerisation and attenuate ligand-independent HR.

5.2.4.Cell-death mediated by RPS4^{TIR}-NLRC4 requires EDS1 and an intact SH dimer-interface

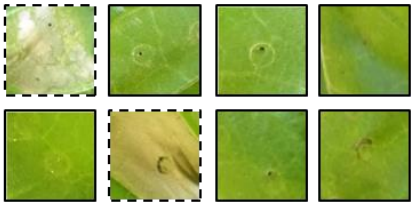
The TIR/CC-NLRC4/NAIP system has potential as an engineered intracellular PRR for plants, but it also has potential as a tool to dissect the mechanism of TNL and CNL signalling. My results using RPS4^{TIR}-NLRC4 strongly suggest that induced proximity of the RPS4^{TIR} is sufficient to initiate an immune response. This has advantages for studying the initiation of TIR/CC signalling over TIR/CC-GFP fusions, which have been used in the past (e.g. Krasileva et al., 2010), because oligomerisation is ligand-dependent, and is probably not concentration-dependent (GFP dimerisation is favoured at concentrations >5 mg mL-1; Ward, 2005). Furthermore, the structure of the 10-, 11-, and 12-mer NLRC4/NAIP inflammasome is well-characterised.

To better characterise RPS4^{TIR}-NLRC4 as a tool, I performed experiments to further characterise the mechanism. First, I tested the requirement of an intact RPS4 SH dimer interface. I co-expressed RPS4^{TIR}-NLRC4-HF with a mutated TIR-dimer interface (RPS4$^{TIR;SH/AA}$-NLRC4-HF) with NAIP2 or NAIP5, and either PrgJ or FlaA (Figure 5.9a). Leaves infiltrated with RPS4^{TIR} -NLRC4-HF and the correct combination of NAIP/PAMP exhibited HR, but leaves infiltrated with RPS4$^{TIR;SH/AA}$-NLRC4-HF did not (Figure 5.9a), demonstrating that oligomerisation via the NLRC4-NBD was not sufficient for RPS4^{TIR} HR and that the RPS4^{TIR} SH dimer interface is also required. Next, I sought to test if the HR is EDS1-dependent. I co-expressed the appropriate inflammasome components in EDS1-silenced tobacco RNAi lines (made available by Barbara Baker) and did not observe HR (Figure 5.9b). I included MLA^{CC}-NLRC4ΔLRR as a control, which triggered HR in both WT and EDS1-silenced lines (Figure 5.9b).

A nuclear pool of RPS4 has been reported to be required for full HR in recognition of AvrRps4 (Heidrich et al., 2011). Neither RPS4^{TIR} nor NLRC4 have a predicted NLS, so RPS4^{TIR}-NLRC4 might not be in the nucleus. To test this, I expressed RPS4^{TIR}-NLRC4-GFP in *N. benthamiana* with NAIP5 and either FlaA or PrgJ. I also expressed RRS1^{TIR}-NLRC4-GFP. In all the cells that I examined by confocal microscopy, GFP fluorescence in leaves infiltrated with RPS4^{TIR}-NLRC4-GFP was strictly cytoplasmic; no nuclear signal was detected (Figure 5.10a). I have demonstrated that FlaA will trigger formation of an NLRC4/NAIP5 inflammasome-like oligomer but PrgJ will not. Therefore, I examined leaves infiltrated with RPS4^{TIR}-NLRC4-GFP, NAIP5 and either FlaA or PrgJ to see if oligomerisation imposed altered subcellular localisation on the complex, and in preliminary experiments, it appears that it did not (Figure 5.10a). I was also concerned that the induction of HR by assembly of the RPS4^{TIR}-NLRC4-GFP/NAIP5/FlaA complex would affect my observations, so I examined fluorescence of RRS1^{TIR}-NLRC4-GFP in the presence of NAIP5 and FlaA, and its localisation resembled that of RPS4^{TIR}-NLRC4-GFP.

A

RPS4^{TIR}-NLRC4-HF	+	+	-	-
RPS4$^{TIR;SH/AA}$-NLRC4-HF	-	-	+	+
NAIP2-HA	+	-	+	-
NAIP5-HA	-	+	-	+



B

RPS4^{TIR}-NLRC4-HF	+	+	-	-
NAIP1-HA + 4xMyc-YscF	+	-	-	-
NAIP5-HA + 4xMyc-*Ps*FliC	-	+	-	-
RPS4^{TIR}-NLRC4ΔLRR-HF	-	-	+	-
MLA^{CC}-NLRC4ΔLRR-HF	-	-	-	+

Figure 5.9. The RPS4^{TIR} SH dimer-interface and the TIR-required signalling component EDS1 are required for RPS4^{TIR}-NLRC4-mediated HR. A) Nicotiana tabacum was agroinfiltrated with a mix of strains carrying RPS4^{TIR}-NLRC4-HF or a RPS4^{TIR} SH dimer-interface mutant form, RPS4$^{TIR;SH/AA}$-NLRC4-HF, a NAIP and a PAMP, as indicated. NLRC4 and RRS1^{TIR}-NLRC4 were included as controls. Each strain was infiltrated at an OD_{600} of 0.5. Leaves were assessed for cell-death at 3 dpi. Panels indicate representative leaves from at least three replicate infiltrations. A solid outline indicates green tissue and a dashed outline indicates HR. HF, 6xHis.3xFLAG; HA, 6xHA; *Ps*, *Pseudomonas syringae*. B) RPS4^{TIR}-NLRC4-induced HR requires EDS1. *N. tabacum* cv. Petit Gerard (PG) and *N. tabacum* cv. Samsun RNAi-*Nt*EDS1, which is expressing siRNA against EDS1, were agroinfiltrated as indicated. MLA^{CC}-NLRC4△LRR-HF was included as a control, because CC-NLRs do not require EDS1 for signalling.

Cytoplasmic streaming and dynamic aggregates of green fluorescence were observed in all samples (Figure 5.10a). I did not expect aggregates to form in leaves with the combination of $RPS4^{TIR}$-NLRC4-GFP/NAIP5/PrgJ, because these should not form a complex. These aggregates I observed may indicate a high local concentration of monomeric $RPS4^{TIR}$-NLRC4-GFP, or it may indicate that there is some spontaneous $RPS4^{TIR}$-NLRC4-GFP oligomerisation occurring due to a high concentration of the protein (though without triggering HR).

Further controls need to be examined, including $RPS4^{TIR;SH/AA}$-NLRC4-GFP and $RPS4^{TIR}$ fused to NLRC4 with a mutated catalytic surface, which would block NLRC4-NLRC4 interactions. The restriction of HR-inducing $RPS4^{TIR}$-NLRC4-GFP to the cytoplasm supports the conclusions of Sohn et al (2014) but conflicts with the observations that nuclear localisation is required for full RPS4- or $RPS4^{TIR}$-mediated HR (Heidrich et al., 2011; Saucet, 2013). The discrepancies between these studies may be due to quantitative, rather than qualitative, effects on HR suppression.

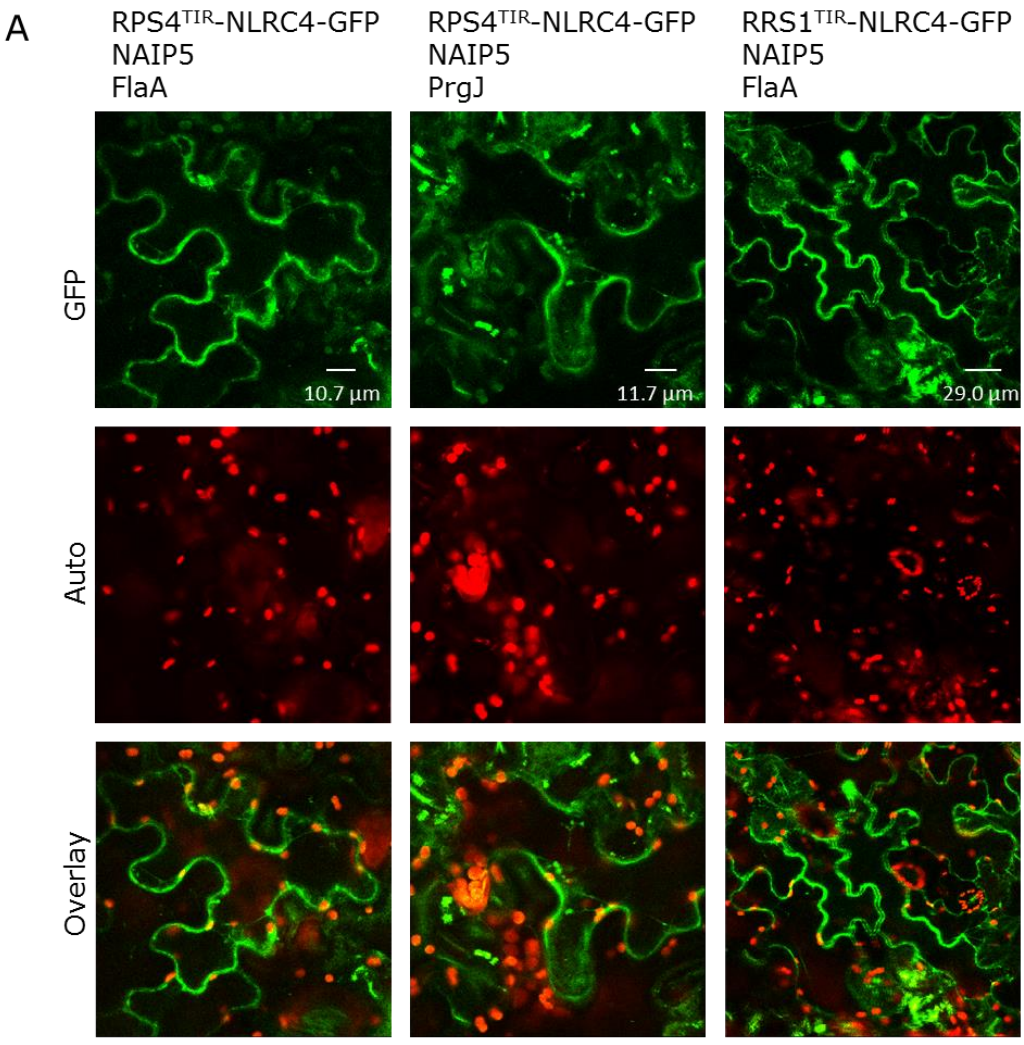
5.3. Discussion

In this chapter, I have reconstituted the NLRC4/NAIP inflammasome in plants. By fusing the TIR domain of RPS4 to the N-terminus of NLRC4 I was able to trigger HR in response to cytoplasmic PAMPs. This system has potential to provide an added layer of pathogen perception and resistance, and a tool for studying the initiation of TIR/CC immune signalling.

5.3.1. Could the NLRC4/NAIP inflammasome provide PAMP-triggered immunity to plants?

$RPS4^{TIR}$-NLRC4/NAIP triggers HR in response to overexpression of intracellular PAMPs, but is it likely that PAMPs from plant-pathogenic bacteria would enter the plant cytoplasm? Intuitively, this seems unlikely, because macrophages phagocytose bacteria, so recognised bacteria are often intracellular. Plants have an additional physical barrier, the cell wall, which may impede intracellular perception of PAMPs. If the NLRC4/PAMP inflammasome will be useful for perception of intracellular bacteria only, then there is a subset of bacterial pathogens that it would be useful to protect against. For example, the causal agents of huanglongbing,

Candidatus Liberibacter asiaticus and *Ca.* L. africanus, the most devastating disease of citrus worldwide, reside intracellularly in the phloem during infection (da Graça and Korsten, 2004).



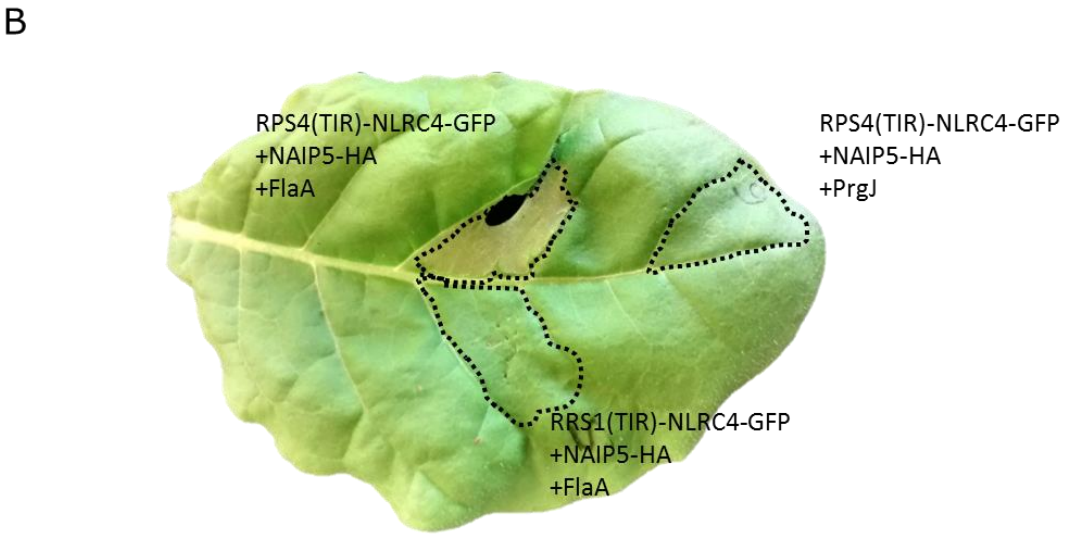


Figure 5.10. RPS4^{TIR}-NLRC4-GFP and RRS1^{TIR}-NLRC4-GFP do not appear to go to the nucleus. A) Panagiotis Moschou operated the microscope and took the images. RPS4^{TIR}-NLRC4-GFP and RRS1^{TIR}-NLRC4-GFP localise to the cell periphery, to transvacuolar strands and in aggregates. No GFP signal was observed in the nucleus, although the nucleus cannot be easily defined in any image or brightfield image. The experiment was to determine the localisation of NLRC4 fusions. The first column, containing images of leaves expressing RPS4^{TIR}-NLRC4-GFP, NAIP5-6xHA and Myc-FlaA, depicts the localisation of RPS4^{TIR}-NLRC4-GFP presumed to be in an "inflammasome-like" complex; in the second column, RPS4^{TIR}-NLRC4-GFP is presumed to be monomeric; in the third column, RRS1^{TIR}-NLRC4-GFP is presumed to be in an inflammasome-like complex, but any protein accumulation or localisation differences that may be triggered by HR should be avoided (although no HR was observed in any leaf at this time-point). Complementary biochemical approaches are required to confirm these presumptions. Images were taken with a Leica SP5 point-scanning confocal microscope with a 111.4 µm pinhole, with 30% excitation at 488 nm (GFP channel) and 46% excitation at 561 nm (auto channel) and a detection filter capturing 500.0 - 545.3 nm (GFP channel) and 570.0 - 682.0 nm (auto channel). A scale line indicates length in µm for each column. Although the magnifications between treatments differ, all images were taken with a HC PL APO CS2 63.0x1.20 WATER UV objective. Column 1 and 2 used a HyD1 gain of 124 and column 3 used a gain of 73. The brightness of each image was enhanced independently (i.e. not to the same levels) to improve image quality while minimising pixel saturation (some aggregated GFP plastids were saturated before brightness was enhanced, but this does not detract from the conclusion of the experiment) using Leica LAS AF Lite software. Images shown are representative of images taken of 2-3 cells and more than 20 cells observed from discs from two *Nicotiana benthamiana* leaves for each combination, infiltrated on the same day and imaged at 2 dpi. GFP, fluorescence of the GFP tag at the C-terminus of NLRC4-constructs; Auto, autofluorescence of plastids at XXX nm; Overlay, the two images above overlaid. Leaves were infiltrated with an equal mix of four *Agrobacterium* strains, each carrying a T-DNA plasmid containing the constructs indicated above each column of panels and p19, at an OD_{600}=0.5 per strain. B) RPS4^{TIR}-NLRC4-GFP triggers HR in *N. tabacum* in the appropriate combination. Strains indicated next to the leaf were agroinfiltrated at an OD_{600}=0.5, each. Image was taken 3 dpi. The infiltrated area is delineated by a dotted line. This was repeated once.

However, studies in bacteria-human pathosystems have established a requirement for TTSS translocation of PAMPs for inflammasome perception, which may also occur in plant-bacterial interactions. How might FlaA and PrgJ be translocated into host cells? The current model proposes that

flagellin is inadvertently translocated across the TTSS into the host cell due to similarity between the TTSS and the flagellar secretion system, stemming from a common protein ancestor (Franchi et al., 2012; Miao et al., 2006, 2007; Wei et al., 2013). Indeed, although pathogens known to trigger pyroptosis via inflammasome activation are phagocytosed, they are enclosed in a host-derived vesicular membrane and do not enter the cytoplasm. *Pseudomonas aeruginosa*, an extracellular pathogen of humans, is also detected by the NAIP5/6 inflammasome (Ince et al., 2015). NAIP5/6 perception of *Salmonella* flagellin requires a functional TTSS and monomeric flagellin, but not a functional flagellar export system (Miao et al., 2006, 2007; Sun et al., 2007). Perception of *Legionella* flagellin requires a functional type IV secretion system (TFSS) (Amer et al., 2006; Molofsky et al., 2006). Deletion of the TTSS greatly reduces flagellin found in the secretome of *E. coli* (Badea et al., 2009). Furthermore, removal of the signal peptide from two *Salmonella* TTSS secreted effectors prevents them from being secreted by the T3SS, but they are instead excreted by the flagellar export system (Lee and Galán, 2004). Recently it was reported that secretion of flagellin in *Citrobacter freundii* requires the type VI secretion system (Liu et al., 2015a). In plants, one study has robustly demonstrated that flagellin is translocated from the bacterial cytoplasm to the plant cytoplasm via the TTSS (Wei et al., 2013). The authors of this study proposed that plant NLRs have not evolved to recognise intracellular PAMPs like mammals have because it would leave them at a disadvantage to necrotrophic bacterial pathogens. It would be exciting to be able to test this hypothesis.

If HR was triggered in response to a bacterial PAMP like flagellin or a TTSS rod protein, would plants become highly susceptible to bacterial necrotrophs? Necrotrophic pathogens often secret toxins in order to kill their host and benefit their infection strategy. Some fungal necrotrophs secrete effectors that trigger an NLR-dependent HR that promotes growth of the pathogen on the plant (Faris et al., 2010; Lorang et al., 2007; Nagy and Bennetzen, 2008; Sweat and Wolpert, 2007; Sweat et al., 2008), although there are no bacterial toxins that have been characterised to function in the same way. Indeed, the term “necrotroph” being applied to bacteria is

controversial due to several features that are uncharacteristic of originally defined fungal necrotrophs (discussed in detail in Kraepiel and Barny, 2016). For example, *Pectobacterium* and *Dickeya* harbour plant pathogens that are often described as necrotrophs. These pathogens do indeed secrete cell-wall degrading enzymes that allow these bacteria to obtain nutrients from dead tissue, but a long-lived latent biotrophic infection of host plants is common prior to the necrotrophic disease-causing phase of infection (which is triggered by quorum sensing mechanisms) (Liu et al., 2008). Perhaps it is more accurate to describe bacterial necrotrophs as hemibiotrophs which have a biotrophic lifestyle prior to a necrotrophic phase of infection Some fungal hemibiotrophs, for example *Phytophthora infestans* and *Magnaporthe oryzae* (Jia et al., 2000; Vleeshouwers et al., 2000), which rapidly spread during the necrotrophic stage, are suppressed by HR in the initial biotrophic phase. The harpin effector protein HrpN from *Erwinia* spp. (and former members in the genera *Dickeya* and *Pectobacterium*) triggers HR in non-host plants but is not associated with an HR in host plants (Dong et al., 1999; Wei et al., 1992). The consensus appears to be that HR is conducive to infection by necrotrophs but can suppress hemibiotrophs if triggered in the initial phases of infection. Installing the RPS4^{TIR}-NLRC4/NAIP in plants will allow us to directly test the hypothesis that bacterial pathogens with at least a partially necrotrophic infection strategy will benefit from a bacterial PAMP-detecting PRR that induces cell death.

5.3.2. The NLRC4/NAIP system is a good tool to study the initiation of CC/TIR signalling

The two main approaches to studying the initiation and propagation of NLR signalling in plants have been to either co-express an effector with its cognate resistance protein (or proteins), or by overexpressing the full-length or the signalling portion of an NLR, which can trigger HR in the absence of an effector. The advantage of the first approach is that signalling presumably occurs whenever a single NLR and cognate effector interact (directly or indirectly), while the second approach typically relies on a relatively high protein abundance threshold before HR is induced. The disadvantage of using the full effector/NLR system to study CC/TIR signalling is that there is such great diversity in recognition mechanisms of effectors and domain

architectures of NLRs that a lot of these factors could obfuscate a universal CC or TIR signalling mechanism. NLRC4/NAIP may be a more amenable approach because it is inducible like the effector/NLR approach, but it also utilises a well-characterised heterologous intramolecular regulation.

In animals, induced proximity or proximity-induced dimerization of NLR-CARDs is sufficient to recruit caspases and initiate immune signalling (Salvesen and Dixit, 1999). In plants, some studies have indicated that this is also the case, but it is difficult to definitively demonstrate this because many NLRs are in a pre-associated homodimer or higher order oligomer in the absence of effectors. I hypothesise that that the induced proximity of $RPS4^{TIR}$ domains is the likely mechanism triggering HR in the RRS1/RPS4 system because HR was observed when $RPS4^{TIR}$-NLRC4 formed a predicted inflammasome-like complex in plants, bringing the N-terminal $RPS4^{TIR}$ domains into close proximity. The NLRC4/NAIP inflammasome is a useful tool to study the initiation of any CC or TIR immune response because it only oligomerises in response to a specific ligand.

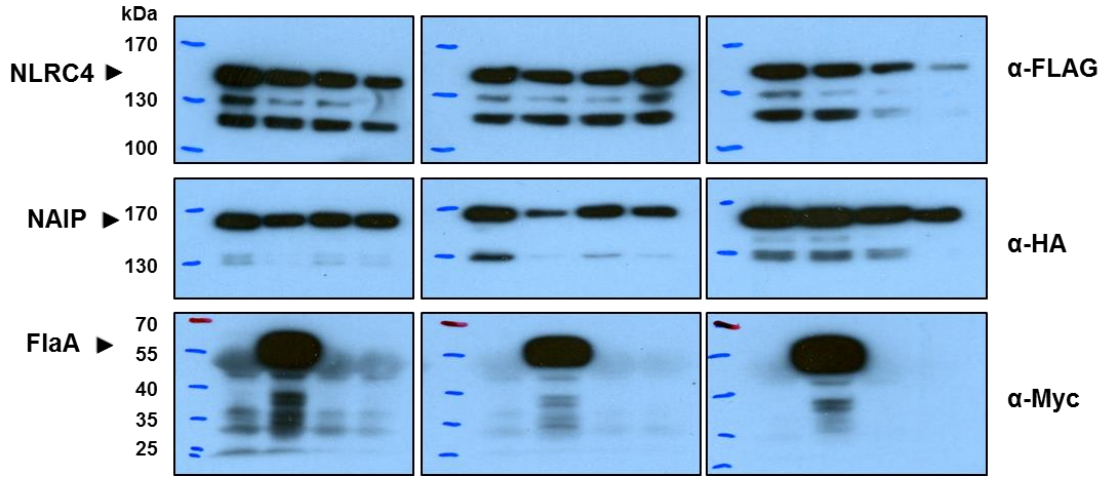
$RPS4^{TIR}$-NLRC4 triggers HR from a cytoplasmic location. I plan to test whether the system works when forced into the nucleus (by including an NLS) or tethered to the plasma membrane. Different CC or TIR domains could be fused to the N-terminus of NLRC4 to determine if they can confer HR when restricted to the cytoplasm. For example, Bai et al (2012) proposed that MLA10-mediated HR is suppressed by nuclear pools of MLA10. In this chapter, MLA^{CC}-NLRC4$\triangle$LRR triggered HR, although MLA^{CC}-NLRC4 conferred ligand-dependent yellowing, only. Domains fused to the N-terminus of NLRC4 will be in close proximity in the inner ring of the inflammasome wheel, according to what we can predict from the wheel-like structure of the NLRC4/NAIP. Would a C-terminal fusion of $RPS4^{TIR}$ to NLRC4 trigger HR, with the TIR domains on the outer ends of the "spokes" of the wheel?

If plant NLR-signalling is initiated via induced-proximity of CC/TIR domains, the principle of NLR-signalling in mammals, we would expect a reciprocal approach to trigger CARD-signalling in mammals. That is, a construct encoding RPS4 lacking its TIR domain, tagged with an N-terminal CARD,

might trigger pyroptosis in mouse or human cells. Other hypotheses could be tested, using different NLRs with their N-terminal signalling domains substituted with CARDs or PYDs. This set of experiments, reciprocal to TIR/CC-NLRC4/NAIP experiments in plants, would confirm a trans-kingdom principle of NLR activation.

5.4. Appendices

	NAIP1					NAIP2					NAIP5				
RPS4^{TIR}-NLRC4-HF	+	+	+	+		+	+	+	+		+	+	+	+	
NAIP-6xHA	+	+	+	+		+	+	+	+		+	+	+	+	
4xMyc-FlaA	-	+	-	-		-	+	-	-		-	+	-	-	
4xMyc-PrgJ	-	-	+	-		-	-	+	-		-	-	+	-	
4xMyc-YscF	-	-	-	+		-	-	-	+		-	-	-	+	



Appendix 5.1. Expression of RPS4^{TIR}-NLRC4-HF, NAIPs and PAMPs from Figure 5.4. Sung Un Huh performed this experiment. SDS-PAGE was performed on all extracts analysed by Blue Native PAGE in Figure 5.3. Gels probed with anti-FLAG and anti-HA antibodies conjugated to HRP used a 6% separating phase, and gels that were probed with anti-Myc used a 10% separating phase. 4×Myc-PrgJ and 4×Myc-YscF were not observed. It was difficult to detect these two ligands in the SDS-PAGE gels of Figure 5.2 and 5.3, too. This experiment was repeated three times.

Chapter 6. General Discussion and Outlook

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6.1. Introduction

Since the first NLRs were cloned in the 1990s (Bent et al., 1994; Bertin et al., 1999; Inohara et al., 1999; Lawrence et al., 1995; Salmeron et al., 1996; Whitham et al., 1994), major progress has been made in the understanding of the mechanisms of NLR perception and activation. The development of model pathosystems has rapidly advanced the refinement of conceptual frameworks that describe host-microbe interactions. In animals, post-activation molecular events are well understood for several model NLRs. In plants, several recognition paradigms (receptor-ligand, guard and decoy models, discussed in detail in section 1.2.2) exist and are supported by strong evidence, but there are knowledge gaps in post-activation molecular events. In addition, the promises of novel synthetic resistance have not been realised. Efforts to engineer useful resistance have been hampered by a lack of understanding of NLR mechanisms, which includes inter- and intra-molecular regulation of activation and inhibition, as well as a restriction in the functionality of some NLRs when transferred to different taxa.

Here, I will discuss my findings in the context of our current understanding of NLR function.

6.2. Engineering synthetic resistance requires better understanding of fundamental mechanisms of NLR function

Efforts to engineer disease resistance have been propelled forward by key fundamental discoveries. For example, the discovery that expression of TMV Coat Protein (CP) in tobacco provides enhanced resistance to TMV (Abel et al., 1986). This breakthrough experiment was developed to understand the mechanism of viral cross protection and to engender this viral resistance in a heritable element. This is the earliest published example of pathogen-derived

resistance (PDR) and is now known to be due to RNA-mediated silencing of viral gene expression.

Another example of fundamental discoveries propelling forward efforts to engineer resistance is antibody-mediated resistance of pathogens. This method was originally developed to provide resistance against artichoke mottled crinkle virus (AMCV) by raising antibodies against AMCV CP in hybridoma cells, cloning the genes for the immunoglobulin chains with the highest affinity for CP, and expressing them in plants (Tavladoraki et al., 1993). The authors acknowledge that "existing data on the crystal structure of the type-member of the tombus group, the tomato bushy stunt virus (TBSV), and on the nucleotide sequence of AMCV coat protein were fundamental" (Tavladoraki et al., 1993).

A thorough understanding of the determinants of specificity of transcription activator-like effectors (TALEs) from *Xanthomonas* strains allowed the manipulation of *R* gene promoters such that, instead of the targeted TALEs activating susceptibility, they would activate resistance (Boch et al., 2014; Hummel et al., 2012; Römer et al., 2009). For example, in incompatible interactions, the TALEs AvrBs3 and AvrXa27 bind to the promoters of the *R* genes Bs3 and Xa27, respectively, to activate transcription and promote resistance (Römer et al., 2007, 2009). Römer et al. (2009) engineered the recognition sequence of AvrXa27 into the promoter of Bs3 and demonstrated in transient assays that a construct containing this chimaeric promoter driving Bs3 conferred recognition of both AvrXa27 and Bs3. This method could be used to expand recognition of novel TALEs with known recognition sequences.

The introgression of *R* genes, many of which were later identified as NLRs, from low-yielding germplasm to cultivars with superior agronomy has been an effective and extensively applied method to manage crop disease. Once NLRs were cloned, it became possible to transfer race-specific resistance to a susceptible plant by transgenically expressing the appropriate NLR (e.g. Horvath et al., 2012; Jones et al., 2014). However, deficits in the understanding of NLR function have undermined programmes to design synthetic NLRs that provide novel resistance recognition capacities. My

thesis highlights some of these problems. For example, fusing additional domains to the C-terminus of RRS1 resulted in autoactive constructs. These setbacks may be overcome through a deeper understanding of RRS1 autoinhibition.

A more complete understanding of RRS1/RPS4 activation would help the design of RRS1-S$^{protease\ trap}$ and engineered R3a alleles. Both approaches generated alleles that triggered an HR-like cell death response when transiently co-expressed with elicitors in tobacco, but neither conferred resistance to their target pathogens (Chapter 4 of this thesis; Segretin et al., 2014). In a follow-up study, the same mutations in R3a that conferred gain-of-function recognition to Avr3a^{EM} in transient expression studies, but not resistance, conferred resistance to *P. infestans* when introduced into I-2 (Giannakopoulou et al., 2015). It is not known why these mutations confer resistance in I-2 but not R3a, but it presents the possibility that introducing NIa cleavage sites in appropriate sites in NLRs other than RRS1 may result in an allele that confers resistance to potyviruses. However, I plan to exhaust all troubleshooting strategies for RRS1-S$^{protease\ trap}$ before seeking out alternatives.

NB-ARC mutations in RxM1, the mutant of Rx that triggered PopMV-dependent trailing necrosis, increased the strength and rapidity of the immune response and trailing necrosis (Harris et al., 2013), demonstrating that a rational approach based on strong foundational knowledge can engineer effective NLRs. Engineering PBS1^{TuMV} has yet to overcome the trailing necrosis barrier, but this may not be due to any mechanism of RPS5 activation but instead be due to the plasma membrane localisation of RPS5/PBS1^{TuMV} (Kim et al., 2016).

I speculated in Chapter 4 that the localisation of RRS1^{TuMV} may affect its function. NLRs in plants have various subcellular localisations, and different requirements for recognition, activation and signalling. RRS1 and RPS4 require nuclear localisation for successful ETI, probably through a requirement for co-localisation with downstream signalling components and effectors (Sohn et al., 2014; Wirthmueller et al., 2007).

Subcellular translocation is required for the function of some NLRs. Upon detection of the effector AVR3a in the cytosol, R3a moves to the endosome (Engelhardt et al., 2012). Suppression of R3a re-localisation suppresses HR, although an autoactive cytosolic mutant of R3a triggers HR without endosomal localisation (Engelhardt et al., 2012). Another example is PVX CP, which is recognised in the cytoplasm and requires an interaction in the cytoplasm between Rx and the cytosolic Ran GTPase-activating protein 2 (RanGAP2) (Sacco et al., 2007; Tameling and Baulcombe, 2007; Tameling et al., 2010). Nucleo-cytoplasmic shuttling of Rx is required for CP recognition; restricting Rx to the cytoplasm or nucleus suppresses activation (Slootweg et al., 2010; Tameling et al., 2010). Once activated and translocated to the nucleus, Rx can bind DNA (Fenyk et al., 2015).

Other NLRs require only cytosolic localisation to confer HR, for example, RGA4/RGA5 (Cesari et al., 2013, 2014b). Furthermore, overexpression of the CC domains of barley MLA10 and the related wheat CNLs Sr33 and Sr50 in the cytoplasm of *N. benthamiana* triggers HR (Bai et al., 2012; Cesari et al., 2016). However, MLA10 and Sr33 confer resistance to their respective pathogens when restricted to the nucleus or cytoplasm, respectively (Bai et al., 2012; Cesari et al., 2016). The differences in localisation requirements may be due to different subcellular targeting of their respective effectors. Important spatial factors contributing to successful effector recognition and signalling most likely involve co-localisation of the target effector with the appropriate NLR, co-localisation of activated NLRs with co-factors and downstream partners, the local concentration of activated NLRs, and the chemical environment of the compartment in which the NLR is located. There are still many fundamental questions that require answers to increase the effectiveness of synthetic resistance approaches.

6.3. Trans-kingdom principles of NLR signalling

Fusing the TIR domain of RPS4 to the N-terminus of NLRC4 creates a functional NLR that triggers cell-death in the presence of the appropriate elicitor. Does this suggest that there is a universal mechanism of NLR activation?

There has been more success in identifying the structural requirements of NLR activation in mammals than plants. Oligomerisation plays a key role in NLR activation. The NLR inflammasomes demonstrate activation after inducible-oligomerisation. The dsDNA-detecting protein AIM2, which is not an NLR and instead consists of a HIN200 dsDNA-binding domain and a PYD, also forms an inflammasome (Lu et al., 2014b). In both cases, oligomerisation brings the signalling domains into close proximity to recruit downstream signalling molecules. The Death Domain (DD) superfamily, which also contains CARD, PYD and Death Effector Domain (DED), contain members outside the NLRs that function via inducible oligomerisation that results in the activation of a caspase family protein (Ferrao and Wu, 2012; Kagan et al., 2014).

The oligomeric apoptosome is formed by the NLR-like Apaf1, CED-4 or Dark, in humans, *Caenorhabditis elegans* or *Drosophila melanogaster*, respectively. The apoptosome is a wheel-like structure, similar to the inflammasome, which contains N-terminal CARDs that recruit Caspase-9 via homotypic interactions at the centre of the wheel. In humans, the apoptosome is stimulated by the interaction between cytochrome c and Apaf1 (Li et al., 1997; Zou et al., 1999). Several TLRs and plant PRRs require inducible oligomerisation for the recruitment of downstream signalling proteins. Induced oligomerisation is a common mechanism of signal activation in innate immunity and has been described in two models: the model of "supramolecular organizing centres (SMOCs)" and the model of signalling by higher-order assemblies (Kagan et al., 2014; Wu, 2013).

After oligomerisation, many animal innate immune receptors recruit signalling molecules directly or via adaptors. In the case of inflammasomes and apoptosomes, Caspase-1 and Caspase-9 are recruited, respectively. After recruitment, many of these signalling molecules assemble in a prion-like way into amyloid-like filaments. This underlying mechanism is widespread but not fully understood. The prion-like formation of innate immune signalling molecules into filaments was first observed with the signalling molecule MAVS (mitochondrial antiviral signalling protein), which is recruited to the viral RNA surveillance receptor RIG-I (Hou et al., 2011). MAVS is recruited to

RIG-I and RIG-I-like complexes, which are induced by viral RNA perception, via homotypic CARD interactions (Takeuchi and Akira, 2008). The RIG-I CARDs form a platform that acts as a nucleation site for MAVS CARDs to form amyloid-like filaments (Hou et al., 2011; Wu et al., 2014; Xu et al., 2014d).

Some viruses can suppress signalling downstream of RIG-I. One response to this is a necroptosis cell death response. Crucial to this are the RIP1 and RIP3 kinases, which when activated form a heteromeric amyloid-like filament (Li et al., 2012). Upon filament aggregation, the RIP kinases auto- and trans-phosphorylate, and RIP3 phosphorylates the downstream protein MLKL (Li et al., 2012). Thereupon, MLKL trimerises and relocates to the plasma membrane, mediating permeabilisation of the membrane (Cai et al., 2014b; Quarato et al., 2016). Similarly to RIG-I, the NLRP3 PYD acts as a nucleation site for ASC-PYD filaments upon NLRP3 inflammasome formation; the ASC CARDs in turn act as nucleation sites for Caspase-1 filament formation (Cai et al., 2014a; Lu et al., 2014a; Man et al., 2014). The NAIP2/NLRC4 inflammasome CARDs nucleate Caspase-1 filaments directly (Zhang et al., 2015b). These recent discoveries suggest that the nucleation of filaments of signalling molecules at N-terminal signalling domains of NLRs might be a universal mechanism of signalling.

The majority of innate immune signalling in animals may be driven by the formation of amyloid-like filaments, but is this process conserved across kingdoms? The fungus *Podospora anserina*, contains the NLR-like STAND protein NWD2, which has a central NACHT domain, a C-terminal WD repeat domain and an N-terminal prion forming domain (PFD) (Daskalov et al., 2012). Upon interaction with an unknown ligand, the PFD of NWD2 interacts with the PFD of the toxic protein HET-S, inducing amyloid templating and HET-S pore formation (Daskalov et al., 2015). In this way, NWD2 and HET-S mediate cell death during non-self recognition. *Chaetomium globosum* contains a HET-S analog called HELLP (Daskalov et al., 2016), which has an N-terminal cell-death-inducing domain termed HeLo-like (HELL) and a C-terminal amyloid motif termed PP. HELLP is in a gene cluster that contains two other PP-containing proteins: a lipase (called SBP) and an NLR-like

protein called PNT1, which presumably regulates HELLP (Daskalov et al., 2016). The PPs of HELLP, SBP and PNT1 are homologous to the prion-forming domains of RIP1 and RIP3 in humans and form amyloid-like filaments *in vitro*. Furthermore, the N-terminal HELL domain has homology with the N-terminal domain of MLKL that is required for protein relocation to the membrane and membrane permeabilisation (Daskalov et al., 2016; Quarato et al., 2016). It appears that amyloid signalling might be a signalling principle in both mammals and fungi, and cell-death inducing proteins share homology between species.

Experiments with the NLRP3 inflammasome provide excellent evidence to support the notion of a universal signalling mechanism in NLRs. The prion domain of the fungal protein HET-S and the N-terminal HET-S-homologous domain of NWD2 can reconstitute NLRP3 signalling when substituted with the PYD domain of ASC and the PYD domain of NLRP3, respectively (Cai et al., 2014a).

Higher-order assembly of complexes to induce signalling is widespread in animal innate immune signalling, and both mammals and fungi utilise amyloid-like filament formation for NLR-like signalling. Do plants employ these mechanisms? It would be advantageous to plants to utilise a similar control on cell death activation. Inducible oligomerisation creates a threshold control that must be reached before cell-death signalling is triggered, safeguarding against inadvertent activation. The formation of open-ended filaments allows rapid signal amplification from even a small number of recognition events and this irreversible process makes it difficult for pathogens to halt signalling. It has been proposed that plants form inflammasome-like signalling platforms (Bentham et al., 2016; Duxbury et al., 2016). However, it has not been demonstrated that a plant NLR complex of a higher order than a dimer is required for activation.

Overexpression of mammalian NLRs, including NLRC4, causes homo-oligomerisation in the absence of elicitor (Damiano et al., 2004; Halff et al., 2012; Kufer et al., 2008; Zurek et al., 2012). However, this was not sufficient to trigger HR in the case of $RPS4^{TIR}$-NLRC4. Either spontaneous homo-oligomerisation occurred at too low a concentration to reach the HR

threshold, or the greater number of RPS4 TIRs in the fully formed RPS4^{TIR}-NLRC4/NAIP inflammasome is required for HR. Overexpression of RPS4-HA and RPS4^{TIR}-HA triggers HR (Swiderski et al., 2009; Zhang et al., 2004); perhaps the CARD-NACHT-LRR in RPS4^{TIR}-NLRC4 prevents recruitment of additional dimers from occurring and triggering HR. Could this be evidence that more than a dimer is required for HR signalling? The forced dimerisation of RPP1-TIR by fusion to YFP suggests that dimerisation is sufficient in that case. However, the local increase in the concentration of TIR domains in these dimers may be sufficient to recruit other TIR-dimers, which subsequently recruit more TIR-dimers until the threshold for signalling is reached. Regardless of the size of the complex, the three stepwise universal aspects of NLR signalling are: autoinhibition of signalling via the N-terminal signalling domain, release of repression by the dual cues of elicitor perception and ADP-for-ATP-exchange, and assembly of a scaffold for signalling (Bentham et al., 2016).

If plants employ amyloid-like filament formation for NLR signalling, what proteins are recruited to the TIR or CC domains to act as adaptors of immune signalling? There are some candidates for TNL signalling. TNLs have an absolute genetic requirement for EDS1, but structural studies have not reported any amyloid aggregations of EDS1 (Wagner et al., 2013). An Arabidopsis TIR-only (AtTIR) protein structurally resembles Myd88, the adaptor for Toll-like receptor-signalling in mammals (Chan et al., 2010). Furthermore, a multitude of Arabidopsis TIR-NB-ARC proteins trigger cell death when overexpressed in *N. benthamiana*, and interact with other NLRs and signalling proteins (Nandety et al., 2013). Therefore, such TIR-containing proteins may function as adaptors for downstream signalling of TNLs. Additionally, the TNLs N, RPS4 and M have splice variants that contain only the TIR or TIR-NB-ARC domains; and it has been proposed that for RPS4 and N, these variants are required for recognition of their cognate effectors (Caplan et al., 2008; Zhang and Gassmann, 2003). The truncated NLR repertoire may directly cooperate with other full length NLRs as adaptors for downstream signalling. A recent computational search of prion domains in Arabidopsis proteins that identified almost 500 candidate proteins identified

a prion domain in AtTIR but did not identify prion domains in RPS4 (Chakrabortee et al., 2016).

A common feature of host-induced cell death is the formation of pores to permeabilise the membrane. Gasdermin D and HET-S can be induced to oligomerise into membrane-permeabilising pores (Daskalov et al., 2014; Kayagaki et al., 2015; Liu et al., 2016; Saupe and Daskalov, 2012; Shi et al., 2015b). The CC_R of RPW8 contains a region homologous to the N-terminal regions of MLKL and HET-S that is responsible for membrane targeting and permeabilisation (Daskalov et al., 2016). This motif is shared with the helper C_RNLs NRG1 and ADR1. Could membrane permeabilisation executed by C_RNLs be involved in HR cell death in cases where helpers are required? The recent finding by my fellow graduate student Baptiste Castel that NRG1 is required for RPS4/RRS1 function provides a path in the lab for further investigation into this question.

Key to understanding unifying concepts of NLR signalling in plants is knowledge of the entire signalling pathway. I have attempted to elucidate the signal pathway of RRS1/RPS4 ETI as a side-project during my PhD. In order to achieve this, I immunoprecipitated epitope tagged RRS1 and RPS4 stably expressed in Arabidopsis to identify downstream signalling components. Proteins that precipitated with RRS1 and RPS4 were identified by mass spectrometry after separation by SDS-PAGE. Analysis and follow-up experiments are ongoing, but I have identified several classes of proteins. Highly represented were proteins from mitochondria and chloroplasts, as well as proteins involved in stress responses, transporters and biosynthesis enzymes (data not shown). I have not yet identified any overrepresented proteins in pre- or post-activation complexes of RRS1 and RPS4. However, two related proteins, glycine rich protein 7 (GRP7) and GRP8, standout as interactors with both RRS1 and RPS4. GRP7 and GRP8 are RNA-binding proteins involved in innate immunity that are both targeted by the effector HopU1 (Fu et al., 2007). GRP7 interacts with translational machinery and the PRRs FLS2 and EFR, as well as their transcripts (Nicaise et al., 2013). Specifically, ADP-ribosylation of GRP7 by HopU1 interferes with its interaction with *FLS2* and *EFR* transcripts, leading to reduced protein

abundance, which subsequently results in a decrease in PTI (Jeong et al., 2011; Nicaise et al., 2013). Could GRP7 and GRP8 be general regulators of innate immune receptor translation and carry out the same role with RRS1 and RPS4? Furthermore, GRP7 and GRP8 contain predicted prion-forming motifs (Chakrabortee et al., 2016). Could these proteins have a second role in amyloid-like filament formation for NLR signalling?

6.4. Conclusions

There are still many avenues left to be explored in NLR signalling and synthetic resistance. Key questions include: What models of NLR activation and signalling provide meaningful hypotheses for future experiments and provide leverage for engineering synthetic resistance? What is the specific role of ATP/ADP in NLR activation? What is the link between subcellular localisation of NLRs and ETI or HR?

The rich diversity of NLR recognition strategies that have been discovered is informing the design of novel NLR detection systems. Structural details of some NLR proteins have revealed the fine control that regulates immune complexes during auto-inhibition and activation. The dissection of these fine-tuned systems should provide valuable new insights for future engineering of synthetic resistance.

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