Toward the understanding of TIR-NB-LRR–mediated immunity; study of the AvrRps4 recognition model in Arabidopsis

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Table of Contents

Abstract

The recognition of pathogen–secreted effectors is a major component of plant innate immunity and is mainly mediated by NB-LRR resistance (R) proteins. However, the mechanisms by which NB-LRR proteins recognize effectors and induce downstream signaling events are poorly known. This PhD work focused on the characterization of TIR-NB-LRR–mediated immnunity in plants. In Arabidopsis, two TIR-NB-LRR encoding *R* genes, *RRS1* and *RPS4,* are in a head-to-head arrangement on chromosome 5. They interact to confer recognition to AvrRps4 and (with the right allele of RRS1) PopP2, two bacterial effectors, from *Pseudomonas syringae* and *Ralstonia solanacearum* respectively.

To dissect AvrRps4–triggered immunity, I focused on the *RRS1*– and *RPS4*–independent AvrRps4 recognition (RRIR) observed in the Ws-2 and Col-0 Arabidopsis accessions. I map-based cloned another pair of *R* genes, *RRS1B* and *RPS4B,* genetically linked and highly similar to *RRS1-RPS4*, responsible for the RRIR. Interestingly, *RRS1B-RPS4B* recognizes AvrRps4 but not PopP2. Using domain swap experiments, I demonstrated that RRS1 exons 5, 6 and 7 specify PopP2 recognition. My data suggest that AvrRps4 and PopP2 interact directly with RRS1 and RRS1B. However, the exact mechanism by which these effectors activate TIR-NB-LRR R proteins remains to be determined. Overexpressing the N-terminal domain of *RPS4*, *RPS4*$^{TIR+80}$, activates cell death in plants and I demonstrated that it requires a nuclear localization. Similarly, the C-terminal part of RPS4B comprises a nuclear localization signal and is required for *RRS1B-RPS4B*–dependent AvrRps4 recognition. Interestingly, RPS4^{TIR+80}–mediated cell death can be suppressed by co-expressing *RRS1*^{TIR}. Using a proteomic approach, I showed that TIR domains and full length RRS1, RPS4, RRS1B and RPS4B can associate *in planta* forming distinct heterodimers. However, I showed that, despite their homologies, these R proteins only function with their respective pair partner for effector recognition and/or downstream signaling activation.

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Major abbreviations

Avr: Avirulence

CC: Coiled-coil

cfu: colony forming unit

DNA: Deoxyribonucleic

EDS1: Enhanced disease susceptibility 1

ETI: Effector–triggered immunity

gDNA: genomic DNA

GFP: Green fluorescent protein

HR: Hypersensitive response

LRR: Leucine rich repeat

NB: Nucleotide binding

NES: Nuclear export signal

NLS: Nuclear localization signal

OD: Optical density

PAMP: Pathogen–associated molecular pattern

Pf: *Pseudomonas fluorescens*

Pst: *Pseudomonas syringae* pv *tomato*

PTI: PAMP–triggered immunity

R: Resistance

RPS4: Resistance to *Pseudomonas syringae* 4

RRS1: Resistance to *Ralstonia solanacearum* 1

RT-PCR: Reverse transcription polymerase chain reaction

TIR: Toll/interleukin-1 receptor/Resistance protein

TTSS: Type three secretion system

TTSE: Type three secreted–effector

WT: Wild type

1 General introduction

1.1 Introduction

Plants are eukaryotic organisms that first appeared on earth approximately 400 million years ago. They have since evolved and diversified and now display a wide range of forms, structures and biological mechanisms. Plants are constantly challenged by biotic (related to living organisms) and abiotic (independent of living organisms) stresses. To protect themselves, they have acquired complex mechanisms to detect invaders, activate signaling pathways and establish defenses.

A myriad of micro-organisms such as viruses, bacteria, oomycetes and fungi, and also insect and nematodes, can parasitize plants (Agrios, 1988). These microbes are considered to be pathogens when they cause diseases and disorders (such as leaf spot, stem canker, crown gall or root rot) in the invaded plant. Establishment of infection by a successful pathogen is described as a compatible relationship between the pathogen (virulent) and the host (susceptible). However, plants can exhibit defense mechanisms that prevent pathogen invasion and prevent disease; the resulting absence of infection is called an incompatible interaction between the pathogen (avirulent) and the host (resistant). In plant–pathogen interactions, there are various ways to define resistance. The term “non-host resistance” is used to describe resistance in which all accessions of a plant species are resistant to a specific pathogen. The term “host resistance” is used when the pathogen is able to infect a particular species but some accessions are resistant (Mysore and Ryu, 2004).

To protect themselves against pathogen infection, plants have developed different strategies involving physical barriers, the production of toxins and the

action of an immune system. The defenses can be constitutive such as leaf cuticle, cell wall, lignin and the biosynthesis of some secondary metabolites known as “phytoanticipins”; these belong to the passive defense system (Heath, 2000). However, many defenses are induced by the recognition of the pathogen (or “non self”), and/or by the detection of pathogen-induced host’s target modification (or “modified self”). These trigger a range of signaling pathways, which induce multiple cellular responses in order to eliminate or restrict the enemy. Plants differ from mammals in that their immune system lacks circulating defender cells and adaptive immunity (for example, lymphocyte cells producing antibodies). However, like vertebrate and invertebrate animals, plants show “innate immunity”.

1.2 Microbial molecular patterns and plant resistance

1.2.1 Perception of conserved microbial molecules in plants

Plants can recognize highly conserved microbial proteins or structures known as microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs) through specific pattern recognition receptors (PRRs) (Postel and Kemmerling, 2009). This is described as the first layer of the plant immune system. Several bacterial PAMPs such as lipopolysaccharide (LPS), harpins, and cold-shock protein have been identified (Alfano and Collmer, 2004; Dow *et al.*, 2000; Felix and Boller, 2003; He *et al.*, 1993; Zeidler *et al.*, 2004). Among the most characterized, flagellin (or its active epitope flg22), a component of the bacterial flagellum, is recognized by the leucine-rich repeat (LRR) receptor-like kinase (RLK) flagellin-sensing 2 (FLS2) (Felix *et al.*, 1999; Gomez-Gomez and Boller, 2000). Similarly, elongation factor Tu (EF-Tu), the most abundant and conserved protein in the bacterial cytoplasm, is perceived (or its minimal motif elf18) by the LRR-RLK EFR (EF-Tu Receptor) (Kunze *et al.*, 2004; Zipfel *et al.*, 2006). In rice and *Arabidopsis*, lysin motif (LysM) proteins (CeBiP in rice and CERK1 in *Arabidopsis*) are required for the perception of

fungal chitin (the main building block of fungal cell walls) (Kaku *et al.*, 2006; Miya *et al.*, 2007; Wan *et al.*, 2008). The RLK Xa21 from rice carries 23 extracellular LRRs and an intracellular kinase domain (Song *et al.*, 1995). This kinase is capable of autophosphorylation which is suggested to participate in the recruitment of downstream signaling proteins (Liu *et al.*, 2002a).

1.2.2 Activation of plant innate immunity by PRRs

Many hemibiotrophic and biotrophic pathogens such as bacteria, fungi and oomycetes, invade and grow in the leaf apoplast. It is then important for the plants to evolve extracellular mechanisms of perception. In fact, FLS2, EFR, CeBiP and CERK1 are plasma membrane proteins and act as cell surface receptors. They carry in the N-terminus an extracellular domain (LRR in FLS2 and EFR; LysM in CeBiP and CERK1) which is proposed to recognize the PAMP elicitor, a transmembrane domain and a C-terminal intracellular domain (Serine/Threonine kinase domain in FLS2, EFR and CERK1) (Gomez-Gomez and Boller, 2000; Kaku *et al.*, 2006; Miya *et al.*, 2007; Zipfel *et al.*, 2006). Likewise, the Toll-Like Receptor (TLR) class of mammal PRRs comprises membrane-bound receptors (Mogensen, 2009). They contain an extracellular LRR domain, a transmembrane domain but differ in the cytosolic domain. The intracellular domain of TLRs have homology with the Toll/interleukin-1 receptor/Resistance protein (TIR) domain (O'Neill and Bowie, 2007; Bowie and O'Neill, 2000). Following microbial compound perception by the LRR domain, TLR TIR domains activate downstream signaling by interacting with other TLRs and cytosolic TIR-containing adaptors such as MyD88 (Akira *et al.*, 2006; Ozinsky *et al.*, 2000; O'Neill *et al.*, 2003).

Interestingly, plant PRRs also form complexes to activate downstream signaling (Aker and de Vries, 2008). The receptor BAK1 (BRI1-Associated receptor Kinase1) interacts with BRI1 (Brassinosteroid Insensitive1) for brassinosteroid

perception and downstream signaling (Li *et al.*, 2002; Nam and Li, 2002). BAK1 has also been shown to interact *in vivo* with FLS2 (Chinchilla *et al.*, 2007; Heese *et al.*, 2007). It has been proposed that flagellin elicitation tightens the interaction between FLS2 and BAK1 that leads to the transphophorylation of the kinase domains, which may initiate the signal transduction (Boller and Felix, 2009). BAK1 is also required for EF-Tu–triggered responses and it interacts with EFR in a ligand–dependent manner (Roux *et al.*, 2011; Chinchilla *et al.*, 2007; Schwessinger *et al.*, 2011).

Recognition of PAMPs by the corresponding PRRs initiates PAMP-triggered immunity (PTI) (Jones and Dangl, 2006). PTI is composed of a set of signaling events and defense responses occurring quickly after elicitation (Nicaise *et al.*, 2009); it involves activation of mitogen-activated protein kinase (MAPK) signaling cascades (Pitzschke *et al.*, 2009; Meng and Zhang, 2013), production of reactive oxygen species (ROS) (Felix *et al.*, 1999), callose deposition (Gomez-Gomez *et al.*, 1999), defense gene transcription (Eulgem *et al.*, 2004; Navarro *et al.*, 2004) and synthesis of anti-microbial compounds (Nurnberger *et al.*, 2004). PTI–associated defense responses do not eradicate pathogen colonization but do impede extension of its spread (Glazebrook *et al.*, 1997a).

1.3 Suppression of PTI by pathogen effectors

As part of the ongoing struggle between pathogens and plants, pathogens have evolved mechanisms that enable them to suppress defenses and notably PTI in order to assure host plant colonization (He *et al.*, 2007). Bacterial pathogens have developed a highly specialized protein delivery apparatus called the type three secretion system (TTSS) (Galan and Collmer, 1999) to translocate virulence factors (referred as effectors) directly into host cells (Collmer *et al.*, 2002). Likewise, fungal and oomycete pathogens secrete effector proteins via a hyphal tip or haustorium, a specialized infection structure invaginating the host cell (de Jonge *et al.*, 2011; Bozkurt *et al.*, 2012). Fungal and oomycete effectors either stay in the apoplast or translocate into the plant cell notably by the presence of an RxLR motif for oomycetes effectors (Whisson *et al.*, 2007; Dou *et al.*, 2008; Song *et al.*, 2009). Among the pathogen effectors identified, only a few have a known function (Lewis *et al.*, 2009; Stassen and Van den Ackerveken, 2011). It is suggested that effectors are required to enable pathogens to manipulate host cells in order to acquire nutrient and suppress host immune responses including PTI. Among the best characterized examples, the *Pseudomonas syringae* effector AvrPto is secreted into the plant cytosol and suppresses basal defense by inhibiting FLS2 and EFR (Hauck *et al.*, 2003; He *et al.*, 2006; Xiang *et al.*, 2008). This leads to effector-triggered susceptibility (ETS) (Jones and Dangl, 2006).

1.4 Pathogenic effectors and plant resistance

1.4.1 The “gene-for-gene” relationship

Interestingly, plants have developed a specific recognition system to ensure resistance and overcome ETS. In 1971, in describing the “gene-for-gene” concept, Flor states that: “for each gene that conditions resistance in the host there is a

corresponding gene that conditions pathogenicity in the parasite" (Flor, 1971). Hence, the product of a resistance (*R*) gene in a plant recognizes a pathogen effector required for virulence and this interaction leads to resistance. The gene coding such an effector is called an avirulence (*Avr*) gene because it conditions the avirulence of the pathogen in the host. This phenomenon is also referred as Effector-Triggered Immunity (ETI) (Jones and Dangl, 2006).

ETI involves accumulation of salicylic acid (SA), expression of pathogenesis-related (PR) genes and production of reactive oxygen species (ROS) (Hammond-Kosack and Jones, 1996). Effector recognition by a resistance protein can also trigger a rapid and high amplitude response known as the hypersensitive response (HR) (Agrios, 1988). The HR is characterized by a programmed cell death (PCD) at the infection site (Hofius *et al.*, 2007). However, it was shown that ETI does not necessarily require HR to prevent disease (Gassmann, 2005). Similarly, non-host resistance (NHR) is not necessarily associated with HR and is proposed to be divided in two types. In type 1 NHR, there are no visible symptoms whereas type II NHR is associated with a HR (Mysore and Ryu, 2004). The dichotomy between PTI and ETI can be exaggerated, because plant resistance is a continuum of immune receptors recognizing specific ligands and activating defense with different timing and amplitude (Katagiri and Tsuda, 2010; Thomma *et al.*, 2011).

1.4.2 Plant resistance proteins

The first isolated *R*–gene, *Pto* from tomato, confers resistance to the bacterium *Pseudomonas syringae* pv. *tomato* (*Pst*) carrying *AvrPto* (Martin *et al.*, 1993). 15 years later, over 70 plant *R*–genes had been cloned and provide efficient resources for resistance against pathogens (Liu *et al.*, 2007). Currently, *R*–genes are divided in classes according to their structural homologies (Hammond-Kosack and Jones, 1997; Dangl and Jones, 2001a).

Pto is a resistance gene representing a class by itself. It encodes a cytoplasmic Ser/Thr kinase that recognizes AvrPto and initiates signal transduction (Martin *et al.*, 1993). *Pto* requires the tomato NB-LRR (see later) protein PRF (*Pseudomonas* Resistance and Fenthion sensitivity) to trigger disease resistance (Salmeron *et al.*, 1996).

Another class comprises the Receptor-Like Proteins (RLPs) consisting of an extracellular LRR and a small cytoplasmic domain without any obvious motif. This class includes tomato *Cf-2*, *Cf-4*, *Cf-5* and *Cf-9* genes that confer resistance to the fungal pathogen *Cladosporium fulvum* (Joosten and de Wit, 1999).

The nucleotide-binding (NB) and LRR proteins (NB–LRRs) represent the largest family of plant *R*–genes. NB–LRRs exhibit domains that have structural homologies with proteins that mediate immune responses in animals (Creagh and O'Neill, 2006). NB-LRR *R* genes are divided into two subclasses according to their respective N-terminus domain. The first subclass is represented by R proteins with a Toll/interleukin-1 receptor (TIR) and the second by these with a Coiled-Coil (CC) domain (Collier and Moffett, 2009).

1.4.3 NB-LRR resistance proteins

Plant NB-LRR resistance proteins, also called “nibblers” (Takken and Tameling, 2009), are important factors of ETI; they are subsequently the principal targets in resistance gene identification (Meyers *et al.*, 2003). This family is divided into two main groups: the CC-NB-LRRs and the TIR-NB-LRRs.

The CC domain consists of two or more alpha–helices (Nooren *et al.*, 1999) and is expected to play a role in homo- and hetero-dimerization of protein complexes (Landschulz *et al.*, 1988). The CC is an oligomerization domain for many proteins such as structural proteins, motor proteins and transcription factors. The

CC-NB-LRR class of resistance genes includes the *Arabidopsis RPS2*, *RPM1* and *RPS5*, the pepper *Bs2* (resistance to *Xantomonas campestri*) and the potato *Rx* (resistance to potato virus X) (Martin *et al.*, 2003).

The TIR domains of the plant *R* proteins carry homology to the *Drosophila* Toll receptor (Hashimoto *et al.*, 1988) or to the cytoplasmic domain of the human interleukin-1 receptor (Sims *et al.*, 1989). Toll like receptors (TLR) and TIR proteins are involved in mammalian innate immunity (Uematsu and Akira, 2007; Burch-Smith and Dinesh-Kumar, 2007). The crystal structure of the Arabidopsis AtTIR, a peptide that contains only a TIR domain, revealed an αD-helix (αD3) which is specific to several plant TIR-containing proteins (Chan *et al.*, 2010). Moreover, the integrity of this domain appears to be important in TIR-NB-LRR function (Swiderski *et al.*, 2009). There are 145 predicted genes that carry a TIR domain in the *Arabidopsis* genome and out of these, 51 do not have a LRR domain (Meyers *et al.*, 2003). Several TIR-NB-LRR resistance genes have been identified in plants. The *Nicotiana glutinosa N* gene confers resistance to tobacco mosaic virus (Whitham *et al.*, 1994). The flax (*Linum usitatissimum*) *L6* gene provides resistance against the rust fungus *Melampsora lini* that carries the *AvrL567* avirulence gene (Lawrence *et al.*, 1995). The *Arabidopsis RPP5* gene confers resistance to *Hyaloperonospora arabidopsidis* (*Hpa*) (Parker *et al.*, 1997). Other TIR-NB-LRRs have been identified by their capability to provide resistance to bacterial pathogens. For instance, *Resistance to Pseudomonas syringae 4* (*RPS4*) from *Arabidopsis* accession Wassilewskija (Ws-0) gives resistance to *Pseudomonas syringae* carrying the avirulence gene *AvrRps4* (Hinsch and Staskawicz, 1996). The avirulence gene *PopP2* from *Ralstonia solanacearum* is recognized in *Arabidopsis* accession Niederzenz (Nd-1) by *RRS1* and triggers resistance (Deslandes *et al.*, 1998). *RRS1* is a particularly interesting TIR-NB-LRR due to the presence of an additional WRKY domain in its C-terminus (Deslandes *et al.*, 2002a). The WRKY domain is defined by a conserved amino acid

sequence starting with WRKYGQK. This domain is found in the transcription factor protein family, in one or two copies, and binds DNA at a sequence motif (T)(T)TGAC(C/T), known as the W box. WRKY domain-containing proteins have been shown to play crucial roles in regulating plant defence responses (Journot-Catalino *et al.*, 2006).

The NB domain of *R* genes shows homologies with adenosine triphosphatases (ATPases) like APAF–1 (for apoptotic protease-activating factor-1) in humans (Zou *et al.*, 1997) and CED–4 (for *Caenorhabditis elegans* death-4 protein) in the nematode *Caenorhabditis elegans* (Vaux, 1997). This region is enlarged by an ARC domain; a conserved motif within APAF-1, R proteins and CED-4. Based on 3D modeling, the NB-ARC domain can be divided in three sub-domains: the NB forming with a P-loop and Walker motifs, the ARC1 consisting of a four-helix bundle and the ARC2 presenting a winged-helix fold (Leipe *et al.*, 2004; Takken *et al.*, 2006). The NB-ARC domain belongs to the class of STAND (for Signal Transduction ATPases with Numerous Domains) proteins which are involved in PCD regulation (Leipe *et al.*, 2004; Danot *et al.*, 2009). It is proposed that the NB-ARC binds and hydrolyzes ATP and this phenomenon is of importance for NB-LRR-mediated downstream signaling resistance (Tameling *et al.*, 2002; Takken *et al.*, 2006). In fact, mutation in the NB-ARC domain of *RPS2*, *N* and *RPM1* prevents their function (Tao *et al.*, 2000; Dinesh-Kumar *et al.*, 2000; Tornero *et al.*, 2002). On the other hand, different point mutations in the NB-ARC domain can result in autoactive forms of the R protein (Tameling *et al.*, 2006; van Ooijen *et al.*, 2008). Supporting an NB-ARC domain role for downstream signaling, overexpression of the Rx NB domain triggers cell death in *N. tabacum* (Rairdan *et al.*, 2008).

The LRR domain is a tandem repetition of 20 – 29 amino acid (AA) consensus with an internal conserved 11-residue portion LxxLxLxx(N/C)xL (x can be any AA) (Kobe and Kajava, 2001). The LRR is implicated in protein–protein

interactions. Various plant LRR-containing proteins exist with diverse biological functions and cellular localizations. The pathogen-effector recognition by the LRR domain could initiate the R protein activation for downstream signalling (Hulbert *et al.*, 2001). In addition, the LRR domain might modulate activation by forming intramolecular interactions within the R protein complex (Bendahmane *et al.*, 2002). There is some evidence that LRR might be required for downstream signaling (Warren *et al.*, 1998). However, other studies show LRR deleted R proteins are able to trigger cell death (Tao *et al.*, 2000; Swiderski *et al.*, 2009; Rairdan *et al.*, 2008)

1.4.4 R protein activation by Avr product recognition

Recognition of an Avr protein by a corresponding R protein can be mediated by direct protein–protein interaction. The simplest model for this recognition is that it occurs as direct receptor/ligand interaction (Keen, 1990). *Magnaporthe grisea* effector AvrPita is recognized in rice by the predicted CC-NB-LRR resistance gene *Pi-ta* (Bryan *et al.*, 2000). A yeast two-hybrid analysis showed that AvrPita and Pi-ta interact directly (Jia *et al.*, 2000); but this interaction has not been demonstrated *in planta* yet. Arabidopsis *RRS1* is a TIR-NB-LRR resistance gene carrying a C-terminus WRKY domain (Deslandes *et al.*, 2002a). *RRS1* confers disease resistance to the bacterial pathogen *Ralstonia solanacearum* expressing the effector PopP2 (Deslandes *et al.*, 1998). RRS1 and PopP2 directly interact in a yeast split ubiquitin two-hybrid system (Deslandes *et al.*, 2003). This interaction has been supported *in planta* where these molecules colocalize and interact in the nucleus (Deslandes *et al.*, 2003; Tasset *et al.*, 2010)(Williams, Sohn *et al*., unpublished data). Another example is the polymorphic *Melamspora lini* (flax rust) effectors *AvrL567* recognized by the flax L5, L6 and L7 proteins (Dodds *et al.*, 2004). Two AvrL567 alleles from different strains directly interact with L5 and L6 respectively in a yeast two hybrid experiment (Dodds *et al.*, 2006). Most of these *R-Avr* direct interactions have been demonstrated *in vitro* and need to be confirmed *in planta*.

Also, to integrate these direct interactions in a dynamic context, it will be important to know if the recognition and R protein activation goes through direct receptor/ligand perception or if the R protein is activated by being the target of the effector activity.

More examples of indirect *Avr-R* interactions have been reported so far, supporting the detection of effector activity by R proteins. Indirect recognition of an Avr protein by an R protein was introduced as the “guard hypothesis” (Van der Biezen and Jones, 1998; Dangl and Jones, 2001a). It was proposed that an R protein (guard) detects an Avr-mediated modification of a plant target (guardee) and this “modified self” recognition results in ETI. *RPM1* is a CC-NB-LRR resistance gene in Arabidopsis (Innes *et al.*, 1993a; Grant *et al.*, 1995) with the capacity to recognize indirectly two distinct *P. syringae* effectors, AvrRpm1 and AvrB (Bisgrove *et al.*, 1994). RIN4 (for RPM1 interacting protein4) interacts with RPM1 but also with AvrRpm1 and AvrB (Mackey *et al.*, 2002). AvrRpm1 and AvrB promote phosphorylation of RIN4 and this is predicted to activate RPM1, so RPM1 is the guard of RIN4. Recently, it was shown that RPM1 activation partially requires the phosphorylation of RIN4 by RIPK, a RIN4-interacting receptor-like protein kinase which is targeted by AvrB (Liu *et al.*, 2011). AvrRpt2, a *P. syringae* effector, is a cysteine protease that targets and cleaves RIN4 (Axtell *et al.*, 2003; Kim *et al.*, 2005; Chisholm *et al.*, 2005). RIN4 interacts with the CC-NB-LRR resistance protein RPS2 (the other guard of RIN4) in Arabidopsis in absence of AvrRpt2 (Mackey *et al.*, 2003). The cleavage of RIN4 by AvrRpt2 is detected by RPS2 which activates defense downstream signaling (Mackey *et al.*, 2003; Axtell and Staskawicz, 2003). Interestingly, RIN4 is a negative regulator of plant basal defenses and the biological reasons for these effectors to target this protein are still not totally clear (Mackey *et al.*, 2002; Liu *et al.*, 2009). RIN4 is proposed to interact with plama membrane H+-ATPases AHA1 and AHA2 to regulate stomatal aperture during PTI (Liu *et al.*,

2009). Stomata are common entry points for bacterial, fungal and oomycete pathogens. However, PAMP perception generally induces stomatal closure (Melotto *et al.*, 2006). One hypothesis is that pathogen effectors, such as AvrRpm1 and AvrRpt2, target RIN4 in order to manipulate stomatal aperture to facilitate pathogenesis (Liu *et al.*, 2009). Another example of indirect recognition is illustrated by the effector AvrPphB from *P. syringae* pv. *phaseolicola* and the CC-NB-LRR protein RPS5 guarding the receptor-like cytoplasmic kinase PBS1 (for *avrPphB* Susceptible1) (Simonich and Innes, 1995; Warren *et al.*, 1998). AvrPphB is a cysteine protease (Shao *et al.*, 2002) that cleaves PBS1 and other kinases involved in PTI such as BIK1 and PBS1-like (PBL) kinases (Zhang *et al.*, 2010; Shao *et al.*, 2003). Cleavage of PBS1 by AvrPphB activates RPS5, releasing downstream signaling for plant defense responses (Warren *et al.*, 1999; Shao *et al.*, 2003; Ade *et al.*, 2007). Pto and AvrPto interact directly but to trigger resistance, the CC-NB-LRR *PRF* is required (Salmeron *et al.*, 1996; Tang *et al.*, 1996). It can be considered that the PRF protein recognizes indirectly AvrPto, corresponding to a typical guard model (Mucyn *et al.*, 2006). No guard model example involving a TIR-NB-LRR resistance protein has yet been reported.

1.5 General model of NB-LRR resistance protein activation for downstream signaling

In plant cells, NB-LRR proteins are supposed to be under internal structural negative control to prevent any inappropriate activation in absence of the Avr protein, thus avoiding unwanted HR. The intramolecular interactions between the R-protein domains may generate a specific folding state and function as an on/off switch (Moffett *et al.*, 2002; Belkhadir *et al.*, 2004; Takken *et al.*, 2006; Lukasik and Takken, 2009; Takken and Tameling, 2009). In the absence of the pathogen, the R protein is autoinhibited in an “OFF” state with an ADP bound to the NB-ARC

domain. During pathogen infection, the C-terminus part of the LRR domain can detect the presence/activity of an effector. It changes the LRR interaction with the ARC domain creating an open form of the protein; termed “intermediate” state. The ADP is then exchanged with an ATP triggering a second conformational change, releasing the interaction between the TIR/CC, the NB and the C-terminus of the LRR. As a result, the R protein becomes in the “ON” state and thereby activates downstream signaling. A growing body of evidence shows that oligomerisation of R proteins occurs upon Avr activation and this seems to be partialy mediated via dimerisation of the CC or TIR domains (Mestre and Baulcombe, 2006; Bernoux *et al.*, 2011; Maekawa *et al.*, 2011; Takken and Goverse, 2012). Finally, ATP hydrolysis leads to the reconfiguration into the initial “OFF” state. While some information is available to describe resistance gene activation, nearly nothing is known about the mechanisms of R protein-mediated defense activation.

1.6 *R* genes suppressor screens

Following *R* gene discovery, another interest was to find how these genes activate plant defenses. Many forward genetics programs were carried out to identify components involved in *R* gene signaling. These studies were mainly performed in *Arabidopsis thaliana* (Nishimura and Dangl, 2010). Several genes were found to be required for *R* gene-triggered disease resistance but their action throughout the signaling is elusive.

EDS1 (for Enhanced Disease Susceptibility 1) has been identified from a mutational analysis of the Arabidopsis ecotype Wassilewskija (Ws-0) (Parker *et al.*, 1996). Ws-0 carries several *RPP* (for Resistance to *P. parasitica*) genes that confer resistance to different isolates of the biotrophic oomycete *Hyaloperonospora arabidopsidis* (downy mildew) (Parker *et al.*, 1993; Holub *et al.*, 1994; Holub, 1996; Reignault *et al.*, 1996). The Ws-0 *eds1* mutant exhibits enhanced susceptibility to *P.*

parasitica isolates. *EDS1* maps to the bottom of chromosome 3 (Parker *et al.*, 1996) and encodes a lipase-like protein (Falk *et al.*, 1999). It is required for most TIR-NB-LRR-disease resistances (Aarts *et al.*, 1998). EDS1 can homodimerize or can heterodimerize with PAD4 or SAG101 (Feys *et al.*, 2001; Feys *et al.*, 2005). However, contradictory results exist concerning the possible formation of a ternary complex between these three defense co-regulators (Rietz *et al.*, 2011; Zhu *et al.*, 2011).

PAD4 (for PhytoAlexin Deficient 4) was identified through a mutational screen for susceptibility to a *P. syringae* pv. *maculicola* (Glazebrook *et al.*, 1996) and maps on chromosome 3. PAD4 is required for disease resistance against *P.syringae* carrying AvrRps4 (Rusterucci *et al.*, 2001). However, RPS2– and RPM1– mediated resistances do not require PAD4 (Glazebrook *et al.*, 1997b; Glazebrook and Ausubel, 1994; Tsuda *et al.*, 2009). The PAD4 sequence revealed similarities with triacyl glycerol lipase proteins and other esterases (Jirage *et al.*, 1999).

SAG101 (for Senescence-Associated Gene 101) was discovered as a gene induced during senescence (He *et al.*, 2001) and also through a proteomic approach as an interactor with EDS1 (Feys *et al.*, 2001). The SAG101 sequence also presents similarities with plant lipases. SAG101 is indispensable for EDS1-mediated disease resistance (Feys *et al.*, 2005). Interestingly, EDS1, PAD4 and SAG101 form nuclear and cytoplasmic complexes that are required for the class of the TIR-NB-LRRs. However, their role in *R* gene-mediated resistance is unclear. Recently, using co-immunoprecipitation (CoIP) and fluorescence resonance energy transfer-fluorescence lifetime imaging (FRET-FLIM), EDS1 was reported to interact with the *P. syringae* effectors AvrRps4 and HopA1 as well as RPS4 and RPS6, two R proteins recognizing AvrRps4 and HopA1 respectively (Bhattacharjee *et al.*, 2011; Hinsch and Staskawicz, 1996; Heidrich *et al.*, 2011; Kim *et al.*, 2009). These

authors proposed that effector–mediated EDS1 complex modification triggers EDS1–guarding R protein activation. This hypothesis is debatable, as other data are inconsistent with EDS1-AvrRps4 direct interaction (Sohn *et al.*, 2012).

In contrast, most of the CC-NB-LRRs require NDR1 and not EDS1 to mediate resistance. NDR1 (for Non-race-specific Disease Resistance) has been identified in a fast-neutron-mutagenesis screening in Col-0 for loss of resistance against *Pst* DC3000 carrying *AvrB* (Century *et al.*, 1995). Interestingly, the Col-0 *ndr1-1* mutant was also susceptible to *Pst* DC3000 carrying *AvrRpm1*, *AvrRpt2* and *AvrPphB* compared to Col-0 wild type. The amino acid sequence analysis of NDR1 predicts a putative C terminal glycosyl-phosphatidyl-inositol (GPI) anchor and an N terminal transmembrane domain suggesting its membrane-associated localization (Century *et al.*, 1997). Moreover, NDR1 has been shown to interact directly with RIN4 and this interaction is required for *RPS2* downstream signaling (Day *et al.*, 2006). It is proposed that NDR1 participates in association with some *R* protein to transduce the elicitor signal.

This specific requirement for either EDS1 complex or NDR1 suggests that the two classes of NB-LRRs differentially evolved in their mechanisms to activate plant defenses (Aarts *et al.*, 1998). Moreover, this support the idea that TIR and CC domains are more involved in signaling rather than in recognition. However, a growing body of evidence shows that the dichotomy between *EDS1* and *NDR1* requirement for TIR-NB-LRR and CC-NB-LRR is obsolete. Resistance to the turnip crinckle virus (TCV) mediated by the CC-NB-LRR *HRT* (HR to TCV infection) in Arabidopsis requires *EDS1* (Dempsey *et al.*, 1997; Chandra-Shekara *et al.*, 2004). Similarly, *RPS2*–mediated resistance to *Pst* DC3000 (AvrRpt2) is compromised in an *eds1 sid2* double mutant but not in the *eds1* or *sid2* (see later) single mutants (Venugopal *et al.*, 2009).

In contrast to EDS1 and NDR1, RAR1, SGT1 and HSP90 are involved in resistance mediated by both classes of NB-LRR R proteins. These three proteins interact with each other to form a dynamic chaperone complex targeting R proteins (Shirasu, 2009). The role of these chaperones is speculated to be to participate in the assembly, stability and accumulation of R proteins and to maintain intra- and inter-molecular interactions for specific signaling complexes (Shirasu and Schulze-Lefert, 2003; Belkhadir *et al.*, 2004; Lukasik and Takken, 2009). These chaperones are in association with the SCF (for SKP1, Cullin, F-box protein) ubiquitin ligase complex that mediates protein degradation via the 26S proteasome (Deshaies, 1999). SGT1 interacts with SKP1, one of the components of this complex (Kitagawa *et al.*, 1999), which could make the link for ubiquitination of HSP90 protein targets (Zhang *et al.*, 2008). This could involve degradation of improperly folded R proteins. Alternatively, recruitment of the ubiquitination complex could lead to the degradation of negative regulators of *R* gene resistance responses (Liu *et al.*, 2002c)

Important consecutive suppressor screens were carried out to identify components that function downstream of R protein activation. An ethyl methansulfonate (EMS) mutagenesis screen performed in Arabidopsis allowed the identification of SA induction deficient 2 (SID2) (Nawrath and Metraux, 1999). *SID2* encodes an isochorismate synthase involved in the SA biosynthesis (Wildermuth *et al.*, 2001). The *sid2* mutant accumulates less SA and is more susceptible to *Pst* DC3000 (EV), *Pst* DC3000 (AvrRpt2), *Pst* DC3000 (AvrRpm1) and *Hpa* (Nawrath and Metraux, 1999; Tsuda *et al.*, 2009). Non-Expressor of PR1 (NPR1) is a positive regulator of salicylic acid (SA)-mediated pathogenesis related (PR) gene expression such as *PR1* and *PR5* (Cao *et al.*, 1994). The SA is involved in defense responses triggered by biotrophic pathogens whereas the jasmonic acid (JA)-dependent responses are activated by necrotrophic pathogens (Thomma *et al.*, 2001). NPR1 localizes into the nucleus (Kinkema *et al.*, 2000) and seems to act as an activator of

transcription factor after SA-dependent cellular redox potential modification (Zhou *et al.*, 2000; Zhang *et al.*, 1999). The *npr1-1* mutant is impaired in *PR* gene expression and shows loss of SA pretreatment-dependent disease resistance to *P. syringae*. A genetic screen was conducted in order to identify suppressors of *npr1-1* phenotype (Li *et al.*, 1999). The identified *snc1-1* mutant (for Suppressor of *npr1-1*, Constitutive) shows a plant dwarf phenotype associated with leaf cell death, constitutive accumulation of SA and expression of PR1, and increased resistance against *H. arabidopsidis* and *P. syringae* (Li *et al.*, 2001). *SNC1* is a TIR-NB-LRR gene mapped in the *RPP4* gene cluster of Col-0. In the *snc1-1* mutant, a point mutation between the NB-ARC and the LRR domains renders SNC1 constitutively active (Zhang *et al.*, 2003). The *snc1-1*-mediated resistance pathway is EDS1-dependent supporting the role of EDS1 in TIR-NB-LRR signaling (Li *et al.*, 2001). Studies were conducted to identify additional loss-of-function mutations that suppress autoimmune responses triggered by snc1. Examples of several Modifiers of snc1-1 (MOS) genes were identified. *MOS2* encodes a nucleus localized protein that displays one G patch and two KOW motifs which bind RNA. *MOS3* encodes a protein with homology to human nucleoporin, *MOS6* an importin α and *MOS7* has homologies with human nucleoporin Nup88 suggesting the importance of cytoplasm-nucleoplasm trafficking for defense response activation (Zhang *et al.*, 2005; Palma *et al.*, 2005; Cheng *et al.*, 2009). The mutants *mos2-1*, *mos3-1* and *mos7-1* are compromised in *RPM1*-, *RPS4*- and *RPP4*-associated disease resistance but also in basal resistance. The role played by the ubiquitination pathway in *R* gene-mediated innate immunity has been underlined throughout the identification of the *mos5* mutant which carries a 15 bp deletion in UBA1, an ubiquitin-activating enzyme (Goritschnig *et al.*, 2007). In the *mos5* mutant only the *snc1*- and *RPS2*-mediated disease resistance is compromised suggesting that only certain R proteins are dependent on functional ubiquitination machinery for downstream signaling.

Plant immune components localize in specific cell organelles to realize their function (Padmanabhan and Dinesh-Kumar, 2010). The cytoplasm-nucleoplasm trafficking is a sophisticated process in plant signaling regulation and transcriptional reprogramming (Garcia and Parker, 2009; Caplan *et al.*, 2008). Cloning of *RRS1* revealed a classical nuclear localization signal (NLS) and a WRKY domain in its C-terminus (Deslandes *et al.*, 2002a). Its confirmed localization and interaction with PopP2 in the nucleus suggests a direct role in transcriptional reprogramming (Deslandes *et al.*, 2003). Nuclear accessibility is required for several cytoplasmic R proteins for defense response activation. The R proteins N and RPS4 display a classical NLS but present nucleo-cytoplamic localization in the absence of the pathogen. Upon perception of Avr protein, p50 and AvrRps4 respectively, their nuclear accumulation is required for defense activation (Burch-Smith *et al.*, 2007; Wirthmueller *et al.*, 2007). Similarly, SNC1 protein tagged with GFP is present mostly in the nucleus. In the *mos7-1* mutant, nuclear accumulation of SNC1-GFP is reduced which correlates with a loss of disease resistance (Cheng *et al.*, 2009). The EDS1-PAD4-SAG101 also transits through the nucleus and this translocation is required for *N*, *RPS4*, *snc1* and *Rx* to trigger defense responses. Similarly, MLA1 and MLA10 need to reach the nucleus for immune response activation even in absence of a classical NLS in their sequences (Shen *et al.*, 2007). The CC domain of MLA10 interacts with the transcription factor *Hv*WRKY1 which appeared to be a repressor of basal defenses (Shen *et al.*, 2007). WRKY1 binds DNA and sequesters the transcription factor MYB6, a positive reulator of defense, by direct interaction (Chang *et al.*, 2013). In barley, the CC-NB-LRR MLA10 recognizes the effector Avra10 from *B. graminis* (Ridout *et al.*, 2006). Data suggest that activation of MLA10 by Avra10 recognition triggers interaction of its CC domain with WRKY1. This interaction releases WRKY1 DNA binding as well as the WRKY1-MYB6 complex allowing MYB6 to activate defense (Chang *et al.*, 2013). This supports an emerging

model where R proteins interefere with transcriptional repressor or activator for defense genes regulation.

1.7 Dynamics in plant-pathogen interactions

Disease resistance in the host population places strong selection on pathogens to evolve new genotypes in order to avoid recognition by plants. Pathogens can acquire new effectors to restore virulence and counter ETI. The effector AvrPtoB from *P. syringae* suppresses the PCD initiated by the AvrPto-Pto recognition in *Nicotiana benthamiana* (Abramovitch *et al.*, 2003; Mucyn *et al.*, 2006). Another example is HopF2, an effector from *P. syringae*, which represses *RPS2*-mediated resistance after AvrRpt2 recognition. HopF2 targets RIN4 and inhibits AvrRpt2-mediated RIN4 degradation (Wilton *et al.*, 2010). This dynamic co-evolution in plant-pathogen interactions is represented in a “zigzagzig” model (Jones and Dangl, 2006). Development of new molecular and cellular strategies, known as “arm race” (Jones and Dangl, 2006; de Wit, 2007; Boller and He, 2009), will decide either plant or pathogen succeeds in the system. Hence, acquisition of a resistance/virulence factor by one protagonist will apply a selection pressure on the other to evolve and to circumvent the adaptation. Finally, plants and pathogens are in constant co-evolution.

1.8 The *RRS1-RPS4 R* gene system

1.8.1 The *AvrRps4-RPS4* gene-for-gene model

In 1996, Hinsch and Staskawicz identified *RPS4* (resistance to *Pseudomonas syringae* 4), a *R* gene in Ws-2 that confers disease resistance by recognizing the *Avr* gene *AvrRps4*, from *P. syringae* pathovar *pisi*. AvrRps4 is a protein of 221 amino acids carrying a N-terminal domain sufficient for effector delivery into plant cell via the TTSS (Sohn *et al.*, 2007). After secretion, AvrRps4 is

cleaved in between glycine 133 and glycine 134. AvrRps4 processing *in planta* requires a KRVY motif (amino acid position 135-138) which is essential for its avirulence function (Sohn *et al.*, 2009). Interestingly, AvrRps4 suppresses PTI and promotes *Pst* DC3000 growth in Arabidopsis Col-0 *rps4-2* (Sohn *et al.*, 2009). Recently, AvrRps4 C-terminal (amino acids 136-221) structure has been solved and revealed a 2 antiparallel α helices (Sohn *et al.*, 2012). Mutation of several amino acids present in negatively charged patches abolished AvrRps4 recognition in Arabidopsis. However, the mechanism of recognition remains unknown. It is still unclear if the AvrRps4 interaction with EDS1 is the trigger for RPS4 activation; EDS1 is proposed to be guarded by RPS4 (Bhattacharjee *et al.*, 2011; Heidrich *et al.*, 2011; Sohn *et al.*, 2012). *RPS4* was mapped on chromosome 5 of Arabidopsis using a cross between the resistant accession Ws-2 and the susceptible accession RLD to *Pst* DC3000 (*AvrRps4*). Structural characterization showed that *RPS4* is a 4109 bps gene comprising five exons and four introns, that encodes a predicted protein of 1217 amino acids with a molecular weight of 138 kiloDalton (kDa). RPS4 protein belongs to the TIR-NB-LRR family (Gassmann *et al.*, 1999). Transcriptional analysis showed that full length *RPS4* mRNA is produced in the susceptible RLD but amino acid substitutions in the sequence compared to Col-0 seem to render the protein inactive. The Col-0 and Ler RPS4 show polymorphism in the protein sequence but this doesn't appear to alter disease resistance. RLD complemented with Col-0 or Ler *RPS4* allele presents *Pst* DC3000 (*AvrRps4*) disease resistance (Gassmann *et al.*, 1999; Zhang and Gassmann, 2003). It has also been demonstrated that two unique amino acid changes, N195D and Y950H, are responsible for nonfunctional RPS4 in RLD. RT-PCR revealed unspliced *RPS4* mRNA variants at intron 2 and 3 predicted to generate shorter protein (563 and 660 aa respectively) due to in frame STOP codons (Gassmann *et al.*, 1999). Removal of intron 2, 3 and both together from the Ler *RPS4* allele transmitted to RLD abrogate

the *Pst* DC3000 (*AvrRps4*) disease resistance indicating that alternative *RPS4* transcripts are essential for resistance (Zhang and Gassmann, 2003).

An intriguing publication uncovered a resistance to *Pst* DC3000 (*AvrRps4*) in RLD (Kwon *et al.*, 2004). An ethyl methane sulfonate (EMS) mutagnenesis screen in RLD has permitted the identification of two suppressors of *rps4*-RLD (*srfr*). These *srfr1* and *srfr3* mutants are susceptible to *Pst* DC3000 but resistant to *Pst* DC3000 (*AvrRps4*) and this *Avr* recognition is *rps4*-RLD independent (Kwon *et al.*, 2004). They don't appear to be autoimmune mutants since no lesions were observable prior to infection, they are susceptible to *Pst* DC3000 WT and no constitutive expression of *PR1* were detected. Later publication showed both mutations in *srfr1* and *srfr3* were in the same gene and therefore renamed *srfr1-1* and *srfr1-2* respectively (Kwon *et al.*, 2009). Authors proposed SRFR1 as a negative regulator (transcriptional repressor) of AvrRps4-triggered disease resistance. Segregation analysis suggests the presence of a new dominant *R* gene in RLD recognizing *AvrRps4* but not able to overcome the threshold required to trigger resistance in the WT RLD background due to the SRFR repressors (Kwon *et al.*, 2004). Interestingly, map-based cloning of *snc1-5*, an Arabidopsis Col-0 autoimmune mutant, revealed a mutation in SRFR1 (therefore *snc1-5* was named *srfr1-3*) (Li *et al.*, 2010). This mutant showed higher accumulation of the SNC1 transcript and protein which in turn activates defense resulting in Arabidopsis dwarf phenotype. Similarly, RPS4 transcript and protein levels were higher in the *snc1-r1 srfr1-3* compared to Col-0 WT (Li *et al.*, 2010). Consistent with this result, *snc1-11 srfr1-4* double mutant showed enhanced resistance to *Pst* DC3000 WT and *Pst* DC3000 AvrRps4 (Kim *et al.*, 2010). Altogether, these data support a negative regulation role of SRFR1 on R proteins. Surprisingly, RLD *srfr1-1* mutant did not present an autoimmune phenotype which is proposed to be due to the absence of a functional SNC1 (Kim *et al.*, 2010). Differential AvrRps4-dependent HR phenotypes can be observed in

different Arabidopsis accessions. Leaf infiltration of *Pst* DC3000 (*AvrRps4*) triggers HR in Ws-0 and Ler but not in Col-0 (Gassmann et al., 1999; Gassmann 2005). The presence of a negative regulator of AvrRps4-dependent HR in Col-0 is suggested by the authors.

In an Agrobacterium transient assay, expression of *RPS4* triggers an *AvrRps4*-independent cell death in *Nicotiana tabacum* (Zhang *et al.*, 2004). This HR phenotype is dependent on *EDS1*, *SGT1* and *HSP90*. To assess the contribution to HR of each RPS4 domain, transient expression of truncated *RPS4* forms was performed. As a result, the TIR and the NB domain were indispensible to observe a HR. Also, the TIR-NB was the smallest truncated protein able to trigger an HR. Furthermore, Swiderski *et al*. (2009) reported that *RPS4*$^{TIR+80}$ (RPS4 exon 1 plus the first 80 amino acids from exon 2) transient expression triggers a rapid and strong cell death in *N. tabacum* (Swiderski *et al.*, 2009). An *A. thaliana* Col-0 *pTA7002::RPS4*$^{TIR+80-HA}$ stable line has been generated expressing *RPS4*$^{TIR+80-HA}$ under the control of a dexamethasone-inductible promoter (Swiderski *et al.*, 2009). Dexamethasone-induced *RPS4*$^{TIR+80-HA}$ expression triggers cell death in Col-0 in the absence of AvrRps4. Interestingly, RPS4$^{TIR+80-HA}$ triggers cell death in Col-0 whereas *AvrRps4* recognition by *RPS4* full length doesn't. Likewise, stable Col-0 lines overexpressing RPS4 exhibit a dwarf phenotype (Wirthmueller *et al.*, 2007). Therefore, the absence of HR to AvrRps4 in Col-0 could relate to the amount of R protein. Similarly to RPS4 full length, RPS4$^{TIR+80-HA}$ -triggered cell death is dependent on *EDS1*, *SGT1* and *HSP90* in *N. tabacum*. Substitution analysis in RPS4^{TIR+80} revealed different HR phenotype from loss to increase of cell death (Swiderski *et al.*, 2009). It is interesting to note that important substitutions for variation in cell death intensity are situated in the αD3-helix of the TIR domain, a structural component specific for plant TIR domains (Chan *et al.*, 2010; Bernoux *et al.*, 2011).

Research based on the characterization of RPS4-triggered immunity has been carried out. Following the prediction of an NLS in the C-terminus of RPS4, its subcellular localization was studied (Wirthmueller *et al.*, 2007). Proteomic analysis demonstrated pools of RPS4 in the cytoplasm and nucleus. Transient assays using YFP-RPS4 supports this nucleo-cytoplasmic localization. Interestingly, mutations in the NLS showed that RPS4 accumulation in the nucleus is required to confer resistance agains *Pst* DC3000 and to trigger cell death in *N. tabacum*. In the same study, they detected AvrRps4 predominantly in the nonnuclear fraction suggesting that AvrRps4 recognition occurs outside the nucleus (Wirthmueller *et al.*, 2007). However, no major relocalization of RPS4 into the nucleus was noticed after AvrRps4 recognition. Proteomic experiments showed that EDS1 is not required for RPS4 nuclear accumulation and localization which supports its role in active-R protein downstream signaling (Wirthmueller *et al.*, 2007). A recent study investigated the cellular compartmentation of AvrRps4 for recognition (Heidrich *et al.*, 2011). AvrRps4 fused to YFP displayed a nucleo-cytoplasmic distribution. It was reported that overexpression of AvrRps4 fused to a nuclear export signal (NES) partially abolishes the dwarf phenotype that normally is seen in Arabidopsis overexpressing AvrRps4 WT. Additionally, *Pst* DC3000 AvrRps4-NES growth was significantly increased compared to *Pst* DC3000 AvrRps4 in Col-0. Altogether, these results support that AvrRps4 recogniton takes place mostly in the nucleus. However, it is still unclear if AvrRps4 requires a cytosolic phase for protein processing and recognition.

1.8.2 The *PopP2-RRS1* gene-for-gene model

RRS1 (resistance to *Ralstonia solanacearum* 1) has first been described as a recessive TIR-NB-LRR providing resistance to *R. solanacearum* GMI1000 carrying PopP2 (Deslandes *et al.*, 1998; Deslandes *et al.*, 2002a; Deslandes *et al.*, 2003). The type three secreted effector PopP2 is a protein of 488 amino acids with

homology to the cysteine protease YopJ (Staskawicz *et al.*, 2001; Orth *et al.*, 2000). The catalytic core residue Cys321 is required for PopP2 autoacetylation and avirulence (Tasset *et al.*, 2010). PopP2 and RRS1 interact in the nucleus (Deslandes *et al.*, 2003). Remarkably, the RRS1 Col-5 allele does not activate defense despite its interaction with PopP2 suggesting that the interaction is not the only requirement for PopP2 recognition. Interestingly, the RRS1 Col-5 (RRS1-S) is 90 amino acids shorter than RRS1 Nd-1 (RRS1-R) which recognizes PopP2. However, the exact molecular mechanism of RRS1 activation by PopP2 is still unknown. An unusual feature of RRS1 is the presence of a C-terminal WRKY domain. WRKY domains are specific to transcription factors. This motif is able to bind DNA on a sequence called W box (TTGACC/T) (Rushton *et al.*, 1996). WRKY transcription factors regulate negatively or positively plant defense responses (Journot-Catalino *et al.*, 2006; Eulgem and Somssich, 2007). In a screen for loss of tolerance to low humidity, a mutant presenting necrotic lesions named *slh1* (sensitive to low humidity) was isolated (Noutoshi *et al.*, 2005). The *slh1* mutation corresponds of a Leucine insertion within the RRS1 WRKY domain. This insertion was proposed to disrupt proper RRS1 folding making it an autoactive form. Remarkably, the *slh1* WRKY domain shows significantly reduced DNA binding capacity (Noutoshi *et al.*, 2005). So far, the relevance of RRS1 WRKY domain DNA binding for immunity remains elusive. Interestingly, only three TIR-NB-LRR-WRKY encoding loci have been predicted in the Arabidopsis genome (*RRS1*, *At5g45050* and *At4g12020*). It is suggested that a specialized TIR-NB-LRR-WRKY R protein could allow a shortcut in the ETI pathway, from the *avr* sensing to the defence gene activation (Rushton *et al.*, 2010).

1.8.3 The multiple effector recognitions by the *RRS1-RPS4 R* gene pair

Recently, a role of *RRS1* in *AvrRps4*-triggered resistance has been reported (Narusaka *et al.*, 2009; Birker *et al.*, 2009). In Arabidopsis chromosome V, *RPS4*

and *RRS1* are arranged in an inverted head-to-head configuration. They are divergently transcribed sharing a short promoter sequence of 264 bp. Interestingly, they are both required for resistance against *Colletotrichum higginsianum*, *R. solanacearum* (*PopP2*) and *Pst* DC3000 (*AvrRps4*) (Narusaka *et al.*, 2009; Birker *et al.*, 2009). Pathogen growth studies using Ws-2 *rps4-21* and Ws-2 *rrs1-1* single mutants and Ws-2 *rsp4-21*/*rrs1-1* double mutant suggest that RRS1 and RPS4 function cooperatively for resistance (Narusaka *et al.*, 2009). A growing body of evidence shows that genetically linked or unlinked NB-LRRs are paired for function (Eitas and Dangl, 2010). The *RPP2A* and *RPP2B* are two adjacent TIR-NB-LRR–encoding genes on the Arabidopsis chromosome 4 (Sinapidou *et al.*, 2004). They are both required for resistance againt *Hpa* Cala2. In tobacco, the TIR-NB-LRR N requires the CC-NB-LRR NRG1 to recognize the tobacco mosaic virus protein p50 (Peart *et al.*, 2005). Interestingly, overexpression of RPS4 or NRG1 but not RRS1 or N triggers cell death in *N. tabacum* suggesting that RPS4 and NRG1 signal downstream of their respective pair partner (Zhang *et al.*, 2004; Collier *et al.*, 2011). In rice (*Orysa sativa*), the two genes *Os11gRAG4* and *Os11gRAG5* are in a head-to-head configuration and are both required for resistance against *Magnaporthe Oryzae* carrying *Avr-Pia* (Okuyama *et al.*, 2011). How paired R proteins cooperate is still unknown. It is hypothesized that the oligomerization of paired R proteins is required to form a functional complex for effector recognition and signaling activation. Supporting this hypothesis, recent unpublished results demonstrate that RRS1 and RPS4 TIR domains can dimerize and that RRS1 and RPS4 full length proteins can associate *in planta* (Williams, Sohn *et al*., unpublished data).

1.8.4 *RRS1-RPS4* independent AvrRps4 recognition

In three independent studies, bacterial growth analysis showed reduced *Pst* DC3000 (*AvrRps4*) growth in the *rps4* and *rrs1* mutants compared to *Pst* DC3000 WT (Narusaka *et al.*, 2009; Birker *et al.*, 2009; Kim *et al.*, 2010). Supporting this, an

HR was observed in Ws-2 *rps4-21* and *rrs1-1* mutants infiltrated with *Pseudomonas fluorescence* Pf0-1 (a non pathogenic *P. syringae* strain engineered to produce a TTSS) carrying AvrRps4 (Sohn *et al.*, 2012). This *RRS1*– and *RPS4*–independent AvrRps4 recognition (RRIR) is dependent on *EDS1*. Altogether, these observations suggest that AvrRps4 is recognized by an additional and presumably TIR-NB-LRR R protein(s) in Arabidopsis.

1.9 Aims of this thesis

Numerous studies support the important role of TIR-NB-LRR proteins in plant immunity. Several gene-for-gene relationships have been described involving TIR-NB-LRRs which provide disease resistance against pathogens. However, although some examples of CC-NB-LRR activation after indirect effector recognition are well studied, little is known about TIR-NB-LRR activation. The *AvrRps4/PopP2-RPS4/RRS1* genes-for-genes relationships have been extensively studied. This model is of interest to decipher TIR-NB-LRR involvement in bacteria-plant interactions. Despite much research effort, many essential mechanisms between *AvrRps4/PopP2* recognition and RPS4/RRS1-mediated plant defense activation remain obscure.

The main goal of this thesis is to better understand TIR-NB-LRR–mediated immunity in Arabidopsis. To lead this project, I have focused my research on the *RRS1-RPS4 R* gene pair. Based on the previous studies made in Prof. Jones's laboratory, I investigated $RPS4^{TIR}$ properties for defense activation (**Chapter 3**). Several data support the presence of a new *R* gene in Arabidopsis recognizing *AvrRps4* in a *RRS1*– and *RPS4*–independent manner. I have map-based cloned this additional *R* locus and revealed that it is another TIR-NB-LRR *R* gene pair (**Chapter 4**). Finally I focused on elucidating the functional mechanisms of RRS1-RPS4 compared to these new paired R proteins (**Chapters 5 and 6**).

2 Material and methods

2.1 Plant material

2.1.1 Plant growth

Arabidopsis thaliana, *Nicotiana benthamiana* and *Nicotiana tabacum* seeds were sown on F2 compost and vernalised for 7 days (dark, 4ºC). Seedlings were grown in growth chamber under controlled conditions: 21-23ºC; 10 h light / 14 h dark; 75% humidity for Arabidopsis; 21-23ºC; 16 h light / 8 h dark; 55% humidity for *Nicotiana sp*.. Two weeks old mature seedlings were individually transferred to fresh pots filled with compost mix for Arabidopsis (F2 compost supplemented with grit and systemic insecticide INTERCEPT) or F2 compost for *Nicotiana sp*.. Plants were grown in the same conditions as for seedlings as mentioned above.

2.1.2 *In vitro* seedling growth

Arabidopsis seeds were surface-sterilized for 4 h in a sealed chamber by chlorine gas (produced by mixing 100 mL of a 10% sodium hypochlorite solution with 3 mL of hydrochloric acid at 36% in a 250 mL becker) . Sterilized seeds were sown on Petri dishes (Sterilin) containing GM medium (for 1 L: 4.3 g MS salts, 0.1 g myoinositol, 0.59 g MES, 1 ml 1000X GM vitamin stock, 8 g Bacto agar, pH 5.7. 100 ml of 1000X GM contains 0.1 g thiamine, 0.05 g pyridoxine, 0.05 g nicotinic acid). GM medium was mixed with antibiotics according to the resistance of Arabidopsis transgenic lines used. Dexamethasone treatment was directly applied into the GM medium at the desired concentration by spreading it prior seeds sowing. Seeds plates were sealed with Micropore 3M tape and vernalised for 7 days (dark, 4ºC). Plates were then transferred in tissue culture growth chamber (16 h light, 24°C).

2.1.3 Arabidopsis mutants

The Arabidopsis T-DNA insertion lines used in this study were obtained from the SALK and INRA institutes. T-DNA insertions were confirmed by PCR using a specific primer of the T-DNA left bordure (LBb1.3 primer for SALK lines: 5'ATTTTGCCGATTTCGGAAC3'; LB4 primer for FLAG lines: 5'CGTGTGCCAGGTGCCCACGGAATAGT3') and a gene-specific primer. Gene expression knockout was confirmed by RT-PCR.

2.1.4 Arabidopsis stable transformation

Six to 7 week-old flowering Arabidopsis plants were used for stable transformation following the floral dip method (Clough and Bent, 1998). Arabidopsis flowers were dipped with an *A. tumefaciens* solution at optical density $_{600}$ (OD_{600})=0.5 (2.5 .10^8 colony forming unit (cfu)/mL) (the OD was measured at 600nm using a spectrophotometer, Eppendorf Bio Photometer plus). T1 seeds were recovered from dipped plants, sterilized as mentioned before and selected either on full Arabidopsis soil tray sprayed with phosphinothricin or on selective GM medium containing the appropriate antibiotics.

2.2 Plant pathology assay

2.2.1 Microorganisms used in this study

Strains	Pathovar	Designation	Details
Escherichia coli		DH10B	Used as recipient strain for cloning
Escherichia coli		HB101	Helper for triparental matings carrying pRK2013 plasmid.
Pseudomonas syringae	*tomato*	DC3000	Sequenced *Pst* strain. Rifampicin resistant. Recipient for triparental matings
Pseudomonas fluorescens		Pf0-1 (T3SS)	Non-pathogenic strain engineered with a functional type III secretion system. Chloramphenicol and tetracyclin resistant

Agrobacterium tumefaciens		Agl1	Rifampicin and carbenicillin resistant. Used for stable and transient transformation.
Alternaria brassicicola			Necrotrophic fungus causing black spot disease

2.2.2 Plant leaf infiltration

2.2.2.1 Bacterial cultures

Each bacterial strain was grown on solid or in liquid L medium (For 1 L: 10 g tryptone, 5 g NaCl, 1 g glucose, 5 g yeast extract, pH 7.0; for solid medium, 10 g agar was included) with the appropriate antibiotics. *E. coli* strains were grown in an incubator at 37°C, *Pseudomonas* and *Agrobacterium* strains at 28°C.

2.2.2.2 Arabidopsis infiltration with *Pseudomonas spp*.

Pseudomonas strains were streaked on fresh selective media and grown for 24 h for *P. fluorescens* and 48 h for *P. syringae*. To proceed to infiltration, bacteria were scraped from plate and resuspended into 1 ml of 10 mM $MgCl_2$. The optical density (OD) was measured using a spectrophotometer at 600nm (MBA 2000, Perkin Elmer) and then adjusted according to the type of experiment done (OD_{600}=0.0001 to 1.0,i. e. 5 $.10^4$ to 5 $.10^8$ cfu/mL). For infiltration, 5 to 6 week-old plants leaves were selected and the bacterial suspension was then infiltrated on the abaxial surface of the leaves using 1 ml syringes (Terumo) without needle.

2.2.2.3 Nicotiana sp. infiltration with Agrobacterium tumefaciens

Agrobacterium strains were streaked on fresh selective media and grown for 24 to 48 h. Single colonies were resuspended liquid media and cultured overnight in a shaking incubator (200 rpm, 28°C). Cultures were centrifuged at 3000 rpm for 10 min and bacteria were resuspended in 10 mM $MgCl_2$ supplemented with 10 mM

MES. The samples OD were measured and adjusted to OD_{600}=0.5 (2.5 .10^8 cfu/ml). *Nicotiana sp*. leaves abaxial surface were infiltrated with a needleless syringe at specific sites punctured using a needle.

2.2.3 Quantification of *in planta* pathogen growth

2.2.3.1 Estimation of *Pseudomonas syringae* growth

Arabidopsis leaf disks (each sample equalling 1 cm^2) were collected 3 days after inoculation with bacteria (OD_{600} = 0.001, 5 .10^5 cfu/mL) and then ground in water. Serial dilutions (10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) were then spotted on selective media. After 2 days incubation, bacterial colonies were counted according to the dilution spot and normalized in cfu/cm^2 of plant leaf.

2.2.3.2 Estimation of Alternaria brassicicola growth

A. brassicicola was propagated onto agar plates containing 10% V8® vegetable juice. Four to 5 weeks old Arabidopsis leaves were punctured with a needle and drop-inoculated with *A. brassicicola* suspension at 5 .10^5 conidia/ml at the puncture site. After inoculation, plants were placed in plastic trays covered with a transparent lid at room temperature. Infected leaves were collected 5 dpi and placed into conical centrifuge tube containing water (1ml/leaf). Tubes were shaked vigorously on vortex for 5 min to release conidia and 10 µL of the suspension were deposited on an haemocytometer (Neubauer) for conidia counting using a light microscope (Zeiss Axiophot or Leica DMR).

2.2.4 Ion leakage measurement

The hypersensitive response (HR) in plant is often accompagnied by programmed cell death. During this process, plant cells release their cytoplasmic fluids that contain ions. The measuremement of ion leakage was used to determine

quantitatively the cell death in Arabidopsis. Rapidly after infiltration with *Pf* Pf0-1 strains, Arabidopsis leaf disks were punctured and incubated in distilled water for 30 min. Tubes containing the leaf disks were gently shaken for 30 min. Leaf disks were then transferred into 24-well microtiter plates with 2 leaf disks per well containing 2 ml of distilled water. The conductivity was measured in each well using a conductivitymeter (Horiba, B-173) at specific time points. For each conditions, 4 replicates were tested.

2.3 Molecular biology

2.3.1 List of selective chemicals used in this study

Antibiotic	Stock concentration	Final concentration
Rifampicin	10mg/ml in methanol	100µg/ml
Chloramphenicol	10mg/ml in water	35µg/ml
Carbenicillin	100mg/ml in water	100µg/ml
Kanamycin	50mg/ml in water	25µg/ml
Gentamycin	10mg/ml in water	20µg/ml
Tetracyclin	5mg/ml in 50% ethanol	10µg/ml
Hygromycin	50mg/ml in water	50µg/ml
Spectinomycin	100mg/ml in water	50µg/ml
Phosphinothricin	5mg/ml in water	20µg/ml

2.3.2 List of plasmids used in this study

Designation	Details	Antibiotic resistance
pENTR/D/TOPO	GATEWAY entry vector for CACC directional cloning (Invitrogen)	Kanamycin

pCR8	GATEWAY entry vector for TA cloning (Invitrogen). Recipient for GOLDEN GATE cloning.	Spectinomycin
pBS46	GATEWAY destination vector for bacterial expression	Gentamycin
pVSP61	Broad host range bacterial expression vector	Kanamycin
pTA7002	Binary vector with dexamathasone inducible gene expression	Kanamycin Hygromycin *in planta*
pBGW	GATEWAY destination binary vector	Spectinomycin Phosphinotrycin *in planta*
pK2GW7	GATEWAY destination binary vector. Constitutive 35S gene expression.	Spectinomycin Kanamycin *in planta*
pBAV139	GATEWAY destination binary vector. Constitutive 35S gene expression and C-term HA fusion.	Kanamycin Phosphinotrycin *in planta*
pK7FWG2	GATEWAY destination binary vector. Constitutive 35S gene expression and C-term GFP fusion.	Spectinomycin Kanamycin *in planta*
pH7WGR2	GATEWAY destination binary vector. Constitutive 35S gene expression and N-term RFP fusion.	Spectinomycin Hygromycin *in planta*
pICH86988	GOLDEN GATE destination binary vector	Kanamycin Kanamycin *in planta*

2.3.3 Plant genomic DNA extraction

Arabidopsis genomic DNA (gDNA) was extracted with 3 different methods according to the quality of the gDNA required. The chelating resin Chelex 100 (BioRad) was used to extract quickly gDNA for single genotyping reaction. Briefly, a leaf disk was sampled using a corkborer Nº1 and placed in an Eppendorf tube with 50 µL of chelex powder diluted in water. The plant leaf disk was disrupted in the chelex using a pipette tip. The mixture was vortex 10 s, incubated at 100ºC for 5 min, vortex 10 s and finally centrifuged at 13000 rpm for 1 min. For DNA amplification, 1 µL of the supernatant was used in a polymerase chain reaction

(PCR). In order to purify good quality gDNA for long fragment amplification by PCR, the Epicentre MaterPure and Qiagen DNeasy kits were used. The plant leaf DNA was extracted following the protocols provided in the kits. One microliter of gDNA extract solution was used for PCR.

2.3.4 Polymerase chain reaction

All PCRs were realised using 10-100 ng DNA as template in 20 µL, 30 µL or 50 µL final volume. Each reaction contained: 1X PCR Taq buffer or Phusion buffer, 0.2 mM dNTPs, 5 U/µl Taq DNA polymerase (NEB) or 2.5 U/µl Phusion high-fidelity DNA polymerase (NEB), 10 µM of each primer. The DNA was amplified with successive cycles in a thermocycler (DNA engine PTC225, MJ Research). Cycles include DNA denaturation, primers annealing and elongation steps. Temperatures and times were optimised according to the primers and the length of the amplified product desired.

2.3.5 Plant RNA extraction

Plant tissue samples were collected in Eppendorf tubes and flash-frozen in liquid nitrogen. The plant tissues were quickly ground to a fine powder using a rotating drill pre-cooled in liquid nitrogen. Nine hundred mililiters of TriReagent (Sigma) was added to the powder and the mixture was incubated 5 min at room temperature to disrupt the cells. One hundred mililiters of Bromo-chloropropane was added to the solution. Tubes were shaken by hand and centrifuged at 11400 rpm for 20 min at 4°C. The supernatant was transferred into a new Eppendorf tube and 400 µL of isopropanol was added to the solution to precipitated nucleic acids. Tubes were centrifuged at 14000 rpm for 20 min, the supernatant was discarded and the pellet was washed with 70% ethanol. Tubes were centrifuged at 14000 rpm for 5 min, the supernatant was discarded, the pellet dried for 5 min and resuspended in 20-50 µL of RNase-free water. DNAse treatment was applied to the RNA solution

for 30 min at 37°C according to the DNase I RNase-free protocol (Roche). After treatment, 10% sodium dodecyl sulphate (SDS) and proteinase K were added to the RNA and the preparation was incubated for 15 min at 42°C. RNA were then purified using RNeasy MinElute cleanup kit (Qiagen) and eluted in RNase-free water. Total RNA were quantified using Nanodrop (Thermo scientific, UK).

2.3.6 Reverse Transcription-PCR

In order to obtain cDNAs, 1 µg of purified total RNAs was added in a final volume of 10 µL of RNAse-free water, placed at 70°C for 5 min and transferred on ice. This RNA solution was then mixed with 1 µL of oligodT [30 µM], 2µL dNTP [10 mM], 2 µL DTT [0.1 M], 4 µL of 5X SuperscritpII buffer, 0.5 µL of RNAsine and 0.5µL of Superscript II (Invitrogen). The reverse transcription reaction was operated for 1-1.5 h at 42°C and was followed by an inactivation step of 15 min at 70°C. The cDNAs solution volume was adjusted up to 50 µL and 1 µL was used as template for PCR.

2.3.7 DNA electrophoresis

Every need to check the presence and the length of DNA after experiment (PCR, digestion, DNA and RNA purification) have been realised on electrophoresis principle. DNA samples were loaded, mixed with 1X Orange G loading buffer (from 3X: 70 mL water, 30 mL glycerol, 2 mL 0.5 M EDTA, spatel full of Orange G powder Sigma-Aldrich), in gel containing 1-3% agarose diluted in TAE and ethidium bromide. DNA migration was carried out in electrophoresis tanks with TAE buffer during 10-30 min at 100 V. To estimate DNA fragment length, 100 bp or 1 kb DNA ladder (40 ng/µL; NEB) was also loaded in gel. Exposure of the gel to UV in a UV transilluminator (BIO-RAD) permitted DNA visualization after electrophoresis run.

2.3.8 Purification of DNA from agarose gel

DNA band of interest was visualized on an UV table, excised from the gel using a razor blade and put in an Eppendorf tube. The fragment was then purified using QIAquick spin columns (Qiagen). At final step, volume of elution buffer (12-30 µl) was optimised to have the DNA concentration wanted. Tube containing DNA was stored at -20ºC or directly processed.

2.3.9 DNA cloning

Genes were amplified by PCR and cloned into entry vectors (pENTR/D/TOPO or pCR8) according to the information provided by the manufacturer (Invitrogen). Vectors were transformed into competent cells by electroporation. Positive clones were confirmed by colony PCR and plasmid sequencing. Genes of interest were transferred from entry to destination vectors following the Gateway® (Invitrogen) or the Golden Gate (Engler *et al.*, 2008) technologies.

2.3.10 Gateway® cloning

The Gateway® technology allows the gene transfer by homolgous recombination realized by LR clonase II. The LR reaction was carried out following the procedure described by the manufacturer. Briefly, 150 ng of entry vector, 150 ng of destination vector and 1 µl of LR clonase II enzyme mix were mixed together. Samples were vortexed quickly and incubated at 25ºC for 1 h. Following this step, 0.5 µl of Proteinase K was added to the mixtures and the tubes were placed at 37ºC for 10 min to stop the reaction. Each LR reaction was desalted using sepharose column and transformed into competent cells.

2.3.11 Golden Gate cloning

The Golden Gate technology allows the specific assembly of DNA modules into destination vector by entry vector restriction with type II endonucleases (BsaI and BbsI) and specific modules ligation by T4 DNA ligase based on the 4 bp overhangs created during digestion. Briefly, each entry vector and destination vector were mixed together in a PCR tube at equivalent molecular units. The Golden Gate reaction was composed of the plasmid mix, 1X BSA, 1X T4 DNA ligase buffer, BsaI/BbsI, T4 DNA ligase. The reaction was carried out in a PCR themoblock with the successive steps: 25 times (37ºC for 30 s, 37ºC for 3 min, 16ºC for 4 min), 50º for 5 min, 80ºC for 5 min. Each reaction was desalted using a sepharose column and transformed into competent cells.

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

Plasmids were integrated into competent *E. coli* DH10B or *A. tumefaciens* AgI1 by electroporation. Tubes containing 50 µL of competent cells were thawed on ice directly from -80ºC stock. Electroporation cuvettes 1 mm gap were cooled down on ice for each cell tube. The ligation product was added and mixed with the competent cells with a tip and this mixture was then carefully added to the cuvette. Electroporation was performed using a cell porator (Gene Pulser Xcell, BIO-RAD) with these following conditions: voltage = 1800 V, capacitance = 25 µF, resistance = 200 Ω. Directly after electroporation, cells were recovered by the addition of 300 ml of L media and the incubation in a shaker for 1 h at 37ºC for *E. coli* and at 28 ºC for *A. tumefasciens*. Transformed cells were selected on L agar supplemented with the appropriate antibiotics.

2.3.13 Colony PCR

After cloning, transformants recovered were tested for the correct insert. Individual colonies were picked with a tip and resuspended in PCR tubes containing 50 µl of distilled water. This suspension served as template DNA in a PCR reaction. The amplification was realised using primers specific of the vector combined with primers specific of the cloned gene. Correct clones were grown in liquid culture overnight to be put in collection.

2.3.14 Plasmid purification

Overnight bacterial cultures were centrifuged 10 min at 4000 rpm. The bacteria pellet was used to extract and purify the plasmid (QIAprep Spin Miniprep Kit, Qiagen). Each plasmid prep was eluted in 30-50 µL and stored at -20ºC. Correct sequence of DNA insert was confirmed by sequencing carried out by the GATC Biotech company (http://www.gatc-biotech.com/en/index.html).

2.3.15 Triparental mating

Mating allows to transfer a plasmid carrying a gene of interest in other strains destinated to express it. In this study, matings were performed to transfer plasmid DNA from *E. coli* strains (donor) to *Pseudomonas* strains (recipient). This form of bacterial conjugation needs a third bacteria strain containing the helper plasmid pRK2013 which assist the transfer of the mobilizable plasmid. Overnight bacterial liquid cultures were mixed to a ratio of 3:3:1 (donor:recipient:helper). Each mix was spotted on L medium without antibiotics and incubated for 8 h at 28ºC. After incubation, cells were streaked on selective media and positive colonies were identified by colony PCR.

2.3.16 Plant protein extraction

Plant materials were collected in foil, flash-frozen and ground in a pre-cooled mortar with liquid nitrogen. The powder was rapidly tranferred into conical tube containing extration buffer (glycerol 10%, Tris-HCl pH 7.5 150 mM, EDTA 1 mM, NaCl 150 mM, DTT 10 mM, Nodinet-40 0.2%, Anti-protease tablet Complete EDTA-free RoChe, PVPP 2%) and tubes were placed horizontally on a shaker at 4°C for 20 min. The tubes were then centrifuged 20 min at 5.000 g at 4°C and the supernatant was filtered through Miracloth to a new conical tube. This protein extract was either mixted with 3X SDS loading buffer (glycerol 30%, SDS 3.3%, Tris-HCl 94 mM, bromophenol blue 0.05%, DTT 50 mM) for SDS-PAGE and immunoblot analysis or used for co-immunoprecipitation (Co-IP).

2.3.17 Co-immunoprecipitation

Protein extracts were mixed with agarose beads (anti-FLAG, Sigma; anti-HA, Roche; anti-GFP, Chromotek) previously washed from conservation buffer with GTEN buffer (glycerol 10%, Tris-HCl pH 7.5 150 mM, EDTA 1 mM, NaCl 150 mM). The Co-IP tubes were incubated on a rotator for 2-3 h at 4°C. Tubes were then centrifuged rapidly 3 times for 30 sec at 7000 rpm and placed on ice. Supernatant was discarded and the Co-IP beads were washed with washing buffer (glycerol 10%, Tris-HCl pH 7.5 150 mM, EDTA 1 mM, NaCl 150 mM, DTT 10 mM, Nodinet-40 0.2%, Anti-protease tablet). Tubes were inverted slowly up and down by hand and spinned down again as described above. After 3 washes, the remaining buffer was removed carefully by using a syringe with needle (0.5 x 25 mm Terumo). Samples were boiled for 5 min at 99°C with 3X SDS loading buffer to release the precipitated proteins from the beads. The IP samples were then analysed by SDS-PAGE and immunobloting.

2.3.18 Identification of protein using SDS-PAGE and immunobloting

Protein samples were loaded onto acrylamide gels at different concentration according to the size of the proteins to be detected. Gels are made of an upper acrylamide stacking phase (5% acrylamide) and a running phase (6-12% acrylamide). Proteins were separated by electrophoresis in presence of 1X SDS running buffer (from 10X SDS stock) at 90-150 V. Proteins were then electroblotted on to Immobilon PVDF membrane (Merck Millipore) using wet transfer. After transfer, unspecific sites were blocked by placing the membrane face up in a plastic box containing 5 % milk in TBST (Tris-Buffered Saline with 0.1% Tween) for 1 h at room temperature. Then the membrane was incubated with 1% milk in TBST containing HRP (Horseradish Peroxidase) conjugated antibody (anti-FLAG 1/10000, Sigma; anti-HA 1/2000, Santa Cruz Biotechnology; anti-GFP 1/10000, Santa Cruz Biotechnology) for 1 h at room temperature. The membrane was rinsed 3 times for 15 min in TBST and 1 time for 15 min in TBS. The membrane was finally incubated 5 min with protein face down on to developing reagent (Supersignal West Pico / West Femto, Thermo Scientific). Proteins of interest were visualized by radiography using X-ray film (Fuji) developed in SRX-101A tabletop processor (Konica Minolta).

2.3.19 Site directed mutagenesis

Nuclotide deletion was introduced in *RPS4B*^{RLD} using the QuickChange II XL Site-Directed mutagenesis kit (Agilent). Briefly, the method consist of designing ovelapping primer containing the desired mutation. A rapid 3-steps procedure consisting of mutant strand synthesis, digestion of template and transformation was performed to introduce the deletion in the wild type plasmid clone and restaure the *RPS4B*^{RLD} reading frame (*RPS4B*$^{\Delta RLD}$).

2.4 Cell biology

2.4.1 Sub-cellular protein localisation

Confocal microscopy was used to detect the subcellular localisation of transiently expressed protein fused to fluorescent tags. *A. tumefaciens*–mediated *Nicotiana sp*. transformation was performed for protein overexpression. Leaf disks were sampled from infiltrated area 2 dpi and mounted on microscopy slides with water. The subcellular localisation of proteins of interest was observed by confocal microscopy (Leica DM6000B/TCS SP5, Leica Microsystems). GFP–tagged proteins were observed after excitation of the samples at 488 nm and RFP-taggd proteins were observed after excitation of the samples at 561 nm.

3 Characterization of the RPS4^{TIR+80}–triggered cell death.

3.1 Introduction and objectives

Several *RPS4* truncations have been tested for their ability to trigger cell death in plant. When overexpressed in *N. tabacum*, full length *RPS4* was reported to trigger leaf tissue cell death (Zhang *et al.*, 2004). Among the different truncations tested, RPS4 exon 1 and the sequence encoding the 77 first amino acids (rounded up to 80) of exon 2 was the minimal fragment that triggered a quick and strong cell death when overexpressed in *N. tabacum* (Swiderski *et al.*, 2009). RPS4 exon 1 was at the time believed to encode the whole TIR domain. Therefore, this fragment was designated RPS4^{TIR+80}. After further analysis, I found that the RPS4 TIR domain expands onto the exon 2. Therefore, RPS4^{TIR+80} corresponds in fact to the TIR domain of RPS4. To test RPS4^{TIR+80}–triggered cell death in Arabidopsis, TIR+80 from *RPS4* was cloned into *pTA7002*, a vector allowing a dexamethasone–dependent transcription activation. Stable Col-0 *pTA7002::RPS4*$^{TIR+80}$ showed HR cell death 24 hours after dexamethasone treatment (Swiderski *et al.*, 2009). RPS4^{TIR+80} does not require the presence of its corresponding avirulence genes, like *AvrRps4* or *PopP2,* to trigger cell death. Therefore, RPS4^{TIR+80} is considered to be an autoactive form of RPS4. Moreover, because this truncated protein is sufficient to trigger cell death in plants, the RPS4 TIR domain is likely the domain that activates downstream signalling leading to cell death. This is supported by several reports of TIR domains from various TIR-NB-LRR proteins, RPP1, L6 and L10, that are sufficient to trigger cell death when overexpressed in tobacco or flax (Weaver *et al.*, 2006; Frost *et al.*, 2004; Bernoux *et al.*, 2011). TIR-NB-LRR *R* genes confer resistance to specific plant pathogens. However, the mechanism by which they activate plant defense remains unknown. Using TIR domains as surrogate may

provide an approach to study TIR-NB-LRR–mediated defense signaling. The aim of this chapter was to characterize the RPS4^{TIR+80}–triggered cell death in plants in order to provide new insights into molecular mechanisms underlying the TIR-domain activated plant defenses.

I choose to utilize Col-0 *pTA7002::RPS4*$^{TIR+80}$ as a tool to study the characteristics of RPS4^{TIR}–mediated immune responses activation in Arabidopsis. Therefore, I correlated over a time course RPS4^{TIR+80} transcript and protein accumulation with appearance of the cell death phenotype. This analysis provides the basis for future disease resistance, genetic, transcriptomic and proteomic studies.

A major goal of my project was to identify novel genetic components required for the RPS4^{TIR+80}–mediated cell death in Arabidopsis. For this purpose, a forward genetic suppressor screen was performed and I analysed Col-0 *pTA7002*::*RPS4*$^{TIR+80}$ mutants that were insensitive to the RPS4^{TIR+80} (itr). As metioned in Chapter 1, a suite of genes have been described in the literature to be required for TIR-NB-LRR–triggered immune responses. However, no signaling mechanisms linking *R*-protein activation and defense gene expression has yet been identified. I showed that in all the M3 itr mutants isolated, the absence of cell death correlated with the absence of the RPS4^{TIR+80} protein.

The majority of studies on TIR domain activity are based on cell death and defense gene activation. Therefore, I was interested to determine if the RPS4^{TIR+80} accumulation *in planta* was associated with resistance/susceptibility to different pathogens. I assessed the growth of *Pst* DC3000 and *Alternaria Brassicicola* on Col-0 *pTA7002*::*RPS4*$^{TIR+80}$ after dexamethasone treatment. My data showed that RPS4^{TIR+80}–triggered immune responses in Arabidopsis provide resistance to *Pst* DC3000 and susceptiblity to *A. brassicicola*.

Identification of the sub-cellular localization of RPS4^{TIR+80} to trigger cell death was also of my interest. It has previously been shown that RPS4–mediated cell death in *N. tabacum* requires a RPS4 nuclear localisation provided by a nuclear localisation signal (NLS) peptide on its C-terminal domain (Wirthmueller *et al.*, 2007). To assess if RPS4 TIR domain functions similarly, different constructs of *RPS4*$^{TIR+80}$ fused to a fluorescent protein and different cellular localization signals (nuclear export signal: NES; nuclear localization signal: NLS) were used to identify in which *N. tabacum* cellular compartment RPS4^{TIR+80} is able to trigger cell death. My data suggests that RPS4^{TIR+80} needs to be in the nucleus to trigger cell death.

RPS4 forms a head-to-head gene pair, and functions cooperatively with *RRS1* (Narusaka *et al.*, 2009; Birker *et al.*, 2009). Recent unpublished data demonstrated that the TIR domains of RPS4 and RRS1 homo and heterodimerize (Williams, Sohn, *et al*., unpublished). We were interested to test the properties of RRS1^{TIR} domain in combination with RPS4^{TIR}. We confirmed that RRS1^{TIR} is unable to trigger cell death in *N. tabacum*. Interestingly, using an *A. tumefaciens* co-infiltration of RRS1^{TIR} with RPS4^{TIR} and a RRS1-RPS4$^{TIR-TIR}$ fusion protein, we showed that the RRS1^{TIR} domain suppresses RPS4^{TIR}–triggered cell death in *N. tabacum*.

3.2 Results

3.2.1 Accumulation of RPS4^{TIR+80} triggers cell death and induces *PR1* gene expression in Arabidopsis.

To characterize RPS4^{TIR}–mediated immune response activation in Arabidopsis, a Col-0 stable transgenic line expressing *RPS4*$^{TIR+80}$, tagged with hemagglutinin (HA) and under the control of a dexamethasone-inducible promoter (Col-0 *pTA7002::RPS4*$^{TIR+80-HA}$), was generated previously in the laboratory (Swiderski *et al.*, 2009). I used this transgenic line as a tool to study TIR domain-mediated cell death in plants. In order to define precisely the effects of *RPS4*$^{TIR+80}$ expression in Arabidopsis, the cell death phenotype, gene expression and protein accumulation were assessed over time. Seeds were germinated and grown 7-10 days on GM medium only for Col-0 wild type (WT) and GM medium containing hygromycin at 40 µg/ml for Col-0 *pTA7002::RPS4*$^{TIR+80-HA}$*.* Seedlings were then transferred onto GM medium supplemented with 40 µM of dexamethasone (DEX). Plant tissue were sampled at specific time points following transfer.

Col-0 *pTA7002::RPS4*$^{TIR+80-HA}$ seedlings showed cell death 24 hours to 48 hours after DEX treatment characterized by chlorosis of the cotyledons (**Figure 3.1A**). The chlorosis extended over the whole seedling 5 days after DEX treatment on Col-0 *pTA7002::RPS4*$^{TIR+80-HA}$ seedlings, whereas Col-0 WT seedlings were perfectly viable. Chlorosis is not a typical HR symptom, as usually, HR is associated with tissue collapse.

In an RT-PCR the transcript accumulation of *RPS4*, *RPS4*$^{TIR+80-HA}$ and the controls *PR1* (*Pathogenesis related 1*) and *RPL4* (*Ribosomal Protein L4*) was analysed. Col-0 *pTA7002::RPS4*$^{TIR+80-HA}$ mRNA showed an increase in *RPS4*$^{TIR+80}$ transcript accumulation from 15 minutes after DEX treatment until 2h, followed by

an apparent stabilisation until 72H (**Figure 3.1B**). I then assessed the impact of *RPS4*$^{TIR+80}$ transcript accumulation onto the expression level of RPS4. *RPS4* showed variable expression during the time course. *RPS4* expression can be induced by biotic stress based on the digital northern database (Zimmermann *et al.*, 2004; Toufighi *et al.*, 2005). The assessment of *RPS4* expression profile in this time course should be repeated to draw any conclusion. I then checked *PR1* expression as it is often used as a marker of salicylic acid (SA)–dependent defense (Glazebrook, 2005). *PR1* transcript was detectable 8 hours after DEX treatment suggesting activation of SA-related defense responses. A decrease of expression was noticed at 72 h for all the tested genes, including the control gene *RPL4*.

In parallel, total proteins were extracted for the same time points following DEX treatment (**Figure 3.1C**). Western blot analysis using a HRP–conjugated anti-HA antibody revealed an increasing accumulation of RPS4^{TIR+80} from 2 h to 8 h after DEX treatment. A significant accumulation of the *RPS4*$^{TIR+80}$ transcript was visible by RT-PCR from 1 hour after DEX treatment and the RPS4^{TIR+80} protein accumulated significantly from 4 hours onwards. However, PR1 expression was detected only 8 hours after DEX treatment. This suggests that defense mechanisms were activated by RPS4^{TIR+80} between 4 and 8 hours after DEX treatment. This indicates a time window that could be investigated to decipher the signaling events leading to defense activation.

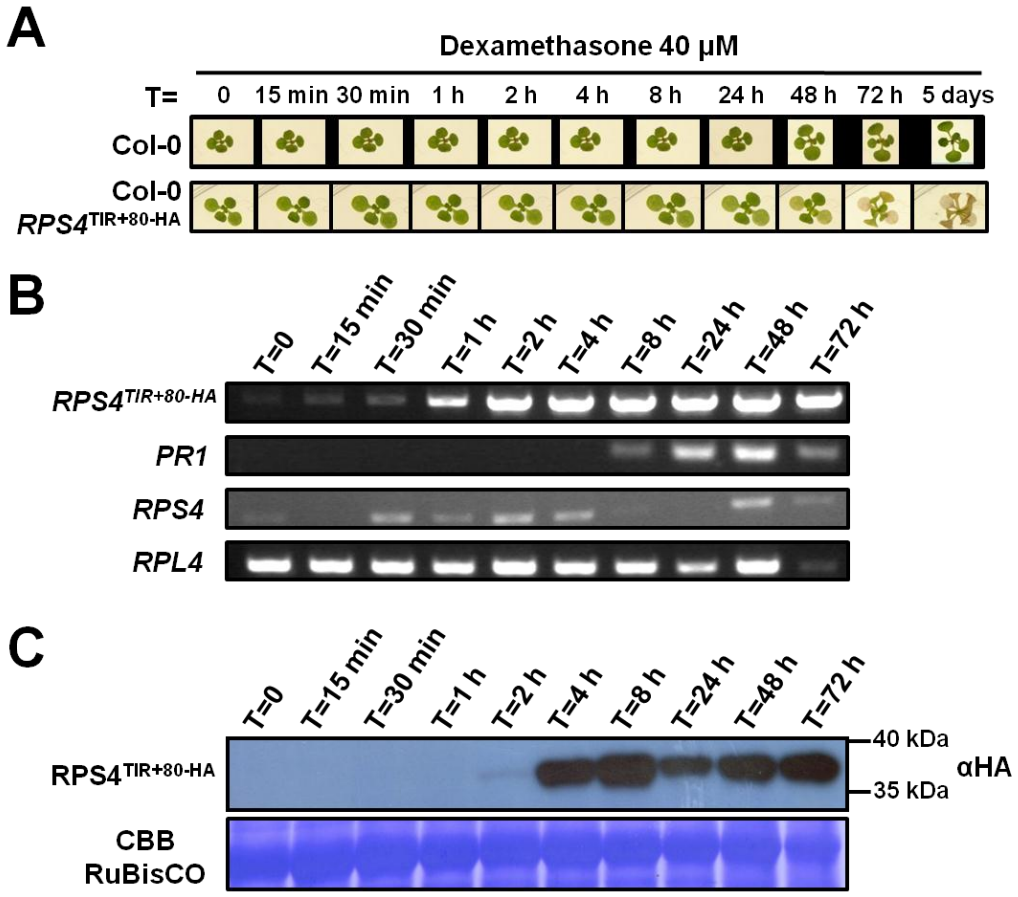

Figure 3.1. Expression and accumulation of RPS4^{TIR+80} induces *PR1* gene expression and cell death in Arabidopsis accession Col-0.

A. Phenotype of Col-0 *pTA7002::RPS4$^{TIR+80-HA}$* seedlings at different time points (t=0, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 24 h, 48 h, 72 h, 5 days) after transfer on GM supplemented with 40 µM dexamethasone. **B**. Expression of *RPL4*, *RPS4^{TIR+80}*, *RPS4*, *PR1* genes in Col-0 *pTA7002::RPS4$^{TIR+80-HA}$* seedlings at different time points (t=0, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 24 h, 48 h, 72 h, 5 dpi) after transfer on GM supplemented with 40 µM dexamethasone. **C**. Western blot showing RPS4^{TIR+80} accumulation in Col-0 *pTA7002::RPS4$^{TIR+80-HA}$* seedlings at different time points (t=0, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 24 h, 48 h, 72 h, 5 dpi) after transfer on GM supplemented with 40 µM dexamethasone. The RPS4^{TIR+80} was fused to an HA (Hemagglutinin) epitope tag and detected with an HRP-conjugated anti-HA antibody. Experiments **B** and **C** have not been replicated.

3.2.2 Analysis of an Arabidopsis EMS mutant population impaired in RPS4^{TIR+80}–triggered cell death.

To further identify novel genetic components that are required for the RPS4^{TIR+80}–mediated cell death in Arabidopsis a forward genetic suppressor screen was performed. Stable T2 Col-0 *pTA7002*::*RPS4*$^{TIR+80}$ seeds were mutagenized with ethyl methanesulfonate (EMS) by a post-doc in the laboratory, Kee Sohn, and I started analyzing M3 mutants which were insensitive to RPS4^{TIR+80} (*itr*). The chosen approach was to cross these *itr* mutants to Arabidopsis *Landsberg erecta* (Ler) accession in order to map-based clone genes required in RPS4^{TIR+80} signaling. I choose Ler accession as it has been used with Col-0 in the past at the TSL for mapping and many molecular markers were available.

Out of the Col-0 *pTA7002*::*RPS4*$^{TIR+80}$ EMS mutagenesis screen, 61 *itr* M3 putative mutants that lost the RPS4^{TIR+80} phenotype were obtained by Kee Sohn. All of them were crossed to Ler in order to generate a segregating population for the mapping. F1 seedling phenotypes were analyzed after transfer onto GM medium supplemented with DEX. The expected restored cell death phenotype (for a recessive mutation) in the F1 populations was not observed except for three crosses with *itr* 46, *itr* 47 and *itr* 81. Western blot analysis of F1 plants revealed presence of RPS4$^{TIR+80-HA}$ protein only in F1 *itr* 46, *itr* 47 and *itr* 81. In parallel, RPS4$^{TIR+80-HA}$ protein detection was performed in all M3 *itr* mutants. Surprisingly, RPS4$^{TIR+80-HA}$ was only detected in *itr* 81 (data not shown). Therefore, I decided to test again the M3 *itr* 46, *itr* 47 and *itr* 81 for absence of cell death phenotype and RPS4$^{TIR+80-HA}$ protein accumulation after DEX treatment. Re-testing these M3 *itr* lines revealed that they were not homogeneous for suppression of cell death phenotype. Only a few seedlings per line died 5 days after DEX treatment (**Figure 3.2A**). To investigate further, I tested the presence of cell death phenotype with RPS4$^{TIR+80-HA}$ protein accumulation in 6 plants for each of the *itr* 46, *itr* 47 and *itr* 81

lines after DEX treatment (**Figure 3.2B**). Altogether, these data demonstrated that the absence of cell death in the tested plants correlates with the absence ot RPS4$^{TIR+80-HA}$ protein accumulation. It would have been necessary to determine if these mutants were impaired in RPS4$^{TIR+80-HA}$ protein stability or if *RPS4$^{TIR+80-HA}$* was subjected to silencing in M1 or M2. Unfortunately, this was not tested as our aim was to identify downstream signaling components. We concluded that the itr mutant phenotype likely arose due to silencing of the transgene, rather than due to an interesting mutation in a signaling gene.

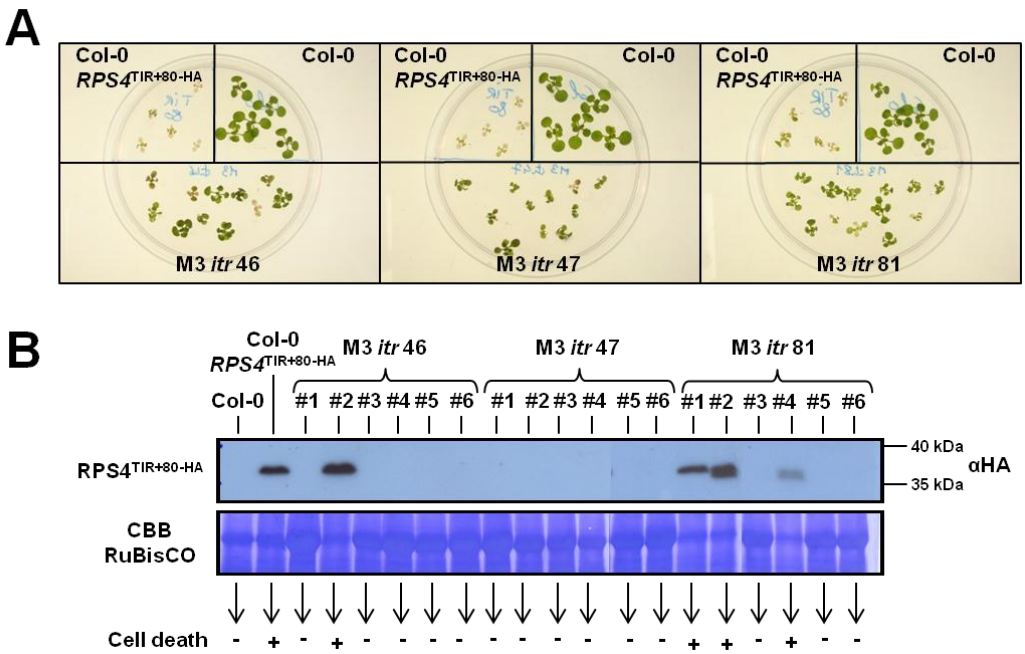

Figure 3.2. The suppression of RPS4^{TIR+80}-triggered cell death in M3 generation of insentive to RPS4^{TIR+80} (itr) mutants correlates with the absence of RPS4$^{TIR+80-HA}$ protein.

A. Phenotype of Col-0, Col-0 *pTA7002::RPS4$^{TIR+80-HA}$* and three different M3 *itr* mutants (lines 46, 47, 81) seedlings, 5 days after transfer on GM supplemented with 40 µM dexamethasone. **B**. Western blot showing RPS4$^{TIR+80-HA}$ protein accumulation in 5 week-old M3 *itr* mutants infiltrated with 40 µM dexamethasone. Protein detection has been performed using an HRP-conjugated anti-HA antibody. Arrows indicate the cell death phenotype observed in each of the corresponding plants after dexamethasone treatment.

3.2.3 The overexpression of RPS4^{TIR+80} in Arabidopsis provides resistance to the hemibiotroph *Pst* DC3000 and susceptiblilty to the necrotroph *Alternaria brassicicola*.

Given that the activation of RPP1A^{TIR}–triggered defense responses has been shown to provide resistance to several *Hpa* isolates but also to *Pst* DC3000 in Arabidopsis (Weaver *et al.*, 2006), I tested whether RPS4^{TIR+80} accumulation was also associated with resistance/susceptibility to different pathogens. I chose two different pathogens with different modes of infection to perform this experiment. *Pst* DC3000 is an hemibiotroph pathogen originally isolated from tomato which is able to infect Arabidopsis (Whalen *et al.*, 1991; Katagiri *et al.*, 2002). *Alternaria brassicicola* is considered as a necrotrophic pathogen that infects many cultivated Brassica species but is unable to infect Arabidopsis (Thomma *et al.*, 1998). Originally, *RPS4* was identified to provide resistance to the biotrophic pathogen *Pseudomonas syringae* carrying AvrRps4 (Hinsch and Staskawicz, 1996). RPS4^{TIR+80}–triggered immune responses are associated with cell death which is characteristic of resistance against biotroph pathogens. Therefore, our hypothesis was that accumulation of RPS4^{TIR+80} in Arabidopsis would arrest *Pst* DC3000 growth, and on the contrary, would elevate susceptibility to *A. brassicicola*.

According to Swiderski *et al*. (2009), leaf tissue collapse was visualized in Col-0 *pTA7002::RPS4*$^{TIR+80-HA}$ 24 hours after DEX treatment (Swiderski *et al.*, 2009). In my hands, DEX infiltration in 5 week-old Col-0 *pTA7002::RPS4*$^{TIR+80-HA}$ leaves triggered mostly chlorosis but also tissue collapse 5 days post infiltration (**Figure 3.3A**). To prevent *RPS4*$^{TIR+80-HA}$-triggered cell death and cholorosis phenotype from interfering with the infection experiments, I decided to use three different DEX concentrations: 0, 1 and 10 µM (**Figure 3.3A**). I could observe leaf chlorosis with DEX treatment at 1 µM.

To test the effect of RPS4^{TIR+80} on *Pst* DC3000 growth, leaves of Arabidopsis Col-0 WT and Col-0 *pTA7002::RPS4*$^{TIR+80-HA}$ were infiltrated with a MgCl2 solution supplemented with different DEX concentrations and *Pst* DC3000 (5 .10^4 cfu/ml). *Pst* DC3000 growth was quantified 3 days post infiltration by colony count. In Col-0 WT, the different DEX concentrations used did not affect *Pst* DC3000 growth (**Figure 3.3B**). In addition, *Pst* DC3000 growth in Col-0 and Col-0 *pTA7002::RPS4*$^{TIR+80-HA}$ was identical in absence of DEX, indicating that the transgenic line behaves as WT (**Figure 3.3B**). However, Col-0 *pTA7002::RPS4*$^{TIR+80-HA}$ plants supported significantly less *Pst* DC3000 growth than Col-0 WT after DEX treatment at 1 and 10 µM (**Figure 3.3B**). This suggests that RPS4^{TIR+80}–triggered immune responses are effective against the hemibiotrophic pathogen *Pst* DC3000.

In order to estimate the growth of *A. brassicicola*, I developed two methods. I created a disease index based on the symptoms observed on Arabidopsis leaves; 0, no symptoms; 1, lesion restricted at the infection site; 2, lesion covers less than 25% of the leaf; 3, lesion expands between 25 and 50% of the leaf with appearance of mycelium; 4, lesion is present between 50 and 75% of the leaf with mycelium; 5, lesion spread from 75% to the entire leaf with mycelium (**Figure 3.3C**). *A. brassicicola* shows a limited growth and is mostly non pathogenic on Arabidopsis. The maximum of disease severity observed on Col-0 WT plants was index 3. However, I observed up to the index 5 of disease severity in Col-0 *pTA7002::RPS4*$^{TIR+80-HA}$ treated with DEX. In parallel, I established a quantitative method based on conidia counting carried out with a light microscope.

Four days after infection, Col-0 WT treated or non treated with DEX showed absence of successful infection, with approximately 80% of the leaves (15 leaves tested in total) with a disease index of 0 and 1 (**Figure. 3.3D**). This

observation correlates with similar amount of conidia counted in these conditions (**Figure 3.3E**). This suggests that DEX treatment does not alter *A. brassicicola* growth on Col-0. In absence of DEX, Col-0 and Col-0 *pTA7002::RPS4*$^{TIR+80\text{-}HA}$ plants exhibited weak symptoms with similar low abundance of conidia (**Figure 3.3D** and **E**) indicating again that the transgenic line behave as WT. In contrast, Col-0 *pTA7002::RPS4*$^{TIR+80\text{-}HA}$ plants showed an increase of symptoms and conidia abundance compared to Col-0 WT after DEX treatment at 1 and 10 µM (**Figure 3.3D** and **E**). This suggests that RPS4^{TIR+80}–triggered defense responses benefit a necrotroph pathogen like *A. brassicicola*.

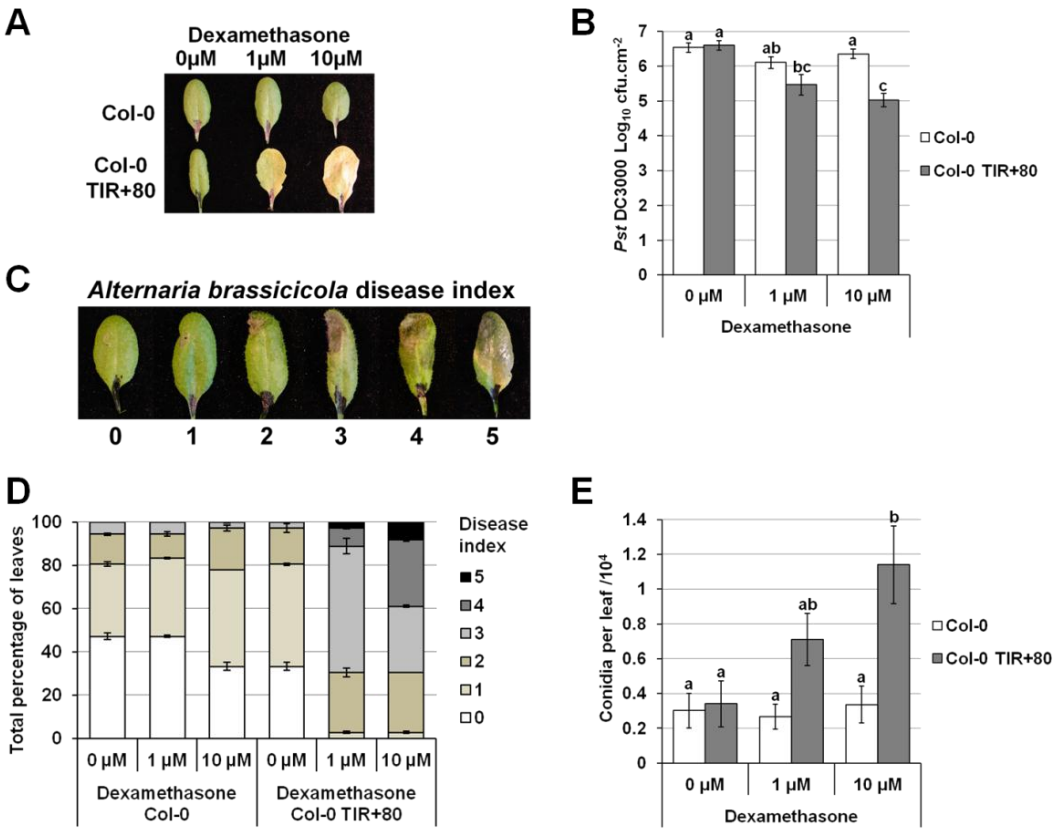

Figure 3.3. RPS4^{TIR+80}-triggered HR in Col-0 decreases growth of the hemibiotroph *Pst* DC3000 and increases growth of the necrotroph *A. brassicicola*.

A. Phenotype of 5 week-old Col-0 and Col-0 *pTA7002::RPS4$^{TIR+80-HA}$* (Col-0 TIR+80) leaves 5 days after dexamethasone infiltration (0, 1 or 10 µM). **B**. *Pst* DC3000 growth at 3 days post infiltration (5.10^4 cfu/ml; OD_{600}=0.0001) in 5 week-old Col-0 and Col-0 *pTA7002::RPS4$^{TIR+80-HA}$* pre-infiltrated with dexamethasone (0, 1, 10 µM). Experiment has been repeated three times with similar results. Samples labelled with the same letter are not statistically different at the 5% confidence level based on Tukey's test. **C**. Visual assessment of disease severity caused by *A. brassicicola* 4 days after drop inoculation (5.10^5 conidia/ml) on 5 wek-old Arabidopsis leaves. The disease index (DI) is based on a 0 to 5 score describing the disease symptoms: 0, no symptoms; 1, lesions are restricted at the infection site; 2, lesions cover less than 25% of the leaf; 3, lesions cover between 25 and 50% of the leaf with apparition of mycelium; 4, lesions cover between 50 and 75% of the leaf with mycelium; 5, lesions cover more than 75% to the entire leaf with mycelium. **D**. *A. brassicicola* growth in 5 week-old Col-0 and Col-0 *pTA7002::RPS4$^{TIR+80-HA}$* plants (18 leaves tested) at 4 days post inoculation (5.10^5 conidia/ml) according to the disease index and different treatments of dexamethasone (0, 1 or 10 µM). **E**. *A. brassicicola* conidia number per leaf at 4 days post inoculation in 5 week-old Col-0 and Col-0 *pTA7002::RPS4$^{TIR+80-HA}$* infiltrated with dexamethasone (0, 1, 10 µM). Experiment has been repeated two times with similar results. Samples labelled with the same letter are not statistically different at the 5% confidence level based on Tukey's test.

3.2.4 Mis-localized RPS4^{TIR+80} have different ability to trigger cell death in *Nicotiana tabacum*.

Determining protein subcellular localization can give important clues towards understanding in which subcellular compartment they function. Despite a lack of information on TIR-NB-LRR–associated downstream signaling components, their mode of function seems tightly linked to their presence in the nucleus (Deslandes and Rivas, 2011). For instance the TIR-NB-LRR N protein requires a nuclear localization to trigger defense after the TMV virus replicase p50 recognition (Burch-Smith *et al.*, 2007). Similarly, RRS1 localized in the nucleus in the presence of PopP2 (Deslandes *et al.*, 2003). Overexpression of *RPS4-YFP* triggers cell death in *N. tabacum* and this requires a functional C-terminal nuclear localization peptide (Wirthmueller *et al.*, 2007). In order to test whether RPS4^{TIR+80}–triggered cell death in *N. tabacum* requires a nuclear localisation of the RPS4^{TIR+80} protein, I constructed a fusion of RPS4^{TIR+80} with FLAG-GFP in the binary vector *pK2GW7*. As described by Swiderski *et al*., (2009) the *A. tumefaciens*–mediated transient expression of RPS4$^{TIR+80\text{-}FLAG\text{-}GFP}$ triggered cell death in *N. tabacum* 3 to 5 days post infiltration. Leaf disks were sampled at 2 days post infiltration for confocal microscopy analysis. A nuclear-cytoplasmic distribution of RPS4$^{TIR+80\text{-}FLAG\text{-}GFP}$ was observed (**Figure 3.4**). In order to understand if the nuclear pool of RPS4$^{TIR+80\text{-}FLAG\text{-}GFP}$ was required for activation of cell death, I introduced different cellular localization peptides to RPS4$^{TIR+80\text{-}FLAG\text{-}GFP}$. Interestingy, the nuclear export signal (NES) excluded RPS4$^{TIR+80\text{-}FLAG\text{-}GFP}$ from the nucleus and abolished the activation of cell death (**Figure 3.4**). To corroborate this results, when fused to a nuclear import signal (NLS), RPS4$^{TIR+80\text{-}FLAG\text{-}GFP}$ was detected mainly in the nucleus and the cell death observed was quicker and stronger than the one observed with RPS4$^{TIR+80\text{-}FLAG\text{-}GFP}$ (**Figure 3.4**). RPS4$^{TIR+80\text{-}FLAG\text{-}GFP}$ carrying mutated NLS and NES (named ‘nls’ and ‘nes’ respectively) showed similar localization and cell death phenotype to the

RPS4$^{TIR+80-FLAG-GFP}$ protein. Western blot analysis of fusion proteins is required to confirm the interpretation of these observations. However, the results obtained support that, like RPS4, RPS4^{TIR+80} triggers cell death through its activity in the nucleus.

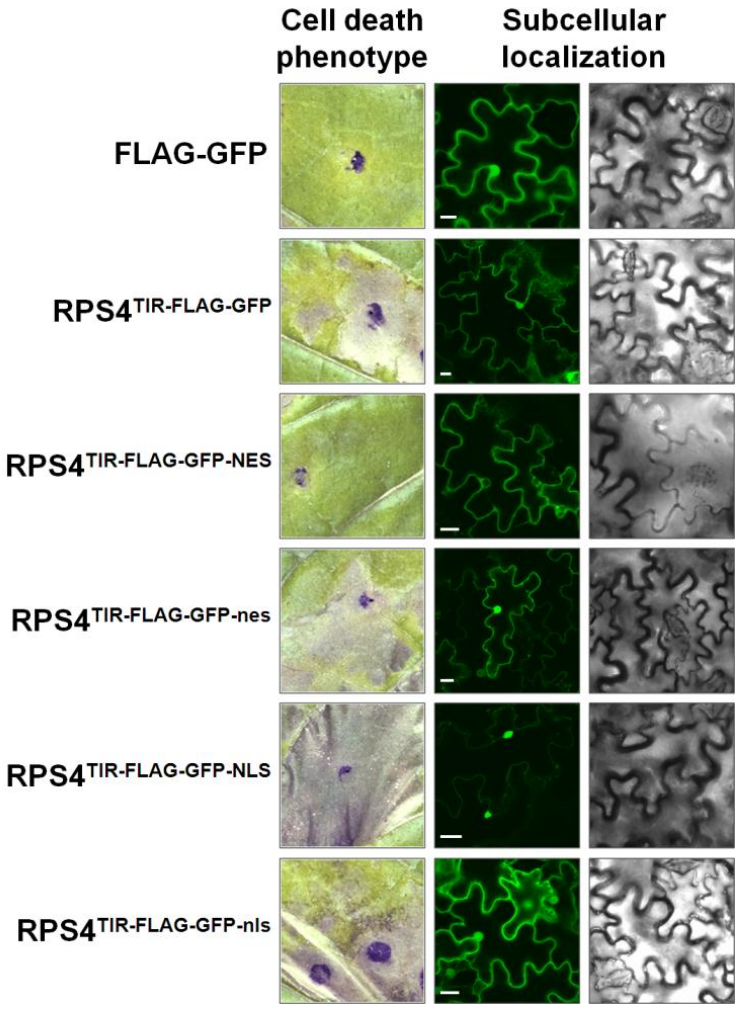

Figure 3.4. The TIR domain of RPS4 requires a nuclear localization to trigger cell death in *N. tabacum*.

Cell death phenotype (left panels) and subcellular localization (centre and right panels) of transiently delivered RPS4^{TIR+80} fused to FLAG-GFP epitope tag and different localization peptides: NES, Nuclear Export Signal (LQLPPLERLTL); nes, mutated version of NES (LQAPPAERATL); NLS, Nuclear Localization Signal (MTAPKKKRK); nls, mutated version of the NLS (LTLLLWSSH). *N. tabacum* leaves were infiltrated with *A. tumefaciens* solution at 2.5.10^8 cfu/ml (OD_{600}=0.5). Images of cell death were taken at 3 days post infiltration and the subcellular localization were assessed using a confocal microscope at 2 days post infiltration. The central panels show the GFP fluorescence and the right panels the bright field. Experiment has been repeated at least three times with similar results. Scale bar = 20 µm.

3.2.5 The RRS1 and RPS4 TIR domain heterodimer complex is unable to trigger cell death in *N. tabacum*.

The N-teminal domains of NB-LRR R proteins are likely to be platforms assembly of protein complexes that activate downstream signaling. In one model, the intramolecular interaction taking place within the R protein in the absence of avirulent effector represses defense activation by the N-terminal domain (Takken *et al.*, 2006). Effector perception by the R protein would disrupt this “off-state” intramolecular configuration, releasing the N-terminal domain. Several biochemical and stuctural studies suggest that R protein N-terminal domains form homodimers for downstream signaling activation (Bernoux *et al.*, 2011; Maekawa *et al.*, 2011).

RPS4 and *RRS1* are both required for the recognition of AvrRps4, PopP2 and a putative effector from *C. higginsianum* (Narusaka *et al.*, 2009; Birker *et al.*, 2009). Recent unpublished data, partially generated in the Jones lab, demonstrated that the TIR domains of RPS4 and RRS1 can form homodimers and heterodimers (Williams, Sohn, *et al*., unpublished). Interestingly, RRS1 and RPS4 TIR domains were co-crystallized using a RRS1-RPS4$^{TIR-TIR}$ fusion protein and the heterodimer interface appeared to be the same as the one identified for homodimerisation (Williams, Sohn, *et al*., unpublished). Using a site-directed mutagenesis approach it was shown that RPS4^{TIR} mutated in dimer interface amino acids loses homo- and heterodimerization and this was correlated with absence of cell death in *N. tabacum* (Williams, Sohn, *et al*., unpublished). In addition, authors showed that RPS4 and RRS1 full length proteins can associate *in planta*. We postulated that, RRS1 and RPS4 interact to cooperate for effector recognition. Therefore, we were interested to test the properties of the RRS1 TIR domain in combination with RPS4 TIR domain for activation of cell death.

I performed the following experiment in collaboration with Yan Ma, a PhD student in the laboratory. We amplified *RRS1*^{TIR} and *RPS4*^{TIR} from Ws-2 gDNA. Using the Golden Gate method, we assembled these TIR domains with a GFP or His, Flag (HF) tag into the overexpression binary vector *pICH86988*. Transiently co-expressed with FLAG-GFP, RPS4$^{TIR-GFP}$ triggered a strong cell death in *N. tabacum* (**Figure 3.5A**). However, RRS1$^{TIR-GFP}$ did not trigger any cell death in *N. tabacum* 5 days post infiltration. Interestingly, RPS4$^{TIR-GFP}$–triggered cell death was suppressed in the presence of RRS1$^{TIR-GFP}$ or RRS1^{TIR-HF}. This indicates that RRS1^{TIR} might repress RPS4^{TIR}–triggered cell death. Therefore, we were interested to test the RRS1-RPS4$^{TIR-TIR}$ fusion used to crystallise the heterodimer, for its ability to trigger cell death. Supporting our previous observation, when transiently overexpressed, the RRS1-RPS4$^{TIR-TIR}$ fusion did not trigger cell death 5 days post infiltration. Here, we tested the exact RRS1-RPS4$^{TIR-TIR}$ fusion amino acid fragment corresponding to the construct used for crystalization the heterodimer (Williams, Sohn, *et al*., unpublished). For biochemical purposes, several amino acids from the N-terminal parts of RRS1 (1 to 5) and RPS4 (1 to 9) TIR domains were deleted in this fusion protein, in order to enable crystal production (Williams, Sohn, *et al*., unpublished). To test if these missing amino acids were the cause of the inability of the RRS1-RPS4$^{TIR-TIR}$ fusion to trigger cell death, I constructed a new fusion protein by replacing the missing amino acids (RRS1-RPS4$^{TIR+-TIR+}$). Similarly to RRS1-RPS4$^{TIR-TIR}$, RRS1-RPS4$^{TIR+-TIR+}$ did not trigger cell death when transiently overexpressed in *N. tabacum* (**Figure 3.5A**). All the TIR-TIR fusion proteins used in this experiment were detected by Western blot 2 days post infiltration (**Figure 3.5B**). Altogether, these results suggest a repressive role of RRS1^{TIR} on RPS4^{TIR}. It would be interesting to examine if other domains of RRS1 and RPS4 can suppress RPS4^{TIR}–triggered cell death.

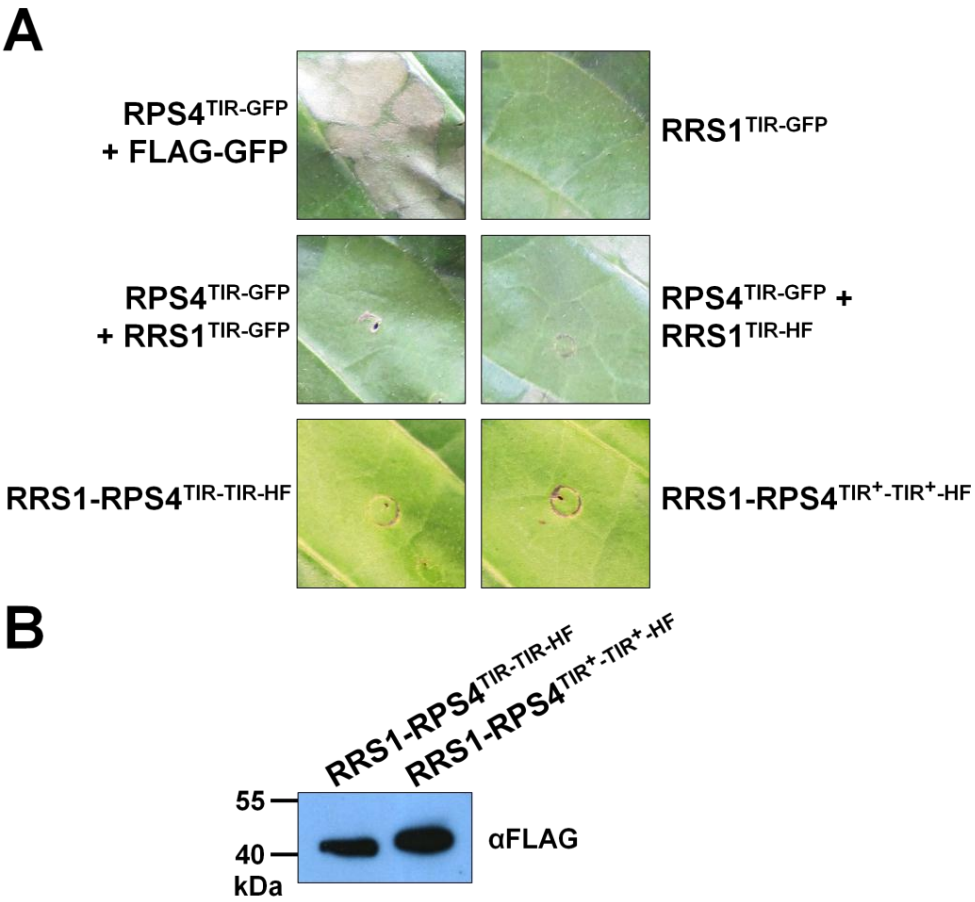

Figure 3.5. RRS1^{TIR} suppresses RPS4^{TIR}-triggered cell death in *N. tabacum*.

A. Cell death phenotype following transient expression of RRS1 and/or RPS4 TIR domains. Constructs were fused to GFP or HisFlag (HF) epitope tags. RRS1-RPS4$^{TIR-TIR}$ and RRS1-RPS4$^{TIR+-TIR+}$ correspond to TIR domains fusions. RRS1-RPS4$^{TIR+-TIR+}$ fusion protein includes the amino acids at each N-terminal parts of RRS1 and RPS4 TIR domains that were missing in the RRS1-RPS4$^{TIR-TIR}$ fusion protein (Williams, Sohn, *et al*., unpublished). *N. tabacum* leaves were infiltrated with *A. tumefaciens* solution at 2.5 .10^{8} cfu/ml (OD_{600}=0.5). For co-infiltrations, *A. tumefaciens* strains were mixed at OD_{600}=0.5 final for each of the strains. Images of cell death were taken at 5 days post infiltration. **B**. Western blot analysis of RRS1-RPS4$^{TIR-TIR-HF}$ and RRS1-RPS4$^{TIR+-TIR+-HF}$, 2 days after infiltration in *N. benthamiana*. Experiment has been repeated at least three times with similar results.

3.3 Discussion

In this **chapter 3**, I investigated different aspects of plant TIR domain–triggered immunity. To perform this analysis, I used the TIR domain of RPS4, an Arabidopsis TIR-NB-LRR studied for the last 20 years.

Studies on RPS4^{TIR} were previously carried out in our laboratory (Swiderski *et al.*, 2009; Zhang *et al.*, 2004). Collectively, these data show that the RPS4^{TIR} domain is sufficient to trigger cell death in *N. tabacum*. They also showed that this requires *EDS1*, *SGT1* and *HSP90*, and that several mutations within the RPS4^{TIR} diminished or amplified the cell death activity (Swiderski *et al.*, 2009).

Through these studies, materials were generated and available in the laboratory at the beginning of my PhD. The Col-0 *pTA7002::RPS4*$^{TIR+80\text{-}HA}$ line is a great tool to investigate TIR domain–mediated immune responses activation as it is possible to manage precisely the induction of the RPS4^{TIR+80} transcription with DEX treatment. I initiated the profiling of the Col-0 *pTA7002::RPS4*$^{TIR+80\text{-}HA}$ line in order to examine over time the cellular events triggered by RPS4^{TIR+80}. Based on a 72 hour time course after DEX treatment, I correlated accumulation of RPS4^{TIR+80} transcript and protein with the appearance of cell death. This experiment would have to be repeated to draw any conclusion about the variations observed in proteins and trancripts levels (**Figure 3.1B**). Nevertheless, it is clear that the RPS4^{TIR+80} transcript is followed by accumulation of the RPS4^{TIR+80} protein which precedes *PR1* transcript detection and then the cell death phenotype (**Figure 3.1A**, **B** and **C**). This profiling provides the basis for future studies. Specially, a transcriptomic analysis could be carried out before and after DEX treatment, compared to Col-0 WT to determine which genes are specificaly regulated by RPS4^{TIR+80}. Moreover, biochemical methods could be employed on the Col-0 *pTA7002::RPS4*$^{TIR+80\text{-}HA}$ line to reveal RPS4^{TIR+80} signaling partners. After DEX treatment, total protein could be extracted

and sujected to an anti-HA immuno precipitation (IP), pulling down RPS4$^{TIR+80-HA}$. Mass spectrometry could be applied on the samples to identify potential RPS4^{TIR+80} interactors. Investigation on the RPS4^{TIR} domain was carried out to understand how TIR domains activate defense but also to understand how the full length RPS4 protein functions in immune response activation. Therefore, the data generated with RPS4^{TIR+80} and RPS4 should be compared to establish any correlation between these two proteins in the activation of plant immunity and confirm that studying TIR is a valid approach to investigating how TIR-NB-LRRs activate plant defense.

Kee Hoon Sohn, a post-doc in the lab undertook a loss of function approach to identify component(s) required for the RPS4^{TIR+80}–activated cell death. He mutagenized with EMS the Col-0 *pTA7002::RPS4*$^{TIR+80-HA}$ line and obtained 61 putative mutants insensitive to RPS4^{TIR+80} (itr). Unfortunately, I showed that the insensitivity to RPS4^{TIR+80} observed in the M3 mutated lines correlated with the absence of RPS4$^{TIR+80-HA}$ protein (**Figure 3.2**). Therefore I did not take forward the study on itr mutants and the map-based cloning of genes required for RPS4^{TIR+80} using the the Col-0 *pTA7002::RPS4*$^{TIR+80-HA}$ line was abandoned.

Following the characterization of the Col-0 *pTA7002::RPS4*$^{TIR+80-HA}$ line, I assessed the impact of RPS4^{TIR+80} on *Pst* DC3000 and *A. brassicicola* growth. After DEX treatment, *Pst* DC3000 did not infect the Col-0 *pTA7002::RPS4*$^{TIR+80-HA}$ line but *A. brassicicola* did (**Figure 3.3**). This supported our hypothesis that RPS4^{TIR+80} would activate defense responses detrimental to a biotroph and favorable to a necrotroph pathogen. However, accumulation RPS4^{TIR+80} protein in triggers Arabidopsis leaf cell death. *Pst* DC3000 is an hemibiotroph pathogen, therefore, it is not unexpected that the cell death triggered by RPS4^{TIR+80} will have a negative impact on its growth. In contrast, *A. brassicicola* is a necrotph pathogen, so the cell death is likely to promote its growth. To conclude, in this experiment, it is difficult to

say if we study the impact on RPS4^{TIR}-triggered immune response or the impact of cell death on these pathogens' growth.

Nuclear localization of NB-LRRs can be necessary for function. For instance, the TIR-NB-LRR proteins N, RPS4 and SNC1 require a nuclear localization to activate cell death (Burch-Smith *et al.*, 2007; Wirthmueller *et al.*, 2007; Zhu *et al.*, 2010). This has also been observed with the CC-NB-LRR MLA10 for defense activation upon Avr_{A10} recognition (Shen *et al.*, 2007). However, cellular compartmentalization for TIR or CC domain to activate cell death was never reported. Using mis-localised RPS4$^{TIR+80GFP}$, I showed that the nuclear pool of RPS4^{TIR+80} was required to activate cell death in *N. tabacum* (**Figure 3.4**). This correlates with the results obtained with RPS4 full length, and supports the relevance of the RPS4^{TIR+80} study. The constructs I generated could be very useful to identify signaling partners of RPS4^{TIR+80} using combined IP and mass spectrometry.

It has been shown that R proteins can oligomerize for function and this requires N-terminal domain dimerization (Mestre and Baulcombe, 2006; Maekawa *et al.*, 2011; Bernoux *et al.*, 2011). As RRS1 and RPS4 associate *in planta* and cooperate for effector recognition, we were interested to investigate the functionality of this heterodimer. The coexpression of RRS1^{TIR} and RPS4^{TIR} in *N. tabacum* did not trigger cell death, neither did a RRS1-RPS4$^{TIR-TIR}$ fusion protein (**Figure 3.5**). Our results suggest that RRS1^{TIR} suppresses the capacity of RPS4^{TIR} to activate downstream signaling. Our current hypothesis is that RRS1 maintains the RPS4-RRS1 heterodimer complex in an off-state. Upon activation by effector recognition, RRS1 will release the complex allowing RPS4 dimerisation for signal transduction.

4 Map–based cloning of the RRS1– and RPS4–independent AvrRps4 recognition (RRIR) locus in Arabidopsis

4.1 Introduction and objectives

In *A. thaliana* accessions, the two *R* genes *RPS4* and *RRS1* are in an inverted head-to-head arrangement on chromosome 5. In resistant accessions, both genes are required for recognition of AvrRps4 and PopP2 from *P. syringae* and *R. solanacearum* respectively but also for resistance to *C. higginsianum* (Narusaka *et al.*, 2009). Using the Col-0, Ws-2 *rps4* and Ws-2 *rps4-21*/*rrs1-1* mutants, it has been shown that an *RRS1*– and *RPS4*–independent AvrRps4–recognition (named RRIR) occurs in both Arabidopsis accessions (Narusaka *et al.*, 2009; Kim *et al.*, 2010). These findings suggest the presence of at least another *R* gene which, unlike *RPS4* and *RRS1*, is specific to AvrRps4 and does not recognize PopP2 or provide resistance to *C. higginsianum*. To fully understand AvrRps4 recognition in Arabidopsis, we decided to map-based clone this putative *R* locus. This method allows the mapping of a locus based on genetic linkage between a phenotype, the chromosomal recombination rate and a DNA sequence polymorphism (Jander *et al.*, 2002). The identification and analysis of this new *R* locus, in addition to *RPS4* and *RRS1*, will provide a better understanding of AvrRps4 and PopP2 recognition and contribute to unravelling ETI in this genes-for-genes interaction.

4.2 Results

4.2.1 An *RRS1*– and *RPS4*–independent AvrRps4 recognition (RRIR) occurs in Arabidopsis.

Based on results obtained by Narusaka *et al*. (2009), Dr Kee Hoon Sohn in the laboratory investigated further the HR triggered by *Pf* Pf0-1 carrying AvrRps4 or PopP2 in Arabidopsis WT and mutants. He showed that infiltration of *Pf* Pf0-1 (AvrRps4) and *Pf* Pf0-1 (PopP2) triggered HR in the Ws-2 WT as well as in the Ws-2 *rps4-21* and *rrs1-1* single mutants and I repeated this observation (**Figure 4.1**) (Sohn *et al.*, 2012). In this experiment, *Pf* Pf0-1 (AvrRps4 KRVY^{AAAA}) was used as a negative control as this AvrRps4 mutant lacks avirulence activity (Sohn *et al.*, 2009). *Pf* Pf0-1 (AvrRps4)–triggered HR was also observed in the Ws-2 *rps4-21*/*rrs1-1* double mutant (**Figure 4.4A**) supporting that *RRS1* is not responsible for the AvrRps4 additional recognition. Infiltration of *Pf* Pf0-1 (PopP2) did not trigger HR in Ws-2 *rps4-21* (**Figure 4.1**) and in Ws-2 *rps4-21*/*rrs1-1* (**Figure 4.4A**). The RRIR is therefore specific to AvrRps4. As expected, RLD did not recognize *Pf* Pf0-1 (AvrRps4) or *Pf* Pf0-1 (PopP2) (Hinsch and Staskawicz, 1996) (**Figure 4.1**).

In order to map-based clone the *R* gene(s) responsible of the RRIR (*RRIR* locus), I generated a Ws-2 *rps4-21* x RLD segregating population. The F1 progeny showed HR to *Pf* Pf0-1 (AvrRps4) (**Figure 4.1**). However, the intensity of HR was variable through experimental repeats compared to the HR observed in the Ws-2 *rps4-21* parent, which always showed strong tissue collapse 24 hours after *Pf* Pf0-1 (AvrRps4) infiltration (**Figure 4.1**). This observation suggests semi-dominance of the *Pf* Pf0-1 (AvrRps4)–triggered HR phenotype in Ws-2 *rps4-21*/RLD heterozygotes. Infiltration of *Pf* Pf0-1 (AvrRps4) did not trigger HR in Ws-0 *eds1-1* (**Figure 4.1**). This suggests that the RRIR could be mediated by a TIR-NB-LRR type

of R protein given that EDS1 is often required for TIR-NB-LRRs (Aarts *et al.*, 1998; Liu *et al.*, 2002b).

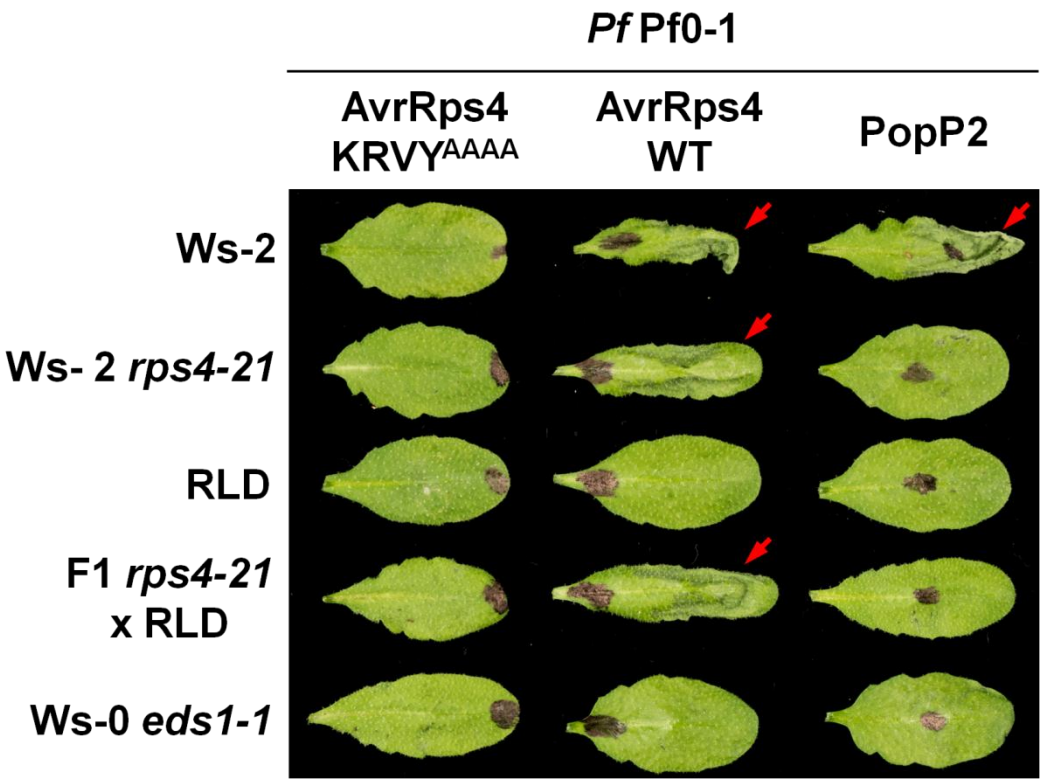

Figure 4.1. AvrRps4 is recognized in an *RPS4*-independent manner in Ws-2 but is not recognized in RLD.

4 to 5-week old Arabidopsis leaves were syringe-infiltrated with a 1.10^8 cfu/ml (OD_{600}=0.2) bacterial solution of *Pf* Pf0-1 carrying either *pVSP61::AvrRps4* KRVY^{AAAA}, *pVSP61::AvrRps4* or *pEDV6::PopP2*. HR phenotypes were assessed 24 hpi. Red arrows indicate leaf collapse due to HR. Experiments have been repeated at least three times with similar results.

4.2.2 Phenotyping and genotyping of a Ws-2 *rps4-21* x RLD segregating population allowed the mapping of the *RRIR* locus at the bottom of Arabidopsis chromosome 5.

In order to map the *RRIR* locus, I analysed the F2 progeny derived from a Ws-2 *rps4-21* x RLD cross, for its response to *Pf* Pf0-1 (AvrRps4). As mentioned earlier, *Pf* Pf0-1 (AvrRps4) triggers a strong HR, described further as [HR] and a weak HR in Ws-2 *rps4-21* x RLD F1, described further as [hr]. I scored the F2 plants for HR symptoms after *Pf* Pf0-1 (AvrRps4) infiltration, scoring [HR] and [hr] phenotypes together as presence of HR. In this F2 population, *Pf* Pf0-1 (AvrRps4)–triggered HR segregated in a 3:1 ratio (601 [HR+hr]:199 [no HR]; χ^2=0.0067, *P*=0.935) (**Table 4.1**). Such a 3:1 ratio suggests that *RRIR* is associated with a single dominant locus. At this stage, I isolated gDNA of the 199 plants showing no response to *Pf* Pf0-1 (AvrRps4) ([no HR]) to map the *RRIR* locus. To test if the *RRIR* locus was homozygous for the RLD allele in the 199 [no HR] F2 selected, I analyzed 10 plants of the progeny of each of the 199 [no HR] F2 (F2:3). Surprisingly, only 48 F2:3 lines did not segregate for the [no HR] phenotype (**Table 4.1**). Such deviation between F2 and F2:3 supports the low penetrance of the HR phenotype which was observed in F1. Therefore, it would have been necessary to test F2:3 from the 601 [HR/hr] plants as well to report the correct F2 ratio.

	F1	F2	F2:3
Ws-2 *rps4-21* x RLD	100% [HR+hr]	601 plants [HR+hr]	
		199 plants [no HR]	151 lines segregating [HR+hr/no HR]
			48 lines non segregating [no HR]
		χ^2 = 0.0067 (*P* = 0.935) for ratio 3 [HR+hr]: 1 [no HR]	χ^2= 0.082 (*P* = 0.775) for ratio 3 segregant: 1 non segregant

Table 4.1. Genetic analysis of the *RRIR* locus mapping population.

The segregating population was obtained by crossing Ws-2 *rps4-21* to RLD. The HR phenotype (indicated in square brackets) was assessed in 4 to 5-week old Arabidopsis plants 24 hours post infiltration with *Pf* Pf0-1 (AvrRps4). The Ws-2 *rps4-21* parent showed [HR] and RLD showed [no HR] to *Pf* Pf0-1 (AvrRps4). The intermediate HR phenotype observed after *Pf* Pf0-1 (AvrRps4) infiltration is described as [hr]. In F2, the probability to have a 3 [HR+hr] : 1 [no HR] ratio is 93.5%. The probability that the F2:3 lines derived from the F2 [no HR] segregated in a 3 segregant : 1 non segregant ratio is 77.5%.

To categorize the type of phenotype segregation in this Ws-2 *rps4-21* x RLD population, I repeated the phenotypic analysis on 48 random F2 plants. This time, I scored the plants very carefully and I separated F2 plants in three categories: either they were showing [HR], [hr] or [no HR] in response to *Pf* Pf0-1 (AvrRps4). After infiltration of *Pf* Pf0-1 (AvrRps4), I observed an approximate 1:2:1 ratio (8 [HR]:28 [hr]:12 [no HR]; χ^2=2, *P*=0.572) (Table 4.2). This segregation suggested that *RRIR* is associated with a single semi-dominant locus. I tested next the HR phenotype segregation in 8 F2:3 plants from each of the 48 F2. Among the F2:3 derived from [HR]– and [hr]–scored F2 (36 F2:3 in total), I identified 4 that did not segregate for [no HR]. Given that these 4 F2:3 lines were derived from 4 F2 plants scored as [hr], a mis-scoring in F2s can be inferred. The other 32 F2:3 comprised 4 non-segregating for the [HR] phenotype and 28 showing a [HR+hr/no HR] segregation. Among the 12 F2 plants scored as [no HR], 5 F2:3 showed a [HR+hr/no HR] segregation and 7 F2:3 did not segregate for [no HR] (Table 4.2). By grouping the F2:3 presenting some [HR] and the one non-segregating for [no HR], one could observe a 3:1 ratio (37 F2:3 with [HR]: 11 F2:3 [no HR]; χ^2=0.111, *P*=0.945).

Altogether, these data suggest a low penetrance of the HR phenotype in the segregating poupulation, but the results are most consistent with the presence of a single locus for the RRIR.

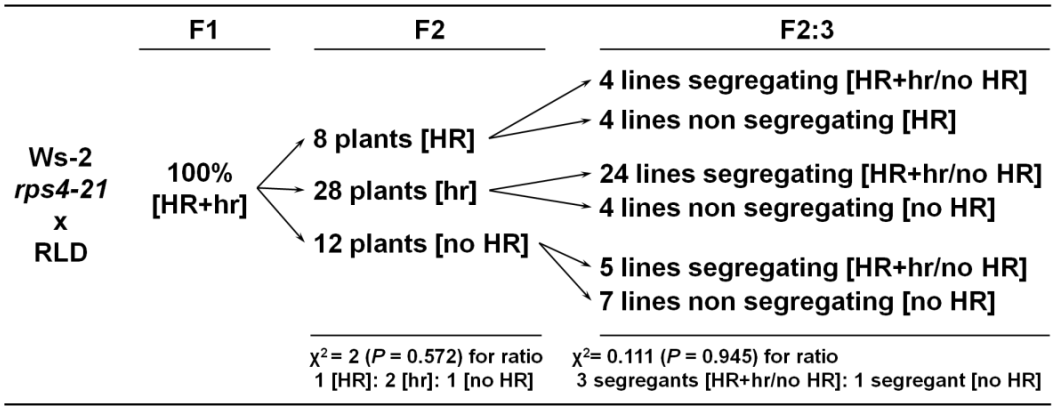

Table 4.2. Investigation of the HR phenotype in the *RRIR* locus mapping population.

The segregating population was obtained by crossing Ws-2 *rps4-21* to RLD. The HR phenotype (indicated in square brackets) was assessed in 4 to 5-week old Arabidopsis plants 24 hours post infiltration with *Pf* Pf0-1 (AvrRps4). The Ws-2 *rps4-21* parent showed [HR] and RLD showed [no HR] to *Pf* Pf0-1 (AvrRps4). Intermediate HR phenotype observed after *Pf* Pf0-1 (AvrRps4) infiltration is described as [hr]. In F2, the probability to have a 1 [HR] : 2 [hr] : 1 [no HR] ratio is 57.2%. For the F2:3 generation, the probability to have a 3 segregating lines for [HR+hr/noHR] : 1 segregating line for [no HR] ration is 94.5%.

The 48 F2 individuals that don't segregate for HR in F2:3 (**Table 4.1**) were used to map the *RRIR* locus. Considering the 3:1 segregation for resistance to *Pst* DC3000 (AvrRps4) observed by Hinsch and Staskawicz (1996) in a RLD x Ws-0 mapping population (Hinsch and Staskawicz, 1996), I assumed that *RRIR* is linked to *RPS4*. Therefore, I directly used genetic molecular markers on chromosome 5 looking for a position where all tested loci are homozygous for an RLD genotype (**Figure 4.2** and **Table S1**). I found Ws-2/RLD distinct molecular markers using the TAIR and the Toronto Marker Tracer websites and I designed others using sequence polymorphism. The *RRIR* locus mapped at 52.5 cM from the marker NGA151 (20 plants tested: 3 plants homozygous Ws-2 and 15 heterozygous) and at 30 cM from the marker K9I9 (20 plants tested: 2 plants homozygous Ws-2, 8 heterozygous) suggesting its position on the lower arm of the chromosome 5 (**Figure 4.2**). I then focused the mapping around *RPS4*. Marker SS007 (designed on *RPS4*) and SS017 mapped 2.1 cM (2 similar recombinants out of 48 F2 plants tested) from the *RRIR* locus confirming the hypothesis that *RRIR* and *RPS4* loci are linked but distinct (**Figure 4.2**). The markers DFR.1 and N5-20408832 mapped respectively 5.7 cM (5 recombinants out of 44 F2 plants tested) and 14.1 cM (13 recombinants out of 46 F2 plants tested) from *RRIR* respectively (**Figure 4.2**). The 2 recombinants (i.e. 2 chromosomes heterozygous for Ws-2 genotype at the marker position) identified with SS017 were also recombinants at the N5-20408832 marker. Interestingly, no recombinants were similarly identified by DFR.1 and SS017. Thus, I concluded that the *RRIR* locus must be between the markers DFR.1 and SS017. Between these two markers, only four TIR-NB-LRR encoding genes are predicted in Col-0 (*TAO1*, *LAZ5*, *At5g45050* and *At5g45060*) according to Meyers et al. (2003) (Meyers *et al.*, 2003). I focused on TIR-NB-LRR encoding genes as they have been shown to preferentially working in an *EDS1*-dependent manner (Aarts *et al.*, 1998). The SS117 marker was directly designed on *At5g45060*, a TIR-NB-LRR in a head-

to-head organization with *At5g45050*. Interestingly, SS117 mapped 0 cM from the *RRIR* locus. This supported *At5g45060* as a candidate for the *RRIR*.

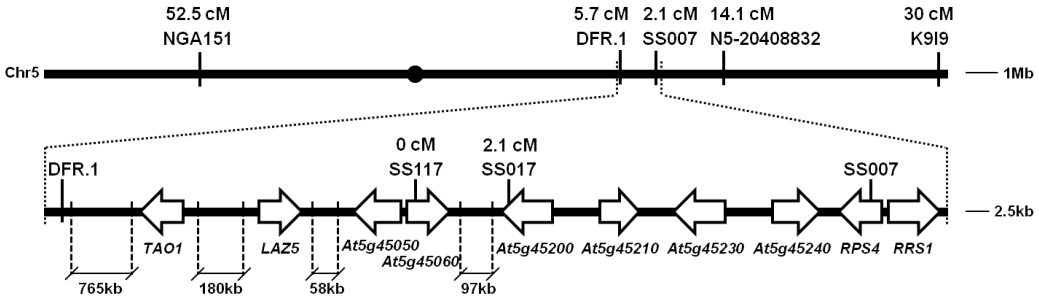

Figure 4.2. The *RRIR* locus is linked to the *RRS1-RPS4* gene pair locus.

Cartoon representing genetic molecular markers and genes on Arabidopsis chromosome 5. Genetic molecular markers are indicated in upper case letters with their corresponding genetic distance (cM) to the *RRIR* locus (based on F2 plants non segregating for the [no HR] phenotype in F2:3). Arrows represent TIR-NB-LRR encoding genes as predicted by Meyers *et al*. (2003) in the region delimited by the genetic molecular marker DFR.1 and SS007. The orientation of the arrow indicates reading frame direction. The physical distance (kb or Mb) is indicated on the right handside of the chromosome, and below (when not to scale).

I was interested to use these mapping data to elucidate the variation of the *Pf* Pf0-1 (AvrRps4)–triggered HR in the Ws-2 *rps4-21* x RLD segregating population. In order to analyze the correlation between the phenotype and the genotype at the *RRIR* locus I backcrossed the F1 (Ws-2 *rps4*-21 x RLD) to RLD. A total of 96 plants derived from this backcross were analyzed for AvrRps4–triggered HR and genotype at the SS117 marker considering it mapped to the *RRIR* locus (**Figure 4.2**). 37 plants showed HR to *Pf* PF0-1 (AvrRps4), whereas 59 plants did not. All 37 [HR] plants were heterozygous at the SS117 marker. Out of the 59 plants showing no HR, 45 were homozygous RLD and 14 were heterozygous at the SS117 marker. Once again, the segregation for [HR] did not follow any expected ratios (1[HR]:1[no HR] for one R gene; 3[HR]:1[no HR] for two R genes) but it approaches to a ratio involving one R gene with an independently segregating negative regulator

(1[HR]:3[no HR]). But, in order to understand the differential responses observed to AvrRps4 in plants heterozygous at SS117 marker, I picked two heterozygous plants, one which showed HR and one which did not, and subsequently tested their progenies. I observed that Ws-2 homozygosity for SS117 correlated with *Pf* Pf0-1 (AvrRps4)–triggered HR whereas RLD homozygozity for SS117 correlated with absence of HR. This result supports our previous assumption about the low penetrance of the HR phenotype and the possible involvement of other loci from RLD for *Pf* Pf0-1 (AvrRps4)–triggered HR in the Ws-2 *rps4-21* x RLD population.

4.2.3 The *RRS1B-RPS4B* gene pair is responsible for the RRIR.

From my mapping results, I identified an interval between the DFR.1 and SS017 markers potentially containing the *RRIR* locus. Considering that the RRIR requires *EDS1*, the four TIR-NB-LRR–encoding genes present in the mapping window (*TAO1*, *LAZ5*, *At5g45050* and *At5g45060*) were selected as candidate genes. TAO1 has been shown to contribute to the disease resistance against *Pst* DC3000 carrying AvrB in Arabidopsis (Eitas *et al.*, 2008). LAZ5 is required for the lesion mimic mutant *acd11* (Palma *et al.*, 2010). *At5g45050* and *At5g45060* were so for uncharacterized. Considering that *RPS4* and *RRIR* loci have the potential to recognize the same effector, AvrRps4, I hypothesized that the *RRIR* locus might have sequence similarities with *RPS4* and/or *RRS1*. Therefore, I compared the amino-acid sequences of the four TIR-NB-LRR candidate genes (*TAO1*, *LAZ5*, *At5g45050* and *At5g45060*) with RPS4 and RRS1 using ClustalW sequence aligment. The closest protein to RPS4 is At5g45060 with 64% identity compared to At5g45050, TAO1 and LAZ5 that present 16%, 22% and 36% of identity respectively. At5g45050 is the closest to RRS1 with 58% of identity compared to At5g45060, TAO1 and LAZ5 that present 18%, 15% and 17% of identity respectively (**Figure S3 and S4**). The amino acid sequence identity between RPS4/At5g45060 and RRS1/At5g45050 is high, and At5g45050 and At5g45060 are the closest homologs of RRS1 and RPS4 respectively within the predicted TIR-NB-LRR proteins (Meyers *et al.*, 2003). Similarly, the high amino-acid sequence identity for RPS4/At5g45060 and RRS1/At5g45050 is correlated with the gene architecture similarities. First, these genes have a similar exon/intron and domain structure (**Figure 4.3A**). Second, like *RPS4* and *RRS1*, *At5g45050* and *At5g45060* are in an inverted head-to-head configuration in the Arabidopsis genome. These observations suggest a duplication of this pair of NB-LRR genes. We found by transformation (see later) that *At5g45050* and *At5g45060* confer AvrRps4 recognition, so they are

hence referred as *RRS1B* and *RPS4B* respectively (**Figure 4.3A**). Genes of each pair are separated by approximately 200 bp (254 bp for *RPS4-RRS1* in Col-0, 264 bp for *RPS4-RRS1* in Ws-2, 232 bp for *RPS4B-RRS1B* in Col-0 and 261 bp for *RPS4B-RRS1B* in Ws-2) suggesting possibly common function and processes regulating transcription within each pair (Li *et al.*, 2006). The promoter and intergenic region between *RPS4-RRS1* and *RPS4B-RRS1B* pairs are only identical at 43% in Ws-2 and 44% in Col-0. Thus, it is hard to say if the two R gene pairs could be similarly regulated transcriptionally.

RRS1 and *RRS1B* are both composed of seven exons encoding proteins of 1288 and 1372 amino-acids respectively. A portion of 55 amino acids encoded by the fourth exon is missing in *RRS1B* compared to *RRS1*. Interestingly, the fifth intron is ten times longer in *RRS1* (around 1 kb) than in *RRS1B* (around 100 bp). A nuclear localization signal (NLS) is predicted on the fifth exon for RRS1 and RRS1B and a bipartite NLS is predicted on the second exon for RRS1 (prediction made using Wolfpsort online software: http://wolfpsort.org/). At the C-terminus, RRS1B is 32 amino-acids longer than $RRS1^{Ws-2}$ which is already 84 aa longer than $RRS1^{Col-0}$. RRS1 and RRS1B both carry a WRKY domain spanned between the sixth and seventh exons. Amino-acid sequence comparisons between $RRS1^{Ws-2}$ and $RRS1B^{Ws-2}$ can be found on **Figure S3**.

RPS4 and *RPS4B* are composed of five exons encoding proteins of 1217 and 1165 amino-acids respectively. The major difference between *RPS4* and *RPS4B* resides in two deletions of 24 and 26 amino-acids in exon 4 and 5 of RPS4B respectively. A bipartite NLS has been predicted for both genes on the fifth exon (prediction made using Wolfpsort online software: http://wolfpsort.org/). Amino-acid sequence comparisons between $RPS4^{Ws-2}$ and $RPS4B^{Ws-2}$ can be found on **Figure S4**.

Altogether, the previous observations revealed *RPS4B-RRS1B* as strong candidates for the *RRIR* locus. In order to test whether *RRS1B-RPS4B* gene pair is the *RRIR* locus, I undertook a gain- and a loss-of-function approach.

The loss-of-function approach consisted in isolating homozygous T-DNA insertion mutants for *RRS1B* and *RPS4B*, generating double mutants and testing for the loss of AvrRps4 recognition in these single and double mutants. One T-DNA insertion line was obtained for *RRS1B* in Col-0 (SALK_001360), designated *rrs1b-1*. Unfortunately, no T-DNA mutants from the Ws-4 FLAG collection were available for *RRS1B*. Two T-DNA insertion lines were obtained for *RPS4B*, FLAG_049F09, designated *rps4b-1* in Ws-4 and SALK_063382, designated *rps4b-2* in Col-0 (**Figure 4.3B**).

The gain-of-function approach consisted in the amplification and the cloning of *RRS1B*, *RPS4B* and the full *RRS1B-RPS4B* pair with their own promoter and terminator from Ws-2 and test whether they could complement RLD for AvrRps4 recognition (**Figure 4.3B**).

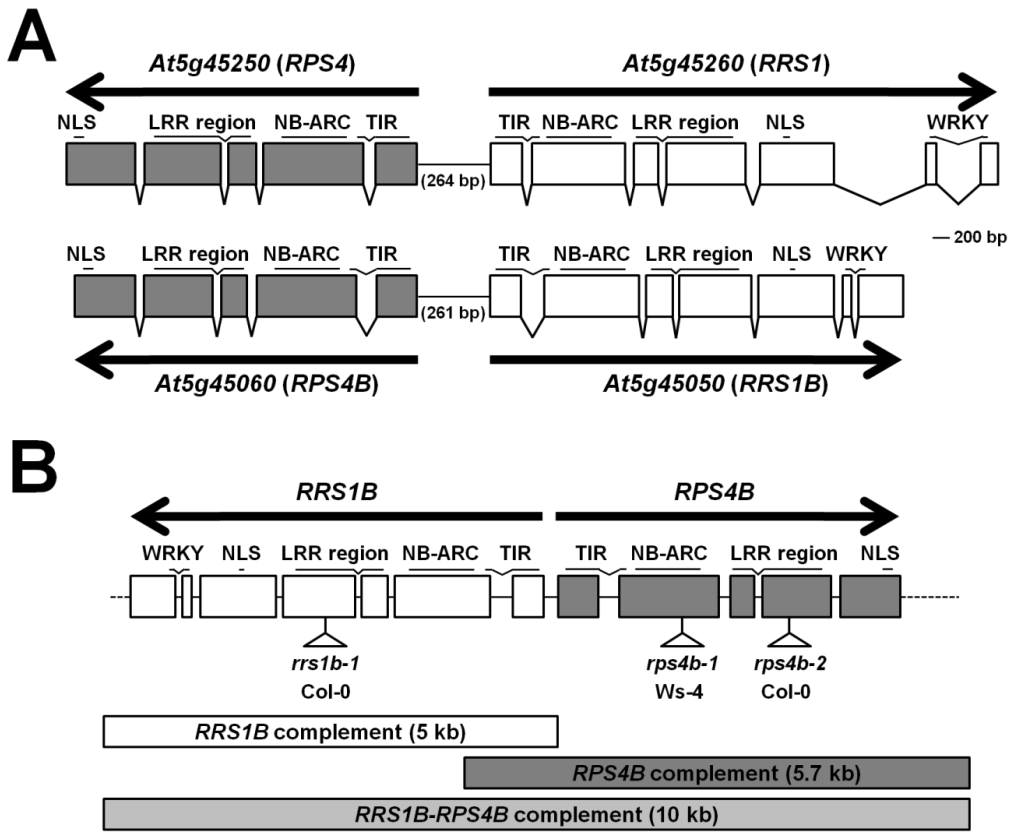

Figure 4.3. Schematic representation of the *RPS4-RRS1* and *At5g45060 (RPS4B)-At5g45050 (RRS1B)* gene pair architectures.

A. Comparison of *RRS1-RPS4 (At5g45260-At5g45250)* and *RRS1B-RPS4B (At5g45050-At5g45060)* gene pair architectures. **B**. Positions of the T-DNA insertions in *RRS1B* and *RPS4B* of the mutant lines used in this study are indicated by white triangle (Col-0 *rrs1b-1*: SALK_001360; Ws-4 *rps4b-1*: FLAG_049F09; Col-0 *rps4b-2*: SALK_063382). The boxes below the gene pair represent gDNA fragments of *RRS1B* and *RPS4B* used for the mutant lines complementation analysis. The exons are depicted as boxes, and the different domains are highlighted on top of the exons/introns (TIR: Toll/interleukin 1 receptor/R protein; NB-ARC: Nucleotide Binding, APAF1, R proteins and CED4; LRR: Leucine Rich Repeat; NLS: Nuclear Localization Signal; WRKY: WRKY DNA binding domain). Black arows indicate the gene reading frame direction.

To test whether *RPS4B* contributes to recognition of AvrRps4 in Ws, I infiltrated *Pf* Pf0-1 (AvrRps4) in several mutants and WT accessions Ws-2 and Ws-4. None of the mutants and WT accessions tested showed HR 24 hours after leaf infiltration with *Pf* Pf0-1 (GUS) (**Figure 4.4A**). As a positive control, I used an unrelated effector, HopA1, which is recognized in Arabidopsis by the TIR-NB-LRR RPS6 (Kim *et al.*, 2009). *Pf* Pf0-1 (HopA1) triggered HR in all the mutants and WT accessions tested except Col-0 (**Figure 4.4A**). The Ws-4 *rps4b-1* mutant showed identical HR and ion leakage level compared to Ws-2 WT after *Pf* Pf0-1 (AvrRps4) infiltration (**Figure 4.4A and B**). I did not detect a loss of PopP2 recognition in the *rps4b-1* either. The Ws-2 *rps4-21* and *rps4-21*/*rrs1-1* mutants showed HR to *Pf* Pf0-1 (AvrRps4) as well as an ion leakage level comparable as Ws-2 WT (**Figure 4.4A and C**). However, the Ws *rps4b-1*/*rps4-21* and *rps4b-1*/*rrs1-1* double mutants did not show HR to *Pf* Pf0-1 (AvrRps4) which correlates with absence of ion leakage in *rps4b-1*/*rps4-21* at 24 hours post infiltration (level similar to *eds1-1*) (**Figure 4.4A and B**). This suggests that *RPS4B* is required for the RRIR in Ws. The Ws *rps4b-1*/*rps4-21* showed HR in response to HopA1 suggesting that HR signaling is still effective in this double mutant. Remarkably, *Pf* Pf0-1 (HopA1) triggered HR in Ws-0 *eds1-1*. This observation correlates with a previous report showing that *EDS1* is only partially required for resistance to *Pst* DC3000 (HopA1) in Ws-0 (Gassmann, 2005). This is surprising as TIR-NB-LRRs were so far supposed to rely entirely on a functional *EDS1* to activate defense responses. Neither AvrRps4, PopP2 or HopA1 triggered HR in Col-0. RLD only showed HR to *Pf* Pf0-1 (HopA1) which is not surprising as RLD is resistant to *Pst* DC3000 (hopA1) (Kim *et al.*, 2009). Interestingly, Col-0 and RLD showed higher ion leakage level compared to *eds1-1* 48 hours after *Pf* Pf0-1 (AvrRps4) (**Figure 4.4C**). This was not observed at 24 hours post infiltration indicating that AvrRps4 can trigger slow and weak cell death–related defense responses in Col-0 but also in RLD.

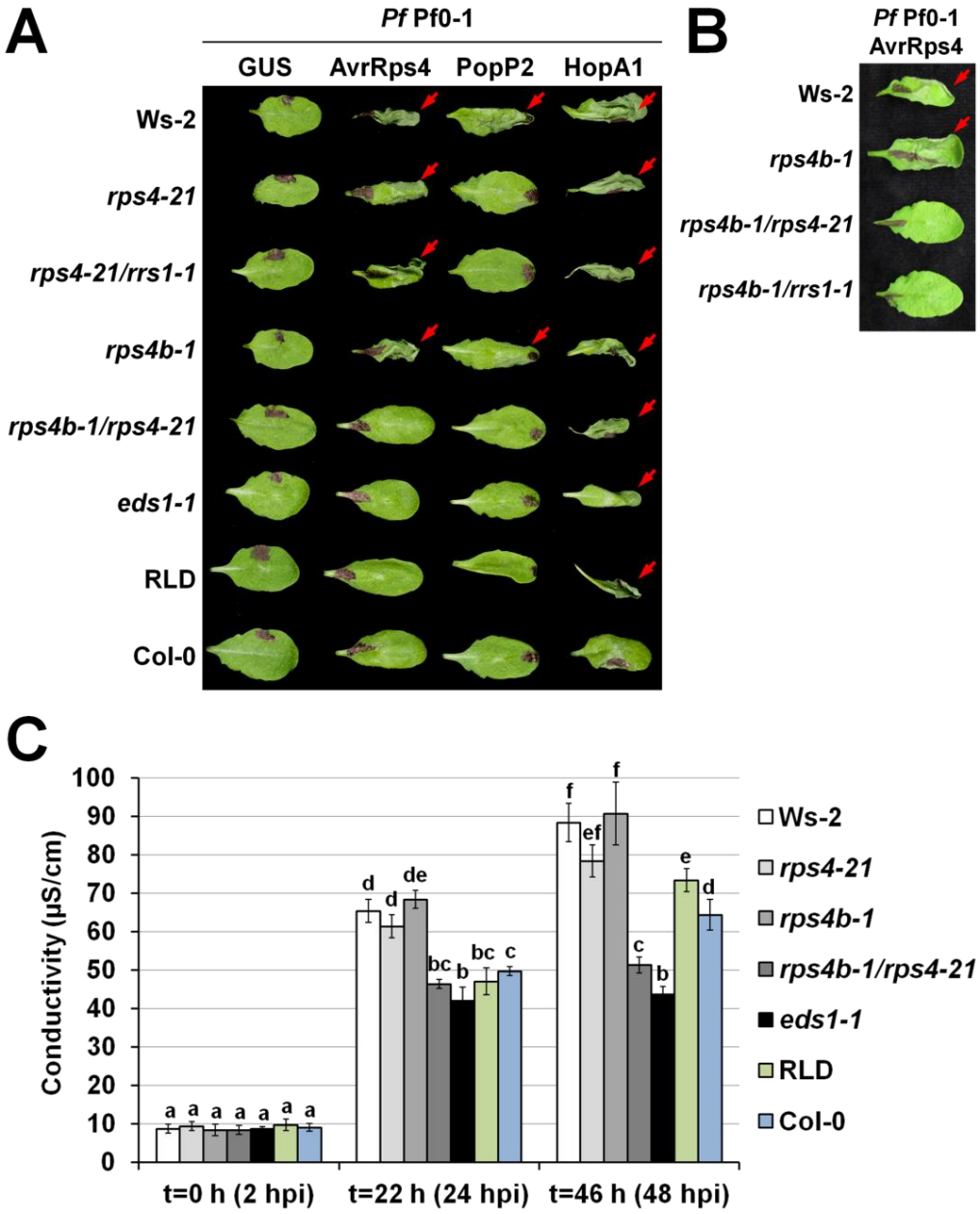

Figure 4.4. *RPS4B* is required for the RRIR.

A-C. Leaves of 4 to 5-week old Arabidopsis plants were syringe-infiltrated with a 1.10^8 cfu/ml (OD_{600}=0.2) bacterial solution of *Pf* Pf0-1 carrying either *pVSP61::GUS*, *pVSP61::AvrRps4*, *pEDV6::PopP2* or *pVSP61::HopA1.* **A-B**. Cell death phenotypes were assessed 24 hpi. Red arrows indicate leaf collapse due to HR. **C**. Ion leakage measurements in leaves infiltrated with *Pf* Pf0-1 *pVSP61*::*AvrRps4* performed at 2, 24 and 48 hours post infiltration (hpi). Samples labelled with the same letter are not statistically different at the 5% confidence level based on Tukey's test. Experiments were repeated at least two times with similar results.

In order to examine whether loss of HR to AvrRps4 correlates with loss of disease resistance, I assessed the growth of *Pst* DC3000 (AvrRps4) in Arabidopsis WT, single and double mutants. AvrRps4 is recognized in Ws-2 and Col-0 restricting *Pst* DC3000 growth. (**Figure 4.5A and B**). Surprisingly, I did not observe significant increases in *Pst* DC3000 (AvrRps4) growth in Ws-2 and Col-0 *rps4* and *rrs1* mutants as reported before (Narusaka *et al.*, 2009; Kim *et al.*, 2010). *Pst* DC3000 (AvrRps4) grew similarly in the Ws-4 *rps4b-1* and Col-0 *rrs1b-1* and *rps4b-2* mutants compared to Ws-4 and Col-0 respectively (**Figure 4.5A and B**). However, *Pst* DC3000 (AvrRps4) grew as well as *Pst* DC3000 (EV) in *rps4b-1*/*rps4-21* and *rps4b-1*/*rrs1-1* mutants (**Figure 4.5A**). This result correlates with the absence of *Pf* Pf0-1 (AvrRps4)–triggered HR in Ws *rps4b-1*/*rps4-21* and *rps4b-1*/*rrs1-1* (**Figure 4.4A and B**). Similarly, Col-0 *rps4b-2*/*rps4-2*, *rrs1b-1*/*rps4-2* and *rrs1b-1*/*rrs1-3* double mutants supported as much *Pst* DC3000 (AvrRps4) growth as *Pst* DC3000 (EV) (**Figure 4.4B**). Altogether, these data indicate that both *RRS1B* and *RPS4B* are required for the RRIR in Arabidopsis accessions Ws and Col-0.

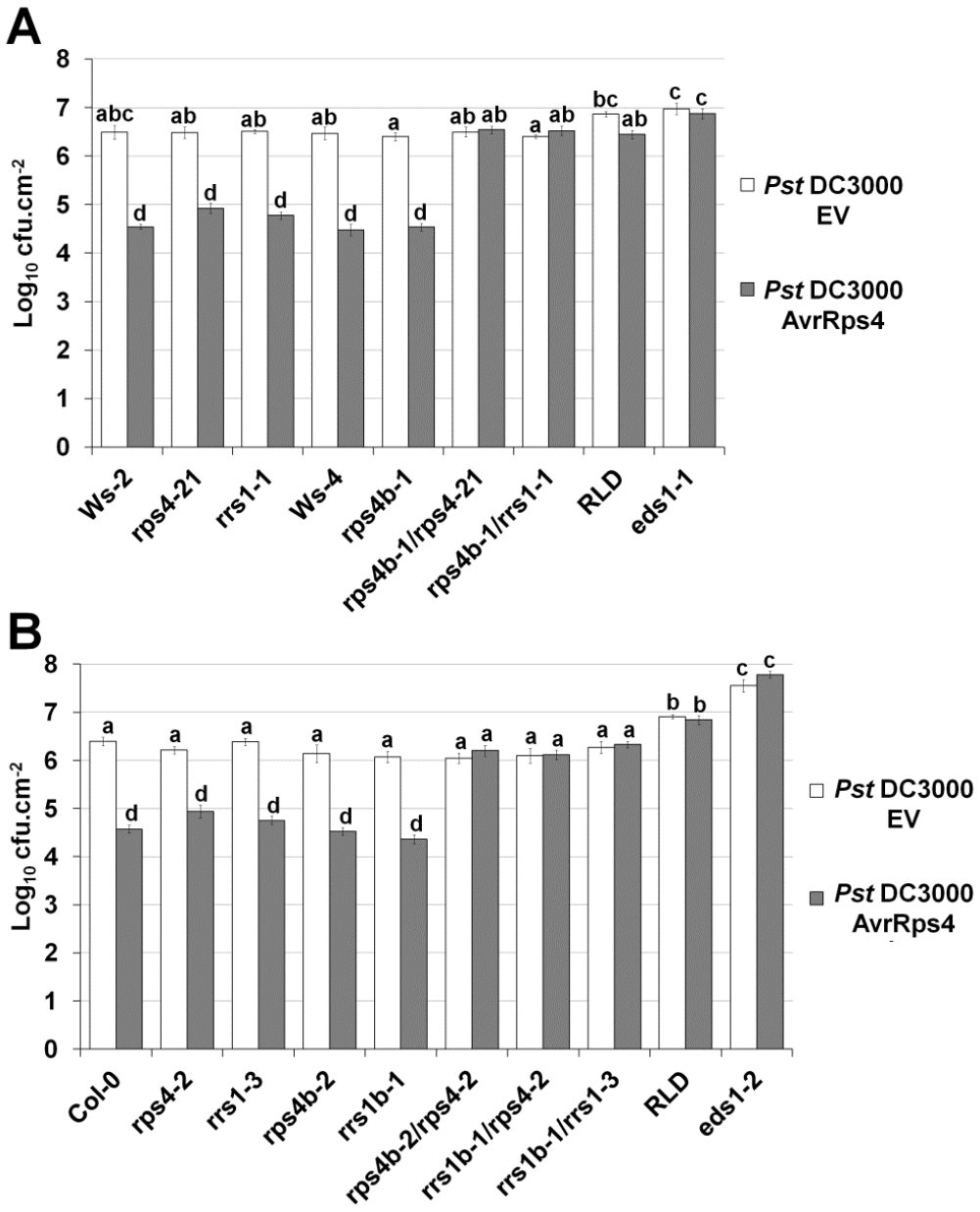

Figure 4.5. *RRS1B* and *RPS4B* are required for the *RRIR* in Ws and Col-0.

A-B. Leaves of 4 to 5-week old Arabidopsis plants were syringe-infiltrated with a 5.10^4 cfu/ml (OD_{600}=0.0001) bacterial solution of *Pst* DC3000 carrying either *pVSP61* empty vector (EV) or *pVSP61::AvrRps4*. Bacterial growth was measured 3 dpi. **A**. Bacterial growth curve in Ws accession. **B**. Bacterial growth curve in Col-0 accession. Samples labelled with the same letter are not statistically different at the 5% confidence level based on Tukey's test. Experiments have been repeated at least three times with similar results.

To check that the results obtained in the single and double mutants were associated with the absence of transcripts, I analyzed mRNA accumulation in the Arabidopsis WT and mutants tested. Using RT-PCR I confirmed the presence of *RPS4*, *RRS1*, *RPS4B* and *RRS1B* transcripts in Ws-2, Ws-4, Col-0, RLD, *eds1-1* and *eds1-2* (**Figure 4.6A and B**). Except for *rps4-21* which has a 5 bp deletion in its sequence causing an open reading frameshift, all the other single and double T-DNA insertion mutants showed absence of the corresponding transcripts (**Figure 4.6A and Table S2**).

It has previously been shown that RPS4 and RRS1 function cooperatively in Arabidopsis. This result was obtained by generating a *rps4-21*/*rrs1-1* double mutant that does not support further increase of *Pst* DC3000 (AvrRps4) growth compared to *rps4-21* and *rrs1-1* single mutants (Narusaka *et al.*, 2009). The single *rps4b* and *rrs1b* mutants did not show increased growth of *Pst* DC3000 (AvrRps4) compared to Arabidopsis WT (**Figure 4.5**). Therefore, generating a *rps4b*/*rrs1b* double mutant would not have been informative to test the cooperation between *RRS1B* and *RPS4B*. However, we hypothesized that, similarly to *RRS1*-*RPS4*, *RRS1B* and *RPS4B* function cooperatively. Considering the high identity between RRS1-RPS4 and RRS1B-RPS4B, I examined if RRS1 could cooperate with RPS4B, and if RRS1B could cooperate with RPS4 for AvrRps4 recognition. To answer this question, I generated the *rps4b-1*/*rrs1-1* and the *rrs1b-1*/*rps4-2* double mutants. In *rps4b-1*/*rrs1-1*, *RPS4* and *RRS1B* transcripts were detected (**Figure 4.5A**). Similarly, in *rrs1b-1*/*rps4-2*, RPS4B and RRS1 transcripts were present (**Figure 4.5B**). I assume that these mRNAs are translated into functional proteins. However, no HR was observed after *Pf* Pf0-1 (AvrRps4) infiltration in Ws *rps4b-1*/*rrs1-1* and no disease resistance was identified in Col-0 *rrs1b-1*/*rps4-2* to *Pst* DC3000 (AvrRps4). In other words, RRS1 with RPS4B (in *rrs1b-1*/*rps4-2*) and RRS1B with RPS4 (in *rps4b-1*/*rrs1-1*) do not cooperate for AvrRps4 recognition. These results

suggest that each *R* gene needs a specific interaction with its own pair partner for function.

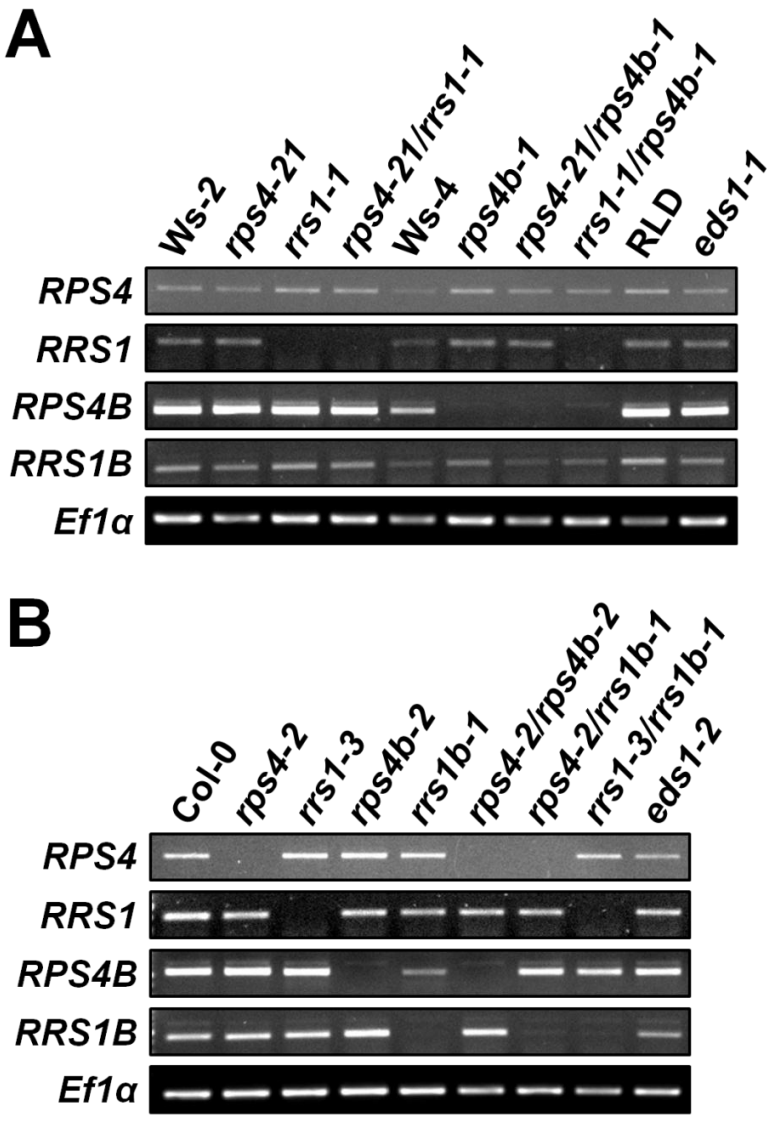

Figure 4.6. Expression analysis of *RPS4*, *RRS1*, *RPS4B* and *RRS1B* in Ws, Col-0, RLD and T-DNA insertion mutants.

A-**B**. RNAs of 4 to 5-week old Arabidopsis plants were extracted from leaves and used to produce cDNAs by reverse transcription. Presence of cDNA for genes of interest was assessed by PCR. **A**. RT-PCR of *Ef1α*, *RPS4*, *RRS1*, *RPS4B* and *RRS1B* in Ws, T-DNA insertion mutants and RLD. **B**. RT-PCR of *Ef1α*, *RPS4*, *RRS1*, *RPS4B* and *RRS1B* in Col-0 and T-DNA insertion mutants.

In parallel, I conducted a gain-of-function approach to assess if *RRS1B*$^{Ws-2}$ and *RPS4B*$^{Ws-2}$ could complement RLD for AvrRps4 responsiveness. I transformed RLD with different DNA fragments showed in **Figure 4.3B**. For each complementation I selected and tested 12 primary T1 transformants. The RLD-derived T3 generation of transformants carrying *RRS1B*$^{Ws-2}$ or *RPS4B*$^{Ws-2}$ did not show HR to *Pf* Pf0-1 (AvrRsp4) (**Figure 4.7**). Given that the *RRS1B*$^{Ws-2}$ and *RPS4B*$^{Ws-2}$ complementation fragments were untagged, it would be necessary to check the expression of these genes in the RLD transformants. However, considering that neither *RRS1B*$^{Ws-2}$ and *RPS4B*$^{Ws-2}$ could complement RLD, and assuming that as *RRS1-RPS4*, *RRS1B* and *RPS4B* act cooperatively, I hypothesized that both *RRS1B* and *RPS4B* were non-functional in RLD. To test this hypothesis, I transformed RLD with the whole *RRS1B-RPS4B*$^{Ws-2}$ pair. Three stable T3 RLD *RRS1B-RPS4B*$^{Ws-2}$ lines (including the line #2-2) showed HR to *Pf* Pf0-1 (AvrRsp4). This result supports the hypothesis that both *RRS1B* and *RPS4B* are likely non-functional in RLD and cannot recognize AvrRps4. However, considering the low penetrance of *Pf* Pf0-1 (AvrRps4)–triggered HR phenotype in the Ws-2 *rps4-21* x RLD population, the RLD complementation for *Pf* Pf0-1 (AvrRsp4)–triggered HR by *RRS1B-RPS4B*$^{Ws-2}$ could be suppressed due to some dominant negative effect of *RRS1B*^{RLD} and *RPS4B*^{RLD} alleles or by the presence of a modifier/negative regulator of *RRS1B-RPS4B*$^{Ws-2}$ activity in RLD background. Therefore, based on this experiment only, it is hard to make firm conclusions about the complementation of RLD by *RRS1*$^{Ws-2}$ and *RPS4B*$^{Ws-2}$ and about the functionality of *RRS1B*^{RLD} and *RPS4B*^{RLD} for AvrRps4 recognition.

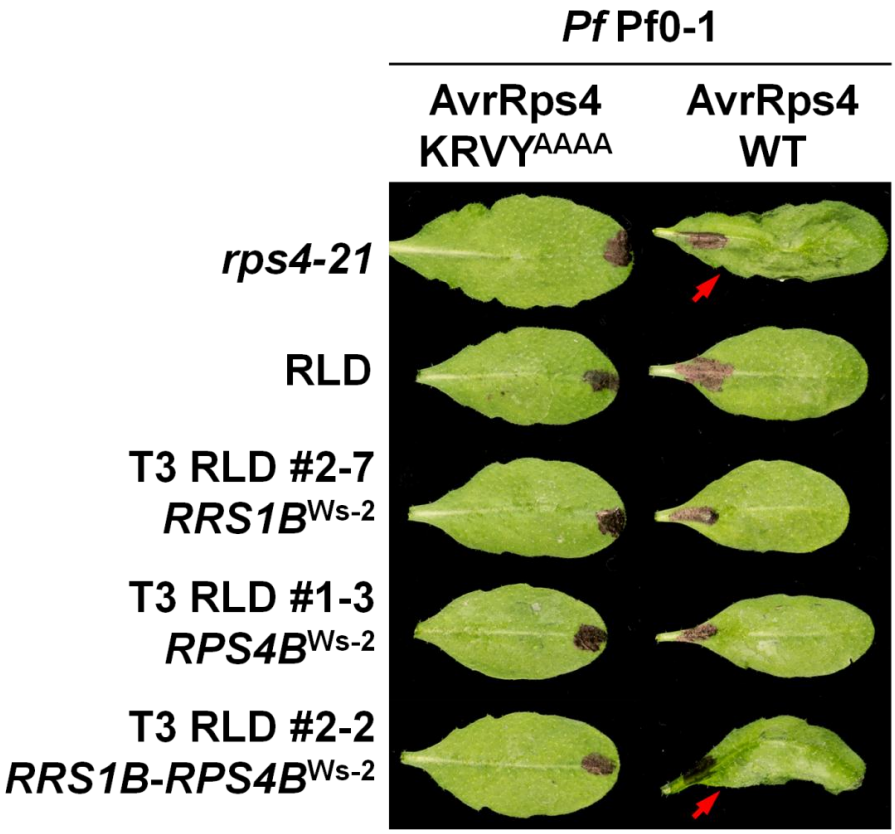

Figure 4.7. *RRS1B-RPS4B*$^{Ws-2}$ confers AvrRps4 recognition in RLD.

Leaves of 4 to 5-week old Ws-2 *rps4-21*, RLD and RLD stable transgenic lines (T3 lines) were syringe-infiltrated with a 1.10^8 cfu/ml (OD_{600}=0.2) bacterial solution of *Pf* Pf0-1 carrying either *pVSP61::AvrRps4* KRVY^{AAAA} *or pVSP61::AvrRps4* WT. RLD was transformed with either *pBGW::RRS1B*$^{Ws-2}$, *pBGW::RPS4B*$^{Ws-2}$ or *pBGW::RRS1B-RPS4B*$^{Ws-2}$. HR phenotypes were assessed 24 hours post infiltration. Red arrows indicate cell death phenotype.

4.2.4 Overexpression of *RRS1B* together with *RPS4B* triggers AvrRps4 recognition and cell death in *N. tabacum*.

The availability of a transient assay is a valuable tool to dissect a biological system. It usually allows a significant gain of time to generate data in comparison to making stably transformed Arabidopsis lines. Efforts have been undertaken in the laboratory to reconstitute AvrRps4 and PopP2 recognition in *Nicotiana sp.* after effector transient co-expression with *RRS1-RPS4* or *RRS1B-RPS4B*. *AvrRps4*, *PopP2*, *RRS1*, *RPS4*, *RRS1B* and *RPS4B* were assembled with a GFP tag (C-terminal fusion) and cloned into the 35S–mediated overepression binary vector. *RRS1*, *RPS4*, *RRS1B* and *RPS4B* were amplified from Ws-2 gDNA. Overexpressed separately, *AvrRps4-GFP*, *PopP2-GFP*, *RRS1-GFP*, *RRS1B-GFP* and *RPS4B-GFP* did not trigger cell death in *N. tabacum* (**Figure 4.8A**). Occasionally, RPS4-GFP triggered cell death to some extent but the tissue collapse usually stopped and never finalized with the complete collapse of the infiltrated plant tissue (**Figure 4.8 A**) (Zhang *et al.*, 2004; Wirthmueller *et al.*, 2007). However, this was not seen with overexpression of RPS4B. The stability of each of these GFP–tagged proteins was verified by western blot using an anti-GFP antibody (data not shown). To test effector recognition by each of the *R* gene pairs, I co-expressed *GFP*, *AvrRps4-GFP* or *PopP2-GFP* together with either *RRS1-GFP* and *RPS4-GFP* or with *RRS1B-GFP* and *RPS4B-GFP*. Five days after infiltration, *AvrRps4-GFP* triggered a strong cell death in tissues expressing both *RRS1-GFP*+*RPS4-GFP* and *RRS1B-GFP*+*RPS4B-GFP* (**Figure 4.8B**). *PopP2-GFP* only triggered cell death when co-expressed with *RRS1-GFP*+*RPS4-GFP* (**Figure 4.8B**). The tissue collapse occuring in *N. tabacum* after AvrRps4 or PopP2 recognition by co-expression of the effectors together with the *R* gene pairs was significantly stronger than the type of necrosis observed by the overexpression of *RPS4-GFP* alone. No cell death was observed when *GFP* was co-expressed with *RRS1-GFP*+*RPS4-GFP* or *RRS1B-*

GFP+RPS4B-GFP (**Figure 4.8B**). Moreover, the weak cell death symptoms that could be observed when *RPS4-GFP* was overexpressed alone disappeared when co-expressed with *RRS1-GFP*. This supports our previous observation that RPS4^{TIR}–triggered HR was suppressed by RRS1^{TIR} (**Chapter 3**, **Figure 3.5**). When *AvrRps4* and *PopP2* were co-expressed with *RRS1-GFP*, *RPS4-GFP*, *RRS1B-GFP* or *RPS4B-GFP* alone, no cell death was observed 5 days after infiltration. Here, the new results obtained correlate with the recognition patterns observed in Arabidopsis i.e. RRS1+RPS4 recognize AvrRps4 and PopP2; RRS1B+RPS4B recognize AvrRps4 but not PopP2. Altogether, these results show that the specific recognition of AvrRps4 and PopP2 by RRS1-RPS4 and RRS1B-RPS4B can be reconstituted in *N. tabacum*.

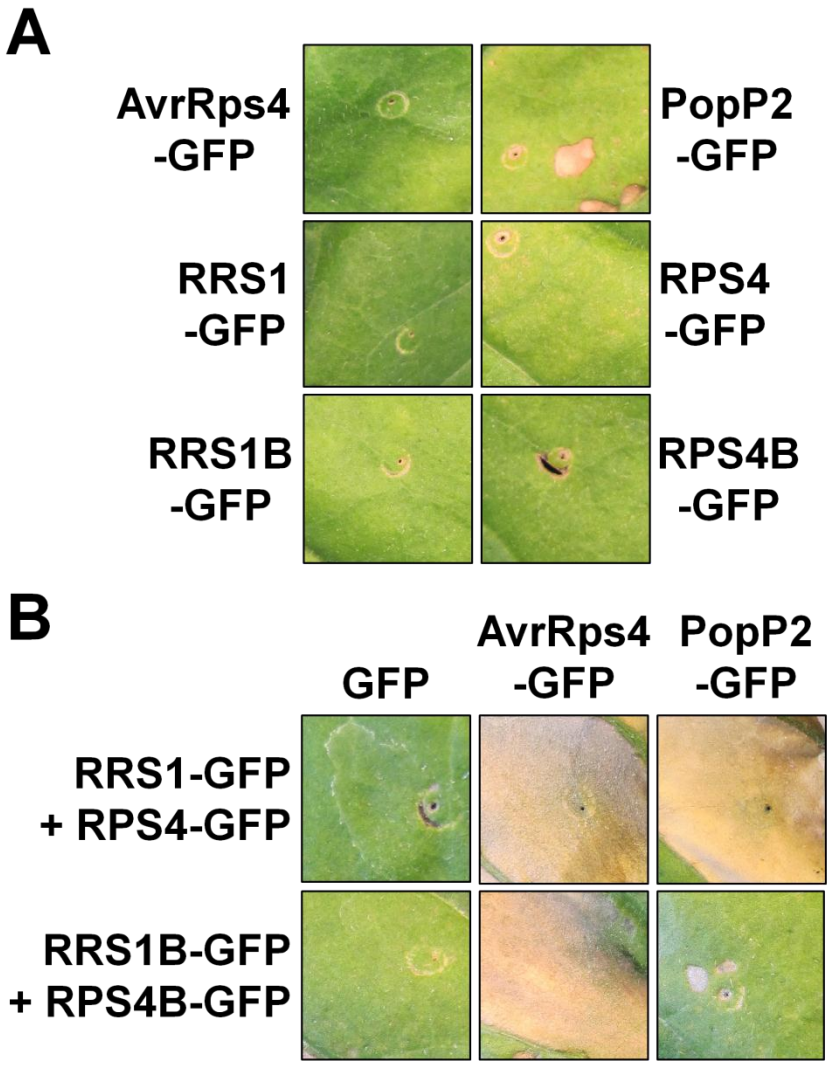

Figure 4.8. AvrRps4 and PopP2 recognition by their cognate *R* gene pairs can be reconstituted in an *A. tumefaciens*–mediated transient assay in *N. tabacum*.

4 to 5 week-old *N. tabacum* leaves were infiltrated with single (**A**) or a mix (**B**) of *A. tumefaciens* Agl1 solutions at 2.5 $.10^8$ cfu/ml (OD_{600}=0.5) each. *RRS1* (*R1*), *RPS4* (*R4*), *RRS1B* (*R1B*) and *RPS4B* (*R4B*) were clones from Ws-2 gDNA into *pICH86988* and fused with a C-terminal GFP tag. *AvrRps4* and *PopP2* were cloned into *pK7FWG2* for C-terminal GFP tagging. Cell death pictures were taken 5 dpi. Experiments have been repeated at least three times with similar results.

I previously showed that transiently delivered RRS1+RPS4 and RRS1B+RPS4B were functional in *N. tabacum* for AvrRps4 and/or PopP2 recognition. Considering that I could not observe cooperation of these R proteins in combination with partner of the other pair in Arabidopsis, I was interested to test if I could repeat this result using this transient assay. *RRS1*, *RPS4*, *RRS1B* and *RPS4B* were amplified from Ws-2 gDNA and assembled 6xHIS, 3xFLAG (HF) tag (C-terminal fusion) into a 35S–mediated overepression binary vector. All the HF fusion proteins were identified by western blot using an anti Flag antibody (**Figure 4.9B**). Similarly to what was observed in **Figure 4.8**, *AvrRps4-GFP* and *PopP2-GFP* triggered cell death when co-expressed with *RRS1-HF*+*RPS4-HF* whereas only AvrRps4-GFP triggered cell death when co-expressed with *RRS1B-HF*+*RPS4-HF* (**Figure 4.9A**). This implies that the HF fusion proteins are functional. I next tested whether AvrRps4 and PopP2 could be recognized with different combination of R proteins. No cell death was observed when *AvrRps4-GFP* and *PopP2-GFP* were co-expressed with either *RRS1-HF*+*RPS4B-HF* or *RRS1B-HF*+*RPS4B-HF*. This correlates with the results obtained in Arabidopsis (**Figure 4.4 and 4.5**) and supports the idea that R proteins require their respective specific pair partner for function.

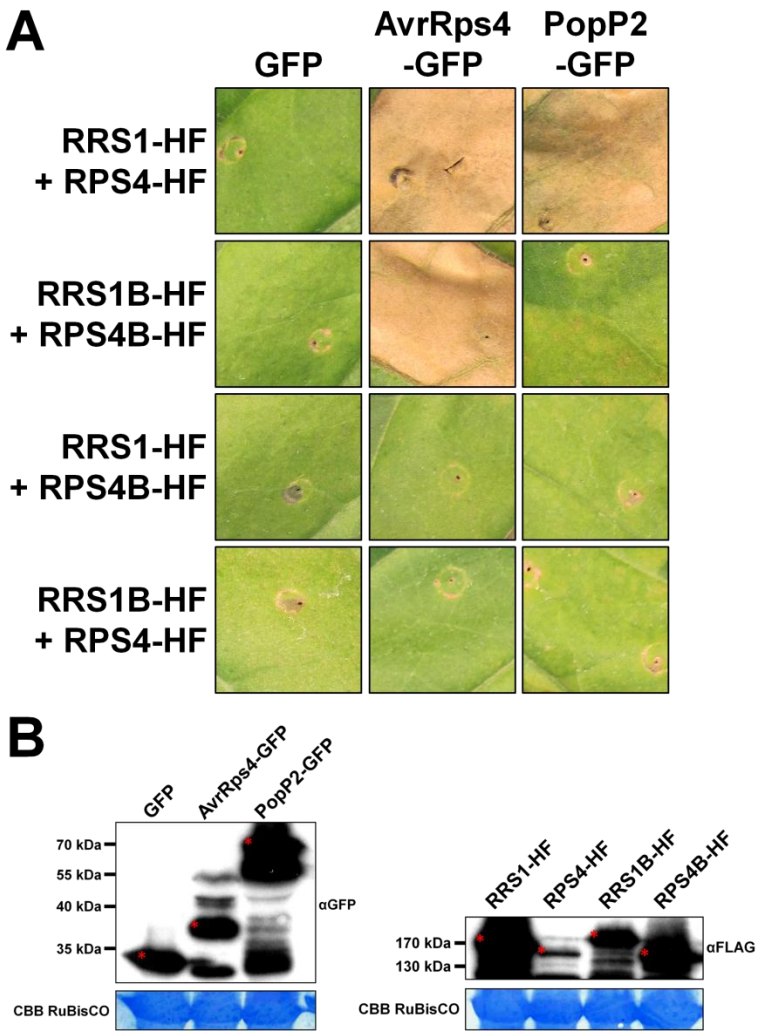

Figure 4.9. RRS1, RPS4, RRS1B and RPS4B function exclusively with their respective pair partner for effector recognition and cell death signalling.

A. 4 to 5 week-old *N. tabacum* leaves were infiltrated with a mix of *A.tumefaciens* solutions at 2.5 .10^8 cfu/ml (OD_{600}=0.5) each. *RRS1*, *RPS4*, *RRS1B* and *RPS4B* were cloned from Ws-2 gDNA into *pICH86988* and fused to a C-terminal HellFire (HF: His-Flag) tag. *AvrRp4* and *PopP2* were cloned into *pK7FWG2* for C-terminal GFP tagging. Cell death pictures were taken 5 dpi. Experiments have been repeated at least three times with similar results. **B**. Western blot analysis of *A. tumefaciens*–mediated transient expression of GFP, GFP–fused effectors and RRS1, RPS4, RRS1B and RPS4B fused to HF, 2 days after infiltration in *N. benthamiana*.

4.3 Discussion

Here, I report the map-based cloning of *RRS1B-RPS4B*, a new *R* gene pair linked to *RRS1-RPS4* on chromosome 5, which is responsible for the *RPS4*– and *RRS1*–independent AvrRps4–recognition (RRIR). In addition to *RRS1-RPS4*, this *R* locus recognizes AvrRps4 in Ws-2 and Col-0 accessions.

In order to map the locus responsible for the RRIR, I used a leaf assay in Arabidopsis, testing for response to *Pf* Pf0-1 (AvrRps4). I generated a mapping population segregating for AvrRps4 recognition using the available Ws-2 *rps4-21* mutant, which recognizes AvrRps4, and the susceptible Arabidopsis accession RLD. All F1 plants arising from this cross recognized *Pf* Pf0-1 (AvrRps4), however a weaker level of leaf tissue collapse was often observed compared to Ws-2 *rps4-21*. Furthermore, I examined carefully the AvrRps4–triggered HR in F2:3 plants to confirm the segregation of the HR phenotype in F2s. This was due to a low penetrance of the AvrRps4-triggered HR phenotype in the Ws-2 *rps4-21* x RLD population. After several attempts, and based on the segregation observed in F2:3 plants, I finally demonstrated a 3:1 segregation in F2 suggesting the presence of a single locus responsible for the RRIR in Ws-2 (**Table 4.2**). The F2 segregation pattern and phenotypic variation between F2 and F3 also support the idea that RLD carries HR modifier(s) of the RRIR. One negative regulator of AvrRps4–triggered immunity, SRFR1, has already been reported in RLD (Kwon *et al.*, 2004). The RLD *srfr1-1* mutant exhibited resistance to *Pst* DC3000 AvrRps4 (Kwon *et al.*, 2009). Likewise, my data suggests that RLD might recognize weakly AvrRps4 as RLD plants show increased ion leakage 48 hours after infiltration with *Pf* Pf0-1 (AvrRps4) compared to Ws *eds1-1* (**Figure 4.4C**). Additionally, two repeats out of four presented a small decrease of *Pst* DC3000 (AvrRps4) growth compared to *Pst* DC3000 (EV) in RLD (**Figure 4.5A**). Altogether, these data suggest that RLD

carries genetic components that positively or negatively regulate AvrRps4–triggered immunity, and which might have introduced a bias for HR scoring in the Ws-2 *rps4-21* x RLD population.

In Col-0, recognition of AvrRps4 does not trigger HR but provides disease resistance to *Pst* DC3000 (**Figure 4.4A**)(Gassmann, 2005). Thus, we can speculate that the defense responses triggered by AvrRps4 might result in the weak ion leakage observed 48 hours after *Pf* Pf0-1 (AvrRps4) infiltration (**Figure 4.4C**). Similarly, Col-0 did not show HR in response to HopA1 even though it is resistant to *Pst* DC3000 (HopA1) (**Figure 4.4A**) (Gassmann, 2005). However, Col-0 displays a robust HR after recognition of other effectors, such as AvrRpt2 and AvrRpm1, due to CC-NB-LRR proteins, RPS2 and RPM1 respectively (Mackey *et al.*, 2002). The reason why Col-0 does not exhibit HR through TIR-NB-LRR–mediated effector recognition remains unclear. Nonetheless, stable Col-0 lines overexpressing *RPS4* show constant activation of defense responses resulting in a dwarf phenotype (Wirthmueller *et al.*, 2007). *RPS4*$^{TIR+80}$ triggers HR when overexpressed in Col-0 (Swiderski *et al.*, 2009). In addition, a mutation in the TIR-NB-LRR SNC1 (*snc1-1*), renders the protein autoactive, also resulting in a Col-0 dwarf phenotype (Zhang *et al.*, 2003). Collectively, these results indicate that effector–independent TIR-NB-LRR–mediated HR can be observed in Col-0. Hypothetically, the quantity of R protein in Col-0 and the derived level of defense activation could be a limiting factor for appearance of HR after TIR-NB-LRR–mediated effector recognition. Alternatively, it was hypothesized that a negative regulator exists in Col-0, *HED1* (HR regulator in *EDS1* pathway), regulating HR to AvrRps4 and HopA1 (Gassmann, 2005), though this negative regulator has not been characterized.

Based on the Ws-2 *rps4-21* x RLD F2 plants which were confirmed in F3 for absence of HR to AvrRps4, I mapped the *RRIR* locus at the bottom of the chromosome 5, close to *RRS1-RPS4* (**Figure 4.2**). The 3:1 segregation for

resistance to *Pst* DC3000 (AvrRps4) observed in the Ws-0 x RLD F2 population used to map *RPS4* indicated the presence of a single *R* locus in Ws-0 (Hinsch and Staskawicz, 1996). This ratio strongly suggests that the *RRIR* locus should be linked to *RPS4* in Ws-0. However, it is still unclear why authors did not map *RRS1B-RPS4B* as well. One explanation could be the negative regulation of *RRS1B-RPS4B*–mediated immunity in RLD that I experienced in my mapping. Based on our hypothesis that the RRIR should be close to *RPS4*, I focused my mapping efforts to chromosome 5. In my final mapping interval, only four TIR-NB-LRRs were identified between the DFR.1 and SS017 genetic markers (**Figure 4.2**). Considering the striking similarities with RRS1-RPS4 (**Figure 4.3**), I decided to prioritize tests of RRS1B-RPS4B over TAO1 and LAZ5.

Using a candidate approach, I generated different double knock-out mutants including *rrs1b* or *rps4b* in combination with *rrs1* or *rps4*. All of them lost AvrRps4 recogntion. Thus, I demonstrated that *RRS1B* and *RPS4B* are both required for the RRIR (**Figure 4.4 and 4.5**). In one of these experiments I tested *Pst* DC3000 (AvrRps4) growth in the Ws-2 and Col-0 *rps4* and *rrs1* single mutants. *Pst* DC3000 (AvrRps4) growth was slightly increased in *rps4* mutants (**Figure 4.5A and B**). However, I could not detect a significant increase of *Pst* DC3000 (AvrRps4) growth in these mutants as reported in previous studies (Narusaka *et al.*, 2009; Birker *et al.*, 2009; Kim *et al.*, 2010). This is likely due to the inherent variations existing between environmental conditions across different research facilities. Even if I occasionally observed a slight increase in *Pst* DC3000 (AvrRps4) growth in *rps4* and *rrs1* mutants, this was not the case for *rps4b* and *rrs1b* mutants (**Figure 4.5**). Similarly, *rps4-21* showed slightly less ion leakage in response to *Pf* Pf0-1 (AvrRps4) compared to Ws WT or *rps4b-1* (**Figure 4.4**). One hypothesis could be that the defense output mediated by RRS1B-RPS4B is weaker than RRS1-RPS4 which reinforces my suggestion about the weak penetrance of the HR phenotype

observed in the Ws-2 *rps4-21* x RLD segregating population. RLD only showed HR to *Pf* Pf0-1 (AvrRps4) when complemented with the full *RRS1B-RPS4B* pair from Ws-2 (**Figure 4.7**). Considering the potential negative effect of RLD genetic background on *RRS1B-RPS4B*–mediated immunity, a critical experiment would be to examine *RRS1B* and *RPS4B* mRNA levels in each of the generated RLD transformants. It is possible that a correlation exists between the abundance of transcript and appearance of HR to AvrRps4. This would also allow me to decipher why none of the RLD transformants carrying $RRS1B^{Ws\text{-}2}$ or $RPS4B^{Ws\text{-}2}$ alone were complemented for AvrRps4 responsivness. Further analysis of *RRS1B-RPS4B* in Col-0 and RLD will be carried out in **chapter 5**.

Interestingly, I observed an HR to *Pf* Pf0-1 (HopA1) in Ws *eds1-1* mutant supporting that *EDS1* is only partially required for HopA1 recognition (**Figure 4.4**)(Gassmann, 2005). It would be interesting to test other mutants impaired in NB-LRR signaling, like *ndr1-1* mutant, alone or combined with *eds1* mutant for loss of HopA1 recognition. This could unravel new specificities in NB-LRR signaling pathways such as a requirement for both EDS1 and NDR1.

Co-expression of *RRS1-RPS4* or *RRS1B-RPS4B* with their respective recognized effectors triggers cell death in *N. tabacum* (**Figure 4.8**). This transient assay in *N. tabacum* may be useful to dissect the molecular mechanisms underlying effector recognition and signaling mediated by these *R* gene pairs. This assay is robust as it recapitulates specifically the effector recognition observed in Arabidopsis (i.e. RRS1+RPS4 recognize AvrRps4 and PopP2; RRS1B+RPS4B only recognize AvrRps4) with a macroscopic phenotypic output (i.e. leaf tissue cell death). This suggests that the downstream signaling pathway(s) induced by AvrRps4 and PopP2 perception through RRS1-RPS4 and RRS1B-RPS4B to activate defense responses are conserved between Arabidopsis and *N. tabacum*. Despite the similarities between RRS1-RPS4 and RRS1B-RPS4B, paralogs from

different pairs do not complement each other for effector recognition (**Figure 4.4, 4.5 and 4.9**). When *RRS1* and *RPS4B* or *RPS4* and *RRS1B* only are expressed under their own promoter in Arabidopsis or overexpressed in *N. tabacum*, they do no cooperate for effector recognition. The pair partnership specificity for function will be investigated in **chapter 6**.

5 Functional analysis of *RRS1B* and *RPS4B* alleles in Ws-2, Col-0 and RLD accessions

5.1 Introduction and objectives

Extensive genetic variation is often found in Arabidopsis and other species for recognition of specific pathogen effectors. The *P. syringae* T3SE AvrRpt2 triggers resistance in Col-0 due to its recognition by *RPS2* (Kunkel *et al.*, 1993). Accession Wü-0 lacks a functional *RPS2* and is susceptible to *Pst* DC3000 (AvrRpt2) (Kunkel *et al.*, 1993). When expressed in *Pst* DC3000, AvrRpm1 activates disease resistance in 7 Arabidopsis accessions out of 15 tested (Innes *et al.*, 1993b). Similarly, AvrRps4 triggers disease resistance to *Pst* DC3000 in 18 of the 19 Arabidopsis accessions tested; RLD is susceptible (Hinsch and Staskawicz, 1996). RLD does not recognize AvrRps4 due to two mutations (N195D and Y950H) in RPS4 (Gassmann *et al.*, 1999; Zhang and Gassmann, 2003). RLD complemented with *RPS4* from Col-0 became resistant to *Pst* DC3000 (AvrRps4) suggesting that *RRS1*^{RLD} is functional (Gassmann *et al.*, 1999). Since RLD is susceptible to *Pst* DC3000 (AvrRps4), it must also lack functional RPS4B and/or RRS1B. In this chapter, I investigated the absence of *RRS1B-RPS4B*–dependent AvrRps4 recognition in RLD, and found that *RPS4B*^{RLD} is truncated causing its unability to recognize AvrRps4.

In Col-0, AvrRps4 triggers disease resistance to *Pst* DC3000 but not HR (Gassmann, 2005). This observation has similarly been reported for HopA1 which, as AvrRps4, is recognized by a TIR-NB-LRR protein, *RPS6* (Gassmann, 2005; Kim *et al.*, 2009). Absence of effector–triggered HR might be a TIR-NB-LRR–specific phenomenon considering that all CC-NB-LRR–mediated recognitions described in Col-0 do trigger HR and confer resistance (Kunkel *et al.*, 1993). However, the molecular basis underlying the absence of HR to AvrRps4 and HopA1 in Col-0 is

still unknown. Here, I first examined the sequence polymorphism of *RRS1B-RPS4B* between Ws-2 and Col-0 in order to identify allelic differences that might be responsible for the absence of HR after AvrRps4 recognition in Col-0. I then undertook a transgenic approach and showed that AvrRps4 triggered HR in Col-0 *rps4-2* carrying *RRS1B*$^{Ws-2}$.

5.2 Results

5.2.1 Ws-2 *RRS1B-RPS4B* gene pair is more similar to RLD than Col-0.

Ws-2, Col-0 and RLD show different phenotypes in response to AvrRps4. Ws-2 shows HR and disease resistance to *Pst* DC3000 (AvrRps4), Col-0 disease resistance but no HR to *Pst* DC3000 (AvrRps4) and RLD does not show HR to AvrRps4 and is susceptible to *Pst* DC3000 (AvrRps4). In order to understand the discrepancies in the responses triggered by the *RPS4*– and *RRS1*–independent AvrRps4–recognition (RRIR) among Ws-2 , Col-0 and RLD, I analyzed *RRS1B* and *RPS4B* sequences in these three accessions (**Figures 5.1**, **S1 and S2**). *RRS1B* and *RPS4B* were amplified from Ws-2 and RLD genomic DNA and sequenced. The Col-0 sequences were uploaded from the TAIR website (http://www.arabidopsis.org/). I then examined internal sequence nucleotide polymorphisms (SNPs) in *RRS1B* and *RPS4B* for the three accessions. I chose *RRS1B*$^{Ws-2}$ and *RPS4B*$^{Ws-2}$ alleles as a reference given that AvrRps4 triggers HR and disease resistance in Ws-2 and can be considered as carrying functional alleles. Compared to *RRS1B*$^{Ws-2}$, only two non synonymous substitutions (S568G and I961V) were identified in *RRS1B*^{RLD}, and a 3 bp deletion caused the absence of the amino acid E362 (**Figure 5.1**). Twenty one amino acid changes were identified in *RRS1B*$^{Col-0}$ compared to *RRS1B*$^{Ws-2}$ (**Figure 5.1 and S1**). Interestingly, most of these non-synonymous substitutions are in the sequence encoding the *RRS1B* TIR domain (**Figure 5.1**). In *RPS4B*^{RLD}, I identified three non-synonymous substitutions

(N264D, C277F and N1053D) and an insertion in the fifth exon causing a frameshift with 4 amino acid changes (D1099E, V1100C, P1101S and V1102C) and an early stop codon (**Figure 5.1**). As a result, RPS4B^{RLD} is missing the C-terminal 63 amino acids including the predicted RPS4B^{Ws-2} NLS (**Figure 5.1**). In *RPS4B*$^{Col-0}$, 39 non-synonymous mutations were identified compared to *RPS4B*$^{Ws-2}$ (**Figure 5.1 and S2**). In contrast to RRS1B^{Col-0}, RPS4B^{Col-0} non-synonymous substitutions were significantly less present in the sequence encoding the RPS4B^{Col-0} TIR domain. To summarize, RRS1B^{RLD} and RRS1B^{Col-0} proteins are identical at 99.8% and 98.5% from RRS1B^{Ws-2} respectively. RPS4B^{RLD} (based on RPS4B^{RLD} protein length) and RPS4B^{Col-0} proteins are identical at 99.6% and 96.7% from RPS4B^{Ws-2} respectively. Among the polymorphisms identified, the truncated RPS4B^{RLD} is the most striking observation that could explain why RRS1B^{RLD}-RPS4B^{RLD} is non functional for AvrRps4 recognition.

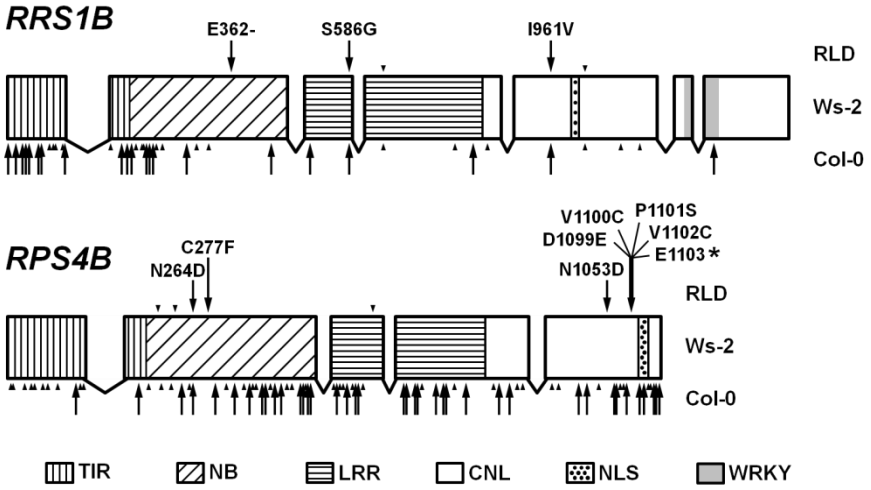

Figure 5.1. Schematic diagram of the RRS1B and RPS4B protein sequences in RLD and Col-0 compared to Ws-2.

Each symbol above and under the diagram indicate changes in RLD and Col-0 alleles respectively compared to Ws-2. Arrows represent amino acid substitutions. Arrowheads represent synonymous mutations. The asterisk represents an early stop codon. TIR: Toll/interleukin 1 receptor/R protein; NB-ARC: Nucleotide Binding, APAF1, R proteins and CED4; LRR: Leucine Rich Repeat; CNL: Coding Non LRR; NLS: Nuclear Localization Signal; WRKY: WRKY DNA binding domain. The amino acid substitutions in $RRS1B^{Col-0}$ from N-terminal to C-terminal are: I2T, V13I, E26K, R29Q, I33V, I68V, E71D, Q115H, H124Q, I129T, L133V, V156I, C157N, L160P, Q220E, D479E, I479M, S586G, S821F, I961V and L1227S. The amino acid substitutions in $RPS4B^{Col-0}$ from N-terminal to C-terminal are: D147G, E192Q, I252T, N264D, S292N, G323R, L358S, T391A, I392T, N424Y, K436I, D532G, I533S, L540V, Y541R, D556N, L577H, Q596H, K599L, A702V, M705T, S727N, E729K, L761V, G770S, A774T, S797L, C890Y, T904A, T1014I, K1024R, G1066D, G1068S, N1090K, R1120I, K1128R, S1150T, A1153P and S1165G.

5.2.2 AvrRps4 triggers cell death in Col-0 plants expressing RRS1B^{Ws-2}.

I next tested whether *RRS1B*$^{Ws-2}$ and *RPS4B*$^{Ws-2}$ could complement Col-0 for *Pf* Pf0-1 (AvrRps4)–triggered HR. I transformed Col-0 *rps4-2* mutant with *RRS1B*$^{Ws-2}$ and *RPS4B*$^{Ws-2}$ genomic DNA sequences cloned into *pBGW* with their own promoter and terminator (as indicated in **Figure 4.3B**). I used Col-0 *rps4-2* to avoid possible effects of AvrRps4 recognition by the *RRS1-RPS4* pair. None of the Col-0 *rps4-2 RPS4B*$^{Ws-2}$ T1s (12 lines in total) obtained and derived T2 plants showed HR after *Pf* Pf0-1 (AvrRps4) infiltration. This suggests that *RPS4B*$^{Ws-2}$ does not complement Col-0 for AvrRps4–triggered HR. However, three Col-0 *rps4-2* *RRS1B*$^{Ws-2}$ lines (T1 and derived T2 plants; 12 lines tested in total) showed HR to *Pf* Pf0-1 (AvrRps4) compared to Col-0 *rps4-2* (**Figure 5.2**). This suggests that polymorphism in *RRS1B*, rather than in *RPS4B*, might contribute to the absence of *Pf* Pf0-1 (AvrRps4)–triggered HR in Col-0. Interestingly, Col-0 *RRS1*$^{Ws-2}$ plants showed HR to *Pf* Pf0-1 (AvrRps4) (Kee Hoon Sohn, personal communication). I hypothesized that a similar allelic difference in *RRS1*$^{Col-0}$ and *RRS1B*$^{Col-0}$ could be responsible for the lack of HR to AvrRps4 in Col-0. However, no conserved mutations within *RRS1*$^{Col-0}$ and *RRS1B*$^{Col-0}$ were identified compared to *RRS1*$^{Ws-2}$ and *RRS1B*$^{Ws-2}$. This implies that different mutations in *RRS1*$^{Col-0}$ and *RRS1B*$^{Col-0}$ could lead to the absence of HR to AvrRps4. Further investigations will be required to answer this specific question. However, it would be necessary to test the level of expression of *RPS4*$^{Ws-2}$ and *RRS1*$^{Ws-2}$ in each of the Col-0 *rps4-2* transformants. Indeed, *RRS1* and *RRS1B* are naturally expressed at a low level in Arabidopsis (http://bar.utoronto.ca/welcome.htm). Analyzing *RRS1B*$^{Ws-2}$ mRNAs in the different Col-0 *rps4-2* RRS1B^{Ws-2} transgenic lines will clarify whether the HR phenotype observed correlates with a presence/absence or a quantitative accumulation of *RRS1B* mRNAs.

Figure 5.2. *RRS1B* from Ws-2 complements Col-0 for AvrRps4–triggered cell death.

Leaves of 4 to 5 week-old Col-0 *rps4-2* and stable transgenic Col-0 *rps4-2* T2 line expressing *RRS1B*$^{Ws-2}$ were syringe-infiltrated with a 1.10^8 cfu/ml (OD_{600}=0.2) bacterial solution of *Pf* Pf0-1 carrying *pVSP61::AvrRps4* WT. Col-0 *rps4-2* was transformed with *A. tumefaciens pBGW:: RRS1B*$^{Ws-2}$. HR phenotypes were observed 24 hpi. HR was observed 3 times in 3 independent transformants.

5.2.3 The C-terminal domain of RPS4B, containing the NLS, is required for its function.

In a previous experiment, I tested wether *RRS1B*$^{Ws-2}$ or *RPS4B*$^{Ws-2}$ or the full *RRS1B*$^{Ws-2}$-*RPS4B*$^{Ws-2}$ pair could complement RLD for AvrRps4 recognition with (**Figure 4.7**). Based on my results, I could not conclude regarding RRS1B^{RLD} or RPS4B^{RLD} functionality for AvrRps4 recognition. Thus, I next tested whether the C-terminal part of RPS4B^{RLD}, which is shorter than RPS4B^{Ws-2} and lacking the RPS4B^{Ws-2} NLS, was required for function. Especially, the NLS could be important for RPS4B to activate defense responses considering its requirement in RPS4 to trigger cell death (Wirthmueller *et al.*, 2007). I cloned *RPS4B* from RLD and Ws-2 genomic DNA into a binary overexpression vector (*pH7WGR2*) which creates a RFP N-terminal fusion after Gateway recombination. These plasmids were then separately transformed into *A. tumefaciens* Agl1 for transient expression assays. Consistent with my previous observation (**Figure 4.8A**), RFP, RFP-RPS4B^{RLD} and RFP-RPS4B^{Ws-2} did not trigger cell death in *N. tabacum* or *N. benthamiana* five days after infiltration. I next determined the subcellular localization of these fusion proteins two days after infiltration in *N. benthamiana* using confocal microscopy. RFP was detected in the nucleus and the cytoplasm (**Figure 5.3**). RFP-RPS4B^{WS-2} localized predominantly in the nucleus with some signal detected in the cytoplasm (**Figure 5.3**). Interestingly, RFP-RPS4B^{RLD} was excluded from the nucleus but present in the cytoplasm (**Figure 5.3**). This indicates that the last 63 amino acids on the C-terminal part of RPS4B^{WS-2} confer nuclear localization to the protein.

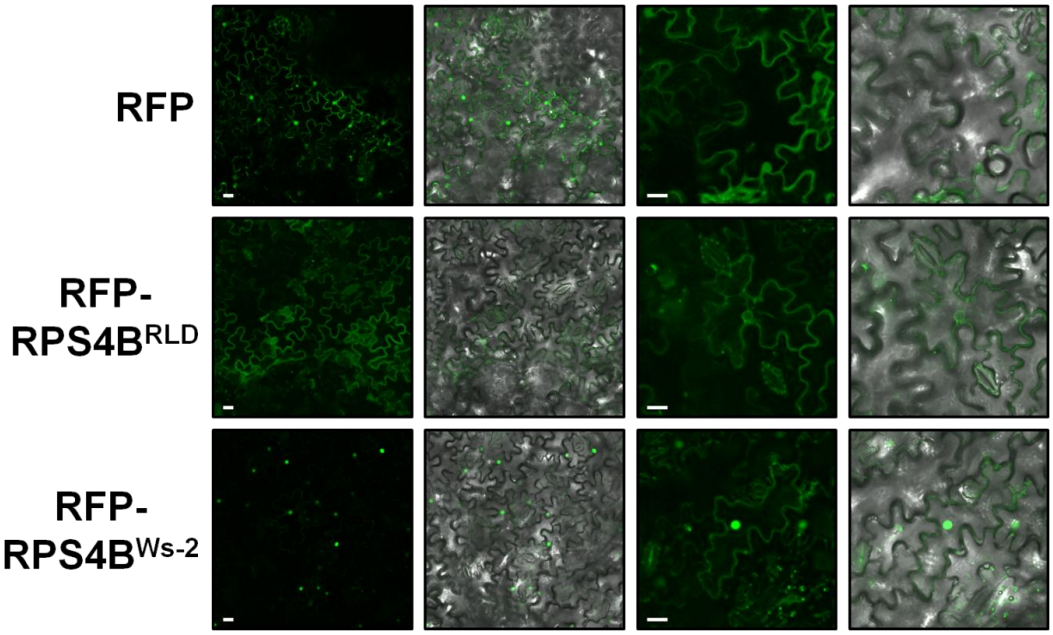

Figure 5.3. RPS4B^{RLD} is excluded from the plant nucleus.

4 to 5 week-old *N. benthamiana* leaves were syringe-infiltrated with *A. tumefaciens pH7WGR2* (RFP), *pH7WGR2::RPS4B*^{RLD} (RFP-RPS4B^{RLD}) or *pH7WGR2::RPS4B*$^{Ws-2}$ (RFP-RPS4B^{Ws-2}) at $2.5.10^8$ cfu/ml (OD_{600}=0.5). The subcellular localization of RFP-tagged proteins was assessed at 2 dpi using confocal microscopy. Scale bar = 20 µm. Experiments were repeated at least three times with similar results.

To test the functionality of *RPS4B*^{RLD} allele in Arabidopsis, I transformed Ws *rps4b-1*/*rps4-21* double mutant with *RPS4B*^{RLD} (with own promoter and terminator). As expected, stable T3 Ws *rps4b-1*/*rps4-21* expressing *RPS4B*^{RLD} did not show HR 24 hours after infiltration with *Pf* Pf0-1 (AvrRps4) whereas Ws *rps4b-1/rps4-21* expressing *RPS4B*$^{Ws-2}$ did (**Figure 5.4**). This results confirms that *RPS4B*$^{Ws-2}$ is required for the RRIR and that *RPS4B*^{RLD} is non-functional. To assess whether the early stop codon in *RPS4B*^{RLD} is deleterious for its function, I removed the insertion in the RPS4B^{RLD} sequence responsible for the frameshift to restore the full length RPS4B^{RLD} protein (RPS4B$^{RLD\Delta}$). As a result, RPS4B^{RLD} is identical to RPS4B^{Ws-2} protein except for N264D, C277F and N1053D substitutions (**Figure 5.1**). The Ws *rps4b-1*/*rps4-21* double mutant expressing *RPS4B*$^{RLD\Delta}$ showed HR to *Pf* Pf0-1 (AvrRps4) 24 hours post infiltration (**Figure 5.4**). This suggests that the C-terminal fragment, present in RPS4B^{Ws-2} but missing in RPS4B^{RLD}, is required for AvrRps4 recognition. It is likely that RPS4B^{RLD} inability to recognize AvrRps4 might be due to its inability to reach the nucleus. It would be necessary to test a *RPS4B*$^{Ws-2}$ allele mutated in the NLS to prove its requirement to trigger HR to *Pf* Pf0-1 (AvrRps4).

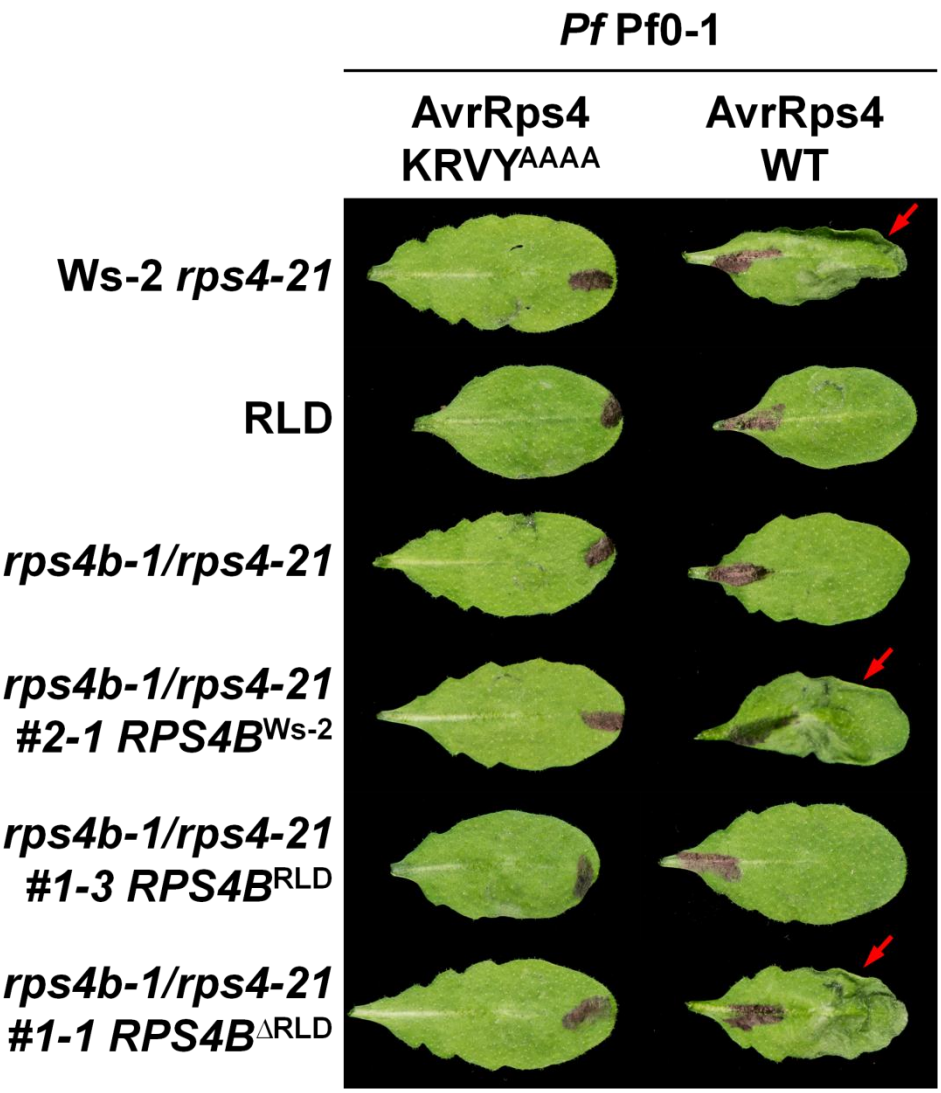

Figure 5.4. The C-terminal extension, naturally missing in RPS4B^{RLD} is required to complement *rps4b-1*/*rps4-21* for AvrRps4–triggered cell death.

Leaves of 4 to 5 week-old Ws-2 *rps4-21*, RLD, Ws *rps4b-1*/*rps4-21* and stable transgenic Ws *rps4b-1*/*rps4-21* T3 lines expressing various *RPS4B* constructs were syringe-infiltrated with a 1.10^8 cfu/ml (OD_{600}=0.2) bacterial solution of *Pf* Pf0-1 *pVSP61::AvrRps4* KRVY^{AAAA} *or pVSP61::AvrRps4* WT. The Ws *rps4b-1/rps4-21* double mutant was transformed with *A. tumefaciens pBGW::RPS4B*$^{Ws-2}$, *pBGW::RPS4B*^{RLD} or *pBGW::RPS4B*$^{\Delta RLD}$ (*RPS4B*$^{\Delta RLD}$ is *RPS4B*^{RLD} with the inserted nucleotide at position 3829 bp removed. This abolishes the *RPS4*^{RLD} frameshift and restores the full coding sequence). Cell death phenotypes were observed 24 hpi. Red arrows indicate leaf collapse due to HR. Experiments were repeated at least three times with similar results.

5.3 Discussion

In this **chapter 5**, I investigated the different *RRS1-RPS4B*–dependent phenotypes observed in Ws-2, Col-0 and RLD in response to AvrRps4. As a starting point, I analyzed the sequence polymorphisms in *RRS1B-RPS4B* between these accessions. Taking *RRS1B-RPS4B* sequences from Ws-2 as a reference, Col-0 contained far more non-synonymous mutations than RLD (**Figure 5.1**). This suggests that Col-0 *RRS1B* and *RPS4B* diverged compared to Ws-2 and RLD alleles. This is interesting given that the Col-0 *RRS1B-RPS4B* pair is able to confer resistance to *Pst* DC3000 (AvrRps4) whereas RLD is not. This suggests that RLD *RRS1B* and/or *RPS4B* alleles carry specific mutation(s) deleterious for AvrRps4 recognition.

In order to understand if *RRS1B-RPS4B*$^{Col-0}$ alleles were responsible for the absence of HR to AvrRps4, I tested whether *RRS1B*$^{Ws-2}$ or *RPS4B*$^{Ws-2}$ could complement Col-0 *rps4-2* for AvrRps4–triggered HR. Only Col-0 *rps4-2 RRS1B*$^{Ws-2}$ transformants showed HR to AvrRps4 (**Figure 5.2**). This suggests that *RRS1B*$^{Col-0}$ is a limiting factor for AvrRps4–triggered HR via RRIR. Considering that HR can be observed in Col-0 when *RPS4* or *RPS4*^{TIR} are overexpressed (Wirthmueller *et al.*, 2007; Swiderski *et al.*, 2009), it would be important to control the amount of RRS1B protein in Col-0 *rps4-2 RRS1B*$^{Ws-2}$ and test whether RRS1B levels are responsible for the HR phenotype. Therefore, as a control, it will be necessary to transform Col-0 *rps4-2* with its own *RRS1B*$^{Col-0}$ allele (with own promoter and terminator) to test whether an additional *RRS1B*$^{Col-0}$ allele can complement the HR to AvrRps4. Ideally, I would test response to AvrRps4 in a Col-0 *rps4-2*/*rrs1b-1* or *rrs1-3*/*rrs1b-1* double mutant complemented with *RRS1B*$^{Ws-2}$ and *RRS1B*$^{Col-0}$. These double mutants are knocked-out for *RRS1B* and therefore should not naturally produce RRS1B protein. Using such double mutants would help to assess exclusively the functionality of the

transformed *RRS1B* alleles. Additionally, they could also be transformed with *RRS1B*^{RLD} to test its functionality. The observation that *RRS1B*$^{Ws\text{-}2}$ complements Col-0 for AvrRps4–triggered HR suggests that *RRS1B* might positively be involved in downstream signaling. From **chapter 3**, we hypothesized that RRS1 is required for a functional recognition complex but suppresses the RPS4–mediated downstream signaling prior to effector recognition. If we consider that RRS1B-RPS4B functions in a similar manner, based on my results, we could imagine that upon AvrRps4 perception, both RRS1B and RPS4B participate to downstream signaling activation.

Strikingly, an insertion in *RPS4B*^{RLD} causes a frameshift and an early stop codon resulting in a truncated protein compared to RPS4B$^{Ws\text{-}2}$. Interestingly, the C-terminal fragment lacking in RPS4B^{RLD} carries a NLS as predicted in RSP4B$^{Ws\text{-}2}$ (**Figure 5.1**). I confirmed that RPS4^{RLD} was excluded from the nucleus whereas RPS4B$^{Ws\text{-}2}$ was mostly nuclear (**Figure 5.3**). The *RPS4B*^{RLD} allele did not complement Ws *rps4b-1*/*rps4-21* for AvrRps4–triggered HR but *RPS4B*$^{Ws\text{-}2}$ did (**Figure 5.4**). In order to determine whether the C-terminus of RPS4B^{RLD} is responsible for the non HR phenotype, I removed the insertion in *RPS4B*^{RLD} allele causing the frameshift. The RPS4B$^{\Delta RLD}$ protein resulted in an identical protein size as RPS4B$^{Ws\text{-}2}$ and it complemented Ws *rps4b-1*/*rps4-21* for AvrRps4 recognition. This suggests that the missing fragment in RPS4B^{RLD} (compared to RPS4B$^{Ws\text{-}2}$) is responsible for its non-functionality. NB-LRR nuclear accumulation can be essential for defense responses activation (Burch-Smith *et al.*, 2007; Wirthmueller *et al.*, 2007). Therefore, we can speculate that RPS4^{RLD} is unable to activate defense due to its (mis)localization. Investigation on RRS1B^{RLD} functionality still need to be carried out.

Considering the importance of TIR dimerization for activation of downstream signaling, full length RPS4 might also dimerize for function. If we consider that

RPS4 and RPS4B function similarly, the activated RPS4B homodimer carries two NLS. In the Ws-2- *rps4-21* x RLD F1, theoretically both RPS4B^{Ws-2} and RPS4B^{RLD} protein should be produced in the cell. We could imagine that a RPS4B^{Ws-2}/RPS4B^{RLD} dimeric complex would not be as efficient as a RPS4B^{Ws-2} homodimer to reach the nucleus. Therefore the defense output observed would be reduced and this could explain the weak HR often observed in the Ws-2 *rps4-21* x RLD population.

In **chapter 4**, I presented a transient assay for AvrRps4 and PopP2 recognition in *N.tabacum*. In parallel to Arabidopsis experiments, I plan to use this assay to test the functionality of *RRS1B-RPS4B* from RLD and Col-0 for AvrRps4 recognition.

6 Functional comparison of RRS1-RPS4 and RRS1B-RPS4B towards effector recognition and activation of downstream signaling

6.1 Introduction and objectives

Following up on the study of RRS1B-RPS4B sequence polymorphisms between various Arabidopsis accessions, I wanted to compare RRS1B-RPS4B and RRS1-RPS4 functions. In **chapter 4**, I demonstrated the high conservation between the two pairs in their sequence and domain architecture, indicating a probable duplication of the *R* locus. The selective advantage to maintain two *R* gene pairs recognizing the same effector, AvrRps4, is intriguing (see **chapter 4**). Potentially both pairs guard a key component within the plant cell that requires extra protection. Therefore, we could assume that two guarding systems might be better than one. Moreover, we could hypothesize that an original *R* gene pair duplicated to create additional recognitions.

It is known that RRS1-RPS4 recognizes AvrRps4, PopP2 and a putative effector from *C. higginsianum* whereas RRS1B-RPS4B recognizes only AvrRps4 (see **chapter 4**). The aim of this chapter is to do comparative functional analyses of these two paralogous *R* gene pairs. The identification of the RRS1B-RPS4B pair provides a unique opportunity to test conserved functionality of paired TIR-NB-LRR proteins. The study of RRS1-RPS4 and RRS1B-RPS4B will enable the description of the mechanisms by which these R proteins act together to recognize AvrRps4 and PopP2 and activate downstream signaling.

Initially I compared AvrRps4 and PopP2 recognition in different Arabidopsis accessions. I identified some accessions that recognize neither, only AvrRps4 or both. This phenotyping, and the analysis of sequence polymorphisms between RRS1-RPS4 and RRS1B-RPS4B enabled the identification of specific residues

required for effector recognition. I tested a suite of AvrRps4 mutants for their ability to be recognized independently of *RPS4* or *RPS4B*. All AvrRps4 mutants tested triggered an HR, except two that weakened *RRS1B-RPS4B*–dependent recognition. I investigated common characteristics between the RRS1/RRS1B and RPS4/RPS4B TIR domains. Despite predicted structural similarities between $RPS4^{TIR}$ and $RPS4B^{TIR}$, only $RPS4^{TIR}$ triggered cell death when overexpressed in *Nicotiana spp*. I was able to show that even though RRS1-RPS4 and RRS1B-RPS4B do not cooperate in effector recognition, their TIR domains can associate *in planta*. Moreover, swapping $RRS1^{TIR}$ and $RRS1B^{TIR}$ does not affect effector recognition by the full-length protein. Interestingly, association of proteins from different pairs could be observed *in planta* using CoIP. Looking into the AvrRps4 and PopP2 recognition, I was able to show that both AvrRps4 and PopP2 associate with RRS1 and RRS1B *in planta*. Swapping exons 5, 6 and 7 from RRS1B into RRS1 retains AvrRps4 but abrogates PopP2 recognition. Finally, I investigated the possible involvement of the three TIR-NB-LRRs carrying a WRKY domain in Arabidopsis basal immunity. I tested a triple mutant of all of these WRKY-carrying TIR-NB-LRRs; surprisingly, this did not result in significant loss/gain of basal resistance to *Pst* DC3000 or HopA1–triggered immunity.

6.2 Results

6.2.1 Several Arabidopsis accessions exhibit AvrRps4 but not PopP2 recognition.

Combining allelic diversity data is a powerful strategy to associate a phenotype to a specific genetic polymorphism (Aranzana *et al.*, 2005; Atwell *et al.*, 2010). I was interested to know if I could identify Arabidopsis accessions that, similarly to the Ws-2 *rps4-21* mutant, recognize AvrRps4 but not PopP2. I tested the MAGIC parental lines for AvrRps4– and PopP2–triggered HR (Kover *et al.*, 2009).

These Arabidopsis accessions have been intercrossed to generate multiple recombinant lines, and are thus very useful genetic resources for gene mapping. Moreover, these accessions have been sequenced and data is publicly available. I infiltrated the 19 MAGIC parents with *Pf* Pf0-1 carrying either AvrRps4 or PopP2. As expected, 24 hours after infiltration, Col-0 did not show HR to *Pf* Pf0-1 (AvrRps4) or (PopP2) whereas Ws-0 did (**Table 6.1**). Interestingly, I could identify 3 accessions, Bur-0, Kn-0 and Tsu-0 that showed HR to *Pf* Pf0-1 (AvrRps4) but not to *Pf* Pf0-1 (PopP2) (**Table 6.1**). This suggests that, in these accessions, at least one of the pairs is recognizing AvrRps4. Therefore, it would be very interesting to study the sequence polymorphism in RRS1-RPS4 and RRS1B-RPS4B to see if there is any common mutation correlating with the absence of PopP2 recognition. Another interesting observation is the absence of HR to *Pf* Pf0-1 (AvrRps4) and *Pf* Pf0-1 (PopP2) in Wu-0. Analysis of RRS1-RPS4 and RRS1B-RPS4B sequences in Wu-0 compared to other accessions that do not recognize AvrRps4 and PopP2 might enable the identification of common SNP that could be associated to the absence of effector recognition. Also, it would be interesting to investigate if the absence of HR correlates with absence of disease resistance to *Pst* DC3000 carrying AvrRps4 or PopP2 or if, as in Col-0, disease resistance can be observed without presence of HR. All the other accessions tested showed HR to *Pf* Pf0-1 (AvrRps4) and to *Pf* Pf0-1 (PopP2) (**Table 6.1**).

Accessions	AvrRps4	PopP2
Bur-0	HR	no HR
Can-0	HR	HR
Col-0	no HR	no HR
Ct-0	HR	HR
Edi-0	hr	hr
Hi-0	HR	HR
Kn-0	HR	no HR
Ler-0	HR	HR
Mt-0	HR	HR
No-0	HR	HR
Oy-0	HR	HR
Po-0	HR	HR
Rsch-4	HR	HR
Sf-2	HR	HR
Tsu-0	HR	no HR
Wil-2	HR	HR
Ws-0	HR	HR
Wu-0	no HR	no HR
Zu-0	hr	hr

Table 6.1. HR cell death phenotype in Arabidopsis MAGIC parent accessions in response to *Pf* Pf0-1 expressing *AvrRps4* or *PopP2*. “hr” indicates a lower degree of tissue collapse observed.

6.2.2 Mutations in AvrRps4 show identical *RPS4*– and *RPS4B*–dependent recognition.

Studies of avirulent proteins can reveal the mechanisms by which they are recognized by their cognate R proteins. Different AvrRps4 mutants have been tested for their ability to trigger a *RPS4*–independent HR in Ws-2 (Sohn *et al.*, 2012) and the crystal structure of the C-terminal fragment of AvrRps4 was determined. Based on this structure, surface residues were revealed and several of them were targeted for mutagenesis. They were chosen based on their probable involvement in protein-protein interaction. Using the Pf *Pf0-1* system, these mutants were tested for their ability to trigger HR in Ws-2 and the *rps4-21* mutant. Two AvrRps4 mutations (E175A and E187A) abolished recognition in Ws-2 and one mutation (L167T) triggered less HR in Ws-2 *rrs1-1* mutant than in Ws-2 (Sohn *et al.*, 2012). The L167 residue was suggested to be especially required for the *RRS1B-RPS4B*–dependent AvrRps4 recognition. With the finding that *RRS1B-RPS4B* is responsible for the RRIR, I used the Ws-4 *rps4b-1* mutant to test if any of the AvrRps4 mutants used by Sohn *et al*. (2012) abolished specifically *RRS1-RPS4*–dependent AvrRps4 recognition. To do so, I infiltrated Arabidopsis leaves with *Pf* Pf0-1 carrying the different AvrRps4 mutants generated by Sohn *et al*. (2012). All the WT and AvrRps4 mutants tested triggered no HR in the *rps4b-1*/*rps4-21* double mutant, suggesting that none of them provided an AvrRps4 gain of function for novel recognition in Arabidopsis. As reported by Sohn *et al*. (2012), AvrRps4 KRVY^{AAAA}, E175A and E187A did not trigger HR in Ws-2. All the other mutations tested triggered HR in *rps4b-1* and Ws-2 suggesting that none of the mutated amino acids were specifically required for *RRS1-RPS4*–mediated AvrRps4 recognition. In the Ws-2 *rps4-21* mutant, the AvrRps4 L167T and E180D still triggered some HR phenotype but weaker than in Ws-2 or Ws-2 *rps4b-1*. This supports previous observation that L167 and E180 are specifically important for *RPS4B*–mediated AvrRps4

recognition, but in my hands, this was more of a quantitative than a qualitative difference (Sohn *et al.*, 2012).

AvrRps4	Ws-2	Ws-2 *rps4-21*	Ws-4 *rps4b-1*	*rps4b-1* /*rps4-21*
WT	HR	HR	HR	no HR
KRVY^{AAAA}	no HR	no HR	no HR	no HR
X138S	HR	HR	HR	no HR
A157E	HR	HR	HR	no HR
R159A	HR	HR	HR	no HR
E163A	HR	HR	HR	no HR
L167T	HR	hr	HR	no HR
E175A	no HR	no HR	no HR	no HR
Q177A	HR	HR	HR	no HR
I180D	HR	hr	HR	no HR
E182A	HR	HR	HR	no HR
P185Q	HR	HR	HR	no HR
A186D	HR	HR	HR	no HR
E187A	no HR	no HR	no HR	no HR
R196A	HR	HR	HR	no HR
R199A	HR	HR	HR	no HR
R213	HR	HR	HR	no HR

Table 6.2. HR cell death observed in Ws-2 wt and mutants 24 hpi with *Pf* Pf0-1 carrying wt and mutated AvrRps4. “hr” indicates a lower degree of tissue collapse observed.

6.2.3 Investigation of RRS1B and RPS4B TIR domains for effector recognition and defense signaling activation.

The two major classes of characterized NB-LRR R plant proteins are defined by their N-terminal domains, the CC and TIR domains (Dangl and Jones, 2001b). These domains are considered to play a crucial role in downstream signaling activation as they can trigger immune responses when overexpressed alone *in planta* (Maekawa *et al.*, 2011; Collier *et al.*, 2011; Frost *et al.*, 2004; Weaver *et al.*, 2006; Swiderski *et al.*, 2009). I next tested whether $RRS1B^{TIR}$ and $RPS4B^{TIR}$ could trigger cell death when overexpressed in *N. tabacum* and *N. benthamiana*. I amplified $RRS1B^{TIR}$ (encoding amino acid 1 to 166) and $RPS4B^{TIR}$ (encoding amino acid 1 to 235) from Ws-2 gDNA and cloned them into *pK7FWG2* (C-terminus GFP tag) using the Gateway technology. These plasmids were transformed into *A. tumefaciens* for transient expression in *Nicotiana spp*. In this experiment, I also used $RRS1^{TIR}$ and $RPS4^{TIR}$ previously cloned in the laboratory and transferred them into *pK7FWG2*. For RRS1 and RPS4 TIR domains, the sequences encoding the first 250 amino acids were used for cloning. As expected, overexpression of $RPS4^{TIR\text{-}GFP}$ triggered cell death in *N. tabacum* and *N. benthamiana* (**Figure 6.1A**). The $RPS4^{TIR}$–triggered cell death could be observed already 2 days after *A. tumefaciens* infiltration. As shown previously, the $RRS1^{TIR\text{-}GFP}$ did not trigger cell death 5 days post infiltration and neither did the $RRS1B^{TIR\text{-}GFP}$. Surprisingly, $RPS4B^{TIR\text{-}GFP}$ did not trigger cell death (**Figure 6.1A**). These results correlate with the overexpression of RPS4B-GFP full length that did not trigger any response at all in *N. tabacum* (**Figure 4.8A**). This implies either that some polymorphism in $RPS4B^{TIR}$ compared to $RPS4^{TIR}$ abolishes its capacity to activate cell death or that *N. tabacum* lacks a component required for $RPS4B^{TIR}$–triggered cell death. The accumulation of proteins was confirmed 2 days post infiltration of *A. tumefaciens*

strains into *N. benthamiana* by immunoblot using an anti-GFP antibody (**Figure 6.1B**).

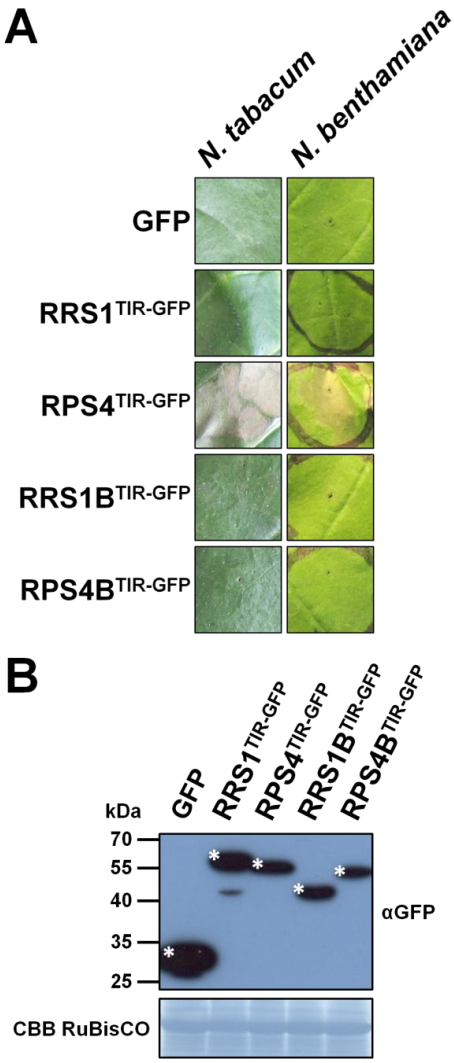

Figure 6.1. Overexpression of RPS4B^{TIR} does not trigger cell death in *Nicotiana spp*.

A. 4 to 5 week-old *N. tabacum* and *N. benthamiana* leaves were syringe-infiltrated with *A. tumefaciens* solutions at OD_{600}=0.5 carrying either *pK7FWG2* (GFP), *pK7FWG2::RRS1*^{TIR} (RRS1$^{TIR-GFP}$), *pK7FWG2::RPS4*^{TIR} (RPS4$^{TIR-GFP}$), *pK7FWG2::RRS1B*^{TIR} (RRS1B$^{TIR-GFP}$) or *pK7FWG2::RPS4B*^{TIR} (RPS4B$^{TIR-GFP}$). Cell death pictures were taken 5 dpi. **B**. Anti-GFP immunoblot showing GFP and GFP-tagged TIR domains accumulation 2 days after *A. tumefaciens*–mediated transient expression in *N. benthamiana*. RRS1^{TIR} and RPS4^{TIR} consist of the first 250 amino acids of the proteins. RRS1B^{TIR} consists of the first 166 amino acids of the protein. RPS4B^{TIR} consists of the first 235 amino acids of the protein. In this experiment, TIR domains DNA sequences were amplified and cloned from the Ws-2 gDNA. Asterisks indicate the expected protein fusion sizes.

Crystallographic data showed that CC and TIR domains from MLA10 and L6 respectively can homodimerize (Maekawa *et al.*, 2011; Bernoux *et al.*, 2011). These authors proposed that this dimerization is required for R protein activation and downstream signaling. Studies have been carried out on the RRS1 and RPS4 TIR domains for dimerization and tertiary structure. Using biochemistry and crystallography, it was shown that $RRS1^{TIR}$ and $RPS4^{TIR}$ can homo- and hetero-dimerize (Williams, Sohn *et al*., unpublished). Based on the crystal structures, a dimeric interface was defined. This interface is identical in the crystal structure of $RRS1^{TIR}$/$RPS4^{TIR}$ heterodimer and individual $RRS1^{TIR}$ and $RPS4^{TIR}$ (indicated by red lines in **Figure 6.2A and C**). Mutations in the dimeric interface prevented TIR-TIR domain interactions, abolished AvrRps4 and PopP2 recognition by RRS1-RPS4 in *N. tabacum* transient assay and suppressed $RPS4^{TIR}$–triggered cell death. In order to investigate $RRS1B^{TIR}$ and $RPS4B^{TIR}$ particularities for function and to examine if similarities could exist with $RRS1^{TIR}$ and $RPS4^{TIR}$, I analyzed the amino acid sequence polymorphism between RRS1/RRS1B and RPS4/RPS4B TIR domains. $RRS1^{TIR}$ and $RRS1B^{TIR}$ consist of 5 parallel β strands surrounded by 5 α helices (**Figure 6.1A**). $RPS4^{TIR}$ and $RPS4B^{TIR}$ are structured similarly with 5 β sheets but 7 α helices instead (**Figure 6.2C**). Indeed, a major difference between RRS1/RRS1B and RPS4/RPS4B TIR domains is the absence of the αD1 and αD2 helices in $RRS1^{TIR}$ and $RRS1B^{TIR}$. The consequences of the absence of αD1 and αD2 helices in $RRS1^{TIR}$ and $RRS1B^{TIR}$ are unclear. Despite amino acid polymorphisms, the overall predicted TIR domain secondary and tertiary structures of RRS1/RRS1B and RPS4/RPS4B appeared similar (**Figure 6.2A, B, C and D**). In the dimeric interface, a few amino acids were defined as essential for dimerization by Williams, Sohn *et al*. (unpublished) (bold letters in sequences in **Figure 6.2A and C**). I next examined if the amino acids involved in the dimeric interface were conserved between $RRS1^{TIR}$-$RPS4^{TIR}$ and $RRS1B^{TIR}$-$RPS4B^{TIR}$. The three chains involved in $RRS1^{TIR}$ dimerization were identical in $RRS1B^{TIR}$ except for D103 (substitution in arginine (R)

for RRS1B^{TIR}). In RPS4^{TIR} the interface involves only 2 amino acid chains. In the first one, RPS4^{TIR} R29 and R30 are substituted in leucine (L) and glycine (G) respectively in RPS4B^{TIR}. These two arginines were critical for RPS4^{TIR} dimerization and cell death activity as well as S33 and H34 which are conserved into RPS4B^{TIR} (Williams, Sohn *et al*., unpublished). Interestingly, when RPS4^{TIR} R30 was mutated to an alanine, RPS4^{TIR} homodimerization was strengthened and the cell death it triggered in *N. tabacum* was stronger. In the second interaction area, the K155 and E160 are conserved in RPS4B^{TIR} but the V159 is substituted into proline (P) which could potentially modify the interaction core. The amino acid chains responsible of the TIR-TIR interaction in RRS1-RPS4 are partially conserved in RRS1B-RPS4B suggesting that interaction could occur at similar interface. However, several substitutions were identified in this interface which might dictate specificity for TIR-TIR interaction between the RRS1-RPS4 and RRS1B-RPS4B.

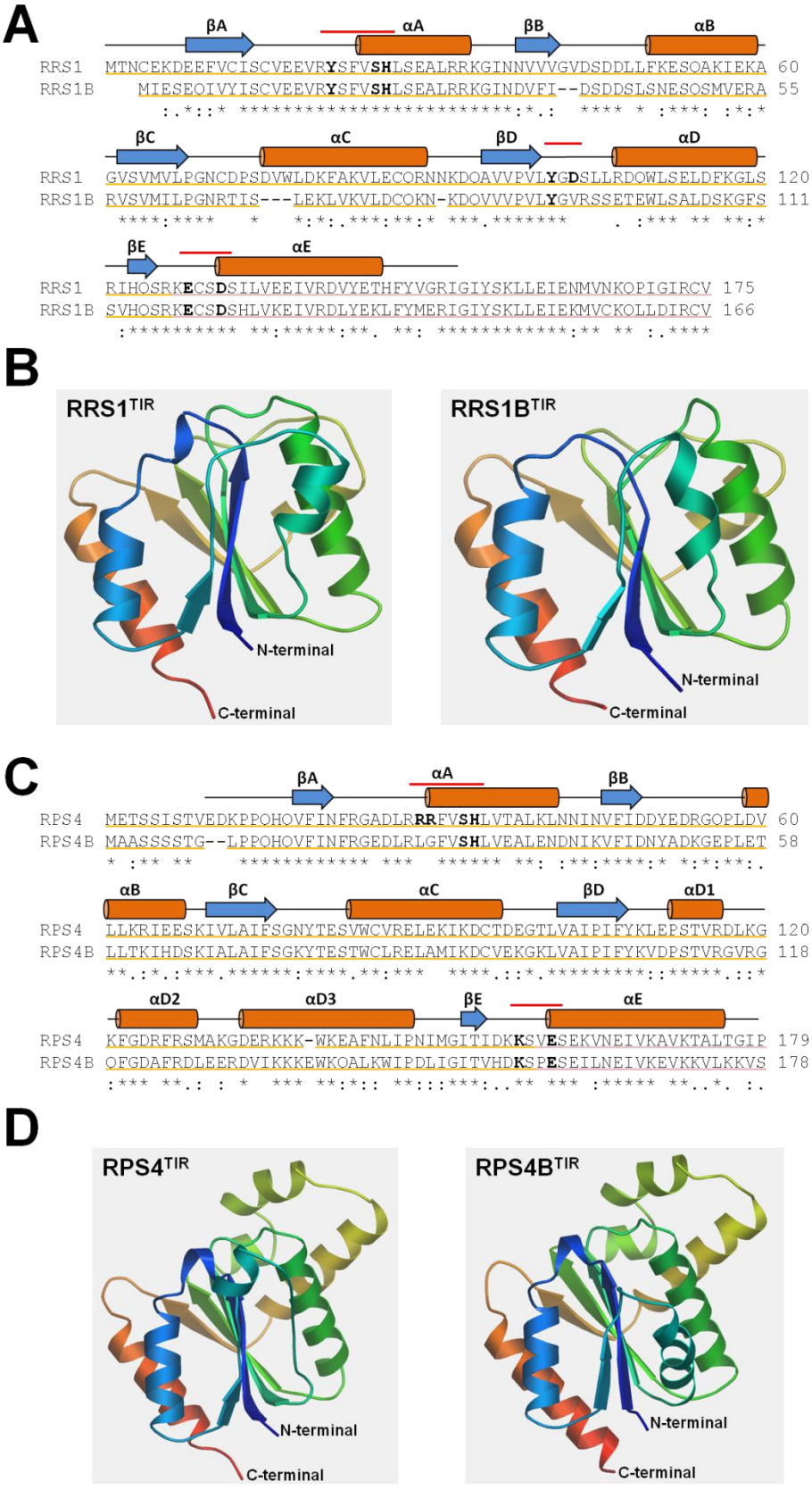

Figure 6.2. Comparison of the predicted secondary and tertiary structures of RRS1, RRS1B, RPS4 and RPS4B TIR domains.

A and **C**. Alignment of the TIR domain amino acid sequences of RRS1 and RRS1B (**A**) and RPS4 and RPS4B (**C**). Amino acids from exon1 are underlined with orange, amino acids form exon2 are underlined with pink. The secondary structure has been predicted using the consensus secondary structure prediction provided by the NPS website from Manchester University (http://www.bioinf.manchester.ac.uk/dbbrowser/bioactivity/NPS2.html). ß sheets are represented by blue arrows and α helices by orange cylinders. Red lines represent the interface of RRS1 and RPS4 TIR dimerization (Williams, Sohn *et al*., unpublished). **B** and **D**. Prediction of the tertiary structure of RRS1 and RRS1B TIR domains (**B**) and RPS4 and RPS4B (**D**). The tertiary structure has been predicted using the SWISSMODEL website (http://swissmodel.expasy.org/). Protein sequences corresponding to Ws-2 alleles were analyzed.

The TIR domains of RRS1 and RPS4 interact in a yeast two hybrid assay and associate *in planta* using CoIP (Williams, Sohn *et al*., unpublished). I next investigated if RRS1B^{TIR} and RPS4B^{TIR} can associate with themselves and with each other *in planta*. Moreover, I also tested if they could associate with RRS1^{TIR} and RPS4^{TIR}. For this experiment, I used the GFP–tagged constructs described previously (**Figure 6.1**) as well as the same TIR domains clones but tagged with HA instead (using the *pBAV139* vector). To test the protein association *in planta*, I co-infiltrated *N. benthamiana* with *A. tumefaciens* strains carrying different tagged TIR domains. I purified proteins from transiently transformed plant tissue two days after infiltration and I performed an anti-HA CoIP on each sample. The two first top blots represent detection of protein in the total extracts and the two bottom blots represent detected proteins after the anti-HA IP (**Figure 6.3**). Confirming previous observation by Williams, Sohn *et al*. (unpublished), RRS1^{TIR-HA} and RPS4^{TIR-HA} pulled down RPS4$^{TIR-GFP}$ and RRS1$^{TIR-GFP}$ respectively (rows 1 and 7). I then tested the CoIP of FLAG-GFP, RRS1B$^{TIR-GFP}$, RPS4B$^{TIR-GFP}$, RRS1$^{TIR-GFP}$ and RPS4$^{TIR-GFP}$ by either RRS1B^{TIR-HA} (rows 2 to 6) or RPS4B^{TIR-HA} (rows 8 to 12). I detected RRS1B$^{TIR-GFP}$, RPS4B$^{TIR-GFP}$, RRS1$^{TIR-GFP}$ and RPS4$^{TIR-GFP}$ signal after CoIP with either RRS1B^{TIR-HA} or RPS4B^{TIR-HA}. After CoIP, a signal could be observed for FLAG-GFP on the blot but the intensity was significantly weaker compared to the signal given by the pulled-down TIR domains. This suggests that RRS1B^{TIR} and RPS4^{TIR} can associate with themselves, with each other and with the RRS1^{TIR} and RPS4^{TIR} *in planta*.

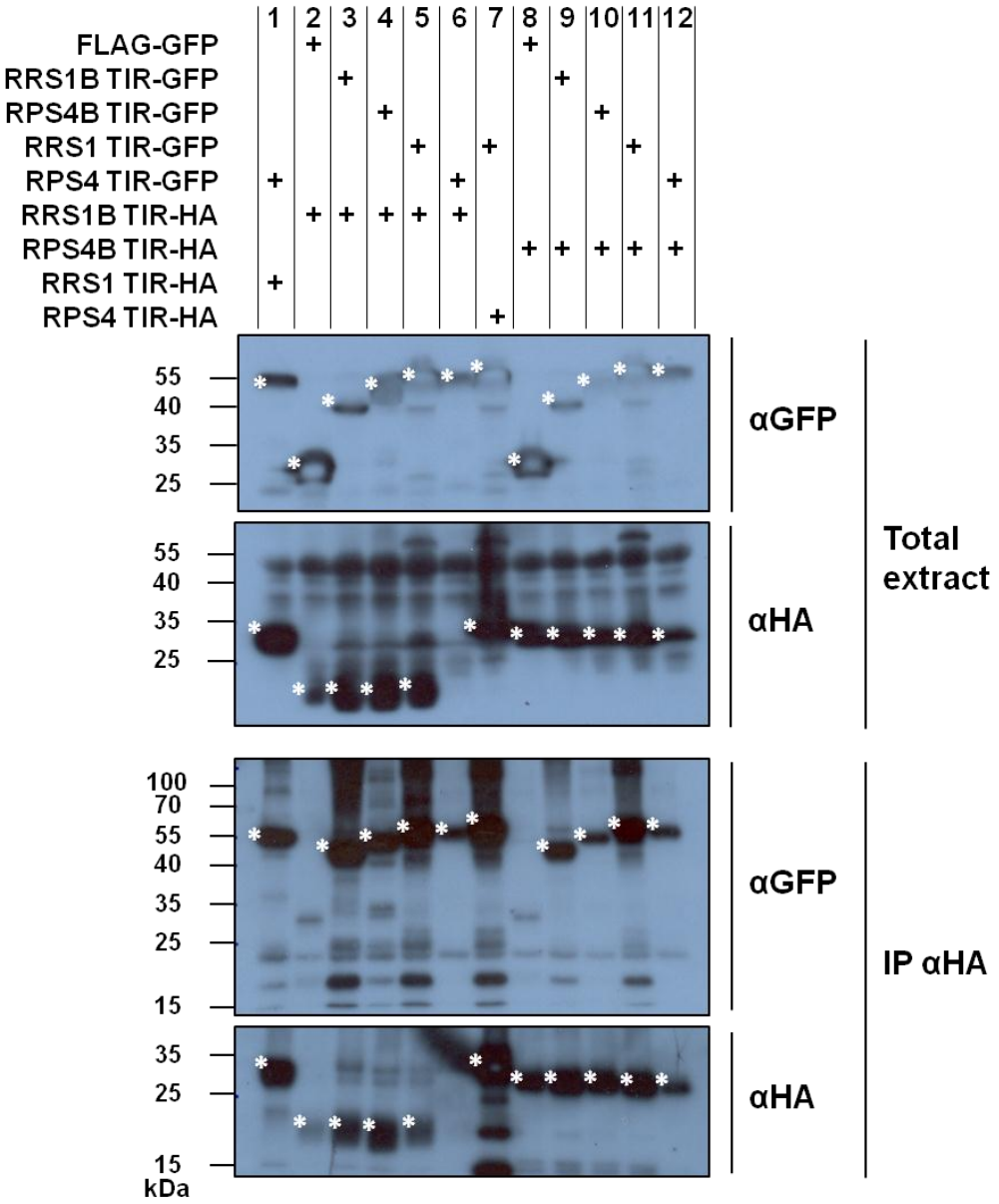

Figure 6.3. TIR domains of RRS1B and RPS4B self-associate, cross-associate and associate with the TIR domains of RRS1 and RPS4.

4 to 5 week-old *N. benthamiana* leaves were syringe-infiltrated with a mix of *A. tumefaciens* solutions at OD_{600}=0.5. Fusion proteins expressed in each mix are indicated by a cross symbol at the top of the figure. Protein samples were purified from plant tissue 2 days after infiltration for immunoblot analysis. Immunoblots show presence of proteins in total extract and after co-immunoprecipitation. In this experiment, the DNA sequences of TIR domains were amplified and cloned from the Ws-2 gDNA. Asterisks indicate the expected size of tagged-proteins. Experiments have been repeated twice with similar results.

The fact that RRS1^{TIR}, RPS4^{TIR}, RRS1B^{TIR} and RPS4B^{TIR} could associate altogether does not correlate with a hypothetical specific protein-protein interaction between pair partners for function. Therefore, I next assessed if the TIR domains were a limiting factor for R protein function with their respective pair partner. To answer this question, I swapped the TIR domains of RRS1^{Ws-2} and RRS1B^{Ws-2}, to get RRS1 (RRS1B^{TIR}-RRS1$^{NB-LRR-WRKY}$) and RRS1B (RRS1^{TIR}-RRS1B$^{NB-LRR-WRKY}$) full length chimeric genes. I then tested these chimeras for AvrRps4 and PopP2 recognition in a transient assay in *N. tabacum*. When co-expressed with *RPS4-HF*, *RRS1-HF* recognized *AvrRps4-GFP* and *PopP2-GFP* resulting in cell death (**Figure 6.4A**). Similar results were obtained when *RPS4-HF* was co-expressed with *RRS1B*^{TIR}*-RRS1*$^{NB-LRR-WRKY}$*-HF*. This implies that RRS1^{TIR} can be replaced by RRS1B^{TIR} without altering AvrRps4 and PopP2 recognition in the presence of RPS4. Transient co-expression of *RPS4B-HF* with *RRS1B-HF* enabled the recognition of AvrRps4 but not PopP2. However, when *RPS4B-HF* was co-expressed with *RRS1*^{TIR}*-RRS1B*$^{NB-LRR-WRKY}$*-HF* and AvrRps4, the cell death phenotype was significantly weaker than with *RRS1B-HF* (**Figure 6.4A**). No cell death was observed when co-expressed with *PopP2*. This suggests that the RRS1B^{TIR} swap by the RRS1^{TIR} might alter RRS1B protein for AvrRps4 recognition in presence of RPS4B. Accumulation of RRS1B^{TIR}-RRS1$^{NB-LRR-WRKY}$HF and RRS1^{TIR}-RRS1B$^{NB-LRR-WRKY}$-HF chimeric proteins was confirmed by immunoblot (**Figure 6.4B**).

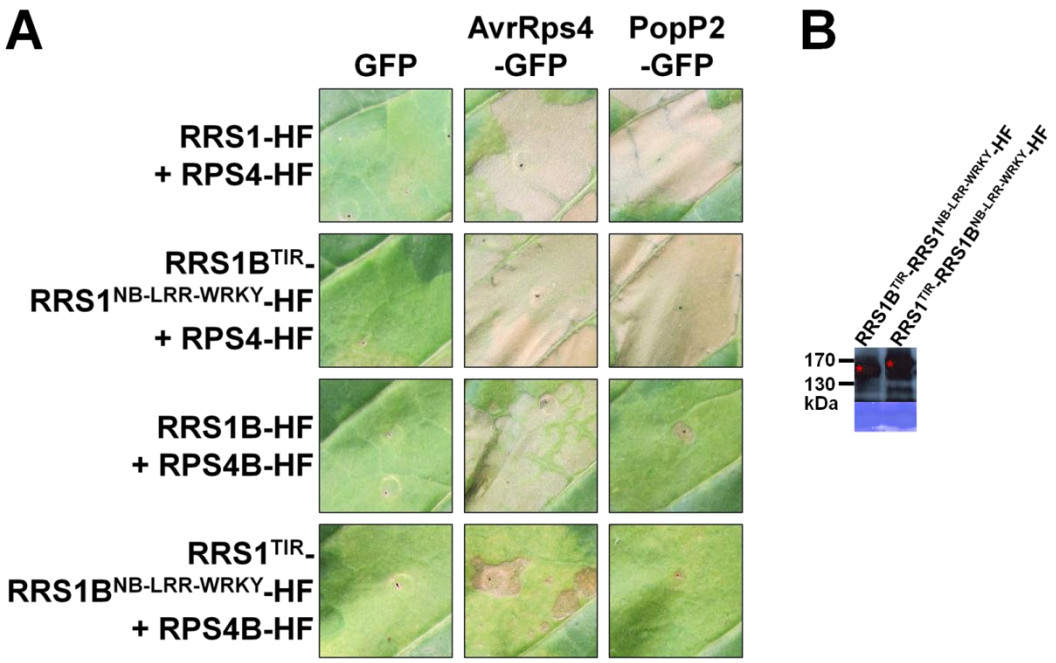

Figure 6.4. Swapping the TIR domains of RRS1 and RRS1B retains effector recognition of the tandem R proteins.

A. 4 to 5 week-old *N. tabacum* leaves were syringe-infiltrated with a mix of *A. tumefaciens* solutions (OD_{600}=0.5). RRS1, RPS4, RRS1B, RPS4B and the chimeras ($RRS1B^{TIR}$-$RRS1^{NB\text{-}LRR\text{-}WRKY}$ and $RRS1^{TIR}$-$RRS1B^{NB\text{-}LRR\text{-}WRKY}$) were tagged with HisFlag (HF); AvrRps4 and PopP2 were tagged with GFP. The chimeras were created by swapping the coding sequences for the first 153 amino acids of RRS1 with the first 144 amino acids of RRS1B in RRS1 and RRS1B proteins. In this experiment, TIR domains were amplified and cloned from the Ws-2 gDNA. Pictures were taken 5 dpi. Experiments have been repeated at least three times with reproducible results. **B**. Western blot analysis of *A. tumefaciens*–mediated transient expression of $RRS1B^{TIR}$-$RRS1^{NB\text{-}LRR\text{-}WRKY}$ and $RRS1^{TIR}$-$RRS1B^{NB\text{-}LRR\text{-}WRKY}$ chimeras fused to HF, 2 days after infiltration in *N. benthamiana*.

6.2.4 Identification of R protein complexes between RRS1, RPS4, RRS1B and RPS4B.

Data obtained in **chapter 4** support the idea that the cooperation for effector recognition between partners of each gene pair is specific and does not extend to genes of the other pair. Similarly to $RRS1^{TIR}$ and $RPS4^{TIR}$, association *in planta* between RRS1 and RPS4 full length proteins has been identified using CoIP (Williams, Sohn *et al*., unpublished). I was interested to test whether RRS1B and RPS4B are able to associate *in planta* and if this association was specific to pair partners. Therefore, I transiently co-expressed *RRS1B-HF* and *RPS4-HF* with either *GFP*, *RRS1-GFP*, *RPS4-GFP*, *RRS1B-GFP* or *RPS4B-GFP* in *N. benthamiana*. I extracted protein 2 days after infiltration. In each total extract samples, I detected either RRS1B-HF or RPS4B-HF using an anti-FLAG antibody. Similarly, I specifically detected GFP, RRS1-GFP, RPS4-GFP, RRS1B-GFP or RPS4B-GFP in total extracts depending on the *A. tumefaciens* strains used (indicated by a sign + I at the top of the **Figure 6.5**). I did not detect RPS4-GFP when coexpressed with *RRS1B-HF*. In a subsequent CoIP on total extracts using anti-FLAG beads, I detected RRS1-GFP, RRS1B-GFP and RPS4B-GFP (**Figure 6.5**). After IP of samples containing RPS4B-HF, I detected only RRS1-GFP and RRS1B-GFP. Collectively these data suggest that, in absence of AvrRps4 and PopP2, proteins from RRS1-RPS4 and RRS1B-RPS4B pairs can associate *in planta*. Notably, RRS1B associates with itself, RPS4B and RRS1; RPS4B associates with RRS1B and RRS1.

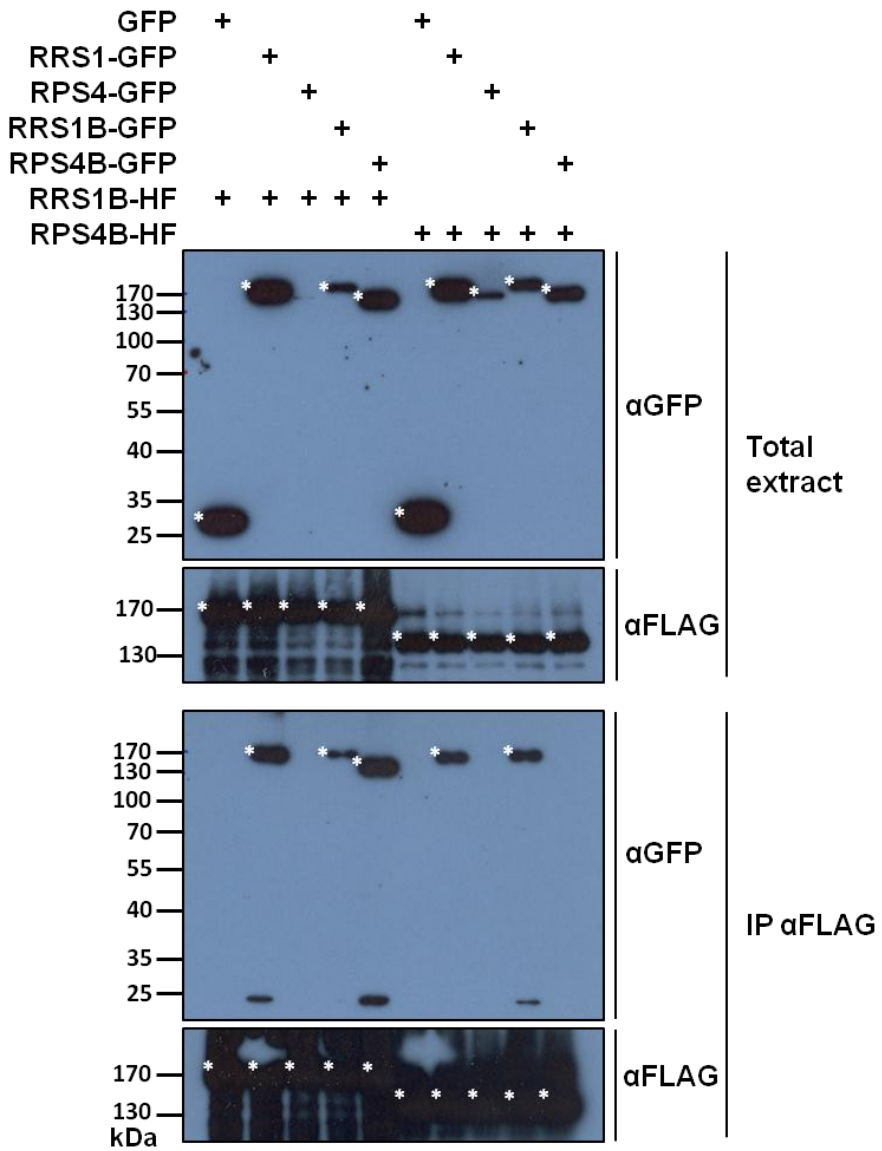

Figure 6.5. RPS4, RRS1, RPS4B and RRS1 can associate *in planta* in the absence of bacterial effector.

4 to 5 week-old *N. benthamiana* leaves were syringe-infiltrated with a mix of *A. tumefaciens* solutions at OD_{600}=0.5. Fusion proteins expressed in each mix are indicated by a cross symbol at the top of the figure. Protein samples were purified from plant tissue 2 days after infiltration for immunoblot analysis. Immunoblots show presence of proteins in total extract and after co-immunoprecipitation. In this experiment, *RRS1*, *RPS4*, *RRS1B* and *RPS4B* were amplified and cloned from the Ws-2 gDNA. Asterisks indicate the expected tagged-protein size. The experiment was not yet repeated.

6.2.5 Investigating the differential recognition of PopP2 by the two different gene pairs, *RRS1-RPS4* and *RRS1B-RPS4B*.

Both RRS1-RPS4 and RRS1B-RPS4B recognize AvrRps4. However, the mechanism by which these two *R* gene pairs recognize AvrRps4 is still unknown. On the other hand, RRS1B-RPS4B does not recognize PopP2 while RRS1-RPS4 does. Despite extensive efforts, the reasons for presence/absence of PopP2 recognition are still unclear. Previous and ongoing research on the differential PopP2 recognition by RRS1 alleles revealed that polymorphism within the C-terminus part of RRS1 might be a determining factor (Deslandes *et al.*, 2002b)(Sarris *et al*., unpublished). I previously examined the amino acid sequence differences between $RRS1^{Ws\text{-}2}$ and $RRS1B^{Ws\text{-}2}$ in exons 5, 6 and 7, and as reported in **chapter 4**, several deletions and amino acid substitutions were identified in this region (**Figure S3**). To assess if the RRS1/RRS1B polymorphism is responsible for the differential PopP2 recognition, I swapped the exons 5, 6 and 7 between RRS1 and RRS1B. Each chimera was assembled with a C-terminus HF tag and tested in an *A. tumefaciens*–mediated *N. tabacum* transient assay. AvrRps4- and PopP2-triggered cell death was observed when *RRS1-HF* was co-expressed with *RPS4-HF* (**Figure 6.6A**). However, when *RPS4-HF* was co-expressed with $RRS1^{Exon\ 1,2,3,4}$-$RRS1B^{Exon\ 5,6,7}$-*HF*, only AvrRps4 triggered cell death, suggesting that the protein is functional. However $RRS1^{Exon\ 1,2,3,4}$-$RRS1B^{Exon\ 5,6,7}$-HF lost the ability to recognize PopP2 suggesting that the RRS1 exons 5, 6 and 7 are essential for PopP2 recognition. Co-expression of *RRS1B-HF* and *RPS4B-HF* triggered cell death in the presence AvrRps4 but not with PopP2 (**Figure 6.6A**). When *RPS4B* was co-expressed with $RRS1B^{Exon\ 1,2,3,4}$-$RRS1^{Exon\ 5,6,7}$-*HF*, no cell death was observed in the presence of AvrRps4 or PopP2. Considering that RRS1 and RRS1B are able to recognize AvrRps4 with their respective partner, this result suggests that $RRS1B^{Exon\ 1,2,3,4}$-$RRS1^{Exon\ 5,6,7}$-HF is non functional at least in combination with RPS4B. Both

RRS1$^{Exon\ 1,2,3,4}$-RRS1B$^{Exon\ 5,6,7}$-HF and RRS1B$^{Exon\ 1,2,3,4}$-RRS1$^{Exon\ 5,6,7}$-HF were detected on immunoblot using an anti-FLAG antibody (**Figure 6.6B**).

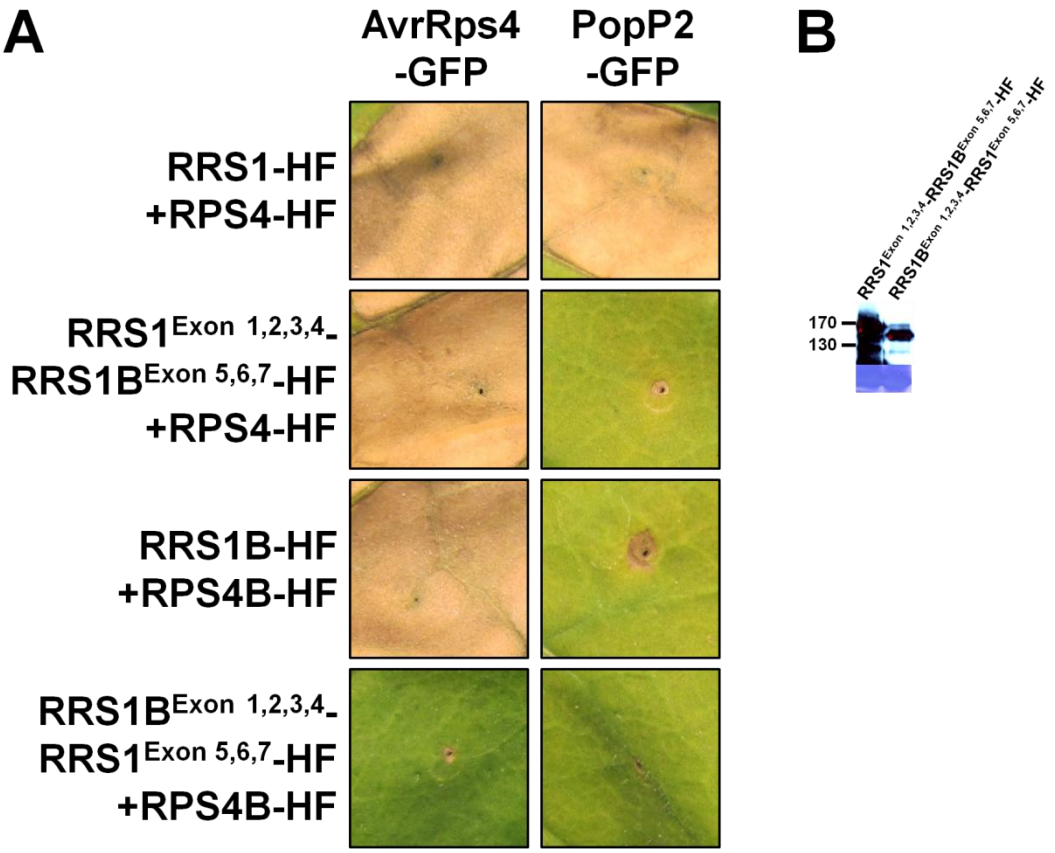

Figure 6.6. The C-terminal part of RRS1 encoded by exons 5, 6 and 7 is required for PopP2 recognition.

A. 4 to 5 week-old *N. tabacum* leaves were syringe-infiltrated with a mix of *A. tumefaciens* solutions at final OD_{600}=0.5. RRS1, RPS4, RRS1B, RPS4B and the chimeras (RRS1$^{Exon\ 1,2,3,4}$-RRS1B$^{Exon\ 5,6,7}$ and RRS1B$^{Exon\ 1,2,3,4}$-RRS1$^{Exon\ 5,6,7}$) were tagged with HisFlag (HF); AvrRps4 and PopP2 were tagged with GFP. The chimeras have been created by swapping coding sequences of the last 469 amino acids of RRS1 and the last 505 amino acids of RRS1B in RRS1 and RRS1B proteins. In this experiment, TIR domains DNA sequences were amplified and cloned from the Ws-2 gDNA. Pictures were taken 5 dpi. Experiments have been repeated at least three times with similar results. **B**. Western blot analysis of *A. tumefaciens*–mediated transient expression of RRS1$^{Exon\ 1,2,3,4}$-RRS1B$^{Exon\ 5,6,7}$ and RRS1B$^{Exon\ 1,2,3,4}$-RRS1$^{Exon\ 5,6,7}$ chimeras fused to HF, 2 days after infiltration in *N. benthamiana*.

Previously, RRS1 and PopP2 were shown to interact in a yeast split ubiquitin assay (Deslandes *et al.*, 2003). CoIP experiments carried out by Kee Hoon Sohn in the laboratory showed that they also associate *in planta*. However, this interaction is not likely to be sufficient for R protein activation as PopP2 was shown to interact with $RRS1^{Col\text{-}5}$ but it does not trigger immune response in Col-5 (Deslandes *et al.*, 2003). Similarly, we observed the association of RRS1 and AvrRps4 *in planta* using CoIP and split YFP (Williams, Sohn *et al*., unpublished). I next examined if RRS1B could associate with AvrRps4 and PopP2 using CoIP. I co-expressed *RRS1-HF* and *RRS1B-HF* with either *GFP*, *AvrRps4-GFP* or *PopP2-GFP* and purified proteins 2 days after infiltration in *N. benthamiana*. I detected each of the specific proteins in the total extract samples (**Figure 6.7** first 2 top panels). I then proceeded to an IP using anti-FLAG beads on total extracts. After IP, I detected RRS1-HF or RRS1B-HF in each specific sample using an anti-FLAG antibody. Interestingly, both RRS1-HF and RRS1B-HF pulled down AvrRps4-GFP and PopP2-GFP but not GFP (**Figure 6.7**). By processing to the reverse IP with anti-GFP beads, I could detect GFP, AvrRps4-GFP and PopP2 but only AvrRps4-GFP and PopP2-GFP pulled down RRS1-HF and RRS1B-HF (**Figure 6.7**). Altogether, these results suggest that similarly to RRS1, RRS1B associates with AvrRps4 and PopP2 *in planta*. Moreover, it supports that interaction of PopP2 with RRS1and RRS1B might not be sufficient for defence activation.

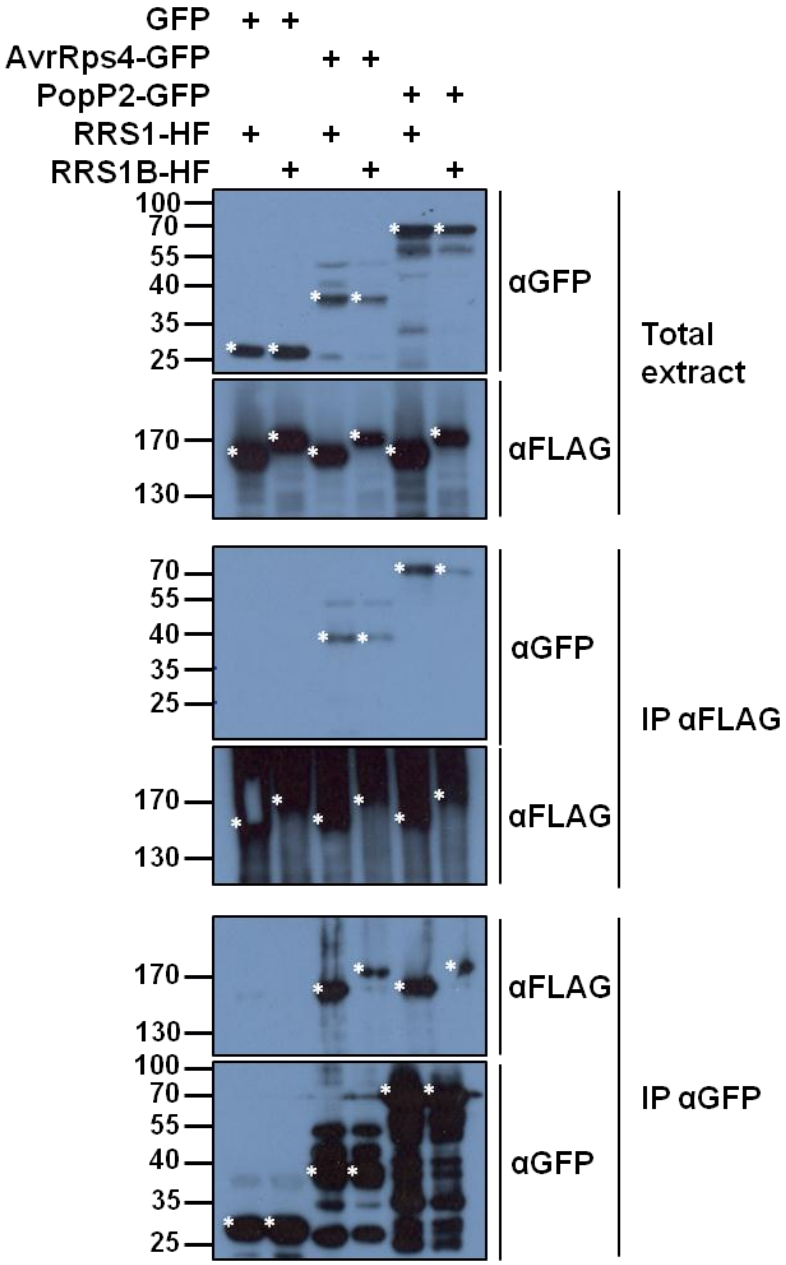

Figure 6.7. RRS1 and RRS1B associate with AvrRps4 and PopP2 *in planta*.

4 to 5 week-old *N. benthamiana* leaves were syringe-infiltrated with a mix of *A. tumefaciens* solutions at OD_{600}=0.5. Fusion proteins expressed in each mix are indicated by a cross symbol at the top of the figure. Protein samples were purified from plant tissue 2 days after infiltration for immunoblot analysis. Immunoblots show presence of proteins in total extract and after co-immunoprecipitation. From total protein extract, parallele immunoprecipitation with anti-FLAG (IP αFLAG) and anti-GFP (IP αGFP) beads were performed. In this experiment, *RRS1* and *RRS1B* were amplified and cloned from the Ws-2 gDNA. Asterisks indicate the expected tagged-proteins size. Experiment has been done once.

6.2.6 Investigation of three TIR-NB-LRR-WRKY R proteins involvement in Arabidopsis immunity.

Within the Arabidopsis TIR-NB-LRR family, several members have been predicted to carry extra motifs. For instance, three members contain a predicted WRKY domain (Meyers *et al.*, 2003), among them RRS1 and RRS1B. The third is *At4g12020,* however the WRKY domain is predicted to be located in the N-terminus of the protein, before the TIR domain. Another peculiarity of *At4g12020* is the predicted mitogen–associated protein kinase kinase kinase domain (MAPKKK) after the LRR domain. One hypothesis is that these WRKY–containing R proteins link effector recognition and defense gene activation. Indeed, many WRKY proteins were identified to be involved in plant defense gene regulation (Eulgem and Somssich, 2007). Interestingly, *At4g12020* is in head-to-head conformation on the Arabidopsis genome with another TIR-NB-LRR encoding gene, *At4g12010*. Considering the particular tandem structure of *At4g12010-At4g12020*, *RRS1B-RPS4B* and *RRS1-RPS4* and the presence of a WRKY domain in one of the partner of each pair, we hypothesized that these 3 *R* gene pairs could be part of a signaling node for positive or negative regulation of immunity. To test this, I took a loss of function approach. I assessed the gain/loss of disease resistance to *Pst* DC3000 (EV) or *Pst* DC3000 (HopA1) in the *At4g12020-1* (SALK_097632) single, the *rrs1b-1*/*rrs1-3* double and the *rrs1b-1*/*rrs1-3*/*At4g12020-1* triple mutants. *Pst* DC3000 (EV) and *Pst* DC3000 (HopA1) were infiltrated in Col-0 WT and in each of the mutants at $5x10^6$ cfu/ml (OD_{600}=0.01) and disease symptoms were estimated 3 days post infiltration. Col-0, *At4g12020-1*, *rrs1b-1*/*rrs1-3* and *rrs1b-1*/*rrs1-3*/*At4g12020-1* presented indistinguishable disease symptoms caused by *Pst* DC3000 (EV) (**Figure 6.8**). Supporting this result, I showed in the previous section that Col-0 and *rrs1b-1*/*rrs1-3* presented the same level of *Pst* DC3000 (EV) growth 3 days post infiltration (**Figure 4.5B**). This suggests that loss of the three Arabidopsis TIR-NB-LRR-

WRKY-containing genes does not influence the basal resistance to the hemibiotrophic pathogen *Pst* DC3000. I was then interested to assess ETI in these mutants. We hypothesized that these three TIR-NB-LRR–carrying WRKY could be involved in other TIR-NB-LRR rather CC-NB-LRR signaling considering that no CC-NB-LRR carrying WRKY were identified in Arabidopsis. Therefore, I chose to test HopA1–triggered immunity as this effector is recognized in Col-0 by the TIR-NB-LRR RPS6 (Kim *et al.*, 2009). Disease provoked by *Pst* DC3000 (HopA1) in Col-0 were largely reduced compared to *Pst* DC3000 (EV) 3 days post infiltration (**Figure 6.8**). Similar results were obtained in *At4g12020-1*, *rrs1b-1*/*rrs1-3* and *rrs1b-1*/*rrs1-3*/*At4g12020-1* suggesting that these mutants are not impaired in HopA1–triggered immunity.

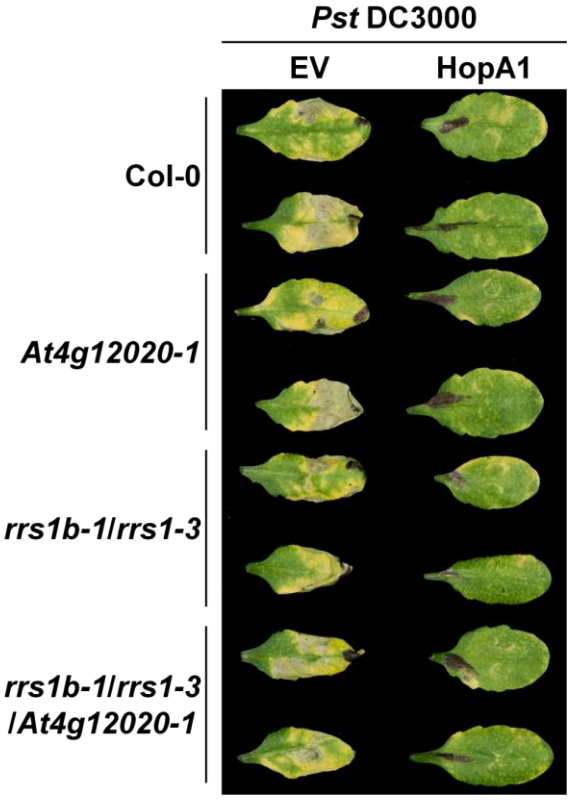

Figure 6.8. The Col-0 triple TIR-NB-LRR-WRKY knockout mutant is not impaired in immunity to either *Pst* DC3000 *pVSP61* or *pML123::HopA1*.

4 to 5 week-old *A. thaliana* leaves were syringe-infiltrated with *Pst* DC3000 carrying *pVSP61* empty vector (EV) or *pML123*::*HopA1* at 5 .10^6 cfu/ml (OD_{600}=0.01). Pictures of infected leaves were taken 3 dpi.

6.3 Discussion

In this chapter, I compared RRS1-RPS4 and RRS1B-RPS4B based on their TIR domains and their recognition of AvrRps4 and PopP2.

First, I tested the recognition of AvrRps4 and PopP2 in the MAGIC parents and could identify three accessions recognizing AvrRps4 but not PopP2 and two that recognized none of them (**Table 6.1**). This phenotypic analysis conducted in Arabidopsis accessions for AvrRps4 and PopP2 recognition set the basis for a SNP study in RRS1-RPS4 and RRS1B-RPS4B. This approach could allow the establishment of a correlation between specific sequence polymorphisms and absence/presence of AvrRps4 and PopP2 recognition.

I investigated the effector recognition mechanism by RRS1-RPS4 and RRS1B-RPS4B. The AvrRps4 mutant screen did not reveal mutations abolishing specifically *RRS1-RPS4*–mediated AvrRps4 recognition (**Table 6.2**). The association of AvrRps4 with RRS1 and RRS1B *in planta* strongly suggests that this interaction might be responsible for the recognition (**Figure 6.7**). An important experiment would be to test the direct interaction of AvrRps4 with RRS1 and RRS1B. Also, it would be interesting to test if the AvrRps4 mutations (E175A and E187A) that do not activate HR in Ws-2 still interact with RRS1 and RRS1B. Two mutations in AvrRps4 (L167T and I180D) gave a weaker HR in an RRS1B-RPS4B–dependent way (**Table 6.2**). Thus, there must be some specificity in the recognition but, so far, we cannot rule out if the AvrRps4 is recognized by a similar or distinct mechanism in RRS1-RPS4 and RRS1B-RPS4B.

Why RRS1B-RPS4B does not recognize PopP2 is intriguing. As shown in **Figure 6.7**, similarly to RRS1, RRS1B interacts with PopP2. It becomes clear that interaction with PopP2 is not the limiting factor for the R protein activation as

RRS1^{Col-5} and RRS1B^{Ws-2} both interact but do not recognize PopP2. Interestingly, PopP2 is predicted to have an acetyl transferase activity and its autoacetylation is required to trigger immune responses in Arabidopsis (Tasset *et al.*, 2010). I showed in **Figure 6.6** that RRS1 exons 5, 6, 7 seem to be essential to recognize PopP2. That orientates future experiments for elucidation of PopP2 recognition to the C-terminus of RRS1 and RRS1B.

How TIR domains function to activate plant defense remains an unsolved problem. RRS1-RPS4 and RRS1B-RPS4B pairs have similar structure and share the recognition of the same effector, AvrRps4. I anticipated that these R protein pairs share similar mechanism for activation and downstream signaling. As TIR domains are believed to play a major role in R protein signaling, I compared RRS1-RPS4 and RRS1B-RPS4B TIR domains. Surprisingly, RPS4B^{TIR} did not trigger cell death in *N. tabacum*. Comparing RPS4^{TIR} and RPS4B^{TIR} sequences and structures could give insight for the reason why RPS4B^{TIR} does not activate cell death when overexpressed in *N*. *tabacum*. Sequence analysis and structure predictions present an overall resemblance in secondary and tertiary conformation of RRS1^{TIR}/RRS1B^{TIR} and RPS4^{TIR}/RPS4B^{TIR}. Similarly, the dimeric interface seems to be quite conserved. However, crystallizing RRS1B^{TIR} and RPS4B^{TIR} would be required to confirm if it is the same as in RRS1^{TIR} and RPS4^{TIR}. Remarkably, despite the polymorphism that exists in the dimeric interface, TIR domains can still associate (**Figure 6.3**). This result should be complemented by a yeast-two-hybrid experiment to prove the direct interaction. To test if these TIR domain associations are specific to RRS1-RPS4 and RRS1B-RPS4B pair, I should include another plant TIR domain in this experiment. Notably, the L6 TIR domain could be used as a control as it is not supposed to interact with RRS1^{TIR} or RPS4^{TIR} (Williams, Sohn *et al*., unpublished). Based on **Figure 3.5** and TIR interaction studies, we can assume that RRS1^{TIR} suppresses RPS4^{TIR}–triggered cell death *via* direct interaction.

Notably, RRS1^{TIR} S25A/H26A variant did not interact with RPS4^{TIR} and did not suppress cell death when co-expressed with RPS4^{TIR} in *N. tabacum* (Williams, Sohn *et al*., unpublished). Considering that RRS1B^{TIR} can associate with RPS4^{TIR}, it would be interesting to test if it also could suppress the RPS4^{TIR}–triggered cell death.

Based on the evident TIR domain dimerization and association of RRS1 and RPS4 *in planta* (Williams, Sohn et al., unpublished), we can assume that the full length proteins dimerize for function. Based on the CoIP results obtained in **Figure 6.3**, I examined if the TIR domains provided partner specificity for function in the context of the full length proteins. Swapping the TIR domain of RRS1^{TIR} with RRS1B^{TIR} retained RRS1 function for effector recognition. In contrast, AvrRps4 recognition was significantly reduced when RRS1B^{TIR} was swapped with RRS1^{TIR}. Also, AvrRps4 recognition was lost when RRS1B$^{Exon\ 5,6,7}$ was swapped with RRS1$^{Exon\ 5,6,7}$. This suggests that RRS1B is less prone to functional alteration than RRS1. RPS4^{TIR}–triggered cell death was suppressed by RRS1^{TIR}. Therefore, we can infer that it is the RPS4^{TIR} homodimerization that is the downstream signaling platform within the protein complex. It would be interesting to test if the RPS4^{TIR} and RPS4B^{TIR} swaps retain AvrRps4 and PopP2 recognition. Finally, to understand if the TIR domain interaction is the determining factor for pair partner function, it would be essential to test the cooperation of RRS1B^{TIR}-RRS1$^{NB\text{-}LRR\text{-}WRKY}$ with RPS4B, RRS1^{TIR}-RRS1B$^{NB\text{-}LRR\text{-}WRKY}$ with RPS4, RRS1B with RPS4B^{TIR}-RPS4$^{NB\text{-}LRR}$ and RRS1 with RPS4^{TIR}-RPS4B$^{NB\text{-}LRR}$ for effector recognition.

Collectively, my data suggest that, at least in the context of RRS1, the TIR domain and exons 5, 6 and 7 are not the limiting factors for the cooperation with RPS4 (**Figure 6.4 and 6.6**). Considering that RRS1 with RPS4B and RRS1B with RPS4 do not function together for effector recognition, I tested if they could associate *in planta*. Surprisingly, CoIP results showed association between R proteins from different pairs (**Figure 6.5**). This experiment needs to be repeated but

suggests so far that it is not a simple protein-protein interaction that produces a functional R protein complex. We can hypothesize that the specific interaction between residues of each pair partner are required for the establishment of functional R protein complexes for effector perception and downstream signaling activation. My data showed that, in the absence of an effector, RRS1B associates with itself and RPS4B. Interestingly, RPS4B associate with RRS1B but not with itself. Additionally, RPP1 does not associate with RRS1 *in planta* and should therefore be used as a control in this experiment (Williams, Sohn *et al*., unpublished). However, considering the repressive role of $RRS1^{TIR}$ on $RPS4^{TIR}$, we can imagine that, in the absence of effector, RRS1B and RPS4B heterodimerize. After effector recognition, conformational changes in the complex would release RRS1B/RPS4B interaction allowing RPS4B to homodimerize and activate immune responses. This experiment needs to be repeated in the presence of AvrRps4 to see if, following our previous hypothesis, RPS4 and RPS4B homodimerize only after effector recognition.

During these studies, I became interested in WRKY-containing TIR-NB-LRRs. This unique feature is displayed by only 3 genes in Arabidopsis, *At4g12020*, *RRS1B* and *RRS1*. The reason why these R proteins carry a WRKY domain is unclear. One hypothesis is that they could be involved in regulating basal resistance. They would be then targeted by pathogen effectors, like AvrRps4 and PopP2. In turn they would be guarded by the respective partner, like RPS4B and RPS4. Therefore, I tested loss of basal resistance in several mutants. I could not observe any increase of disease caused by *Pst* DC3000 in *At4g12020-1*, *rrs1b-1/rrs1-3* or *At4g12020-1*/*rrs1b-1*/*rrs1-3* compared to Col-0 WT (*At4g12020-1* knock-out was confirmed by RT-PCR). I also tested if these three R proteins could play a central role in TIR-NB-LRR mediated immunity. However, HopA1 triggered resistance to *Pst* DC3000 in *At4g12020-1*, *rrs1b-1/rrs1-3*, *At4g12020-1*/*rrs1b-1*/*rrs1-*

3 and Col-0 WT. Despite the requirement of a quantitative assay, these data suggest that these TIR-NB-LRRs carrying WRKY domains are not involved in basal defense, at least against *Pst* DC3000, nor in ETI, at least for HopA1–triggered immunity. Even if the $RRS1^{WRKY}$ has been shown to bind DNA (Noutoshi *et al.*, 2005), there is no direct proof of involvement of RRS1-RPS4 in transcription machinery. The reasons for the presence of WRKY domains in TIR-NB-LRR still need to be elucidated.

7 General discussion and outlook.

In the last two decades, extensive efforts were expended towards the identification of *R* genes and the understanding of R proteins. *R* genes represent a formidable natural resource for crop protection against pathogens carrying the corresponding *Avr* genes. This interaction has been described as the “gene-for-gene” resistance model (Flor, 1971). The plant NB-LRR R proteins are potent receptors of pathogen invasion and defense activation. Recognition of pathogen secreted–effectors by NB-LRRs results in defense activation, leading to resistance. In order to dissect plant-pathogen interactions, researchers study plant resistance and plant disease. A significant number of studies therefore focus on plant receptors and signaling components as well as on the pathogen infection structures and effectors (Dodds and Rathjen, 2010). In order to decipher the functional mechanism of NB-LRR R proteins, various questions arise: How are effectors recognized by R proteins? What are the roles of the different R protein domains? What are the molecular events and particularly the intra- and inter-molecular interactions taking place prior to and upon effector recognition? What are the signaling pathways involved? And finally, what is the plant defense output limiting the pathogen growth?

During my PhD, I focused my research on plant immunity. I chose the RRS1-RPS4/AvrRps4/PopP2 model to study ETI. This system is unique as it involves multiple paired R proteins cooperating for the recognition of several effectors from unrelated plant pathogens. Therefore, I would like to refer to it as a “genes-for-genes” model. In this context, the data gathered during this PhD project contributed to the effort of describing and understanding TIR-NB-LRR–mediated immunity in plants.

7.1 Towards understanding the "genes-for-genes" interaction: cloning new *R* genes

Despite resistance breeding programs, pathogens are still a threat for crops. Therefore, to ensure stable food production, it is essential to understand and control diseases. A crucial step is the discovery of new genetic resources for disease resistance. Such research can be performed using wild plant species or a plant model like Arabidopsis. *RPS4* was identified in Arabidopsis in a screen for recognition of AvrRps4, an effector from *Pseudomonas syringae* pv. *pisi* that causes bacterial blight of pea (*Pisum sativum*) (Hinsch and Staskawicz, 1996). Similarly, *RRS1* was isolated from Arabidopsis after screens for genetic resistance against *Ralstonia solanacearum* (Deslandes *et al.*, 1998). Following the cloning of *RRS1*, it was determined that it recognizes the *R. solanacearum* effector PopP2 (Deslandes *et al.*, 2003). Interestingly, *RPS4* and *RRS1* are arranged in a head-to-head conformation in the Arabidopsis genome and function cooperatively for ETI (Narusaka *et al.*, 2009; Birker *et al.*, 2009). Study of this particular R protein pair system represents a unique opportunity to shed light on a new resistance phenomenon. The evolutionary mechanism for physical gene pairing associated with the functional cooperation of RRS1 and RPS4 is remarkable but still needs to be elucidated in more details.

In order to fully understand this "genes-for-genes" system I decided to identify the genetic basis for the *RRS1*– and *RPS4*–independent AvrRps4 recognition (RRIR) in Arabidopsis. Using a population derived from the cross between Ws-2 *rps4-21* mutant and RLD, I mapped the *RRIR* locus at the bottom of chromosome 5, close to *RRS1-RPS4*. A loss-of-function approach confirmed that the major candidate locus *RRS1B-RPS4B* was responsible for the RRIR. Strikingly, *RRS1B-RPS4B* is highly similar to *RRS1-RPS4,* in gene architecture and protein

domain composition. The evolutionary advantage for the plant to carry two *R* gene pairs recognizing the same effector, AvrRps4, is intriguing. We could hypothesize that the duplication of such an *R* gene pair would reduce the effect of purifying selection and increase the potential for one of the pairs to evolve a new effector recognition ability. Indeed RRS1-RPS4 recognize AvrRps4, PopP2 and an unknown effector from *C. higginsianum* whereas RRS1B-RPS4B recognizes only AvrRps4 (**Figure 7.1A**). Considering this, a possible scenario would be that *RRS1B-RPS4B* duplicated to create *RRS1-RPS4*. A future discovery might uncover effector(s) recognized by *RRS1B-RPS4B* but not by *RRS1-RPS4*. To test this possibility, the Arabidopsis Ws-2 *rps4-21*, Ws-4 *rps4b-1* and Ws *rps4b-1*/*rps4-21* mutants could be useful to test different Arabidopsis non-host pathogens and perhaps identify new effectors recognized by *RRS1-RPS4* and/or *RRS1B-RPS4B*. On the other hand, developing two similar recognition systems might be a strategy for the plant to ensure the efficient protection of an important cellular complex generally targeted by pathogen effectors. Therefore we could hypothesize that RRS1-RPS4 and RRS1B-RPS4B guard the same AvrRps4 target. Another hypothesis is that RRS1 and RRS1B, guarded by RPS4 and RPS4B respectively, could behave as decoys for effectors (van der Hoorn and Kamoun, 2008).

Conversely, RLD carries mutations in *RPS4* and *RPS4B* abolishing the recognition mediated by *RRS1-RPS4* and *RRS1B-RPS4B*. Interestingly, Col-0 displays more substitutions in *RRS1B-RPS4B* than RLD compared to Ws-2 and still retains AvrRps4 recognition. Again, the adaptive advantage (or apparent lack of disadvantage) for RLD to have these two *R* gene pairs non-functional for AvrRps4 and PopP2 recognition remains obscure. Possibly, the substitutions observed in *RRS1-RPS4*^{RLD} and *RRS1B-RPS4B*^{RLD} enable the recognition of other unknown pathogen effector(s). Otherwise, we could imagine that, if RLD evolved separately from pathogens recognized by *RRS1-RPS4* and/or *RRS1B-RPS4*, shutting down

these two *R* gene pairs could enhance fitness. Another hypothesis, is that mutating these *R* gene pairs would have been a bottleneck in RLD evolution to avoid any hybrid incompatibilty effect (Bomblies and Weigel, 2007).

7.2 Effector recognition and R protein activation

How effectors are recognized and activate TIR-NB-LRRs remains unclear. Several TIR-NB-LRRs have been shown to recognize an effector directly and this direct interaction is supposed to trigger R protein activation (Deslandes *et al.*, 2003; Dodds *et al.*, 2006; Krasileva *et al.*, 2010; Ravensdale *et al.*, 2012). The direct effector recognition appears to be a general mechanism for TIR-NB-LRR in contrast to CC-NB-LRRs which often recognize effectors indirectly (Jones and Dangl, 2006). The exact mechanism by which RRS1-RPS4 and RRS1B-RPS4B recognize effectors is unknown. Similarly to other TIR-NB-LRRs, a growing body of evidence shows that RRS1 and RRS1B interact directly with AvrRps4 and PopP2. However, the exact mechanisms by which AvrRps4 and PopP2 are recognized still remain to be elucidated. We can speculate that the direct interaction of AvrRps4 with RRS1 and RRS1B triggers R protein intra-molecular modifications leading to defense activation. The AvrRps4 mutations E175A and E187A abolish recognition and it will be interesting to test if they lost the interaction with RRS1 and RRS1B. Moreover, we still need to assess if RRS1-RPS4 and RRS1B-RPS4B recognize AvrRps4 by the same mechanism. An important challenge will be to identify which RRS1 and RRS1B domain(s) interact with these effectors and how these R proteins get activated. Using a domain swap experiment between RRS1 and RRS1B, I showed that exons 5, 6 and 7 from RRS1 were required for PopP2 recognition but did not affect AvrRps4 recognition. The role of the C-terminal part of RRS1 and RRS1B has not yet been elucidated but we could speculate that it is targeted by pathogen effectors which in turn activate the R protein complex. Each partner of RRS1-RPS4

and RRS1B-RPS4B pairs are required for ETI. Based on my results and other unpublished data, these proteins might form heterodimer complexes in a resting state prior to effector perception (**Figure7.1B**). Additionally, heterodimeric complexes can be formed with partners from different pairs prior to effector recognition. However, such complexes might not be functional for AvrRps4 and PopP2 recognition and/or downstream signaling activation. Indeed using a combination of double mutants in Arabidopsis and a transient assay in *N. tabacum*, I showed that partners are specific to their own pair for function. However, we cannot discard the hypothesis that heterocomplexes of R proteins from different pairs might be functional for other unknown effector recognition. The next critical experiment will be to identify the components of the protein complex(es) formed after effector recognition.

7.3 R protein–mediated plant defense activation

The mechanism by which TIR-NB-LRRs activate plant defense remains largely an open question. Therefore, extensive studies were dedicated to investigate function of TIR-NB-LRR domains. Dissecting the function of the various domains revealed that the TIR domain in particular is able to activate cell death on its own when overexpressed *in planta* (Weaver *et al.*, 2006; Swiderski *et al.*, 2009). Construction of the Col-0 *pTA7002::RPS4*$^{TIR+80}$ has enabled us to correlate the RPS4^{TIR+80}–triggered cell death with resistance to *Pst* DC3000 and susceptiblity to *A. brassicicola*. Furthermore, RPS4^{TIR+80} requires a nuclear localization to activate cell death, as does RPS4 (Wirthmueller *et al.*, 2007). Consistent with this observation, the C-terminal part of RPS4B, which carries a NLS, is required for defense activation. Altogether, these data strongly support the importance of the TIR-NB-LRR nuclear localization for defense activation. Interestingly, not all the TIR domains identified in the TIR-NB-LRR repertoire have the capability to induce

defense when overexpressed alone. This has been shown with RPP2^{TIR}, RRS1^{TIR}, RRS1B^{TIR} and RPS4B^{TIR} (Swiderski *et al.*, 2009). The lack of uniformity in NB-LRR fragment–mediated cell death needs further investigation but suggests so far that, the mechanisms of TIR-NB-LRR downstream signaling pathways activation might diverge. Swapping domains from RRS1 into RRS1B seems to alter RRS1B function. Addtionally, heterodimeric complexes of R proteins from different pairs do not appear to be functional. Altogether, this indicates that despite the similarity in motif prediction, TIR-NB-LRRs evolved particular intra- and inter-molecular specificity for function.

Considering the effector interaction with RRS1 and RRS1B, it is unclear how these two TIR-NB-LRR-WRKY participate in R complex activation and if they are also involved in downstream signaling. In particular, the function of the WRKY domain still needs to be elucidated. Based on the observation that RRS1^{TIR} supresses RPS4^{TIR}–triggered cell death, we can speculate that the heterodimeric RRS1 maintains RPS4 in an inactive state prior to effector recognition. Considering that RPS4^{TIR} alone is able to activate cell death, we speculate that it works downstream of RRS1. Additionally and similarly to L6^{TIR}, RPS4^{TIR} requires homodimerization to activate cell death. In absence of AvrL567, L6 does not self-associate (Bernoux *et al.*, 2011). After AvrL567 recognition, it is proposed that L6 homodimerize, notably through the TIR domain, for defense activation. Thus, we also hypothesize that after AvrRps4 recognition by RRS1 and RRS1B, RPS4 and perhaps RPS4B homodimerize, at least through the TIR domain association, for downstream signaling activation (**Figure 7.1C**). However, after effector recognition, it is still unclear whether RRS1 completely dissociates from RPS4, enabling RPS4 to homodimerize or if RRS1 releases only the interaction with RPS4^{TIR} and stays in association with RPS4, forming a complex oligomer.

Many techniques exist to control disease in crops. Unfortunately, they are mostly based on chemical application. Therefore, from an environmental perspective, it appears preferable to isolate natural resistance genes and deploy them in the field. Though a significant acreage of genetically modified crops has been planted on most continents, there is a continuing debate about the ethics and environmental effects of this technology. Genetic modification is a very useful tool to quickly engineer resistant crop species against specific pathogens (Tai *et al.*, 1999; Lacombe *et al.*, 2010). Recently, it has been shown that transfer of *RRS1-RPS4* confers disease resistance in *Brassicaceae*, *Solanaceae* and *Cucurbitaceae* to *Colletotrichum spp*, *R. solanacearum* (PopP2) and *Pst* DC3000 (AvrRps4) (Narusaka *et al.*, 2013). We could imagine that, in the future, *RRS1B* and *RPS4B* will also serve as contributors for crop protection against pathogens.

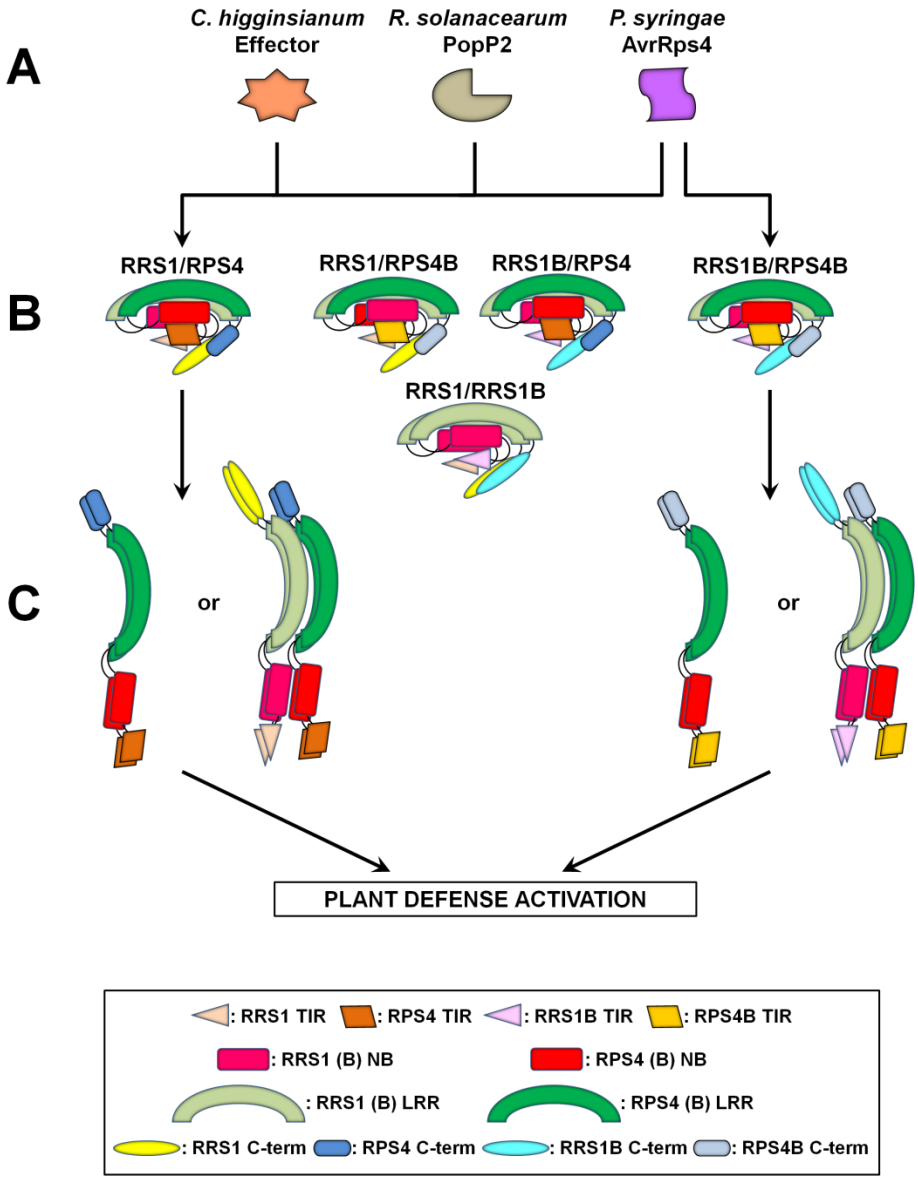

Figure 7.1. RRS1-RPS4 and RRS1B-RPS4B "genes-for-genes" working model.

Three different pathogens, *Colletotrichum higginsianum*, *Ralstonia solanacearum* and *Pseudomonas syringae* secrete effectors into plant cells. (**A**). In the resting state, RRS1, RPS4, RRS1B and RPS4B form a variety of heterodimers. Only the RRS1/RPS4 heterodimer is capable of *C. higginsianum* effector and *R. solanacearum* PopP2 recognition. Both RRS1/RPS4 and RRS1B/RPS4B recognize AvrRps4 from *P. syringae* (**B**). These respective effector recognitions lead to the activation of the R protein complex involving modifications in the inter- and intra-molecular interactions. Ultimately, the homodimerisation of RPS4 and RPS4B serves as a signaling platform for plant defense activation (**C**). It is still not clear whether RRS1 and RRS1B have a role in the signaling activation complex. All the other combinations of R protein from different pairs do not trigger immunity. We hypothesize that this could be due to the inabillity of such a complex to recognize the effector, or that the unspecific inter-molecular interactions prevent the complex from being unleashed, or finally that the protein complex is non-functional for downstream signaling activation.

8 Appendix

Marker name	Associated locus	Gene position	Marker type	Ws-2 *rps4-21* fragment size	RLD fragment size	Forward primer	Reverse primer
NGA151	*At5g14480*	4,669,929 bp	SSLP	102 bp	120 bp	CAGTCTAAAAGCGAGAGTATGATG	GTTTTGGGAAGTTTTGCTGG
DRF.1	*At5g42800*	17,164,364 bp	CAPS (BsaA1)	609+534 bp	609+318+216 bp	TGTTACATGGCTTCATACCA	AGATCCTGAGGTGAGTTTTTC
SS117	*At5g45060*	18,182,038 bp	CAPS (Cla1)	365 bp	140+225 bp	ATCCATCAGAAAGTATTGACACTCT	ACGCAGTAGACCCTTATGAGTTT
SS017	*At5g45200*	18,290,332 bp	CAPS (Xma1)	400 bp	163+237 bp	GCACTCCACATTTATCTTCACTAA	AACTCACCGCTACATGTAACTAAGA
SS007	*rps4-21*	18,323,976 bp	CAPS (Sal1)	186 bp	95+91 bp	TAAGCTACCATTGAAAGAAGTTCG	TTAACCATTCACAAAAGCAATCAACAG
N5-20408832	*At5g50130*	20,408,832 bp	CAPS (Nde1)	400 bp	280+120 bp	TTGCCAGATTCTGCTCTCAA	TCATTTTCTCTATCAGCATTTCTGT
K9I9-3	*At5g67480*	26,932,090 bp	SSLP	190 bp	180 bp	CATGCCTTTGATCACAGTGGAAGC	ACATCCTTGTAAGCTCTCCTTATA

Table S1. List of the Arabidopsis chromosome 5 genetic molecular markers used for mapping of the *RRIR* locus.

In this study, Single Sequence Lengh Polymorphism (SSLP) and Cleaved Amplified Polymorphic Sequence (CAPS) molecular makers were used for genotyping.

Gene	Fragment size	Forward primer	Reverse primer
RPL4	≈350bp	GGAGAAGTTTGAGAAGCCCAAGAC	CCACACTTGAAGCAATTGGAACTACG
Ef1α	≈250bp	CAGGCTGATTGTGCTGTTCTTA	GTTGTATCCGACCTTCTTCAGG
PR1	≈200bp	ATACACTCTGGTGGGCCTTACG	TACACCTCACTTTGGCACATCC
TIR+80	≈910bp	ATGGAGACATCATCTATTTCCACTGT	CTGAGCAGCGTAATCTGGAA
RPS4	≈960bp	CAGTTTCAAAGCCTTTGGCCCGTA	TCAGAAATTCTTAACCGTGTGCATG
RRS1	≈420bp	GGTAAAGAAATCCTCCATGGACAA	AGATGAGGCAGAGGTAGTTATGG
RPS4B	≈530bp	GATGCACGTAAACACTACAATGAGG	TGCCTCAAGAGAACTGTTGTTG
RRS1B	≈440bp	GTTTGGTAGCACCACTTATTGC	AAACATTTGGATCAGTGCGG

Table S2. List of primers used to identify genes transcripts by RT-PCR.

```
RRS1B Ws-2      MIESEQIVYISCVEEVRYSFVSHLSEALRRKGINDVFIDSDDSLSNESQSMVERARVSVM 60
RRS1B RLD       MIESEQIVYISCVEEVRYSFVSHLSEALRRKGINDVFIDSDDSLSNESQSMVERARVSVM 60
RRS1B Col-0     MTESEQIVYISCIEEVRYSFVSHLSKALQRKGVNDVFIDSDDSLSNESQSMVERARVSVM 60
                * **********:************:**:***:************************** 

RRS1B Ws-2      ILPGNRTISLEKLVKVLDCQKNKDQVVVPVLYGVRSSETEWLSALDSKGFSSVHQSRKEC 120
RRS1B RLD       ILPGNRTISLEKLVKVLDCQKNKDQVVVPVLYGVRSSETEWLSALDSKGFSSVHQSRKEC 120
RRS1B Col-0     ILPGNRTVSLDKLVKVLDCQKNKDQVVVPVLYGVRSSETEWLSALDSKGFSSVHHSRKEC 120
                *******:**:*************************************************:***** 

                                                                  Kinase 1a
RRS1B Ws-2      SDSHLVKEIVRDLYEKLFYMERIGIYSKLLEIEKMVCKQLLDIRCVGIWGMPGIGKTTLA 180
RRS1B RLD       SDSHLVKEIVRDLYEKLFYMERIGIYSKLLEIEKMVCKQLLDIRCVGIWGMPGIGKTTLA 180
RRS1B Col-0     SDSQLVKETVRDVYEKLFYMERIGIYSKLLEIEKMINKQPLDIRCVGIWGMPGIGKTTLA 180
                ***:**** ***:**********************: ** ******************** 

RRS1B Ws-2      KAVFDQMSGEFDAHCFIEDYTKAIQEKGVYCLLEEQFLKQNAGASGTVTKLSLLRDRLNN 240
RRS1B RLD       KAVFDQMSGEFDAHCFIEDYTKAIQEKGVYCLLEEQFLKQNAGASGTVTKLSLLRDRLNN 240
RRS1B Col-0     KAVFDQMSGEFDAHCFIEDYTKAIQEKGVYCLLEEQFLKENAGASGTVTKLSLLRDRLNN 240
                ************************************:******************** 

                      Kinase 2                    Kinase 3a
RRS1B Ws-2      KRVLVVLDDVRSPLVVESFLGGFDWFGPKSLIIITSKDKSVFRLCRVNQIYEVQGLNEKE 300
RRS1B RLD       KRVLVVLDDVRSPLVVESFLGGFDWFGPKSLIIITSKDKSVFRLCRVNQIYEVQGLNEKE 300
RRS1B Col-0     KRVLVVLDDVRSPLVVESFLGGFDWFGPKSLIIITSKDKSVFRLCRVNQIYEVQGLNEKE 300
                ************************************************************ 

RRS1B Ws-2      ALQLFSLCASIDDMAEQNLHEVSMKVIKYANGHPLALNLYGRELMGKKRPPEMEIAFLKL 360
RRS1B RLD       ALQLFSLCASIDDMAEQNLHEVSMKVIKYANGHPLALNLYGRELMGKKRPPEMEIAFLKL 360
RRS1B Col-0     ALQLFSLCASIDDMAEQNLHEVSMKVIKYANGHPLALNLYGRELMGKKRPPEMEIAFLKL 360
                ************************************************************ 

RRS1B Ws-2      KECPPAIFVDAIKSSYDTLNDREKNIFLDIACFFQGENVDYVMQLLEGCGFFPHVGIDVL 420
RRS1B RLD       K-CPPAIFVDAIKSSYDTLNDREKNIFLDIACFFQGENVDYVMQLLEGCGFFPHVGIDVL 419
RRS1B Col-0     KECPPAIFVDAIKSSYDTLNDREKNIFLDIACFFQGENVDYVMQLLEGCGFFPHVGIDVL 420
                * ************************************************************ 

RRS1B Ws-2      VEKSLVTISENRVRMHNLIQDVGRQIINRETRQTKRRSRLWEPCSIKYLLEDKEQNENDE 480
RRS1B RLD       VEKSLVTISENRVRMHNLIQDVGRQIINRETRQTKRRSRLWEPCSIKYLLEDKEQNENDE 479
RRS1B Col-0     VEKSLVTISENRVRMHNLIQDVGRQIINRETRQTKRRSRLWEPCSIKYLLEDKEQNENEE 480
                ************************************************************:* 

RRS1B Ws-2      QKTTFERAQVPEEIEGIFLDTSNLSFDIKHVAFDNMLNLRLFKIYSSNPEVHHVNNFLKG 540
RRS1B RLD       QKTTFERAQVPEEIEGIFLDTSNLSFDIKHVAFDNMLNLRLFKIYSSNPEVHHVNNFLKG 539
RRS1B Col-0     QKTTFERAQVPEEIEGMFLDTSNLSFDIKHVAFDNMLNLRLFKIYSSNPEVHHVNNFLKG 540
                ***************:************************************************ 

RRS1B Ws-2      SLSSLPNVLRLLHWENYPLQFLPQNFDPIHLVEINMPYSQLKKLWSGTKDLEMLKTIRLC 600
RRS1B RLD       SLSSLPNVLRLLHWENYPLQFLPQNFDPIHLVEINMPYSQLKKLWGGTKDLEMLKTIRLC 599
RRS1B Col-0     SLSSLPNVLRLLHWENYPLQFLPQNFDPIHLVEINMPYSQLKKLWGGTKDLEMLKTIRLC 600
                ***********************************************.************ 

RRS1B Ws-2      HSQQLVDIDDLLKAQNLEVVDLQGCTRLQSFPATGQLLHLRVVNLSGCTEIKSFPEIPPN 660
RRS1B RLD       HSQQLVDIDDLLKAQNLEVVDLQGCTRLQSFPATGQLLHLRVVNLSGCTEIKSFPEIPPN 659
RRS1B Col-0     HSQQLVDIDDLLKAQNLEVVDLQGCTRLQSFPATGQLLHLRVVNLSGCTEIKSFPEIPPN 660
                ************************************************************ 

RRS1B Ws-2      IETLNLQGTGIIELPLSIVKPNYRELLNLLAEIPGLSGVSNLEQSDLKPLTSLMKISTSY 720
RRS1B RLD       IETLNLQGTGIIELPLSIVKPNYRELLNLLAEIPGLSGVSNLEQSDLKPLTSLMKISTSY 719
050Col-0        IETLNLQGTGIIELPLSIVKPNYRELLNLLAEIPGLSGVSNLEQSDLKPLTSLMKISTSY 720
                ************************************************************ 

RRS1B Ws-2      QNPGKLSCLELNDCSRLRSLPNMVNLELLKALDLSGCSELETIQGFPRNLKELYLVGTAV 780
RRS1B RLD       QNPGKLSCLELNDCSRLRSLPNMVNLELLKALDLSGCSELETIQGFPRNLKELYLVGTAV 779
RRS1B Col-0     QNPGKLSCLELNDCSRLRSLPNMVNLELLKALDLSGCSELETIQGFPRNLKELYLVGTAV 780
                ************************************************************ 
```

```
RRS1B Ws-2      RQVPQLPQSLEFFNAHGCVSLKSIRLDFKKLPVHYTFSNCSDLSPQVVNDFLVQAMANVI 840
RRS1B RLD       RQVPQLPQSLEFFNAHGCVSLKSIRLDFKKLPVHYTFSNCSDLSPQVVNDFLVQAMANVI 839
RRS1B Col-0     RQVPQLPQSLEFFNAHGCVSLKSIRLDFKKLPVHYTFSNCFDLSPQVVNDFLVQAMANVI 840
                ************************************** ****************** 

RRS1B Ws-2      AKHIPRERHVTGFSQKTVQRSSRDSQQELNKTLAFSFCAPSHANQNSKLDLQPGSSSMTR 900
RRS1B RLD       AKHIPRERHVTGFSQKTVQRSSRDSQQELNKTLAFSFCAPSHANQNSKLDLQPGSSSMTR 899
RRS1B Col-0     AKHIPRERHVTGFSQKTVQRSSRDSQQELNKTLAFSFCAPSHANQNSKLDLQPGSSSMTR 900
                ************************************************************ 

RRS1B Ws-2      LDPSWRNTLVGFAMLVQVAFSEGYCDDTDFGISCVCKWKNKEGHSHRREINLHCWALGKA 960
RRS1B RLD       LDPSWRNTLVGFAMLVQVAFSEGYCDDTDFGISCVCKWKNKEGHSHRREINLHCWALGKA 959
RRS1B Col-0     LDPSWRNTLVGFAMLVQVAFSEGYCDDTDFGISCVCKWKNKEGHSHRREINLHCWALGKA 960
                ************************************************************ 

RRS1B Ws-2      IERDHTFVFFDVNMRPDTDEGNDPDIWADLVVFEFFPVNKQRKPLNDSCTVTRCGVRLIT 1020
RRS1B RLD       VERDHTFVFFDVNMRPDTDEGNDPDIWADLVVFEFFPVNKQRKPLNDSCTVTRCGVRLIT 1019
RRS1B Col-0     VERDHTFVFFDVNMRPDTDEGNDPDIWADLVVFEFFPVNKQRKPLNDSCTVTRCGVRLIT 1020
                :************************************************************ 

RRS1B Ws-2      AVNCNTSIENISPVLSLDPMEVSGNEDEEVLRVRYAGLQEIYKALFLYIAGLFNDEDVGL 1080
RRS1B RLD       AVNCNTSIENISPVLSLDPMEVSGNEDEEVLRVRYAGLQEIYKALFLYIAGLFNDEDVGL 1079
RRS1B Col-0     AVNCNTSIENISPVLSLDPMEVSGNEDEEVLRVRYAGLQEIYKALFLYIAGLFNDEDVGL 1080
                ************************************************************ 

RRS1B Ws-2      VAPLIANIIDMDVSYGLKVLAYRSLIRVSSNGEIVMHYLLRQMGKEILHTESKKTDKLVD 1140
RRS1B RLD       VAPLIANIIDMDVSYGLKVLAYRSLIRVSSNGEIVMHYLLRQMGKEILHTESKKTDKLVD 1139
RRS1B Col-0     VAPLIANIIDMDVSYGLKVLAYRSLIRVSSNGEIVMHYLLRQMGKEILHTESKKTDKLVD 1140
                ************************************************************ 

                                                                  WRKY domain
RRS1B Ws-2      NIQSSMIATKEIEITRSKSRRKNNKEKRVVCVVDRGSRSSDLWVWRKYGQKPIKSSPYPR 1200
RRS1B RLD       NIQSSMIATKEIEITRSKSRRKNNKEKRVVCVVDRGSRSSDLWVWRKYGQKPIKSSPYPR 1199
RRS1B Col-0     NIQSSMIATKEIEITRSKSRRKNNKEKRVVCVVDRGSRSSDLWVWRKYGQKPIKSSPYPR 1200
                ************************************************************ 

RRS1B Ws-2      SYYRCASSKGCFARKQVERSRTDPNVLVITYISEHNHPFPTLRNTLAGSTRSSSSKCSDV 1260
RRS1B RLD       SYYRCASSKGCFARKQVERSRTDPNVLVITYISEHNHPFPTLRNTLAGSTRSSSSKCSDV 1259
RRS1B Col-0     SYYRCASSKGCFARKQVERSRTDPNVSVITYISEHNHPFPTLRNTLAGSTRSSSSKCSDV 1260
                ************************* ********************************** 

RRS1B Ws-2      TTSASSTVSQDKEGPDKSHLPSSPASPPYAAMVVKEEDMEQWDNMEFDVDVEEDTFIPEL 1320
RRS1B RLD       TTSASSTVSQDKEGPDKSHLPSSPASPPYAAMVVKEEDMEQWDNMEFDVDVEEDTFIPEL 1319
RRS1B Col-0     TTSASSTVSQDKEGPDKSHLPSSPASPPYAAMVVKEEDMEQWDNMEFDVDVEEDTFIPEL 1320
                ************************************************************ 

RRS1B Ws-2      FPEDTFADMDKLEENSQTMFLSRRSSGGNMEAQGKNSSDDREVNLPSKILNR 1372
RRS1B RLD       FPEDTFADMDKLEENSQTMFLSRRSSGGNMEAQGKNSSDDREVNLPSKILNR 1371
RRS1B Col-0     FPEDTFADMDKLEENSQTMFLSRRSSGGNMEAQGKNSSDDREVNLPSKILNR 1372
                ************************************************** 
```

Figure S1. Multiple protein sequence alignment using CLUSTALW

***** (asterisk) indicates positions which have a single, fully conserved residue.
: (colon) indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix.
. (period) indicates conservation between groups of weakly similar properties - scoring =< 0.5 in the Gonnet PAM 250 matrix.
Domains are highlighted on top of the sequence alignment by rectangular boxes.

```
RPS4B Ws-2      MAASSSSTGLPPQHQVFINFRGEDLRLGFVSHLVEALENDNIKVFIDNYADKGEPLETLL  60
RPS4B RLD       MAASSSSTGLPPQHQVFINFRGEDLRLGFVSHLVEALENDNIKVFIDNYADKGEPLETLL  60
RPS4B Col-0     MAASSSSTGLPPQHQVFINFRGEDLRLGFVSHLVEALENDNIKVFIDNYADKGEPLETLL  60
                ************************************************************

RPS4B Ws-2      TKIHDSKIALAIFSGKYTESTWCLRELAMIKDCVEKGKLVAIPIFYKVDPSTVRGVRGQF 120
RPS4B RLD       TKIHDSKIALAIFSGKYTESTWCLRELAMIKDCVEKGKLVAIPIFYKVDPSTVRGVRGQF 120
RPS4B Col-0     TKIHDSKIALAIFSGKYTESTWCLRELAMIKDCVEKGKLVAIPIFYKVDPSTVRGVRGQF 120
                ************************************************************

RPS4B Ws-2      GDAFRDLEERDVIKKKEWKQALKWIPDLIGITVHDKSPESEILNEIVKEVKKVLKKVSLE 180
RPS4B RLD       GDAFRDLEERDVIKKKEWKQALKWIPDLIGITVHDKSPESEILNEIVKEVKKVLKKVSLE 180
RPS4B Col-0     GDAFRDLEERDVIKKKEWKQALKWIPGLIGITVHDKSPESEILNEIVKEVKKVLKKVSLE 180
                *************************.**********************************
                                                                 Kinase 1a
RPS4B Ws-2      GSQKVVSVDPSESIDTLSSVGGEKDKTFGIKQRLKELEEKLDLVKYKGTRVIGVVGMPGI 240
RPS4B RLD       GSQKVVSVDPSESIDTLSSVGGEKDKTFGIKQRLKELEEKLDLVKYKGTRVIGVVGMPGI 240
RPS4B Col-0     GSQKVVSVDPSQSIDTLSSVGGEKDKTFGIKQRLKELEEKLDLVKYKGTRVIGVVGMPGI 240
                **********:*************************************************

RPS4B Ws-2      GKTTLVKELYKIWQGKFSRYALINQIRGKSNNFRLECLPTLLLEKLLPELNSPQLDSIEE 300
RPS4B RLD       GKTTLVKELYKIWQGKFSRYALIDQIRGKSNNFRLEFLPTLLLEKLLPELNSPQLDSIEE 300
RPS4B Col-0     GKTTLVKELYKTWQGKFSRYALIDQIRGKSNNFRLECLPTLLLEKLLPELNNPQLDSIEE 300
                ********** ***********:************ **************.********
                          Kinase 2                          Kinase 3a
RPS4B Ws-2      PYKTHKGLLRERKVLVVLDDVSGREQIYALLGKYDLHSKHEWIKDGSRIIIATNDISLLK 360
RPS4B RLD       PYKTHKGLLRERKVLVVLDDVSGREQIYALLGKYDLHSKHEWIKDGSRIIIATNDISLLK 360
RPS4B Col-0     PYKTHKGLLRERKVLVVLDDVSRREQIYALLGKYDLHSKHEWIKDGSRIIIATNDISSLK 360
                ********************* ********************************** **

RPS4B Ws-2      GLVHDTYVVRQLNHRDGLQLFRYHAFHYDQTIPPKVDFMKLSDEFVHYARGHPLALKILG 420
RPS4B RLD       GLVHDTYVVRQLNHRDGLQLFRYHAFHYDQTIPPKVDFMKLSDEFVHYARGHPLALKILG 420
RPS4B Col-0     GLVHDTYVVRQLNHRDGLQLFRYHAFHYDQATPPKVDFMKLSDEFVHYARGHPLALKILG 420
                ******************************: ****************************

RPS4B Ws-2      RELNEKNMKHWETKLKILAQSPTTYIGEVVQVSYDELSLAQKDAFLDIACFRSQDVDYVE 480
RPS4B RLD       RELNEKNMKHWETKLKILAQSPTTYIGEVVQVSYDELSLAQKDAFLDIACFRSQDVDYVE 480
RPS4B Col-0     RELYEKNMKHWETKLIILAQSPTTYIGEVVQVSYDELSLAQKDAFLDIACFRSQDVDYVE 480
                *** *********** ************************************************

RPS4B Ws-2      SLLVSSDPGSAEAIKALKNKFLIDTCDGRVEMHDLLYRFSRELDLKASTQGDIKQRRLWL 540
RPS4B RLD       SLLVSSDPGSAEAIKALKNKFLIDTCDGRVEMHDLLYRFSRELDLKASTQGDIKQRRLWL 540
RPS4B Col-0     SLLVSSDPGSAEAIKALKNKFLIDTCDGRVEMHDLLYRFSRELDLKASTQGGSKQRRLWV 540
                ************************************************. ******:

RPS4B Ws-2      YQDIINVQQKTMGAADVRGIFLDLSEVKVETSLDRELFKNMRNLRYLKLYNSHCPQECKT 600
RPS4B RLD       YQDIINVQQKTMGAADVRGIFLDLSEVKVETSLDRELFKNMRNLRYLKLYNSHCPQECKT 600
RPS4B Col-0     RQDIINVQQKTMGAANVRGIFLDLSEVKVETSLDREHFKNMRNLRYLKLYNSHCPHECLT 600
                 **************:******************* ******************:** *

RPS4B Ws-2      NNKINMPDGLELPLKEVRCLHWLKFPLEELPNDFDPINLVDLKLPYSEIERLWDGVKDTP 660
RPS4B RLD       NNKINMPDGLELPLKEVRCLHWLKFPLEELPNDFDPINLVDLKLPYSEIERLWDGVKDTP 660
RPS4B Col-0     NNKINMPDGLELPLKEVRCLHWLKFPLEELPNDFDPINLVDLKLPYSEIERLWDGVKDTP 660
                ************************************************************

RPS4B Ws-2      VLKWVDLNHSSKLCSLSGLSKAQNLQRLNLEGCTSLESLRDANLMSLKTLTLSNCSNFKE 720
RPS4B RLD       VLKWVDLNHSSKLCSLSGLSKAQNLQRLNLEGCTSLESLRDANLMSLKTLTLSNCSNFKE 720
RPS4B Col-0     VLKWVDLNHSSKLCSLSGLSKAQNLQRLNLEGCTSLESLRDVNLTSLKTLTLSNCSNFKE 720
                ****************************************.** ***************
```

```
RPS4B Ws-2      FPLIPESLEALYLDGTSISQLPDNVGNLKRLVLLNMKDCKLLETIPTCVGELKALQKLVL 780
RPS4B RLD       FPLIPESLEALYLDGTSISQLPDNVGNLKRLVLLNMKDCKLLETIPTCVGELKALQKLVL 780
RPS4B Col-0     FPLIPENLKALYLDGTSISQLPDNVGNLKRLVLLNMKDCKVLETIPTCVSELKTLQKLVL 780
                ******.*:*********************************:********.***:******

RPS4B Ws-2      SGCSKLKEFPEINKSSSKILLLDGTSIKTMPQLPSVQYLCLSRNDHLIYLPAGINQVSQL 840
RPS4B RLD       SGCSKLKEFPEINKSSSKILLLDGTSIKTMPQLPSVQYLCLSRNDHLIYLPAGINQVSQL 840
RPS4B Col-0     SGCSKLKEFPEINKSSLKILLLDGTSIKTMPQLPSVQYLCLSRNDHLIYLPAGINQVSQL 840
                *************** ********************************************

RPS4B Ws-2      TRLDLKYCTKLTYVPELPPTLQYLDAHGCSSLKNVAKPLARIMSTVQNHCTFNFTNCGNL 900
RPS4B RLD       TRLDLKYCTKLTYVPELPPTLQYLDAHGCSSLKNVAKPLARIMSTVQNHCTFNFTNCGNL 900
RPS4B Col-0     TRLDLKYCTKLTYVPELPPTLQYLDAHGCSSLKNVAKPLARIMSTVQNHYTFNFTNCGNL 900
                ************************************************ **********

RPS4B Ws-2      EQATKEEITSYAQRKCQLLSDARKHYNEGSEALFSTCFPGCEVPSWFGHEAVGSLLQRKL 960
RPS4B RLD       EQATKEEITSYAQRKCQLLSDARKHYNEGSEALFSTCFPGCEVPSWFGHEAVGSLLQRKL 960
RPS4B Col-0     EQAAKEEITSYAQRKCQLLSDARKHYNEGSEALFSTCFPGCEVPSWFGHEAVGSLLQRKL 960
                ***:********************************************************

RPS4B Ws-2      LPHWHDKRLSGIALCAVVSFPDSQDQLSCFSVTCTFKIKAEDKSWVPFTCPVGTWTREGN 1020
RPS4B RLD       LPHWHDKRLSGIALCAVVSFPDSQDQLSCFSVTCTFKIKAEDKSWVPFTCPVGTWTREGN 1020
RPS4B Col-0     LPHWHDKRLSGIALCAVVSFPDSQDQLSCFSVTCTFKIKAEDKSWVPFTCPVGIWTREGN 1020
                ************************************************** ******

RPS4B Ws-2      KKDKIESDHVFIAYISSPHSIRCLEEKNSDKCNFSEASLEFTVTSGTGGIGVFKVLKCGL 1080
RPS4B RLD       KKDKIESDHVFIAYISSPHSIRCLEEKNSDKCDFSEASLEFTVTSGTGGIGVFKVLKCGL 1080
RPS4B Col-0     KKDRIESDHVFIAYISSPHSIRCLEEKNSDKCNFSEASLEFTVTSDTSGIGVFKVLKCGL 1080
                ***:****************************:************.*.************
                                                          NLS bipartite
RPS4B Ws-2      SLVYENDKNNNSSLEAKYDVPVEVSFQEPEHGIMEEERYRNKRRSDDKRPKKKRKTKRDD 1140
RPS4B RLD       SLVYENDKNNNSSLEAKYECSC---------------------------------------- 1102
RPS4B Col-0     SLVYENDKNKNSSLEAKYDVPVEVSFQEPEHGIMEEERYINKRRSDDRRPKKKRKTKRDD 1140
                *********:********: .

RPS4B Ws-2      IMIISTVTQSCVASVNARIEDKVTS 1165
RPS4B RLD       ------------------------
RPS4B Col-0     IMIISTVTQTCVPSVNARIEDKVTG 1165
```

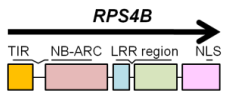

Figure S2. Multiple protein sequence alignment using CLUSTALW

***** (asterisk) indicates positions which have a single, fully conserved residue.
: (colon) indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix.
. (period) indicates conservation between groups of weakly similar properties - scoring =< 0.5 in the Gonnet PAM 250 matrix.
Domains are highlighted on top of the sequence alignment by rectangular boxes.

```
RRS1   MTNCEKDEEFVCISCVEEVRYSFVSHLSEALRRKGINNVVVGVDSDDLLFKESQAKIEKA 60
RRS1B    MIESEQIVYISCVEEVRYSFVSHLSEALRRKGINDVFI--DSDDSLSNESQSMVERA 55
           :.*::* ************************:*.:  **** * :***: :*:*

RRS1   GVSVMVLPGNCDPSDVWLDKFAKVLECQRNNKDQAVVPVLYGDSLLRDQWLSELDFKGLS 120
RRS1B  RVSVMILPGNRTIS---LEKLVKVLDCQKN-KDQVVVPVLYGVRSSETEWLSALDSKGFS 111
        ****:****   *   *:*:.***:**:* ***.*******    . :*** ** **:*

RRS1   RIHQSRKECSDSILVEEIVRDVYETHFYVGRIGIYSKLLEIENMVNKQPIGIRCVGIWGM 180
RRS1B  SVHQSRKECSDSHLVKEIVRDLYEKLFYMERIGIYSKLLEIEKMVCKQLLDIRCVGIWGM 171
        :********** **:*****:**. **: ************:** ** :.*********

       Kinase 1a
RRS1   PGIGKTTLAKAVFDQMSSAFDASCFIEDYDKSIHEKGLYCLLEEQLLPGND---ATIMKL 237
RRS1B  PGIGKTTLAKAVFDQMSGEFDAHCFIEDYTKAIQEKGVYCLLEEQFLKQNAGASGTVTKL 231
       *****************. *** ****** *:*:***:*******:*  *    .*: **

                    Kinase 2                    Kinase 3a
RRS1   SSLRDRLNSKRVLVVLDDVRNALVGESFLEGFDWLGPGSLIIITSRDKQVFCLCGINQIY 297
RRS1B  SLLRDRLNNKRVLVVLDDVRSPLVVESFLGGFDWFGPKSLIIITSKDKSVFRLCRVNQIY 291
       * ******.***********..** **** ****:** *******:**.** ** :****

RRS1   EVQGLNEKEARQLFLLSASIKEDMGEQNLQELSVRVINYANGNPLAINVYGRELKGKKKL 357
RRS1B  EVQGLNEKEALQLFSLCASI-DDMAEQNLHEVSMKVIKYANGHPLALNLYGRELMGKKRP 350
       ********** *** *.*** :**.****:*:*::**:****:***:*:***** ***:

        NLS bipartite
RRS1   SEMETAFLKLKRRPPFKIVDAFKSTYDTLSDNEKNIFLDIACFFQGENVNYVIQLLEGCG 417
RRS1B  PEMEIAFLKLKECPPAIFVDAIKSSYDTLNDREKNIFLDIACFFQGENVDYVMQLLEGCG 410
       .*** ******. **  :***:**:****.*.*****************:**:*******

RRS1   FFPHVEIDVLVDKCLVTISENRVWLHKLTQDIGREIINGETVQIERRRRLWEPWSIKYLL 477
RRS1B  FFPHVGIDVLVEKSLVTISENRVRMHNLIQDVGRQIINRETRQTKRRSRLWEPCSIKYLL 470
       ***** *****:*.********* :*:* **:**:*** ** * :** ***** ******

RRS1   EYNEHKANGEPKTTFKRAQGSEEIEGLFLDTSNLRFDLQPSAFKNMLNLRLLKIYCSNPE 537
RRS1B  EDKEQNENDEQKTTFERAQVPEEIEGIFLDTSNLSFDIKHVAFDNMLNLRLFKIYSSNPE 530
       * :*:: *.* ****:*** .*****:******* **::  **.*******:***.****

RRS1   VHPVINFPTGSLHSLPNELRLLHWENYPLKSLPQNFDPRHLVEINMPYSQLQKLWGGTKN 597
RRS1B  VHHVNNFLKGSLSSLPNVLRLLHWENYPLQFLPQNFDPIHLVEINMPYSQLKKLWSGTKD 590
       ** * ** .*** **** ***********: ******* ***********:***.***:

RRS1   LEMLRTIRLCHSHHLVDIDDLLKAENLEVIDLQGCTRLQNFPAAGRLLRLRDVNLSGCIK 657
RRS1B  LEMLKTIRLCHSQQLVDIDDLLKAQNLEVVDLQGCTRLQSFPATGQLLHLRVVNLSGCTE 650
       ****:*******::**********:****:*********.***:*:**:** ****** :

RRS1   IKSVLEIPPNIEKLHLQGTGILALPVSTVKPNHRELVNFLTEIPGLSEASKLER-----L 712
RRS1B  IKSFPEIPPNIETLNLQGTGIIELPLSIVKPNYRELLNLLAEIPGLSGVSNLEQSDLKPL 710
       ***. *******.*:******: **:* ****:***:*:*:****** .*:**:     *

RRS1   TSLLESNSSCQDLGKLICLELKDCSCLQSLPNMANLDLN-VLDLSGCSSLNSIQGFPRFL 771
RRS1B  TSLMKISTSYQNPGKLSCLELNDCSRLRSLPNMVNLELLKALDLSGCSELETIQGFPRNL 770
       *** : .:* *: *** ****:*** *:*****.**:*  .*******.*::****** *


RRS1   KQLYLGGTAIREVPQLPQSLEILNAHGSCLRSLPNMANLEFLKVLDLSGCSELETIQGFP 831
RRS1B  KELYLVGTAVRQVPQLPQSLEFFNAHG----------------------CVSLKSIR--- 805
       *:*** ***:*:*********::****                     * .*::*:
```

```
RRS1   RNLKELYFAGTTLREVPQLPLSLEVLNAHGSDSEKLPMHYKFNNFFDLSQQVVNDFFLKT 891
RRS1B  -----------------------------LDFKKLPVHYTFSNCSDLSPQVVNDFLVQA 835
                                      * :***:**.*.*  *** ******::::

RRS1   LT--YVKHIPRG----------------YTQELINKAPTFSFSAPSHTNQNATFDLQPGS 933
RRS1B  MANVIAKHIPRERHVTGFSQKTVQRSSRDSQQELNKTLAFSFCAPSHANQNSKLDLQPGS 895
       ::   .*****                  :*: :**: :***.****:***:.:******

RRS1   SVMTRLNHSWRNTLVGFGMLVEVAFPEDYCDATDFGISCVCRWSNKEGRSCRIERNFHCW 993
RRS1B  SSMTRLDPSWRNTLVGFAMLVQVAFSEGYCDDTDFGISCVCKWKNKEGHSHRREINLHCW 955
       * ****: *********.***:***.*.*** *********:*.****:* * * *:***

        NLS
RRS1   APGKVVPKVRKDHTFVFSDVNMRPSTGEGNDPDIWAGLVVFEFFPINQQTKCLNDRFTVT 1053
RRS1B  ALGKAIER---DHTFVFFDVNMRPDTDEGNDPDIWADLVVFEFFPVNKQRKPLNDSCTVT 1012
       * **.: :   ****** ******.*.*********.********:*:* * ***  ***
                                                  NLS

RRS1   RCGVRVINVATGNTSLENISLVLSLDPVEVSG---YEVLRVSYDDLQEMDKVLFLYIASL 1110
RRS1B  RCGVRLITAVNCNTSIENISPVLSLDPMEVSGNEDEEVLRVRYAGLQEIYKALFLYIAGL 1072
       *****:*.... ***:**** ******:****    ***** * .***: *.******.*

RRS1   FNDEDVDFVAPLIAG-IDLDVSSGLKVLADVSLISVSSNGEIVMHSLQRQMGKEILHGQS 1169
RRS1B  FNDEDVGLVAPLIANIIDMDVSYGLKVLAYRSLIRVSSNGEIVMHYLLRQMGKEILHTES 1132
       ******.:******. **:*** ******  *** ********** * ********* :*

RRS1   ----MLLSDCESSMTEN-LSDVPKKEKKHRESKVKKVVSIP--AIDEGDLWTWRKYGQKD 1222
RRS1B  KKTDKLVDNIQSSMIATKEIEITRSKSRRKNNKEKRVVCVVDRGSRSSDLWVWRKYGQKP 1192
            *:.: :***  .   ::.:.:.::::.* *:**.:   .  ..***.*******

        WRKY domain
RRS1   ILGSRFPRGYYRCAYKFTHGCKATKQVQRSETDSNMLAITYLSEHNHPRPTKRKALADST 1282
RRS1B  IKSSPYPRSYYRCAS--SKGCFARKQVERSRTDPNVLVITYISEHNHPFPTLRNTLAGST 1250
       * .* :**.*****   ::** * ***:**.**.*:*.***:****** ** *::**.**

RRS1   RSTSSSICSAITTSASSRVFQNKDEPNKPHLPSSSTPPGNAAVLFKMTDMEEFQDNMEVD 1342
RRS1B  RS-SSSKCSDVTTSASSTVSQDKEGPDKSHLPSSPASPPYAAMVVKEEDMEQWDN-MEFD 1308
       ** *** ** :****** * *:*: *:*.*****.:.*  **::.*  ***:::: **.*

RRS1   NDVVD-TRTLALFPEFQHQPE---EEYPWSTFFDY-------------------------- 1373
RRS1B  VDVEEDTFIPELFPEDTFADMDKLEENSQTMFLSRRSSGGNMEAQGKNSSDDREVNLPSK 1368
        ** : *    ****  .      ** . : *:.

RRS1   ----
RRS1B  ILNR 1372
```

Figure S3. Protein sequence alignment of Ws-2 RRS1 and Ws-2 RRS1B using ClustalW.

***** (asterisk) indicates positions which have a single, fully conserved residue.
: (colon) indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix.
. (period) indicates conservation between groups of weakly similar properties - scoring =< 0.5 in the Gonnet PAM 250 matrix.
Domains are highlighted on top of the sequence alignment by rectangular boxes.

```
RPS4   METSSISTVEDKPPQHQVFINFRGADLRRRFVSHLVTALKLNNINVFIDDYEDRGQPLDV 60
RPS4B  MAASSSSTG--LPPQHQVFINFRGEDLRLGFVSHLVEALENDNIKVFIDNYADKGEPLET 58
       * :** **    ************ ***  ****** **: :**:****:* *:*:**:.

RPS4   LLKRIEESKIVLAIFSGNYTESVWCVRELEKIKDCTDEGTLVAIPIFYKLEPSTVRDLKG 120
RPS4B  LLTKIHDSKIALAIFSGKYTESTWCLRELAMIKDCVEKGKLVAIPIFYKVDPSTVRGVRG 118
       **.:*.:***.******:****.**:***  ****.::*.*********::*****.::*

RPS4   KFGDRFRSMAKGDERKKK-WKEAFNLIPNIMGITIDKKSVESEKVNEIVKAVKTALTGIP 179
RPS4B  QFGDAFRDLEERDVIKKKEWKQALKWIPDLIGITVHDKSPESEILNEIVKEVKKVLKKVS 178
       :*** **.: : *  *** **:*:: **::*:**:..** *** :***** **..*. :.

RPS4   PEGSHNAV-VGALGNSNAGTSSGDKKHETFGNEQRLKDLEEKLDRDKYKGTRIIGVVGMP 238
RPS4B  LEGSQKVVSVDPSESIDTLSSVGGEKDKTFGIKQRLKELEEKLDLVKYKGTRVIGVVGMP 238
        ***::.* *..  . :: :* *.:*.:*** :****:******  ******:*******

       Kinase 1a
RPS4   GIGKTTLLKELYKTWQGKFSRHALIDQIRVKSKHLELDRLPQMLLGELS-KLNNPHVDNL 297
RPS4B  GIGKTTLVKELYKIWQGKFSRYALINQIRGKSNNFRLECLPTLLLEKLLPELNSPQLDSI 298
       *******:***** *******:***:*** **:::.*: ** :** :*  :**.*::*.:

                    Kinase 2                        Kinase 3a
RPS4   KDPYS----QLHERKVLVVLDDVSKREQIDALREILDWIKE---GKEGSRVVIATSDMSL 350
RPS4B  EEPYKTHKGLLRERKVLVVLDDVSGREQIYALLGKYDLHSKHEWIKDGSRIIIATNDISL 358
       ::**.     *:************ **** **    *  .:    *:***:::***.*:**

RPS4   TNGLVDDTYMVQNLNHRDSLQLFHYHAFIDDQANPQKKDFMKLSEGFVHYARGHPLALKV 410
RPS4B  LKGLVHDTYVVRQLNHRDGLQLFRYHAFHYDQTIPPKVDFMKLSDEFVHYARGHPLALKI 418
       :***.***:*::*****.****:****  **: * * ******: **************:

RPS4   LGGELNKKSMDHWNSKMKKLAQSPSPNIVSVFQVSYDELTTAQKDAFLDIACFRSQDKDY 470
RPS4B  LGRELNEKNMKHWETKLKILAQSPTTYIGEVVQVSYDELSLAQKDAFLDIACFRSQDVDY 478
       ** ***:*.*.**::*:* *****:. * .*.*******: ********************

RPS4   VESLLASSDLGSAEAMSAVKSLTDKFLINTCDGRVEMHDLLYKFSREIDLKASNQDGSRQ 530
RPS4B  VESLLVSSDPGSAEAIKALK---NKFLIDTCDGRVEMHDLLYRFSRELDLKASTQGDIKQ 535
       *****.*** *****:.*:*   :****:**************:****:*****.*.. :*

RPS4   RRLWLHQHIIKGGIINVLQNKMKAANVRGIFLDLSEVEDETSLDRDHFINMGNLRYLKFY 590
RPS4B  RRLWLYQDIIN-----VQQKTMGAADVRGIFLDLSEVKVETSLDRELFKNMRNLRYLKLY 590
       *****:*.**:     * *:.* **:************: ******: * ** ******:*

RPS4   NSHCPQECKTNNKINIPDKLKLPLKEVRCLHWLKFPLETLPNDFNPINLVDLKLPYSEME 650
RPS4B  NSHCPQECKTNNKINMPDGLELPLKEVRCLHWLKFPLEELPNDFDPINLVDLKLPYSEIE 650
       ***************:** *:***************** *****:*************:*

RPS4   QLWEGDKDTPCLRWVDLNHSSKLCSLSGLSKAEKLQRLNLEGCTTLKAFPHDMKKMKMLA 710
RPS4B  RLWDGVKDTPVLKWVDLNHSSKLCSLSGLSKAQNLQRLNLEG------------------ 692
       :**:* **** *:********************::********

RPS4   FLNLKGCTSLESLPEMNLISLKTLTLSGCSTFKEFPLISDNIETLYLDGTAISQLPMNME 770
RPS4B  ------CTSLESLRDANLMSLKTLTLSNCSNFKEFPLIPESLEALYLDGTSISQLPDNVG 746
             ******* : **:********.**.*******.:.:*:******:***** *:

RPS4   KLQRLVVLNMKDCKMLEEIPGRVGELKALQELILSDCLNLKIFPEIDISFLNILLLDGTA 830
RPS4B  NLKRLVLLNMKDCKLLETIPTCVGELKALQKLVLSGCSKLKEFPEINKSSSKILLLDGTS 806
       :*:***:*******:** **  ********:*:**.* :** ****: *  :*******:

RPS4   IEVMPQLPSVQYLCLSRNAKISCLPVGISQLSQLKWLDLKYCTSLTSVPEFPPNLQCLDA 890
RPS4B  IKTMPQLPSVQYLCLSRNDHLIYLPAGINQVSQLTRLDLKYCTKLTYVPELPPTLQYLDA 866
       *:.*************** ::  **.**.*:***. *******.** ***:**.** ***
```

```
RPS4   HGCSSLKTVSKPLARIMPTEQNHSTFIFTNCENLEQAAKEEITSYAQRKCQLLSYARKRY 950
RPS4B  HGCSSLKNVAKPLARIMSTVQNHCTFNFTNCGNLEQATKEEITSYAQRKCQLLSDARKHY 926
       *******.*:*******.* ***.** **** *****:**************** ***:*

RPS4   NGGLVSESLFSTCFPGCEVPSWFCHETVGSELEVKLLPHWHDKKLAGIALCAVVSCLDPQ 1010
RPS4B  NEG--SEALFSTCFPGCEVPSWFGHEAVGSLLQRKLLPHWHDKRLSGIALCAVVSFPDSQ 984
       * *  **:*************** **:*** *: *********:*:*********  *.*

RPS4   DQVSRLSVTCTFKVKDEDKSWVPYTCPVGSWTRHGGGKDKIELDHVFIGYTSCPHTIKCH 1070
RPS4B  DQLSCFSVTCTFKIKAEDKSWVPFTCPVGTWTREGNKKDKIESDHVFIAYISSPHSIRCL 1044
       **:* :*******:* *******:*****:***.*. ***** *****.* *.**:*:*

RPS4   EEGNSDECNPTEASLKFTVTGGTSENGKYKVLKCGLSLVYAKDKDKNSALETKYDMLIGK 1130
RPS4B  EEKNSDKCNFSEASLEFTVTSGTGGIGVFKVLKCGLSLVYENDKNNNSSLEAKYD----- 1099
       ** ***:** :****:****.**.  * :*********** :**::**:**:***

                                                    NLS bipartite
RPS4   SFQETSEGVDGRVKKTKGKYVMPVEKNFQETTEGVDGRVKKKKKTRMDNGRPKKKQRSGR 1190
RPS4B  ----------------------VPVEVSFQEPEHGIMEEERYRNKRRSDDKRPKKKRKTKR 1138
                            :*** .***. .*:  . : ::* * *: *****::: *

RPS4   DDNQTRMQVELQ-EGNINSVIMHTVKNF 1217
RPS4B  DDIMIISTVTQSCVASVNARIEDKVTS- 1165
       **      *  .  ..:*: * ..*..
```

Figure S4. Protein sequence alignment of Ws-2 RPS4 and Ws-2 RPS4B using ClustalW.

***** (asterisk) indicates positions which have a single, fully conserved residue.
: (colon) indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix.
. (period) indicates conservation between groups of weakly similar properties - scoring =< 0.5 in the Gonnet PAM 250 matrix.
Domains are highlighted on top of the sequence alignment by rectangular boxes.

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