Recognition of bacterial effector AvrRps4 in Arabidopsis

Inaugural-Dissertation

zur

Erlangung des Doktorgrades

der Mathematisch-Naturwissenschaftlichen Fakultät

der Universität zu Köln

vorgelegt von

Katharina Elisabeth Heidrich

aus Berlin

Köln, 2012

Die vorliegende Arbeit wurde am Max-Planck-Institut für Pflanzenzüchtungsforschung in Köln in der Abteilung für Molekulare Phytopathologie (Direktor: Prof. Dr. P. Schulze-Lefert) angefertigt.

Berichterstatter:	Prof. Dr. Paul Schulze-Lefert
	Prof. Dr. Martin Hülskamp
	Dr. Frank Takken
Prüfungsvorsitzender:	Prof. Dr. Ulf-Ingo Flügge
Tag der Disputation:	25. Mai 2011

Was wir wissen, ist ein Tropfen;
was wir nicht wissen, ein Ozean.

Isaac Newton

Publications

Birker D, Heidrich K, Takahara H, Narusaka M, Deslandes L, Narusaka Y, Reymond M, Parker JE, O'Connell R (2009). A locus conferring resistance to *Colletotrichum higginsianum* is shared by four geographically distinct Arabidopsis accessions. Plant Journal; 60(4):602-13

Table of Contents

ABBREVIATIONS

-	fused to (in the context of gene/protein fusion constructs)
° C	degree Celsius
Avr	avirulence
bp	base pair(s)
C-terminal	carboxy-terminal
CaMV	cauliflower mosaic virus
CC	coiled-coil
cDNA	complementary DNA
CFP	cyan fluorescent protein
cfu	colony forming unit
CLSM	confocal laser scanning microscopy
d	day(s)
dATP	deoxyadenosinetriphosphate
dCTP	deoxycytidinetriphosphate
DEPC	diethylpyrocarbonate
Dex	dexamethasone
dH2O	deionised water
ddH2O	deionised, distilled water
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleosidetriphosphate
dpi	day(s) post infection
dTTP	deoxythymidinetriphosphate
EDTA	ethylenediaminetetraacetic acid
ET	ethylene
ETI	effector-triggered immunity
EtOH	ethanol
Fig.	Figure
FLIM	fluorescence lifetime imaging microscopy

FP	fluorescent protein
FRET	Förster resonance energy transfer
f. sp.	forma specialis
g	gram(s)
gDNA	genomic DNA
GFP	green fluorescent protein
h	hour(s)
hpi	hour(s) post infection/induction
HR	hypersensitive response
HRP	horseradish peroxidase
JA	jasmonic acid
kb	kilobase(s)
kDa	kiloDalton(s)
l	litre
LRR	leucine-rich repeats
m	milli
M	molar (mol/l)
µ	micro
MAMP	microbe-associated molecular patterns
MAPK	mitogen-activated protein kinase
MCS	multiple cloning site
min	minute(s)
mM	millimolar
mRNA	messenger ribonucleic acid
MW	molecular weight
mYFP	monomeric YFP
NB	nucleotide binding site
NES	nuclear export signal
nes	non-functional nuclear export signal
ng	nanogram
NLR	NOD-like receptor
NLS	nuclear localization signal
nls	non-functional nuclear localization signal
nm	nanometer

NOD	nucleotide-binding oligomerization domain
NPC	nuclear pore complex
N-terminal	amino-terminal
Nup	nucleoporin
OD	optical density
ORF	open reading frame
p35S	35S promoter of CaMV
PAA	polyacrylamide
PAGE	polyacrylamide gel-electrophoresis
PAMP	pathogen-associated molecular pattern
PCR	polymerase chain reaction
PEPC	phosphoenolpyruvate carboxylase
pH	negative decimal logarithm of the H+ concentration
PR	pathogenesis related
PRR	PAMP/pattern recognition receptor
Pst	*Pseudomonas syringae* pv. *tomato*
PTI	PAMP-triggered immunity
pv.	pathovar
R	resistance
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	rounds per minute
RPM	resistance to *Pseudomonas syringae* pv. *maculicola*
RPP	resistance to *Peronospora parasitica*
RPS	resistance to *Pseudomonas syringae*
RT	room temperature
RT-PCR	reverse transcription-polymerase chain reaction
SA	salicylic acid
SAR	systemic acquired resistance
SDS	sodium dodecyl sulphate
sec	second(s)
T3SS	type III secretion system
TBS	Tris buffered saline
T-DNA	transfer DNA

TIR	*Drosophila* Toll and mammalian interleukin-1 receptor
TLR	Toll-like receptor
Tris	Tris-(hydroxymethyl)-aminomethane
U	unit
UV	ultraviolet
V	Volt(s)
v/v	volume per volume
WT	wild-type
w/v	weight per volume
Y2H	yeast two hybrid
YFP	yellow fluorescent protein

SUMMARY

Plants, like animals, are constantly exposed to attack by pathogens such as viruses, bacteria, fungi or oomycetes that have different life styles and infection strategies. Unlike animals, plants lack a somatic adaptive immune system and rely on cell-autonomous events and the innate immune system. Successful pathogens have evolved effector proteins that can suppress plant defense responses. To counteract the activities of pathogen effectors, plants have evolved resistance (R) proteins that specifically recognize these effectors either through direct interaction or through perception of effector manipulation of the host cell. TIR-type Nucleotide-binding Leucine-rich-repeat (TIR-NB-LRR) receptors invariably require EDS1 to confer resistance. Activation of effector-triggered immunity usually involves a rapid influx of calcium ions, a burst of reactive oxygen species, activation of MAPKs, synthesis of SA, reprogramming of gene expression and often a hypersensitive response (HR). However, it is not known whether the HR is an essential component of disease resistance mechanisms in plants. Deciphering the molecular mechanisms by which bacterial effectors contribute to disease and are recognized by resistant hosts is important for our understanding of host-pathogen communication and co-evolution. It is also important to establish how activated TIR-type R proteins molecularly connect to *EDS1*-dependent defense induction.

The work presented here characterizes the mechanism by which the *Pseudomonas syringae* effector AvrRps4 is recognized by its cognate TIR-NB-LRR-type receptor proteins RPS4 and RRS1 and how this leads to subsequent defense signalling in the host plant *Arabidopsis thaliana* (hereafter named Arabidopsis). Nuclear localization of RPS4 is required for its resistance function but it remained unclear how and where in the host cell AvrRps4 is perceived by its cognate R proteins. Increased export of AvrRps4 from the nucleus by fusion to a nuclear export sequence (NES) fails to induce a full immune response. In contrast, AvrRps4 targeted to the nucleus through the addition of a nuclear localization sequence (NLS) triggers full resistance, measured by limitation of bacterial growth. Notably, AvrRps4 targeted to the nucleus induces resistance but fails to elicit a host cell death response. Restriction of bacterial multiplication can thus be uncoupled from induction of cell death. Collectively, the data suggest that at least one essential step of RPS4/RRS1-mediated AvrRps4 recognition leading to resistance occurs in the nucleus. Results from genetic and molecular analysis of RPS4 and RRS1 modes of action suggest that these R proteins function cooperatively in the nucleus in recognizing AvrRps4.

To further dissect AvrRps4 function and recognition through RPS4 and RRS1, interaction partners of AvrRps4 *in planta* were analyzed. AvrRps4 physically interacts with the central immune regulator EDS1 in the nucleus suggesting that EDS1 might be the virulence target of AvrRps4. Furthermore, EDS1 forms a complex with RPS4 and RRS1 in the nucleus. These data provide a potential direct link between TIR-NB-LRR-type receptors and *EDS1*-dependent defense induction and suggest that important recognition and signalling events can occur inside the plant nucleus.

ZUSAMMENFASSUNG

Pflanzen und Tiere werden ständig von Krankheitserregern, wie z.B. Viren, Bakterien und Pilzen angegriffen. Im Gegensatz zu Tieren besitzen Pflanzen kein humorales Immunsystem, sondern nur ein angeborenes Immunsystem. Krankheitserreger verfügen über Effektorproteine, welche die pflanzliche Immunantwort unterdrücken können. Um dem entgegenzuwirken, haben Pflanzen Resistenzproteine entwickelt, die entweder Effektorproteine direkt erkennen können oder deren Funktionen in der Wirtszelle. Eine bestimmte Gruppe dieser Resistenzproteine, TIR-NB-LRR Rezeptoren, benötigen das Pflanzenprotein EDS1 zur Aktivierung der Immunantwort. Aktivierung der Resistenzantwort durch die Erkennung eines Effektorproteins führt zu einem Einstrom von Kalziumionen in die Zelle, gefolgt von der lokalen Freisetzung radikaler Sauerstoffspezies und oft zur Aktivierung des programmierten Zelltods in den angegriffenen Zellen. Allerdings wird die Aktivierung des programmierten Zelltods nicht immer für die Resistenz benötigt. Um ein besseres Verständnis der Co-Evolution von Krankheitserregern und Pflanzen zu erlangen, ist es wichtig, die molekularen Mechnismen der Erkennung von Effektorproteinen sowie deren Funktionen in der Wirtszelle zu untersuchen. Desweiteren ist bisher unbekannt, wie TIR-NB-LRR Rezeptoren nach ihrer Aktivierung die EDS1-abhängige Immunantwort auslösen.

Diese Arbeit beschäftigt sich mit den molekularen Mechanismen der Erkennung des Effektorproteins AvrRps4 von *Pseudomonas syringae* durch die TIR-NB-LRR Rezeptorproteine RPS4 und RRS1 in der Wirtspflanze *Arabidopsis thaliana*. Eine Lokalisierung von RPS4 im Zellkern wird benötigt, um eine effektive Immunantwort auszulösen. Bisher ist nicht bekannt, in welchem Zellkompartiment die Erkennung von AvrRps4 stattfindet. Ein verstärkter Export von AvrRps4 aus dem Zellkern korreliert mit einer schwächeren Immunantwort der Pflanze wohingegen der Import von AvrRps4 in den Zellkern Resistenz auslöst. Interessanterweise führt der Import von AvrRps4 in den Zellkern zu einer vollständigen Resistenz der Pflanze, jedoch ohne das dafür programmierter Zelltod ausgelöst werden muss. Aus diesen Ergebnissen lässt sich schliessen, dass zumindest ein essentieller Schritt zur Erkennung von AvrRps4 im Zellkern stattfindet. Weitere Ergebnisse deuten darauf hin, dass die beiden Rezeptorproteine RPS4 und RRS1 kooperativ arbeiten um Resistenz gegenüber AvrRps4 zu vermitteln.

Um einen besseres Verständnis für die Funktion von AvrRps4 in der Pflanze und die Erkennung durch RPS4 und RRS1 zu bekommen, wurden die Interaktionspartner von AvrRps4 untersucht. AvrRps4 interagiert mit EDS1 im Zellkern der Pflanze, was darauf

hindeutet, dass EDS1 das Target (Zielprotein) von AvrRps4 sein könnte. Desweiteren wurde eine Interaktion von EDS1 mit RPS4 und RRS1 im Zellkern beobachtet. Diese Ergebnisse weisen darauf hin, dass sowohl die Erkennung von Effektorproteinen als auch die Aktivierung der Immunantwort im Zellkern der Pflanze stattfinden können.

1 INTRODUCTION

Plants, like animals, are constantly exposed to attack by multiple pathogens such as viruses, bacteria, fungi or oomycetes that have different life styles and infection strategies (Dangl and Jones, 2001). Plant pathogens can be broadly divided into those that kill the host and feed on dead plant material (necrotrophs) and those that require a living host to complete their life cycle (biotrophs). However, disease is rather rare in nature due to the existence of a sophisticated multi-layered immune system. Unlike animals, plants lack a somatic adaptive immune system and rely on cell-autonomous events and the innate immune system. The innate immune systems of both plants and animals consist of two layers (Jones and Dangl, 2006). The first layer is activated upon recognition of conserved molecules common to many classes of microbes, including potentially non-pathogens. The second responds to pathogen virulence proteins either directly or through their effects on host targets. Although the innate immune systems of animals and plants share common features and analogous regulatory modules, the two immune systems are likely the consequence of convergent evolution (Ausubel, 2005).

1.1 First layer- recognition of conserved microbial patterns

Plants possess passive and active defenses against pathogen attack. Preformed physical and biochemical barriers such as epidermal wax layers, the cell wall and apoplastic antimicrobial compounds, represent initial obstacles to infection that provide resistance against a wide range of potential plant pathogens (Heath, 2000). When pathogens are successful in overcoming these passive defenses, plants rely on active immunity to restrict pathogen proliferation.

The first active layer of the plant immune system is based on the recognition of slowly evolving microbial-or pathogen-associated molecular patterns (MAMPs or PAMPs). These are conserved molecules, which are characteristic to certain classes of microbes and usually essential for the life cycle of the microbe, such as flagellin (Jones and Dangl, 2006). PAMPs are sensed at the plant cell surface by pattern-recognition receptors (PRRs), which leads to induction of PAMP-triggered immunity (PTI). PTI involves MAPK (mitogen-activated protein kinases) activation, production of reactive oxygen species (ROS), transcriptional re-programming and hormone biosynthesis but does usually not include programmed cell death

at the infection site, named the hypersensitive response (HR) (Segonzac and Zipfel). So far characterized plant PRRs are transmembrane proteins belonging to the receptor-like kinase (RLK) or receptor-like protein (RLP) families. RLKs are structurally defined by the presence of a ligand-binding extracellular leucine-rich repeat (LRR) domain and a cytoplasmic kinase signalling domain. In animals, PAMPs are recognized by plasma-membrane resident Toll-like receptors (TLRs). These receptors are characterized by an extracellular LRR domain and an intracellular Toll/Interleukin-1 receptor (TIR) protein-protein interaction domain that initiates a defense signalling cascade through interaction with signalling adaptors such as MyD88, which also have TIR domains (Hayashi et al., 2001; Ausubel, 2005; Kawai and Akira, 2007). In plants, the best characterized PRRs are FLS2 (FLAGELLIN SENSING 2) that perceives bacterial flagellin in most plant species and EFR (EF-TU RECEPTOR) that recognizes the elongation factor Tu (EF-Tu) in *Brassicaceae* species (Gomez-Gomez and Boller, 2000; Zipfel et al., 2006). Furthermore, the LysM-RLK CERK1 (CHITIN ELICITOR RECEPTOR KINASE 1) was indentified in Arabidopsis that is involved in perception of fungal chitin (Miya et al., 2007). Recently, the ligand of the rice PRR Xa21, which confers immunity to most strains of *Xanthomonas oryzae* pv. *oryzae,* was identified as the secreted protein Ax21 (Chen et al., ; Lee et al., 2009b). A sulphated peptide corresponding to the first 17aa of Ax21 is sufficient to trigger *Xa21*-mediated resistance. In contrast to the conserved PAMPs flagellin and EF-Tu, Ax21 shows only a narrow distribution and is restricted to *Xanthomonas* strains (Thomma et al.). Thus, the strict definition of PAMPs and PRRs as being conserved between species does not always hold since EFR is restricted to *Brassicaceae* and Xa21 provides race-specific resistance to *Xanthomonas*.

In addition to these pattern recognition receptor systems, another class of surveillance system recognizes plant-derived molecules known as DAMPs (damage-associated molecular patterns): DAMPs are endogenous molecules that appear in the intercellular space in response to the damage caused by a pathogen attack, e.g. cell wall fragments or effectors derived from cytoplasmic proteins (Lotze et al., 2007; Matzinger, 2007; Boller and Felix, 2009). One hypothesis is that the integration of PAMP and DAMP responses is critical to mount robust PAMP-triggered immunity. This signal integration might help plants to distinguish between pathogenic and non-pathogenic microbes.

1.2 Pathogen effectors- enhancers of virulence

Successful pathogens have to avoid or actively suppress recognition of their PAMPs, given the ability of plants to recognize a multitude of PAMPs. Bacterial pathogens have acquired a needle-like type III secretion system (T3SS) which enables them to deliver effector proteins into plant cells to suppress plant defense responses (Alfano and Collmer, 2004). Plant pathogens such as the hemi-biotrophic bacterium *Pseudomonas syringae* deliver 15-35 effectors per strain into host cells (Chang et al., 2005). The vast majority of the *P. syringae* DC3000 effectors can suppress ETI and many of them also PTI (Guo et al., 2009). This suggests an overlap in the signalling machinery between PTI and ETI (Katagiri and Tsuda, 2010). A well characterized example for an effector protein that suppresses PTI is the *P. syringae* bipartite protein AvrPtoB. The amino terminus suppresses flagellin responses through its kinase-targeting domain and its carboxy-terminal E3 ligase domain can tag interacting kinase proteins with ubiquitin to direct them for degradation (Rosebrock et al., 2007; Gimenez-Ibanez et al., 2009).

In addition to suppression of plant defenses, some effectors may promote nutrient leakage or pathogen dispersal (Badel et al., 2002; Jones and Dangl, 2006). One example of an alternative bacterial effector strategy is given by the transcription activator-like (TAL) effectors of *Xanthomonas* spp., which are transcription factors that induce the expression of specific host genes, some of which contribute to symptom development (Kay and Bonas, 2009).

In addition to effector proteins, pathogens can produce small molecules that mimic plant hormones. Some *P. syringae* strains produce coronatine, a mimic of a jasmonic acid precursor that suppresses salicylic-acid (SA)-mediated defense to biotrophic pathogens and induces stomatal opening, thereby facilitating pathogen entry into the apoplast (Brooks et al., 2005; Melotto et al., 2006). Plants protect themselves from virulent pathogens by exerting the so-called `basal resistance` which describes the residual, effector-suppressed level of PAMP-triggered immunity (Jones and Dangl, 2006).

1.3 Second layer- NB-LRR receptor mediated immunity

To counteract the activities of pathogen effectors, plants have evolved resistance (R) proteins that specifically recognize these effectors. In the absence of a matching R protein (compatible interaction) the effector exerts its virulence function, whereas recognition by its cognate R protein (incompatible interaction) results in a rapid defense response that overrides effector-

suppression of PTI, named effector-triggered immunity (ETI) (Jones and Dangl, 2006; Dodds and Rathjen). In this case, the recognized effector is called an avirulence (Avr) protein. The major differences between PTI and ETI result not from differences in the nature of the signalling machinery but are caused by a different timing and amplitude of the response (Katagiri and Tsuda, 2010).

Activation of effector-triggered immunity induces a rapid influx of calcium ions, a burst of reactive oxygen species, activation of MAPKs, synthesis of SA, reprogramming of gene expression and often a hypersensitive response (HR) (Dodds and Rathjen, 2010). Induction of this programmed cell death is thought to restrict pathogen colonization (Jones and Dangl, 2006). However, it is not clear whether the HR is an essential component of disease resistance mechanisms in plants. For instance, resistance mediated by the potato R protein Rx to *Potato virus X* (PVX) involves the suppression of virus accumulation without an HR (Bendahmane et al., 1999). Recently, it was shown that two type I metacaspases, AtMC1 and AtMC2, antagonistically control programmed cell death in Arabidopsis (Coll et al., 2010). Mutation of AtMC1 could nearly eliminate the hypersensitive cell death response induced by plant intracellular immune receptor, but did not lead to enhanced pathogen proliferation, suggesting that HR is not always required for resistance.

1.3.1 Direct and indirect recognition of pathogen effectors

NB-LRR proteins can recognize pathogen effectors either directly through physical interaction (Figure 1A) or indirectly by detecting modifications caused by these effectors on host targets. A direct interaction of an effector with its cognate R protein is consistent with the gene-for-gene hypothesis proposed by Flor (Flor, 1971). In the case of direct recognition, effector and R proteins tend to show high levels of sequence polymorphism between alleles of the host and pathogen populations, reflecting the coevolutionary arms race between host and pathogen (Stukenbrock and McDonald, 2009). Direct recognition has been reported only for few cases (Jia et al., 2000; Deslandes et al., 2003; Dodds et al., 2006) and given that the Arabidopsis Col-0 genome encodes only ~150 *NB-LRR* genes in contrast to thousand of pathogen effectors, direct interaction is most likely not the most prevalent mode of recognition (Meyers et al., 2003).

Currently, three different models have been proposed that could explain indirect recognition mechanisms of an effector protein by its cognate R protein. The `guard` model postulates that R proteins function as `guards` of target host proteins (`guardee`) and are activated upon

effector-induced modifications of a target protein (Figure 1B) (Dangl and Jones, 2001). An extension of the guard hypothesis is described in the `decoy` model (van der Hoorn and Kamoun, 2008). In this model, a host protein that mimics the real virulence target of an effector can act as a decoy and participate solely in effector perception (Figure 1B). A further modification of the guard model is the bait-and-switch model, which describes a two-step recognition event (Figure 1C). First, an effector interacts with the target host protein associated with an NB-LRR which in turn facilitates direct recognition of the pathogen effector (Collier and Moffett, 2009).

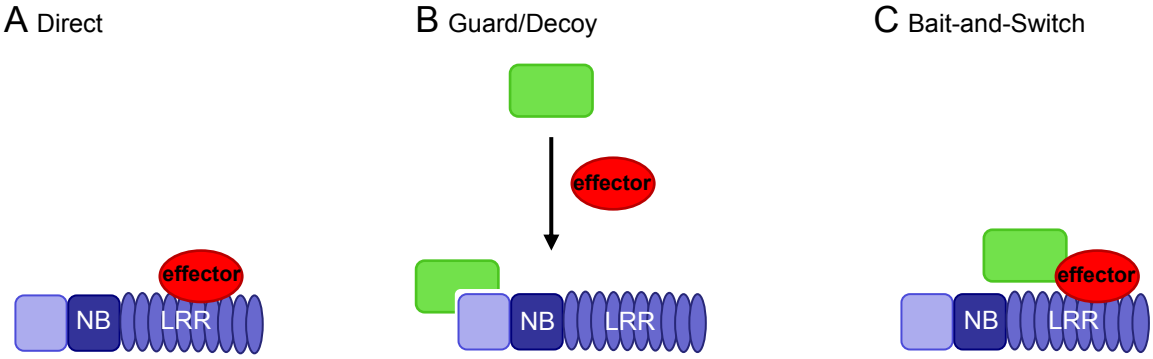


Fig. 1: Recognition mechanisms of pathogen effectors through plant resistance proteins. (A) In a direct recognition mechanism, the effector (red) physically interacts with the NB-LRR resistance protein (blue) which leads to defense signalling. **(B)** In the guard or decoy model, the effector modifies a host protein (green) that is either its virulence target (guard model) or a structural mimic of the target protein (decoy model). Effector-induced alterations of this host protein are recognized by the receptor. **(C)** In the bait-and-switch model, the effector physically interacts with a host protein which in turn leads to direct recognition through the R protein. Adapted from Dodds and Rathjen, 2010.

One example for an indirect recognition mechanism is Arabidopsis RIN4 (RPM1 INTERACTING PROTEIN 4), a plasma-membrane-associated protein that negatively regulates basal host defense responses (Mackey et al., 2002; Mackey et al., 2003; Desveaux et al., 2007). Two unrelated type III effectors, AvrRpm1 and AvrB associate with and induce phosphorylation of RIN4. Recently, it was shown that RIPK, a receptor-like kinase, can phosphorylate RIN4 in response to these bacterial effectors (Liu et al., 2011). However, it is not clear whether AvrB can also directly phosphorylate RIN4. RIN4 phosphorylation activates the RPM1 resistance protein (RESISTANCE TO P. SYRINGAE PV. MACULICOLA 1) (Chung et al., 2011). A third effector, AvrRpt2 is a cysteine protease that cleaves RIN4 which leads to de-repression of the RPS2 (RESISTANCE TO P. SYRINGAE 2) NB-LRR protein (Axtell and Staskawicz, 2003; Mackey et al., 2003). Activation of RPS2 requires the GPI-anchored NDR1 protein, and RIN4 interacts with NDR1 (Day et al., 2006).

These examples illustrate how plants use NB-LRR proteins to guard against pathogens that deploy effectors to inhibit defense induction.

1.3.2 Structures and functions of resistance proteins

Some plant *R* genes encode membrane bound proteins with an extracellular leucine-rich repeat (LRR) domain, either with or without an intracellular kinase domain. The corresponding Avr proteins are secreted into the apoplastic space during infection, where they may be detected (Dangl and Jones, 2001). However, the majority of known *R* genes encode intracellular proteins with an LRR (leucine-rich repeat) domain and a NB (nucleotide-binding) domain. The NB domain is part of a larger domain termed NB-ARC that shows homology to cell death effectors such as the human Apaf-1 (APOPTOSIS ACTIVATING FACTOR 1) and *Caenorhabditis elegans* CED4 (CELL DEATH 4) (Dangl and Jones, 2001). Proteins that have an NB-ARC domain are evolutionary related to the mammalian nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), many of which also function in innate immunity (Takken et al., 2006). Both plant and animal NB-LRR receptors belong to the STAND (signal transduction ATPases with numerous domains) group of NTPases. ATP hydrolysis of the NB-ARC domain is thought to trigger a conformational change leading to receptor activation and signal transduction (Takken et al., 2006). To prevent damage due to inappropriate activation, R proteins must be under tight negative control. This is thought to be accomplished by intramolecular interactions between the various subdomains of the R protein. Pathogen perception is proposed to release this autoinhibition, enabling the conformational changes required to activate defence signalling (Lukasik and Takken, 2009; Bernoux et al., 2011).

The LRR domain of plant R proteins has a role in determining specificity towards an effector and direct effector-LRR interactions have been reported (Jia et al., 2000; Krasileva et al., 2010). Extensive amino acid variation occurs in this domain as a result of strong diversifying selection (Rafiqi et al., 2009). However, in the *L* class of flax rust resistance genes, diversifying selection also acts on the N-terminal receptor domain indicating that these residues are likely co-evolving with the corresponding LRR domain to provide specificity (Luck et al., 2000). Also, the tobacco (*Nicotiana tabacum*) NB-LRR receptor N recognizes the helicase domain of *Tobacco Mosaic Virus* (TMV) replicase protein, termed p50, through association with its N-terminal domain (Burch-Smith et al., 2007).

Plant NB-LRR proteins are subdivided into two classes based on their N-terminal domain: they carry either a coiled coil (CC) domain or a TIR domain with homology to *Drosophila* Toll and mammalian Interleukin-1 receptors (Meyers et al., 2003). In animal TLRs, PAMP perception induces homo-dimerization of the cytoplasmic TIR domain which provides a new scaffold that can bind to adapter proteins and activates defense signalling (Tapping, 2009). Overexpression of the TIR domain of plant R proteins can trigger an effector-independent cell death response suggesting that the TIR domain of plants is also involved in mediating R protein signalling (Frost et al., 2004; Swiderski et al., 2009). Recently, it was shown that homo-dimerization of the TIR domain of L6 strongly correlates with autoactivity *in planta*, suggesting that this is a key event in defense signalling (Bernoux et al., 2011). Also for the MLA CC domain it has been recently shown that this domain forms homo-dimers and that homo-dimerization is required for autoactivity *in planta* (Maekawa et al., 2011).

In contrast to plants, mammalian NB-LRR proteins carry either N-terminal caspase recruitment (CARD) or pyrin domains that link activated NB-LRR receptors to both activation of nuclear factor κB (NF-κB) and the caspase I complex leading to production of proinflammatory cytokines (Strober et al., 2006; Ting et al., 2006). Mammalian NOD receptors recognize only highly conserved microbe-associated molecules (e.g. peptidoglycan) in contrast to species-specific effectors recognized by plant NB-LRRs. For vertebrates, the evolution of the adaptive immune system may have obviated the need for pathogen-specific PRRs or may have allowed their disappearance (Ausubel, 2005).

1.3.3 Components required for R protein-mediated resistance

Genetic screens for loss of resistance in plants have identified several components required for the function of NB-LRR receptors. Mutations in the cochaperone HSP90 (HEAT SHOCK PROTEIN 90) compromise resistance triggered by the CC-NB-LRR receptors RPM1 and RPS2 (Hubert et al., 2003; Takahashi et al., 2003). Furthermore, it has been demonstrated that RAR1 (REQUIRED FOR Mla12 RESISTANCE 1) and SGT1 (SUPPRESSOR OF G-2 ALLELE OF SKIP1) are co-chaperones of HSP90 in the folding of NB-LRR proteins, and are required for efficient defense activation (Shirasu and Schulze-Lefert, 2000; Belkhadir et al., 2004; Shirasu, 2009). Each of these three proteins can independently interact with one another and it is thought that RAR1, SGT1 and HSP90 operate together as a cytosolic co-chaperone for NB-LRR function (Nishimura and Dangl, 2010).

The signal transduction pathway downstream of activated NB-LRR receptors is poorly understood. TIR-and CC-type NB-LRR receptors employ different signalling components. *EDS1* (*ENHANCED DISEASE SUSCEPTIBILITY 1*), which encodes a lipase-like protein, is required for the function of *TIR-NB-LRR* class *R* genes (Parker et al., 1996; Falk et al., 1999; Feys et al., 2001), whereas *NDR1* (*NON-RACE SPECIFIC DISEASE RESISTANCE 1*) encoding a plasma membrane-localized protein is essential for the function of most, but not all, CC-NB-LRR class *R* genes (McDowell et al., 2000; Day et al., 2006). In addition to its role in TIR-NB-LRR mediated resistance, EDS1 is an essential component of basal resistance to virulent biotrophic and hemi-biotrophic pathogens (Falk et al., 1999). It forms complexes in the cytoplasm and nucleus with its defense co-regulators PAD4 (PHYTOALEXIN DEFICIENT 4) and SAG101 (SENESCENCE ASSOCIATED GENE 101) (Feys et al., 2001; Feys et al., 2005). *EDS1* and *PAD4* function upstream of pathogen-induced SA accumulation, but their expression is also enhanced by exogenous applications of SA indicating that defense induction involves a SA-associated positive feedback loop that may potentiate resistance (Shirasu et al., 1997; Falk et al., 1999; Jirage et al., 1999). EDS1 was shown to signal downstream of activated TIR-NB-LRR receptors and upstream of the transcriptional reprogramming of defense genes and cell death induction (Rusterucci et al., 2001; Wirthmueller et al., 2007; Garcia et al., 2010). A balanced nuclear and cytoplasmic distribution of EDS1 is required for the coordinated induction and repression of particular defense-related genes and the establishment of an appropriate immune response (Garcia et al., 2010). However, it is not yet clear what intermediaries connect EDS1 and activated TIR-NB-LRRs.

1.3.4 Subcellular localization of NB-LRR immune receptors and pathogen effectors

The establishment of an appropriate immune response requires the spatial and temporal regulation of its immune components (Deslandes and Rivas, 2011). Recent studies on the localization of defense components showed that many subcellular organelles play a role in orchestrating a successful immune response (Padmanabhan and Dinesh-Kumar, 2010). Especially protein translocation to the nucleus constitutes an important level for the regulation of the defense response (Garcia and Parker, 2009).

Several R proteins have been found to localize to the nucleus, including N, MLA, RPS4, and snc1 (SUPPRESSOR OF NPR1-1, CONSTITUTIVE 1), and their nuclear localization is required for proper functioning (Burch-Smith et al., 2007; Shen et al., 2007; Wirthmueller et

al., 2007; Cheng et al., 2009). The tobacco (*Nicotiana tabacum*) TIR-NB-LRR protein N confers resistance against *Tobacco Mosaic Virus* (TMV) through recognition of the viral helicase protein p50. N has a nucleo-cytoplasmic distribution and interfering with its nuclear localization renders the receptor non-functional in triggering resistance (Burch-Smith et al., 2007). N associates with transcription factors via its LRR domain and thus provides a link of receptor function to transcriptional reprogramming of host cells (Shen and Schulze-Lefert, 2007). Nuclear activity was also shown for the barley CC-NB-LRR protein MLA. Shen et al (2007) could show that nuclear localization of MLA10 is required for race-specific resistance towards the powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (*Bgh*). The nuclear pool of MLA10 associates with two WRKY transcription factors that function as negative regulators of resistance and the MLA10-*Hv*WRKY2 interaction abolishes WRKY repressor function, enabling rapid defense gene induction (Shen et al., 2007). These data suggest a relatively short pathway between R protein activation and downstream transcriptional reprogramming.

The importance of subcellular localization of effector proteins for recognition through their cognate R proteins has been shown only in few cases. A recent study by Slootweg et al (2010) investigated the role of the subcellular distribution of the *Potato Virus X* (PVX) coat protein (CP) for the function of its cognate receptor protein. PVX CP is recognized in potato (*Solanum tuberosum*) by the CC-NB-LRR receptor Rx1 that shows a nucleo-cytoplasmic distribution (Slootweg et al., 2010). Forcing PVX CP exclusively to the nucleus showed that Rx1 needs to be activated in the cytoplasm and that the CP is not able to activate Rx1 in the nucleus (Slootweg et al., 2010). Another example is the *Xanthomonas campestris* pv. *vesicatoria* effector avrBs3 that requires functional NLSs for induction of HR on pepper carrying the *R* gene *Bs3* (VandenAckerveken et al., 1996). However, these studies solely utilized HR induction as an indicator of recognition.

In eukaryotes, trafficking between the cytoplasm and the nucleus occurs via the nuclear pore complexes (NPCs) consisting of approximately 30 nucleoporins that form channels through the nuclear envelope (Cook et al., 2007; Meier, 2007; Xu and Meier, 2008). Proteins larger than 50kDa cannot passively diffuse through the nuclear pore and thus require specific transporters. Nuclear import is mediated by importin α which recognizes nuclear localization sequences (NLS) consisting of a short stretch of basic residues (three to six Lys/Arg) and forms a heterodimer with importin β. This allows an energy-dependent, Ran-mediated translocation of the proteins through the nuclear pore (Gorlich et al., 1995). Nuclear export is

directed via exportins that recognize Leucine-rich nuclear export sequences (NES) (Haasen et al., 1999; Hutten and Kehlenbach, 2007).

The importance of nucleocytoplasmic trafficking for both basal resistance and R protein-mediated resistance was underlined by a suppressor screen that identified components required for the Arabidopsis *snc1* mutant phenotype. *Snc1* contains a gain-of-function mutation in the NB-LRR linker region of a TIR-NB-LRR receptor resulting in an autoactivated defense response and increased resistance to several pathogens (Li et al., 2001; Zhang et al., 2003). In a *snc1* suppressor screen, mutations in *MOS3, MOS6* and *MOS7* (for *MODIFIER OF SNC1*) were identified (Palma et al., 2005; Zhang and Li, 2005; Cheng et al., 2009). *MOS3* and *MOS7* encode homologs of the nucleoporins Nup96 and Nup88 and *MOS6* encodes importin α3. Mammalian Nup88 promotes nuclear retention of NFκB transcription factors thereby regulating the immune reponse (Xylourgidis et al., 2006). Similarly, MOS7 is required for the nuclear accumulation of EDS1 and the autoactivated snc1 protein, suggesting that regulation of the nuclear accumulation of some defense components is crucial for orchestrating an appropriate immune response (Cheng et al., 2009).

1.4 The Arabidopsis TIR-NB-LRR receptors RPS4 and RRS1

RPS4 (*RESISTANCE TO P. SYRINGAE 4*) was identified in Arabidopsis due to its ability to confer resistance to *P. syringae* pv. *tomato* (*Pst*) expressing the effector protein AvrRps4 (Hinsch and Staskawicz, 1996). The *RPS4* locus (At5g45250) was mapped to a disease resistance gene cluster on chromosome 5 in a cross between Arabidopsis accession RLD (susceptible) and Ws-0 (resistant) (Gassmann et al., 1999). *RPS4* encodes a TIR-NB-LRR receptor that in addition to the TIR and NB-ARC domains harbors 15 degenerate LRRs consisting of 24 aa each and C-terminal 318 aa that do not show homology to other known proteins. The non-functional *rps4* allele from RLD encodes a protein that contains five amino-acid changes compared to the functional Col-0 protein (Gassmann et al., 1999). A T-DNA insertion line in Col-0, designated *rps4-2,* was shown to be more susceptible to *Pst* AvrRps4 compared to Col-0 but did not show compromised basal resistance to virulent bacteria (Wirthmueller et al., 2007).

Consistent with other *TIR-NB-LRR R* genes from different plant species, *RPS4* produces alternative transcripts with truncated open reading frames encoding mainly the TIR and NBS domains and a combination of full length and alternative transcripts is required for RPS4 function (Gassmann et al., 1999; Zhang and Gassmann, 2003). As mentioned for other TIR-

NB-LRR receptors, the TIR domain of RPS4 is likely to have a signalling role during resistance responses since transient expression of the TIR domain plus 80 aa of the NB-ARC domain in *Nicotiana benthamiana* leads to cell death (Swiderski et al., 2009).
As a member of the TIR-NB-LRR class of R proteins, RPS4 shows complete dependence on *EDS1*. Constitutive RPS4 overexpression in stable transgenic Arabidopsis lines induces dwarfism resembling an auto-activated defense response, which is *EDS1*-dependent (Wirthmueller et al., 2007). Moreover, overexpression of RPS4 in *N. benthamiana* triggers AvrRps4-independent cell death that is dependent on *NbEDS1*, *NbSGT1* and *NbHSP90* orthologs (Zhang et al., 2004). How activated RPS4 links molecularly to *EDS1*-dependent defense induction is not known.
RPS4 contains a bipartite NLS in the C-terminus and a subpool of functional RPS4 was shown to localize to nuclei, whereas the majority of RPS4 protein is associated loosely with endomembranes (Wirthmueller et al., 2007). Mutation of the NLS and thus depletion of the nuclear RPS4 pool correlates with loss of RPS4 function (Wirthmueller et al., 2007). However, the exact roles of nuclear or the endomembrane pools of RPS4 in AvrRps4-triggered resistance are not known.

Arabidopsis *RRS1* (*RESISTANCE TO R. SOLANACEARUM 1*) was identified by its ability to confer resistance to the bacterial strain *Ralstonia solanacearum* GMI1000 (Deslandes et al., 2002). The *RRS1* locus (At5g45260) maps to a disease resistance gene cluster on chromosome 5 where *RPS4* is also located (Deslandes et al., 2002). *RRS1* encodes an unusual TIR-NB-LRR-type receptor harbouring a C-terminal WRKY transcription factor domain (Deslandes et al., 2002; Eulgem and Somssich, 2007). According to the Rosetta stone principle (Enright and Ouzounis, 2001), the existence of such a chimeric protein is an indication that interaction with transcription factors may be part of the R protein signalling mechanism, as was shown for MLA10 (Shen et al., 2007). A single amino acid insertion in the RRS1 WRKY domain in the *slh1* (*SENSITIVE TO LOW HUMIDITY 1*) mutant causes a loss of DNA-binding activity and results in autoactivation of defense responses and hypersensitive cell death (Noutoshi et al., 2005). This suggests that RRS1, as most WRKY transcription factors, acts as a negative regulator of defense gene induction (Noutoshi et al., 2005; Eulgem and Somssich, 2007).
The *RRS1* gene exhibits a high level of sequence polymorphism between Arabidopsis accessions and different allelic variants appear to be required to confer resistance to different pathogens (Birker et al., 2009; Narusaka et al., 2009). Based on resistance to *R. solanacearum*, the Col-0 *RRS1* allele is termed *RRS1-S* (for susceptible) and the Nd-1 allele

RRS1-R (for resistant). In comparison to RRS1-R, RRS1-S contains several amino acid substitutions in the TIR-NB-LRR region and a 90 amino acid deletion at its C-terminus after the WRKY domain (Deslandes et al., 2002). RRS1-R recognizes the *R. solanacearum* GMI1000 type III effector PopP2, which belongs to the YopJ/AvrRxv protein family that is conserved in both mammalian and plant pathogens (Staskawicz et al., 2001; Deslandes et al., 2003). PopP2 contains a predicted NLS and localizes to the plant nucleus where it physically interacts with RRS1-R and RRS1-S (Tasset et al., 2010). Thus, the susceptibility of Col-0 to *R. solanacearum* GMI1000 cannot be explained by the lack of interaction between PopP2 and RRS1-S. PopP2 is able to stabilize RRS1-R and RRS1-S protein accumulation *in planta* probably through inhibition of their proteasome-dependent degradation (Tasset et al., 2010). As for other members of the YopJ/AvrRxv protein family, PopP2 displays acetyl-transferase activity and is able to auto-acetylate. RRS1-R is able to perceive this enzymatic activity leading to the activation of a resistance response whereas RRS1-S might be unable to perceive PopP2 acetyl-transferase activity (Tasset et al., 2010).

In addition to AvrRps4-triggered resistance, RPS4 was shown to be required for resistance against *R. solanacearum* and the hemibiotrophic fungus *Colletotrichum higginsianum* (Birker et al., 2009; Narusaka et al., 2009). Similarly, RRS1 mediates resistance not only to *R. solanacearum* PopP2 but is involved as well in the immune response against *Pst* AvrRps4 and *C. higginsianum* (Birker et al., 2009; Narusaka et al., 2009). Thus, both TIR-NB-LRR receptors were found to confer resistance to three different pathogens. As already mentioned, the *RRS1* gene displays high sequence polymorphisms and different allelic variants are required to confer resistance to different pathogens. Accession Col-0 harbours the *RRS1-S* allele and is susceptible to *R. solanacearum* and *C. higginsianum* but resistant to *Pst* AvrRps4 (Birker et al., 2009; Narusaka et al., 2009). The molecular basis for the allelic dependency on recognition capability is not known. In the Arabidopsis genome, *RPS4* and *RRS1* display a head-to-head (inverted) tandem arrangement separated by a small intergenic region (254bp) (Gassmann et al., 1999). This region contains potential cis-regulatory motifs arranged in opposite orientations: the E2F consensus sequence, which can be bound by E2F transcription factors, and Element II, both of which are characteristic of plant bidirectional promoters (Birker et al., 2009; Dhadi et al., 2009). It was found that homologs of *RPS4* are physically paired with homologs of *RRS1* in Arabidopsis, and that these gene pairs also display a head-to-head arrangement with variable spacings (Gassmann et al., 1999). The evolutionary conservation of homologous gene pairs in a head-to-head arrangement might allow

cooperative action of these genes since this might lead to a transcriptional co-regulation and thus balanced levels of the protein pair (Narusaka et al., 2009). It was shown that mutation of both *RPS4* and *RRS1* genes in Ws-0 does not lead to increased susceptibility to any of the three pathogens compared with each single mutant indicating that these two NB-LRR receptors might function closely together (Narusaka et al., 2009). Resistance mediated by pairs of *NB-LRR* genes is an emerging theme in the field (Eitas and Dangl, 2010). The two NB-LRR receptors RPP2A and RPP2B are both required to confer isolate-specific resistance to *Hyaloperonospora arabidopsidis* and are thought to function cooperatively (Sinapidou et al., 2004). This is in contrast to the case of *TAO1*, a *TIR-NB-LRR* gene that, together with RPM1, additively contributes to resistance against *Pst* expressing AvrB (Eitas et al., 2008).
It was shown in several reports that a single *R* gene can confer resistance to multiple pathogens. For instance, the tomato *Mi* resistance gene mediates resistance to three different types of pest (Nombela et al., 2003). However, *RPS4* and *RRS1* so far represent the only pair of *R* genes that induces dual resistance against three distinct pathogens. Analysis of RPS4 and RRS1 functions and interactions might provide deeper insights into the molecular mechanisms required for pathogen perception and reveal common components involved in resistance to diverse pathogens.

1.5 The *Pseudomonas syrinage* type III effector AvrRps4

AvrRps4 was cloned from the plant pathogenic bacterial strain *Pseudmonas syringae* pv. *pisi* based on its ability to induce HR on Arabidopsis accession Po-1 (Hinsch and Staskawicz, 1996). From DNA hybridization experiments, it was shown that *AvrRps4* is present in all *P. syringae* pv. *pisi* strains tested as well as in *P. syringae* pv. *glycinea* and pv. *phaseolicola* (Hinsch and Staskawicz, 1996). AvrRps4 is secreted into the plant cell by the bacterial T3SS, where it is then processed into a smaller form (Sohn et al., 2009). The C-terminal 88 amino acids are sufficient to induce resistance. A virulence activity of AvrRps4 could be demonstrated since expression of AvrRps4 in Arabidopsis plants lacking *RPS4* promotes susceptibility of *Pst* bacteria and can suppress PTI (Sohn et al., 2009). *In planta* processing is required for this virulence but not for the avirulence activity of AvrRps4 (Sohn et al., 2009). The KRVY motif at the N-terminus of cleaved AvrRps4 is required for both virulence and avirulence function (Sohn et al., 2009).
As mentioned previously, AvrRps4 is recognized by the two TIR-NB-LRR receptors RPS4 and RRS1. Recently, it was shown that the *TIR-NB-LRR* gene *SNC1* also contributes to

AvrRps4-triggered immunity in the absence of *RPS4* at a certain temperature (Kim et al., 2010). In contrast to RPS4/RRS1-mediated resistance, *RPS4* and *SNC1* contribute additively to AvrRps4-triggered resistance (Kim et al., 2010).
Thus, AvrRps4 recognition seems to involve a complex mechanism involving several receptor proteins. Molecular and genetic dissection of this recognition mechanism could provide further insights into immune receptor signalling networks.

1.6 Thesis aims

Deciphering the molecular mechanisms by which bacterial effectors are recognized by resistant hosts is important for our understanding of host-pathogen communication and co-evolution. Although R proteins were first reported as plant immune receptors more than 15 years ago, how these proteins activate downstream defense responses is largely unknown. Identification of potential downstream signalling partners in plants will enhance our knowledge of plant immunity. In Arabidopsis, the *P. syringae* effector AvrRps4 is recognized by two TIR-NB-LRR-type receptors RPS4 and RRS1. *RPS4* and *RRS1* represent the only *R* gene pair so far characterized that induces resistance to three distinct pathogens. Analysis of RPS4 and RRS1 function might thus provide deeper insights into the molecular mechanisms required for pathogen perception and reveal common components and mechanisms involved in resistance to diverse pathogens. Nuclear localization of RPS4 is required for its resistance function but it remained unclear how and where in the host cell AvrRps4 is perceived by its cognate R proteins. The importance of subcellular localization of effector proteins for recognition through their cognate R proteins has been shown only in few cases.
Therefore, the aims of my thesis were to: (1) analyze in which subcellular compartment AvrRps4 induces a resistance response, (2) determine the modes of action of RPS4 and RRS1 in response to AvrRps4 and (3) identify a potential virulence target of AvrRps4 in the host and dissect the molecular mechanism of resistance signalling through RPS4 and RRS1.
To explore in which subcellular compartment AvrRps4 recognition takes place, transgenic Arabidopsis lines were generated expressing AvrRps4 fused to a functional or mutated nuclear localization (NLS) or nuclear export signal (NES) and tested for defense activation. Also, *P. syringae* strains expressing AvrRps4-NLS or -NES variants were tested for induction of resistance leading to restriction of bacterial growth. To determine the modes of action of RPS4 and RRS1 in response to AvrRps4, a knockdown line of *RRS1* in Col-0 *rps4* mutant background was generated and analyzed in terms of resistance activation to bacteria

expressing AvrRps4. Furthermore, transgenic lines overexpressing functional epitope-tagged RPS4 protein in the *rrs1* mutant background were generated to assess to which extent RPS4 function might be dependent on *RRS1*. Finally, an interaction between RPS4 and RRS1 *in planta* was determined. In order to identify potential virulence targets of AvrRps4 and to dissect resistance signalling in response to AvrRps4, interaction partners of AvrRps4 *in planta* were analyzed.

2 RESULTS

2.1 Nuclear localization of AvrRps4 is required to induce full disease resistance

2.1.1 Enforced localization of AvrRps4 to a specific cellular compartment

The *P. syringae* pv. *pisi* effector AvrRps4 is translocated to the plant cell via the type III secretion system (Sohn et al., 2009). It was shown that AvrRps4 is cleaved *in planta* by an unknown protease but not in bacteria or yeast and that only the C-terminus induces a defense response in resistant Arabidopsis genotypes or backgrounds (Sohn et al., 2009). AvrRps4 is recognized by the two TIR-NB-LRR-type receptors RPS4 and RRS1 (Birker et al., 2009; Narusaka et al., 2009). RPS4 localizes to nuclei and is associated with endomembranes and depletion of the nuclear RPS4 pool correlates with loss of RPS4 function (Wirthmueller et al., 2007). RRS1 was shown to localize exclusively to the plant nucleus when coexpressed with its elicitor PopP2 (Deslandes et al., 2003).

In order to determine in which subcellular compartment AvrRps4 is recognized, I created a set of constructs of the full length AvrRps4 protein C-terminally fused to mYFP and a signal that alters its localization in the plant cell. Since AvrRps4 is cleaved *in planta*, only the C-terminus would be directed to a specific subcellular compartment by the localization signal. To target AvrRps4 C-terminus to the nucleus, it was fused to the SV40 Large T-antigen monopartite nuclear localization signal (Lanford and Butel, 1984; Haasen et al., 1999). The PKI nuclear export signal (Wen et al., 1995) was used to promote AvrRps4 C-terminus export from the nucleus to the cytoplasm. As controls, fusions were made with mutated, non-functional versions of these targeting signals (nes and nls). The constructs were first transiently expressed by *Agrobacterium*-mediated infiltration in *N. benthamiana* leaves. Optimal fluorescence levels for microscopic imaging were reached 2 to 3 days after infiltration. AvrRps4-mYFP showed a nuclear and cytoplasmic distribution in *N. benthamiana* epidermal cells although a putative nuclear localization sequence was not predicted using the PSORT program (Nakai and Horton, 1999). The AvrRps4 C-terminus fused to mYFP (37kDa) is below the size exclusion limit of ~50kDa of the nuclear pore

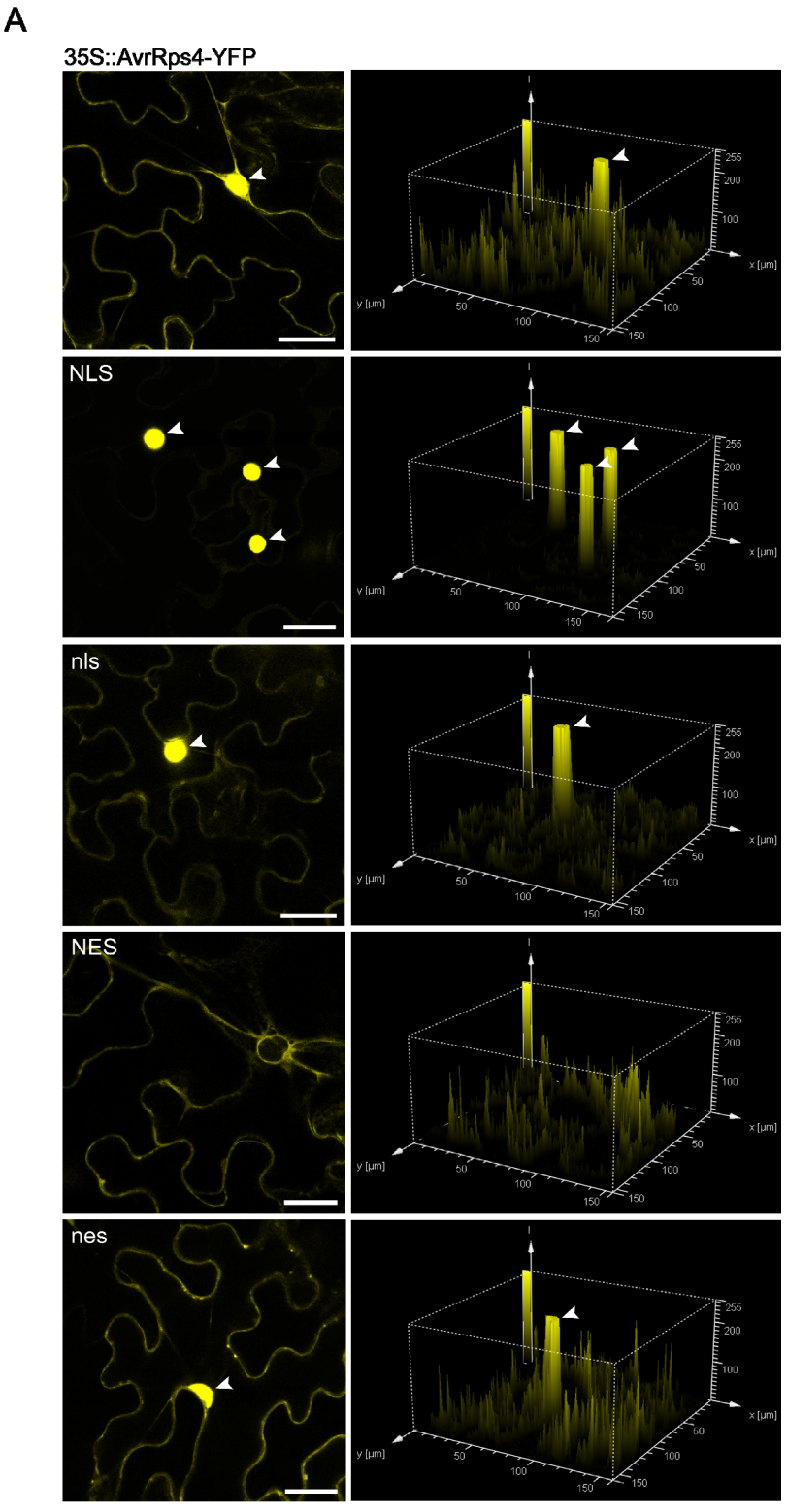

A
35S::AvrRps4-YFP
NLS
nls
NES
nes
B
35S::AvrRps4-YFP
-
-NLS
-nls
-NES
-nes
α-GFP
Ponceau S

Fig. 2: Enforced localization of AvrRps4 to a specific cellular compartment.
(A) Subcellular localization of YFP-tagged AvrRps4 constructs fused to functional (big letters) or mutated (small letters) NLS or NES. Confocal images of representative cells were taken at 72 hours after *Agrobacterium* infiltration in *N. benthamiana* epidermal cells. Right panel shows 3D plot of the image in the left panel. The transient expression assay was repeated three times with similar results. Arrowheads indicate nuclei. Scale bars= 25µm. **(B)** Immunoblot analysis showing expression of AvrRps4 fusion proteins. AvrRps4-mYFP-NES protein is not detectable. Samples were taken 72hpi from leaf tissue. Membrane was probed with α-GFP antibody. Ponceau staining was performed to ensure equal loading.

complex and might thus passively diffuse into the nucleus (Xu and Meier, 2008). This is in contrast to the results of Wirthmueller et al (2007), who showed only cytoplasmic localization of AvrRps4-HA in a biochemical nuclear fractionation experiment (Wirthmueller et al., 2007). This discrepancy might be due to the method since AvrRps4-HA might have leaked out of the nuclei during the fractionation procedure and was thus not detectable in the nuclear fraction.

Confocal microscopy imaging of the constructs expressed from the constitutive CaMV 35S-overexpression promoter in *N. benthamiana* confirmed that the NES and NLS sequences were able to redirect the localization of the C-terminus of AvrRps4 although the fusion proteins are small enough to passively diffuse between cytoplasm and nucleus (Figure 2A, left panel). When fused to the mutated nls sequence, AvrRps4-mYFP-nls gave stronger nuclear accumulation than AvrRps4-mYFP suggesting that the mutation of one amino acid is not sufficient to completely disrupt the NLS sequence function. Fluorescence signals in cytoplasm and nucleus were quantified by 3D plot analysis. This further supported the functionality of localization sequences in *N. benthamiana* (Figure 2A, right panel).

Protein accumulation of mYFP-fusions 3 days after infiltration was analyzed by immunoblot analysis (Figure 2B). The AvrRps4-mYFP-NES fusion protein was not detectable on a Western Blot probed with α-GFP antibody although fluorescence was visible (Figure 2A). This might be due to a conformational change of YFP that prevents exposure of the epitope. Furthermore, cleavage of AvrRps4-mYFP fusion proteins into an N- and a C-terminal part was confirmed, although not all protein was cleaved, probably due to the overexpression of AvrRps4 fusion proteins.

Transient expression of AvrRps4 fusion proteins in *N. benthamiana* did not lead to cell death induction since AvrRps4 is not recognized in *N. benthamiana*. Also, coexpression of AvrRps4-mYFP and RPS4-YFP did not affect the weak cell death induced by RPS4-YFP alone (data not shown).

I was interested to determine the effects of directed mis-localisation of AvrRps4 in its host plant Arabidopsis in which AvrRps4 is recognized and triggers a defense response. For that, I generated several independent transgenic lines that stably express AvrRps4 C-terminally

fused to a functional or mutated NLS or NES sequence by transforming the overexpression (p35S) constructs that were already characterized in *N. benthamiana* to a Col-0 *eds1-2* background. The *eds1-2* mutant is defective in downstream signalling after activation of TIR-NB-LRR-type intracellular R proteins and thus enabled me to first analyse protein expression and localization of AvrRps4 C-terminus without induction of a defense response. Transgenic lines were then selected that express AvrRps4 fusion proteins to similar levels but do not massively overexpress it.

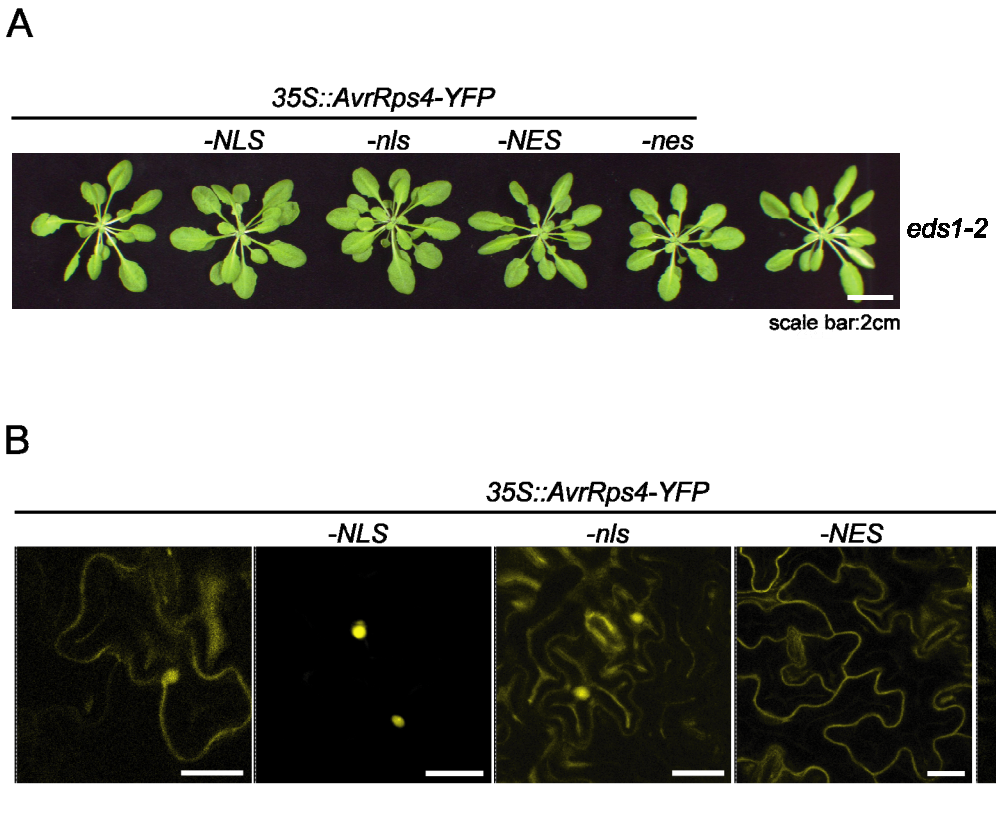


Fig. 3: Stable overexpression of AvrRps4 fusion proteins in Arabidopsis *eds1-2* plants.
(A) Transgenic T2 plants overexpressing YFP-tagged AvrRps4 fused to functional or mutated NLS or NES in Col-0 *eds1-2* do not show any morphological defects. Picture was taken of 4 week old plants. **(B)** Confocal microscopy of transgenic Arabidopsis lines show that localization tags are also functional in Arabidopsis. Images of representative cells were taken of 3 week old plants.

Stable overexpression of AvrRps4 fusions in Col-0 *eds1-2* background did not lead to a morphological defect (Figure 3A). Confocal microscopy of AvrRps4-mYFP in these lines showed that the NLS and NES localization sequences are also functional in Arabidopsis (Figure 3B).

2.1.2 AvrRps4 needs to localize to the plant nucleus to induce a full resistance response

To establish in which subcellular compartment AvrRps4 induces a resistance response, I crossed the segregating T2 generation of the stable transgenic lines overexpressing AvrRps4 fusion proteins in *eds1-2* background with Col-0 to introduce an *EDS1* allele. The F1 progeny of multiple transgenics were analyzed in terms of their growth inhibition as a marker of resistance activation when grown at 22°C under short day conditions (see Methods section).

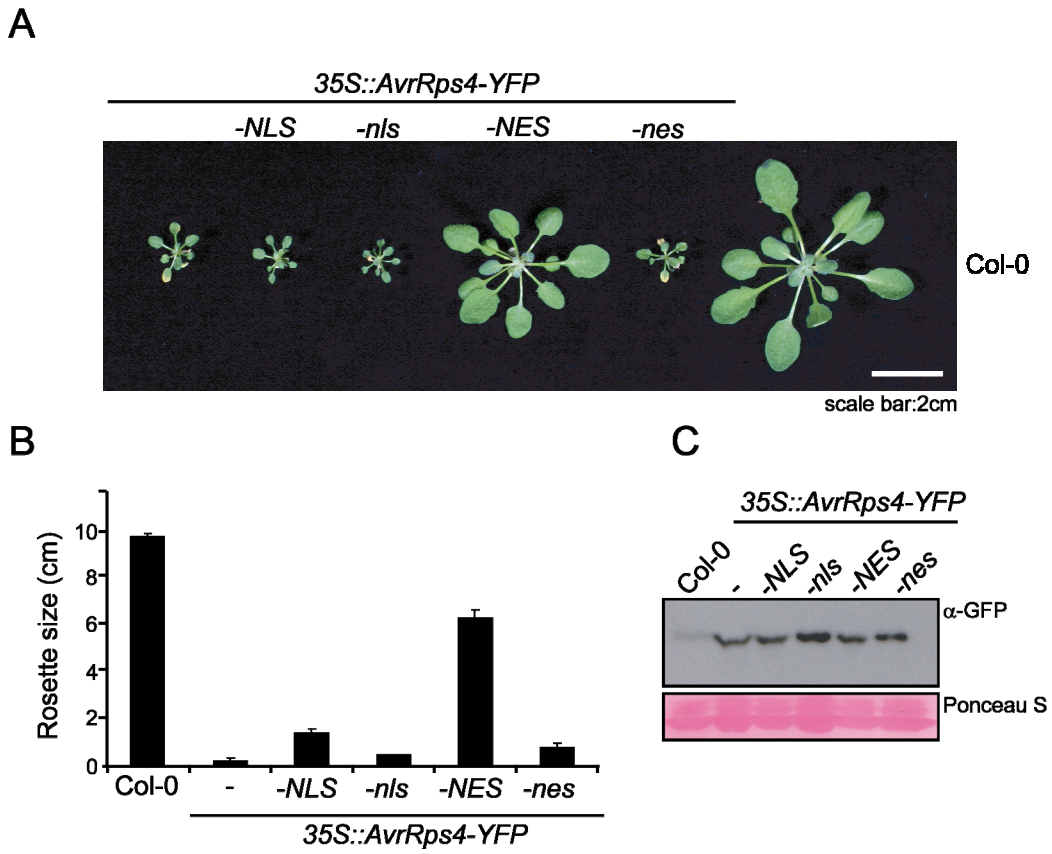


Fig. 4: AvrRps4 needs to localize to the plant nucleus to induce a full resistance response.
(A) Stable overexpression of AvrRps4-mYFP and AvrRps4-mYFP-NLS/nls/nes in an *EDS1* containing background induces strong dwarfism. Overexpression of AvrRps4-YFP-NES does not result in dwarfism, although plants are smaller than Col-0 wildtype plants. Pictures were taken of 4 week old F1 plants. **(B)** Quantitative measurement of plant size of 5 week old F1 plants. Diameter of plant rosettes was measured. **(C)** Equal accumulation of AvrRps4-mYFP fusion proteins in F1 plants was verified by immunoblot analysis. Membrane was probed with α-GFP antibody and a Ponceau staining was performed to ensure equal sample loading.

The stable overexpression of AvrRps4-mYFP in an *EDS1* containing background caused severely dwarfed plants that died after approximately six weeks when grow under short day conditions at 22°C. Also, overexpression of nuclear accumulated AvrRps4 C-terminus in

AvrRps4-mYFP-NLS lines caused strong dwarfism and eventually death (Figure 4A, B). Interestingly, overexpression of only cytoplasmic localized AvrRps4-mYFP-NES did not cause severe dwarfism although these plants were smaller than Col-0 wt plants (Figure 4A, B). This phenotype is not due to different protein expression levels of AvrRps4 fusion proteins as shown by immunoblot analysis (Figure 4C). Although the AvrRps4-mYFP-NES fusion protein was not detectable in immunoblot analysis when transiently expressed in *N. benthamiana* plants (Figure 2B), it was detectable in the stable Arabidopsis transgenics. These results indicate that the AvrRps4 C-terminus needs to localize to the plant nucleus to induce a strong resistance response as measured by inhibition of plant growth. The reduced plant size of *35S::AvrRps4-mYFP-NES* Col-0 plants compared to Col-0 wt plants might be due to the fact that the AvrRps4 fusion protein needs to reside for a short time in the plant nucleus before it is exported to the cytoplasm. This may be sufficient to induce a partial defense response that results in growth inhibition. Alternatively, it cannot be excluded that extra-nuclear AvrRps4 also contributes to full resistance.

2.1.3 Delivery of AvrRps4 to the plant cell via the bacterial type III secretion system

To avoid prolonged overexpression of a bacterial protein in the host plant Arabidopsis, I followed a complementary approach in which the AvrRps4 variants were secreted via the bacterial type III secretion system. This approach allowed AvrRps4 analysis under more natural conditions since the bacteria expressing AvrRps4 variants had to gain excess to the plant by entry through the stomata. Furthermore, AvrRps4 was not constitutively overexpressed in the host plant but could be expressed under control of its native promoter. For this, I generated *P. syringae* pv. *tomato* (*Pst*) DC3000 bacteria expressing AvrRps4 variants by using the effector detector vector system (EDV) that contains the native promoter of *AvrRps4* (vector kindly provided by J. Jones group; Sainsbury Laboratory, Norwich, UK). I cloned full length *AvrRps4* into it, C-terminally fused to an HA-tag and a functional or mutated NLS or NES sequence. I used the NES sequence of the HIV Rev protein (Wen et al., 1995) for these constructs since fusions of AvrRps4 to the PKI NES sequence were not detectable by immunoblot analysis (Figure 2B). Introduction of AvrRps4-HA fused to a functional or mutated NLS or NES sequence to *Pst* DC3000 did not alter bacterial growth rates in bacterial growth medium (data not shown). To rule out the possibility that the attachment of a localization tag might interfere with AvrRps4 secretion via the T3SS, I measured AvrRps4 delivery in a secretion assay in type III secretion induction media

(Mudgett and Staskawicz, 1999). I was able to detect similar levels of uncleaved AvrRps4 fusion proteins in the cellular lysate and secreted fraction by immunoblot analysis (Figure 5B).

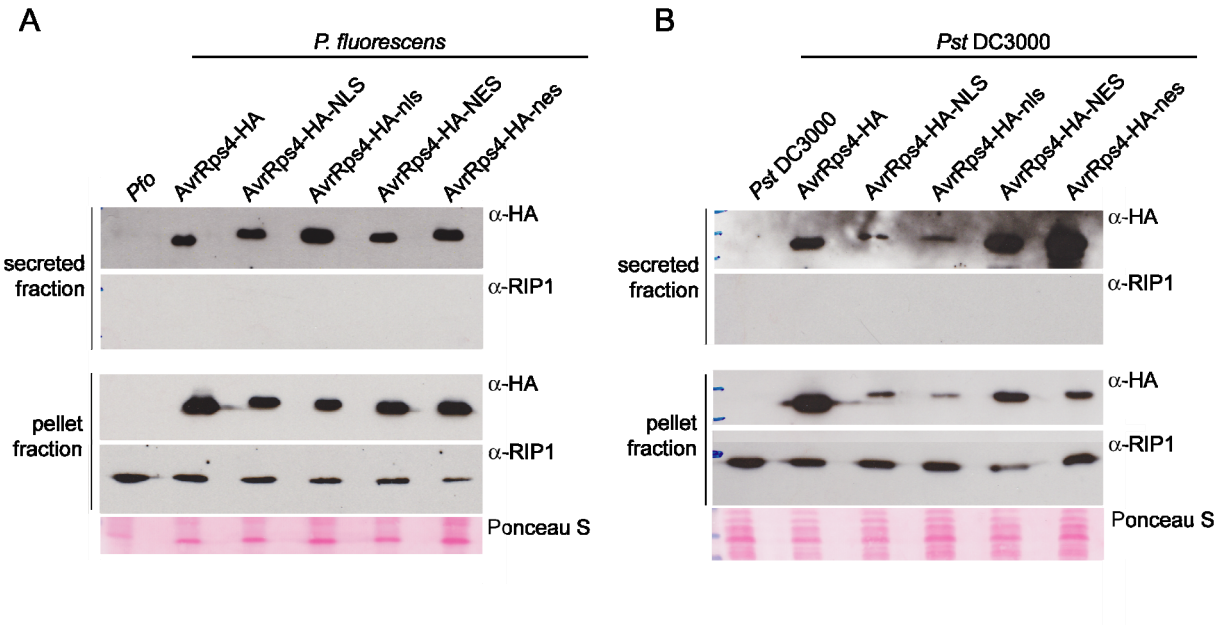


Fig. 5: *Pst* DC3000 and *Pfo* bacteria express and secrete HA-tagged AvrRps4 variants.
(A) Secretion assay of *P. fluorescens* strains expressing AvrRps4-HA fused to a functional or mutated NLS or NES confirms that AvrRps4 fusion proteins are expressed and secreted to similar levels. Membranes were probed with α-HA and α-RIP1 antibody and a Ponceau staining was performed to ensure equal sample loading. **(B)** Secretion assay of *Pst* DC3000 strains expressing AvrRps4-HA fused to a functional or mutated NLS or NES confirms that AvrRps4 fusion proteins are expressed and secreted. AvrRps4-HA-NLS/nls proteins are expressed and secreted to lower amounts compared to AvrRps4-HA. Membranes were probed with α-HA and α-RIP1 antibody and a Ponceau staining was performed to ensure equal sample loading.

2.1.4 Bacteria expressing AvrRps4-NES induce a weaker resistance response

In order to determine if enforced localisation of the AvrRps4 C-terminus to a specific subcellular compartment might alter its recognition, resistant Col-0 plants that contain functional *RPS4* and *RRS1* alleles (Gassmann et al., 1999; Birker et al., 2009; Narusaka et al., 2009), were spray infected with *Pst* DC3000 expressing AvrRps4 variants ($7x10^7$ cfu/ml). The Col-0 *eds1-2* mutant was included as measure of full bacterial growth potential. As a control for successful infection, *Pst* DC3000 wt bacteria were also inoculated. A similar entry rate of bacteria to the plant was ensured by measuring bacterial growth 4h after spray infection (hpi) (data not shown).

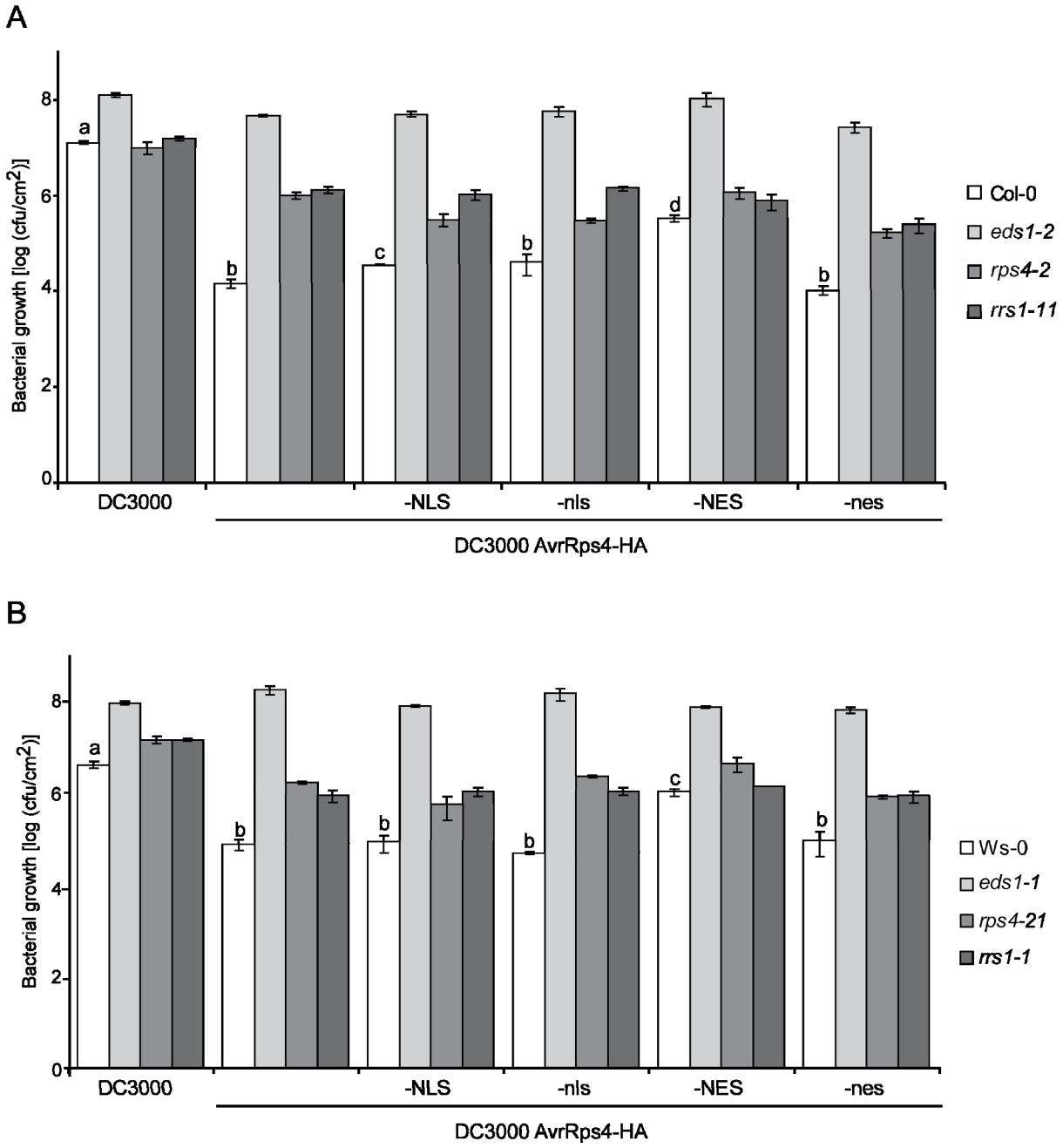


Fig. 6: Bacteria expressing AvrRps4-NES induce a weaker resistance response.
(A) Five week old plants of the indicated genotypes were spray-infected with *Pst* DC3000 expressing AvrRps4 variants. Bacterial titers 3 days post infection (dpi) are depicted. Bacterial entry was determined at 4hpi and was similar for all genotypes and bacterial strains but is not shown here due to space limitations. Error bars represent standard error of 3 biological replicates. Characters a-d indicate significant differences in growth (Student`s t-test: p= 0.05). The experiment was repeated twice with similar results. **(B)** Five week old plants of the indicated genotypes were spray-infected with *Pst* DC3000 expressing AvrRps4 variants. Bacterial titers 3 days post infection are depicted. Bacterial entry was determined at 4hpi and was similar for all genotypes and bacterial strains but is not shown here due to space limitations. Error bars represent standard error of 3 biological replicates. Characters a-d indicate significant differences in growth (Student`s t-test: p= 0.05). The experiment was repeated twice with similar results.

Pst DC3000 bacteria expressing AvrRps4-HA-NLS induced a similar degree of resistance in Col-0 as AvrRps4-HA at 3 days after spray-infection (dpi) (Figure 6A). By contrast, bacteria

expressing AvrRps4-HA-NES grew to >10-fold higher levels. Bacterial proliferation in Col-0 *eds1-2* mutant was similar for all bacterial strains. This result suggests that bacteria expressing AvrRps4-HA-NES induce a weaker resistance response.

I also tested growth of *Pst* DC3000 expressing AvrRps4 variants in Ws-0 and Ws-0 *eds1-1* mutant backgrounds since a different allelic recognition variant of *RRS1* is present in Ws-0 accession (Birker et al., 2009; Narusaka et al., 2009). Furthermore, Ws-0 might harbour different proteins involved in AvrRps4 recognition that are not present in Col-0 since components of the plant immune system, such as *R* genes, show high levels of polymorphism between natural populations (Van der Hoorn et al., 2002; Todesco et al., 2010).

I found that Ws-0 plants are more susceptible to *Pst* DC3000 in general since I inoculated these plants with half the dose of bacteria compared to Col-0 but obtained similar growth rates after 3dpi. Infection with the same concentration of bacteria as used for Col-0 resulted in heavy disease that made it difficult to observe subtle differences in susceptibility (data not shown). As in Col-0, *Pst* DC3000 bacteria expressing AvrRps4-HA-NES could proliferate to significantly higher levels than AvrRps4-HA in Ws-0 background (Figure 6B). Thus, the reduced resistance response to bacteria expressing AvrRps4-HA-NES is not accession-dependent.

Taken together with the results in the stable AvrRps4 overexpression lines, this strongly indicates that AvrRps4 C-terminus needs to accumulate in the plant nucleus to induce a full resistance response.

2.1.5 RPS4 and RRS1 are both required to confer resistance to mis-localized AvrRps4 variants

To further investigate the mechanism of AvrRps4 recognition in Arabidopsis, I tested if the localization of AvrRps4 to a specific subcellular compartment might reduce the need for RPS4 or RRS1 in resistance. One possibility could be that enforced nuclear localization of AvrRps4 might abolish the requirement of RPS4 for resistance induction. A possible scenario is that RPS4 might be activated in the cytoplasm through recognition of the cytoplasmic AvrRps4 pool and then translocates into the nucleus to activate downstream components

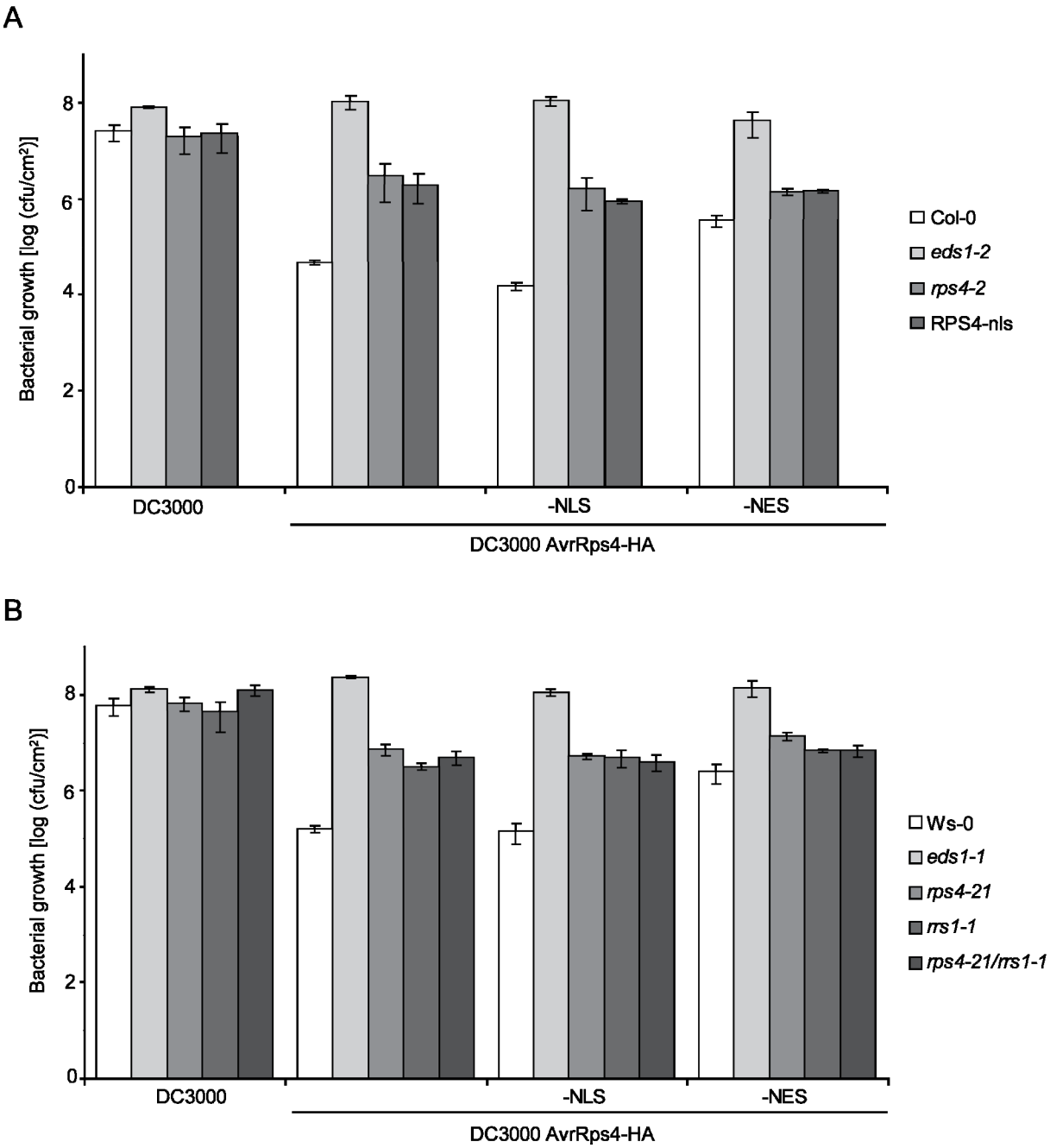


Fig. 7: RPS4 and RRS1 are both required to confer resistance to mis-localized AvrRps4 variants.
(A) Five week old plants of the indicated genotypes were spray-infected with *Pst* DC3000 expressing AvrRps4 variants. Bacterial titers 3 days post infection are depicted. Bacterial entry was determined at 4hpi and was similar for all genotypes and bacterial strains but is not shown here due to space limitations. Error bars represent standard error of 3 biological replicates. The experiment was repeated twice with similar results. **(B)** Five week old plants of the indicated genotypes were spray-infected with *Pst* DC3000 expressing AvrRps4 variants. Bacterial titers 3 days post infection are depicted. Bacterial entry was determined at 4hpi and was similar for all genotypes and bacterial strains but is not shown here due to space limitations. Error bars represent standard error of 3 biological replicates. The experiment was repeated twice with similar results.

involved in the defense response. Enforced nuclear accumulation of AvrRps4 might already activate the nuclear downstream defense components and circumvent the need for RPS4 to translocate into the nucleus for activation of a defense response. In this case, the *rps4* mutant

would not be more susceptible to bacteria expressing AvrRps4-HA-NLS. To analyze this hypothesis, I infected *rps4* and *rrs1* mutants in the Col-0 and Ws-0 backgrounds with *Pst* DC3000 expressing the different AvrRps4 variants. Both *R* gene mutants showed an impaired resistance response to *Pst* DC3000 expressing AvrRps4-HA in Col-0 and Ws-0 (Figure 5A, B). No significant changes in bacterial proliferation compared to *Pst* DC3000 AvrRps4-HA were observed in *rps4* and *rrs1* mutants when infected with *Pst* DC3000 expressing either AvrRps4-HA-NLS or AvrRps4-HA-NES in both accessions (Figure 6 A, B). Thus, enforced nuclear localization of AvrRps4 still requires RPS4 for defense induction. Furthermore, it can be concluded that enforced localization of AvrRps4 to a specific compartment does not reduce the need for both RPS4 and RRS1 in resistance.

It was shown that RPS4 needs to accumulate in the nucleus to trigger immunity through activation by AvrRps4 and depletion of the nuclear RPS4 pool by mutation of its intrinsic NLS sequence correlates with loss of RPS4 resistance (Wirthmueller et al., 2007). Nuclear localized AvrRps4 might compensate or abolish the need for RPS4 nuclear accumulation for resistance. Another possibility might be that the extra-nuclear RPS4 pool might recognize cytoplasmic enriched AvrRps4-NES and induce a defense response. To investigate this hypothesis, I tested bacterial growth of *Pst* DC3000 AvrRps4-HA-NLS and AvrRps4-HA-NES in an RPS4-nls mutant. In the RPS4-nls mutant, four lysine residues that constitute the core basic regions of the bipartite NLS were substituted with alanines resulting in an exclusion of RPS4 from nuclei (Wirthmueller et al., 2007). RPS4-nls mutant plants showed a similar degree of enhanced susceptibility to bacteria expressing AvrRps4 as the *rps4-2* mutant (Figure 7A) which correlated with published results (Wirthmueller et al., 2007). No significant differences in bacterial proliferation compared to *Pst* DC3000 AvrRps4 were observed in this mutant when infected with *Pst* DC3000 AvrRps4-HA-NLS or AvrRps4-HA-NES (Figure 7A). Thus, the hypothesis that cytoplasmic RPS4 can recognize cytoplasmic localized AvrRps4 can be rejected since RPS4-nls plants do not show enhanced resistance to *Pst* AvrRps4-HA-NES. Nuclear localization of RPS4 is required to trigger immunity to nuclear and cytoplasmic localized AvrRps4.

An *rps4-21/rrs1-1* double null mutant was generated by Narusaka et al (2009) by crossing of the two single mutants in the Ws-0 background (Narusaka et al., 2009). No significant difference could be observed in pathogen growth of *Ralstonia solanacearum, Colletotrichum higginsianum* and *Pst* AvrRps4 in the double mutant compared to the single mutants suggesting that RPS4 and RRS1 function cooperatively in resistance to these pathogens (Narusaka et al., 2009). I tested whether RPS4 and RRS1 function additively or cooperatively

in response to mis-localized AvrRps4. The modes of action of RPS4 and RRS1 might be altered when AvrRps4 is enforced to a specific subcellular compartment. For this, I spray-infected the *rps4-21/rrs1-1* double mutant with *Pst* DC3000 AvrRps4-HA, NLS or NES and compared susceptibility with the *rps4-21* and *rrs1-1* single mutants. Figure 7B shows that growth of *Pst* DC3000 AvrRps4-HA was similar in the double and the single mutants, confirming published results (Narusaka et al., 2009). There were no significant differences in bacterial growth of *Pst* DC3000 expressing AvrRps4-NLS or NES in the *rps4-21/rrs1-1* double mutant compared to the single mutants (Figure 7B). This result further indicates that enforced localization of AvrRps4 to a specific subcellular compartment does not compromise the need for RPS4 and RRS1 and that the modes of action of RPS4 and RRS1 are not altered upon AvrRps4 mis-localization.

2.1.6 RPS4 and RRS1 are required for AvrRps4-triggered cell death induction

The above data suggest that enforced nuclear export of the AvrRps4 C-terminus induces a weaker RPS4/RRS1-dependent resistance response. It has been widely viewed in the field that induction of effector triggered immunity through activation of R proteins correlates with cell death induction. The localized cell death is thought to stop bacterial proliferation (Chisholm et al., 2006). I therefore tested whether reduced resistance to AvrRps4-NES correlates with reduced cell death induction.

For this, I introduced the vectors encoding *AvrRps4-HA* fused to a functional or mutated NLS or NES sequence into the saprophytic bacterial strain *Pseudomonas fluorescens* (*Pfo*), a nonpathogen of Arabidopsis that does not cause disease on Arabidopsis (Huang et al., 1988). Introduction of a cosmid that contains the type III secretion apparatus allows this strain to secrete effector proteins into plant cells (van Dijk et al., 2002; Guo et al., 2009). First, I validated in a secretion assay that all AvrRps4 variants are secreted from *Pfo* (Figure 5A).

Infiltration of *Pfo* expressing AvrRps4-HA in Col-0 and Ws-0 leaves induced strong cell death which was visible as necrosis in the infiltrated area at 24hpi (Figure 8B). The cell death induction is completely dependent on *EDS1* since the *eds1* mutant in Col-0 and Ws-0 background did not exhibit necrosis upon AvrRps4 delivery. I quantified cell death induction by monitoring ion leakage through conductivity measurements after *Pfo* infiltration. AvrRps4-mediated cell death induction measured by ion leakage started at 8hpi. Interestingly,

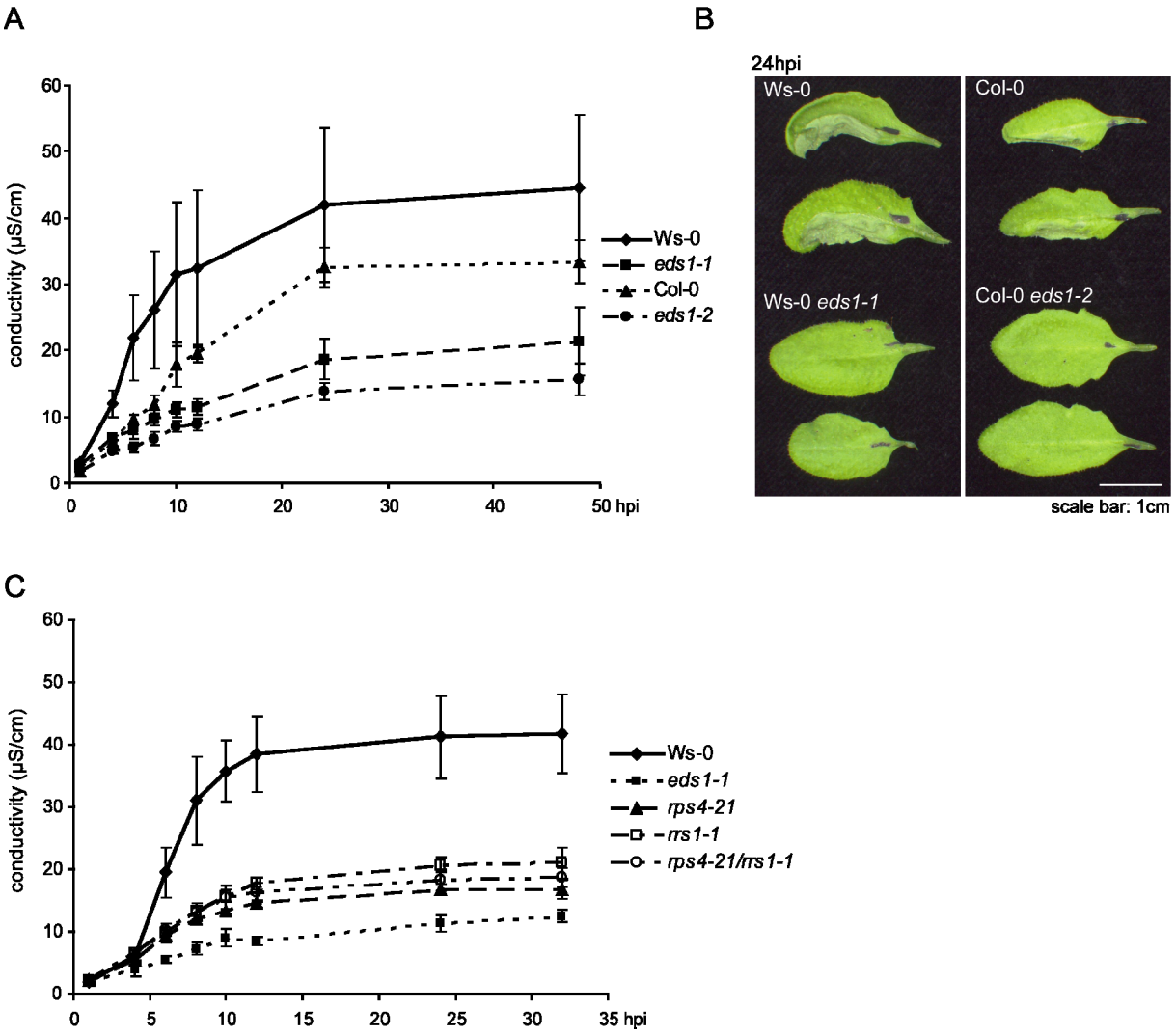


Fig. 8: RPS4 and RRS1 are required for AvrRps4-triggered cell death induction.
(A) Four week old plants of the indicated genotypes were infiltrated with *Pfo* expressing AvrRps4-HA and conductivity was measured at the indicated time points. Error bars represent standard deviation of 4 biological replicates. The experiment was repeated twice with similar results. **(B)** Lower half of leaves of 4 week old plants were infiltrated with *Pfo* expressing AvrRps4-HA. Pictures were taken 24hpi and show necrosis induction through AvrRps4 secretion that is *EDS1*-dependent. **(C)** Four week old plants of the indicated genotypes were infiltrated with *Pfo* expressing AvrRps4-HA and conductivity was measured at the indicated time points. Error bars represent standard deviation of 4 biological replicates. The experiment was repeated twice with similar results.

a stronger ion leakage response was observed in Ws-0 accession than in Col-0 upon *Pfo* AvrRps4-HA infiltration (Figure 8A). This was surprising given the fact that Col-0 is generally more resistant to *Pst* DC3000 AvrRps4 than Ws-0 (see Figure 7; Ws-0 was infected with half the dose of bacteria compared to Col-0). Thus, cell death induction and resistance do not necessarily correlate in all cases. The *eds1* mutants in Col-0 and Ws-0 background only produced background levels of ion leakage (Figure 8A). This further supports the specificity of measured conductivity to AvrRps4-triggered cell death. Natural populations of Arabidopsis show high levels of polymorphisms in components of the immune system reflecting the

adaptation to spatially restricted pathogens (van der Hoorn et al, 2002; Todesco et al, 2010). For instance harbour Col-0 and Ws-0 different allelic recognition variants of *RRS1* (Narusaka et al, 2009; Birker et al, 2009). These natural variations might cause the differences in cell death induction after AvrRps4 infiltration in Ws-0 and Col-0.

To elucidate the role of RPS4 and RRS1 in AvrRps4-triggered cell death induction, I measured ion leakage upon AvrRps4 delivery in the *rps4-21* and *rrs1-1* single and double mutants in the Ws-0 background. Both the single and double mutant exhibited strongly reduced cell death, although this was greater than in *eds1-1* leaves (Figure 8C). I was not able to detect an additive effect of the *rps4-21/rrs1-1* double mutant in this assay. Thus, the cell death measurements in these mutants correlate with the phenotypes observed in bacterial growth assays, since the *rps4-21* and *rrs1-1* mutants display enhanced bacterial growth of *Pst* DC3000 AvrRps4 and reduced cell death upon AvrRps4 delivery. Furthermore, the absence of an additive phenotype of the *rps4-21/rrs1-1* double mutant in terms of bacterial growth (Figure 7B) is reflected in the extent of AvrRps4-triggered cell death.

2.1.7 AvrRps4 targeted to the nucleus fails to elicit a cell death response

To establish whether the mis-localized AvrRps4 variants are still capable of inducing a cell death response, I measured ion leakage of Ws-0 plants after *Pfo* delivery. AvrRps4 induces strong cell death detectable from 6hpi (Figure 9). Cytoplasmic localized AvrRps4-HA-NES produces an attenuated cell death response which correlates with its reduced resistance induction. The mutated AvrRps4-nes variant that displays AvrRps4-like distribution in the plant when fused to YFP (Figure 2A) also induced ion leakage equivalent to AvrRps4-HA when infiltrated via *Pfo*. Surprisingly, I found that nuclear localized AvrRps4-HA-NLS fails to elicit a cell death response even though it leads to full resistance in Arabidopsis (Figure 9). *Pfo* delivery of AvrRps4-HA-nls caused intermediate levels of ion leakage, correlating with its distribution in the plant cell. Note, AvrRps4-mYFP-nls exhibited stronger nuclear accumulation than AvrRps4-mYFP (Figure 2A).

In conclusion, these data suggest that increased nuclear export of AvrRps4 C-terminus correlates with a reduced resistance response and attenuated cell death induction. These results further suggest an uncoupling of cell death induction and bacterial growth since AvrRps4-NLS induces resistance but fails to induce cell death.

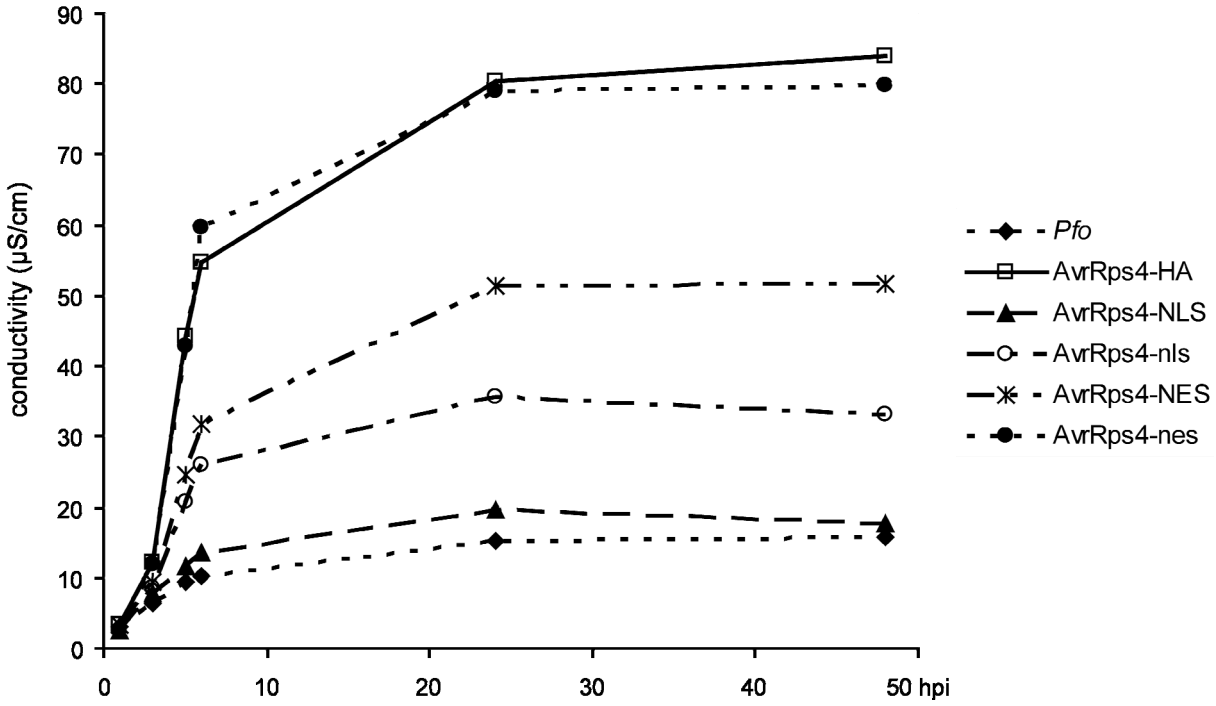


Fig. 9: Nuclear localized AvrRps4 fails to elicit a cell death response.
Four week old Ws-0 plants were infiltrated with *Pfo* expressing AvrRps4-HA fused to a functional or mutated NLS or NES and conductivity was measured at the indicated time points. The experiment was repeated twice with similar results.

2.1.8 Transcriptional reprogramming induced by AvrRps4-NLS

Based on the data obtained, nuclear localized AvrRps4 effectively induces a resistance response but fails to induce host cell death. In order to determine the cause of what seems to be a discrepancy, I analyzed defense gene expression after AvrRps4-HA-NLS delivery. It was previously shown that transcriptional reprogramming after infection with *Pst* DC3000 AvrRps4 bacteria is almost completely *EDS1*-dependent (Bartsch et al., 2006; Garcia et al., 2010). Central well characterized defense genes were induced in wildtype plants but not in *eds1* mutants. I selected some of these *EDS1*-dependent defense marker genes for my transcriptional analysis.

For this, I spray-infected Col-0 plants with *Pst* DC3000, *Pst* DC3000 AvrRps4-HA and AvrRps4-HA-NLS and took samples for RNA extraction from aerial tissue at 0, 8 and 24hpi. I chose these time points since most *EDS1*-dependent genes are induced or repressed at 8h after spray-infection with *Pst* DC3000 AvrRps4 (Garcia et al., 2010). By choosing a later time point (24hpi) I wanted to asses if transcriptional reprogramming might be delayed after infection with *Pst* DC3000 AvrRps4-NLS. Quantitative transcription profiles of selected defense marker genes were analyzed using *UBIQUITIN* as internal control.

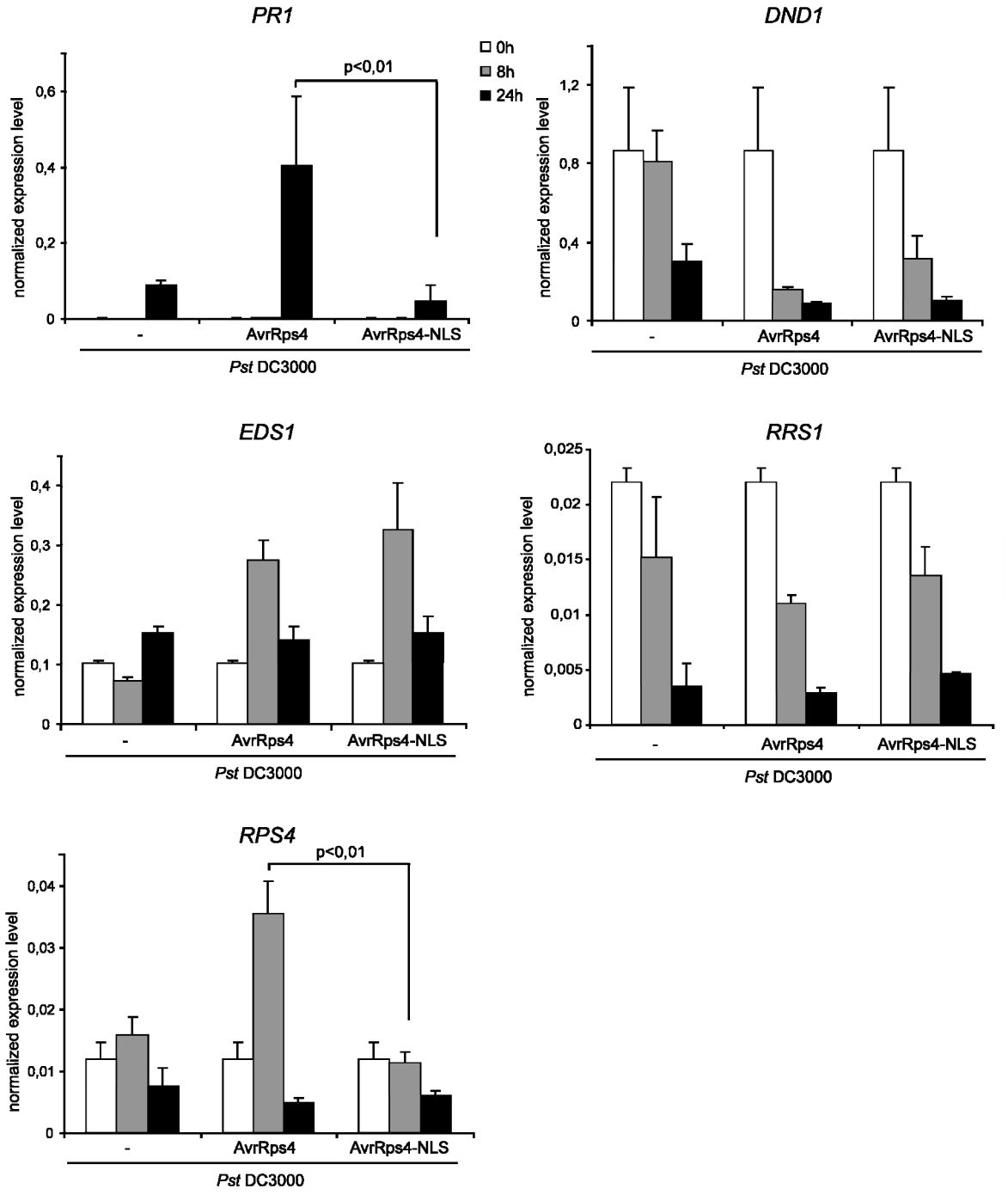


Fig. 10: Transcriptional reprogramming induced by AvrRps4-NLS.
Four week old Col-0 plants were spray-infected with *Pst* DC3000 wt or expressing AvrRps4-HA or AvrRps4-NLS. Leaf samples were collected at 0 (white bars), 8 (grey bars) and 24 (black bars) hpi. Transcript levels were determined and normalized using the internal control *UBIQUITIN*. Error bars represent standard deviation of 4 technical replicates. $p<0.01$ indicates significant differences calculated with Student`s t-test. The experiment was repeated twice with similar results.

Transcript levels of *PR1*, a commonly used defense marker gene (Laird et al., 2004), and *EDS1* itself, which is rapidly induced in response to avirulent bacteria (Bartsch et al., 2006; Garcia et al., 2010), were analyzed. Furthermore, the downregulated gene *DND1*, encoding a nucleotide-gated ion channel whose mutation results in resistance in the absence of HR, was

tested (Yu et al., 1998). Also, gene expression of *RPS4* and *RRS1* was analyzed. Figure 10 shows *PR1* induction 24h after infection with *Pst* DC3000 AvrRps4-HA but not with AvrRps4-NLS or *Pst* DC3000. Similarly, *RPS4* expression was induced at 8h after infection with *Pst* DC3000 AvrRps4-HA but not with AvrRps4-HA-NLS. By contrast, *EDS1* gene expression was induced by AvrRps4-HA and AvrRps4-HA-NLS and *DND1* and *RRS1* genes were both repressed by AvrRps4-HA and AvrRps4-HA-NLS. The induction of *PR1* and *RPS4* by AvrRps4-HA but not by AvrRps4-HA-NLS correlates with the cell death measurements whereas gene expression of *EDS1*, *DND1* and *RRS1* does not correlate. It is interesting that not all defense genes tested behave in the same manner. There might be specific sets of genes that are induced or repressed for a resistance response and others whose gene products are associated with cell death induction. More genes have to be tested in future experiments, e.g. genes that encode components involved in cell death in plants.

Taken together, these results show that enhanced nuclear export of AvrRps4 C-terminus causes attenuated resistance in the plants as measured by restriction of bacterial proliferation and growth of Arabidopsis transgenics overexpressing AvrRps4-NES. Also, AvrRps4-NES induces intermediate levels of cell death. This suggests that nuclear localization of AvrRps4 is required to induce a full immune response. Alternatively, a shuttling of AvrRps4 between the cytoplasm and the nucleus is needed for resistance. The intermediate effect of AvrRps4-NES might have several reasons. On the one hand, the AvrRps4-NES fusion protein needs to reside for a short time in the plant nucleus before it is exported to the cytoplasm. This may be sufficient to induce a partial defense response. On the other hand, it cannot be excluded that extra-nuclear AvrRps4 also contributes to full resistance. Furthermore, an intra-nuclear component might inhibit cell death induced by AvrRps4-NES.

2.2 Modes of action of RPS4 and RRS1

2.2.1 Regulation of *RPS4* and *RRS1* gene expression

The two *TIR-NB-LRR* genes *RPS4* and *RRS1* display a head-to-head (inverted) tandem arrangement in the Arabidopsis genome separated by a small intergenic region (254bp) (Gassmann et al., 1999). This region contains potential cis-regulatory motifs arranged in opposite orientations: the E2F consensus sequence, which can be bound by E2F transcription factors, and Element II, both of which are characteristic of plant bidirectional promoters (Figure 11A) (Dhadi et al., 2009). Genes regulated by bidirectional promoters are more likely to be co-ordinately expressed than random pairs of genes (Engstrom et al., 2006).

To investigate whether *RPS4* and *RRS1* are co-regulated, I analysed their expression profiles after spray infection with virulent *Pst* DC3000 or avirulent *Pst* DC3000 AvrRps4 bacteria. Samples for RNA extraction were taken at 0h, 8h and 24h after infection from aerial tissue. I chose these timepoints since Garcia et al (2010) showed that most defense-related genes are upregulated 8h after spray infection with avirulent Pst DC3000. Transcript levels were determined by qRT-PCR and normalized using the internal control *UBIQUITIN*. *RPS4* mRNA levels were induced more than 3-fold at 8h after infection with avirulent *Pst* DC3000 AvrRps4 but not with virulent bacteria (Figure 11B). At 24h after infection, *RPS4* mRNA levels were back to uninduced state. Surprisingly, *RRS1* mRNA levels were repressed ~2-fold at 8h and ~5-fold at 24h after infection with virulent or avirulent bacteria (Figure 11B). However, quantitative transcription profile data should be interpreted with caution since both genes are expressed to extremely low levels.

In order to determine whether a mutation in *RRS1* affects *RPS4* expression and *vice versa*, I analyzed *RPS4* gene expression in Col-0 *rrs1-11* as well as *RRS1* gene expression in the Col-0 *rps4-2* mutant background by qRT-PCR. *RPS4* gene expression levels were comparable in Col-0 and *rrs1-11* backgrounds (Figure 11C). Similarly, *RRS1* gene expression was not altered in *rps4-2* mutant compared to Col-0 wildtype.

In summary, these results indicate that i) *RPS4* and *RRS1* seem not to be co-regulated since *RPS4* is induced whereas *RRS1* is repressed upon infection with avirulent bacteria and ii) that there is no epistasis conferred by either mutation on the expression of the other gene.

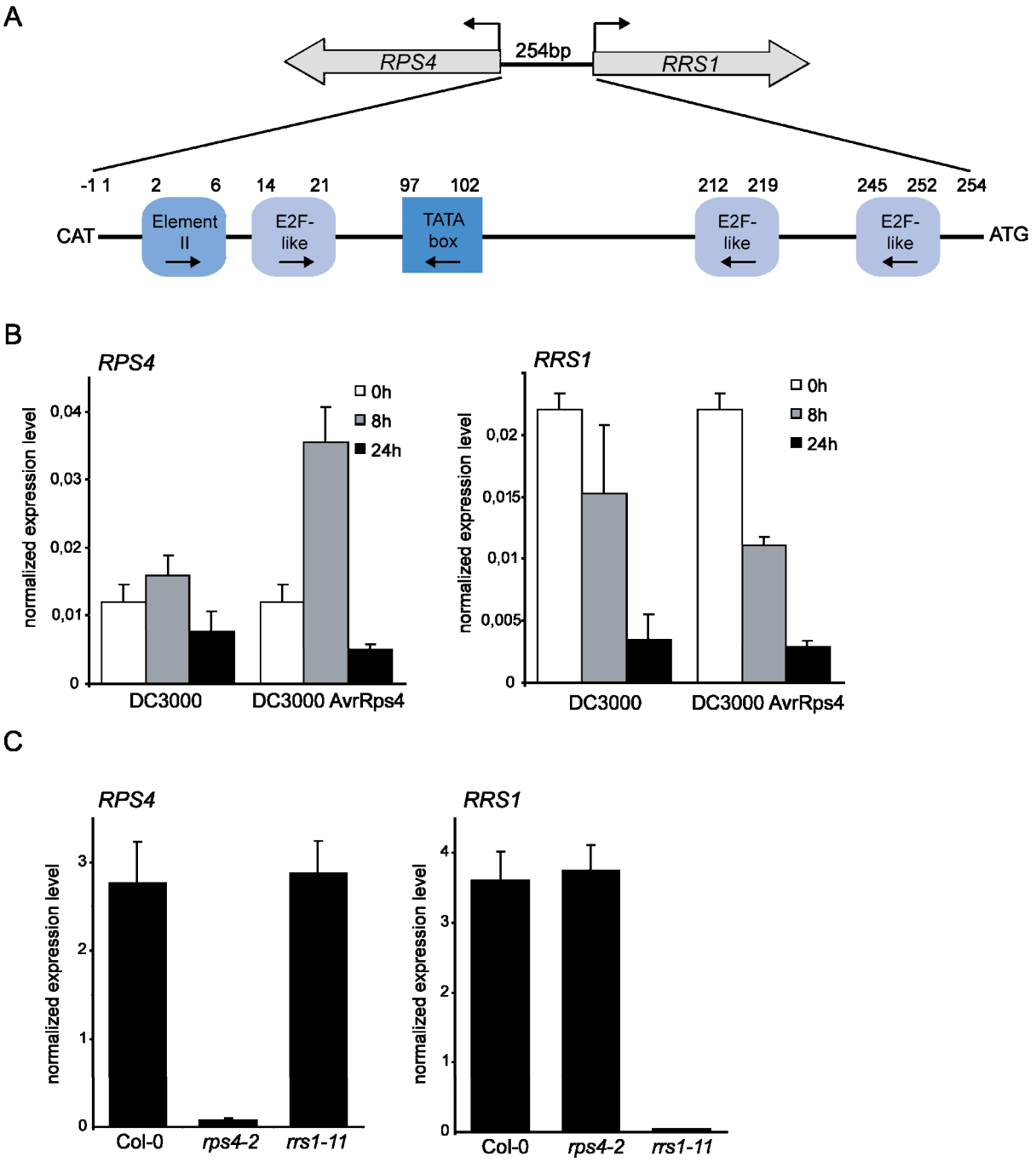
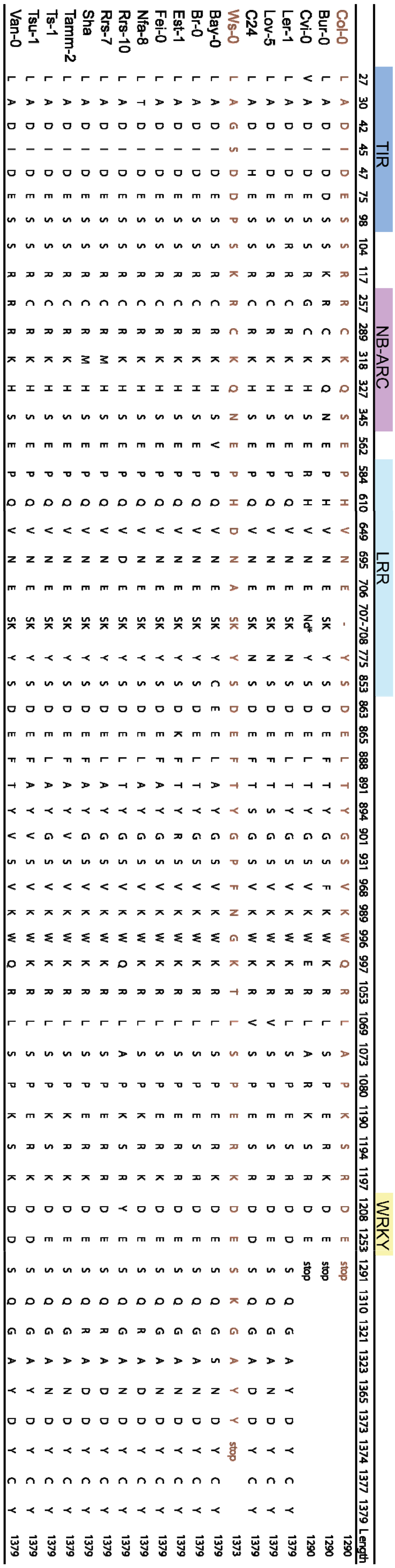


Fig. 11: Regulation of *RPS4* and *RRS1* gene expression.
(A) Schematic representation of the Arabidopsis locus encoding *RPS4* and *RRS1*. Nucleotide positions are based on the Col-0 reference genome sequence. Two potential regulatory elements were located at positions 2–6 (GCCCA; Element II) and 14–21 (TTTCCCGG; high similarity to E2F consensus sequence), corresponding to the upstream sequence of *RRS1*. Two other putative *cis*-elements at positions 219–212 (TTTCGCCG) and 252–245 (TTTCGCGC) showed high homology to the E2F consensus sequence, corresponding to the upstream sequence of *RPS4* (inverse direction). One putative TATA box was identified at positions 102–97 (TATTTA), corresponding to the *RPS4* orientation, but none was found in the *RRS1* orientation. Adapted from Birker et al, 2009. **(B)** Four week old Col-0 plants were spray-infected with *Pst* DC3000 wt or expressing AvrRps4-HA. Leaf samples were collected at 0 (white bars), 8 (grey bars) and 24 (black bars) hpi. Transcript levels of *RPS4* and *RRS1* were determined and normalized using the internal control *UBIQUITIN*. Error bars represent standard deviation of 4 technical replicates. The experiment was repeated twice with similar results. **(C)** Quantitative transcript levels of *RPS4* and *RRS1* in the depicted genotypes. Leaf samples were collected from untreated, four week old plants and transcript levels were normalized to *UBIQUITIN*. Error bars represent standard deviation of 4 technical replicates. The experiment was repeated twice with similar results.

A



B

	Pst AvrRps4	*R. solanacearum*	*C. higginsianum*
Col-0	R	S	S
Ws-0	R	R	R

Fig. 12: Amino acid sequence variations in RRS1 and recognition specificities in different accessions.
(A) Depicted are amino acid sequence variations in RRS1 proteins. Amino acid substitutions among 19 Arabidopsis accessions are shown. TIR (Toll/Interleukin-1 receptor), NB-ARC (nucleotide-binding adaptor shared by APAF-1, R proteins, and CED-4), LRR (leucine-rich repeat), WRKY (WRKY-containing DNA-binding domain). Nd: not detected. Adapted from Narusaka et al, 2009 **(B)** Table showing either resistance (R) or susceptibility (S) of Col-0 and Ws-0 accessions to the denoted pathogens.

2.2.2 Evidence that RPS4 and RRS1 act cooperatively

The two adjacent *R* genes *RPS4* and *RRS1* confer resistance to three distinct pathogens with different infection strategies and virulence mechanisms. Natural variation analysis of *RRS1* in Arabidopsis accessions revealed distinct amino acid polymorphisms as illustrated in Figure 12A. Dependent on their resistance response to *Ralstonia solanacearum*, they are named *RRS1-R* (for resistant) and *RRS1-S* (for susceptible). *RRS1* alleles from Col-0 (*RRS1-S*) contain a premature stop codon at S1291 resulting in an 83-aa deletion after the WRKY domain compared to Ws-0 (*RRS1-R*) (Figure 12A) (Narusaka et al., 2009). This region might be important for resistance against *R. solanacearum* and *C. higginsianum* since Col-0 plants are susceptible to both pathogens whereas Ws-0 plants are resistant (Figure B). However, this region is not required for resistance to *Pst* AvrRps4 since Col-0 plants are resistant (Gassmann et al., 1999; Birker et al., 2009). Thus, the precise recognition mechanism of *Pst* AvrRps4 is probably different from that of *R. solanacearum* and *C. higginsianum*.
As mentioned earlier, RPS4 and RRS1 seem to work cooperatively in Ws-0 (Chapter 2.1; Figure 7B). I was interested in analyzing a potential cooperativity between RPS4 and RRS1 in the Col-0 background since this accession is resistant to *Pst* AvrRps4 and features of RPS4 resistance (e.g. nuclear activity) and associated EDS1-dependent defense reprogramming have been characterized in Col-0 (Wirthmueller et al., 2007; Garcia et al., 2010). Because *RRS1* and *RPS4* are tightly linked, the generation of a double mutant in Col-0 by crossing of the individual single mutants would be extremely difficult and time consuming. Thus, I generated stable transgenic Arabidopsis lines in which *RRS1* is silenced in an *rps4-2* mutant background. For this, I ordered a commercially available AGRIKOLA Gateway entry plasmid that contains a gene-specific sequence tag of *RRS1* (165bp) designed to be as specific as possible to the cognate gene (http://bccm.belspo.be/db/lmbp_gst_clones). I then performed a double LR clonase reaction into pHellsgate destination vector that is designed for the expression of hairpin RNAs in transgenic plants (http://www.pi.csiro.au/RNAi/vectors.htm).

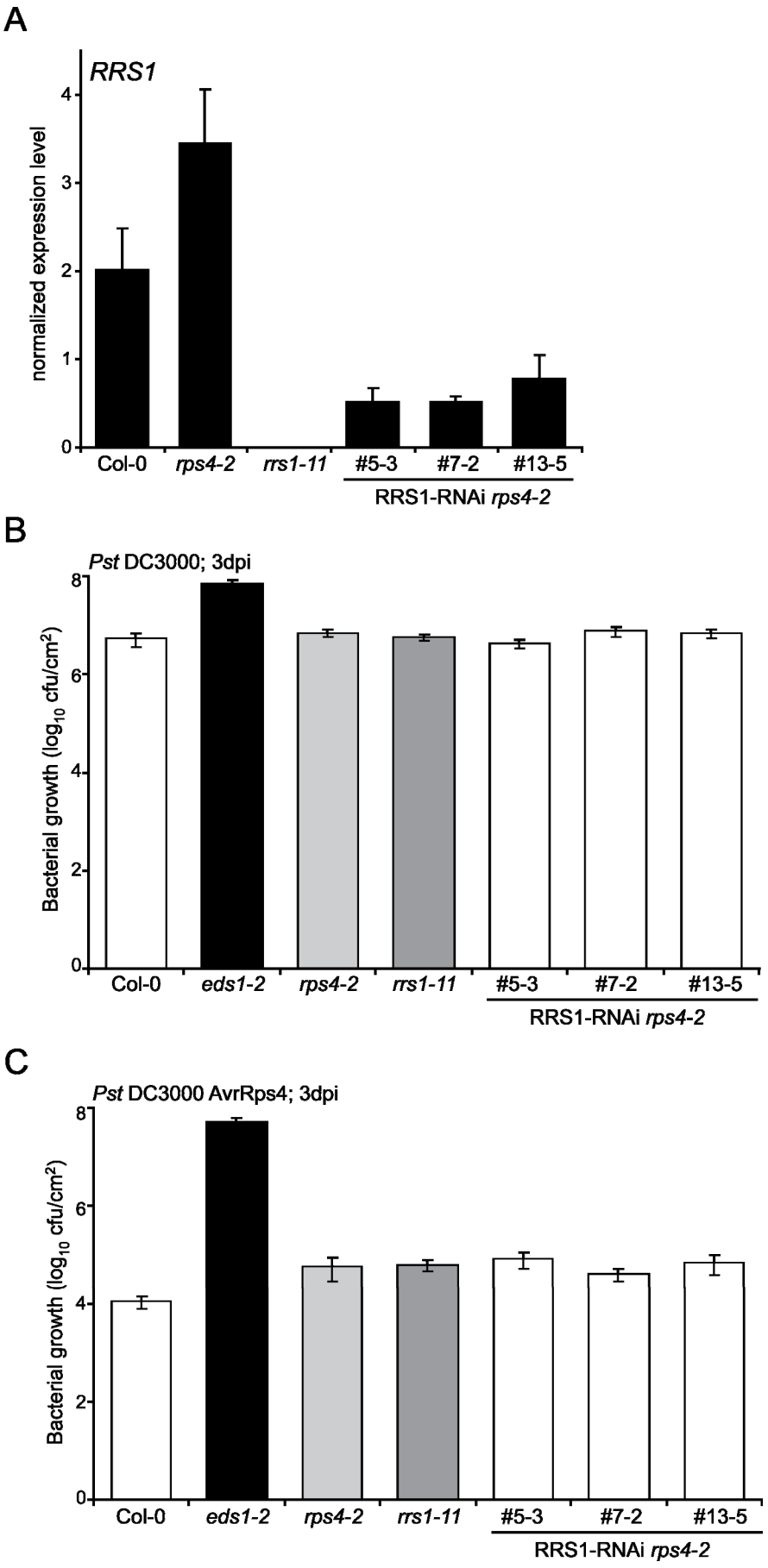

A
RRS1
normalized expression level
0
1
2
3
4
Col-0
rps4-2
rrs1-11
#5-3
#7-2
#13-5
RRS1-RNAi rps4-2
B
Pst DC3000; 3dpi
Bacterial growth (log10 cfu/cm2)
0
2
4
6
8
Col-0
eds1-2
rps4-2
rrs1-11
#5-3
#7-2
#13-5
RRS1-RNAi rps4-2
C
Pst DC3000 AvrRps4; 3dpi
Bacterial growth (log10 cfu/cm2)
0
2
4
6
8
Col-0
eds1-2
rps4-2
rrs1-11
#5-3
#7-2
#13-5
RRS1-RNAi rps4-2

Fig. 13: Silencing of *RRS1* in the Col-0 *rps4-2* mutant.
(A) Quantitative transcription profile of *RRS1* in T2 *RRS1-RNAi* lines. Samples for RNA extraction were taken from aerial tissue from 3 week old plants. *UBIQUITIN* was used as internal control. Error bars represent the standard deviation of 4 technical replicates. The experiment was repeated twice with similar results. **(B)** Four week old plants of the indicated genotypes were spray-infected with *Pst* DC3000. Bacterial titers 3 dpi are depicted. Bacterial entry was determined at 4hpi and was similar for all genotypes but is not shown here due to space limitations. Error bars represent standard error of 3 biological replicates. The experiment was repeated twice with similar results. **(C)** Four week old plants of the indicated genotypes were spray-infected with *Pst* DC3000 AvrRps4. Bacterial titers 3 dpi are depicted. Bacterial entry was determined at 4hpi and was similar for all genotypes but is not shown here due to space limitations. Error bars represent standard error of 3 biological replicates. The experiment was repeated twice with similar results.

Initially, I tested if the silencing construct was functional in Arabidopsis by analysing *RRS1* gene expression in three independent T2 transgenic lines, referred to as *RRS1-RNAi* lines. Figure 13A shows quantitative transcript levels of *RRS1* normalized to *UBIQUITIN*. *RRS1* gene expression was downregulated by 7-fold in the *RRS1-RNAi* lines compared to the *rps4-2* mutant background (Figure 13A). This indicates that silencing of *RRS1* was successful. Nevertheless, the *RRS1-RNAi* lines had low residual *RRS1* expression which might be sufficient for *RRS1* function.

To assess whether RRS1 and RPS4 are acting additively or cooperatively in Col-0 background, I spray infected the same three independent T2 *RRS1-RNAi* lines with *Pst* DC3000 AvrRps4 and measured bacterial growth at 3 days after infection. I analyzed susceptibility of T2 lines since *RRS1* gene expression showed attenuated silencing in homozygous T3 lines maybe due to silencing of the *RRS1-RNAi* construct (data not shown). A similar entry rate of bacteria to the plant was ensured by measuring bacterial growth 4h after spray infection (data not shown). The Col-0 *eds1-2* mutant was included as a measure of full bacterial growth potential and the *rps4-2* and *rrs1-11* mutants as references for a potential additive effect. As a control for successful infection, *Pst* DC3000 bacteria were infected. Growth of virulent *Pst* DC3000 was similar in all *RRS1-RNAi* lines compared to Col-0, indicating that the infection was successful and that the silencing of *RRS1* has no effect on basal resistance (Figure 13B). Susceptibility of all three *RRS1-RNAi* lines to *Pst* DC3000 AvrRps4 was similar to that exhibited by the *rps4-2* and *rrs1-11* single mutants since all mutants showed a 5-10-fold increase in bacterial numbers compared to Col-0 (Figure 13C). The absence of an additive susceptible phenotype of the *RRS1-RNAi* lines compared to *rps4-2* in response to *Pst* DC3000 AvrRps4 suggests that RRS1 and RPS4 also function cooperatively in Col-0 background. Nonetheless, the residual *RRS1* expression in *RRS1-RNAi* lines might be sufficient for AvrRps4 recognition.

2.2.3 Overexpression of RPS4 induces an autoimmune phenotype that is *RRS1*-dependent

The expression of R proteins is tightly regulated in order to prevent inappropriate activation of defense responses. Plant defense responses need to be tightly regulated to prevent autoimmunity which is detrimental to growth and development of the plant. It was shown in several studies that the overexpression of an *R* gene leads to constitutive activation of defense responses (Oldroyd and Staskawicz, 1998; Moffett et al., 2002).

RPS4 expression under control of its own promoter was close to the detection limit. Thus, transgenic lines constitutively expressing *RPS4-HA-StrepII* (*RPS4-HS*) under control of the strong CaMV 35S overexpression promoter were generated in the signalling-deficient *eds1-2* background and then crossed to Col-0 to introduce a functional *EDS1* allele (Wirthmueller et al., 2007). The overexpression of *RPS4* in Col-0 leads to reduced plant size which is indicative of a constitutively activated plant defense (Figure 14A, C). The stunted morphology is completely dependent on *EDS1* since *35S::RPS4-HS* lines in *eds1-2* background show wildtype morphology demonstrating that the *RPS4* overexpression signal is transduced in an *EDS1*-dependent manner (Figure 14A, C) (Wirthmueller et al., 2007).

Since the above and published results (Narusaka et al., 2009) suggest that the *R* gene pair *RPS4* and *RRS1* functions cooperatively to confer resistance to three different pathogens, I was interested in testing whether the *RPS4* overexpression phenotype depends on the presence of *RRS1*. To investigate this, I generated a *35S::RPS4-HS* line in an *rrs1-11* background by crossing the *rrs1-11* mutant to the *35S::RPS4-HS* Col-0 line (Wirthmueller et al., 2007). I selected F3 lines homozygous for the *RPS4* transgene and *rrs1-11* and analyzed *RRS1* gene expression by qRT-PCR to ensure that they were *RRS1* null mutants (Figure 15; right panel). Figure 14A shows the developmental phenotype of one representative homozygous F3 line in comparison to *35S::RPS4-HS* in Col-0 and *eds1-2*. The stunted phenotype of *35S::RPS4-HS* Col-0 line depended on the presence of *RRS1* since the *35S::RPS4-HS rrs1-11* line displayed a wildtype-like morphology. To exclude the possibility that the wildtype-like growth of the *35S::RPS4-HS rrs1-11* line is caused by reduced RPS4 protein accumulation, I compared RPS4 steady state levels in all three genetic backgrounds. RPS4 protein levels were similar in all lines (Figure 14B). Therefore, the stunted morphology of *RPS4* overexpressing plants in Col-0 depends on the presence of *RRS1*. This further supports the hypothesis that RPS4 and

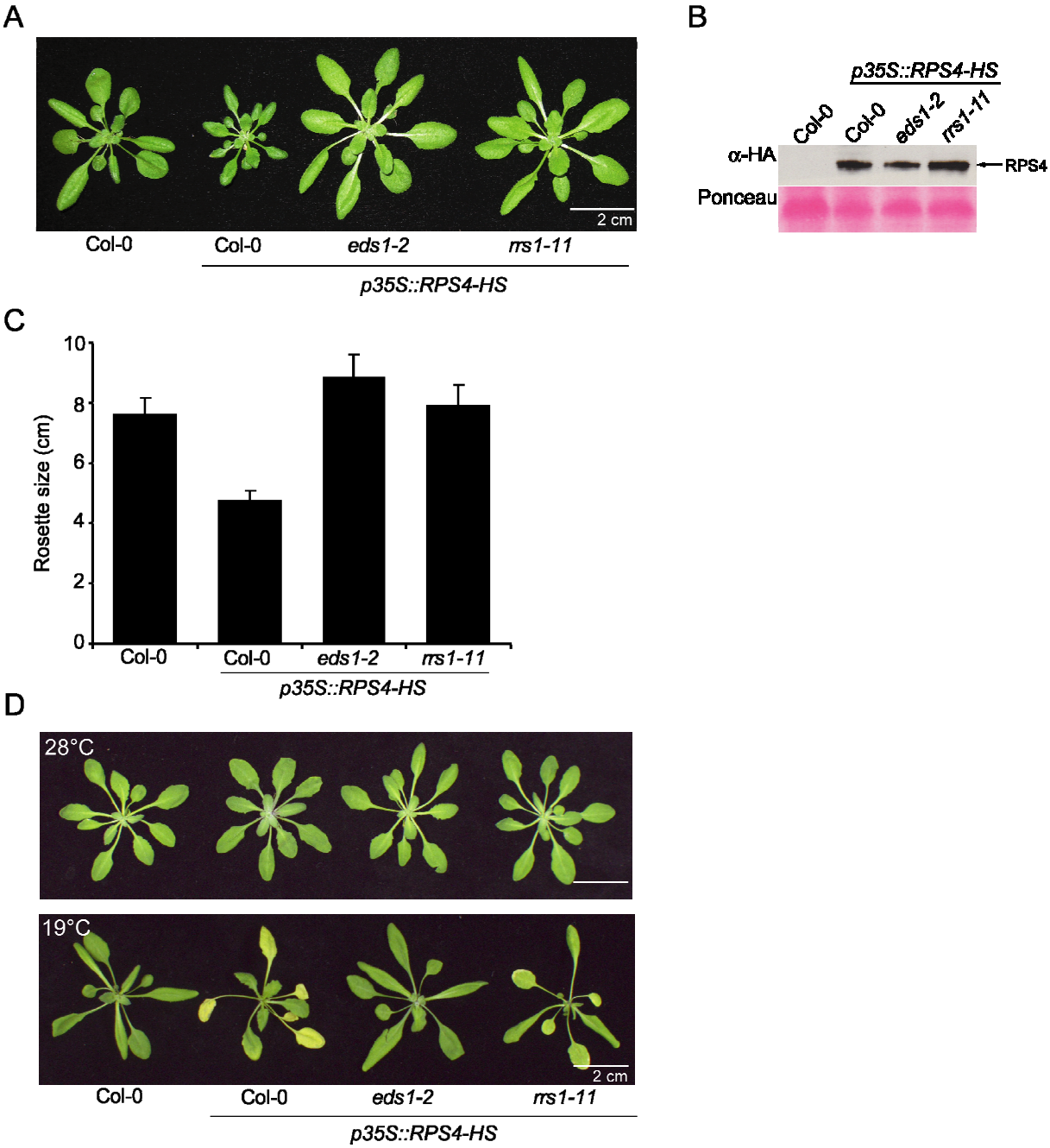


Fig. 14: Constitutive *RPS4* overexpression induces dwarfism that is *EDS1*- and *RRS1*-dependent.
(A) Constitutive RPS4 overexpression causes a dwarfed phenotype that is *EDS1*- and *RRS1*-dependent. Picture was taken from 4 week old plants grown at 22°C. **(B)** RPS4 protein accumulation is similar in all RPS4 overexpression lines. Immunoblot analysis was performed of total leaf extract taken from 4 week old plants. Membrane was probed with α-HA antibody. A Ponceau staining was performed to ensure equal sample loading. **(C)** Quantitative measurement of plant size of 4 week old plants. Diameter of plant rosettes was measured. **(D)** Dwarfism of *35S::RPS4-HS* Col-0 is suppressed at high temperature. Upper picture: Growth phenotype of RPS4 overexpression lines at 28°C. Picture was taken from plants grown at 28°C for 4 weeks. Lower picture: Shifting of plants to low temperature (19°C) induces leaf yellowing in *35S::RPS4-HS* Col-0 and partially in *35S::RPS4-HS rrs1-11* . Plants were grown at 28°C for 4 weeks before shifting them to 19°C. Picture was taken after 6d at 19°C.

RRS1 act cooperatively in Col-0 and suggests that RRS1 operates coincidently or downstream of activated (deregulated) RPS4.

2.2.4 Constitutive defense gene activation caused by RPS4 overexpression is partially *RRS1*-dependent

It is known that elevated temperature can inhibit plant defense responses (Whitham et al., 1996; Wang et al., 2009b). Recently, it was shown that R proteins are a major temperature-sensitive component in plant immunity and that constitutive activated defense responses caused by autoactive R proteins can be suppressed at elevated temperature (Zhu et al., 2009). The stunted phenotype of RPS4 overexpressing plants can be suppressed by growing plants at elevated temperature (J. Parker group, unpublished). Figure 14D shows the growth phenotypes of *35S::RPS4-HS* in Col-0, *eds1-2* and *rrs1-11* mutant backgrounds compared to Col-0 when grown at 28°C revealing that the stunted phenotype can indeed be suppressed at this temperature. When shifted to 19°C, this suppression effect is abolished and *35S::RPS4-HS* Col-0 plants develop leaf yellowing indicative of cell death induction visible from 6 days at 19°C (Figure 14D). The RPS4-induced chlorosis was partially *RRS1*-dependent since *35S::RPS4-HS rrs1-11* plants displayed weak leaf yellowing. The temperature shift from 28°C to 19°C of the *35S-RPS4-HS* Col-0 line resembles defense activation through *Pst* AvrRps4 infection since several well characterized *EDS1*-dependent defense marker genes are up- or downregulated after temperature shift that are also induced or repressed after *Pst* AvrRps4 infection (J. Parker group, unpublished; Garcia et al, 2010; Bartsch et al, 2006). Among the upregulated genes are components of SA biosynthesis and signalling (*ICS1, PBS3, CBP60*) (Wildermuth et al., 2001; Okrent et al., 2009; Wang et al., 2009a), as well as *FMO1*, a positive regulator of an SA-independent branch of EDS1 signalling (Bartsch et al., 2006; Mishina and Zeier, 2006). *EDS1* and *PR1*, a commonly used defense marker gene (Laird et al, 2004), were also among the *EDS1*-dependent upregulated genes. Furthermore, the downregulated gene *ERECTA*, encoding a receptor-like kinase required for resistance to *R. solanacearum* (Godiard et al., 2003), also displayed *EDS1*-dependency. I made use of this temperature shift system to analyse whether attenuated defense gene reprogramming in *35S::RPS4-HS rrs1-11* plants could be the cause of the suppression of the stunted phenotype. For this, I grew Col-0, *35S::RPS4-HS* in Col-0, *eds1-2* and *rrs1-11* plants for 4 weeks at 28°C before shifting to 19°C. Samples for RNA extraction were taken from aerial tissue directly before and 1h, 2h, 4h, 8h and 24h after temperature shift. I chose early as well as late time

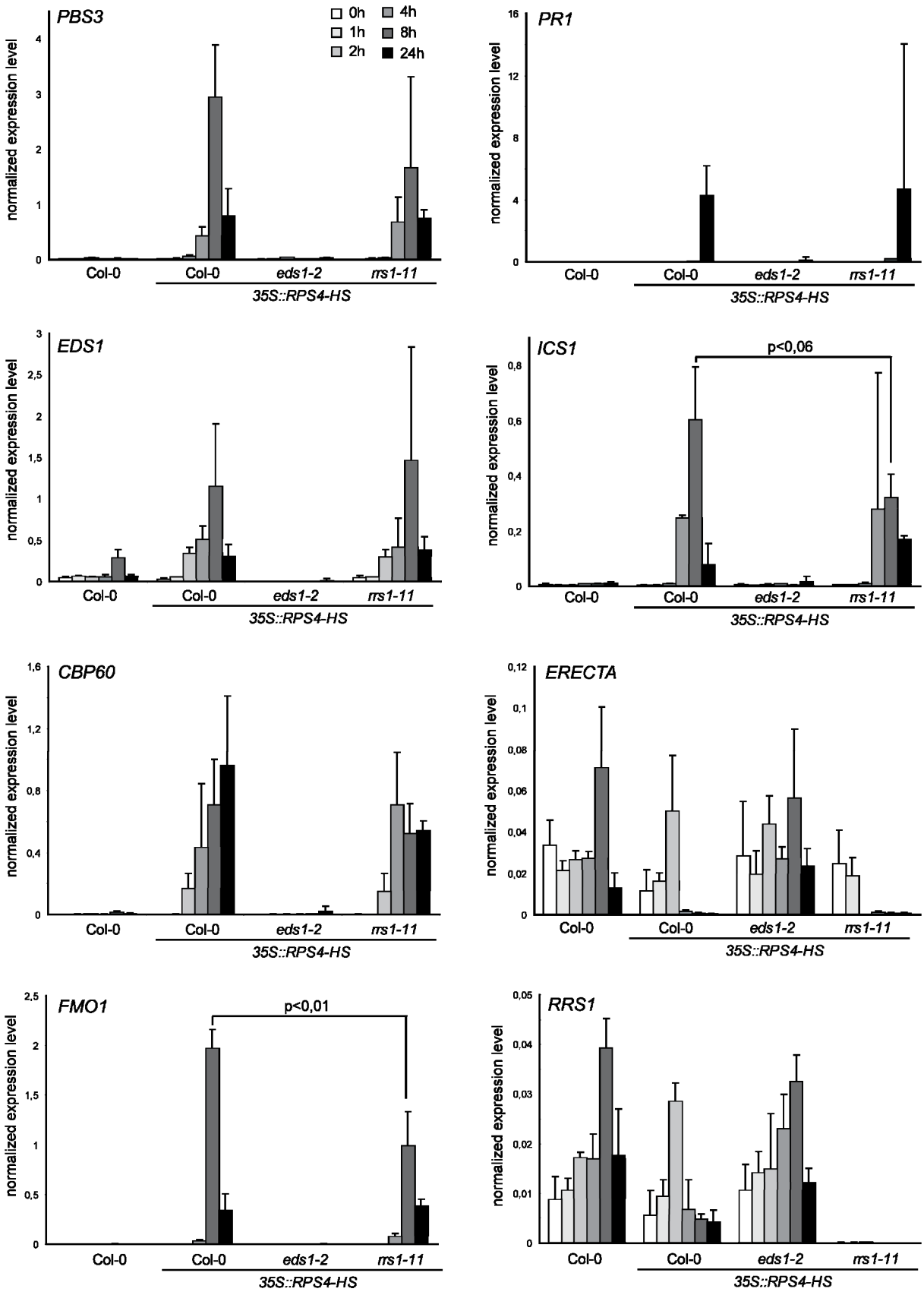


Fig. 15: Constitutive defense gene activation of RPS4 overexpression is only partially *RRS1*-dependent.
Four week old plants of the indicated genotypes were shifted from 28°C to 19°C. Leaf samples were collected at 0, 2, 4, 8 and 24 hpi. Transcript levels were determined and normalized using the internal control *UBIQUITIN*. Error bars represent standard deviation of 3 biological replicates. $p<0.01$ indicates significant differences calculated with Student`s t-test.

points after the temperature shift to assess if *RRS1* might be either required for i) initial induction or repression of defense genes, ii) timing of transcriptional reprogramming or iii) maintenance of induction or repression through RPS4. Transcriptional induction or repression started at 2 to 4h after the temperature shift and reached a maximum at 8h (Figure 15). The transcriptional reprogramming of defense genes was faster compared to bacterial spray-infection experiments probably due to the fact that avirulent bacteria have to enter the leaves before a defense response is activated. The expression of all genes tested was as expected strongly *EDS1*-dependent. However, most of the genes tested were not strongly *RRS1*-dependent. Rather, a quantitative difference in gene expression in *35S::RPS4-HS* Col-0 compared to *35S::RPS4-HS rrs1-11* was observed for certain genes. For example, transcript levels of *FMO1* and *ICS1* were significantly reduced in *rrs1-11* mutant background. A clear explanation for the *RRS1*-dependency of the stunted morphology of *35S::RPS4-HS* Col-0 cannot be provided by this analysis because only a limited number of genes were tested. It is possible that the threshold for the transcriptional reprogramming of defense genes might be different than for the suppression of the stunted morphology. Alternatively, *RRS1* might regulate a specific subset of genes that are induced upon RPS4 activation. Also, *RRS1* mutation might have a quantitative effect on transcriptional reprogramming as it seems the case for *FMO1* and *ICS1*. A gene expression microarray of *35S-RPS4-HS* line in Col-0 compared to *rrs1-11* background might provide deeper insights into which genes are *RRS1*-dependent or if *RRS1* is required for the timing or maintenance of gene induction through *RPS4*.

2.2.5 Increased *RPS4* levels cannot compensate for loss of *RRS1* in bacterial resistance

Overexpression of *RPS4* in Col-0 leads to a dwarfed phenotype which is dependent on *RRS1* (Figure 14A). To further position *RRS1* in the *RPS4*-mediated resistance pathway, I tested whether *RPS4* overexpression can compensate for loss of *RRS1* in resistance to *Pst* bacteria. First, I analyzed growth of virulent *Pst* DC3000 bacteria in the *35S::RPS4-HS* in the Col-0, *eds1-2* and *rrs1-11* backgrounds. As control for full bacterial growth potential, the *eds1-2* mutant was included as well as *rrs1-11*, which displayed enhanced susceptibility to *Pst* AvrRps4 as described in Section 2.1.5. Plants were grown for 4 weeks at 28°C in order to avoid developmental differences that might affect pathogen growth. After spray-infection, plants were kept at 23°C for 3 days. As shown in Figure 16A, the *35S::RPS4-HS* Col-0 line

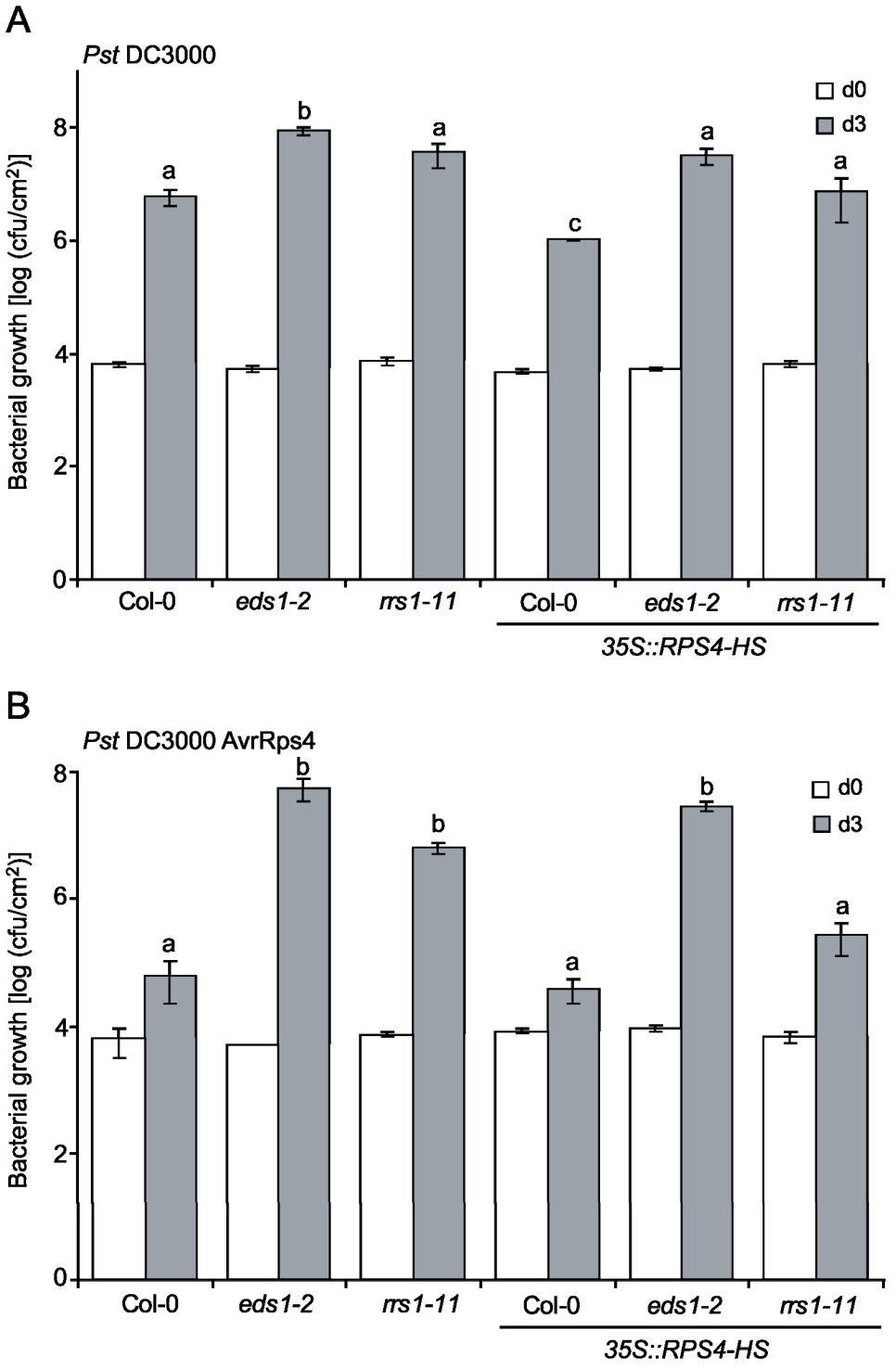


Fig. 16: RPS4 overexpression cannot compensate for loss of RRS1.
(A) Four week old plants grown at 28°C of the indicated genotypes were spray-infected with *Pst* DC3000. Bacterial titers 3 dpi are depicted. Bacterial entry was determined at 4hpi and was similar for all genotypes but is not shown here due to space limitations. Error bars represent standard error of 3 biological replicates. The experiment was repeated twice with similar results. **(B)** Four week old plants grown at 28°C of the indicated genotypes were spray-infected with *Pst* DC3000 AvrRps4. Bacterial titers 3 dpi are depicted. Bacterial entry was determined at 4hpi and was similar for all genotypes but is not shown here due to space limitations. Error bars represent standard error of 3 biological replicates. Characters a-c indicate significant differences calculated by Student`s t-test. The experiment was repeated twice with similar results.

exhibited increased resistance to virulent *Pst* DC3000 bacteria suggesting that the RPS4 deregulation response after the temperature shift increases basal resistance to virulent bacteria. The RPS4-activated defense response was not observed in *35S::RPS4-HS rrs1-11* since these plants were as susceptible as Col-0 wt plants (Figure 16A). Next, I tested growth of avirulent *Pst* DC3000 AvrRps4 bacteria. *35S::RPS4-HS* Col-0 plants exhibited slightly increased resistance compared to Col-0 wt plants that was not statistically significant but was reproducible in independent repeats (Figure 16B). By contrast, overexpression of *RPS4* in *eds1-2* did not render plants more resistant than *eds1-2* plants confirming that *EDS1* functions downstream of activated *RPS4* (Wirthmueller et al., 2007). *35S::RPS4-HS rrs1-11* plants showed increased susceptibility compared to *35S::RPS4-HS* Col-0 plants which was also not statistically significant but reproducible and plants were not as susceptible as *rrs1-11*. It is notable that susceptibility of the *rrs1-11* mutant to *Pst* AvrRps4 was increased in this assay compared to bacterial infections of plants grown at 22°C (Figure 6). This suggests that RRS1-mediated resistance is temperature-dependent. Taken together, these results indicate that *RPS4* still partially functions in the absence of *RRS1* but not as effectively as in the presence of *RRS1*. Thus, the overexpression of one of the two *R* genes cannot fully compensate for the absence of the other. It might be possible that both R proteins have distinct functions in the cell and are not completely working cooperatively.

2.2.6 TIR domains of RPS4 and RRS1 physically interact

In animals, perception of PAMPs is mediated by the extracellular LRR region of Toll-like receptors (TLR). This induces homo-dimerization of the cytoplasmic TIR domain which provides a new scaffold that can bind to adapter proteins and initiate a defense response (Tapping et al, 2009). Recently, it has been shown that the TIR domain of plant R proteins also has signalling function (Bernoux et al., 2011). The TIR domain of flax L6 self-associates in yeast and *in vitro*. This homo-dimerization of L6 TIR domain strongly correlates with autoactivity *in planta* suggesting that this is a key event in TIR domain signalling (Bernoux et al., 2011). Furthermore, Bernoux et al (2011) were able to redefine the minimal functional region of the TIR domain required for defense signalling.

Our collaborators M. Bernoux and P. Dodds (CSIRO Canberra) analyzed whether the functional TIR domains of RPS4 and RRS1, which consist of the first exon plus 24 aa, are able to associate in yeast. Functional TIR domains were determined by aligning the minimal region required for autoactivity of L6 with RPS4 and RRS1. The TIR domains of *RRS1-R*

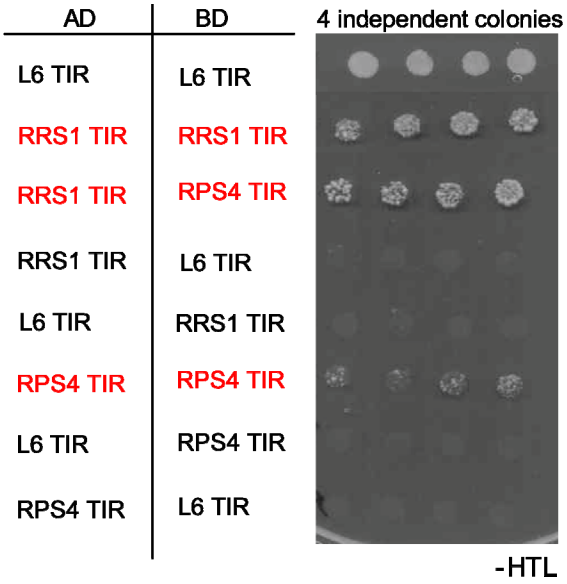


Fig. 17: Homo- and hetero-domerization of TIR domains of RPS4 and RRS1.
Yeast-Two-hybrid analysis of the TIR domains of L6, RRS1 and RPS4. Four independent colonies of each tested combination were grown for four days at 30°C on SC media -His-Try-Leu (-HTL). AD= GAL4 Activation Domain; BD= GAL4 DNA Binding Domain. Yeast-Two-hybrid analysis was performed by Maud Bernoux (CSIRO, Canberra).

(Nd-0 allele) and *RRS1-S* (Col-0 allele) do not harbour nucleotide polymorphisms. Both homo- and hetero-dimerization of TIR RPS4 and TIR RRS1 could be observed (Figure 17). As a negative control, the TIR domain of L6 was used which did not hetero-dimerize with the TIR domain of RPS4 or RRS1 (Figure 17). This suggests that the interaction of the TIR domains of RPS4 and RRS1 is specific. Nonetheless, a further control of a functional TIR domain of another Arabidopsis R protein should be included since L6 is a flax R protein that shows higher sequence polymorphisms to RPS4 and RRS1 compared to other Arabidopsis R proteins.

To test whether the interaction of the TIR domains of RPS4 and RRS1 can occur *in planta*, we performed FRET/FLIM experiments of protein domains transiently expressed in *N. benthamiana* in collaboration with L. Deslandes and C. Tasset (CNRS/INRA Toulouse). This method has several advantages compared to other interaction assays since it allows the analysis of potential interactions in living plant cells, in contrast to Yeast-Two-Hybrid, and is more reliable than the BiFC (Bimolecular Fluorescence Complementation) method. In this quantitative noninvasive approach, the Förster resonance energy transfer (FRET) between a donor (CFP; cyan fluorescent protein) and an acceptor (YFP; yellow fluorescent protein) fluorophore is measured. If these two proteins interact, the transfer of energy from the donor to the acceptor decreases the fluorescence lifetime (FLIM; average time that a molecule remains in its excited state prior returning to its basal state) of the donor fluorophore. The

relative difference of lifetime is a measure of FRET efficiency. Several controls have to be included to make sure that the reduction in lifetime of the donor is not due to nonspecific transfer of energy between the two fluorophores. To this end, lifetime of the donor upon coexpression with free YFP is measured. In the assay, the TIR domain of RRS1 was fused at its C-terminus to CFP and the TIR domain of RPS4 to YFP (data not shown). Both fusion proteins were expressed under the control of the constitutive 35S promoter via *Agrobacterium*-mediated infiltration in *N. benthamiana* leaves. At 36h after infiltration, fluorescence lifetime of the donor was measured upon coexpression of the fusion proteins compared to expression of the donor alone. Preliminary experiments showed that the CFP lifetime was reduced upon coexpression of both fusion proteins compared to expression of TIR-RRS1-CFP alone. This experiment was performed only once and has to be repeated to be able to calculate whether the reduction in CFP lifetime is significant. Taken together, these data point to a physical interaction of the TIR domains of RPS4 and RRS1 inside the plant nucleus. Nevertheless, further controls have to be included to make sure that the reduction in TIR-RRS1-CFP lifetime is not due to nonspecific transfer of energy between the two fluorophores. To this end, lifetime of TIR-RRS1-CFP upon coexpression with free YFP should be measured.

Since the TIR domain of R proteins is thought to mediate resistance signalling, the interaction of RPS4 TIR and RRS1 TIR might be a critical early event for activation and signalling and required for the cooperative action of the two R proteins. The homo- and hetero-dimerization of the TIR domains might reflect formation of a multi-protein complex consisting of several RPS4 and RRS1 molecules required for AvrRps4 recognition *in planta*. An important aspect that should be addressed in the future is whether the full length R proteins also interact. Accessibility of the TIR domain resembles an activated conformation of the R protein and addition of NB-ARC or NB-ARC-LRR domains might inhibit TIR domain interactions. If the full length proteins interact as well might answer the question if RPS4 and RRS1 are interacting in their activated or resting state. In addition, it would be highly informative to assess whether this interaction occurs only in the plant nucleus since at least one critical step of AvrRps4 recognition takes place in the nucleus. Another important aspect that should be addressed is whether the interaction is a consequence of AvrRps4 recognition to induce subsequent defense signalling.

2.3 AvrRps4 complex formation with immune regulators

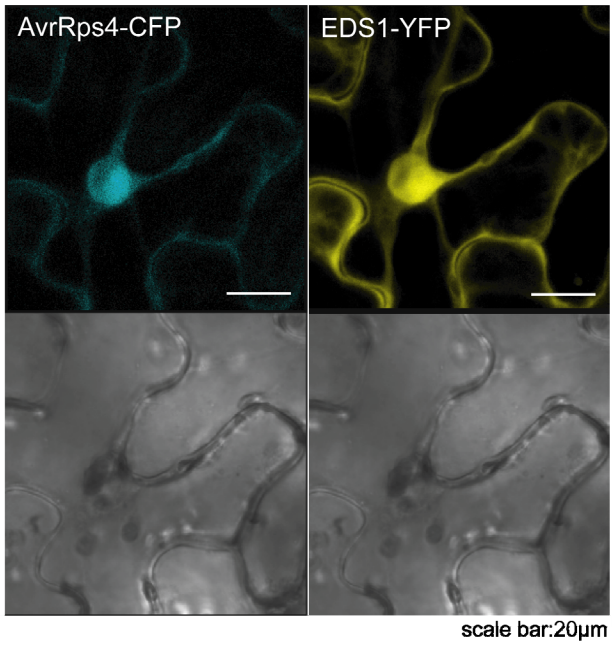
2.3.1 Nuclear AvrRps4 physically interacts with the central immune regulator EDS1

Recognition of a pathogen effector through direct interaction with its corresponding plant Resistance (R) protein has been shown only for few cases (Jia et al., 2000; Deslandes et al., 2003; Dodds et al., 2006). Another scenario is indirect recognition of an effector, which would conform to the guard hypothesis. The guard hypothesis proposes that an R protein guards a host protein that is targeted by an effector to enhance pathogen virulence. Effector-induced alterations of the target protein are perceived by R proteins so that they become activated and induce a defense response (Collier and Moffett, 2009).

The mode of recognition of AvrRps4 by RPS4 and RRS1 is not known. Also, the virulence target of AvrRps4 was not yet identified. To gain more information about the function of AvrRps4 *in planta*, we analyzed if AvrRps4 physically interacts with known components of AvrRps4-triggered immunity. In collaboration with L. Deslandes and C. Tasset (CNRS/INRA Toulouse), I performed FRET/FLIM experiments after *Agrobacterium*-mediated expression of candidate proteins in *N. benthamiana*. All genes tested were under control of the constitutive CaMV 35S overexpression promoter and protein expression of all fusion proteins was verified on a Western Blot probed with α-GFP antibody. First, I analyzed a potential interaction of AvrRps4 with the central immune regulator EDS1 since AvrRps4-triggered resistance depends on *EDS1* (Wirthmueller et al., 2007). For that, I generated constructs of AvrRps4 C-terminally fused to CFP and EDS1 fused to YFP. Since AvrRps4 is cleaved *in planta* and is C-terminally tagged, only interactions of the AvrRps4 C-terminus are analyzed. Upon transient coexpression of AvrRps4-CFP and EDS1-YFP in *N. benthamiana*, both proteins showed a nucleo-cytoplasmic distribution (Figure 18A). Lifetime of the donor was measured in nuclei since I showed that AvrRps4 needs to localize to the nucleus to induce a full resistance response. Also, the nuclear pool of EDS1 was shown to have an essential role in immunity and reprogramming defense gene expression (Garcia et al., 2010). Furthermore, measurement of CFP lifetime in the nucleus is easier compared to measurements in the cytoplasm since fluorescence is usually higher in nuclei and less diffuse. The average CFP lifetime in nuclei expressing AvrRps4-CFP was 2.30ns (Figure 18B). A significant reduction of the average CFP lifetime to 1.98ns was recorded in nuclei coexpressing the AvrRps4-CFP

and EDS1-YFP fusion proteins which corresponds to a FRET efficiency of 14%. This indicates that the AvrRps4 C-terminus and EDS1 physically interact in the nucleus when transiently coexpressed in *N. benthamiana*.

A



B

Donor	Acceptor	Lifetime	p value	nuclei	FRET
AvrRps4-CFP	-	2.30 ns	-	29	
AvrRps4-CFP	EDS1-YFP	1.98 ns	0.0011	27	13,8%
AvrRps4-CFP	MLA1-YFP	2.21 ns	-	10	-

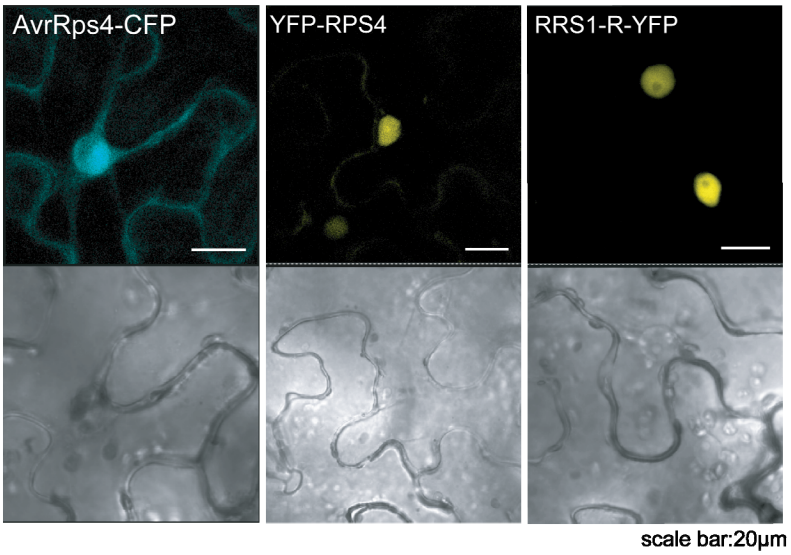
Fig. 18: AvrRps4 physically interacts with the central immune regulator EDS1.
(A) Subcellular localization of AvrRps4-CFP and EDS1-YFP transiently expressed in *N. benthamiana*. Confocal images of representative cells were taken 48h after *Agrobacterium* infiltration. Experiment was repeated twice with similar results. **(B)** FLIM Measurements showing that AvrRps4 physically interacts with EDS1 in the nucleus of *N. benthamiana* epidermal cells. Lifetime= mean lifetime in nanoseconds; p value= probability value; nuclei= total number of nuclei measured; FRET= FRET efficiency percentage (E= 1- τDA/τD) was calculated by comparing the lifetime of the donor in the presence of the acceptor (τDA) with its lifetime in the absence of the acceptor (τD). Experiment was repeated twice with similar results.

As negative control, MLA1-YFP (material kindly provided by T. Maekawa, MPIPZ Cologne), a CC-NB-LRR R protein, was coexpressed with AvrRps4-CFP. This did not result in a significant decrease in CFP lifetime, indicating that the interaction of AvrRps4 and EDS1 is specific (Figure 18B). Based on these data, one possible hypothesis is that EDS1 is the virulence target of AvrRps4.

2.3.2 Evidence for AvrRps4 physical interaction with RPS4 and RRS1

To gain more information about how AvrRps4 is recognized by its cognate R proteins, either directly or indirectly, we tested if AvrRps4 physically interacts with RPS4 or RRS1. In these experiments, the *RRS1-R* allele was used for interaction studies. In future experiments, the *RRS1-S* allele should be included since this allelic variant confers resistance to *Pst* AvrRps4 (Birker et al., 2009; Narusaka et al., 2009).

A



B

Donor	Acceptor	Lifetime	FRET
AvrRps4-CFP	-	2.11 ns	
AvrRps4-CFP	YFP-RPS4	1.97 ns	10%
AvrRps4-CFP	RRS1-R-YFP	1.81 ns	10%

Fig. 19: AvrRps4 physically interacts with its cognate R proteins, RPS4 and RRS1.
(A) Subcellular localization of AvrRps4-CFP, YFP-RPS4 and RRS1-R-YFP transiently expressed in *N. benthamiana*. Confocal images of representative cells were taken 48h after *Agrobacterium* infiltration. Experiment was performed only once. **(B)** FLIM Measurements showing that AvrRps4 physically interacts with RPS4 and RRS1-R in the nucleus of *N. benthamiana* epidermal cells. Lifetime= mean lifetime in nanoseconds; FRET= FRET efficiency percentage (E= 1- τDA/τD) was calculated by comparing the lifetime of the donor in the presence of the acceptor (τDA) with its lifetime in the absence of the acceptor (τD). Experiment was performed only once.

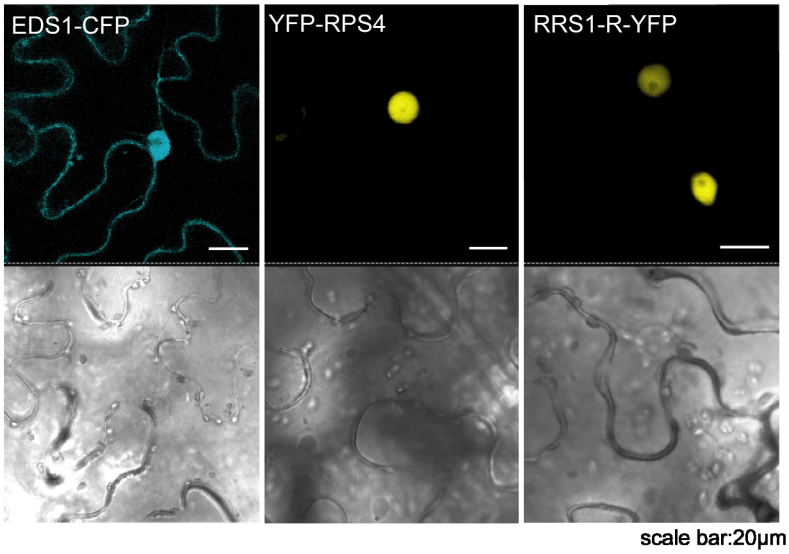
Upon coexpression with RRS1-R-YFP or YFP-RPS4, the nucleo-cytoplasmic distribution of AvrRps4-CFP was not altered (Figure 19A). RRS1-R-YFP showed exclusive nuclear localization and YFP-RPS4 was nuclear and extra-nuclear localized (Figure 19A). Lifetime of

the donor was measured in nuclei since the nuclear pool of RPS4 is required for its function (Wirthmueller et al., 2007) and RRS1-R exclusively localized to nuclei. The average CFP lifetime in nuclei expressing AvrRps4-CFP was 2.11ns (Figure 19B). Upon coexpression with YFP-RPS4, CFP lifetime was reduced to 1.97ns which corresponds to 10% FRET efficiency. A significant reduction of the average CFP lifetime to 1.81ns was recorded in nuclei coexpressing AvrRps4-CFP and RRS1-R-YFP fusion proteins which also corresponds to 10% FRET. This experiment was performed only once and needs to be repeated with further controls. Also, it was not possible to perform a statistical analysis of these data due to a limited number of nuclei tested in order to analyze if the reduction in lifetime is significant. These results suggest that AvrRps4 C-terminus is directly recognized by RPS4 and RRS1, although FRET does not reveal with complete confidence whether an interaction is direct or through an intermediate. Lifetime of the donor decreases if donor and acceptor molecules are less than 10nm apart. It is thus possible that AvrRps4 does not directly interact with RPS4 and RRS1 but rather that this interaction is mediated by another protein, such as EDS1. Despite years of research, the mechanism how EDS1 connects with TIR-NB-LRR receptors is not known. Therefore, we tested whether RPS4 and RRS1 are also physically interacting with EDS1.

2.3.3 Nuclear EDS1 physically interacts with RPS4 and RRS1

To test whether RPS4 and RRS1 are interacting with EDS1, EDS1-CFP was coexpressed with either RRS1-YFP or YFP-RPS4 in *N. benthamiana* and lifetime of CFP was measured. The average CFP lifetime in nuclei expressing EDS1-CFP was 2.56ns (Figure 20B). A significant reduction of the average CFP lifetime to 2.04ns was recorded in nuclei coexpressing the EDS1-CFP and YFP-RPS4 fusion proteins which corresponds to a FRET efficiency of 20%. Upon coexpression of EDS1-CFP with RRS1-R-YFP, CFP lifetime was reduced to 2.20ns, corresponding to a FRET efficiency of 14%. As negative control, EDS1-YFP was coexpressed with PopP2-CFP which did not result in a significant decrease in CFP lifetime suggesting that the interaction of EDS1 with RPS4 and RRS1-R is specific. The *Ralstonia solanacearum* effector PopP2 is recognized by RRS1-R and also localizes to the nucleus which makes it an appropriate negative control. Furthermore, the absence of an interaction between EDS1 and PopP2 indicates that the observed interaction of AvrRps4 and EDS1 is specific. Protein expression of none of the combinations tested resulted in a cell death reaction of the plant at the infiltration site.

A



B

Donor	Acceptor	Lifetime	p value	nuclei	FRET
EDS1-CFP	-	2.56 ns		25	
EDS1-CFP	YFP-RPS4	2.04 ns	0.0003	26	20.3%
EDS1-CFP	RRS1-R-YFP	2.20 ns	0.007	25	14.1%
PopP2-CFP	-	2.33 ns		20	
PopP2-CFP	EDS1-YFP	2.24 ns	0.43	25	4.0%

Fig. 20: EDS1 physically interacts with the R proteins RPS4 and RRS1.
(A) Subcellular localization of EDS1-CFP, YFP-RPS4 and RRS1-R-YFP transiently expressed in *N. benthamiana*. Confocal images of representative cells were taken 48h after *Agrobacterium* infiltration. Experiment was repeated once with similar results. **(B)** FLIM Measurements showing that EDS1 physically interacts with RPS4 and RRS1-R in the nucleus of *N. benthamiana* epidermal cells. Lifetime= mean lifetime in nanoseconds; p value= probability value; nuclei= total number of nuclei measured; FRET= FRET efficiency percentage (E= 1- τDA/τD) was calculated by comparing the lifetime of the donor in the presence of the acceptor (τDA) with its lifetime in the absence of the acceptor (τD). Experiment was repeated once with similar results.

Another interesting observation we made is that AvrRps4 protein seems to be stabilized by RPS4, RRS1-R and EDS1. Upon transient *Agrobacterium*-mediated coexpression of AvrRps4-CFP with either YFP-RPS4, RRS1-R-YFP or EDS1-YFP in *N. benthamiana*, AvrRps4 protein steady state accumulation increased compared to expression of AvrRps4-CFP alone (Figure 21A). This stabilization effect seems to be specific for AvrRps4-interacting proteins since coexpression of MLA1-YFP did not have a stabilizing effect (Figure 21A). Furthermore, we found that EDS1 protein also seems to be stabilized through coexpression with either RPS4 or RRS1-R (Figure 21B). Immunoblot analysis of total protein

extracts showed higher accumulation of EDS1-CFP in the presence of YFP-RPS4 and RRS1-R-YFP. This effect appears specific for EDS1-interacting proteins since coexpression of MLA1-YFP did not have an effect on EDS1 protein accumulation (Figure 21B). It was recently shown that PopP2 stabilizes accumulation of its interacting partners RRS1-R and RRS1-S *in planta* (Tasset et al, 2010). The authors suggest that the interaction of PopP2 and RRS1-R/S may block a molecular mechanism that leads to RRS1-R/S proteasome-dependent degradation. It has still to be determined whether this is also the case for the stabilization effect of AvrRps4 and EDS1 protein accumulation.

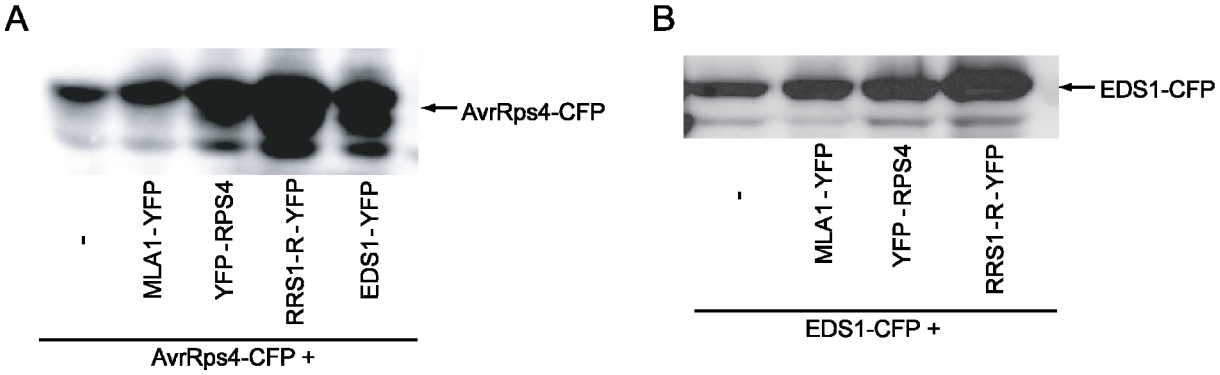


Fig. 21: AvrRps4 and EDS1 proteins are stabilized by coexpression of their interacting partners.
(A) Immunoblot analysis showing that AvrRps4-CFP protein is stabilized upon coexpression with YFP-RPS4, RRS1-R-YFP and EDS1-YFP. Fusion proteins were transiently expressed via *Agrobacterium* in *N. benthamiana* epidermal cells. Leaf samples were harvested 48hpi and subjected to immunoblot analysis with α-GFP antibody. Experiment was repeated twice with similar results. **(B)** Immunoblot analysis showing that EDS1-CFP protein is stabilized upon coexpression with YFP-RPS4 and RRS1-R-YFP. Fusion proteins were transiently expressed via *Agrobacterium* in *N. benthamiana* epidermal cells. Leaf samples were harvested 48hpi and subjected to immunoblot analysis with α-GFP antibody. Experiment was repeated twice with similar results.

Taken together, the data show potential physical interaction between AvrRps4 and EDS1 as well as between EDS1 and RPS4 or RRS1-R in nuclei. An interaction of AvrRps4 with RPS4 and RRS1-R is suggested by the FRET/FLIM study although it is not clear whether this interaction is mediated by an intermediate, such as EDS1. Also, *N. benthamiana* harbours an EDS1 homolog that might mediate the interaction of AvrRps4 and RPS4 or RRS1. Since Col-0 harbours the RRS1-S allele which is sufficient to confer resistance to *Pst* AvrRps4, it would be interesting to assess in future experiments if RRS1-S also interacts with AvrRps4 and EDS1. Also, CFP lifetime was measured only in plant nuclei, although EDS1 and AvrRps4 also localize to the cytoplasm and RPS4 to endomembranes. In follow-up experiments it would be interesting to assess if these interactions are also taking place in other subcellular compartments. Another important aspect to take into account is that AvrRps4 is cleaved *in planta* by an unknown protease into an N- and a C-terminal part, whereas only the C-terminus

induces a resistance response. CFP was fused C-terminally to AvrRps4 so that only interactions of AvrRps4 C-terminus were analyzed. In future experiments, interactions of AvrRps4 N-terminus should be explored as well to shed light on AvrRps4 function in the plant cell.

2.3.4 Verification of AvrRps4-EDS1 interaction in Arabidopsis

The FRET/FLIM interaction studies were performed upon transient expression of the fusion proteins in *N. benthamiana*. It is crucial to verify these interactions in Arabidopsis, since transient expression of Arabidopsis proteins in *N. benthamiana* is an artificial system. In a first experiment, I wanted to verify the interaction of AvrRps4 and EDS1 by performing a Co-Immunoprecipitation (Co-IP) in stable transgenic Arabidopsis lines. For that, I used lines expressing *AvrRps4-HA* under the control of the Dexamethasone-inducible promoter in Col-0 background. Material for Co-IP was harvested 2 days after Dexamethasone induction since AvrRps4 expression is at its maximum at this timepoint (data not shown). As negative controls for this experiment, I used lines expressing *TIR1-HA-StrepII* (*TIR1-HS*), an auxin receptor, under control of its native promoter in Col-0 as well as Col-0 wt plants that do not express the HA-epitope tag. Co-IP was performed on total plant extract of ~3 week old plants with α-HA antibody. Input and eluate fractions were subject to SDS-PAGE and Western Blot (Figure 22).

Figure 22 shows that the pulldown of AvrRps4-HA as well as TIR1-HS was successful since both proteins show a strong enrichment in the eluate fraction. In order to determine if EDS1 was co-immunoprecipitated, I probed the fractions with α-EDS1 antibody and could detect a strong signal in the eluate of *pDex::AvrRps4-HA* but no signal in the negative controls *pTIR1::TIR1-HS* and Col-0. This result indicates that AvrRps4 and EDS1 are also part of the same complex in Arabidopsis. However, EDS1 protein levels were higher in the input fraction of *pDex::AvrRps4-HA* compared to *pTIR1::TIR1-HS* and Col-0 since AvrRps4 expression in resistant plants induces EDS1 accumulation. In future experiments, EDS1 protein levels in the different input fractions should be adjusted, e.g. by inducing EDS1 accumulation through *Pst* AvrRps4 infection of the control lines.

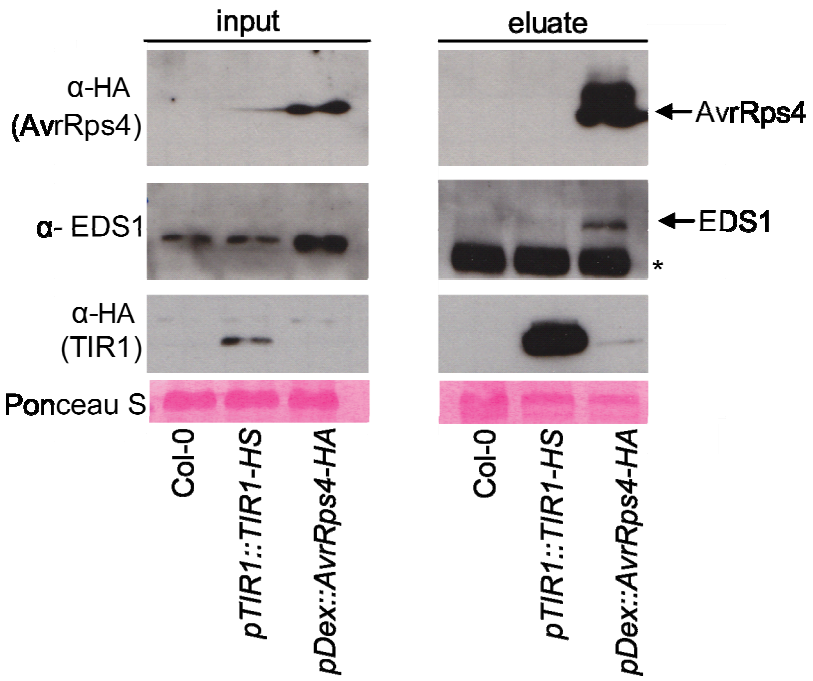


Fig. 22: Co-Immunoprecipitation of AvrRps4 and EDS1 from transgenic Arabidopsis plants.
Co-IP with α-HA agarose beads from total extracts of wildtype Col-0, *pTIR1::TIR1-HS* Col-0 and *pDex::AvrRps4-HA* Col-0 plants. Crude extracts (left panel, input) and immunoprecipitated protein (right panel, eluate) were detected with α-HA and α-EDS1 antibodies. Ponceau staining was performed to ensure equal loading. Asterisk= unspecific band.

2.3.5 Verification of EDS1-RPS4 interaction in Arabidopsis

To verify the interaction of RPS4 and EDS1 in Arabidopsis, I used *p35S::RPS4-HS* Col-0 plants that were grown at 28°C for ~3 weeks and then shifted to 19°C to induce a resistance response (Wirthmueller et al., 2007). The temperature shift from 28°C to 19°C of the *35S-RPS4-HS* Col-0 line resembles defense activation through *Pst* AvrRps4 infection since several well characterized *EDS1*-dependent defense marker genes are up- or downregulated after temperature shift that are also induced or repressed after *Pst* AvrRps4 infection (Bartsch et al., 2006; Garcia et al., 2010). Plant material for Co-IP was harvested 8h after temperature shift. RPS4 is mainly associated with endomembranes but also localizes to the plant nucleus (Wirthmueller et al., 2007). To address the question in which subcellular compartment RPS4 and EDS1 may interact, I performed a Co-IP of the soluble and microsomal fractions. For this, the microsomal fraction was separated by ultracentrifugation from the soluble cell extract and from both fractions a pulldown with α-HA antibody was performed. As negative control, the *pTIR1::TIR1-HS* line was included. Furthermore, I included the *pDex::AvrRps4-HA* line in order to test whether AvrRps4 and EDS1 are interacting as well in the microsomal fraction.

Figure 23 shows that EDS1 protein could be detected in the total, soluble and in small amounts in the microsomal fraction of the input. AvrRps4 protein was also detected in all fractions whereas RPS4 accumulated in the total and microsomal fraction and only to very low amounts in the soluble fraction. TIR1 was found in the total and soluble fraction of the input.

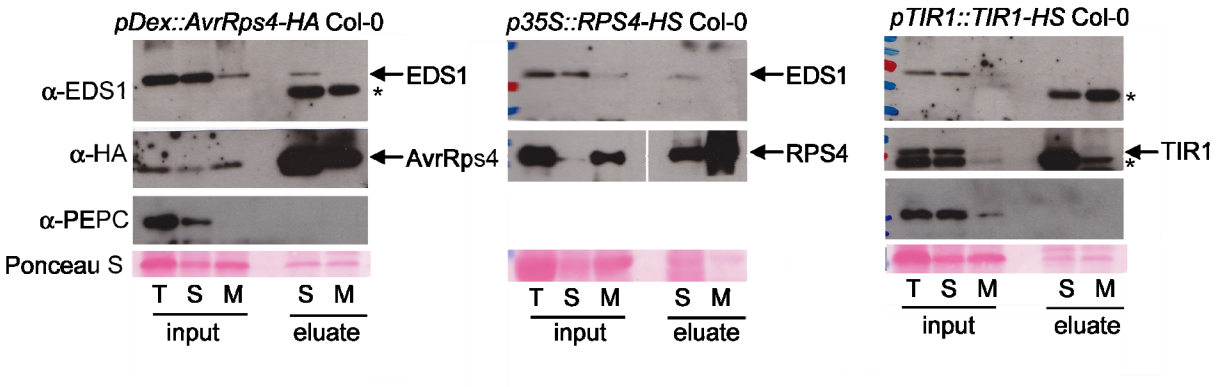


Fig. 23: Co-Immunoprecipitation of AvrRps4 with EDS1 and EDS1 with RPS4 from transgenic Arabidopsis plants. Co-IP with α-HA agarose beads from soluble and microsomal fractions of *pDex::AvrRps4-HA* Col-0*, p35S::RPS4-HS* Col-0 and *pTIR1::TIR1-HS* Col-0 plants. Immunoblot analysis was performed of total (T), soluble (S) and microsomal (M) fractions of the input and immunoprecipitated proteins of the soluble (S) and microsomal (M) fractions (eluate). Membranes were probed with α-HA, α-PEPC and α-EDS1 antibodies. Ponceau staining was performed to ensure equal loading. Asterisk= unspecific band.

As a marker for the cleanness of the microsomal fraction, the soluble protein PEPC was used. RPS4 protein was purified from the soluble and microsomal fraction. Interestingly, I was able to co-purify EDS1 together with RPS4 from the soluble fraction. Furthermore, AvrRps4 accumulated in both the soluble and microsomal fraction and EDS1 co-purified with AvrRps4 in the soluble fraction. The negative control TIR1 was found in the soluble fraction but EDS1 did not co-immunoprecipitate with this protein.

Taken together, I could show that EDS1 and RPS4 associate in one complex in the soluble fraction of Arabidopsis leaf extracts. Furthermore, I could confirm the Co-IP of AvrRps4 and EDS1 in the soluble fraction. In our FRET/FLIM experiments, an interaction of EDS1 and RPS4 as well as EDS1 and AvrRps4 was measured in the plant nucleus. To verify these interactions in the nucleus, a Co-IP of the nuclear fraction should be performed. However, total extract contains nuclear protein as well (data not shown) so that the co-purification of EDS1 and AvrRps4 from total plant extract might be a result of a nuclear interaction.

Another question that should be addressed is whether AvrRps4 and RPS4 are interacting in the absence of EDS1 or if this interaction is mediated by EDS1. So far, attempts to co-purify AvrRps4 and RPS4 in Arabidopsis leaf extracts were unsuccessful (data not shown). Another important aspect that should be addressed in future experiments is the dynamics of these

interactions. Is EDS1 the virulence target of AvrRps4 or is this interaction a consequence of AvrRps4 recognition by RPS4 and RRS1? Are EDS1 and RPS4 interacting before or after pathogen stimulus? Furthermore, it would be informative to know if AvrRps4, EDS1 and RPS4 are part of a higher order complex or if they are part of separate complexes that may even reside in different subcellular compartments.

3 DISCUSSION

3.1 Nuclear localization of AvrRps4 is required to induce full disease resistance

Induction of an immune response against infection by microbial pathogens is a highly dynamic process that requires the spatial and temporal regulation of defense components (Deslandes and Rivas, 2011). Many recent publications emphasize the role of nucleo-cytoplasmic partitioning in regulation of an appropriate resistance response (Burch-Smith et al., 2007; Shen and Schulze-Lefert, 2007; Wirthmueller et al., 2007; Garcia et al., 2010; Slootweg et al., 2010). Several effector proteins from diverse pathogens are targeted to the plant nucleus (Szurek et al., 2002; Deslandes et al., 2003; Schornack et al., 2010). Despite recent advances, little is known about the influence of the subcellular localization of an effector protein on the initiation of a resistance response. Therefore, the aim of this part of my thesis was to analyze in which subcellular compartment AvrRps4 induces a resistance response and how this relates to RPS4 distribution and signalling.

3.1.1 Enhanced nuclear export of AvrRps4 causes attenuated resistance

A C-terminal fusion to mYFP revealed that AvrRps4 localizes to the cytoplasm and the nucleus when expressed *in planta* (Figure 2A). Addition of a functional or mutated NLS or NES to AvrRps4-mYFP could redirect the AvrRps4 C-terminus to the respective compartment in *N. benthamiana* and Arabidopsis plants (Figure 2A). Stable expression of these AvrRps4 constructs under control of the constitutive CaMV 35S promoter in *eds1-2* plants did not cause morphological defects (Figure 3A). By contrast, the stable overexpression of AvrRps4-mYFP and AvrRps4-mYFP-NLS in an *EDS1* containing background caused severely dwarfed plants that eventually died (Figure 4A). The *EDS1*-dependency of this phenotype suggests that it is caused by an activated immune response that is induced upon AvrRps4 overexpression. Enhanced nuclear export of AvrRps4 in AvrRps4-mYFP-NES overexpressing lines did not cause severe dwarfism although these plants were smaller than Col-0 wt plants.

These results indicate that the AvrRps4 C-terminus needs to localize to the plant nucleus to induce a strong resistance response, as measured by inhibition of plant growth. Nevertheless,

constitutive overexpression of a bacterial protein in the host plant might lead to secondary consequences not directly related to effector recognition. To circumvent this, I followed a complementary approach in which the AvrRps4 variants were secreted via the bacterial type III secretion system. *Pst* DC3000 bacteria expressing AvrRps4-HA-NES grew to >10-fold higher levels than bacteria expressing AvrRps4-HA in the accessions Ws-0 and Col-0 (Figure 6A,B). Furthermore, I could show that the reduced resistance response to AvrRps4-HA-NES also correlates with an attenuated cell death response (Figure 9).

Taken together, these results show that enhanced nuclear export of the AvrRps4 C-terminus causes attenuated resistance but not full susceptibility in the host. The residual resistance response to AvrRps4-NES might have several reasons: i) the AvrRps4-NES fusion protein resides for a short time in the plant nucleus before it is exported to the cytoplasm and this may be sufficient to induce a partial defense response or ii) the cytoplasmic AvrRps4 C-terminus might also contribute to full resistance or iii) a shuttling of AvrRps4 C-terminus between the cytoplasm and the nucleus might be required to induce a full resistance response. So far, none of these possibilities can be excluded. I favour the first hypothesis since a NES sequence promotes export of nuclear proteins to the cytoplasm which implies that AvrRps4 needs to reside briefly in the nucleus before it is exported. This would suggest that at least one essential step of RPS4/RRS1-mediated AvrRps4 recognition occurs in the nucleus.

Little is known about how the subcellular localization of an effector protein influences the initiation of resistance in the host. A recent study investigated the role of the subcellular distribution of the *Potato Virus X* (PCX) coat protein (CP) for the function of its cognate receptor protein (Slootweg et al., 2010). PVX CP is recognized in potato (*Solanum tuberosum*) by the CC-NB-LRR receptor Rx1 that has a nucleo-cytoplasmic distribution, as does the elicitor. Forcing PVX CP to the nucleus abolished activation of Rx1 suggesting that Rx1 needs to be activated in the cytoplasm where elicitor recognition also takes place (Slootweg et al., 2010). Another study showed that the tobacco (*Nicotiana tabacum*) resistance protein N recognizes its cognate elicitor, the *Tobacco Mosaic Virus* (TMV) protein p50 as well in the cytoplasm (Burch-Smith et al., 2007). However, these studies solely utilized the HR (localized programmed cell death) induction as an indicator of recognition and measure elicitor recognition upon transient *Agrobacterium*-mediated expression in the host plant. This could lead to potential artefacts since it might not resemble the localization of the elicitor proteins in a natural infection situation. More evidence on how localization of an effector influences the recognition through its cognate R protein comes from the *Ralstonia solanacearum* effector PopP2. This bacterial protein is recognized in resistant Arabidopsis

accessions through the nuclear localized TIR-NB-LRR receptor RRS1-R (Deslandes et al., 2002). PopP2 contains a predicted NLS and localizes to the plant nucleus where it physically interacts with RRS1-R (Tasset et al., 2010). Deletion of the PopP2 NLS prevents its own nuclear accumulation as well as the nuclear localization of RRS1-R and does not trigger RRS1-R-mediated defense (Deslandes et al., 2003; Lahaye, 2004).

Analogous to these results, one possibility could be that AvrRps4-NES prevents nuclear accumulation of RRS1 and thereby abolishes RRS1-mediated resistance. The localization of RRS1 upon coexpression with AvrRps4-NES has to be determined in future experiments. Another possibility is that AvrRps4 might interact with a host protein or its cognate R proteins in the cytoplasm and a subsequent shuttling of this complex into the nucleus might induce a complete resistance response. A further scenario is that AvrRps4 C-terminus targets a host protein in the nucleus and thus activates RPS4 and RRS1 which is consistent with the guard hypothesis. Alternatively, AvrRps4 might directly interact with RPS4 and/or RRS1 in the nucleus which leads to resistance activation. The enforced nuclear export of AvrRps4 C-terminus might prevent its interaction with its target or cognate R proteins which may explain why nuclear localization of AvrRps4 is required to induce full resistance.

3.1.2 AvrRps4 targeted to the nucleus induces resistance but fails to elicit a cell death response

The AvrRps4 C-terminus targeted to the nucleus induces resistance as shown by growth inhibition of plants constitutively overexpressing AvrRps4-mYFP-NLS as well as by restriction of multiplication of bacteria expressing AvrRps4-HA-NLS (Figure 4, 6). Surprisingly, I found that AvrRps4-HA-NLS fails to elicit a cell death response even though it leads to full resistance (Figure 9). Quantitative transcription profiling after pathogen infection revealed that the genes *PR1* and *RPS4* were induced by AvrRps4-HA but not by AvrRps4-HA-NLS infection (Figure 10). By contrast, *EDS1* gene expression was induced by both AvrRps4-HA and AvrRps4-HA-NLS and *DND1* and *RRS1* genes were both repressed by infection with the AvrRps4 variants. It is interesting that all defense genes tested show transcriptional reprogramming after infection with AvrRps4-HA but only a subset with AvrRps4-HA-NLS. Thus, there might be specific sets of genes that are induced or repressed for a resistance response and others whose gene products are associated with cell death induction. Transcriptional upregulation of *PR1* expression is widely used as a marker for SA-promoted resistance activation and was found to be upregulated after infection with avirulent

bacteria (Bartsch et al., 2006). However, the data suggest that resistance can be uncoupled from *PR1* induction since AvrRps4-HA-NLS induces resistance but *PR1* levels are not induced. Alternatively, *PR1* expression might be induced earlier after infection with AvrRps4-HA-NLS and might already be back to uninduced state at 24h. Therefore, in future experiments, it is important to analyze transcriptional reprogramming very early after infection and to compare the timings of transcriptional reprogramming and cell death induction. Furthermore, more genes have to be analyzed including ones whose gene products are associated with cell death, for example by performing a gene expression microarray. Also, it would be highly informative to measure SA levels in plants infected with bacteria expressing AvrRps4-HA or AvrRps4-HA-NLS since SA plays a role in ETI whereas the exact function of PR1 is not known (Mishina and Zeier, 2007).

Taken together, these results suggest an uncoupling of cell death induction and bacterial growth restriction since AvrRps4 targeted to the nucleus induces resistance but fails to elicit a cell death response. Induction of HR is thought to restrict pathogen colonization (Jones and Dangl, 2006). However, it is not clear whether an HR is an essential component of disease resistance mechanisms in plants. Recently, it was shown that two type I metacaspases, AtMC1 and AtMC2, antagonistically control programmed cell death in Arabidopsis (Coll et al., 2010). AtMC1 positively regulates cell death whereas AtMC2 is a negative regulator of cell death. Mutation of *AtMC1* can almost completely suppress induction of HR through intracellular immune receptors but does not lead to increased susceptibility of the plant. Thus, these results also show an uncoupling of HR and pathogen growth restriction. This observation is reminiscent to the analysis of the potato Rx receptor protein that mediates resistance to *Potato Virus X* (PVX) without the induction of HR, a phenomenon named `extreme resistance` (Bendahmane et al., 1999). The authors argue that extreme resistance occurs if resistance is activated early in the infection cycle and if there is a high affinity between the elicitor and its receptor. In a different study it was shown that Rx is also capable of inducing an HR response depending on the viral strain and host genetic background. This further supports the idea that there is no qualitative difference between Rx-mediated and other NB-LRR-mediated resistance, but rather that the response is very rapid (Baures et al., 2008).

Based on these results, it is possible that recognition of AvrRps4-NLS induces a defense response that is extremely rapid and robust, arresting the bacteria before cell death occurs. If *PR1* expression is indeed induced earlier after infection with bacteria expressing AvrRps4-NLS, this would support this hypothesis. It might be informative to analyze if AvrRps4-NLS is capable of inducing a systemic acquired resistance (SAR) response. SAR leads to broad-

spectrum resistance towards otherwise virulent pathogens (Durrant and Dong, 2004). For many years it was anticipated that HR is required to induce an effective SAR response (Durrant and Dong, 2004). However, recent results show that extreme resistance mediated by Rx leads to induction of SAR against subsequent TMV infections without cell death being required (Liu et al., 2010). If AvrRps4-NLS is able to induce SAR might indicate if AvrRps4-NLS triggered resistance is caused by a similar mechanism as the Rx-mediated extreme resistance.

A possible explanation may be that during a natural infection of the plant with *Pseudomonas* bacteria, AvrRps4 C-terminus enters the nucleus at later stages of the infection process. Forcing AvrRps4 C-terminus to the nucleus already from the beginning of the infection process by fusing it to a NLS sequence might induce a very rapid and robust defense response without HR being required. Unfortunately, due to technical constraints, it is not yet possible to visualize the secretion of an effector protein into the plant cell in lifetime.

Another possibility is that enforced nuclear localization of the AvrRps4 C-terminus might enhance its affinity to a nuclear receptor early in the infection process which induces a rapid defense response resulting in resistance before cell death occurs. Alternatively, a shuttling of the AvrRps4 C-terminus between the nucleus and the cytoplasm might be required to induce a full cell death response. Finally, AvrRps4-NLS might interfere with a cell death inducing factor in the nucleus and thus suppress cell death induction. Whatever might be the precise mechanism of AvrRps4 recognition, the AvrRps4 mis-localization experiments show that nuclear AvrRps4 can fully suppress bacterial proliferation without cell death being required.

3.1.3 RPS4 and RRS1 are both required to confer resistance to mis-localized AvrRps4

I further tested whether recognition of mis-localized AvrRps4 still requires RPS4 and RRS1 for resistance by analyzing the susceptibility of the *rps4* and *rrs1* mutants in Col-0 and Ws-0 accessions (Figure 6A,B). One possibility could be that enforced nuclear localization of AvrRps4 might abolish the requirement of RPS4 for resistance induction. A possible scenario is that RPS4 might be activated in the cytoplasm through recognition of the cytoplasmic AvrRps4 pool and then translocates into the nucleus to activate downstream components involved in the defense response. Enforced nuclear accumulation of AvrRps4 might already activate the nuclear downstream defense components and circumvent the need for RPS4 to translocate into the nucleus for activation of a defense response.

When infected with *Pst* DC3000 expressing either AvrRps4-HA-NLS or AvrRps4-HA-NES no significant changes in bacterial proliferation compared to *Pst* DC3000 AvrRps4-HA were observed in *rps4* and *rrs1* mutants in both accessions (Figure 6A,B). This suggests that the reduced resistance response to bacteria expressing AvrRps4-HA-NES depends on RPS4 and RRS1. Furthermore, these results indicate that enforced localization of AvrRps4 to a specific subcellular compartment does not circumvent the need for either RPS4 or RRS1 in resistance. They also show that both allelic variants of *RRS1* in Col-0 and Ws-0 are involved in the resistance response to mis-localized AvrRps4 variants.

I also tested whether RPS4 and RRS1 function additively or cooperatively in response to mis-localized AvrRps4 variants by infecting the *rps4-21/rrs1-1* double mutant in Ws-0 background with bacteria expressing the different AvrRps4 variants (Figure 7B). It was previously shown that mutation of both *R* genes in Ws-0 did not have an additive effect in susceptibility to *R. solanacearum*, *C. higginsianum* or *Pst* AvrRps4 suggesting that RPS4 and RRS1 function cooperatively in response to these distinct pathogens (Narusaka et al., 2009). No significant differences in susceptibility of the double mutant compared to the single mutants were observed when infected with AvrRps4-HA, AvrRps4-HA-NLS or AvrRps4-HA-NES (Figure 7B). This indicates that the modes of action of RPS4 and RRS1 are not altered upon AvrRps4 mis-localization and suggests that RPS4 and RRS1 function cooperatively in response to nuclear or cytoplasmic enriched AvrRps4.

Depletion of the nuclear RPS4 pool by mutation of its intrinsic C-terminal NLS correlates with loss of RPS4 resistance to *Pst* AvrRps4 (Wirthmueller et al., 2007). Upon infection with *Pst* DC3000 expressing either AvrRps4-HA, AvrRps4-HA-NLS or AvrRps4-HA-NES, I could not observe significant differences in susceptibility of the RPS4-nls mutant (Figure 7A). This suggests that nuclear localization of RPS4 is required to trigger immunity to nuclear and cytoplasmic localized AvrRps4. The nuclear RPS4 pool might thus be required for its signalling function but not for recognition of AvrRps4. Alternatively, mutation of the NLS could alter the biochemical properties of RPS4 and thus affect the resistance-inducing function of RPS4. Results obtained from transient expression of YFP-RPS4-NES in *N. tabacum* argue against this hypothesis since this construct that contains a non-mutated NLS failed to induce HR (L. Wirthmueller, unpublished).

Taken together, these results indicate that both *RPS4* and *RRS1* are required to confer resistance to mis-localized AvrRps4 and that the reduced resistance response to bacteria expressing AvrRps4-HA-NES depends on *RPS4* and *RRS1*. Furthermore, enforced localization of AvrRps4 to a specific subcellular compartment does not affect the modes of

action of RPS4 and RRS1 which seem to function cooperatively. Forcing AvrRps4 into the nucleus induces full RPS4/RRS1-mediated resistance. Thus, recognition of AvrRps4 through RPS4 and RRS1 in the cytoplasm and subsequent shuttling of the protein complex into the nucleus for induction of resistance can be excluded. This is in contrast to Rx1-mediated resistance since forcing PVX CP to the nucleus abolished activation of Rx1 suggesting that the receptor needs to be activated in the cytoplasm (Slootweg et al., 2010).

3.1.4 Model of AvrRps4-triggered resistance

Based on my results in the context of published data, I suggest the following model to describe a possible mechanism of AvrRps4-triggered resistance. Initially, AvrRps4 is secreted into the plant cell via the type III secretion system. In the cell, it is cleaved by an unknown protease into an N-terminal and a C-terminal part and only the C-terminus that consists of 88 aa induces a resistance response (Sohn et al., 2009). Cleavage of AvrRps4 protein is not required to induce resistance since a mutant of AvrRps4 (R112L) that is not cleaved still induces an immune response (Sohn et al., 2009). Rather, a nuclear localization of the AvrRps4 C-terminus is needed for resistance induction since AvrRps4 C-terminus targeted to the cytoplasm fails to induce a full resistance response. The non-cleaved mutant protein of AvrRps4 (R112L) still localizes to the nucleus and the cytoplasm when transiently expressed in *N. benthamiana* (data not shown) suggesting that cleavage of AvrRps4 protein does not alter its subcellular localization in the cell. I found that the N-terminus of AvrRps4 localizes exclusively to the cytoplasm in Arabidopsis when fused to mYFP (Supplementary Figure 2). In preliminary experiments, I observed that the N-terminus might have a virulence activity which I measured as enhancement of virulence when bacteria express the AvrRps4 N-terminus (Supplementary Figure 1). Thus, the N-terminus of AvrRps4 might execute its virulence activity in the cytoplasm and the C-terminus might induce a resistance response in the nucleus. Unexpectedly, the nuclear localized AvrRps4 C-terminus failed to elicit a cell death response although it induced full resistance as measured by restriction of bacterial growth. Thus, cell death induction is not the end point of resistance as it is not required for resistance to nuclear localized AvrRps4. This is reminiscent of the extreme resistance to TMV mediated by the NB-LRR receptor Rx that is characterized by a rapid and robust resistance response without HR induction. Thus, the AvrRps4 C-terminus targeted to the nucleus might induce a rapid resistance response which suggests that under natural conditions in which an HR is observed, the C-terminus enters the nucleus at a later stage of infection. The exact

mechanism of nuclear import of the AvrRps4 C-terminus is not known since it does not harbour an intrinsic NLS for active transport. Instead, it might passively diffuse into the nucleus as it is below the size exclusion limit of the nuclear pore complex. Another possibility is that the AvrRps4 C-terminus is transported through the nuclear pores by a `piggyback` mechanism together with a nuclear targeted host protein (Garcia and Parker, 2009). This in turn induces a resistance response mediated by RPS4 and RRS1 since both receptors are required to confer resistance to nuclear localized AvrRps4 C-terminus. A possible scenario is that resistance is activated upon direct interaction of RPS4 and /or RRS1 with AvrRps4 in the nucleus. Alternatively, AvrRps4 might target a host protein in the nucleus which leads to activation of RPS4 and RRS1 and defense induction.

3.2 Modes of action of RPS4 and RRS1

Early research in plant pathology revealed the gene-for-gene relationship in which a pathogen avirulence (*Avr*) gene is recognized by its corresponding Resistance (*R*) gene (Flor, 1971). Further characterization of several R and Avr proteins suggests that resistance is often not activated upon a simple ligand-receptor association as predicted by the gene-for-gene model. Recent advances indicate that an emerging theme in the field is resistance mediated by pairs of *NB-LRR* genes (Eitas and Dangl, 2010). However, *RPS4* and *RRS1* represent the only *R* gene pair so far that induces resistance to three distinct pathogens, namely *Colletotrichum higginsianum*, *Ralstonia solanacearum* and *Pst* AvrRps4 (Gassmann et al., 1999; Deslandes et al., 2002; Birker et al., 2009; Narusaka et al., 2009). Analysis of RPS4 and RRS1 function might provide deeper insights into the molecular mechanisms required for pathogen perception and might reveal common principles underlying resistance to diverse pathogens.

3.2.1 Evidence that RPS4 and RRS1 act cooperatively

The modes of action of pairs of *NB-LRR* genes in resistance can be very different. The two NB-LRR receptors RPP2A and RPP2B are both required to determine isolate-specific resistance to *Hyaloperonospora arabidopsidis* and are thought to function cooperatively (Sinapidou et al., 2004). RPP2A and RPP2B may become activated by multiple effector proteins since there was no evidence that they recognize a single effector. By contrast, the *TIR-NB-LRR* gene *TAO1* contributes additively, with *RPM1*, to resistance to *Pst* expressing the effector protein AvrB (Eitas et al., 2008). RPS4 and RRS1 are both activated upon perception of the *R. solanacearum* effector PopP2, the *P. syringae* effector AvrRps4 and a so far unknown effector protein from *C. higginsianum* (Gassmann et al., 1999; Deslandes et al., 2003; Birker et al., 2009; Narusaka et al., 2009). Examples of resistance mediated by pairs of NB-LRR receptors are not restricted to TIR-type receptors since the CC-NB-LRR pair Pi5-1 and Pi5-2 mediates resistance to a specific isolate of *Magnaporthe oryzae* (Lee et al., 2009a).
For *RPS4* and *RRS1* it was shown that mutation of both genes in Ws-0 does not lead to increased susceptibility compared to the single mutants to all three pathogens indicating that these two NB-LRR receptors might function cooperatively (see Chapter 2.1.5) (Narusaka et al., 2009). I also found that both *RPS4* and *RRS1* are involved in cell death induction by AvrRps4 and that mutation of both genes did not lead to decreased cell death suggesting a cooperative action (Figure 8C). I analyzed the potential cooperativity between RPS4 and

RRS1 in Col-0 since this accession is resistant to *Pst* AvrRps4 and harbours a different allelic recognition variant of *RRS1* that might affect the mode of action of RPS4 and RRS1 (Birker et al., 2009; Narusaka et al., 2009). Furthermore, RPS4-mediated resistance and associated *EDS1*-dependent defense reprogramming have been characterized in Col-0 (Wirthmueller et al., 2007; Garcia et al., 2010).

Silencing of *RRS1* in a Col-0 *rps4-2* background via RNAi effectively reduced *RRS1* mRNA (Figure 13A). Nevertheless, the *RRS1-RNAi* lines had low residual *RRS1* expression. Susceptibility of the *RRS1-RNAi* lines to *Pst* AvrRps4 was similar to that exhibited by the *rps4-2* and *rrs1-11* single mutants (Figure 13C). The absence of an additive susceptible phenotype suggests that RPS4 and RRS1 also function cooperatively in Col-0 background. Nonetheless, the residual *RRS1* expression in *RRS1-RNAi* lines might be sufficient for RRS1 function in AvrRps4-triggered resistance.

Further evidence for a cooperative action between the RPS4 and RRS1 NB-LRR receptors came from stable transgenic Arabidopsis lines overexpresing RPS4 in an *rrs1-11* mutant background. Constitutive overexpression of RPS4 in Col-0 led to reduced plant size which is indicative of a constitutively activated plant defense that is caused by transcriptional reprogramming of *EDS1*-dependent defense genes (Figure 14, 15) (Wirthmueller et al., 2007). The stunted morphology caused by RPS4 overexpression was completely dependent on *EDS1* since *p35S::RPS4-HA-StrepII* (*35S::RPS4-HS*) *eds1-2* plants showed wildtype-like morphology demonstrating that the RPS4 overexpression signal is transduced in an *EDS1*-dependent manner (Figure 14) (Wirthmueller et al., 2007). Reduced plant size in *35S::RPS4-HS* Col-0 was also *RRS1*-dependent since *35S::RPS4-HS rrs1-11* displayed wildtype-like growth although RPS4 protein accumulation was similar in all genetic backgrounds (Figure 14A, B).

Taken together, these data suggest that RRS1 operates coincidently or downstream of activated RPS4 since removal of one of the receptors seems to abolish the function of the other at least in terms of growth inhibition. In future experiments, it will be interesting to analyze whether all allelic variants of *RRS1* operate in a cooperative manner with *RPS4* or whether the different resistance capabilities of the *RRS1* variants are caused by different modes of action with *RPS4*. The head-to-head arrangement of *RPS4* and *RRS1* genes was also found for the closest *RPS4* and *RRS1* homologs (Gassmann et al., 1999). Evolutionary conservation of homologous gene pairs in a head-to-head arrangement might allow a cooperative action of these genes and thus might indicate that all allelic variants of *RRS1* might function in a cooperative manner with *RPS4* alleles.

For the Ws-0 accession it has been shown that RPS4 and RRS1 act cooperatively in response to *R. solanacearum*, *C. higginsianum* and *Pst* AvrRps4 (Narusaka et al., 2009). It is not known yet if all allelic variants of *RRS1* act cooperatively with *RPS4* in response to all three pathogens.

A cooperative function of two NB-LRR receptors might have several reasons. The two NB-LRR receptors RPP2A and RPP2B function cooperatively to confer isolate-specific resistance to *Hyaloperonospora arabidopsidis* (Sinapidou et al., 2004). RPP2A has an unusual structure since it contains two potential but incomplete TIR-NB domains and a short LRR domain that lacks conserved motifs (Sinapidou et al., 2004). By contrast, RPP2B encodes a typical TIR-NB-LRR protein but requires RPP2A for pathogen recognition. Thus, the cooperative action of RPP2A and RPP2B might be caused by their structural features as they may provide a recognition or signalling function lacked by either partner protein (Sinapidou et al., 2004). Since *RPS4* and *RRS1* both encode functional R proteins this is probably not the cause for their potential cooperativity. One possibility might be that RPS4 and RRS1 act together at the DNA for transcriptional reprogramming of defense genes. RPS4 might regulate the association and dissociation of the RRS1 WRKY transcription factor domain with the DNA and thus regulate induction or repression of particular defense genes. In future experiments, it will be important to examine RRS1 binding sites on the DNA and to analyze whether RPS4 regulates RRS1 binding or dissociation to particular sites on the DNA.

The cooperative activity of pairs of NB-LRR receptors might be beneficial for the plant since this allows a higher diversity of recognition capabilities of a specific R protein. The combination of R proteins might allow recognition of several effector proteins which could explain how the relatively small number of R proteins can mediate recognition of a large set of pathogen effectors. Consistent with this, RPS4 and RRS1 are able to recognize three different effector proteins of diverse pathogens.

3.2.2 TIR domains of RPS4 and RRS1 physically interact

Further evidence for a potential cooperative action of RPS4 and RRS1 came from a Yeast-Two-Hybrid study. Our collaborators M. Bernoux and P. Dodds (CSIRO Canberra) found that the functional TIR domains of RPS4 and RRS1 homo- and hetero-dimerize in yeast (Figure 17). Furthermore, in preliminary experiments, we found that the TIR domains of RPS4 and RRS1 also interact in the plant nucleus as measured by FRET/FLIM analysis of transiently expressed protein domains in *N. benthamiana* (in collaboration with L. Deslandes and C.

Tasset; CNRS/INRA Toulouse). In future experiments, further controls have to be included to make sure that the reduction in TIR-RRS1-CFP lifetime is not due to nonspecific transfer of energy between the two fluorophores.

The TIR domains of plant R proteins are thought to have a signalling role in the plant immune response as was mostly measured by cell death induction (Frost et al., 2004; Swiderski et al., 2009; Krasileva et al., 2010; Bernoux et al., 2011). Recently, it was shown that the homo-dimerization of the TIR domain of the flax L6 NB-LRR receptor correlates with autoactivity *in planta*, suggesting that this is a key event in defense signalling (Bernoux et al., 2011). Another study showed that the TIR-NB-LRR receptor N from tobacco oligomerizes upon coexpresion with the elicitor p50 (Mestre and Baulcombe, 2006). This is reminiscent of animal PAMP-triggered immunity. Perception of PAMPs in animals is mediated by the extracellular LRR domain of Toll-like receptors (TLRs). This induces homo-dimerization of the intracellular TIR domain which provides a new scaffold that can bind to adapter proteins, such as MyD88 and initiate a defense response (Tapping, 2009). Thus, the homo-dimerization of the TIR domains of RPS4 and RRS1 might be a critical early event for activation and signalling of a defense response and might represent an interacting surface for downstream signalling components. Indeed it was previously shown that the TIR domain of RPS4 has a role in the cell death signalling pathway since overexpression of the functional TIR domain triggers an effector-independent cell death response in the plant (Swiderski et al., 2009). It was observed that the L6 TIR domain homo-dimerization is of low affinity and readily reversible *in vitro* suggesting that transient low-affinity oligomerization may be required for the regulation of R proteins as this might prevent inappropriate defense activation (Bernoux et al., 2011).

The hetero-dimerization of the TIR domains of RPS4 and RRS1 might reflect formation of a multi-protein complex consisting of several RPS4 and RRS1 molecules *in planta*. The formation of such a multi-protein complex might be required for AvrRps4 recognition or defense signalling and is a further indication for the cooperative activity of RPS4 and RRS1. The TIR sequence used for the interaction studies described here is from the *RRS1-R* allele from Nd-0 accession but this does not have sequence polymorphisms compared to the *RRS1-S* allele from Col-0. It would be interesting to analyze whether the TIR domains of all RRS1 allelic variants can form homo- and hetero-dimers with the RPS4 TIR domain. This might indicate if the mode of action of RPS4 and RRS1 is the same in response to all three distinct pathogens since different allelic RRS1 variants are required for resistance to different pathogens.

Another question that should be addressed in future experiments is whether the full length R proteins are also physically interacting. This might answer the question if RPS4 and RRS1 are interacting in their activated or resting state and thus if the interaction is the cause or consequence of AvrRps4 recognition. Accessibility of the TIR domain resembles an activated conformation of the R protein and addition of NB-ARC or NB-ARC-LRR domains might inhibit TIR domain interactions (van Ooijen et al., 2007). It is not clear whether the interaction of RPS4 and RRS1 TIR domains is a functional requirement. In mammals, it was shown that NLRs can form hetero-oligomers (Damiano et al., 2004). The interaction of NLRP1 and NOD2 for example is required for the defense induction upon perception of bacterial peptidoglycan (Pan et al., 2007).

Furthermore, it would be interesting to assess if the interaction of the TIR domains is restricted to the plant nucleus or might also occur in the cytosol. RRS1 was so far shown to localize exclusively to the nucleus when coexpressed with the *R. solanacearum* effector PopP2 but subcellular distribution of RRS1 when expressed alone could not yet be determined. RPS4 in contrast exhibits also endomembrane localization in addition to its nuclear accumulation and the endomembrane-associated pool of RPS4 might have a distinct function in immunity and possibly be part of other resistance complexes. However, it was shown that RPS4 needs to localize to the nucleus to induce a resistance response (Wirthmueller et al., 2007) which suggests that the hetero- and homo-dimerization of the TIR domains might be restricted to the plant nucleus. Nevertheless, it is not yet clear whether RPS4 is activated in the nucleus. However, since a shuttling of RPS4 to the nucleus after pathogen infection was not observed (Wirthmueller et al., 2007), it is more likely that RPS4 is also activated in the nucleus.

3.2.3 Evidence that RPS4 and RRS1 have distinct functions in plant immunity

Not all my data support simple cooperativity between RPS4 and RRS1. Constitutive overexpression of RPS4 in Col-0 leads to reduced plant size that was *RRS1*-dependent (Figure 14A). The reduced plant size is indicative of a constitutively activated plant defense and quantitative transcriptional profiling revealed a transcriptional reprogramming of defense genes in this line (Figure 15). As expected, the expression of all genes tested was strongly *EDS1*-dependent (Figure 15). However, most of the genes tested were not strongly *RRS1*-dependent. Rather, a quantitative difference in expression was observed for certain genes. A clear explanation for the *RRS1*-dependency of the stunted morphology of *35S::RPS4-HS* Col-

0 could not be provided by this analysis because only a limited number of genes were tested. Based on the obtained data different scenarios are possible: i) RRS1 might regulate a specific subset of genes whose products are important for the stunted phenotype but were not tested here or ii) *rrs1* mutation might have a quantitative effect on transcriptional reprogramming of genes so that a certain threshold for the induction of the stunted phenotype is not reached. This is supported by the existing data. In this case, the threshold for the transcriptional reprogramming of defense genes might be different than for the suppression of the stunted morphology. A gene expression microarray of *35S::RPS4-HS* line in Col-0 compared to *rrs1-11* background should provide deeper insight to which genes are *RRS1*-dependent or whether *RRS1* is required for the timing or maintenance of defense gene induction or repression through RPS4.

To further position *RRS1* in the *RPS4*-mediated resistance pathway, I tested whether increased RPS4 levels can compensate for loss of *RRS1* in bacterial resistance. In response to virulent *Pst* DC3000 bacteria, the *35S::RPS4-HS* Col-0 line exhibited enhanced resistance suggesting that the basal resistance is increased due to RPS4 activation (Figure 16A). An increased basal resistance response was not observed for the *35S::RPS4-HS rrs1-11* line suggesting that a certain threshold of defense activation depends on *RRS1*. In response to *Pst* AvrRps4 bacteria, *35S::RPS4-HS rrs1-11* plants displayed increased susceptibility compared to *35S::RPS4-HS* Col-0 plants which was not statistically significant but reproducible (Figure 16B). However, *35S::RPS4-HS rrs1-11* plants were not as susceptible as *rrs1-11*. This suggests that *RPS4* still partially functions in the absence of *RRS1* but not as effectively as in the presence of *RRS1*. Thus, overexpression of one of the two R proteins cannot fully compensate for the loss of the other which suggests that they might have distinct attributes for the immune response. The generation of lines overexpressing RRS1 in an *rps4-2* mutant background might help to further dissect RPS4 and RRS1 functions.

Further evidence for the absence of a cooperative action came from the gene expression analysis of *RPS4* and *RRS1* genes after pathogen challenge. *RPS4* gene expression was induced after infection with *Pst* AvrRps4 whereas *RRS1* gene expression was repressed (Figure 11B). Thus, *RPS4* and *RRS1* do not seem to be co-regulated which suggests that RPS4 and RRS1 might not function in an entirely cooperative manner.

Taken together, these results suggest a more complex relationship between RPS4 and RRS1 than simple cooperativity. Overexpression of RPS4 initiates a defense response in the absence of RRS1 activation which might suggest that RPS4 operates downstream of RRS1. Alternatively, overexpression of one R protein might overcome the requirement for the

partner NB-LRR receptor. This is reminiscent of the NB-LRR pair N and NRG1 since overexpression of NRG1 induces a resistance response without N being required (Peart et al., 2005). It is also possible that the overexpression system does not resemble the natural situation of RPS4 and RRS1 function even though all activated defense responses were *EDS1*-dependent.

A different scenario is that a certain threshold of RPS4 and RRS1 protein accumulation is needed to induce resistance. For a cooperative action of two proteins, a strict stoichiometry of both proteins in the cell is required. Overexpression of RPS4 completely alters the stoichiometric relations of RPS4 and RRS1 in the cell which might disrupt their modes of action under natural conditions.

3.2.4 Model for RPS4 and RRS1 modes of action

Based on the obtained data, it is not possible to formulate a clear hypothesis for RPS4 and RRS1 function. In the following section, I will discuss hypothetical scenarios and will put them into the context of published data.

The presence of a WRKY transcription factor domain of RRS1 suggests that one function of RRS1 is the regulation of defense gene expression through direct association with the DNA. Indeed, a DNA binding activity of RRS1 has been shown (Noutoshi et al., 2005). Mutation of one amino acid in the WRKY domain in the *slh1* mutant leads to incapability of RRS1 to bind to DNA. This mutant shows constitutive activated defense response suggesting that RRS1 acts as a repressor of defense genes as suggested for many WRKY transcription factors (Eulgem and Somssich, 2007). However, a transcriptional null mutant of *RRS1* does not show constitutive activated defense. One possibility could be that in the complete absence of RRS1 protein, another WRKY transcription factor takes over RRS1 function and suppresses defense gene induction. A redundant function has indeed been shown for some WRKY transcription factors (Xu et al., 2006).

A possible scenario is that RPS4 regulates the dissociation of the RRS1 WRKY domain from the DNA after AvrRps4 perception and thus induces defense activation. Alternatively, RPS4 might also redirect RRS1 to other sites on the DNA to regulate defense genes. The hetero-dimerization of RPS4 and RRS1 TIR domains in the nucleus would be consistent with this model. The accessibility of the TIR domain resembles the activated state of the R protein which suggests that RPS4 and RRS1 are interacting in their activated state. This in turn might induce RRS1 dissociation off the DNA or a redirection to other sites on the DNA and

subsequent reprogramming of defense gene expression. However, it is equally possible that the hetero-dimerization of the TIR domains induces a downstream signalling response that mediates RRS1 dissociation off the DNA or binding to other sites on the DNA. Thus, in an *rps4* mutant, RRS1 might stay associated with the DNA which prevents defense activation and results in susceptibility of the plant.

Interestingly, the *rps4-21/rrs1-1* mutant still allows residual resistance activation upon infection with *Pst* AvrRps4. This suggests that another R protein is involved in AvrRps4 recognition and resistance signalling which is probably another TIR-NB-LRR-type R protein since AvrRps4-triggered immunity is completely *EDS1*-dependent. Recently, it was shown that the *TIR-NB-LRR* gene *SNC1* contributes to AvrRps4-triggered resistance in the absence of *RPS4* at a certain temperature (Kim et al., 2010). However, susceptibility of *rps4-2/snc1-1* was not complete suggesting that additional factors contribute to AvrRps4-triggered immunity. A double mutant of *rrs1-11/snc1-1* should be analyzed in the future.

These results suggest that *RPS4* and *SNC1* function in parallel to provide resistance at 22°C. The authors further showed that RPS4 and SNC1 both interact with SRFR1 (SUPPRESSOR OF *rps4*-RLD) in the microsomal compartment. SRFR1 is a negative regulator of RPS4-mediated resistance possibly through its interaction with the co-chaperone SGT1 (Li et al., 2010). Mutations in *SRFR1* also activate SNC1. In a resting state, SRFR1 might form a complex with RPS4 and SNC1 in the microsomal compartment and negatively regulate RPS4 and SNC1 function by preventing their nuclear translocation or maintaining them in an inactive conformation. To induce resistance, both RPS4 and SNC1 need to localize to the nucleus (Wirthmueller et al., 2007). This is supported by another publication where it was shown that the autoactive mutant of SNC1 (snc1) has a function in the nucleus since mutation of a Nup88 homolog, *MOS7*, reduced snc1 nuclear levels and diminished snc1 function (Cheng et al., 2009). SNC1 interacts via its TIR domain with the transcriptional co-repressor TPR1 (Zhu et al., 2010). TPR1 associates with histone deacetylase 19 *in vivo* and represses negative regulators of immunity that are *EDS1*-dependent thus probably activating the immune response. Similarly, RPS4 interacts via its TIR domain with RRS1, as well a proposed negative regulator of immunity, which might regulate defense gene expression.

A possible scenario of RPS4/RRS1-mediated resistance might be that RPS4 is part of different multi-protein complexes before and after pathogen stimulus and in different subcellular compartments. Before pathogen stimulus, RPS4 might reside in a complex with SRFR1 and SNC1 in the microsomal compartment that maintains RPS4 in an inactive state. After pathogen stimulus, the nuclear pool of RPS4 is required to induce a resistance response.

RPS4 might either regulate RRS1 association or dissociation of the DNA or redirect RRS1 to particular binding sites on the DNA which leads to a reprogramming of defense genes.

3.3 AvrRps4 complex formation with immune regulators

Pathogen effectors can be recognized by plant Resistance (R) proteins through different mechanisms. Recognition of an effector protein through direct interaction with its corresponding plant R protein has been shown only for a few cases (Jia et al., 2000; Deslandes et al., 2003; Dodds et al., 2006). In the case of direct recognition, effector and R proteins tend to show high levels of sequence polymorphism between alleles of the host and pathogen populations, reflecting the coevolutionary arms race between host and pathogen (Stukenbrock and McDonald, 2009). Another possibility is the indirect recognition of an effector which would conform to the guard hypothesis. The guard hypothesis proposes that an R protein guards a host protein that is targeted by an effector to enhance pathogen virulence. Effector-induced alterations of the target protein are perceived by R proteins so that they become activated and induce a defense response (Collier and Moffett, 2009). The mode of recognition of AvrRps4 by RPS4 and RRS1 is not known. Also, the virulence target of AvrRps4 was not yet identified. Analysis of AvrRps4 interaction partners *in planta* might provide deeper insights to the mechanism of AvrRps4 recognition and function in the plant.
Although R proteins were first reported as plant immune receptors more than 15 years ago, how these proteins activate downstream defense responses is largely unknown. Identification of potential downstream signalling partners in plants will greatly enhance our knowledge of plant immunity. The analysis of AvrRps4 targets *in planta* and how these activate downstream resistance responses should provide deeper insights to this topic.

3.3.1 Nuclear AvrRps4 interacts with the central immune regulator EDS1 and its cognate R proteins RPS4 and RRS1

In collaboration with L. Deslandes and C. Tasset (CNRS/INRA Toulouse), I performed FRET/FLIM experiments after *Agrobacterium*-mediated expression of components of AvrRps4-triggered immunity in *N. benthamiana*. I found that a significant reduction of the average CFP lifetime occurred in nuclei coexpressing AvrRps4-CFP and EDS1-YFP (Figure 18). This suggests that the AvrRps4 C-terminus and EDS1 physically interact in the plant nucleus. I could confirm that the AvrRps4 C-terminus and EDS1 are part of the same complex in Arabidopsis by co-purifying AvrRps4-HA and EDS1 from total plant extract (Figure 22). Based on these data, it is possible that EDS1 is the virulence target of AvrRps4.

Furthermore, I found that coexpression of AvrRps4-CFP with RRS1-R-YFP or YFP-RPS4 also led to a significant reduction in CFP lifetime in the nucleus suggesting that AvrRps4 C-terminus is part of one complex with RPS4 and RRS1-R in the plant nucleus (Figure 19). So far, attempts to co-purify AvrRps4 and RPS4 from Arabidopsis were not successful (data not shown). The interaction of AvrRps4 with RPS4 and RRS1-R might be mediated by an intermediate, such as EDS1. For example, in the FRET/FLIM experiments, the *N. benthamiana* EDS1 homolog bridges the interaction of AvrRps4 with RPS4 and RRS1-R. Another interesting observation we made is that AvrRps4 protein seems to be stabilized by RPS4, RRS1-R and EDS1. Upon transient *Agrobacterium*-mediated coexpression of AvrRps4-CFP with either YFP-RPS4, RRS1-R-YFP or EDS1-YFP in *N. benthamiana*, AvrRps4 protein steady state accumulation increased compared to expression of AvrRps4-CFP alone (Figure 21A). A similar observation was made for the TIR-NB-LRR receptor N that is stabilized upon coexpression with its cognate elicitor p50 (Mestre and Baulcombe, 2006). Furthermore, we found that EDS1 protein also seems to be stabilized through coexpression with either RPS4 or RRS1-R (Figure 21B). It was recently shown that PopP2 stabilizes accumulation of its interacting partners RRS1-R and RRS1-S *in planta* (Tasset et al., 2010). The authors suggest that the interaction of PopP2 and RRS1-R/S may block a molecular mechanism that leads to RRS1-R/S proteasome-dependent degradation. It has still to be determined whether this is also the case for the stabilization effect of AvrRps4 and EDS1 protein accumulation.

In future experiments, the *RRS1-S* allele should be analyzed as well since this allelic variant confers resistance to *Pst* AvrRps4 with the Col-0 *RPS4* allele (Birker et al., 2009; Narusaka et al., 2009). Another open question that should be addressed is how RPS4 and RRS1 can recognize several distinct effectors. An interaction of EDS1 with PopP2 was not found in FRET/FLIM experiments, suggesting that the recognition mechanism of PopP2 is different from the recognition of AvrRps4.

An important aspect that has to be taken into account is that AvrRps4 is cleaved *in planta* whereas only the C-terminal part induces a resistance response (Sohn et al., 2009). Since the fusion proteins of AvrRps4 for FRET/FLIM analysis and Co-IP were C-terminally fused to either a fluorophore or the HA-epitope, only interactions of the AvrRps4 C-terminus were analyzed. In future experiments, interactions of the AvrRps4 N-terminus should be explored as well since preliminary data show that the N-terminus might have a virulence activity (Supplementary Figure 1). How this would connect molecularly to C-terminal recognition would be a point of interest.

3.3.2 EDS1 physically interacts with RPS4 and RRS1 in the plant nucleus

In order to analyze further whether the interactions of AvrRps4 C-terminus with RPS4 and RRS1-R are direct or mediated by EDS1, I explored a physical interaction between RPS4 or RRS1-R and EDS1. In FRET/FLIM experiments, I found that EDS1 physically interacts with RPS4 and RRS1-R in the plant nucleus (Figure 20). An interesting observation in the FRET/FLIM experiments was made when EDS1-CFP was coexpressed with either YFP-RPS4 or RRS1-R-YFP. Without EDS1 coexpression, the YFP fluorescence was evenly distributed in the nucleus whereas upon EDS1 coexpression, YFP fluorescence was detectable in speckles at the nuclear periphery. This observation is reminiscent of a study describing the localization of the NLRP3 inflammasome before and after immune induction (Zhou et al., 2011). Resting NLRP3 localizes to the endoplasmic reticulum whereas upon inflammasome activation, NLRP3 localizes to the perinuclear space. A possible scenario might be that EDS1 redirects the localization of RPS4 and RRS1 to the nuclear periphery possibly to regulate defense gene expression: It is a widely accepted model that subnuclear localization of chromatin is important for gene expression. Active genes have been found to be associated with nuclear pores (Heessen and Fornerod, 2007; Dillon, 2008) and nuclear pore proteins were found to be involved in transcriptional regulation (Mendjan et al., 2006).

To confirm whether interaction of EDS1 and RPS4 occurs in Arabidopsis, I performed a Co-IP of *p35S::RPS4-HA-StrepII* (*35S::RPS4-HS*) Col-0 plants after temperature shift and found that EDS1 and RPS4 are present in the same complex in the soluble plant extract (Figure 23). Temperature shift of the *35S::RPS4-HS* Col-0 line from 28°C to 19°C resembles a defense activation which suggests that EDS1 and RPS4 are interacting after RPS4 activation. Nevertheless, it is not clear whether the temperature shift completely resembles an infection with *Pst* AvrRps4. Ideally, in future experiments it has to be determined whether this interaction occurs before and/or after infection with *Pst* AvrRps4. Also, the interaction of EDS1 with RRS1-R and RRS1-S has to be measured. The difference in the recognition capabilities of the *RRS1* allelic variants might be caused by different binding affinities to EDS1 due to the amino acid sequence variation of RRS1-R and RRS1-S. Furthermore, it would be interesting to analyze whether EDS1 interacts with the TIR domains of RPS4 and RRS1 since the TIR domain of R proteins has a function in defense signalling (Swiderski et al., 2009; Bernoux et al., 2011).

Since defense signalling mediated by TIR-NB-LRR-type receptors is *EDS1*-dependent, the finding of an interaction of EDS1 with RPS4 and RRS1 might indicate that this is the link to

signal emission. EDS1 might have the function of a signalling adapter mediating signal transmission. In mammals, signal transduction mediated by NLRs depends on the recruitment of specific adapter proteins. Intriguingly, some NLRs are part of higher order signalling complexes together with adapter proteins, termed inflammasomes (Schroder and Tschopp). The best characterized inflammasome to date is the NLRP3 inflammasome that consists of NLRP3, the adapter ASC and caspase-1. Upon NLRP3 activation, NLRP3 oligomerizes which mediates an interaction with the adapter protein ASC that in turn leads to activation of caspase-1 (Schroder and Tschopp). Thus, this higher order complex provides a molecular platform that controls immune activation.

RPS4 and RRS1 might be part of a similar `resistasome` higher order complex with EDS1 as adapter protein. Upon pathogen stimulus, the TIR domains of RPS4 and RRS1 might form homo- and hetero-oligomers which mediates their interaction with EDS1. EDS1 in turn might regulate defense gene expression through association with RPS4 and RRS1 and transcription factors. Analysis of the dynamics of this potential `resistasome` complex should provide further insights to the mechanism of AvrRps4-triggered resistance. Convergence of numerous TIR-NB-LRR receptor activities on EDS1 raises the question whether EDS1 might act as an adapter for all TIR-NB-LRRs. Since the TIR domain is thought to be required for signalling, this implies that EDS1 interacts with multiple TIR domains of diverse TIR-NB-LRR receptors. Given that the sequence of the TIR domain is diverse among different receptors, a direct interaction with EDS1 is unlikely for all cases. The differential responses mediated by distinct NB-LRR receptors might be explained in part by the selective usage of adapter proteins as it is the case for animal receptors (Akira et al., 2006).

In a recent study, it was shown that the TIR-NB-LRR gene SNC1 contributes to AvrRps4-triggered immunity in the absence of RPS4 at 22°C (Kim et al., 2010). Furthermore, the authors found that RPS4 and SNC1 both interact with SRFR1 in the microsomal compartment and hypothesize that this negatively regulates RPS4 and SNC1 function. Based on these results, another possibility is that RPS4 cooperates with SNC1 in response to AvrRps4. Since both receptors need to localize to the nucleus for resistance function (Wirthmueller et al., 2007; Cheng et al., 2009), a physical interaction of RPS4 and SNC1 should be explored in this compartment. Furthermore, an interaction of EDS1 and SNC1 should be measured since SNC1-mediated resistance is *EDS1*-dependent (Li et al., 2001).

Nevertheless, the data suggest that defense signalling in plants might as well be mediated by higher order complexes as it is the case for animal innate immunity. A systems biology approach using mathematical modelling of crystallography, biochemistry and protein

interaction data further supports that the cell is to a large extent regulated by highly discrete yet dynamic protein complexes in which individual proteins can make several interactions (Gibson, 2009).

3.3.3 Possible mechanisms of AvrRps4 recognition and resistance induction

Based on the obtained data, three different models might explain the observed interactions (Figure 24). The described mechanisms of AvrRps4 recognition only refer to the C-terminus of AvrRps4 since only interactions of the AvrRps4 C-terminus were analyzed. In the first scenario, EDS1 is the virulence target of AvrRps4 in the plant cytoplasm and forms a pre-recognition complex with AvrRps4 in this compartment (Figure 24A). This interaction induces a relocalization of EDS1 into the nucleus with AvrRps4 being transported by a `piggyback` mechanism with EDS1 import. In the plant nucleus, an interaction of EDS1/AvrRps4 with RPS4 and RRS1 occurs which activates both R proteins. This in turn might lead to *EDS1*-dependent defense gene reprogramming. This model is consistent with results from Garcia et al (2010) that show increased EDS1 steady state accumulation in the nucleus after infection with *Pst* AvrRps4. Furthermore, conditional release of EDS1 to nuclei correlated with transcriptional reprogramming of *EDS1*-dependent defense genes (Garcia et al., 2010). A similar mechanism was proposed for the tobacco TIR-NB-LRR receptor N that mediates resistance to the replicase p50 protein of *Tobacco Mosaic Virus* (TMV). The elicitor p50 interacts in the cytoplasm with NRIP1. This pre-recognition complex is recognized by N through direct association with its TIR domain. Upon activation, cytoplasmic N is either translocated to the nucleus or activates the N nuclear pool that mediates resistance (Burch-Smith et al., 2007; Caplan et al., 2008).

In the second scenario, EDS1 is constitutively guarded by RPS4 and RRS1 in the plant nucleus (Figure 24B). AvrRps4 enters the nucleus through an unknown mechanism or simple diffusion and targets EDS1 for virulence promotion. This in turn activates RPS4 and RRS1 and leads to *EDS1*-dependent defense gene reprogramming. In contrast to the first scenario, EDS1 resides here in a complex with RPS4 and RRS1 already before the pathogen stimulus. The association of the R proteins with their guardee might keep them in an inactive state until pathogen effectors activate them. This is reminiscent of the plasma-membrane localized RIN4 protein that is guarded by the two R proteins, RPM1 and RPS2, (Mackey et al., 2002; Mackey et al., 2003) illustrating that the same effector target can be monitored by different R proteins.

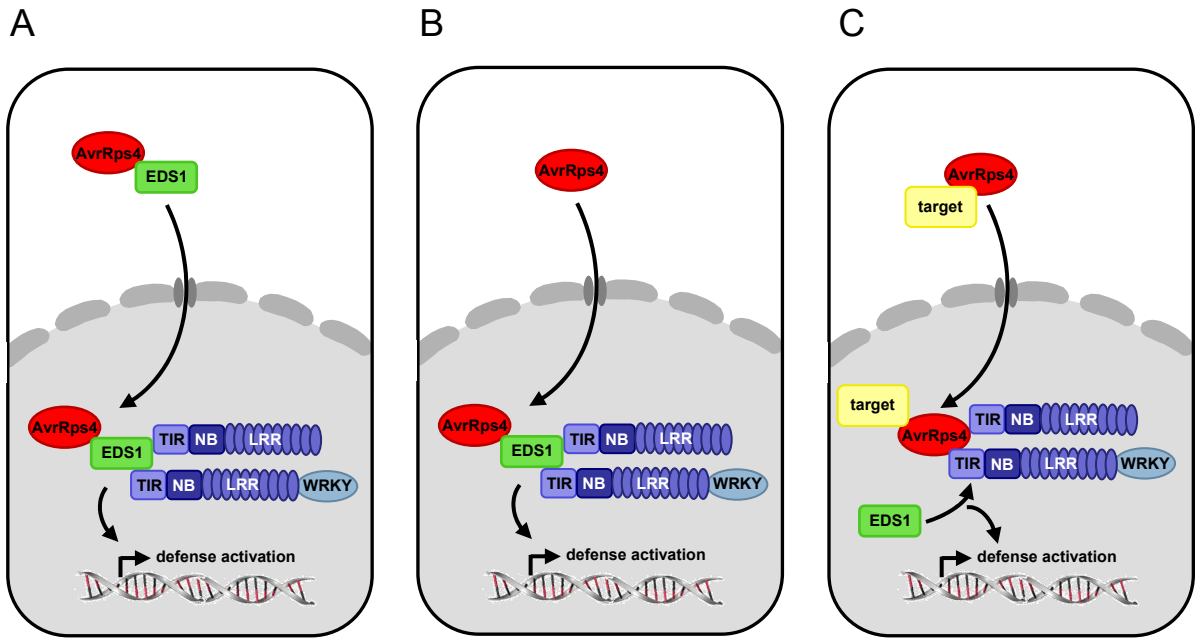


Fig. 24: Models of AvrRps4 C-terminus recognition and defense induction. (A) AvrRps4 C-terminus targets EDS1 in the plant cytoplasm which leads to the formation of a pre-recognition complex that is re-localized to the nucleus. In the nucleus, EDS1 interacts with RPS4 and RRS1 which in turn activates both R proteins and leads to defense gene reprogramming. **(B)** EDS1 is guarded by RPS4 and RRS1 in the nucleus. AvrRps4 C-terminus enters the nucleus and targets EDS1. This subsequently activates RPS4 and RRS1 and leads to activation of a resistance response. **(C)** AvrRps4 C-terminus targets an unknown protein in the nucleus which leads to its direct recognition by RPS4 and RRS1. EDS1 is recruited to this complex to mediate resistance activation.

The first two scenarios are consistent with the guard hypothesis since AvrRps4 is recognized not through direct interaction with its cognate R proteins but through its interaction with EDS1. However, it is not known whether AvrRps4 modifies EDS1 protein which in turn leads to activation of RPS4 and RRS1. EDS1 might also function as a `decoy` and exclusively be required for perception of AvrRps4. This is a rather unlikely scenario since EDS1 is required for resistance mediated by most TIR-NB-LRRs and has a role in basal defense (Parker et al., 1996; Aarts et al., 1998; Feys et al., 2001; Wirthmueller et al., 2007). Furthermore, mutation of a `decoy` has by definition no effect on immunity in the absence of the cognate R protein whereas mutation of *EDS1* renders plants hypersusceptible to virulent and avirulent biotrophic and hemi-biotrophic pathogens (Parker et al., 1996; Aarts et al., 1998; Feys et al., 2001; van der Hoorn and Kamoun, 2008).

In the third scenario, AvrRps4 has an unknown virulence target either in the cytoplasm which leads to subsequent transport together with the target into the nucleus or in the plant nucleus (Figure 24C). In the nucleus, it is directly recognized through RPS4 and RRS1 which are then activated upon AvrRps4 interaction. This in turn leads to a direct interaction of RPS4 and RRS1 with EDS1 which promotes proximity of AvrRps4 to EDS1. In this scenario,

interaction of AvrRps4 and EDS1 is the consequence of AvrRps4 recognition whereas in the other two scenarios, AvrRps4 targets EDS1 which leads to resistance activation. A straightforward way to test which possibility might be most fitting, is to test whether the KRVY mutant of AvrRps4 interacts with EDS1. The AvrRps4 KRVY mutant has the amino acids KRVY at the beginning of the AvrRps4 C-terminus substituted with alanine and does not induce a defense response (Sohn et al., 2009). An interaction of EDS1 with this AvrRps4 mutant might discriminate whether AvrRps4 and EDS1 are interacting as a consequence of recognition. Consistent with all three models are my results showing that nuclear localization of AvrRps4 is required to induce a full defense response (Chapter 3.1).

The role of EDS1 as a defense signalling `hub` makes it an attractive target for pathogen effectors since interfering with EDS1 function is highly incapacitating for the plant innate immune system. If this were the case, EDS1 would be expected to be targeted by several distinct effector proteins and this is yet unknown.

3.4 Future perspectives

The results of this study showed that the *P. syringae* effector AvrRps4 needs to localize to the nucleus to induce a full RPS4/RRS1-mediated resistance response. There is evidence that RPS4 and RRS1 function cooperatively in response to AvrRps4. One hypothesis is that RPS4 regulates RRS1 binding to particular sites on the DNA and thus mediates defense gene reprogramming. A gene expression microarray of Arabidopsis lines overexpressing RPS4 in Col-0 and the *rrs1-11* mutant backgrounds in the temperature shift system will reveal whether RRS1 is required for the timing or maintenance of defense gene reprogramming through RPS4. In addition, Chromatin Immunoprecipitation (ChIP) experiments in combination with a whole genome microarray should reveal RRS1 binding sites to the DNA and clarify whether RRS1 association or dissociation to particular sites is regulated by RPS4 or the downstream regulator EDS1. Both homo- and hetero-dimerization of the TIR domains of RPS4 and RRS1 was observed in the plant nucleus which might indicate the formation of a multi-protein complex for resistance. In future experiments, the dynamics of this interaction should be explored.

I showed that EDS1 can form a complex with RPS4 or RRS1 in the nucleus in transient expression assays. These data provide a potential direct link between TIR-NB-LRR-type receptors and *EDS1*-dependent defense induction and suggest that important recognition and signalling events can occur inside the plant nucleus. However, it is crucial to analyze the dynamics of this potential `resistasome` complex to determine whether formation of particular interactions is required for effector recognition or downstream signalling. Also, certain proteins might be part of distinct complexes in different subcellular compartments. For example, RPS4 is forms distinct associations in the microsomal and nuclear compartments (Wirthmueller et al., 2007; Kim et al., 2010) but the dynamics and functional importance of these different configurations for resistance signalling still have to be explored. Affinity purification of RPS4 and subsequent mass spectrometric analysis of its interaction partners from different subcellular compartments before and after pathogen stimulus should reveal the dynamics of these complexes and provide a link to gene expression reprogramming dynamics (Seebacher and Gavin, 2011). This will help us to understand how the fine control of cellular reprogramming is established and which events or molecules are decisive downtream components of ETI.

Furthermore, I showed that AvrRps4 C-terminus interacts with EDS1 in the nucleus, suggesting that EDS1 might be the virulence target of AvrRps4. Being a major regulator of

defense, EDS1 is an attractive target for pathogen effectors. Therefore, it will be interesting to analyze whether more effectors of diverse pathogens target EDS1. Also, potential targets of the N-terminus of AvrRps4 in the plant cytoplasm should be identified since preliminary data suggest that the N-terminus might have virulence activity. For these experiments, Arabidopsis lines expressing HA- and YFP-tagged AvrRps4 N-terminus that were generated within this study will be useful. This will improve our knowledge on AvrRps4 function in the host cell.

4 MATERIALS AND METHODS

4.1 Materials

4.1.1 Plant materials

4.1.1.1 *Arabidopsis thaliana* (hereafter Arabidopsis)

Arabidopsis wild-type, mutant, and transgenic lines used in this study are listed in Table 4.1, 4.2, and 4.3, respectively.

Table 4.1: Wild-type Arabidopsis lines used in this study

Accession	Abbreviation	Original Source
Columbia	Col-0	J. Dangl[a]
Wassilewskija	Ws-0	K. Feldmann[b]
RLD	RLD-0	W. Gassmann[c]

[a] University of North Carolina, Chapel Hill, NC, USA
[b] University of Arizona, Tucson, AZ, USA
[c] University of Missouri-Columbia, Columbia, MO, USA

Table 4.2: Mutant Arabidopsis lines used in this study

Mutant allele	Accession	Mutagen	Reference/ Source
eds1-2	Col-0 /(L*er*)[a]	FN	Bartsch et al., 2006
eds1-1	Ws-0	EMS	Parker et al., 1996
rps4-2	Col-0	T-DNA	Wirthmueller et al., 2007
rps4-21	Ws-0	T-DNA	K. Shirasu[b]
rrs1-11	Col-0	T-DNA	L. Deslandes[c]
rrs1-1	Ws-0	T-DNA	Y. Narusaka[d]
rps4-21/rrs1-1	Ws-0	T-DNA	Y. Narusaka[d]

[a] L*er eds1-2* allele introgressed into Col-0 genetic background, 8th backcrossed generation
[b] RIKEN Plant Science Center, Tsurumi, Yokohama 230-0045, Japan
[c] Laboratoire des Interactions Plantes-Microorganismes (LIPM), UMR CNRS-INRA, Castanet Tolosan, France
[d] Research Institute for Biological Sciences, 7549-1 Yoshikawa, Kibityuo, Kaga-gun, Okayama, Japan

FN: fast neutron; EMS: ethyl methane sulfonate; T-DNA: Transfer-DNA

Table 4.3: Transgenic *Arabidopsis* lines used in this study

Line	Accession	Construct	Reference/ Source
Dex-AvrRps4-HA	Col-0	*pDex::AvrRps4-HA*	Mackey et al, Cell 2003
TIR1-HS	Col-0	*pTIR1::TIR1-HA-StrepII*	Zhang et al., PNAS 2008
35S-RPS4-HS	Col-0	*p35S::RPS4-HA-StrepII*	Wirthmueller et al., 2007
35S-RPS4-HS eds1-2	Col-0	*p35S::RPS4-HA-StrepII*	Wirthmueller et al., 2007
RPS4-nls rps4-2	Col-0	*pRPS4::RPS4-HA-StrepII-nls*	Wirthmueller et al., 2007

4.1.1.2 *Nicotiana benthamiana*

Nicotiana benthamiana (310A) plants were obtained from T. Romeis (MPIZ, Cologne) and used for transient *Agrobacterium*-mediated transformation of leaf tissues.

4.1.2 Pathogens

Arabidopsis plants were infected with isogenic *Pseudomonas syringae* pv. *tomato* strains (DC3000) expressing different *Pseudomonas* effector proteins.

4.1.2.1 *Pseudomonas syringae pv. tomato* (*Pst*)

Pseudomonas syringae pv. *tomato* (*Pst*) strain DC3000 was obtained from R. Innes (Indiana University, Bloomington Indiana, USA) and used throughout this study.

Table 4.4: *Pseudomonas syringae* pv. *tomato* (*Pst*) strains generated in this study

Name	Strain	Construct
AvrRps4-HA	DC3000	*EDV6::pAvrRps4::AvrRps4-HA*
AvrRps4-HA-NLS	DC3000	*EDV6::pAvrRps4::AvrRps4-HA-NLS*
AvrRps4-HA-nls	DC3000	*EDV6::pAvrRps4::AvrRps4-HA-nls*
AvrRps4-HA-NES	DC3000	*EDV6::pAvrRps4::AvrRps4-HA-NES*
AvrRps4-HA-nes	DC3000	*EDV6::pAvrRps4::AvrRps4-HA-nes*

4.1.2.2 *Pseudomonas fluorescens* (*Pfo*)

Pseudomonas fluorescens (*Pfo*) strain was obtained from J. Dangl (University of North Carolina, Chapel Hill, NC, USA).

Table 4.5: *Pseudomonas fluorescens* (*Pfo*) strains generated in this study

Name	Strain	Construct
AvrRps4-HA	*Pfo*	*EDV6::pAvrRps4::AvrRps4-HA*
AvrRps4-HA-NLS	*Pfo*	*EDV6::pAvrRps4::AvrRps4-HA-NLS*
AvrRps4-HA-nls	*Pfo*	*EDV6::pAvrRps4::AvrRps4-HA-nls*
AvrRps4-HA-NES	*Pfo*	*EDV6::pAvrRps4::AvrRps4-HA-NES*
AvrRps4-HA-nes	*Pfo*	*EDV6::pAvrRps4::AvrRps4-HA-nes*

4.1.3 Bacterial strains

4.1.3.1 Escherichia coli strains

All *E. coli* strains were obtained from Invitrogen™ (Karlsruhe, Germany).

DH5α
Genotype: F- Φ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_{k-}, m_{k+})

*pho*A *sup*E44 λ- *thi*-1 *gyr*A96 *rel*A1

DH10B
Genotype: F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *end*A1 *ara*Δ139 Δ(*ara*, *leu*)7697 *gal*U *gal*K λ- *rps*L (Str^R) *nup*G

DB3.1
Genotype: F- *gyr*A462 *end*A Δ(*sr*1-*rec*A) *mcr*B *mrr hsd*S20 (r_B- m_B-) *sup*E44 *ara*14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20 (Str^R) *xyl*5 λ- *leu mtl*1

4.1.3.2 *Agrobacterium tumefaciens* strains

For stable transformation of *Arabidopsis* and transient expression in *N. benthamiana*, DNA constructs were transformed in *Agrobacterium tumefaciens* strain GV3101 carrying the helper plasmid pMP90 (with resistance to Rifampicin and Gentamycin) or the helper plasmid pMP90RK (with resistance to Rifampicin, Kanamycin and Gentamycin) (Koncz and Schell, 1986).

4.1.4 Vectors

The following vectors have been used or were generated in this study:

pEDV6	Gateway®-compatible vector carrying AvrRps4 N-terminus under control of the native promoter (kindly provided by Kee Hoon Sohn; Sainsbury Lab, Norwich)
pEDV6-AvrRps4-HA-NLS/nls/NES/nes	Bacterial expression vectors carrying AvrRps4 full length genomic sequence under control of the native promoter with C-terminal HA- tag and functional or non-functional localization signals; cloned into pEDV6 via PstI/XhoI
pEDV6-AvrRps4-N-terminus-HA	Bacterial expression vector carrying AvrRps4 N-terminus (aa 1-133) genomic sequence under control of the native promoter with C-terminal HA- tag; cloned into pEDV6 via PstI/XhoI
pXCSG-mYFP	Binary Gateway® destination vector for expression of a fusion protein under control of 35S promoter with a C-terminal mYFP tag
pXCSG-mYFP-NLS/nls/NES/nes	Binary Gateway® destination vectors for expression of a fusion protein under control of 35S promoter with a C-terminal mYFP tag carrying a functional or non-functional localization signals
pXCSG-AvrRps4-mYFP	Binary Gateway® destination vector for expression of AvrRps4 full length under control

	of 35S promoter with a C-terminal mYFP tag
pXCSG-AvrRps4-mYFP-NLS/nls/NES/nes	Binary Gateway® destination vectors for expression of AvrRps4 full length under control of 35S promoter with a C-terminal mYFP tag carrying a functional or non-functional localization signals
pXCSG-AvrRps4-N-terminus-mYFP	Binary Gateway® destination vector for expression of AvrRps4 N-terminus (aa 1-133) under control of 35S promoter with a C-terminal mYFP tag
pXCSG-AvrRps4-N-terminus-GGAA-mYFP	Binary Gateway® destination vector for expression of AvrRps4 N-terminus (aa 1-133) with G132 and G133 substituted with Alanine under control of 35S promoter with a C-terminal mYFP tag
pENTRY-RRS1-RNAi	AGRIKOLA Gateway® entry plasmid that contains a gene-specific sequence tag of *RRS1* (165bp)
pHellsgate	Binary Gateway® destination vector containing two Gateway cassettes that is designed for the expression of hairpin RNAs
pHellsgate-RRS1-RNAi	Binary Gateway® destination vector for the expression of hairpin RNAs of a gene-specific sequence tag of *RRS1*; cloned by double LR clonase reaction

4.1.5 Oligonucleotides

Primers used in this study are listed in Table 2.4. Oligonucleotides were purchased from Sigma-Aldrich (Deisenhofen, Germany), Operon (Cologne, Germany) or Metabion (Martinsried, Germany). Lyophilised primers were resuspended in ddH_2O to a final concentration of 100 pmol/µl (= 100 µM). Working solutions were diluted to 10 pmol/µl (=10 µM).

Table 4.6: Oligonucleotides used in this study

Name	Purpose	Sequence (5`→ 3`)
Cloning		
KH1-NES-XbaI	cloning pXCSG-mYFP-NES	AGTCTAGAGCTCTTACAGTGTTAGTCTTTCCAAAG GTGGTAATTGGAGCTTGTACAGCTCGTCCATGCCG
KH2-nes-XbaI	cloning pXCSG-mYFP-nes	AGTCTAGAGCTCTTACAGTGTTGCTCTTTCTGCAGG TGGTGCTTGGAGCTTGTACAGCTCGTCCATGCCG
AvrRps4-HA-XhoI-rev	cloning pEDV6-AvrRps4-HA	GATCTAGAGCTCTCATGCGTAATCAGGAACATCGT
AvrRps4-HA-NLS-XhoI-rev	cloning pEDV6-AvrRps4-HA-NLS	AGTCTAGAGCTCTTATCCTCCAACCTTTCTCTTCTT CTTAGGTGCGTAATCAGGAACATCG

AvrRps4-HA-nls-XhoI-rev	cloning pEDV6-AvrRps4-HA-nls	AGTCTAGAGCTCTTATCCTCCAACCTTTCTCTTCGTCTTAGGTGCGTAATCAGGAACATCG
AvrRps4-HA-NES-XhoI-rev	cloning pEDV6-AvrRps4-HA-NES	AGTCTAGAGCTCTTACAGTGTTAGTCTTTCCAAAGGTGGTAATTGGAGTGCGTAATCAGGAACATCG
AvrRps4-HA-nes-XhoI-rev	cloning pEDV6-AvrRps4-HA-nes	AGTCTAGAGCTCTTACAGTGTTGCTCTTTCTGCAGGTGGTGCTTGGAGTGCGTAATCAGGAACATCG
qRT-PCR		
RPS4 fw	qRT-PCR	CGGCTGCTCAACTTTTAAGG
RPS4 rev	qRT-PCR	GGCCTGGAATTTCCTCTAGC
RRS1 fw	qRT-PCR	CCACTAAACGCAAGGCTCTC
RRS1 rev	qRT-PCR	CTCCTCCATGTCCGTCATTT
UBIQ fw	qRT-PCR	AGATCCAGGACAAGGAGGTATTC
UBIQ rev	qRT-PCR	CGCAGGACCAAGTGAAGAGTAG
EDS1fw	qRT-PCR	CGAAGACACAGGGCCGTA
EDS1 rev	qRT-PCR	AAGCATGATCCGCACTCG
CBP60 fw	qRT-PCR	GGCGAGAAGTGAAGCTTTTG
CBP60 rev	qRT-PCR	GCGAAAATCCTTGACGGTTA
PBS3 fw	qRT-PCR	ACACCAGCCCTGATGAAGTC
PBS3 rev	qRT-PCR	CCCAAGTCTGTGACCCAGTT
ICS1 fw	qRT-PCR	TTCTGGGCTCAAACACTAAAAC
ICS1 rev	qRT-PCR	GGCGTCTTGAAATCTCCATC
FMO1 fw	qRT-PCR	GTTCGTGGTTGTGTGTACCG
FMO1 rev	qRT-PCR	TGTGCAAGCTTTTCCTCCTT
ERECTA fw	qRT-PCR	CATGGCCCTACGAAGAAAAA
ERECTA rev	qRT-PCR	TGGACGACTTCACGTCTCTG
DND1 fw	qRT-PCR	CTCCCATGGTGGTTCCTCTA
DND1 rev	qRT-PCR	ATCGATCCCAGTCGTTTGTC
PR1 for	qRT-PCR	TTCTTCCCTCGAAAGCTCAA

PR1 rev	qRT-PCR	AAGGCCCACCAGAGTGTATG
Genotyping		
rps4-mutR	detection of rps4-21 mutation	TTAACCATTCACAAAAGCAATCAACAG
rps4-mutF	detection of rps4-21 mutation	TAAGCTACCATTGAAAGAAGTTCG
JL271	detection of rrs1-1 mutation	ACATGAAGCCATTTACAATTGAATATATCC
RRS1-CR	detection of rrs1-1 mutation	TGATGGGTTTACAGTTTGGGGAGGACTGGTAATTG
NRS2-S8	detection of rrs1-1 mutation	TACAAAAAGCCATTAGAGATGTATCAGTATGCTAC C
W2-52-400F	detection of rrs1-11 mutation	TGTAGAAGAGATTGTGAGAGATGT
Fish 1rev	detection of rrs1-11 mutation	CTGGGAATGGCGAAATCAAGGCATC
RRS1 fw	genotyping rrs1-11 mutation homozygous	GGCTCGTCAACTTTTCTTGC
RRS1 rev	genotyping rrs1-11 mutation homozygous	CCTGAGCACGTTTGAAGGTT
Sequencing		
Agri 51	sequencing of pHellsgate insert	CAA CCA CGT CTT CAA AGC AA
Agri 56	sequencing of pHellsgate insert	CTG GGG TAC CGA ATT CCT C
Agri 64	sequencing of pHellsgate insert	CTT GCG CTG CAG TTA TCA TC
Agri 69	sequencing of pHellsgate insert	AGG CGT CTC GCA TAT CTC AT

4.1.6 Enzymes

4.1.6.1 Restriction endonucleases

Restriction enzymes were purchased from New England Biolabs (Frankfurt, Germany) unless otherwise stated. Enzymes were supplied with 10x reaction buffer which was used for restriction digests.

4.1.6.2 Nucleic acid modifying enzymes

Standard PCR reactions were performed using home made *Taq* DNA polymerase. To achieve high accuracy, *Pfu* polymerases were used when PCR products were generated for cloning. Modifying enzymes and their suppliers are listed below:

Taq DNA polymerase	home made
PfuTurbo® DNA polymerase	Stratagene® (Heidelberg, Germany)
T4 DNA ligase	Roche (Mannheim, Germany)

Alkaline Phosphatase, shrimp	Roche (Mannheim, Germany)
SuperScript™ II RNase H- Reverse Transcriptase	Invitrogen™ (Karlsruhe, Germany)
Gateway™ LR Clonase™ Enzyme mix	Invitrogen™ (Karlsruhe, Germany

4.1.7 Chemicals

Laboratory grade chemicals and reagents were purchased from Sigma-Aldrich (Deisenhofen, Germany), Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany), Invitrogen™ (Karlsruhe, Germany), Serva (Heidelberg, Germany), and Gibco™ BRL® (Neu Isenburg, Germany) unless otherwise stated.

4.1.8 Antibiotics (stock solutions)

Ampicillin (Amp)	100 mg/ml in ddH_2O
Carbenicillin (Carb)	50 mg/ml in ddH_2O
Gentamycin (Gent)	15 mg/ml in ddH_2O
Kanamycin (Kan)	50 mg/ml in ddH_2O
Rifampicin (Rif)	100 mg/ml in DMSO
Spectinomycin	10 mg/ml in ddH_2O
Tetracycline (Tet)	10 mg/ml in 70 % ethanol

Stock solutions (1000x; 100x for Spectinomycin) stored at -20° C. Aqueous solutions were sterile filtrated.

4.1.9 Media

Media were sterilised by autoclaving at 121° C for 20 min. For the addition of antibiotics and other heat labile compounds the solution or media were cooled to 55° C. Heat labile compounds were sterilised using filter sterilisation units prior to addition.

Escherichia coli media: Luria-Bertani (LB) broth or agar plates; SOC
Pseudomonas syringae media: NYG broth or agar plates
Agrobacterium tumefaciens media: YEB broth or agar plates
Arabidopsis thaliana media: MS (Murashige and Skoog).

4.1.10 Antibodies

Listed below are primary and secondary antibodies used for immunoblot detection.

Table 4.7: Primary antibodies used in this study

Antibody	Source	Dilution	Reference
α-EDS1	rabbit polyclonal	1:500	S. Rietz; J. Parker[a]
α-PEPC	rabbit polyclonal	1:500	Rocklands, Gilbertsville, PA, USA
α-Histone H3 (ab1791)	rabbit polyclonal	1:5000	Abcam (Cambridge, UK)
α-HA 3F10	rat monoclonal	1:2000	Roche (Mannheim, Germany)
α-GFP	mouse monoclonal	1:2000	Roche (Mannheim, Germany)

α-RIP1	rabbit polyclonal	1:2000	F. Kaschani and R. van der Hoorn; unpublished

[a] Max-Planck-Institute for Plant Breeding Research, Carl-von-Linné-Weg 10, 50829 Cologne, Germany

Table 4.8: Secondary antibodies used in this study

Antibody	Feature	Dilution	Source
goat anti-rabbit IgG-HRP	horseradish peroxidase conjugated	1:5000	Santa Cruz (Santa Cruz, USA)
goat anti-mouse IgG-HRP	horseradish peroxidase conjugated	1:5000	Santa Cruz (Santa Cruz, USA)
goat anti-rat IgG-HRP	horseradish peroxidase conjugated	1:5000	Santa Cruz (Santa Cruz, USA)

4.1.11 Buffers and solutions

Buffers and solutions are displayed in the following listing. Buffers and solutions not displayed in this listing are denoted with the corresponding methods. All buffers and solutions were prepared using Milli-Q water. Buffers and solutions for molecular biological experiments were autoclaved or sterilised using filter sterilisation units.

DEPC-H_2O
Diethylpyrocarbonate 0.1 % in H_2O
Shake vigorously, let stand O/N and autoclave 30 min.

DNA extraction buffer

Tris	200 mM
NaCl	250 mM
EDTA	25 mM
SDS	0.5 %
pH 7.5 (HCl)	

DNA gel loading dye (6x)

Sucrose	4 g
EDTA (0.5 M)	2 ml
Bromphenol blue	25 mg
dH_2O to 10 ml	

PCR reaction buffer (10x)

Tris	100 mM
KCl	500 mM
MgCl2	15 mM
Triton X-100	1 %
pH 9.0	

Ponceau S
Ponceau S working solution was prepared by dilution of ATX Ponceau S concentrate (Fluka) 1:5 in H_2O.

Running buffer (10x)

Tris	30.28 g
Glycine	144.13 g
SDS	10 g
dH_2O to 1000 ml	
Do not adjust pH.	

Sample buffer (2x)	Tris	0.125 M
	SDS	4 %
	Glycerol	20 % (v/v)
	Bromphenol blue	0.02 %
	Dithiothreitol (DTT)	0.2 M
	pH 6.8	
TAE buffer (50x)	Tris	242 g
	EDTA	18.6 g
	Glacial acetic acid	57.1 ml
	dH_2O to 1000 ml	
	pH 8.5	
TBS-T buffer	Tris	10 mM
	NaCl	150 mM
	Tween 20	0.05 %
	pH 7.5 (HCl)	
TE buffer	Tris	10 mM
	EDTA	1 mM
	pH 8.0 (HCl)	
Transfer buffer (10x)	Tris	58.2 g
	Glycine	29.3 g
	SDS (10 %)	12.5 ml
	dH_2O to 1000 ml	
	pH 9.2	
	Before use dilute 80 ml 10x buffer with 720 ml H_2O and add 200 ml methanol.	

4.2 Methods

4.2.1 Maintenance and cultivation of Arabidopsis plants

Arabidopsis seeds were sown directly onto moist compost (Stender, Schermbeck, Germany) containing 10 mg l-1 Confidor® WG 70 (Bayer, Germany). Seeds were vernalised at 4° C for 48 h in the dark covered with a propagator lid. Afterwards the seeds were transferred to a plant growth chamber and maintained under short day conditions (10 h photoperiod, light intensity of approximately 200 µEinsteins m^{-2} sec^{-1}, 22° C and 65 % humidity). 3-5 days post germination, propagator lids were removed. To obtain progeny, three week old plants were transferred to long day conditions (16 h photoperiod) and allowed to flower. To collect seeds, aerial tissue was enveloped with a paper bag and sealed with tape at its base until siliques were ripe.

4.2.2 Generation of Arabidopsis F_1 and F_2 progeny

To emasculate individual flowers, fine tweezers and a magnifying-glass were used. Only flowers that had a well-developed stigma but immature stamen were used for crossing to

prevent self-pollination. Fresh pollen from three to four independent donor stamens was dabbed onto each single stigma. F_1 seed were harvested from mature siliques and allowed to dry. F_1 seeds were grown as described above and allowed to self pollinate. Produced F_2 seeds were collected and stored.

4.2.3 *Agrobacterium*-mediated stable transformation of Arabidopsis (floral dip)

The method for *Agrobacterium*-mediated stable transformation of Arabidopsis was adapted from (Clough and Bent, 1998). Approximately 10 Arabidopsis plants were grown in 9 cm square pots (3 pots for each transformation) under short day conditions for 4 - 5 weeks. To induce flowering, the plants were shifted to 16 h photoperiod conditions. First inflorescence shoots were cut off as soon as they emerged to induce the growth of more inflorescences. When plants had a maximum number of young flower heads, plants were used for transformation. For that, *Agrobacterium* was streaked out onto selective YEB plates containing appropriate antibiotics and was grown at 28 °C for 3 days. Bacteria were scraped off the plates and resuspended in 5 % sucrose to OD_{600} ~ 0.8. Silwet L-77 (Lehle seeds, USA) at 500 µl/l was added as surfactant. Plants to be transformed were inverted in the cell-suspension ensuring all flower heads were submerged. Plants were agitated slightly to release air bubbles and left in the solution for approximately 5 sec. Plants were removed and dipping was repeated as before. Plants were placed overnight into the glasshouse away from direct light covered in plastic bags and sealed with tape. Afterwards, bags were removed and pots were moved to direct light and left to set seed.

4.2.4 Arabidopsis seed surface sterilization

Previous to *in vitro* growth of Arabidopsis, seeds were sterilized. Seeds were distributed in 1.5 ml open microcentrifuge tubes inside a desiccator jar together with a beaker containing 100 ml of 12 % hypochlorite solution ("chlorine bleach"). 10 ml of 37 % HCl was added directly into the hypochlorite solution to allow chlorine gas to be produced. The lid of the desiccator was immediately closed and vacuum was generated by connecting to a vacuum pump and incubated for 4 – 8 h. After the sterilisation period, seeds were quickly closed and removed from the jar, then placed in a sterile hood and let stand for 15 min in the opened vessel. Sterilized seed were spread out on suitable culture media and vernalized for 48 h at 4°C in the dark. Alternatively, the seeds were sterilized using columns from DNA/RNA prep kits by subsequent incubations and washes with ethanol 70% for 2 min followed by ethanol 95% for 1 min. Afterwards, tubes were centrifuged for 1 min to remove all ethanol and seeds were dried under the sterile hood.

4.2.5 Maintenance of *P. syringae* pv. *tomato* and *Pseudomonas fluorescens* cultures

Pseudomonas syringae pv. *tomato* and *Pseudomonas fluorescens* strains were streaked onto selective NYG agar plates from -80° C DMSO or glycerol stocks. Streaked plates were incubated at 28° C for 72 h before storing at 4° C and refreshed weekly.

4.2.6 *P. syringae* pv. *tomato* growth assay

P. syringae cultures of the denoted strains were started from bacteria grown on NYG agar plates. One day before infection, bacterial strains were restreaked on NYG agar plates containing the appropriate antibiotics and incubated ON at 28°C. For spray-infection, the

concentration of bacteria was adjusted to 1 x 10^7 cfu/ml in 10 mM MgCl2 containing 0.04 % Silwet L-77 (Lehle seeds, USA) if not otherwise stated. For bacterial growth assays, single pots with five plants grown under short day conditions for 4-5 weeks, were used. Two hours before spray-infection, plants were watered and kept under a dH_2O-humidified lid to allow opening of stomata. Plants were spray-infected with a dispenser and kept under a dH_2O-humidified lid for 3 hours. Day zero (d0) samples were taken 3-4 hours after spray-infection by using a cork borer (d= 0.6 cm). 3 parallel samples each with 3 leaf discs were taken from 5 independent plants and transferred to a 1.5 ml centrifuge tube, resulting in a total excised area of ~1 cm^2. Bacterial titers were determined by shaking leaf discs from infected leaves in 10 mM $MgCl_2$ supplemented with 0,01% Silwet L-77 at 28°C for 1 h (Tornero and Dangl, 2001; Garcia et al., 2010). 20µl of the resulting bacterial suspension were plated on NYG agar plates containing the appropriate antibiotics and incubated at 28°C for 48 h before colonies were counted. Day three (d3) samples were taken in an identical manner to that of d0. For each sample a dilution series ranging between 10^{-1} and 10^{-7} was made and 20 µl aliquots from each dilution were spotted sequentially onto a single NYG agar plate containing the appropriate antibiotics. Bacterial plates were incubated at 28° C for 48 h before colony numbers were determined.

4.2.7 Infiltration assays of *Pseudomonas fluorescens*

One day before infection, bacterial strains were restreaked on NYG agar plates containing the appropriate antibiotics and incubated ON at 28°C. The concentration of bacteria was adjusted to OD_{600} 0.3 in 10 mM $MgCl_2$. Leaves of 4 to 5 week old plants were infiltrated with a needle-less syringe. 24 h after infiltration, HR symptom formation was analyzed visually.

4.2.8 Ion leakage experiments

Pseudomonas fluorescens wt bacteria or expressing AvrRps4 variants were restreaked on NYG agar plates containing the appropriate antibiotics and incubated ON at 28°C. The concentration of bacteria was adjusted to OD_{600} 0.3 in 10 mM $MgCl_2$. Leaves of 4 to 5 week old plants were infiltrated with a needle-less syringe. After infiltration, 12 leaf discs were collected using a cork borer (d=0.6 cm) and washed in a petri dish containing 30 ml H_2O for 30 min. 4 parallel samples of 3 leaf discs were transferred in one well of a 24-well microtiter plate with 3 ml H_2O. To determine ion leakage, the conductivity of 60µl of each sample was measured with the conductivity meter Horiba Twin cond B-173. In the beginning, ion leakage was measured every 2 hours until 12 hpi and then again at 24 hpi and 48 hpi.

4.2.9 Secretion assay

Protocol for secretion assay was adapted from (Mudgett and Staskawicz, 1999). Briefly, *Pst* DC3000 or *P. fluorescens* strains were grown overnight on NYG agar plates containing the appropriate antibiotics at 28°C. Cells were diluted to 1x10^8 cells/ml in NYG medium containing antibiotics. Cultures were grown overnight at 28°C in a rotary shaker until late log-phase growth. Cells were collected by centrifugation at 3000g and washed with minimal media containing 50 mM KPO_4 pH5.7; 7.6 mM $(NH_4)_2SO_4$; 1.7 mM $MgCl_2$; 1.7 mM NaCl; 10 mM fructose and 10 mM mannitol. Cells were then diluted to 2x10^8 cells/ml in minimal media lacking antibiotics and grown for 14 h at 21°C in a rotary shaker. After centrifugation at 3000 g, supernatants were filtered through 0.2µm sterile filters (Nalgene) to remove residual bacteria. 100 µl 2x Laemmli-buffer was added to the pellet fraction and boiled for 5 min prior to immunoblot analysis. The supernatant fraction was precipitated by adding 10 µl StrataClean beads and incubated for 15 min on a rotating wheel. After centrifugation at 1000

g for 3 min, 100 µl 2x Laemmli-buffer was added and samples were boiled for 5 min before loading them on a SDS-PAGE. Western blots were probed with α-HA antibody for detection of AvrRps4 fusion proteins and α-RIP1 (antibody against *Pst* DC3000 lipoprotein PSPTO_4211; F. Kaschani and R. van der Hoorn, unpublished) for analyzing bacterial contamination of the supernatant fraction.

4.2.10 Triparental Mating

The *E. coli* HB101 (pRK2013) helper strain, donor *E. coli* pEDV6 strain and the recipient *Pst* strain were plated on agar plates with appropriate antibiotics and incubated overnight. Bacterial strains were cultured overnight in LB (*E. coli*) and NYGA (*Pst*) medium. OD_{600} was measured, diluted to 0.2-0.3, and cultures were further incubated for 3 hours. Bacterial density of cultures was measured again and cultures were centrifuged at 3000 rpm for 10 min at room temperature. The bacterial pellets were washed with H_2O and centrifuged again at 3000 rpm for 10 min. The pellets were resuspended in H_2O to a final concentration of OD_{600}= 0.8. 100 µl of donor, helper and recipient strain were mixed and centrifuged at 3000 rpm for 10 min. The bacterial pellet was resuspended in 100 µl H_2O and streaked on NYGA agar plates without antibiotics and incubated at 28°C overnight. Bacteria were streaked on NYGA plates with appropriate antibiotics and incubated for 2-3 days. Three independent single colonies were re-streaked on plates with appropriate antibiotics and incubated for 2-3 days and tested by colony-PCR.

4.2.11 Biochemical methods

4.2.11.1 Arabidopsis total protein extraction for immunoblot analysis

Total protein extracts were prepared from 3- to 5-week-old plant materials and frozen in liquid nitrogen. Samples were homogenized 2 x 15 sec to a fine powder using a Mini-Bead-Beater-8TM (Biospec Products) and 1.2 mm stainless steel beads (Roth) in 2 ml centrifuge tubes. After the first 15 sec of homogenisation samples were transferred back to liquid nitrogen and the procedure was repeated. 150 µl of 2x SDS-PAGE sample buffer was added to 50 mg sample on ice. Subsequently, samples were boiled for 5 min while shaking at 700 rpm in an appropriate heating block. If not directly loaded onto SDS PAGE gels, samples were stored at -20° C.

4.2.11.2 Co-Immunoprecipitation from total plant extract

Plant material was ground in YS buffer (50 mM Tris pH 7,5; 150 mM NaCl; 1 mM EDTA; 10% glycerol; 5 mM DTT; 1x protease inhibitor cocktail). The extract was centrifuged at 2000 g for 15 min at 4°C. 30 µl pre-cleared protein G Sepharose was added to the supernatant and incubated at 4°C for 30 min. The samples were subjected to centrifugation at 1000 g for 3 min. 10 µl anti-HA Agarose was added to the supernatant and incubated on a rotating wheel for 2 h at 4°C. The samples were centrifuged for 3 min at 1000 g. The beads were washed three times with YS buffer and subsequently boiled in 2x Laemmli-buffer for 5min before analyzing the immunoprecipitates by immunoblot assay.

4.2.11.3 Microsomal fractionation and subsequent Co-Immunoprecipitation

Microsomal and soluble fractions were prepared according to (Heese et al., 2007). Briefly, plant materials were ground in Buffer H (50 mM HEPES pH 7.5; 250 mM sucrose; 15 mM EDTA; 5% glycerol; 0,5% polyvinylpyrrolidone containing 3 mM DTT and 1x protease inhibitor cocktail (Sigma)). The extracts were filtered through two layers of buffer H pre-wet miracloth and centrifuged at 2000 g for 15 min at 4°C to remove cell debris and nuclei. The supernatant was further subjected to ultracentrifugation at 100,000 g (Optima™ MAX-E ultracentrifuge, Beckmann Coulter, USA) to separate the soluble and microsomal fractions. The pellet was resuspended in buffer H containing 1x protease inhibitor cocktail. The non-ionic detergent Igepal CA-630 (Sigma) was added to 0,2% and 1% final concentration to the soluble and microsomal fractions respectively. The extracts were precleared by incubation for 30 min with 25 µl protein G agarose (Millipore). The samples were centrifuged at 16000g at 4°C. The supernatants were incubated overnight with 20 µl anti-HA Agarose beads (Sigma). The beads were washed three times with buffer H containing 0,2% Igepal CA-630 and the immunoprecipitates were analyzed by immunoblot assay with anti-HA antibody.

4.2.11.4 Denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Denaturing SDS-PAGE was carried out using Mini-PROTEAN 3 system (BioRad). Tris-Glycine polyacrylamide (PAA) gels were prepared according to standard precedures (Sambrook et al., 1989) and poured between two glass plates and overlaid with 500 µl of 50 % isopropanol. After gels were polymerized the alcohol overlay was removed and the gel surface rinsed with dH_2O. Stacking gel was poured onto the top of the resolving gel, a comb was inserted and the gel was allowed to polymerize. In this study, resolving gels used were 6, 10 or 15 % polyacrylamide and stacking gel 5 % polyacrylamide. Gels prepared were of 0.75, 1.0 or 1.5 mm thickness. After removing the combs, each PAA gel was placed into the electrophoresis tank and submerged in 1x Tris-glycine electrophoresis running buffer (25 mM Tris, 250 mM glycine pH 8.3, 0.1% SDS). A pre-stained molecular weight marker (Precision plus protein standard dual color, BioRad) and denatured protein samples were loaded onto the gel and run at 90 V (stacking gel) and 110-130 V (resolving gel) until the desired separation was reached.

4.2.11.5 Immunoblot analysis (Western Blot)

Proteins that had been resolved on PAA gels were transferred to Hybond™-ECL™ nitrocellulose membrane (Amersham Biosciences) after gels were released from the glass plates and stacking gels have been removed. PAA gels and membranes were pre-equilibrated in 1x transfer buffers for 10 min on a rotary shaker. The blotting apparatus (Mini Trans-Blot® Cell, BioRad) was assembled according to the manufacturer instructions. Transfer was carried out at 100 V for 80 min. Equal loading of gels was tested by staining with Ponceau S for 5 min before rinsing in copious volumes of deionised water. Ponceau S stained membranes were scanned and thereafter washed for 5 min in TBS-T before membranes were blocked for 1 h at room temperature in TBS-T containing 5% (w/v) non-fat dry milk (Roth). The blocking solution was removed and membranes were washed briefly with TBS-T. Incubation with primary antibodies was carried out overnight by slowly shaking on a rotary shaker at 4° C in TBS-T supplemented with 3% (w/v) non-fat dry milk. Afterwards, the primary antibody solution was removed and membranes were washed 3 x 15 min with TBS-T at room temperature on a rotary shaker. Secondary antibodies were diluted 1:5000 in TBS-T containing 2% (w/v) non-fat dry milk. Membranes were incubated in the secondary antibody solution for 1 h at room temperature on a rotary shaker. The antibody solution was removed and membranes were washed as described above. This was followed by chemiluminescence

detection using the SuperSignal® West Pico Chemimuminescent kit or a 9:1 - 1:1 mixture of the SuperSignal® West Pico Chemimuminescent- and SuperSignal® West Femto Maximum Sensitivity-kits (Pierce) according to the manufacturer instructions. Luminescence was detected by exposing the membrane to photographic film (BioMax light film, Kodak).

4.2.12 Molecular biological methods

4.2.12.1 Isolation of genomic DNA from Arabidopsis (Quick prep for PCR)

This procedure yields a small quantity of poorly purified DNA. However, the DNA is of sufficient quality for PCR amplification. Leaf samples were taken by closing the cap of a 1.5 ml microcentrifuge tube onto a leaf to clip out a section of tissue and 400 µl of DNA extraction buffer were added. To grind the tissue, a micropestle was used until the tissue was well mashed. The solution was centrifuged at maximum speed for 5 min in a bench top microcentrifuge and 300 µl supernatant were transferred to a new tube. To precipitate the DNA, 1 volume of isopropanol was added and centrifuged at maximum speed for 5 min in a bench top microcentrifuge. The supernatant was discarded carefully. The pellet was washed with 750 µl of 70 % ethanol and dried for 5 min at 45 °C. Finally the pellet was dissolved in 100 µl dH_2O and 0.5 - 2 µl of the solution were used for PCR.

4.2.12.2 Isolation of total RNA from Arabidopsis

Total RNA was prepared from 3- to 6-week-old plant material. RNA was either extracted with QIAGEN Plant Rneasy Kit or according to the following protocol: Liquid nitrogen frozen samples (approximately 100 mg) were homogenized 2 x 15 sec to a fine powder using a Mini- Bead-Beater-8TM (Biospec Products) and 1.2 mm stainless steel beads (Roth) in 2 ml centrifuge tubes. After the first 15 sec of homogenisation samples were transferred back to liquid nitrogen and the procedure was repeated. 1 ml of TRI® Reagent (Sigma) was added and samples were homogenised by vortexing for 1 min. For dissociation of nucleoprotein complexes the homogenised sample was incubated for 5 min at room temperature. 0.2 ml of chloroform was added and samples were shaken vigorously for 15 sec. After incubation for 3 min at room temperature samples were centrifuged for 15 min at 12000 g and 4° C. 0.5 ml of the upper aqueous, RNA containing phase were transferred to a new microcentrifuge tube. RNA was precipitated by adding 0.5 volumes of isopropanol and incubation for 10 min at room temperature. Afterwards, samples were centrifuged for 10 min at 12000 g and 4°C and the pellet was washed by vortexing in 1 ml of 75 % ethanol. Samples were again centrifuged for 5 min at 7500 g and 4° C, pellets were air dried for 10 min and dissolved in 50 µl DEPC-H_2O. All RNA extracts were adjusted to the same concentration with DEPC-H_2O and samples were stored at -80° C.

4.2.12.3 Polymerase chain reaction (PCR)

Standard PCR reactions were performed using home made Taq DNA polymerase. For cloning of PCR products Pfu or Phusion polymerases were used according to the manufacturers instructions. All PCRs were carried out using a PTC-225 Peltier thermal cycler (MJ Research).

4.2.12.4 Reverse transcription-polymerase chain reaction (RT-PCR)

SuperScript™ II RNase H- Reverse Transcriptase (Invitrogen) was used for first strand cDNA synthesis by combining 1 - 2 µg template total RNA, 1 µl oligo dT18X (0.5 µg/µl, X standing for a variable nucleotide), 5 µl dNTP mix (each dNTP 2.5 mM) in a volume of 13.5 µl (deficit made up with DEPC-H_2O). To destroy secondary structures, the sample was incubated at 65° C for 10 min before cooling on ice. For reverse transcription, 4 µl of 5x reaction buffer (supplied with the enzyme), 2 µl of 0.1 M DTT and 0.5 µl reverse transcriptase was added to a final volume of 20 µl. The reaction was incubated at 42° C for 60 min before the enzyme was heat inactivated at 70° C for 10 min.

4.2.12.5 Quantitative Real Time-PCR (qRT-PCR)

Quantitative RT-PCR experiments were performed in an iQ5 Real-Time PCR Detection System (Bio-Rad). Brilliant SYBR Green QPCR Core Reagent (Stratagene) was used as dye. Experiments were performed using three independent biological samples if not otherwise mentioned. Relative transcript levels were calculated using the iQ5 Optical System Software (Version 2.0). As internal reference, *Ubiquitin* (At4g05320) transcript levels were used.

4.2.12.6 Plasmid DNA isolation from bacteria

Standard alkaline cell lysis minipreps of plasmid DNA were carried out using the Macherey-Nagel plasmid prep kit according to the manufacturer's instructions. Larger amounts of plasmid DNA were isolated using QIAGEN Midi preparation kits.

4.2.12.7 Restriction endonuclease digestion of DNA

Restriction digests were carried out using the recommended manufacturer's instructions. Usually, reactions were carried out using 1 µl of restriction enzyme per 20 µl reaction. All digests were carried out at the appropriate temperature for a minimum of 1h.

4.2.12.8 DNA ligations

Usually, DNA ligations were carried out at 16° C overnight in a total volume of 15 µl containing 1 µl T4 DNA ligase (1 U/µl; Roche), ligation buffer (supplied by the manufacturer), 25 - 50 ng vector and 3- to 5-fold molar excess of insert DNA for sticky and blunt end ligations. In some cases ligations were performed for 1 - 3 h at room temperature.

4.2.12.9 Agarose gel electrophoresis of DNA

Agarose gel electrophoresis was used to separate DNA fragments. Gels consisted of 1 – 2 % (w/v) SeaKem® LE agarose (Cambrex, USA) in TAE buffer and agarose was dissolved by heating in a microwave. Molten agarose was cooled to 50° C before 2.5 µl of ethidium bromide solution (10 mg/ml) was added. The agarose was poured and allowed to solidify before being placed in TAE in an electrophoresis tank. 6x DNA loading buffer were added to the DNA samples before loading onto an agarose gel. Separated DNA fragments were visualised by placing the gel on a 312 nm UV transilluminator and photographed.

4.2.12.10 Isolation of DNA fragments from agarose gels

DNA fragments were excised from a separating agarose gel with a clean razor blade and extracted using the Macherey-Nagel gel extraction kit according to the manufacturer's protocol.

4.2.12.11 Site specific recombination of DNA in Gateway®-compatible vectors

LR reactions between the entry clone and a Gateway® destination vector were performed by incubating the reaction for 1 h at room temperature before 0.5 µl proteinase K solution (supplied with the kit) was added. Reactions were incubated at 37° C for 10 min before completely transformed into *E. coli* strain DH10B.

4.2.12.12 DNA sequencing and sequence analysis

DNA sequences were determined by the "Automatische DNA Isolierung und Sequenzierung" (ADIS) service unit at the MPIPZ on Applied Biosystems (Weiterstadt, Germany) Abi Prism 377 and 3700 sequencers using Big Dye-terminator chemistry. Sequence data were analysed using SeqMan™ II version 5.00 (DNASTAR, Madison, USA), EditSeq™ version 5.00 (DNASTAR, Madison, USA) and Clone Manager 6 version 6.00 (Scientific and Educational software, USA).

4.2.12.13 Transformation of chemically competent *E. coli* cells

To 50 µl aliquot of chemically competent cells, 10 to 25 ng of ligated plasmid DNA (or ~ 5 µl of ligated mix from 10 µl ligation reaction) was added and incubated on ice for 30 min. The mixture was heat-shocked for 90 sec at 42° C and immediately put on ice for 2 min. 500 µl of SOC or LB medium was added to the microcentrifuge tube and incubated at 37° C for 1 h on a rotary shaker. The transformation mixture was centrifuged for 5 min at 1500 g, resuspended in 50 µl LB medium and plated onto selective media plates.

4.2.12.14 Transformation of electro-competent *A. tumefaciens* cells

Aliquots of 40 µl of electro-competent *A. tumefaciens* cells were thawed on ice. 50 ng of plasmid DNA was added and transferred to an electroporation cuvette on ice (2 mm electrode distance; Eurogentec, Seraing, Belgium). The BioRad Gene Pulse™ apparatus was set to 25 µF, 2.5 kV and 400 Ω. The cells were pulsed once at the above settings for a second, the cuvette was put back on ice and immediately 1 ml of YEB medium was added to the cuvette. Cells were quickly resuspended by slowly pipetting and cuvettes were incubated for 3 h at 28° C and 600 rpm. A 5 µl fraction of the transformation mixture was plated onto selective YEB agar plates.

4.2.13 Transient plant transformations

4.2.13.1 *Agrobacterium*-mediated transient transformation of tobacco leaves

Prior to *A. tumefaciens* infiltration the following media were prepared:

Induction medium (1 l):		Infiltration medium:
K_2HPO_4	10.5 g	MES 10 mM

KH_2PO_4	4.5 g
$(NH_4)_2SO_4$	1.0 g
NaCitrate·$2H_2O$	0.5 g
$MgSO_4$ (1M)	1.0 ml
Glucose	1.0 g
Fructose	1.0 g
Glycerol	4.0 ml
MES	10.0 mM
pH 5.6	
autoclave	

$MgCl_2$ 10 mM
pH 5.3 - 5.5
Prior to use add 150 µg/ml Acetosyringone.

Prior to use add appropriate antibiotics and 50 µg/ml Acetosyringone (3',5'-Dimethoxy-4'-hydroxyacetophenone).

Cultures were grown in liquid YEB (including appropriate antibiotics) at 28 °C overnight. Afterwards, the cultures were spun down and bacteria were resuspended in 5 ml induction medium. Cultures were grown further for another 4 - 6 h. Bacteria were spun down and the pellet was resuspended in infiltration medium to an OD_{600} of 0.4. The bacterial solution was then left at room temperature for 1 - 3 h. Young *N. benthamiana* plants were watered a few hours before infiltrating and incubated under a propagator lid. Healthy, fresh looking leaves were infiltrated with a needle-less syringe on the underside. Samples of infiltrated leaf areas for protein extractions or microscopy were taken 2 - 3 d after infiltration.

4.2.14 Confocal laser scanning microscopy (CLSM)

Detailed analysis of intracellular fluorescence was performed by confocal laser scanning microscopy using a Leica TCS SPS AOBS (Leica, Wetzlar, Germany) based on an Axiovert microscope equipped with an Argon ion laser as an excitation source. YFP tagged proteins were excited by a 514 nm laser line. YFP fluorescence was selectively detected by using an HFT 514 dichroic mirror and BP 535 – 590 band pass emission filter. Images were analysed with Leica Lite software. 3D images of fluorescence were generated using the 3D plot feature of the Leica Lite software.

4.2.15 FLIM measurements and data analysis

Fluorescence lifetime of the donor was experimentally measured in the presence and absence of the acceptor. FRET efficiency (E) was calculated by comparing the lifetime of the donor in the presence (τDA) or abscence (τD) of the acceptor: E = 1 2 (τDA)/(τD). FLIM measurements were performed using a multiphoton FLIM system coupled to a streak camera (Krishnan et al., 2003). The light source was a mode-locked Ti: sapphire laser (Tsunami, model 3941; Spectra-Physics), pumped by a 10-W diode laser (Millennia Pro; Spectra-Physics), delivering ultrafast femtosecond pulses with a fundamental frequency of 80 MHz. A pulsepicker (model 3980; Spectra-Physics) was used to reduce the repetition rate to 2 MHz. All the experiments were performed at l = 820 nm, the optimal wavelength to excite CFP in multiphoton mode while minimizing the excitation of YFP (Chen and Periasamy, 2004). The power delivered at the entrance of the FLIM optics was 14 mW. All images were acquired with a 360 oil immersion lens (Plan Apo 1.4 numerical aperture, IR) mounted on an inverted microscope (Eclipse TE2000E; Nikon) coupled to the FLIM system. The fluorescence emission was directed back out into the detection unit through a short-pass filter (l < 750 nm). The FLIM unit was composed of a streak camera (Streakscope C4334; Hamamatsu

Photonics) coupled to a fast and high-sensitivity CCD camera (model C8800-53C; Hamamatsu) (Krishnan et al., 2003; Biener et al., 2005). For each nucleus, average fluorescence decay profiles were plotted and lifetimes were estimated by fitting data with biexponential function using a nonlinear least squares estimation procedure with Origin 7.5 software (OriginLab) (Bernoux et al., 2008).

REFERENCES

Aarts, N., Metz, M., Holub, E., Staskawicz, B.J., Daniels, M.J., and Parker, J.E. (1998). Different requirements for *EDS1* and *NDR1* by disease resistance genes define at least two *R* gene-mediated signaling pathways in *Arabidopsis*. Proceedings of the National Academy of Sciences of the United States of America **95,** 10306-10311.

Akira, S., Uematsu, S., and Takeuchi, O. (2006). Pathogen recognition and innate immunity. Cell **124,** 783-801.

Alfano, J.R., and Collmer, A. (2004). Type III secretion system effector proteins: double agents in bacterial disease and plant defense. Annu Rev Phytopathol **42,** 385-414.

Ausubel, F.M. (2005). Are innate immune signaling pathways in plants and animals conserved? Nat Immunol **6,** 973-979.

Axtell, M.J., and Staskawicz, B.J. (2003). Initiation of RPS2-specified disease resistance in *Arabidopsis* is coupled to the AvrRpt2-directed elimination of RIN4. Cell **112,** 369-377.

Badel, J.L., Charkowski, A.O., Deng, W.L., and Collmer, A. (2002). A gene in the Pseudomonas syringae pv. tomato Hrp pathogenicity island conserved effector locus, hopPtoA1, contributes to efficient formation of bacterial colonies in planta and is duplicated elsewhere in the genome. Mol Plant Microbe Interact **15,** 1014-1024.

Bartsch, M., Gobbato, E., Bednarek, P., Debey, S., Schultze, J.L., Bautor, J., and Parker, J.E. (2006). Salicylic acid-independent ENHANCED DISEASE SUSCEPTIBILITY1 signaling in Arabidopsis immunity and cell death is regulated by the monooxygenase FMO1 and the Nudix hydrolase NUDT7. The Plant cell **18,** 1038-1051.

Baures, I., Candresse, T., Leveau, A., Bendahmane, A., and Sturbois, B. (2008). The Rx gene confers resistance to a range of potexviruses in transgenic Nicotiana plants. Mol Plant Microbe Interact **21,** 1154-1164.

Belkhadir, Y., Subramaniam, R., and Dangl, J.L. (2004). Plant disease resistance protein signaling:NBS-LRR proteins and their partners. Curr. Opin. Plant Biol. **7,** 391-399.

Bendahmane, A., Kanyuka, K., and Baulcombe, D.C. (1999). The Rx gene from potato controls separate virus resistance and cell death responses. The Plant cell **11,** 781-792.

Bernoux, M., Timmers, T., Jauneau, A., Briere, C., de Wit, P.J., Marco, Y., and Deslandes, L. (2008). RD19, an Arabidopsis cysteine protease required for RRS1-R-mediated resistance, is relocalized to the nucleus by the Ralstonia solanacearum PopP2 effector. The Plant cell **20,** 2252-2264.

Bernoux, M., Ve, T., Williams, S., Warren, C., Hatters, D., Valkov, E., Zhang, X., Ellis, J.G., Kobe, B., and Dodds, P.N. (2011). Structural and Functional Analysis of a Plant Resistance Protein TIR Domain Reveals Interfaces for Self-Association, Signaling, and Autoregulation. Cell Host Microbe **9,** 200-211.

Birker, D., Heidrich, K., Takahara, H., Narusaka, M., Deslandes, L., Narusaka, Y., Reymond, M., Parker, J.E., and O'Connell, R. (2009). A locus conferring resistance to Colletotrichum higginsianum is shared by four geographically distinct Arabidopsis accessions. Plant J **60,** 602-613.

Boller, T., and Felix, G. (2009). A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. Annu Rev Plant Biol **60,** 379-406.

Brooks, D.M., Bender, C.L., and Kunkel, B.N. (2005). The Pseudomonas syringae phytotoxin coronatine promotes virulence by overcoming salicylic acid-dependent defences in Arabidopsis thaliana. Mol Plant Pathol **6,** 629-639.

Burch-Smith, T.M., Schiff, M., Caplan, J.L., Tsao, J., Czymmek, K., and Dinesh-Kumar, S.P. (2007). A novel role for the TIR domain in association with pathogen-derived elicitors. PLoS biology **5,** e68.

Caplan, J.L., Mamillapalli, P., Burch-Smith, T.M., Czymmek, K., and Dinesh-Kumar, S.P. (2008). Chloroplastic protein NRIP1 mediates innate immune receptor recognition of a viral effector. Cell **132,** 449-462.

Chang, J.H., Urbach, J.M., Law, T.F., Arnold, L.W., Hu, A., Gombar, S., Grant, S.R., Ausubel, F.M., and Dangl, J.L. (2005). A high-throughput, near-saturating screen for type III effector genes from Pseudomonas syringae. Proceedings of the National Academy of Sciences of the United States of America **102,** 2549-2554.

Chen, X., Chern, M., Canlas, P.E., Jiang, C., Ruan, D., Cao, P., and Ronald, P.C. A conserved threonine residue in the juxtamembrane domain of the XA21 pattern recognition receptor is critical for kinase autophosphorylation and XA21-mediated immunity. The Journal of biological chemistry **285,** 10454-10463.

Cheng, Y.T., Germain, H., Wiermer, M., Bi, D., Xu, F., Garcia, A.V., Wirthmueller, L., Despres, C., Parker, J.E., Zhang, Y., and Li, X. (2009). Nuclear pore complex component MOS7/Nup88 is required for innate immunity and nuclear accumulation of defense regulators in Arabidopsis. The Plant cell **21,** 2503-2516.

Chisholm, S.T., Coaker, G., Day, B., and Staskawicz, B.J. (2006). Host-microbe interactions: shaping the evolution of the plant immune response. Cell **124,** 803-814.

Chung, E.H., da Cunha, L., Wu, A.J., Gao, Z., Cherkis, K., Afzal, A.J., Mackey, D., and Dangl, J.L. (2011). Specific threonine phosphorylation of a host target by two unrelated type III effectors activates a host innate immune receptor in plants. Cell Host Microbe **9,** 125-136.

Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J **16,** 735-743.

Coll, N.S., Vercammen, D., Smidler, A., Clover, C., Van Breusegem, F., Dangl, J.L., and Epple, P. (2010). Arabidopsis type I metacaspases control cell death. Science (New York, N.Y **330,** 1393-1397.

Collier, S.M., and Moffett, P. (2009). NB-LRRs work a "bait and switch" on pathogens. Trends in plant science **14,** 521-529.

Cook, A., Bono, F., Jinek, M., and Conti, E. (2007). Structural biology of nucleocytoplasmic transport. Annu Rev Biochem **76,** 647-671.

Damiano, J.S., Oliveira, V., Welsh, K., and Reed, J.C. (2004). Heterotypic interactions among NACHT domains: implications for regulation of innate immune responses. Biochem J **381,** 213-219.

Dangl, J.L., and Jones, J.D.G. (2001). Plant pathogens and integrated defence responses to infection. Nature **411,** 826-833.

Day, B., Dahlbeck, D., and Staskawicz, B.J. (2006). NDR1 interaction with RIN4 mediates the differential activation of multiple disease resistance pathways in Arabidopsis. The Plant cell **18,** 2782-2791.

Deslandes, L., and Rivas, S. (2011). The plant cell nucleus: A true arena for the fight between plants and pathogens. Plant Signal Behav **6**.

Deslandes, L., Olivier, J., Theulieres, F., Hirsch, J., Feng, D.X., Bittner-Eddy, P., Beynon, J., and Marco, Y. (2002). Resistance to Ralstonia solanacearum in Arabidopsis thaliana is conferred by the recessive RRS1-R gene, a member of a novel family of resistance genes. Proceedings of the National Academy of Sciences of the United States of America **99,** 2404-2409.

Deslandes, L., Olivier, J., Peeters, N., Feng, D.X., Khounlotham, M., Boucher, C., Somssich, I., Genin, S., and Marco, Y. (2003). Physical interaction between RRS1-R, a protein conferring resistance to bacterial wilt, and PopP2, a type III effector

targeted to the plant nucleus. Proceedings of the National Academy of Sciences of the United States of America **100,** 8024-8029.

Desveaux, D., Singer, A.U., Wu, A.J., McNulty, B.C., Musselwhite, L., Nimchuk, Z., Sondek, J., and Dangl, J.L. (2007). Type III effector activation via nucleotide binding, phosphorylation, and host target interaction. PLoS Pathog **3,** e48.

Dhadi, S.R., Krom, N., and Ramakrishna, W. (2009). Genome-wide comparative analysis of putative bidirectional promoters from rice, Arabidopsis and Populus. Gene **429,** 65-73.

Dillon, N. (2008). The impact of gene location in the nucleus on transcriptional regulation. Dev Cell **15,** 182-186.

Dodds, P.N., and Rathjen, J.P. (2010). Plant immunity: towards an integrated view of plant-pathogen interactions. Nat Rev Genet **11,** 539-548.

Dodds, P.N., Lawrence, G.J., Catanzariti, A.M., Teh, T., Wang, C.I., Ayliffe, M.A., Kobe, B., and Ellis, J.G. (2006). Direct protein interaction underlies gene-for-gene specificity and coevolution of the flax resistance genes and flax rust avirulence genes. Proceedings of the National Academy of Sciences of the United States of America **103,** 8888-8893.

Durrant, W.E., and Dong, X. (2004). Systemic acquired resistance. Annual Review of Phytopathology **42,** 185-209.

Eitas, T.K., and Dangl, J.L. (2010). NB-LRR proteins: pairs, pieces, perception, partners, and pathways. Current opinion in plant biology **13,** 472-477.

Eitas, T.K., Nimchuk, Z.L., and Dangl, J.L. (2008). Arabidopsis TAO1 is a TIR-NB-LRR protein that contributes to disease resistance induced by the Pseudomonas syringae effector AvrB. Proceedings of the National Academy of Sciences of the United States of America **105,** 6475-6480.

Engstrom, P.G., Suzuki, H., Ninomiya, N., Akalin, A., Sessa, L., Lavorgna, G., Brozzi, A., Luzi, L., Tan, S.L., Yang, L., Kunarso, G., Ng, E.L., Batalov, S., Wahlestedt, C., Kai, C., Kawai, J., Carninci, P., Hayashizaki, Y., Wells, C., Bajic, V.B., Orlando, V., Reid, J.F., Lenhard, B., and Lipovich, L. (2006). Complex Loci in human and mouse genomes. PLoS Genet **2,** e47.

Enright, A.J., and Ouzounis, C.A. (2001). Functional associations of proteins in entire genomes by means of exhaustive detection of gene fusions. Genome biology **2,** RESEARCH0034.

Eulgem, T., and Somssich, I.E. (2007). Networks of WRKY transcription factors in defense signaling. Current opinion in plant biology **10,** 366-371.

Falk, A., Feys, B.J., Frost, L.N., Jones, J.D., Daniels, M.J., and Parker, J.E. (1999). EDS1, an essential component of *R* gene-mediated disease resistance in Arabidopsis has homology to eukaryotic lipases. Proceedings of the National Academy of Sciences of the United States of America **96,** 3292-3297.

Feys, B.J., Moisan, L.J., Newman, M.A., and Parker, J.E. (2001). Direct interaction between the Arabidopsis disease resistance signaling proteins, EDS1 and PAD4. EMBO Journal **20,** 5400-5411.

Feys, B.J., Wiermer, M., Bhat, R.A., Moisan, L.J., Medina-Escobar, N., Neu, C., Cabral, A., and Parker, J.E. (2005). Arabidopsis SENESCENCE-ASSOCIATED GENE101 stabilizes and signals within an ENHANCED DISEASE SUSCEPTIBILITY1 complex in plant innate immunity. The Plant cell **17,** 2601-2613.

Flor, H.H. (1971). Current status of the gene-for-gene concept. Annual Review of Phytopathology **9,** 275-296.

Frost, D., Way, H., Howles, P., Luck, J., Manners, J., Hardham, A., Finnegan, J., and Ellis, J. (2004). Tobacco transgenic for the flax rust resistance gene L expresses

allele-specific activation of defense responses. Mol Plant Microbe Interact **17,** 224-232.

Garcia, A.V., and Parker, J.E. (2009). Heaven's Gate: nuclear accessibility and activities of plant immune regulators. Trends in plant science **14,** 479-487.

Garcia, A.V., Blanvillain-Baufume, S., Huibers, R.P., Wiermer, M., Li, G., Gobbato, E., Rietz, S., and Parker, J.E. (2010). Balanced nuclear and cytoplasmic activities of EDS1 are required for a complete plant innate immune response. PLoS Pathog **6,** e1000970.

Gassmann, W., Hinsch, M.E., and Staskawicz, B.J. (1999). The Arabidopsis RPS4 bacterial-resistance gene is a member of the TIR-NBS-LRR family of disease-resistance genes. Plant J **20,** 265-277.

Gibson, T.J. (2009). Cell regulation: determined to signal discrete cooperation. Trends Biochem Sci **34,** 471-482.

Gimenez-Ibanez, S., Hann, D.R., Ntoukakis, V., Petutschnig, E., Lipka, V., and Rathjen, J.P. (2009). AvrPtoB targets the LysM receptor kinase CERK1 to promote bacterial virulence on plants. Curr Biol **19,** 423-429.

Godiard, L., Sauviac, L., Torii, K.U., Grenon, O., Mangin, B., Grimsley, N.H., and Marco, Y. (2003). ERECTA, an LRR receptor-like kinase protein controlling development pleiotropically affects resistance to bacterial wilt. Plant J **36,** 353-365.

Gomez-Gomez, L., and Boller, T. (2000). FLS2: an LRR recpetor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. Mol. Cell **5,** 1003-1011.

Gorlich, D., Vogel, F., Mills, A.D., Hartmann, E., and Laskey, R.A. (1995). Distinct functions for the two importin subunits in nuclear protein import. Nature **377,** 246-248.

Guo, M., Tian, F., Wamboldt, Y., and Alfano, J.R. (2009). The majority of the type III effector inventory of Pseudomonas syringae pv. tomato DC3000 can suppress plant immunity. Mol Plant Microbe Interact **22,** 1069-1080.

Haasen, D., Kohler, C., Neuhaus, G., and Merkle, T. (1999). Nuclear export of proteins in plants: AtXPO1 is the export receptor for leucine-rich nuclear export signals in *Arabidopsis thaliana*. Plant Journal **20,** 695-705.

Hayashi, F., Smith, K.D., Ozinsky, A., Hawn, T.R., Yi, E.C., Goodlett, D.R., Eng, J.K., Akira, S., Underhill, D.M., and Aderem, A. (2001). The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature **410,** 1099-1103.

Heath, M.C. (2000). Nonhost resistance and nonspecific plant defenses. Current opinion in plant biology **3,** 315-319.

Heese, A., Hann, D.R., Gimenez-Ibanez, S., Jones, A.M., He, K., Li, J., Schroeder, J.I., Peck, S.C., and Rathjen, J.P. (2007). The receptor-like kinase SERK3/BAK1 is a central regulator of innate immunity in plants. Proceedings of the National Academy of Sciences of the United States of America **104,** 12217-12222.

Heessen, S., and Fornerod, M. (2007). The inner nuclear envelope as a transcription factor resting place. EMBO Rep **8,** 914-919.

Hinsch, M., and Staskawicz, B. (1996). Identification of a new Arabidopsis disease resistance locus, RPs4, and cloning of the corresponding avirulence gene, avrRps4, from Pseudomonas syringae pv. pisi. Mol Plant Microbe Interact **9,** 55-61.

Huang, H.C., Schuurink, R., Denny, T.P., Atkinson, M.M., Baker, C.J., Yucel, I., Hutcheson, S.W., and Collmer, A. (1988). Molecular cloning of a Pseudomonas syringae pv. syringae gene cluster that enables Pseudomonas fluorescens to elicit the hypersensitive response in tobacco plants. J Bacteriol **170,** 4748-4756.

Hubert, D.A., Tornero, P., Belkhadir, Y., Krishna, P., Takahashi, A., Shirasu, K., and Dangl, J.L. (2003). Cytosolic HSP90 associates with and modulates the Arabidopsis RPM1 disease resistance protein. Embo Journal **22,** 5679-5689.

Hutten, S., and Kehlenbach, R.H. (2007). CRM1-mediated nuclear export: to the pore and beyond. Trends Cell Biol **17,** 193-201.

Jia, Y., McAdams, S.A., Bryan, G.T., Hershey, H.P., and Valent, B. (2000). Direct interaction of resistance gene and avirulence gene products confers rice blast resistance. EMBO Journal **19,** 4004-4014.

Jirage, D., Tootle, T.L., Reuber, T.L., Frost, L.N., Feys, B.J., Parker, J.E., Ausubel, F.M., and Glazebrook, J. (1999). Arabidopsis thaliana PAD4 encodes a lipase-like gene that is important for salicylic acid signaling. Proceedings of the National Academy of Sciences of the United States of America **96,** 13583-13588.

Jones, J.D., and Dangl, J.L. (2006). The plant immune system. Nature **444,** 323-329.

Katagiri, F., and Tsuda, K. (2010). Understanding the plant immune system. Mol Plant Microbe Interact **23,** 1531-1536.

Kawai, T., and Akira, S. (2007). TLR signaling. Semin Immunol **19,** 24-32.

Kay, S., and Bonas, U. (2009). How Xanthomonas type III effectors manipulate the host plant. Curr Opin Microbiol **12,** 37-43.

Kim, S.H., Gao, F., Bhattacharjee, S., Adiasor, J.A., Nam, J.C., and Gassmann, W. (2010). The Arabidopsis resistance-like gene SNC1 is activated by mutations in SRFR1 and contributes to resistance to the bacterial effector AvrRps4. PLoS Pathog **6,** e1001172.

Koncz, C., and Schell, J. (1986). The promoter of the TL -DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. Molecular General Genetics **204,** 383-396.

Krasileva, K.V., Dahlbeck, D., and Staskawicz, B.J. (2010). Activation of an Arabidopsis resistance protein is specified by the in planta association of its leucine-rich repeat domain with the cognate oomycete effector. The Plant cell **22,** 2444-2458.

Lahaye, T. (2004). Illuminating the molecular basis of gene-for-gene resistance; Arabidopsis thaliana RRS1-R and its interaction with Ralstonia solanacearum popP2. Trends in plant science **9,** 1-4.

Laird, J., Armengaud, P., Giuntini, P., Laval, V., and Milner, J.J. (2004). Inappropriate annotation of a key defence marker in Arabidopsis: will the real PR-1 please stand up? Planta **219,** 1089-1092.

Lanford, R.E., and Butel, J.S. (1984). Construction and characterization of an SV40 mutant defective in nuclear transport of T antigen. Cell **37,** 801-813.

Lee, S.K., Song, M.Y., Seo, Y.S., Kim, H.K., Ko, S., Cao, P.J., Suh, J.P., Yi, G., Roh, J.H., Lee, S., An, G., Hahn, T.R., Wang, G.L., Ronald, P., and Jeon, J.S. (2009a). Rice Pi5-mediated resistance to Magnaporthe oryzae requires the presence of two coiled-coil-nucleotide-binding-leucine-rich repeat genes. Genetics **181,** 1627-1638.

Lee, S.W., Han, S.W., Sririyanum, M., Park, C.J., Seo, Y.S., and Ronald, P.C. (2009b). A type I-secreted, sulfated peptide triggers XA21-mediated innate immunity. Science (New York, N.Y **326,** 850-853.

Li, X., Clarke, J.D., Zhang, Y., and Dong, X. (2001). Activation of an EDS1-mediated R-gene pathway in the snc1 mutant leads to constitutive, NPR1-independent pathogen resistance. Mol Plant Microbe Interact **14,** 1131-1139.

Li, Y., Li, S., Bi, D., Cheng, Y.T., Li, X., and Zhang, Y. (2010). SRFR1 negatively regulates plant NB-LRR resistance protein accumulation to prevent autoimmunity. PLoS Pathog **6**.

Liu, J., Elmore, J.M., Lin, Z.J., and Coaker, G. (2011). A receptor-like cytoplasmic kinase phosphorylates the host target RIN4, leading to the activation of a plant innate immune receptor. Cell Host Microbe **9,** 137-146.

Liu, P.P., Bhattacharjee, S., Klessig, D.F., and Moffett, P. (2010). Systemic acquired resistance is induced by R gene-mediated responses independent of cell death. Mol Plant Pathol **11,** 155-160.

Lotze, M.T., Zeh, H.J., Rubartelli, A., Sparvero, L.J., Amoscato, A.A., Washburn, N.R., Devera, M.E., Liang, X., Tor, M., and Billiar, T. (2007). The grateful dead: damage-associated molecular pattern molecules and reduction/oxidation regulate immunity. Immunol Rev **220,** 60-81.

Luck, J.E., Lawrence, G.J., Dodds, P.N., Shepherd, K.W., and Ellis, J.G. (2000). Regions outside of the leucine-rich repeats of flax rust resistance proteins play a role in specificity determination. The Plant cell **12,** 1367-1377.

Lukasik, E., and Takken, F.L. (2009). STANDing strong, resistance proteins instigators of plant defence. Current opinion in plant biology.

Mackey, D., Holt, B.F., Wiig, A., and Dangl, J.L. (2002). RIN4 interacts with Pseudomonas syringae type III effector molecules and is required for RPM1-mediated resistance in Arabidopsis. Cell **108,** 743-754.

Mackey, D., Belkhadir, Y., Alonso, J.M., Ecker, J.R., and Dangl, J.L. (2003). *Arabidopsis* RIN4 is a target of the type III virulence effector AvrRpt2 and modulates RPS2-mediated resistance. Cell **112,** 379-389.

Maekawa, T., Cheng, W., Spiridon, L.N., Toller, A., Lukasik, E., Saijo, Y., Liu, P., Shen, Q.H., Micluta, M.A., Somssich, I.E., Takken, F.L., Petrescu, A.J., Chai, J., and Schulze-Lefert, P. (2011). Coiled-Coil Domain-Dependent Homodimerization of Intracellular Barley Immune Receptors Defines a Minimal Functional Module for Triggering Cell Death. Cell Host Microbe **9,** 187-199.

Matzinger, P. (2007). Friendly and dangerous signals: is the tissue in control? Nat Immunol **8,** 11-13.

McDowell, J.M., Cuzick, A., Can, C., Beynon, J., Dangl, J.L., and Holub, E.B. (2000). Downy mildew (Peronospora parasitica) resistance genes in Arabidopsis vary in functional requirements for NDR1, EDS1, NPR1 and salicylic acid accumulation. Plant J **22,** 523-529.

Meier, I. (2007). Composition of the plant nuclear envelope: theme and variations. J Exp Bot **58,** 27-34.

Melotto, M., Underwood, W., Koczan, J., Nomura, K., and He, S.Y. (2006). Plant stomata function in innate immunity against bacterial invasion. Cell **126,** 969-980.

Mendjan, S., Taipale, M., Kind, J., Holz, H., Gebhardt, P., Schelder, M., Vermeulen, M., Buscaino, A., Duncan, K., Mueller, J., Wilm, M., Stunnenberg, H.G., Saumweber, H., and Akhtar, A. (2006). Nuclear pore components are involved in the transcriptional regulation of dosage compensation in Drosophila. Molecular cell **21,** 811-823.

Mestre, P., and Baulcombe, D.C. (2006). Elicitor-mediated oligomerization of the tobacco N disease resistance protein. The Plant cell **18,** 491-501.

Meyers, B.C., Kozik, A., Griego, A., Kuang, H.H., and Michelmore, R.W. (2003). Genome-wide analysis of NBS-LRR-encoding genes in Arabidopsis. The Plant cell **15,** 809-834.

Mishina, T.E., and Zeier, J. (2006). The Arabidopsis flavin-dependent monooxygenase FMO1 is an essential component of biologically induced systemic acquired resistance. Plant physiology **141,** 1666-1675.

Mishina, T.E., and Zeier, J. (2007). Pathogen-associated molecular pattern recognition rather than development of tissue necrosis contributes to bacterial induction of systemic acquired resistance in Arabidopsis. Plant J **50,** 500-513.

Miya, A., Albert, P., Shinya, T., Desaki, Y., Ichimura, K., Shirasu, K., Narusaka, Y., Kawakami, N., Kaku, H., and Shibuya, N. (2007). CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America **104,** 19613-19618.

Moffett, P., Farnham, G., Peart, J., and Baulcombe, D.C. (2002). Interaction between domains of a plant NBS-LRR protein in disease resistance-related cell death. Embo Journal **21,** 4511-4519.

Mudgett, M.B., and Staskawicz, B.J. (1999). Characterization of the Pseudomonas syringae pv. tomato AvrRpt2 protein: demonstration of secretion and processing during bacterial pathogenesis. Mol Microbiol **32,** 927-941.

Nakai, K., and Horton, P. (1999). PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. Trends Biochem Sci **24,** 34-36.

Narusaka, M., Shirasu, K., Noutoshi, Y., Kubo, Y., Shiraishi, T., Iwabuchi, M., and Narusaka, Y. (2009). RRS1 and RPS4 provide a dual Resistance-gene system against fungal and bacterial pathogens. Plant J **60,** 218-226.

Nishimura, M.T., and Dangl, J.L. (2010). Arabidopsis and the plant immune system. Plant J **61,** 1053-1066.

Nombela, G., Williamson, V.M., and Muniz, M. (2003). The root-knot nematode resistance gene Mi-1.2 of tomato is responsible for resistance against the whitefly Bemisia tabaci. Mol Plant Microbe Interact **16,** 645-649.

Noutoshi, Y., Ito, T., Seki, M., Nakashita, H., Yoshida, S., Marco, Y., Shirasu, K., and Shinozaki, K. (2005). A single amino acid insertion in the WRKY domain of the Arabidopsis TIR-NBS-LRR-WRKY-type disease resistance protein SLH1 (sensitive to low humidity 1) causes activation of defense responses and hypersensitive cell death. Plant J **43,** 873-888.

Okrent, R.A., Brooks, M.D., and Wildermuth, M.C. (2009). Arabidopsis GH3.12 (PBS3) conjugates amino acids to 4-substituted benzoates and is inhibited by salicylate. The Journal of biological chemistry **284,** 9742-9754.

Oldroyd, G.E., and Staskawicz, B.J. (1998). Genetically engineered broad-spectrum disease resistance in tomato. Proceedings of the National Academy of Sciences of the United States of America **95,** 10300-10305.

Padmanabhan, M.S., and Dinesh-Kumar, S.P. (2010). All hands on deck-the role of chloroplasts, endoplasmic reticulum, and the nucleus in driving plant innate immunity. Mol Plant Microbe Interact **23,** 1368-1380.

Palma, K., Zhang, Y., and Li, X. (2005). An importin alpha homolog, MOS6, plays an important role in plant innate immunity. Curr Biol **15,** 1129-1135.

Pan, Q., Mathison, J., Fearns, C., Kravchenko, V.V., Da Silva Correia, J., Hoffman, H.M., Kobayashi, K.S., Bertin, J., Grant, E.P., Coyle, A.J., Sutterwala, F.S., Ogura, Y., Flavell, R.A., and Ulevitch, R.J. (2007). MDP-induced interleukin-1beta processing requires Nod2 and CIAS1/NALP3. J Leukoc Biol **82,** 177-183.

Parker, J.E., Holub, E.B., Frost, L.N., Falk, A., Gunn, N.D., and Daniels, M.J. (1996). Characterization of *eds1*, a mutation in Arabidopsis suppressing resistance to *Peronospora parasitica* specified by several different *RPP* genes. The Plant cell **8,** 2033-2046.

Peart, J.R., Mestre, P., Lu, R., Malcuit, I., and Baulcombe, D.C. (2005). NRG1, a CC-NB-LRR protein, together with N, a TIR-NB-LRR protein, mediates resistance against tobacco mosaic virus. Curr Biol **15,** 968-973.

Rafiqi, M., Bernoux, M., Ellis, J.G., and Dodds, P.N. (2009). In the trenches of plant pathogen recognition: Role of NB-LRR proteins. Seminars in cell & developmental biology **20,** 1017-1024.

Rosebrock, T.R., Zeng, L., Brady, J.J., Abramovitch, R.B., Xiao, F., and Martin, G.B. (2007). A bacterial E3 ubiquitin ligase targets a host protein kinase to disrupt plant immunity. Nature **448,** 370-374.

Rusterucci, C., Aviv, D.H., Holt, B.F., Dangl, J.L., and Parker, J.E. (2001). The disease resistance signaling components *EDS1* and *PAD4* are essential regulators of the cell death pathway controlled by *LSD1* in Arabidopsis. The Plant cell **13,** 2211-2224.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. (NY: Cold Spring Harbor).

Schornack, S., van Damme, M., Bozkurt, T.O., Cano, L.M., Smoker, M., Thines, M., Gaulin, E., Kamoun, S., and Huitema, E. (2010). Ancient class of translocated oomycete effectors targets the host nucleus. Proceedings of the National Academy of Sciences of the United States of America **107,** 17421-17426.

Schroder, K., and Tschopp, J. The inflammasomes. Cell **140,** 821-832.

Seebacher, J., and Gavin, A.C. (2011). SnapShot: Protein-Protein Interaction Networks. Cell **144,** 1000-1000 e1001.

Segonzac, C., and Zipfel, C. Activation of plant pattern-recognition receptors by bacteria. Curr Opin Microbiol **14,** 54-61.

Shen, Q.H., and Schulze-Lefert, P. (2007). Rumble in the nuclear jungle: compartmentalization, trafficking, and nuclear action of plant immune receptors. The EMBO journal **26,** 4293-4301.

Shen, Q.H., Saijo, Y., Mauch, S., Biskup, C., Bieri, S., Keller, B., Seki, H., Ulker, B., Somssich, I.E., and Schulze-Lefert, P. (2007). Nuclear activity of MLA immune receptors links isolate-specific and basal disease-resistance responses. Science (New York, N.Y **315,** 1098-1103.

Shirasu, K. (2009). The HSP90-SGT1 chaperone complex for NLR immune sensors. Annu Rev Plant Biol **60,** 139-164.

Shirasu, K., and Schulze-Lefert, P. (2000). Regulators of cell death in disease resistance. Plant molecular biology **44,** 371-385.

Shirasu, K., Nakajima, H., Rajasekhar, V.K., Dixon, R.A., and Lamb, C. (1997). Salicylic acid potentiates an agonist-dependent gain control that amplifies pathogen signals in the activation of defense mechanisms. The Plant cell **9,** 261-270.

Sinapidou, E., Williams, K., Nott, L., Bahkt, S., Tor, M., Crute, I., Bittner-Eddy, P., and Beynon, J. (2004). Two TIR:NB:LRR genes are required to specify resistance to Peronospora parasitica isolate Cala2 in Arabidopsis. Plant J **38,** 898-909.

Slootweg, E., Roosien, J., Spiridon, L.N., Petrescu, A.J., Tameling, W., Joosten, M., Pomp, R., van Schaik, C., Dees, R., Borst, J.W., Smant, G., Schots, A., Bakker, J., and Goverse, A. (2010). Nucleocytoplasmic distribution is required for activation of resistance by the potato NB-LRR receptor Rx1 and is balanced by its functional domains. The Plant cell **22,** 4195-4215.

Sohn, K.H., Zhang, Y., and Jones, J.D. (2009). The Pseudomonas syringae effector protein, AvrRPS4, requires in planta processing and the KRVY domain to function. Plant J **57,** 1079-1091.

Staskawicz, B.J., Mudgett, M.B., Dangl, J.L., and Galan, J.E. (2001). Common and contrasting themes of plant and animal diseases. Science (New York, N.Y **292,** 2285-2289.

Strober, W., Murray, P.J., Kitani, A., and Watanabe, T. (2006). Signalling pathways and molecular interactions of NOD1 and NOD2. Nat Rev Immunol **6,** 9-20.

Stukenbrock, E.H., and McDonald, B.A. (2009). Population genetics of fungal and oomycete effectors involved in gene-for-gene interactions. Mol Plant Microbe Interact **22,** 371-380.

Swiderski, M.R., Birker, D., and Jones, J.D. (2009). The TIR domain of TIR-NB-LRR resistance proteins is a signaling domain involved in cell death induction. Mol Plant Microbe Interact **22,** 157-165.

Szurek, B., Rossier, O., Hause, G., and Bonas, U. (2002). Type III-dependent translocation of the *Xanthomonas* AvrBs3 protein into the plant cell. Molecular Microbiology **46,** 13-23.

Takahashi, A., Casais, C., Ichimura, K., and Shirasu, K. (2003). HSP90 interacts with RAR1 and SGT1 and is essential for RPS2-mediated disease resistance in Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America **100,** 11777-11782.

Takken, F.L., Albrecht, M., and Tameling, W.I. (2006). Resistance proteins: molecular switches of plant defence. Current opinion in plant biology **9,** 383-390.

Tapping, R.I. (2009). Innate immune sensing and activation of cell surface Toll-like receptors. Semin Immunol **21,** 175-184.

Tasset, C., Bernoux, M., Jauneau, A., Pouzet, C., Briere, C., Kieffer-Jacquinod, S., Rivas, S., Marco, Y., and Deslandes, L. (2010). Autoacetylation of the Ralstonia solanacearum effector PopP2 targets a lysine residue essential for RRS1-R-mediated immunity in Arabidopsis. PLoS Pathog **6,** e1001202.

Thomma, B.P., Nurnberger, T., and Joosten, M.H. Of PAMPs and Effectors: The Blurred PTI-ETI Dichotomy. The Plant cell **23,** 4-15.

Ting, J.P., Kastner, D.L., and Hoffman, H.M. (2006). CATERPILLERs, pyrin and hereditary immunological disorders. Nat Rev Immunol **6,** 183-195.

Todesco, M., Balasubramanian, S., Hu, T.T., Traw, M.B., Horton, M., Epple, P., Kuhns, C., Sureshkumar, S., Schwartz, C., Lanz, C., Laitinen, R.A., Huang, Y., Chory, J., Lipka, V., Borevitz, J.O., Dangl, J.L., Bergelson, J., Nordborg, M., and Weigel, D. (2010). Natural allelic variation underlying a major fitness trade-off in Arabidopsis thaliana. Nature **465,** 632-636.

Tornero, P., and Dangl, J.L. (2001). A high-throughput method for quantifying growth of phytopathogenic bacteria in Arabidopsis thaliana. Plant J **28,** 475-481.

van der Hoorn, R.A., and Kamoun, S. (2008). From Guard to Decoy: a new model for perception of plant pathogen effectors. The Plant cell **20,** 2009-2017.

Van der Hoorn, R.A., De Wit, P.J., and Joosten, M.H. (2002). Balancing selection favors guarding resistance proteins. Trends in plant science **7,** 67-71.

van Dijk, K., Tam, V.C., Records, A.R., Petnicki-Ocwieja, T., and Alfano, J.R. (2002). The ShcA protein is a molecular chaperone that assists in the secretion of the HopPsyA effector from the type III (Hrp) protein secretion system of Pseudomonas syringae. Mol Microbiol **44,** 1469-1481.

van Ooijen, G., van den Burg, H.A., Cornelissen, B.J., and Takken, F.L. (2007). Structure and function of resistance proteins in solanaceous plants. Annu Rev Phytopathol **45,** 43-72.

VandenAckerveken, G., Marois, E., and Bonas, U. (1996). Recognition of the bacterial avirulence protein AvrBs3 occurs inside the host plant cell. Cell **87,** 1307-1316.

Wang, L., Tsuda, K., Sato, M., Cohen, J.D., Katagiri, F., and Glazebrook, J. (2009a). Arabidopsis CaM binding protein CBP60g contributes to MAMP-induced SA accumulation and is involved in disease resistance against Pseudomonas syringae. PLoS Pathog **5,** e1000301.

Wang, Y., Bao, Z., Zhu, Y., and Hua, J. (2009b). Analysis of temperature modulation of plant defense against biotrophic microbes. Mol Plant Microbe Interact **22,** 498-506.

Wen, W., Meinkoth, J.L., Tsien, R.Y., and Taylor, S.S. (1995). Identification Of a Signal For Rapid Export Of Proteins From the Nucleus. Cell **82,** 463-473.

Whitham, S., McCormick, S., and Baker, B. (1996). The N gene of tobacco confers resistance to tobacco mosaic virus in transgenic tomato. Proceedings of the National Academy of Sciences of the United States of America **93,** 8776-8781.

Wildermuth, M.C., Dewdney, J., Wu, G., and Ausubel, F.M. (2001). Isochorismate synthase is required to synthesize salicylic acid for plant defence. Nature **414,** 562-565.

Wirthmueller, L., Zhang, Y., Jones, J.D., and Parker, J.E. (2007). Nuclear accumulation of the Arabidopsis immune receptor RPS4 is necessary for triggering EDS1-dependent defense. Curr Biol **17,** 2023-2029.

Xu, X., Chen, C., Fan, B., and Chen, Z. (2006). Physical and functional interactions between pathogen-induced Arabidopsis WRKY18, WRKY40, and WRKY60 transcription factors. The Plant cell **18,** 1310-1326.

Xu, X.M., and Meier, I. (2008). The nuclear pore comes to the fore. Trends in plant science **13,** 20-27.

Xylourgidis, N., Roth, P., Sabri, N., Tsarouhas, V., and Samakovlis, C. (2006). The nucleoporin Nup214 sequesters CRM1 at the nuclear rim and modulates NFkappaB activation in Drosophila. J Cell Sci **119,** 4409-4419.

Yu, I.C., Parker, J., and Bent, A.F. (1998). Gene-for-gene disease resistance without the hypersensitive response in Arabidopsis dnd1 mutant. Proceedings of the National Academy of Sciences of the United States of America **95,** 7819-7824.

Zhang, X.C., and Gassmann, W. (2003). RPS4-mediated disease resistance requires the combined presence of RPS4 transcripts with full-length and truncated open reading frames. The Plant cell **15,** 2333-2342.

Zhang, Y., and Li, X. (2005). A putative nucleoporin 96 Is required for both basal defense and constitutive resistance responses mediated by suppressor of npr1-1,constitutive 1. The Plant cell **17,** 1306-1316.

Zhang, Y., Dorey, S., Swiderski, M., and Jones, J.D. (2004). Expression of RPS4 in tobacco induces an AvrRps4-independent HR that requires EDS1, SGT1 and HSP90. Plant J **40,** 213-224.

Zhang, Y.L., Goritschnig, S., Dong, X.N., and Li, X. (2003). A gain-of-function mutation in a plant disease resistance gene leads to constitutive activation of downstream signal transduction pathways in *suppressor of npr1-1*, *constitutive 1*. The Plant cell **15,** 2636-2646.

Zhou, R., Yazdi, A.S., Menu, P., and Tschopp, J. (2011). A role for mitochondria in NLRP3 inflammasome activation. Nature **469,** 221-225.

Zhu, Y., Qian, W., and Hua, J. (2009). Temperature modulates plant defense responses through NB-LRR proteins. PLoS Pathog **6,** e1000844.

Zhu, Z., Xu, F., Zhang, Y., Cheng, Y.T., Wiermer, M., Li, X., and Zhang, Y. (2010). Arabidopsis resistance protein SNC1 activates immune responses through association with a transcriptional corepressor. Proceedings of the National Academy of Sciences of the United States of America **107,** 13960-13965.

Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, J.D., Boller, T., and Felix, G. (2006). Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts Agrobacterium-mediated transformation. Cell **125,** 749-760.

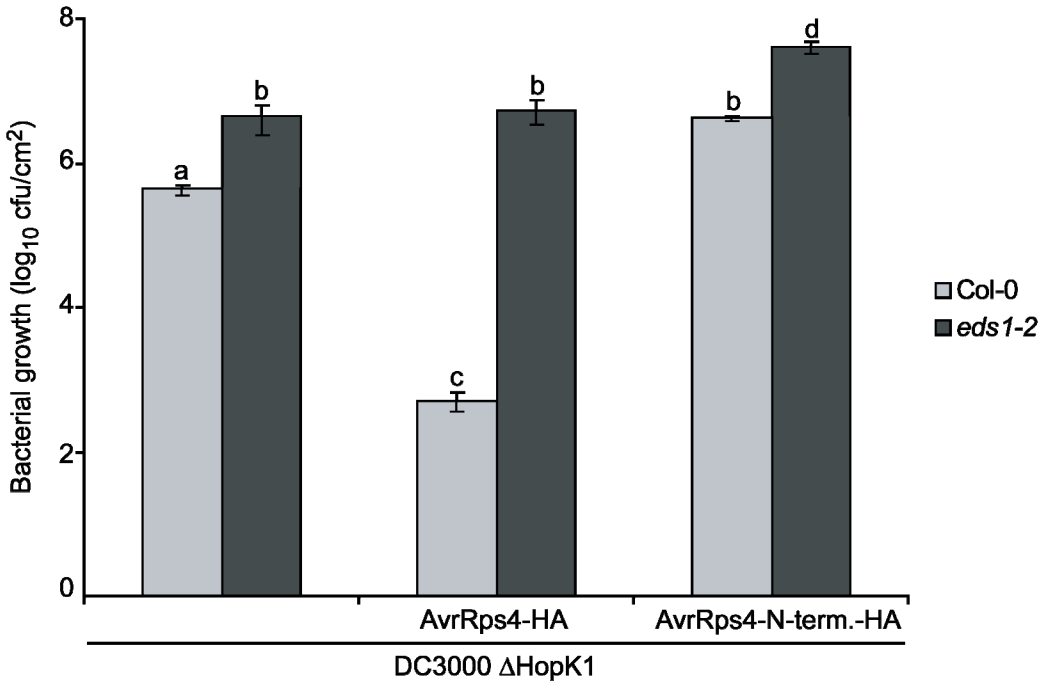
Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von auf Seite I angegebenen Teilpublikationen - noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Paul Schulze-Lefert betreut worden.

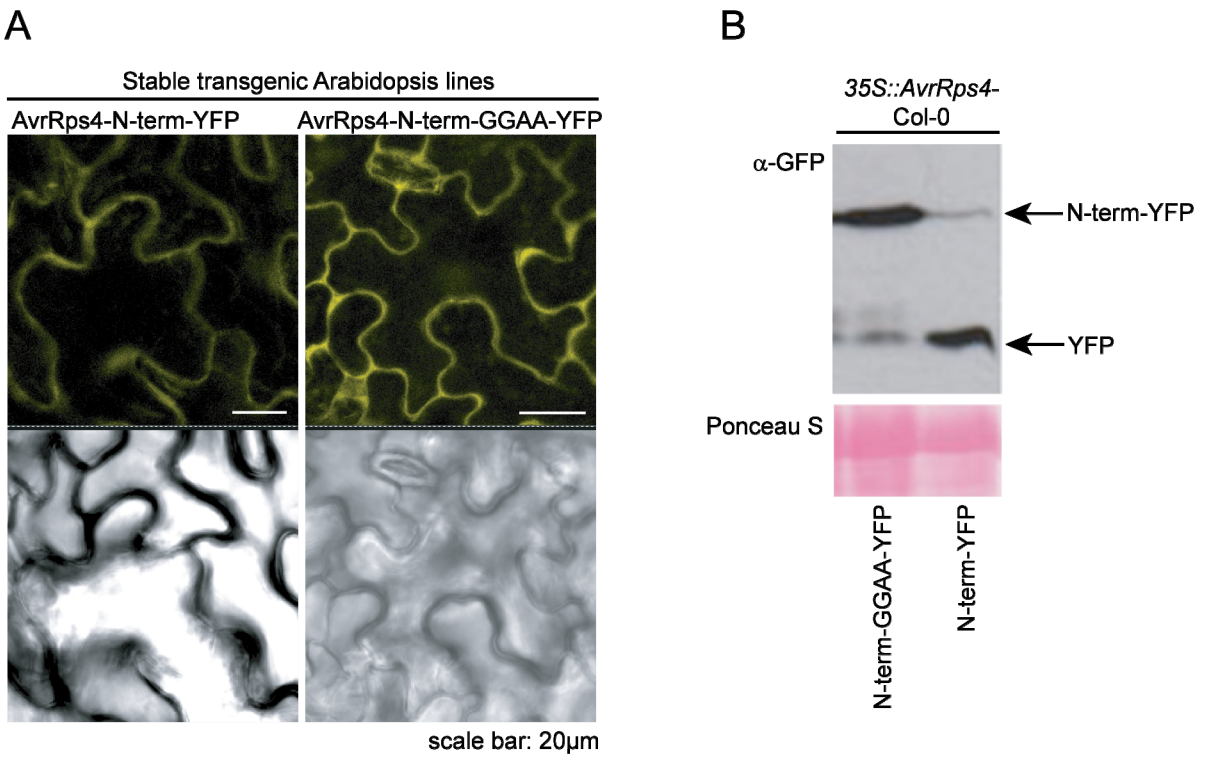
Köln, 28. März 2011 _____

Katharina Heidrich

Supplementary



Supplementary Fig. 1: Potential virulence activity of the N-terminus of AvrRps4.
Four week old plants were spray-infected with DC3000ΔHopK1 wt, AvrRps4-HA and AvrRps4 N-terminus-HA. The bacterial strain DC3000ΔHopK1 has a deletion in the DC3000 AvrRps4 homolog HopK1. Depicted is bacterial growth at 3 dpi. Letters indicate significant differences calculated by Sudent`s t-test. The experiment was repeated twice showing inconsistent results.



Supplementary Fig. 2: Localization of the N-terminus of AvrRps4 in stable transgenic Arabidopsis lines. **(A)** Confocal images of three week old T2 plant expressing AvrRps4-N-terminus-YFP and AvrRps4-N-terminus-GGAA-YFP in under control of CaMV 35S promoter in Col-0 background. The last two amino acids of the N-terminus, G132 and G133 were mutated to Alanine since these amino acids might be required for the cleavage of AvrRps4 protein *in planta*. The localization of AvrRps4 N-terminus in Arabidopsis upon mutation of G132 and G133 is not altered. **(B)** Immunoblot analysis of the stable transgenic Col-0 lines depicted in (A). Samples for Western Blot were taken from three week old plants and the membrane was probed with α-GFP antibody. Mutation of G132 and G133 to Alanine prevents that the YFP-tag is cleaved off.