Positioning the *Arabidopsis* TIR-NB-LRR immune receptor RPS4 in EDS1-dependent defence

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Abstract

In contrast to mammals, plants lack a humoral immune system and thus rely entirely on cell-autonomous innate immune defences to combat plant pathogens. While plant innate immunity is effective against the majority of invaders, successful pathogens have evolved effector proteins to overcome or suppress plant defences. In a process of co-evolution plants have, in turn, developed intracellular resistance (R) proteins that detect pathogen effectors or the consequences of their actions. Intracellular R proteins possess a central nucleotide binding (NB) domain and C-terminal leucine rich repeats (LRRs) that mediate effector recognition. Effector-induced activation typically leads to generation of reactive oxygen species, localised programmed cell death (hypersensitive response, HR) and restriction of pathogen colonisation. TIR-type NB-LRR receptors possess an N-terminal domain with sequence homology to the *Drosophila* Toll and mammalian Interleukin-1 receptors. In contrast to other NB-LRR R proteins, TIR-type receptors specifically require the lipase-like EDS1 protein to confer resistance. *Arabidopsis* EDS1 protein localises to the cytoplasm and the nucleus and functions together with salicylic acid (SA) in a positive amplification loop to induce local and systemic defences. It is not yet known how TIR-type receptors molecularly connect to and activate EDS1/SA signalling. Thus elucidating the subcellular localisation of TIR-NB-LRR R proteins is crucial to understand their function in EDS1/SA-dependent defence.

The work presented here characterises the *Arabidopsis* TIR-NB-LRR receptor RPS4 that recognises the *Pseudomonas syringae* effector protein AvrRps4 in terms of subcellular localisation and genetic interaction with *EDS1*. RPS4 protein localises to both endocellular membranes and the nucleus. Nuclear import requires a nuclear localisation signal (NLS) in the C-terminal domain of the receptor. Interference with RPS4 nuclear trafficking results in loss of RPS4-mediated resistance. Moreover, nuclear localisation of RPS4 is required for activation of defence gene expression downstream of AvrRps4 recognition that most likely occurs outside the nucleus. EDS1 functions as an intrinsic signal transducer in RPS4-mediated immunity and is essential for transcriptional reprogramming of defence genes upon RPS4 activation. The results presented suggest that activated RPS4 connects intimately to transcriptional regulation of defence genes in an EDS1-dependent manner and thus indirectly support a nuclear role of EDS1 in plant immunity.

Zusammenfassung

Im Gegensatz zu Säugetieren besitzen Pflanzen kein humorales Immunsystem. Pflanzliche Pathogenresistenz basiert auf immanenten, zellautonomen Immunantworten, die ein effektives Abwehrsystem gegen die Mehrzahl von Pflanzenschädlingen bilden. Adaptierte Phytopathogen verfügen jedoch über Effektorproteine, welche pflanzliche Immunreaktionen unterdrücken können. In einem co-evolutiven Prozess entwickelten Pflanzen intrazelluläre Resistenzproteine (R Proteine), die entweder Effektorproteine direkt oder effektorvermittelte Modifikationen von anderen pflanzlichen Proteinen erkennen. Charakteristisch für intrazelluläre R Proteine ist eine zentrale Nukleotidbindedomäne (NB) und C-terminale Leucin-reiche Sequenzwiederholungen (leucine rich repeats, LRRs), die Spezifität für das komplementäre Effektorprotein vermitteln. Aktivierung eines R Proteins durch den entsprechenden Effektor führt zur lokalen Freisetzung von radikalen Sauerstoffspezies sowie zur Aktivierung eines auf die Infektionsstelle begrenzten, programmierten Zelltodprogramms (hypersensitive response, HR). Das Zusammenspiel dieser lokalen Reaktionen ist in der Regel ausreichend, um den Infektionsversuch des Pathogens zu stoppen. TIR-NB-LRR Rezeptoren besitzen eine N-terminale Domäne mit Sequenzhomologie zum *Drosophila* Toll-Rezeptor und Interleukin-1 Rezeptoren von Säugetieren. Im Gegensatz zu anderen R Proteinen der NB-LRR Gruppe ist die durch TIR-NB-LRR Rezeptoren vermittelte Pathogenresistenz abhängig von EDS1, einem pflanzenspezifischen Protein mit Sequenzhomologie zu eukaryotischen Lipasen. Das *Arabidopsis* EDS1 Protein liegt sowohl im Cytoplasma als auch im Zellkern vor und vermittelt lokale und systemische Resistenz im Zusammenspiel mit Salicylsäure (SA). Dieser SA-abhängige Resistenzmechanismus unterliegt einer positiven feed-back Kontrolle und ist ein zentraler Bestandteil der Potenzierung von NB-LRR R Protein-vermittelten Immunsignalen. Die subzelluläre Lokalisation von TIR-NB-LRR Rezeptoren ist weitgehend unbekannt. Außerdem ist nicht untersucht, wie R Proteine des TIR-Typs nach ihrer Aktivierung EDS1/SA-abhängige Immunreaktionen auslösen. Lokalisationsstudien mit NB-LRR Proteinen der TIR-Gruppe sind daher ein wichtiger Schritt zum Verständnis der durch sie vermittelten EDS1/SA-abhängigen Pathogenresistenz.

Thema dieser Arbeit ist die Bestimmung der subzellulären Lokalisation des TIR-NB-LRR Rezeptors RPS4 aus *Arabidopsis*, der das *Pseudomonas syringae* Effektorprotein AvrRps4 erkennt. Zudem wird in genetischen Analysen die Position von RPS4 im *EDS1*-abhängigen Signaltransduktionsweg spezifiziert. RPS4 ist sowohl in der Endo-

membranfraktion als auch im Zellkern zu detektieren. Der Zellkernimport wird durch eine Importsequenz (nuclear localisation signal, NLS) in der C-terminalen Domäne von RPS4 vermittelt und Verminderung der nukleären RPS4 Konzentration führt zum Verlust der RPS4-induzierten Immunität. Nukleo-cytoplasmatischer Transfer von RPS4 ist Vorraussetzung für die Aktivierung von Abwehrgenen im Zellkern und liegt daher unterhalb der effektorvermittelten Aktivierung von RPS4. In Übereinstimmung mit diesem Ergebnis liegt der AvrRps4 Effektor als lösliches Protein im Cytoplasma, nicht jedoch im Zellkern vor. EDS1 fungiert als intrinsisches Signalprotein in der RPS4-vermittelten Immunreaktion und ist essentiell für die transkriptionale Aktivierung von Abwehrgenen nach RPS4 Aktivierung. Die Ergebnisse legen nahe, dass EDS1-abhängige, RPS4-vermittelte Immunität räumlich und funktional eng mit nukleären Transkriptionsregulatoren verbunden ist. Indirekt implizieren diese Resultate daher auch eine zentrale Rolle von EDS1 im Zellkern in der TIR-NB-LRR Rezeptor-vermittelten Resistenz.

Table of abbreviations

-	fused to (in the context of gene/protein fusion constructs)
° C	degree Celsius
Avr	avirulence
bp	base pair(s)
Bgh	*Blumeria graminis* forma specialis *hordei*
C	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
cM	centimorgan
d	day(s)
dATP	deoxyadenosinetriphosphate
dCTP	deoxycytidinetriphosphate
DEPC	diethylpyrocarbonate
dGTP	deoxyguanosinetriphosphate
dH_2O	deionised water
ddH_2O	deionised, distilled water
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleosidetriphosphate
DTT	dithiothreitol
dTTP	deoxythymidinetriphosphate
EDS1	Enhanced Disease Susceptibility 1
EDTA	ethylenediaminetetraacetic acid
ET	ethylene
EtOH	ethanol

Fig.	Figure
FLIM	fluorescence lifetime imaging microscopy
fp	fluorescent protein
f. sp.	forma specialis
g	gram
g	gravity constant (9.81 ms^{-1})
GFP	green fluorescent protein
GST	glutathione S transferase
GUS	β-glucuronidase reporter
h	hour(s)
His6	sextuple histidine tag
HR	hypersensitive response
HRP	horseradish peroxidase
kb	kilobase(s)
kDa	kiloDalton(s)
l	litre
LRR	leucine-rich repeats
m	milli
M	molar (mol/l)
µ	micro
MCS	multiple cloning site
min	minute(s)
mM	millimolar
mRNA	messenger ribonucleic acid
MW	molecular weight
N	amino-terminal
NB	nucleotide binding site
ng	nanogram
nm	nanometer
NOS	Nopaline synthase
OD	optical density
OE	over-expressor line
ORF	open reading frame
35S	35S promoter of CaMV

PAA	polyacrylamide
PAD4	Phytoalexin Deficient 4
PAMP	pathogen-associated molecular pattern
PCR	polymerase chain reaction
PAGE	polyacrylamide gel-electrophoresis
pH	negative decimal logarithm of the H^+ concentration
PR	pathogenesis related
PRR	PAMP/pattern recognition receptor
Pst	*Pseudomonas syringae* pv. *tomato*
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
SAG101	Senescence Associated Gene 101
SAR	systemic acquired resistance
SDS	sodium dodecyl sulphate
sec	second(s)
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
VIGS	virus induced gene silencing
vir	virulence

v/v	volume per volume
WT	wild-type
w/v	weight per volume
YFP	yellow fluorescent protein

1 Introduction

As in animals, plants successfully combat a wide range of pathogens and pests such as viruses, bacteria, fungi, oomycetes and insects (Dangl and Jones, 2001). Whereas necrotrophic pathogens feed on dead plant tissue, biotrophs are able to subvert photosynthesis products advantageous for their own replication from living plant cells. In spite of a plethora of pathogens, disease is a rather rare case in nature. Work of the past decade has revealed the evolution of a sophisticated multi-layered plant immune system that detects and combats biotrophic pathogens at different stages of the infection process (Jones and Dangl, 2006). However, it is poorly understood how cues from different plant immunity layers are integrated and how an adequate defence response comprising transcriptional reprogramming, changes in redox homeostasis but also production and directed delivery of antimicrobial compounds, is achieved.

1.1 Non-host resistance – A first barrier for non-adapted pathogens

Each plant species is resistant to a wide range of potential plant pathogens, an observation referred to as species-level resistance or non-host resistance. The reason for this effective protection is that most pathogen colonisation attempts are stopped early in pathogenesis at the levels of cell wall penetration (oomycetes and fungi) or apoplast colonisation (bacteria) due to the inability of non-adapted pathogens to overcome constitutive barriers. These barriers are of both physical and chemical nature and comprise epidermal wax layers, the plant cell wall, toxic secondary metabolites and the a basic apoplastic pH (Heath, 2000). The importance of cell wall integrity in host defence is poorly understood. However, mutations in the *Arabidopsis POWDERY MILDEW RESISTANCE 5 (PMR5)* and *PMR6* genes encoding a protein of unknown function and a pectate lyase, respectively, indicate that wild type cell wall composition is crucial for successful infection with the adapted powdery mildew pathogen *Golovinomyces chicoacearum* (Vogel et al., 2002; Vogel et al., 2004). Passive and constitutive species level resistance mechanisms have been termed “type I” non-host resistance (Holub and Cooper, 2004). Inducible plant responses (type II) also contribute to non-host resistance. The active character of type II responses is underpinned by the fact that many depend on secondary metabolites such as salicylic acid (SA) or the hormones jasmonic

acid (JA) and ethylene (ET) (van Loon et al., 2006). Generally, SA is required for resistance towards biotrophs whereas mutations in the JA/ET pathway often compromise resistance to necrotrophs. One of the earliest cellular reactions to attempted pathogen penetration is a rearrangement of the actin cytoskeleton followed by redistribution of secretory pathway organelles towards the site of penetration (Schmelzer, 2002; Takemoto et al., 2003). Analysis of the *Arabidopsis penetration 1* (*pen1*) mutant, that has reduced penetration resistance towards the non-adapted powdery mildew *Blumeria graminis* f. sp. *hordei*, provides substantial evidence for a role of directed vesicular transport in cell wall defence (Collins et al., 2003). *PEN1* encodes a plasma membrane resident syntaxin that focally accumulates at penetration sites and may serve as a docking point for vesicles delivering cell wall materials or compounds detrimental to the pathogen (Schulze-Lefert, 2004b).

Consistent with the idea of focal accumulation of antimicrobial compounds, pathogen recognition often leads to callose deposition, a local reinforcement of cell walls. Callose deposition requires the callose synthase PMR4 (Jacobs et al., 2003; Nishimura et al., 2003). Counter intuitively, *pmr4* mutants are more resistant to a wide range of adapted powdery mildew fungi and oomycete (*Hyaloperonospora parasitica, Hp*.) strains suggesting that cell wall reinforcement, although generally thought to be a protective mechanism might be exploited by adapted pathogens to overcome cell wall based defences (Schulze-Lefert, 2004b). Furthermore *pmr4* resistance requires functional SA metabolism and thus seems to be linked to central stress signalling compounds. Similarly, mutations in the cellulose synthase gene *CONSTITUTIVE EXPRESSION OF VSP1* (*CEV1*) lead to constitutive expression of JA/ET-dependent defence marker genes and broad range resistance to different pathogens (Ellis and Turner, 2001; Ellis et al., 2002). Thus plants might possess a cell wall integrity surveillance system that directly or indirectly impinges on central stress or defence pathways (Schmelzer, 2002; Collins et al., 2003; Schulze-Lefert, 2004b).

Inducible type II non-host resistance relies on the recognition of pathogens via perception of pathogen-associated molecular pattern (PAMPs) (Nürnberger and Lipka, 2005). As in animals, plants have evolved the ability to differentiate “self” from “non-self”, culminating in a surveillance system of pattern recognition receptors (PRRs) sensing conserved molecular patterns that are danger signals of microbial invasion (Ausubel, 2005; Nürnberger and Lipka, 2005). The characterisation of different PAMPs perceived by plant cells revealed that PAMPs are usually i) highly conserved and ubiquitous molecules within a genus of microbes ii) fulfil a function essential for the pathogen and iii) are absent from the host plant (Felix et al., 1999; Brunner et al., 2002; Felix and Boller, 2003; Kunze et al., 2004).

The currently best characterised PAMP/PRR pair in plants is the *Arabidopsis* FLAGELLIN SENSING 2 (FLS2) receptor that recognises an N-terminal 22 amino acid epitope (flg22) from flagellin of several Gram-negative bacterial species (Felix et al., 1999; Gomez-Gomez and Boller, 2000; Gomez-Gomez et al., 2001; Zipfel et al., 2004; Chinchilla et al., 2006; Robatzek et al., 2006). Like animal PRRs of the Toll and Interleukin 1-like transmembrane receptors (TLRs), FLS2 has an extracellular leucine rich repeat (LRR) region and an intracellular signalling domain (Gomez-Gomez and Boller, 2000). In contrast to the mammalian TLR5 flagellin receptor that signals through intracellular homotypic TIR domain interactions with MyD88 (Hayashi et al., 2001) FLS2 requires the activity of its intracellular kinase domain for flg22-triggered responses (Gomez-Gomez et al., 2001). Also, TLR5 and FLS2 recognise different epitopes of the conserved flagellin molecule suggesting that PRRs in these different lineages are the consequence of convergent evolution (Felix et al., 1999; McDermott et al., 2000; Eaves-Pyles et al., 2001; Ausubel, 2005). Activation of PRRs by their cognate PAMPs results in cytosolic and nuclear calcium fluxes, accumulation of reactive oxygen species (ROS) and nitric oxide (NO) production (Zhang and Klessig, 2001; Nakagami et al., 2005; Garcia-Brugger et al., 2006). Signal transduction by activated PRRs involves MAPK cascades culminating in transcriptional reprogramming of defence associated genes at least in part regulated by the WRKY group of transcription factors (Asai et al., 2002; Ülker and Somssich, 2004; Ichimura et al., 2006; Shen et al., 2007; Suarez-Rodriguez et al., 2007). Different PRRs appear to share MAPK cascades exemplified by signal transduction from *Arabidopsis* FLS2 and the Elongation-factor TU-receptor (EFR) (Zipfel et al., 2006). In summary, PRR-mediated PAMP perception provides plants with an effective defence system against pathogens that are able to overcome passive plant barriers.

Given the ability of plants to recognise a multitude of PAMPs, successful pathogens have to either avoid or actively suppress recognition of their PAMPs. Examples of both strategies have been reported. The bacterial plant pathogens *Agrobacterium tumefaciens* and *Ralstonia solanacearum* as well as symbiotic *Rhizobium* species possess functional flagellins that are not effectively recognised by FLS2 (Felix et al., 1999; Pfund et al., 2004; Sun et al., 2006). Adapted biotrophic pathogens have further evolved mechanisms to actively suppress PRR-mediated plant defences (Mudgett, 2005; Kamoun, 2006). *Pseudomonas syringae* pv. *tomato* produces the phytotoxin coronatine to suppress plant defences induced by flagellin perception (Brooks et al., 2005; Li et al., 2005). In addition, *P. syringae* and other bacterial plant pathogens inject effector proteins directly into the host cell by means of their needle-like type three secretion systems (T3SS) (Alfano and Collmer, 2004; Büttner and Bonas, 2006).

The mechanism of interference with host defences has been recently elucidated for some *P. syringae* effector proteins delivered through the T3SS. For example the type three effector AvrPtoB, a E3 ubiquitin ligase, acts as a general suppressor of cell death in *Nicotiana benthamiana* counteracting the host's programmed cell death response (Janjusevic et al., 2006). Another effector AvrPto was shown to suppress the formation of callose containing papillae at the plant cell wall (Hauck et al., 2003). Yet other bacterial effectors perturb the plant's SUMOylation system or seem to directly affect transcription of host genes (Hauck et al., 2003; Schornack et al., 2006). Importantly, suppression of the host's PRR-mediated defence by a pathogen's effector repertoire is not absolute. The isolation of immunosuppressed *enhanced disease susceptibility* (*eds*) mutants revealed that even susceptible plants are able to attenuate pathogen growth to some extent (Cao et al., 1994; Glazebrook et al., 1996; Parker et al., 1996). The term “basal defence” is commonly used to define this residual, effector-suppressed level of PRR-mediated resistance (Abramovitch and Martin, 2004). Many *eds* mutants that are hypersusceptible to primarily biotrophic pathogens such as *Hp* or *P. syringae* have defects in both pathogen-inducible upregulation of SA and plant defences induced by elevated SA levels (Cao et al., 1994; Shah, 2003; Wang et al., 2005). In contrast, inducible responses involving the plant hormones JA and ET are generally effective against necrotrophs (Glazebrook, 2005). Antagonism between the SA and JA/ET pathways has been proposed as a regulatory mechanism to orchestrate plant defences towards biotrophs and necrotrophs (Feys and Parker, 2000; Brodersen et al., 2006).

1.2 NB-LRR receptor mediated immunity

Acquisition of effector proteins by pathogens resulted in a co-evolutionary adaptation of a second resistance layer superimposed on PRR-mediated defence by host plants (Chisholm et al., 2006; Tiffin and Moeller, 2006). Resistant plant cultivars evolved resistance (*R*) genes that encode receptors specific for pathogen effectors (gene-for-gene hypothesis) (Flor, 1971; Ellis et al., 2000). In contrast to non-host resistance, which acts at the species level, resistance conferred by *R* genes tends to be cultivar-specific. This selectivity might be explained by differences in environmental factors within the distinct cultivar habitats, the limited number of *R* genes present in plant genomes and fitness costs of effective *R* gene mediated defence (Ronald, 1998; Brown, 2003; Tian et al., 2003; Meyers et al., 2005).

In absence of a matching R protein (compatible interaction) the effector exerts its virulence function, whereas recognition by its cognate plant receptor (incompatible interaction) results in a rapid defence response that overrides effector-suppressed PRR-mediated defences (Nimchuk et al., 2003; Nürnberger et al., 2004; Chisholm et al., 2006). By definition the recognised effector in an incompatible interaction is referred to as avirulence (Avr) protein.

Effector-mediated activation of R proteins induces an increase in cytosolic calcium, depolarisation of the plasma membrane, a localised ROS burst, NO production and the activation of phospholipases – a pattern of cellular responses that shows a significant overlap with those following PRR activation (Nimchuk et al., 2003; Nürnberger et al., 2004; Andersson et al., 2006; Garcia-Brugger et al., 2006). In many cases effector recognition results in a localised programmed cell death (hypersentive response, HR) and it has been speculated that the ROS burst and the HR represent direct antimicrobial defences although there is also compelling evidence for signalling functions (Rusterucci et al., 2001; Nimchuk et al., 2003; Lorrain et al., 2004). The local HR leads to induction of systemic acquired resistance (SAR) which is active throughout the plant and results in broad spectrum resistance towards otherwise virulent pathogens (Durrant and Dong, 2004). However, recent results question the role of the HR in systemic resistance and argue that PRR activation is sufficient to induce SAR (Mishina and Zeier, 2007).

Although the gene-for-gene hypothesis is compatible with a direct interaction between R proteins and their respective effectors direct recognition has been reported for only few effectors (Jia et al., 2000; Deslandes et al., 2003; Dodds et al., 2006). Other well characterised R proteins evolved to associate with targets of pathogen effectors and are activated indirectly by effector-induced alterations of these target proteins (Innes, 2004). This indirect recognition scenario is formulated in the guard hypothesis predicting R proteins to function as "guards" of target proteins ("guardees") (Van der Biezen and Jones, 1998b). Since many host proteins that function in PRR-mediated immunity might be common targets modified by more than one effector, the guard hypothesis could explain how plants recognise a plethora of effectors with a limited set of R proteins. An example for the indirect recognition paradigm is the *Arabidopsis* RIN4 (RPM1 INTERACTING PROTEIN 4) that functions as a negative regulator of PRR-mediated resistance towards *P. syringae* (Kim et al., 2005). RIN4 is phosphorylated by AvrB and AvrRpm1, two sequence-unrelated *P. syringae* effectors, and phosphorylated RIN4 activates the RPM1 resistance protein (RESISTANCE TO P. SYRINGAE PV. MACULICOLA 1) (Mackey et al., 2002). A third *P. syringae* effector, AvrRpt2, functions as a cysteine protease and promotes bacterial virulence by eliminating a

number of host proteins, among these RIN4 (Axtell et al., 2003; Axtell and Staskawicz, 2003; Lim and Kunkel, 2004). In turn a second R protein, RPS2 (RESISTANCE TO P. SYRINGAE 2), stably associates with RIN4 *in planta* and is activated by AvrRpt2-induced elimination of RIN4 (Axtell and Staskawicz, 2003). Although the precise function of RIN4 in resistance to *P. syringae* needs to be determined, this example illustrates that effector-mediated suppression of PRR-mediated resistance and host surveillance appear to converge on a limited number of target proteins.

Some R proteins span the plasma membrane and detect their cognate effectors in the apoplast, while others are intracellular receptors (Dangl and Jones, 2001). Except for a few members, intracellular R proteins combine a central NB (nucleotide binding) domain with C-terminal leucine rich repeats (LRRs) (Dangl and Jones, 2001). The NB domain is part of a larger region termed NB-ARC due to its conservation in human APOPTOSIS ACTIVATING FACTOR 1 (APAF1) and *Caenorhapditis elegans* CELL DEATH 4 (CED4) (Traut, 1994; van der Biezen and Jones, 1998a; Takken et al., 2006). A similar NB-LRR domain combination is found in mammalian PRRs of the CATERPILLAR (Caspase recruitment domain, transcription enhancer, R/purine-binding, pyrine, lots of leucine repeats) family (Ting et al., 2006). Both plant and animal NB-LRR receptors belong to the STAND (signal transduction ATPases with numerous domains) group of NTPases characterised by a proposed switch function of the NB domains for receptor signal transduction (Leipe et al., 2004). Indeed ATP or GTP binding has been demonstrated for plant and animal NB-LRR proteins and is dependent on the conserved NB domain P-loop motif (also referred to as kinase-1a or Walker A motif) (Harton et al., 1999; Tameling et al., 2002). ATP hydrolase activity has been reported for two NB-LRR receptors (Tameling et al., 2002). A functional P-loop motif is required for receptor activation, a process that includes effector-induced changes in intramolecular interactions and the formation of receptor oligomers (Linhoff et al., 2001; Moffett et al., 2002; Mestre and Baulcombe, 2006; Rairdan and Moffett, 2006; Ade et al., 2007).

Residues in the LRR domain of plant R proteins are the main determinant of specificity for an effector and direct effector-LRR interactions have been reported (Jia et al., 2000; Shen et al., 2003; Dodds et al., 2006; Ueda et al., 2006). However, in some cases amino acids in the variable N-terminal receptor domains contribute to the specificity indicative of intramolecular domain interactions (Luck et al., 2000). Although the N-terminal regions of NB-LRR receptors show less overall conservation, a number of different annotated domains have been identified and there is a preference for protein-protein interaction domains. Many

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 culminating in production of proinflammatory cytokines (Strober et al., 2006; Ting et al., 2006).

In contrast, most plant NB-LRR proteins either have an N-terminal domain with a predicted coiled coil fold (CC class) or carry a TIR (Toll and Interleukin 1 receptor) domain with significant sequence homology to *Drosophila* Toll and human TLRs (Whitham et al., 1994; Meyers et al., 2003). As in human TLRs, plant TIR domains undergo homotypic interactions although the role of dimerisation in TIR-NB-LRR receptor function remains to be determined (Mestre and Baulcombe, 2006). Functional studies of the NB domain led to a hypothesis predicting that the ADP-bound state of R proteins represents the inactive state (Takken et al., 2006). In absence of the cognate effectors the LRR domain is thought to associate with the ARC2 region of the NB-ARC domain as has been shown for the several NB-LRR receptors (Moffett et al., 2002; Leister et al., 2005; Ade et al., 2007). The model further predicts that effector recognition (either direct or indirect) leads to a reorientation of the LRR domain which in turn induces conformational changes in the NB-ARC region. The induced "open" conformation of the NB-ARC domain would lead to release of ADP and favour ATP binding. The ATP-bound active state of the receptor would then induce downstream signalling, possibly through receptor oligomerisation and the recruitment of downstream signalling components. Finally, ATPase activity of the NB-ARC domain might revert the receptor to its inactive state through ATP hydrolysis (Takken et al., 2006).

Forward genetic screens for compromised NB-LRR receptor-mediated immunity in *Arabidopsis* and tobacco identified components of the eukaryotic chaperone machinery. Mutations in *HSP90* (*HEAT SHOCK PROTEIN 90*) compromise resistance to *P. syringae* triggered by the CC-NB-LRR receptors RPM1 and RPS2 (Hubert et al., 2003; Takahashi et al., 2003). Also, HSP90 function is essential for resistance to tobacco mosaic virus (TMV) in *N. benthamiana* conferred by N, a TIR-type NB-LRR receptor (Lu et al., 2003). These genetic dependencies are supported by yeast two hybrid and *in planta* interactions between R proteins and HSP90 indicating that NB-LRR receptors are clients of the core HSP90/HSC70 (HEAT SHOCK COGNATE 70) machinery (Hubert et al., 2003; Liu et al., 2004; de la Fuente van Bentem et al., 2005).

Further genetic screens revealed that RAR1 (REQUIRED FOR MLA12 SPECIFIED RESISTANCE) and SGT1 (SUPPRESSOR OF G2 ALLELE OF SKP1), two proteins conserved among eukaryotes that have molecular features of co-chaperones (Schulze-Lefert,

2004a), are required for function of some but not all NB-LRR receptors (Austin et al., 2002; Muskett et al., 2002; Tornero et al., 2002b; Liu et al., 2004; Azevedo et al., 2006). Loss of RAR1 leads to depleted NB-LRR receptor levels endorsing a direct or indirect chaperoning function and it is generally assumed that loss of race-specific resistance in *rar1* plants is a consequence of diminished NB-LRR protein levels (Tornero et al., 2002b; Belkhadir et al., 2004b; Bieri et al., 2004; Holt et al., 2005). However, *rar1* mutants also display attenuated PRR-mediated resistance, have reduced SA levels and respond faster to flg22 treatment with callose depositions (Holt et al., 2005; Shang et al., 2006 and J. Kaur & J. Parker, unpublished). These observations strongly suggest additional roles of RAR1 in PRR-mediated resistance.

RAR1 and SGT1 proteins interact in the yeast-2-hybrid system and associate *in planta* (Azevedo et al., 2002; Liu et al., 2002). Both proteins bind to distinct domains of HSP90 supporting a possible function as co-chaperones (Takahashi et al., 2003). Like its yeast and human homologues plant SGT1 associates with subunits of SCF (Skp1/Cullin/F-box) type E3 ubiquitin ligase complexes and the COP9 signallosome, that functions in protein degradation via the 26S proteasome (Wei and Deng, 1999; Azevedo et al., 2002; Liu et al., 2002; Liu et al., 2004). Thus SGT1 might link NB-LRR receptor function to control of receptor turnover or degradation. Indeed NB-LRR protein stabilising and degrading functions have been found for RAR1 and SGT1, respectively, although it remains to be determined whether this antagonistic scenario holds true for other NB-LRR receptors (Holt et al., 2005; Azevedo et al., 2006). NB-LRR protein levels need to be tightly regulated to avoid inappropriate triggering of cell death (Axtell and Staskawicz, 2003; Bieri et al., 2004). Thus it appears conclusive that NB-LRR receptor turnover is regulated by the HSP90/HSC70 chaperone machinery in association with the co-chaperones RAR1 and SGT1 (Schulze-Lefert, 2004a).

Genetic analysis of *SGT1* in *Arabidopsis* is complicated by the presence of two *SGT1* genes, *SGT1a* and *SGT1b* (Azevedo et al., 2002). Only *sgt1b* alleles have been identified in forward genetic screens suggesting a predominant function of SGT1b in NB-LRR receptor-mediated defence (Austin et al., 2002; Tor et al., 2002). The *sgt1a/sgt1b* double mutant is an early embryo lethal pointing to essential developmental functions of SGT1a and SGT1b (Azevedo et al., 2006). Also, SGT1a and SGT1b have partially redundant functions since both single mutants are viable and *SGT1a,* if over-expressed, can function in NB-LRR receptor-mediated defence (Azevedo et al., 2006).

The signal transduction pathway downstream of activated plant NB-LRR receptors is poorly understood. Forward genetics screens revealed that TIR- and CC-type NB-LRR

receptors predominantly employ different signalling components (Aarts et al., 1998). Whereas mutations in *EDS1* encoding a protein with sequence homology to eukaryotic lipases compromise local resistance conferred by TIR- type receptors, many CC-NB-LRR proteins require the plasma membrane-anchored NDR1 protein to induce resistance (Parker et al., 1996; Aarts et al., 1998; Feys et al., 2001; Peart et al., 2002; Hu et al., 2005). Some notable exceptions to this paradigm have been found. For example the *Arabidopsis HRT* gene encodes a CC-NB-LRR receptor and yet genetically requires *EDS1* to counteract viral spread (Chandra-Shekara et al., 2004). However, this study did not investigate whether it was an early *EDS1* signalling function or a later *EDS1*-dependent potentiation of SA-mediated defences that led to suppression of virus replication (see below). Also broad spectrum powdery mildew resistance conferred by RPW8 proteins that have CC and putative transmembrane domains depends on *EDS1*. However, the two RPW8 proteins confer broad spectrum resistance and lack NB and LRR domains (Xiao et al., 2001). Thus RPW8 proteins might use signal transduction pathway distinct from NB-LRR receptors.

In spite of differences in CC- and TIR-type receptors, activation of both receptor types eventually culminates in transcriptional regulation of a core set of SA biosynthesis and SA signalling defence genes (Shah, 2003). Forward genetic screens revealed that EDS1 and the sequence-related PAD4 (PHYTOALEXIN DEFICIENT 4) proteins function upstream of and in synergy with SA to amplify signals from NB-LRR receptors (Glazebrook et al., 1996; Parker et al., 1996; Glazebrook et al., 1997; Zhou et al., 1998; Jirage et al., 1999; Feys et al., 2001). Elevated SA levels induce effective resistance against a range of biotrophic pathogens and exogenous application of SA is sufficient to activate transcription of TIR-type *R* genes, *EDS1*, *PAD4* and central defence markers such as *PR1* (*PATHOGENESIS RELATED 1*) (Shirano et al., 2002; Shah, 2003). Based on these results it has been proposed that initial signals from NB-LRR receptors activate a positive feedback loop involving SA, EDS1 and PAD4 that leads to heightened resistance (Feys et al., 2001). SA-induced redox changes further cause dissociation of cytoplasmic NPR1 (NONEXPRESSER OF *PR* GENES 1) oligomers. Monomeric NPR1 translocates to the nucleus where it acts in concert with TGA class transcription factors as a central inducer of systemic immunity (Kinkema et al., 2000; Dong, 2004).

Importantly, genetic analysis by Rusterucci et al. (2001) demonstrated that apart from its potentiating function EDS1 plays an additional role in signalling from TIR-type NB-LRR receptors. Using the *lsd1* (*LESIONS SIMULATING DISEASE RESISTANCE RESPONSES*) mutant that has a reduced threshold for cell death induced by SA, ROS or NB-LRR receptors

(Dietrich et al., 1994; Jabs et al., 1996) the authors found that *EDS1* is dispensable for local cell death induced by the CC-NB-LRR receptor RPM1. However *lsd1*-induced spreading of the cell death response is completely *EDS1*-dependent. Conversely, *EDS1* is required for both local and spreading cell death initiated by the TIR-type RPS4 receptor pointing out a TIR-receptor-specific EDS1 signalling function at the infection site. Based on pathogen infection assays Rusterucci et al. (2001) further positioned EDS1 upstream of the local HR.

Nevertheless, the molecular functions of EDS1 and PAD4 proteins in both local and systemic resistance have not been unravelled (Wiermer et al., 2005). Biochemical analysis revealed that EDS1 forms homodimers but also interacts directly with PAD4 and a third lipase-like protein SAG101 (SENESCENCE ASSOCIATED GENE 101) (Feys et al., 2001; Feys et al., 2005). *pad4* mutants are partially compromised in TIR-type receptor-mediated immunity whereas *sag101* single mutants do not show an *eds* phenotype. However the *pad4/sag101* double mutant phenocopies *eds1* plants, suggesting that PAD4 and SAG101 have partially redundant functions and might regulate EDS1 signalling (Feys et al., 2005). Although all three proteins show significant homology to eukaryotic lipases no lipolytic activity has been detected so far and conserved amino acid residues in EDS1 predicted to be essential for lipase activity do not interfere with its function in disease resistance (S. Rietz & J. Parker, unpublished results).

EDS1 and PAD4 proteins localise to both the cytoplasm and the nucleus whereas SAG101 protein is entirely nuclear (Feys et al., 2005). The subcellular localisation of most TIR-NB-LRR receptors has not been determined and thus the intriguing question of how signals from activated TIR-type receptors connect to the EDS1/SA signalling node remains to be answered. In a recent publication Burch-Smith et al. (2007) used functional fluorescence protein-tagged versions of the tobacco N TIR-NB-LRR receptor to show that N localises to the nucleus and the cytoplasm. Combining these studies with an assay for N-mediated resistance to TMV the authors further established that the N protein requires nuclear localisation in order to mount an effective antiviral defence. These results tally with a previous report by Shen et al. (2007) demonstrating that nuclear localisation of the barley MLA10 CC-NB-LRR receptor is essential for race-specific resistance towards *Bgh*. The same study revealed a potential link to PRR-mediated resistance since nuclear MLA10 interacts with a WRKY transcription factor that functions as a suppressor of PRR-mediated defences (Shen et al., 2007). Thus one potential mechanism that NB-LRR receptors might employ to activate defences is sequestration or inactivation of negative regulators of PAMP-induced resistance. This idea is supported by microarray transcript profiling data revealing that

transcriptional responses to PAMPs and those induced by effector recognition strongly overlap and mainly differ in timing and altitude of the response (Tao et al., 2003; Caldo et al., 2004). The N and MLA10 reports further demonstrate that combining functional studies with subcellular localisation analysis is a valuable approach to elucidate NB-LRR receptor function.

1.3 The *Arabidopsis* TIR-NB-LRR receptor RPS4

The *RPS4* locus conferring resistance to *P. syringae* strains expressing AvrRps4 was initially mapped to a ~15 cM interval on chromosome 5 using a cross between *Arabidopsis* accessions RLD (susceptible) and Ws-0 (resistant) (Hinsch and Staskawicz, 1996). A second study (Gassmann et al., 1999) delimited the genetic region to ~2.2 cM and showed that RLD susceptibility towards *P. syringae* AvrRps4 was complemented by expression of the Col-0 At5g45250 gene encoding a TIR-NB-LRR receptor. In addition to the conserved TIR and NB domains RPS4 possesses 15 degenerate LRRs and a 320 amino acid C-terminal domain that lacks sequence homology to proteins of known function. Analysis of the non-functional RLD *rps4* gene revealed that RLD *rps4* is transcribed and encodes a protein that differs in only five of the 1217 amino acids from functional Col-0 RPS4, none of them being conserved in other TIR-NB-LRR R proteins.

Like other TIR-type receptors *RPS4* gives rise to truncated transcripts encoding the TIR-NB domains and various parts of the LRR region (Gassmann et al., 1999; Jordan et al., 2002) and a later study suggested that a combination of full length *RPS4* and truncated transcripts is required for full RPS4-mediated resistance (Zhang and Gassmann, 2003). As a member of the TIR-NB-LRR family RPS4 shows an absolute dependence on *EDS1* and RPS4-triggered resistance is partially compromised in *pad4* mutants (Aarts et al., 1998; Feys et al., 2001). Mutations in *RAR1* affect RPS4 function and unexpectedly this effect is much more pronounced in L*er* compared to Col-0 (Muskett et al., 2002; Tornero et al., 2002b). In contrast, mutations in *SGT1b* or *SGT1a* do not attenuate RPS4 function (Austin et al., 2002; Azevedo et al., 2006).

AvrRps4 is one of a few characterised effectors recognised by a TIR-NB-LRR protein (Hinsch and Staskawicz, 1996; Erickson et al., 1999; Rehmany et al., 2005). Thus it is possible to trigger RPS4-mediated defence responses either by infiltration of *P. syringae*

strains expressing AvrRps4 or by inducible expression of *AvrRps4* in *Arabidopsis* (Mackey et al., 2003). Also, RPS4 is a *bona fide* TIR-type receptor with genetic dependence on *EDS1, RAR1* and SA. For these reasons I chose the RPS4/AvrRps4 system to analyse biochemically the TIR-NB-LRR receptor and its *EDS1*-dependent function in disease resistance.

1.4 Thesis aims

Determining the subcellular localisation of TIR-NB-LRR receptors is a prerequisite to understand their function in race-specific resistance. Furthermore, proteins like EDS1 that are candidate signal transducers of plant immune receptors need to be positioned in TIR-NB-LRR receptor signalling. It is not yet understood how activated TIR-type receptors impinge on the transcriptional machinery and which mechanisms lead to elevated SA concentrations.

When this work was started almost no data on subcellular localisation of TIR-NB-LRR proteins or their modes of function were available (Deslandes et al., 2003) and only two receptors have been analysed since (Weaver et al., 2006; Burch-Smith et al., 2007). RPS4 resistance in particular was characterised only genetically (Gassmann et al., 1999; Kwon et al., 2004; Zhang et al., 2004). A first aim of this study was therefore to generate transgenic *Arabidopsis* lines expressing functional epitope-tagged RPS4 protein in an *rps4* mutant background. To assess whether *EDS1* or *RAR1*, that are genetically required by RPS4, directly affect RPS4 protein levels or localisation these lines were to be crossed into *eds1* and *rar1* null mutant backgrounds, respectively. Although RPS4-mediated disease resistance is *EDS1*-dependent (Aarts et al., 1998) the precise position of *EDS1* in TIR-NB-LRR receptor signal transduction was unknown when this work was initiated. To dissect RPS4-triggered EDS1 signalling, transgenic lines over-expressing functional epitope-tagged RPS4 protein in *EDS1* and *eds1* mutant backgrounds were generated. These transgenics were expected to be in a constitutive RPS4 signalling state and thus would allow me to position where *eds1* interferes with TIR-NB-LRR receptor signalling. The availability of *P. syringae* strains expressing *AvrRps4* and a chemically inducible *AvrRps4* expression system allowed me to monitor changes in RPS4 protein levels and subcellular accumulation upon pathogen challenge or AvrRps4 expression.

2 Materials and Methods

The Materials and Methods section is subdivided into two parts. In the first part **(2.1)** materials used throughout this study, including plant lines, pathogens, bacterial strains, chemicals, enzymes, media, buffers and solutions are listed. Methods applied in this work are described in the second part **(2.2)**.

2.1 Materials

2.1.1 Plant materials

2.1.1.1 *Arabidopsis thaliana*

Arabidopsis wild-type and mutant lines use in this study are listed in Table 2.1 and 2.2, respectively.

Table 2.1. Wild-type *Arabidopsis* accessions used in this study

Accession	Abbreviation	Original Source
Columbia	Col-0	J. Dangl[a]
Landsberg-*erecta*	L*er*	Nottingham *Arabidopsis* Stock Centre[b]
RLD	RLD-0	W. Gassmann[c]

[a]University of North Carolina, Chapel Hill, NC, USA
[b]Nottingham, UK
[c]University of Missouri-Columbia, Columbia, MO, USA

Table 2.2. 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)
eds1-2	L*er*	FN	(Falk et al., 1999)
eds1-1	Ws-0	EMS	(Parker et al., 1996)
rps4-2	Col-0	T-DNA	SALK collection
rps4-3	Col-0	T-DNA	GABI-Kat collection
rps4-10	L*er*	Ds3(GT)	CSHL collection
rar1-28	Col-0	EMS	(Tornero et al., 2002b)
rar1-13	L*er*	EMS	(Muskett et al., 2002)
sgt1b^{eta3}	Col-0	EMS	(Gray et al., 2003)
sgt1b-3	L*er*	EMS	(Austin et al., 2002)
rar1-13/sgt1b-3	L*er*	EMS/EMS	P. Muskett[b], unpublished

[a]L*er eds1-2* allele introgressed into Col-0 genetic background, 8th backcrossed generation, referred to as "*eds1*" in this study
[b]Max-Planck-Institute for Plant Breeding Research, Carl-von-Linné-Weg 10, 50829 Cologne, Germany

EMS: ethylmathane sulfonate; FN: fast neutron; T-DNA: transfer-DNA; Ds3(GT): gene trap insertion

Table 2.3. Transgenic *Arabidopsis* lines used in this study

Line	Accession	Construct	Reference/Source
BON1-3HA	Col-0	*pBON1-BON1-3HA*	(Hua et al., 2001)
RPM1-myc	Col-0	*pRPM1-RPM1-myc*	(Boyes et al., 1998)
ER-GFP	Col-0	*35S-GFP-HDEL*	(Matsushima et al., 2003)

2.1.1.2 *Nicotiana benthamiana*

Nicotiana benthamiana (310A) plants expressing the *N* resistance gene were obtained from T. Romeis (MPIZ, Cologne) and used for transient *Agrobacterium*-mediated transformation (2.2.8.1) of leaf tissues.

2.1.1.3 *Nicotiana tabacum*

N. tabacum cv. *Petit Havana SR1* plants used for *Agrobacterium*-mediated transient expression (2.2.8.1) were obtained from A. Lautscham (MPIZ, Cologne).

2.1.2 Pathogens

Arabidopsis plants were infected with isogenic *Pseudomonas syringae* pv. *tomato* strains (DC3000) expressing different *Pseudomonas* effector proteins as specified in section 2.1.2.1.

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

Pseudomonas syringae pv. *tomato* (*Pst*) strain DC3000 harbouring either the empty vector pVSP61 or expressing the *Pseudomonas syringae* pv. *pisi* effector AvrRps4 from the same plasmid (Hinsch and Staskawicz, 1996) were obtained from R. Innes (Indiana University, Bloomington Indiana, USA) and used throughout this study.

2.1.3 Bacterial strains

2.1.3.1 *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

2.1.3.2 *Agrobacterium tumefaciens* strains

DNA constructs for stable transformation of *Arabidopsis thaliana* plants (2.2.3) and transient expression in *Nicotiana benthamiana* or *Nicotiana tabacum* (2.2.8.1) were transformed in *Agrobacterium tumefaciens* strain GV3101 carrying the helper plasmids specified in Table 2.3.

Table 2.3. ***Agrobacterium tumefaciens*** **strains used for stable and transient transformations**

Binary vector[a]	***Agrobacterium*** **strain**	**Helper plasmid (resistance)**	**Reference**
pGreen0229	GV3101	pSOUP (Tet)	(Koncz and Schell, 1986; Hellens et al., 2000)
pJH20-YFP-GW	GV3101	pMP90 (Rif, Gent)	(Koncz and Schell, 1986)
pXCSG-mYFP	GV3101	pMP90RK (Rif, Kan, Gent)	(Koncz and Schell, 1986)

[a]described in section 2.1.4

Tet: Tetracycline; Rif: Rifampicin; Gent: Gentamycin; Kan: Kanamycin

2.1.4 Vectors

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

pENTR4 — Entry vector for the Gateway® system with MCS for restriction enzyme-based cloning of DNA constructs (Invitrogen™)
Antibiotic resistance: Kanamycin (50 µg/ml)

pET42a(+) — *E. coli* expression vector used for subcloning NdeI fragments and site directed mutagenesis (2.2.7.4) (Novagen™)
Antibiotic resistance: Kanamycin (50 µg/ml)

pGreenII0229-35S-RPS4-HA — Binary vector obtained from Stephan Dorey (Sainsbury Laboratory, Norwich, UK) used to generate pGreenII0229-35S-RPS4-HA-StrepII (2.2.7.11)
Antibiotic resistance: Kanamycin (50 µg/ml)

pGreenII0229-OP-RPS4-HA — Binary vector obtained from Stephan Dorey (Sainsbury Laboratory, Norwich, UK) used to generate pGreenII0229-OP-RPS4-HA-StrepII (2.2.7.11)
Antibiotic resistance: Kanamycin (50 µg/ml)

pGreenII0229-35S-RPS4-HA-StrepII	Binary vector based on pGreenII0229-35S-RPS4-HA. The HA epitope tag sequence and the *Nos* 3' terminator were replaced by a construct encoding HA and StrepII epitope tags fused to 1623 bp of *RPS4* 3' regulatory sequence. The vector was used for stable transformation of *Arabidopsis thaliana* plants (2.2.3) (Hellens et al., 2000) Antibiotic resistance: Kanamycin (50 µg/ml)
pGreenII0229-OP-RPS4-HA-StrepII	Binary vector based on pGreenII0229-OP-RPS4-HA. The HA epitope tag sequence and the *Nos* 3' terminator were replaced by a construct encoding HA and StrepII epitope tags fused to 1623 bp of *RPS4* 3' regulatory sequence. The vector was used for stable transformation of *Arabidopsis thaliana* plants (2.2.3) (Hellens et al., 2000) Antibiotic resistance: Kanamycin (50 µg/ml)
pGreenII0229-OP-RPS4^{nls}-HA-StrepII	pGreenII0229-OP-RPS4-HA-StrepII carrying a mutated RPS4 NLS. The vector was used for stable transformation of *Arabidopsis thaliana* plants (2.2.3) (Hellens et al., 2000) Antibiotic resistance: Kanamycin (50 µg/ml)
pENTR4-RPS4	Gateway®-compatible entry vector carrying the *RPS4* coding sequence including the termination codon cloned as a NcoI/BamHI fragment into the pENTR4 MCS. Antibiotic resistance: Kanamycin (50 µg/ml)
pENTR4-RPS4^{nls}	Gateway®-compatible entry vector carrying the *RPS4* coding sequence including the termination codon and the mutated RPS4 NLS in pENTR4. Antibiotic resistance: Kanamycin (50 µg/ml)

pENTR4-RPS4-NES and pENTR4-RPS4-nes	Gateway®-compatible entry vectors carrying the *RPS4* coding sequence and C-terminal 60 bp sequences encoding either a functional (NES) or a mutated non-functional (nes) motif (Wen et al., 1995). Cloned into the pENTR4 MCS via NcoI/XhoI.
pJH20-GW	Gateway®-compatible binary over-expression vector obtained from Yoshiteru Noutoshi (RIKEN Tsukuba Inst., Japan) used to generate pJH20-YFP-GW (2.2.7.14) Antibiotic resistance: Kanamycin (50 µg/ml) and Spectinomycin (100 µg/ml)
pJH20-YFP-GW	Gateway®-compatible binary over-expression vector pJH20-GW carrying an N-terminal YFP tag. Used for transient over-expression in *Nicotiana spec.*(2.2.8.1), map in Appendix. Antibiotic resistance: Kanamycin (50 µg/ml) and Spectinomycin (100 µg/ml)

2.1.5 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). Start and Stop codons are highlighted in red, sequences encoding epitope tags are shown in green and recognition sited for restriction endonucleases are underlined. Lyophilised primers were resuspended in ddH_2O to a final concentration of 200 pmol/µl (= 200 µM). Working solutions were diluted to 10 pmol/µl (=10 µM).

Table 2.4. Oligonucleotides used in this study

Primer	Sequence (5´ → 3´)	Characteristics	Purpose
PM105	AGAGGATCCTATCCATACGATGTTCCAGATTATGCT GTCGGCGCCGGTTGGTCTCATCCTCAATTTGAAAAA TGAGTGGGAGCCCTGTCAAG	HA-StrepII epitope tag BamHI fwd.	**cloning & mutagenesis**
PM106	CTTGGATCCATAGCTGAAGCAACT	Col-0 *RPS4* 3' region BamHI rev.	
LW10	ACGGCTGTAGTTCGCTGAAG	*RPS4* CDS upstream of internal NdeI fwd.	
LW114	GATGGAAGAGTAAATAAA**GCAGCA**AAAACAAGAATGGATAAT	mutagenesis RPS4 K1172A and K1173A fwd.	
LW115	CCGTCCATTATCCATTCTTGTTTT**TGCTGC**TTTATTTACTCTTCCA TC	mutagenesis RPS4 K1172A and K1173A rev.	
LW116	ATAATGGACGGCCA**GCAGCA**AAGCAGAGATCAGGAAGAGATG	mutagenesis RPS4 K1182A and K1183A fwd.	
LW117	ATCTCTTCCTGATCTCTGCTT**TGCTGC**TGGCCGTCCATTATC	mutagenesis RPS4 K1182A and K1183A rev.	
LW119	CTTTCTAGAATGGTGAGCAAGGGCGAGG	*YFP* XbaI fwd.	
LW120	TCGCCCGGGCGTGTCAATAATATC	*ccdB* gene internal XmaI rev.	
LW128	CCGGATCCTCAGAAATTCTTAACCGTGTGC	*RPS4* 3' incl. stop BamHI rev.	
LW137	TACTCGAGCTACTTGTTAATATCAAGTCCAGCCAACTTAAGAGC AAGCTCGTTCTTGTACAGCTCGTCCATGAAATTCTTAACCGTGTG C	*RPS4* 3' NES XhoI rev.	
LW138	TACTCGAGCTACTTGTTAGCATCTGCTCCAGCTGCCTTAAGAGCA GCTCGTTCTTGTACAGCTCGTCCATGAAATTCTTAACCGTGTGC	*RPS4* 3' nes XhoI rev.	

Primer	Sequence (5´ → 3´)	Characteristics	Purpose
LW10	ACGGCTGTAGTTCGCTGAAG	*RPS4* intron 4 flanking fwd.	**RT-PCR**
LW21	ACAAGCGGCTGACTTGATCT	*RPS4* intron 4 flanking rev.	
actin-F	TGCGACAATGGAACTGGAATG	*ACTIN* Takahashi et al. (2003) fwd.	
actin-R	GCTTTTTAAGCCTTTGATCTTGAGA	*ACTIN* Takahashi et al. (2003) rev.	
LB1a	TGGTTCACGTAGTGGGCCATCG	SALK T-DNA left border primer	***rps4* T-DNA insertions**
LN45	ATATTGACCATCATACTCATTGC	GABI-Kat T-DNA left border primer	
LW49	GTTACCGACCGTTTTCATCCCTA	CSHL gene trap Ds3 insertion	
PM109	TTGTCCAAGTTAAACCATCCT	flanking *rps4-2* insertion fwd.	
PM110	GAGAGATTTGACTGCACTCATT	flanking *rps4-2* insertion rev.	
LW4	AATACCACCGGAGGGAAGTC	flanking *rps4-3* insertion fwd.	
LW27	TCGAATTCCAATGATCCA	flanking *rps4-3* insertion rev.	
LW23	GCTTCAGAGAGGTGGCTCACG	flanking *rps4-10* insertion fwd.	
LW15	CGGTGGCTTGTCTTCCA	flanking *rps4-10* insertion rev.	

fwd.: forward; rev.: reverse

2.1.6 Enzymes

2.1.6.1 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.

2.1.6.2 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

2.1.7 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.

2.1.8 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.

2.1.9 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

LB (Luria-Bertani) broth

Tryptone	10.0	g/l
Yeast extract	5.0	g/l
NaCl	5.0	g/l

pH 7.0

For LB agar plates 1.5 % (w/v) agar was added to the above broth.

SOC

Tryptone	20.0	g/l
Yeast extract	5.0	g/l
NaCl	10.0	mM
KCl	2.5	mM
$MgCl_2$	10.0	mM
$MgSO_4$	10.0	mM
Glucose	20.0	mM

pH 7.0

Pseudomonas syringae media

NYG broth

Peptone	5.0	g/l
Yeast extract	3.0	g/l
Glycerol	20	ml/l

pH 7.0

For NYG agar plates 1.5 % (w/v) agar was added to the above broth.

Agrobacterium tumefaciens media

YEB

Beef extract	5.0	g/l
Yeast extract	1.0	g/l
Peptone	5.0	g/l
Sucrose	5.0	g/l
1M $MgSO_4$	2.0	ml/l

pH 7.2

For YEB agar plates 1.5 % (w/v) agar was added to the above broth.

2.1.10 Antibodies

Listed below are primary and secondary antibodies used for immunoblot detection (2.2.6.6).

Primary antibodies

Antibody	**Source**	**Dilution**	**Reference**
α-EDS1	rabbit polyclonal	1:500	S. Rietz[a]
α-RPS4 (NB)	rabbit polyclonal	1:500	Y. Zhang[b] and this study
α-BIP	rabbit polyclonal	1:2000	C. Koncz[a]
α-PR1	rabbit polyclonal	1:5000	X. Dong[c]
α-Histone H3 (ab1791)	rabbit polyclonal	1:5000	Abcam (Cambridge, UK)
α-Hsc70 (plant, cytosolic)	mouse monoclonal	1:10000	Stressgen (Victoria, Canada)
α-HA 3F10	rat monoclonal	1:2000	Roche (Mannheim, Germany)
α-GFP	mouse monoclonal	1:2000	Roche (Mannheim, Germany)

[a]Max-Planck-Institute for Plant Breeding Research, Carl-von-Linné-Weg 10, 50829 Cologne, Germany
[b]Sainsbury Laboratory, Colney Lane, Norwich, NR4 7UH, UK
[c]Duke University, Durham, NC 27708, USA

Secondary antibodies

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)

2.1.11 Buffers and solutions

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

DEPC-H_2O	Diethylpyrocarbonate	0.1	% in H_2O
	Shake vigorously, let stand O/N and autoclave 30 min.		
DNA extraction buffer (Quick prep)	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		
Ethidium bromide stock solution	Ethidium bromide	10	mg/ml H_2O
	Dilute 1:40000 in agarose solution		

Honda buffer	Ficoll 400	5	g
	Dextran T40	10	g
	Sucrose	27.38	g
	Tris	0.606	g
	$MgCl_2$	0.407	g
	dH_2O to 200 ml		
	pH 7.4		
	Before use add 10 mM DTT and protease inhibitor cocktail for plant cell and tissue extracts (Sigma).		
Bulk phase system (aqueous 2-phase separation)	20% (w/w) Dextran T-500	18.6	g
	40% (w/w) PEG 3350	9.3	g
	Sucrose	6.778	g
	0.2 M KH_2PO_4 pH 7.8	1.5	ml
	2 M KCl	90	µl
	dH_2O to 60 g		
Phase mixture (aqueous 2-phase separation)	20% (w/w) Dextran T-500	2.232	g
	40% (w/w) PEG 3350	1.116	g
	Sucrose	0.61	g
	0.2 M KH_2PO_4 pH 7.8	135	µl
	2 M KCl	8.2	µl
	dH_2O to 5.4 g		
Lactophenol trypan blue	Lactic acid	10	ml
	Glycerol	10	ml
	dH_2O	10	ml
	Phenol	10	g
	Trypan blue	10	mg
	Before use dilute 1:1 in ethanol.		

PCR reaction buffer (10x)	Tris	100	mM
	KCl	500	mM
	$MgCl_2$	15	mM
	Triton X-100	1	%
	pH 9.0		
	Stock solution was filter sterilised and used for homemade *Taq* DNA polymerase.		
Ponceau S	Ponceau S working solution was prepared by dilution of ATX Ponceau S concentrate (Fluka) 1:5 in H_2O.		
SDS-PAGE:			
Resolving gel buffer (4x)	Tris	1.5	M
	pH 8.8 (HCl)		
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		
Stacking gel buffer (4x)	Tris	0.5	M
	pH 6.8 (HCl)		
Water-saturated n-butanol	N-butanol	40	ml
	dH_2O	10	ml

	Combine in a 50 ml Falcon tube and shake. Allow phases to separate. Use the top phase to overlay SDS-polyacrylamide gels.		
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)		
Immunoblotting:			
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.		

2.2 Methods

2.2.1 Maintenance and cultivation of *Arabidopsis* plants

Arabidopsis seeds were germinated by sowing directly onto moist compost (Stender, Schermbeck, Germany) containing 10 mg l^{-1} Confidor® WG 70 (Bayer, Germany). Seeds were covered with a propagator lid and vernalised at 4° C for 48 h in the dark. Subsequently the seeds were transferred to a controlled environment 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). Propagator lids were removed 3-5 days post germination. To obtain progeny three week old plants were transferred to long day conditions (16 h photoperiod) and allowed to flower. To collect seed aerial tissue was enveloped with a paper bag and sealed with tape at its base until siliques shattered.

2.2.2 Generation of *Arabidopsis* F_1 and F_2 progeny

Fine tweezers and a magnifying-glass were used to emasculate an individual flower. To prevent self-pollination, only flowers that had a well-developed stigma but immature stamen were used for crossing. Fresh pollen from three to four independent donor stamens was dabbed onto each single stigma. Mature siliques containing F_1 seed were harvested and allowed to dry. Approximately five F_1 seeds per cross were grown as described above and allowed to self pollinate. Produced F_2 seeds were collected and stored.

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

This method for *Agrobacterium*-mediated stable transformation of *Arabidopsis* is based on the floral dip protocol described by Clough and Bent (1998). Approximately 10 - 15 *Arabidopsis* plants were grown in 9 cm square pots (3 pots for each transformation) under short day conditions for 4 - 5 weeks. Then the plants were shifted to 16 h photoperiod conditions to induce flowering. First inflorescence shoots were cut off as soon as they emerged to induce the growth of more inflorescences. Plants were used for transformation when they did not have pods but maximum number of young flower heads. *Agrobacterium* was streaked out onto selective YEB plates containing appropriate antibiotics (see Table 2.3) and was grown at 28 °C for 3 days. A 20 ml ON culture was prepared in selective YEB

medium and cultured at 28 °C in an orbital shaker. The next day 200 ml YEB broth with appropriate antibiotics was inoculated with all of the overnight culture and grown overnight at 28° C in an orbital shaker until OD_{600} > 1.6. Cultures were spun down at 5000 rpm for 10 min at room temperature and the pellet was resuspended in 5 % sucrose to OD_{600} ~ 0.8. Silwet L-77 (Lehle seeds, USA) at 500µl/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. Excess inoculum was removed by dabbing of inflorescences onto kitchen roll. Plants were then placed into plastic bags, sealed with tape and placed overnight into the glasshouse away from direct light. Bags were removed and pots were moved to direct light and left to set seed.

2.2.4 Maintenance of *P. syringae* pv. *tomato* cultures

Pseudomonas syringae pv. *tomato* strains described in 2.1.2.2 were streaked onto selective NYG agar plates containing Rifampicin (100 µg/ml) and Kanamycin (50 µg/ml) from -80° C DMSO stocks. Streaked plates were incubated at 28° C for 72 h before storing at 4° C and refreshed weekly.

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

P. syringae cultures of the denoted strains (see 2.1.2.2) were started from bacteria grown on NYG agar plates in 20 ml NYG broth with Rifampicin (100 µg/ml) and Kanamycin (50 µg/ml). The 20 ml cultures were incubated overnight at 28° C and 160 rpm in a rotary shaker. 2.5 ml of the overnight cultures were used to inoculate 50 ml of NYG broth in 300 ml Erlenmeyer flasks supplemented with antibiotics. The flasks were incubated at 28° C and 160 rpm in a rotary shaker for 3 h. An ideal OD_{600} reading at this time point should be 0.2. The bacteria were transferred to sterile 50 ml Falcon tubes and pelleted at 4600 rpm for 10 min at 20° C (Heraeus Multifuge 3_{S-R}). The bacterial pellet was resuspended in 40 ml of sterile 10 mM $MgCl_2$, and the culture was centrifuged as above. The supernatant was removed and the bacteria were resuspended in 20 ml of sterile 5 mM $MgCl_2$. For vacuum-infiltration the concentration of bacteria was adjusted to 5 x 10^4 cfu/ml in 600 ml of 5 mM $MgCl_2$ containing 0.002 % Silwet L-77 (Lehle seeds, USA). Single pots with five plants, grown under short day conditions (see 2.2.1) for five weeks, were routinely used for bacterial growth assays. Two

hours before vacuum-infiltration, plants were watered and kept under a dH_2O-humidified lid. Vacuum infiltration of the plants was accomplished by inverting the pots and carefully submerging all leaf material in 600 ml of diluted bacterial suspension in a plastic desiccator. Vacuum was applied and maintained within the desiccator for 2 min before being gradually released. Plants were removed from the desiccator and remaining non-infiltrated leaves were removed. The excess of bacterial solution was washed off by inverting the pots and gently agitating them in water.

Day zero (d_0) samples were taken one hour after infiltration by using a cork borer (∅ 0.55 cm) to excise and transfer 4 leaf discs from 4 independent plants to a 1.5 ml centrifuge tube, resulting in a total excised area of 1 cm^2. This was repeated with a second batch of 4 leaf discs from 4 independent plants. The discs were then ground with a plastic pestle in 100 µl of sterile 10 mM $MgCl_2$. Subsequently, 900 µl of sterile 10 mM $MgCl_2$ were added (10^{-1} dilution) and 100 µl of each sample were plated onto NYG agar (Rifampicin 100 µ/ml; Kanamycin 50 µg/ml). Day three (d_3) samples were taken in an identical manner to that of d_0 except that 4 leaf discs from 5 independent plants per infiltrated genotype were taken in triplicates. For each sample a dilution series ranging between 10^{-1} and 10^{-7} was made and 15 µl aliquots from each dilution were spotted sequentially onto a single NYG agar plate (Rifampicin 100 µg/ml, Kanamycin 50 µg/ml). All bacteria plates were incubated at 28° C for 48 h before colony numbers were determined.

2.2.6 Biochemical methods

2.2.6.1 *Arabidopsis* total protein extraction for immunoblot analysis

Total protein extracts were prepared from 3- to 5-week-old plant materials. Liquid nitrogen frozen samples were homogenized 2 x 15 sec to a fine powder using a Mini-Bead-Beater-8™ (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. Samples were stored at -20° C if not directly loaded onto SDS-PAGE gels.

2.2.6.2 Nuclear fractionation for immunoblot analysis

Nuclear fractionations were performed according to the protocol described by Kinkema *et al*. (2000), which is based on that described by Xia *et al*. (1997), with minor modifications: 1.5 g fresh weight of unchallenged leaf tissues grown under short day conditions (see 2.2.1) were homogenised in 3 ml Honda buffer using a pre-cooled mortar and pestle and then filtered through a 62 µm (pore size) nylon mesh. Triton X-100 (10% working solution) was added to a final concentration of 0.5 % and the solution was slowly mixed by swirling. The mixture was incubated on ice for 15 min. The extract was then centrifuged at 1500 *g* for 5 min. An aliquot of the nuclei-depleted fraction was saved and the pellet washed by gentle resuspension in 2.5 ml Honda buffer containing 0.1 % Triton X-100. The sample was centrifuged again at 1500 *g* for 5 min. The pellet was resuspended in 2.5 ml Honda buffer and 620µl ml aliquots were transferred to 1.5 ml microcentrifuge tubes. The preparations were centrifuged at 100 *g* for 5 min to pellet starch and cell debris. The supernatants were transferred to new microcentrifuge tubes and centrifuged at 2000 *g* for 5 min to pellet the nuclei. Nuclear pellets were resuspended in 200 µl 2 x SDS-PAGE sample buffer, boiled for 10 min, and pooled. The nuclear and nuclei-depleted fractions were run on 15%-12%-8% step gradient SDS-PAGE gels (see 2.2.12.5). Nuclear and nuclei-depleted extracts were loaded in a 1:2.5 volume ratio on the gel corresponding to a 16-fold over-representation of the nuclear pool. α-HSC70 and α-histone H3 antibodies (2.1.10) were used as cytosolic and nuclear markers, respectively.

2.2.6.3 Isolation of microsomal membranes

To isolate microsomal membranes 0.5 g of 4-week-old leaves grown in short day conditions (see 2.2.1) were homogenised in liquid nitrogen. The ground tissue was thawed under further homogenisation in 1 ml extraction buffer (100 mM Tris-HCl pH 7.5, 12% sucrose, 1 mM EDTA, 5 mM DTT and 1x protease inhibitor cocktail for plant cell and tissue extracts (Sigma)). The homogenate was passes through two layers of Miracloth (Calbiochem). The filtrate was transferred to a microcentrifuge tube and centrifuged at 2000 *g* and 4°C for 15 min in a bench top centrifuge to remove cell debris and nuclei. 100 µl of the supernatant were kept as a crude extract fraction whilst 600 µl of the supernatant were transferred to an ultracentrifugation tube (Beckmann) and centrifuged for 1 h at 100.000 x g and 4° C (Optima™ MAX-E ultracentrifuge, Beckmann Coulter, USA). 600 µl supernatant were kept as a soluble fraction and the pellet was washed with extraction buffer. After washing, the pellet was resuspended in 600 µl of extraction buffer containing 1% (v/v) Triton X-100 using

an ultrasonic bath. One volume of 2x SDS-PAGE sample buffer was added to the all fractions and samples were boiled for 5 min to denature proteins. Samples were stored at -20° C.

2.2.6.4 Aqueous two phase separation of *Arabidopsis* microsomes

Purification of *Arabidopsis* plasma membranes was performed as described by Larsson et al. (1987) with all volumes scaled down to 20%. A 60 g bulk phase system was prepared to provide fresh upper and lower phases (see 2.1.11). After temperature equilibration at 4 °C the phase system was mixed thoroughly in a 300 ml separating funnel and phases were allowed to settle ON at 4 °C. The next day upper and lower phases were collected and stored at 4 °C until further usage.

The complete plasma membrane purification was carried out at 4 °C. 7.5 g of *Arabidopsis* leaf material was ground using a pre-cooled mortar and pestle in 15 ml of extraction buffer (0,5 M sucrose, 50 mM HEPES-KOH pH 7.5, 5 mM ascorbic acid, 1 mM DTT and protease inhibitor cocktail for plant cell and tissue extracts (Sigma)). The extract was filtered through two layers of Miracloth® (Calbiochem) and the filtrate was centrifuged at 2500 x g and 4 °C for 10 min. Microsomal membranes were prepared from 4 ml of the supernatant by ultra-centrifugation for 1 h at 100.000 x g and 4 °C. The pellet was resuspended in 1 ml of microsomal buffer (0.33 M sucrose, 3 mM KCl, 5 mM KH_2PO_4, 1x protease inhibitor cocktail) by grinding in a small mortar at 4 °C. The solution was then added to a 5.4 g phase mixture (see 2.1.11) in a 15 ml Falcon tube to obtain a final weight of 7.2 g. The phase system was thoroughly mixed by 30 inversions and subsequently phases were separated by centrifugation at 1500 x g and 4 °C for 5 min. The upper phase (U_0) was carefully transferred to a new tube and extracted again with fresh lower phase as above to obtain U_1. In the same manner the lower phase (L_0) was re-extracted with fresh upper phase to yield L_1. Phases U_1 and L_1 were diluted with microsomal buffer 2x and 10x, respectively, and then centrifuged at 100.000 x g and 4 °C for 45 min to pellet the membranes. The supernatant was removed and membrane pellets were boiled in 500 µl of 2x SDS-PAGE sample buffer (2.1.11). Samples were stored at -20 °C. The purity of the plasma membrane fraction was determined by immunoblot analysis (2.2.6.6) using an α-BIP polyclonal antiserum (provided by C. Konzc, MPIZ Cologne, see 2.1.10) as an endomembrane marker. A transgenic line expressing an HA-tagged version of the plasma membrane associated BON1 protein (Hua et al., 2001) was processed in parallel and used to estimate the efficiency of plasma membrane purification.

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

Denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the Mini-PROREAN® 3 system (BioRad) and discontinuous polyacrylamide (PAA) gels. Gels were made fresh on the day of use according to the manufacturer instructions. Resolving gels were poured between to glass plates and overlaid with 500 µl of water-saturated n-butanol or 50 % isopropanol. After gels were polymerised for 30 – 45 min the alcohol overlay was removed and the gel surface was rinsed with dH_2O. Excess water was removed with a filter paper. A stacking gel was poured onto the top of the resolving gel, a comb was inserted and the gel was allowed to polymerise for 30 - 45 min. In this study, 8%, 15% and 8%-12%-15% step gradient resolving gels were used, overlaid by 4 % stacking gels. Gels were 0.75 mm or 1.5 mm in thickness.

Table 2.4. Formulation for different percentage resolving gels

Component[a]	7.5 % resolving gel	10 % resolving gel
dH_2O	4.82 ml	4.1 ml
Resolving gel buffer	2.5 ml	2.5 ml
10 % SDS	0.1 ml	0.1 ml
30 % Acrylamide/Bis solution, 29:1 (BioRad)	2.5 ml	3.3 ml
TEMED (BioRad)	5.0 µl	5.0 µl
10 % APS[b]	75 µl	75 µl

Table 2.5. Constituents of a protein stacking gel

Component[a]	4 % stacking gel
dH_2O	6.1 ml
Stacking gel buffer	2.5 ml
10 % SDS	0.1 ml
30 % Acrylamide/Bis solution, 29:1 (BioRad)	1.3 ml
TEMED (BioRad)	10 µl
10 % APS[b]	100 µl

[a]Add in stated order

[b]Store at -20° C

If protein samples were not directly extracted in 2x SDS-PAGE sample buffer (see 2.1.11) proteins were denatured by adding 1 volume of 2x SDS-PAGE sample buffer to the protein sample followed by boiling for 5 min.

After removing the combs under running water, each PAA gel was placed into the electrophoresis tank and submerged in 1x running buffer. A pre-stained molecular weight marker (Precision plus protein standard dual colour, BioRad) and denatured protein samples were loaded onto the gel and run at 80 - 100 V (stacking gel) and 100 – 150 V (resolving gel) until the marker line suggested the samples had resolved sufficiently.

2.2.6.6 Immunoblot analysis

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 were removed with a scalpel. In order to detect AvrRps4 protein a PVDF membrane (Roche) was used. PAA gels and membranes were pre-equilibrated in 1x transfer buffers for 10 min on a rotary shaker and the blotting apparatus (Mini Trans-Blot® Cell, BioRad) was assembled according to the manufacturer instructions. Transfer was carried out at 100 V for 70 min. The transfer cassette was dismantled and membranes were checked for equal loading 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. For antibody details see 2.1.10. Next morning the primary antibody solution was removed and membranes were washed 3 x 15 min with TBS-T at room temperature on a rotary shaker. Bound primary antibodies were detected using horseradish peroxidase (HRP)-conjugated goat anti-rabbit, goat anti-mouse or goat anti-rat secondary antibodies (for antibody details see 2.1.10) 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 by slowly rotating. 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 - 3:1 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).

2.2.7 Molecular biological methods

2.2.7.1 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. If preps are to be used over a long period of time, they should be frozen in aliquots. The aliquot in use should be stored at 4° C. The cap of a 1.5 ml microcentrifuge tube was closed onto a leaf to clip out a section of tissue and 400 µl of DNA extraction buffer were added. A micropestle was used to grind the tissue in the tube 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. 1 volume of isopropanol was added to precipitate DNA 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 10 mM Tris-HCl pH 8.0 and 0.5 - 2 µl of the solution were used for PCR.

2.2.7.2 Isolation of total RNA from *Arabidopsis*

Total RNA was prepared from 3- to 6-week-old plant materials. Liquid nitrogen frozen samples (approximately 50 mg) were homogenized 2 x 15 sec to a fine powder using a Mini-Bead-Beater-8^{TM} (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 an 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 and RNA was precipitated by adding 0.5 volumes of isopropanol and incubation for 10 min at room temperature. Subsequently, samples were centrifuged for 10 min at 12000 *g* and 4° C. The supernatant was removed 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. Samples were stored at -80° C.

2.2.7.3 Polymerase chain reaction (PCR)

Standard PCR reactions were performed using home made *Taq* DNA polymerase while for cloning of PCR products *Pfu* or *Pfx* polymerases were used (see 2.1.6.2) according to the manufacturer instructions. All PCRs were carried out using a PTC-225 Peltier thermal cycler (MJ Research). A typical PCR reaction mix and thermal profile is shown below.

Reaction mix (20 µl total volume):

Component[a]	**Volume**
Template DNA (genomic or plasmid)	0.1 - 20 ng
10 x PCR reaction buffer	2 µl
dNTP mix (2.5 mM each: dATP, dCTP, dGTP, dTTP)	2 µl
Forward primer (10 µM)	1 µl
Reverse primer (10 µM)	1 µl
Taq DNA polymerase (4U/µl)	0.5 µl
Nuclease free water	to 20 µl total volume

Thermal profile

Stage	**Temperature (°C)**	**Time period**	**No. of cycle**
Initial denaturation	94	2 min	1 x
Denaturation	94	30 sec	
Annealing	50 - 60	30 sec	23 - 36
Extension	72	1 min per kb	
Final extension	72	3 min	1 x

2.2.7.4 Site directed mutagenesis

Site directed mutagenesis was basically performed as described in the instruction manual of the QuickChange® site-directed mutagenesis kit of Stratagene®.

PCR reaction mix (25 µl total volume):

Component	Volume
Template plasmid (20 ng/µl)	1 µl
10 x *PfuTurbo*® reaction buffer	2.5 µl
dNTP mix (2.5 mM each: dATP, dCTP, dGTP, dTTP)	2.5 µl
Primer 1 (10 µM)	1.4 µl
Primer 2 (10 µM)	1.4 µl
PfuTurbo® DNA polymerase (2.5 U/µl)	0.5 µl
Nuclease free water	to 25 µl total volume

Thermal profile:

Stage	Temperature (°C)	Time period	No. of cycle
Initial denaturation	94	1 min	1 x
Denaturation	94	30 sec	
Annealing	55	1 min	12 - 18
Extension	72	2 min per kb	
Final extension	72	10 min	1 x

After the PCR, 0.5 µl *Dpn*I (20 U/µl) were added to the reaction mix to digest methylated, non-mutated, parental DNA and to select for mutation-containing synthesised DNA. The reaction was incubated for 1 h at 37° C before the endonuclease was heat-inactivated at 65° C for 20 min. 3 µl of the reaction mixture, containing the circular, nicked vector DNA with the desired mutations were then transformed into DH10B cells and plated on LB agar containing the appropriate antibiotic.

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

RT-PCR was carried out in two steps. SuperScript™ II RNase H^- Reverse Transcriptase (Invitrogen) was used for first strand cDNA synthesis by combining 1 - 1.5 µg template total RNA, 1 µl oligo $dT_{18}V$ (0.5 µg/µl, V standing for an 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). The sample was incubated at 65° C for 10 min to destroy secondary structures before cooling on ice.

Subsequently the reaction was filled up to a total volume of 20 µl by adding 4 µl of 5x reaction buffer (supplied with the enzyme), 2 µl of 0.1 M DTT and 0.5 µl reverse transcriptase. The reaction was incubated at 42° C for 60 min before the enzyme was heat inactivated at 70° C for 10 min. For subsequent normal PCR, 1 µl of the above RT-reaction was used as cDNA template. As template total RNA for the reverse transcription reaction was not DNase treated, a control reaction for each RNA preparation was performed in which the reverse transcription reaction was incubated without reverse transcriptase enzyme (enzyme replaced by equal volume of DEPC-H_2O) to check in the following PCR for contamination by genomic DNA.

2.2.7.6 Plasmid DNA isolation from bacteria

Standard alkaline cell lysis minipreps of plasmid DNA were carried out using the GFX™ micro plasmid prep kit from Amersham Biosciences according to the manufacturer's instructions. Larger amounts of plasmid DNA were isolated using Qiagen Midi preparation kits.

2.2.7.7 Restriction endonuclease digestion of DNA

Restriction digests were carried out using the recommended manufacturer's conditions. Typically, reactions were carried out in 0.5 ml tubes, using 1 µl of restriction enzyme per 20 µl reaction. All digests were carried out at the appropriate temperature for a minimum of 60 min.

2.2.7.8 DNA ligations

Typically, DNA ligations were carried out overnight at 16° C 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 overnight at 4° C, overnight at room temperature or for 1 - 3 h at room temperature.

2.2.7.9 Agarose gel electrophoresis of DNA

DNA fragments were separated by agarose gel electrophoresis in gels consisting of 1 – 2 % (w/v) SeaKem® LE agarose (Cambrex, USA) in TAE buffer. Agarose was dissolved in TAE buffer 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 pored and allowed to

solidify before being placed in TAE in an electrophoresis tank. DNA samples were loaded onto an agarose gel after addition of 2 µl 6x DNA loading buffer to 10 µl PCR- or restriction-reaction. Separated DNA fragments were visualised by placing the gel on a 312 nm UV transilluminator and photographed.

2.2.7.10 Isolation of DNA fragments from agarose gels

DNA fragments separated by agarose gel electrophoresis were excised from the gel with a clean razor blade and extracted using the QIAEX®II gel extraction kit (Qiagen) according to the manufacturer's protocol.

2.2.7.11 Generation of binary vectors for expression of epitope-tagged RPS4 proteins

The plasmids pGreenII0229-OP-RPS4-HA and pGreenII0229-35S-RPS4-HA (obtained from Stephan Dorey, Sainsbury Laboratory, Norwich, UK) were used to generate binary vectors for stable transformation of *Arabidopsis thaliana*. pGreenII0229-OP-RPS4-HA carries a 4.9 kb Col-0 genomic fragment comprised of 511 bp *RPS4* 5' regulatory sequence (own promoter, OP) and the *RPS4* genomic sequence lacking the termination codon. pGreenII0229-35S-RPS4-HA carries the Col-0 *RPS4* sequence lacking the stop codon under control of the constitutive CaMV 35S promoter. In both plasmids the *RPS4* genomic construct is fused to a C-terminal HA-epitope tag followed by the *NOS* 3' regulatory sequence. The *HA-Nos* 3' region was excised from both plasmids as a BamHI fragment and both vectors were re-ligated to create pGreenII0229-OP-RPS4 and pGreenII0229-35S-RPS4. In order to fuse the HA-StrepII epitope tags and the endogenous *RPS4* 3' regulatory sequence to *RPS4* primer PM105 (5'-AGAGGATCCTATCCATACGATGTTCCAGATTATGCTGTCGGCGCCGGTTGGTC TCATCCTCAATTTGAAAAATGAGTGGGAGCCCTGTCAAG-3') encoding a combination of the HA-StrepII epitope tags was used as forward primer together with the reverse primer PM106 (5'-CTTGGATCCATAGCTGAAGCAACT-3') to amplify 1697 bp of *RPS4* 3' regulatory sequence (*RPS4* terminator) with flanking BamHI sites from Col-0 genomic DNA (bases encoding epitope tags in green, stop codons red and BamHI restriction sites underlined). The HA-StrepII-terminator fusion was then cloned via BamHI into pGreenII0229-35S-RPS4 and pGreen-OP-RPS4 to create pGreenII0229-35S-RPS4-HA-StrepII and pGreenII0229-OP-RPS4-HA-StrepII (see Appendix).

In order to create pGreenII0229-OP-RPS4^{nls}-HA-StrepII carrying the mutated NLS region an NdeI 2365 bp fragment was excised from pGreenII0229-OP-RPS4-HA-StrepII. The NdeI fragment comprising parts of the *RPS4* coding sequence, the HA-StrepII encoding

region and 1357 bp of the *RPS4* terminator (see Appendix) was subcloned into pET42a(+) for site directed mutagenesis (see 2.2.7.4). Primers LW114 and LW115 were used to make amino acid changes K1172A and K1173A (LW114: 5'-GATGGAAGAGTAAATAAA**GCAGCA** AAAACAAGAATGGATAAT-3', LW115: 5'-CCGTCCATTATCCATTCTTGTTTT **TGCTGC**TTTATTTACTCTTCCATC-3', bases encoding alanine exchanges in bold). The mutated sequence was used as a template in a second round of mutagenesis with primers LW116 and LW177 to exchange K1182A and K1183A (LW116: 5'-ATAATGGACGGCCA**GCAGCA**AAGCAGAGATCAGGAAGAGA TG-3', LW117: 5'-ATCTCTTCCTGATCTCTGCTT**TGCTGC**TGGCCGTCCATTATC-3', bases encoding alanine exchanges in bold). The NdeI fragment carrying the quadruple lysine to alanine exchange was cloned back into pGreenII0229-OP-RPS4-HA-StrepII to generate pGreenII0229-OP-RPS4^{nls}-HA-StrepII.

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

In order to create RPS4 entry clones for the Gateway® system the pENTR4 vector was used to clone genomic *RPS4* (and *RPS4* variants). To transfer the fragment of interest into gene expression constructs, an LR reaction between the entry clone and a Gateway® destination vector (see also 2.2.7.14 for the generation of destination vectors) was performed.

Basic LR reaction approach:

LR reaction buffer (5x)	1	µl
Entry clone	70	ng
Destination vector	70	ng
LR clonase™ enzyme mix	1	µl
TE buffer	to 5	µl

Reactions were incubated 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 (see 2.1.3.1).

2.2.7.13 Generation of Gateway®-compatible RPS4 entry clones

To generate RPS4 entry clones pENTR4-RPS4 and pENTR4-RPS4^{nls} the Col-0 *RPS4* coding sequence and the *RPS4* sequence harbouring the mutated NLS (see 2.2.7.11) were isolated as NcoI/BamHI fragments from the corresponding pGreenII0229 clones and ligated into the

MCS of the pENTR4 vector. To add a termination codon to *RPS4* a 936 bp fragment was amplified by PCR using primers LW10 (5'-ACGGCTGTAGTTCGCTGAAG-3') and LW128 (5'-CCGGATCCTCAGAAATTCTTAACCGTGTGC-3', termination codon in red, BamHI restriction site underlined). The PCR product was digested with NdeI/BamHI, re-ligated into the template vector and the ligation mix was transformed into *E. coli*. Since these clones still contained the pENTR4 *ccdB* gene the ligation mix was transformed into *E. coli* DB3.1 cells. Positive clones were identified and the *ccdB* gene was excised by BamHI/NotI. The 3' NotI and BamHI ends of the pENTR4-RPS4 fragments were filled using the Pfu polymerase. The pENTR4-RPS4 fragments were self-ligated and transformed into *E. coli* DH10B cells to select for clones that had lost the *ccdB* gene.

Entry clones pENTR4-RPS4-NES and pENTR4-RPS4-nes are based on the pENTR4-RPS4 plasmid. To generate RPS4-NES and RPS4-nes fusions a 1161 bp product was PCR amplified from pENTR4-RPS4 using primers LW10/LW137 (NES) and LW10/LW138 (nes), respectively (LW10: 5'-ACGGCTGTAGTTCGCTGAAG-3', LW137: 5'-TACTCGAGCTACTTGTTAATATCAAGTCCAGCCAACTTAAGAGCAAGCTCGTTC TTGTACAGCTCGTCCATGAAATTCTTAACCGTGTGC-3', LW138: TACTCGAG CTACTTGTTAGCATCTGCTCCAGCTGCCTTAAGAGCAAGCTCGTTCTTGTACAGCT CGTCCATGAAATTCTTAACCGTGTGC, termination codons in red, sequences encoding NES/nes in green and XhoI restriction sites underlined). The PCR products were digested with NdeI/XhoI and cloned back into pENTR4-RPS4.

2.2.7.14 Generation of YFP-tagged RPS4 constructs for transient expression in *Nicotiana spec.*

A pBIN19 Gateway®-compatible variant (pJH20-GW) carrying the CaMV 35S promoter and terminator was modified to introduce an N-terminal YFP-tag. To this end the YFP coding sequence was PCR amplified as a fusion with part of the Gateway® cassette from template pENSG-YFP-GW (Parker lab, general stock collection) using primers LW119 (5'-CTTTCTAGAATGGTGAGCAAGGGCGAGG-3') and LW120 (5'-TCGCCCGGG CGTGTCAATAATATC-3'). The 2050 bp PCR product was cloned into pJH20-GW via XbaI and XmaI to generate pJH20-YFP-GW.

2.2.7.15 DNA sequencing

DNA sequences were determined by the "Automatische DNA Isolierung und Sequenzierung" (ADIS) service unit at the MPIZ on Applied Biosystems (Weiterstadt, Germany) Abi Prism 377 and 3700 sequencers using Big Dye-terminator chemistry.

2.2.7.16 DNA sequence analysis

Sequence data were analysed mainly 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).

2.2.7.17 Preparation of chemically competent *E. coli* cells

Media and solutions required for preparation of rubidium chloride *E. coli* chemically competent cells:

ΦB:		TFB1:		TFB2:	
Yeast extract	0.5 %	KAc	30 mM	MOPS	10 mM
Tryptone	2 %	$MnCl_2$	50 mM	$CaCl_2$	75 mM
$MgSO_4$	0.4 %	RbCl	100 mM	RbCl	10 mM
KCl	10 mM	$CaCl_2$	10 mM	Glycerol	15 %
pH	7.6	Glycerol	15 %	sterile-filter	
autoclave		pH	5.8		
		sterile-filter			

5 ml of an *E. coli* strain DH10B over night culture grown in ΦB was added to 400 ml of ΦB and shaken at 37° C until the bacterial growth reached an OD_{600} 0.4 - 0.5. Cells were cooled on ice and all following steps were carried out on ice or in a 4° C cold room. The bacteria were pelleted at 5000 *g* for 15 min at 4° C. The pellet was gently resuspended in 120 ml ice-cold TFB1 solution and incubated on ice for 10 min. The cells were pelleted as before and carefully resuspended in 16 ml ice-cold TFB2 solution. 1.5 ml eppendorf reaction tubes containing 50 µl aliquots of cells were frozen in liquid nitrogen and stored at -80° C until use.

2.2.7.18 Transformation of chemically competent *E. coli* cells

A 50 µl aliquot of chemically competent cells was thawed on ice. 10 to 25 ng of ligated plasmid DNA (or ~ 5 µl of ligated mix from 10 µl ligation reaction) was mixed with the aliquot and incubated on ice for 30 min. The mixture was heat-shocked for 30 sec at 42° C and immediately put on ice for 1 min. 500 µl of SOC 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 broth and plated onto selective media plates.

2.2.7.19 Preparation of electro-competent *A. tumefaciens* cells

The desired *Agrobacterium* strain was streaked out onto YEB agar plate containing adequate antibiotics and grown at 28° C for two days. A single colony was picked and a 5 ml YEB culture, containing appropriate antibiotics, was grown overnight at 28° C. The whole overnight culture was added to 200 ml YEB (without antibiotics) and grown to an OD_{600} of 0.6. Subsequently, the culture was chilled on ice for 15 – 30 min. From this point onwards bacteria were maintained at 4° C. Bacteria were centrifuged at 6000 *g* for 15 min and 4° C and the pellet was resuspended in 200 ml of ice-cold sterile water. Bacteria were again centrifuged at 6000 *g* for 15 min and 4° C. Bacteria were resuspended in 100 ml of ice-cold sterile water and centrifuged as described above. The bacterial pellet was resuspended in 4 ml of ice-cold 10 % glycerol and centrifuged as described above. Bacteria were resuspended in 600 µl of ice-cold 10 % glycerol. 40 µl aliquots were frozen in liquid nitrogen and stored at -80° C.

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

50 ng of plasmid DNA was mixed with 40 µl of electro-competent *A. tumefaciens* cells, 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 re-suspended by slowly pipetting and transferred to a 2 ml microcentrifuge tube. The tube was incubated for 3 h in an Eppendorf thermomixer at 28° C and 600 rpm. A 5 µl fraction of the transformation mixture was plated onto selection YEB agar plates.

2.2.8 Transient plant transformations

2.2.8.1 *Agrobacterium*-mediated transient transformation of tobacco leaves

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

Induction medium (1 l):

K_2HPO_4	10.5 g
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

Prior to use add appropriate antibiotics

and 50 µg/ml Acetosyringone (3´,5´-Dimethoxy-4´-hydroxyacetophenone).

Infiltration medium:

MES	10 mM
$MgCl_2$	10 mM
pH	5.3 - 5.5

Prior to use add 150 µg/ml Acetosyringone.

4 ml overnight cultures were grown in liquid YEB (including appropriate antibiotics) at 28 °C. The culture was spun down, bacteria were resuspended in 5 ml induction medium and grown further for another 4 - 6 h. Cultures 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* or *N. tabacum* plants were watered a few hours before infiltrating healthy, fresh looking leaves with a needle-less 1 ml syringe on the underside. Samples of infiltrated leaf areas for protein extractions were taken 2 - 3 d after infiltration.

2.2.9 Localisation studies using confocal laser scanning microscopy (CLSM)

Detailed analysis of intracellular fluorescence was performed by confocal laser scanning microscopy using a Zeiss LSM 510 META microscopy system (Zeiss, Germany) based on an Axiovert inverted 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. A Plan-Apochromat 20x/0.75 lens was used for scanning of leaves. Images were acquired in the multichannel tracking mode and analysed with Zeiss LSM510 software

3 Results

The study is subdivided into two parts. Section **3.1** describes the analysis of three *rps4* T-DNA insertion mutants and their phenotype in plant disease resistance towards avirulent *P. syringae* expressing AvrRps4. Also, the generation of transgenic *Arabidopsis thaliana* plants expressing epitope-tagged RPS4 either under control of its own 5' regulatory sequence or the constitutive CaMV 35S promoter is presented. A selected *RPS4* over-expression line was used to further position *EDS1* in RPS4-mediated disease resistance. The aim of the second section (**3.2**) was to elucidate the subcellular localisation of endogenous and functional epitope-tagged RPS4 proteins. It combines biochemical fractionation methods with *P. syringae* pathology assays and transient expression of RPS4 variants in tobacco to correlate RPS4 subcellular localisation with function.

3.1 Susceptibility of *rps4* mutants to *P. syringae* AvrRps4 and positioning of *EDS1* in RPS4-mediated resistance

3.1.1 Susceptibility of *rps4* mutants to *P. syringae* AvrRps4

Some publications suggest that RLD is a natural *rps4* mutant (Gassmann et al., 1999; Zhang and Gassmann, 2003), while other findings demonstrate that RLD plants also have lower basal resistance to virulent *P. syringae* compared to Col-0 or L*er* (Ton et al., 1999). I thus first compared RLD plants to Col-0 and an *eds1* mutant in Col-0 genetic background (Bartsch et al., 2006) in terms of resistance to virulent and avirulent *P. syringae* ± AvrRps4. Fig. 3.1A shows that RLD plants allow significantly more growth of virulent *P. syringae* compared to Col-0 (t-test, α=0.05). In fact, bacterial growth in RLD was similar to hypersusceptible *eds1* plants in Col-0 background. Surprisingly, when infiltrated with *P. syringae* AvrRps4 RLD plants were found to strongly suppress the growth of the avirulent strain (Fig 3.1 A). Since in RLD avirulent bacteria grew to ~200 fold lower titres than the isogenic virulent *P. syringae* strain, AvrRps4 is recognised in RLD plants. In order to confirm the genetic background the RLD *rps4* allele was sequenced (data not shown) and found to be consistent with the published annotation (Gassmann et al., 1999). In summary, the bacterial growth assay in Fig. 3.1A revealed that i) RLD plants allow more growth of virulent *P. syringae* compared to Col-0 and ii) that AvrRps4 is recognised in this accession. Consequently, RLD plants were

not used as an *rps4* mutant background in this study. Instead, I characterised T-DNA insertion lines that were predicted to have an interrupted *RPS4* coding sequence. Lines *rps4-2* (SALK_057697) and *rps4-3* (GABI_130F04) were obtained from the SALK and GABI-Kat collections, respectively, and are in Col-0 background (Alonso et al., 2003; Rosso et al., 2003). A third line, *rps4-10* (CSHL_GT6567), from the CSHL gene trap collection (Martienssen, 1998) in L*er* background was characterised to not restrict the analysis to the Col-0 accession. *rps4-2* and *rps4-3* carry T-DNA insertions in the second exon of *RPS4* encoding the NB domain (Fig. 3.1B). In *rps4-10* a promoterless *GUS* gene trap Ds3 construct is inserted after the 7th base of *RPS4* (Fig. 3.1B). All insertions were confirmed by sequencing the T-DNA/Ds3 flanking regions and individual plants homozygous for the insertions were identified (data not shown). Conventional reverse transcription PCR revealed the absence of *RPS4* transcript in all three insertion lines around *RPS4* intron 4 confirming that the insertions led to a loss of *RPS4* mRNA (Fig. 3.1C).

When tested for their ability to restrict the growth of *P. syringae* AvrRps4, *rps4-2* and *rps4-3* allowed 1-1.5 logs more growth than wild type Col-0 plants (Fig. 3.1D left panel). However, in four independent experiments titres of *P. syringae* AvrRps4 in *rps4* mutants were significantly lower than those of the isogenic virulent *P. syringae* strain in Col-0 plants (Fig. 3.1D middle panel; t-test, α=0.05), demonstrating that *rps4* plants retain partial resistance towards bacteria expressing AvrRps4. RPS4-independent recognition of AvrRps4 was even more pronounced in the *rps4-10* mutant that was as resistant as L*er* wild type plants when challenged with avirulent *P. syringae* (Fig. 3.1D right panel). Taken together these findings show that the Col-0 and *Ler* accessions possess other receptors besides RPS4 that are able to recognise AvrRps4 and induce partial (Col-0) or full (L*er*) quantitative resistance. Bacterial titres of virulent and avirulent *P. syringae* were similar in *eds1* mutants (Fig. 3.1D middle panel). Thus, RPS4-independent recognition requires *EDS1,* strongly suggesting that the additional receptor(s) mediating AvrRps4 recognition belong to the TIR-NB-LRR class of plant R proteins (Aarts et al., 1998; Feys et al., 2001).

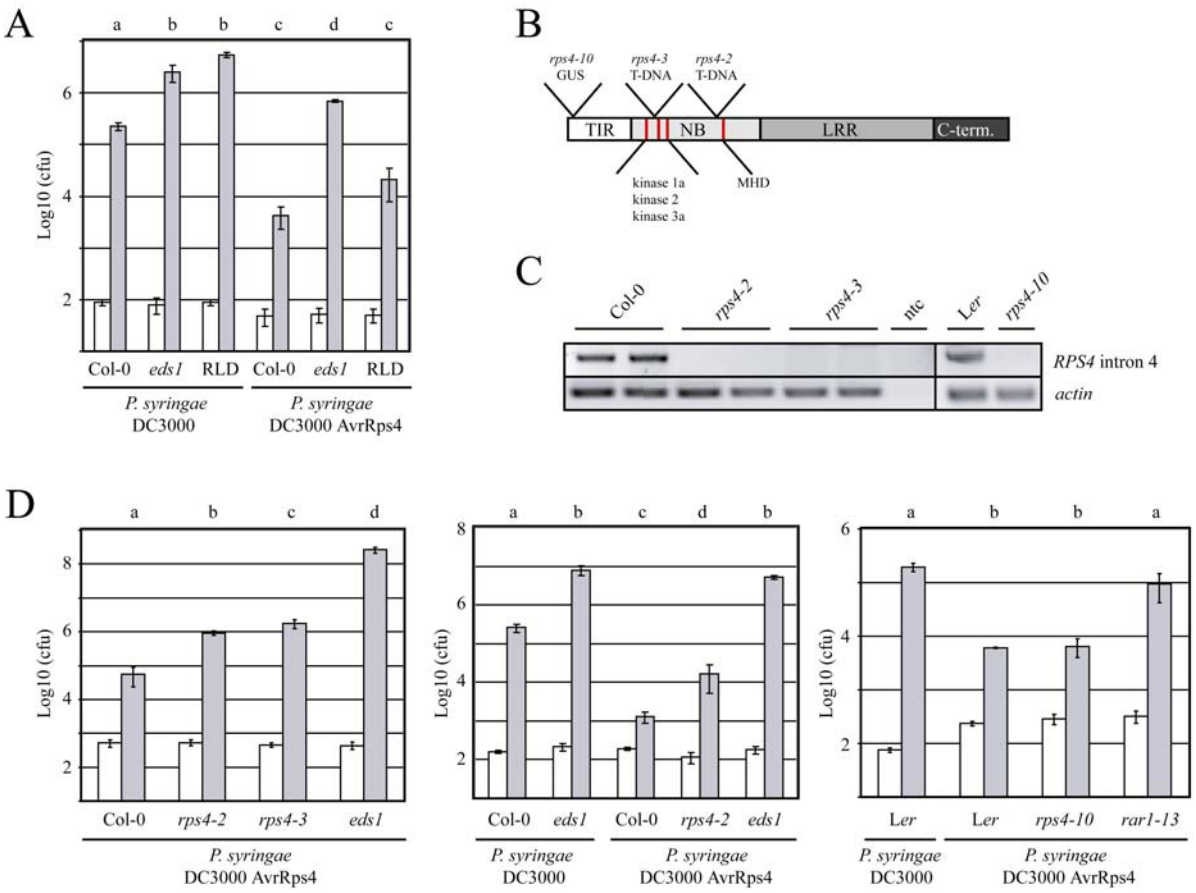

Fig. 3.1. Ability of natural and transgenic *rps4* mutants to restrict growth of *P. syringae* ±AvrRps4

(A) Five week old plants of the indicated genotypes were infiltrated with *P. syringae* ± AvrRps4 as marked below at 5x10^4 CFU/ml (2.2.5) and bacterial titres were determined 1 h (white bars) and 72 h (grey bars) post infiltration. Error bars represent the standard deviation of two (1h) and four (72 h) repeated samplings of five individual plants. Characters a-d indicate significant differences in growth at 72 h (t-test; α=0.05). This experiment was repeated three times with consistent results.

(B) Domain structure of RPS4 protein. Vertical red bars represent the highly conserved kinase-1a, kinase-2, kinase-3a and MHD motifs of the NB domain (van der Biezen and Jones, 1998a). T-DNA and *GUS*-reporter construct insertion loci are indicated above.

(C) *RPS4* transcript levels in the T-DNA insertion lines *rps4-2*, *rps4-3* and the *GUS*-reporter construct insertion line *rps4-10* in comparison to Col-0 and L*er* wild type plants. Data are representative of two independent RNA extractions.

(D) Five week old plants of the indicated genotypes were infiltrated with *P. syringae* ± AvrRps4 as marked below at 5x10^4 CFU/ml (2.2.5) and bacterial titres were determined 1 h (white bars) and 72 h (grey bars) post infiltration. Error bars represent the standard deviation of two (1h) and four (72h) repeated samplings of five individual plants. Characters a-d indicate significant differences in growth at 72h (t-test; α=0.05). Data are representative of two to four independent experiments.

3.1.2 Epitope-tagged RPS4 protein is functional

To confirm that the enhanced susceptibility to avirulent *P. syringae* observed in Col-0 *rps4* mutants was due to the loss of *RPS4* function I transformed *rps4-2* with the Col-0 genomic *RPS4* allele. The construct I used was under transcriptional control of the *RPS4* promoter and C-terminally fused to a double HA-StrepII epitope tag (pGreenII0229-OP-RPS4-HA-StrepII, see 2.2.7.11). *Arabidopsis rps4-2* plants were transformed by the floral dip method (see 2.2.3) and four homozygous T_3 lines (RPS4-HS #1 to #4) with a single transgene insertion were selected. In order to analyse the functionality of the RPS4-HA-StrepII fusion protein the transgenic RPS4-HS lines were tested for their susceptibility to *P. syringae* AvrRps4. As shown in Fig 3.2 A (left panel) all four transgenic lines restricted bacterial growth to the same extents as Col-0 wild type plants demonstrating that RPS4-HA-StrepII protein is functional. I also confirmed that expression of the RPS4-HA-StrepII fusion protein did not result in enhanced resistance towards virulent *P. syringae* (Fig. 3.2A right panel). Thus resistance induced by RPS4-HA-StrepII was specific to avirulent bacteria. I concluded that RPS4-HA-StrepII protein mimics the function of endogenous RPS4 and complements the *rps4-2* mutant phenotype. As shown in the immunoblot in Fig. 3.2B, all four selected transgenic lines accumulated full length RPS4-HA-StrepII protein (predicted MW 138 kDa). Line RPS4-HS #2 was chosen for all subsequent experiments since it expressed the RPS4-HA-StrepII fusion protein to levels sufficient for biochemical analysis.

3.1.3 Over-expression of *RPS4* induces *EDS1*-dependent constitutive defence gene expression

Since many *Arabidopsis* R proteins accumulate to very low levels (Boyes et al., 1998) and functional RPS4-HA-StrepII protein expressed under its own 5' and 3' regulatory sequence was close to the detection threshold of immunoblots I generated transgenic lines constitutively expressing *RPS4-HA-StrepII* under control of the strong CaMV 35S promoter. To this end *rps4-2* mutants were transformed with the binary plasmid pGreenII0229-35S-RPS4-HA-StrepII (see 2.2.7.11). From a total of 50 transformed plants in two independent transformations only six Basta® resistant individuals could be recovered. All confirmed T_1 plants showed severe stunting, developed spontaneous lesions and died within three to four weeks after germination (Fig. 3.3A). This lethal phenotype was consistent with previous reports describing attempts to over-express *R* genes in general and *RPS4* in particular (Oldroyd and Staskawicz, 1998; Gassmann et al., 1999; Weaver et al., 2006).

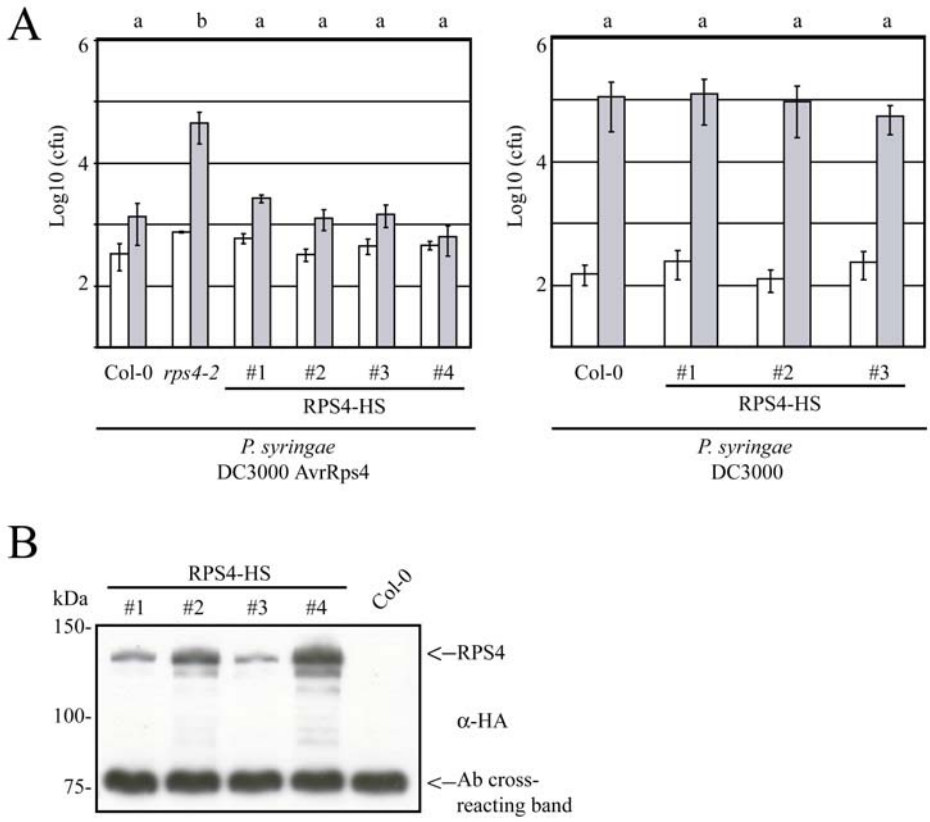

Fig. 3.2. *RPS4-HA-StrepII* construct complements the *rps4-2* mutant phenotype and the encoded fusion protein can be detected in immunoblots.

(A) Five week old plants of the indicated genotypes were infiltrated with *P. syringae* ± AvrRps4 at $5x10^4$ CFU/ml (2.2.5) and bacterial titres were determined 1 h (white bars) and 72 h (grey bars) post infiltration. Error bars represent the standard deviation of two (1h) and four (72h) repeated samplings of five individual plants. Characters a and b indicate significant differences in growth at 72h (t-test; α=0.05). RPS4-HS: Independent transgenic lines expressing the *RPS4-HA-StrepII* construct under control of the *RPS4* promoter. Data are representative of three independent experiments.

(B) Immunoblot probed with α-HA monoclonal antibody to detect RPS4-HA-StrepII protein in total protein extracts following separation by SDS-PAGE in four independent transgenic lines. The cross-reacting 75 kDa band demonstrates equal protein transfer onto the membrane.

In order to overcome this problem I generated *RPS4* over-expression lines in the signalling-deficient *eds1* background. The stunted phenotype was completely suppressed by the *eds1* mutation since twelve characterised T_1 plants were indistinguishable from Col-0 or *eds1* individuals (Fig. 3.3A). Four independent T_3 lines carrying a single transgene insertion were selected and the RPS4-HA-StrepII levels were analysed by immunoblot. Fig 3.3B shows the RPS4-HA-StrepII protein levels in the four selected transgenic lines in comparison to line

RPS4-HS #2 which expresses the RPS4-HA-StrepII fusion protein under control of the *RPS4* promoter. As expected, different protein levels were obtained most likely due to different transgene insertion loci. Line RPS4-HS^{OE} #1 expressed epitope-tagged RPS4 to similar levels as line RPS4-HS #2 whereas no signal was detected in RPS4-HS^{OE} #2 (Fig. 3.3B). These two lines accumulated RPS4-HA-StrepII to lower amounts than those expected for the strong CaMV 35S promoter most likely due to RNA silencing of the transgene (Finnegan and McElroy, 1994). In contrast, RPS4-HS^{OE} lines #3 and #4 accumulated RPS4-HA-StrepII to considerably higher levels than RPS4-HS #2. Thus, line RPS4-HS^{OE} #3 was chosen for further analysis.

To test whether the stunting and lethal phenotypes observed in *RPS4* over-expressing plants in *rps4-2/EDS1* background were due to elevated RPS4 levels, line RPS4-HS^{OE} #3 was crossed with Col-0 to introduce a functional *EDS1* allele. Genetically confirmed F_1 plants showed various degrees of stunting (data not shown) but viable individuals could be recovered. 178 F_2 individuals were categorised by eye as "stunted" or "wild type-like" plants. 26.41% (47 out of 178 plants) were categorised as "stunted". This finding was inconsistent with the assumption that the presence of one functional *EDS1* allele and one copy of the *RPS4^{OE}* construct were sufficient to induce reduced plant size (Table 3.1; scenario A).

Table 3.1. Correlation between the stunted phenotype in F_2 (RPS4-HS OE #3 x Col-0) and possible genetic scenarios

scenario	Stunted genotype(s)	% stunted individuals expected	% stunted individuals observed	X^2	p
A	*EDS1/x RPS4^{OE}/x*	56.25	26.41	64.400	0.000
B	*EDS1/eds1 RPS4^{OE}/RPS4^{OE}* and *EDS1/EDS1 RPS4^{OE}/--*	31.25	26.41	1.950	0.163
C	*EDS1/EDS1 RPS4^{OE}/RPS4^{OE}*	6.25	26.41	123.000	0.000

However, the observed numbers correlated with an outcome in which stunted plants were homozygous for *EDS1* and carried at least one copy of the *RPS4^{OE}* construct or were *EDS1/eds1* heterozygous but homozygous for *RPS4^{OE}* (Table 3.1; scenario B; expected: 31.25%; X^2=1.95; p=0.163). To further analyse the observed segregation pattern 20 "stunted" and 20 "wild type-like" individuals were genotyped. All 20 individuals categorised as "stunted" carried the *RPS4^{OE}* construct and were either heterozygous (7 plants) or homozygous (13 plants) for *EDS1*. Seventeen out of 20 individuals with "wild type"

morphology either lacked the *RPS4*^{OE} construct (11 plants) or were homozygous for the *eds1* null allele (9 plants). These results were consistent with the observed F_2 segregation pattern and demonstrate a correlation between the stunted phenotype and *RPS4* over-expression in an *EDS1* genetic background. Significantly, one F_3 line homozygous for both *EDS1* and the *RPS4*^{OE} construct recovered the phenotype observed with *RPS4* over-expressing T_1 transgenics in *rps4-2/EDS1* genetic background (Fig. 3.3A). The finding that the double homozygous F_3 *RPS4*^{OE}*/EDS1* line is viable but much smaller than F_2 plants indicates that the stunted phenotype might be influenced by the *RPS4* and/or *EDS1* gene dosage. Although more F_3 lines need to be analysed, these data are consistent with the hypothesis that *RPS4* over-expression in a signalling-competent *EDS1* background leads to reduced plant size and development of spontaneous lesions.

This phenotype is indicative of a constitutively activated plant defence system (Stokes et al., 2002), although resistance to *P. syringae* could not be assessed due to the small plant size. Instead, I determined protein levels of the SA-inducible defence marker PR1 (Uknes et al., 1992). As shown in Fig. 3.3E, the *RPS4*^{OE}*/EDS1* homozygous F_3 line had elevated PR1 levels compared to unchallenged Col-0 and *eds1* control plants. PR1 accumulation was completely *EDS1*-dependent since no PR1 immunoblot signal could be detected in RPS4-HS^{OE} #3 (Fig. 3.3E). Thus stunted morphology and spontaneous lesion formation induced by *RPS4* over-expression in *EDS1* plants correlated with constitutive expression of a SA-inducible defence marker. These results demonstrate that the *RPS4* over-expression signal is transduced in an *EDS1*-dependent manner to activate defence responses downstream of *EDS1* and SA.

To further position *EDS1* in the RPS4-mediated resistance pathway I tested whether *RPS4* over-expression line RPS4-HS^{OE} #3 showed increased resistance to *P. syringae*. As shown in Fig. 3.3D, over-expression of *RPS4* in an *eds1* background did not render plants more resistant to virulent *P. syringae* demonstrating that *EDS1* function in RPS4-mediated resistance cannot be overcome by *RPS4* over-expression. Even in the presence of the cognate effector AvrRps4, line RPS4-HS^{OE} #3 was as susceptible as the *eds1* control (Fig. 3.3D). These findings demonstrate that *EDS1* functions downstream of activated RPS4 and correlate well with the *EDS1*-dependent stunted phenotype and constitutive PR1 expression in *RPS4-HA-StrepII* over-expression lines. This finding is significant since it shows that it is not the strength of the R protein trigger that defines *EDS1* dependence or independence.

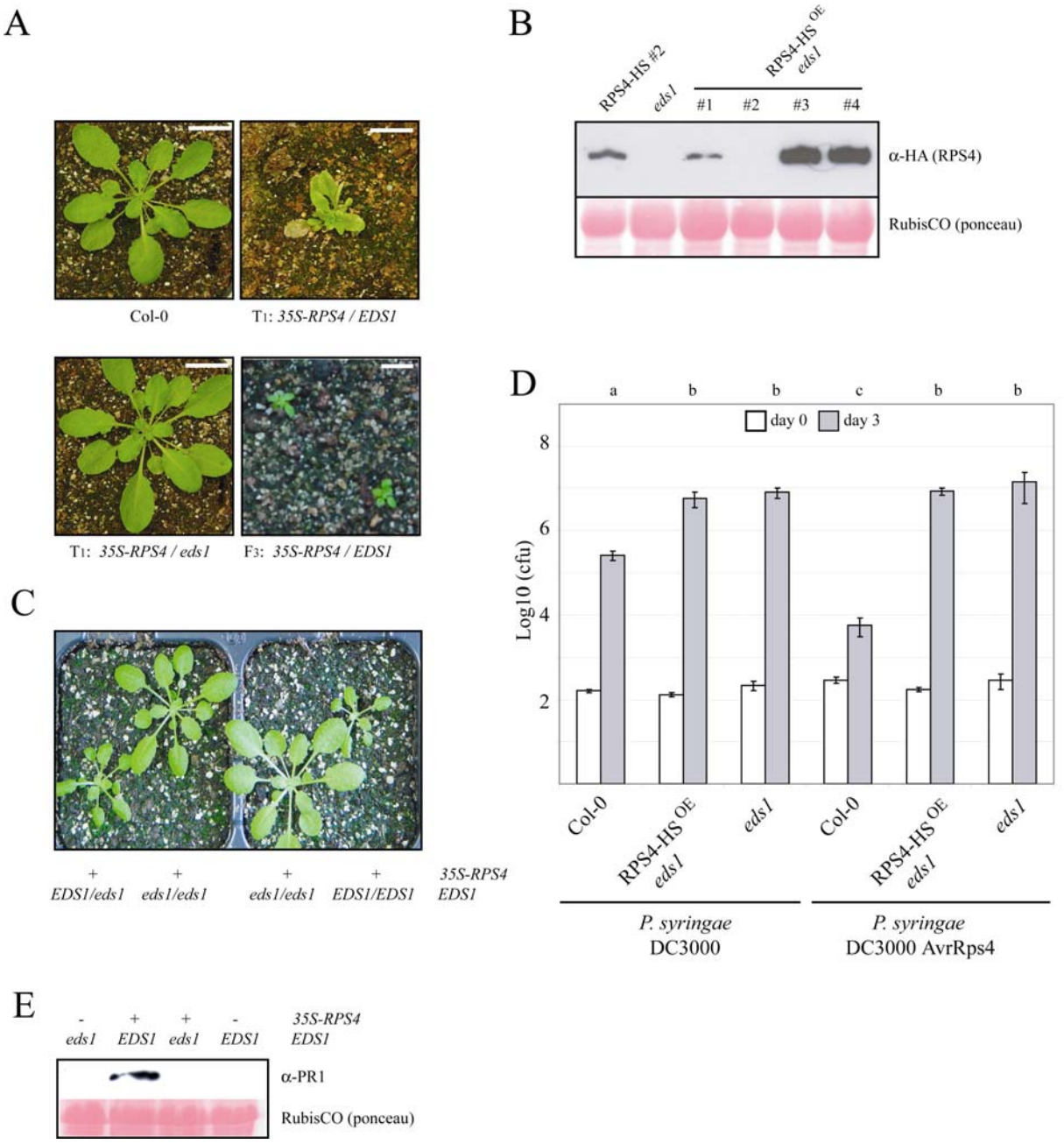

Fig. 3.3. *EDS1* functions as an intrinsic downstream signal transducer downstream of activated RPS4 protein.

(A) Pictures of three week old plants. Genotypes indicated below. Over-expression of *RPS4-HA-StrepII* results in a stunted stature and this phenotype is fully *EDS1*-dependent. The white bar corresponds to 1.5 cm in all pictures.

(B) Immunoblot showing RPS4-HA-StrepII protein levels in four independent transgenic lines expressing *35S-RPS4-HA-StrepII* in comparison to line RPS4-HS #2 expressing the same construct under control of the *RPS4* promoter. Total protein extracts were separated by SDS-PAGE and probed with α-HA antibody. The Ponceau S stain demonstrates equal protein transfer onto the membrane.

(C) Four individuals of the segregating F_2 population from the cross RPS4-HS^{OE} #3 x Col-0. Genotype indicated below. Plants over-expressing *RPS4-HA-StrepII* appear smaller than plants expressing the transgene in *eds1* background.

(D) Five week old plants of the indicated genotypes were infiltrated with *P. syringae* ± AvrRps4 (as shown below) at 5x10^4 CFU/ml (2.2.5) and bacterial titres were determined 1 h (white bars) and 72 h (grey bars) post infiltration. Error bars represent the standard deviation of two (1h) and four (72h) repeated samplings of five individual plants. Characters a-c indicate significant differences in growth at 72h (t-test; α=0.05). The experiment was repeated two times with consistent results.

(E) Immunoblot probed with α-PR1 antibody. *RPS4-HA-StrepII* over-expression leads to PR1 accumulation only in the presence of EDS1. The Ponceau S stain demonstrates equal protein transfer onto the membrane.

3.1.4 *EDS1* functions downstream of activated RPS4 and is essential for transcriptional reprogramming of defence genes

Given the result that *EDS1* functions downstream of RPS4 I made use of published microarray transcript profiling data (Bartsch et al., 2006) to analyse where *eds1* blocks RPS4 signalling. The micorarray profiling experiment of M. Bartsch compared the responses to *P. syringae* AvrRps4 in Wassilewskija (Ws-0) wild type plants with *eds1-1* mutants at 3 and 6 h post infiltration. I focused on the 6 h time point since very few differences were observed at 3 h post infiltration (Bartsch et al., 2006) and AvrRps4 protein was found to be secreted within 4 h after *P. syringae* infiltration (Kee Hoon Sohn & J. Jones, Sainsbury laboratory, Norwich, UK unpublished).

Using a significance threshold of 30-fold transcript change (~1.5 log) I first compared the response of Ws-0 wild type with *eds1* mutants. Compared to the mock control (10 mM $MgCl_2$) 116 genes were induced in Ws-0 whereas 17 were repressed. In *eds1* mutants only two genes were induced and one gene was repressed and none of these three were among the differentially expressed genes in wild type (results not shown). Since the 17 downregulated transcripts encode proteins of unknown functions and the absolute transcript values were low I concentrated on the 116 genes upregulated in an *EDS1*-dependent manner. The upregulated transcripts included several well characterised defence marker genes such as *FMO1* (Bartsch et al., 2006), *NUDT6* (M. Bartsch & J. Parker, unpublished), *PAD4* (Glazebrook et al., 1996), *BAP1* (Yang et al., 2006), *EDS5* (Rogers and Ausubel, 1997) and *PBS3* (Warren et al., 1999) (Table 3.2), demonstrating that central defence genes were induced in wild type plants but not *eds1* mutants. This finding shows that *eds1* plants strongly differ in their transcriptional response to *P. syringae* AvrRps4. I also considered that by comparing $MgCl_2$ *vs. P. syringae* AvrRps4 infiltration several transcriptional responses such as recognition of PAMPs, changes induced by bacterial effectors and RPS4-induced signalling were included in the gene sets. The precise contribution of EDS1 to PAMP-induced responses is not known. However, flg22-

triggered immune responses are not compromised in *eds1* mutants (Zipfel et al., 2004) and the *EDS1/PAD4/SAG101* resistance layer can be genetically separated from preinvasion resistance to *Bgh* (Lipka et al., 2005). Still it was possible that the observed transcript changes were mainly due to PAMP perception or were consequences of bacterial effectors. I therefore compared the list of 116 *EDS1*-dependent upregulated genes with another microarray experiment that analysed the transcript changes of Col-0 plants to virulent *P. syringae* at 7 h post infiltration (Thilmony et al., 2006). Since transcript changes in 50 randomly selected genes were very similar in both experiments (data not shown), I concluded that a comparison of the two microarray data sets was most likely valid. At the 7 h time point 31 genes were induced by virulent bacteria. Only six of these overlapped with the 116 genes upregulated in an *EDS1*-dependent manner in response *to P. syringae* AvrRps4 (Table 3.2). Again transcript changes of these 6 overlapping genes were very similar in both experiments, validating the comparability of the two microarray profiling experiments.

Table 3.2. Upper part: Transcript changes of central defence marker genes upregulated in an *EDS1*-dependent manner 6 h post infiltration with *P. syringae* (*Ps*) AvrRps4. Lower part: Transcript changes of genes induced by both avirulent and virulent *P. syringae.*

Atg code	gene	Fold change Ws-0 *Ps* AvrRps4	Fold change *eds1-1 Ps* AvrRps4	Fold change Col-0 virulent *Ps*
At1g19250	*FMO1*	+152.3	-9.0	+10.8
At2g04450	*NUDT6*	+49.0	+1.2	+1.5
At3g52430	*PAD4*	+60.8	-1.5	+6.9
At3g61190	*BAP1*	+35.7	-1.7	+6.3
At4g39030	*EDS5*	+49.6	+1.9	+5.2
At5g13320	*PBS3*	+394.0	-2.2	+11.5
At5g61900	*BON1*	+60.3	+2.1	+4.3
At1g35910	--	+78.6	-3.2	+88.1
At1g76640	--	+205.2	+9.8	+346.3
At3g11480	--	+81.1	+16.0	+476.2
At3g15500	--	+31.1	+2.2	+40.2
At4g24110	--	+49.9	+9.0	+49.9
At5g67080	*MEKK19*	+97.1	+3.4	+61.2

In conclusion the transcript profiling data suggest that the majority of the 116 genes upregulated in an *EDS1*-dependent manner are not part of the response to PAMPs or *P. syringae* effectors but are induced by RPS4 signalling. Thus transcriptional reprogramming of defence genes is blocked at a very early stage in *eds1* mutants.

3.1.5 Summary of the *rps4* mutant phenotype and positioning of *EDS1* in RPS4-mediated resistance

The data presented in section **3.1** demonstrate that RPS4 is not the only TIR-NB-LRR protein able to recognise the *P. syringae* effector protein AvrRps4. In contrast to published data (Hinsch and Staskawicz, 1996; Gassmann et al., 1999), I observed clear AvrRps4 recognition in the proposed natural *rps4* mutant RLD (Fig. 3.1A). Furthermore, three independent *rps4* T-DNA insertion lines exhibited no (r*ps4-10*; L*er*) or only moderately increased (*rps4-2* and *rps4-3*; Col-0) susceptibility to *P. syringae* AvrRps4 (Fig. 3.1D). Taken together these results indicate that in both accessions, L*er* and Col-0, additional TIR-NB-LRR proteins recognise AvrRps4. Expression of epitope-tagged genomic *RPS4* in *rps4-2* plants under transcriptional control of the *RPS4* 5' and 3' genomic sequence fully restored resistance to *P. syringae* in an AvrRps4-specific manner (Fig. 3.2A), confirming that the moderately increased susceptibility of *rps4-2* mutants was indeed due to loss of *RPS4* function. Full length epitope-tagged RPS4 protein could be detected in immunoblots using a α-HA antibody (Fig. 3.2B).

Over-expression of epitope-tagged RPS4 protein resulted in stunted plant morphology, development of spontaneous lesions and constitutive PR1 accumulation (Fig. 3.3). This phenotype, indicative of a constitutively activated defence system, was fully dependent on *EDS1* (Fig. 3.3). *RPS4* over-expression in an *eds1* background did not result in increased resistance to *P. syringae* AvrRps4 (Fig. 3.3D). This finding demonstrates that *EDS1* is an intrinsic signal transducer functioning downstream of activated RPS4 protein. Furthermore, I could deduce from published microarray profiling data that loss of *EDS1* leads to a very early block of RPS4 signalling since virtually all transcript changes observed in wild type were not altered in *eds1* mutants (Table 3.2).

3.2 Subcellular localisation of endogenous and functional epitope-tagged RPS4 protein

Since the *RPS4-HA-StrepII* construct driven by the *RPS4* promoter complemented the *rps4-2* mutant phenotype (Fig. 3.2A), line RPS4-HS #2 was used for subcellular localisation analysis. When this work was initiated no protein data for RPS4 were available. Also, almost no other TIR-NB-LRR receptor was characterised in terms of subcellular localisation (Deslandes et al., 2003). Thus, determining RPS4 subcellular localisation was a first step to understand where and how TIR-NB-LRR proteins function within the cell and whether they directly associate with EDS1 or its signalling partners PAD4 and SAG101.

3.2.1 RPS4 protein associates with endomembranes

I first tested whether RPS4 is a soluble R protein or associates with organelles or membranes. As shown in the immunoblot in Fig. 3.4A, RPS4-HA-StrepII protein from total leaf extracts could be detected in pellets of 2000 x g, 5000 x g and 100.000 x g spins but not in the soluble fraction following the 100.000 x g ultracentrifugation step. By contrast, EDS1 protein was entirely soluble, a finding that is in accordance with previous results (Wiermer, 2005). A transgenic line expressing a functional triple HA-tagged version of the BONZAI1 (BON1) protein served as a control for a membrane associated protein (Hua et al., 2001). The results presented in Fig. 3.4A demonstrate that RPS4 is not a soluble R protein.

Since the RPS4 primary sequence lacks candidate transmembrane regions (Gassmann et al., 1999; Horton et al., 2006), I further characterised the association with membranes. Fig. 3.4B shows that RPS4-HA-StrepII protein recovered in the supernatant of the 2000 x g spin was partially soluble in buffers containing either 2M urea or 1% (v/v) Triton-X 100. Also, increasing the NaCl concentration of the buffer from 150 to 500 mM brought almost all RPS4-HA-StrepII protein to the soluble fraction, indicative of an ionic interaction with membranes (Fig. 3.4B). Since the well characterised CC-NB-LRR receptors RPM1, RPS2 and RPS5 associate with the plasma membrane (Boyes et al., 1998; Belkhadir et al., 2004b; Holt et al., 2005), I analysed whether this is the case for RPS4. Aqueous two-phase purification of *Arabidopsis* plasma membranes revealed that RPS4-HA-StrepII associates with endomembranes and not the plasma membrane (Fig. 3.4C).

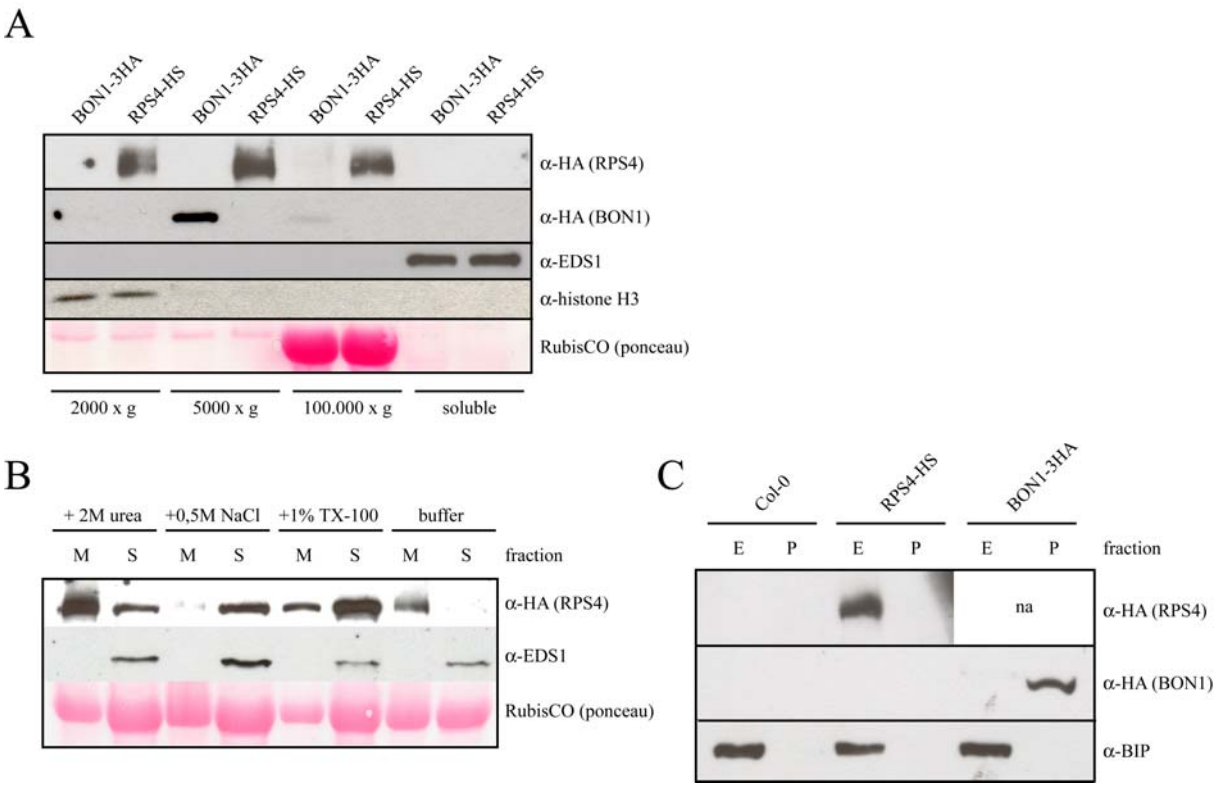

Fig. 3.4. Detection of functional RPS4-HA-StrepII protein in immunoblots following fractionation of *Arabidopsis* protein extracts and protein separation by SDS-PAGE. Data are representative of two independent experiments.

(A) Total protein extracts of line RPS4-HS #2 and a transgenic line expressing BON1-3HA (control) were subjected to incremental centrifugation steps (shown below) and fractions were analysed by immunoblotting with the indicated antibodies following separation by SDS-PAGE. The Ponceau S stain shows the separation of the large (55 kDa) RubisCO subunit.

(B) Microsomes of line RPS4-HS #2 were resuspended in extraction buffer alone or the same buffer supplemented with urea, NaCl or Triton X-100 at the indicated concentrations. Samples were subjected to ultracentrifugation to determine the solubility of RPS4 protein under different buffer conditions. Supernatant and pellet fraction were separated by SDS-PAGE and probed with α-HA and α-EDS1 antibodies. S: soluble fraction. M: membrane fraction.

(C) Aqueous two phase separation of *Arabidopsis* microsomes was carried out at a polymer concentration of 6.2% (w/v) following the protocol described in Larsson et al. (1987). Endomembrane (E) and plasma membrane (P) fractions of Col-0 wild type, RPS4-HS #2 and BON1-3HA transgenic lines were analysed by SDS-PAGE and subsequent immunoblotting. na: not assayed (due to strong unspecific signal of the BON1-3HA transgenic line in the higher molecular weight range).

The monomeric form of the BON1-3HA protein served as a control for a plasma membrane-associated protein (Hua et al., 2001) and BIP was used as an endomembrane marker. I concluded that functional RPS4-HA-StrepII protein associates with endocellular membranes and this interaction is most likely of an ionic character. RPS4-HA-StrepII protein also co-fractionated with the ER marker BIP in sucrose density centrifugations of *Arabidopsis*

microsomes indicating that it might localise to the ER (data not shown). However, since specific markers for Golgi and vacuolar compartments were not available association with other endomembranes could not be ruled out.

3.2.2 A pool of RPS4 protein associates with nuclei

Fig. 3.4A shows that functional RPS4-HA-StrepII protein was also found in the 2000 x g pellet. This was not due to incomplete homogenisation of the extract because EDS1 and BON1-3HA proteins were not detected in this fraction. Since the 2000 x g pellet contained a mixture of cell wall debris, organelles and nuclei (see α-histone H3 blot in Fig. 3.4A) I used subcellular localisation prediction software to search for signal peptides that could mediate secretion or transport to organelles or nuclei. Analysis using the WoLF PSORT subcellular localisation prediction software (Horton et al., 2006) revealed that RPS4 carries a putative bipartite nuclear localisation sequence (NLS) in the C-terminal extension domain (amino acids K1171 to R1187; KKKKTRMDNGRPKKKQR; core basic motifs underlined, Fig. 3.5A). Alternatively, the motifs KKKK and KKQR could function as monopartite NLS since they fit to the loose K[K/R]X[K/R] consensus sequence of monopartite import signals (Lange et al., 2006). In contrast, no indication for organellar or secretion targeting signals were found in the RPS4 sequence.

Purification of nuclei from unchallenged *Arabidopsis* leaf tissue revealed that RPS4-HA-StrepII protein was indeed found in both the nuclear fraction and the supernatant from which nuclei were removed (Fig. 3.5B). In order to validate the success of nuclear extraction the same immunoblot was probed with α-histone H3 antibody, a nuclear marker protein. Contaminations with cytosolic proteins could be ruled out because only small amounts of HSC70 and EDS1, both predominantly cytosolic proteins (Welch and Feramisco, 1982; Bae et al., 2003; Wiermer, 2005), were detected in the nuclear fraction (Fig. 3.5B). Furthermore, the plasma membrane-associated BON1-3HA (Hua et al., 2001) and a GFP variant with an ER retention signal (Matsushima et al., 2003) were not enriched in the nuclear fraction. These results show that a pool of functional RPS4-HA-StrepII protein is associated with nuclei. Also a functional epitope-tagged variant of the RPM1 CC-NB-LRR receptor (RPM1-myc) was found in nuclear extracts although, when compared to RPS4-HA-StrepII partitioning, a lower proportion of RPM1-myc was present in the nuclear fraction. This is interesting because so far RPM1 was assumed to be entirely plasma membrane-associated (Boyes et al., 1998). Consistent with a nuclear localisation, RPM1 protein carries a putative monopartite (K448 to

R451) and bipartite (R717 to R733) NLS (Horton et al., 2006). Although R proteins are expressed at very low levels (Boyes et al., 1998), nuclear localisation of endogenous RPS4 protein could be confirmed using an antiserum raised against the RPS4 NB domain (Fig. 3.5C) whereas no specific RPS4 signal could be distinguished in total protein preparations due to unspecific background (data not shown). Significantly, the α-RPS4 antiserum detected a double band of ~140 kDa in Col-0 and L*er* wild type extracts of which the upper band corresponds to full length RPS4 protein (predicted MW 138 kDa) since it could not be detected in the corresponding T-DNA insertion lines *rps4-2* and *rps4-10* (Fig. 3.5C).

I also probed RLD nuclear extracts with the α-RPS4 antiserum because the *Arabidopsis* ecotype RLD has been proposed to be a natural *rps4* mutant (Hinsch and Staskawicz, 1996). As shown in Fig. 3.5D, the upper band corresponding to full length RPS4 protein was present in RLD nuclear extracts indicating that RLD RPS4 protein accumulates to levels comparable to those in resistant Col-0 and L*er* accessions. This finding is consistent with a previous report demonstrating that the RLD *rps4* locus is transcribed (Gassmann et al., 1999) and my finding that RPS4 in RLD is functional (Fig. 3.1A). Having a workable α-RPS4 antiserum in hand I tested line RPS4-HS #2 for nuclear RPS4-HA-StrepII levels in comparison to Col-0 endogenous RPS4 levels. The immunoblot in Fig. 3.5E demonstrates that RPS4-HA-StrepII protein in RPS4-HS #2 accumulated to slightly higher levels than endogenous Col-0 RPS4. Thus, the selected transgenic line did not highly over-express epitope-tagged RPS4 protein. This finding, together with the *P. syringae* growth assays (Fig. 3.2A) demonstrating functionality of the *RPS4-HA-StrepII* construct, strengthens the relevance of localisation studies performed with line RPS4-HS #2.

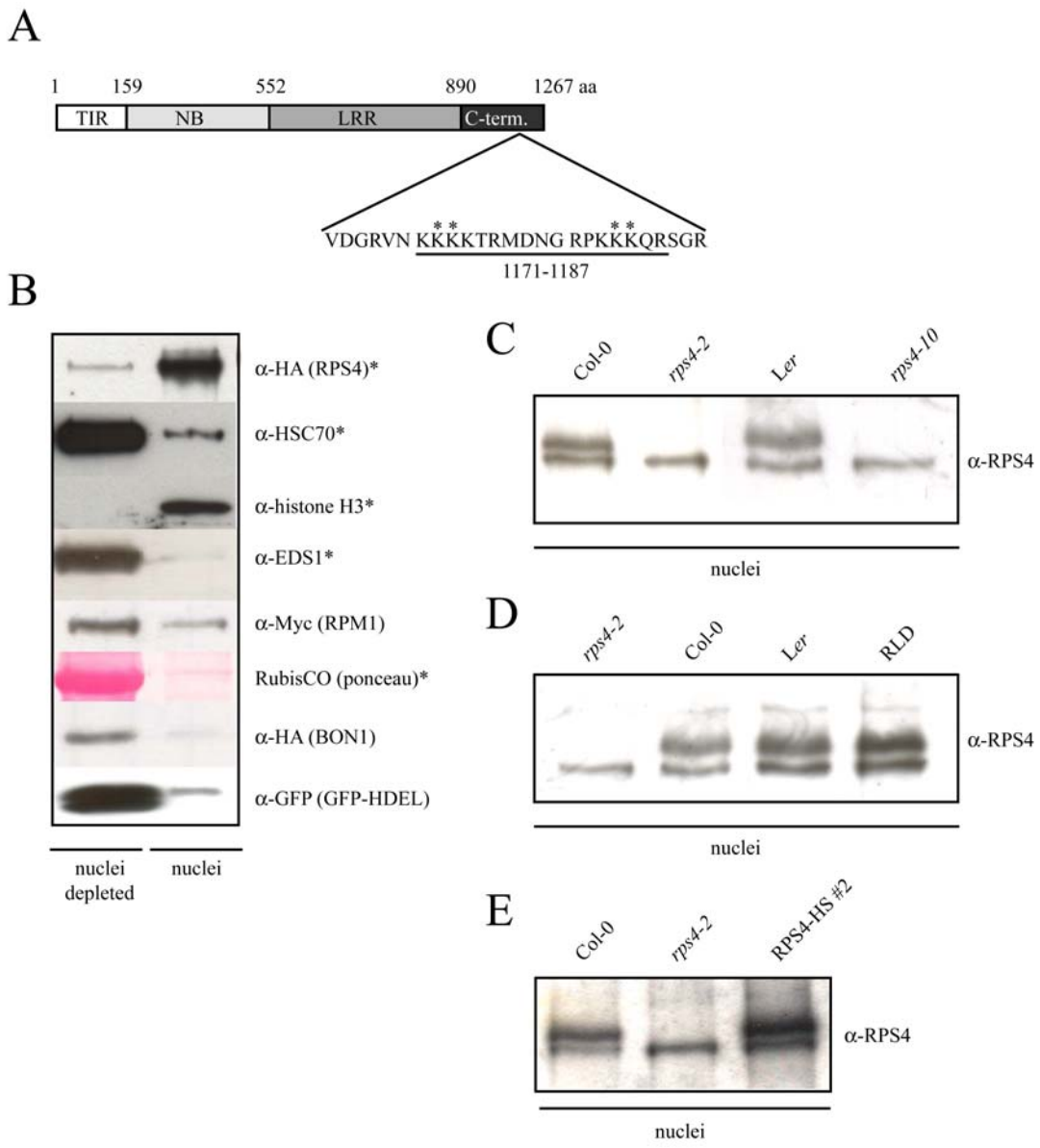

Fig. 3.5. RPS4 protein possesses a bipartite NLS and a pool of RPS4 protein associates with nuclei.

(A) Domain structure of RPS4 protein. The bipartite NLS (amino acids 1171 to 1187) in its C-terminal extension domain is highlighted. aa: amino acids. The asterisks indicate the four core lysine residues predicted to be required for NLS function.

(B) A pool of RPS4 associates with nuclei. Nuclear extracts were prepared from line RPS4-HS #2 (indicated by an asterisk) and the transgenic RPM1-myc, BON1-3HA and GFP-HDEL lines. Nuclei-depleted and nuclear fractions were separated by SDS-PAGE and analysed by immunoblot. α-HSC70 and α-histone H3 antibodies served as cytoplasmic and nuclear markers, respectively. Note that the described protocol (2.2.6.2) results in a ~16 fold over-representation of nuclear proteins compared to the non-nuclear fraction. The Ponceau S stain shows the separation of the large (55 kDa) RubisCO subunit. This experiment was repeated three times with consistent results.

(C) Immunoblot of nuclear extracts prepared from Col-0 and L*er* wild type and the *rps4-2* and *rps4-10* mutant lines probed with α-RPS4 antiserum raised against the RPS4 NB domain. The upper band corresponds to full length RPS4 protein (predicted MW: 138 kDa).

(D) Immunoblot as in (C) but nuclear extracts prepared from *rps4-2* mutant and Col-0, L*er* and RLD wild type plants.

(E) Immunoblot as in (C) but nuclear extracts prepared from *rps4-2* mutant, Col-0 and transgenic line RPS4-HS #2 expressing *RPS4-HA-Str*epII under transcriptional control of the *RPS4* promoter in *rps4-2* background.

3.2.3 The C-terminal cleavage fragment of AvrRps4 locates to the host cytoplasm

Having determined the subcellular localisation of RPS4 I tested whether the *P. syringae* effector AvrRps4 that activates RPS4 co-localises to nuclei or the endomembrane system. The hydrophilic 24 kDa AvrRps4 protein is processed after secretion to the host cell in a plant-specific manner to yield a 11 kDa C-terminal cleavage product ($AvrRps4^C$) that is sufficient and necessary for recognition by RPS4 (K. Hoon Sohn & J. Jones, personal communication). To study AvrRps4 subcellular localisation I made use of a transgenic line transformed with a Dexamethasone (Dex)-inducible *AvrRps4-HA* fusion construct (Mackey et al., 2003).

As reported before, $AvrRps4^C$ could be detected on immunoblots at 24 h after Dex application (time course experiment not shown) (Mackey et al., 2003). The *Dex-AvrRps4-HA* construct was crossed into line RPS4-HS #2 expressing functional RPS4-HA-StrepII protein in *rps4-2* and *rps4-2/eds1* genetic backgrounds. I then tested whether inducible expression of *AvrRps4-HA* triggers an *EDS1*-dependent cell death as it would be expected since AvrRps4 is recognised by RPS4. As shown in Fig. 3.6A, staining with Lactophenol Trypan blue, a diazo dye that is preferentially retained by dead cells, revealed stained cells in *EDS1* but not *eds1* genetic background 4 d after Dex application. This timing correlated with the first necrotic lesions being visible in *EDS1* background from 5 d after Dex spraying whereas *eds1* plants stayed symptomless at 4 d (Fig. 3.6A) and throughout a 7 d time course (data not shown). Thus *EDS1* is essential for AvrRps4-induced cell death, a result that is consistent with previous reports demonstrating that *EDS1* functions upstream of the programmed cell death triggered by TIR-NB-LRR receptors (Aarts et al., 1998; Feys et al., 2001; Rusterucci et al., 2001).

Immunoblot analysis revealed that $AvrRps4^C$ was present at 24 h post Dex application in both *EDS1* and *eds1* plants, although levels were consistently higher in *eds1* mutants (Fig. 3.6B). This difference in protein levels might be due to the fact that *EDS1* cells respond with programmed cell death resulting in protein degradation. The immunoblot in Fig. 3.6B further shows that the $AvrRps4^C$ protein accumulated in the non-nuclear fraction. Given the high expression levels obtained with the Dex-inducible promoter (Aoyama and Chua, 1997;

Mucyn et al., 2006) and the low molecular weight of the C-terminal AvrRps4^{C} cleavage product (11 kDa), that is far below the size exclusion limit of the nuclear pore complex (~40 kDa) (Ribbeck and Gorlich, 2001), it is surprising that no epitope-tagged AvrRps4^{C} was found in nuclei. Fig. 3.6B also reveals that endomembrane *vs.* nuclear partitioning of RPS4-HA-StrepII protein was not affected by AvrRps4-HA expression at the 24 h time point. This finding was corroborated in a time course experiment following an infection with *P. syringae* AvrRps4 (data not shown).

Cell fractionation by ultracentrifugation demonstrated that AvrRps4^{C} does not associate with host membranes but is soluble (Fig. 3.6C). The α-EDS1 and α-PEN1 antibodies served as soluble and microsomal markers, respectively. In summary these experiments suggest that the major pools of RPS4 and the cognate effector AvrRps4^{C} do not co-localise to a specific subcellular compartment. They further indicate that AvrRps4^{C} recognition might be via an indirect mechanism as has been reported for the *P. syringae* effectors AvrRpm1, AvrB, AvrRpt2 and AvrRps5 (Mackey et al., 2002; Mackey et al., 2003; Shao et al., 2003; Ade et al., 2007).

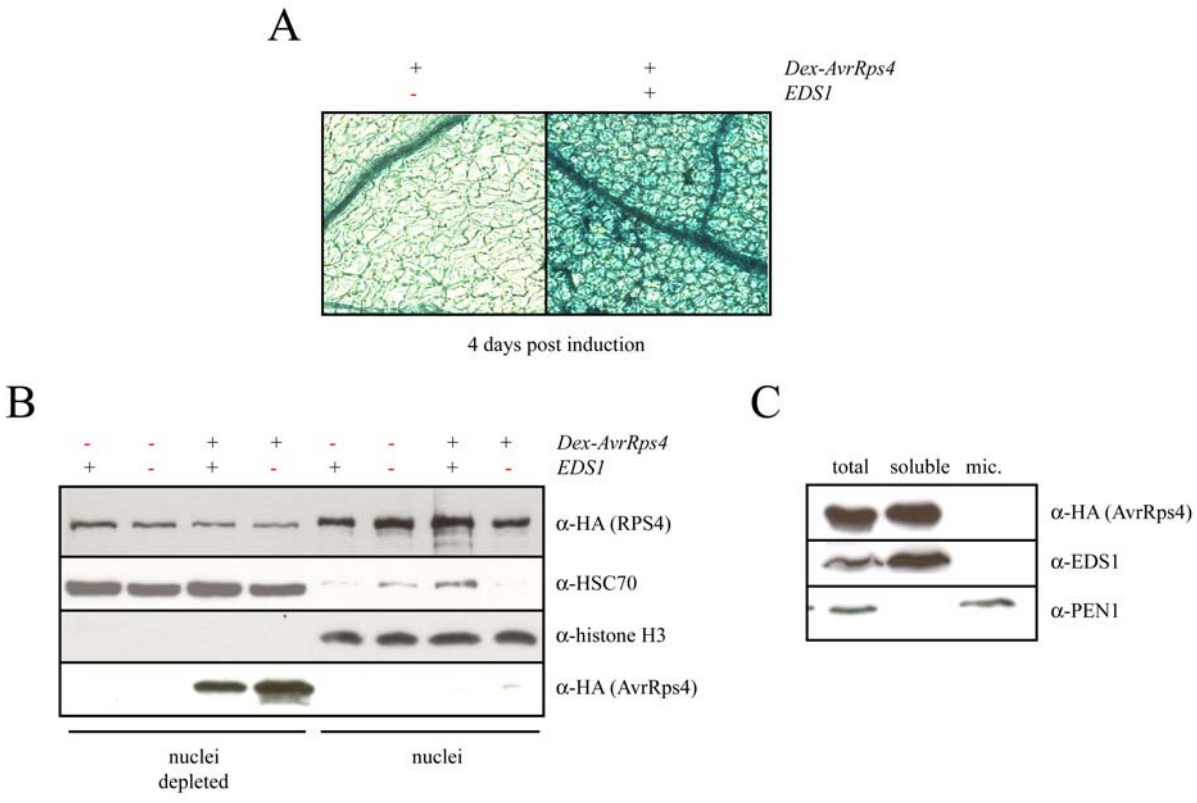

Fig. 3.6. AvrRps4^C, the processed form of the *P. syringae* effector AvrRps4, triggers an *EDS1*-dependent cell death response and localises to the host cell cytoplasm when over-expressed.

(A) Lactophenol Trypan blue stained leaf tissue of transgenic lines expressing *AvrRps4-HA* from a Dex-inducible promoter in *EDS1* and *eds1* genetic backgrounds. Tissue was stained 4 d post induction of protein expression with 30 µM Dex. Pictures are representative of two experiments.

(B) Immunoblot showing the nuclear vs. non-nuclear separation of RPS4-HA-StrepII and AvrRps4^C 24 h after induction of *AvrRps4-HA* expression with 30 µM Dex. Plants that did not carry the *Dex-AvrRps4-HA* construct served as controls. α-HSC70 and α-histone H3 antibodies served as cytoplasmic and nuclear markers, respectively. This experiment was repeated two times with consistent results.

(C) Total protein extracts of the Dex-inducible AvrRps4-HA line were subjected to ultracentrifugation 24 h post induction with 30 µM Dex. Total, soluble and microsomal fractions were separated by SDS-PAGE and probed in an immunoblot with α-HA antibody. Antisera specific for EDS1 and PEN1 served as controls for soluble and membrane proteins, respectively. Data are representative of two independent extractions.

However, since AvrRps4^C was expressed from the strong Dex-inducible promoter and accumulated to high levels in the host cell I cannot unambiguously rule out that there are a limited number of AvrRps4^C binding sites in the nucleus or an endomembrane compartment that might be important for AvrRps4 ^{C} recognition by RPS4.

3.2.4 The RPS4 NLS is required for nuclear import and resistance to *P. syringae* AvrRps4

The existence of a nuclear RPS4 pool together with the finding that AvrRps4^C does not co-localise to nuclei suggests that AvrRps4^C is recognised outside the nucleus and raises the question whether RPS4 nuclear localisation is required for its function in disease resistance. In order to test this, four lysine residues constituting the two core basic motifs of the bipartite NLS were substituted by alanines (K1172A, K1173A, K1184A and K1185A; see Fig. 3.5A). I chose to substitute all four core lysine residues predicted to be required for karyopherin binding since the two basic amino acid stretches KKKK (amino acids 1171 to 1174) and KKQR (amino acids 1184 to 1187) in the NLS are predicted to function individually as strong monopartite import signals (Lange et al., 2006). The *RPS4^{nls}-HA-StrepII* construct was tested for functionality by transformation into *rps4-2* plants. Three independent transgenic T_3 lines that accumulated the RPS4^{nls}-HA-StrepII protein to similar levels as the complementing line RPS4-HS #2 were selected (Fig. 3.7A). The expression levels observed in these lines indicate that the quadruple lysine to alanine exchange did not affect RPS4 stability. However, when nuclei were isolated from the three transgenic lines (RPS4-HS^{nls} #1 – #3) the nuclear RPS4 pool was depleted (Fig. 3.7B) demonstrating that RPS4 amino acids 1171-1187 comprise a

functional NLS. I tested the ability of transgenic lines RPS4-HS^{nls} #1 – #3 to restrict growth of *P. syringae* AvrRps4.

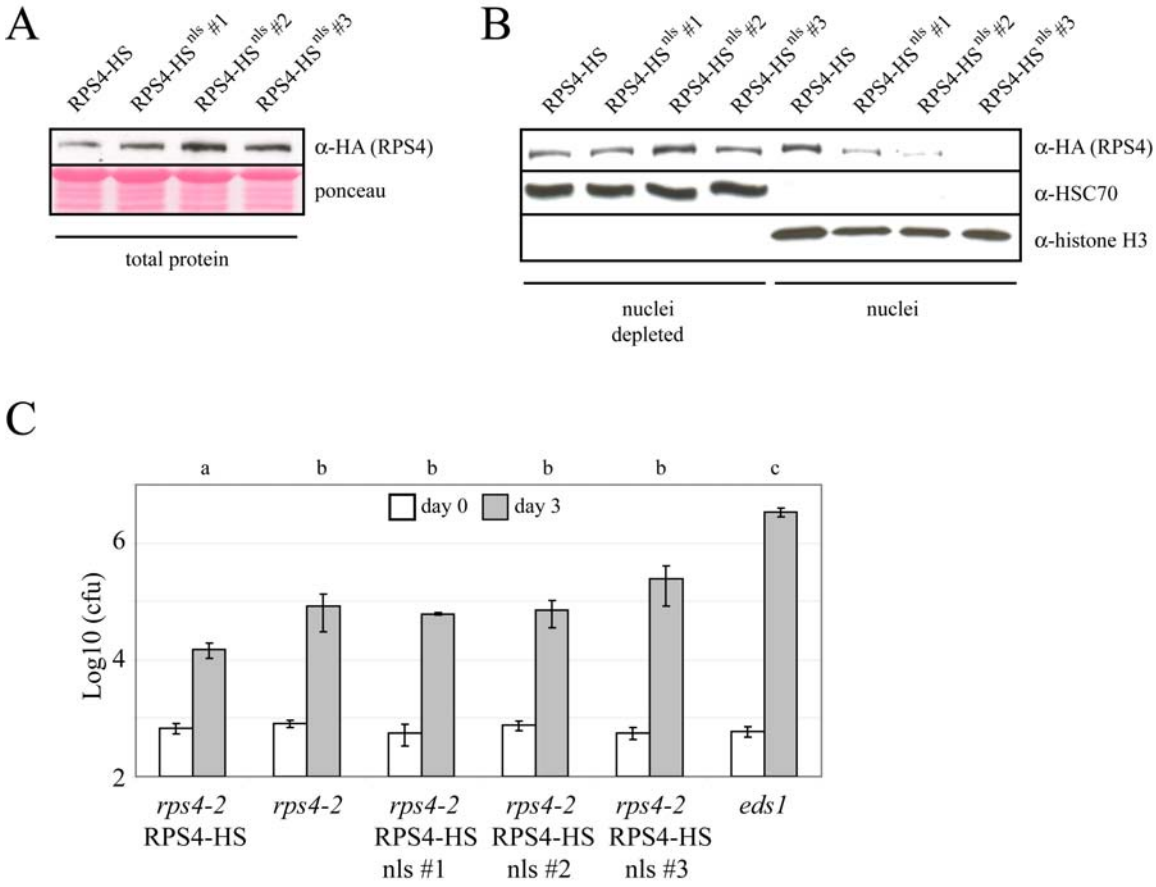

Fig. 3.7. A quadruple lysine to alanine exchange in the RPS4 NLS depletes the nuclear RPS4 pool and leads to loss of RPS4-mediated resistance towards *P. syringae* AvrRps4. Experiments (A)-(C) were repeated three times with consistent results.

(A) Total protein extracts were prepared from lines RPS4-HS #2 and three independent transgenic lines expressing RPS4^{nls}-HA-StrepII protein carrying the mutated NLS. Extracts were separated by SDS-PAGE and subjected to immunoblotting with α-HA antibody. The Ponceau S stain indicates equal protein transfer onto the membrane.

(B) Nuclear extracts were prepared from line RPS4-HS #2 and the three transgenic lines expressing RPS4^{nls}-HA-StrepII protein with the mutated NLS. Nuclei-depleted and nuclear fractions were separated by SDS-PAGE and analysed by immunoblotting using α-HA antibody. α-HSC70 and α-histone H3 antibodies served as cytoplasmic and nuclear markers, respectively.

(C) Five week old plants of the indicated genotypes expressing the RPS4 constructs given below were infiltrated with *P. syringae* AvrRps4 at 5x10^4 CFU/ml (2.2.5) and bacterial titres were determined 1 h (white bars) and 72 h (grey bars) post infiltration. Error bars represent the standard deviation of two (1h) and four (72h) repeated samplings of five individual plants. Characters a-c indicate significant differences in growth at 72h (t-test; α=0.05).

As shown in Fig. 3.7C, bacterial growth in all three lines was not significantly different from the growth in *rps4-2* plants (t-test, α=0.05). Thus there is a correlation between depletion of the nuclear RPS4 pool and loss of RPS4-mediated resistance suggesting that a functional NLS is required for RPS4-triggered defence. However, from these results it cannot be ruled out that amino acids K1172, K1173, K1184 or K1185 are necessary for other RPS4 sub-functions, such as AvrRps4 recognition or signalling (see below).

3.2.5 RPS4 nuclear localisation is required for AvrRps4-independent cell death induced by *RPS4* over-expression in tobacco

Since cell fractionation methods can produce artefacts I sought to corroborate my biochemical nuclear localisation experiments with a non-invasive imaging technique and to this end used a transient *Agrobacterium*/tobacco expression system to test the localisation and functionality of the YFP-tagged RPS4 constructs. Over-expression of *RPS4* in *Nicotiana tabacum* triggers an AvrRps4-independent cell death response that requires a functional NB domain P-loop motif necessary for efficient ATP binding (Tameling et al., 2002; Zhang et al., 2004; Takken et al., 2006). Furthermore, cell death induced by *RPS4* over-expression in *Nicotiana benthamiana* is dependent on *N. benthamiana EDS1*, *SGT1* and *HSP90* homologues and thus robustly mimics the genetic requirements of effector-induced resistance mediated by TIR-NB-LRR proteins (Zhang et al., 2004). Although expressed under the strong 35S promoter, only moderate fluorescence signals could be obtained with YFP-RPS4 protein expressed in *N. benthamiana,* probably due to activation of a cell death programme that was manifested by necrosis of the infiltrated area at 3-4 d post infiltration (results not shown) (Zhang et al., 2004). Consistent with this idea the necrosis was completely abolished in a *N. benthamiana* line stably silenced for *NbEDS1* (*hpEDS1*; kindly provided by S. Schornack and T. Lahaye, University of Halle, Germany) and YFP-RPS4 accumulated to detectable levels at 3 d after *Agrobacterium* infiltration (Fig. 3.8A).

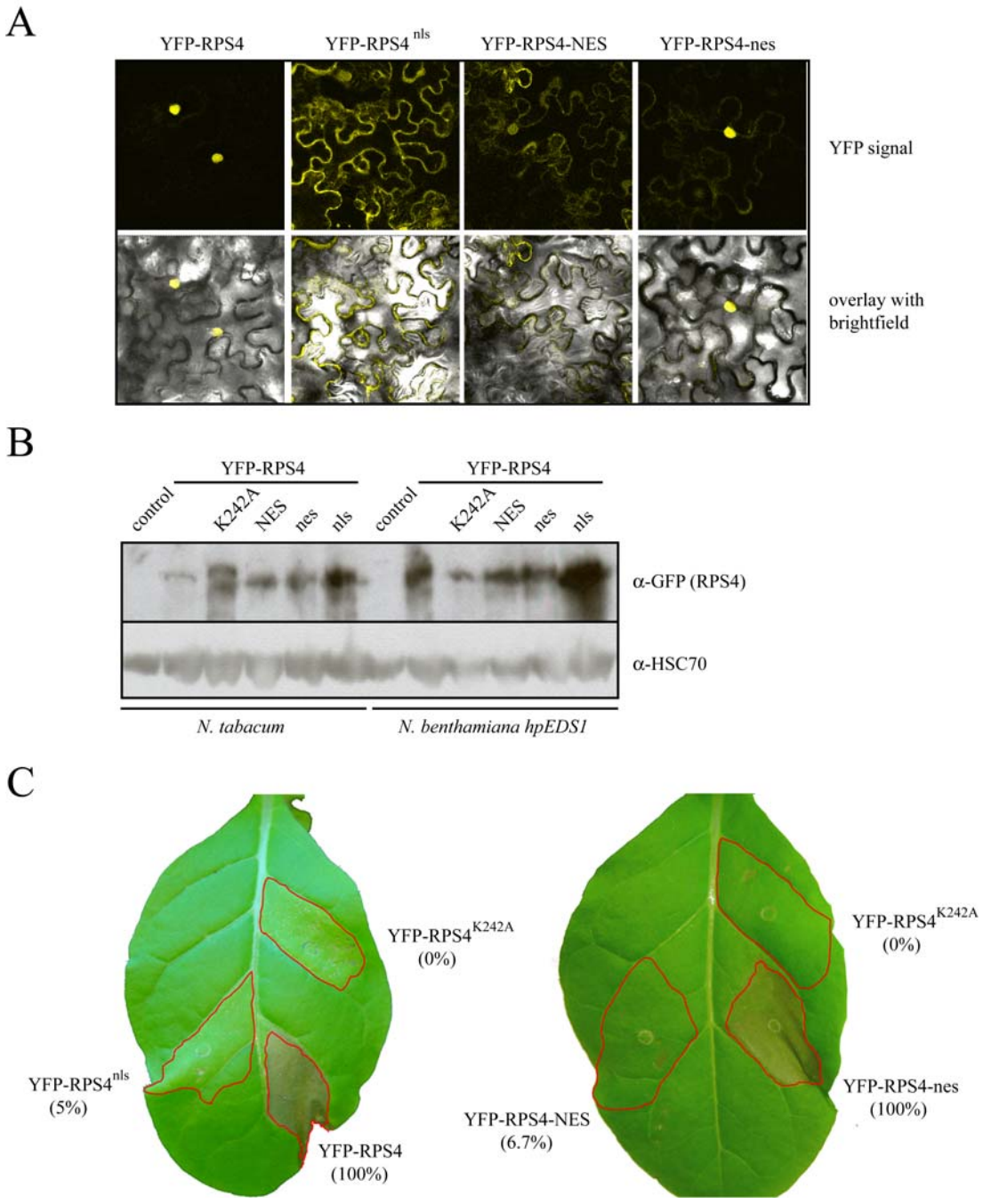

Fig. 3.8. RPS4 nuclear localisation is required for AvrRps4-independent cell death induced by *RPS4* over-expression in tobacco.

(A) Subcellular localisation of YFP-tagged RPS4 constructs over-expressed in a *N. benthamiana* line stably silenced for *NbEDS1*. Confocal images of representative cells were taken at 72 h after *Agrobacterium* infiltration. Cells were divided into z-stacks and the section with the strongest nuclear signal is shown. In case of the RPS4^{nls} mutant only weak nuclear YFP signals could be observed. The transient expression assay was repeated three times with consistent results.

(B) Immunoblot demonstrating that all YFP-RPS4 fusion proteins were expressed in *N. tabacum* and *N. benthamiana hpEDS1* at 40 h post *Agrobacterium* infiltration. RPS4 amino acid K242 located in the conserved P-loop motif is proposed to be required for efficient ATP binding. "NES" and

"nes" denote fusions of a nuclear export signal and a non-functional version of the same signal to the RPS4 C-terminus, respectively. "nls" refers to the RPS4 construct carrying the quadruple lysine to alanine exchange in the NLS. The α-HSC70 blot indicates the protein levels transferred onto the membrane. Plants over-expressing *GUS* were used as negative controls. This experiment was repeated two times with consistent results.

(C) HR eliciting phenotype of YFP-RPS4 fusion constructs transiently expressed in *N. tabacum*. *Agrobacteria* expressing the indicated constructs were syringe-infiltrated into the leaf areas surrounded in red and pictures were taken at 44 h post infiltration. The RPS4 K242A P-loop mutant served as a negative control (Zhang et al., 2004). The number of triggered HRs for each construct is given in percent of a total of 60 infiltrated leaves.

Over-expressed YFP-tagged RPS4 protein was detected mainly inside tobacco nuclei (Fig. 3.8A). The lack of a strong non-nuclear YFP-RPS4 signal in tobacco differed from my results obtained with cell fractionation in *Arabidopsis* that suggested that only a minor proportion of RPS4 was found in nuclei (Fig. 3.7A and B). However the karyopherin-mediated import process is dependent on both the affinity of the NLS-karyopherin interaction and the concentration of the cargo in the cytoplasm (Hodel et al., 2006; Timney et al., 2006) Thus, over-expression of YFP-RPS4 most likely leads to an increased import of the fusion protein into nuclei. Consistent with this assumption, the quadruple lysine to alanine exchange strongly diminished nuclear localisation of over-expressed YFP-RPS4^{nls} (Fig. 3.8A).

Since the mutations in the NLS might affect RPS4 functions other than its localisation I also fused a 20 amino acid nuclear export sequence (NES) and a mutated non-functional control sequence (nes), that lacks two leucine and one alanine residues required for export, to YFP-RPS4 (Wen et al., 1995; Shen et al., 2007). Fusion of the NES but not the mutated nes resulted in a partial relocalisation of YFP-RPS4 protein from nuclei to the exterior (Fig. 3.8A), although a clear YFP signal from the nucleus was retained. Scanning through z-stacks of nuclei in YFP-RPS4-NES expressing cells revealed that the fusion protein was still inside the nucleus rather than forming a rim around it (data not shown).

I concluded that the nuclear export rates mediated by the NES are below the import rates obtained with the endogenous RPS4 NLS. Since the HR induced by *RPS4* over-expression is much stronger in *N. tabacum* as opposed to *N. benthamiana* (Zhang et al., 2004), I tested the YFP-RPS4 constructs for functionality by transient expression in *N. tabacum*. I first confirmed that all fusion proteins were expressed by immunoblot analysis. As shown in Fig. 3.8B, all YFP-RPS4 fusion constructs could be detected at 40 h after *Agrobacterium* infiltration in *N. tabacum* and *N. benthamiana hpEDS1*. This time point was chosen because the HR in *N. tabacum* became visible from 44 h on (data not shown). The immunoblot in Fig. 3.8B further demonstrates that protein levels at 44 h post infiltration were

similar in both tobacco subspecies and that all proteins except the YFP-RPS4^{nls} mutant, which gave a stronger signal, accumulated to similar levels.

When tested for functionality (Fig. 3.8C), transient expression of YFP-RPS4 induced a strong HR in *N. tabacum* in 100% (60 out of 60) of the infiltrated leaf sections. In contrast, the K242A mutant lacking a functional NB domain P-loop motif failed to do so in all infiltrated leaves (0%) as it has been reported for HA-tagged RPS4 variants (Zhang et al., 2004). A strongly reduced ability to elicit the HR (6.7%; 4 out of 60 leaf sections) was observed with over-expressed YFP-RPS4-NES (Fig. 3.8C). In contrast, the non-functional YFP-RPS4-nes construct induced the HR as efficiently as YFP-RPS4. Thus, the reduced activity of the YFP-RPS4-NES fusion construct was not due to addition of 20 amino acids to the RPS4 C-terminus. As further shown in Fig. 3.8C, and in accordance with my *Arabidopsis* data, the quadruple lysine to alanine substitution (Fig. 3.5A) within the RPS4 NLS had a reduced HR efficiency (5%; 3 out of 60 leaf sections) in *N. tabacum*.

These results demonstrate that RPS4 nuclear localisation is required for AvrRps4-independent cell death induced by *RPS4* over-expression. Importantly, the YFP-RPS4-NES protein carries the endogenous RPS4 NLS but facilitating nuclear export from the nucleus by addition of the NES still interfered with its HR-inducing activity (Fig. 3.8C). Thus the loss of RPS4 function observed with the YFP-RPS4^{nls} construct is most likely due to depletion of the nuclear RPS4 pool and not the result of a general loss of receptor function caused by substitution of the four lysine residues. Also, the fact that both YFP-RPS4-NES and YFP-RPS4^{nls} were able to trigger the HR in rare cases (Fig. 3.8C) excludes a complete loss of RPS4 function. The occasional HR in these particular leaves could be explained by differences in expression levels or additional stresses imposed on single plants. The YFP-RPS4-NES construct was partially retained within the nucleus (Fig. 3.8A) and yet did not efficiently induce the HR in *N. tabacum* (Fig. 3.8B). This could mean that alterations of the RPS4 nuclear import/export rates already might be sufficient to abolish its function. However, since protein levels in *Agrobacterium*-mediated transient expression experiments usually increase from the second until the fifth day (Cazzonelli and Velten, 2006) there could have been less YFP-RPS4-NES protein at the 44 h time point and this might have been excluded more efficiently from nuclei.

The findings that RPS4 nuclear localisation is required for AvrRps4-independent cell death in tobacco (Fig. 3.8C) and that AvrRps4^{C} does not localise to the nucleus (Fig. 3.6B) strongly suggest that interference with RPS4 nuclear localisation in *Arabidopsis* blocks RPS4-

mediated defence downstream of AvrRps4 recognition. This further suggests that AvrRps4 recognition and RPS4 signalling could be spatially separated events in the infected host cell.

3.2.6 Neither EDS1 nor RAR1 are important assembly factors of RPS4

The absolute dependency of TIR-NB-LRR proteins on *EDS1* and the compromised RPS4-mediated resistance in *rar1* mutants (Muskett et al., 2002; Tornero et al., 2002b) prompted me to test RPS4 protein levels and localisation in *eds1* and *rar1* mutant backgrounds. To this end line RPS4-HS #2 was crossed to *rar1-28* and *eds1* null mutants (both in Col-0 genetic background). As shown in Fig. 3.9A, levels of RPS4-HA-StrepII protein were not altered in *eds1* or *rar1-28* mutants. Also, I did not observe consistent changes in endomembrane *vs.* nuclear partitioning of functional RPS4 protein in healthy leaf tissues (Fig. 3.9A). Thus, EDS1 protein is not required for RPS4 accumulation arguing against the idea that loss of TIR-NB-LRR receptor function in *eds1* mutants could be due to a role of EDS1 in stabilising these receptors. Also, in contrast to the *Arabidopsis* CC-NB-LRR receptors RPM1, RPS2 and RPS5 (Tornero et al., 2002b; Belkhadir et al., 2004b; Holt et al., 2005) RAR1 is dispensable for the accumulation of RPS4-HA-StrepII (Fig. 3.9A).

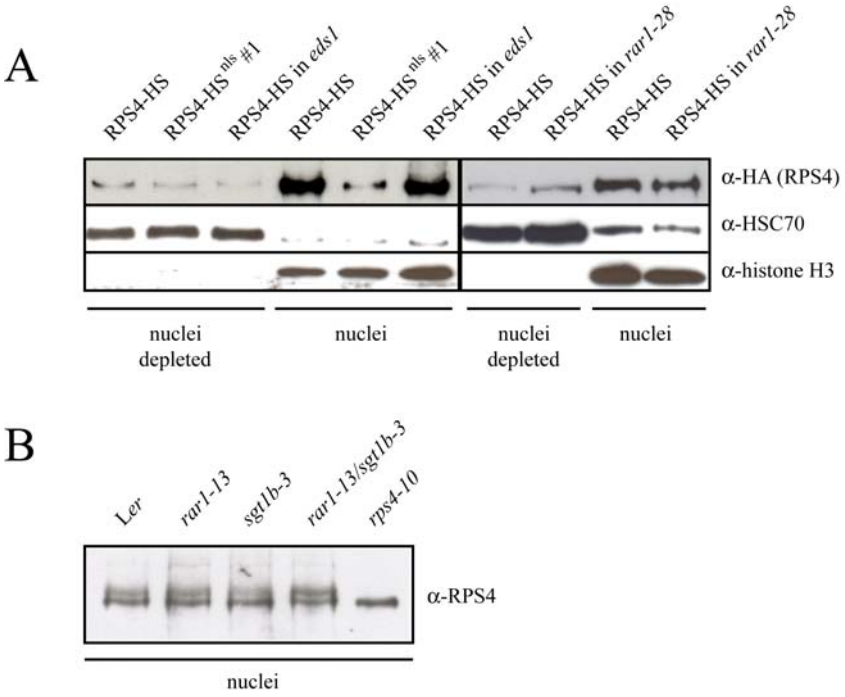

Fig. 3.9. RPS4 levels and partitioning of nuclear *vs.* endomembrane RPS4 protein are not affected in *eds1* and *rar1* mutants. Experiments (A) and (B) were repeated two times with consistent results.

(A) Nuclear extracts were prepared from line RPS4-HS #2 and the same line crossed into *eds1* and *rar1-28* mutant backgrounds, respectively. Nuclei-depleted and nuclear fractions were separated by SDS-PAGE and analysed by immunoblot using α-HA antibody. A transgenic line expressing RPS4-HA-StrepII with a non-functional NLS served as an internal control for depletion of the nuclear RPS4 pool (see Fig. 3.7B). α-HSC70 and α-histone H3 antibodies served as cytoplasmic and nuclear markers, respectively.

(B) Immunoblot of nuclear extracts prepared from L*er* wild type and *rps4-10, rar1-13, sgt1b-3* and *rar1-13/sgt1b3* mutants separated by SDS-PAGE and probed with α-RPS4 antiserum raised against the RPS4 NB domain. The upper band corresponds to full length RPS4 protein (predicted MW: 138 kDa).

Since RPS4-mediated resistance in L*er* shows a stronger genetic dependence on *RAR1* compared to Col-0 (compare Tornero et al. (2002b) and Muskett et al. (2002)), I tested whether endogenous nuclear RPS4 protein was depleted in *rar1-13* (L*er* background). As shown in Fig. 3.9B, nuclear RPS4 was stable in *rar1-13*. Since antagonistic roles of RAR1 and SGT1b in controlling NB-LRR immune receptor levels have been proposed (Holt et al., 2005), I also determined endogenous RPS4 levels in the *sgt1b-3* null mutant. However loss of SGT1b protein did not affect RPS4 levels consistently nor were RPS4 levels altered in the *rar1-13/sgt1b-3* double mutant (Fig. 3.9B). Thus, although functional RPS4 protein accumulates to wild type levels, RPS4 cannot signal in the absence of RAR1. This result suggests that RAR1 has additional functions apart from its stabilising effect on CC-NB-LRR

receptors in *Arabidopsis* immunity. In summary, I could demonstrate that loss of RPS4-mediated resistance in *eds1* and *rar1* mutants is not due to depleted RPS4 protein levels or depletion of the nuclear RPS4 pool prior to pathogen challenge.

3.2.7 Summary of subcellular localisation of endogenous and functional epitope-tagged RPS4 protein

Cell fractionation revealed that functional epitope-tagged RPS4 protein associates with endomembranes (Fig. 3.4A and C) but also nuclei (Fig. 3.5B and C). Membrane associated RPS4 protein was released in buffers of high ionic strength indicative of an ionic interaction with an endomembrane compartment (Fig. 3.4B). The presence of a nuclear RPS4 sub-pool could be substantiated by immunoblot analysis using an antiserum raised against endogenous RPS4 protein (Fig. 3.5C). These experiments further revealed that RLD RPS4 protein does not differ from functional Col-0 and L*er* RPS4 in terms of accumulation or nuclear localisation (Fig. 3.5D).

An over-expressed epitope-tagged form of the *P. syringae* effector AvrRps4 localises to the host cell cytoplasm and thus does not overlap in localisation with the subcellular compartments harbouring RPS4 protein. I could show that the putative NLS in the C-terminal extension domain of RPS4 functions as a NLS *in vivo* (Fig. 3.7B). Substitution of the four core lysine residues in the NLS by alanines diminished nuclear RPS4 levels and impaired RPS4-mediated resistance in *Arabidopsis* (Fig. 3.7C). When transiently expressed in *N. tabacum* the non-functional RPS4^{nls} also led to a strongly decreased efficiency (5% compared to 100% with the functional NLS) in eliciting AvrRps4-independent cell death (Fig. 3.8C). Facilitating nuclear export of RPS4 in the transient expression system resulted in a similar reduction in HR efficiency of 6.7% (Figure 3.8 C). The NES/nes strategy further corroborated that loss of RPS4^{nls} function is most likely due to depletion of the nuclear RPS4 pool and not the consequence of amino acid substitutions in the NLS. Finally, *Arabidopsis* RPS4 protein accumulation and localisation was not affected in *eds1*, *rar1* or *sgt1b* mutant backgrounds demonstrating that neither EDS1 protein nor the co-chaperone like RAR1 or SGT1b proteins are essential for RPS4 accumulation in unchallenged leaf tissues (Fig. 3.9).

4 Discussion

4.1 Susceptibility of *rps4* mutants to *P. syringae* AvrRps4 and positioning of *EDS1* in RPS4-mediated resistance

The TIR-NB-LRR group of R proteins confers race-specific resistance to diverse pathogens such as oomycetes, bacteria and viruses (Whitham et al., 1994; Botella et al., 1998; Gassmann et al., 1999). A striking feature of TIR-NB-LRR receptors in different species such as *Arabidopsis*, tobacco and tomato is their absolute dependence on *EDS1* to induce local and systemic resistance (Aarts et al., 1998; Feys et al., 2001; Rusterucci et al., 2001; Peart et al., 2002; Hu et al., 2005). In contrast, *EDS1* is dispensable for local but not systemic resistance mediated by CC-NB-LRR receptors. Neither the molecular function of EDS1 protein nor its precise position in TIR-NB-LRR receptor signalling were known when this work was initiated. Since RPS4 and AvrRps4 are one of a few cognate TIR-type receptor/effector pairs characterised (Hinsch and Staskawicz, 1996; Erickson et al., 1999; Rehmany et al., 2005) and RPS4 constitutes a classical *EDS1*-dependent TIR-NB-LRR protein (Gassmann et al., 1999), this system was suitable to search for a molecular connection between TIR-type NB-LRR receptors and the EDS1 signalling node. However, the precise localisation of intracellular TIR-NB-LRR receptors and their cognate effector proteins was unknown. The aim of this thesis was therefore to characterise RPS4, as a typical TIR-type NB-LRR protein, in terms of subcellular localisation, mechanisms of AvrRps4 recognition, RPS4 signalling and possible molecular connections to EDS1 or other proteins required for RPS4-mediated resistance. The results achieved in this study will be summarised, evaluated and discussed within the scope of the recent literature.

4.1.1 Susceptibility of *rps4* mutants to *P. syringae* AvrRps4

The *RPS4* locus was initially identified in a screen for *Arabidopsis* accessions that had lost the ability to respond with an HR to infiltration of a high dose of *P. syringae* pv. *pisi* expressing the type three effector AvrRps4 (Hinsch and Staskawicz, 1996). In contrast to the study by Hinsch and Staskawicz (1996) and later publications by Walter Gasman's group (Gassmann et al., 1999; Zhang and Gassmann, 2003), I observed clear recognition of AvrRps4 in RLD

evident from reduced growth of *P. syringae* AvrRps4 compared to growth of the isogenic virulent strain (Fig. 3.1A). This phenotype was consistent with bacterial strains and RLD seeds obtained from W. Gassmann (University of Missouri-Columbia, MO, USA) ruling out seed or bacterial strain contaminations. AvrRps4 recognition in RLD plants was also observed by Kee Hoon Sohn (J. Jones lab, Sainsbury laboratory, Norwich UK, personal communication) demonstrating that this phenotype is reproducible under different experimental conditions. Based on data obtained in this study, I propose that at least under my infection conditions RLD plants do recognise AvrRps4 implicating that accession RLD is not a natural *rps4* mutant.

This idea is consistent with previous findings showing that, i) the RLD *RPS4* allele is transcribed (Gassmann et al., 1999), ii) the RLD RPS4 protein possesses only two amino acids (D195 and H950) that are not shared with the functional Col-0 or L*er* RPS4 proteins and are not conserved in other TIR-NB-LRR receptors (Gassmann et al., 1999), and iii) nuclear RLD RPS4 protein accumulates to similar levels as L*er* and Col-0 RPS4 (Figure 3.5D). When tested for resistance to virulent *P. syringae* I found that RLD plants reproducibly allowed more growth of virulent bacteria than Col-0 plants (Fig. 3.1A) suggesting that genetic differences between the two accessions influence resistance to *P. syringae* under my infection conditions. This result is in contrast to publications from W. Gasman's group (Kwon et al., 2004) showing almost identical bacterial growth rates in Col-0 and RLD but is consistent with a report by Ton et al. (1999) demonstrating that RLD plants are impaired in induced systemic resistance and generally allow more growth of *P. syringae* than Col-0. Thus RLD resistance to virulent and avirulent *P. syringae* seems to vary with different experimental conditions. Nevertheless, my data strongly suggest that under conditions used in this study AvrRps4 is recognised in RLD and hence RLD is not a natural *rps4* mutant.

The two characterised Col-0 *rps4* T-DNA insertion lines *rps4-2* and *rps4-3* allowed 1-1.5 logs more growth of *P. syringae* AvrRps4 than wild type plants (Fig. 3.1C, left panel). This result demonstrates that Col-0 RPS4 recognises AvrRps4. The gene-for-gene hypothesis predicts that growth levels of *P. syringae* AvrRps4 in *rps4* mutants should be as high as the levels obtained with the isogenic virulent strain on Col-0, because AvrRps4 can no longer be recognised (Flor, 1971). Also, it is possible that AvrRps4 contributes to the virulence of *P. syringae* as has been shown for other bacterial effector proteins (Mudgett, 2005). In this case one would expect even higher multiplication levels of *P. syringae* AvrRps4 compared to the virulent strain in host plants that cannot recognise AvrRps4. However, when the growth of *P. syringae* AvrRps4 in *rps4-2* and *rps4-3* was compared to growth of the virulent strain in

Col-0 it became evident that both T-DNA insertion lines supported less growth of avirulent *P. syringae* (Fig. 3.1D, middle panel; data not shown for *rps4-3*). This difference was even more pronounced in the L*er rps4-10* gene trap insertion line that was not significantly more susceptible than L*er* wild type plants (Fig. 3.1D, right panel; t-test, α=0.05). Hence there is redundancy in AvrRps4 recognition in Col-0 and L*er rps4* mutants.

In view of this result one might question whether the three *rps4* insertion lines are null mutants. As shown in Fig. 3.1C, no *RPS4* transcript could be detected in any of the *rps4* mutants using primers flanking intron 4 which is located downstream of the T-DNA and Ds3-*GUS* element insertions in the region encoding the LRR domain (Gassmann et al., 1999). Similar results were obtained with a primer set flanking intron 2 which is located in the region encoding the NB-LRR transition (data not shown). Furthermore, nuclear RPS4 protein could not be detected in *rps4-2*, *rps4-3* or *rps4-10* (Fig. 3.5C; data not shown for *rps4-3*). Although *rps4-2* and *rps4-3* might still accumulate a truncated RPS4 protein consisting of the TIR domain and parts of the NB domain (to levels below the immunoblot detection limit), these hypothetical RPS4 variants would most likely be non-functional since the T-DNA insertion in both mutants disrupts the NB domain that is essential for NB-LRR receptor function (Dinesh-Kumar et al., 2000; Tao et al., 2000; Bendahmane et al., 2002; Tameling et al., 2002; Tornero et al., 2002a; Zhang et al., 2004). Moreover, in the case of the L*er* gene trap insertion line *rps4-10* the *RPS4* reading frame is disrupted after the second codon and yet *rps4-10* is as resistant as L*er* wild type plants (Fig. 3.1B and D right panel). In summary, these data show that all three *rps4* insertion lines are null mutants and thus AvrRps4 recognition is mediated by at least one other receptor than RPS4 in the Col-0 and L*er* accessions.

Recognition of AvrRps4 by more than one R protein constitutes an exception from the gene-for-gene hypothesis (Flor, 1971). Recently, Janjusevic et al. (2006) reported that site-directed mutants of the *P. syringae* effector AvrPtoB, that have lost ubiquitin E3-ligase activity, are recognised by a putative second R protein in *pto* mutant tomato plants. Thus redundant effector recognition appears to be an infrequent exception from the gene-for-gene paradigm.

4.1.2 Expression of *RPS4-HA-StrepII* complements the *rps4-2* mutant phenotype

In order to test whether the slightly increased susceptibility to *P. syringae* AvrRps4 (Fig. 3.1D, left panel) was due to loss of *RPS4* function I aimed to complement the *rps4-2* mutant by expression of epitope-tagged *RPS4* under its own promoter. The complementation test for

P. syringae growth in Fig. 3.2A demonstrates that expression of *RPS4-HA-StrepII* complemented the *rps4-2* mutant phenotype but did not lead to increased resistance towards virulent *P. syringae*. Thus the RPS4-HA-StrepII fusion construct robustly mimics the function of endogenous RPS4 protein. As shown in Fig. 3.2B, the RPS4-HA-StrepII fusion protein could be detected in total protein extracts on immunoblots using the α-HA antibody. Immunoblot analysis using the α-RPS4 antiserum confirmed that the selected transgenic line accumulated nuclear RPS4-HA-StrepII to levels not more than five times higher than Col-0 endogenous RPS4 protein (Fig. 3.5E) demonstrating that epitope-tagged RPS4 was not strongly over-expressed. Taken together, the *P. syringae* growth assays (Fig. 3.2A) and expression of the *RPS4-HA-StrepII* transgene at levels close to wild type *RPS4* in line RPS4-HS #2 (Fig. 3.5E) assure that biochemical analysis using this transgenic line most likely reflects the features of endogenous RPS4 protein.

4.1.3 *EDS1* has an intrinsic signalling function downstream of activated RPS4 protein

Attempts to over-express *R* genes often result in transgenic plants with stunted morphology (Stokes et al., 2002). These plants constitutively express central defence genes such as *PR1* and show heightened resistance to not only avirulent but also virulent pathogens, indicative of a constitutively activated defence system (Oldroyd and Staskawicz, 1998; Tang et al., 1999; Stokes et al., 2002; Weaver et al., 2006). This constitutive defence phenotype, also named "R protein overdose effect" (Tao et al., 2000), is thought to result either from the out-titration of negative regulators of R proteins (Belkhadir et al., 2004a; Zhang et al., 2004) or could be the result of the limited chaperoning capacity of the cell leading to a pool of improperly folded R protein, that is in a signalling active conformation (Takken et al., 2006).

In view of these published results the finding that *RPS4-HA-StrepII* over-expression lines in *rps4-2* mutant background were only rarely recovered is not surprising. Since the generation of constitutive *RPS4* over-expression lines was a valuable tool for the biochemical analysis of "activated" RPS4 protein, I characterised *35S-RPS4-HA-StrepII* lines in the TIR-NB-LRR signalling deficient *eds1* background (Fig. 3.3A). *RPS4-HA-StrepII* over-expressing lines in an *eds1* background showed no obvious developmental defects and were indistinguishable by size from Col-0 wild type and *eds1* plants (Fig. 3.3A). This finding suggests that the lethal phenotype observed in T_1 plants over-expressing the same construct in *rps4-2* background was due to the functional *EDS1* allele, consistent with *EDS1* being

essential for TIR-type NB-LRR receptor-mediated signalling (Aarts et al., 1998; Feys et al., 2001). Introduction of functional *EDS1* by crossing over-expression line RPS4-HS^{OE} #3 with Col-0 resulted in a F_2 population segregating for wild type-like individuals and plants that were smaller than Col-0 (Fig. 3.3C). Genotyping 20 individuals revealed an absolute correlation between *RPS4* over-expression in the *EDS1* background and reduced plant size. Furthermore, the dwarf phenotype was even more pronounced in the first double homozygous *RPS4^{OE}/EDS1* line selected (Fig. 3.3A). Conversely, 17 out of 20 plants classified as "wild type-like" either lacked the *RPS4^{OE}* construct or were homozygous for the *eds1* mutation. The remaining three plants carried the *RPS4^{OE}* construct and were heterozygous for *EDS1*. RPS4-HA-StrepII and EDS1 protein accumulation in these three lines was confirmed by immunoblot analysis and found not to be altered compared to the parental lines (data not shown). Thus the penetrance of the *RPS^{OE}/EDS1* genotype is lower than 100%.

However, the observed F_2 segregation pattern (26.41% dwarfed individuals) is inconsistent with the assumption that the sole presence of the *RPS^{OE}* construct is sufficient to induce stunting (expected: 75%; X^2=224, p=0.000) but fits to a scenario in which (*EDS1/eds1 RPS4^{OE}/RPS4^{OE}*) and (*EDS1/EDS1 RPS4^{OE}*/--) plants are stunted (expected: 31.25%; X^2=1.950, p=0.163; Table 3.1). Given the proposed lethal phenotype of *RPS4-HA-StrepII* over-expression in *rps4-2* background in T_1 plants, it was unexpected that double homozygous *EDS1/EDS1 RPS4^{OE}/RPS4^{OE}* F_3 plants were viable although much smaller in size. Since the *RPS4-HA-StrepII* expression levels in T_1 plants were not directly compared to those in the selected over-expression line RPS4-HS^{OE} #3 this difference might be due to lower transgene expression levels in RPS4-HS^{OE} #3. In summary, the segregation pattern in the F_2 population of the RPS4-HS^{OE} #3 x Col-0 cross showed a strong correlation between smaller plant size and the combined presence of the *RPS4^{OE}* construct and a functional *EDS1* allele. This finding confirms that the genetic requirement for *EDS1* in *RPS4*-mediated pathogen defence (Aarts et al., 1998; Feys et al., 2001) holds true for constitutively signalling over-expressed RPS4-HA-StrepII protein and is consistent with a recently published experiment with a truncated variant of the RPP1 TIR-NB-LRR receptor (Weaver et al., 2006).

Positioning EDS1 protein in TIR-type receptor mediated defence is complicated by its diverse functions in plant immunity and abiotic stress responses (Wiermer et al., 2005; Ochsenbein et al., 2006). A distinct role in signalling downstream of activated TIR-NB-LRR receptors but upstream of the HR has been proposed based on mutant studies (Rusterucci et al., 2001). Importantly, this proposed "early" function of EDS1 is specific to TIR-NB-LRR

receptors whereas a second function in amplification of immune receptor signals in combination with SA and PAD4 is common to R proteins from different structural classes and chemical inducers of SAR (Rusterucci et al., 2001; Mateo et al., 2004; Wiermer et al., 2005). From these experiments it cannot be ruled out that activated TIR-type NB-LRR receptors *per se* signal in an EDS1-independent manner but that these initial signals require further amplification by an EDS1-dependent mechanism to trigger defence gene expression. This idea is further substantiated by microarray transcript profiling of RPM1- and RPS4-triggered transcript changes showing that the CC-type NB-LRR receptor RPM1 induces stronger alterations in gene expression compared to RPS4 (Bartsch et al., 2006). Since *R* gene expression levels need to be tightly regulated in plants (Bieri et al., 2004) I reasoned that over-accumulation of RPS4-HA-StrepII protein should be sufficient to overcome a possible signal amplifying function of EDS1.

If this was the case *RPS4-HA-StrepII* over-expression in *eds1* background should induce SA-dependent defence markers and render line RPS4-HS^{OE} #3 more resistant to virulent and/or avirulent *P. syringae*. However as shown in Fig. 3.3D, RPS4-HS^{OE} #3 supported growth of *P. syringae* to the same extent as the *eds1* mutant demonstrating that no resistance could be induced in the absence of EDS1. Since it remains to be elucidated whether the "overdose" effect observed in *R* gene over-expression lines closely mimics pathogen effector-induced activation of R proteins, I also tested line RPS4-HS^{OE} #3 for resistance towards avirulent *P. syringae* AvrRps4. As shown in Fig. 3.3D, even in presence of the cognate *P. syringae* effector AvrRps4 and high levels of RPS4-HA-StrepII there was no increase in resistance in line RPS4-HS^{OE} #3 compared to *eds1*. By contrast, over-expression of *RPS4-HA-StrepII* in an *EDS1* background led to development of spontaneous lesions and constitutive PR1 expression, demonstrating that the levels of RPS4-HA-StrepII accumulation were sufficient to induce constitutive defence responses if relayed by EDS1 (Fig. 3.3).

These results demonstrate that even a strong TIR-NB-LRR trigger remains fully EDS1-dependent and thus EDS1 functions as an intrinsic signal transducer in RPS4-mediated resistance. This result supports the model proposed by Rusterucci et al. (2001), reinforcing the idea of a distinct EDS1 signalling function downstream of TIR-NB-LRR receptors that can be separated from its SA-dependent signal potentiating function in later stages of plant defence. Interestingly, microarray transcript profiling (Table 3.2 this study and Bartsch et al., 2006) revealed that the *eds1-1* mutation suppresses almost all early transcript changes that can be observed at the same time point in RPS4 signalling plants. Thus in terms of transcriptional

reprogramming, EDS1 constitutes a very early and crucial signal transduction node downstream of activated TIR-NB-LRR receptors. However, many rapid signal transduction cascades function on protein levels and are activated through protein-protein interactions, posttranslational modifications or changes in subcellular localisation that cannot be identified by transcriptional profiling (Kinkema et al., 2000; Hoecker, 2005; Chow and McCourt, 2006; Garcia-Brugger et al., 2006; Oldroyd and Downie, 2006). Therefore it will be interesting to elucidate how activated TIR-NB-LRR receptors induce transcriptional changes required for efficient activation of plant defences.

4.2 Subcellular localisation of endogenous and functional epitope-tagged RPS4 protein

Elucidating the subcellular localisation of NB-LRR receptors is crucial to understand mechanisms of effector recognition and downstream signalling. For example the *Arabidopsis* RIN4 protein that is a molecular target of at least three *P. syringae* effector proteins is a plasma membrane associated protein (Mackey et al., 2002; Mackey et al., 2003; Belkhadir et al., 2004b). Significantly, the *P. syringae* effectors AvrB, AvrRpm1 and AvrRpt2 co-localise to the plasma membrane (Nimchuk et al., 2000; Mackey et al., 2003) and so do the CC-NB-LRR receptors RPM1 and RPS2 that monitor effector-mediated RIN4 phosphorylation and proteolysis, respectively (Boyes et al., 1998; Axtell and Staskawicz, 2003; Mackey et al., 2003). Moreover, the GPI-anchored NDR1 protein that is required for resistance mediated by RPM1 and RPS2 is plasma membrane localised and interacts via its cytoplasmic N-terminal domain with RIN4 (Aarts et al., 1998; Day et al., 2006). Two other recent publications highlight the importance of combined function/localisation analysis of plant immune receptors. Shen et al. (2007) demonstrated that the CC-type NB-LRR receptor MLA10 requires nuclear localisation to confer race-specific resistance towards *Bgh*. Using a similar approach, Burch-Smith et al. (2007) reported that nuclear localisation of the tobacco TIR-NB-LRR receptor N, but not its cognate effector TMV p50 is necessary for N-mediated resistance towards TMV. In view of these recent findings it is crucial to precisely determine (or carefully re-evaluate) the subcellular localisation of *Arabidopsis* NB-LRR receptors in order to understand their function in plant immunity.

4.2.1 RPS4 protein associates with endocellular membranes

Using a crude total plant extract separation protocol based on incremental centrifugation forces, I detected functional epitope-tagged RPS4 protein in the 2000 x g, 5000 x g and 100.000 x g fractions (Fig. 3.4A). No RPS4-HA-StrepII protein was found in the supernatant of the 100.000 x g spin demonstrating that RPS4 is not a soluble R protein. This finding further implies that the major pools of RPS4 and EDS1 do not co-localise within a subcellular compartment arguing against a hypothetical stable RPS4/EDS1 protein complex in unchallenged plant tissue. The majority of epitope-tagged RPS4 was found in the 5000 and 100.000 x g fractions indicative of an association with membranes (Fig. 3.4A). However, RPS4-HA-StrepII partitioning was not identical to that of the plasma membrane marker BON1-3HA that was preferentially enriched in the 5000 x g pellet (Fig. 3.4A) and RPS4-HA-StrepII protein but not BON1-3HA was found in the 2000 x g pellet. Taken together this separation suggests that a sub-pool of RPS4-HA-StrepII is membrane-associated but that epitope-tagged RPS4 might localise to more than one subcellular compartment.

Since the RPS4 primary amino acid sequence does not possess putative transmembrane regions (Gassmann et al., 1999), I reasoned that RPS4 might associate with membranes either due to posttranslational modifications or indirectly via protein-protein interactions. Manual scanning of the RPS4 primary sequence together with subcellular prediction algorithms (Horton et al., 2006) revealed that RPS4 lacks strong myristoylation, palmitoylation or prenylation motifs. The results summarised in Fig. 3.4B and C demonstrate that RPS4 associates with endomembranes in a salt-sensitive manner. A similar charge-dependent association with membranes has been found for the barley CC-NB-LRR MLA1 receptor (Bieri et al., 2004). In addition, the MLA1 membrane association was fully reversible suggesting that the increase in soluble MLA1 protein at high salt concentrations is not due to disruption of organelles. Importantly, in my experiments the nuclear RPS4 pool was depleted from extracts at 2000 x g prior to the preparation of microsomes and thus the soluble RPS4 protein detected was not the result of nuclear envelope disruption. I concluded that functional RPS4-HA-StrepII protein associates with endocellular membranes possibly in an indirect way, e. g. through protein-protein interactions with integral or peripheral endomembrane proteins.

Recently, a myc-tagged version of the TIR-NB-LRR receptor RPP1 has been reported to localise to the endomembrane compartment (Weaver et al., 2006). In contrast to RPS4, the RPP1 protein carries an additional hydrophobic N-terminal domain and based on N-terminal deletion mutants Weaver et al. (2006) proposed that this domain is required for RPP1

membrane association. However, the interpretation of these results is complicated by strong differences in the accumulation of epitope-tagged RPP1 variants and the use of different promoters (Weaver et al., 2006). Also, a nuclear pool of RPP1 protein might have been missed by Weaver et. al due to early depletion of nuclei by the described extraction procedures. Taken together, the RPS4 localisation data obtained in this study and the analysis of functional RPP1 could suggest that the TIR domain itself is sufficient for association with endocellular membranes since it is the most striking difference to the plasma membrane-associated RPM1, RPS2 and RPS5 CC-type NB-LRR receptors (Boyes et al., 1998; Belkhadir et al., 2004b; Holt et al., 2005). However, functional epitope-tagged tobacco TIR-NB-LRR N protein was found to be completely soluble (Burch-Smith et al., 2007) arguing against the idea that the TIR domain alone mediates endomembrane association. Thus TIR-NB-LRR protein subcellular localisation might be determined by more subtle differences in the primary amino acid sequence, posttranslational modifications or differential associations with host proteins.

4.2.2 A pool of RPS4 protein localises to nuclei and nuclear localisation requires a functional NLS

Epitope-tagged RPS4 protein was not only present in the 5000 x g and 100.000 x g pellets as would be expected for a membrane associated protein, but was also detected in the 2000 x g fraction (Fig. 3.4A; compare to membrane marker BON1-3HA). The 2000 x g pellet contains cell walls, organelles and any material insoluble in the extraction buffer. As evident from the α-histone H3 immunoblot in Fig. 3.4A, nuclei were also pelleted at 2000 x g. This co-fractionation of RPS4 and nuclei was interesting since the WoLF PSORT algorithm (Horton et al., 2006) identified a putative bipartite NLS between RPS4 amino acids K1171 and R1187 (Fig. 3.5A). Although the WoLF PSORT prediction scans for a rather loose pattern ($B_2[N_{10}]B_{3/5}$; two basic amino acids (B), ten residue spacer, followed by a basic motif with at least three basic residues within five) exemplified by the *Xenopus* nucleoplasmin NLS (Robbins et al., 1991), protein blast analysis revealed that a similar NLS-like sequence is conserved in mammalian HMG-BOX transcription factors of the BBX class (data not shown) although the functional relevance of this sequence has not been demonstrated. When nuclear extracts were prepared from line RPS4-HS #2, epitope-tagged RPS4 protein could be detected in both the nuclear and the nuclei-depleted fraction (Fig. 3.5B). This finding is consistent with

the initial crude separation (Fig. 3.4A) and supports the idea that RPS4 amino acids K1171 to R1187 might constitute a NLS. Importantly, lysine to alanine substitutions of residues K1172, K1173, K1184, and K1185, which constitute the core basic regions in the putative NLS, diminished nuclear localisation of RPS4^{nls}-HA-StrepII in three independent transgenic lines (Fig. 3.7B) and in the *N. tabacum* transient expression system (Fig. 3.8B). These results demonstrate that RPS4 amino acids K1171 to K1187 contain a functional NLS and RPS4 protein is imported to nuclei in a NLS-dependent manner.

As shown by the immunoblot in Fig. 3.5B, nuclear extracts were not strongly contaminated with cytoplasmic, plasma membrane or endomembrane proteins (markers: HSC70 and EDS1 as predominantly cytoplasmic proteins; BON1-3HA for plasma membrane and GFP-HDEL for the endomembrane compartment; (Welch and Feramisco, 1982; Hua et al., 2001; Bae et al., 2003; Matsushima et al., 2003; Wiermer, 2005). Localisation artefacts due to the HA-StrepII epitope tag or expression levels that differed from endogenous RPS4 protein could be ruled out since i) endogenous RPS4 protein could be detected in Col-0 and L*er* nuclear extracts but not in the *rps4* T-DNA insertion lines *rps4-2* and *rps4-10* (Fig. 3.5C), and ii) RPS4-HA-StrepII protein in line RPS4-HS #2 accumulated to levels comparable to Col-0 endogenous RPS4 (Fig. 3.5E). Taken together these results demonstrate that a proportion of RPS4 protein distinct from the endomembrane pool is present in nuclei. Since the nuclear fraction in Fig. 3.5B is 16-fold over-represented and it was not clear whether proteins from the nucleoplasm leaked out during the extraction procedure, the actual ratio of nuclear *vs.* endomembrane RPS4 protein could not be determined. However the finding that exclusion of RPS4-HA-StrepII from nuclei did not result in a detectable increase in the non-nuclear pool (Fig. 3.7B) suggests that only a small amount of RPS4 protein is nuclear, at least in unchallenged leaf extracts. In contrast to previous results (Wiermer, 2005) I found only a weak signal for nuclear EDS1 protein. This could be due to different efficiencies in nuclear extractions or due to the fact that the nuclear fraction in previous immunoblots was even 50-fold over-represented (Wiermer, 2005).

Although the proportions of RPS4 and EDS1 proteins that localise to nuclei remain to be determined, it should be noted that both proteins co-localise to nuclei. Elucidating whether RPS4 and EDS1 interact directly or are part of a multi protein complex in the nucleus is not trivial, because initial experiments indicate that the robustness of the nuclear envelope complicates biochemical analysis and the protein levels required for fluorescence microscopy studies obviously trigger cell death due to elevated RPS4 protein expression levels (data not shown). I tested for direct interactions between EDS1 and different RPS4 domains (TIR, NB,

LRR-CT, TIR-NB) *in vitro* using proteins recombinantly expressed as soluble GST or His6 fusions in *E. coli*, but found no interaction. Also, negative results from yeast two hybrid experiments argue against a direct association of EDS1 and RPS4 (data not shown). I also reasoned that a possible EDS1/RPS4 interaction might be indirect. However, EDS1 could not be identified in immuno-purified RPS4-HA-StrepII extracts (data not shown). Finally I also addressed by FLIM (fluorescence lifetime imaging microscopy) analysis whether both proteins interact in tobacco nuclei when over-expressed in *N. benthamiana hpEDS1*, but could not find a significant reduction in lifetime of CFP-EDS1 in the presence of RPS4-YFP (data not shown). Thus although RPS4-triggered defence genetically requires *EDS1* (Fig. 3.3) there seems to be no direct or indirect stable interaction between the two proteins in unchallenged plant tissues.

4.2.3 Cytoplasmic localisation of the C-terminal cleavage fragment of *P. syringae* AvrRps4

For some R proteins such as three flax L TIR-NB-LRR variants, a direct interaction between the R protein and the cognate effector has been proposed, based mainly on yeast-two-hybrid analysis (Jia et al., 2000; Deslandes et al., 2003; Dodds et al., 2006). For other R protein/effector pairs the indirect recognition scenario predicted by the guard hypothesis (Van der Biezen and Jones, 1998b) could be experimentally verified (Mackey et al., 2002; Belkhadir et al., 2004b; Rooney et al., 2005; Mucyn et al., 2006; Ade et al., 2007). I investigated the subcellular localisation of AvrRps4-HA protein by Dex-inducible expression in *Arabidopsis* in order to test whether RPS4 and AvrRps4 co-localise. AvrRps4 is processed in *Arabidopsis* and the C-terminal cleavage product is required and sufficient for RPS4-mediated recognition (K. H. Sohn & J. Jones, unpublished data).

As shown in Fig. 3.6A, I found that Dex-inducible expression of *AvrRps4-HA* results in cell death that requires *EDS1*. The immunoblot in Fig. 3.6C demonstrates that the AvrRps4^{C} cleavage product is a soluble protein and thus localises to the host cell cytoplasm. As further shown in Fig. 3.6B, AvrRps4^{C} does not localise to nuclei. Given the low molecular weight of the C-terminal AvrRps4^{C} cleavage product (11 kDa), that is far below the size exclusion limit of the nuclear pore complex (~40 kDa) (Ribbeck and Gorlich, 2001), it was surprising that epitope-tagged AvrRps4^{C} is excluded from nuclei. Assuming a hypothetical virulence function of AvrRps4 it seems likely that AvrRps4^{C} associates with host proteins (Mudgett, 2005). If AvrRps4^{C} was part of a multi protein complex, the exclusion from nuclei

could be due to the total molecular weight of the complex. Consistent with this idea TAP-tagged AvrRps4^{C} migrates between 80 and 110 kDa in size exclusion chromatography experiments (J. Jones, unpublished data). Alternatively, exclusion of AvrRps4^{C} could be a consequence of its hydrophilic properties that could impede passive diffusion through the hydrophobic nuclear pore (Ribbeck and Gorlich, 2002). Whatever the molecular mechanism, these data suggest that in contrast to RPS4, AvrRps4^{C} is not actively imported to the nucleus. Thus the major pools of RPS4 and its cognate effector do not co-localise within a specific subcellular compartment. This in turn implicates an indirect mechanism of AvrRps4 recognition, an idea that is consistent with the finding that AvrRps4 associates with other host proteins (J. Jones, unpublished data), which could be potential targets of this bacterial effector.

The immunoblot in Fig. 3.6B also shows that expression of AvrRps4-HA does not result in gross changes of RPS4-HA-StrepII levels nor does it affect nuclear *vs*. endomembrane partitioning of epitope-tagged RPS4 at the 24 h time point. I also confirmed that pressure infiltration of *P. syringae* AvrRps4 at high titres does not affect RPS4 protein levels or localisation (results not shown). In contrast to my findings, the nuclear levels of the barley MLA10 CC-NB-LRR receptor increase upon infection with an avirulent *Bgh* isolate (Shen et al., 2007) although the relevance of this shift in localisation remains to be elucidated.

4.2.4 RPS4 nuclear localisation is required for activation of AvrRps4-triggered and effector-independent defence

The finding that RPS4 localises to nuclei (Fig. 3.5), but its cognate effector AvrRps4 is not found in this same compartment (Fig. 3.6B) prompted me to test whether the nuclear RPS4 pool has a biological function apart from AvrRps4 recognition. To this end I analysed resistance to *P. syringae* AvrRps4 in lines RPS4^{nls}-HS #1 - #3 that have depleted nuclear RPS4 levels (Fig. 3.7B). As shown in Fig. 3.7C, none of the three transgenic lines complemented the *rps4-2* phenotype. Hence there is a correlation between depletion of the nuclear RPS4 pool and loss of resistance to avirulent *P. syringae* AvrRps4. One might argue that the substitutions in the NLS could alter the biochemical properties of RPS4 and thus affect the resistance-inducing functions of RPS4. However, the results obtained with transiently expressed RPS4 variants in tobacco argue against this possibility, since nuclear depletion of the YFP-RPS4-NES construct that carries the non-mutated NLS also failed to induce the HR (Fig. 3.8C). By using a very similar but non-functional mutated nes sequence

(Wen et al., 1995; Shen et al., 2007), I could further confirm that loss of function observed with YFP-RPS4-NES was not due to addition of the C-terminal NES tag (Fig. 3.8C). However, the YFP-RPS4^{nls} and the YFP-RPS4-NES constructs retained a low HR efficiency (5% and 6.7%, respectively) demonstrating that in principle they are capable of triggering cell death. In summary the results obtained with both *Arabidopsis* and the finding that RPS4 nuclear localisation is required for the AvrRps4-independent HR in tobacco suggest that a nuclear RPS4 pool is necessary for its signalling function but not for recognition of AvrRps4. Thus effector recognition and RPS4 signalling are likely to be spatially separated events.

This result is reminiscent to analysis of the tobacco N TIR-NB-LRR receptor that recognises its cognate viral effector TMV p50 in the cytoplasm but requires translocation to the nucleus in order to induce anti-viral defences (Burch-Smith et al., 2007). It is not yet clear where the barley MLA10 protein, which also requires nuclear localisation in order to confer resistance to avirulent *B. graminis* isolates, recognises the AVR_{A10} effector and whether recognition is direct or indirect (Shen et al., 2007). Preliminary results indicate that MLA10 and AVR_{A10} do not form a stable complex in the nucleus (Q. Shen & P. Schulze-Lefert, unpublished) strengthening the idea that activated NB-LRR receptors might induce defence responses in the plant nucleus after encountering their cognate avirulence determinants in different cellular compartments. Since pathogen infection or expression of AvrRps4 did not affect nuclear RPS4 levels, I speculate that there might be constitutive shuttling of RPS4 between the nucleus and an endomembrane compartment that remains to be precisely defined. In this scenario nuclear import of activated RPS4 protein would be the trigger to induce defence gene expression.

4.2.5 Possible roles of EDS1 and RAR1 in RPS4-mediated resistance

As shown by the immunoblot in Fig. 3.9A, loss of EDS1 or RAR1 did not have obvious effects on RPS4 protein levels or nuclear trafficking, at least in unchallenged tissues. Thus a direct link between RPS4 accumulation or nuclear localisation and EDS1 or RAR1 cannot be established. For EDS1 this finding is consistent with a signalling function downstream of RPS4 (Fig. 3.3). Since loss of EDS1 blocks the vast majority of pathogen-induced transcript changes (Table 3.2), EDS1 could function as major transcriptional inducer of defence genes. This hypothesis is substantiated by preliminary results indicating that facilitating EDS1 nuclear export rates partially compromises *R* gene-mediated resistance (A. Garcia & J. Parker,

unpublished). Thus RPS4 and most likely its signal transducer EDS1 require nuclear localisation to induce resistance. These findings raise the question whether nuclear EDS1 and RPS4 proteins act synergistically to induce transcript changes or if *EDS1* itself might be a primary transcriptional target of activated RPS4 (Fig. 4.1). Other genes like *RAR1* and *PAD4* that affect RPS4-mediated resistance could also be part of this regulon. Fig. 4.1 shows a simplified hypothetical model of how activated RPS4 protein might induce defence gene expression in an EDS1-dependent manner.

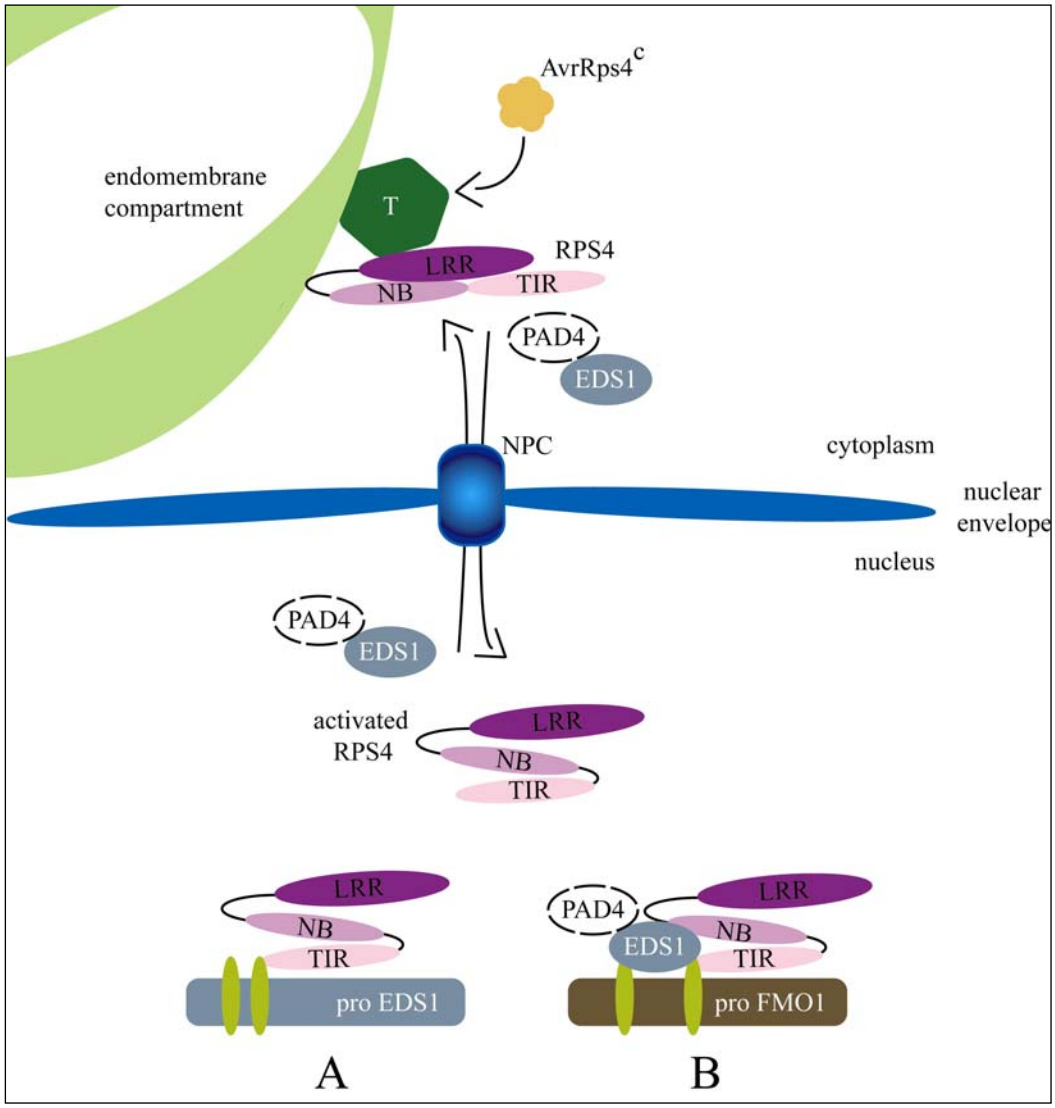

Fig. 4.1. Simplified, hypothetical model of EDS1-dependent RPS4 signal transduction based on data obtained in this study and transcript profiling microarrays (Bartsch et al., 2006). The processed form of the *P. syringae* effector AvrRps4 localises to the cytoplasm when over-expressed (Fig. 3.6). The model assumes that AvrRps4 modifies a putative host target (T) that associates with endocellular membranes and is guarded by RPS4. RPS4 senses the effector-induced modification of the host target (guard hypothesis). Karyopherin-mediated nuclear import of activated RPS4 activates defence gene expression in an EDS1-dependent manner (Figs. 3.3, 3.7 and 3.8). Two

possible scenarios for RPS4-mediated defence gene induction are shown. Model A predicts that RPS4, possibly via interaction with transcription factors (green), induces *EDS1* transcription. Elevated EDS1 protein levels might then, most likely in association with PAD4 and so far unidentified transcription factors, orchestrate transcriptional reprogramming of early defence genes. Model B assumes that activated RPS4 and EDS1 act synergistically to induce transcriptional reprogramming of early defence genes (*FMO1* shown as example). Full defence gene activation requires PAD4. Thus PAD4 and possibly SAG101 that form nuclear heterodimers with EDS1 might positively modify EDS1 function. Since DNA binding has not been demonstrated for RPS4, EDS1, PAD4 or SAG101 this process might involve yet unidentified positive or negative regulators of transcription (green). Note that this simplified model is partially based on transcript data and thus does not incorporate possible signal transduction events on protein levels upstream of transcriptional reprogramming.

AvrRps4^{C} modifies a host protein (hypothetical target, T) that resides in or is associated with an endomembrane compartment. Target modification results in activation of RPS4 that is shuttling between the nucleus and endocellular membranes. Nuclear import of activated RPS4 leads to defence gene activation in an EDS1-dependent manner. The RPS4 TIR domain can be considered as the "effector" domain since its over-expression is sufficient to induce AvrRps4-independent cell death in *N. tabacum* (Y. Zhang & J. Jones, unpublished and this study, data not shown).

Since EDS1 functions downstream of RPS4, activated nuclear RPS4 might boost *EDS1* transcription (Fig. 4.1A). Elevated EDS1 protein levels might then activate early defence genes such as *FMO1*. Consistent with this idea, *EDS1* is transcriptionally upregulated early in *R* gene-mediated resistance and EDS1 protein levels increase within 8-12 h after *P. syringae* AvrRps4 infection (Aarts et al., ; Feys et al., 2001). It is worth mentioning that *EDS1* was upregulated 15.9-fold at 6 h in the microarray transcript profiling experiment analysed in this study, although this value was below the applied threshold of 30 fold transcript change. In a not mutually exclusive model, activated RPS4 and steady state nuclear EDS1 proteins might act synergistically to induce transcription of early defence genes (Fig. 4.1B).

It is not known whether RPS4 or EDS1 bind to chromatin, but both proteins lack obvious DNA binding domains. Thus defence gene activation might involve other transcription factors (green in Fig. 4.1) that could function as positive or negative regulators of gene expression. Notably, these hypothetical factors have not been identified in forward genetic screens and thus might function redundantly or be essential for plant viability. Loss of PAD4 function partially suppresses RPS4-induced transcript changes (data not shown). Hence PAD4, in a partially redundant manner with SAG101, seems to be required for full defence

gene activation (Feys et al., 2005; Wiermer, 2005). Since PAD4 and SAG101 form nuclear heterodimers with EDS1, both proteins might regulate EDS1 activity.

To distinguish between models A and B in Fig 4.1 the *RPS4-HA-StrepII* over-expression construct will be crossed to a transgenic line harbouring a $_{Pro}$*EDS1-GUS* (*EDS1* promoter fused to a *GUS* reporter gene) construct (Wiermer, 2005). Analysis of the F_2 population will reveal whether activated RPS4 is able to induce *EDS1* transcription in the absence or presence of EDS1 protein. The model in Fig. 4.1 further predicts that activated RPS4 associates either directly or indirectly with promoter regions of genes upregulated early in defence towards *P. syringae,* a hypothesis that can be tested by chromatin immunoprecipitation experiments (ChIP). Notably, the simplified model presented in Fig. 4.1 is partially based on transcript data. Thus possible signal transduction events on protein level upstream of transcriptional reprogramming should be considered.

The finding that *rar1* does not affect pre-recognition RPS4 levels or RPS4 localisation is surprising since a stabilising function for RAR1 is well documented for *Arabidopsis* and barley CC-NB-LRR receptors (Tornero et al., 2002b; Belkhadir et al., 2004b; Bieri et al., 2004; Holt et al., 2005). It cannot not be ruled out that RPS4 protein in *rar1-28* is in a signalling incompetent fold but stable and thus not distinguishable from RPS4 in *RAR1* plants on immunoblots. However, the finding that *rar1* null mutants retain wild type RPS4 levels argues for additional roles of RAR1 in plant defence besides its proposed co-chaperoning function for CC-NB-LRR receptors (Schulze-Lefert, 2004a).

Consistent with this idea indications of additional RAR1 functions in basal defence have been reported. Holt et al. (2005) found that *rar1* mutants are moderately impaired in basal defence towards virulent *P. syringae* strains and *Hp* isolates. The authors argued that this phenotype was due to a concerted instability of numerous NB-LRR receptors activating weak resistance responses (Holt et al., 2005). However, it is difficult to experimentally corroborate this hypothesis and the results obtained in this study argue against the model proposed by Holt et al. Work by Shang et al. (2006) provides evidence for a negative regulatory role of RAR1 in PAMP-triggered immunity. Conversely, dwarfism, spontaneous cell death and callose deposition caused by loss of the *Arabidopsis* MAPKK MEKK1 are dependent on *RAR1* and SA (Ichimura et al., 2006). Interestingly, *rar1* mutants also have reduced steady state SA levels (J. Kaur & J. Parker, unpublished) and loss of RAR1 affects *EDS1* transcript levels, resulting in reduced EDS1 protein accumulation (S. Betsuyaku & J. Parker, unpublished). Thus there is good evidence for molecular RAR1 effects on PRR-mediated resistance, possibly in close connection to the EDS1/SA positive feedback loop. The

reported depletion of CC-NB-LRR receptors in *rar1* mutants could either be independent of RAR1 functions in PAMP-triggered immunity or could be a secondary effect of depleted SA levels – a hypothesis that remains to be tested. It will also be interesting to see whether the stabilising effect of RAR1 is specific to CC-type immune receptors or also applies to some TIR-NB-LRR proteins.

4.3 The nucleus in plant defence

Until recently a role for the nucleus in plant defence has mainly been drawn from the analysis of transcript changes accompanying the induction of local and systemic resistance (Maleck et al., 2000; Tao et al., 2000). Several structural classes of proteins that function in transcriptional reprogramming upon pathogen attack have been identified (Fan and Dong, 2002; Eulgem, 2005; Journot-Catalino et al., 2006; Kaminaka et al., 2006; Xu et al., 2006). Whereas the FLS2 PRR employs one or more MAPK cascades to transduce defence activating signals to the nucleus (Asai et al., 2002; Ichimura et al., 2006; Meszaros et al., 2006), a molecular link between intracellular R proteins and the transcriptional machinery has been sought after.

In this respect, the recently reported interaction between the MLA10 NB-LRR receptor and a WRKY transcription factor (Shen et al., 2007) supports the notion of a direct link between NB-LRR-type R proteins and the defence-associated transcriptional machinery (Dangl, 2007). The MLA10-WRKY interaction also revealed a molecular link between components of race-specific and PRR-mediated resistance, supporting the hypothesis that *R* gene-mediated resistance manifests an early and intense activation of general defence mechanisms (Tao et al., 2000). The characterisation of the RRS1 protein, a natural fusion between a TIR-NB-LRR receptor and a WRKY transcription factor (Deslandes et al., 2003), and the necessity for N protein nuclear localisation in TMV resistance (Burch-Smith et al., 2007) add further evidence for a conserved intimate molecular connection between the transcriptional machinery and activated NB-LRR receptors.

The results obtained in this study support and extend the recent research on NB-LRR receptor function in the nucleus. The identification of a functional NLS in RPS4 (Fig. 3.5, 3.7 and 3.8) and the presence of putative NLS in approximately two thirds of all *Arabidopsis* TIR-NB-LRR receptors (data not shown) suggests that several NB-LRR proteins might rely

on the karyopherin-mediated nuclear transport system. In contrast, MAL10 and N lack strong NLS, indicating that R proteins use different mechanisms to translocate to nuclei. Both strategies used in this study to deplete the nuclear RPS4 pool resulted only in a partial redistribution of RPS4 from the nucleus to exterior cell compartments (Fig. 3.7B and 3.8A). Thus it seems possible that already minor alterations of import or export rates affect RPS4 function, suggesting that constitutive bidirectional shuttling rather than the nuclear localisation *per se* is important for RPS4 function.

A suppressor screen using the constitutive TIR-NB-LRR signalling *snc1* mutant identified several components of karyopherin-mediated transport such as a putative nucleoporin 96 (Zhang and Li, 2005) and an importin-α homologue (Palma et al., 2005). The results strongly suggest that karyopherin-mediated shuttling is required for TIR-NB-LRR mediated resistance and PAMP-triggered immunity. It appears striking that several forward genetic screens for suppressors of TIR-NB-LRR signalling identified only a limited set of genes (Glazebrook, 2001). This phenomenon could be explained by a very direct signal transduction pathway of NB-LRR immune receptors. This emerging rapid signal transduction would make sense in terms of plant-pathogen co-evolution, since pathogen effectors are most likely able to target many of the conserved host signalling cascades such as (de-)phosphorylation (Bretz et al., 2003; Espinosa et al., 2003), SUMOylation (Hotson et al., 2003) or ubiquitination (Abramovitch et al., 2006). This might have created evolutionary pressure on host plants to evolve new signal transduction pathways that are able to rapidly activate adequate defence responses.

In contrast to plant NB-LRR receptors, the homologous CATERPILLAR class of mammalian immune receptors senses the presence of PAMPs or endogenous “danger” signals (Strober et al., 2006; Ting et al., 2006). In spite of this functional difference there are similarities in events leading to NB-LRR receptor activation in plants and animals. The LRR domain plays a crucial function in ligand sensing and negative regulation of receptor activity in the absence of PAMPs/effectors (Bertin et al., 1999; Bendahmane et al., 2002; Moffett et al., 2002; Girardin et al., 2003; Inohara et al., 2003; Zhang et al., 2003; Belkhadir et al., 2004a). NB-LRR receptor-mediated immunity in both lineages requires NTP binding and in some cases hydrolase activity (Harton et al., 1999; Tameling et al., 2002). As for plant NB-LRR R proteins, there is substantial evidence that activated CATERPILLARs form receptor oligomers which recruit downstream signal transducers. For example NOD1 and NOD2, intracellular sensors of bacterial peptidoglycan in epithelial and antigen presenting cells, associate via homotypic CARD-CARD domain interactions with the adapter protein RICK

(receptor-interacting Ser/Thr kinase) (Kobayashi et al., 2002). Activation of RICK results in ubiquitin-mediated degradation of IKKγ, the major inhibitor of the NF-κB pathway, leading to translocation of NF-κB transcription factors to the nucleus (Abbott et al., 2004; Zhou et al., 2004). Cryopyrin, another mammalian NB-LRR receptor sensing for danger signals associated with invasion of intracellular bacteria, associates via adapter proteins with procaspase-1 and promotes formation of the active caspase-1 complex (Kanneganti et al., 2006; Mariathasan et al., 2006). Although the localisation of cryopyrin is not known, processing of procaspase-1 has been proposed to occur in the nucleus (Mao et al., 1998).

Another well studied example of CATERPILLAR signal transduction is the master regulator of class II major histocompatibility complex (MHC) gene expression, CIITA. CIITA is essential for the transcription of *MHCI* and *II* genes and loss of function mutations result in deficiency of T-cell-mediated and humoral immune responses (Ting et al., 2006). CIITA binds GTP via its NB domain and GTP-binding is essential for nucleo-cytoplasmic CIITA trafficking (Harton et al., 1999; Linhoff et al., 2001). Nuclear CITTA functions as a co-activator of *MHC* gene transcription by undergoing direct protein-protein interactions with transcription factors and components of the polymerase II complex (Zhu et al., 2000; Ting et al., 2006).

Thus, while at least one CATERPILLAR protein functions on transcript level, others, like NOD1 and NOD2, activate conserved immune signalling cascades. Importantly, the precise subcellular localisation of most plant and animal NB-LRR proteins needs to be determined. Even more enigmatic remains in which subcellular compartments activated NB-LRR proteins initiate signalling. So far, the characterisation of a few plant and animal NB-LRR receptors suggests that there is no conserved downstream signal transduction pathway (Ausubel, 2005; Ting et al., 2006). However, the fact that both lineages employed the conserved STAND receptor family to evolve NB-LRR immune receptors (Leipe et al., 2004) suggests that mechanisms of receptor activation and early signal transduction events such as recruitment of adapter proteins might also be conserved.

4.4 Perspectives

The findings presented in this study together with recent publications from other groups (Burch-Smith et al., 2007; Shen et al., 2007) hint to a conserved signalling function of NB-LRR receptors in the nucleus. It is tempting to speculate that plant immune receptors, in concert with

transcription factors, directly impinge on defence gene expression. Re-evaluation of yeast two hybrid results that are available for many characterised NB-LRR receptors (Quirino et al., 2004; Al-Daoude et al., 2005) could lead to the identification of NB-LRR interacting transcription factors (Holt et al., 2002; Liu et al., 2004). ChIP experiments, in combination with whole genome microarrays (ChIP-ChIP) or massive parallel sequencing, should reveal whether NB-LRR proteins are part of multi-protein complexes that reside on promoter sequences of central plant defence genes. For these experiments *Arabidopsis* lines expressing functional HA-tagged RPS4 protein in *rps4*, *rps4/eds1*, *rps4/rar1* and *rps4/sgt1b* mutant backgrounds that have been generated within this study will be useful.

Furthermore, the Dex-inducible *AvrRps4* expression system allows simultaneous triggering of the RPS4 pathway, circumventing possible timing effects and the biological variations associated with pathogen infection assays. Thus, ChIP experiments could compare the untriggered and AvrRps4-triggered state of the RPS4 pathway. Analysis of microarray data already revealed that *eds1* blocks RPS4 signalling at a stage upstream of transcriptional reprogramming (Table 3.2). Further microarray profiling experiments comparing the consequences of *RPS4* over-expression and/or Dex-inducible expression of *AvrRps4* in *EDS1* and *eds1* backgrounds will shed light on early transcriptional changes upon RPS4 activation. More directly it will be tested by quantitative RT-PCR whether the *RPS4* over-expression in *EDS1* background leads to an upregulation of *EDS* transcript compared to Col-0. However this analysis might be complicated by the existence of the EDS1/SA positive feedback loop or effects of ROS on *EDS1* transcript levels (Feys et al., 2001; Rusterucci et al., 2001; Ochsenbein et al., 2006). Thus a more conclusive experiment would be to cross the *RPS4-HA-StrepII* over-expression line in *eds1* background to a transgenic line harbouring a $_{Pro}$*EDS1-GUS* fusion construct (in *EDS1* and *eds1* backgrounds) to test whether activated RPS4 protein impinges on *EDS1* transcription rates and, if true, whether this requires EDS1 protein.

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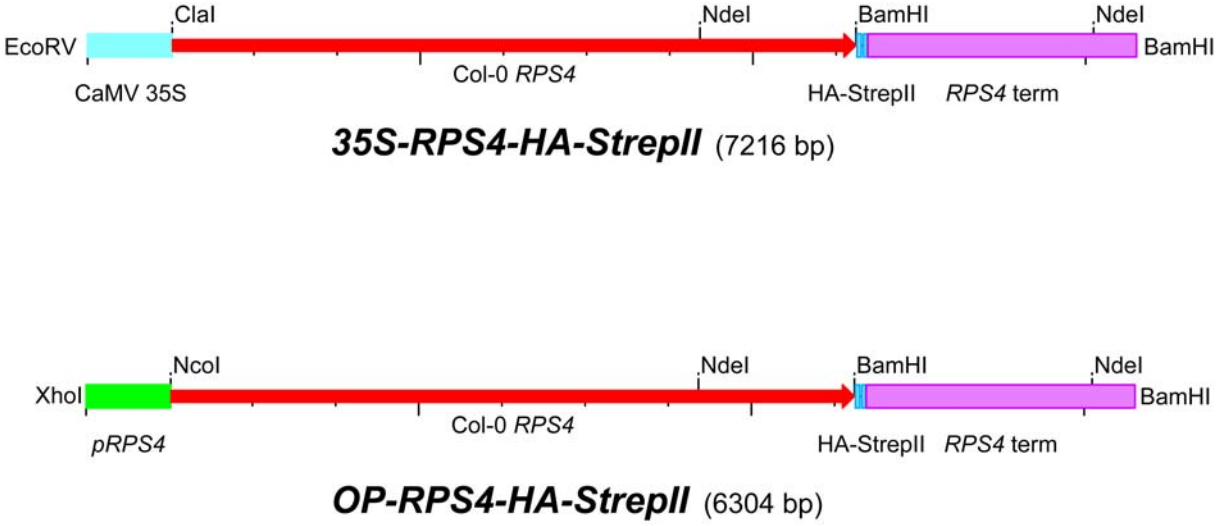
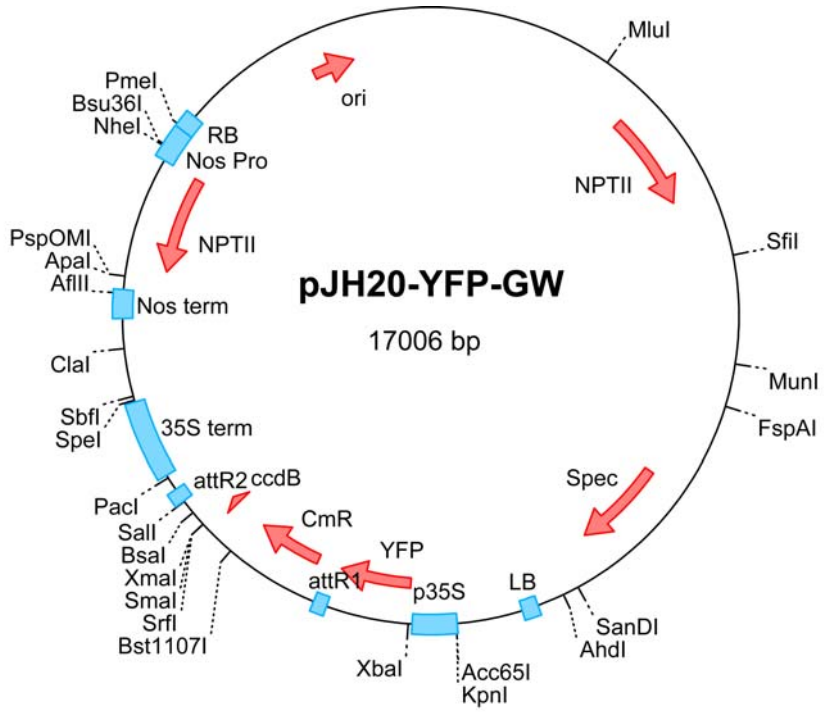


***RPS4-HA-StrepII* expression cassettes generated within this study in pGreenII0229 (Hellens et al., 2000). The upper map shows the *35S-RPS4-HA-StrepII* construct cloned via EcoRV and BamHI into the MCS of pGreenII0229. The lower map depicts the same construct under transcriptional control of 511 bp of *RPS4* 5' regulatory sequence ("own promoter", OP). The *OP-RPS4-HA-StrepII* construct was cloned into pGreenII0229 via XhoI and BamHI. Col-0 *RPS4*: genomic *RPS4* (At5g45250) without termination codon; HA-StrepII: region encoding C-terminal epitope tags followed by a stop codon; *RPS4* term: 1619 bp of *RPS4* 3' regulatory sequence.**



Plasmid map of the pJH20-YFP-GW over-expression vector generated within this study. This Gateway®-compatible vector was used for transient over-expression of YFP-tagged RPS4 variants in tobacco. Spec: Spectinomycin resistance; YFP: N-terminal yellow fluorescent protein tag; CmR: Chloramphenicol resistance; ccdB: negative selection marker; NPTII: Neomycinphosphotransferase; ori: origin of replication; LB: T-DNA left border; p35S: CaMV 35S promoter; attR1: attachment site R1; attR2: attachment site R2; 35S term: CaMV 35S terminator; Nos term: Noplinesynthase terminator; Nos Pro: Nopalinesynthase promoter; RB: T-DNA right border.

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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 den 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.

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