Molecular mechanisms of NLR pair-mediated immunity in Arabidopsis

马晏

Yan Ma

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Abstract

Central to plant survival is the ability to activate immunity upon pathogen perception. Plants deploy immune receptors to recognise specific pathogen-derived molecules (effectors) and to trigger defence. These receptors usually recognise a specific effector, but some work in pairs and can detect multiple effectors. The Arabidopsis RRS1-R/RPS4 receptor pair forms an immune complex, conferring recognition of two distinct bacterial effectors, AvrRps4 and PopP2. A paralogous pair linked to RRS1/RPS4, designated as RRS1B/RPS4B, only recognises AvrRps4. My work has revealed that both pairs detect AvrRps4 via an integrated WRKY domain of RRS1 or RRS1B, which mimics the effector's host targets: the WRKY transcription factors (TF). It has also been shown that the WRKY TF-targeting PopP2 is also perceived by the RRS1-R WRKY domain. Together, we suggest that RRS1 (or RRS1B) with the WRKY domain fusion has evolved to protect defence-regulating WRKY proteins from being attacked by effectors. These integrated domains of immune receptors are becoming popular targets for synthetic resistance engineering. However, one of the biggest challenges is to avoid auto-activity while enabling new recognition capacity when manipulating the integrated domains.

To better understand how these receptors operate to convert effector perception into defence activation, I investigated the dynamic molecular interactions in the pre-activation complex, and those that change upon effector perception. I found that RRS1-R/RPS4 complex is negatively regulated by the WRKY domain during pre-activation, and effector-triggered activation is likely mediated by de-repression of the WRKY domain. After effector-triggered RRS1 de-repression, the activation signal is transduced to RPS4. Domain swaps between RRS1-R/RPS4 and RRS1B/RPS4B have revealed the key interaction required for this transduction is between RRS1 domain 4 and the RPS4 C-terminal domain. Furthermore, I discovered possible distinct domain-domain interactions that enable AvrRps4- and PopP2-triggered activation. The mechanistic insights into complex auto-inhibition and activation described in this thesis will prove valuable for many other cooperative immune receptor systems.

Contents

List of Publications

This thesis includes material from the published works below.
* Indicates that these authors contributed equally.

*Williams, Simon J and *Sohn, Kee H and *Wan, Li and *Bernoux, Maud and Sarris, Panagiotis F and Segonzac, Cecile and Ve, Thomas and **Ma, Yan** and Saucet, Simon B and Ericsson, Daniel J and Casey, Lachlan W and Lonhienne, Thierry and Winzor, Donald J and Zhang, Xiaoxiao and Coerdt, Anne and Parker, Jane E and Dodds, Peter N and Kobe, Bostjan and Jones, Jonathan DG **Structural basis for assembly and function of a heterodimeric plant immune receptor**. *Science* 344.6181 (2014), 299–303.

*Saucet, Simon B and ***Ma, Yan** and Sarris, Panagiotis F and Furzer, Oliver J and Sohn, Kee H and Jones, Jonathan DG **Two linked pairs of Arabidopsis TNL resistance genes independently confer recognition of bacterial effector AvrRps4**. *Nature communications* 6 (2015), 6338

*Sarris, Panagiotis F and *Duxbury, Zane and *Huh, Sung U and ***Ma, Yan** and *Segonzac, Cécile and Sklenar, Jan and Derbyshire, Paul and Cevik, Volkan and Rallapalli, Ghanasyam and Saucet, Simon B and Wirthmueller, Lennart and Menke, Frank L and Sohn, Kee H and Jones, Jonathan DG **A Plant Immune Receptor Detects Pathogen Effectors that Target WRKY Transcription Factors**. *Cell* 161.5 (2015), 1089–1100.

*Duxbury, Zane and ***Ma, Yan** and *Furzer, Oliver and Huh, Sung Un and Cevik, Volkan and Jones, Jonathan DG and Sarris, Panagiotis F **Pathogen perception by NLRs in plants and animals: Parallel worlds**. *BioEssays* 38.8 (2016), 769–781.

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Yan Ma
The Sainsbury Laboratory, Norwich
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1 Introduction

1.1 Overview of plant-pathogen interactions

Plants constantly face the threat of various pathogenic microorganisms, including fungi, bacteria, viruses, oomycetes and nematodes. These pathogens use host-derived nutrients to grow and multiply at the expense of plant health, causing diseases. Yet each plant species is only susceptible to a relatively small number of pathogens, indicating a largely successful plant defence system that fends off numerous invaders [1]. This durable resistance against a wide range of potential pathogens, known as non-host resistance, is common to all plant species [2, 3].

Effective non-host resistance relies on both preformed and inducible defence responses. Preformed structural and chemical barriers (e.g. cuticle layers, cell walls, antimicrobial secondary metabolites) provide constitutive protection at the plant surface and apoplast, whereas inducible defence is often triggered upon the perception of pathogen invasions at the cell wall-plasma membrane interface [4]. Plants can detect physical and molecular changes caused by these invasion attempts, either via recognising exogenous signals as 'non-self' or by monitoring the modified 'self'. The endogenous molecules released upon pathogen invasion are referred to as damage- or danger-associated molecular patterns (DAMPs)[5, 6]. The typical non-self signatures referred to as pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs), are recognised by trans-membrane pattern recognition receptors (PRRs) in plants and in other multicellular organisms [6–8]. Perception of PAMPs by PRRs activates a chain of signalling events leading to a defence response, termed PAMP- or pattern-triggered immunity (PTI) [9].

During evolution, PTI can be overcome by certain pathogens deploying virulence factors (effectors), which can suppress plant defence and render the plant species susceptible to pathogen colonisation [9]. Such plant species are considered hosts. Specific host-pathogen interactions exert strong selection pressure on the hosts, selecting for the evolution

of host resistance within a susceptible plant species. Host resistance relies on plant resistance genes (R genes) that confer specific recognition of pathogen effectors, and activate effector-triggered immunity (ETI) [9, 10]. Plant R genes usually encode intracellular immune receptors called NLRs (Nucleotide-binding domain and Leucine-rich Repeat-containing), analogous to animal intracellular NLR proteins that mediate innate immunity [11]. The first characterised effectors were called "avirulence (AVR) proteins" because they were recognised by an NLR encoded by a specific R gene, resulting in disease resistance and the loss of pathogen virulence [12].

The host-pathogen co-evolutionary dynamics therefore can be exemplified by the interactions between highly polymorphic plant R genes, which encode NLRs, and fast diversifying AVR genes, which encode pathogen effectors, that are selected to evade recognition while retaining function. The "gene-for-gene" hypothesis [13] describes a simple one-to-one relationship between AVR and R genes that genetically determine the outcome of a plant-pathogen interaction. Matching of AVR and R genes leads to host resistance (incompatible interaction), while mismatching leads to disease (compatible interaction). Since then, increased understanding of effector-triggered immunity has revealed that in addition to one-to-one, there are many-to-one, one-to-many or many-to-many AVR-R relationships, thus requiring a more elaborate "genes-for-genes" model [14]. Recent insights on convergent targeting of host proteins by effectors and the cooperation of R proteins for resistance function also emphasise the need for a greater understanding of the protein-protein interaction network in immunity [14–17].

Overall, the plant innate immune system is thought to primarily comprise PTI and ETI. PTI is activated by largely conserved microbe elicitors (PAMPs), while ETI is activated by specific pathogen effectors. The downstream defence responses of PTI and ETI are similar (e.g. changes in gene expression), with the ETI response being quantitatively faster and stronger and usually involving localised cell death called the hypersensitive response (HR) [10, 18]. However, the boundaries between PTI and ETI are blurred at elicitor recognition level, as PAMPs and effectors, PRRs and R proteins sometimes could not be exclusively defined [19, 20]. Furthermore, possible interplays and cross-talk between PTI and ETI defence signalling make it more complex to dissect the output of plant resistance. Therefore the identification of immediate downstream components that convey signals

of PRR and NLR activation may help us better understand the similarities and differences between PTI and ETI. On the other hand, the overall disease outcome also depends on the lifestyle of the pathogen (biotrophy, necrotrophy or hemibiotrophy), the developmental status of the plants, and the environmental elements [1]. For future plant immunity research, finer insights into the molecular processes as well as a broader perspective integrating developmental and environmental elements are needed.

1.2 Pathogen recognition is the key to immunity

Lacking the mobile immune cells found in animals, plants rely on the innate immunity of each cell to recognise pathogens and mount defence responses. The effective pathogen recognition via the trans-membrane and intracellular receptors compensates for the lack of an adaptive immune system in plants. On the other hand, pathogens evolve counter strategies to dodge plant perception and suppress immunity. Therefore, pathogen recognition is the key to plant immunity, and recognition specificity usually defines the specificity of plant-pathogen interactions.

1.2.1 *Recognition of PAMPs by PRRs triggers immunity*

Microbial pathogens need to breach the plant cell wall and sometimes the plasma membrane to gain access to host resources for successful colonisation. While the infectious pathogens are invading the apoplast, they shed PAMPs, which are recognised by PRRs. PAMPs are usually considered as conserved and indispensable for the microbes, serving as molecular signatures for non-self perception of a whole class of microbes in both plants and animals [21, 22].

Plants are capable of recognising a wide range of PAMPs derived from bacteria, fungi and oomycetes [6, 21]. Well studied bacterial PAMPs include flagellin and elongation factor Tu (Ef-Tu), which are recognised by the leucine-rich repeat receptor kinases FLS2 (Flagellin-Sensing 2) and EFR (Ef-Tu Receptor) respectively [23–26]. Flagellin, the main component of the flagellum, is present in almost all flagellated bacteria, including non-pathogenic ones. The epitope flg22 (the N-terminal 22 amino acids of flagellin), corresponding to a region of flagellin required for bacterial virulence and mobility, is sufficient to trigger FLS2-dependent PTI in *Arabidopsis*

thaliana and many other higher plants [23]. More recently, a flagellin epitope (flgII-28) of *Pseudomonas syringae* distinct from flg22 was found to be specifically recognised in *Solanaceae* species by an additional unknown receptor, anticipated as FLS3 (Flagellin-Sensing 3) [27, 28].

Similarly, the Ef-Tu epitope elf18 if N-acetylated is as potent as the full length Ef-Tu to trigger EFR-dependent defence in *Brassicaceae* plants [25]. These examples illustrate the precise targeting of plant perception to the essential core of PAMPs to hinder the rapid pathogen mutations that would otherwise lead to the loss of PAMP recognition. However in some cases, bacteria are able to mutate residues within the recognised epitope to dodge recognition, exhibiting variations in PAMP sequences (e.g. flg22, flgII-28) and the subsequent plant perception [20, 28–31]. Recent evidence suggests that plants may counteract pathogen mutations by evolving receptors to recognise different epitopes within the PAMP, such as flgII-28, CD2-1 (epitopes of flagellin) and Efa50 (an epitope of Ef-Tu) [25, 32, 33]. The diversity found in the PRR-PAMP system suggests a host-pathogen co-evolution more specific than previously suspected [20].

Other characterised bacterial PAMPs include: structural components such as lipopolysaccharides (LPS) and peptidoglycan (PGNs), proteinaceous PAMPs such as harpins and cold shock proteins, microbial nucleic acids such as non-methylated CpG DNA [21, 34–36]; though many corresponding PRRs for these PAMPs are yet to be identified. Fungal chitin is recognised by lysine motif-containing (LysM) proteins *Os*CeBiP and *Os*CERK1 in rice (*Oryza sativa*), and *At*CERK1 in *Arabidopsis thaliana* [37, 38]. Furthermore, LysM proteins such as *Os*LYP4 and *Os*LYP6 in rice, and *At*LYM1 and *At*LYM3 in Arabidopsis directly perceive bacterial PGNs to trigger immunity [39–41]. Interestingly, PGN perception induces complex formation of PGN-receptors with CERK1 to initiate downstream defence signalling, indicating the dual role of CERK1 in chitin- and PGN-triggered immunity [41].

In addition to direct pathogen recognition (PAMP recognition), pathogen invasions can also trigger the production of host-derived compounds termed DAMPs, which signal "danger" in the cell to activate defence response. For example, cell wall integrity is closely monitored by various mechanosensors for physical damage, and by receptors that can detect released plant cell wall fragments during pathogen attack [4, 42]. One such DAMP receptor that contributes to plant immunity is the wall-associated kinases

(WAKs), which monitor the integrity of pectin by sensing the presence of oligogalacturonides [43]. However, for many other cell wall integrity sensors, the detailed mechanisms of ligand perception and signalling during plant defence remain obscure.

1.2.2 *Pathogen-secreted effectors facilitate invasion*

Adapted pathogens usually deliver a suite of effectors into the plants, which promotes pathogen virulence and results in effector-triggered susceptibility (ETS) in host plants [9]. The effector protein family is evolutionarily diverse and exhibits a range of functions targeting different aspects of eukaryotic cellular processes [44]. Recent studies revealed the convergent targeting of effectors from distinct plant pathogens to highly connected host protein 'hubs' [16, 45]. Through mimicking or manipulating host cellular functions, effectors often suppress host defence (PTI or ETI) to promote pathogen colonisation. Fungi and oomycete pathogens typically secrete effectors into the biotrophic interface, of which some remain in the apoplast and some are translocated into the host cytosol, to suppress host defence [46]. Additionally, some of these effectors may also play structural roles during haustorial formation; others may contribute to nutrient release into apoplast during infection.

Bacterial T3SS is a key virulence feature

Diverse gram-negative bacteria use the type III secretion system (T3SS), which forms a needle-like structure, to inject effector proteins into the host cytoplasm. Many of the most important plant diseases are caused by T3SS bacteria, best exemplified by *Pseudomonas syringae*, *Ralstonia solanacearum* and *Xanthomonas spp.* that are responsible for bacterial spot, blight and wilt diseases on a range of crops [47]. T3SS is essential to bacterial virulence, as T3SS deletion from a virulent strain leads to full induction of PTI and the failure of pathogen colonisation in otherwise susceptible plants. However, the virulence function is carried out by its cargo, the T3SS effectors.

Effectors share overlapping functions

Single strains of *Pseudomonas spp.* can deliver ~30 effectors into plant cells, where they function in a diverse and redundant manner. Genome-enabled

bioinformatics and effector mutation screens in *P. syringae* pv. *tomato* (*Pst*) DC3000 and other strains provide valuable insights into the functional composition of the effector repertoire [48]. Although single effector mutations seldom produce significant reduction of virulence, multiple deletions can reveal the importance of redundant effectors. For example, while double knockout of AvrE and HopM1 at the conserved effector locus (CEL) of *P. syringae* severely reduces virulence, single knockouts have no apparent effect on pathogenicity [49]. Similarly, overlapping effector function is illustrated by unrelated *P. syringae* effectors AvrPto and AvrPtoB, which both target the FLS2-BAK1[1] complex to suppress PTI [50, 51]. In fact, Alan Collmer's work on *Pst* DC3000 has revealed that no single effector is necessary or sufficient for pathogen full virulence, implying both redundancy and cooperativity of the effectors [48].

[1] **BAK1:** BRI1-Associated Kinase 1 was identified as a co-receptor of brassinosteroid insensitive 1 (BRI1).

The evolutionary advantage for pathogens to maintain effector redundancy is not immediately apparent in a single host, as it may increase the risk of host recognition. However, because a single effector can sometimes limit host range (due to ETI), having redundant effectors could be crucial for the pathogen to flexibly adapt to a range of hosts. Perhaps as an outcome of counteracting plant recognition, effectors redundantly targeting the same plant protein sometimes use different modes of action. For instance, *P. syringae* effectors AvrB, AvrRpm1, AvrRpt2 and HopF2 all target RPM1-INTERACTING PROTEIN 4 (RIN4), but adopt different molecular strategies: AvrB or AvrRpm1 phosphorylates RIN4 via host kinases; AvrRpt2 cleaves RIN4; whereas HopF2 protects RIN4 from cleavage [52–55] (see later sections for more details).

Do pathogens of different lifestyle tend to deploy specific effectors to modulate certain functions of the host? Or do effectors from distinct pathogens share largely overlapping functions? Wide-scale yeast-two-hybrid analyses of Arabidopsis proteins and pathogen effectors from three different kingdoms suggest that the answer is the latter [16, 45]. These data suggest that independently evolved effectors convergently target a limited set of host protein hubs, which are the heart of highly connected protein-protein interaction networks of defence-related or key cellular processes. Mukhtar et al. [16] postulated that pathogens have evolved effectors to target protein hubs for effective network manipulation. One example is that the *P. syringae* effector AvrPphB and *Xanthomonas campestris* effector AvrAC both target a key PTI-regulating kinase BIK1 (Botrytis-Induced Kinase 1), for

cleavage (AvrPphB) or for uridylylation (AvrAC) [56, 57]. Recent studies also revealed that effectors of distinct pathogens, *P. syringae* (AvrRps4) and *R. solanacearum* (PopP2) convergently target host WRKY transcription factors for immune suppression [58, 59].

T3SS effectors suppress host defence

Many T3SS effectors contribute to virulence by targeting different components of the PTI pathways to suppress plant defence response. The PRR receptor complex at the plasma membrane (PM) is a major target for PTI inhibition: the FLS2-BAK1 complex is targeted by AvrPto and AvrPtoB; BIK1, which is required for FLS2 signalling, is targeted by AvrPphB and *Xanthomonas* effector AvrAC. Many of the above mentioned effectors, including AvrPto, AvrPphB, AvrB, AvrRpm1 and HopF2, exploit the host myristoylation machinery for targeting to the PM where PTI signalling initiates [60]. Interestingly, this behaviour of incorporating eukaryotic myristoylation motifs into bacterial effectors appears specific to plant pathogens [44]. Effectors target other signalling components of PTI. For example, MAPK (Mitogen-activated protein kinase) is dephosphorylated and suppressed by *Pst* DC3000 effector HopAI1. Moreover, some effectors are able to suppress the PAMP-responsive microRNA pathway to inhibit PTI signalling [61], and others can interfere with plant hormone signalling to suppress defence [47].

Vesicle trafficking is important for plant defence responses as it is implicated in delivering various antimicrobial compounds (e.g. pathogenesis related (PR) proteins) and components for cell wall reinforcement to the site of infection [62]. *Pst* DC3000 effectors HopM1 and AvrE as well as *Xanthomonas* effector XopJ have been shown to target vesicle trafficking pathways [49, 63]. HopM1 causes degradation of a guanine nucleotide exchange (GEF) factor named *At*MIN7, which is thought to regulate G protein-mediated vesicle trafficking, via host proteasome [49]. An interesting parallel to animal pathogens is that several bacterial effectors act as GEFs (e.g. *Salmonella* effector SopE and SptP) to manipulate host G proteins involved in cytoskeletal regulation [44].

A group of transcription-activator like effectors (TALEs) from *Xanthomonas spp.* has evolved to directly manipulate host gene expression. Significantly, it was found that the central repeat domain of each TAL effector codes for a precise DNA target sequence. Decoding of these DNA binding mo-

tifs enables the accurate engineering of specific DNA-targeting machinery, which has since become a powerful tool for genome editing [64, 65]. TALEs evolve to activate host susceptibility genes, and yet plants have hijacked this mechanism for defence activation. Plant R gene *Bs3* demonstrates this remarkable countermeasure: It incorporates the binding sequence of AvrBs3 into its promoter; so instead of activating the virulence promoting genes, AvrBs3 activates Bs3 and ETI [66, 67].

Pathogens with avirulence effectors that trigger ETI are under selection to evolve new strategies that can suppress ETI [9]. Indeed, several *P. syringae* effectors have been reported to specifically suppress host recognition of another effector(s). For example, RIN4 cleavage and degradation triggered by effector AvrRpt2 suppresses AvrB- and AvrRpm1-mediated activation of RPM1 [52–54]. When AvrB or AvrRpm1 induces phosphorylation of RIN4 via the host kinase RIPK (RPM1-interacting protein kinase), RPM1 senses the conformational change of RIN4 (following phosphorylation at Threonine 166) and triggers defence [68, 69]. A recent study has revealed that AvrPphB can suppress RPM1 recognition of AvrB via cleaving RIPK [70]. Intriguingly, this AvrPphB-mediated ETI suppression is specific to AvrB but not to AvrRpm1, suggesting that RIPK is not required for AvrRpm1 phosphorylation of RIN4 [70]. Consistent with this, the *ripk* knockout does not impair AvrRpm1-triggered resistance in Arabidopsis [68]. Furthermore, while host R protein RPS2 can recognise AvrRpt2-induced RIN4 cleavage, effector HopF2 can interfere with this RIN4 cleavage, and thereby suppress RPS2 activation [55].

Besides deploying new effectors to counteract ETI, pathogens are capable of 'upgrading' effectors to defy their own recognition. For instance, while the N-terminus of AvrPtoB can be recognised by the plant kinase Fen, resulting in programmed cell death in resistant plants, the C-terminus of AvrPtoB remarkably mimics E3 ubiquitin ligase and targets Fen for proteasomal degradation in susceptible plants [71, 72]. In other words, the acquisition of the C-terminal E3 ligase domain 'upgrades' the avirulent AvrPtoB, enabling the suppression of its own ETI through Fen degradation. Altogether, these examples demonstrate the the intricacy of evolutionary arms race between the plant recognition mechanism and pathogen suppression of immunity.

T3SS effectors evolve multiple functions

What domains and motifs of an effector are responsible for its virulence function(s)? Despite the immense diversity of effector functions, they seem to possess a relatively small subset of domains and motifs that have defined functions in virulence. The N-terminal part of most T3SS effectors contains a 15-25 aa signal sequence essential for type III secretion, followed by a longer downstream chaperone-binding domain. Consequently the N-terminal amino acid composition have been used to identify potential effectors. However, although the N-terminal secretion signals are often functionally interchangeable between effectors, they lack apparent consensus sequences [44, 73].

Effectors are often multifunctional proteins. In fact, each effector protein can contain functionally distinct domains or motifs, which appear to evolve independently and combine to form chimeric effectors, a common effector phenomenon called 'chimerization'. Stavrinides et al. [74] found a higher percentage of chimeric effectors (32%) amongst all T3SS effector families compared to other protein families. The evolutionary advantage of effector chimerization is possibly to enable rapid emergence of new effectors to maintain diversity while conserving basic virulence functions. For example, *P. syringae* effectors HopD1 and HopAO1 share a homologous N-terminal domain, while each possessing a unique C-terminal domain that allows them to perform distinct biological functions [47, 75]. Similarly, AvrRps4 and HopK1 from *P. syringae* with distinct C-termini also share an N-terminal domain, which is believed to target the effectors to the host chloroplast [76]. SptP exemplifies such a chimeric effector from the animal pathogen *Salmonella*, which is composed of a N-terminal domain similar to *Pseudomonas* ExoS, and a C-terminal region homologous to YopH [44]. Since different domains within an effector can carry out distinct and unrelated functions, the effector repertoire can be viewed as a mosaic of functional domains combined together.

Another driving force behind effector evolution is hypothesised to be horizontal gene transfer (HGT). Some evidence suggests that bacterial effector genes have distinct G/C base composition [77] and produce unique phylogenies [47] compared to the rest of the bacterial genome. It provides an attractive hypothesis where pathogens may acquire foreign genetic materials, which they mix and match via genomic shuffling to rapidly produce

fresh effector repertoires. Bacterial conjugation and DNA transfer via viruses might assist HGT, however, exactly how HGT occurs is unclear.

Fungi and oomycetes secrete effectors to aid invasion

Fungi such as rice blast fungus *Magnaporthe oryzae*, and oomycetes such as the potato late blight pathogen *Phytophthora infestans* are eukaryotic filamentous pathogens that pose serious threats to crops. Fungi and oomycetes are known to secrete effectors at different invasion stages, and the effectors function in extracellular spaces (apoplast) as well as in diverse subcellular compartments after translocation into the host cytosol [46]. Fungal effectors often carry a signal peptide for secretion. Oomycete effectors sometimes carry characteristic motifs associated with host translocation (e.g. RXLR motif of *P. infestans* effectors) after the signal peptide. However, very few conserved motifs exist to allow predictions of effector function. It remains a challenge to dissect the functions of fungi and oomycete effectors, especially with the diverse nature of their spatiotemporal deployment.

Most fungi and oomycete effectors are host-induced and expressed at different stages associated with pathogen transitions [46]. Some evidence suggests that effectors can be deployed even before pathogen penetration [78]. Kleemann et al. [78] showed that distinct sets of *Colletotrichum higginsianum* candidate effectors are delivered in successive waves: one before penetration and another before and during penetration [78]. The tomato leaf mould fungus *Cladosporium fulvum*, which proliferates in extracellular spaces, typically deploys apoplastic effectors to combat host defence. Other pathogens such as *M. oryzae*, *P. infestans* and *C. higginsianum* invade host cells with intracellular hyphae, and secrete apoplastic effectors predominantly at the biotrophic interaface [46].

To defend against fungal or oomycete pathogens, plants secrete chitinases to breakdown chitin, a major component of the fungal cell wall. *C. fulvum* effector Avr4, a chitin binding effector, is able to protect the fungal cell wall from plant chitinases [79]. Chitin fragments released by chitinases can also be recognised as PAMPs to trigger PTI. The *C. fulvum* LysM-containing effector Ecp6, however, binds chitin oligosaccharides with high affinity, which is believed to out-compete chitin-binding PRRs to reduce fungal recognition [80]. Apoplastic LysM effectors are also used by other fungal

pathogens (e.g. slp1 from *M. oryzae*) to suppress chitin-induced PTI. Another *C. fulvum* apoplastic effector Avr2 inhibits the cysteine protease RCR3 in tomato. However, the Avr2-mediated modification of RCR3 is recognised by the plant transmembrane receptor-like resistance protein Cf-2, triggering defence [81]. Similar resistance proteins Cf-4 and Cf-9 recognise *C. fulvum* Avr4 and Avr9 respectively, via their extracellular membrane-anchored LRR domain [82–84].

Some effectors are translocated into the host cell after secretion. For example, the *P. infestans* cytoplasmic effector Avr3a stabilises a host E3 ubiquitin ligase (CMPG1) to block defence-related cell death and promote biotrophic growth in potato [85]. Another cytoplasmic effector Avrblb2 of *P. infestans* accumulates around the special feeding structure, termed the haustorium, to prevent the secretion of a tomato immune protease C14 [86]. Interestingly, the *Uromyces fabae* cytoplasmic effector Rtp1, which also accumulates around the haustoria, was suggested to play a structural role during infection [87]. Kemen et al. [87] reported that Rtp1 forms amyloid-like filaments *in vitro*, and may stabilise and protect the haustoria structurally.

Additionally, some fungal and oomycete effectors can contribute to nutrient release into the apoplast during infection [9, 88]. Furthermore, a recent study revealed that the root pathogen *Fusarium oxysporum* is able to secrete a virulence peptide reminiscent of the plant RALF (rapid alkalinization factor) to hijack host regulatory pathways and increase infection [89]. Overall, better understanding of the dynamic functions of fungi and oomycete effectors would provide valuable insights to plant-pathogen interaction.

1.2.3 *Recognition of effectors by NLRs triggers immunity*

To counteract the virulence activity of pathogen effectors, plants have evolved R proteins dedicated to recognise specific effectors and to trigger defence. They either directly recognise effectors through physical interaction, or indirectly via recognising the effector-triggered modification of a host protein. Most plant R proteins contain a characteristic NB-ARC domain[2] and a leucine rich repeat (LRR) domain, thus are called NLR proteins. Generally, plant NLRs can be divided into two classes based on their N-terminal signalling domain: The TNLs, which contain an N-terminal

[2] **NB-ARC domain:** Nucleotide-Binding domain shared by human apoptotic regulator APAF-1, plant R proteins and CED-4 from *C.elegans*.

TIR (Toll/Interleukin-1 receptor/Resistance protein) domain; and the CNLs, which contain an N-terminal CC (coiled-coil) domain. Similarly, animal NLRs are comprised of a nucleotide binding NACHT domain[1], a LRR domain and an N-terminal signalling domain, which is often a PYRIN domain (PYD) or a caspase recruitment domain (CARD). Together, plant and animal NLRs form part of a structural class termed STAND[2] [90]. Accumulating evidence is revealing interesting functional parallels between the NLRs from the two kingdoms. In this section, I will mainly focus on the diverse mechanisms plant NLRs used to maximise their recognition capacity and to protect various cellular processes from pathogen effectors. Based on well-studied examples, several models for NLR-mediated effector recognition have been proposed, including the receptor-ligand model, the guard hypothesis and the decoy model, although none of the current models is able to adequately describe all recognition mechanisms.

NLR proteins can recognise their ligands through direct binding

Some NLR receptors directly interact with their effector ligands to activate defence, as proposed by the 'receptor-ligand' model. Interestingly, the majority of these direct recognition examples are demonstrated for fungi or oomycete effectors. For instance, rice CNL Pi-ta recognises the rice blast fungus (*M. oryzae*) effector Avr-Pita through direct interaction [91]. In addition, flax TNLs L and M interact with cognate effectors AvrL567 and AvrM from the flax rust fungus *Melampsora lini* respectively [92, 93]. Likewise, the recognition of the downy mildew (*Hyaloperonospora arabidopsidis*) effector ATR1 by Arabidopsis TNL RPP1 is also mediated by direct interaction [94]. The high level of observed polymorphism and the presumed flexibility of the LRR domain makes it a logical candidate site for effector interaction; it is also the site of interaction between many cell surface PAMP detectors and their ligands [95]. Indeed, the NLRs RPP1 and Pi-ta interact with their cognate effectors via their LRR domains. However, in some cases, the N-terminal TIR or CC domain of NLRs can interact with the AVR ligand [96, 97]. The receptor-ligand model focuses on the necessity but not the sufficiency of the AVR/R interaction. Therefore we should not exclude the requirement

[1] **NACHT domain:** NAIP (Neuronal Apoptosis Inhibitor Protein), C2TA (MHC class 2 Transcription Activator), HET-E (incompatibility locus protein from *Podospora anserina*) and TP1 (telomerase-associated protein).

[2] **STAND:** Signal Transduction ATPase with Numerous Domains. This class of P-loop NTPases includes the AP-ATPases (e.g. APAF-1, CED-4, and plant R proteins) and NACHT NTPases.

for AVR/R subcellular localisation and the roles of accessory proteins during effector detection and NLR signalling.

NLR proteins can indirectly recognise effectors via "guarding" their host targets

In most cases, however, plant NLR proteins do not directly interact with their cognate effectors. To explain the observed indirect recognition, the 'Guard hypothesis' was proposed, in which an R protein "guards" (ie monitors the integrity of) the virulence target (guardee) of the effector to activate defence after detection of effector-induced modifications [98]. This model is best exemplified by the guardee RIN4, which is targeted by multiple *P. syringae* effectors and is guarded by two NLRs. RPM1 and RPS2 (NLR proteins) constitutively associate with RIN4 to detect the AvrB- or AvrRpm1-mediated phosphorylation and AvrRpt2-mediated protease activity respectively [52, 53]. The convergent effector targeting on RIN4 implies a central role of RIN4 in immunity and/or pathogenicity. So far, studies suggest that RIN4 is a versatile immune regulator, playing both positive and negative roles in various defence pathways [99, 100].

For example, FLS2 or EFR-induced phosphorylation of RIN4 at residue S141 enhances PTI signalling; and AvrB- and AvrRpm1-mediated phosphorylation of RIN4 T166 could inhibit this PTI-promoting S141 phosphorylation of RIN4 [100]. In addition, HopF2 that ribosylates RIN4 may also promote pathogen virulence via inhibiting the accumulation of S141 phosphorylated RIN4 to suppress PTI [69]. On the other hand, the NO_3-induced (NOI) domain containing fragments of RIN4, which are released upon AvrRpt2 cleavage, are capable of suppressing PTI [101]. Also, RIN4 is proven to assist pathogenicity through enhancing stomata opening [99]. Perhaps the multifunctional nature of RIN4 in a highly interconnected immune network makes it an attractive effector target, and a hub that is worth guarding.

In an elaboration of the guard model, van der Hoorn and Kamoun proposed that plants could evolve guarded decoys that had lost their original functionality and now only functioned as "effector baits" [102]. These decoy proteins, possibly evolved from duplications of the effector target gene, may divert the selection pressure from the real effector target, and specialise in enabling effector perception [10]. This model is consistent with the protein kinase Pto, which activates tomato NLR Prf upon detection of

P. syringae effectors AvrPto and AvrPtoB [103, 104]. As it plays no critical role during basal defence, Pto is thought to be a decoy that mimics the kinase domains of the effector host targets, such as FLS2 and BAK1 [50, 51]. Another decoy protein kinase PBS1 (AvrPphB-Susceptible 1) activates the NLR RPS5 upon its cleavage by the cysteine protease effector AvrPphB [105, 106]. PBS1 closely resembles a group of PTI-regulating RLCKs (Receptor Like Cytoplasmic Kinases) such as BIK1 and PBL1 (PBS1-like kinase 1) that are targeted by AvrPphB [56]. It should be noted that although Pto and PBS1 are decoys of immune kinases, their kinase activity is not lost and is required for ETI activation [106–108] . The decoy protein ZED1, on the other hand, is a pseudokinase that functions to detect the acetylation by *P. syringae* effector HopZ1a and to activate the associating NLR ZAR1 [109]. Interestingly, ZAR1 forms a complex with another pseudokinase RKS1, which specifically recruits the kinase PBL2, when PBL2 is uridinylated by *Xanthomonas* effector AvrAC [57]. PBL2 has been shown to be a decoy of the AvrAC target, BIK1; and the kinase activity of PBL2 is not required for AvrAC-triggered immunity [57]. These examples demonstrate that whether the 'guardee' is a true decoy (functional mimic) that only participates in effector recognition is not entirely dependent on the loss of ancestral biochemical function. Altogether, the guard and decoy models describe efficient mechanisms by which a plant can use a limited repertoire of NLRs to recognise a multitude of pathogens via specifically guarding a limited number of host proteins.

NLR proteins evolve to integrate domains that can detect effectors

Certain plant NLRs integrate atypical domains into the canonical TIR-NB-ARC-LRR or CC-NB-ARC-LRR structure. Recently, many of these integrated domains have been associated with a specialised function in effector detection, expanding our view of NLR recognition mechanisms [58, 59, 110–113]. Detailed functional analyses of two NLR pairs in particular, the Arabidopsis RRS1/RPS4 and the rice RGA4/RGA5, have led to the proposition of the 'integrated decoy' model [112]. In this model, Cesari et al proposed that the atypical domains of NLRs are likely to be decoys of the true effector host targets, incorporated into the NLR structure for effector sensing. Arabidopsis TNL RRS1 may have integrated such a C-terminal WRKY domain, homologous with the family of WRKY transcription factors. It has been recently reported that bacterial effectors PopP2 from *R. solanacearum* and AvrRps4 from *P.syringae* interact with Arabidopsis TNL RRS1 via its integrated WRKY

domain [58, 59]. Since both AvrRps4 and PopP2 associate with several host WRKY proteins, presumably to suppress their function in immunity, it was proposed that RRS1 integrates a structural mimic of the effector targets (WRKY proteins) for effector detection [58, 59]. RRS1 forms a constitutive complex with another TNL RPS4, which is adjacent and divergently transcribed from RRS1 [114, 115]. Unlike RRS1, RPS4 has a canonical TIR-NB-ARC-LRR structure, and acts as a signalling partner for RRS1 [115, 116]. Similarly, the rice CNL pair RGA4/RGA5 functions to recognise two *M. oryzae* effectors, AVR1-CO39 and Avr-Pia, via an integrated heavy metal-associated (HMA) domain at the C-terminus of RGA5 [110]. The HMA domain is also integrated into the rice CNL Pikp-1, between its CC and NB-ARC domain, which functions to directly interact with the *M. oryzae* effector AVR-PikD [113]. Again, Pikp-1 requires its pair partner Pikp-2, to activate defence. Rice HMA domain-containing protein Pi21 is a disease susceptibility factor [117]. It was therefore hypothesised that these integrated HMA domains are decoys of potential HMA-containing host proteins targeted by effectors for pathogen virulence [112].

Recent studies have revealed the widespread occurrence of atypical domains in NLR proteins in various plant genomes [118, 119]. Most of these domains appear to have a distinct evolutionary history compared to the rest of the NLR domains, indicating a relatively recent fusion event that has combined them to make the chimera NLR [118].

Where do these extraneous domains originally come from? One explanation in line with the integrated decoy model states that these domains derive from duplicated effector host targets, which subsequently fuse into the NLR structure [112, 120]. During the course of evolution, the integrated domain would evolve specifically towards effector detection and might lose its ancestral function (becoming a decoy). However, Wu et al. [120] pointed out that some domains might retain their ancestral functions possibly to assist immunity, acting more like integrated 'guardees' rather than 'decoys'; and thus the broader term 'sensor domain' is more suitable. After the fusion event, the NLRs equipped with an effective 'effector sensor' would be selected for, and retained. Supporting this theory, Saucet et al. [121] has identified a possible pre-WRKY fusion ancestor of RRS1 and its paralog RRS1B, present in *Brassica rapa* and *Arabidopsis lyrata*, but lost in *Arabidopsis thaliana* [121]. Interestingly, the orthologs of RRS1 or RRS1B in *A. lyrata* and *Capsella rubella* appear to be further duplicated, showing evidence of selec-

tive advantage of the WRKY fusion [121]. One of the advantages could be the rapid acquisition of new effector recognition capacity. Additionally, the physical fusion of the guard to a guardee (or decoy) would ensure adaptive co-evolution and genetic co-segregation.

Some domains are observed to be integrated more often than others, with protein kinase, WRKY and BED domain among the most common [118, 119]. WRKY transcription factors and various protein kinases are often associated with plant immunity, therefore could be important nodes for the plants to guard against the effectors. Recently, a BED-domain containing ZBED protein in rice has been shown to mediate resistance towards *M. oryzae* [119]. Intriguingly, 32 NLRs in poplar (*Populus trichocarpa*) have been identified to carry the BED domains [122], indicating possible targeting of BED-domain proteins in poplar by effectors. Some of these integrated domains have been identified in NLRs with known functions: wheat stem rust resistance protein Rpg5 in barley has an integrated kinase domain [123]; Arabidopsis RRS1 and RRS1B both integrate a C-terminal WRKY domain [114, 121]; and rice Xa1, with integrated BED domains, recognises an unknown bacterial blight effector [124]. Conceivably, many other NLR fusions may be functional effector sensors that use similar strategies.

With the observations of effectors converging on important cellular hubs to suppress immunity [16, 45], it is plausible that NLR proteins would evolve to incorporate hub-mimicking domains to ensure efficient host protection, and to maximise their gain of recognition capacity. In line with this is the discovery of a RIN4-like NOI domain fused into the rice CNL Pi5-2, which pairs with Pi5-1 [125, 126]. Additionally, the CNL AetRGA2a has a C-terminal integrated domain, which mimics the Exo70 protein, a component of the exocyst complex [127]. Exo70 plays a crucial role in polarised exocytosis (vesicle trafficking) that is important for plant immunity (discussed previously). It is also guarded by rice CNL Pii to detect the *M. oryzae* effector Avr-Pii [128]. Exo70 protein is also guarded in Arabidopsis, as the loss of function mutant *exo70b1* triggers constitutive defence activation dependent on a TIR-NB-ARC-only protein (TN2) [129]. Many other integrated domains of NLRs do not yet to have any defined role in immunity (or susceptibility), such as the LIM domain of Arabidopsis CHS3 [130] and the brevis radix domains of RLM3 [131]. The implications of integrated domains resembling important cellular targets of effectors could provide a new source to identify immune components previously unknown to us [118, 119, 126]. Moreover,

increasing insights into the recognition and activation of fusion NLRs will open up the possibility to engineer new or expanded resistance capacities via manipulating the integrated domains.

NLR proteins often cooperate to function

Many NLRs require another NLR to function. Some are genetically linked pairs that work closely with each other, such as RRS1/RPS4, RRS1B/RPS4B, RGA4/RGA5 and Pikp-1/Pikp-2 [110, 113, 114, 121]. Interestingly, functional specialisations of pair partners are implicated in all above examples: typically one partner executes defence signalling (termed 'the executor'), and the other senses the effector through an integrated domain specialised for detection (termed 'the sensor'). In fact, pair partners exhibiting differential domain architectures are frequently observed: Arabidopsis TNLs RPP2A/RPP2B [132] and CHS3/CSA1 [130], wheat CNLs LR10/RGA2 [133] and melon Fom-1/Prv [134] are all pairs with only one member containing additional domains, yet no function has been ascribed to them. Presumably, co-evolution of the linked pair partners, especially those that function together in a complex, could result in increasingly specific cooperation. The paralogous pairs RRS1/RPS4 and RRS1B/RPS4B demonstrate such specificity, as inappropriate pairings of RRS1/RPS4B and RRS1B/RPS4 are non-functional [121].

On the other hand, some NLRs cooperate with genetically unlinked NLRs, and often with less specificity. For example, the TNL N in tobacco (*Nicotiana tabacum*) requires a CNL NRG1 to recognise p50 of the tobacco mosaic virus (TMV) [135]. Another example of a TNL and a CNL that function together is the Arabidopsis TAO1 (TNL) and RPM1 (CNL), which additively provide full resistance to *P. syringae* carrying AvrB [136]. However, although required by RPM1 for AvrB recognition, TAO1 is able to function independently from RPM1, suggesting partial cooperation of the NLRs [136]. Tomato NLR Prf, which functions in complex with the kinase Pto, requires the NLRs NRC2a/b and NRC3 [137]. In addition, three redundant CNLs (ADR1, ADR1-L1 and ADR1-L2) are shown to be required for the function of several unrelated Arabidopsis NLRs [138, 139]. These NLRs, which assist one or more NLRs to function, are often referred to as helper NLRs. Helper NLRs are perceived to act downstream of the 'sensor' NLR that detect the effectors, mirroring the role of the 'executor' in genetically linked pairs. Several helper

NLRs appear to co-evolve with a specific clade of NLRs, with which they may cooperate with broad specificity [137, 140]. Nevertheless, it is not simple to define whether the cooperation between helpers and sensors or between pair partners would evolve to become more specific or more general, as various elements could steer the evolution differently.

1.3 Activation and signalling: how do plant receptors convert recognition to defence?

1.3.1 *PTI signalling and responses*

The best understood PTI signalling and response are demonstrated in the FLS2-mediated pathway. Upon flg22 elicitation, FLS2-flg22 recruits the co-receptor BAK1 in a stable complex, resulting in auto-phosphorylation of both FLS2 and BAK1 [141]. *bak1* mutants are impaired in their flg22 response, which render them susceptible to *Pseudomonas syringae* [142]. BAK1 is an LRR-RLK (Receptor-Like Kinase) that has been shown to regulate the brassinosteroid receptor kinase BRI1, which functions in plant development and growth [142, 143]. In fact, BAK1 or related SERK proteins have been implicated to form complexes with several other PRRs to mediate their activation [144, 145].

The receptor-like cytoplasmic kinases (RLCKs) are the direct substrates of PRR complexes to activate downstream PTI responses [95, 145]. One such RLCK, BIK1, associates with both FLS2 and BAK1 in the absence of flg22, and flg22 treatment leads to the phosphorylation and disassociation of BIK1 [56]. Interestingly, single and/or double mutants of BAK1 and BIK1 result in salicylic acid (SA)-dependent cell death, suggesting that their activities may be protected against effector suppression [146]. The release of BIK1 is believed to bring about the activation of PTI signalling. Importantly, BIK1 directly phosphorylates the NADPH oxidase RBOHD [147, 148], which is responsible for the rapid production of apoplastic reactive oxygen species (ROS), a hallmark of PTI.

FLS2 also interacts constitutively with the DENN-domain protein SCD1 (Stomata Cytokinesis-Defective 1), which has been shown to positively regulate FLS2-medieated signalling (such as ROS burst) [149]. SCD1 plays a role in endocytosis during cytokinesis and cell expansion [150]. This is consistent with the hypothesis that endocytosis and degradation of PRRs after ligand

activation may serve to prevent constitutive signalling, and also to recycle and refresh PRRs at the PM [151].

Downstream of PAMP perception, at least two separate MAPK (Mitogen Activated Protein Kinase) cascades are activated, which subsequently reprogram a set of immune related genes via phosphorylating WRKY transcription factors. In addition, PAMP perception triggers a calcium (Ca^{2+}) burst that activates CDPKs (Calcium-Dependent Protein Kinases), which can also turn on transcription factors. In *N. benthamiana*, CDPKs phosphorylate the NADPH oxidase RbohB to induce ROS burst [152]. ROS and nitric oxide (NO) serve as important signalling messengers and antimicrobial compounds during PTI. At later stage, callose deposition, strengthening of the cell wall and limiting water and nutrients availability all contribute to restricting pathogen growth.

1.3.2 *NLR activation and signalling: Intra- and inter-molecular regulation is essential for a robust immune response*

The multi-domain NLRs accomplish dual roles of pathogen perception and signal initiation for the activation of defence. Canonical NLRs consists of an N-terminal CC or TIR motif as the scaffolding and signalling domain; a central nucleotide-binding NB-ARC domain as the 'molecular switch'; and a C-terminal LRR as the regulatory domain [153, 154]. NLR-mediated defence activation often leads to cell death, so activation of NLRs must be both resilient to inappropriate stimuli to avoid ectopic activation, and sensitive to specific pathogenic stimuli. How do hosts fine-tune their NLRs at rest and then activate them with great versatility? As discussed in previous sections, increasing evidence shows that combinatorial cooperation of NLRs and the fusion of non-canonical domains may greatly enhance the NLR repertoire [17, 155]. Current models for canonical NLR activation in plants provide the foundation for understanding individual domain activity, but need expanding in light of recent findings of NLR pairings and non-canonical NLRs.

The NB-ARC domain is required for ATP/ADP binding.

The conserved NB-ARC domain of plant NLRs is believed to act as a molecular switch regulated by nucleotide binding and ATP hydrolysis activity [153, 154]. Similarly, the structurally related NACHT domains of mammalian NLR proteins also directly bind nucleotides and have ATPase activity [156,

157]. The architecturally and functionally similar APAF-1 undergoes intramolecular conformational changes when activated, with the subsequent replacement of ADP to ATP at the NB-ARC domain [158]. This is proposed to be the core mechanism of NLR activation [153, 159, 160].

In plants, supporting evidence comes from mutational analysis: when mutated, conserved motifs in the NB-ARC domain essential for nucleotide binding (P-loop, MHD[3]) or for hydrolysis (Walker-B) often lead to loss-of-function or auto-activity of the NLR [116, 129, 161–165]. However, direct structural evidence demonstrating changes from a closed ADP-bound 'OFF' state to an open ATP-bound 'ON' state is lacking for plant NLRs. Recent studies on two CNLs, potato Rx1 and tomato I-2, have linked the NLR activation with the newly discovered DNA-binding activity of the NB-ARC domains [166, 167]. These findings implicate a possible direct role of active NLR proteins on DNA, bending or melting the DNA, which may contribute to defence activation [166, 167]. However, it remains to be seen if a binding partner is required to provide some DNA-binding specificity. Furthermore, an 'Equilibrium-based switch' model was recently proposed based on a study of flax TNLs L6 and L7. This model proposes that NLRs exist in an equilibrium between 'ON' and 'OFF' states, and effectors bind to the 'ON' state to stabilise the active conformation and shift the equilibrium towards activation [168].

[3] **MHD:** conserved methionine histidine aspartate motif present at the carboxy-terminus of ARC2.

Recent studies of mouse NLRC4, using crystallography and cryo-electron microscopy, have resolved this NLR structure in both an inactive state and in an active oligomer complex [169–172]. Structural comparison revealed a dramatic rearrangement of the subdomains in the NACHT, creating a new oligomerisation surface crucial for NLRC4 activation.

Although ADP was implicated to be critical for maintaining a closed conformation of NLRC4 [169], it is unclear whether ADP/ATP exchange is the cause or consequence of activation [171, 173].

In plants, several examples indicate that ADP/ATP exchange is not always required for NLR activation. Rice CNL Pb1 (Panicle blast 1), lacking the P-loop motif for ADP/ATP binding, provides durable resistance to a broad range of *M. oryzae* races [174]. In the paired NLR systems of Arabidopsis RRS1/RPS4 and rice RGA4/RGA5, for the immune complex to activate, an intact P-loop is only required in one of the partners (RPS4 and RGA4), but is dispensable in the other partner that detects effectors (RRS1 and RGA5) [112,

116]. This reflects the functional specialisation of the pair partners, each adopting a separate role as sensor and executor respectively. As an immune complex may only need one NB-ARC switch to execute defence activation, the NB-ARC function of those NLRs specialised in effector sensing (e.g. RRS1 and RGA5) would become redundant. Intriguingly, this redundancy of P-loop activity seems to coincide with the integration of extraneous domains, which are often observed in paired NLRs, although not exclusively [15, 112, 119, 126]. Similar to the pairs, 'helper NLRs' that function in a complex with other NLRs also show examples of a relaxed requirement for a functional P-loop: one such example is ADR1-L2, which retains its helper function even with a mutated P-loop [138, 163]. However, this is not the case for all plant helper NLRs. In parallel, the animal NLR NAIP5 does not require ATP binding activity to initiate NAIP5-NLRC4 inflammasome assembly; although unlike ADR1-L2, NAIP5's role is to sense the elicitor [175].

Auto-inhibition of NLRs is mediated by multiple domains

Intra- and inter-molecular interactions are important to regulate the fine balance between auto-inhibition and activation of NLRs. To stabilise the 'off' state at the resting stage, NLRs are likely to form a compact conformation, involving multiple interactions between domains. Various domain surfaces of NB, ARC2 (subdomains of NB-ARC) and the LRR N-terminus have been shown required to retain the 'off' conformation, as domain swaps or specific point mutations in them often trigger constitutive activation [176, 177]. The N-terminal domains (TIR or CC) are often predicted to be in close proximity with the LRR C-termini, and may also structurally regulate the ATP/ADP exchange at the NB-ARC domain [154, 168, 178]. Accordingly, a simple model emerges for auto-inhibition that the N-terminal domain and the LRR domain enclose the central NB-ARC domain, inhibiting active ATP/ADP exchange and defence activation [153, 154, 179].

LRR domains of NLRs have been shown to play an important regulatory role, to prevent inappropriate activation, and to transduce the effector-activation signal to de-repress the immune receptor complex. In the absence of empirical structural knowledge, predictions of several plant LRR domains (from Lr10, Rx1, Gpa2) helped to generate representative structural models, which suggest a bipartite-functioning LRR, with the N-termini contributing to NLR auto-inhibition, and the C-termini mediating pathogen

recognition [179–181]. In these models, the positively charged LRR N-terminal region associates with a negatively charged region of the NB-ARC to stabilise the closed conformation in an inactive NLR [154]. This is exemplified by RPS5, in which the first 4 LRRs are required for the LRR association with the NB-ARC in order to keep the NLR inactive [182]. Consistently, LRR dis-association due to interface disruption via domain swap or residue mutations also causes auto-activation of some NLRs [180, 181]. In addition, deletion of LRR domains in many plant NLRs results in auto-activity [183–185]. This is also true for several animal NLRs; for example, deletion of the LRR domain of NLRC4 or NOD1 results in constitutive defence activation (Caspase1-dependent cell death) in the absence of the elicitors [175, 186]. The crystal structure of NLRC4 revealed that interaction between the LRR and the nucleotide-binding domain (NBD), as well as interactions between the NBD and a C-terminal subdomain of the NACHT (called HD2) sterically prevented oligomerisation of NLRC4, providing an explanation for the auto-inhibitory mechanism of the LRR [169].

Nevertheless, LRR interaction with the NB-ARC has recently been shown to positively regulate NLR activity: In the auto-active maize Rp1 (CNL) proteins, the interaction of the LRR with the NB-ARC de-represses CC domain via releasing NB-ARC/CC association [165]. Another example demonstrating the positive role of the LRR domain is that the expression of Rx NB-ARC domain with auto-active mutations requires the presence of the LRR to trigger full host defence [178].

On the other hand, domain swaps between Rx and its homologue Gpa2 revealed the key role of LRR C-termini in pathogen recognition specificity [153, 178]. This LRR-driven specificity is also observed for flax L5 and L6, which perceive variants of flax rust effector AvrL567 [187]. Consistant with its function, the highly variable structure of LRR C-termini exhibits strong diversifying selection, possibly under the pressure of ever-changing pathogen effectors [98, 188] . Although the exact role of LRRs during pathogen perception awaits clarification, at least in some cases, LRRs can act as a sensor for effector-induced perturbations [179].

With the recently apparent function of integrated domains in NLR proteins in effector sensing, it is logical to ask: how do they convert the effector perception into NLR activation? As with the multi-functional LRR domain, it is conceivable that the integrated domains may also function

as both an effector-sensor and a regulatory module. Consistent with this hypothesis, mutating or acetylating the WRKY domain of RRS1 is sufficient to trigger effector-independent cell death when transiently or stably co-expressed with RPS4 in *N. tabacum* [58, 59]. This suggests that the WRKY domain negatively regulates the RRS1/RPS4 immune complex when in a resting state, and pathogen recognition via the WRKY domain releases this negative regulation, activating immunity. It would be interesting to test whether the fusion domain in other paired NLRs (HMA domain in RGA4/RGA5 and Pikp-1/Pikp-2, and the LIM domain of CHS3/CSA1) also functions similarly to RRS1 WRKY domain as an auto-inhibitory and sensor domain. Intriguingly, the N-terminal 'sensor domain' of mouse NLRP1b also contributes to auto-inhibition, as the N-terminal cleavage by anthrax lethal factor activates NLRP1b and initiates inflammasome assembly [189].

Last but not least, auto-inhibition maintained by domain-domain interactions is also essential for competent activation upon elicitor perception, as the activation requires appropriate de-repression. Indeed, NLR residues essential for auto-inhibition and activation often overlap. However, the fact that mutations can lead to either constitutive activation or loss-of-function indicates auto-inhibition and activation require unique aspects of domain-domain interactions. By shuffling polymorphic sites between Rx1 and Gpa2, Slootweg et al. [181] have revealed that residues on different interfaces of the ARC2 domain, ARC2-LRR and ARC2-NB interfaces, are involved in effector-dependent and -independent activation respectively [181]. This shows how a single domain could play different roles during auto-inhibition and activation via interactions at different interfaces.

The N-terminal TIR/CC domain of NLRs is important for signalling and oligomerisation

The majority of plant NLRs seem to rely on their N-terminal domains for defence signalling [140]. Transient overexpression of the TIR domains of many TNLs (e.g. RPS4, RPP1, L10, L6) and the CC domains of some CNLs (e.g. MLA, NRG1, ADR1) induces cell death [94, 140, 168, 185, 190]. In these systems, TIR or CC domains are often viewed as the minimal functional unit for cell death induction [159, 191]. It is often observed that the TIR/CC of the executor component of an NLR pair (e.g. RPS4) or the helper NLR (e.g. ADR1, NRG1), which is directly involved in signalling, triggers cell

death when expressed as a truncated protein, or is at least essential for cell death mediated by ectopic NLR expression [135, 138, 185]. It is possible that cell death triggered by the overexpression of CC or TIR domains is an ectopic activation that differs from authentic NLR immune signalling. However, not all N-terminal domains of functional NLRs trigger cell death when overexpressed, for example, the sensor NLR RRS1 does not.

TIR/CC domains also function as scaffolds, facilitating the formation of NLR complexes. For example, self-association of the CC domain is required for homo-dimerisation and activation of the barley CNL MLA10 [192]. Alternatively, hetero-dimerisation of the N-terminal domains is necessary to build the functional NLR-pair complexes RPS4/RRS1 (TIR/TIR) and RGA4/RGA5 (CC/CC) [111, 115]. On the basis of transient co-expression in *Nicotiana spp.* followed by co-immunoprecipitation, these NLR pair complexes appear to be assembled prior to effector activation. Inactive immune complexes likely adopt a closed conformation that sequesters the signalling surface of TIR/CC domains. Consistently, structural models predict a spatial proximity of TIR/CC to LRR C-terminus in the absence of the effectors [154]. Upon effector activation, conformational changes occur that might allow the recruitment of downstream signalling components as part of defence activation. It has recently been shown that the CC domain of Rp1 directly interacts with its NB-ARC domain, and this interaction prevents the CC-domain triggered defence activation [165].

Some animal NLRs multimerise to form a wheel-like structure, termed the inflammasome [170, 171, 173, 193, 194]. Inflammasome assembly promotes close proximity of the NLR N-terminal domains, which provide the activation platform for signalling components [195, 196]. As with the animal NLRs, the N-terminal domain (CC or TIR) of plant NLRs is shown to be crucial for oligomerisation-dependent defence signalling. Crystal structures of the CC domain of barley MLA10 and the TIR domain of Flax L6 have revealed the in solution status of CC/CC or TIR/TIR homo-dimers, which are sufficient to trigger defence signalling *in planta* [190, 197]. Mutations in the CC/CC or TIR/TIR interface abolish both dimerisation and signalling activity, supporting the idea that N-terminal domain oligomerisation is required for defence activation [154]. Furthermore, Bernoux et al. [197] revealed a conserved patch on the L6 TIR/TIR interface required for dimerisation, suggesting a possible common feature of TIR-dependent dimerisation of TNLs [197]. Nevertheless, whether oligomerisation is a mechanism common to all plant NLR

activation, and whether plant NLRs form high molecular-weight multimers resembling the animal inflammasomes, remain open questions.

Recent structural studies revealed that at the centre of the animal NLR inflammasome "wheel", the N-terminal domains recruit adaptor proteins to transduce the signal [170–172]. Some data suggest that the adaptor proteins, such as caspase-1 and ASC, are able to polymerise into filaments upon inflammasome formation, probably for rapid signal amplification [196, 198, 199]. Conceivably, plant NLRs could use a similar mechanism to recruit signalling adaptors via their N-terminal domains. Induced proximity of N-terminal domains of plant NLRs or their associated adaptors may be sufficient to initiate defence signalling. The observation that increasing the local concentration of TIR or CC domains by transient overexpression triggers effector-independent cell death supports this hypothesis [159, 165, 185, 191, 200].

In addition, the RPP1^{WsB} TIR domain when fused to a GFP tag, which is prone to forming homo-dimers at high concentrations, is sufficient to induce TIR domain self-association and cell death in transient assays [94]. In contrast, the RPP1^{WsB} TIR-domain fusion to a monomeric GFP does not trigger cell death [94]. Likewise Rp1 CC domain also depends on a GFP fusion for cell death induction [165]. More recently, Schreiber et al (2016) has demonstrated that the auto-activity of the RPP1 TIR domain is correlated with its self-association ability in solution [201]. Intriguingly, on the L6 TIR domain, residues required for signalling activities have been identified independently from the ones required for dimerisation, suggesting that the TIR domain plays a role in defence signalling after NLR activation (possibly in adaptor recruitment) [197]. Altogether, oligomerisation of the N-termini of plant NLRs is likely to play a role in signalling.

Truncated NLRs in Arabidopsis, such as TIR-NB-ARC (TN) and TIR-unknown domain (TX), are able to trigger defence activation when transiently overexpressed in both *N. tabacum* and Arabidopsis [202, 203]. Although functions of these truncated NLRs are largely unknown, they have been shown to directly interact with NLR proteins and pathogen effectors, implying their role in immunity [203]. Interestingly, Chan et al. [204] observed that an Arabidopsis TIR-only protein structurally resembles Myd88, the adaptor for Toll-like receptor-signalling in mammals [204]. This suggest that TIR-containing proteins may function as adaptors for TNL signalling in plants. It is possible that

the truncated NLR repertoire may directly cooperate with other full length NLRs as adaptors for downstream signalling.

However, despite the effort to identify N-terminal domain interactors, few candidates so far have shown the potential to be signalling components that may act immediately downstream of plant NLRs [179]. These N-terminal domain interactors are often diverse and sometimes highly specific, which is conflicting with the concept of a common downstream signalling pathway for plant NLRs. In fact, TIR domains have been implied as the specificity determinant in some TNLs against flax rust, and the CC domain of Lr10, which also plays a role in recognition specificity, shows greater sequence diversity than the LRR domain [168, 180]. Such diversity observed in plant N-terminal domains may be an indication that the plant NLR signalling pathway can be more complex than the animal NLR system. Overall for plant NLRs, the mechanism of N-terminal domain triggered cell death and the role of the N-terminal domain in defence signalling awaits elucidation.

Subcellular localisation of the NLR immune complex is important for defence signalling

Increasing evidence suggests that appropriate subcellular localisation of immune components, including the immune receptors, cognate effectors and their interactors, is crucial for the full establishment of defence response [205]. Nuclear accessibility is essential for several NLRs to activate defence (e.g. N, MLA, SNC1, Pb1, RPS4); and blocking their nuclear localisation render these receptors non-functional. More often than not, the nuclear localisation of these NLR proteins is required for their interactions with transcription factors (TFs) to activate defence. For example, upon pathogen perception, the increased nuclear pool of barley MLA proteins (CNLs) can interact with both negative and positive immune TFs to activate defence [206, 207]. The active MLA10 (also MLA1) interacts with a defence activator MYB6 via its CC domain to release MYB6 from the repressor WRKY1, and MYB6 subsequently binds DNA to activate defence genes [207]. The rice CNL Pb1 activates defence via binding and protecting the positive TF WRKY45 from degradation [174, 208]. The tobacco TNL, N, associates with transcription factor SPL6 via its LRR domain [209]. Another TNL, SNC1, associates with transcriptional co-repressor TPR1 (Topless Related 1) via its TIR domain, and TPR1 suppresses the expression of the negative defence regulators DND1 and DND2 [210]. In

addition, SNC1 as well as RPS4 interacts with the positive immune regulator TF bHLH84 [211].

For some NLRs, nucleocytoplasmic trafficking and the dual distribution are required for their full functions (e.g. MLA, N, Rx and Rp1), indicating NLR activities in distinct subcellular compartments [96, 192, 200, 210, 212, 213]. For the nucleocytoplasmically distributed MLA10, forced cytoplasmic localisation (MLA10-NES) results in cell death induction but with no restriction of pathogen growth; whereas forced nuclear-localisation (MLA10-NLS) is sufficient to provide disease resistance, but fails to trigger cell death [192, 206]. These findings suggest that cytoplasmic and nuclear pools of MLA10 mediate cell death and disease resistance signalling respectively, and proper coordination of the signalling pathways are important to achieve full defence responses. Another example demonstrating the importance of dual subcellular localisation is the maize auto-active Rp1-D21 protein, as targeting Rp1-D21 predominantly to either the nucleus or the cytoplasm abolishes HR when transiently expressed in *N. benthamiana* [200]. Furthermore, confined co-expression of Rp1-D21-NLS (in the nucleus) and Rp-D21-NES (in the cytoplasm) can not provide full activity, showing that nucleocytoplasmic trafficking is essential for the cell death induction of Rp1-D21 [200]. What is mediating the nucleocytoplasmic trafficking of NLRs? It has been reported that proper nucleocytoplasmic distribution of Rx1 requires Ran GTPase-activating protein2 (RanGAP2) [212] and the nucleocytoplasmic translocation of EDS1/PAD4/SAG101 seems to be required for the functions of several NLRs (e.g. N, SNC1) [210, 213].

Co-localisation of the R proteins and their cognate effectors in certain cellular compartments is also important for the activation of defence. Recent studies have confirmed that the co-localisation and direct interaction of RRS1 with either PopP2 or AvrRps4 occurred in the nucleus, and forced exclusion of either the NLR protein or the effector can abolish defence signalling [58, 116, 214]. Recognition of potato virus X (PVX) coat protein (CP) by Rx on the other hand requires co-localisation to the cytoplasm, as forced nuclear localisation of CP fails to activate Rx.

Nevertheless, some NLR proteins, such as RPM1 and RPS2 are shown exclusively localised at the plasma membrane for effector detection and signalling initiation [215, 216]. How these NLRs activate defence signalling in the nucleus is still unknown. Overall, NLRs often co-localise with their

cognate effectors and/or their host targets, and sometimes move to specific subcellular compartments to perform different branches of immune functions. Future studies should reveal where pathogens are perceived in the cell and in which subcellular compartment the different branches of the defence system are activated by NLRs.

Several signalling components are required for plant NLR-triggered immunity

In plants, studies have identified several signalling components that seemed to act downstream of different groups of NLRs. For example, EDS1 (Enhanced Disease Susceptibility 1) is a lipase-like protein that is required for the immune signalling of all known TNLs, and has been shown to interact with the TNLs RPS4, RPS6 and SNC1 [213, 217]. It has been reported that pathogen effectors disrupt the interactions between EDS1 and certain TNLs, which might allow EDS1 to function downstream [217]. Recent structural studies demonstrated that EDS1 forms heterodimers with PAD4 (PhytoAlexin Deficient 4) or the structurally related SAG101 (Senescence-Associated Gene 101) , which are indispensable for EDS1 function [218]. EDS1, PAD4 and SAG101 form nuclear and cytoplasmic complexes and have been implicated in bridging NLR defence signalling in different cellular compartments (cytoplasm and nucleus) [213]. EDS1 also interacts with SRFR1, a negative regulator of immunity, which interacts and suppresses a subset of TCP-family transcription factors [219–222]. These interactions may contribute to signal transduction in a subset of TNLs.

Some CNLs localise to the plasma membrane (PM) and require NDR1 for immune signalling (e.g. RPS2, RPM1, and RPS5) [223, 224]. NDR1 (non-race-specific disease resistance 1) undergoes glycosylphosphatidyl-inositol and glycosylation for PM anchoring [225]. NDR1 has been demonstrated to associate with RIN4, which is required for RPM1 and RPS2-mediated resistance [226]. The *ndr1-1* mutant is susceptible to *Pst* DC3000 carrying AvrRpm1, AvrRpt2 and AvrPphB, and overexpression of *NDR1* results in enhanced resistance to *Pst* DC3000 [225]. Interestingly, Knepper et al. [227] suggest that NDR1 may function like a mammalian integrin protein, mediating the adhesion of plasma membrane and cell wall to prevent fluid loss during pathogen infection [227]. However, how this protein participates in the activation of effector-triggered immunity is still unclear.

HSP90 (heat shock protein 90) and its co-chaperones RAR1 (Required for MLA12 Resistance 1) and SGT1 (Suppressor of the G2 allele of Skp1) form dynamic complexes to assist the functioning of both TNLs and CNLs [228]. These chaperones are important for the assembly and stabilisation of NLR proteins so they maintain proper intra- and inter-molecular interactions for their signalling competence [176, 229–231]. In addition, chaperone proteins are shown to associate with the SCF (SKP1, Cullin, F-box protein) E3 ubiquitin ligase complex that mediates proteasomal degradation of target proteins [232]. For example, SGT1 was shown to interact with the SCF complex in yeast, which results in the ubiquitination and degradation of many signalling proteins [233, 234]. Silencing of SGT1 affects the accumulation of NLR proteins, suggesting that SGT1 regulates the stability of NLRs, and may also degrade improperly folded NLRs via the ubiquitination pathway [235]. Regulation of the NLR accumulation level is essential, as overexpression of NLRs may trigger harmful constitutive defence activation. It is also possible that the HSP90/SGT1 (or HSP90/RAR1) complex may employ ubiquitin-mediated protein degradation of immune suppressors to help activate plant defence [236].

1.4 The NLR pair RRS1/RPS4 as a model to study plant defence

1.4.1 *Arabidopsis dual resistance genes, RRS1 and RPS4, prevent infection by distinct pathogens*

Arabidopsis *RPS4* (Resistance to *P. syringae* 4) was found to be genetically required for the resistance against *Pst* expressing AvrRps4, and it was mapped as a single dominant locus on chromosome 5 (*At5g45250*) in a cross between the susceptible accession RLD and the resistant accession Ws-0 [237, 238]. *RRS1* (Resistance to *R. solanacearum* 1) was independently identified by its ability to confer resistance to *R. solanacearum* GMI1000 carrying the effector PopP2, and was mapped to chromosome 5 (*At5g45260*) closely linked to *RPS4* [214, 239, 240]. Interestingly, *RRS1* segregates as a recessive locus for *R. solanacearum* resistance: the F1 is susceptible in a cross between the resistant Nd-1 (carrying a *RRS1-R* allele) and the susceptible Col-0 (carrying a *RRS1-S* allele) [239]. Allelic variation of RRS1 is observed in various Arabidopsis accessions, and these polymorphisms seem to determine their

resistance to *R. solanacearum* (PopP2) [114]. In this thesis, *RRS1-R* refers to the resistance allele of Ws-2, and *RRS1-S* refers to the susceptible allele of Col-0.

It was later discovered that *RRS1* and *RPS4* are both genetically required for resistance against two bacterial pathogens, *Pst* (AvrRps4) and *R. solanacearum* (PopP2), and also the fungal pathogen *C. higginsianum* [114, 241, 242]. The *rps4* or *rrs1* single mutant is sufficient to render the plant susceptible to *R. solanacearum* and *C. higginsianum*, each phenocopies the double mutant, suggesting that *RPS4* and *RRS1* function cooperatively rather than additively [114]. It should be noted that mutants that abolish the RRS1/RPS4 function in Ws-2 and Col-0 still show resistance to *Pst* (AvrRps4), indicating an additional R protein may recognise AvrRps4 independently of RRS1/RPS4 [114, 241, 243]. An additional gene pair conferring AvrRps4 recognition, designated as *RRS1B/RPS4B*, was discovered in the Jones lab [121]. Following up this discovery, I report analyses of RRS1B/RPS4B and RRS1/RPS4 in Chapter 3. The specific allelic requirement of *RRS1* for *R. solanacearum* (PopP2) resistance seems to hold true for the immunity against *C. higginsianum*, but is not required for *Pst* (AvrRps4): RPS4/RRS1-R confers resistance to the three pathogens, whereas RPS4/RRS1-S is only effective against *Pst* (AvrRps4) [114, 241, 242]. The molecular basis for the allelic determination of recognition specificity is still under investigation. I will address and discuss the possible specific determinants for PopP2 and AvrRps4 recognition capability mainly in Chapter 5.

RPS4 and *RRS1* are arranged in the genome in a head-to-head orientation separated by a small intergenic region (264bp), which possibly functions as a bidirectional promoter [238]. This head-to-head arrangement is commonly observed for functional gene pairs (e.g.RGA4/RGA5, Pik-1/Pik-2), which maybe a signature of transcriptional co-regulation and close cooperation of the pair partners [110, 113, 114]. Interestingly, many *RPS4* homologs are paired with *RRS1* homologs in a similar inverted arrangement with variable intergene distances [238], suggesting their co-evolution as a linked pair.

The Jones lab showed that transient co-expression of RRS1 and RPS4 via *Agrobacterium tumefaciens*-mediated transformation in *N. tabacum* confers specific recognition of effector proteins (AvrRps4 and PopP2), triggering HR [58, 115]. In contrast, the expression of a single R protein with either effector

fails to trigger HR, consistent with their cooperative function. Importantly, this transient system in *N. tabacum* recapitulates the allelic-dependent variation of effector recognition capability observed in Arabidopsis, and also does not display ectopic cell death when treated with non-recognisable effector mutants [58, 115]. These data and many other evidence suggest that for RRS1 and RPS4 this heterologous system is a viable tool to study their native functions in Arabidopsis. This assay is frequently used in this thesis for functional studies of R proteins, and is often referred to as the *N. tabacum* (tobacco) transient assay or agro-infiltration assay.

Remarkably, Arabidopsis RRS1 and RPS4 have recently been shown to function in two other *Brassicaceae*, *Brassica rapa* and *Brassica napus*, and also in two *Solanaceae*, *Nicotiana benthamiana* and tomato (*Solanum lycopersicum*), conferring disease resistance [244–246]. In addition to RRS1/RPS4's function to protect *Brassicaceae* against *C. higginsianum*, the gene pair can also prevent *Colletotrichum orbiculare* infection in cucumber (*Cucurbitaceae*) [244, 245]. However, cognate effectors in these fungal species recognised by RRS1/RPS4 have yet to be identified.

1.4.2 *The modular structure of TNLs RRS1 and RPS4*

RPS4 encodes a TIR-NB-LRR receptor with a C-terminal 338 aa that does not show homology to other known functional protein domains. This RPS4 C-terminal domain is abbreviated as CTD in this thesis **Figure 1.1**. *RPS4* produces alternative transcripts, which encode various truncated proteins corresponding to the TIR and TIR-NB domains, and these alternative splicing varients in combination with full-length transcripts are required for RPS4 function [238, 247]. During pathogen-induced defence activation, the total mRNA level and the alternative splicing of RPS4 are both increased, confirming their importance in defence [248]. Compared to the functional $RPS4^{Col\text{-}0}$, the non-functional $RPS4^{RLD}$ (encoded by *RPS4* allele from RLD) contains five amino acid changes, of which two (N195D and Y950D) are responsible for it's loss of function [238]. Molecular functions of these specific residues in functional RPS4 have not been demonstrated.

RRS1 encodes a TIR-NB-LRR with an atypical C-terminal WRKY domain, which resembles the WRKY family of transcription factors [240]. WRKY transcription factors play crucial roles in regulating plant defence [249]. The *slh1* (Sensitive to low humidity 1) mutant of RRS1-R, with a single leucine

insertion in the WRKY domain, exhibits constitutive defence activation and a dwarf phenotype in Arabidopsis [250]. In turn, a popular hypothesis emerged that RRS1 may negatively regulate defence via directly binding to the DNA, and the disruption of WRKY domain DNA binding (in the *slh1* mutant) would trigger defence activation. Furthermore, due to its chimeric structure, RRS1 has been proposed to be a 'Rosetta stone' that can combine the function of recognition, signalling and defence activation into one unit [251, 252]. Recent evidence from the Jones lab suggest that the loss of RRS1 DNA binding is not sufficient to activate defence: Unlike RRS1-R*slh1*, RRS1-S*slh1* that also loses DNA binding activity does not trigger auto-immunity [58]. Whether RRS1 activation depends its DNA binding activity will be discussed in detail in Chapter 4 and Chapter 5 with further evidence.

Comparison of *RRS1-S* with *RRS1-R* reveals a pre-mature stop codon in the S allele, leading to an 83-90 amino acid (aa) deletion (depending on the accessions) at its C-terminus after the WRKY domain [240], and the C-terminal extension is crucial for PopP2 recognition by RRS1-R [58]. In this thesis, the shorter C-terminal extension (21 aa) after the WRKY 'HNH' motif in RRS1-S^{Col-0} and the longer extension (104 aa) in RRS1-R $^{Ws-2}$ are termed the domain 6 of RRS1 **Figure 1.1**, and abbreviated as DOM6-S or DOM-R respectively. The roles of the WRKY and DOM6 in effector recognition and immune complex activation will be explored in Chapter 4 and Chapter 5. Similar to the *slh1* mutant, Narusaka et al (2016) recently reported that mutations of a leucine zipper (LZ) motif located between the LRR and WRKY domain in RRS1-R^{Ws-2} triggers RPS4-dependent auto-immunity in Arabidopsis and in *N. benthamiana* [253]. This LZ motif of RRS1 seems to be conserved in many different Arabidopsis accessions [253], however the contribution of this motif to RRS1 activity is still unclear. Besides the LZ motif, no function has been ascribed to the 322 aa between the LRR and the WRKY domain of RRS1, and in this thesis we define this domain of RRS1 as domain 4, or DOM4 **Figure 1.1**. Functional studies regarding domain 4 of RRS1 will be included in Chapter 5.

Furthermore, wild type (WT) RRS1 has been reported to have a dominant negative effect on its auto-immune allele *slh1*, as co-expression of RRS1-WT with RRS1-R*slh1*/RPS4 suppresses HR induction in tobacco transient assays [116], and the *slh1* mutant is recessive in crosses to RRS1-R or RRS1-S. This mirrors the recessiveness of *RRS1-R* (for PopP2 recognition) in heterozygotes with RRS1-S [239, 250]. Interestingly, using similar transient

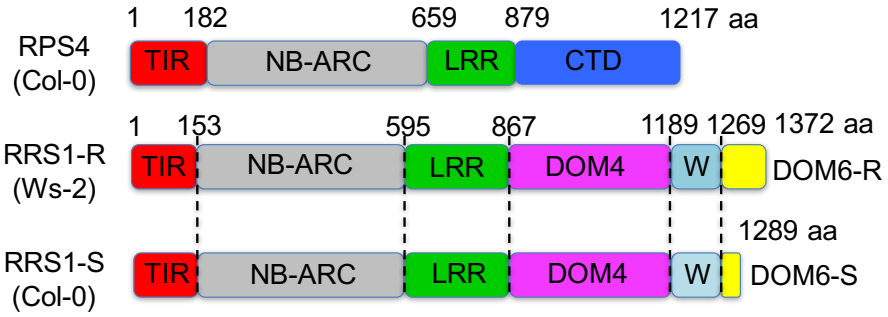

Figure 1.1: Domain architecture of RPS4, RRS1-R and RRS1-S with defined boundaries. The six domains of RRS1-R^{Ws-2} & RRS1-S^{Col-0} (TIR, NB-ARC, LRR, Domain 4 (DOM4), WRKY, Domain 6 (DOM6)) and the four domains of $RPS4^{Col-0}$ (TIR, NB-ARC, LRR, C-terminal domain (CTD)) are defined. Numbers indicate the amino acid positions for domain boundaries.

assays, Narusaka et al. [253] showed that RRS1 cannot completely abolish the HR triggered by the LZ mutant of RRS1-R coexpressed with RPS4, suggesting the *RRS1-R LZ* mutant maybe semi-dominant. However, it is also possible that by mixing two *Agrobacterium* strains, transient co-expression of RRS1-WT and RRS1-R LZ mutant may allow certain cells to express only the RRS1-R LZ mutant, thus explaining the residual HR. The P-loop motif of RRS1 is required for its dominant negative effect [116]. In Chapter 5, I will further explore the requirement of RRS1 for suppressing its auto-active alleles, hopefully to learn more about the recessiveness of *RRS1*.

1.4.3 *RRS1/RPS4 recognises effectors AvrRps4 and PopP2 in the nucleus*

Both RRS1 and RPS4 contain a predicted nuclear localisation signal (NLS) [240, 254], however their functions in the nucleus have not been clearly characterised. Wirthmueller et al. [254] showed that RPS4 is distributed between the endomembrane (cytoplasm) and the nuclei, and that nuclear accumulation of RPS4 requires the NLS and is important for defence activation and resistance to *Pst* AvrRps4 [254]. AvrRps4 shows a nucleocytoplasmic distribution, and forced nuclear export of AvrRps4 (via fusing to NES) partially suppresses cell death responsiveness and resistance in Arabidopsis, suggesting that nuclear co-localisation of AvrRps4 and RPS4 is required for defence activation [213]. Although the cytoplasmic fraction of AvrRps4 has been suggested to specifically mediate cell death signalling (independent of the resistance signalling) [213], later reports showed that forced nuclear

localisation of AvrRps4-NLS does not abolish cell death [116]. Overall, these results suggest that the nucleus is the main site where AvrRps4 recognition and subsequent signalling occurs. It remains possible that a sub-pool of AvrRps4 may be detected in the cytoplasm, yet the defence signalling needs to be carried out in the nucleus. As RPS4 does not re-localise after effector treatment, EDS1 has been proposed to be the signalling coordinator between the cytoplasm and the nucleus [213, 217].

RRS1 has been observed to co-localise with the effector PopP2 in the nucleus [214]. The N-terminal NLS signal of PopP2 is required for the nuclear localisation of the RRS1/PopP2 complex, and deletion leads to the nuclearcytoplasmic distribution of RRS1/PopP2 complex [214]. However, PopP2 with the NLS deletion (149-448aa) is still recognised by RRS1-R/RPS4 [116], suggesting that nuclear targeting of PopP2 is not necessary for it to be recognised. Perhaps after RRS1 perceives PopP2 (149-448aa) in the cytoplasm, it can relocate PopP2 into the nucleus for defence activation. On the other hand, PopP2(149-448aa)-NES forced to the cytoplasm is not recognised and fails to trigger RRS1-R/RPS4 mediated resistance [116]. To summarise, it seems PopP2 recognition also requires nuclear co-localisation with its receptor similar to AvrRps4. Indeed, Sarris et al. [58] confirmed that the receptor complex RRS1/RPS4 associates with both AvrRps4 and PopP2 in the nucleus using Bimolecular Fluorescence Complementation (BiFC) assay.

1.4.4 *The knowns and unknowns: T3SS effectors AvrRps4 and PopP2*

AvrRps4 was identified from the *Pseudmonas syringae* pv. *pisi*, and it is a 221 aa protein with an N-terminal domain sufficient for its delivery into the plant cell by the T3SS [237, 255]. After secretion, AvrRps4 is cleaved *in planta* between G133 and G134, and the processed C-terminal 88 amino acids are necessary and sufficient to induce resistance, referred to as AvrRps4 C-term in this thesis [256]. Nevertheless, this *in planta* processing is not necessary for AvrRps4-triggered immunity, as the processing-deficient mutant AvrRps4-R112L is still recognised [256]. The crystal structural of the AvrRps4 C-term has revealed an anti-parallel α-helical coiled coil, with a prominent negative charged patch predicted to be important for protein-protein interaction [243]. Interestingly, residues E187 and E175 on the negative patch

as well as the KRVY motif (135-138 aa) at the N-terminus of AvrRps4 C-term are essential for AvrRps4 recognition [243, 256]. In addition, a surface exposed residue L167 of AvrRps4, which is on a distinct patch from E187 and E175, is shown to be specifically required for RRS1- and RPS4- independent recognition [243]. Chapter 3 will focus on the recognition mechanism of AvrRps4, and will address the reason why these residues are required for its avirulence.

Virulence activity of AvrRps4 was demonstrated where expression of AvrRps4 in Arabidopsis lacking RPS4 promotes susceptibility to *Pst* DC3000 [256]. Both the *in planta* cleavage and the exposed KRVY motif after the cleavage are required to achieve this full virulence [256]. Interestingly, it has been shown that AvrRps4 is targeted to the chloroplast for processing via a possible N-terminal chloroplast transit peptide, which is shared with effector HopK1 [69]. This result indicates that although the recognition occurs in the nucleus, AvrRps4 may perform some of its virulence functions in the chloroplast. Furthermore, as with many other multifunctional effectors, the N-terminal and C-terminal cleavage products of AvrRps4 could play distinct roles in different cellular compartments.

R. solanacearum T3SS effector PopP2 belongs to the YopJ protein family that is present in both mammalian and plant pathogens [214, 257]. PopP2 has been shown to physically interact with RRS1-R and RRS1-S, and subsequently stabilizing the R proteins possibly via preventing their proteasomal degradation [214, 257]. PopP2 also interacts with a cysteine protease RD19 (Responsive to dehydration 19) required for PopP2-triggered immunity, which is transported into the nucleus in the presence of PopP2 [258]. The role of RD19 during defence activation is not known. PopP2 is an active acetyl-transferase, and the catalytic core residue C321 is essential for the virulence and avirulence function of PopP2 [257]. Recent studies showed that PopP2 contributes to *R. solanacearum* virulence via acetylating host WRKY transcription factors, which abolishes their DNA binding [58, 59]. Le Roux et al. [59] also demonstrate that delivery of PopP2 by the non-pathogenic strain *Pseudomonas fluorescens* PfO-1 can suppress PTI in *N. benthamiana* [59]. PopP2 also acetylates the WRKY domain of both RRS1-R and RRS1-S, which leads to its recognition by RRS1-R/RPS4 [58, 59]. The link between the virulence activity and its recognition will be detailed in Chapter 4.

1.4.5 *How does the RRS1/RPS4 immune complex operate?*

RRS1 and RPS4 associate to form a functional immune complex that detects effector and activates defence [58, 115, 121]. The TIR domains of RRS1 and RPS4 can form homo- and heter-dimers, and the crystal structure of RRS1-RPS4 TIR-TIR heterodimer demonstrates the physical interaction between the two proteins [115]. TIR domain hetero-interaction mediated by the conserved SH motif is essential for effector recognition by the RRS1/RPS4 complex, although SH-AA mutants of full-length RRS1 and RPS4 are still able to associate [115]. A long standing observation is that RPS4 or RPS4 TIR domain can trigger effector-independent cell death when over-expressed, implicating it's function in defence signalling [185, 254]. Williams et al. [115] further demonstrate that homo-dimerisation of RPS4 TIR domains is required for this signalling activity. Moreover, P-loop mutation in RPS4, but not in RRS1, renders the RRS1/RPS4 immune complex non-functional for effector recognition [115], confirming the role of RPS4 as an executor. Chapter 3 will investigate the functional implications of TIR domain homo- and hetero-oligomerisation during the immune complex assembly and activation, not only for RRS1/RPS4, but also for RRS1B/RPS4B. Specific pairing between cognate partners is required for the functioning of immune complexes RRS1/RPS4 and RRS1B/RPS4B, I will explore in Chapter 3 the domain components required for this specificity. Chapter 4 and Chapter 5 will reveal the distinct roles of RRS1 and RPS4 within the immune complex, with Chapter 4 focusing on recognition mechanisms for AvrRps4 and Chapter 5 focusing on immune complex activation after effector recognition. The background and project aim will be individually introduced in each of the result chapters.

In summary, the Arabidopsis R gene pair *RRS1/RPS4* together with its paralogous pair *RRS1B/RPS4B* serves as a good system to study NLR mechanisms. The chimeric nature of RRS1 and RRS1B, which is shared with many other fusion NLRs, provides a unique opportunity to uncover new NLR functional mechanisms. Variations in the NLR pairs' ability to perceive different effectors (AvrRps4, PopP2) allow us to explore the recognition specificity and NLR activation in a dynamic context. How paired NLR proteins work as a single immune complex is an interesting and important problem. It is a challenge to study the dynamic molecular interactions that convert effector recognition to defence activation, but dissecting their operating

mechanisms will provide valuable insights for many other cooperative NLR systems. The molecular details of auto-inhibition and effector-triggered activation of this iconic NLR pair would provide a foundation for future synthetic resistance engineering.

2 Materials and Methods

2.1 Materials

2.1.1 *Plant Materials*

Arabidopsis thaliana

Wild type or mutant *Arabidopsis thaliana* (hereafter Arabidopsis) lines used in this study are in Col-0 (Columbia) or Ws-2 (Wassilewskija) accession. All mutant lines are listed in **(Table 2.1)**. Seeds were sown directly on compost and plants were grown under controlled conditions: 21 °C; 10 hours light and 14 hours dark (short days); 75% humidity.

Mutant allele	Accession	Reference/Source
rrs1-3	Col-0	[121], SALK-061602
rps4-2	Col-0	[254], SALK-057697
rrs1b-1	Col-0	[121], SALK-001360
rps4b-2	Col-0	[121], SALK-063382
rrs1-3/rrs1b-1	Col-0	[121]
rps4-2/rrs1b-1	Col-0	[121]
rps4-2/rps4b-2	Col-0	[121]
eds1-2	Col-0	[259]
rrs1-1	Ws-2	[114]
rps4-21	Ws-2	[241]
rps4b-1	Ws-2	[121], FLAG-049F09
rrs1-1/rps4b-1	Ws-2	[121]
rps4-21/rps4b-1	Ws-2	[121]

Table 2.1: Mutant Arabidopsis lines used in this study. T-DNA insertion lines obtained from the SALK and INRA institutes are given IDs.

Nicotiana spp.

Nicotiana benthamiana and *Nicotiana tabacum* (cultivar 'Petit Gerard') were used for transient *Agrobacterium tumefaciens*-mediated transformation of leaf tissues. Seeds were sown directly on compost and plants were grown under controlled conditions: 24 °C; 16 hours light and 8 hours dark (long days); 55% humidity.

2.1.2 *Antibiotics*

Antibiotic	Stock concentration	Working concentration
Carbenicillin (Carb)	100 mg/mL in H_2O	100 µg/mL
Chloramphenicol (Cam)	10 mg/mL in H_2O	30 µg/mL
Gentamycin (Gen)	10 mg/mL in H_2O	20 µg/mL
Kanamycin (Kan)	50 mg/mL in H_2O	50 µg/mL
Rifampicin (Rif)	10 mg/mL in methanol	50 µg/mL
Spectinomycin (Spt)	100 mg/mL in H_2O	50 µg/mL
Tetracycline (Tet)	5 mg/mL in 50% ethanol	10 µg/mL

Table 2.2: Antibiotics used in this study.

Stock solutions were stored at -20 °C, except for Rifampicin, which was stored at 4 °C. Working concentrations indicate the final concentrations used in selective media.

2.1.3 *Bacterial strains*

Pseudomonas spp.

Pseudomonas syringae pv. tomato strain DC3000 (*Pst* DC3000) and *Pseudomonas fluorescens* strain Pf0-1 (*Pf* Pf0-1) were used. *Pst* DC3000 is a sequenced *Pst* strain, and is Rifampicin (Rif) resistant. *Pf* Pf0-1 was engineered with a functional T3SS for effector delivery [260], and is Chloramphenicol (Cam) and Tetracyclin (Tet) resistant. All *Pseudomonas* strains carrying various constructs used in this study were obtained from Kee Hoon Sohn (previous member of the lab), detailed cloning information were described in [243] and [116]. Constructs were introduced into *Pst* DC3000 and *Pf* Pf0-1 using standard triparental mating methods described in [255].

Strain	Construct	Antibiotic resistance
Pst DC3000	*pVSP61* (empty vector)	Rif/Kan
Pst DC3000	*pVSP61::avrRps4*	Rif/Kan
Pf Pf0-1	*pBBR1MCS-5::avrRps4*	Cam/Tet/Gen
Pf Pf0-1	*pBBR1MCS-5::avrRps4-KRVYAAAA*	Cam/Tet/Gen
Pf Pf0-1	*pBBR1MCS-5::avrRps4-E187A*	Cam/Tet/Gen
Pf Pf0-1	*pBBR1MCS-5::avrRps4-L167T*	Cam/Tet/Gen
Pf Pf0-1	*pBBR1MCS-5::avrRps4-L167S*	Cam/Tet/Gen
Pf Pf0-1	*pEDV6::popP2*	Cam/Tet/Gen
Pf Pf0-1	*pEDV6::popP2-C321A*	Cam/Tet/Gen
Pf Pf0-1	*pML123::hopA1*	Cam/Tet/Gen

Table 2.3: *Pseudomonas* strains used in this study

Escherichia coli

E. coli strains DH10B and DH5α were used in this study for cloning purposes.

DH5α genotype: F- Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK-, mK+) *pho*A *sup*E44 λ- *thi*-1 *gyr*A96 *rel*A1 [261]

DH10B genotype: F- *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L *nup*G λ- [262]

Agrobacterium tumefaciens

A. tumefaciens strains GV3101 and Agl1 were used in this study for transient transformation of *Nicotiana spp.*. The strain Agl1 is Rifampicin and Carbenicillin resistant. The strain GV3101 carrying the helper plasmid pMP90 is Rifampicin and Gentamycin resistant [263].

2.1.4 *Oligonucleotides (Primers)*

Lyophilised primers were resuspended in ddH_2O to a final concentration of 100 µM and stored at -20 °C. The working solutions were diluted to 10 µM. Primers used for polymerase chain reaction (PCR) to amplify R gene fragments are listed in **(Table 2.7)**, and the ones used for quantitative reverse transcription-PCR (qRT-PCR) are listed in **(Table 2.4)**.

2.2 Methods

2.2.1 *Molecular biological methods*

Isolation of genomic DNA from Arabidopsis

Arabidopsis genomic DNA (gDNA) was extracted using Qiagen DNeasy Plant Mini kit following manufacturer's instructions. Briefly, 4–5-week-old plant leaves were ground into fine powder using mortar and pestle in liquid nitrogen. Samples were then lysed and homogenized through the QIAshredder spin column. By passing the samples through the DNeasy Mini spin column, the DNA selectively binds to the silica membrane while contaminants pass through. Remaining contaminants and enzyme inhibitors were removed by wash steps. Genomic DNA was then eluted in water, and stored at -20 °C.

Polymerise chain reaction (PCR)

Standard PCR reactions (e.g. for colony PCR) were performed using Taq DNA polymerase (NEB, Qiagen). For cloning of PCR products, Phusion high-fidelity DNA polymerase (NEB) were used. Reaction mix was made following manufacturer's instructions. Depending on the experiment, plasmid DNA, genomic DNA or cDNA was used as template. PCR reactions were carried out in a thermocycler (DNA engine PTC225, MJ Research), following standard protocol instructions for cycles and thermal conditions. Annealing temperatures and elongation time were optimised based on primer properties and the desired amplifying fragments' size for each experiment.

Isolation of total RNA from Arabidopsis

Leaves of 5-week-old Arabidopsis were snap frozen in liquid nitrogen for RNA extraction. Samples were ground into fine powder either by using mortar and pestle or by shaking in a 2 mL centrifuge tubes with 1.2 mm stainless steel beads. 1 mL of TRI reagent (Sigma) was added to (~100 mg) samples and homogenised by vortexing. After incubation at room temperature for 5 min (for dissociation of nucleo-protein complexes), samples were centrifuged at 12000 *g* for 5 min at 4 °C. One hundred µL of BCP (1-bromo 3-chloropropane, Sigma) was then added to each sample, which was shaken

vigorously for 15 seconds. After incubation for 10 min at room temperature, samples were centrifuged at 12000 *g* for 15 min at 4 °C. The RNA containing upper aqueous phase (~0.5 mL) were then transferred into a new eppendorf tube.

RNA was precipitated by adding 0.5 volumes of isopropanol and 0.5 volumes of high salt precipitation solution (0.8 M sodium citrate and 1.2 M NaCl), and mixed well by turning the tubes. After incubation at room temperate for 5 min, samples were centrifuged for 15 min at 12 at 12000 *g* at 4 °C. The RNA pellets were washed with 1.5 mL of 75 % ethanol, centrifuged for 5 min at 7500 *g* at 4 °C.

Pellets were air dried at room temperature for 5–10 min, and re-suspended in DEPC-treated H_2O. To remove DNA from the RNA samples, DNAse treatment was applied according to the DNase I (RNase-free) protocol (Roche). After the treatment, proteinase K were added to the RNA and incubated for 15 min at 42 °C to deactivate DNAse I. RNA was then purified using Qiagen RNeasy columns following the manufacturer's instructions. Integrity of RNAs was assessed by electrophoresis. RNA concentration was quantified using Nanodrop (Thermo scientific, UK). RNA was then stored at -80 °C.

Quantitative reverse transcription-PCR (qRT-PCR)

First strand cDNA was synthesised from 1–2 µg purified total RNA using SuperScript II Reverse Transcriptase (Invitrogen). Reverse transcription reaction mix was made with oligodT following manufacturer's instructions. Note that the sample mixture was incubated at 70 °C for 10 min to disrupt secondary structures, and then transferred on ice. Then the reaction mix was incubated at 42 °C for 75 min and transferred back on ice. The cDNA was adjusted to 50-100 µl and stored at -80 °C. Quantitative RT-PCR were performed in the CFX96 Thermal Cycler (Bio-Rad) using real time PCR SYBR Green JumpStart Taq ReadyMix (Sigma) as dye. Specific defence marker gene primers used in this study were listed in **(Table 2.4)**.

Each reaction (~50 ng cDNA per reaction) was performed in triplicates and the average threshold cycle (Ct) was used to quantify relative gene expression. The relative expression values of defense marker genes were determined using the comparative Ct method (ΔΔCt) with *Ef1α* (*At5g60390*) as a reference for normalization.

Gene	Size	Forward Primer	Reverse Primer
EF1α (*AT5G60390*)	150 bp	TGAGCACGCTCTTCTTGCTTTCA	GTTGTATCCGACCTTCTTCAGG
SARD1 (*AT1G73805*)	202 bp	CCGATATGCGAAGTTATGAAAGC	AGTGGCTCGCAGCATATTGTT
SID2 (*AT1G74710*)	208 bp	CAATTGGCAGGGAGACTTACG	GAGCTGATCTGATCCCGACTG
EDS5 (*AT4G39030*)	194 bp	ACCTTTCTTCATGGCGTTGTCT	ATTGAAATCCGACGAGAACGA
PAD4 (*AT3G52430*)	188 bp	TGTTCTTTTCCCCGGCTTATC	ATAGAAGCCAAAGTGCGGTGA

Table 2.4: List of primers used for qRT-PCR experiments in this study

Golden Gate Cloning

The Golden Gate technology enables a single-step assembly of multiple DNA fragments into a destination vector, utilising the activities of a type IIS endonuclease (*BsaI* was used in this study) and T4 DNA ligase [264] [265] [266]. The Type IIS endonucleases cleave outside their recognition sites, creating 4 base pair (bp) overhangs after digestion. Therefore the Type IIS recognition site is designed to be distal to the cleavage site in both the inserts and destination vectors, such that digestion & ligation can remove the recognition sequence, allowing seamless assembly.

For ease of interpretation, the DNA fragments (or insert-containing plasmids) are termed the modules, the destination vectors are termed the acceptors, and the one-pot one-step digestion & ligation reaction is termed the dig-lig reaction. For each assembly, a unique set of compatible 4 bp overhangs at the junctions of all modules and the acceptor are designed to ensure the assembly in a desired order. It should be noted that all modules and acceptor plasmids must be otherwise free of internal recognition sites for the enzyme (e.g. *BsaI*), and the removal of these sites are called "domestication". The dig-lig reaction composition and cycles are shown in **Table 2.5 & Table 2.6**.

Each 20 µL reaction was desalted using a sepharose column, of which 1-5 µL was transformed into competent *E. coli* cells.

Golden gate assembly of chimeric proteins

Domain boundary definitions In Chapter 5, six domains of RRS1-R and RRS1B (TIR, NB-ARC, LRR, Domain 4 (DOM4), WRKY, Domain 6 (DOM6)) and four domains of RPS4 and RPS4B (TIR, NB-ARC, LRR, C-terminal domain

Components	volume per 20 µL reaction
100 ng/µL Acceptor plasmid	1 µL
Modules (inserts), each adjusted to 2:1 molar concentration of insert:acceptor	1 µL per module
20 U/µL *BsaI* (ThermoFisher)	1 µL
400 U/µL T4 DNA ligase (NEB)	1 µL
10x CutSmart Buffer (NEB)	2 µL
10 mM ATP	2 µL

Table 2.5: Digestion-ligation reaction composition dH_2O was added to bring the total volume to 20 µL.

Temperatures	Duration	Cycles
40 °C	10 min	
16 °C	10 min	x3
50 °C	10 min	x1
80 °C	20 min	x1
16 °C	∞	

Table 2.6: Digestion-ligation reaction cycles

(CTD)) are defined with their swapping breakpoint amino acid labelled in **Table 5.1**. Ws-2 alleles of RRS1-R, RRS1B and RPS4B and Col-0 allele of RPS4 were used. TIR, NB-ARC and LRR domains are generally defined based on sequence homologies and domain boundaries characterised in other plant TNLs, and the WRKY domain of RRS1 and RRS1B in Chapter 5 is defined from 22-25 aa before the WRKYGQK motif at EKK (RRS1-R) or NNK (RRS1B) till the end of the zinc finger motif HNH. The domains with no predicted function (based on homology) are subsequently defined as RRS1 or RRS1B Domain 4 (between LRR and the WRKY domain), RRS1 or RRS1B Domain 6 (the C-terminal amino acids after the defined WRKY domain) and RPS4 or RPS4B CTD (the C-terminal domain after the LRR) respectively. The exact domain boundaries were chosen for cloning and swapping purposes so that the breakpoint amino acids are identical between RRS1-R & RRS1B and RPS4 & RPS4B, and also are designed to minimise potential disruption of predicted structural and functional motifs.

Chimera assembly Using the Golden Gate shuffling method, each single domain was cloned as a module, and together assembled into full-length R genes with varying domain combinations from the A pair (RRS1, RPS4) or the B pair (RRS1B, RPS4B) according to these defined boundaries. Each domain module was generated by PCR-amplifying fragments from genomic DNA materials with primers containing the *BsaI* recognition site and a specific 4 bp overhang, and then cloned into the pCR8/GW/TOPO vector (ThermoFisher). **Table 2.7** lists the primers and cloning information for all domain modules. Note that prior to domain fragments amplification, the RRS1, RPS4, RRS1B, RPS4B gDNA templates were 'domesticated' i.e. removal of internal *BsaI* sites. The 4 bp overhangs at the domain junctions are designed that they match between the A pair and B pair modules. The 3 nucleotides within the 4 bp overhangs that code for the swapping breakpoint amino acids were highlighted in **Figure 5.1**.

Each full-length chimeric (or wild type) R gene was assembled with a C-terminal tag module *pICSL50001* containing the HF tag (6xHis 3xFlag) into a binary vector (*pICSL86922* or *pICSL86977*), which has a built-in 35S (CaMV) promoter. These acceptor vectors are compatible for *BsaI* digestion, accepting insert modules with overhangs starting with AATG and ending with GCTT. More information about Golden Gate compatible plasmids and cloning techniques can be found on [`http://synbio.tsl.ac.uk/`]. Experimental procedures for Golden Gate assembly are described in the previous section.

Transformation of competent bacterial cells and selection

Plasmids or ligation products were transformed into electro-competent *E. coli* (DH10B, DH5α) or *A. tumefaciens* (AgI1, GV3101) by electroporation. 50 µl of competent cells were thawed on ice before adding 1-2 µL plasmid DNA (~200-500 ng/µL) or 5 µL ligation products and mixed well. The mixture was then transferred into a pre-cooled electroporation cuvettes (1 mm gap) and kept on ice. Electroporation was performed using a cell porator (Gene Pulser Xcell, Bio-Rad) with following conditions: for *E. coli*, voltage = 1800 V, capacitance = 25 µF, resistance = 200 Ω; for *A. tumefaciens*, voltage = 2400 V, capacitance = 25 µF, resistance = 200 Ω. Immediately after the electroporation, cells were recovered by adding of 250 µL of L-medium

Domain modules	Amino acid No.(aa)	Nucleotide No. (bp)	Over-hangs 5'-3'		Primers (5'-3')
			RRS1^{Ws-2} (R1)		
R1-TIR	1-153	1-568	AATG	F	CACCGGTCTCTAATGACCAATTGTGAAAAGGATG
	153aa	568bp		R	TACGGTCTCATCCAATTCGTCCAACATAAAAG
R1-NB-ARC	154-595	569-1974	TGGA	F	CACCGGTCTCTTGGAATCTATTCGAAGCTGCT
	442aa	1406bp		R	TACGGTCTCAGGTTCCACCCCAAAGTTTCTG
R1-LRR	596-867	1975-2883	AACC	F	CACCGGTCTCTAACCAAGGTAAGCAATCTCT
	272aa	909bp		R	TACGGTCTCAGTAGCTTCTCCGAGTCAGAAC
R1-DOM4	868-1189	2884-4036	CTAC	F	CACCGGTCTCTCTACCTATGCATTACAAGTTC
	322aa	1153bp		R	TCCGGTCTCACTTTTTGGTACGTCAGACA
R1-WRKY	1190-1269	4037-5959	AAAG	F	CGCCGGTCTCTAAAGTAAGTTCTCTTTTTCTATC
	80aa	1923bp		R	TCCGGTCTCAATGGTTATGCTCAGATAGGTA
R1R-DOM6	1270-1373	5960-6271	CCAT	F	CAAGGTCTCTCCATCCACGGCCCACTAAA
	104aa	312bp		R	TAGGTCTCACGAACCATAATCGAAGAATGTTGACCAAGG
			RRS1B^{Ws-2} (R1B)		
R1B-TIR	1-144	1-499	AATG	F	CACCGGTCTCTAATGATCGAGAGTGAGCAAATC
	144aa	499bp		R	TCCGGTCTCATCCAATTCGTTCCATATAA
R1B-NB-ARC	145-588	500-1914	TGGA	F	CACCGGTCTCTTGGAATCTACTCGAAGCTG
	444aa	1415bp		R	TACGGTCTCAGGTTCCACTCCAAAGTTTCT
R1B-LRR	589-811	1915-2671	AACC	F	CACCGGTCTCTAACCAAGGTAAGCAATCTCA
	223aa	757bp		R	TACGGTCTCAGTAGCTTCTTGAAGTCCAAAC
R1B-DOM4	812-1162	2672-3802	CTAC	F	CACCGGTCTCTCTACCAGTGCATTACACATT
	351aa	1131bp		R	TACGGTCTCACTTTCGGCGACTCTTTGAAC
R1B-WRKY	1163-1237	3803-4190	AAAG	F	CGCCGGTCTCTAAAGTATGTTGCTTAGTTT
	75aa	388bp		R	TACGGTCTCAATGGTTATGCTCAGAGATGT
R1B-DOM6	1238-1372	4191-4598	CCAT	F	CAAGGTCTCTCCATCCATTCCCCACTCTAC
	135aa	408bp		R	TAGGTCTCACGAACCTCTATTCAGAATTTTACTAG
			RPS4^{Col-0} (R4)		
R4-TIR	1-182	1-694	AATG	F	CACCGGTCTCTAATGGAGACATCATCTATTTCC
	182aa	694bp		R	TATGGTCTCATCCCTCCGGTGGTATTCC
R4-NB-ARC	183-659	695-2318	GGGA	F	CAATGGTCTCTGGGAAGTCACAACGCCGT
	477aa	1624bp		R	TGCCGGTCTCAGTGTGTCCTATTACAGAAAA
R4-LRR	660-879	2319-2977	ACAC	F	CAACGGTCTCTACACCATGCTTAAGGTGGGT
	220aa	659bp		R	TCCGGTCTCATGGAACTGATGTAAGACTCGT
R4-CTD	880-1217	2978-4113	TCCA	F	CACCGGTCTCTTCCAGAGTTTCCACCAAAT
	338aa	1136bp		R	TATGGTCTCACGAACCGAAATTCTTAACCGTGTG
			RPS4B^{Ws-2} (R4B)		
R4B-TIR	1-181	1-777	AATG	F	CAAAGGTCTCTAATGGCGGCGTCGTCGTCCT
	181aa	777bp		R	TACGGTCTCATCCCTCCAATGAAACTTTCTTCA
R4B-NB-ARC	182-659	778-2404	GGGA	F	CAAGGTCTCTGGGAAGCCAAAAGGTGGTGTC
	478aa	1627bp		R	TACCGGTCTCAGTGTATCCTGAGCAGTAAAA
R4B-LRR	660-855	2405-2991	ACAC	F	CACCGGTCTCTACACCAGTCTTAAAATGGGT
	196aa	587bp		R	TACCGGTCTCATGGAACATATGTAAGCTTCG
R4B-CTD	856-1165	2992-4030	TCCA	F	CAAAGGTCTCTTCCAGAGTTGCCACCAACTC
	310aa	1039bp		R	TACCGGTCTCACGAACCACTAGTGACTTTATCTTCTA

Table 2.7: List of primers used for cloning domain modules of RRS1, RRS1B, RPS4 and RPS4B. Forward (F) and Reverse (R) primers used to amplify each domain from genomic DNA of *BsaI*-domesticated *RRS1*$^{Ws-2}$, *RRS1B*$^{Ws-2}$, *RPS4*$^{Col-0}$ and *RPS4B*$^{Ws-2}$ are listed. The primers highlight *BsaI* recognition sequences (blue) and cleavage sites (bold & underlined). Amino acid numbers and nucleotide numbers indicate the boundaries and the size of each domain. The 4 bp overhang at the 5′ end of each domain is shown.

followed by incubating in a shaker for 1 h at 37 °C for *E. coli* and at 28 °C for *A. tumefasciens*.

Transformed cells were then selected on L agar plates supplemented with the appropriate antibiotics **Table 2.2**. Positive clones can also be selected using blue/white section if vector plasmids contained the lacZ cassette. For blue-white selection, IPTG (0.5 mM) and X-gal (40 µg/mL) were spread onto agar plates prior to adding bacterial cells. Plates were incubated at 37 °C for *E. coli* overnight, or at 28 °C for *A. tumefasciens* for 2–3 days to select for single colonies.

Plasmid purification and confirmation

The cultures of bacterial clones carrying desired DNA plasmids were either grown from single colonies on a selective agar plate, or re-cultured from a frozen glycerol stock (details see below). Cultures were grown in liquid L-medium in a shaking incubator (200 rpm, at 37 °C overnight for *E.coli*; 200 rpm, at 28 °C 2 days for *A. tumefaciens*). Cells were collected by centrifugation for 10 min at 3500 rpm, and then lysed and purified using QIAprep Spin Miniprep Kit (Qiagen) following manufacturer's instructions. Each preparation was eluted in 40 µl dH_2O and stored at -20 °C.

Successful DNA insertions into the plasmids were checked by restriction enzyme digestion analysis. Plasmid sequences, especially the DNA inserts, were then confirmed by illumina sequencing using services provided by the GATC Biotech company [`http://www.gatc-biotech.com/en/index.html`]. Correct plasmids were re-transformed to obtain single colonies, and then cultured and stored as glycerol stocks at -80 °C: 800 µL bacterial liquid culture made from a single colony plus 400 µL 60% glycerol.

2.2.2 *Bacterial infection and infiltration assays*

P. syringae pv. tomato (Pst) growth assay

Pst DC3000 strains carrying denoted constructs were grown on selective L-medium agar plates for 48 hours (h) at 28 °C. Bacteria were harvested from plates and re-suspended in 10mM $MgCl_2$, and adjusted to OD_{600}=0.001 [$5x10^5$ colony forming unit (cfu)/mL] for infiltration. Leaves of 5-week-old Arabidopsis plants were hand-infiltrated with needle-less syringes. Infected leaf samples were harvested 3 days post infiltration (dpi) using a cork borer

(d = 0.6 cm), resulting in 1 cm^2-sized leaf discs. For each condition, six leaf discs from 4 independent plants were randomly sampled, and ground in 10 mM $MgCl_2$. The suspensions were then serially diluted (10^2, 10^3, 10^4, 10^5), and spotted (each spot=10 µl) on selective L agar plates to grow at 28 °C for 48 h before colony counting. The bacterial growth (cfu/cm^2) was estimated according to the average colony counts per unit of sample leaf area, normalised to respective dilution factors.

Infiltration (HR) assays of P. fluorescens (Pf)

Pf Pf0-1 strains carrying denoted constructs were grown on selective L-medium agar plates for 24 h at 28 °C. Bacteria suspension in 10mM $MgCl_2$ were adjusted to OD_{600}=0.2 (1x 10^8 cfu/mL) for infiltration. Leaves of 5-week-old Arabidopsis plants were hand-infiltrated and kept for 20–24 h for HR symptom development (leaf tissue collapse).

Agrobacterium-mediated transient transformation of N. tabacum and N. benthamiana

A. tumefaciens strains carrying denoted binary constructs were streaked on selective L-medium agar plates and grown for 24-48 h. From single colonies, cultures were grown in liquid L-medium for 24 h in a shaking incubator (200 rpm, 28 °C). Cells were harvested by centrifugation and re-suspended at OD_{600}=0.5 (2.5x10^8 cfu/mL) in infiltration medium (10mM $MgCl_2$, 10mM MES, pH 5.6). For co-expression, each bacterial suspensions carrying individual constructs was adjusted to OD_{600}=0.5 in the final mix for infiltration. Leaf sections of 5-week-old *N. tabacum* or leaves of *N. benthamiana* were infiltrated with 1 mL needle-less syringe. The infiltrated *N. benthamiana* leaves were taken off at 2 dpi for total protein extraction and co-immunoprecipitation. *N. tabacum* cell death or HR phenotypes were photographed at 4–5 dpi.

2.2.3 *Biochemical methods*

Protein extraction from plant cells

Protein samples were prepared from *N. benthamiana* 48 h after *Agrobacterium*-mediated transformation. Plant materials were flash-frozen and ground in a pre-cooled mortar with liquid nitrogen. Total proteins were

extracted in GTEN buffer (10% glycerol, 150 mM Tris-HCl pH 7.5, 1 mM EDTA, 150 mM NaCl) supplemented extemporaneously with 10 mM DTT, 0.2% (v/v) Nodinet-40, anti-protease tablet (Complete EDTA-free RoChe) and 2% PVPP. Homogenisation was achieved by mixing on a rotator for 20 min at 4 °C.

The lysates were then centrifuged for 20 min at 5000 *g* at 4 °C, and the supernatant was filtered through Miracloth. This total protein extract was either mixed with 3xSDS loading buffer (30% glycerol, 3.3% SDS, 94 mM Tris-HCl pH 6.8, 0.05% (vol/vol) bromophenol blue) supplemented with 10 mM DTT for SDS-PAGE and immunoblot analysis as input samples or used for immunoprecipitation.

Co-immunoprecipitation (Co-IP) from total plant extract

Immunoprecipitations were conducted on 1.5 mL of filtered extract incubated with 30 µl agarose beads (α-FLAG M2, Sigma; α-GFP, Chromotek) for 2 h at 4 °C under gentle agitation. Antibodies-coupled agarose beads were then collected via centrifugation at 7000 rpm for 30 seconds in 1.5 mL tubes, followed by removal of supernatant. Beads were washed three times in the washing buffer (GTEN buffer supplemented with 10 mM DTT, 0.2% Nodinet-40 and Anti-protease tablet), and re-suspended in SDS-loading buffer supplemented with 10 mM DTT. The immunoprecipitated protein samples were released from the beads and denatured by boiling at 96 °C for 10 min. Proteins were separated by SDS-PAGE and analysed by immunoblotting.

SDS-PAGE and immunoblot analysis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to separate protein samples according to their molecular weight (in kilodalton, kDa). Tris-Glycine polyacrylamide (PAA) gels were prepared with 5% polyacrylamide for stacking gel, and 8, 10 or 12% polyacrylamide for resolving gels in this study. Denatured protein samples were separated by electrophoresis in 1xSDS running buffer (0.1% SDS) firstly at 90 V (stacking gel) and then at 130 V (resolving gel) for as long as appropriate for protein separation. The pre-stained protein ladder (PageRuler, ThermoFisher) was used as molecular weight marker from 10 to 180 kDa.

Proteins on the gels were than transferred to an immobilon-P PVDF membrane (Merck Millipore) using a semi-dry transfer apparatus supplied by Trans-Blot Turbo (Bio-Rad). Membrane was blocked for 1 h at room temperature or overnight at 4 °C in TBST (Tris-Buffered Saline with 0.1% Tween) containing 5% (w/v) non-fat dry milk. Membrane incubation with Horseradish Peroxidate (HRP) conjugated antibodies (α-FLAG M2, 1:10000 dilution, Sigma; α-GFP, 1:10000 dilution, Santa Cruz Biotechnology) was carried out in TBST supplemented with 5% milk by gentle agitation at room temperature for 1 h. The membrane was then rinsed 3 times each time for 10 min in TBST, and once in TBS (Tris-Buffered Saline) for 10 min. Chemiluminescence detection for a protein of interest was carried out firstly by incubating the membrane with developing reagent (SuperSignal West Pico & West Femto), and then exposing it to X-ray film (Fuji) or imaging using ImageQuant LAS 4000 (Life Sciences).

2.2.4 *Cell biology*

Multi-colour bimolecular fluorescence complementation (BiFC) assays

For transient expression, *A. tumefacien* strains carrying BiFC constructs were used for infiltration of *N. benthamiana* leave sections at OD_{600}=0.5. Based on vectors published in [267], Golden Gate compatible modules for multi-color BiFC tagging were constructed. The N- and/or C- termini of the protein was tagged with the C-terminal part of CFP (cCFP) as a bait, and the N-terminal part of either Venus (nVenus) or Cerulean (nCerulean) as a prey respectively (e.g. cCFP:D456-R:nVenus, cCFP:D456-R:nCerulean). Interactions of the cCFP with nVenus tags generate yellow fluorescence (YFP), and interactions of cCFP with nCerulean tags generate blue fluorescence (CFP). In this study, effector proteins (or their mutant controls) used for co-expression with R proteins in the BiFC assays were C-terminally tagged with mCherry. All control experiments were conducted on different sections of the same leaf, with each experiments replicated independently for at least 3 times.

For microscopic analyses, leaf sections were sampled 2 dpi with water as imaging medium. Live-cell imaging with a Leica DM6000B/TCS SP5 laser-scanning confocal microscope (Leica microsystems, Bucks, UK), using a 63x (water immersion) objective. Fluorescence emissions were collected between 465–485 nm for CFP (shown in blue), 680–700 nm for chlorophyll

autofluorescence (shown in red), 530–550 nm for YFP (shown in yellow), and 580–620 nm for mCherry (shown in red). Image analysis was performed with Fiji [http://fiji.sc/Fiji].

3

Two linked pairs of Arabidopsis TNL resistance genes independently confer recognition of bacterial effector AvrRps4

Notes: Figures and results presented in this chapter include my contributions to Williams et al (2014) [115] and Saucet et al (2015) [121] unless stated otherwise. As an author of [115, 121], I have obtained copyright permission to use the figures and captions. Simon Saucet's (SS) contribution is acknowledged as appropriate.

3.1 Introduction and Chapter aim

In Arabidopsis accessions Ws-2 and Col-0, the recognition of *P. syringae* effector AvrRps4 requires both RRS1 and RPS4. Only in Ws-2, RRS1-R and RPS4 confer resistance to *R. solanacerum* effector PopP2. However, AvrRps4 recognition was retained in both Col-0 and Ws-2 after mutating *RRS1* or *RPS4* [114, 241, 243, 268]. Furthermore, this residual AvrRps4 recognition is also EDS1-dependent [121, 223], suggesting an additional TNL protein confers RRS1- and RPS4-Independent AvrRps4 Recognition (RRIR). Saucet et al (2015) identified a pair of TNL encoding genes (*At5g45050*/*At5g45060*) on chromosome 5 linked to *RRS1/RPS4*, designated as RRS1B/RPS4B, which are responsible for the RRIR. For simplicity, hereafter RRS1/RPS4 are referred to as the A pair, and RRS1B/RPS4B as the B pair.

RRS1B and *RPS4B* are divergently transcribed and in a head-to-head configuration similar to *RRS1* and *RPS4* **(Figure 3.1)**. Moreover, the B pair proteins closely resemble the exon/intron architecture and domain arrangement of the A pair proteins **(Figure 3.1)**. However, RRS1B/RPS4B only confers recognition of AvrRps4 but not of PopP2. This is supported by the genetic evidence that *RRS1-R* mutant Ws-2 *rrs1-1*, which carries *RRS1B/RPS4B*, shows the complete loss of PopP2 recognition and HR **(Figure 3.2A)**. Like *RRS1/RPS4*, both *RRS1B* and *RPS4B* are genetically required for RRIR. Mutations of either *RRS1B* (Col-0 *050-1*) or *RPS4B* (Col-0 *060-2*, Ws-2 *060-1*) completely abolish the residual AvrRps4 responsiveness and disease resis-

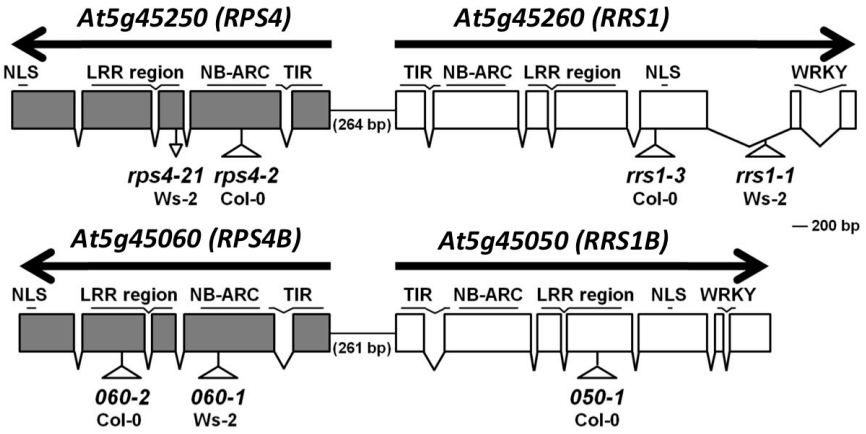

Figure 3.1: Schematic representation of *RRS1/RPS4* and *RRS1B/RPS4B* gene pairs. Black arrows indicate the reading frame direction. Boxes depict exons, with encoding domains highlighted on the top: TIR, Toll/Interleukin-1 receptor/R protein; NB-ARC, Nucleotide binding, APAF1, R proteins and CED4; LRR, leucine-rich repeat; WRKY, WRKY transcription factor family DNA binding domain; NLS, nuclear localisation signal. White triangles indicate the positions of T-DNA insertions for various mutant lines. *At5g45050*/*At5g45060* are abbreviated as *050* and *060*respectively. Figure was generated by SS, adapted from[121] Fig 2b.

tance in *RRS1* or *RPS4* mutant backgrounds **(Figure 3.2A,B)**. In other words, any double mutants that compromises both A and B pair functions loses the ability to recognise and respond to AvrRps4. Using transient assays in tobacco (*N. tabacum*), Saucet et al (2015) has shown that co-expression of RPS4B and RRS1B confers recognition and HR when co-delivered with AvrRps4, but not with PopP2 **(Figure 3.3A)** [121].

This chapter will investigate the molecular mechanisms of how the immune receptor pair RRS1B/RPS4B functions to recognise AvrRps4 and then triggers defence, in comparison with RRS1/RPS4.

3.2 RRS1B and RPS4B associate and function together

RRS1 and RPS4 have been reported to associate in an immune complex for effector perception and defence activation [115]. Using co-immunoprecipitation (Co-IP) we (YM and SS independently) showed that without any effectors, RRS1B and RPS4B also associate *in planta* when transiently co-expressed

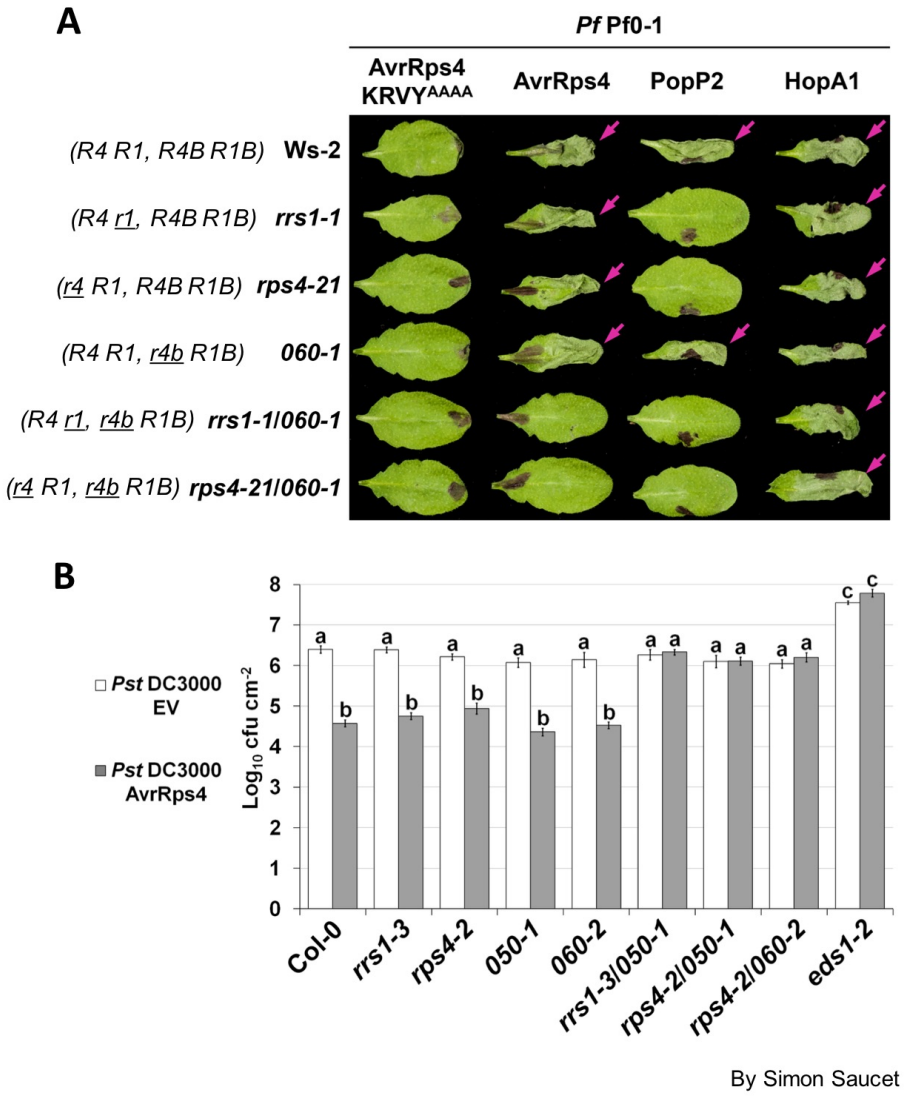

Figure 3.2: *RRS1B* and *RPS4B* are both required for the RRIR. (**A**) HR assays in Ws-2, Ws-2 single and double mutants using leaf infiltration with *Pf* Pf0-1 secreting AvrRps4-KRVYAAAA, AvrRps4, PopP2 or HopA1. HR phenotypes were assessed 24 h post infiltration (hpi). Magenta arrows indicate leaves showing HR. (**B**) Bacterial growth of *Pst* DC3000 carrying either pVSP61 empty vector (EV) or secreting AvrRps4 in Col-0, Col-0 single and double mutants. Bacterial growth was measured 3 days post infiltration (dpi). *RRS1B* mutant of Col-0 is shown as *050-1*, *RPS4B* mutants are shown as *060-1* or *060-2* in Ws-2 and Col-0 respectively. Means ± standard error of four replicates per sample are given. Samples with different letters are statistically different at the 5% confidence level based on Tukey's test. Figure was generated by SS, adapted from [121] Fig 2c,d.

in *N. benthamiana* **(Figure 3.3B)**. This indicates that similar to RRS1/RPS4, the RRS1B and RPS4B may also associate in an immune complex prior to pathogen perception. However, we cannot exclude that the pre-activation immune complex detected using the over-expressing transient systems in *N. benthamiana* may not accurately reflect the complex formation status in Arabidopsis.

Given that RRS1B/RPS4B are the closest paralogs of RRS1/RPS4 in Arabidopsis (~70% identity), it is interesting that inappropriate combinations of RRS1 and RPS4B, or RRS1B and RPS4 are non-functional. Genetic evidence in Arabidopsis shows that Ws-2 *rrs1-1/rps4b-1* (RRS1B/RPS4 combination) does not give HR to *P. fluorescens* (*Pf*) Pf0-1 secreting AvrRps4 **(Figure 3.2A)**. Likewise Col-0 *rps4-2/rrs1b-1* (RRS1/RPS4B combination) is susceptible to *P. syringae* pv *tomato* (*Pst*) DC3000 carrying AvrRps4 **(Figure 3.2B)**. Consistently, the combinations of RRS1/RPS4B or RRS1B/RPS4 co-expression in *N. tabacum* transient assays failed to show HR in response to any effectors, AvrRps4 or PopP2 **(Figure 3.3A)**. These data demonstrate that each R protein must pair with its cognate partner to assemble functional immune complexes.

To investigate whether inappropriate combinations of R proteins lose the ability to form complexes, I carried out Co-IP experiments to test the R protein associations between the inappropriate pairings (RRS1B/RPS4 and RRS1/RPS4B). We found that RRS1B-GFP Co-IPs with RPS4-FLAG, and RRS1-GFP Co-IPs with RPS4B-FLAG **(Figure 3.3B)**. However, the intensity of the Co-IP signals is higher between the appropriate pair partners (lane 2,6) than between the inappropriate partners (lane 3,5) **(Figure 3.3B)**. Therefore I infer that the complexes formed between RRS1B and RPS4 or between RRS1 and RPS4B are less stable than the authentic pair complexes (RRS1/RPS4 and RRS1B/ RPS4B). The lack of stability may compromise the function of the mis-pairing complexes. However, the complete loss of function is unlikely to be fully explained by weaker affinity between the full-length R proteins. The most likely explanation is that complex activation requires specific domain-domain interactions between pair partners, and incompatible domain-domain interactions within the inappropriate pairings lead to non-functionality. However, it is also possible that the weaker associations between inappropriate pairings observed using the over-expressing transient system in *N. benthamiana* are non-authentic, and do not exist in Arabidopsis.

Collectively, our data suggest immune complexes are formed preferably between respective partners, and only these appropriate pairing complexes (RRS1/RPS4 and RRS1B/RPS4B) enable effector recognition and defence signalling.

3.3 TIR domains of RRS1B/RPS4B and RRS1/RPS4 associate *in planta*

Many lines of evidence suggest that TIR/TIR interactions are important for TNL function [115, 197]. Williams et al (2014) [115] showed that the TIR domains of RRS1 and RPS4 physically associate. Crystal structures of the TIR domains in homo-dimeric or hetero-dimeric complexes reveal a common TIR/TIR interaction interface, stabilised by conserved histidine and adjacent serine residues, termed the SH motif. Alanine substitutions of the SH motif in RPS4 TIR domain (S33A/H34A) and in RRS1 TIR domain (S25A/H26A) disrupt TIR/TIR interaction, and impair effector recognition of the RRS1/RPS4 complex. Therefore, TIR/TIR interactions are essential to assemble a functional RRS1/RPS4 complex for effector-triggered immunity.

The predicted TIR domains of RRS1B (1–144 aa) and RPS4B (1–181 aa), consisting of complete 5 α helices, each share 64.58% and 61.67% identity and similar predicted topologies with the TIR domains of RRS1 (1–153 aa) and RPS4 (1–182 aa) respectively [269]. Interestingly, the SH motif is conserved in both RPS4B and RRS1B TIR domains. Furthermore, structural modelling based on the crystal structure of the RRS1 and RPS4 TIR/TIR hetero-dimer predicts a similar TIR/TIR interface for the RRS1B/RPS4B TIR domains stabilised by the SH motifs (data not shown). This suggests that the TIR domains of the B pair (RRS1B/RPS4B) may interact and function similarly to those of the A pair (RRS1/RPS4).

It has been shown that the TIR domains of the A pair interact in a yeast two hybrid (Y2H) assay, and associate *in planta* after Co-IP [115]. Furthermore, both assays (Y2H and Co-IP) demonstrated specificity of the heterotypic interaction between the TIR domains of RRS1 and RPS4, as they did not interact with L6 or RPP5 TIR domains [115]. To investigate whether the TIR domains of the B pair associate with each other *in planta*, and whether the association is specific within the pair, I carried out Co-IP experiments. TIR domain containing regions of RRS1 (1–175 aa), RPS4 (1–

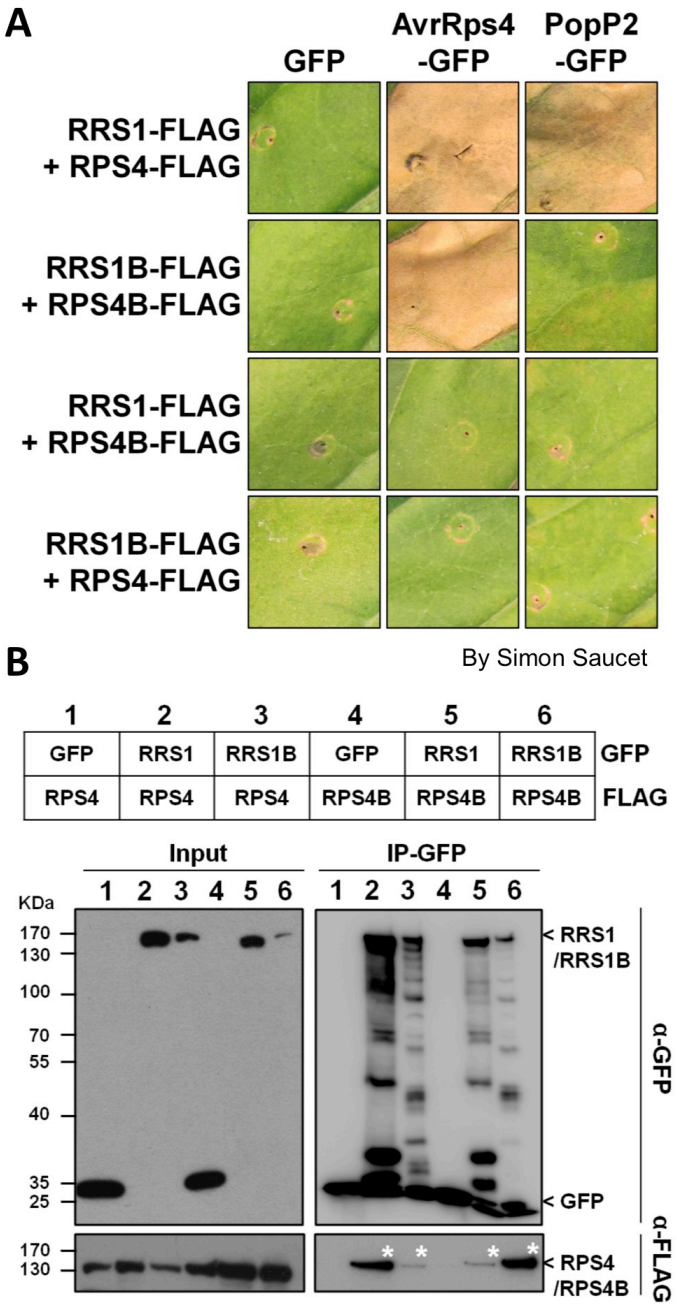

Figure 3.3: Corresponding pair partner association *in planta* is required for function. (**A**) Transient assays in *N. tabacum* leaves using *A. tumefaciens* transformation (agro-infiltration) show that inappropriate pair combinations fail to respond to effectors. Each leaf section was co-infiltrated to express a different combination of R genes together with GFP, *avrRps4-GFP* or *PopP2-GFP*. *RRS1*, *RPS4*, *RRS1B* and *RPS4B* were cloned from Ws-2 gDNA and fused to a C-terminal FLAG tag. Cell death pictures were taken 5 dpi. (**B**) Co-IP analysis shows the associations within and between RRS1/RPS4 and RRS1B/RPS4B pair proteins. *RRS1*, *RPS4*, *RRS1B* and *RPS4B* were fused to either a C-terminal FLAG or GFP tag. The combination of fusion proteins in each sample (C-terminal tag indicated on the right) is listed in the panel with a corresponding number (1–6). Immunoblots show the presence of proteins in total extracts (Input) and after IP with α-GFP beads (IP-GFP). Asterisks indicate the presence of protein bands after Co-IP. Figure adapted from [121] Fig 5; **A** was generated by SS.

235 aa), RRS1B (1–166 aa) and RPS4B (1–235 aa), designated as RRS1^{TIR}, RPS4^{TIR}, RRS1B^{TIR} and RPS4B^{TIR}, were cloned from Ws-2 gDNA and fused to either a C-terminal FLAG or GFP tag. After agro-infiltration in *N. benthamiana*, RRS1B^{TIR}-FLAG Co-IPs with both RPS4B^{TIR}-GFP and RPS4^{TIR}-GFP, likewise RPS4B^{TIR}-FLAG Co-IPs with both RRS1B^{TIR}-GFP and RRS1^{TIR}-GFP **(Figure 3.4)**. The results show that, similar to full-length proteins, RRS1B^{TIR} and RPS4B^{TIR} hetero-dimerise and also associate with RPS4^{TIR} and RRS1^{TIR} respectively *in planta* . As mis-pairing TIR domains hetero-dimerise with similar affinities to the authentic pairing, it suggests that heterotypic TIR/TIR associations are not specific between these two pairs.

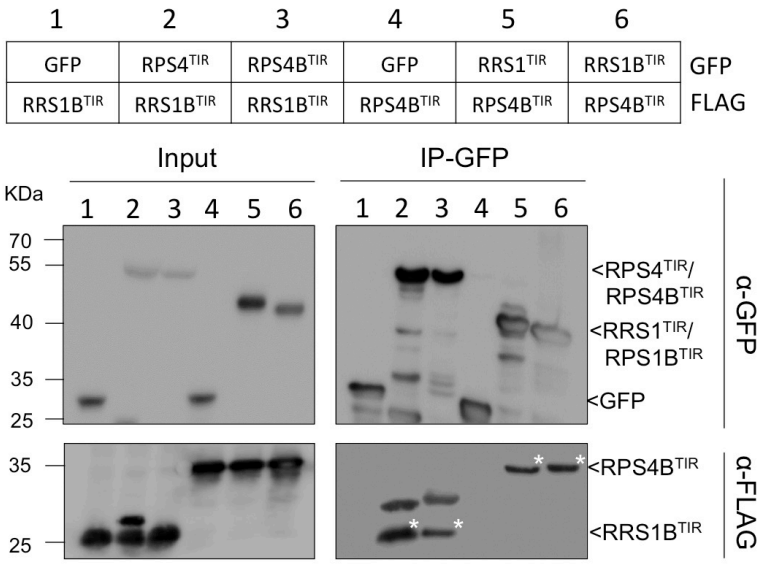

Figure 3.4: TIR domains of RRS1B and RPS4B associate *in planta*. This Co-IP analysis shows the associations of TIR domains from respective or different pair partners. TIR domain containing regions of RRS1(1–175 aa), RPS4(1–235 aa), RRS1B(1–166 aa) and RPS4B(1–235 aa) designated as RRS1^{TIR}, RPS4^{TIR}, RRS1B^{TIR} and RPS4B^{TIR}, were cloned from Ws-2 gDNA and fused to a C-terminal FLAG or GFP tag. The combination of fusion proteins in each sample (C-terminal tag indicated on the right) is listed in the panel with a corresponding number (1 - 6). Immunoblots show the presence of proteins in total extracts (Input) and after IP with α-GFP beads (IP-GFP). Asterisks indicate the presence of protein bands after Co-IP. Published in [121] Fig 6b.

TIR domains are considered to play a crucial role in defence activation as many of them can trigger effector-independent defence when over-expressed alone *in planta* [140, 159, 185, 270, 271]. I found that over-expression of RPS4^{TIR}-GFP but not RRS1^{TIR}-GFP triggered cell death, whereas neither RPS4B^{TIR}-GFP nor RRS1B^{TIR}-GFP triggered cell death in tobacco **(Fig-**

ure 3.5A,C) [115, 121]. Homotypic oligomerisations of some TIR domains, including RPS4^{TIR}, have shown to be essential to initiate defence signalling (e.g. L6) [115, 197]. Kee Hoon Sohn and others have demonstrated that mutations (S33A, H34A and S33A/H34A) that disrupt homo-dimerisation of RPS4^{TIR} completely abolished cell death signalling in tobacco [115]. On the other hand, the RPS4$^{TIR(R30A)}$ variant, which promotes stronger homo-dimerisation, induced a stronger cell death in tobacco compared to the RPS4^{TIR} wild type [115]. Although homo-dimerisation of RPS4^{TIR} is required for cell death signalling, the ability to homo-dimerise does not guarantee signalling competence: RRS1^{TIR} forms a homo-dimer, but fails to trigger cell death. Williams et al. [115] pointed out that the major differences between TIR domains of RRS1 and RPS4 is in the αD-helical region, where RRS1^{TIR} contains one helix and RPS4^{TIR} contains three. Sequence analysis and structural modelling suggest that RRS1B^{TIR} contains one αD helix whereas RPS4B^{TIR} contains three αD helices, similar to RRS1^{TIR} and RPS4^{TIR} respectively [269]. Therefore it is puzzling that RPS4B^{TIR}, with high structural similarities to RPS4^{TIR}, fails to trigger cell death in tobacco **(Figure 3.5A,C)**.

Interestingly, I found co-expression of RRS1^{TIR}-GFP suppressed RPS4^{TIR}-GFP induced cell death, whereas the S25A/H26A loss-of-hetero-dimerisation variant of RRS1$^{TIR(SHAA)}$-GFP did not **(Figure 3.5A)** [115]. Because a stronger hetero-dimeric interaction compared to homo-dimeric interactions of RPS4 and RRS1 TIR domains was observed using Co-IP and Y2H [115], we infer that the inactive hetero-dimer outcompetes the formation of the active RPS4^{TIR} homo-dimer thus suppressing cell death. Additionally, we (YM and SS independently) found that the RRS1-RPS4 TIR-TIR tandem fusion, which links RRS1^{TIR} (6-153 aa) and RPS4^{TIR} (10–178 aa) with a five-residue linker (GSGGS) to enable crystallisation [115], remained signalling inactive when transiently expressed in tobacco **(Figure 3.5B)**. However, the RRS1-RPS4 TIR(SHAA)-TIR fusion, introduced with the non-dimerising S25A/H26A mutations into RRS1^{TIR}, did not restore RPS4^{TIR} cell death signalling in tobacco **(Figure 3.5B)**. We suspected that the short 5 aa linker fused to the N-terminus of RPS4^{TIR} might restrict its movement away from the RRS1^{TIR}, posing steric hindrance to cell death signalling. Therefore I constructed a new tandem protein which links RRS1^{TIR} (6-153 aa) and RPS4^{TIR} (10-178 aa) with a longer 13 aa linker, designated as RRS1-RPS4 TIR^{+}-TIR^{+}. While the RRS1-RPS4 TIR^{+}-TIR^{+} fusion did not trigger cell death in tobacco, the mutant RRS1-RPS4 TIR^{+}(SHAA)-TIR^{+} did **(Figure 3.5B)**.

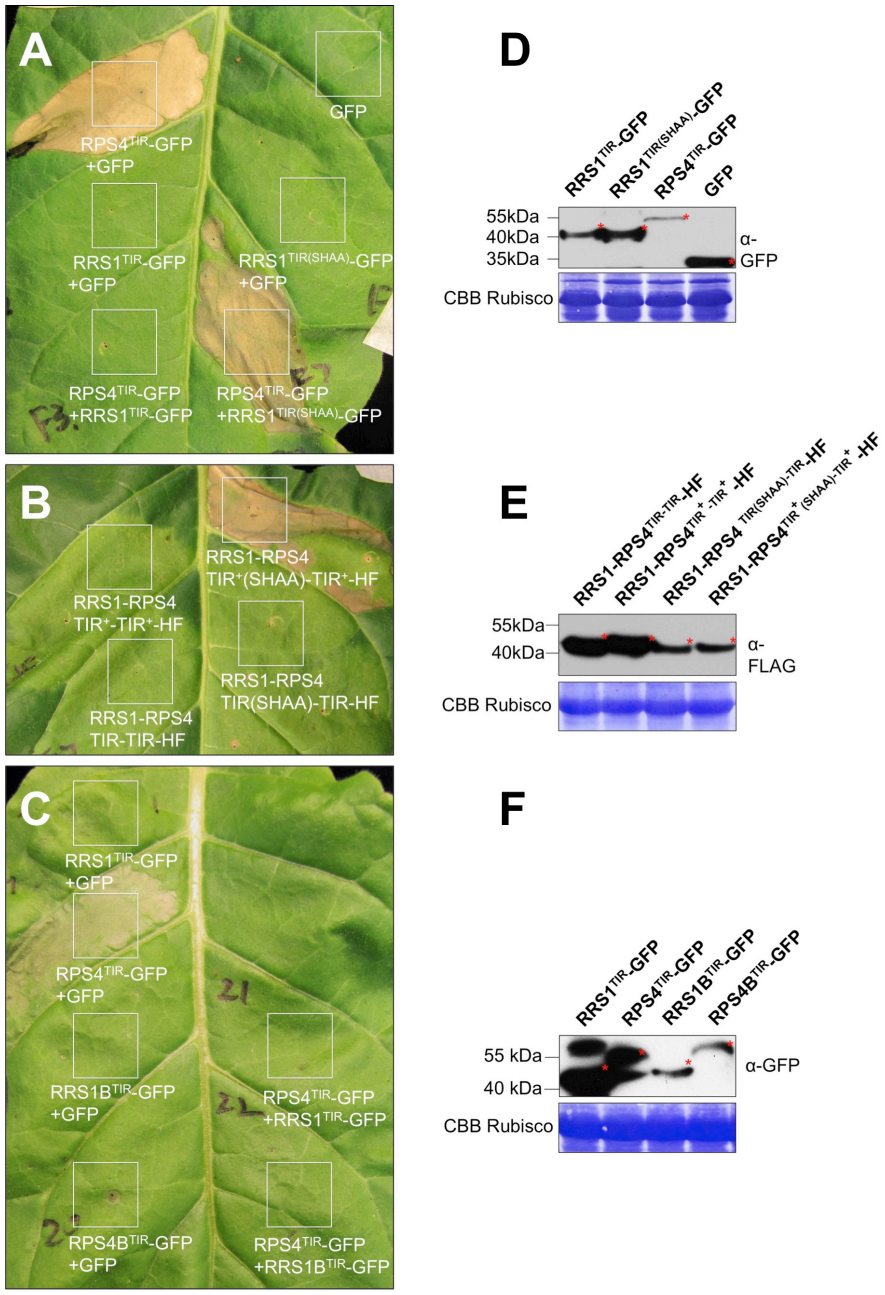

Figure 3.5: TIR domain of RRS1 or RRS1B suppresses RPS4 TIR domain-triggered cell death. (**A**-**C**) Transient assays in *N. tabacum* leaves using agro-infiltration assess cell death activities of various TIR domains and their combinations. *RRS1*, *RPS4*, *RRS1B* and *RPS4B* TIR domains or their mutant variants were expressed individually or as TIR-TIR fusions. Each leaf section was indicated by a white square with corresponding infiltrated samples labelled. Pictures were taken 5 dpi. (A) RRS1^{TIR}-GFP (1–175 aa) suppresses RPS4^{TIR}-GFP (1–235 aa)-induced cell death. Mutations in the SH motif of RRS1$^{TIR(SHAA)}$ abolish the suppression activity. (B) RRS1-RPS4 TIR-TIR and RRS1-RPS4 TIR^{+}-TIR^{+} tandem fusions, C-terminally tagged with 6xHis3xFlag (HF), do not trigger cell death. Mutations in the SH motif of RRS1^{TIR}, restores cell death activity of RRS1-RPS4 TIR^{+}(SHAA)-TIR^{+}-HF but not RRS1-RPS4 TIR(SHAA)-TIR-HF. (C) Neither RRS1B^{TIR}-GFP (1–166 aa) nor RPS4B^{TIR}-GFP (1–235 aa) triggers cell death. RRS1B^{TIR}-GFP suppresses RPS4^{TIR}-GFP-triggered cell death similarly to RRS1^{TIR}-GFP. (**D-F**) Immunoblot detection of TIR domain expression at 2 dpi in *N. benthamiana* leaves corresponds to those used in (A-C) respectively. TIR domains were tagged with GFP or HF as indicated. Commassie blue (CBB) staining of the large subunit of RuBisCo served as loading control. Asterisks indicate the presence of expected protein bands. Figure panels A and C adapted from [115] Fig 2D, and [121] Fig 6A.

Consistent with **Figure 3.5A**, these new fusion phenotypes suggest that RRS1^{TIR} suppresses RPS4^{TIR} by forming a poised stable hetero-dimer. When the mutant RRS1$^{TIR(SHAA)}$ failed to hetero-dimerise, RPS4^{TIR} was left free to homo-dimerise thus triggering cell death. Interestingly, despite their lack of activity, RRS1-RPS4 TIR-TIR-HF and RRS1-RPS4 TIR(SHAA)-TIR-HF accumulate at higher levels compared to RRS1-RPS4 TIR^{+}-TIR^{+}-HF and RRS1-RPS4 TIR^{+}(SHAA)-TIR^{+}-HF in Western blots **(Figure 3.5E)**. This suggests that the longer linker used between the TIR-TIR tandems possibly de-stabilises the RRS1 and RPS4 TIR/TIR hetero-dimer. On the other hand, the longer linker of RRS1-RPS4 TIR^{+}(SHAA)-TIR^{+} may also provide extra flexibility for RPS4^{TIR} to form homo-oligomers away from the RRS1$^{TIR(SHAA)}$, contributing to cell death signalling. Furthermore, the S25A/H26A mutations of RRS1^{TIR} do not affect protein accumulation, both when introduced into RRS1^{TIR} expressed alone **(Figure 3.5D)** and into the fusions **(Figure 3.5E)**, demonstrating that the lack of suppression activity of RRS1$^{TIR(SHAA)}$ was not due to less accumulation.

As I previously showed that RRS1B^{TIR} can hetero-dimerise with RPS4^{TIR}, I next investigated whether RRS1B^{TIR} is able to interfere with RPS4^{TIR}-triggered cell death. Indeed, I found that co-expression of RRS1B^{TIR} can suppress RPS4^{TIR}-triggered cell death **(Figure 3.5C)**. While the protein accumulation level of RRS1B^{TIR} is lower compared to RRS1^{TIR} **(Figure 3.5F)**, their suppression activity is comparable, indicating the hetero-dimer of RRS1B^{TIR}/RPS4^{TIR} is equally stable compared to RRS1^{TIR}/RPS4^{TIR}. This phenotype is consistent with our biochemical evidence that the non-pairing TIR domains (RRS1B^{TIR}/RPS4^{TIR} and RRS1^{TIR}/RPS4B^{TIR}) Co-IP as well as the authentic pairing **(Figure 3.4)**.

3.4 TIR swaps between R protein pairs retain function

We (YM and SS) next assessed if, despite associations of TIR domains between non-paired R proteins, the TIR domains contribute to the specificity of R protein function with each respective pair partner. To answer this question, we (YM and SS) constructed chimeras in which RRS1$^{Ws\text{-}2}$ and RRS1B$^{Ws\text{-}2}$ TIR domains were exchanged, and similarly with RPS4$^{Ws\text{-}2}$ and RPS4B$^{Ws\text{-}2}$ TIR domains. Here we designated the 4 domains of RRS1 and RPS4 (TIR, NB, LRR and C-Terminal Domain (CTD)) as “AAAA” and of RRS1B

and RPS4B as "BBBB", defining the TIR domain-swapped full-length chimeric proteins as RRS1(BAAA), RRS1(ABBB), RPS4(ABBB), and RPS4(BAAA) **(Figure 3.6A)**. Note that in Chapter 4, the C-Terminal Domain (CTD) of $RRS1^{Ws-2}$ and $RRS1B^{Ws-2}$ will be further divided into three separate domains: the 4^{th} domain C-terminal of the LRR domain (Domain 4, or DOM4), a WRKY DNA binding domain (WRKY), and the 6^{th} domain extended from the C-terminal end of the WRKY domain (DOM6) **(Figure 5.1)**. These TIR-swapped chimeras were tested in combination with wild type R proteins for AvrRps4 and PopP2 recognition using tobacco transient assays. Similar to the wild type combination RRS1(AAAA) + RPS4(AAAA), both RRS1(BAAA) + RPS4(AAAA) and RRS1(AAAA) + RPS4(BAAA) are able to recognise AvrRps4 and PopP2, triggering strong cell death **(Figure 3.6B)**. On the other hand, RRS1(ABBB) + RPS4(BBBB) and RRS1(BBBB) + RPS4(ABBB) respond only to AvrRps4, phenocopying the wild type RRS1(BBBB) + RPS4(BBBB) **(Figure 3.6B)**. The accumulations of chimeric proteins and effectors were confirmed by immunoblots **(Figure 3.6C,D)**.

These results show that the exchange of TIR domains from paralogous R genes does not compromise AvrRps4 or PopP2 recognition. We therefore infer that the TIR domains do not determine the pairing specificity for function, and domain-domain interactions other than TIR-TIR must account for the pairing specificity of RRS1/RPS4 and RRS1B/RPS4B.

We (YM and SS) also characterised additional domain swaps between $RRS1^{Ws-2}$ and $RRS1B^{Ws-2}$, with the breakpoint at the junction of exon 4 and exon 5. In $RRS1^{Ws-2}$ and $RRS1B^{Ws-2}$, exons 5, 6 and 7 encode the WRKY domain plus the C-terminal amino acids (DOM6) and ~260 amino acids between the LRR domain and the WRKY domain (part of DOM4), which altogether corresponds to a C-terminal part of the CTD defined in this chapter. These swaps were designated as RRS1(AAAB) and RRS1B(BBBA), and tested for responsiveness to AvrRps4 and PopP2 in the presence of either RPS4 or RPS4B. Neither of the RRS1(AAAB) or RRS1(BBBA) chimeras conferred recognition of AvrRps4 or PopP2 **(Figure 3.7A)**. Accumulation of the chimeric proteins was confirmed by Western blots, suggesting that the lack of cell death was not due to a lack of protein accumulation **(Figure 3.7B)**. The loss of effector responsiveness in RRS1(BBBA)+ RPS4B(BBBB) indicates the importance of the RRS1B C-terminal region during effector-triggered defence activation: it might be required for specific interactions with RPS4B during activation. It is also possible that these C-terminal swaps have impaired the

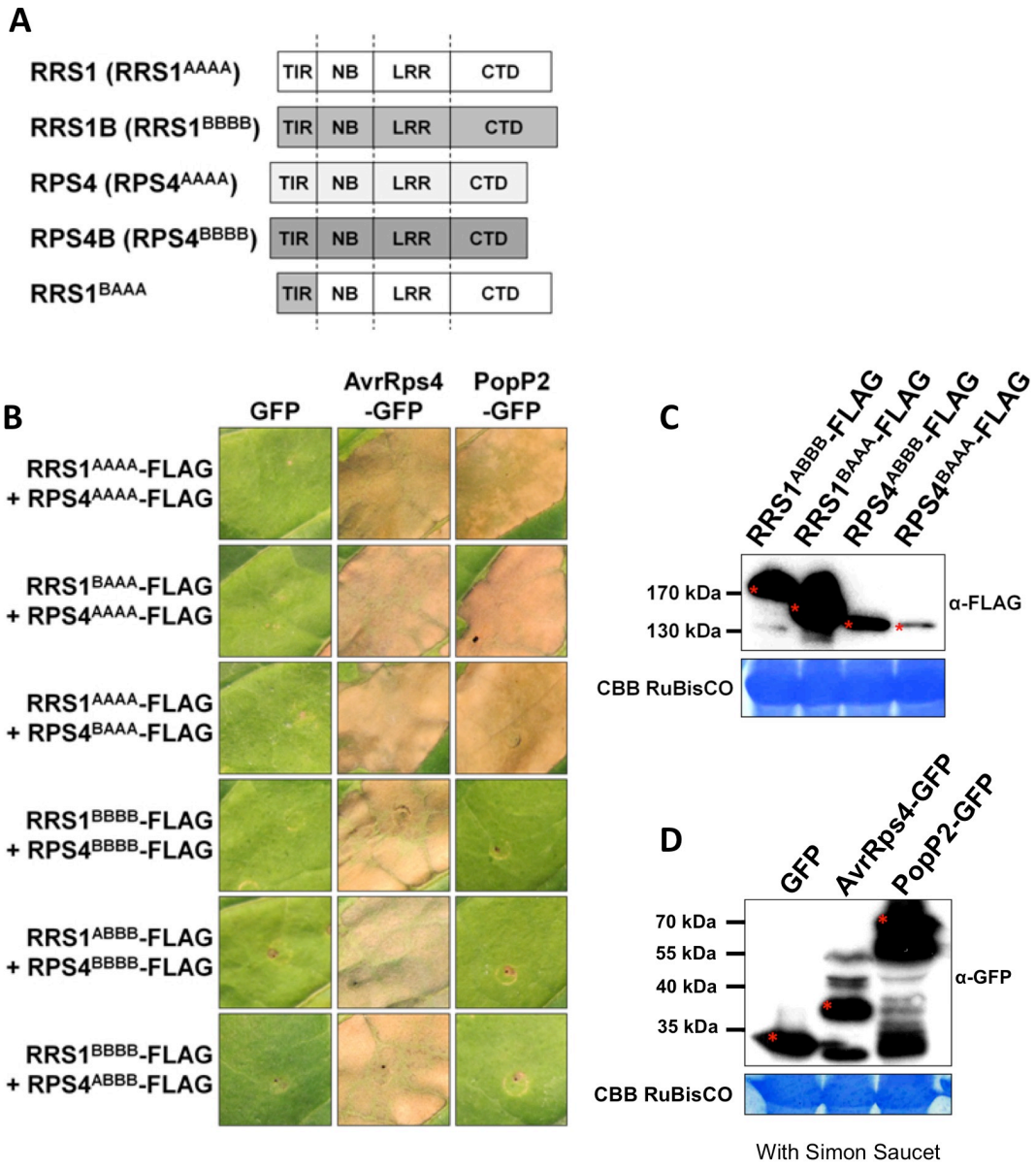

Figure 3.6: TIR domain swaps between RRS1/RPS4 and RRS1B/RPS4B retain function (**A**) Schematic representation of RRS1, RRS1B, RPS4, RPS4B from Ws-2, and a chimera RRS1(BAAA), showing domain structures: RRS1 or RRS1B as TIR-NB-LRR-CTD (C-Terminal Domain); RPS4 or RPS4B as TIR-NB-LRR-CTD. Each domain is indicated as an 'A' or 'B', depending on which pair they belong to: RRS1/RPS4 ('A' pair), RRS1B/RPS4B ('B' pair). (**B**) Transient assays in *N. tabacum* leaves using agro-infiltration assess the effector responsiveness of the TIR domain-swapped chimeras in combination with their cognate partners. Each leaf section was co-infiltrated to express a different combination of wild type and chimeric proteins with TIR exchanged between 'A' and 'B' pair proteins together with GFP, *avrRps4*-GFP or *popP2*-GFP. Pictures were taken 5 dpi. (**C-D**) Immunoblot detection of (C) Chimeric protein accumulation, RRS1(ABBB), RRS1(BAAA), RPS4(ABBB) and RPS4(BAAA)(with C-terminal FLAG tag); as well as (D) effector protein accumulation, AvrRps4 and PopP2 (with C-terminal GFP tag) at 2 dpi in *N. benthamiana* leaves. Coomassie blue (CBB) staining of the large subunit of RuBisCO served as loading control. Asterisks indicate the presence of expected protein bands. Published in [121] Fig 6C,D; experiments done together with SS.

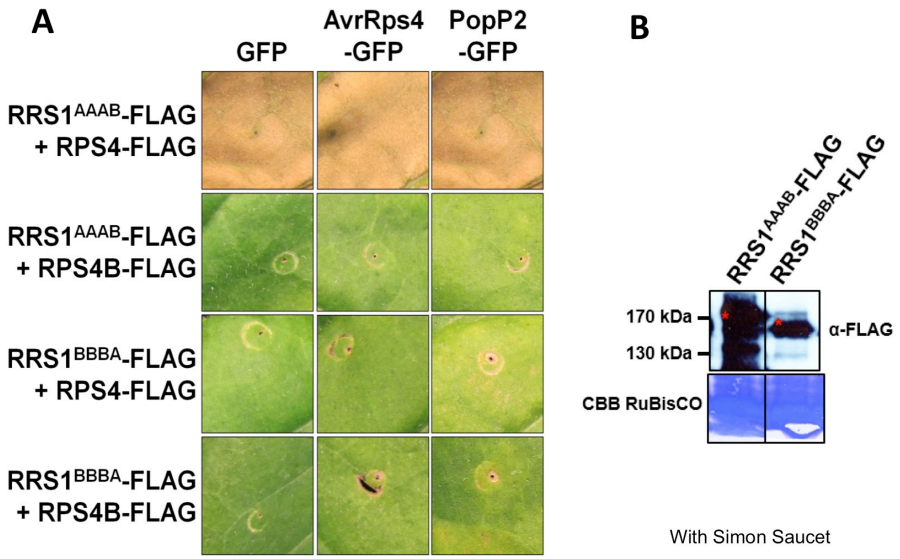

Figure 3.7: The RRS1(AAAB) chimera is auto-active in combination with RPS4 but not with RPS4B. (**A**) Transient assays in *N. tabacum* leaves using agro-infiltration assess the functionality of CTD-swapped RRS1 and RRS1B chimeras. Each leaf section was co-infiltrated to express a different combination of RPS4 or RPS4B with the chimeric proteins RRS1(AAAB) and RRS1(BBBA) ($RRS1^{Ws\text{-}2}$ and $RRS1B^{Ws\text{-}2}$ exchanging a WRKY-containing C-terminal part, encoded by exons 5,6 and 7) together with GFP, *avrRps4*-GFP or *popP2*-GFP. Pictures were taken 5 dpi. (**B**) Immunoblot detection of chimeric proteins RRS1(AAAB) and RRS1(BBBA) (with C-terminal FLAG tag) accumulation at 2 dpi in N. *benthamiana* leaves. Coomassie blue (CBB) staining of the large subunit of RuBisCO served as loading control. Asterisks indicate the presence of expected protein bands. Published in [121] Fig S15; experiments done together with SS.

integrity of the domain(s), disrupting proper folding of the chimeric proteins, thus rendering them non-functional. On the other hand, RRS1(AAAB) in combination with RPS4(AAAA) triggers constitutive cell death in the absence of an effector **(Figure 3.7A)**. This suggests that the integrity and appropriate interactions in the C-terminal regions of these proteins might be crucial for auto-regulation in a pre-activation immune complex. In contrast, RRS1(AAAB) in combination with RPS4B(BBBB) fails to trigger this effector-independent cell death, implying that RPS4(AAAA) is specifically required for RRS1(AAAB)-triggered auto-activity **(Figure 3.7A)**.

3.5 RRS1B associates with AvrRps4 and PopP2 *in planta*

Williams et al (2014) showed that the R protein complex formed by RRS1-R^{Ws-2} and RPS4 when co-expressed can associate with AvrRps4 or PopP2 *in planta* using Co-IP. Because RRS1-R and RPS4 are able to interact before effector perception, the paired R proteins are proposed to detect the effectors as a RRS1-R/RPS4 complex [115]. RRS1-R^{Nd-1} alone was also shown to interact with PopP2 in a yeast split-ubiquitin assay (Y2H) [214]. However, interaction with the R protein is not sufficient for defence activation, as PopP2 also interacts with RRS1-S^{Col-5} but does not trigger an immune response in Col-5 [214].

I examined whether RRS1B/RPS4B as a complex can associate with AvrRps4 and also PopP2 *in planta*, even though RRS1B/RPS4B cannot recognise PopP2. I co-expressed $RRS1^{Ws-2}$-FLAG + $RPS4^{Ws-2}$-FLAG and $RRS1B^{Ws-2}$-FLAG + $RPS4B^{Ws-2}$-FLAG, with either GFP, AvrRps4-GFP or PopP2-GFP in *N. benthamiana*. After IP with α-GFP beads, AvrRps4-GFP and PopP2-GFP but not the negative control GFP were able to pull-down RRS1B-FLAG and RPS4B-FLAG in a complex, likewise pulling down RRS1-FLAG and RPS4-FLAG together **(Figure 3.8B)**.

Collectively, the data suggest that effectors are perceived by RRS1 or RRS1B in their respective pre-formed complexes with RPS4 or RPS4B. However, I cannot exclude that AvrRps4 or PopP2 associates with RRS1 on its own, and promotes the recruitment of RPS4 to form a more stable hetero-complex (likewise for RRS1B). Interestingly, after IP with effectors the signal intensity was much stronger for RRS1 and RPS4 compared to RRS1B and

RPS4B **(Figure 3.8B)**, indicating the RRS1/RPS4 complex may have stronger affinity for both effectors than the RRS1B/RPS4B complex. This result also suggests that RRS1B/RPS4B associates *in planta* with AvrRps4 as strongly as with PopP2, despite the lack of activation of the latter.

Considering the high intensity of RRS1 protein bands compared to RPS4 after AvrRps4 and PopP2 immunoprecipitation, we (YM and SS independently) then tested whether $RRS1^{Ws\text{-}2}$ and $RRS1B^{Ws\text{-}2}$ alone are sufficient for effector association. Indeed, we found that both $RRS1^{Ws\text{-}2}$-GFP and $RRS1B^{Ws\text{-}2}$-GFP are sufficient to Co-IP with either AvrRps4-GFP or PopP2-GFP, but not with GFP **(Figure 3.8A)**. Like in complex with their partner, $RRS1^{Ws\text{-}2}$ also showed a stronger association with AvrRps4 or PopP2 compared to $RRS1B^{Ws\text{-}2}$. Altogether, our results show that, similar to RRS1-S/RPS4[214], protein-protein association of PopP2 with RRS1B/RPS4B complex can be detected, but this is not sufficient for defence activation.

3.6 AvrRps4-induced defence gene expression require either RPS4 or RPS4B

Given that two paired R genes provide resistance to a single effector, I investigated the quantitative contributions of *RRS1B/RPS4B* to AvrRps4-triggered immunity compared to *RRS1/RPS4* by measuring defence gene induction using quantitative RT-PCR. Based on previous studies, I selected defence marker genes that are specifically regulated by AvrRps4 and PopP2 in a RRS1/RPS4-dependent manner at early stages of immunity, namely *SARD1*, *SID2*, *PAD4* and *EDS5* [116]. Genotypes Ws-2, Ws-2 *rps4-2*, Ws-2 *rps4b-1*, and Ws-2 *rps4-2/rps4b-1* were infiltrated with *Pf*Pf0-1 carrying AvrRps4 or AvrRps4-KRVYAAAA mutant. The Ws-2 mutant lines used in this section are abbreviated as *rps4-2*, *rps4b-1* and *rps4-2/rps4b-1*. Primers used to amplify these genes, together with the expected PCR fragment sizes were listed in **Table 2.4**. Six hours post-infiltration, all selected defence marker genes compared to H_2O treatment were consistently more induced in Ws-2, *rps4-21* or *rps4b-1* after infiltration with *Pf* Pf0-1 (AvrRps4) than with *Pf* Pf0-1 (AvrRps4-KRVYAAAA) **(Figure 3.9)**. This pattern of a more pronounced fold-induction of AvrRps4-dependent defence genes in Ws-2, *rps4-21* or *rps4b-1* was also consistent across the three independent biological replicates **(Figure 3.10)**.

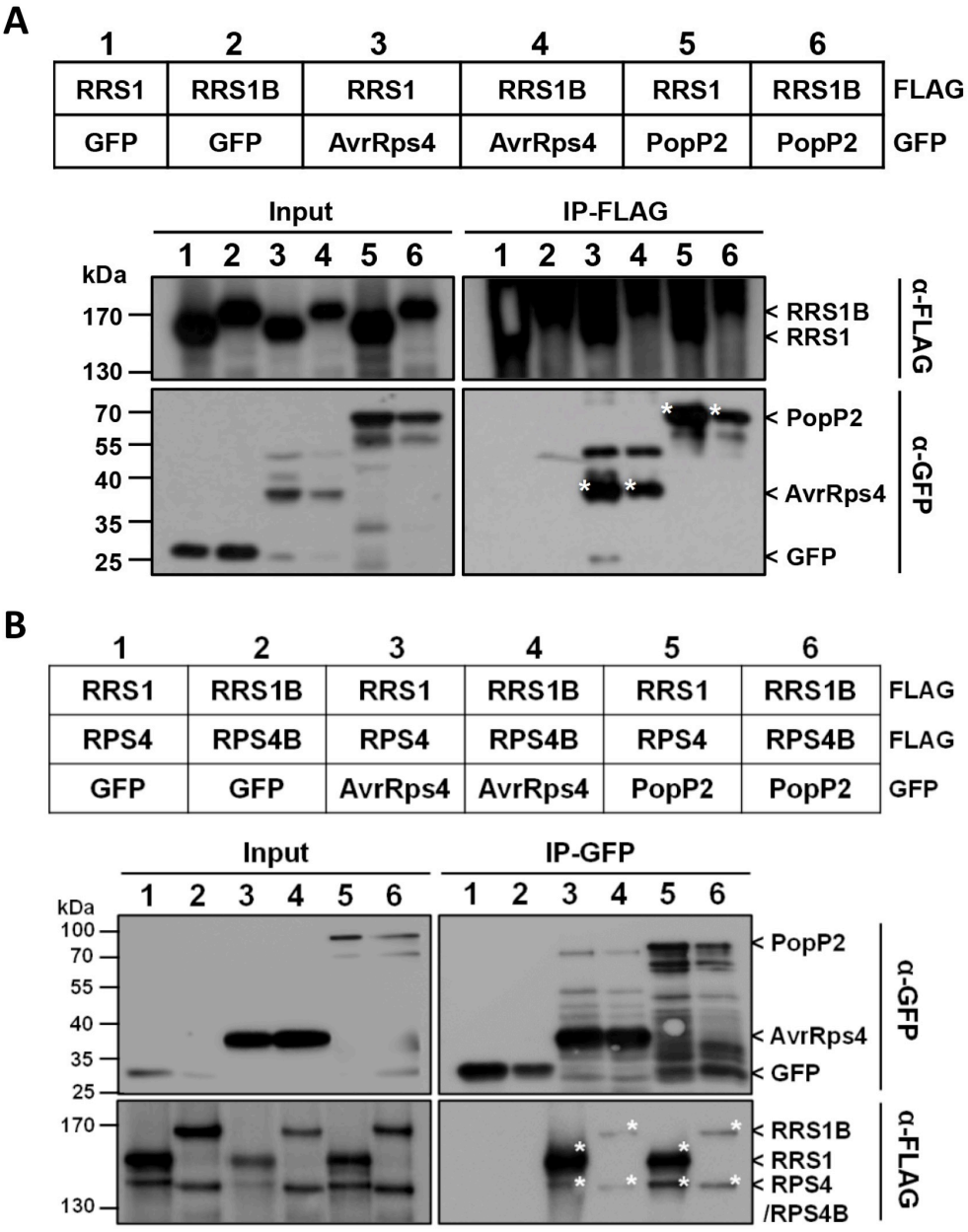

Figure 3.8: RRS1B and RRS1B/RPS4B complex associate with AvrRps4 and PopP2 *in planta*. This Co-IP analysis shows the associations of RRS1 and RRS1B with AvrRps4 and PopP2 (**A**), and association of RRS1/RPS4 and RRS1B/RPS4B complexes with AvrRps4 and PopP2 (**B**). All R genes were cloned from Ws-2 gDNA. The combination of fusion proteins in each sample (C-terminal tag indicated on the right) is listed in the panel with a corresponding number (1 to 6). Immunoblots show the presence of proteins in total extracts (Input) and after IP with α-FLAG beads (IP-FLAG). Asterisks indicate the presence of protein bands after Co-IP. Published in [121] Fig 7, S16; experiments done together with SS.

Assuming that each pair functions independently, these data indicate that RRS1B/RPS4B (in *rps4-21*) and RRS1/RPS4 (in *rps4b-1*) activate a similar set of defence genes upon AvrRps4 recognition, and are therefore likely to share downstream signalling mechanisms. In the single knockout mutants tested (*rps4-21* and *rps4b-1*), there was no consistent pattern of quantitative differences between RRS1/RPS4- or RRS1B/RPS4B-dependent defence gene induction triggered by AvrRps4. For example, RRS1/RPS4 in *rps4b-1* contributes to higher induction of *EDS5* and *SID2*, but lower induction of *SARD1* and indistinguishable induction of PAD4 compared to RRS1B/RPS4B in *rps4-21* **(Figure 3.9 & Figure 3.10)**. This indicates that RRS1/RPS4 and RRS1B/RPS4B activate similar sets of defence genes.

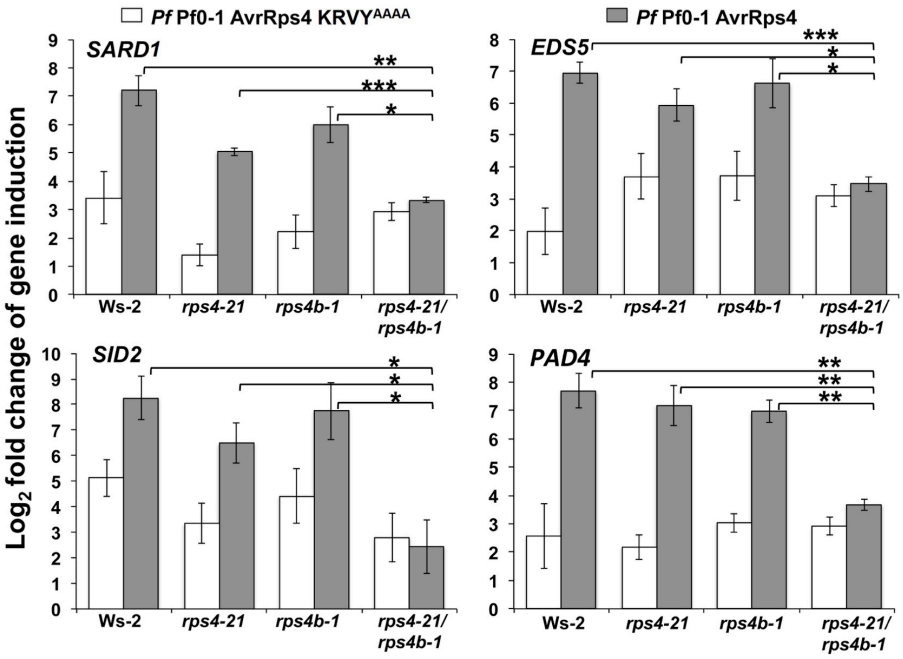

Figure 3.9: Loss of *RPS4* and *RPS4B* completely abolishes AvrRps4-triggered defence gene induction in Ws-2. Quantitative RT-PCR showing fold-change of *SARD1*, *SID2*, *EDS5* and *PAD4* gene expression. Primer information see (Table 2.4) in the appendix. Transcript levels were estimated in leaves of five week old Ws-2, Ws-2 *rps4-21* and *rps4b-1* single and Ws-2 *rps4-21/rps4b-1* double mutants infiltrated with either H_2O, *Pf* Pf0-1 secreting AvrRps4-KRVYAAAA or AvrRps4. RNA was extracted from samples taken at 6 hpi for cDNA synthesis. Results of quantitative RT-PCR for selected defence marker genes were first normalised to *EF1α*, then calculated as log_2-scaled fold change compared to H_2O treatment. Means ± SE of 3 biological replicates per sample are given. Differences that are statistically significant are indicated (Student's t-test $P<=0.05^{*}$, $P<=0.01^{**}$, $P<=0.001^{***}$). Published in [121] Fig 4

Although occasionally the presence of both RRS1/RPS4 and RRS1B/RPS4B in Ws-2 gave a higher AvrRps4-triggered defence gene induction compared

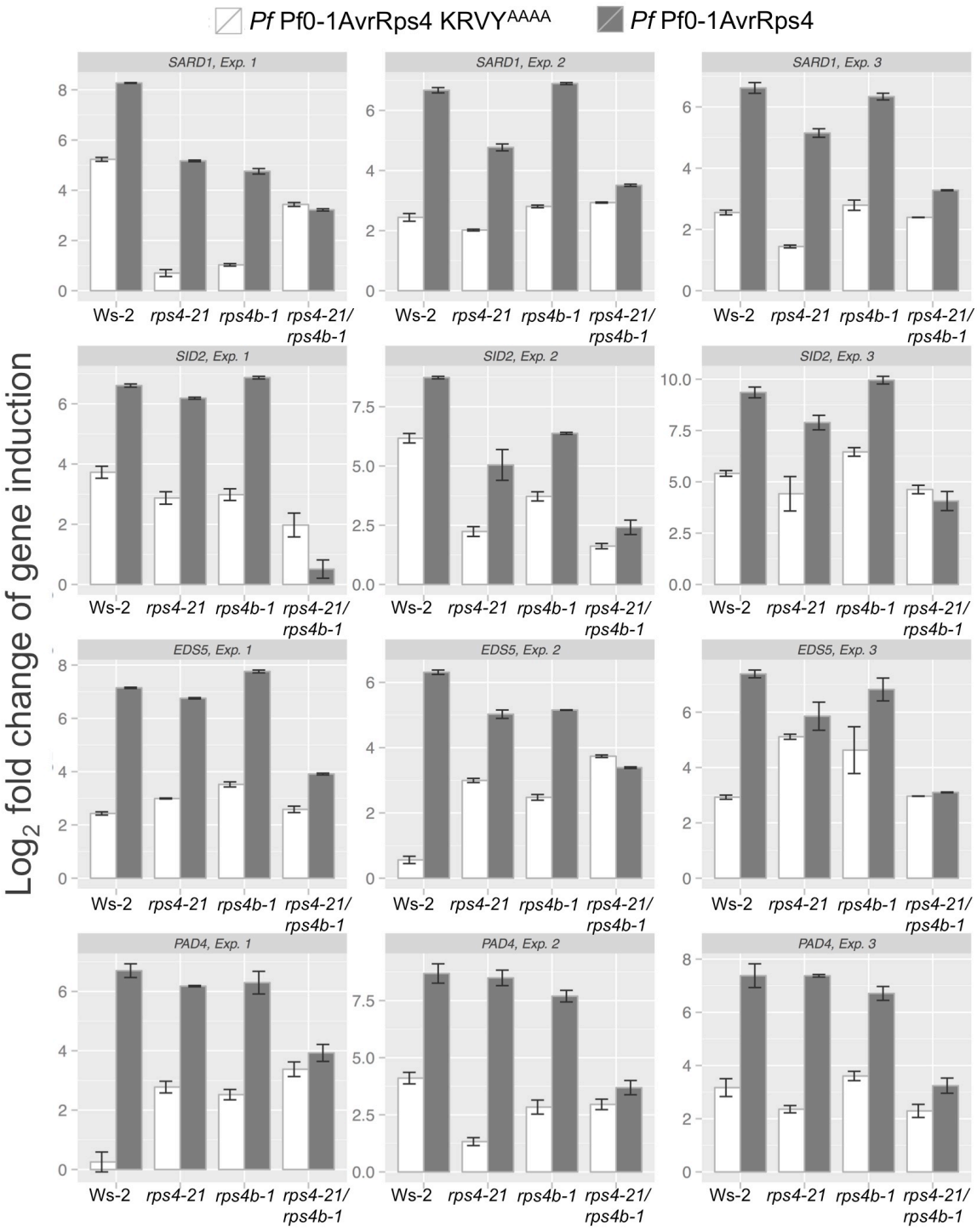

Figure 3.10: Loss of *RPS4* and *RPS4B* completely abolishes AvrRps4-triggered defence gene induction in Ws-2 — biological repeats. Details as for Figure 3.9. Means ± SE of 3 technical replicates per sample are given. Each column represents an independent biological experiment (indicated by Exp. 1, 2 and 3). Published in [121] Fig S8

to either *rps4-21* or *rps4b-1* in a single experiment **(Figure 3.10)**, overall I could not observe greater gene induction in Ws-2 compared to either single mutants **(Figure 3.9)**. I infer that RRS1/RPS4 and RRS1B/RPS4B independently activate defence genes to a level adequate for resistance in response to AvrRps4. As together RRS1B/RPS4B and RRS1/RPS4 do not contribute to a greater defence gene induction and a stronger resistance, it suggests the two pairs function redundantly for AvrRps4 resistance.

In the *rps4-21/rps4b-1* double mutant, fold induction of defence genes triggered by *Pf* Pf0-1 (AvrRps4) is not different to *Pf* Pf0-1 (AvrRps4-KRVYAAAA), but is significantly lower than in AvrRps4-treated Ws-2 or either single mutants **(Figure 3.9 & Figure 3.10)**. This means AvrRps4-triggered defence gene induction is fully dependent on functional RRS1B/RPS4B and/or RRS1/RPS4, which is consisent with the loss of resistance to *Pst* DC3000 (AvrRps4) observed in the double mutants **(Figure 3.2)**. These data on defence gene regulation provide a quantitative view of how the two R gene pairs work individually for AvrRps4-triggered immunity.

3.7 Discussion

In this chapter, I have revealed functional insights into an *R* gene pair *RRS1B/RPS4B* that resembles and is closely linked to *RRS1/RPS4*. *RRS1B/RPS4B* confers recognition of AvrRps4 but not PopP2. Like RRS1 and RPS4, both RRS1B and RPS4B are required for effector recognition. We found that each of these R proteins cooperates specifically with their respective partner, and each pair acts as a single functional unit for defence. Evidence so far supports the presence of pre-activation heteromeric complexes formed between the paired proteins prior to effector perception. Although partners from different pairs can also form heteromeric complexes, these inappropriate pair complexes are less stable and not functional for AvrRps4 and PopP2 recognition and/or downstream signalling activation **(Figure 3.3)**. However, we cannot exclude that the inappropriate pair complexes observed are the result of protein over-expression in a transient system, which might not occur under native expression. Altogether, this indicates that despite the similarity in motif prediction, TNL pairs evolved particular inter-molecular specificity for function (pairing specificity).

Conceivably, non-deleterious mutations accumulated independently in each pair after duplication, and those that promote stronger association and cooperation between pair partners (potentially different in each pair) would be selected for over time, driving the specificity of pairing. In Saucet et al 2015 [121], a probable common ancestor of both A and B pair was uncovered in *A. thaliana* relatives *Arabidopsis lyrata* and *Brassica rapa*. Furthermore, distinct orthologous pairs matching either the A pair or the B pair were also found in *A. lyrata* and *Capsella rubella*, showing evidence of further duplications after the divergence of A and B pair in species related to *A. thaliana* [121]. This suggests that there is a selective advantage to maintaining both pairs. One of the advantages could be that R gene pair duplications create redundancy that might reduce the effect of purifying selection and increase the potential for the evolution of new functions [272, 273]. We speculate that at some point after duplication, one of the pairs (RRS1-R/RPS4) evolved new effector recognition capacity (PopP2 or *Colletotrichum* resistance), becoming functionally distinct to RRS1B/RPS4B. Additionally, developing two or more similar recognition systems might enable the plant to maximise protection of an important cellular complex generally targeted by pathogen effectors [121].

TIR domain interactions are essential to build a functional effector recognition complex of RRS1/RPS4, and the structural similarities to RRS1B/RPS4B TIR domains indicate similar roles in complex assembly. These TIR domains exhibit a level of specificity for heteromeric interactions, but not between the paralogous pairs. For example, RRS1 and RPS4 TIR domains do not interact with TIR domains from the TNLs L6 or RPP5 [115], but can associate with RPS4B and RRS1B TIR domains respectively. Consistently, we found the TIR domain exchanges between pairs do not impair pair function, therefore are not responsible for the pairing specificity **(Figure 3.3)**. It has been shown that domains other than the TIRs also associate to help forming the pair complex [115], one or more of which must be responsible for the pairing specificity. Additional domain swap experiments between the pairs may help us better understand this specificity, and this is studied in Chapter 5.

Expression of the TIR domain from RPS4 but not RRS1 triggers effector-independent defence, indicating the role of RPS4 in defence signalling. As co-expression of RRS1^{TIR} suppresses RPS4^{TIR}-triggered auto-activity **(Figure 3.5)**, it can be proposed that RRS1 plays a regulatory role to keep RPS4

inactive in the absence of effectors. Upon effector recognition, RRS1 may de-repress RPS4, allowing RPS4 TIR domain to homo-oligomerise, which mediates defence signalling. The fact that the P-loop mutation of RPS4 but not RRS1 abolishes effector recognition [115], is evidence for the separation of roles of RRS1 and RPS4, conceivably in effector recognition and defence signalling respectively.

I also observed that RRS1B^{TIR} is able to suppress RPS4^{TIR}-triggered cell death **(Figure 3.5)**, and can functionally replace RRS1^{TIR} in the RRS1(BAAA) chimera **(Figure 3.6)**. This indicates RRS1B may play a similar role in the B pair complex compared to RRS1 in the A pair complex. However, to our surprise, the presumed signalling partner RPS4B, did not show signalling activity when its TIR domain is expressed in *N. tabacum* **(Figure 3.5)**. It is likely that either polymorphisms in RPS4B^{TIR} compared to RPS4^{TIR} abolish its ability to recruit downstream signalling components essential for defence activation, or that *N. tabacum* lacks the essential components required for RPS4B^{TIR}-triggered cell death. However, the RPS4B TIR domain is signalling competent when swapped into RPS4(BAAA), triggering cell death after effector recognition **(Figure 3.6)**. It is also possible that cell death triggered by the over-expression of TIR domains is an ectopic activation that differs from authentic NLR immune signalling.

More generally, we still don't fully understand the mechanism of TIR or CC domains during defence signalling, as the N-terminal domains of many functional TNL or CNL proteins cannot initiate effector-independent cell death. For those N-terminal domains (TIR or CC) that can trigger cell death (such as RPS4 TIR), homo-oligomerisation appears to be essential for the cell death signalling. We infer that the N-terminal domains of some plant NLRs act as protein scaffolds for immune complex assembly, and the multimerisation of NLRs enables close proximity of their N-terminal domains to initiate defence signalling. This model is based on the animal inflammasomes formed of NLR multimers, which act as a platform that enables the recruitment of components essential for signal transduction via the NLR N-terminal domain. Thus we speculate that plants NLRs also may form high order NLR multimers upon effector detection, resembling the animal inflammasomes. Alternatively, if the N-terminal domains of plant NLRs do not activate defence directly, they may play important roles during the effector-triggered inter-domain rearrangements that are essential for NLR activation. The exchanges of TIR or CC domains with other NLR proteins

may give us clues about the general functional mechanism of these N-terminal domains, as to whether they are more conserved for signalling or more specific for inter-domain interaction.

Several TNLs have been shown to recognise an effector directly and this direct interaction is presumed to trigger R protein activation [92, 94, 187, 214]. RRS1 alone is able to associate with effectors, and it perceives the effector, resulting in conformational changes that activates RPS4. As RRS1B resembles RRS1, and is also able to associate with effectors alone, we infer it has a similar role in effector recognition. An important clue is that both RRS1 and RRS1B carry WRKY domains in their C-termini, which are absent in their putative common ancestor in *A. lyrata* or *B. rapa* [121]. Intriguingly, evolutionary analysis suggests that the WRKY domain in RRS1, RRS1B and their other orthologs share a common origin from *WRKY35* [121]. WRKY domain-containing proteins play crucial roles in regulating plant defence [274, 275], and thus are likely to be virulence targets of pathogen effectors. It is plausible that RRS1 or RRS1B incorporated the WRKY domain for the perception of effectors that target the WRKY proteins. I will explore more of this aspect in Chapter 4.

We found that the integrity of the C-terminal part of RRS1 is important for maintaining auto-inhibition, as co-expression of the RRS1(AAAB) chimera with RPS4 triggers effector-independent HR. RRS1B(BBBA) however, does not trigger auto-activity when co-expressed with RPS4B. This suggests RRS1B may act a bit differently compared to RRS1-R during activation, and could help to explain why the B pair does not recognise PopP2. Furthermore, as PopP2 associates with both RRS1B and RRS1-S (which also does not confer recognition of PopP2), it maybe the conformational changes triggered after PopP2 association involving the C-terminal domains that defines recognition capability. Finer domain swaps of RRS1-R and RRS1B at their C-termini maybe able to identify the domain that specifies PopP2 recognition. It should be noted that exon boundaries are not the best way for swapping, as they might not reflect domain boundaries. Chapter 5 will include domain swaps with properly defined domain boundaries. It is also possible that the full length protein reconfiguration rather than a specific region would define PopP2 recognition specificity, which would not be revealed by single domain swaps.

Even though the current understanding is that both R protein pairs form pre-activation complexes for function, we cannot exclude that a proportion of these R proteins may exist as monomers in the resting state. An 'Equilibrium-based switch' model was recently proposed based on a study of flax TNLs L6 and L7 [168]. This model proposes that NLRs exist in an equilibrium between 'ON' and 'OFF' states, and effectors bind to the 'ON' state to stabilise the active conformation and shift the equilibrium towards activation [168]. We propose a possible scenario where the R proteins may exist in an equilibrium between poised complexes and inactive monomers, and effector perception shifts the balance towards complex formation, and may even drive higher order oligomerisation for defence activation [276]. Effectors can be perceived by monomers and complexes, because we observed that effectors associate with the RRS1/RPS4 and RRS1B/RPS4B complexes as well as with RRS1 and RRS1B alone **(Figure 3.8)**. However, to prove this model we need to find evidence in Arabidopsis showing that effector-activated RRS1 or RRS1B forms more stable complexes with RPS4 or RPS4B compared to the inactive ones.

To summarise, in this chapter we show that the RRS1B/RPS4B largely resembles the RRS1/RPS4 pair structurally and functionally. However, we also uncover within the two pairs the pairing specificity and different recognition capacity for PopP2, suggesting potentially distinctive features of their activation mechanisms. The RRS1/RPS4 and RRS1B/RPS4B gene pairs will be further investigated in later chapters to try to unravel the molecular and structural requirements for pairing specificity and how an interaction with AvrRps4 or PopP2 is converted into defence activation.

4

Bacterial effector AvrRps4 is detected by RRS1 and RRS1B via their WRKY domains

Notes: Figures and results presented in this chapter include my contributions to Sarris et al (2015) [58] unless stated otherwise. Copy Right Transfer has been obtained from the publisher. Contributions by Panagiotis F. Sarris (PS), Zane Duxbury (ZD), Sung Un Huh (SH), Cecile Segonzac (CS), Lennart Wirthmueller (LW), Hannah Brown (HB) and Volkan Cevik (VC) are acknowledged as appropriate.

4.1 Introduction and Chapter aim

The molecular mechanism of how the RRS1-R/RPS4 complex perceives distinct effectors (AvrRps4 and PopP2) was not well understood. PopP2 was previously shown to interact with RRS1-R [115, 121, 214], but the interacting domain of RRS1-R was not defined. However, PopP2 also interacts with RRS1-S or RRS1B, but this interaction is insufficient to activate defence [121, 214]. PopP2 is an acetyltransferase of the YopJ-like effector family, and its enzymatic activity is essential for its recognition [257]. The catalytically dead mutant PopP2-C321A fails to activate defence via RRS1-R, suggesting that PopP2 recognition may involve acetylation of RRS1-R protein by PopP2. PopP2 and PopP2-C321A associate with both RRS1-R and RRS1-S in the nucleus with comparable affinity [58, 115, 257].

CS and others in Sarris et al. [58] were able to detect acetylation of both RRS1-R and RRS1-S using an anti-acetyl-lysine antibody after transient co-expression with only the catalytically active PopP2 in *N. benthamiana*. Later using mass spectrometry, ZD and others identified four PopP2-acetylated lysine (K) residues in and nearby the WRKY domains of RRS1-R and RRS1-S, including both lysines (K^1 and K^2) in the WRK^1YGQK^2 motif **(Figure 4.1A)** [58]. Typically conserved in all WRKY transcription factors, the WRKYGQK motif followed by a zinc-finger motif is essential for the DNA binding activity of these WRKY proteins [277]. In Arabidopsis, more than 70% of WRKY transcription factors are implicated in defence [274, 278]. Using similar methods,

the companion paper [59] independently showed that PopP2 acetylates the WRKYGQK motif of RRS1-R WRKY domain, but predominantly at K^2 (K1221 in RRS1-R). To determine the effect of PopP2 acetylation at K^1 and K^2, ZD tested the activities of RRS1-R and RRS1-S WRKYGQK acetyl-lysine mimic mutants (WR**Q**YGQK, WRKYG**Q** or WR**Q**YGQ**Q**) when transiently co-expressed with RPS4 in *N. tabacum* leaves [58]. His results showed that acetyl-lysine mimic alleles of RRS1-R, but not RRS1-S, trigger RPS4-dependent cell death **(Figure 4.1B,C)**, which recapitulates PopP2-triggered defence activation [58]. Furthermore, RRS1-R mutants WRKYGQ**Q** and WR**Q**YGQ**Q** show stronger HR compared to RRS1-R WR**Q**YGQK when co-expressed with RPS4 **(Figure 4.1B)**, suggesting that PopP2-triggered acetylation on RRS1-R K^2 is more strongly recognised [58]. These data indicate that the WRKY domain of RRS1 is the biochemical target of PopP2.

Post docs PS and SH also demonstrated that the WRKY domain is indispensable for RRS1 and PopP2 interaction: When they replaced the RRS1 WRKY domain by the bacterial LexA DNA-binding domain [279], which has no known target in plant DNA, PopP2 fail to Co-IP with RRS1-R(WRKY/LexA) or RRS1-S(WRKY/LexA) [58]. Additionally, we (SH and YM independently) also showed that exons 6 and 7 (E67) of RRS1-R, which encodes the WRKY and domain 6 (DOM6), is sufficient to Co-IP with PopP2 or PopP2-C321A **(Figure 4.3F)** [58].

AvrRps4 recognition by RRS1/RPS4 or RRS1B/RPS4B is also not well understood. AvrRps4 is cleaved *in planta* releasing a C-terminal fragment, AvrRps4(C-term), containing residues Gly134–Gln221 that displays an anti-parallel coiled-coil structure [243]. Mutation of the Lys135-Arg-Val-Tyr138 (KRVY)-motif to four alanine residues (AvrRps4-KRVYAAAA), or Glu187 to alanine (AvrRps4-E187A), abolishes AvrRps4-triggered HR and immunity [243, 256]. In Chapter 3, I showed that AvrRps4 associates with RRS1 or RRS1B to activate the pre-existing complexes of RRS1/RPS4 or RRS1B/RPS4B. This chapter aims to unravel the detailed mechanism of AvrRps4 perception by RRS1 and RRS1B. In parallel with the study of PopP2 perception [58], this chapter will also explore the significance of the non-canonical WRKY domain (of RRS1 and RRS1B) during effector perception and defence activation.

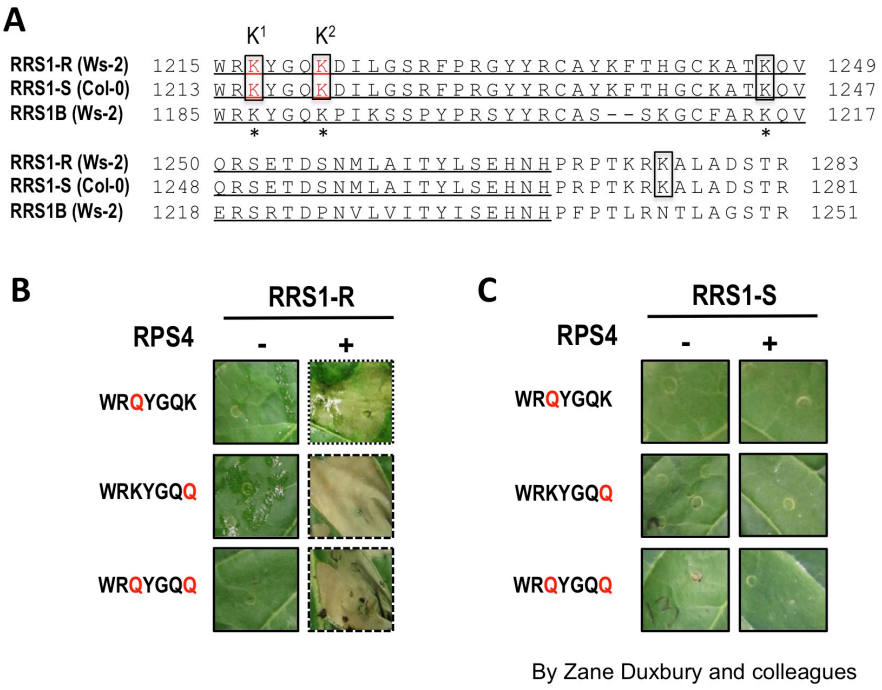

Figure 4.1: PopP2 acetylation of lysines in the RRS1-R WRKY domain activates defence (**A**) Acetylated lysine (K) residues in and around the WRKY domain (underlined) of RRS1-R and RRS1-S are indicated by boxes. Acetylated Ks of RRS1 WRKY domain that are conserved in RRS1B WRKY domain are indicated by asterisks. Acetylated Ks subjected to substitutions used in (B) and (C) are highlighted in red. (**B-C**) Transient assay in *N. tabacum* leaves using *A. tumefaciens* transformation (agro-infiltration) shows the cell death activity of acetyl-lysine mimic alleles (K to Q) of RRS1-R (C) but not RRS1-S (B) when co-expressed with RPS4. Each leaf section was infiltrated to co-express RRS1-S mutants (K1215Q, K1219Q and K1215Q/K1219Q) and RRS1-R mutants (K1217R, K1221R and K1217R/K1221R) with or without RPS4. Photographs were taken 4 dpi. This was repeated three times with similar results. Agro-infiltrations resulting in HR are bordered by a dashed line. Data for this figure were generated by ZD and colleagues, figure panels are adapted from [58] Fig 2D,5B,6B.

4.2 AvrRps4 associates with RRS1 WRKY domain, but also weakly elsewhere

Given that PopP2 is detected via the RRS1-R WRKY domain, I hypothesized that the WRKY domain might also be essential for AvrRps4 perception. I investigated if AvrRps4 Co-IPs with exons 6 and 7 (E67) of RRS1 or RRS1B, which encode the WRKY domain and a variable DOM6. The 55 aa WRKY domain defined from WRKY to HNH of RRS1-R and RRS1-S are identical, which shares 67.9% identity with the WRKY domain of RRS1B **(Figure 4.2A)**. RRS1-R E67 (E67-R) and RRS1B E67 (E67-B) carry longer DOM6-R and DOM6-B, which are comprised of 104 and 135 aa respectively, whereas RRS1-S E67 (E67-S) carries a 21 aa DOM6-S **(Figure 4.2A)**. Co-IP experiments verified that E67-R, E67-S **(Figure 4.2B)** and E67-B **(Figure 4.9)** are able to associate with AvrRps4 [58]. In contrast, E67 derivatives in which the WRKY domain was replaced by the bacterial LexA DNA binding domain, E67-S(WRKY/LexA) and E67-R(WRKY/LexA), do not Co-IP with AvrRps4 **(Figure 4.2B)** [58]. These results suggest that the WRKY domains of RRS1 and RRS1B can bind AvrRps4.

Nevertheless, SH and colleagues showed that full length RRS1-R(WRKY/LexA) or RRS1-S(WRKY/LexA) still co-IPs with AvrRps4, indicating that the WRKY domain is not the only binding domain for AvrRps4 [58] **(Figure 4.2C)**. I found that AvrRps4 co-IPs with RRS1-R exons 1-5 (E12345), which lacks the WRKY domain and domain 6, confirming that additional RRS1 domain(s) in E12345 associate with AvrRps4 **(Figure 4.2D)**. It should be noted that AvrRps4 association with E12345 is weaker compared to with E67-R **(Figure 4.2D)**. Interestingly, a non-recognizable AvrRps4 mutant, AvrRps4-E187A, associates more weakly with E67-R compared to type (WT) AvrRps4, whilst showing comparable affinity with E12345 **(Figure 4.2D)**. This indicates that AvrRps4 association with RRS1 WRKY domain is important to AvrRps4-triggered defence.

4.3 PopP2 acetylation of RRS1-S or RRS1B WRKY domain inhibits AvrRps4 recognition

PopP2 acetylation of key lysine residues in the RRS1 WRKY domain changes the biochemical properties of the domain, and subsequently abolishes its DNA binding ability [58, 59]. To elucidate the role of the WRKY domain

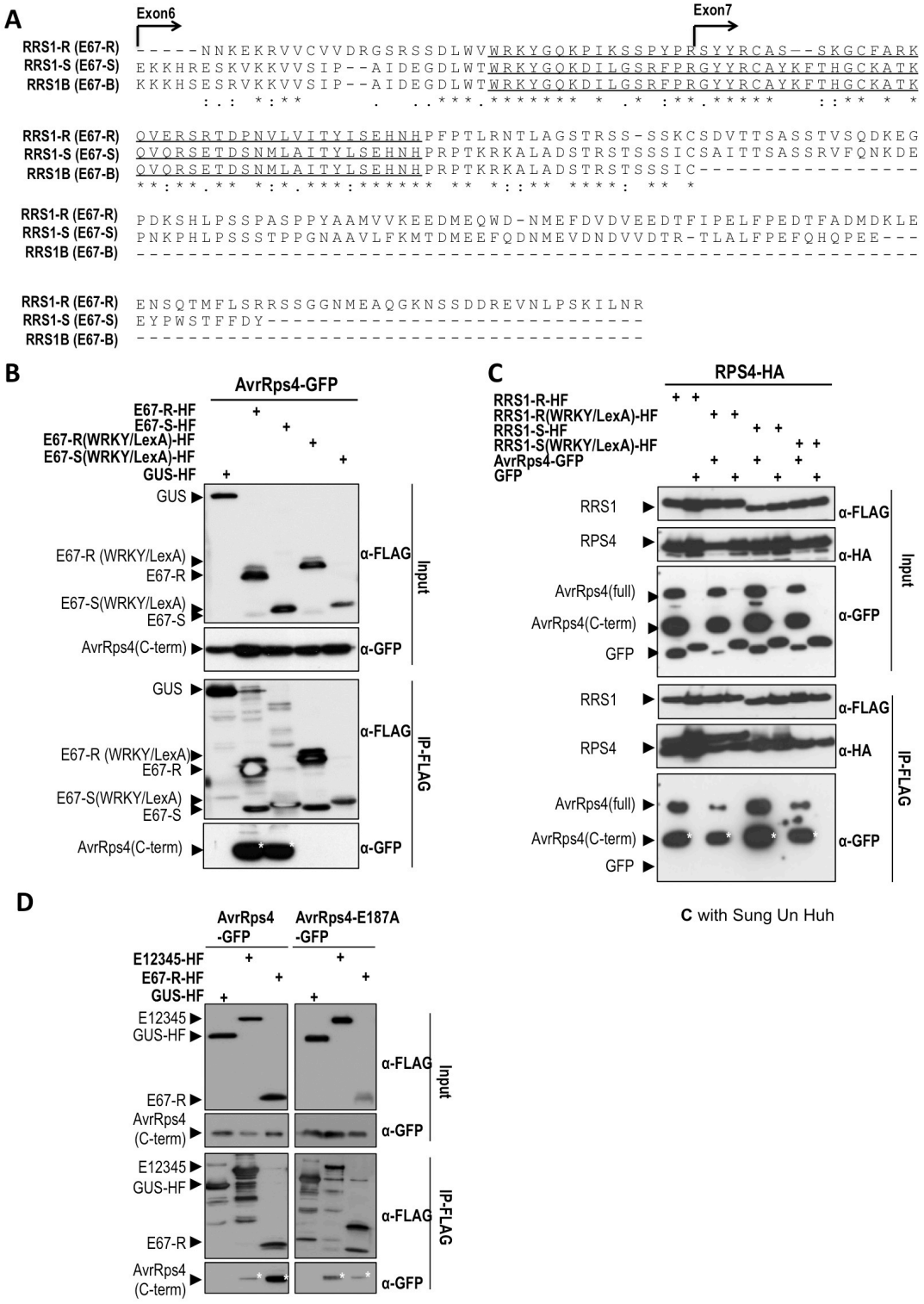

Figure 4.2: AvrRps4 associates with RRS1 WRKY domain, but also weakly elsewhere (**A**) Alignment of the amino acid sequences encoded by exon 6 and 7 (E67) of RRS1-R, RRS1-S and RRS1B. Arrows mark the start positions of exons 6 and 7. The WRKY domain is marked by underline. The Alignment was generated by Clustal Omega. (**B**) Co-IP assay shows AvrRps4-GFP completely loses interaction with E67-R(WRKY/LexA)-HF and E67-R(WRKY/LexA)-HF. (**C**) Co-IP assay shows that AvrRps4-GFP interacts with full length RRS1-R(WRKY/LexA)-HF and RRS1-S(WRKY/LexA)-HF. (**D**) Co-IP assay shows the association of AvrRps4-GFP or AvrRps4-E187A-GFP with the gene product exons 1-5 of RRS1-R (E12345-HF) and E67-R-HF. HF tag is 6xHis 3xFlag. Figure panels B, C, D are adapted from [58] Fig 5C, S5C, D.

during AvrRps4 perception, I tested whether the acetylation of RRS1-S WRKY domain by PopP2 compromises the recognition of AvrRps4. Using transient assays in tobacco, I showed that leaf sections co-expressing RRS1-S and RPS4 respond only to AvrRps4, but not to PopP2, showing cell death **(Figure 4.3A)**. Significantly, this AvrRps4-triggered RRS1-S/RPS4 dependent cell death is suppressed by the co-expression with PopP2, but not with PopP2-C321A **(Figure 4.3A)**.

In addition, I tested if delivering PopP2 interferes with disease resistance to AvrRps4-carrying bacterial strains conferred by RRS1-S/RPS4 in Arabidopsis Col-0. *P. fluorescens* (*Pf*) Pf0-1 carrying T3SS was used to deliver PopP2 or the PopP2-C321A mutant. Consistent with the transient assay results as above, I demonstrated that Pf0-1 (PopP2), but not Pf0-1 (PopP2-C321A), restores growth of *Pst* DC3000 (AvrRps4) to a level comparable to *Pst* DC3000 (EV) + Pf0-1 (PopP2) **(Figure 4.3C)**. PopP2-C321A associates with RRS1-S [58, 115, 257], but this association is insufficient to suppress AvrRps4 recognition and resistance. Therefore, I infer that the competition between AvrRps4 and PopP2 for WRKY domain binding cannot explain the attenuation of AvrRps4 responsiveness by PopP2, and it is likely the enzymatic function of PopP2 (acetylation of RRS1-S) that causes the attenuation. Likewise, PopP2-dependent acetylation of RRS1-S compromised the resistance in Arabidopsis Col-0 against *Pst* DC3000(AvrRps4).

RRS1B also associates with PopP2 (shown in Chapter 3), and both the full-length RRS1B and the WRKY domain containing E67-B can be acetylated by PopP2 (SH, unpublished). Therefore, we (YM and colleagues) speculate that PopP2 acetylates similar lysine residues of RRS1B WRKY domain that are targeted in RRS1-R and RRS1-S **(Figure 4.1A)**. PopP2 is not recognised by RRS1B/RPS4B [121]. Here I confirmed that RRS1B and RPS4B co-expression shows no responsiveness to PopP2 in *N. tabacum* leaves **(Figure 4.3B)**; and Pf0-1 (PopP2) can not trigger cell death in the leaves of Arabidopsis Ws-2 *rrs1-1/rps4-21* **(Figure 4.3D)**. I hypothesised that if AvrRps4 is recognised via the WRKY domain of RRS1B, then PopP2 acetylation of RRS1B might attenuate AvrRps4 recognition by RRS1B/RPS4B. Indeed, tobacco transient assay showed that AvrRps4-triggered RRS1B/RPS4B-dependent cell death is suppressed (although not completely abolished) by PopP2, but not affected by PopP2-C321A **(Figure 4.3B)**. Consistently in Arabidopsis, Pf0-1 (AvrRps4) triggers cell death in Ws-2 *rrs1-1/rps4-21*, which is dependent

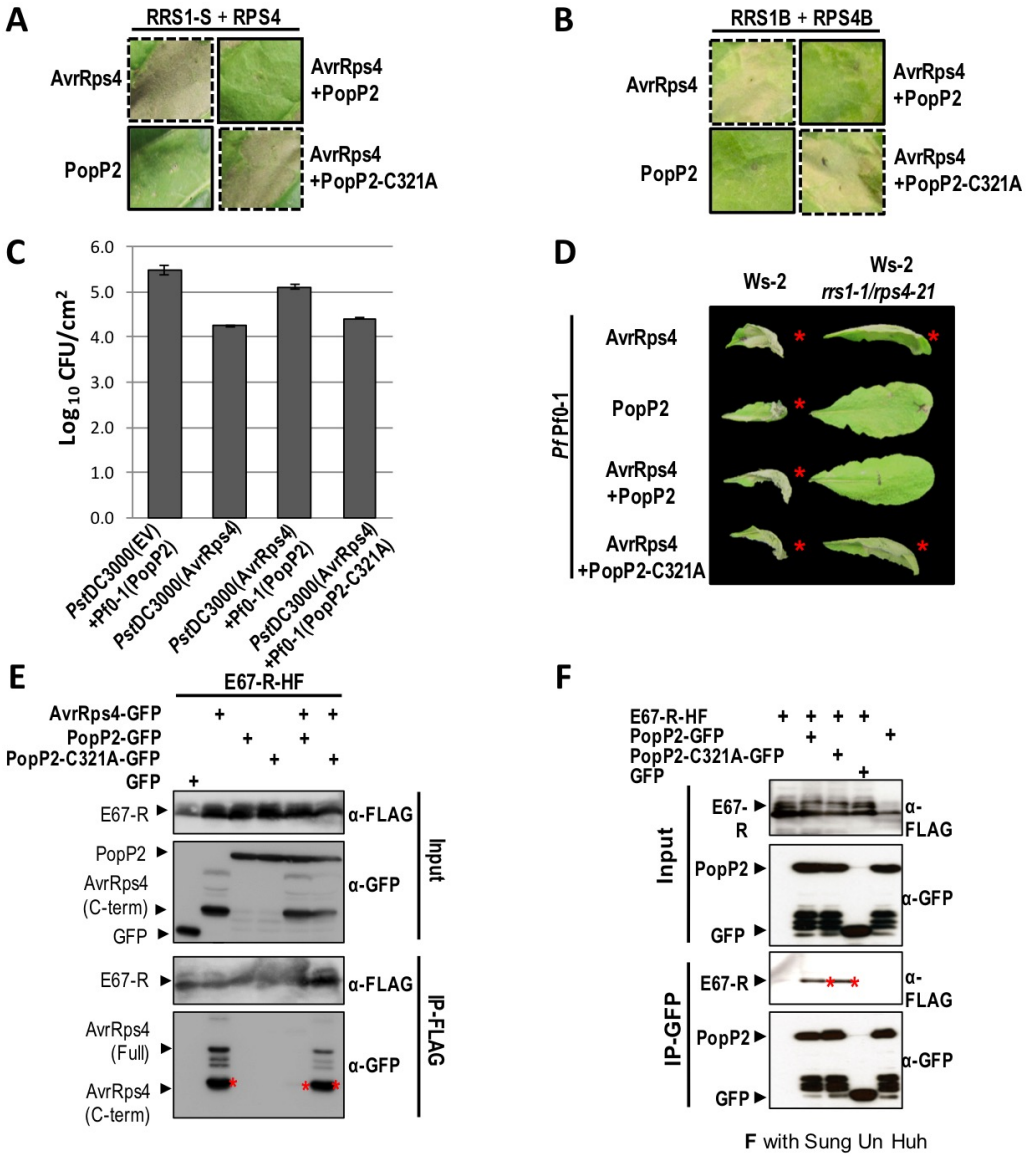

Figure 4.3: PopP2 acetylation of the RRS1-S and RRS1B WRKY domain inhibits AvrRps4 recognition

(**A** & **B**) Transient assays in *N. tabacum* leaves using agro-infiltration show that PopP2, but not PopP2-C321A, inhibits AvrRps4 recognition by the RRS1-S/RPS4 complex (A) and the RRS1B/RPS4B complex (B). Each leaf section co-expresses RRS1-S/RPS4 or RRS1B/RPS4B with individual effectors or their combinations. Photographs were taken 4 dpi. Agro-infiltrations resulting in HR are bordered by a dashed line. (**C**) Bacterial growth assays show that PopP2, but not PopP2-C321A, inhibits resistance of Arabidopsis Col-0 to *Pst* DC3000 carrying AvrRps4. Histogram shows the growth of *Pst* DC3000 carrying empty vector (EV) or AvrRps4, measured 3 dpi in Arabidopsis leaves. (*Pf*) Pf0-1 carrying PopP2 or PopP2-C321A were co-infiltrated with *Pst* DC3000 strains. Means ± SD of three replicates per sample are given. (**D**) HR assays in Arabidopsis using *Pf* Pf0-1 to deliver effectors, show that PopP2, but not PopP2-C321A, inhibits cell death response of Ws-2 *rrs1-1/rps4-21* to AvrRps4. Leaves showing HR are indicated with asterisks. HR phenotypes were assessed 24 hpi. (**E** & **F**) (E) Co-IP assays show PopP2-GFP, but not PopP2-C321A-GFP, inhibits AvrRps4-GFP association with E67-R-HF after transient co-expression in *N.benthamiana*. (F) Co-IP assays show PopP2-GFP and PopP2-C321A-GFP associate with E67-R-HF after transient co-expression in *N.benthamiana*. Immunoblots show the presence of proteins in total extracts (input) and after IP. Asterisks indicate the expected protein bands. There experiments were repeated at least three times with similar results. Figure panels A, C, E, F were published in [58] Figure 4, S4E; experiment for F was done by SH and YM independently.

on AvrRps4 recognition by RRS1B/RPS4B, is suppressed by co-infiltration of Pf0-1 (PopP2), but not by Pf0-1 (PopP2-C321A) **(Figure 4.3D)**.

Additionally, in Ws-2, when RRS1-R/RPS4 and RRS1B/RPS4B are present, either Pf0-1 (PopP2) or Pf0-1 (AvrRps4) can be recognised and trigger cell death; and co-infiltration of Pf0-1 (PopP2) and Pf0-1 (AvrRps4) does not suppress cell death **(Figure 4.3D)**. Similarly, co-expression of PopP2 and AvrRps4 triggers strong cell death in the tobacco leaf section expressing RRS1-R and RPS4 **(Figure 4.4A)**. These results indicate that the presence of AvrRps4 does not interfere with PopP2 recognition by RRS1-R/RPS4. Furthermore, I tested the suppression activity of PopP2 on other R protein-mediated effector-triggered cell death using transient assay in *N. tabacum*. **Figure 4.4B** illustrates that PopP2 does not suppress the cell death triggered by [4]WRR4^{Col-0} recognition of [5]Avr-WRR4 [280]. Likewise, post doc PS showed that PopP2 also does not suppress the cell death triggered by the recognition of ATR1 by RPP1^{WsB} [58]. Thus, I can rule out that PopP2-mediated suppression of AvrRps4 recognition by RRS1-S/RPS4 is due to the general HR-suppressing activity of PopP2. Altogether, I favour the hypothesis that PopP2 acetylation of the RRS1-S or RRS1B WRKY domain causes the loss of its affinity for AvrRps4, and thus the loss of AvrRps4 recognition.

[4] **WRR4^{Col-0}:** White Rust Resistance protein 4, a TIR-NB-LRR from Arabidopsis Col-0.

[5] **Avr-WRR4:** A candidate effector recognised by WRR4 identified by Volkan Cevik from *Albugo candida* race Nc-2 (unpublished).

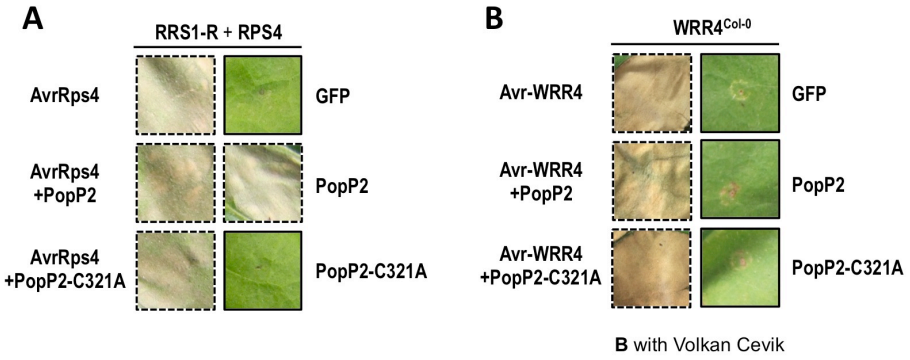

Figure 4.4: AvrRps4 does not interfere with PopP2 recognition, and PopP2 specifically inhibits AvrRps4 recognition (**A**) Transient assays in *N. tabacum* leaves using agro-infiltration show that AvrRps4 does not interfere the HR triggered by PopP2 recognition via RRS1-R/RPS4. (**B**) Transient assays in *N. tabacum* leaves using agro-infiltration show that PopP2 does not suppress the HR triggered by WRR4^{Col-0} recognition of Avr-WRR4. Each leaf section was co-infiltrated to express a combination of R genes (shown on top) and effectors (labelled left or right of each square). Photographs were taken 4 dpi. Agro-infiltrations resulting in HR are bordered by a dashed line. These experiment were repeated at least three times with similar results.

To test this hypothesis, I investigated if PopP2 acetylation interferes with AvrRps4 association with the RRS1 WRKY domain using Co-IP assays. Results show that the association between E67-R and AvrRps4 is greatly reduced in the presence of PopP2, but not in the presence of PopP2-C321A **(Figure 4.3E)**. PopP2 and PopP2-C321A associations with E67-R are much weaker compared to AvrRps4, therefore are not visible in **(Figure 4.3E)**. In an independent Co-IP with a longer exposure time, we (SH and YM independently) are able to detect the associations of E67-R with both PopP2 and PopP2-C321A in equal affinity **(Figure 4.3F)**. This strongly suggests that PopP2-mediated interference is not due to competition for binding, but is dependent on its function to acetylate. Post doc SH detected acetylation of E67-R by PopP2, but not by mutant PopP2-C321A, using the anti-acetyl-lysine antibody [58]. PopP2 specifically acetylates lysine residues (K^1 and K^2) in the RRS1 WRKY domain **(Figure 4.1A)** [58], and this acetylation blocks AvrRps4 binding, implying that AvrRps4 directly interacts with the WRKY domain of RRS1.

4.4 WRKY domain mutants that lose recognition of AvrRps4 fail to interact with AvrRps4

Given that PopP2 targets K^1 and K^2 of the RRS1 WRK^{1}YGQK^2, we (YM and ZD) investigated the importance of K^1 and K^2 during AvrRps4 recognition. ZD showed that RRS1-S acetyl-lysine mimic (Q) substitution of either K^1 (WR**Q**YGQK) or K^2 (WRKYGQ**Q**) completely abolishes AvrRps4 responsiveness when co-expressed with RPS4 in tobacco leaves **(Figure 4.5B)** [58]. This suggests that acetylation of either K^1 or K^2 of the RRS1-S WRKY domain is sufficient to deter AvrRps4 recognition, which explains the suppression of the RRS1-S/RPS4-mediated AvrRps4 recognition by PopP2. ZD also showed that when K^1 or K^2 of RRS1-R WRKYGQK was replaced with an arginine (R), a lysine mimic that cannot be acetylated, both RRS1-R WRKYGQ**R** and WR**R**YGQK are not autoimmune when co-expressed with RPS4 **(Figure 4.5A)** [58].

Interestingly, RRS1-R WRKYGQ**R**, but not WR**R**YGQK, loses the ability to recognise AvrRps4, implying the crucial role of K^2 for AvrRps4 detection **(Figure 4.5A)** [58]. In comparison, the contribution of K^1 to AvrRps4 recognition is disrupted by a Q substitution **(Figure 4.5B)** but not an R substitution **(Figure 4.5A)**. Furthermore, I showed that PopP2 acetylation of

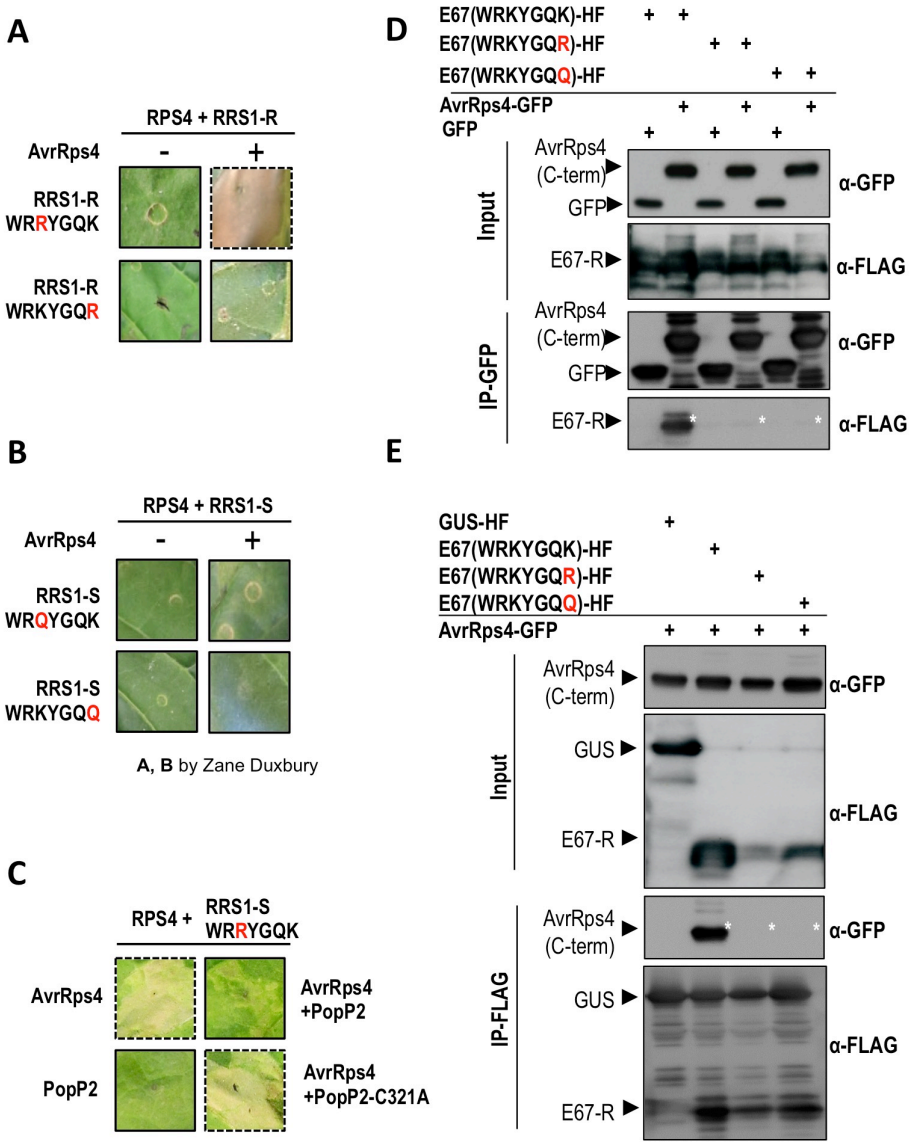

Figure 4.5: WRKY Domain lysine residues mutants that lose recognition of AvrRps4 fail to associate with AvrRps4 (**A-C**) Transient assays in *N. tabacum* leaves show the AvrRps4 recognition phenotypes of RRS1-R mutants (K1217R, K1221R) (A) and RRS1-S mutants (K1215Q, K1219Q) (B) when co-expressed with RPS4. (C) Transient assays show that PopP2, but not PopP2-C321A can suppress AvrRps4-triggered HR in RRS1-S (K1215Q) and RPS4 co-expressing *N. tabcum* leaves. Photographs were taken 4 dpi. Agro-infiltrations resulting in HR are bordered by a dashed line. This was repeated at least three times with similar results. (**D-E**) Co-IP assays show AvrRps4-GFP associates with (E67)-R-HF, but not with E67-R(WRKYGQR)-HF or E67-R(WRKYGQQ)-HF mutants. Immunoblots show the presence of proteins in total extracts (input) and after IP (E). Asterisks indicate expected protein bands. Data for panels A and B were generated by ZD. Figure panels A, B, D, E are adapted from [58] Fig 5, S5.

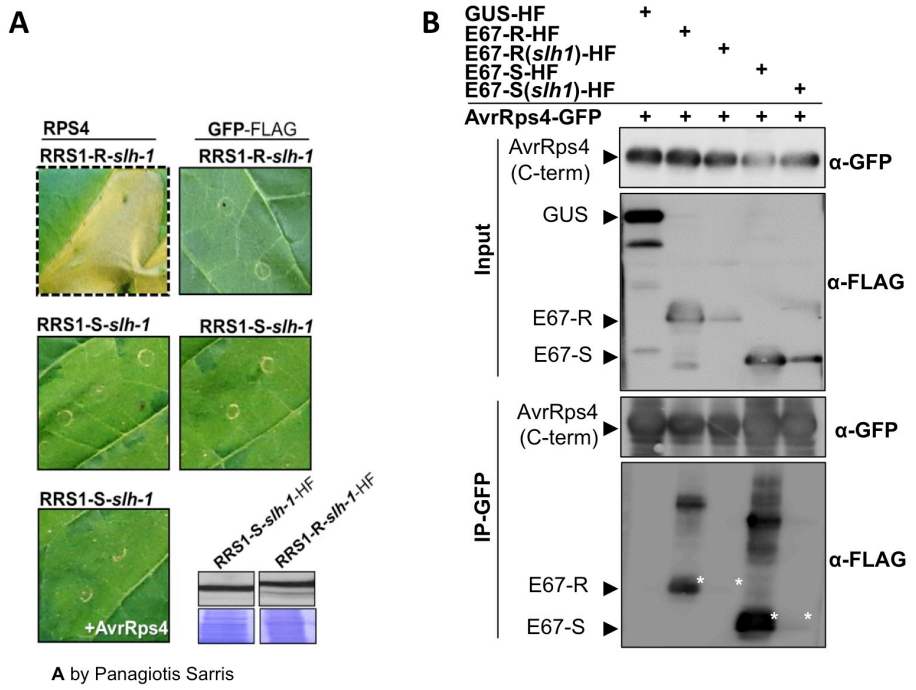

Figure 4.6: RRS1-S (*slh1*) that lose recognition of AvrRps4 fail to associate with AvrRps4 via the WRKY domain (**A**) Transient assays in *N. tabacum* leaves show that RRS1-R(*slh1*), but not RRS1-S(*slh1*) mutant, triggers RPS4-dependent auto-activity. The RRS1-S(*slh1*) and RPS4 co-expression is not able to induce AvrRps4 responsiveness. Agro-infiltrations resulting in HR are bordered by a dashed line. Immunoblot shows equal protein expression of RRS1-R(*slh1*) and RRS1-S(*slh1*). CBB staining shows equal loading. (**B**) Co-IP assay shows that *slh1* mutants of both E67-R-HF and E67-S-HF lose interaction with AvrRps4-GFP. Immunoblots show the presence of proteins in total extracts (input) and after IP. Asterisks indicate expected protein bands. Panel A was generated by PS. Figure panels were published in [58] Fig S5.

the K^2 residue of RRS1-S WRK**R**YGQK can reduce AvrRps4 responsiveness in RRS1-S WRK**R**YGQK + RPS4 combination **(Figure 4.5C)**. These observations suggest that recognition of AvrRps4 is sensitive to subtle changes at the K^2 position of the WRK^{1}YGQK^2 motif in RRS1-R and RRS1-S. Therefore, I infer that K^2 must be crucial for the WRKY domain to interact with AvrRps4. Indeed, I co-expressed AvrRps4 with E67-R and their K^2 mutant variants and found that AvrRps4 Co-IPs with E67-R but not with E67 WRKYGQ**R** or WRKYGQ**Q** mutants **(Figure 4.5D,E)**.

Furthermore, like PopP2 acetylation of the RRS1-S WRKY domain, PS showed that RRS1-S(*slh1*), with a leucine insertion in the WRKY domain, loses the ability to recognise AvrRps4 **(Figure 4.6A)** [58]. Unlike RRS1-R(*slh1*), PS discovered that RRS1-S(*slh1*) does not confer RPS4-dependent

activation of cell death after transient expression in tobacco **(Figure 4.6A)** [58]. I found that both E67-R(*slh1*) and E67-S(*slh1*) fail to Co-IP with AvrRps4, suggesting a single leucine insertion in the WRKY domain abolishes affinity for AvrRps4 **(Figure 4.6B)**.

In summary, the RRS1 WRKY domain interacts with AvrRps4, and mutant forms of the WRKY domain that compromise responsiveness to AvrRps4 also compromise binding. Therefore, AvrRps4 perception must require binding to the WRKY domain of RRS1.

4.5 WRKY domain interactions with AvrRps4 are necessary but not sufficient for the perception of AvrRps4

I next investigated whether AvrRps4 mutants lose the ability to be recognised due to their lack of affinity with the WRKY domain of RRS1 or RRS1B. E67-R interacts very weakly with AvrRps4-E187A, but maintains strong affinity with AvrRps4-KRVYAAAA comparable to that of AvrRps4-WT in Co-IP **(Figure 4.7A & Figure 4.9)**. This suggests that binding to the RRS1 WRKY domain is necessary but not sufficient for AvrRps4 recognition. I infer that E187 is required for AvrRps4 interaction with the RRS1 WRKY domain, whereas the KRVY motif is irrelevant for WRKY domain binding, but is required for interacting with other components or domains required for recognition.

Structural modelling of possible RRS1 WRKY domain and AvrRps4(C-term) interactions places K^2 of RRS1 WRK^1YGQK^2 and E187 & E175 of AvrRps4 at the interface **(Figure 4.8A)**. The strutural model of RRS1 WRKY domain was predicted by Phyr2 based on WRKY1, and AvrRps4(C-term) was obtained from the published crystal structure (PDB ID:4B6X) [243]. Like E187, the E175 is a surface exposed residue of AvrRps4 that is required for full RRS1/RPS4- and RRS1B/RPS4B-mediated AvrRps4 responsiveness in Arabidopsis [243]. The N-terminal 20 aa of the AvrRps4(C-term) was not observed in the electron density, therefore the KRVY motif at the N-terminal end was not shown in the crystal structure [243]. However, as the N-terminus of AvrRps4(C-term) is at the opposite end of the predicted WRKY/AvrRps4 interface **(Figure 4.8A)**, it is possible that the KRVY motif is involved in interactions other than with the WRKY domain. Although AvrRps4 and AvrRps4-E187A differ

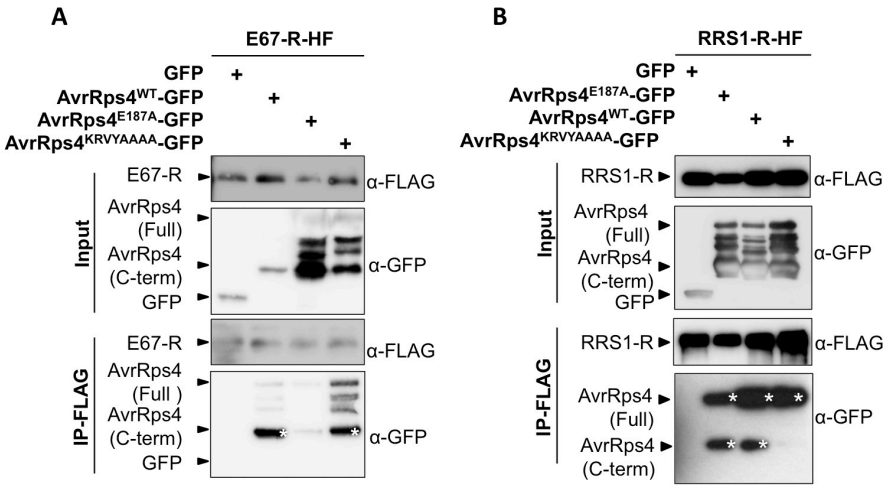

Figure 4.7: Associations of AvrRps4 mutants with the WRKY domain and the full-length protein of RRS1-R (**A**) Co-IP assay shows that AvrRps4-E187A-GFP, but not AvrRps4-KRVYAAAA-GFP, exhibits reduced association with E67-R-HF compared to AvrRps4-WT-GFP (wild type). (**B**) AvrRps4-WT-GFP, AvrRps4-E187A-GFP or AvrRps4-KRVYAAAA-GFP but not GFP Co-IP with RRS1-R-HF in the absence of RPS4-HA. Immunoblots show the presence of proteins in total extracts (input) and after IP. Asterisks indicate expected protein bands. Figure panels were published in [58] Fig S1E, S5E.

in their interaction with the WRKY domain **(Figure 4.7A & Figure 4.9)**, they show similar affinity with the full-length RRS1-R **(Figure 4.7B)**. This can be explained by AvrRps4 and AvrRps4-E187A interacting equally with RRS1 E12345 **(Figure 4.2D)**. I speculate that a distinct surface of AvrRps4, which is not involved in interaction with the WRKY domain, mediates the interaction with RRS1 E12345.

Modelling of RRS1B WRKY domain and AvrRps4(C-term) interactions predicts a similar (but not identical) interface to that of RRS1 WRKY/AvrRps4(C-term), with K^2 of RRS1B WRK^1YGQK^2 and AvrRps4 E187 & E175 at the interface **(Figure 4.8A)**. This model is consistent with the observation that PopP2 acetylation of RRS1B WRKY domain suppresses AvrRps4 recognition **(Figure 4.3B, D)**. I show that AvrRps4 also Co-IPs with RRS1B E67-B, but with much weaker affinity compared to RRS1-R E67-R **(Figure 4.9)**, confirming the possible AvrRps4/RRS1B-WRKY interaction. AvrRps4 mutants' associations with E67-B exhibit a similar pattern compared to E67-R, but with much weaker signals overall: AvrRps4-KRVYAAAA Co-IPs strongly with E67-B, while AvrRps4-E187A fails to associate with E67-B **(Figure 4.9)**.

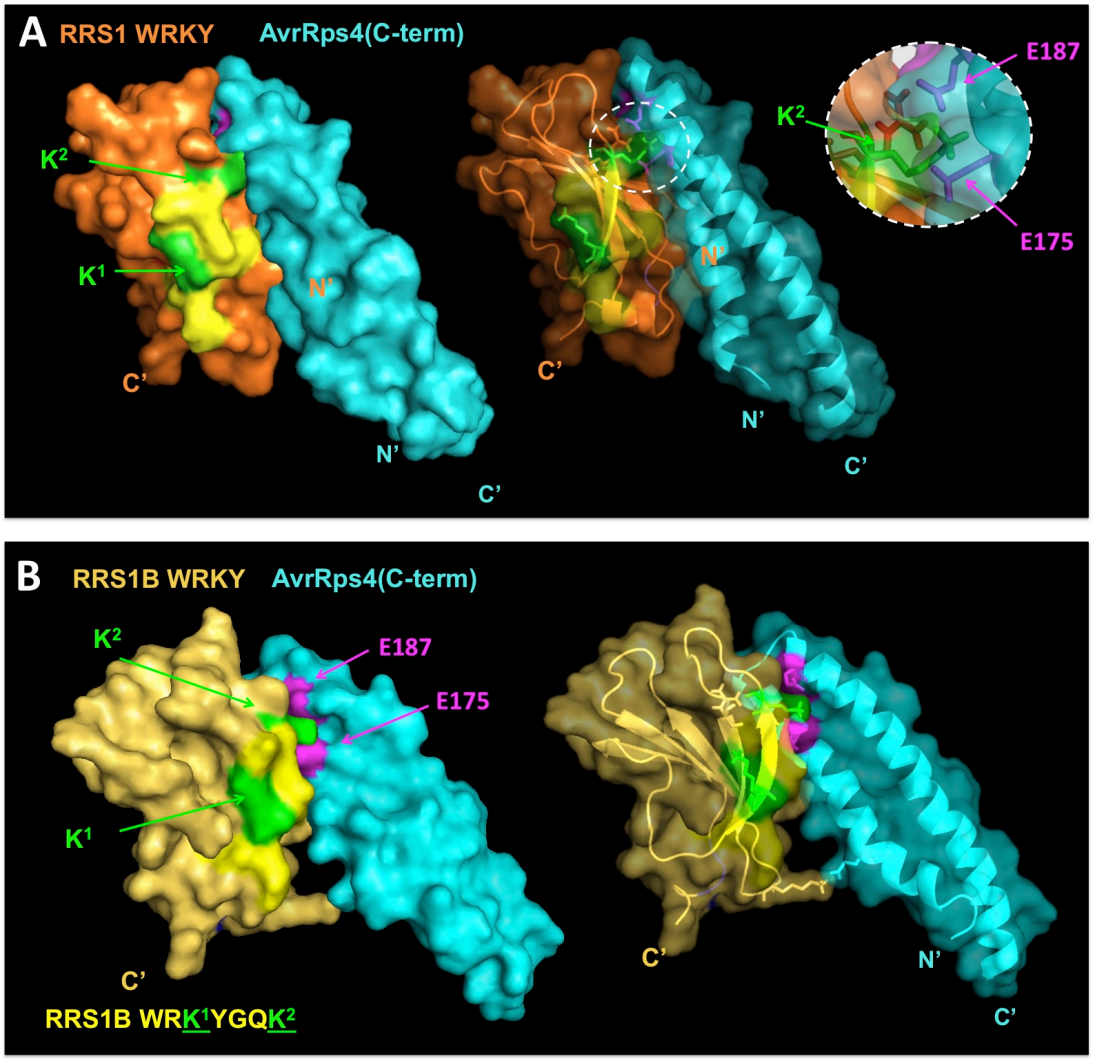

Figure 4.8: Structural modelling of possible interactions between AvrRps4(C-term) and the WRKY domain of RRS1-R and RRS1B Protein-protein interaction modelling by ClusPro [281–283] predicts the most possible interactions between AvrRps4(C-term) and RRS1-R WRKY domain (**A**) and between AvrRps4 and RRS1B WRKY domain (**B**). (**A**) shows a close up view of RRS1-R and AvrRps4 (C-term) interface. Structural models of the RRS1-R (Ws-2) WRKY domain (Ser1196-Thr1273), shown in orange, and RRS1BWs-2 WRKY domain (Glu1166-Thr1241), shown in yellow, were predicted with 100% confidence by Phyr2 [284] using the crystal structure of WRKY1 with 5 β strands(PDB ID:c2aydA) as their template. AvrRps4(C-term) structure (PDB ID:4B6X) shown in cyan was previously determined by [243]. Solid molecular surface was presented (on the right) as well as ribbons illustrating the secondary protein structure (on the left). Amino acids predicted at the interface were shown as sticks, with lysine (K) residues of RRS1-R or RRS1B WRKY motif highlighted in green, and glutamic acid (E) residues of AvrRps4 highlighted in magenta.

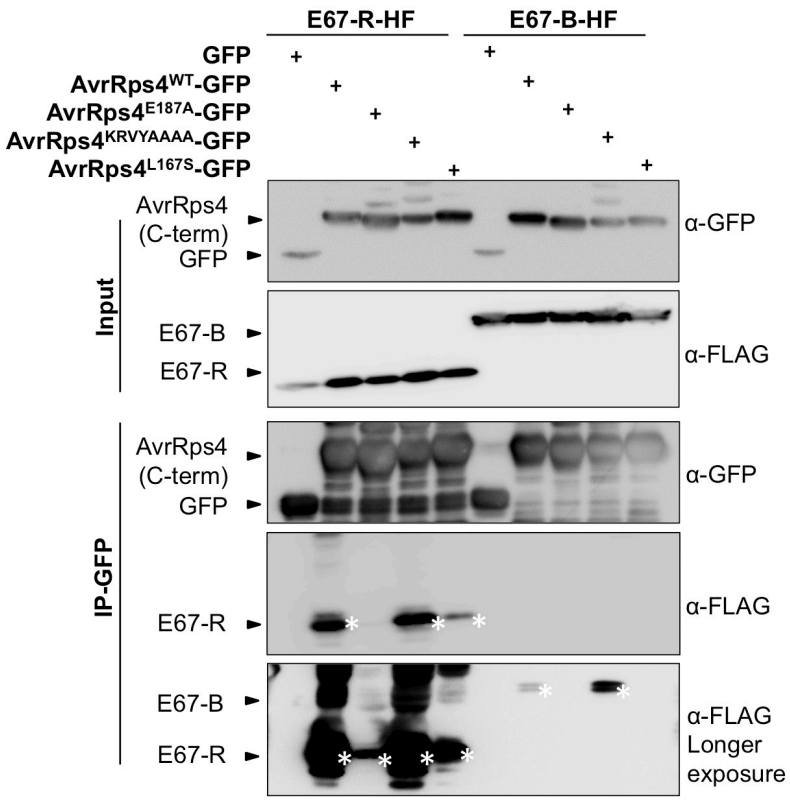

Figure 4.9: Associations of AvrRps4 mutants with the WRKY domain and RRS1-R and RRS1B show a similar pattern Co-IP assay shows that AvrRps4-E187A-GFP and AvrRps4-L167S-GFP, but not AvrRps4-KRVYAAAA-GFP show reduced association with E67-R-HF and E67-B-HF when compared to AvrRps4-WT-GFP. Immunoblots show the presence of proteins in total extracts (input) and after IP. Asterisks indicate expected protein bands.

Sequence analysis of AvrRps4 natural variants revealed that the L167 residue is under positive selection, and L167T or L167S variants manage to evade recognition by RRS1B/RPS4B, but not RRS1/RPS4 [243]. I validated that Pf0-1 (AvrRps4-L167T) and Pf0-1 (AvrRps4-L167S) are only recognised in the Arabidopsis lines with functional RRS1-R/RPS4 (Ws-2 and Ws-2 *rps4b-1*), showing cell death; whereas RRS1B/RPS4B (in Ws-2 *rrs1-1* and Ws-2 *rps4-21*) fails to show responsiveness **(Figure 4.10A)**. Consistently, tobacco transient assay showed that AvrRps4-L167T and AvrRps4-L167S show weak cell death response when co-expressed with RRS1-R and RPS4, but they do not respond to RRS1B/RPS4B **(Figure 4.10B)**. I also established that AvrRps4-L167T is more weakly recognised by RRS1-R/RPS4 compared to AvrRps4-L167S in Arabidopsis and in transient assays **(Figure 4.10A, B)**.

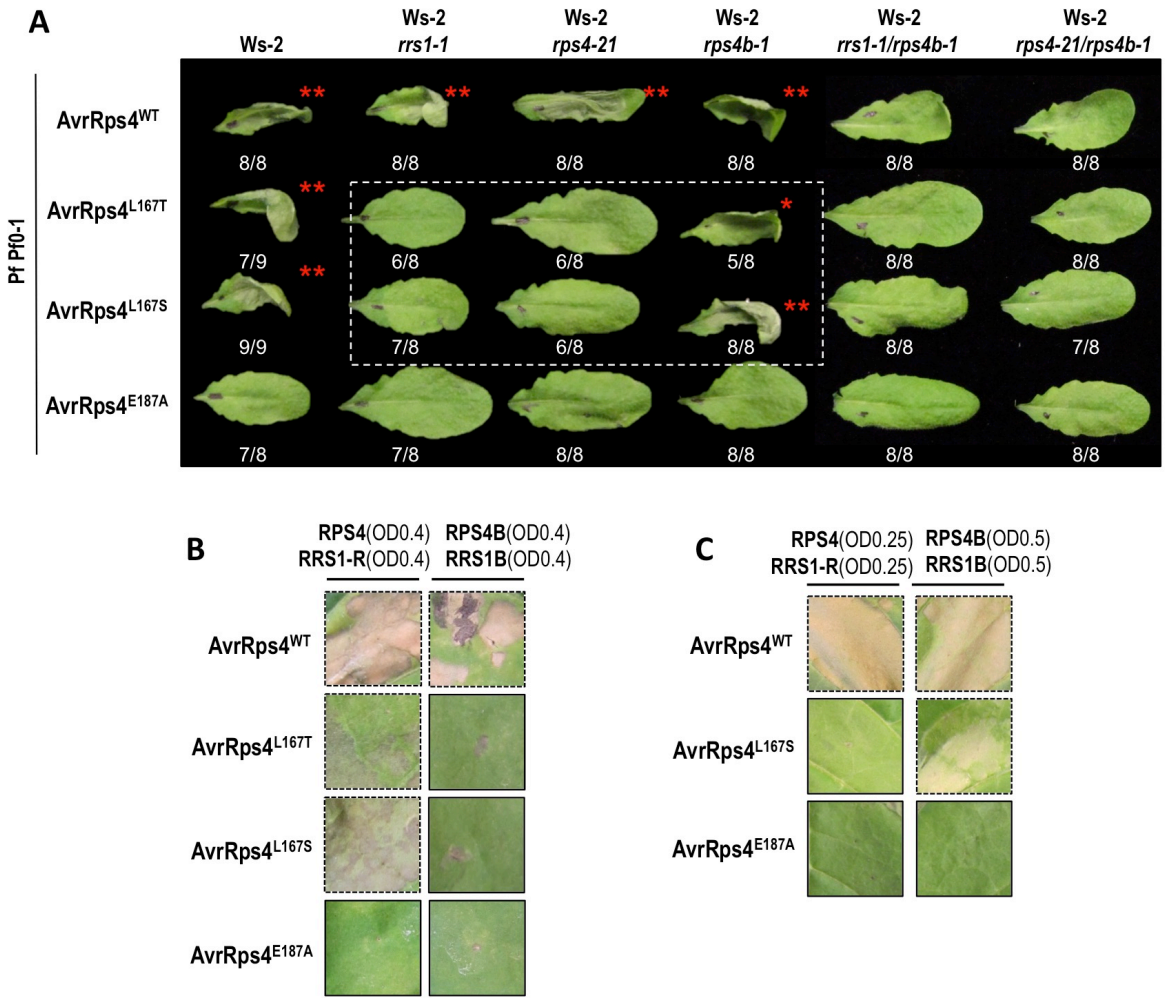

Figure 4.10: AvrRps4 L167T and L167S variants are weakly recognised by RRS1-R/RPS4 and RRS1B/RPS4B
(**A**) HR assays in Arabidopsis show that *Pf* Pf0-1 carrying AvrRps4-L167T and AvrRps4-L167S trigger cell death only in Ws-2 and Ws-2 *rps4b-1*, but not in Ws-2 *rrs1-1* and Ws-2 *rps4-21* (highlighted in the box). *Pf* Pf0-1 carrying AvrRps4-WT and AvrRps4-E187A were used as positive and negative control respectively. No AvrRps4 responsiveness were shown in Ws-2 *rrs1-1/rps4b-1* and Ws-2 *rps4-21/rps4b-1* double mutants. Leaves showing HR are indicated with asterisks, number of asterisks correlate with the strength of HR. Fractions indicate the number of leaves displaying the shown phenotype over the total number of infiltrated leaves. HR phenotypes were assessed 24 hpi. (**B-C**) Transient assays in *N. tabacum* leaves show that AvrRps4-L167S responsiveness positively correlates with the infiltration concentrations of *A. tumefaciens* carrying R genes. (B) When all *R* genes are infiltrated at equal concentrations (OD=0.4), RRS1-R/RPS4 shows a weak HR to AvrRps4-L167S and an even weaker HR to AvrRps4-L167T; but RRS1B/RPS4B shows no HR to AvrRps4-L167S or AvrRps4-L167T. (C) However, when RRS1-R and RPS4 are infiltrated at (OD=0.25), they lose HR in response to AvrRps4-L167S. In contrast, RRS1B and RPS4B when infiltrated at (OD=0.5) restore the HR response to AvrRps4-L167S. AvrRps4-WT and AvrRps4-E187A were used as positive and negative controls respectively. Photographs were taken 4 dpi. Agro-infiltrations resulting in HR are bordered by a dashed line. These experiments were repeated twice with similar results.

As the WRKY domain interaction is necessary for AvrRps4 perception, I hypothesised that L167 of AvrRps4 maybe specifically required for interaction with the WRKY domain of RRS1B but not RRS1-R. Co-IP experiments showed that AvrRps4-L167S fails to associate with E67-B, but maintains association with E67-R **(Figure 4.9)**. E67-R Co-IPs with AvrRps4-L167S more weakly compared to AvrRps4-WT, but more strongly compared to AvrRps4-E187A **(Figure 4.9)**. However, I cannot exclude that the observed lack of AvrRps4-L167S and E67-B association is due to the initial weak association between AvrRps4-WT and E67-B. To determine whether the lack of AvrRps4-L167S responsiveness by RRS1B/RPS4B is due to a quantitative weakening or a qualitative loss of recognition, I increased the infiltration concentrations of Agrobacterium strains carrying RRS1B and RPS4B in transient assays to test for AvrRps4-L167S responsiveness. When both RRS1B- and RPS4B-carrying strains were infiltrated at OD=0.5, AvrRps4-L167S triggers a weaker cell death response compared to AvrRps4-WT **(Figure 4.10C)**, which was not observed when RRS1B and RPS4B were both expressed at OD=0.4 as above in **(Figure 4.10B)**. This demonstrated that the weaker AvrRps4-L167S recognition by RRS1B/RPS4B can be revealed by artificially increasing the concentration of the transiently expressed R proteins. In contrast, the lower OD=0.25 (compared to OD=0.4 in **(Figure 4.10B)**) for both RRS1-R- and RPS4-carrying strains completely suppress AvrRps4-L167S but not AvrRps4-WT responsiveness **(Figure 4.10C)**. These results reveal that AvrRps4-L167 is important for the interactions with the WRKY domain of both RRS1-R and RRS1B, and mutation at L167 weakens AvrRps4/WRKY associations, therefore impairing its recognition by RRS1-R/RPS4 and RRS1B/RPS4B.

Altogether, the positive correlation between the recognisability of many AvrRps4 variants and their affinity with the WRKY domain strongly suggest that AvrRps4 is recognised via interacting with the WRKY domain of RRS1 and RRS1B. However, this interaction is not sufficient to activate defence, as AvrRps4-KRVYAAAA interacts with the WRKY domain but is not recognised.

4.6 AvrRps4 associates with WRKY transcription factors involved in plant immunity

AvrRps4 interacts with the WRKY domain of RRS1 and RRS1B, and these interactions are required for activation of immunity. [58, 59] showed that PopP2

also interacts with and acetylates the WRKY domain of RRS1, the latter of which is essential for PopP2-triggered defence. These evidence suggest that the non-canonical WRKY domain of RRS1 might function to detect effectors that would otherwise target the WRKY-domain containing transcription factors and impair their contribution to plant immunity. To determine whether AvrRps4 associates with WRKY transcription factors, AvrRps4-GFP was transiently co-expressed with several C-terminally HF-tagged WRKY proteins. The WRKY proteins were selected because of their possible involvement in plant defence. We (YM and ZD) showed that AvrRps4 Co-IPs with WRKY41, WRKY70, WRKY33 and WRKY60 **(Figure 4.11A, B)**, but not with WRKY22 **(Figure 4.11C)**. Previously, [16] showed that AvrRps4 interacts with WRKY41 in Y2H, implying that AvrRps4 might interfere with WRKY transcription factors via direct interaction. In parallel, ZD showed that PopP2-C321A-GFP Co-IPs with all the chosen WRKY proteins and WRKY41, WRKY70 and WRKY33, but not WRKY60 can be acetylated by PopP2 [58]. These results suggest that both AvrRps4 and PopP2 target a subset of WRKY proteins that are implicated in plant immunity. Therefore, we (YM and colleagues) propose that the RRS1/RPS4 immune complex has evolved to detect effectors that interfere with the function of WRKY transcription factors.

4.7 Discussion

4.7.1 *AvrRps4 is perceived via the WRKY domain*

In this chapter, I report that AvrRps4 associates with the WRKY domains of RRS1 and RRS1B. Although AvrRps4 associates with other domains of RRS1, its association with the WRKY domain is the trigger that is crucial for activation of defence. PopP2 acetylation of the WRKY domains in RRS1-S and RRS1B compromises their capacity to recognise AvrRps4 **(Figure 4.3)**. Moreover, substitution of K^2 in the RRS1-S WRK^1YGQK^2 motif with an acetyl-lysine mimic (Q), or with an R, abolishes AvrRps4 recognition and WRKY domain binding, emphasising the importance of K^2 during effector recognition **(Figure 4.5)**.

Protein-protein interaction modelling predicts that K^2 of RRS1 or RRS1B WRKY domain directly interacts with the E187 & E175 residues of AvrRps4 **(Figure 4.8)**. In turn, I established that mutating E187 to alanine significantly reduces AvrRps4's affinity with RRS1 or RRS1B WRKY domain **(Fig-**

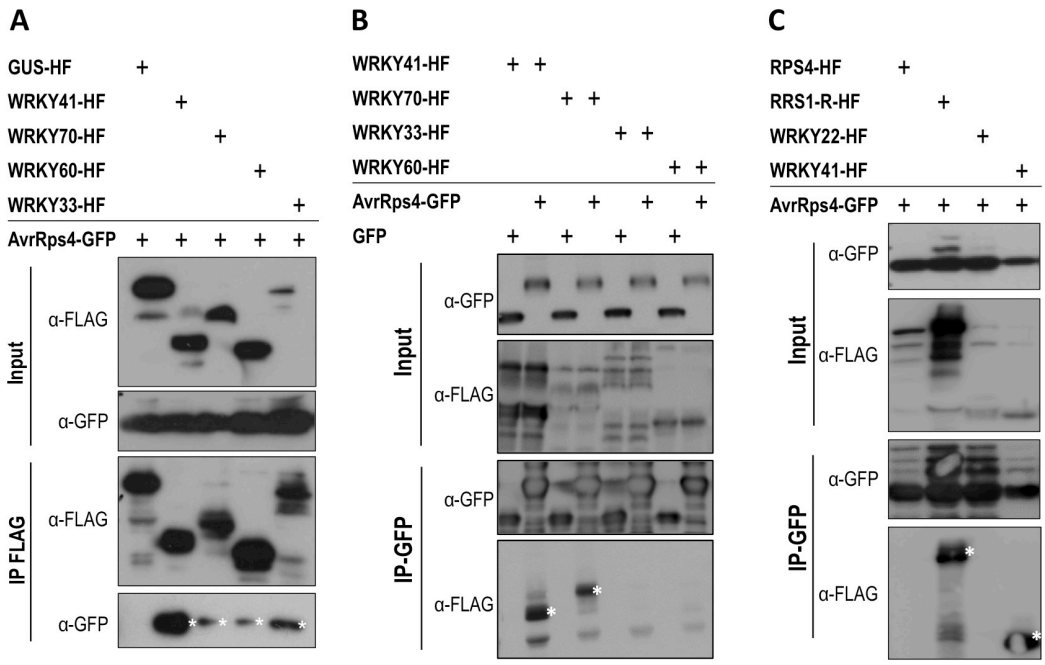

Figure 4.11: AvrRps4 associates with several WRKY transcription factors (**A-C**) Co-IP assays assess the association between AvrRps4-GFP and WRKY41-HF, WRKY70-HF, WRKY60-HF, WRKY33-HF and WRKY22-HF. Immunoblots show the presence of proteins in total extracts (input) and after IP. Asterisks indicate expected protein bands. Figure panels A and B were published in [58] Fig S7C, S6; experiments were done together with ZD.

ure 4.7A), explaining the loss of AvrRps4-E187A recognition. Overall, it seems that mutations that reduce the association between AvrRps4 and the WRKY domain, also weaken the resulting cell death responses, except for AvrRps4-KRVYAAAA. KRVY substitutions to AAAA completely abolish AvrRps4 recognition, but do not comprise WRKY domain interaction **(Figure 4.7A)**, implying that additional properties besides WRKY domain interaction must be required for AvrRps4 recognition. So far, I showed that various mutations in AvrRps4 interfere with the recognition by RRS1/RPS4 and likewise by RRS1B/RPS4B, indicating that similar properties of AvrRps4 are required for its perception by both pairs.

Sohn et al. [243] suggested that L167 of AvrRps4 is specifically required for RRS1B/RPS4B recognition but not by RRS1/RPS4, yet I have shown that RRS1B/RPS4B can recognise AvrRps4-L167S if the R proteins are expressed at high enough concentrations in the transient assay **(Figure 4.10C)**. Therefore, within the limits of the transient assay, it seems that the strength of AvrRps4-L167S responsiveness is determined by its affinity with either WRKY domain and the concentration of the R proteins.

4.7.2 *PopP2 inhibits AvrRps4 recognition via acetylating the WRKY domain*

In parallel to my findings that AvrRps4 is perceived by the WRKY domain, [58, 59] revealed that PopP2 also interacts with and acetylates the WRKY domain to activate RRS1-R/RPS4-mediated defence. Together, our work unveiled a plant immune receptor pair that detects two distinct bacterial effectors via a WRKY domain of one receptor, while the other activates defence upon such detection.

PopP2 directly interacts with RRS1 [214], and this interaction is dependent on the WRKY domain [58], suggesting a direct PopP2/WRKY domain interaction. Direct interaction between AvrRps4 and WRKY41 was first reported in Y2H screens [16], and various clues imply that AvrRps4 also directly interacts with the WRKY domains of RRS1 or RRS1B. For example, E187 and E175, which are crucial for AvrRps4 recognition, are located at a prominent electro-negative surface of AvrRps4 [243], and also mapped to the predicted interface with the WRKY domain **(Figure 4.8)**. Structural comparison of AvrRps4 and the coiled-coil domain of PRK1 kinase (HR1b) suggested that the negative surface of AvrRps4 might be directly involved in protein-protein interaction: the equivalent AvrRps4 residues (E187 and E175) on the negative surface of HR1b were shown to directly interact with Rho GTPase (Rac1) in the HR1b/Rac1 complex [243, 285].
PopP2 acetylation of lysines (especially K^2) blocks AvrRps4 perception via the WRKY domain, strongly suggesting that AvrRps4 interact with K^2 of the WRKY domain.

Although PopP2 has a weaker affinity with the WRKY domain compared to AvrRps4 **(Figure 4.3E, F)**, I infer that PopP2 acetylation possibly precedes AvrRps4 binding, and is therefore able to suppress AvrRps4 recognition. This is consistent with my observation that AvrRps4 cannot interfere with PopP2 recognition by RRS1-R/RPS4 **(Figure 4.4A)**. Several examples demonstrated that some pathogens, such as *P. syringae* [53, 286] and *Blumeria graminis* [287], secrete effectors that interfere with the recognition of other effectors. However, the suppression of AvrRps4 recognition by PopP2 is perhaps not likely to occur naturally, because they are delivered by a root and a leaf pathogen respectively. Therefore, I infer that rather than a collaboration of different pathogens, PopP2 suppression of AvrRps4 recognition is likely to be a consequence of them both targeting similar WRKY proteins, and

thereby being perceived by a common WRKY domain of RRS1. This coincidence however has proven a powerful tool to understand the mechanisms of effector perception via an R protein domain mimicking common effector host targets.

4.7.3 *The integrated WRKY domain functions to detect effectors*

Our discovery (YM, ZD and colleagues) of AvrRps4 and PopP2 both interacting with other Arabidopsis WRKY proteins implies that RRS1 and RRS1B have evolved with a WRKY domain and thus can hijack effectors that target WRKY transcription factors for immune suppression. In other words, the WRKY domains of RRS1 and RRS1B act as a bait for pathogen effectors. As discussed in Chapter 3, a probable common ancestor of both RRS1 and RRS1B lacks the WRKY domain [121], suggesting that RRS1/RPS4 and RRS1B/RPS4B evolved from a duplication event after the integration of a WRKY domain. These duplicated RRS1/RPS4-like proteins with an integrated WRKY domain are maintained or have further expanded in several relatives of *A. thaliana* [121], implying the evolutionary advantage of the WRKY domain fusion. Furthermore, Sarris et al (2016) [118] highlighted the recurrent integration of WRKY transcription factor into NLRs, which appear to have emerged independently in several lineages of plants, emphasising the significance of WRKY proteins for plant immunity. More broadly, incorporations of such atypical domains into NLRs are shown to be widespread in plants, and 'hub proteins' convergently targeted by many effectors are suggested to be highly enriched in NLR fusions [16, 118, 119, 126].

Intriguingly, these NLRs that perceive effectors via extraneous domains are often found to partner with a classic NB-LRR protein, which functions for defence signalling [15, 110, 112, 126]. As with RRS1/RPS4, other examples include the *RGA4/RGA5* and *Pik-1/Pik-2* gene pairs in rice, in which *RGA5* and *Pik-1* each incorporates a heavy metal-associated domain (HMA, also known as RATX1) that interacts with recognised effectors [110, 112, 113]. However, these effectors have not been reported to target other HMA-domain proteins involved in or required for disease resistance.

Hypothetically, these NLR fusion domains might have evolved from duplication of effector targets, followed by incorporation into the NLR protein. After the fusion event, the extraneous domain may evolve exclusively

towards effective pathogen detection, and as a consequence losing the ancestral biochemical activity, thus becoming an 'integrated decoy'[112]. A frequently asked question is "Are all integrated domains of plant NLRs true decoys?" Wu et al (2015) [120] pointed out that some integrated domains are not necessarily decoys, as they might maintain their ancestral function in the effector-targeted pathway as well as effector sensing.

The integrated WRKY domains of RRS1 and RRS1B preserve all the signatures of a functional WRKY transcription factor (WRKYGQK and Zn^{2+}-binding motifs), and are shown to bind a W-box DNA element [58, 59]. However, *rrs1-1/rrs1b-1* double mutants did not show enhanced susceptibility to pathogens lacking the Avrs, and so far there is no evidence that RRS1 or RRS1B contribute to basal defence. I hypothesise that the DNA-binding motifs of these integrated WRKY domains are required for effector detection, and thus the retained DNA-binding activity; and as duplicates of an effector-targeted WRKY protein, their biochemical activities are possibly dispensable for immunity. Nevertheless, whether integrated WRKY domains of RRS1 and RRS1B are true decoys remains to be seen. In contrast, some integrated WRKY domains in NLRs are found to have lost the conserved residues critical for DNA binding, suggesting that they might have become 'decoys' [118]. It will be interesting to see if these 'decoy' WRKY fusions perceive a different groups of effectors that do not target the key DNA-binding residues.

4.7.4 *Is DNA binding via the WRKY domain relevant for RRS1/RPS4 complex activation?*

If the W-box DNA binding activity of the RRS1 WRKY domain is not required for basal defence, could it be regulating AvrRps4- or PopP2-triggered defence activation? RRS1-R (*slh1*), in which the leucine insertion in the WRKY domain abolishes its DNA binding, was reported to trigger constitutive defence activation in the presence of RPS4 [58, 250]. In addition, [58, 59] showed that PopP2 acetylation of the RRS1-R WRKY domain also disrupts DNA binding, and subsequently triggers defence. This correlation between the loss of WRKY domain DNA binding and the activation of RRS1-R/RPS4 complex, has led to the belief that the DNA binding property of the WRKY domain is required for the activation of RRS1/RPS4. On the contrary, DNA-binding deficient mutants RRS1-S(*slh1*) and RRS1-R($K^1$221R or K^2R), along with PopP2-acetylated RRS1-S do not confer constitutive activa-

tion of defence [58, 59], demonstrating that the loss of W-box DNA binding is insufficient for defence activation.

Additionally, using electrophoretic mobility shift (EMSA) assays, postdoc SH showed that AvrRps4 activates defence without interfering with the DNA binding of the RRS1 WRKY domain [58]. Overall, it seems the DNA binding status of RRS1 WRKY domain does not determine the activation (or inactivation) of the RRS1/RPS4 complex. Therefore, I propose that it is likely the intra- and inter-molecular changes triggered by effector detection at the WRKY domain activate the complex, and I will explore this further in Chapter 5.

The K^2 residue of the WRKY motif is located at the interface with DNA; how then does AvrRps4 bind to K^2 without disrupting the DNA binding? Some WRKY proteins form dimers [206, 275, 288, 289], and it is possible that AvrRps4 may interact with only one molecule in a hypothetical WRKY domain dimer of RRS1, allowing the other WRKY domain to still bind DNA. Our attempts (LW & HB and YM) at obtaining the co-crystal structure of AvrRps4 and the WRKY domain aim to elucidate the nature of this interaction. Nevertheless, we cannot exclude that AvrRps4 may only bind to a proportion of RRS1 molecules to activate defence, leaving the unbound molecules free to interact with DNA.

Unlike PopP2, which disrupts the DNA binding capacity of several WRKY transcription factors [58, 59], AvrRps4 binding may interfere with WRKY protein function by other means. I infer that AvrRps4 and PopP2 essentially have different modes of action, and perhaps are likely to trigger defence via the WRKY domain of RRS1 slightly differently. Comparisons of AvrRps4- and PopP2-triggered activation will be discussed in Chapter 5.

To conclude, our data (YM and colleagues) imply a general mode of evolution of new immune recognition capacities: NLR receptor pairs are selected in which one member integrates a domain that enables perception of effectors via mimicking their host targets, while the other activates defence upon such perception.

5

De-repression of the immune receptor complex RRS1/RPS4 by distinct effectors, AvrRps4 and PopP2

5.1 Introduction and Chapter aim

So far, our work (YM, colleagues and collaborators) has revealed that the paired TNLs RRS1-R and RPS4 form an immune complex to detect effectors via an integrated WRKY domain of RRS1-R, which mimics the effector's host targets, WRKY transcription factors [58, 59, 115]. My work also suggests that the paralogous TNL pair RRS1B/RPS4B operates similarly to detect the effector AvrRps4 [121]. The next big question is "how do these immune complexes convert effector recognition into defence activation?" The 'molecular switch' model stresses the importance of intra-molecular reconfigurations for NLR activation [153, 154]. In addition, by analogy with mammalian NLR mechanisms, NLR homo- or hetero-oligomerization (via inter-molecular interaction) may be an important element of plant NLR activation. Therefore, detailed molecular studies on the changes in intra- and inter-molecular interactions before and after effector elicitation are needed to understand the operating mechanisms of NLRs. Specifically, this chapter will investigate these molecular changes in an immune pair complex with a typical integrated domain, which has not been attempted before.

The role of the RRS1 WRKY domain as the effector 'sensor' implies that it is also the domain that initiates activation. Several mutations in the WRKY domain (e.g. *slh1*, K1221Q) have been shown to constitutively activate the RRS1-R/RPS4 immune complex [58, 59, 250]. As the DNA binding activity of the WRKY domain is unlikely to directly activate defence (discussed in Chapter 4), the WRKY domain may regulate immune complex activity. In this chapter, I will look into the function of the WRKY domain in the pre-activation state and during activation via exploring its interactions with neighbouring domains.

How then does the effector detection via RRS1 WRKY domain activate RPS4? A simple hypothesis is that the WRKY domain perceives the effectors, starting a chain of molecular changes in RRS1 to pass on the activation signal to the executor RPS4. The mechanism of a sensor (RRS1) activating an executor (RPS4) in an immune complex is likely to be via specific domain-domain interactions. Consistent with this idea, the immune complexes formed of inappropriate pairings (RRS1/RPS4B and RRS1B/RPS4) do not support effector-triggered activation, indicating that specific domain-domain interactions between cognate pair partners are essential for activation **Figure 3.3**[121].

I carried out systematic domain swapping experiments between RRS1-R and RRS1B, and between RPS4 and RPS4B in an attempt to identify domain(s) that might determine the pair partner compatibility. **Figure 5.1** illustrates the domain boundaries for swapping or deletions in this study: the six domains of RRS1-R and RRS1B (TIR, NB-ARC, LRR, Domain 4 (DOM4), WRKY, Domain 6 (D6)) and the four domains of RPS4 and RPS4B (TIR, NB-ARC, LRR, C-terminal domain (CTD)) are defined with their swapping breakpoint amino acid labelled. Ws-2 alleles of RRS1-R, RRS1B and RPS4B and Col-0 allele of RPS4 were used. When not specified, these alleles are considered the defaults throughout this chapter. The exact domain boundaries were chosen for cloning and swapping purposes so that the breakpoint amino acids are identical between RRS1-R & RRS1B, and RPS4 & RPS4B, and also are designed to minimise potential disruption of predicted structural and functional motifs (details see 2.2.1). Using the golden gate shuffling method [264–266], each single domain was cloned and together assembled into full-length R genes with varying domain combinations according to the defined boundaries. For simplicity, the chimeric proteins are represented with the domains from the A pair proteins (RRS1-R and RPS4) abbreviated as "A"s, and the domains from the B pair proteins (RRS1B and RPS4B) abbreviated as "B"s. For example, RPS4(AAAB) represents a chimera of RPS4 TIR-NB-ARC-LRR carrying a CTD from RPS4B. All the wild type and chimeric R proteins in this chapter are expressed under 35S promoter and C-terminally tagged with HF (6xHis 3xFlag). This chapter will focus on the functional analyses of some of these domain-swapped chimeras that reveal key domain-domain interactions involved in auto-inhibition and activation.

A remaining puzzle is that PopP2 is not recognised by RRS1-S/RPS4 and RPS4B/RRS1B, which are fully functional to recognise AvrRps4 [121, 214]. It

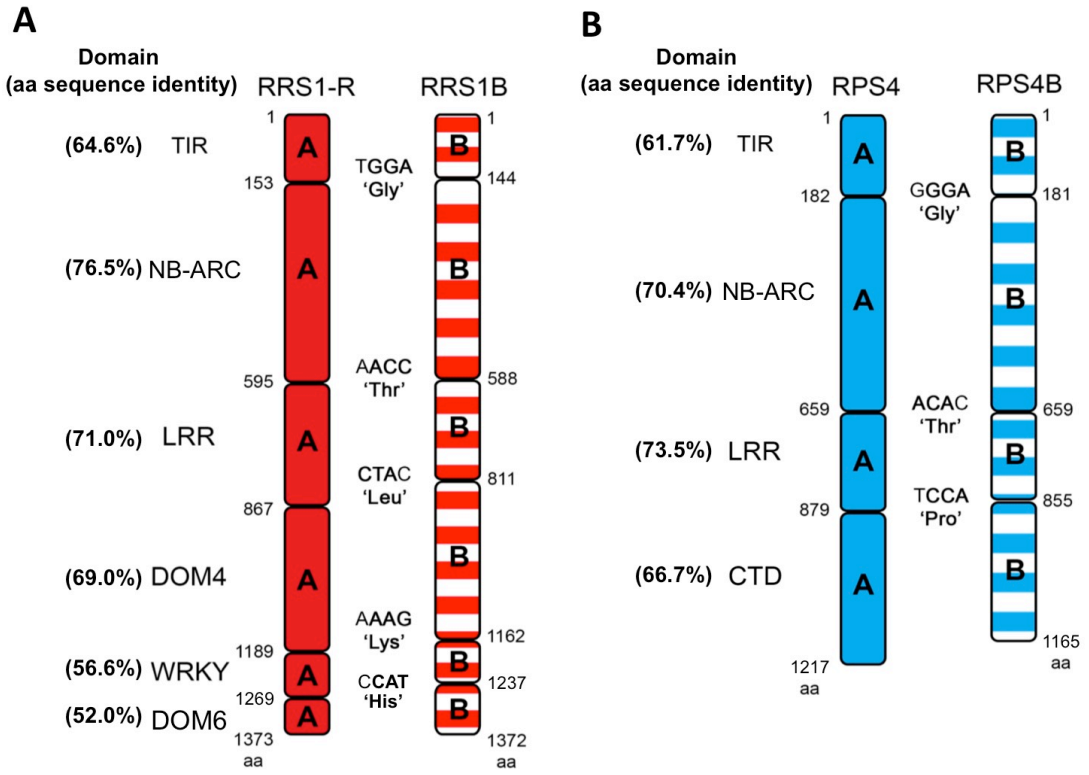

Figure 5.1: Schematic representation of RRS1-R & RRS1B and RPS4 & RPS4B domain structures. The six domains of RRS1-R^{Ws-2} & RRS1B^{Ws-2} (TIR, NB-ARC, LRR, Domain 4 (DOM4), WRKY, Domain 6 (DOM6)) (**A**) and the four domains of RPS4^{Col-0} & RPS4B^{Ws-2} (TIR, NB-ARC, LRR, C-terminal domain (CTD)) (**B**) are defined. Numbers indicate the amino acid positions for domain boundaries. Domains from the A pair proteins (RRS1-R & RPS4) are abbreviated as "A"s and shown in solid colours; and domains from the B pair proteins (RRS1B & RPS4B) are abbreviated as "B"s and shown in coloured stripes. RRS1-R & RRS1B are shown in red, RPS4 & RPS4 are shown in blue. Percentages indicate the amino acid (aa) sequence identity for each domain of 'A' and 'B'. The breakpoint amino acids are identical between RRS1-R & RRS1B and RPS4 & RPS4B, which are shown in the middle as 'Gly' Glycine, 'Thr' Threonine, 'Leu' Leucine, 'Lys' Lysine, 'His' Histidine, and 'Pro' Proline. The golden gate assembly of individual domains utilises the matching 4 bp over-hangs, in which the 3 nucleotides code for the breakpoint amino acid (highlighted bold). More details of cloning see 2.2.1.

begs the question whether PopP2 activates the immune complex differently from AvrRps4. The fact that PopP2 binds and acetylates the WRKY domain of RRS1-S (and possibly RRS1B) in the same manner compared to RRS1-R indicates that something extra is needed for PopP2 responsiveness [58, 59]. PS in Jones lab has reported that the extra C-terminal 83 aa of RRS1-R (C83) compared to RRS1-S determines PopP2 recognition capacity, because fusing the C83 to RRS1-S is sufficient to restore PopP2 responsiveness [58]. However it is still a mystery how the RRS1-R DOM6, especially the C83, enables RRS1-R/RPS4 to respond to PopP2. For clarity, DOM6 of RRS1-R, RRS1-S and RRS1B in this study are labelled as DOM6-R (104 aa), DOM6-S (21 aa) and DOM6-B (135 aa) respectively. Here I hypothesise that the extended C-terminal domain of RRS1-R assists specific inter-domain reconfigurations in RRS1-R that may allow PopP2 to activate the immune complex in a distinct manner compared to AvrRps4.

In this chapter, I will investigate the possible domain-domain interactions specifically involved in PopP2-triggered activation, utilising the point mutations identified in a suppressor screen [116]. The genetic screen has identified various mutations that suppress *slh1* immunity in Arabidopsis accession Nossen (No-0), termed the *sushi (suppressor of slh1 immunity)* mutants, most of which are in RRS1 and RPS4 [116]. ZD and OF later helped to expand the *sushi* mutant list: totalling 9 mutations in RRS1 (with 2 in DOM4) and 38 mutations in RPS4 (with 10 in CTD), excluding early stop codons (unpublished). These point mutations are good indicators of important domains and residues during activation, and in combination with the domain swap analyses, enabled more detailed insights into the functional mechanisms of the RRS1/RPS4 immune complex.

5.2 WRKY domain of RRS1 is the key to maintain RRS1/RPS4 auto-inhibition

To investigate the role of the WRKY domain and its neighbouring domains (DOM4 and DOM6), I carried out sequential domain deletions from the C-terminal end of RRS1-R, and analysed the function of the truncated RRS1-R proteins using tobacco transient assays **(Figure 5.2)**. Consistent with the published data [58], I first confirmed that the C83 deletion of RRS1-R abolishes PopP2 but not AvrRps4 recognition when co-expressed with RPS4, phenocopying the function of RRS1-S **(Figure 5.2)**. Similarly, RRS1-RΔD6,

with the entire DOM6-R deleted, only shows responsiveness to AvrRps4, and is not constitutively active **(Figure 5.2)**. Together these data imply that the DOM6 of RRS1-R is dispensable for AvrRps4 recognition, but is essential for PopP2 recognition.

While RRS1-RΔD6 is fully functional for AvrRps4 perception, a further deletion of the WRKY domain (Domain 5) results in effector-independent constitutive HR of the RRS1-RΔD56 + RPS4 combination **(Figure 5.2)**. Likewise, RRS1-SΔD56 + RPS4 is also auto-active when co-expressed in tobacco (data not shown). Therefore the absence of the WRKY domain in RRS1 is sufficient to activate defence, implying its role as a negative regulator of the immune complex, contrary to the belief that the WRKY domain is involved in downstream defence signalling. Consistently, RRS1-R WRKY domain when swapped with either the non-related LexA DNA binding domain, or the related RRS1B WRKY domain (56.6% aa identity) causes RPS4-dependent auto-activity, indicating that the WRKY domain is specifically required to keep RRS1-R inactive **(Figure 5.3)**.

Furthermore, I showed that the auto-activity of RRS1-RΔD56 relies on DOM4 of RRS1-R, since the further deletion of DOM4 renders RRS1-RΔD456 non-autoactive and non-responsive to any effectors **(Figure 5.2)**. The importance of DOM4 during activation is also demonstrated by the DOM4 swaps between RRS1 and RRS1B: Both RRS1(AAABAA) + RPS4 and RRS1B(BBBABB) + RPS4B combinations fail to respond to effectors in tobacco transient assays **(Figure 5.10B,F)**. From these results, a simple model emerges that in the absence of the effectors, the WRKY domain is negatively regulating DOM4 to keep RRS1 inactive, and that upon loss of that negative regulation, DOM4 plays a role in activating the complex, likely via interactions with RPS4.

Interestingly, the replacement of the RRS1-R C83 by the C115 of RRS1B (forming RRS1-R(AAAAA^{+}B)), or the exchange of the entire DOM6-R (104 aa) by DOM6-B (135 aa) (forming RRS1-R(AAAAAB)), also leads to RPS4-dependent auto-activity **(Figure 5.3)**. Similarly, the RRS1B chimeras with the C-terminal amino acids swapped with RRS1-R are autoimmune when co-expressed with RPS4B **(Figure 5.13F)**. These data suggest that the compatibility of WRKY and DOM6 in RRS1 or RRS1B are also important for immune complex auto-inhibition. Nevertheless, the deletion of C83 or DOM6-R does not automatically de-repress RRS1-R , whilst the addition of RRS1B

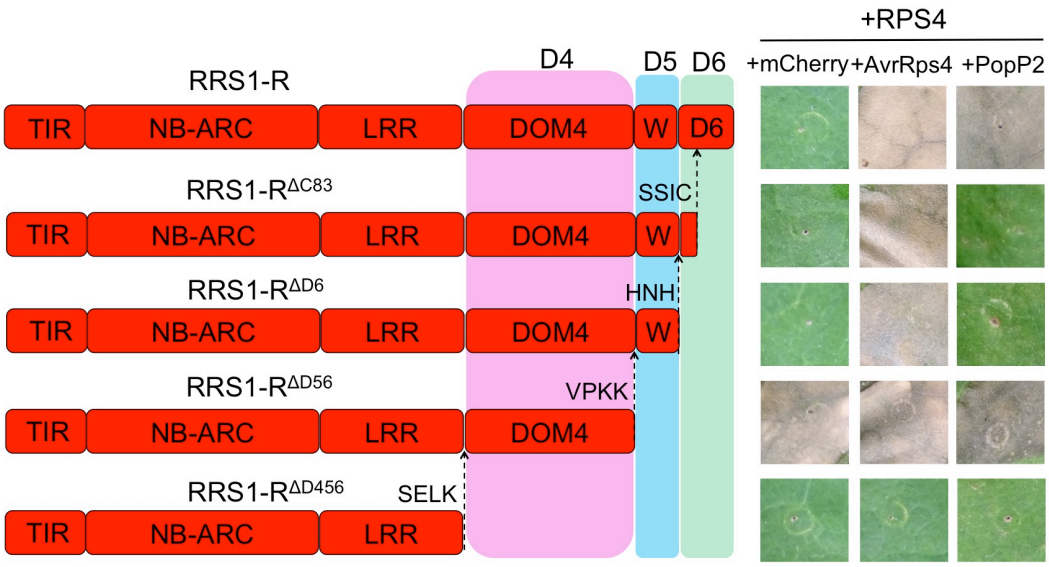

Figure 5.2: The WRKY domain negatively regulates Domain 4 (DOM4) to keep RRS1 inactive Transient assays in *N. tabacum* leaves using *A. tumefaciens* transformation (agro-infiltration) show that successive deletions of DOM6, WRKY domain, and DOM4 of RRS1-R alter its function for effector responsiveness and auto-inhibition. Each leaf section was co-infiltrated to express RPS4 and a truncated RRS1-R or a full-length RRS1-R (left of each row) in combination with mCherry, AvrRps4:mCherry or PopP2:mCherry (top of each column). Schematic diagram shows the domain structure of the full-length RRS1-R and various truncated RRS1-R derived from successive deletions: RRS1-RΔC83, deletion of C-terminal 83 amino acids; RRS1-RΔD6, deletion of DOM6-R (shown as D6); RRS1-RΔD56, deletion of WRKY (shown as W) and DOM6-R; RRS1-RΔD456, deletion of DOM4, WRKY and DOM6-R. Amino acid sequences indicate the border where the deletions terminate. Cell death pictures were taken 4 dpi. These experiments were repeated at least three times with similar results.

C-terminal amino acids leads to the auto-activity of RRS1-R(AAAAA^{+}B) or RRS1-R(AAAAAB). One possible explanation could be that an incompatible DOM6 may interfere with WRKY domain's negative regulation on DOM4, and thereby triggers activation (see 5.6 for more details).

In addition, replacing the WRKY and DOM6 domain simultaneously with the equivalent domains from RRS1B, or with the WRKY domain and C-terminal amino acids from WRKY41 results in auto-activity of RRS1-R(AAAABB) and RRS1-R(AAAAW41) respectively **(Figure 5.3)**. With their respective compatible C-terminal amino acids intact, it is possible that these WRKY domains fail to suppress DOM4 in these chimeric proteins, thus activating RRS1-R. It should also be noted that the WRKY domains of WRKY41 and RRS1B are functional DNA binding domains, and therefore should enable the DNA binding of the chimeras carrying these domains similarly to RRS1-R. Yet all of these chimeras [e.g. RRS1-R(AAAABB), RRS1-R(AAAAW41)] are able to activate defence possibly without being dislodged from the DNA, supporting that DNA binding activity *per se* (or the loss of it) does not determine the immune complex activity.

5.3 WRKY domain associates with Domain 4 of RRS1 in the absence of the effectors

I next investigated whether the WRKY domain associates with DOM4 of RRS1 to suppress its activity in the pre-activation state. Co-IP experiments showed that the DOM4 is able to associate with both the WRKY domain and WRKY & DOM6 of RRS1-R (DOM56-R) after transient co-expression in *N. benthamiana* leaves **(Figure 5.4A)**. Consistently, BiFC data suggest that the N- and C-termini of RRS1-R DOM456, i.e. DOM4 and DOM6-R, are in close proximity in the absence of effectors **(Figure 5.4B)**. It is also possible that these BiFC signals are produced by inter-molecular interactions, meaning that the N-terminus (DOM4) of one molecule could associate with the C-terminus (DOM6) of another molecule. A test for DOM456-R's ability to self-associate may be a simple way to rule out that possibility. To clarify directional interactions (head-to-head or head-to-tail), future experiments would require testing a series of combinations of N- or C-terminally tagged DOM456 (e.g cCFP:DOM456-R + DOM456:nVenus; cCFP:DOM456-R + nVenus:DOM456-R). Overall, these data are consistent with the model that RRS1-R DOM456 is

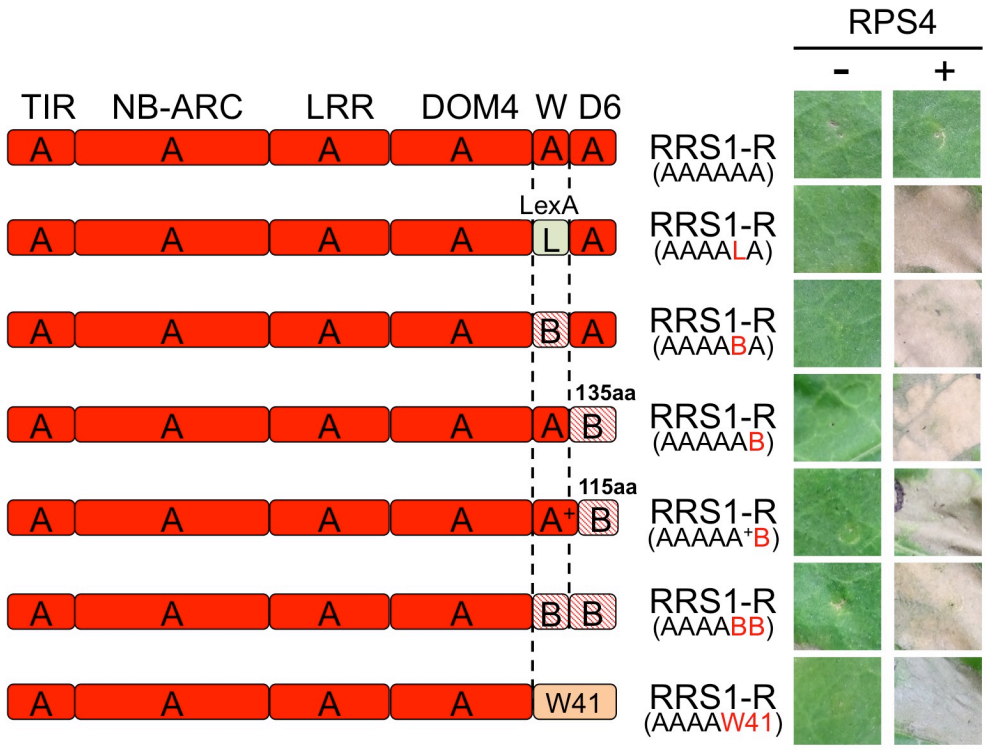

Figure 5.3: Replacement of the WRKY domain and/or Domain 6 (D6) in RRS1-R causes RPS4-dependent autoactivity Transient assays in *N. tabacum* leaves using agro-infiltration shows the HR activities of chimeric RRS1-R proteins in the presence of RPS4, when the RRS1 WRKY and/or DOM6 were replaced by other domains. Each leaf section was infiltrated to express a wild type or a chimeric RRS1-R (left of each row) with or without the presence of RPS4 (indicated as + or -). Schematic diagram shows the domain structure of the wild type RRS1-R and various RRS1-R chimeras. These RRS1-R chimeras are represented with domains from RRS1-R shown as 'A's, domains from RRS1B shown as 'B's, the bacterial LexA DNA binding domain [279] shown as 'L' and the WRKY domain and C-terminal amino acids derived from *At*WRKY41 (123–313 aa) shown as 'W41'. The borders for domain replacement are consistent with the definition in Figure 5.1, except for RRS1-R(AAAAA$^+$B) which has the RRS1-R C83 replaced by the C-terminal 115 aa from RRS1B. Pictures were taken 5 dpi. These experiments were repeated at least three times with similar results.

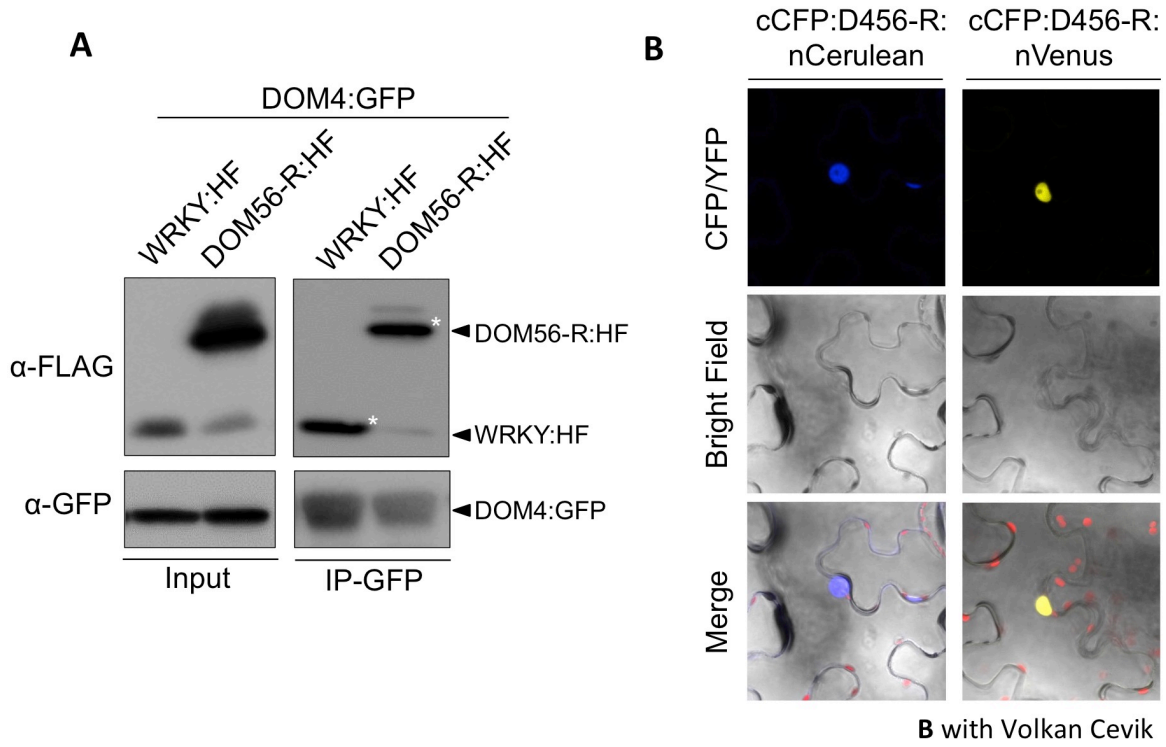

Figure 5.4: RRS1-R WRKY & DOM6 (DOM56-R) associates with DOM4 in the absence of effectors (**A**) The Co-IP assay shows that both WRKY:HF and DOM56-R:HF associate with the DOM4:GFP of RRS1-R after *A. tumefaciens*-mediated transient co-expression in *N.benthamiana* leaves. Immunoblots show the presence of proteins in total extracts (input) and after IP with α-GFP beads (IP-GFP). Asterisks indicate expected protein bands. (**B**) BiFC assays indicate close proximity of N- and C-termini of RRS1-R domains 4,5 and 6 (DOM456-R), i.e. DOM4 and DOM6-R. DOM456-R was N-terminally tagged with cCFP and also C-terminally tagged with either nVenus or nCerulean. When the N- and C-termini of DOM456 are adjacent, the interaction of cCFP and nCerulean tags create CFP (blue signal), and the interactions of cCFP and nVenus tags creates YFP (yellow signal). Agro-infiltration of cCFP:DOM456-R:nCerulean and cCFP:DOM456-R:nVenus in *N. benthamiana* leaves show CFP and YFP signals respectively in the nuclei. The labels on the left indicate the channel imaged. Images were recorded 48 hpi. At least three biological replicates were performed showing consistent results.

kept in a closed conformation in resting state, which is likely maintained by WRKY/DOM4 association.

The next question is whether the auto-activities of several RRS1-R WRKY domain mutants are caused by the lack of WRKY/DOM4 associations. Using Co-IP assays, I assessed the associations of RRS1-R DOM4 with DOM56-R carrying various mutations including auto-active mutations *slh1* and K1221Q (K^2Q), and non-autoactive mutation K1221R (K^2R)), and found that all tested mutants of DOM56-R Co-IP with DOM4 **(Figure 5.5)**. Compared to DOM56-R:HF and DOM56-R(K^2R):HF (lanes 1 & 3), DOM56-R*slh1*:HF and DOM56-R(K^2Q):HF (lanes 2 & 4) pull down less DOM4:GFP after IP with FLAG beads,

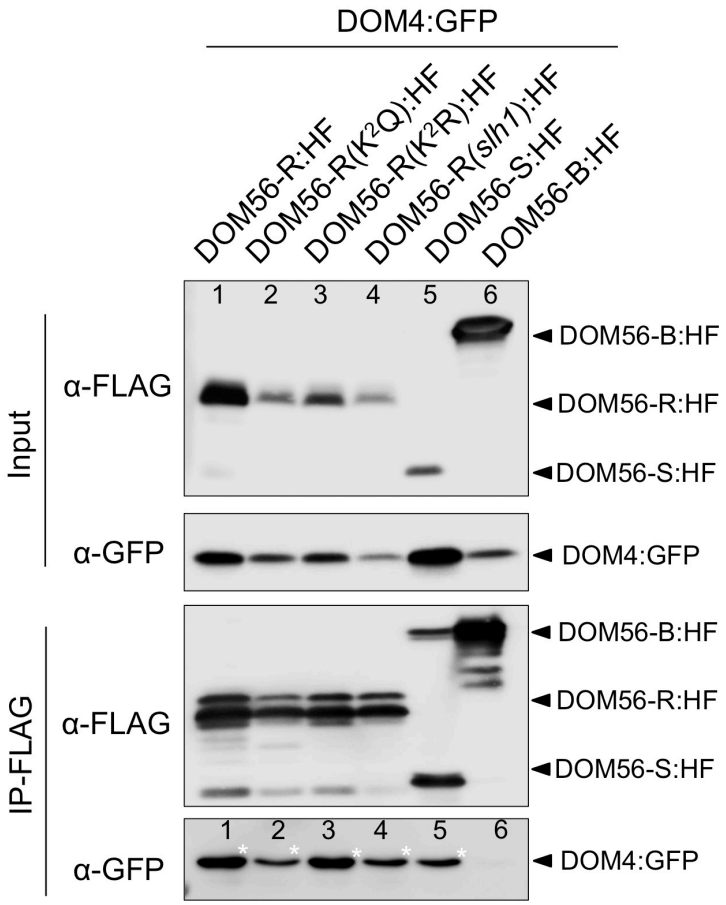

Figure 5.5: RRS1B WRKY & DOM6 (DOM56-B) fails to associate with DOM4 of RRS1-R
This Co-IP assay assesses the associations between DOM4:GFP (RRS1-R) and different alleles or mutants of DOM56:HF. DOM56-R, DOM56-S and DOM56-B are WRKY & DOM6 from RRS1-R, RRS1-S and RRS1B respectively. K^2Q (K1221Q) and *slh1* are mutations in the RRS1-R WRKY domain that lead to auto-activity, and K^2R (K1221R) is a mutation of RRS1-R that impairs effector responsiveness but does not lead to auto-activity. Immunoblots show the presence of proteins in total extracts (input) and after IP with α-FLAG beads (IP-FLAG). Asterisks indicate expected protein bands. Independent experiments have shown consistent results in that auto-active variants of DOM56-R (DOM56-R(K^2Q) and DOM56-R(*slh1*)) Co-IP with DOM4 more weakly, but are less stable compared to DOM56-R. A representative result is shown here.

suggesting weaker association **(Figure 5.5)**. However, as those DOM56-R carrying auto-active mutations are often less stable, showing less accumulations in the input (lanes 2 & 4), it is difficult to compare their strength of association **(Figure 5.5)**. In addition, DOM56-S with an identical WRKY and a shorter DOM6-S (21 aa) from RRS1-S, also accumulates less and Co-IPs more weakly with DOM4 (lane 5) compared to DOM56-R (lane 1) **(Figure 5.5)**. In contrast, DOM56-B, consisting of WRKY domain and DOM6-B (135 aa) from RRS1B, fails to Co-IP with DOM4 of RRS1 (lane 6) **(Figure 5.5)**. This lack of association between RRS1 DOM4 and DOM56-B provides an explanation for the auto-activity of RRS1-R(AAAABB) similar to RRS1-RΔD56: As DOM56-B fails to pose a negative regulation on RRS1-R DOM4 in RRS1-R(AAAABB), it phenocoypies the deletion of DOM56-R in RRS1-RΔD56.

I infer that the complete dissociation of DOM4/DOM56 can trigger RRS1 activation. It is possible that the auto-active mutations in the WRKY domain can cause a change of DOM4 and DOM56 association that is sufficient to de-repress RRS1-R without a complete dissociation. Additionally, I found that *in trans* co-expression of DOM56-R cannot suppress RRS1-RΔD56 + RPS4-triggered HR in *N. tabacum* **(Figure 5.6A)**, suggesting that proper folding of the full-length RRS1-R is required to maintain the auto-inhibition. Similarly, *in trans* co-expression of DOM4 and RRS1-RΔD456 cannot reconstitute the auto-activity of RRS1-RΔD56 + RPS4 in *N. tabacum* **(Figure 5.6B)**. These data reveal the highly intricate connections between the domains to maintain auto-inhibition and to activate the immune complex.

5.4 Do effectors de-repress RRS1 via disrupting WRKY domain association with Domain 4?

In Chapter 4 I showed that AvrRps4 association with the WRKY domain of RRS1 is required for its recognition. Co-IP data **(Figure 5.7A,B)** suggest that the presence of AvrRps4 destabilizes DOM4/WRKY and DOM4/DOM56-R associations (without completely abolishing them). As a negative control, the non-recognisable AvrRps4-E187A with much weaker affinity to the WRKY domain shows less interference to these associations **(Figure 5.7A,B)**. Consistent results are shown using BiFC assays: Co-expression of AvrRps4:mCherry, but not 35S:mCherry or AvrRps4-E187A:mCherry, with cCFP:D456-R:nVenus inhibits the BiFC signal in the nuclei **(Figure 5.7D)**. Therefore, it is likely that AvrRps4 de-represses RRS1 via disrupting DOM4/WRKY association, and

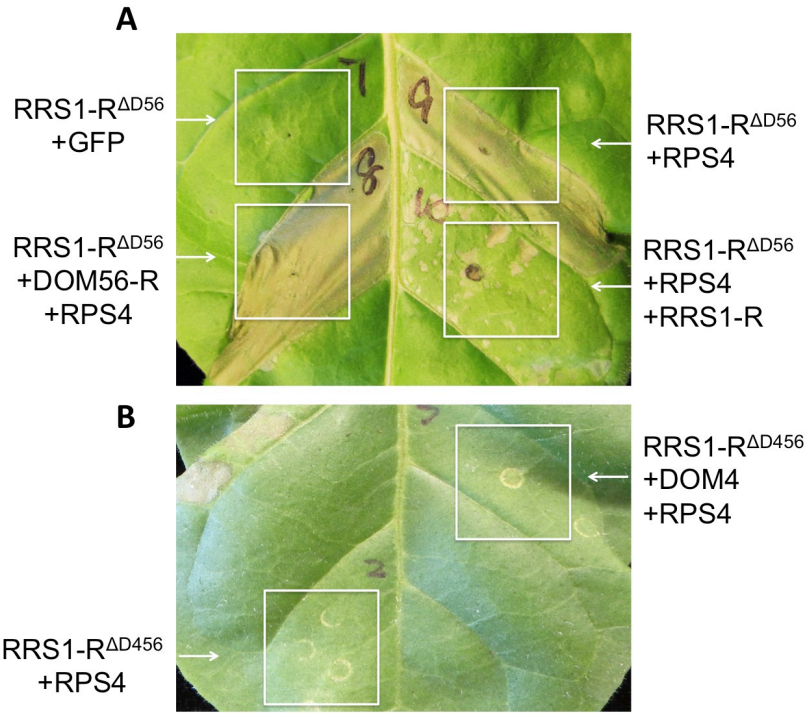

Figure 5.6: RRS1-RΔD56/RPS4-triggered cell death cannot be suppressed by co-expression of DOM56-R *in trans* (**A**) Transient assays in *N. tabacum* leaves using agro-infiltration show that RRS1-RΔD56 + RPS4-triggered HR (top right) cannot be suppressed by co-expressing DOM56-R (bottom left), but can be partially suppressed by co-expressing RRS1-R (bottom right). Pictures were taken 4 dpi. (**B**) Agro-infiltration in *N. tabacum* leaves show that RRS1-RΔD456 + RPS4 does not trigger HR (left), and *in trans* co-expression of RRS1-R DOM4 (DOM4) with RRS1-RΔD456 + RPS4 does not recover HR (right). Each leaf section infiltrated was indicated by a white square with corresponding infiltrated samples labelled. Pictures were taken 4 dpi. These experiments were repeated three times with similar results.

inadequate disruption by AvrRps4-E187A may explain its lack of recognition. On the other hand, as AvrRps4-KRVYAAAA mutant associates similarly strongly with the WRKY domain compared to the wild type (see Chapter 4), the ability to disrupt the DOM4/WRKY association by AvrRps4-KRVYAAAA may not be sufficient to activate defence. Preliminary results suggest that AvrRps4-KRVYAAAA disrupts DOM4/WRKY association as much as the wild type (data not shown).

Unlike AvrRps4, PopP2 did not show significant interference to DOM4/DOM56 association using similar assays (Co-IP and BiFC) **(Figure 5.7C, E)**. In both assays, the enzymatically inactive PopP2-C321A resembles PopP2, showing little effect of disrupting the DOM4/DOM56 association **(Figure 5.7C, E)**. This suggests that the lack of PopP2-C321A recognition cannot be easily explained by these results. It should be noted however, compared to PopP2-C321A:mCherry and 35S:mCherry, the co-expression of PopP2:mCherry seems to stabilise DOM56-R, resulting in more accumulation in the input (lane 4) **(Figure 5.7C)** which could result in a slightly greater suppression of DOM56-R/DOM4 associations by PopP2:mCherry. Nevertheless, DOM56-R(K^2Q), which mimics PopP2 acetylation of the WRKY reside K1221, also does not show complete loss of affinity with DOM4 **(Figure 5.5)**. Overall, these data suggest that PopP2 acetylation of the WRKY domain does not lead to disassociation of DOM4/DOM56. While AvrRps4 de-represses RRS1 via disrupting WRKY domain association with DOM4, PopP2 may activate the immune complex slightly differently. This will be discussed further in 5.6.

5.5 De-repressed RRS1 activates RPS4 via enabling DOM4 and CTD interaction.

Many lines of evidence suggest that RPS4 executes defence activation downstream of RRS1 effector sensing, and that the weak constitutive activity of RPS4 is attenuated in the pre-activation complex by RRS1 [58, 115, 116]. Here I show that RRS1-RΔD56 requires a signalling competent RPS4 to trigger cell death: The P-loop mutant RPS4(K242A) and the TIR domain mutant RPS4(SHAA) that disrupts TIR-TIR association both fail to activate cell death when co-expressed with RRS1-RΔD56 **(Figure 5.8)**. In contrast, the P-loop mutation K185A of RRS1-RΔD56 does not affect the cell death response in the presence of a functional RPS4 **(Figure 5.8)**. Consistent with the model of

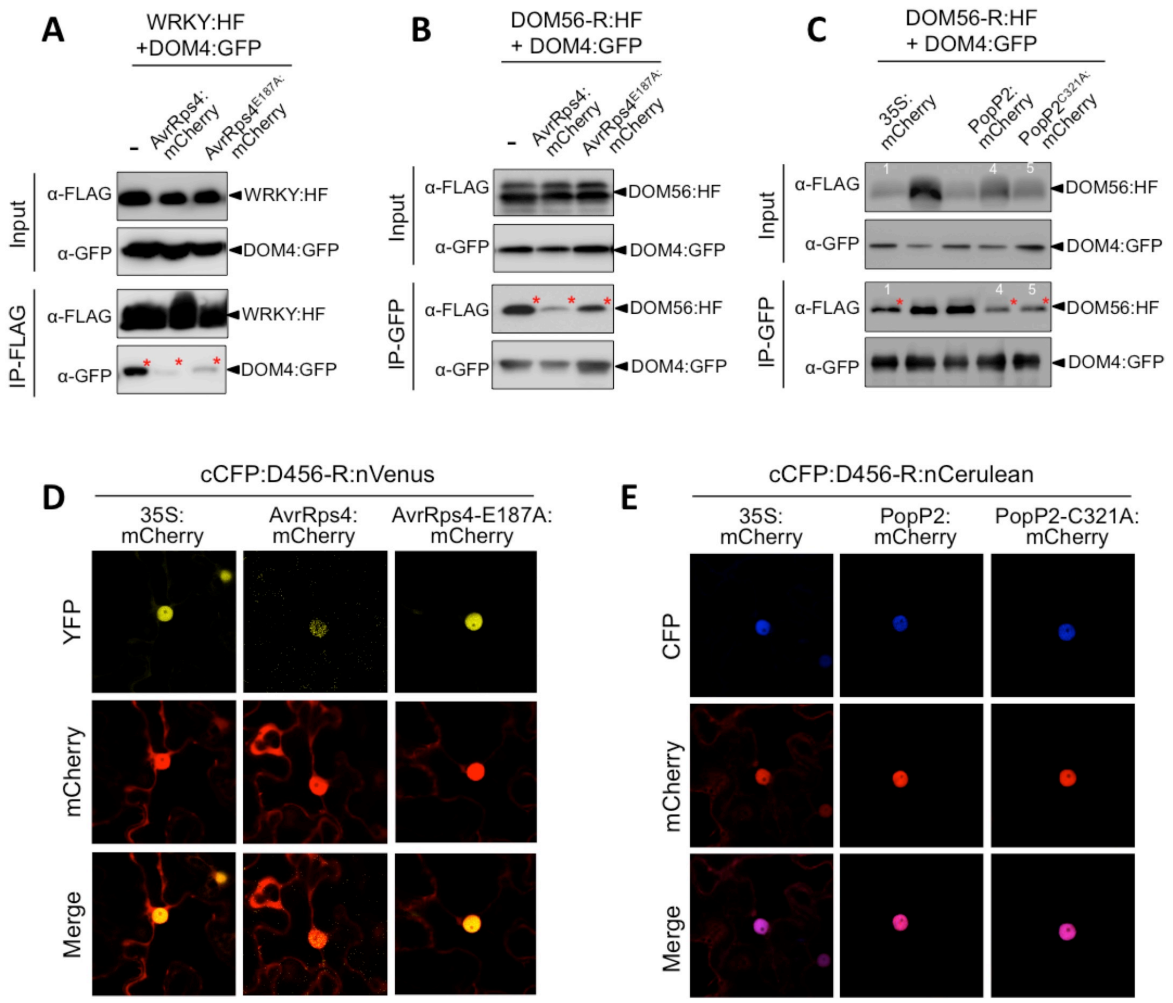

Figure 5.7: AvrRps4 de-represses RRS1-R via disrupting DOM56-R association with DOM4 yet PopP2 shows much weaker interference to DOM56-R/DOM4 association (**A-C**) Co-IP assays assess the effector interference to the associations of DOM4:GFP/WRKY:HF and DOM4:GFP/DOM56-R:HF by co-expressing AvrRps4:mCherry or PopP2:mCherry. AvrRps4:mCherry but not PopP2:mCherry shows disruption of these associations. 35S:mcherry and effector mutants AvrRps4-E187A:mCherry and PopP2-C321A:mCherry were used as negative controls. DOM4, WRKY and DOM56-R are from RRS1-R. Immunoblots show the presence of proteins in total extracts (input) and after IP. Asterisks indicate expected protein bands. (**D-E**) BiFC assays assess the effector interference to the BiFC signals of cCFP:DOM456-R:nVenus or cCFP:DOM456-R:nCerulean by transiently co-expressing AvrRps4:mCherry or PopP2:mCherry in *N. benthamiana* leaves. Co-expression of AvrRps4:mCherry but not PopP2:mCherry suppresses the BiFC signal, showing disruption of N- and C-termini associations of DOM456-R. 35S:mcherry and effector mutants AvrRps4-E187A:mCherry and PopP2-C321A:mCherry were used as negative controls. The accumulation of the effectors or controls are shown in the mCherry (RFP) channel. The labels on the left indicate the channel imaged. Images were recorded 48 hpi. At least three biological replicates were performed showing consistent results. The functionality of the effector proteins (or their mutant controls) were confirmed using tobacco transient assays.

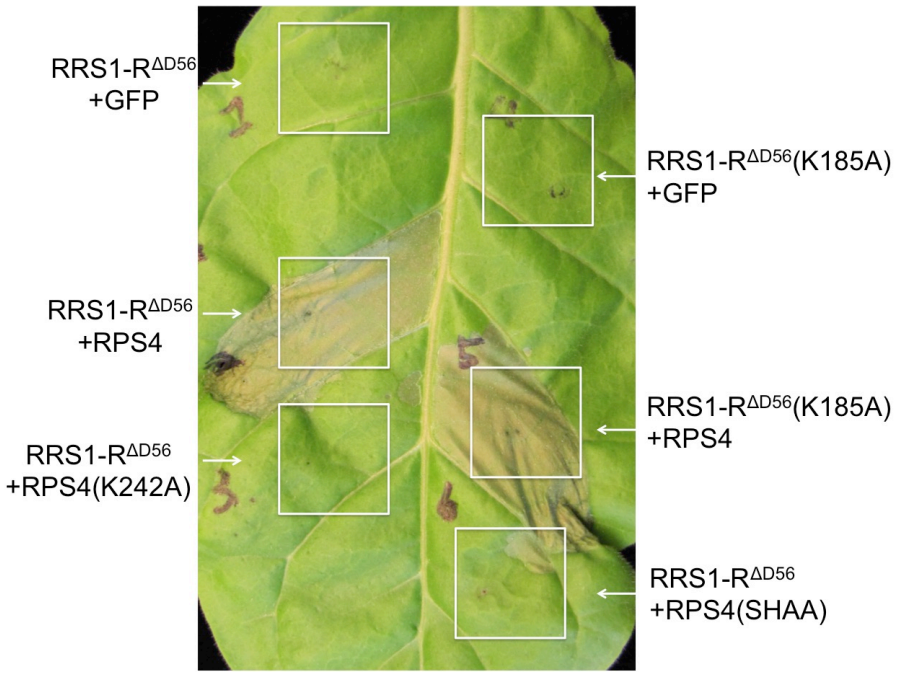

Figure 5.8: A functional P-loop motif of RPS4, but not RRS1-RΔD56, is required for the RRS1-RΔD56/RPS4-triggered cell death Transient assays in *N. tabacum* leaves using agro-infiltration show that the RPS4 P-loop mutant, RPS4(K242A), or the TIR domain mutant, RPS4(SHAA), fail to trigger HR when co-expressed with RRS1-RΔD56. Co-expression of RPS4 with the RRS1-R P-loop mutant, RRS1-R(K185A), triggers HR. Each leaf section infiltrated was indicated by a white square with corresponding infiltrated samples labelled. Pictures were taken 4 dpi. This was repeated three times with similar results.

RRS1 and RPS4 being the sensor and executor respectively, these evidence suggest that the de-repressed RRS1 must transduce the activation signal to RPS4 to enable defence signalling.

Figure 5.9 suggests that CTD of RPS4 is essential to sense the de-repression of RRS1. Partial truncation of CTD (RPS4ΔExon5, with the C-terminal 266 aa of CTD deleted), CTD swap with RPS4B [(RPS4(AAAB)], and multiple *sushi* mutations in CTD (C887Y, S914F, G952E, G997E) all impair RPS4's ability to activate cell death in the presence of RRS1-RΔD56. Note that these *sushi* mutants were generated from $RPS4^{No-0}$, and wild type $RPS4^{No-0}$ is functionally identical to RPS4col-0 **(Figure 5.2, Figure 5.9)**, sharing 99.6% identity. Interestingly, compared to C887Y and S914F, RPS4 mutations G952E and G997E have a stronger impairment of the RPS4 + RRS1-RΔD56-triggered HR **(Figure 5.9)**, suggesting that G952 and G997 might be in an important region(s) of CTD. The RPS4-dependent RRS1-RΔD56 auto-activity is proposed to be caused by the lack of WRKY domain suppression of RRS1

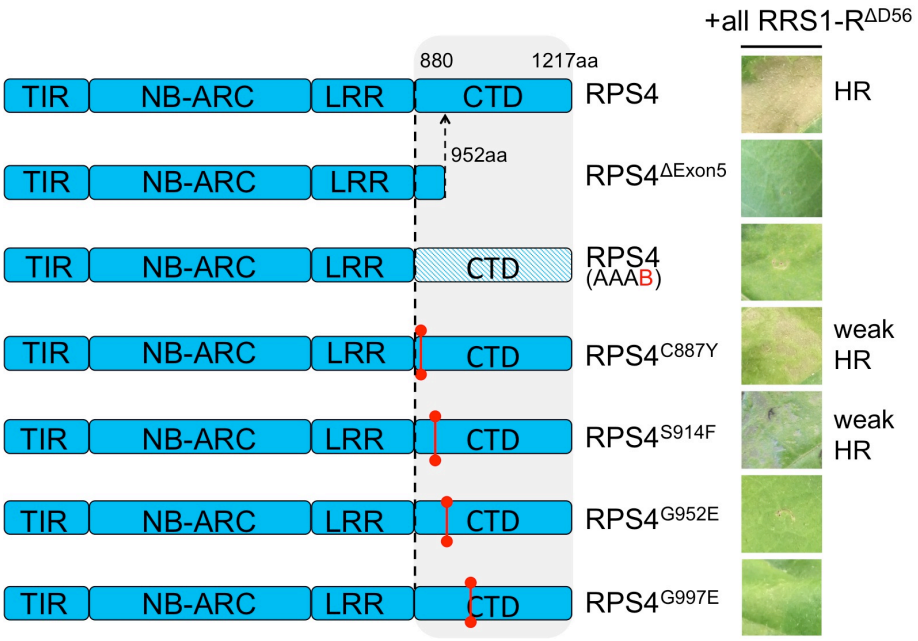

Figure 5.9: C-terminal domain (CTD) of RPS4 is essential to sense the de-repression of RRS1 to activate defence Transient assays in *N. tabacum* leaves using agro-infiltration show that changes in RPS4 CTD impair the HR activity of RPS4/RRS1-RΔD56. Each leaf section was co-infiltrated to express RRS1-RΔD56 with a CTD variant or mutant of RPS4 (indicated on the left). $RPS4^{No\text{-}0}$ (Nossen), which shares 99.6% identity with RPS4Col-0, was used as a positive control (row 1). HR activity was abolished when the CTD of RPS4Col-0 was partially truncated (RPS4ΔExon5: deletion of the C-terminal 266 aa of CTD), or replaced by CTD of RPS4B (RPS4(AAAB)). Single *sushi* mutations in the CTD of $RPS4^{No\text{-}0}$ either reduce the HR activity [RPS4(C887Y), RPS4(S914F)] or completely abolish the HR [RPS4(G952E), RPS4(G997E)]. Schematic diagram shows the domain structure of the wild type RPS4 and various RPS4 CTD variants or mutants. The amino acid numbers indicate the border of CTD and where the deletion terminates. Cell death pictures were taken 4 dpi. These experiments were repeated at least three times with similar results.

DOM4. This would then suggest that the sensing by RPS4 CTD of the de-repressed DOM4 is as an essential step for complex activation.

I carried out domain swap experiments to test whether matching DOM4 and CTD from the A and B pairs are required for effector-triggered activation **(Figure 5.10)**. As mentioned previously, DOM4 swaps between RRS1-R and RRS1B results in no response to AvrRps4 when co-expressed with their cognate pair partners **(Figure 5.10B,F)**. Likewise, a CTD swap of RPS4B(BBBA) co-expressed with RRS1B fails to respond to AvrRps4. These data suggest that compatible DOM4 and CTD pairing is important for complex activation **(Figure 5.10G)**.

However, RPS4(AAAB) + RRS1-R are functional to recognise AvrRps4 **(Figure 5.10C)**, even though RPS4(AAAB) + RRS1-RΔD56 fails to trigger auto-activity **(Figure 5.9)**. The presence of the DOM56 in RRS1-R compared to RRS1-RΔD56 must explain this discrepancy. It is possible that DOM56-R is able to compensate for the incompatible DOM4-A/CTD-B via interacting with RPS4 (will be discussed further in 5.6). On the other hand, the combinations where DOM4 and CTD are from a matching pair [RRS1-R(AAABAA) + RPS4(AAAB) and RRS1B(BBBABB) + RPS4B(BBBA)] are also non-functional for AvrRps4 recognition **(Figure 5.10D,H)**. This indicates that having matching DOM4/CTD alone is not sufficient to activate defence, implying that appropriate reconfigurations of, and interactions between, additional domains triggered by DOM4/CTD interaction are required for activation.

To investigate whether DOM4 and CTD associate *in planta*, I carried out Co-IP assays after transiently co-expressing DOM4:GFP and CTD:HF in *N. benthamiana* leaves **(Figure 5.11A)**. I found that all DOM4 and CTD combinations from A and B pair associate, but did not observe a stronger association between matching pairs (lanes 3 & 5) compared with the inappropriate partners (lanes 4 & 6) **(Figure 5.11A)**. In comparison, the negative control GUS:HF does not Co-IP with DOM4-A:GFP or DOM4-B:GFP (lanes 1 & 2) **(Figure 5.11A)**. Therefore the lack of function of certain DOM4 and CTD swaps cannot be simply explained by the lack of DOM4/CTD association affinity.

Furthermore, Co-IP data in **Figure 5.11B** showed that DOM4 association with CTD can be reduced by co-expressing DOM56-R, suggesting that DOM4/DOM56-R is able to compete with DOM4/CTD association. Conceivably, in the pre-activation immune complex, DOM56-R maintains auto-inhibition via suppressing DOM4/CTD association. For activation, we hypothesise that the de-repressed RRS1, with the DOM56's negative regulation on DOM4 alleviated, enables DOM4 to interact with RPS4 CTD **(Figure 5.14)**.

5.6 Distinct DOM4/CTD interfaces may be involved in AvrRps4- and PopP2-triggered activation

So far, my data suggest that AvrRps4 de-represses RRS1 via disrupting DOM56 and DOM4 association, which subsequently allows DOM4 to acti-

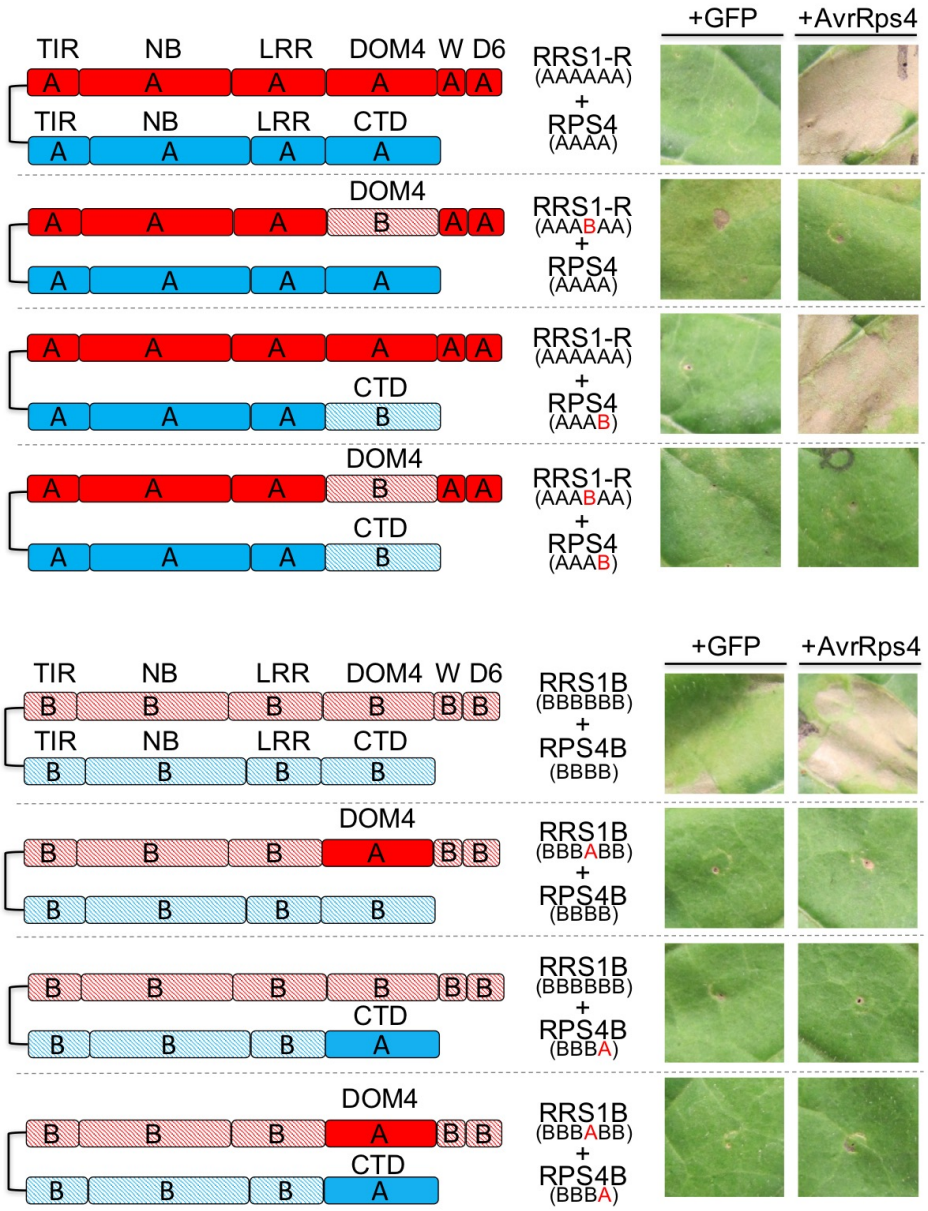

Figure 5.10: DOM4 and CTD compatibility is important for AvrRps4-triggered immune complex activation (**A-H**) Transient assays in *N. tabacum* leaves using agro-infiltration show that DOM4 swaps between RRS1-R and RRS1B (B & F) and CTD swaps between RPS4 and RPS4B (C & G) often impair the AvrRps4 responsiveness by RRS1-R/RPS4 (A) and RRS1B/RPS4B (E). (C) shows an exception that RRS1-R + RPS4(AAAB) shows HR in the presence of AvrRps4, but not GFP. (D) and (H) show that the combinations of chimeras RRS1-R(AAABAA) + RPS4(AAAB) and RRS1B(BBBABB) + RPS4B(BBBA), where DOM4 and CTD are from matching pairs do not trigger HR when co-expressed with AvrRps4. All tested R protein pair combinations are not auto-active when co-expressed with GFP. Schematic diagram shows the domain structure of all the combinations infiltrated, either the chimeric or wild type proteins. Chimeras are represented with domains from RRS1-R or RPS4 shown as 'A's, domains from RRS1B or RPS4B shown as 'B's. Cell death pictures were taken 4 dpi. These experiments were repeated at least three times with similar results.

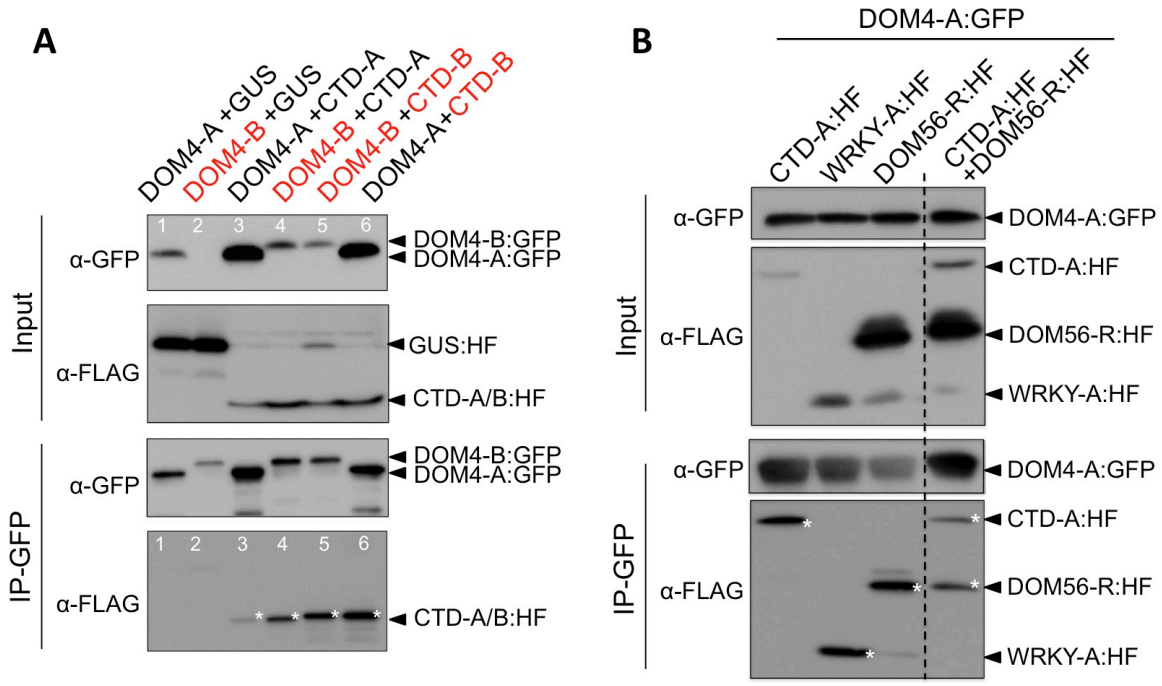

Figure 5.11: DOM4 of RRS1 or RRS1B associates with CTD of RPS4 or RPS4B (**A**) This Co-IP assay shows the associations of DOM4:GFP and CTD:HF from A or B pair proteins after *A. tumefaciens*-mediated transient co-expression in *N.benthamiana* leaves. DOM4 from RRS1B and CTD from RPS4B are labelled as DOM4-B and CTD-B and highlighted in red; DOM4 from RRS1 and CTD from RPS4 are labelled as DOM4-A and CTD-A. GUS:HF was used as a negative control. All combinations of DOM4:GFP and CTD:HF associate, but DOM4-A:GFP or DOM-B:GFP does not associate with GUS:HF. (**B**) This Co-IP assay shows that the associations of DOM4-A:GFP and CTD-A:HF is reduced in the presence of DOM56-R:HF. DOM4-A:GFP associates with each of the CTD-A:HF, WRKY:HF and DOM56-R:HF, and the association affinity of DOM4-A:GFP/CTD-A:HF and DOM4-A:GFP/DOM56-R:HF were both reduced when DOM4-A:GFP, DOM56-R:HF and CTD-A:HF were co-expressed together. Immunoblots show the presence of proteins in total extracts (input) and after IP with α-GFP beads (IP-GFP). Asterisks indicate expected protein bands.

vate RPS4 via DOM4/CTD association **(Figure 5.14)**. A remaining question is, how does PopP2 de-repress RRS1-R without displacing the DOM56-R away from DOM4? Via examining several RRS1 DOM4 and RPS4 CTD mutants (identified in the *sushi* screen [116]) for their ability to recognise effectors, I found residues that are specifically required for PopP2-triggered activation, but not for AvrRps4-triggered activation **(Figure 5.12)**.

When co-expressed with RPS4 in *N. tabacum*, RRS1-R DOM4 mutants S983F and E1070K both show reduced PopP2 responsiveness (weak HR), whilst responding strongly to AvrRps4 (HR) **(Figure 5.12A)**. In addition, RRS1-R co-expressed with RPS4(C887Y), an RPS4 CTD mutant, is functional to recognise AvrRps4, but exhibits similar partial loss of PopP2 recognition (weak HR) **(Figure 5.12B, C)**. Other RPS4 CTD mutants tested (S914F, G952E, G997E) lose the function to recognise both effectors (AvrRps4 and PopP2) in combination with RRS1-R, suggesting that these residues are necessary for activation triggered by either effector **(Figure 5.12B)**.

As the DOM4(RRS1)/CTD(RPS4) interaction is important for activation, it is intriguing that PopP2 recognition requires specific residues in DOM4 and in CTD. An attractive hypothesis is that these specific residues may represent a distinct DOM4/CTD interaction interface that only occurs during PopP2-triggered activation. In line with this hypothesis, the partial PopP2 responsiveness observed in RRS1-R + RPS4(C887Y) or RRS1-R(S983F) + RPS4 can be completely abolished when the two mutants are combined [RRS1-R(S983F) + RPS4(C887Y)] **(Figure 5.12C)**. Importantly, RRS1-R(S983F) + RPS4(C887Y) is fully functional to recognise AvrRps4, showing strong HR, proving that the loss of function is specific to PopP2 **(Figure 5.12C)**. Preliminary data showed a similar result for the combination of RRS1-R(E1070K) + RPS4(C887Y). Therefore, I infer that the specific residues in DOM4 and CTD cooperate (possibly via interaction) to enable a PopP2-triggered complex activation distinct from AvrRps4.

Furthermore, we know that the DOM6-R of RRS1-R is specifically required for PopP2 recognition, but not for AvrRps4 recognition **(Figure 5.2)** [58]. Conceivably, DOM6-R enables PopP2-triggered activation via distinct inter-domain reconfigurations compared to those triggered by AvrRps4. The observation that RRS1-RΔD56 + RPS4(AAAB) fails to trigger HR, whereas RRS1-R*slh1* + RPS4(AAAB) triggers HR, suggests that DOM56-R*slh1* of RRS1-R*slh1* enables complex activation despite the incompatible DOM4-A/CTD-B

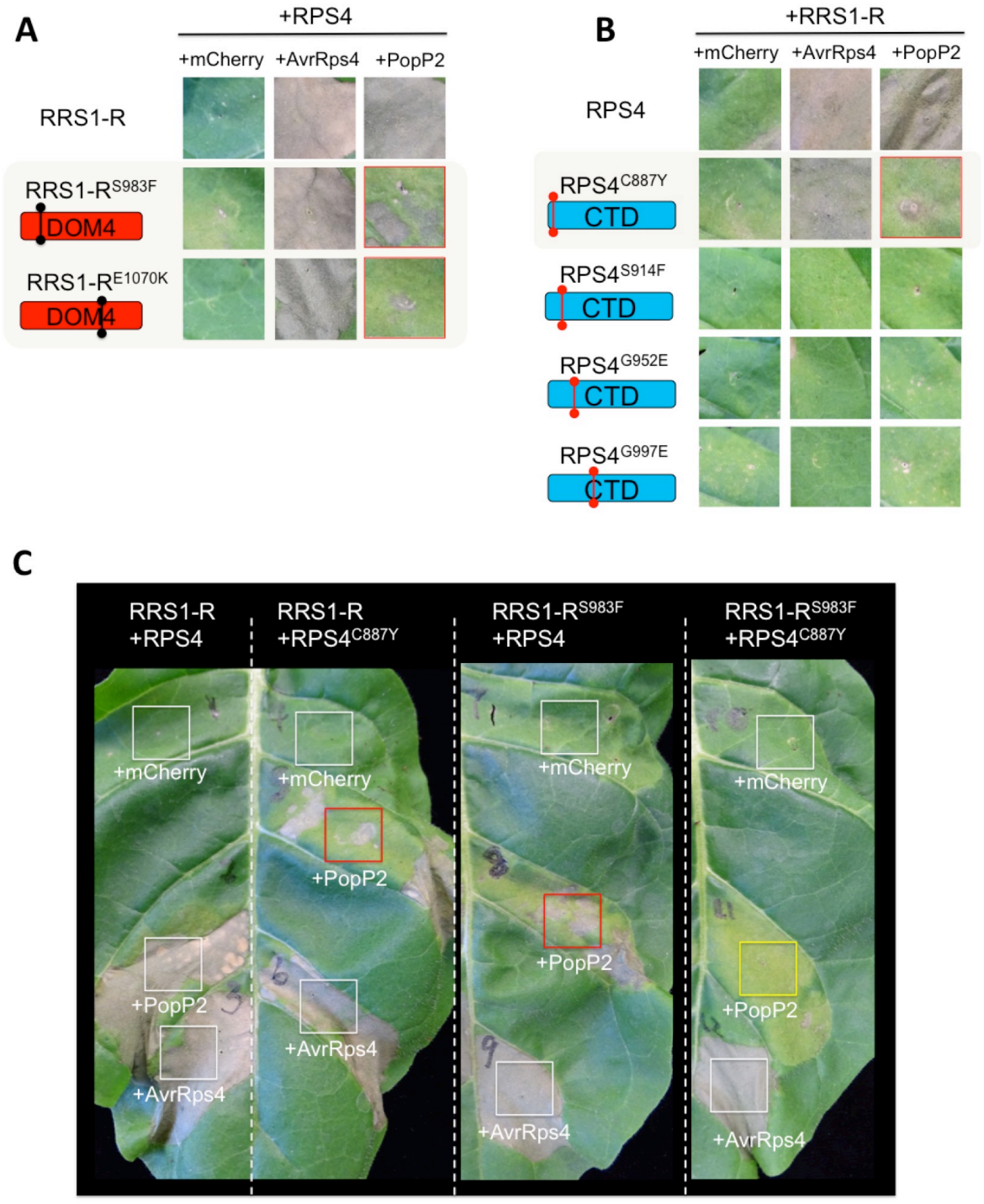

Figure 5.12: Interaction between specific residues in RRS1-R DOM4 and RPS4 CTD is likely required for PopP2 but not for AvrRps4 responsiveness (**A**) Transient assays in *N. tabacum* leaves using agro-infiltration show that when co-expressed with RPS4, RRS1-R DOM4 *sushi* mutants S983F and E1070K partially suppress PopP2-triggered HR, but do not affect AvrRps4-triggered HR. (**B**) Similar transient assays show that when co-expressed with RRS1-R, RPS4 CTD *sushi* mutant C887Y partially suppresses PopP2-triggered HR, but does not affect AvrRps4-triggered HR. Other RPS4 CTD *sushi* mutants (S914F, G952E, G997E) abolish the responsiveness of both AvrRps4 and PopP2. (**C**) Transient assays show that the partial PopP2 responsiveness in RRS1-R + RPS4(C887Y) and in RRS1-R(S983F)+ RPS4 were completely abolished in RRS1-R(S983F) + RPS4(C887Y) (yellow square). RRS1-R(S983F) + RPS4(C887Y) still shows HR in response to AvrRps4. Each leaf section infiltrated was indicated by a square with corresponding infiltrated samples labelled: R proteins on top of each panel, mCherry or effectors below each square. RRS1-R + RPS4 was used as positive controls to compare with the *sushi* mutants, mCherry was used as a negative control for effector responsiveness. Pictures were taken 4 dpi. These experiments was repeated twice with similar results.

combination **(Figure 5.13A,B)**. This is consistent with what was described in 5.5 that RRS1-R + RPS4(AAAB) is functional to recognise AvrRps4 **(Figure 5.10C)**. To further investigate whether specifically the DOM6-R in DOM56-R is enabling this activity, I compared the activities of RRS1-R(AAAABB) and RRS1-R(AAAABA) in combination with RPS4(AAAB) using tobacco transient assays **(Figure 5.13C,D)**. Although both chimeras are autoimmune when co-expressed with RPS4(AAAA) **(Figure 5.3)**, only RRS1-R(AAAABA) triggers HR with RPS4(AAAB) **(Figure 5.13C,D)**, suggesting that DOM6-R might be specifically required.

More evidence of the contribution of DOM6-R (especially C83) to activation is shown by the auto-activity of RRS1B(BBBBB^{+}A) + RPS4B **(Figure 5.13F)**. This chimera has replaced the C115 of RRS1B with C83 from RRS1-R. In contrast, RRS1BΔC115 + RPS4B is not autoimmune and can recognise AvrRps4 **(Figure 5.13E)**. Similarly, RRS1-R(AAAAA^{+}B) + RPS4 is autoactive, but RRS1-RΔC83 + RPS4 is not **(Figure 5.13G,H)**, showing that DOM6-B also promotes activation. As postulated before, these auto-activities may be caused by the addition of incompatible C-terminal amino acids, which could interfere with the WRKY/DOM4 association essential for auto-inhibition. In hindsight, taking into account that DOM6-R is able to compensate for DOM4/CTD mismatching in **Figure 5.13B,D**, DOM6-R is likely to promote activation via assisting or modulating DOM4/CTD association.

Considering all these data, I propose a model in which PopP2 acetylation of the WRKY domain might displace DOM6-R, which allows DOM6-R to assist DOM4/CTD association **(Figure 5.14)**. Alternatively, the affinity of DOM6-R for DOM5 (WRKY) may increase when the WRKY domain is acetylated. If DOM6-R can rebind DOM4 after PopP2 elicitation, this might explain how PopP2 is able to de-repress RRS1 without completely dissociating DOM56 from DOM4 **(Figure 5.14)**. Because PopP2 is likely to require a specific DOM4/CTD interface to activate defence, the hypothesised DOM6-R/DOM4 association could help to create this new interface. The new interface is not involved in AvrRps4-triggered activation, and thus distinguishes the activation models of the two effectors **(Figure 5.14)**.

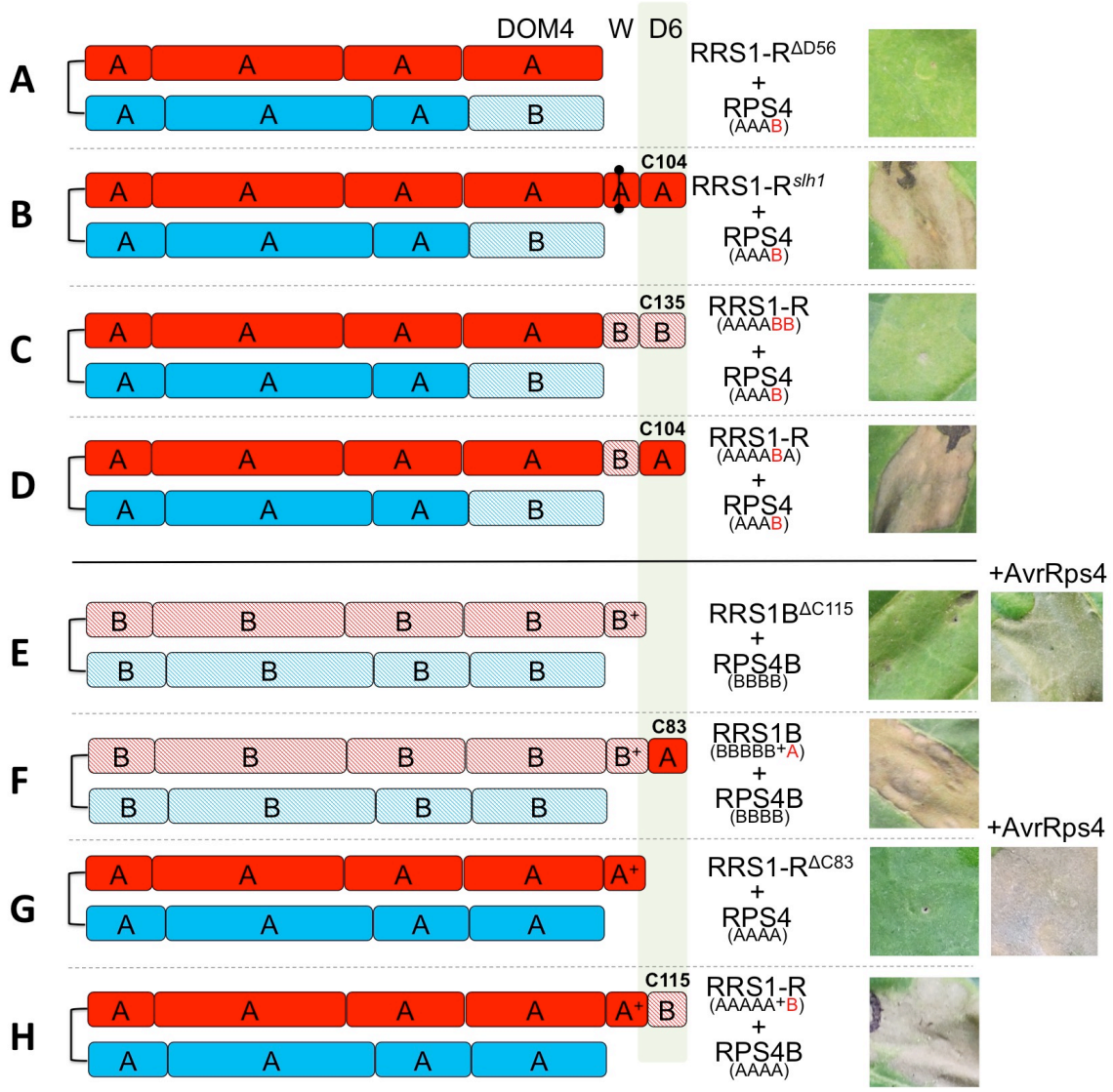

Figure 5.13: DOM6 of RRS1-R contributes to immune complex activation. (**A-D**) Transient assays in *N. tabacum* leaves using agro-infiltration show that the presence of DOM6 in RRS1-R enables functionality of the incompatible DOM4-A/CTD-B combination. This incompatibility was shown by the lack of HR in RPS4(AAAB) + RRS1-RΔD56 (A) in contrast to the auto-active RPS4(AAAA) + RRS1-RΔD56 (Figure 5.2). Each leaf section was co-infiltrated to express RPS4(AAAB) with an RRS1-R mutant or chimera (indicated on the left). The numbers of amino acids in DOM6 were labelled. (**E-H**) Transient assays show that when co-expressed with cognate pair partners, RRS1-R or RRS1B with amino acids in DOM6 deleted [RRS1BΔC115, C-terminal 115aa deletion (E);RRS1-RΔC83, C-terminal 83 aa deletion (G)], do not show auto-activity, and retain AvrRps4 responsiveness. In contrast, adding back the C-terminal amino acids from the other pair [RRS1B(BBBBB$^+$A), RRS1BΔC115 adds C83 from RRS1-R (F); RRS1-R(AAAAA$^+$B), RRS1-RΔC83 adds C115 from RRS1B (H)] causes auto-activity when co-expressed with respective partners. Schematic diagram shows the domain structure of all the combinations infiltrated, either the chimeric or truncated proteins. WRKY domain and DOM6 are abbreviated as WRKY and D6 respectively. Pictures were taken 4 dpi. These experiments were repeated at least three times with similar results.

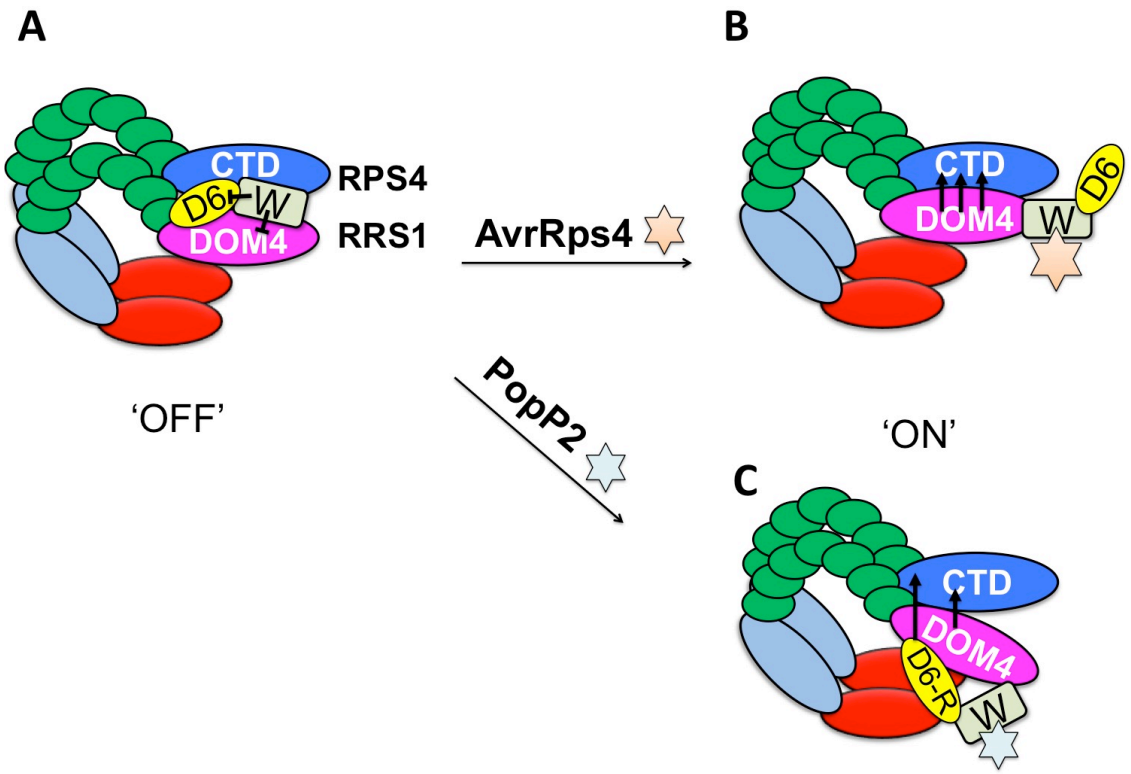

Figure 5.14: Working model of RRS1/RPS4 immune complex activation triggered by effectors AvrRps4 and PopP2. This model summarises the probable distinct intra- and intra-molecular reconfigurations triggered by AvrRps4 and PopP2 to convert an inactive ('OFF') RRS1/RPS4 immune complex into an active one ('ON'). (**A**)In the absence of the effectors, the immune complex stays 'OFF' to avoid ectopic defence activation. This auto-inhibition requires the RRS1 WRKY domain (WRKY) to negatively regulate DOM4, which prevents RRS1 DOM4 from activating RPS4. The WRKY domain may also negatively regulate DOM6 (D6), an allele of which can promote activation. (**B**) AvrRps4 de-represses RRS1 via binding to the WRKY domain and disrupting DOM4 and WRKY & DOM6 (D6) association. The de-repressed RRS1 enables DOM4 to interact with RPS4 CTD, and thereby activates RPS4. (**C**) PopP2 de-represses RRS1-R without completely dissociating DOM4 from WRKY & DOM6-R (D6-R). The hypothesis is that PopP2 de-represses D6-R via acetylating the WRKY domain, which allows D6-R to rebind DOM4. This subsequently facilitates RRS1-R DOM4 to associate with RPS4 CTD at a new interface, and thereby activates RPS4.

5.7 Domain swaps between the A and B pair proteins reveal additional domains important to assemble a functional immune complex

5.7.1 *NB-ARC domain of RPS4 negatively regulates its signalling activity*

In Chapter 3, I have reported that over-expression of RPS4 TIR domain, but not RPS4B TIR domain, triggers effector-independent cell death in tobacco. Here I confirm that full-length RPS4, but not RPS4B, is able to activate constitutive HR when over-expressed in tobacco **(Figure 5.15A)**. This RPS4-triggered cell death is fully suppressed by RRS1 co-expression **(Figure 5.15A)**. In addition, $RPS4^{TIR}$-triggered HR is also suppressed by co-expression of $RRS1^{TIR}$ **(Figure 3.5A)**. These data suggest that in a pre-activation immune complex, RRS1 negatively regulates RPS4's signalling activity to avoid ectopic defence activation in the absence of effectors.

As the TIR domain of RPS4 is crucial for its signalling activity, it is intriguing that RPS4(BAAA), with the RPS4 TIR domain replaced by RPS4B TIR, fails to activate constitutive cell death **(Figure 5.15A)**. The protein accumulation of RPS4(BAAA) was shown in Figure 3.6C. Strikingly, when the NB-ARC domain of RPS4 is swapped with RPS4B, RPS4(ABAA) becomes strongly auto-active (often observed to be stronger than RPS4-triggered HR), and this HR cannot be completely suppressed by RRS1 **(Figure 5.15A)**. RPS4(ABAA) resembles the activity of RPS4 TIR domain, as the $RPS4^{TIR}$ also triggers strong HR which cannot be suppressed by RRS1 **(Figure 5.15A)**. These results imply that the NB-ARC domain of RPS4 negatively regulates its TIR domain, contributing to RPS4 auto-inhibition. The incompatible RPS4B NB-ARC domain of RPS4(ABAA) fails to suppress $RPS4^{TIR}$, which could allow $RPS4^{TIR}$ to oligomerise and activate cell death more easily. This provides an explanation why the phenotype of RPS4(ABAA) mimics the removal of NB-ARC-LRR-CTD from RPS4. In addition, I show that the LRR and the CTD swap with RPS4B, forming RPS4(AABA) and RPS4(AAAB) respectively, both impair RPS4's ability to trigger constitutive cell death, indicating that these domains might also contribute to RPS4 signalling **(Figure 5.15A)**. The capacity for accumulation of inactive chimeric proteins [RPS4(AABA), RPS4(AAAB), RPS4(BBBA)] is shown in **Figure 5.15B**, indicating any lack of signalling is not due to instability.

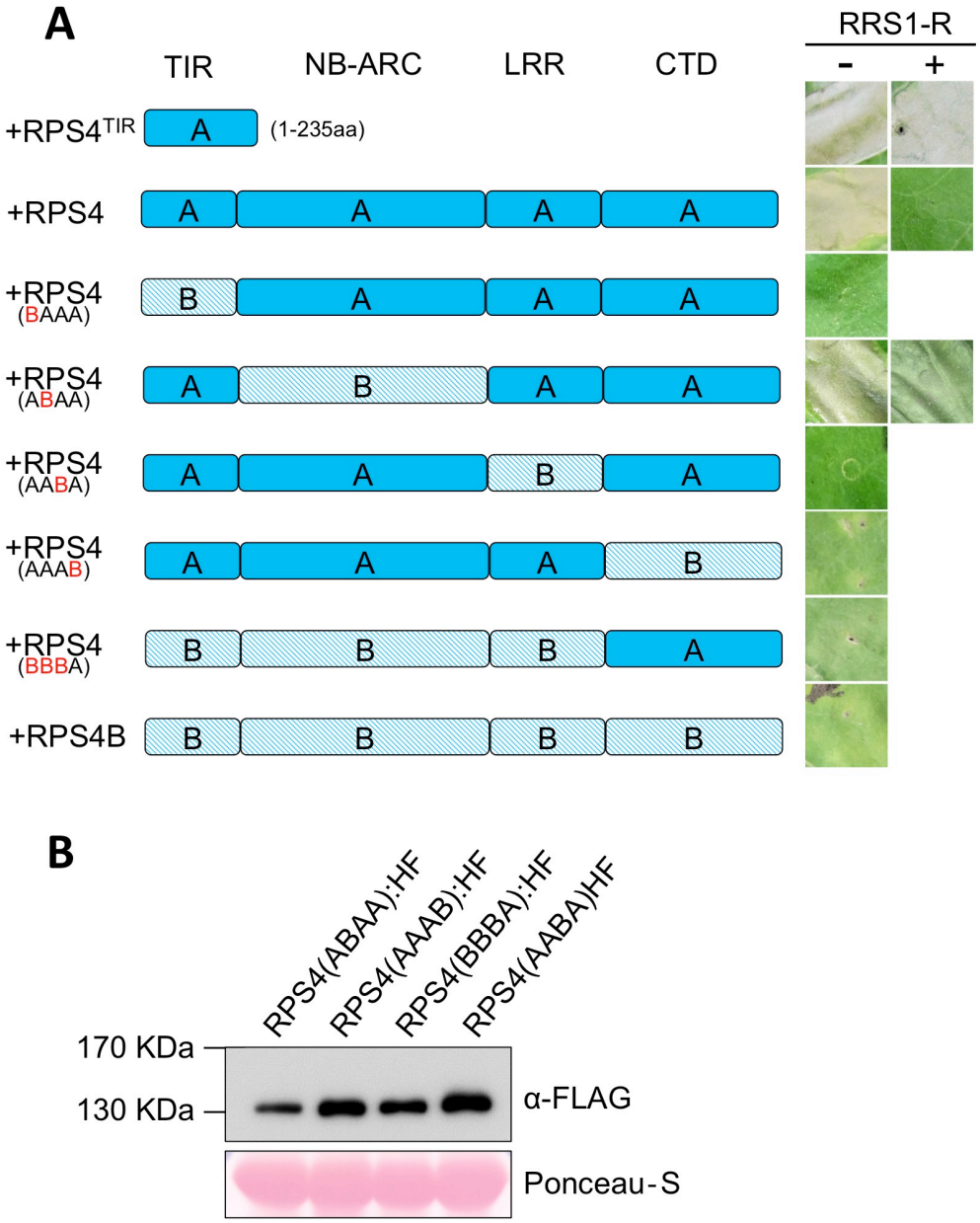

Figure 5.15: Replacement of RPS4 NB-ARC domain with that of RPS4B leads to strong effector-independent HR when over-expressed in *N. tabacum* (**A**) The transient assays using agro-infiltration assess the ability of RPS4 chimeras (RPS4(BAAA), RPS4(ABAA), RPS4(AABA), RPS4B(BBBA)) and RPS4B to trigger effector-independent HR when over-expressed in *N. tabacum* leaves. TIR domain (1-235aa) and full-length RPS4 were used as positive controls. Over-expression of $RPS4^{TIR}$, RPS4 and RPS4(ABAA) triggers HR. Suppression of these HR by co-expressing RRS1-R at 1:1 ratio in the infiltration inoculum was also assessed. Only RPS4-triggered HR was completely suppressed by RRS1-R, RPS4(ABAA)-triggered HR was partially suppressed, and $RPS4^{TIR}$-triggered HR was not affected. Schematic diagram shows the domain structure of wild type and chimeric RPS4 proteins. Cell death pictures were taken 5 dpi. Note that these RPS4-triggered effector-independent HR are sometimes affected by the high humidity in the growth chamber (where most other transient HR assays were conducted), and therefore were assessed in the lab. These experiments were repeated three times with similar results. (**B**) Immunoblot detection of the expression of RPS4 chimeras at 2 dpi in *N. benthamiana* leaves. All chimeras used here were tagged with HF. Ponceau-S staining of the large subunit of RuBisCo served as loading control.

In summary, these data show that in the pre-activation complex, RPS4 is kept in a signalling inactive state by both inter- and intra-molecular interactions. More specifically, RRS1 negatively regulates RPS4 in the absence of effectors, possibly in part via forming a more stable TIR-TIR hetero-dimer to suppress $RPS4^{TIR}$ activity. The NB-ARC domain of RPS4 also suppresses RPS4 activation, likely via interacting with its TIR domain . Furthermore, while the other domains of RPS4 (LRR and CTD) may not directly suppress $RPS4^{TIR}$, they are required for the signalling activity of full-length RPS4. I infer that upon sensing the effector-activated RRS1, changes in LRR- and CTD-mediated domain-domain interactions enable RPS4 de-repression.

5.7.2 *NB-ARC domain of RRS1 is required for its dominant negative suppression of auto-active RRS1 variants*

RRS1 has been reported to suppress the auto-activity of RRS1-R*slh1* + RPS4 in tobacco transient assays [116]. Here I confirm that co-expression of either RRS1-R or RRS1-S suppresses the HR triggered by RRS1-R*slh1* + RPS4 in tobacco **(Figure 5.16)**. Similarly, RRS1-RΔD56 + RPS4 auto-activity is partially attenuated by RRS1-R co-expression after 4 dpi **(Figure 5.6 A)**. Note that after 3 dpi, there was still complete suppression of HR . In contrast, the paralogous RRS1B, which shares 68.9% identity with RRS1-R, shows no such suppression **(Figure 5.16)**.

To investigate which domain(s) of RRS1 (but not RRS1B) enables its dominant negative suppression, I tested all the non-autoactive RRS1-R chimeric proteins with individual domains swapped with RRS1B, for their suppression of RRS1-R*slh1* + RPS4 in tobacco. Interestingly, only the NB-ARC domain swap [i.e. RRS1-R(ABAAAA)] completely loses the suppression activity, while all the other single domain swaps tested [i.e. RRS1-R(BAAAAA), RRS1-R(AABAAA), RRS1-R(AAABAA)] are able to show suppression, although not always as strong as RRS1-R **(Figure 5.16)**. Consistent with RRS1-R(ABAAAA), the importance of the NB-ARC domain is also reflected by the RRS1 P-loop mutant, RRS1-R(K185A), which fails to suppress the HR triggered by RRS1-R*slh1* + RPS4 [116], and HR triggered by RRS1-RΔD56 + RPS4 (data not shown). Nevertheless, RRS1B NB-ARC domain contains a functional P-loop motif, suggesting that to explain the lack of suppression activity of RRS1-R(ABAAAA), additional properties of the RRS1 NB-ARC domain (which are not present in RRS1B NB-ARC domain) must be involved.

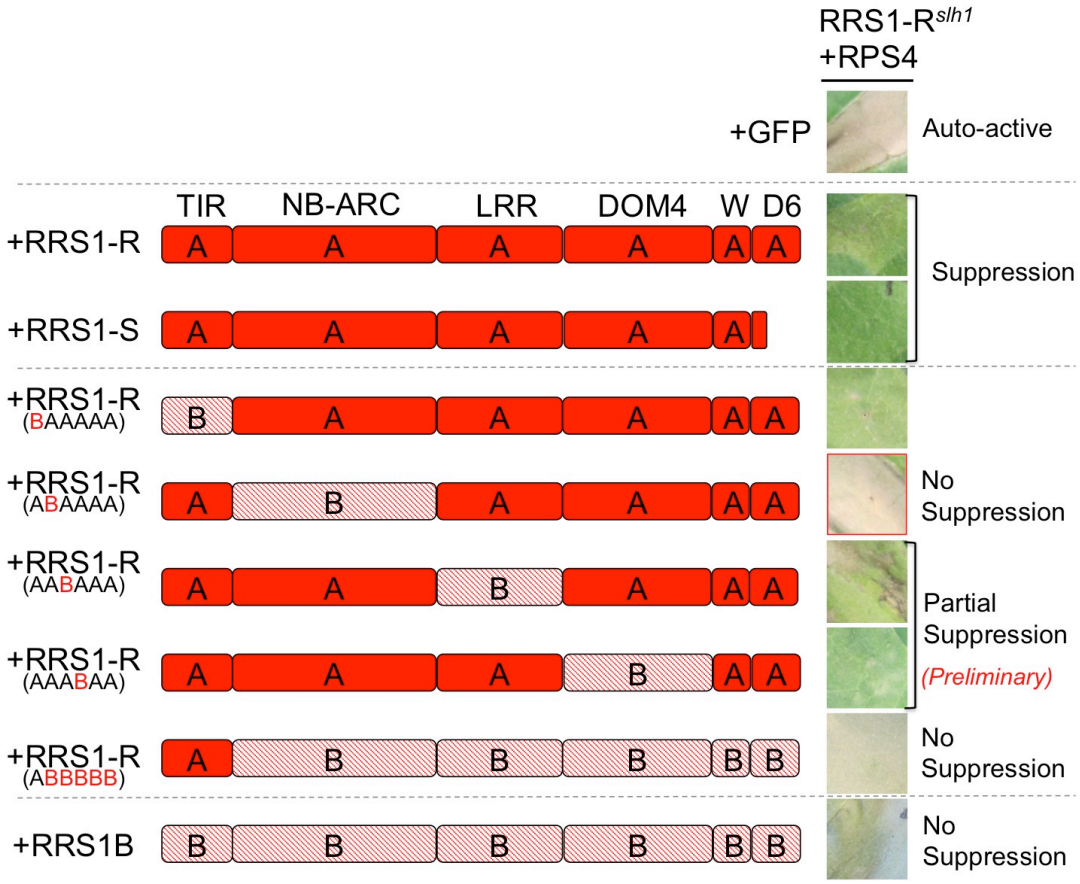

Figure 5.16: The NB-ARC domain of RRS1-R strongly contributes to its dominant negative suppression on RRS1-R*slh1* auto-activity. The transient assays using agro-infiltration assess the dominant negative suppression of RRS1-R*slh1*/RPS4 by non-autoactive RRS1-R chimeras [RRS1-R(BAAAAA), RRS1-R(ABAAAA), RRS1-R(AABAAA), RRS1-R(AAABAA), RRS1B(ABBBBB)] and RRS1B when co-expressed in *N. tabacum* leaves. The ratio of agrobacteria strains carrying RRS1-R*slh1*, RPS4 and one RRS1 chimera (or wild type RRS1) was 1:1:1 in the infiltration inoculum. As controls, the auto-activity triggered by RRS1-R*slh1*/RPS4 is not suppressed by co-expression with GFP (HR), but completely suppressed by RRS1-R and RRS1-S (no HR). Co-expression of RRS1B or RRS1B(ABBBBB) along with RRS1-R(ABAAAA) do not show suppression, showing strong HR resembling that of RRS1-R*slh1* + RPS4 + GFP. RRS1-R(AAABAA) and RRS1-R(AABAAA) exhibit partial suppression. Schematic diagram shows the domain structure of wild type and chimeric RRS1 and RRS1B proteins. Cell death pictures were taken 4 dpi. Except for RRS1-R(AAABAA) (labelled as preliminary), these experiments were repeated three times with similar results.

One explanation could be that RPS4 preferably associates with an inactive RRS1 over an auto-active variant, thus allowing the inactive RRS1/RPS4 complexes to out-compete the auto-active ones. Conceivably, this RPS4 binding preference may be determined by the properties of the RRS1 NB-ARC domain (e.g. accessibility, affinity with RPS4). It is plausible that the NB-ARC domain of RRS1B has a weaker affinity with RPS4, which prevents RRS1-R(ABAAAA) to form stable complexes with RPS4, and thus impairs its suppression activity. However, biochemical evidence is needed to support this hypothesis, and whether this hypothesis explains the genetic recessiveness of *slh1* and RRS1-R (for PopP2 recognition) remains to be seen.

5.8 Discussion

5.8.1 *Chapter summary*

How plant immune receptor complexes operate to convert effector perception into defence activation is poorly understood. In this chapter, I have investigated the mechanisms of how effectors AvrRps4 and PopP2 activate the RPS4/RRS1 receptor complex via targeting the integrated WRKY domain of RRS1. Data so far suggest possible distinct intra- and inter-domain reconfigurations involved in AvrRps4- and PopP2-triggered complex activation. Deletion of RRS1 WRKY domain activates effector-independent cell death in the presence of RPS4, indicating that the RRS1/RPS4 complex is likely activated by effector-dependent alleviation of the WRKY domain's negative regulation. Co-IP and BiFC experiments then showed that AvrRps4 disrupts WRKY domain association with the DOM4 of RRS1. Various data suggest that DOM4/CTD interactions are crucial for the immune complex activation: Domain swaps, truncations, and point mutations in DOM4 and/or CTD often result in the loss of effector responsiveness. Therefore, I proposed that the AvrRps4-derepressed DOM4 of RRS1 activates RPS4 via interacting with the CTD of RPS4.

However, for PopP2-triggered activation, disassociation of DOM4/DOM56-R was not observed, indicating an alternative activation pathway. Consistent with this idea, I found specific residues in RRS1-R DOM4 and in RPS4 CTD that when mutated only suppress PopP2 recognition but not AvrRps4. Additionally, DOM6-R of RRS1-R is also specifically required for PopP2 recognition. Altogether, these data support a model where distinct DOM4/CTD

interfaces are involved in AvrRps4- and PopP2-triggered activation, and the latter requires the DOM6-R **(Figure 5.14)**.

5.8.2 *RRS1 DOM4 may possess distinct interfaces mediating auto-inhibition and activation.*

I have reported that DOM4 of RRS1 can associate with both WRKY (RRS1) and CTD (RPS4) using Co-IP **(Figure 5.11B)**. Conceivably, distinct DOM4 surfaces might be involved during activation (mediated by DOM4/CTD association) and auto-inhibition (mediated by DOM4/WRKY association). I have shown that there are DOM4 residues that are important for activation, probably via interacting with CTD. Firstly, RRS1-R(AAABAA) + RPS4(AAAA) is non-functional, implying that the amino acid changes in DOM4-B (69% sequence identify to DOM4-A) must diminish DOM4-B's ability to activate RPS4 CTD-A. Secondly, DOM4 mutations S983F and E1070K in RRS1-R do not cause auto-activity when co-expressed with RPS4, but reduce PopP2 responsiveness. This suggests that these DOM4 residues (S983 and E1070) are not required for DOM4/WRKY interaction, but instead are important for activating RPS4 upon PopP2 perception.

RRS1 DOM4/WRKY association is important for complex auto-inhibition, because disruption of this association either by removing the WRKY, or by replacing RRS1-R WRKY domain with other domains (e.g. LexA, WRKY-B) lead to effector-independent defence activation. It is likely that mutations in DOM4 at the DOM4/WRKY interface could also lead to the loss of WRKY interaction, and thus de-repress RRS1. However, I have yet to identify the surface of DOM4 that is essential for this DOM4/WRKY interaction. Interestingly, Narusaka et al (2016) recently reported that mutations of a leucine zipper (LZ) motif in the DOM4 of RRS1-R triggers RPS4-dependent autoimmunity in Arabidopsis and in *N. benthamiana* [253]. I speculate that this auto-immunity could be a result of DOM4/WRKY disruption, that is the LZ motif [6] of RRS1 DOM4 may be required for its interaction with the WRKY domain. Moreover, with some variations compared that of RRS1, an LZ motif [7] is also found in RRS1B DOM4 [253], which perhaps associates with DOM56-B. However, whether DOM4-B Co-IPs with DOM56-B has yet to be tested. The lack of DOM4-A/DOM56-B association **(Figure 5.5)** implies the co-evolution of DOM4 and DOM56 , resulting in specific interactions within each pair. It would be interesting to test whether DOM4-B and DOM56-A

[6] **The LZ motif in RRS1-R DOM4:** LRVSYDDLQEMD-KVLFLYIASL

[7] **The LZ motif in RRS1B DOM4:** LRVRYAGLQEIYKALFLY-IAGL

are also unable to interact, and whether the polymorphisms in the LZ motif in DOM4 are important for compatible interactions.

Nevertheless, as preliminary data suggest that RRS1BΔD56 + RPS4B does not trigger cell death in tobacco, whether RRS1B is de-repressed in the same way as RRS1 is still unclear. Alternatively, this suggests that the removal of negative regulation in the RRS1B/RPS4B complex might not be sufficient for activation.

5.8.3 *Co-evolution of RRS1 DOM4 and RPS4 CTD*

DOM4 or CTD swaps between the A and B pair proteins often result in inactive immune complexes that cannot respond to effectors **(Figure 5.10)**, implying that the two domains from respective pairs co-evolved to enable the pair partners to cooperate. The observation that DOM4 and CTD from compatible and incompatible pairs all Co-IP **(Figure 5.11)** suggests that functional DOM4/CTD interactions may require the interaction of specific residues at their interfaces, and affinity *per se* is insufficient for functionality. I hypothesise that these residues may have co-evolved independently in two separate pairs, and over time the domain-domain cross-talk became specific within each pair, resulting in domains that are not interchangeable.

By comparing the analyses of domain swaps and the *sushi* mutants, I have found some indications of where these residues required for functional DOM4/CTD interactions might be. Using tobacco transient assays, I showed that RRS1 DOM4 residues (S983 and E1070K) may interact with residue C887 of RPS4 CTD to allow specifically the PopP2-triggered activation, as *sushi* mutations in both DOM4 (S983F or E1070K) and CTD (C887Y) completely block PopP2 responsiveness, but do not affect AvrRps4. Further experiments such as testing HR responses to Pf0-1 (AvrRps4) and Pf0-1 (PopP2) in the Arabidopsis stable transgenic line, Ws-2 *rrs1/rrs1b* expressing RRS1-R(S983F or E1070K) and RPS4 (C887Y), would confirm the effect of these mutations on PopP2 and AvrRps4 responsiveness.

Interestingly, S983 and E1070 of RRS1-R DOM4 are conserved in RRS1B DOM4 (S945 and E1029), whereas the residue C887 of RPS4 CTD is changed to Y863 at the equivalent position in RPS4B CTD, mimicking the *sushi* mutation RPS4(C887Y). In addition, RPS4B also shows polymorphisms at the positions where two other *sushi* mutations of RPS4 CTD (S914F and G952E), which are C890 and E928 in RPS4B respectively. C887Y, S914F,

G952E of RPS4 suppress (C887Y and S914F partially, G952E completely) the HR activity of RPS4 + RRS1ΔD56 **(Figure 5.9)**, suggesting that they are important for DOM4-A/CTD-A compatibility. Conceivably, the equivalent amino acids changed in RPS4B CTD could be responsible for the non-functionality of the DOM4-A/CTD-B interaction in RPS4(AAAB) + RRS1ΔD56. To test this, I could introduce mutations in CTD-B of RPS4(AAAB) to match CTD-A, namely Y863C, C890S and E928G, to see if this recovers RPS4(AAAB) + RRS1ΔD56-triggered HR.

Intriguingly, the homologous sequences of DOM4 and CTD are found in other paired TNLs such as CHS3/CSA1 [130] and several other TNLs that are arranged in a head-to-head orientation, such as *At4g12010/At4g12020*, *At4g19530/At4g19520*, *At3g51570/At3g51560* and *At4g36150/At4g36140*. This pattern implies a conserved coupling of DOM4-like and CTD-like domains in paired NLRs, which might serve as a 'bridge' for one partner (e.g. the sensor) to transmit an activation signal to the other (e.g. the executor). Therefore, DOM4/CTD interactions may be the key for the cooperative function of many TNL pairs.

5.8.4 *DOM56 of RRS1-R is necessary for auto-inhibition, but can also promote activation via DOM6-R*

Auto-activity caused by the truncation of DOM56-R implies that it negatively regulates the immune complex, and is not necessary for downstream activation of cell death. However other evidence suggests that DOM56-R, in particular DOM6-R, plays a role during activation **(Figure 5.13)**. The difference between RRS1-R*slh1* and RRS1-RΔD56 in their ability to activate RPS4(AAAB), in which the former can and the latter cannot, suggests that DOM56-R*slh1* promotes activation. However, it is puzzling given DOM56-R*slh1* can still weakly Co-IP with DOM4 **(Figure 5.5)**, that this DOM56-R*slh1*/DOM4 association should partially inhibit activation. One explanation could be that the WRKY domain suppresses DOM6-R in pre-activation state, and the de-repressed DOM6-R of DOM56-R*slh1* is able to engage in interactions that compensate the lack of functionality of the DOM4-A/CTD-B combination. Consistent with this hypothesis, I showed that when co-expressed with RPS4(AAAB), DOM6-R of the chimera RRS1-R(AAAABA), but not DOM6-B of RRS1-R(AAAABB), enables activation. Conceivably, the DOM6-R is de-repressed in RRS1-R(AAAABA), as WRKY-B may fail to suppress DOM6-R.

Biochemical evidence of possible WRKY/DOM6 associations in compatible combinations, and lack of association in incompatible combinations, is needed to support this hypothesis. Alternatively, it is possible that DOM6-R but not DOM6-B is able to promote this activity. A simple test for cell death activity of RRS1-R(AAAAAB) + RPS4(AAAB) in tobacco would clarify that.

Furthermore, I have shown evidence that suggests de-repressed DOM6 can activate the immune complex when it is incompatible with the WRKY domain. This is true for both DOM6-R and DOM6-B, because RRS1-R(AAAAAB) and RRS1B(BBBBBA) are both auto-active when co-expressed with RPS4 and RPS4B respectively. This auto-activity cannot be explained by the lack of negative regulation, as deletions of the C-terminal amino acids (e.g. RRS1-RΔD6, RRS1-RΔC83 and RRS1BΔC115) do not lead to auto-activity. Therefore, I infer that DOM6 when not suppressed by the WRKY domain can contribute to activation.

Intriguingly, while RRS1-R:HF and RRS1-S:HF (HF, 56 aa) are non-autoactive and fully functional, RRS1-R or RRS1-S when fused to a C-terminal tag with higher molecular weight, such as GFP (239 aa) and nCerulean (172 aa), triggers auto-activity in the presence of RPS4 in tobacco (data not shown). Conceivably, the underlying mechanisms of tag-dependent auto-activity may resemble those of the DOM6-swapped chimeric proteins of RRS1-R and RRS1B. This similarity is best exemplified by the auto-activities of RRS1-S:nCerulean and RRS1-R(AAAAA^{+}B):HF [RRS1-R(AAAAA^{+}) is identical to RRS1-S}, which differ only in their additional C-terminal amino acids sequences: nCerulean (172 aa) and DOM6-B:HF (171 aa). This implies that the additional C-terminal amino acids of RRS1 may trigger activation in a sequence independent manner, such as by imposing steric hindrance to WRKY/DOM4 association. We could test this via fusing different lengths of neutral sequences (such as multiple 'NAAIRS') to the C-terminal end of RRS1, which may reveal a minimal length required for auto-activity [290]. Ultimately, obtaining and then comparing functional and auto-active DOM456 crystal structures, e.g. DOM456-R(AAA) and DOM456(AAB), would help us to elucidate the role of DOM56 and DOM6 in inhibition and activation.

5.8.5 *PopP2 de-represses RRS1-R via DOM6-R-enabled inter-domain reconfigurations*

Truncation of DOM6-R from RRS1-R reveals the difference between the AvrRps4- and PopP2-triggered immune complex activation **(Figure 5.2)**. DOM6-R is specifically required for PopP2 but not AvrRps4 responsiveness by RRS1-R/RPS4. Acetylation of the WRKY domain by PopP2 somehow de-represses DOM6-R/WRKY. Besides DOM6-R, PopP2-triggered activation also involves specific residues in RRS1-R DOM4 and RPS4 CTD **(Figure 5.12)**. Combining the role of the three domains involved, I envisage that the PopP2-derepressed DOM6-R may associate with DOM4, creating a new interface for DOM4 to interact with RPS4 CTD **(Figure 5.14)**. Alternatively, DOM6-R only interacts with acetylated WRKYGQK, modulating DOM56 interactions with DOM4.

These models are consistent with DOM6-R's ability to enable the DOM4-A/CTD-B functionality. The proposed DOM4/DOM6-R association after PopP2 elicitation is consistent with the BiFC signal observed for cCFP:DOM456-R:nCerulean treated with PopP2 **(Figure 5.7)**. Furthermore, this may help to explain the lack of PopP2 responsiveness in RRS1-S/RPS4: After PopP2 acetylation of the WRKY domain of RRS1-S, the de-repressed DOM6-S (21 aa) may be too short to rebind DOM4. Future experiments should test for BiFC signals of cCFP:DOM456-S:nCerulean with and without PopP2.

5.8.6 *Interactions that assist the transition to an active RPS4*

In RPS4, the NB-ARC domain was found to have the most abundant number of missense *sushi* mutations, totalling 18 out of 38, indicating its importance for RPS4 function [116]. We already know that the P-loop motif of RPS4 is required for both effector-dependent and -independent activation of defence [115, 254]. In this chapter, I have shown that the RPS4 NB-ARC domain may also play a negative regulatory role, as the NB-ARC exchange results in an enhanced cell death activity of RPS4(ABAA) compared to RPS4. Since the RPS4(ABAA) phenotype is similar to $RPS4^{TIR}$-triggered cell death in that it could not be suppressed by RRS1, I speculate that this domain swap de-represses the RPS4 TIR domain to allow signalling activity. Thus sequence polymorphisms in RPS4B NB-ARC may impair proper TIR/NB-ARC association, leading to de-repression and autoactivity of RPS4(ABAA).

For plant NLR proteins, the NB-ARC domain has been reported to regulate the signalling activity of the N-terminal domain. For example, the NB-ARC of flax L6 (TNL) negatively regulates the L6 TIR domain-triggered HR, as successive addition of sub-domains (i.e. NB, ARC1, ARC2) cumulatively reduces the HR phenotype [197]. In addition, allele-specific negative interactions between the TIR and NB-ARC domains also prevent effector-triggered activation of L7 [168]. Similarly, for maize Rp1 (CNL), the CC domain-triggered HR is suppressed by its NB-ARC domain via CC/NB-ARC interaction [165].

In contrast to the negative role of NB-ARC observed for L6 and Rp1, the NB-ARC1 of RPP1 (TNL) seems to play a positive role [201]. It has been shown that induced RPP1 TIR domain oligomerisation promotes effector-independent cell death, as tagging non-autoactive RPP1^{WsB} TIR with a self-associating GFP leads to HR [94]. Schreiber et al. [201] recently reported that the RPP1 TIR-oligomerisation is likely facilitated by its NB-ARC1 domain, which can self-associate. Interestingly, it was also revealed that the ARC2 of RPP1 negatively regulates that activity, as TIR-NB-ARC1-ARC2 cannot self-associate and is not auto-active . Thus, these recent results demonstrate the complexity of the regulatory relationship between an NLR's N-terminal domain and NB-ARC domain, and that my speculation of the negative regulatory activity of the RPS4 NB-ARC domain may be premature without performing truncations and finer domain swaps. In particular, domain swaps between the RPS4 and RPS4B NB, ARC1 and ARC2 sub-domains would be valuable experiments, and may reveal whether the NB-ARC of RPS4 operates more similarly to L6 or RPP1.

For L6 and RPP1, the LRR domain is responsible for direct effector perception. The LRR domain of RPS4 is not directly involved in effector perception, as perception is via the WRKY domain of RRS1. My preliminary data show that when RPS4-LRR is swapped with RPS4B, forming RPS4(AABA), RPS4(AABA) + RRS1-R becomes non-functional for defence activation upon effector recognition (data not shown). Additionally, the LRR swap (RPS4(AABA)) inhibits the effector-independent cell death activity of RPS4, and likewise for the CTD swap RPS4(AAAB). It is possible that the LRR domain and CTD of RPS4 mediate the conformational changes leading to RPS4 activation.

I have identified the CTD as a domain of RPS4 that senses the activation signal from RRS1 via interacting with RRS1 DOM4. Conceivably, the CTD/DOM4 interaction may mediate the de-repression of the RPS4 TIR domain by alleviating the NB-ARC negative regulation together with the LRR domain. Also, effector-induced changes of the RRS1 and RPS4 TIR domains oligomerisation status are important for the transition to an active RPS4 [115]. Nevertheless it is probable that other domains of RRS1 and RPS4 also interact and take part in these immune complex reconfigurations. Thus the detailed molecular events leading to RPS4 activation in the RRS1/RPS4 complex remain to be elucidated.

6 General Discussion and future perspectives

6.1 Studying NLR function in a pair complex

Over the past decade, a new paradigm has emerged where functionally diverged NLRs work as a pair to regulate immunity in both plants and mammals. In human and mouse, the NLR "NAIPs" function to directly recognise pathogen-derived ligands (PAMPs) and then oligomerise with NLRC4 (another NLR) to initiate defence signalling [175, 276]. The mechanisms of the interaction between the NAIPs, specialised as 'sensors', and the NLRC4, specialised to transduce immune signals, have been well studied [170, 171, 173, 291, 292]. Though many plant NLRs have been reported to cooperate with another NLR for function [15, 112, 138], only a few have been recently studied in detail as functional pairs (e.g. Rice CNL pairs RGA4/RGA5 and Pik-1/Pik-2). In collaboration with colleagues, my PhD work on the Arabidopsis TNL pairs RRS1/RPS4 and RRS1B/RPS4B has provided novel insights and has advanced the mechanistic understanding of these pairs, which will contribute to the study of paired NLRs in the future.

A common theme of studied plant NLR pairs is the separation of roles as sensor and executor. For RGA4/RGA5 and RRS1/RPS4 in particular, the sensors RGA5 and RRS1 directly interact with pathogen effectors, and the executors RGA4 and RPS4 respectively are responsible for cell death signalling [58, 59, 110, 111, 115]. The functional distinction between these sensors and executors are demonstrated by the following. First, RPS4 and RGA4, but not RRS1 or RGA5, can trigger effector-independent cell death [111, 254]. Secondly, the P-loop motifs of RPS4 and RGA4 are essential for defence activation, while those of RRS1 and RGA5 are dispensable [111, 115]. Last but not least, in contrast to RPS4 and RGA4, both RRS1 and RGA5 have incorporated an atypical domain C-terminal to the canonical TIR-NB-ARC-LRR and CC-NB-ARC-LRR structures (WRKY and HMA domain respectively), which is specialised for effector detection [58, 59, 110, 112]. Given that RPS4/RRS1 and RGA4/RGA5 form pre-activation immune complexes, in which RGA5 and RRS1 can suppress the auto-activity of RGA4

and RPS4 respectively [112, 115], a popular view in the field is that the immune complex activation is driven by the effector-triggered alleviation of the sensors' negative regulation on the executors **(Figure 6.1A)**.

However, for the following reasons, I think that there are more subtleties than this simple model. Since the effector binding does not disrupt the sensor/executor association for either RGA4/RGA5 or RRS1/RPS4 (in transient assays in *N. benthamiana*), the effector-triggered defence activation must be carried out by an immune complex. A conundrum arises that if the executor alone is sufficient to activate defence, why doesn't the presumed negative regulator (the sensor) disassociate completely from the executor to allow more efficient activation? One might argue that maintaining an immune complex during activation could be useful for a rapid re-inhibition to avoid harmful and unnecessary prolonged defence signalling, or perhaps to also allow multiple rounds of activation.

In addition, by comparing the RGA4/RGA5 and the RRS1/RPS4 systems, and also with the Pik-1/Pik-2 and RPS4B/RPS1B systems, I think that there are more clues as to why the so called sensor and executor may activate defence as a complex. Despite the apparent similarities of the RGA4/RGA5 and the RRS1/RPS4 systems, there might be intrinsic differences between them. For example, the RGA4- and RPS4-mediated effector-independent cell death are different, and I infer that the differences are either quantitative or qualitative for the following reasons. Firstly, RGA4 is strongly auto-active and needs RGA5 to keep it in check [111], while RPS4 is not auto-active when expressed under its native promoter in the absence of RRS1 [254]. The evidence supporting this is that silencing of *RGA5* in rice protoplasts leads to RGA4 de-repression and cell death [111], whereas the *rrs1* or *rrs1/rrs1b* double knockout in Arabidopsis does manifest an auto-active phenotype [121]. Secondly, the strong auto-activity of RGA4 correlates with a degenerated MHD motif TYG, as mutations of RGA4 TYG to MHD lead to the loss of effector-independent and -dependent activity [111]. However RPS4 has a conserved MHD motif [161], and only triggers auto-activity when over-expressed and when negative regulation is removed e.g. as shown by RPS4(ABAA) Chapter 5. It would be interesting to test if an MHD mutant enhances RPS4 auto-activity and whether RRS1 could (or could not) suppress it. This would help clarify whether the conserved RPS4 MHD motif accounts for its weaker cell death signalling compared to RGA4.

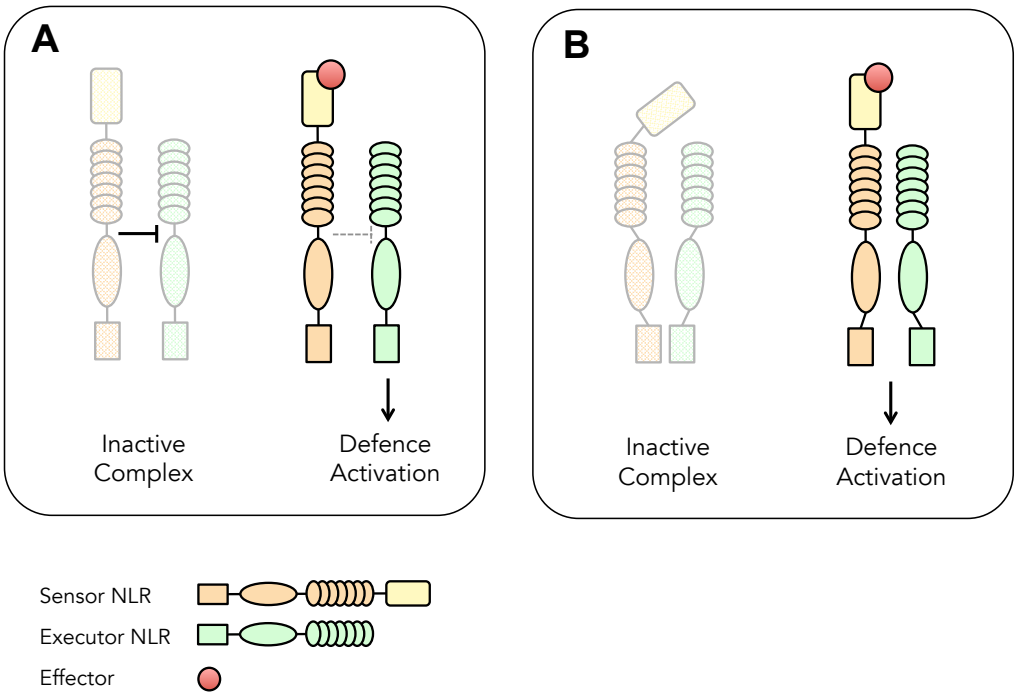

Figure 6.1: Conceptual activation models of a paired NLR complex (**A**) One model describes that in the absence of an effector, the sensor NLR negatively regulates the executor NLR to keep the complex inactive. Upon effector perception via the sensor NLR, the executor NLR is de-repressed, which thereby triggers defence activation. (**B**) An alternative model describes that the inactive complex is maintained via various inter-and intra-molecular interactions between the sensor and the executor NLR. Effector-binding to the sensor NLR triggers immune pair complex reconfiguration that leads to defence activation. Here the sensor NLR refers to the NLR carrying an integrated domain (yellow) that directly perceives effectors.

Alternatively, RGA4 and RPS4 may trigger cell death with different mechanisms, as they operate in distinct cellular compartments. RGA4 is localised in the cytoplasm and does not re-localise into the nucleus upon activation [111]. In contrast, RPS4-triggered cell death requires nuclear localisation, as RPS4*nls* (mutation in its nuclear localisation signal) fails to trigger HR [254]. Nevertheless, we should not exclude that a similar process (or downstream components) could mediate cell death induction in either the nuclear or cytoplasmic fractions.

The most striking difference is that RGA5 with the HMA domain deleted is still able to suppress RGA4, implying that HMA is dispensable for this suppression; whereas the WRKY domain of RRS1 is required for auto-inhibition, as RRS1ΔD56 without the WRKY domain triggers auto-activity with RPS4 (Chapter 5). Importantly, the auto-activity of RRS1ΔD56 + RPS4 is not due

to the lack of RPS4 suppression, as the experimental condition in which the former was assessed does not allow the HR activity triggered by RPS4 alone. Also the further deletion of DOM4 (RRS1ΔD456) with RPS4 no longer triggers HR in the same condition (Chapter 5). An additional evidence is that the SHAA mutation in RRS1-R*slh1*, which abolishes TIR-TIR interactions, diminishes the HR when co-expressed with RPS4 [116], implying that RRS1-R*slh1*/RPS4-triggered autoimmunity is not simply caused by the removal of RPS4 suppression. Together these data strongly suggest that a de-repressed RRS1 positively contributes to RPS4 defence signalling in a complex. Intriguingly, there is some evidence showing that RPS4-triggered autoimmunity partially depends on RRS1: Arabidopsis dwarfism and constitutive defence gene induction caused by stably over-expressing RPS4 is reduced in the absence of RRS1 and RRS1B [293, 294]. Furthermore, unpublished data in our lab (SH, VC, PS et al) suggest that RRS1 stabilises RPS4, and RPS4 homo-oligomerisation requires RRS1. To conclude, from these data I infer that there is a requirement for RPS4/RRS1 and other NLR pairs to be in a complex to activate defence, and activation via the executor is not equivalent to the removal of the sensors' repression, but rather via conformational changes of the whole complex **(Figure 6.1B)**.

Beyond this, it is likely that not all executors can initiate cell death response on their own. In analogy to the RPS4/RRS1 and RGA4/RGA5 pairs, RRS1B and Pikp-1 are the sensors of their respective pairs that interact with the effectors via their integrated WRKY and HMA domains, and RPS4B and Pikp-2 are candidate executors that play a role in signalling. However, unlike RGA4 and RPS4, transient over-expression of Pikp-2 or RPS4B does not trigger constitutive cell death in *Nicotiana spp.*, and activation requires the co-expression of both pair partners together with the cognate effectors [113] (Chapter 5). Therefore, for RPS4B/RRS1B and Pikp-1/Pikp-2, without excluding that the presumed 'executors' may still be important for defence signalling, activation must not be a simple de-repression. More generally, the clear cut distinction of sensor and executor functions could be an over-simplification for many paired NLRs. Though it is yet unclear whether all NLR pairs form immune complexes, classifying their functions into strict 'sensor' and 'executor' could be constraining our understanding of how a probable immune pair complex activates defence. Thus I think we should not study the partners of the pairs independently of each other, but rather as a whole package for their activation and signalling function in the future.

6.2 Implications of atypical NLRs carrying additional domains

The work in this thesis has helped to unravel the mechanism of integrated WRKY domains as potential decoys for effector host targets. Both effectors AvrRps4 and PopP2 target other Arabidopsis WRKY proteins possibly for immune suppression [58, 59]. Thus it is no coincidence that they are being perceived via the atypical WRKY domain embedded in a NLR structure [58, 121]. We inferred that these plant NLRs have incorporated domains mimicking the effector targets as a means to detect effectors. Similarly, the rice RGA4/RGA5 pair functions to recognise two *M. oryzae* effectors via an integrated HMA domain in RGA5 [110]. A review of these two NLR pairs in particular has led to the advocacy of the 'integrated decoy' model [112]. However, as Wu et al. [120] pointed out, some domains might retain their ancestral functions possibly to assist immunity, and thus the broader term 'sensor domain' is more suitable.

Following on from these discoveries, the perception is that the atypical domains in NLRs are a potential hallmark for the sensor function. Interestingly, many integrated domains are found in paired NLRs, although not exclusively [112, 118, 119]. Typically one member has a canonical structure, and the other possesses an integrated domain. Besides the ones already described, there are others: Arabidopsis TNLs RPP2A/RPP2B [132] and CHS3/CSA1 [130], rice CNLs Pi5-1/Pi5-2 [125], wheat CNLs LR10/RGA2 [133], barley RGA1/Rpg5 [295] and melon Fom-1/Prv [134] (for details of their integrated domains see Chapter 1). Why then are integrated domains often found in pairs? I hypothesise that when multiple NLRs evolve to function as one unit for defence activation, redundancy arises in those duplicated domains (e.g. NB-ARC, LRR). As a consequence, in paired NLR systems, relaxed evolutionary constraints on individual NLR domains may provide flexibility for the integration of extra domains. This can explain the frequent occurrences of integrated domains in paired NLRs and the subsequent functional specialisation of pair partners. Above I contemplated on how integration of extraneous domains could happen; this leaves the important questions of which domains to integrate and why are they maintained through evolution?

As discussed in Chapter 4, important targets for effectors could be popular for integration. One important target for effectors might be the WRKY TF proteins which have expanded during the evolution of higher plants as a crucial defence regulator [275], and 70% of Arabidopsis WRKY proteins are implicated in defence [274, 278]. The evolutionary advantage of the WRKY domain fusion is suggested by the duplicated RRS1/RPS4-like proteins with an integrated WRKY domain that are maintained or have further expanded in several relatives of *A. thaliana* [121]. Indeed, recent studies have highlighted the recurrent integration of WRKY domains into NLRs, which appear to have emerged independently in several lineages of plants [118, 119]. Besides the WRKY domain, these studies also revealed a widespread tendency for integrated domains in plant NLR proteins, with kinase and BED domains also among the most common [118, 119]. It is worth noting that the elements or processes initiating the integration in the first place could also influence the integration frequency. Intriguingly, WRKY TF have evolutionary links with transposons such as mutator elements that resembles the element domain of BEAF and DREF proteins [296], and such elements could have mediated their integrations into NLRs.

More broadly, incorporations of such atypical domains might mirror the convergent targeting of plant pathogen effectors to hub proteins so that important nodes would be well protected [16, 45, 126]. As mentioned in Chapter 1, an advantage for domain integration into NLRs could be to enable the rapid acquisition of new effector recognition capacity. Additionally, the physical fusion of the sensor domain to the NLR structure would ensure adaptive co-evolution and genetic co-segregation allowing stability.

6.3 How does effector binding trigger NLR activation?

In certain direct recognition systems, the affinity between the NLR sensor domain and the effectors correlate with recognition capacity. This positive correlation has been reported for Pikp-1/AvrPik and RGA5/AvrPia, where either the polymorphisms in the HMA domain or the variations in the effectors affect binding and recognition [110, 113]. My work on AvrRps4 perception via the WRKY domain of RRS1 or RRS1B has revealed a similar pattern to some degree (Chapter 4). At the predicted AvrRps4/WRKY interaction interface, mutating E187 to A in AvrRps4, or mutating K1221 to R or Q

in RRS1-R significantly reduces affinity, which in turn abolishes recognition. Also the weaker responsiveness of AvrRps4 L167 variants in RRS1/RPS4 and RRS1B/RPS4B correlates with weaker effector/WRKY associations. However, I showed that the lack of AvrRps4-L167S-triggered HR in RRS1B/RPS4B can be restored by increasing the R protein concentrations expressed in tobacco transient assays, indicating affinity is not the only limiting factor for recognition. Natural variation analysis by Sohn et al. [243] has reported L167 as the only residue under positive selection in AvrRps4. Conceivably, AvrRps4 L167 variants modulate interactions with WRKY domains such that they evade host recognition without the trade-off in virulence and thus have been selected for.

However, strong affinity is not always sufficient for recognition. For example, AvrRps4-KRVYAAAA strongly associates with the WRKY domain of RRS1 (Chapter 4), but is not recognised. In addition, both RRS1-S and RRS1B associate with PopP2, and PopP2-C321A, but do not confer recognition (Chapter 3) [214]. By studying these systems, we have revealed there is more to recognition than affinity *per se*, and therefore propose that the NLR domains other than the sensor domain could also determine recognition capacity. In particular, the extended DOM6 of RRS1-R compared to RRS1-S enables PopP2 recognition, even though it is not required for effector binding [58]. It is worth noting that DOM6 (amino acids C-terminal of the WRKY domain) also represents the most polymorphic region of RRS1-R and RRS1B, with 52% identity **(Figure 5.1)**. Besides DOM6-R, my work suggests that other residues in DOM4 (RRS1) and CTD (RPS4) also contribute to PopP2 recognition specificity, and it is possible that RRS1B/RPS4B lacks these domain interactions necessary for PopP2 responsiveness. I believe these insights would be useful to help understand the mechanisms of *C. higginsianum* resistance mediated by RRS1-R/RPS4, but not by RRS1-S/RPS4 or RRS1B/RPS4B [114, 253]. Our observation is not unique; TIR domain polymorphisms between flax L6 and L7 determine their strength of resistance towards flax rust carrying AvrL567, despite the TIR domains not being required for effector interaction [168, 191]. The overall implication is that effector binding is not the whole story; how binding triggers NLR activation is the key.

Here I consider possible underlying mechanisms that could assist conversion of effector binding into activation: Effector binding could (1) compete with NLR domains for binding surfaces and disrupt interactions; (2)

bridge contacts between NLR domains to stabilise interactions; (3) promote NLR interaction with other downstream immune components; (4) stabilise the active form of the NLR; (5) induce a change in the oligomerisation status from an inactive NLR complex into an active one.

In the RRS1/RPS4 system, I have shown that AvrRps4 disrupts DOM4/WRKY association, while PopP2 likely disrupts DOM6 and enables new contacts of DOM6/DOM4/CTD for immune complex activation. Beyond this current model, as AvrRps4 also interacts with domain(s) of RRS1 other than the WRKY (Chapter 4), it is possible that AvrRps4 also bridges new interactions. In hindsight, this could explain why the KRVY motif is important, despite not being required for WRKY domain binding. This motif might be essential for mediating new interactions for the transition to an active complex. Alternatively, the KRVY motif may be important to promote RRS1/RPS4 interaction with other immune molecules.

Bernoux et al. [168] proposed an 'Equilibrium-based switch' model in which NLRs exist in an equilibrium between 'ON' and 'OFF' states, and effectors shift the balance towards activation via binding and stabilising the active NLR. We know that PopP2 stabilises RRS1 [214, 257], and I have seen that co-expression of PopP2 or AvrRps4, but not their mutants, enhances WRKY domain accumulation *in planta* (western data not shown). How might such stabilisation contribute to activation? Consider an expansion of the 'equilibrium model', in which there is a balance between unbound NLR monomers and pre-activation complexes. The effector binds and stabilises the complex, reducing breakdown into monomers, which shifts the equilibrium towards active complexes. Alternatively, effector binding to a pre-activation complex might promote the formation of higher order complexes, which provide a platform for defence signalling. Effector-induced oligomerisation has been described for the TNL tobacco N, which forms TIR-dependent oligomers upon perception of TMV p50 protein [297]. Recently it has been reported that RPP1 (TNL) also shows ligand (ATR1) dependent oligomerisation [201]. On the other hand, several CNLs, exemplified by Arabidopsis RPS5 and tomato Prf and MLA1 exist as oligomers in the absence of effectors [159, 184, 298]. The effector-induced oligomerisation may be a phenomenon more general to TNLs than CNLs, however many more examples are needed. For the NLRs that bind effectors as pre-existing complexes, whether there are effector-inducible changes in the number of monomers in any oligomeric complex remains to be seen.

6.4 Does unleashing the N-terminal domain of an NLR lead to defence activation?

The N-terminal domains of plant NLRs (CC or TIR) are perceived to be crucial for defence signalling [140, 185]. As many TIR or CC domains are sufficient for cell death induction, and their signalling activities are often oligomerisation-dependent, parallels can be drawn between the plant and animal NLRs in terms of their signalling mechanisms [276]. In animals, some NLRs multimerise to form a wheel-like inflammasome structure that imposes proximity on their N-terminal domains, creating a platform for the recruitment of downstream signalling adaptors [170, 171, 173, 193, 194]. At the centre of the inflammasome "wheel", the NLR N-terminal domains bound to these adaptors can initiate nucleation, forming filaments that are important for defence signalling [198, 199, 276, 299]. The speculation is that the plant NLRs may form large complexes similar to the animal inflammasomes. How the plant NLR N-terminal domains mediate defence signalling, and how this process is regulated before and after effector elicitation are important unanswered questions in this field.

As discussed in Chapter 5, my data suggest that RPS4, especially the TIR domain, is being negatively regulated by multiple interactions in the pre-activation complex. I inferred that disruption of these possible negative regulations could assist the transition to an active RPS4 in the immune complex, conceivably via exposing the RPS4 TIR domain surface required for homo-oligomerisation. One of the negative regulations could be via the RRS1 TIR domain, which directly interacts with RPS4 TIR and can inhibit $RPS4^{TIR}$-triggered effector-independent cell death (Chapter 3). Crystal structures of RRS1 and RPS4 TIR domains revealed a common TIR-TIR interaction interface at α-helices A and E for either homo- or hetero-dimerisation [115]. Interestingly, this interface differs from that of the L6 TIR domain homodimer spanning α-helix D to α-helix E, with the two being 90° away from each other [197]. Furthermore, additional residues required for defence signalling have been identified on the L6 TIR domain distinct from those at the dimerisation interface [197], suggesting that other interfaces are required for signalling. With conserved patches resembling either RPS4-like or L6-like interfaces found in other TIR domains of plant TNLs [197, 204], we could speculate that both interfaces are important for TIR domain-mediated defence signalling.

Additionally, the NLR N-terminal domains are regulated by intra-molecular interactions. For L6, L7 (TNL) and Rp1 (CNL), the negative interactions between NB-ARC and N-terminal domains inhibit the self-association of the latter [165, 168, 197]. In contrast, the NB-ARC1 of RPP1 promotes TIR-NB-ARC1 to self-associate, and facilitates cell death signalling [201]. The NB-ARC domain of RPS4 as a whole is likely to negatively regulate the TIR domain as the NB-ARC deletion in RPS4^{TIR} or the NB-ARC swap RPS4(ABAA) enhances auto-activity (Chapters 3 & 5). However, future experiments need to clarify the regulatory role of individual NB-ARC sub-domains.

Notwithstanding these data, whether all N-terminal domains directly mediate defence signalling remains to be seen. And it is puzzling that the N-terminal domains of many other functional TNL or CNL proteins cannot initiate effector-independent cell death. One possible explanation is that the properties required for cell death signalling by only an N-terminal domain could be different from it acting within a full-length protein or in an immune complex. Altogether, the evidence suggest that the N-terminal domains of plant NLRs are usually closely regulated, and their activities are important for effector-triggered defence activation.

6.5 Summary and Outlook

My work presented in this thesis aimed to tease apart the fine-tuned recognition specificities between different effectors and different R gene alleles. I have successfully demonstrated: how AvrRps4 recognition is independently conferred by two TNL pairs; how this recognition is mediated by an integrated WRKY domain; and how the immune complex is de-repressed by distinct effectors, AvrRps4 and PopP2. Studying how paired NLR proteins work as a single immune complex was, and remains, an interesting and important problem. I have studied the dynamic molecular interactions that convert effector recognition to defence activation, and this will provide valuable insights for many other cooperative NLR systems. More quantitative and detailed insight will require more refined techniques. To study the changes of intricate domain-domain interactions and to analyse affinity differences, Surface Plasmon Resonance (SPR) and Fluorescence Resonance Energy Transfer (FRET) can be useful. To understand the overall domain organisation, and to visualise possible oligomeric changes, full-length NLR structural insights via X-ray crystallography and high-resolution cryo-electron microscopy are cru-

cial. Ultimately such mechanistic insights of complex auto-regulation and activation would provide valuable knowledge for future immune receptor engineering. The ability to design new R genes that could recognise any pathogen effector based on its host target would underpin a revolution in crop disease control.

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