# Intracellular dynamics of *Arabidopsis* EDS1 immune regulatory complexes

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# ABBREVIATIONS

| | |
|---|---|
| - | fused to (in the context of gene/protein fusion constructs) |
| °C | degree Celsius |
| Avr | avirulence |
| BTH | benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester |
| CC | coiled-coiled |
| cDNA | complementary DNA |
| CFP | cyan fluorescent protein |
| cfu | colony forming unit |
| CLSM | confocal laser scanning microscopy |
| C-terminal | carboxy terminal |
| d | day(s) |
| DEX | dexamethasone |
| $dH_2O$ | deionised water |
| DMSO | dimethylsulfoxide |
| DNA | deoxyribonucleic acid |
| dpi | day(s) post inoculation |
| DTT | dithiothreitol |
| EDS1 | Enhanced Disease Susceptibility 1 |
| EDTA | ethylenediaminetetraacetic acid |
| EEE | excess excitation energy |
| ET | ethylene |
| ETI | effector-triggered immunity |
| ETS | effector-triggered susceptibility |
| FP | fluorescent protein |
| FRET | fluorescence resonance energy transfer |
| f. sp. | forma specialis |
| FW | fresh weight |
| g | gram(s) |
| gDNA | genomic DNA |
| GA | gibberellic acid |
| GFP | green fluorescent protein |

| | |
|---|---|
| GR | glucocorticoid receptor hormone binding domain |
| h | hour(s) |
| hpi | hour(s) post inoculation |
| HR | hypersensitive response |
| HRP | horseradish peroxidase |
| HSL | hormone sensitive lipase |
| JA | jasmonic acid |
| kb | kilobases(s) |
| kDa | kiloDalton(s) |
| l | litre(s) |
| log | decimal logarithm |
| LRR | leucine-rich repeat |
| m | milli |
| M | molar (mol/l) |
| µ | micro |
| MAMP | microbe-associated molecular patterns |
| MAPK | mitogen-activated protein kinase |
| min | minute(s) |
| mRNA | messenger RNA |
| MW | molecular weight |
| mYFP | monomeric YFP |
| *nahG* | bacterial SA-degrading enzyme salicylate hydroxylase |
| NB | nucleotide binding |
| NES | nuclear export signal |
| nes | non-functional nuclear export signal |
| NLR | NOD-like receptor |
| NLS | nuclear localization signal |
| nls | non-functional nuclear localization signal |
| NOD | nucleotide-binding oligomerization domain |
| NPC | nuclear pore complex |
| N-terminal | amino terminal |
| Nup | nucleoporin |
| OD | optical density |
| PAA | polyacrylamide |

| | |
|---|---|
| PAD4 | Phytoalexin Deficient 4 |
| PAGE | polyacrylamide gel-electrophoresis |
| PAMP | pathogen-associated molecular pattern |
| PCR | polymerase chain reaction |
| PEPC | phosphoenolpyruvate carboxylase |
| pH | negative decimal logarithm of the $H^+$ concentration |
| prom35S | 35S promoter of Cauliflower Mosaic Virus |
| PRR | PAMP/pattern recognition receptor |
| *Pst* | *Pseudomonas syringae* pv. *tomato* |
| PTI | PAMP-induced immunity |
| pv. | pathovar |
| R | resistance |
| RatA | Ratjadone A |
| RLK | receptor-like kinase |
| RNA | ribonucleic acid |
| ROS | reactive oxygen species |
| rpm | revolutions per minute |
| *RPM* | resistance to *Pseudomonas syringae* pv. *maculicola* |
| *RPP* | resistance to *Peronospora parasitica* |
| *RPS* | resistance to *Pseudomonas syringae* |
| TIR | *Drosophila* Toll and mammalian interleukin-1 receptor |
| SA | salicylic acid |
| SAG101 | Senescence Associated Gene 101 |
| SAR | systemic acquired resistance |
| sec | second(s) |
| SDS | sodium dodecyl sulfate |
| *SNC1* | *suppressor of npr1-1, constitutive 1* |
| TBS | Tris buffered saline |
| T-DNA | transfer DNA |
| TLR | Toll-like receptor |
| Tris | Tris-(hydroxymethyl)-aminomethane |
| UV | ultraviolet |
| V | Volt(s) |
| v/v | volume per volume |

| | |
|---|---|
| w/v | weight per volume |
| Y2H | yeast two hybrid |
| YFP | yellow fluorescent protein |

# SUMMARY

Plants are resistant to most microbial pathogens due to the presence of an effective and multi-layered defense system. *Arabidopsis* ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) and its interacting partners PHYTOALEXIN DEFICIENT 4 (PAD4) and SENESCENCE ASSOCIATED GENE 101 (SAG101) constitute an important regulatory node in disease resistance. Together they are essential for basal defense against invasive biotrophic and hemi-biotrophic pathogens, programmed cell death conditioned by TIR-type NB-LRR immune receptors and accumulation of the phenolic hormone salicylic acid (SA). It was previously shown that the central component EDS1 forms molecularly and spatially distinct complexes with PAD4 and SAG101 in the cytoplasm and the nucleus of healthy plants. The fate of these complexes during defense responses and the relevance of their distribution were not known. The work presented here shows that no major redistribution of EDS1 occurs at late stages of infection or in immune-deregulated backgrounds since its steady-state levels increase in both the nuclear and cytoplasmic compartments. By contrast, enrichment in nuclear EDS1 was observed during early stages of TIR-NB-LRR mediated responses, suggesting that the nuclear pool of EDS1 has a role in rapid signal relay to this compartment after specific pathogen recognition. Treatment of *Arabidopsis* protoplasts expressing EDS1-YFP with nuclear export inhibitors revealed that EDS1 is able to shuttle between the nucleus and the cytoplasm, further suggesting a role for EDS1 in relaying important information between both compartments. To gather information about the functional significance of EDS1 distribution and discriminate cytosolic from nuclear functions, I examined whether manipulation of EDS1 localization affects its functionality. Transgenic plants were generated in which a nuclear export signal (NES), the hormone binding domain of the rat glucocorticoid receptor (GR) or a nuclear localization signal (NLS) were attached to a functional EDS1-YFP fusion and expressed in an *eds1* null mutant background. EDS1 nuclear exclusion through the presence of the NES or cytosolic tethering by the GR led to reduced basal, TIR-NB-LRR-mediated and systemic resistance. Conversely, allowing nuclear accumulation of EDS1-YFP-GR fusion protein through dexamethasone treatment enhanced resistance. Results also pointed to a role of cytosolic EDS1 in modulating plant cell death that may relate to the balance of reactive oxygen species and SA. Strikingly, constitutive driving of EDS1 to the nucleus by addition of an NLS results in a dose-dependent developmental reprogramming that likely reflects the need for rigorous control of nuclear EDS1 levels. In sum, these studies point to a key nuclear activity of EDS1 complexes in regulating plant immune responses that may link immune receptor activation to gene expression outputs.

# ZUSAMMENFASSUNG

Pflanzen sind durch ein effektives und mehrschichtiges Abwehrsystem gegenüber den meisten mikrobiellen Pathogenen resistent. In *Arabidopsis* stellen ENHANCED DISEASE SUSCEPTIBILITY (EDS1) und seine Interaktoren PHYTOALEXIN DEFICIENT 4 (PAD4) and SENESCENCE ASSOCIATED GENE 101 (SAG101) einen wichtigen regulatorischen Knotenpunkt in der Pathogenabwehr dar. Zusammen sind sie essentiell für die basale Abwehr von invasiven biotrophen und hemibiotrophen Pathogenen, für TIR-type NB-LRR Immunrezeptor-abhängigen programmierten Zelltod und für die Akkumulation des phenolischen Pflanzenhormons Salicylsäure (SA). Dieser Arbeit vorhergehend wurde gezeigt, dass die zentrale Komponente EDS1 molekulare und räumlich getrennte Komplexe mit PAD4 und SAG101 im Zytoplasma und dem Zellkern gesunder Pflanzen bildet. Das Schicksal dieser Komplexe während der Abwehrantwort und die Bedeutung ihrer zellulären Verteilung waren bisher nicht bekannt. Die hier präsentierte Arbeit zeigt, dass keine bedeutende Umverteilung von EDS1 während der späten Phase der Infektion oder in immunologisch deregulierten genetischen Hintergründen erfolgt, da die steady-state Mengen im Nukleus und im Zytoplasma ansteigen. Im Gegensatz dazu wurde eine Anreicherung von nukleären EDS1 während der frühen Phase der TIR-NB-LRR-vermittelten Antwort beobachtet, was auf eine Rolle des nukleären EDS1-Pools im schnellen Signaltransfer zu diesem Zellkompartment nach spezifischer Pathogenerkennung hindeutet. Behandlung von EDS1-YFP expremierenden Arabidopsis-Protoplasten mit nukleären Exportinhibitoren zeigte, dass EDS1 zwischen dem Kern und dem Zytoplasma pendelt, was weiterhin auf eine Rolle von EDS1 in der Weiterleitung von wichtigen Informationen zwischen beiden Zellkompartimenten hinweist. Um Informationen über die funktionelle Bedeutung der EDS1-Verteilung zu erhalten und um zytosolische von nukleären Funktionen zu unterscheiden, untersuchte ich, ob die Manipulation der EDS1-Lokalisation die Funktionalität beeinflusst. Transgene Pflanzen wurden generiert, in denen ein nukleäres Exportsignal (NES), eine hormonbindende Domäne des Glucocorticoid Rezeptors (GS) von Ratte, oder ein nukleäres Lokalisationssignal (NLS) an eine funktionelle EDS1-YFP Fusion angefügt waren und in einem *eds1* Nullmutantenhintergrund expremiert wurden. Nukleärer EDS1 Ausschluss durch die Anwesenheit von NES, oder über GR zytosolisch fixiertes EDS1, führte zu reduzierter basaler, TIR-NB-LRR-vermittelter und systemischer Resistenz. Umgekehrt erlaubte die durch Dexamethasonbehandlung vermittelte nukleäre Akkumulation von EDS1-YPF-GR

Fusionsproteinen eine verstärkte Resistenz. Weitere Ergebnisse wiesen auf eine Rolle von zytosolischem EDS1 in der Modulierung von Pflanzenzelltod hin, welcher sich möglicherweise dem Verhältnis von reaktiven Sauerstoffspezies zu SA zuordnen lässt. Interessanterweise resultierte die konstante Zuführung von EDS1 in den Nukleus durch die Anfügung eines NLS in einer dosisabhängigen Umprogrammierung der Entwicklung, was wahrscheinlich den Bedarf einer strengen Kontrolle von nukleären EDS1-Leveln widerspiegelt. Insgesamt weisen diese Studien auf eine nukleäre Schlüsselrolle von EDS1-Komplexen in der Regulierung von pflanzlichen Immunantworten hin, indem sie die Aktivierung von Immunrezeptoren mit dem Output der Genexpression verbinden.

# 1 INTRODUCTION

Plants are continuously exposed to above- and below-ground attack by a diverse range of potential pathogens that have different life styles and infection strategies. Bacterial pathogens, for example, rely on natural openings, e.g. stomata and hydathodes, or wounds for entry into plant tissues. For successful infection, bacteria need to proliferate in the apoplastic space and deploy means to gain access to the host cell and nutrients. Some fungi and oomycetes form feeding structures (haustoria) that are still surrounded by an intact host cell plasma membrane and extend hyphae on top of, between or through plant cells. However, disease is the exception and not the rule due to the presence of a highly effective, multi-layered plant immune system (Jones and Dangl, 2006). Plants can also benefit from being host to certain microorganisms, for example in symbiotic relationships with rhizobia or mycorrhiza. Therefore, it is essential that in addition to preexisting physical and chemical barriers, plants express efficient recognition systems to monitor the environment and mount appropriate inducible immune responses.

## 1.1 The plant immune system

Unlike mammals, plants lack mobile defender cells and an adaptive immune system, and therefore must rely on the germ line-encoded capacity for innate immunity of each cell and generation of mobile signals that travel from the infection site to systemic tissues to combat pathogens. The first line of active defense is generally exhibited by an entire plant species to all variants of a non-adapted pathogen species and is commonly referred to as ‘non-host’ resistance (Nürnberger and Lipka, 2005). The infrequent host range shifts reflect that non-host resistance is usually robust and durable. This is believed to be the consequence of the activities of several successive protective layers. The first layer of inducible resistance is mediated by plasma membrane resident-pattern recognition receptors (PRRs) that recognize highly conserved pathogen- or, more accurately, microbe-associated molecular patterns (PAMPS or MAMPS). So far characterized PRRs in plants belong to the family of receptor-like kinases (RLKs), such as the leucine-rich repeat (LRR)-RLKs FLS2 that intercepts bacterial flagellin (Gomez-Gomez and Boller, 2000) and EFR that recognizes bacterial EF-Tu (Zipfel et al.,

2006), or the LysM-RLK CERK1 receptor for fungal chitin oligosaccharides (Miya et al., 2007). PAMP recognition triggers so-called PAMP-triggered immunity (or PTI) which typically involves ion fluxes, the activity of MAPKs (mitogen activated protein kinases), and production of ROS (reactive oxygen species) and the hormone ethylene (ET), among other responses (Schwessinger and Zipfel, 2008). PTI is usually very efficient in halting pathogen growth without macroscopic disease symptoms. Successful pathogens are equipped with mechanisms to evade early detection or actively suppress recognition of their PAMPs, thereby making host colonization possible. These adapted pathogens are able to deliver effector molecules inside the host cell that interfere with PTI, at the level of perception, signaling, or defense action, resulting in effector-triggered susceptibility (ETS). In these cases the plant is said to fall within the 'host' range of the pathogen, the pathogen is denoted virulent, and the interaction is classified as 'compatible' (Chisholm et al., 2006). Plants protect themselves from such virulent pathogens by exerting so-called 'basal resistance' that helps them to contain the infection. Basal resistance could be understood as the remaining level of PTI after suppression by effector molecules (PTI minus ETS) (Jones and Dangl, 2006). Mutants compromised in this crucial protective layer become hypersusceptible to normally virulent pathogens, a phenotype often described as 'enhanced disease susceptibility' (Glazebrook et al., 1996).

A further layer of the plant immune system acts largely inside the cell and is mediated by cultivar-specific resistance (*R*) genes. *R* genes encode receptors specific for pathogen effector molecules which are in this context called 'avirulence' factors (gene-for-gene hypothesis (Flor, 1971)). This layer of immunity is known as R-mediated resistance or effector-triggered immunity (ETI) and leads to an 'incompatible interaction' between the host and the 'avirulent' pathogen. R proteins can recognize their cognate effectors directly or intercept their actions by monitoring their host cellular targets (Dangl and Jones, 2001). The latter scenario of R-mediated recognition is elaborated in the 'guard hypothesis' which is conceptually similar to the models proposed for the perception of 'modified self' by recognition of 'damage-associated molecular patterns' (DAMPS) or danger signals in the mammalian immune system (Matzinger, 2007). In plants, *R*-gene protein products mostly belong to a class of intracellular NB-LRR proteins, named after their characteristic central nucleotide binding (NB) and C-terminal leucine rich repeat (LRR) domains. NB-LRR receptors are subdivided based on their N-terminal domains: some possess a TIR (Toll/Interleukin-1 Receptor) domain with homology to the intracellular signaling domains of

Drosophila Toll and mammalian Interleukin-1 receptors (TIR-NB-LRRs) and others contain a coiled-coiled (CC) domain (CC-NB-LRRs) (Dangl and Jones, 2001). R protein structure determines different genetic requirement for downstream signaling components: TIR-NB-LRR type show greater dependence on *EDS1* (*ENHANCED DISEASE SUSCEPTIBILITY1*) while CC-NB-LRR type generally require *NDR1* (*NONRACE-SPECIFIC DISEASE RESISTANCE1*) (Century et al., 1995; Parker et al., 1996; Aarts et al., 1998). R-mediated recognition results in rapid activation of defense responses that arrests pathogen spread. This type of resistance is typically accompanied by a localized oxidative burst, the development of hypersensitive response (HR)-cell death at the site of attempted pathogen penetration, alterations in hormone levels and activation of salicylic acid (SA)-dependent signaling pathway (Dangl and Jones, 2001).

Accumulation of the plant phenolic hormone salicylic acid (SA) is a key component of the activation of plant disease resistance (Loake and Grant, 2007). It was early noticed that exogenous application of SA or aspirin induce local and systemic disease resistance against different pathogen species in plants (White, 1979; Malamy et al., 1990; Metraux et al., 1990). The central role of SA in defense against pathogens was further supported by the observation that transgenic plants expressing the bacterial SA-degrading enzyme salicylate hydroxylase (*nahG*) or mutant plants impaired in SA accumulation became hypersusceptible to pathogen infection (Gaffney et al., 1993; Hunt et al., 1997; Wildermuth et al., 2001; Nawrath et al., 2002). The majority of pathogen-induced SA is synthesized via ISOCHORISMATE SYNTHASE (ICS) most likely in the chloroplasts (Wildermuth et al., 2001) and is subsequently modified *in planta* by glucosylation and/or methylation (Seskar et al., 1998; Dean et al., 2005; Park et al., 2007). Recent studies suggest that SA modification to an amino acid conjugate may also be a critical step in SA metabolism and have a role in disease resistance (Staswick et al., 2002; Zhang et al., 2007). SA-dependent defense signaling is important for the onset of a long-lasting and broad-spectrum disease resistance in uninfected tissues referred to as ‘systemic acquired resistance’ (SAR), probably through the generation of a mobile signal able to trigger defense in systemic tissues (Vlot et al., 2008).

NB-LRR mediated resistance is effective against pathogens that grow on living host cells at least partly during their life cycles (biotrophs or hemi-biotrophs) but generally not against pathogens that kill host cells to colonize tissues (necrotrophs). Resistance to necrotrophic pathogens is dependent on pathways activated by the hormones jasmonic acid

(JA) and ET which can interact extensively with SA-activated responses, often in an antagonistic manner (Glazebrook, 2005). Mutational and transcriptional analyses in *Arabidopsis* have revealed extensive overlap in the genetic requirements of the different defense mechanisms (Tao et al., 2003). Furthermore, there is evidence suggesting that weak effector-triggered immunity also underlies non-host resistance (Jones and Dangl, 2006).

Animals detect microbial agents through recognition of their PAMPs or MAMPs by innate immune receptors, also known as PRRs (Ishii et al., 2008). The best characterized examples are the Toll-like receptors (TLRs) of insects and mammals which are transmembrane proteins containing a LRR ectodomain and a cytoplasmic TIR domain. Mammalian TLR gene family consists of at least 13 members and the encoded proteins recognize microbial signatures as well as damaged host cell components (Kawai and Akira, 2007). TLR ligand recognition can take place either at the cell surface, such as TLR4 recognition of lipopolysaccharides and TLR5 recognition of flagellin, or within vesicular compartments, such as TLR9 recognition of bacterial CpG DNA motifs in endolysosomes (Poltorak et al., 1998; Hemmi et al., 2000; Hayashi et al., 2001; Latz et al., 2004; Nishiya and DeFranco, 2004; Chockalingam et al., 2008). Upon activation, TLRs trigger downstream signaling by recruiting and interacting with other TIR-containing adaptors such as MyD88 (Kawai and Akira, 2007). Another major group of animal innate immune sensors contains intracellular NOD-like receptors (NLRs) characterized by a central nucleotide-binding oligomerization domain (NOD), a C-terminal ligand-recognition domain and a variable N-terminal signaling domain. The modular structure of NLRs is related to plant NB-LRR proteins and the high level of conservation of these associated modules can be seen even in phylogenetically primitive organisms (Chen et al., 2008). Although TLRs and NLRs have different subcellular localizations and engage different signaling pathways, they share some of their ligands and interact at the level of signaling cascades, thereby enabling synergistic activation of innate immune responses (Ishii et al., 2008). Some of the responses triggered by TLRs and NLRs are the activation of cell death programs and of signaling cascades involving MAPKs and the transcription factor Nuclear Factor-κ B (NFκB) that result in defense gene induction. Despite the similarities among the components of plant and animal innate immune systems, current evidence suggests that these pathogen recognition systems arose through convergent evolution rather than common phylogeny (Ausubel, 2005). It is likely that the protein domains involved have been recruited

independently in different evolutionary lineages due to their biochemical properties that make them especially well suited for these pathways.

## 1.2 The plant EDS1 regulatory node

*Arabidopsis* EDS1 (ENHANCED DISEASE SUSCEPTIBILITY1), together with its interacting partners PAD4 and SAG101, plays a central role in plant disease resistance which seems to be conserved among flowering plants (Liu et al., 2002; Peart et al., 2002; Hu et al., 2005; Wiermer et al., 2005; Chong et al., 2008). *EDS1* was originally identified in a screen for *Arabidopsis* mutants defective in *R*-gene mediated resistance to the obligate biotrophic oomycete pathogen Hyaloperonospora parasitica (Parker et al., 1996) and was found to be a necessary component of resistance triggered by TIR-NB-LRR *R* genes (Aarts et al., 1998). Also, *eds1* mutants are hypersusceptible to virulent pathogens, in the absence of obvious specific recognition, indicating a role for EDS1 in basal defense (Parker et al., 1996). *Pad4* (PHYTOALEXIN DEFICIENT 4) was similarly identified as an *Arabidopsis* mutant with enhanced disease susceptibility to a virulent isolate of the bacteria *Pseudomonas syringae* (Glazebrook et al., 1996). EDS1 and PAD4 proteins interact in plant tissues and, consistent with a cooperative function in defense signaling, are required by the same spectrum of R genes although the resistance suppression caused by *pad4* mutation is less complete than that caused by *eds1* (Feys et al., 2001). Evidence that EDS1 and PAD4 act downstream of R protein activation was gathered from the examination of deregulated TIR-NB-LRR genes, in that constitutive activation of defense responses by these *R* genes is blocked when combined with *eds1* or *pad4* mutations (Li et al., 2001; Shirano et al., 2002; Zhang et al., 2003). Interestingly, the atypical *R* gene loci *RPW8* coding for CC transmembrane proteins that confer broad spectrum resistance to powdery mildew and the CC-NB-LRR HRT that intercepts turnip crinkle virus also require *EDS1* and *PAD4* to confer disease resistance (Chandra-Shekara et al., 2004; Xiao et al., 2005). It is possible that the role of EDS1 and PAD4 in RPW8- and HRT-conditioned resistance is related to their function in basal resistance. More recently, *Arabidopsis SAG101* (*SENESCENCE ASSOCIATED GENE 101*) was identified as encoding an additional in vivo EDS1 interactor that signals within EDS1-dependent resistance (Feys et al., 2005). The role of *SAG101* in disease resistance is genetically partially redundant with that of

PAD4, and *pad4/sag101* double mutants show defects in TIR-NB-LRR-mediated and basal resistance that are equivalent to those present in *eds1* (Feys et al., 2005). Furthermore, a role for EDS1 and partners in non-host resistance has also been demonstrated. *Arabidopsis eds1* mutant plants allowed reproduction of pathogens that naturally infect *Brassica oleracea* (Parker et al., 1996) and supported an increased penetration of non-host powdery mildew isolates (Yun et al., 2003; Zimmerli et al., 2004; Lipka et al., 2005). Notably, the *pad4/sag101* double mutant (significantly more so than *eds1* plants) permitted invasive growth and sporulation of grass and pea powdery mildews (Lipka et al., 2005). Therefore, EDS1 and the combined activities of PAD4 and SAG101 constitute a major resistance layer to host and non-host pathogens where they restrict post-invasive pathogen growth (Wiermer et al., 2005).

Close inspection of *eds1* and *pad4* null mutant phenotypes revealed some differences. *EDS1* and *PAD4* appear to be equally required for SA accumulation and SA itself induces their expression as part of a positive feedback loop which seems important for defense amplification (Falk et al., 1999; Jirage et al., 1999; Feys et al., 2001). Nevertheless, while the *eds1* mutation completely abolishes the appearance of HR-associated cell death and allows unrestricted growth of the pathogen, *pad4* mutant plants are able to develop HR lesions but pathogens often grow beyond the initial infection site giving rise to a trailing necrotic phenotype where dead cells accompany pathogen growth (Feys et al., 2001). This trailing necrosis phenotype is also observable in other *Arabidopsis* SA-deficient mutants, such as *sid2* (Nawrath and Metraux, 1999). Furthermore, both *EDS1* and *PAD4* genes are required for the runaway cell death observed in the *lsd1* (*lesions simulating disease resistance1*) mutant upon pathogen or superoxide treatments (Rusterucci et al., 2001). LSD1 codes for a zinc-finger protein that negatively regulates superoxide-dependent activation of cell death (Dietrich et al., 1997). Hence, while both genes are involved in the processing of reactive oxygen species (ROS)-derived signals during biotic and photooxidative stress signaling necessary for *lsd1*-conditioned runaway cell death, EDS1 exerts a distinct activity in TIR-NB-LRR triggered defense necessary for HR initiation (Feys et al., 2001; Rusterucci et al., 2001; Mateo et al., 2004). Nevertheless, the differences observed between *eds1* and *pad4* null mutant phenotypes in HR initiation could be due to *PAD4* and *SAG101* genetic redundancy. In *pad4/sag101* mutant plants the initiation of HR-associated cell death conditioned by TIR-NB-LRRs is abolished, which also means that EDS1 function in defense depends on the combined activities of PAD4 and SAG101 (Feys et al., 2005). Since SA-deficient plants initiate an HR which is,

however, ineffective at halting pathogen growth within the site of invasion, it is still unclear what is actually responsible of restricting pathogen colonization. It is possible that the ability of the SA signaling pathway to generate mobile signals is important for 'alerting' neighboring cells of the attempt of invasion and hence for containing the infection of both virulent and avirulent pathogens. It is worth mentioning at this point, that *eds1* and *pad4* mutants show defects in generating and perceiving a SAR-inducing mobile signal (L. Jorda and J. Parker, unpublished; (Truman et al., 2007)).

EDS1, PAD4, and to a lesser extent, SAG101 share a domain of homology to eukaryotic lipases (Figure 1) (Falk et al., 1999; Jirage et al., 1999; He and Gan, 2002). Embedded in the lipase homology domains of EDS1 and PAD4 are the Ser-Asp-His catalytic triad amino acids of acyl hydrolases. Some features of the lipase homology domain, including the catalytic triad and the GxSxG motif around the active serine, are related to the consensus sequence of the hormone-sensitive lipase (HSL) family (Osterlund, 2001). The rice GIBBERELLIN INSENSITIVE DWARF1 (GID1) protein with similarity to HSL family members was recently identified as a soluble nuclear receptor for the phytohormone gibberellic acid (GA) (Ueguchi-Tanaka et al., 2005). The GID1 GA-binding pocket corresponds to the substrate-binding site of HSLs and single amino acid exchange within the GxSxG motif in *gid1-1* abolishes GA-binding activity and confers a severe GA-insensitive phenotype (Ueguchi-Tanaka et al., 2005; Shimada et al., 2008). Notably, OsGID1 presents a substitutions of the His catalytic residue and no hydrolase enzymatic activity could be found for the protein although this does not compromise GA signaling. A form of SAG101 was reported to have acyl hydrolase activity *in vitro* (He and Gan, 2002). Nevertheless, an *in vivo* lipase activity for EDS1 and interacting partners remains elusive (S. Rietz and J. Parker, unpublished) and it is anticipated that EDS1 complexes function in binding and/or passaging of another type of signal molecule. The three proteins share a second domain of unknown function, denoted EP for its first description in EDS1 and PAD4 proteins (Figure 1) (Feys et al., 2001).

A combination of cell fractionation, coimmunoprecipitation and fluorescence resonance energy transfer (FRET) experiments have demonstrated that EDS1, PAD4 and SAG101 form molecularly and spatially distinct associations in the nucleus and cytoplasm of *Arabidopsis* leaf cells (Feys et al., 2001; Feys et al., 2005). EDS1 and PAD4 were shown to interact in yeast two-hybrid (Y2H) assays and in both healthy and infected plant cells by co-immunoprecipitation (Feys et al., 2001). Higher levels of protein in infected tissue correlated

with higher amounts of co-immunoprecipitable protein, indicating that the physical association of EDS1 and PAD4 is retained after pathogen challenge and also that their affinity does not increase massively during the defense response. Both proteins localize to the cytosol and the nucleus but the absence of a FRET signal between EDS1- and PAD4-fluorescent proteins precluded a direct determination of the subcellular compartment where the interaction takes place (Feys et al., 2005). SAG101 protein is localized in the nucleus where it interacts with EDS1 (Feys et al., 2005). FRET and Y2H analyses also showed the existence of homodimeric EDS1 complexes which appear to be exclusively cytosolic (Feys et al., 2001; Feys et al., 2005). Size exclusion chromatography revealed the existence of a higher order PAD4 complex which in principle could contain dimeric EDS1, EDS1 and SAG101, or EDS1 and an unidentified component (Feys et al., 2005). Close inspection of the protein sequences revealed two possible bipartite nuclear localization signals (NLS) (double lysine (K) motif at amino acid positions 366 and 440) in EDS1 and a potential monopartite NLS (KKKK, amino acids 48 to 51) in SAG101 (Falk et al., 1999; Feys et al., 2005) (Figure 1).

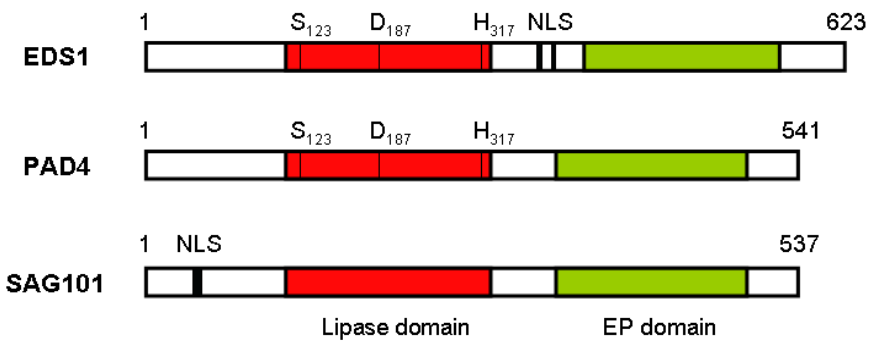


**Figure 1.1. Schematic representation of the domain architecture of *Arabidopsis* EDS1, PAD4 and SAG101 proteins.** The lipase domain is shown in red and the EP domain in green. The putative catalytic triad in EDS1 and PAD4 sequences composed of a Ser (S), Asp (D) and His (H) residues are highlighted in the lipase domain. EDS1 predicted bipartite NLS in amino acid positions 366 and 440 and SAG101 predicted monopartite NLS in amino acids 48 – 51 are highlighted. Numbers indicate amino acid positions.

Importantly, EDS1, PAD4 and SAG101 are mutually stabilized by their interacting partners. EDS1 is necessary for protein accumulation of PAD4 and SAG101 in leaves, and steady state EDS1 protein levels are reduced in *pad4* and *sag101* mutant backgrounds (Feys et al 2005). These stabilizing effects seem to be expressed mostly at the post-transcriptional level, although *PAD4* transcript accumulation is reduced in *eds1* plants and mutations in *PAD4* partially compromise pathogen- or SA-induced *EDS1* expression (Feys et al., 2001). Pathogen

challenge triggers *EDS1* and *PAD4* transcriptional upregulation which correlates with the induction of EDS1-dependent genes (Feys et al., 2001; Bartsch et al., 2006). Nevertheless, accumulation of *EDS1* transcripts cannot account for the whole activation of downstream events since EDS1-dependent genes are still further induced after pathogen challenge in transgenic lines constitutively expressing EDS1 and PAD4 (Gobbato *et al*, manuscript in preparation). This suggests that other modes of regulation, besides transcriptional activation, are important for the response and that a pathogen-derived trigger is needed to fully activate EDS1 signaling. In line with the notion of post-transcriptional regulation of EDS1 pathway activities, it was postulated that the dynamics of the interactions and subcellular distribution of EDS1 and its signaling partners in nuclei and cytoplasm may have an important role in defense activation.

## 1.3 Principle features of nucleocytoplasmic transport

All eukaryotic cells have a physical separation between the nuclear genomic material and the rest of the cellular components due to the presence of the double lipid bilayer of the nuclear envelope. Nucleocytoplasmic trafficking of macromolecules is therefore an essential process that allows the required nuclear export of RNA molecules to the cytoplasm and the entry of proteins synthesized in the cytosol (Xu and Meier, 2008). Entrance and exit from the nucleus occurs via nuclear pore complexes (NPCs) which are large proteinaceous structures consisting of approximately 30 nucleoporin proteins (Nups) that form channels spanning the nuclear envelope (Rout et al., 2000; Cronshaw et al., 2002; Xu and Meier, 2008). Molecules up to 60 kD can passively diffuse through the NPC, but the translocation of molecules larger than 50 kD generally requires the aid of transport receptors. Importins and exportins, collectively named Karyopherins (Kaps), are the transport receptors involved in nuclear import and export that recognize exposed NLS or nuclear export signals (NES), respectively (Gorlich et al., 1995; Fornerod et al., 1997; Stade et al., 1997). After recognition and binding of their protein cargoes, the transport receptors engage in multiple, low-affinity interactions with NPC proteins that allow the movement of the receptor-cargo complex across the channel. The mechanism of translocation remains controversial and poorly understood, and evidence gathered so far suggest that the NPC permeability barrier could be either physical or energetic or both (Terry

et al., 2007). The small GTPase Ran (Ras-related nuclear protein) is also required for nucleocytoplasmic trafficking (Kadowaki et al., 1993; Moore and Blobel, 1993). An asymmetric distribution of Ran-GTP versus Ran-GDP maintains the directionality of transport: nuclear Ran-GTP is required by the transporters for dissociation of imported cargoes and the binding of export cargoes, whereas cytoplasmic Ran-GTP to GDP conversion is required for the dissociation of the exported cargoes (Gorlich et al., 1996).

Active nuclear import of proteins generally begins with the binding of the cargo's NLS by importin α which then bridges the interaction with importin β that is responsible of docking the ternary complex to the NPC (Gorlich et al., 1995). Once inside the nucleus, importins interact with Ran-GTP and release their cargoes (Gorlich et al., 1996). The cycle is completed when importin-RanGTP complexes are transported back to the cytoplasm and Ran-GTP to GDP hydrolysis is stimulated by Ran-GTPase activating protein (RanGAP) (Gorlich et al., 1996). There are other mechanisms of NLS-dependent nuclear import, since some proteins are capable of direct binding to importin β and do not need importin α for their nuclear import (Palacios et al., 1997; Henderson and Eleftheriou, 2000). The amino acid sequences of several NLSs are known and are usually composed of one or two stretches of basic residues. Nevertheless, there are still many NLSs that do not correspond to the consensus rule and many nonfunctional sequences that match the consensus. It was recently shown, by screening a random peptide library, that importin α from plants and humans can recognize a broader range of peptides than previously known (Kosugi et al., 2009). NLS-independent translocation of signaling proteins into the nucleus has also been described either through direct interaction with nucleoporins or through the use of novel nuclear translocation signals (Xu and Massague, 2004; Chuderland et al., 2008). In the case of nuclear export, several nuclear export receptors have been identified, such as exportin 1 (CRM1/XPO1) that mediates the export of proteins containing a leucine-rich NES (Haasen et al., 1999). XPO1 binds to the NES-containing cargo cooperatively with RanGTP in the nucleus, resulting in a ternary complex that docks to the NPC through the interaction between the transport receptor and nucleoporins. After translocation to the cytoplasm, hydrolysis of Ran-GTP takes place and XPO1 enters the nucleus on its own (Merkle, 2004). Different NES have been identified which vary profoundly in their export efficiency and led to the conclusion that nuclear export rate depends on the signal activity and accessibility (Henderson and Eleftheriou, 2000).

The *Arabidopsis* genome encodes nine importin α proteins (Bhattacharjee et al., 2008) and at least 17 predicted importin β-like proteins (Bollman et al., 2003). The presence of multigene families in various plants suggests that plant importins could have some specificity in their substrates and functions. Indeed, several mutant screens have identified nucleoporins and other components of the nucleocytoplasmic trafficking machinery as crucial for plant growth, development and interaction with the environment (Meier and Brkljacic, 2008). Surprisingly, the defects observed in these mutants were mild and specific for certain pathways, further suggesting cargo specificity for the nuclear transport components. Support to this hypothesis came with the cloning of three importin α genes from rice that are differentially expressed in different tissues and under different light conditions (Jiang et al., 2001). Among them, the rice importin α1b was shown to be specifically involved in the nuclear import of constitutive photomorphogenic 1 (COP1) (Jiang et al., 2001). A recent paper suggests a similar scenario for the *Arabidopsis* importin α family (Bhattacharjee et al., 2008). Plant transformation by *Agrobacterium tumefaciens* involves the transport of T-DNA to the host's nucleus, and the accompanying proteins of the T-complex, VirD2 and VirE2, interact with several members of the *Arabidopsis* importin α familiy. However, only mutations in *IMPa4* resulted in deficiencies in *Agrobacterium*-mediated transformation (Bhattacharjee et al., 2008). How the specificity of importins for their cargoes is determined is still not fully understood. Whether the specificity resides in the NLS sequence, is influenced by the expression patterns of the corresponding cargoes and transporters, or is determined by a combination of various factors remains to be clarified.

## 1.4 Nucleocytoplasmic trafficking and immunity

Recent findings suggest an important role for dynamic protein import into the nucleus and activities happening in the nuclear compartment in plant disease resistance signaling. These key pieces of information may help understand how pathogen recognition is translated into downstream activation of defense genes. A simplified cartoon representing the identified components of the nucleocytoplasmic transport machinery that play a role in plant-microbe interactions is depicted in Figure 1.2.

### 1.4.1 *Nucleocytoplasmic trafficking of immune receptors*

Some recent reports point to nuclear activities for plant R proteins (Burch-Smith et al., 2007; Shen et al., 2007; Wirthmueller et al., 2007). Tobacco TIR-NB-LRR N protein confers resistance to tobacco mosaic virus (TMV) upon recognition of the p50 helicase domain of the TMV replicase protein (Whitham et al., 1994; Erickson et al., 1999). Both N and p50 display nucleocytoplasmic distribution, and interfering with the nuclear localization of N (but not of p50) by translational fusion of a NES rendered the receptor non-functional (Burch-Smith et al., 2007). N interacts with members of the squamosa promoter-like (SPL) family of plant specific transcription factors that are required for TMV disease resistance (Shen and Schulze-Lefert, 2007), thereby providing a possible link between N-mediated recognition and transcriptional activation of defense genes. The *Arabidopsis* RPS4 TIR-NB-LRR receptor, conditioning resistance to *P. syringae* bacteria expressing AvrRps4 (Gassmann et al., 1999), distributes between endomembranes and the nucleus in healthy and AvrRps4-triggered tissues (Wirthmueller et al., 2007). Mutagenesis of a bipartite NLS in RPS4 sequence was shown to interfere with its nuclear localization and compromised RPS4-mediated defense to *P. syringae* (Wirthmueller et al., 2007). N- and RPS4-conditioned resistance responses require EDS1 signaling to trigger defense responses (Liu et al., 2002; Wirthmueller et al., 2007). Importantly, the RPS4 requirement for EDS1 appears not to be at the level of receptor stability or subcellular distribution but as a downstream positive signal transducer necessary for transcriptional reprogramming (Wirthmueller et al., 2007). Nevertheless, the molecular mechanism underlying RPS4-mediated activation of defense genes is not known and it remains to be elucidated whether it is mediated by an immediate role of EDS1 in transcriptional reprogramming.

A direct role of an R protein in transcriptional activation is suggested by the unusual domain structure of *Arabidopsis* TIR-NB-LRR protein RRS1, which contains a C-terminal DNA-binding motif that is characteristic of the WRKY family of plant specific transcription factors (Deslandes et al., 2002). The RRS1 variant RRS1-R confers disease resistance to the bacterial pathogen *Ralstonia solanacearum* expressing the effector PopP2 (Deslandes et al., 2002; Deslandes et al., 2003). RRS1 and PopP2 were shown to interact and co-localize in plant nuclei, although nuclear signal of fluorescent protein-tagged RRS1 was only observed in the presence of the effector (Deslandes et al., 2003). Hence, it is possible that RRS1-PopP2

interaction either stabilizes RRS1 or triggers its nuclear localization, activating the WRKY domain to bind target gene promoters. Consistent with this idea, the WRKY domain of SLH1, which is identical in sequence and function to RRS1-R but in a different *Arabidopsis* ecotype, is capable of binding DNA W-boxes (Noutoshi et al., 2005).

Important information on R protein nuclear activities was also obtained from analyses of CC-NB-LRR receptors. One report is of barley MLA CC-NB-LRR receptors that recognize isolate-specific effectors of the grass powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (Ridout et al., 2006). Shen et al (2007) observed specific nuclear enrichment of MLA1 during an incompatible interaction of barley with *B. graminis* expressing the cognate effector $Avr_{A1}$. The same study further showed that compromising the nuclear accumulation of another MLA receptor, MLA10, through a NES-protein fusion abolished MLA10-mediated disease resistance (Shen et al., 2007). In the presence of $Avr_{A10}$, nuclear MLA10 associates with two WRKY transcription factors that function as negative regulators of barley basal resistance to virulent *B. graminis* and the MLA10-*Hv*WRKY2 interaction interferes with WRKY repressor activity enabling rapid defense gene induction (Shen et al., 2007). Furthermore, the potato CC-NB-LRR protein Rx, mediating resistance to Potato Virus X (PVX) by recognizing the viral coat protein (Bendahmane et al., 1995), interacts with a Ran GAP, RanGAP2, from *Nicotiana benthamiana* and *Solanum tuberosum* (Sacco et al., 2007; Tameling and Baulcombe, 2007). RanGAP2 was demonstrated to be specifically required for the extreme resistance conferred by Rx, suggesting a role for nucleocytoplasmic trafficking in the activation of Rx-conditioned disease resistance (Sacco et al., 2007; Tameling and Baulcombe, 2007). Rx has been detected in the nucleus, so is possible that RanGAP2 influences the nuclear import of Rx itself or other resistance-related components necessary for Rx function (Tameling and Baulcombe, 2007).

In mammals, the major histocompatibility complex (MHC) class II transactivator CIITA, belonging to the NLR/CATERPILLER class of intracellular immune receptors, is the only characterized member to date that localizes to the nucleus (Chen et al., 2008). CIITA is the master regulator of MHC class II gene expression, regulating the transcription of all MHC class II genes and other genes encoding proteins important for antigen presentation. Not being a DNA-binding protein itself, evidence suggests that CIITA coordinates the assembly of acetylases, methylases and transcription factors on MHC class II promoters (Ting et al., 2006). Mutagenesis of the CIITA LRR domain impaired both CIITA nuclear localization and in vivo recruitment of CIITA to the MHC-II promoter binding complex (Hake et al., 2000). These data

present interesting possibilities for the nuclear activities of plant R proteins in defense-associated transcriptional reprogramming.

### 1.4.2 *Nucleocytoplasmic trafficking of immune signaling regulators*

There are some examples illustrating that nucleocytoplasmic trafficking of plant immune regulators, other than R proteins, is important for defense activation in plants. Specifically, the importance of restraining transcriptional activators in the cytoplasm or in an inactive state in the nuclei has emerged in plants as a mechanism of signaling regulation. For example, the negative regulator of cell death LSD1 has recently been shown to interact with the transcription factor AtbZIP10 which is a positive regulator of ROS-induced cell death and defense against *H. parasitica* (Kaminaka et al., 2006). AtbZIP10 is able to shuttle between the nucleus and cytoplasm and LSD1 interaction is likely to antagonize AtbZIP10 functions in disease resistance through its cytosolic retention (Kaminaka et al., 2006). The authors present genetic evidence suggesting that LSD1 antagonizes other positive regulators of cell death besides AtbZIP10, raising the possibility that LSD1 may function as a scaffold protein controlling the localization of various interacting proteins. A similar scenario was proposed for another regulator of plant immunity, the MAP kinase MPK4 (Qiu et al., 2008). *Arabidopsis* MPK4 is a repressor of SA-dependent resistance and a positive regulator of JA/ET-dependent gene expression (Petersen et al., 2000). MPK4, its substrate MKS1 and the transcription factor WRKY33 were shown to form a ternary complex in nuclei of healthy *Arabidopsis* tissue (Andreasson et al., 2005; Qiu et al., 2008). Flagellin treatment or *P. syringae* infection trigger MPK4 activation, dissociation of WRKY33 from the complex and activation of defense gene expression through WRKY33 binding of promoters of target genes, such as *PAD3* (Qiu et al., 2008). Furthermore, a regulated nucleocytoplasmic distribution of NPR1 (NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1) is key for SA-mediated activation of signal transduction leading to SAR (Cao et al., 1994; Kinkema et al., 2000). The *NPR1* gene encodes a protein with ankyrin repeats and a BTB/POZ domain and plants mutated in this gene fail to respond to various SAR-inducing agents (Cao et al., 1994; Cao et al., 1997). In resting conditions, NPR1 is retained in the cytoplasm as an oligomer maintained through the formation of intermolecular disulfide bonds facilitated by the function of the oxidant S-nitrosoglutathione. SAR induction involves a biphasic change in the cellular redox potential

that results in thioredoxin-mediated NPR1 reduction causing its monomerization and accumulation in the nucleus (Mou et al., 2003; Tada et al., 2008). NPR1 nuclear accumulation is essential for its function, since it interacts with TGA transcription factors and activates defense gene expression in this compartment (Zhang et al., 1999; Despres et al., 2000; Kinkema et al., 2000).

The reprogramming of transcriptional outputs by controlling nuclear entry or release of transcriptional regulators is reminiscent of the regulation of NFκB transcription factors in animals and seems to be an efficient strategy to regulate defense gene expression common to plants and animals. The activity of NFκB family members is regulated by its association with inhibitor IκB proteins and its translocation from the cytoplasm to the nucleus (Hayden and Ghosh, 2008). The traditional model of IκB function posits that IκBα sequesters NFκB in the cytoplasm, thereby preventing its nuclear localization and binding of target gene promoters. However, the situation was shown to be more dynamic and, despite having a steady-state localization that appears almost exclusively cytosolic, IκB/NFκB complexes are continuously shuttling between the nucleus and the cytoplasm (Carlotti et al., 2000; Huang et al., 2000). Degradation of IκB alters the dynamics of nucleocytoplasmic trafficking and favors NFκB nuclear localization where it promotes transcription of target genes (Hayden and Ghosh, 2008). Recent findings show that the onset of apoptosis in mammalian cells is associated with a reduction in nuclear RanGTP levels; the dissipated RanGTP gradient across the nuclear envelope leads to a cytoplasmic retention of NLS-containing proteins and inhibition of NFκB nuclear import (Wong et al., 2009).

### 1.4.3 *Nuclear transport machinery in host-microbe interactions*

Additional evidence for the role of nuclear export/import in pathogen defense responses, came from a genetic screen designed to identify components required for the *Arabidopsis snc1* (*suppressor of npr1-1, constitutive 1*) 'auto-immune' mutant phenotype. *Snc1* plants carry a gain-of-function mutation that results in an amino acid exchange in the NB-LRR linker region of a TIR-NB-LRR-type receptor and constitutive activation of defense responses (Li et al., 2001; Zhang et al., 2003). An *snc1* suppressor screen led to the identification of mutants in *MOS6* (*MODIFIER OF SNC1, 6*) that codes for Importin α3, and in *MOS3* and *MOS7* which are homologs of vertebrate genes encoding the nucleoporins Nup96 and Nup88, respectively

((Palma et al., 2005; Zhang and Li, 2005); Cheng et al., submitted). Besides interfering with *snc1* activation of defense responses, plants carrying mutations in these *MOS* genes exhibit enhanced disease susceptibility to virulent pathogens and partial loss of resistance mediated by other *R* genes. Interestingly, mouse Nup96 and Drosophila Nup88 are also selectively required for immune responses (Uv et al., 2000; Faria et al., 2006), which suggests that the recruitment of certain Nups for immunity is highly conserved. In a screen for suppressors of *auxin-resistant1* (*axr1*) mutant, the mutants *suppressor of auxin resistance1* (*sar1*) and *sar3* were identified (Parry et al., 2006). AXR1 is a subunit in the RUB-activating enzyme required for the normal function of $SCF^{TIR1}$, which is involved in the degradation of the Aux/IAA family of transcriptional repressors presumably in the nucleus (del Pozo et al., 2002). *SAR1* and *SAR3* encode proteins with similarity to nucleoporins and their mutants are thought to restore partial auxin sensitivity to *axr1* mutant plants by reducing the nuclear import of Aux/IAA proteins (Parry et al., 2006). SAR3 encodes the Nup88 homologue previously identified as MOS3. It would be interesting to determine whether the *mos* mutants show defects in the nucleocytoplasmic distribution of plant immune regulators or in the nuclear export of their coding mRNAs. The possibility that they affect SNC1 nuclear accumulation is an interesting option. Nevertheless, it remains to be determined whether SNC1 needs to enter the nucleus to accomplish its function. Interestingly, components of the nucleocytoplasmic transport machinery are also required for beneficial plant-microbe associations, as is the case of Nup85 and Nup133 which have roles in the establishment of rhizobial and fungal symbioses in *Lotus japonicus* (Kanamori et al., 2006; Saito et al., 2007).

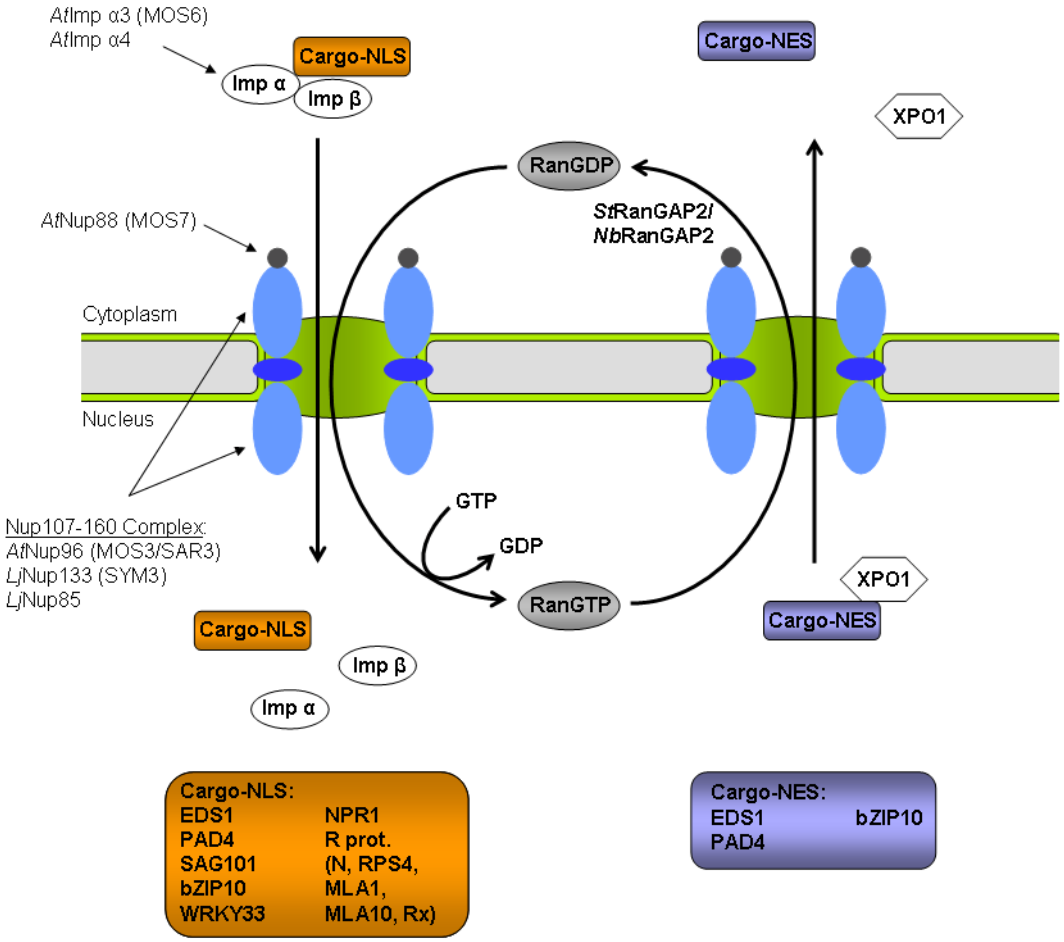


**Figure 1.2. Schematic diagram of the nucleocytoplasmic trafficking machinery, highlighting the identified components that have a role in plant-microbe interactions.** The identification of plant homologues of animal nuclear pore complex (NPC) components (Nucleoporins; Nups) indicate they may transport immune regulatory proteins. *Arabidopsis thaliana* (*At*) Nup96 homologue (MOS3) and *At*Nup88 (MOS7) have a role in plant immunity (Zhang and Li, 2005; Wiermer et al., 2007) and *Lotus japonicus* (*Lj*) Nup133 (SYM3) and *Lj*Nup85 are important for fungal and bacterial symbioses (Kanamori et al., 2006; Saito et al., 2007). Nuclear import is initiated by the interaction between NLS containing cargoes and the transport receptors Importin α (Imp α) and Imp β in the cytoplasm. *At*Imp α3 (MOS6) is involved in disease resistance (Palma et al., 2005) and *At*Imp α4 is important for *Agrobacterium*-mediated *Arabidopsis* transformation (Bhattacharjee et al., 2008). Aided by RanGTP, the imported complex dissociates in the nuclear side of the NPC. Nuclear RanGTP also promotes the formation of complexes of NES containing cargoes and exportins (XPO1). After the nuclear export of the complex, RanGTP hydrolysis in the cytoplasm triggers the release of exported cargo from XPO1-containing complexes. The hydrolysis of RanGTP in the cytoplasm is promoted by Ran GTPase Activating Proteins (RanGAP) and Ran Binding Proteins (RanBP). RanGAP2 from *Solanum tuberosum* and *Nicotiana benthamiana* interact with the CC-NB-LRR Rx and play a role in Rx-conditioned resistance (Sacco et al., 2007; Tameling and Baulcombe, 2007). Based on recent reports, possible cargoes for nuclear import (Cargo-NLS) or export (Cargo-NES) are included.

## 1.5 Thesis aims

In plants, certain pathogen effectors and host immune receptors localize to and function in the nucleus suggesting that activities in this compartment are important for appropriate defense activation. Understanding the mode of action of immune receptors and how pathogen recognition is transduced to transcriptional reprogramming and triggering of programmed cell death is of major interest. *Arabidopsis* EDS1 and its interacting partners PAD4 and SAG101 constitute a central regulatory node in plant disease resistance against biotrophic and hemi-biotrophic pathogens (Wiermer et al., 2005). They are necessary for resistance conferred by TIR-NB-LRR-type of immune receptors and for basal defenses that restrict colonization by host-adapted pathogens. It was recently shown that the TIR-NB-LRR receptor RPS4 functions in the nuclei and signals entirely through EDS1 which functions as a downstream positive signal transducer necessary for transcriptional reprogramming and triggering of resistance (Wirthmueller et al., 2007). Nevertheless, the molecular mechanism underlying RPS4-mediated activation of defense genes is not known.

EDS1 forms molecularly and spatially distinct complexes with PAD4 and SAG101 in the cytoplasm and the nuclei of healthy *Arabidopsis* leaf cells (Feys et al., 2005). The aim of this study was to: (1) assess whether a change in the subcellular distribution of EDS1 complexes in response to a pathogen stimulus is important for defense signal transmission, (2) determine where in the cell EDS1 activity is required for its role in defense activation and (3) discriminate nuclear versus cytoplasmic roles for EDS1 protein. Using confocal laser fluorescence microscopy and biochemical fractionation of cells the nucleocytoplasmic partitioning of EDS1 and PAD4 was analyzed during the activation of defense responses and in a constitutive resistance background. To assess the functional significance of EDS1 distribution in the cell, stable transgenic plants were generated in which a nuclear export signal (NES), the hormone binding domain of the rat glucocorticoid receptor (GR) or a nuclear localization signal (NLS) were attached to EDS1. Examining where in the cell EDS1 distributes and carries its function during defense responses would allow me to understand specifically the role of EDS1 in plant defense activation, provide a link between pathogen recognition and activation of disease resistance, and inform more broadly on the mobility of intracellular signaling systems in plant sensing of and response to its environment.

# 2 MATERIALS AND METHODS

## 2.1 Materials

### 2.1.1 Plant materials

*Arabidopsis thaliana* wild-type and mutant lines used in this study are listed in Table 2.1 and 2.2, respectively. *Nicotiana benthamiana* (310A) plants were obtained from T. Romeis (MPIZ, Cologne) and used for transient *Agrobacterium*-mediated transformation of leaves.

**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*-0 | Nottinghan *Arabidopsis* Stock Center[b] |
| **Wassilewskija** | Ws-0 | K. Feldmann[c] |

[a]University of North Carolina, Chapel Hill, NC, USA

[b]Nottingham, UK

[c]University of Arizona, Tucson, AZ, USA

**Table 2.2 Mutant *Arabidopsis* lines used in this study**

| Gene | Accession | Mutagen | Reference/Source |
|---|---|---|---|
| *eds1-1* | Ws-0 | EMS | Parker et al., 1996 |
| *eds1-2* | L*er*-0 | FN | Falk et al., 1999 |
| *eds1-2* | Col-0 (L*er*-0)[a] | FN | Bartsch et al., 2006 |
| *pad4-1* | Col-0 | EMS | Glazebrook et al., 1997 |
| *sag101-1* | Col-0 | dSpm | Feys et al., 2005 |
| *eds1-2/sag101-1* | Col-0 | FN/dSpm | J. Parker[b], unpublished |
| *pad4-1/sag101-1* | Col-0 | EMS/dSpm | Feys et al., 2005 |
| *rps4-2* | Col-0 | T-DNA | Wirthmueller et al., 2007 |
| *snc1* | Col-0 | EMS | Li et al., 2001 |
| *snc1*/Col *eds1-2* | Col-0 | EMS/FN | J. Parker[b], unpublished |

[a]L*er eds1-2* allele introgressed into Col-0 genetic background, 8th backcrossed generation, referred to as Col *eds1-2* in this study.

[b]Max Planck Institute for Plant Breeding Research, Cologne, Germany.

EMS: ethyl methane sulfonate; FN: fast neutron; dSpm: defective Suppressor-mutator; T-DNA: Transfer DNA

### 2.1.2 Pathogens

*Arabidopsis* plants were inoculated with different isolates of the oomycete pathogen *Hyaloperonospora parasitica*, listed in Table 2.3, with different types of interaction with *Arabidopsis* ecotypes, listed in Table 2.4. Inoculations of *Arabidopsis* plants with the bacterial pathogen *Pseudomonas syringae* were also performed. *P. syringae* pv. *tomato* (*Pst*) strain DC3000 harbouring either the empty broad host range vector pVSP61 (Innes et al., 1993) or expressing the following effectors from the same plasmid: *P. syringae* pv. *pisi* effector AvrRps4 (Hinsch and Staskawicz, 1996) and *Pst* DC3000 expressing AvrRpt2 (Whalen et al., 1991) were used throughout this study. The *Pst* isolates were originally obtained from R. Innes (Indiana University, Bloomington, Indiana, USA).

**Table 2.3 *Hyaloperonospora parasitica* isolates used in this study**

| Isolate | Original source | Reference |
|---|---|---|
| **Cala2** | Oospore infection of a single seedling | (Holub et al., 1994) |
| **Emwa1** | Oospore infection of a single seedling | (Holub et al., 1994) |
| **Noco2** | Oospore infection of a single seedling | (Parker et al., 1993) |

**Table 2.4 Interaction between *Hyaloperonospora parasitica* isolates and *Arabidopsis* ecotypes used in this study**

| ***Arabidopsis*** | ***Hyaloperonospora parasitica* isolate** | | |
|---|---|---|---|
| **ecotype** | **Cala2** | **Emwa1** | **Noco2** |
| **Col-0** | Incompatible (*RPP2*)* | Incompatible (*RPP4*) | Compatible |
| **L*er*** | Compatible | Incompatible (*RPP4* and *RPP8*) | Incompatible (*RPP5*) |
| **Ws-0** | Incompatible (*RPP1A*) | Compatible | Incompatible (*RPP1*) |

*Genetic analysis of *Arabidopsis* segregating populations has allowed the identification, mapping and cloning of ecotype specific resistance genes conferring recognition of specific *H. parasitica* isolates, denominated *Resistance to Peronospora Parasitica* (*RPP*) genes.

### 2.1.3 Other bacterial strains

For standard cloning the *Escherichia coli* strains DH10B and TOP10 were used. The *E. coli* strain DB3.1, which is resistant to the *ccdB* gene, was used for cloning Gateway Donor and Destination vectors. All *E. coli* strains were obtained from Invitrogen™ (Karlsruhe, Germany). For stable transformation of *Arabidopsis* and transient expression in *N. benthamiana*, DNA constructs were transformed in *Agrobacterium tumefaciens* strain GV3101 carrying the helper plasmid pMP90 (with resistance to Rifampicin and Gentamycin) or the helper plasmid pMP90RK (with resistance to Rifampicin, Kanamycin and Gentamycin) (Koncz and Schell, 1986).

### 2.1.4 Media

Sterile media was used for the growth of bacteria and *in vitro* cultivation of *Arabidopsis thaliana* as follows:

*Escherichia coli* media: Luria-Bertani (LB) broth or agar plates; SOC

*Pseudomonas syringae* media: NYG broth or agar plates

*Agrobacterium tumefaciens* media: YEB broth or agar plates

*Arabidopsis thaliana* media: MS (Murashige and Skoog).

### 2.1.5 Chemicals and antibiotics

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. Antibiotics were used in the following concentrations: Ampicillin (Amp) 100 µg/ml, Carbenicillin (Carb) 50 µg/ml, Gentamycin (Gent) 15 µg/ml, Kanamycin (Kan) 50 µg/ml, Rifampicin (Rif) 50 µg/ml. All antibiotic stock solutions were prepared as 1000x concentrated in dd$H_2O$, except for Rif which is soluble in DMSO.

### 2.1.6 Vectors

The following vectors were used or generated in this study:

| | |
|---|---|
| promEDS1-gEDS1ΔStop / pENTR™/D-TOPO® | Entry vector containing promEDS1-gEDS1 without Stop codon |
| pXCS-HisHA | (Witte et al., 2004), vector backbone used to generate pXCSG-mYFP and pXCG-mYFP vectors, with or without C-terminal localization signal |
| pXCSG-mYFP | Binary Gateway® destination vector for expression of a fusion protein with a C-terminal mYFP tag under control of prom35S |
| pXCG-mYFP | Binary Gateway® destination vector for expression of a fusion protein with a C-terminal mYFP tag under control of their native promoters |
| pXCSG-mYFP-NLS/nls/NES/nes | Binary Gateway® destination vectors for expression of a fusion protein under control of prom35S with a C-terminal mYFP tag carrying a functional or non-functional localization signals |
| pXCG-mYFP-NLS/nls/NES/nes | Binary Gateway® destination vectors for expression of a fusion protein under control of their native promoter with a C-terminal mYFP tag a carrying functional or non-functional localization signal |
| pBIΔGR | (Simon et al., 1996), binary vector for expression of a fusion protein under control of prom35S with a C-terminal GR domain |

### 2.1.7 Oligonucleotides

Listed below are the primers used in this study that were synthesized by Invitrogen. Lyophilised primers were resuspended in nuclease-free water to a final concentration of 100 µM and working stocks were diluted to 10 µM.

**Table 2.5. Oligonucleotides used in this study**

| Primer | Sequence (5' → 3') | Purpose |
|---|---|---|
| AG1:YFPNLS (rev) | AGTCTAGAGCTC**TTA**TCCTCCAACCTTTCTCTTCTTCTTAG GCTTGTACAGCTCGTCCATGCCG | To attach NLS to YFP C-terminus |
| AG2:YFPNES (rev) | AGTCTAGAGCTC**TTA**AATATCAAGTCCAGCCAACTTAAGA GCAAGCTTGTACAGCTCGTCCATGCCG | To attach NES to YFP C-terminus |
| AG3:YFPNLSm (rev) | AGTCTAGAGCTC**TTA**TCCTCCAACCTTTCTCTTCGTCTTAG GCTTGTACAGCTCGTCCATGCCG | To attach nls to YFP C-terminus |
| AG4:YFPNESm (rev) | AGTCTAGAGCTC**TTA**AGCATCAGCTCCAGCCGCCTTAAGA GCAAGCTTGTACAGCTCGTCCATGCCG | To attach nes to YFP C-terminus |
| AG5:EDS1 (fwd) | AAAGTGGTGGATCACGCTTC | EDS1 gDNA |
| AG6:EDS1 (fwd) | ATCACGCTTCCCAAAAATCA | |
| AG7:YFP (rev) | GCTGAACTTGTGGCCGTTTA | YFP |
| AG8:YFP (rev) | GAACTTCAGGGTCAGCTTGC | |
| MW15 (fwd) | ATCCCCGGG**ATG**GTGAGCAAGGGCGAGGAGC | |
| MW16 (rev) | AGTCTAGAGCTC**TTA**CTTGTACAGCTCGTCCATGC | |
| GY7-YFP-F (fwd) | TTGATGGGTCTAGAG**ATG**GTGAGCAAGGGCGAGGAGCTG | YFP |
| GY8-EDS1-F (fwd) | GATGGGTCTAGAG**ATG**GCGTTTGAAGCTCTTACCGGAATC | EDS1 cDNA |
| GY11-YFP-R (rev) | TGGATCCTCTAGATCCTTGTACAGCTCGTCCATGCCGAG | YFP |
| LN38 (fwd) | ATGACGCACAATCCCACTATCCTTCGCA | prom35S |
| PPM-R (rev) | AGCGAAACCCTATAAGAA | 35S terminator |
| AG9:105/E2 | ACACAAGGGTGATGCGAGACA | *eds1-2* mutant detection |
| EDS4 | GGCTTGTATTCATCT TCTATCC | |
| EDS6 | GTGGAAACCAAATTTGACATTAG | |
| MW73 (fwd) | TGGTTTTGAAGTCAGTTACG | *snc1* mutant detection |
| MW74 (rev) | CAAGTTGAGATCGGTTGG | |
| dSpm11 | GGTGCAGCAAAACCCACACTTTTACTTC | *sag101-1* mutant detection |
| BF52 | CACGCGTCCGAAGATCTTGGAGATACATA | |
| BF53 | ACTTCCGGGTGTTCATAAACTCGGTCAAG | |

Start or Stop codons are marked in bold. Restriction enzymes sites are underlined.

### 2.1.8 Enzymes

Restriction enzymes were purchased from New England Biolabs (Frankfurt/Main, Germany) and MBI-Fermentas (St. Leon-Rot, Germany). Enzymes were supplied with a 10x reaction buffer which was used for restriction digestions following manufacturer recommendations. Standard PCR reactions were performed using home made *Taq* DNA polymerase. High accuracy *Pfu* DNA polymerases were used when PCR products were generated for cloning or sequencing. Nucleic acid modifying enzymes used for PCR and cloning were purchased from Stratagene® (Heidelberg, Germany), Roche (Mannheim, Germany) or Invitrogen™ (Karlsruhe, Germany).

### 2.1.9 Antibodies

Primary and secondary antibodies used for immunoblot detection are listed below.

**Table 2.6. Primary antibodies**

| Antibody | Source | Dilution | Source |
|---|---|---|---|
| α-EDS1 | rabbit polyclonal | 1:500 in TBS-T + 2% (w/v) milk | S. Rietz and J. Parker[a] |
| α-PAD4 | rabbit polyclonal | 1:500 in TBS-T | S. Rietz and J. Parker[a] |
| α-GFP | mouse polyclonal | 1:2500 in TBS-T + 2% (w/v) milk | Roche (Mannheim, Germany) |
| α-Histone H3 (ab1791) | rabbit polyclonal | 1:5000 in TBS-T + 5% (w/v) milk | Abcam (Cambridge, UK) |
| α-PEPC | rabbit polyclonal | 1:15000 in TBS-T + 2% (w/v) milk | Rockland (Gilbertsville, PA, USA) |

[a]Max Planck Institute for Plant Breeding Research, Cologne, Germany.

**Table 2.7. Secondary antibodies**

| Antibodies | 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) |

[*]Dilutions were made in TBS-T with same milk concentration than primary antibody.

## 2.2 Methods

### 2.2.1 Plant methods

#### 2.2.1.1 Plant growth conditions

*Arabidopsis thaliana* seeds were germinated on soil by direct sowing onto moist compost (Stender, Schermbeck, Germany) containing 10 mg/l Confidor® WG 70 (Bayer, Germany). After sowing, seeds were covered with a propagator lid and stratified at 4°C for 24 h in the dark. Subsequently, plants were grown in controlled environment chambers under a 10 hr light regime (150-200 $\mu E/m^2s$) at 22°C and 65% relative humidity (short day conditions), unless otherwise stated. Propagator lids were removed 3-5 days post germination. To obtain progeny 3-week-old plants were transferred to long day conditions (16 h photoperiod; long day conditions) and allowed to flower. Seeds were collected from dried siliques. To grow *Arabidopsis* in MS media, seeds were surface sterilized and sown in half-strong MS plates. Sealed plates were stratified at 4°C for 24 h in the dark and then transferred to controlled environment chambers (similar conditions than above).

#### 2.2.1.2 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.

#### 2.2.1.3 *Arabidopsis* seed surface sterilization

Seeds were sterilized for *in vitro* growth of *Arabidopsis*. Seeds were placed in 1.5 ml open microcentrifuge tubes inside an desiccator jar together with a beaker containing 100 ml of 12 % hypochlorite solution (“chlorine bleach”). Subsequently, 10 ml of 37 % HCl was added directly into the hypochlorite solution to allow chlorine gas to be produced. The lid of the desiccator was immediately closed and vacuum was generated by a connection to a vacuum pump until an air tight seal was obtained. This was let stand for 4 – 8 h. After the sterilisation

period, the desiccator jar was opened carefully under a fume hood and seeds were quickly closed and removed from the jar. Seeds were placed in a sterile hood and let stand for 15 min in the opened vessel. Sterilized seed were stored for several days at 4° C or directly plated out on suitable culture media. Alternatively the seeds were sterilized using columns from DNA/RNA prep kits by subsequent incubations and washes with ethanol 70% for 2 min followed by ethanol 95% for 1 min. Afterwards, tubes were centrifuged for 1 min to remove all ethanol and seeds were dried under the sterile hood.

#### 2.2.1.4 *Agrobacterium*-mediated stable transformation of *Arabidopsis* by floral dip

The protocol used for *Agrobacterium*-mediated stable transformation of *Arabidopsis* is based on the floral dip method (Clough and Bent, 1998). Approximately 9 *Arabidopsis* plants were grown in 9 cm square pots under short day conditions for 3-4 weeks and then transferred to long day conditions to induce flowering. First inflorescence shoots were cut to induce the growth of more inflorescences. Plants were used for transformation when a maximum number of young flower heads were present. Transformed *Agrobacterium* was streaked onto selective YEB plates containing the appropriate antibiotics and grown at 28°C for approximately 2-3 days. The content of the plate was collected and dissolved in 30 ml YEB liquid media to an $OD_{600}$ of approximately 2. The bacterial solution was then mixed with 120 ml of solution with 5% sucrose and 0.03% Silwet L-77 (Lehle seeds, USA) to a final $OD_{600}$ of 0.4. Plants to be transformed were inverted and all inflorescences submerged in the bacterial suspension for 5-10 seconds with gentle agitation. Dipped plants were then covered with a plastic bag to maintain high humidity and placed away from direct light for 24 hrs. Afterwards, bags were removed and pots transferred to long-day conditions and left to set seeds.

#### 2.2.1.5 Glufosinate selection of *Arabidopsis* transformants on soil

Seeds collected from floral-dipped plants were densely sown on soil and germinated as described before. Once cotyledons were fully opened but before true leaves appeared, approximately 10 d after sowing, young seedlings were sprayed with 0.1 % (v/v) Basta® (the commercial product of glufosinate). This treatment was repeated twice on a two day basis. Only transgenic *Arabidopsis* plants carrying the phosphinothricin acetyltransferase (*PAT*) gene that confers glufosinate-resistance survived while untransformed plants died.

#### 2.2.1.6 Lactophenol trypan blue staining

Lactophenol trypan blue staining was used to visualize *H. parasitica* mycelium and necrotic plant tissue (Koch and Slusarenko, 1990). Trypan blue stock solution was diluted with 1 volume of ethanol 95% before use. Leaf material was placed in a 15 ml Sarstedt tube (Nümbrecht, Germany), covered with lactophenol trypan blue and placed in boiling water bath for 2 min. For destaining, the solution was replaced with chloral hydrate (2.5 g/ml $dH_2O$) and incubated overnight with shaking. Leaf material was left in 70% glycerol for at least 2-3 hours before mounting onto glass microscope slides with 70% glycerol and examined using a light microscope.

#### 2.2.1.7 Pathogen maintenance and pathology assays

*H. parasitica* isolates were maintained as mass conidiosporangia cultures on leaves of their genetically susceptible *Arabidopsis* ecotypes over a 7 d cycle (see 2.1.2). *H. parasitica* inoculations were done on 2-week-old plants by spray-inoculation with *H. parasitica* conidiospores ($4x10^4$ spores/ml). To determine pathogen conidiospore numbers, replicate samples of 300-500 mg of infected tissue were harvested at the indicated times after inoculation, vortexed in water and counted in a Neubauer chamber on a light microscope. Plant cell necrosis and development of *H. parasitica* hyphae in leaf tissue was monitored by lactophenol trypan blue staining. *P. syringae* virulent and avirulent DC3000 strains used (described in 2.1.2) were maintained by streaking onto selective NYG agar plates, incubation in 28°C for 48 h and stored at 4°C. *P. syringae* pv. *tomato* inoculations were performed on 4-5 week-old plants by hand infiltration with a bacterial suspension of $1x10^5$ cfu/ml in 10 mM $MgCl_2$ or spray inoculation with a bacterial suspension of $1x10^5$ cfu/ml in 10 mM $MgCl_2$ supplemented with 0,04% Silwet L-77. *In planta* bacterial titers were determined by shaking leaf discs from infected leaves in 10 mM $MgCl_2$ supplemented with 0,01% Silwet L-77 at 28°C for 1 h (Tornero and Dangl, 2001). The resulting bacterial suspensions were serially diluted and spots of 20 µl per dilution were grown on selective NYG agar medium at 28°C. Systemic acquired resistance assays were performed as described (Maldonado et al., 2002). SAR was monitored by comparing the growth of virulent *Pst* DC3000 in plants induced for SAR, to growth in uninduced plants (mock-treated). Three to four-week-old plants were induced by hand-infiltration of two leaves with avirulent *Pst* AvrRpt2 ($1x10^6$ cfu/ml in 10 mM $MgCl_2$) or mock-inoculated with 10 mM $MgCl_2$ and three days later challenged by inoculation of systemic

leaves with virulent *Pst* DC3000 ($1x10^5$ cfu/ml). Bacterial titers were determined as previously described.

#### 2.2.1.8 Generation of *Arabidopsis* protoplasts and shuttling assay

*Arabidopsis* leaf meshophyll protoplasts were prepared according to Asai et al 2000, with some modifications. Protoplasts were prepared from leaves of 4-week-old plants, grown in normal light/dark regime. Leafs were cut in strips and placed in enzyme solution (0.4 M Mannitol; 20 mM KCl; 20 mM MES pH 5.7) with 1.5% cellulase (Onozuka R-10, Merck, Darmstadt, Germany) and 0.4% Macerozyme (R-10, Serva, Heidelberg, Germany). Solution was vacuum infiltrated for 3 minutes, incubated for 30 minutes with the pressure in the vacuum desiccator, and after that for 2 hrs at 65-80 rpm on a platform shaker, in light and at room temperature. The protoplast solution was filtered through a 62 µm nylon mesh and centrifuged at 500 rpm for 3 minutes. The protoplast pellet was washed with W5 solution (154 mM NaCl, 25 mM $CaCl_2 \cdot 2H_2O$, 5 mM KCl, 2 mM MES pH 5.7) and centrifuged again. Protoplast pellet was resuspended in Mannitol solution (0.4 M Mannitol, 15 mM $MgCl_2 \cdot 6H_2O$, 4 mM MES, pH 5.8). The nuclear export inhibitor Ratjadone A (Alexis Biochemicals) was dissolved in methanol (10 ng/µl) and added to the protoplast containing solution at a final concentration of 15 ng/ml. Control sample was mock treated-with an equal concentration of methanol.

#### 2.2.1.9 *Agrobacterium*-mediated transient transformation of *Nicotiana benthamiana* leaves

*Agrobacterium* containing the constructs of interest were cultured overnight at 28°C in 4 ml of selective liquid YEB media containing the appropriate antibiotics. The culture was spun down, bacteria resuspended in 5 ml selective induction medium containing 50 µl/ml Acetosyringone and grown further for 4-6 h. Cultures were spun down and the pellet was resuspended in infiltration medium (10 mM MES, 10 mM $MgCl_2$, pH 5.3-5.5) to an $OD_{600}$ of 0.4. The bacterial solution was let stand at room temperature for 1-3 h. Young *N. benthamiana* leaves were hand-infiltrated with a needle-less 1 ml syringe on the underside. Infiltrated leaf areas were used for microscopy or protein extracts 2 d after infiltration.

#### 2.2.1.10 Transient transformation of *Arabidopsis* leaves by particle bombardment

The biolistic particle delivery is a transient transformation method that uses helium pressure to introduce DNA coated on gold or tungsten particles (microcarriers) into living cells. Detached 3 week-old *Arabidopsis* leaves were placed on 1% agar plates containing 85 µM benzimidazole and incubated in a light chamber at 22°C, 2 h prior to the bombardment. The preparation of gold microcarriers for 10 bombardments was carried out as follows: 30 mg of gold microcarriers (1.0 µm diameter, BioRad) were transferred into a 1.5 ml microcentrifuge tube and 1 ml 70% ethanol was added. The suspension was vortexed for 3-5 min on a platform vortexer and then let stand for 15 min to sediment the microcarriers. Tubes were spinned 5 sec and the supernatant removed. The pellet was rinsed 3 times with 1 ml $dH_2O$ through vortexing and sedimentation of the particles, as described. After washing, 500 µl sterile glycerol (50% v/v in $dH_2O$) was added to obtain a concentration of 60 mg/ml of microcarriers (at this step microcarriers can be stores at 4°C for up to 2 weeks). Subsequently, microcarriers were coated with DNA 1 h before using. For this, they were vortexed for 5 min in a platform vortexer to disrupt agglomerated particles. 50 µl of the microcarrier suspension were pipetted while vortexing and transferred to a new 1.5 microcentrifuge tube. While vigorously vortexing, the DNA mixture containing 5 µg DNA, 50 µl 2.5 M $CaCl_2$ and 20 µl 0.1 M spermidine was added. Coated microcarriers were spun down for 2 sec, the supernatant discarded and washed with 70% ethanol. After vortexing and pelleting the coated microcarriers, a similar wash step was performed with 100% ethanol. The coated microcarriers were resuspended in 50 µl 100% ethanol and kept on ice until use. Seven macrocarriers (BioRad) were placed inside the macrocarrier holder of the Hepta Adapter™ (BioRad). 6 µl aliquots of DNA-coated microcarriers were removed from the suspension while vortexing and transferred to each of the seven macrocarriers. After complete evaporation of the ethanol, the Hepta Adapter™ was placed inside the BioRad particle delivery system (Biolistic PDS-1000/He™) and a vacuum of 27 mm Hg was applied. Rupture discs bursting at a pressure of 900 psi were used in the bombardment process. The bombarded leaves were kept in a light chamber at 22° C and fluorescence microscopy were carried out 24 - 48 h after transfection.

#### 2.2.1.11 Microscopy

Light microscopy was performed in an Axiovert 135 TV (Zeiss, Germany) connected to a Nikon DXM1200 Digital Camera. For fluorescence microscopy, *Arabidopsis thaliana* leaves

were mounted in distilled water with 0,01% Tween 20 onto object slides. Confocal laser-scanning microscopy was performed with Leica TCS SPS AOBS (Leica, Wetzlar, Germany).

## 2.2.2 Molecular biologal methods

### 2.2.2.1 Isolation of genomic DNA from *Arabidopsis* (Quick prep for PCR)

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 (200 mM Tris pH7.5; 250 mM NaCl; 25 mM EDTA; 0,5% SDS) were added. Tissue was grinded with a micropestle in the tube and incubated for 10-60 min at room temperature. The solution was centrifuged at 13.000 rpm for 5 min and 300 µl supernatant was transferred to a clean tube. One volume of cold isopropanol (-20°C) was added, the solution was mixed and let stand for 15 min to precipitate DNA. Solution was centrifuged at 13.000 rpm for 5 min and pellet washed with 70% ethanol. Dried pellet was resuspended in 100 µl TE buffer (10 mM Tris-HCl pH 8.0; EDTA 1 mM). This protocol yields small quantity of poorly purified DNA but of sufficient quality for PCR amplification. For each PCR reaction 0.5 – 2 µl DNA solution was used.

### 2.2.2.2 Plasmid DNA isolation from bacteria

Standard alkaline cell lysis minipreps of plasmid DNA were performed using the GFX$^{TM}$ micro plasmid prep kit (Amersham Biosciences) according to the manufacturer's instructions. Larger amounts of plasmid DNA were isolated using QIAGEN Plasmid Midiprep kits.

### 2.2.2.3 DNA manipulation

DNA manipulation and cloning were carried out using standard procedures (Sambrook et al 1989). All PCR-amplified fragments used for cloning were sequenced prior to further investigation.

### 2.2.2.4 Generation of binary vectors for *Arabidopsis* stable transformation

To generate binary destination vectors for Gateway® cloning technology (Invitrogen) suitable for protein localization studies, forward primer MW15 (5´-ATCCCCGGGATGGTGAGCAAGGGCGAGGAGC-3´) and reverse primer MW16 (5´-AGTCTAGAGCTCTTACTTGTACAGCTCGTCCATGC-3´) were used to PCR amplify

yellow fluorescent protein (YFP) from vector pMon999 (Shah et al., 2001). PCR products for *YFP* were digested with *Sma*I and *Xba*I and ligated into the binary vector pXCS-HisHA (Witte et al., 2004) digested by *Sma*I and *Xba*I, to result in pXCS-YFP. A Gateway® recombination cassette (reading frame B, blunt *Eco*RV fragment, Invitrogen) was then ligated into the *Sma*I site of pXCS-YFP. Clones with the right orientation of the Gateway® cassette were selected and named pXCSG-YFP. Vector pXCSG-YFP allows the expression of fusion proteins under control of the constitutive cauliflower mosaic virus 35S promoter (prom35S). To allow the expression of fusion proteins under control of their native promoters, vector pXCSG-YFP was digested with *Asc*I and *Xho*I to remove prom35S. *Pfu Turbo*® DNA polymerase (Stratagene®, Heidelberg, Germany) was used to fill-in the restriction enzyme generated DNA overhangs. Subsequently, the linear, blunt-end vectors were re-ligated and the originated vector was named pXCG-YFP. For fusions of YFP to the C-terminus of the protein of interest, LR reaction between an entry clone and the Gateway® destination vector was performed. Genomic L*er EDS1* sequence including 1.4 kb of endogenous promoter and without the stop codon was cloned into the Gateway® entry vector pENTR/D-TOPO (Invitrogen) and an LR reaction was performed between the destination and entry vectors to generate the vector pXCG-promEDS1-gEDS1-YFP. Constructs were transferred to *Agrobacterium tumefaciens* strain GV3101 (pMP90RK) and used to transform Col *eds1-2* plants (Bartsch et al., 2006) using the floral-dip method (Clough and Bent, 1998). The vector used for *Arabidopsis* transformation confers BASTA® (glufosinate-ammonium) resistance and stable homozygous transgenic lines were selected by growing the seedlings on MS plates supplemented with DL-Phosphinothricin (PPT) 100 µg/ml. A representative line, internally referred to as #1-3, was used for further analysis. The localization signals were attached to the C-terminus of mYFP through PCR amplification of vector pcDNA3-mYFP (obtained from Irine Prastio, Howard Hughes Medical Institut). Functional NLS from SV40 (PKKKRKVGG) and non-functional nls (PKKKRKVGG) (Kalderon et al., 1984) were attached using the primer pair MW15 and AG1 (5´-
AGTCTAGAGCTCTTATCCTCCAACCTTTCTCTTCTTCTTAGGCTTGTACAGCTCGTC
CATGCCG-3´) or MW15 and AG3 (5´-
AGTCTAGAGCTCTTATCCTCCAACCTTTCTCTTCGTCTTAGGCTTGTACAGCTCGTC
CATGCCG-3´), respectively. Functional NES from PKI (LALKLAGLDI) and the non-

functional nes (LALKAAGADA) (Wen et al., 1995) were attached using primer MW15 and the reverse primer AG2 (5´-AGTCTAGAGCTCTTAAATATCAAGTCCAGCCAACTTAAGAGCAAGCTTGTACAGCTCGTCCATGCCG-3´) or the reverse primer AG4 (5´-AGTCTAGAGCTCTTAAgcATCAGCTCCAGCCGCCTTAAGAGCAAGCTTGTACAGCTCGTCCATGCCG-3´), respectively. Afterwards, the same strategy previously described was followed for the generation of pXCG-promEDS1-gEDS1-YFP constructs carrying the localization signals in the C-terminus. A similar construct containing L*er* PAD4 promoter region and cDNA was generated and was designated pXCG-pPAD4-cPAD4-mYFP. Constructs were transferred to *A. tumefaciens* strain GV3101 (pMP90RK) and used to transform Col-0 *pad4-1* plants (Glazebrook et al., 1997) by floral dipping. Stable homozygous transgenic lines were generated and a representative line, internally referred to as #DB9g 1-1, was used for further analysis. GR fusions were obtained by using the vector pBI-ΔGR (Simon et al., 1996). Primer pair GY7-YFP-F (TTGATGGGTCTAGAGATGGTGAGCAAGGGCGAGGAGCTG) and GY11-YFP-R (TGGATCCTCTAGATCCTTGTACAGCTCGTCCATGCCGAG) was used to amplify YFP and primer pair GY8-EDS1-F (GATGGGTCTAGAGATGGCGTTTGAAGCTCTTACCGGAATC) and GY11-YFP-R was used to amplify cEDS1::YFP. PCR products were digested with *Xba*I and ligated in the pBI-ΔGR vector on the *Xba*I site. Constructs were transferred to *A. tumefaciens* strain GV3101 (pMP90) and used to transform L*er eds1-2* plants (Falk et al., 1999). Transgenic lines were selected on medium containing Kanamycin and homozygous lines for the transgenes were generated.

## 2.2.3 Biochemical methods

### 2.2.3.1 Protein Expression Analysis

Total protein extracts were prepared from 50 mg leaf tissue from 3- to 5- week-old plants. Liquid nitrogen frozen samples were homogenized by grinding 2 x 20 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. Ground tissue was resuspended in 150 µl of 2x SDS-PAGE loading buffer

(0.125 M Tris-HCl, 4% SDS, 20% Glycerol, 0.02% Bromophenol blue, 0.2 M DTT pH 6.8) and boiled for 8 min with shaking. Subsequently, to remove cell debris and beads, samples were centrifuged for 5 min at full speed and 4°C and supernatant was recovered. Samples were stored at -20°C or directly loaded onto SDS-PAGE gels.

#### 2.2.3.2 Nuclear fractionation for immunoblot analysis

Nuclear fractionations were performed according to Feys et al. (2005). Two grams fresh weight of leaf tissue from 3- to 4-week-old plants were homogenized in 4 ml Honda buffer (2.5% Ficoll 400, 5% Dextran T40, 0.4M Sucrose, 25 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$ and added before use: 5 mM DTT, 1% protease inhibitor cocktail (SIGMA)) and then filtered through a 62 µm (pore size) nylon mesh. Triton X-100 was added to a final concentration of 0.5%, mixed slowly and the mixture was incubated on ice for 15 min. Total fraction aliquot was taken at this point. The extract was centrifuged at 1500 g for 5 min and nuclei-depleted fraction aliquot was taken from the supernatant. The pellet was washed by gentle resuspension in 3 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 3 ml of Honda buffer and transferred to 1.5 ml microcentrifuge tubes. Starch and cell debris were removed by centrifugation at 100 g for 1 min. Supernatants were transferred to new microcentrifuge tubes and nuclei were pelleted by centrifugation at 2000 g for 5 min. Nuclear pellets were resuspended in 150 µl 2x SDS-PAGE loading buffer, and this sample was called nuclei-enriched fraction. Total and nuclei-depleted fractions were mixed with 1 volume of 2x SDS-PAGE loading buffer and all samples were boiled for 8 min with shaking.

#### 2.2.3.3 Denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Denaturing SDS-PAGE was carried out using Mini-PROTEAN® 3 system (BioRad) and discontinuous Tris-Glycine polyacrylamide (PAA) gels were prepared according to standard precedures (Sambrook et al., 1989). Resolving gels were poured between two glass plates and overlaid with 500 µl of water-saturated n-butanol or 50 % isopropanol. After gels were polymerized the alcohol overlay was removed and the gel surface rinsed with $dH_2O$. Stacking gel was poured onto the top of the resolving gel, a comb was inserted and the gel was allowed to polymerize. In this study, resolving gels used were 8, 10 or 12 % polyacrilamide and stacking gel 5 % polyacrilamide. Gels prepared were of 0.75, 1.0 or 1.5 mm thickness. In some

cases, 4-20 % Tris-Glycine PAGEr® precast gradient gels (Lonza Rockland Inc. USA) were used. After removing the combs, each PAA gel was placed into the electrophoresis tank and submerged in 1x Tris-glycine electrophoresis running buffer (25 mM Tris, 250 mM glycine pH 8.3, 0.1% SDS). A pre-stained molecular weight marker (Precision plus protein standard dual color, BioRad) and denatured protein samples were loaded onto the gel and run at 100 V (stacking gel) and 110-150 V (resolving gel) until the desired separation was reached.

#### 2.2.3.4 Immunoblot analysis (Western blotting)

Proteins were electroblotted from the PAA gels to Hybond™-ECL™ nitrocellulose membranes (Amersham Biosciences) for protein gel blot analysis. PAA gels and membranes were pre-equilibrated in transfer buffer (39 mM glycine, 48 mM Tris base pH 8.3, 0.037% SDS, 20% methanol) for 5 min. Blotting apparatus (Mini Trans-Blot® Cell, BioRad) was assembled according to the manufacturer instructions. Transfer was carried out at 110 V for 70 min. Equal loading was monitored by staining membranes with Ponceau S (Sigma-Aldrich). Ponceau S stained membranes were scanned, distained with TBS-T (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween®20, pH 7.5) and blocked for 1 h with sacking at room temperature in TBS-T containing 5% (w/v) milk powder (Roth). After blocking, membranes were washed briefly with TBST-T and incubated with primary antibodies (see 2.1.9) overnight at 4°C with slow shacking. Afterwards, membranes were washed 3 x 15 min with TBS-T and antibody-bound proteins were detected by incubating for 1 h at room temperature with horseradish peroxidase–conjugated secondary antibodies (see 2.1.9). Antibody solution was removed and membranes were washed 3 x 15 min with TBS-T. Detection was performed using the SuperSignal® West Pico chemiluminescence kit alone or in a 9:1 – 3:1 mixture with SuperSignal® West Femto Maximum Sensitivity kit (Pierce) according to the manufacturer instruction. Luminescence was detected by exposing the membranes to a photographic film (BioMax light film, Kodak).

# 3 RESULTS

## 3.1 EDS1 localization in defense-activated tissues

It was previously shown through transient expression of fluorescent protein (FP)-tagged proteins and cellular fractionation experiments that EDS1 and PAD4 localize to the cytosol and the nucleus, whereas SAG101 localizes only to the nuclei of *Arabidopsis* cells (Feys et al., 2005). I aimed to identify where in the cell EDS1 is localized during defense responses and assess whether relocalization or enrichment in a particular compartment is associated with the establishment of resistance. I generated transgenic lines expressing genomic L*er EDS1* sequence without translational stop codon with a C-terminal fusion to the monomeric yellow fluorescent protein (mYFP) under the control of its endogenous promoter (promEDS1:gEDS1-mYFP) in the Col-0 *eds1-2* null mutant background (Bartsch et al., 2006). I obtained stable homozygous lines and confirmed the functionality of EDS1-YFP fusion in *RPP4* (TIR-NB-LRR) mediated resistance to *H. parasitica* isolate Emwa1 (van der Biezen et al., 2002) (see Figure 3.7A) and basal defense to virulent *H. parasitica* isolate Noco2 (data not shown). A representative complementing line with detectable fluorescence was selected. Hereafter this line is referred to as EDS1-YFP.

Fluorescence analyses in healthy epidermal cells of EDS1-YFP stable transgenic plants through confocal laser scanning microscopy (CLSM) showed a similar localization of EDS1 as observed in transient assays (Figure 3.1, left panel). To gather information about the interacting partners, stable transgenic lines expressing L*er PAD4* cDNA sequence with a C-terminal fusion to mYFP under the control of its endogenous promoter (promPAD4:cPAD4-mYFP) were generated in the Col-0 *pad4-1* null mutant background (Glazebrook et al., 1997). Among several independent transgenic lines generated that complemented the *pad4-1* null mutant, I could not detect fluorescence in healthy tissue probably due to low levels of PAD4 accumulation. One representative line was selected for further studies and is hereafter referred to as PAD4-YFP line.

To monitor possible prolonged redistribution of EDS1 protein, I examined EDS1-YFP fluorescence after infection with *H. parasitica* isolate Noco2 which is virulent on Col-0 and

hence is able to colonize the tissue. Figure 3.1 (middle panel) shows a representative CLSM picture 6 d after infection, which reveals enhanced fluorescence in both cytosolic and nuclear compartments. EDS1-YFP total protein levels were also found to be induced upon *H. parasitica* Noco2 infection on a protein gel (Western) blot (see Figure 3.6B). While I could not detect PAD4 fluorescence in healthy tissue, fluorescence was observable in both nuclei and cytosol of *Arabidopsis* leaves 5 d after infection with virulent *H. parasitica* Noco2 (Figure 3.1, right panel), in accordance with the localization observed in transient expression assays of FP-tagged PAD4 (Feys et al., 2005). This result indicates that the PAD4 native promoter sequence used to drive transgene expression is responsive to pathogen infection, as shown for *PAD4* mRNA in previous studies (Jirage et al., 1999; Feys et al., 2001; Bartsch et al., 2006). Furthermore, analysis of fluorescent protein tagged-SAG101 transgenic lines show enhanced fluorescence after infection, with a fluorescence signal confined to the nuclear compartment before and after pathogen treatment (J. Bautor and J. Parker, unpublished).

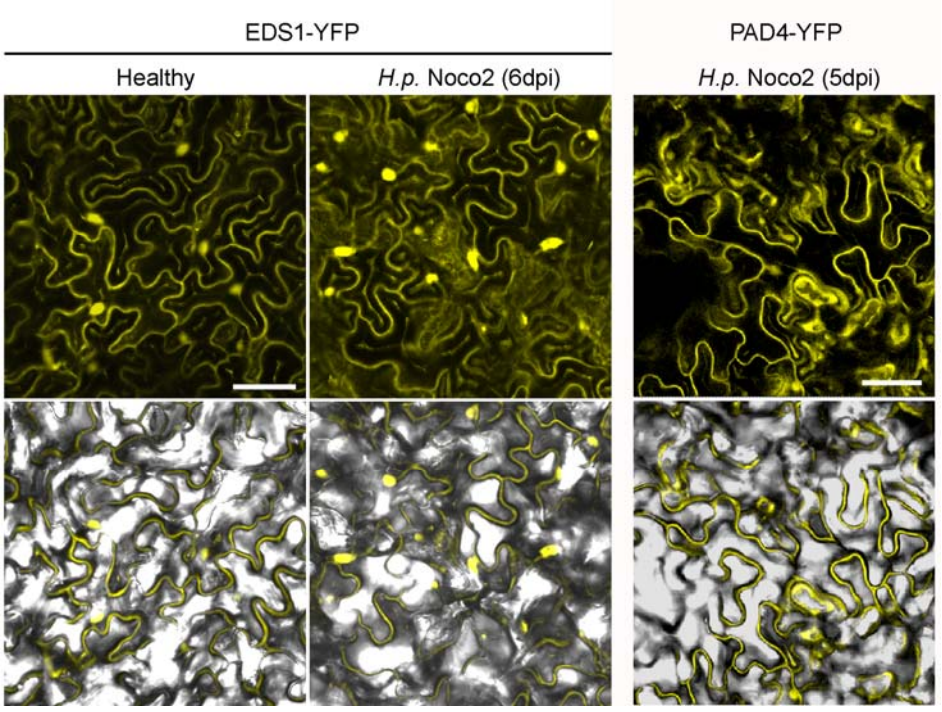


**Figure 3.1. Subcellular distribution of YFP-tagged EDS1 and PAD4 in stable transgenic plants infected with virulent *H. parasitica* isolate Noco2.** Left panel: Confocal images of YFP fluorescence and its overlay with bright field in leaf epidermal cells of a selected EDS1-YFP transgenic line in Col *eds1-2* mutant background. Pictures were taken from healthy leaves or from leaves 6 dpi with virulent *H. parasitica* Noco2. Bar = 15 µm. Right panel: Confocal image of YFP fluorescence and its overlay with bright field in leaf epidermal cells of a selected PAD4-YFP transgenic line in *pad4-1* mutant background, 5 dpi with virulent *H. parasitica* Noco2. Bar = 15 µm.

To further confirm EDS1 localization during basal defense, I studied EDS1 nucleocytoplasmic partitioning in wild-type Col-0 plants 7 d post-infection with *H. parasitica* Noco2 by cell fractionation. I prepared total, nuclei-depleted and nuclei-enriched fractions from leaves of 3-4 week old plants according to Feys et al. (2005), and determined the presence of EDS1 on protein gel blot. In accordance with the fluorescence microscope analysis, Western blots revealed enhanced EDS1 protein levels in total as well as in both the nuclear and cytoplasmic compartments but no major redistribution of EDS1 protein (Figure 3.4). These results suggest that at late stages of infection with a virulent pathogen there is strong upregulation of EDS1 and its signaling partners. This occurs in the same compartments in which these proteins are localized in un-triggered tissue. Thus no prolonged redistribution of EDS1 or PAD4 occurs during basal defense responses.

To study EDS1 localization in a constitutive resistant background in which all cells are probably equally induced, I made use of the immune deregulated mutant *snc1* (in Col-0) in which the EDS1 pathway is activated. Phenotypes associated with *snc1* mutation include dwarfism, constitutively high levels of SA and enhanced resistance to pathogens, all of which are blocked by mutations in *EDS1* or *PAD4* (Li et al., 2001; Zhang et al., 2003). With the generation of the Col *eds1-2* mutant (Bartsch et al., 2006), it was crossed with *snc1* to generate *snc1*/Col *eds1-2* double mutants in full Col-0 background. I was able to confirm that also in this case the *eds1-2* mutation compromised *snc1* phenotypes (see Figures 3.9A and 3.10B) and therefore included the double mutant in our analysis. Consistent with EDS1 pathway being constitutively active, EDS1 total protein levels were highly upregulated in the *snc1* mutant background (Figure 3.2A). I then assessed EDS1 levels in nuclei-depleted and nuclei-enriched fractions. The protein blots revealed enhanced EDS1 levels in both fractions without any obvious redistribution (Figure 3.2A). To examine PAD4 accumulation, α-PAD4 polyclonal antisera was generated and purified by immunoprecipitation against PAD4 produced in *Escherichia coli* (S. Rietz and J. Parker, unpublished). Due to PAD4 low abundance, obtaining a PAD4 signal in a Western blot proved to be difficult. Fortunately, I could observe a PAD4 signal in nuclei-depleted and nuclei-enriched fractions of *snc1* and determine that PAD4 protein levels are enhanced in this auto active mutant in a similar fashion to EDS1 (Figure 3.2A).

I crossed the *snc1*/Col *eds1-2* double mutant with the EDS1-YFP transgenic line in Col *eds1-2* mutant and assessed the effect of *snc1* on EDS1 localization through confocal fluorescence microscopy. As expected, *snc1* auto immunity led to a great enhancement in EDS1 fluorescence without an obvious difference in nucleocytoplasmic partitioning (Figure 3.2B). We could conclude from these analyses that *snc1* phenotype is not associated with a prolonged relocalization of EDS1 or PAD4. A possible explanation would be that changes in protein amounts in one compartment are rapidly equilibrated by a corresponding change in the other protein pool. Recent findings regarding EDS1 localization in *mos7-1* (*modifier of snc1, 7*) plants which carry a mutation in a homologue of human nucleoporin Nup88, show reduced EDS1 protein levels in both nucleocytoplasmic compartments (Cheng et al., submitted). The authors hypothesize that reduced nuclear EDS1 accumulation may contribute to the ability of *mos7-1* mutation to suppress *snc1* and that a direct effect of *mos7-1* mutation on EDS1 nuclear accumulation might be compensated by increased protein degradation in the cytoplasm.

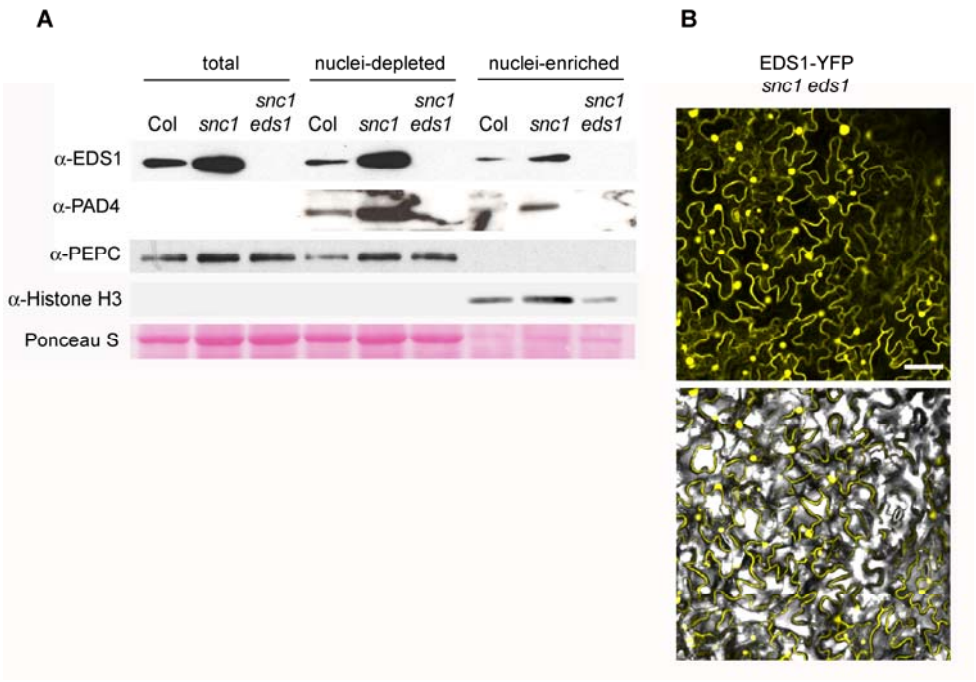


**Figure 3.2. Subcellular distribution of EDS1 and PAD4 in the immune-deregulated mutant *snc1*.**
(A) Protein gel blot analysis of EDS1 and PAD4 in subcellular fractions of unchallenged tissue of wild-type Col, *snc1* single mutant and *snc1*/Col *eds1-2* double mutant plants. Histone H3 and PEPC were used as nuclear and cytosolic markers respectively. Ponceau S staining of the membrane indicates equal loading and transfer onto the membrane. (B) Confocal image of YFP fluorescence and its overlay with bright field in leaf epidermal cells of stable transgenic plants expressing EDS1-YFP in the *snc1*/*eds1-2* mutant background. Bar = 20 µm.

I reasoned that if a transient relocalization of EDS1 takes place which is important for defense activation I may have missed it by examining the constitutive active *snc1* mutant background or advanced stages of infection by virulent *H. parasitica. Arabidopsis* defense responses to *Pseudomonas syringae* are triggered within the first few minutes and hours of inoculation and are stronger during R-mediated responses than during basal defense to the bacteria (Tao et al., 2003). Recently, RPS4 was shown to function in the nucleus and signal entirely through EDS1 (Wirthmueller et al., 2007). RPS4-triggered *EDS1* and *PAD4* transcript accumulation is visible 3 h after infiltration of *Arabidopsis* leaves with *Pst* DC3000 AvrRps4, anticipating or concomitant with the upregulation of target genes (Bartsch et al., 2006). For these reasons I decided to look at EDS1 localization after triggering the TIR-NB-LRR RPS4 by inoculating with *Pseudomonas syringae* pv. tomato (*Pst*) strain DC3000 expressing AvrRps4 (hereafter referred to as *Pst* DC3000 AvrRps4). I triggered RPS4 by spraying with high doses of *Pst* DC3000 AvrRps4 and performed cell fractionation before and up to several hours after spraying with *Pst* DC3000 AvrRps4 and looked at EDS1 total protein amounts and nucleocytoplasmic partitioning on a protein gel blot. These experiments revealed an enrichment in nuclear EDS1 at 2, 3 and 4 hrs after bacterial spraying which was not observed in total or nuclei-depleted fractions (Figure 3.3A and 3.3B). Importantly, EDS1 nuclear enrichment was not present in a sample mock-treated with $MgCl_2$ supplemented with Silwet L-77. Possible causes for the observed specific increase in nuclear accumulation of EDS1 are stabilization of the EDS1 nuclear protein pool, enhanced nuclear retention or enhanced nuclear import. It remains to be tested whether EDS1 nuclear accumulation occurs only during early TIR-NB-LRR-activated responses or also during early stages of CC-NB-LRR-mediated and PAMP-triggered immunity. We are assessing the relevance of this accumulation with respect of the activation of downstream responses, by correlating EDS1 nuclear accumulation with the transcriptional upregulation of EDS1 pathway-target genes.

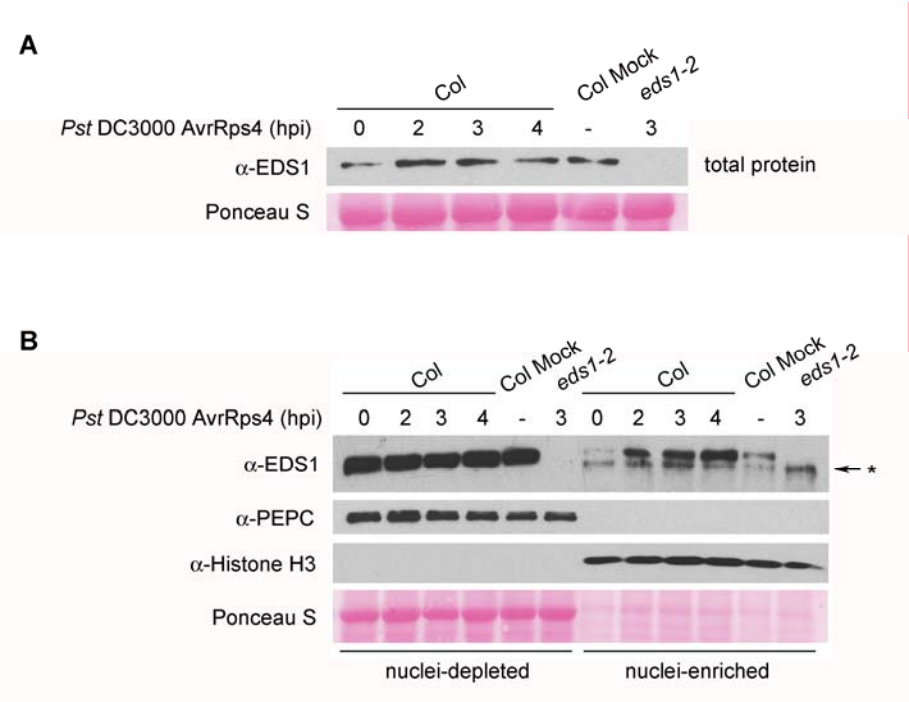


**Figure 3.3. Subcellular distribution of EDS1 during early stages of TIR-NB-LRR (RPS4)-triggered resistance.** Protein gel blot analysis of EDS1 in total (A) and subcellular fractions (B) after spraying with *Pst* DC3000 AvrRps4. Mock treatment of wild-type plants (Col Mock) with $MgCl_2$ containing 0,04% Silwet L-77 was included as control. Histone H3 and PEPC were used as nuclear and cytosolic markers respectively. Ponceau S staining of the membrane indicates equal loading and transfer onto the membrane. The asterisk and arrow indicate a cross-reacting band. Data are representative from two independent experiments.

## 3.2 Role of PAD4 and SAG101 on EDS1 nucleocytoplasmic partitioning

Consistent with EDS1 nuclear localization, protein sequence analysis revealed two putative bipartite nuclear localization signals (see Figure 1.1) (Falk et al., 1999). However, site directed mutagenesis of the conserved basic residues in the consensus sequence coupled with transient expression of a fluorescent protein-tagged fusion, showed no effect on EDS1 nucleocytoplasmic distribution (G. Li and J. Parker, unpublished). This result is not so surprising since there are still many functional NLSs that do not correspond to the consensus rule and many nonfunctional sequences that match the consensus (Kosugi et al., 2009). A recent study further demonstrated that the activity of some NLS require sequences outside the core basic residues (Kosugi et al., 2009). Besides the possibility of not having mutated the right residues, this result raised the option that EDS1 nuclear import depends on a NLS containing protein that would interact with and facilitate the translocation of EDS1 through the nuclear

pore complex to the nuclear compartment. To explore this possibility we assessed the effect of EDS1 known interacting partners, PAD4 and SAG101 on its nucleocytoplasmic distribution. In a previous study, a higher nuclear EDS1-YFP fluorescence was observed when it was cobombarded with SAG101-CFP than when bombarded alone or in combination with PAD4-CFP (Feys et al., 2005). For this reason and the exclusive nuclear localization of EDS1-SAG101 complexes (Feys et al., 2005), I initially speculated that SAG101 protein could have a role in EDS1 nuclear accumulation. However, when EDS1-YFP was bombarded into *sag101-1* or *pad4-1*/*sag101-1* leaves, a proportion of EDS1-YFP fluorescence was still observed in the nuclei suggesting that overexpressed EDS1 does not depend on the presence of the interacting partners to enter the nuclear compartment (Feys et al., 2005). I therefore prepared total, nuclei-enriched and nuclei-depleted cell fractions of healthy *sag101-1* and *pad4-1* single mutant plants and examined EDS1 levels on a protein blot. Both fractions showed a reduction in EDS1 protein comparable to that observed in total protein extracts of wild-type (Figure 3.4); further confirming that EDS1 nucleocytoplasmic partitioning is not grossly affected by loss of each signaling partner in an un-induced state.

To test the role of PAD4 and SAG101 on EDS1 protein levels and partitioning during immune responses, extracts were fractionated from *pad4-1* and *sag101-1* pathogen infected tissue. EDS1 was induced both in *pad4-1* and *sag101-1* single mutants 7 d after infection with virulent *H. parasitica* Noco2 (Figure 3.4), meaning that the reduced EDS1 steady state accumulation observed in the absence of its interacting partners does not affect pathogen inducibility of EDS1 protein. EDS1 partitioning was not affected in the single *pad4-1* or *sag101-1* mutants since a similar ratio to wild-type protein levels was observed in all compartments (Figure 3.4). In the cell fractionation experiments we could observe similar or slightly higher EDS1 nuclear accumulation during basal defenses in the *sag101-1* mutant. These data argue against a role of SAG101 in aiding EDS1 nuclear accumulation. I reasoned that EDS1 nuclear levels may be enhanced in the absence of SAG101 with the possibility of EDS1 taking over a SAG101 nuclear activity, accounting for the weak loss of resistance observable in the single *sag101* mutant background (Feys et al., 2005).

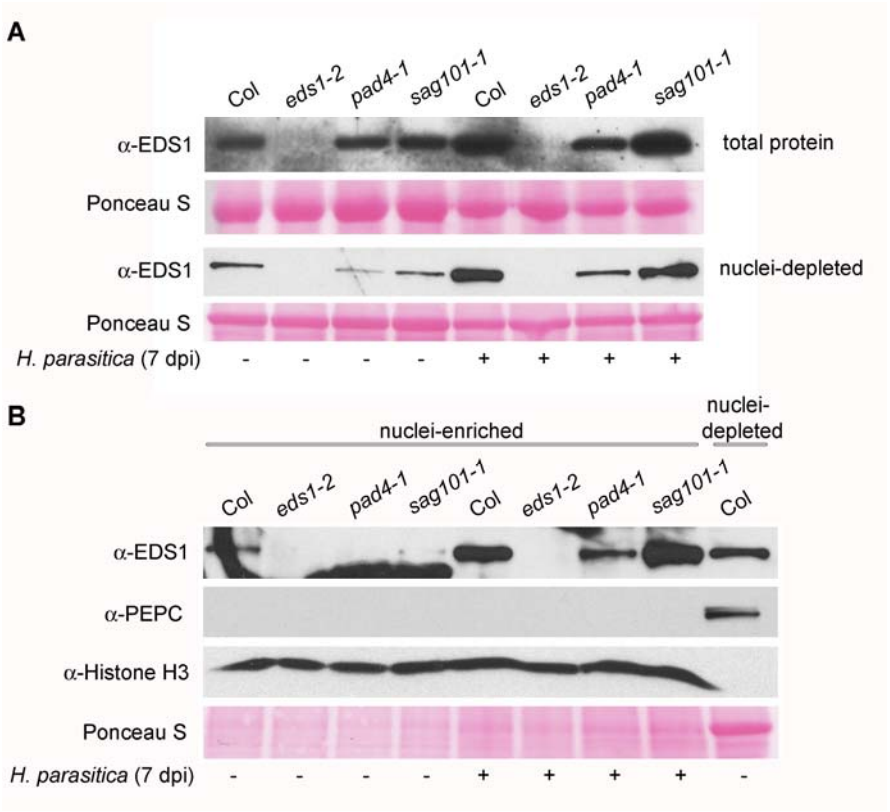


**Figure 3.4. Subcellular distribution of EDS1 in *pad4-1* and *sag101-1* single mutant plants during basal defenses.** Extracts were prepared from 4-week-old healthy plants or 7 dpi with virulent *H. parasitica* Noco2. Protein gel blot analysis of EDS1 in total (top panel), nuclei-depleted (medium panel) and nuclei-enriched (bottom panel) fractions of *pad4-1* and *sag101-1* mutant plants. Histone H3 and PEPC were used as nuclear and cytosolic markers respectively. Ponceau S staining of the membrane indicates equal loading and transfer onto the membrane. Localization analysis in *pad4-1* and *sag101-1* healthy tissues was repeated three times and during basal defense to *H. parasitica* two times, with similar results.

I then studied EDS1 partitioning during RPS4-triggered resistance in *pad4-1* and *sag101-1* single and *pad4-1/sag101-1* double mutants. I prepared cell extracts 3 h after spraying with *Pst* DC3000 AvrRps4 and assessed EDS1 protein levels in the different fractions. Similar to the observations during basal resistance to *H. parasitica*, a similar EDS1 nucleocytoplasmic partitioning was detected in wild-type and single *pad4* and *sag101* mutants (Figure 3.5). As shown in the previous section (Figure 3.3), EDS1 nuclear levels are increased 3 h post inoculation with *Pst* DC3000 AvrRps4 and this is also observed in single *pad4* and *sag101* mutants. By contrast, EDS1 nuclear accumulation was not observed in *pad4-1/sag101-1* double mutant plants. Currently, it is unclear whether RPS4-mediated EDS1 nuclear enrichment is due to protein upregulation taking place preferentially in the nuclear compartment or a mobilization of pre-existing protein. Hence, the absence of *Pst* DC3000

AvrRps4-triggered EDS1 nuclear enrichment in *pad4-1/sag101-1* plants could be explained by a failure in EDS1 pathogen-inducibility or EDS1 nuclear translocation in the absence of both interactors.

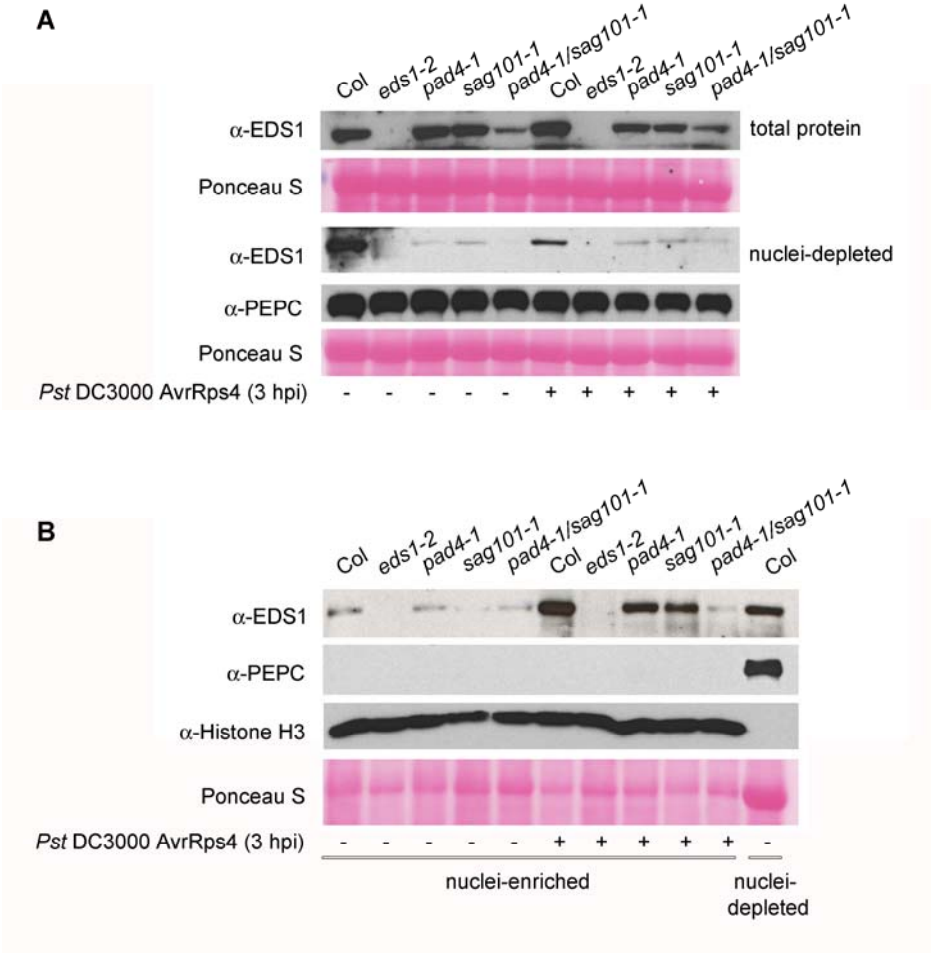


**Figure 3.5. Subcellular distribution of EDS1 in *pad4-1* and *sag101-1* single mutant plants during TIR-NB-LRR (RPS4)-mediated resistance.** Extracts were prepared from 4-week-old plants, unchallenged and 3 hpi with *Pst* DC3000 AvrRps4. Protein gel blot analysis of EDS1 in total (top panel), nuclei-depleted (medium panel) and nuclei-enriched (bottom panel) fractions of *pad4-1* and *sag101-1* single mutant plants and *pad4-1/sag101-1* double mutant plants. Histone H3 and PEPC were used as nuclear and cytosolic markers respectively. Ponceau S staining of the membrane indicates equal loading and transfer onto the membrane. EDS1 localization in *pad4-1* and *sag101-1* mutants after *Pst* DC3000 AvrRps4 inoculation was studied once and must be repeated.

## 3.3 Nuclear exclusion of EDS1 through NES fusion

The data I have shown on EDS1 nuclear enrichment during bacterial defense responses, suggests that nuclear EDS1 might play an important role in EDS1-pathway activation. Furthermore, due to the recent findings regarding nuclear activity of two TIR-NB-LRR proteins (Burch-Smith et al., 2007; Wirthmueller et al., 2007), I hypothesized that nuclear EDS1 is required for race-specific resistance conferred by certain TIR-NB-LRR-type receptors. To explore this hypothesis I decided to interfere with EDS1 nuclear accumulation and assess the consequences on its functionality. Given our failure to identify a functional nuclear localization signal in EDS1 sequence, I decided to target EDS1 to the cytoplasm through fusion of EDS1 to the nuclear export signal (NES) of the mammalian heat-stable inhibitor (PKI) of cAPK ($LALKL_{41}AGL_{44}DI_{46}$) (Wen et al., 1995). I attached the NES to the C-terminus of the gEDS1-mYFP fusion and expressed the fusion under the EDS1 native promoter (promEDS1:gEDS1-mYFP-NES) in the Col *eds1-2* null mutant background (hereafter referred to as EDS1-YFP-NES lines). A control construct was made with a C-terminal non-functional mutated signal (nes, $LALKA_{41}AGA_{44}DA_{46}$) and transformed into Col *eds1-2* (hereafter referred to as EDS1-YFP-nes lines). The effects of these fusions on EDS1 distribution inside the cell was first tested by transient expression in *Arabidopsis* (by particle bombardment) and in *Nicotiana benthamiana* (by agroinfiltration) and detailed analyses of intracellular fluorescence performed by CLSM. For better analysis of the subcellular distribution, a construct driving the expression of mCFP under the cauliflower mosaic virus 35S promoter (prom35S:mCFP) was coexpressed in *N. Benthamiana* which distributed freely in nucleus and cytoplasm. These experiments confirmed the functionality of the NES tag which largely excluded the fusion protein from the nucleus, while the non-functional mutated nes tag allowed nucleocytoplasmic distribution of the fusion protein similar to wild-type localization of EDS1 (data not shown).

Multiple independent stable transgenic lines were generated and lines expressing detectable protein on a Western blot and fluorescence viewed under the confocal microscope were selected for further analysis (Figure 3.6A and C). Figure 3.6C shows representative confocal laser scanning microscopy images that confirm the expected cytoplasmic localization in EDS1-YFP-NES and nucleocytoplasmic distribution in EDS1-YFP-nes transgenic lines.

Analysis of multiple image stacks obtained through confocal microscopy allowed us to conclude that fusion protein was largely excluded from nuclei of healthy tissue of EDS1-YFP-NES lines. As can be seen in Figure 3.6B, the amount of fusion protein expressed in these lines was in the range between wild-type EDS1 levels and the amount of fusion protein in the selected complementing EDS1-YFP line. Line NES #2-10 with a lower level of EDS1 expression was included for analysis in case the efficiency of nuclear export might be insufficient to shunt all EDS1 out of the nuclei in lines with higher expression (NES #2-11 and NES #3-4). Importantly, fusion of the NES to EDS1-YFP did not preclude protein increase after pathogen inoculation (Figure 3.6B). The observed protein induction could be due to transcriptional upregulation or protein stabilization, and would imply either that nuclear EDS1 is not necessary for its own transcriptional or post-transcriptional upregulation. Alternatively, a transient or small residual pool of nuclear EDS1-YFP-NES would be enough to fulfill this function.

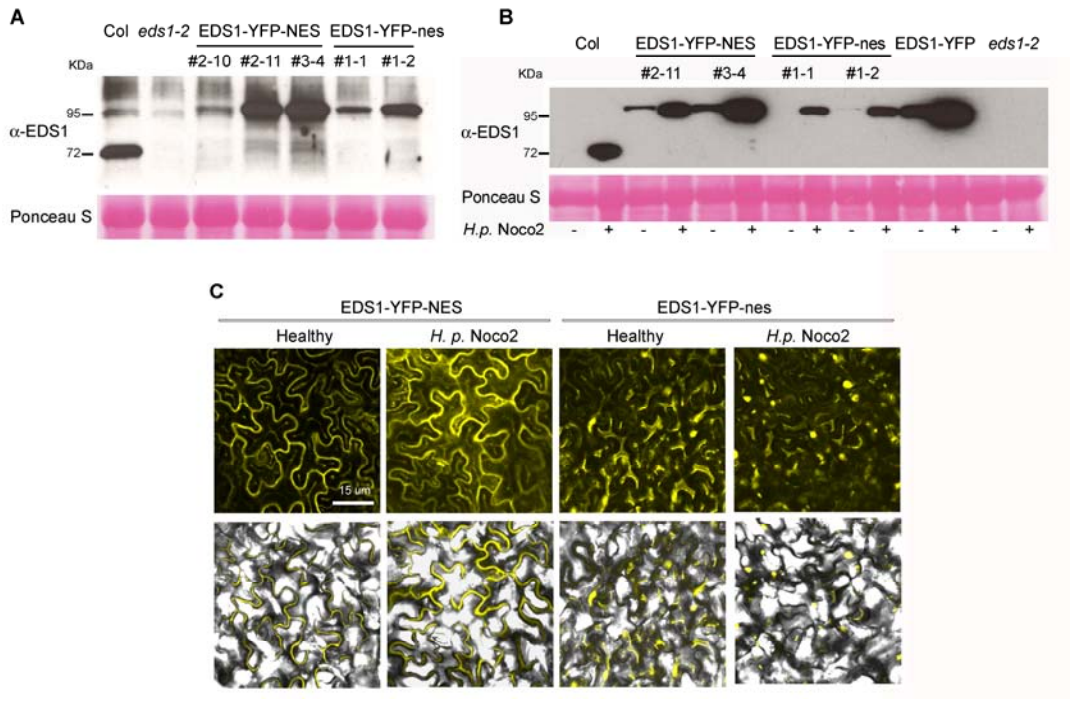


**Figure 3.6. Accumulation and subcellular distribution of EDS1-YFP-NES or EDS1-YFP-nes fusion proteins in healthy and pathogen challenged tissue of independent transgenic lines.** (A) Protein gel blot of total protein extracts from EDS1-YFP-NES/nes transgenic lines probed with anti-EDS1 and anti-GFP antibodies. Protein extracts were prepared from 4-week-old untreated plants. Ponceau S staining of the membrane indicates equal loading and transfer onto the membrane. (B) Protein gel blot of total protein extracts from EDS1-YFP-NES/nes transgenic lines probed with anti-EDS1 antibody. Protein extracts were prepared from 3-week-old plants that were either untreated or 7 dpi with virulent *H. parasitica* Noco2. Ponceau S staining of the membrane indicates equal

loading and transfer onto the membrane. (C) Confocal images of YFP fluorescence, and its overlay with bright field, in leaf epidermal tissue of EDS1-YFP-NES (line #2-11) and EDS1-YFP-nes (line #1-2) transgenic plants. Images were taken from 3-week-old healthy plants or 6 dpi with virulent *H. parasitica* Noco2. Bar = 15 µm.

Homozygous transgenic lines (selected in the $T_3$ generation) were used to assess the ability of EDS1-YFP-NES/nes fusion proteins to complement the *eds1-2* mutant phenotype in defense activation. EDS1-YFP-NES transgenic lines were first tested for their ability to function in TIR-NB-LRR-conditioned resistance. To test RPP4-mediated resistance to *H. parasitica* Emwa1, 2-week-old seedlings were spray-inoculated with a conidiospore suspension and 6 d after inoculation leaves were stained with lactophenol trypan blue to visualize pathogen growth and plant cell death. As shown in Figure 3.7A, the *eds1-2* mutation completely abolished an HR that was visible in wild-type Col-0 plants and allowed pathogen growth and reproduction which is characteristic of its hypersusceptible phenotype. As expected, EDS1-YFP-nes transgenic plants expressing EDS1 fused to the mutated signal, showed complementation and restored the development of HR and resistance as fully as the EDS1-YFP line. Transgenic plants expressing EDS1-YFP-NES fusion proteins showed spreading HR lesions that were not as efficient as wild-type or EDS1-YFP-nes transgenic plants in containing pathogen growth within the initial site of infection. This weakened resistance was manifested as occasional trailing necrosis and appearance of free hyphae accompanied by delayed sporulation 10 d after inoculation. I then inoculated the EDS1-YFP-NES lines with *Pst* DC3000 AvrRps4 to assess the effect of reduced nuclear EDS1 on RPS4-mediated recognition. Bacterial titers in the leaves were counted 1 h and 3 d post infection. The three EDS1-YFP-NES transgenic lines allowed 1 to 3 log units more growth at day 3 after inoculation when compared with wild-type and the control EDS1-YFP-nes lines (Figure 3.7B). Significantly, the line NES #2-10 with similar protein amounts to the line nes #1-1 gave bacterial titers 3 log units higher than that in the nes #1-1 line. The bacterial titer observed in the line NES #2-10 is in the same range as that observed in the Col-0 *rps4-2* null mutant. These results strongly suggest that EDS1 nuclear amounts may be critical for RPS4-triggered immune response.

To analyze the effect of EDS1 mislocalization on basal defense, EDS1-YFP-NES/nes transgenic lines were inoculated with virulent *H. parasitica* Noco2 and pathogen growth was measured by counting conidiospore production on leaves 6 d after infection. Fusions to the mutated versions of the localization signals showed complementation and pathogen growth

within the same range as wild-type Col-0 plants (Figure 3.7C). EDS1-YFP-NES transgenics displayed a mild increased sporulation when compared to wild-type and EDS1-YFP-nes transgenics, thus a partial loss of basal resistance to the oomycete was present (Figure 3.7C). Again, the line NES #2-10 which shows similar protein levels than the line nes #1-1 showed a significant increase in conidiospore production.

To evaluate further whether EDS1 functions in a similar manner at different levels of defense, EDS1-YFP-NES/nes transgenic lines were tested for their ability to function in systemic acquired resistance (SAR). EDS1 has been shown to be essential for SAR due to the inability of *eds1* mutant plants to generate and/or send a SAR-dependent mobile signal (C. Vlot, L. Jorda and J. Parker, unpublished). I tested whether SAR could be induced in EDS1-YFP-NES/nes transgenic lines and control plants, by comparing the growth of virulent bacteria in systemic leaves of SAR-triggered and mock-treated plants. *Arabidopsis* plants were SAR-induced through hand infiltration of two lower leaves with *Pst* DC3000 AvrRpt2 ($1x10^6$ cfu/ml in 10 mM $MgCl_2$) which is recognized in *Arabidopsis* by the CC-NB-LRR protein RPS2, or mock-inoculated with 10 mM $MgCl_2$. At day 3 after primary inoculation, systemic leaves of SAR-induced or mock-inoculated plants were hand infiltrated with virulent *Pst* DC3000 and bacterial growth was counted 3 d after secondary infection. Wild-type plants and EDS1-YFP-nes transgenic lines mounted a successful SAR response, manifested as a 2 log units reduction of *Pst* DC3000 growth in systemic leaves of SAR-triggered plants with respect to the growth observed in mock-treated plants (Figure 3.7D). This reduction was not observable in the *eds1-2* background due to its SAR-deficient phenotype. EDS1-YFP-NES transgenics allowed bacterial growth that was intermediate between wild-type or control EDS1-YFP-nes lines and *eds1-2* mutant background.

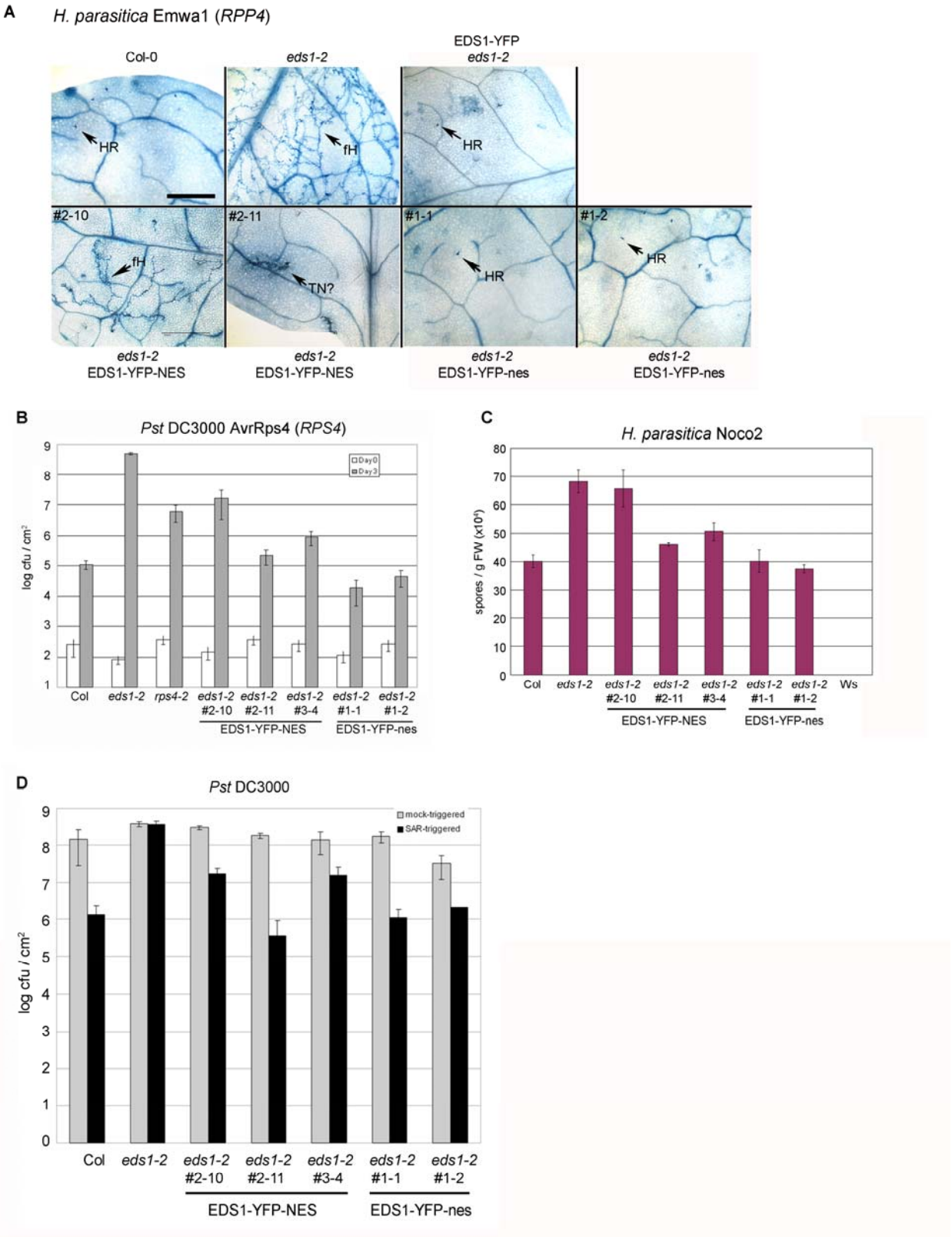
A
H. parasitica Emwa1 (RPP4)
Col-0
eds1-2
EDS1-YFP
eds1-2
HR
fH
HR
#2-10
#2-11
#1-1
#1-2
fH
TN?
HR
HR
eds1-2
EDS1-YFP-NES
eds1-2
EDS1-YFP-NES
eds1-2
EDS1-YFP-nes
eds1-2
EDS1-YFP-nes
B
Pst DC3000 AvrRps4 (RPS4)
log cfu / cm²
Day0
Day3
Col
eds1-2
rps4-2
eds1-2 #2-10
eds1-2 #2-11
eds1-2 #3-4
eds1-2 #1-1
eds1-2 #1-2
EDS1-YFP-NES
EDS1-YFP-nes
C
H. parasitica Noco2
spores / g FW (x10³)
Col
eds1-2
eds1-2 #2-10
eds1-2 #2-11
eds1-2 #3-4
eds1-2 #1-1
eds1-2 #1-2
Ws
EDS1-YFP-NES
EDS1-YFP-nes
D
Pst DC3000
log cfu / cm²
mock-triggered
SAR-triggered
Col
eds1-2
eds1-2 #2-10
eds1-2 #2-11
eds1-2 #3-4
eds1-2 #1-1
eds1-2 #1-2
EDS1-YFP-NES
EDS1-YFP-nes

**Figure 3.7. EDS1 nuclear exclusion through NES fusion leads to partial reduction of resistance.**

(A) Visualization of pathogen growth and dead cells in leaves of 3-week-old plants of the indicated genotypes 6 dpi with avirulent *H. parasitica* Emwa1 through staining with lactophenol trypan blue. HR: hypersensitive response associated cell death; TN: trailing necrosis; fH: free pathogen hyphae. Bar = 500 µm. Data are representative of three independent experiments.

(B) Leaves of 5-week-old plants of the indicated genotypes were hand-infiltrated with *Pst* DC3000 AvrRps4 and bacterial titers were determined 1 h (white bars) or 3 d (grey bars) post infiltration. Values are the average of four samplings from 5 individual plants and error bars represent Standard Error of the mean. Data are representative of three independent experiments.

(C) Pathogen conidiospores were counted on leaves 6 dpi with virulent *H. parasitica* Noco2. Values are the average of 4 replicate samples and error bars represent Standard Error of the mean. Data are representative of two independent experiments.

(D) SAR was analyzed in mock-treated (grey bars) and in *Pst* DC3000 AvrRpt2-infected (black bars) plants by quantifying the systemic growth of virulent *Pst* DC3000. Bacterial titers in systemic leaves were determined in triplicates at 3 dpi and the values represent the average with the corresponding Standard Errors of the mean. This experiment was performed once and must be repeated.

The phenotypes described argue for a weaker complementation of local effector-triggered immunity, basal and systemic defense responses in transgenic plants in which EDS1 nuclear pools are reduced through the fusion of a functional NES, suggesting that increased shunting EDS1 out of the nucleus imposes a detrimental effect on defense activation. The moderate effect observed could be due to the lack of complete nuclear depletion generated by NESs or to a function of cytosolic EDS1 in defense. By comparing the phenotypes of the NES line #2-10 with lower level of transgene expression, with NES lines #2-11 and #3-4, I hypothesize that higher protein levels reduce NES efficiency leading to a more moderate detrimental effect on the activation of resistance responses. It is also possible that the different types of defense responses have different requirements for nuclear EDS1. In particular, the requirement for nuclear EDS1 in R-mediated resistance could be determined by the localization and site of action of the R protein involved. In this study, we examined the functionality of mislocalized EDS1 in RPS4 and RPP4 conditioned defense responses. Nevertheless, RPS4 is the only *Arabidopsis* TIR-NB-LRR protein which has been shown to act in the nucleus and signal entirely through EDS1 for activation of downstream events. For this reason, it is possible that RPS4 requirement for nuclear EDS1 is greater than that of other TIR-NB-LRR proteins which may not function in the nucleus. This idea is consistent with the observation that the line NES #2-10, with lower level of EDS1 protein accumulation, gives similar *Pst* DC3000 AvrRps4 bacterial titers to the *rps4-2* null mutant, indicating that RPS4 response to *Pst* DC3000 AvrRps4 may be disabled by the lack of sufficient nuclear amounts of EDS1.

Another possible explanation for the moderate detrimental effect of EDS1-YFP-NES lines on defense is that a nuclear function of SAG101 is able to complement for the reduced EDS1 nuclear levels. To explore this hypothesis I crossed a selected EDS1-YFP-NES line (#2-11) in the *eds1-2* mutant background to the *eds1-2/sag101-1* double mutant. Plants homozygous for the 3 loci were identified in the $F_4$ generation and were tested in RPP4-mediated resistance to *H. parasitica* Emwa1. Lactophenol trypan blue staining of the leaves 7 d post inoculation revealed no further enhancement of the resistance defect observed in EDS1-YFP-NES lines in the absence of SAG101 (Figure 3.8). These results suggest that SAG101 does not compensate for the removal of nuclear EDS1. For the reasons previously discussed, it would be worth assessing the phenotype of these lines in RPS4-mediated resistance.

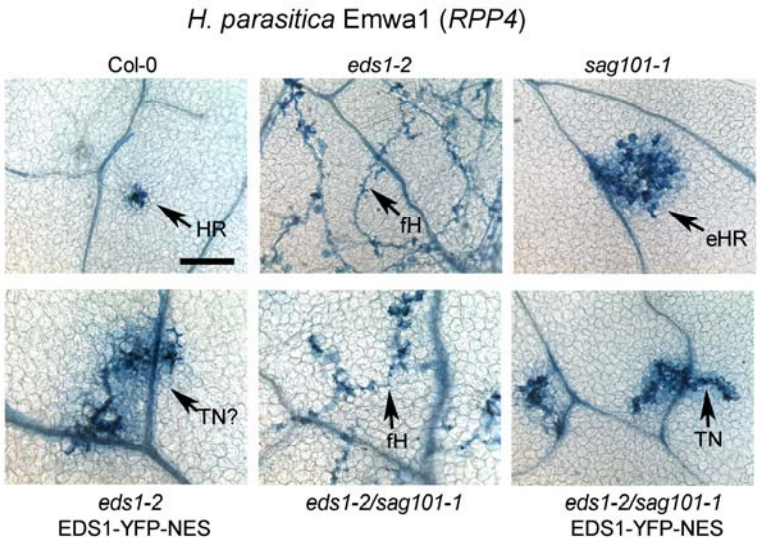


**Figure 3.8. Resistance phenotypes of EDS1-YFP-NES in the *eds1-2/sag101-1* double mutant background and the corresponding controls show that EDS1-YFP-NES partial resistance phenotype is not due to SAG101 nuclear function.** Visualization of pathogen growth and dead cells in leaves of 3-week-old plants 7 dpi with avirulent *H. parasitica* Emwa1 through staining with lactophenol trypan blue. HR: hypersensitive response associated cell death; eHR: enhanced HR; TN: trailing necrosis; fH: free pathogen hyphae. Bar = 200 µm.

## 3.4 Partial complementation of mislocalized EDS1 in snc1 resistance and growth phenotypes

To gain more information about the ability of differentially partitioned EDS1 to complement the *eds1-2* mutation, the promEDS1:gEDS1-mYFP-NES/nes constructs were transformed into the *snc1*/Col *eds1-2* double mutant background. Independent stable transgenic lines were selected for further analyses (referred to as sEDS1-YFP-NES, with the s denoting the *snc1* background). As mentioned before, *snc1* plants contain a gain-of-function mutation in a TIR-NB-LRR gene that results in constitutively high levels of SA, dwarfism and enhanced resistance to virulent *P. syringae* and *Hyaloperonospora parasitica*, all of which are abolished by the *eds1-2* mutation (Li et al., 2001; Zhang et al., 2003). Our hypothesis was that if *mos* mutants suppress *snc1* phenotype through altering EDS1 movement and/or partitioning between the cytosol and the nucleus, the expression of mislocalized EDS1 would have a similar suppressive phenotype. For example, if expression of *snc1* constitutive resistance and growth retardation needs EDS1 in the nuclei, withholding EDS1 in the cytoplasm would not relieve the suppression of an *eds1* mutation. Initial studies showed a difference in the ability of mislocalized EDS1 to complement *snc1* growth and resistant phenotypes (Figure 3.9). sEDS1-YFP-NES transgenic lines resembled morphologically *snc1*/Col *eds1-2* double mutant plants, suggesting an impairment of EDS1-YFP-NES fusion to complement with respect to *snc1* stunted growth phenotype (Figure 3.9A). By contrast, several sEDS1-YFP-NES transgenic lines fully restored *snc1* enhanced resistance to virulent *H. parasitica* Noco2 (Figure 3.9B). As expected, sEDS1-YFP-nes expressing control lines were capable of full complementation in growth and resistance. While we could detect EDS1 fusion protein in the sEDS1-YFP-nes transgenics on a Western blot and by fluorescence microscopy (Figures 3.9C and D), I had difficulties to detect the EDS1-YFP-NES fusion protein in the *snc1/* Col *eds1-2* background (Figure 3.9C).

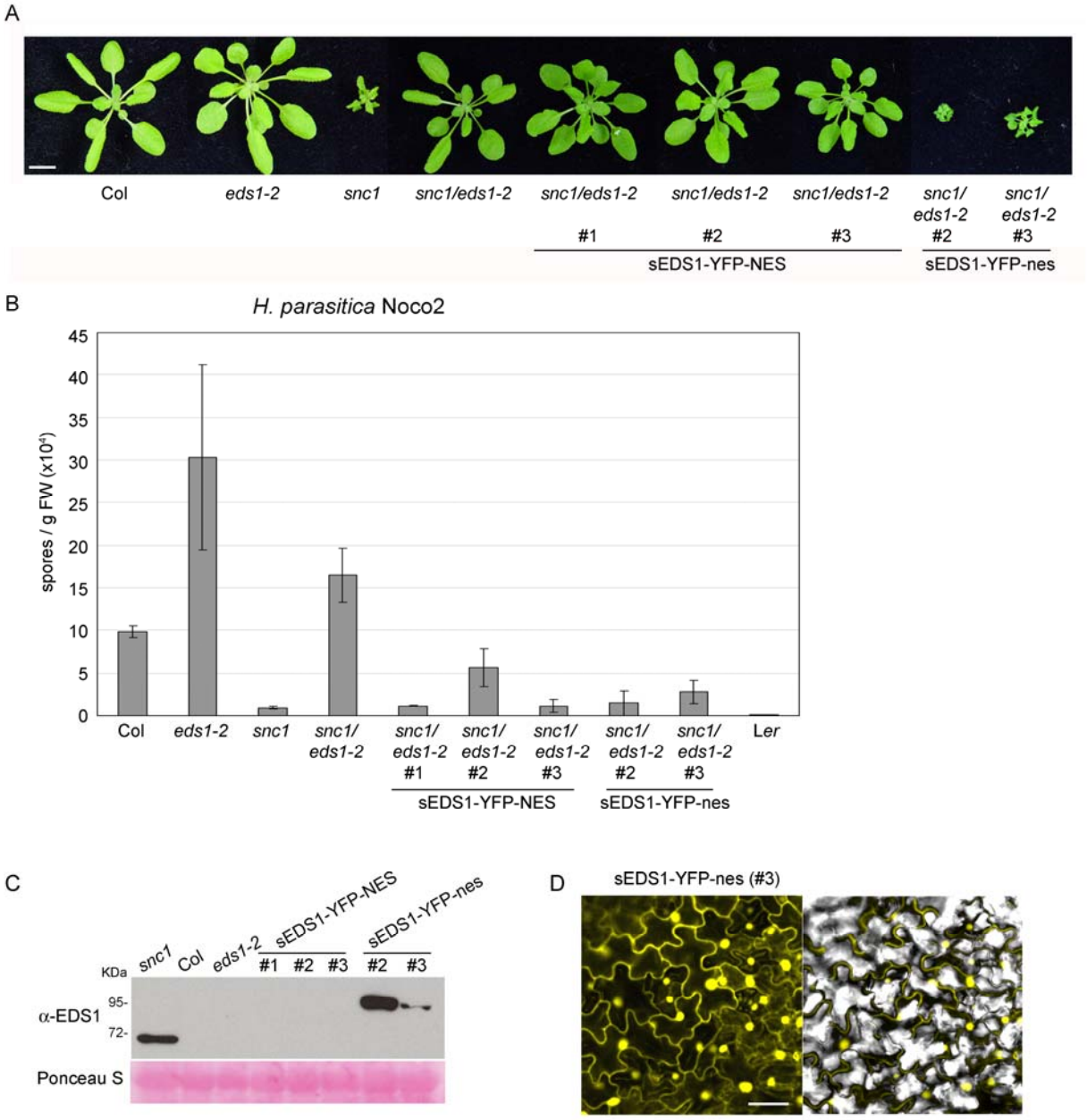


**Figure 3.9. Independent transgenic lines expressing EDS1-YFP-NES in *snc1*/Col *eds1-2* double mutant background show differential complementation of *snc1* growth and resistance phenotypes.**

(A) Representative 4-week-old soil grown plants of the indicated genotypes are shown. Bar = 1 cm.

(B) Pathogen conidiospores were counted on leaves 7 dpi with virulent *H. parasitica* Noco2. Values are the average of 3 replicate samples and error bars represent Standard Error of the mean. Data are representative of two independent experiments.

(C) Difficulty in obtaining detectable signal through protein gel blot and fluorescence microscopy in transgenic lines expressing EDS1-YFP-NES fusion protein. Protein fusion to the non-functional nes presented no problem for detection and localized as expected.

(D) Confocal images of YFP fluorescence in leaf epidermal tissue of sEDS1-YFP-nes transgenic plants in *snc1/eds1-2* double mutant background. Bar = 15 µm.

Rather than attempting to select further sEDS1-YFP-NES transgenics, I decided to cross the already characterized EDS-YFP-NES #2-11 and EDS-YFP-nes #1-2 lines in the single *eds1-2* background to the *snc1*/Col *eds1-2* double mutant, since these lines express detectable EDS1 fusion protein. Homozygous plants for the two mutations and the transgene were identified in the $F_3$ generation and studied further. I could observe clear fluorescence in both lines and confirm the expected localization for the fusion proteins (Figure 3.10A), showing that the enhanced accumulation of EDS1 fusion proteins due to the constitutive activation of EDS1 pathway does not compromise the ability of the NES to exclude the majority of EDS1 from the nuclear compartment. I then examined the growth phenotype of the parental lines and selected crosses. As shown in Figure 3.10B, EDS1-YFP and EDS1-YFP-nes fusions were capable of full complementation for *snc1* growth defect, while EDS1-YFP-NES expressing plants exhibited incomplete complementation. To quantify this effect, I measured the fresh weight of 5-week-old soil grown plants of each line. As expected, *snc1* mutant plants showed drastically reduced growth, manifested as a 35-fold reduction of fresh weight compared to wild-type Col-0 plants (Figure 3.10C). In agreement with the dependence of *snc1* phenotypes on *EDS1*, the growth retardation was substantially alleviated in the *snc1*/Col *eds1-2* double mutant plants which had fresh weights in the range of wild-type. When I examined the transgenic lines obtained through the crosses, I found that EDS1-YFP and EDS1-YFP-nes fusion proteins fully complemented the *eds1-2* mutation and showed similar growth as *snc1* single mutant plants. The analysis of EDS1-YFP-NES expressing plants revealed a small but significant increase in fresh weight with respect to *snc1* plants and *snc1* plants expressing the other two EDS1 fusion proteins. Nevertheless, while the *eds1-2* mutation allowed a 32-fold increase in growth compared with *snc1*, the increase observed in EDS1-YFP-NES expressing *snc1* plants was only 2-fold. Thus, the EDS1-YFP-NES fusion is able to complement almost 95% of EDS1 function in *snc1*-triggered growth retardation.

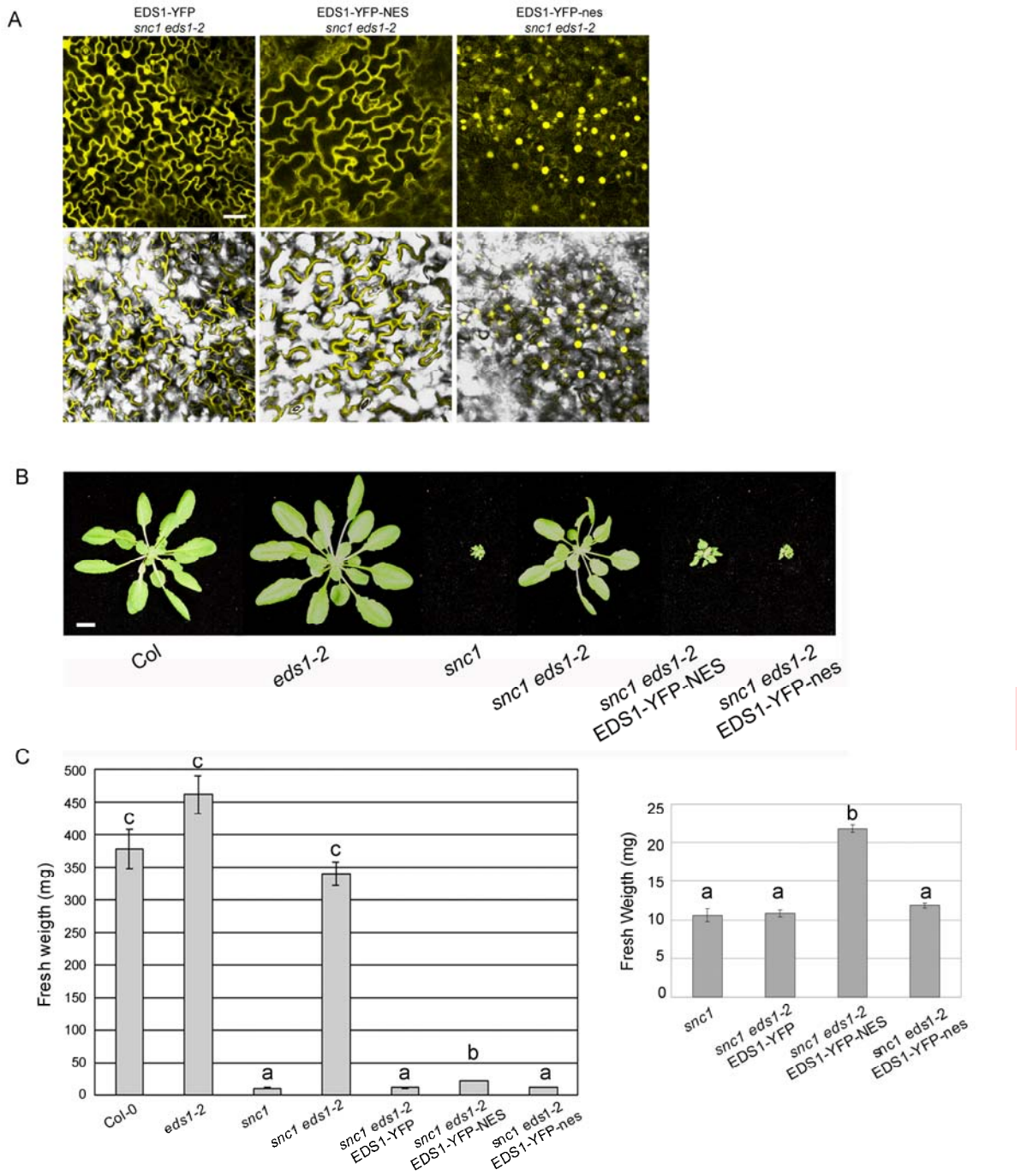


**Figure 3.10. Growth phenotypes in EDS1-YFP-NES/nes lines in the *snc1*/Col *eds1-2* double mutant background, obtained through crosses with transgenic lines with detectable levels of fluorescence.**
(A) Confocal images of YFP fluorescence and its overlay with bright field in leaf epidermal tissue of transgenic plants expressing the indicated EDS1 fusion proteins in the *snc1/eds1-2* double mutant background. Bar = 20 µm.
(B) Representative 5-week-old soil grown plants of the indicated genotypes are shown. Bar = 1 cm.
(C) Average fresh weight (FW) per plant was calculated by measuring the weight of the aerial tissue of 3 5-week-old soil grown plants in 4 independent samplings. Values represent the average and error bars represent the Standard Error of the mean. In the right panel a magnified graph with the FW of smaller genotypes is shown. Characters a, b and c indicate significant differences in FW (t-test; p-value <0,0001)

In a recent report, Wang et al. demonstrated that SA repression of auxin accumulation and auxin-related genes is responsible for the morphological phenotypes observed in SA overaccumulating mutants, such as *snc1* (Wang et al., 2007). From these data and the observed slight increased growth of *snc1*/Col *eds1-2* EDS1-YFP-NES lines, one can speculate that shunting EDS1 out of the nucleus may reduce EDS1 ability to promote SA overaccumulation or SA repression of auxin responses. Interestingly, *snc1* morphological phenotypes were shown to be a recessive trait whereas constitutive expression of resistance was dominant (Li et al., 2001). In the study of Li and colleagues, SA overaccumulation was not confirmed in the heterozygous plants to gain more insight whether the recessiveness of the stunted growth phenotype could be explained by a lower accumulation of SA in the heterozygous plants. One possible explanation is that growth and hyper resistance phenotypes need different SA threshold levels. A mild reduction in SA levels in the heterozygous *snc1* or in *snc1*/Col *eds1-2* plants expressing EDS1-YFP-NES fusion protein could restore wild-type morphology but still be high enough to confer enhanced resistance. Another possibility would be that a signaling molecule other than SA is involved in one of the phenotypes. Measuring SA and auxin levels in the *snc1*/Col *eds1-2* EDS1-YFP-NES lines could help to answer these questions, but the difference in fresh weight is small suggesting that a difference in hormone levels might be difficult to observe. The more pronounced difference observed between the growth of sEDS1-YFP-NES transgenic lines and *snc1* indicate that reduced EDS1 fusion protein accumulation may be responsible for the reduced complementation. Therefore, it may be worth analyzing a cross between *snc1*/Col *eds1-2* and the characterized EDS1-YFP-NES line #2-10 in the Col *eds1-2* background (with lower fusion protein accumulation than the line #2-11).

## 3.5 Nuclear exclusion of EDS1 through a fusion with the Glucocorticoid Receptor Hormone Binding Domain

Another approach to control protein nuclear accumulation makes use of the steroid-binding domain of the mammalian glucocorticoid receptor (GR) (Picard et al., 1988). Proteins fused to GR are retained in the cytoplasm through its association with hsp90 chaperone complex (Dittmar et al., 1997).. In cells treated with the steroid hormone dexamethasone, hsp90 binding is released and the GR domain drives the nuclear localization of the fusion protein through its

two NLSs (Savory et al., 1999). This system has been used with success in plants (Aoyama et al., 1995; Simon et al., 1996). Constructs containing the cDNA sequence of EDS1 driven by the constitutive cauliflower mosaic virus 35S promoter with a translational fusion to YFP followed by the rat GR at the C-terminus (prom35S:cEDS1-YFP-GR, hereafter referred to as EDS1-YFP-GR) were generated by Guangyong Li. He also generated a control construct expressing the fusion protein YFP-GR under the control of 35S promoter (prom35S:YFP-GR, hereafter referred to as YFP-GR). These two constructs were transformed into the L*er eds1-2* mutant background and multiple independent transgenic lines selected. Among these, three independent EDS1-YFP-GR transgenic lines and one YFP-GR transgenic line showing detectable EDS1 fusion protein levels in protein gel blots were chosen for further analysis.

When I monitored total protein extracts for the accumulation of the EDS1 fusion proteins (Figure 3.11A, top panel), I found that expression was not as high as would be expected for a 35S promoter-driven transgene or as that observed in transgenic lines expressing EDS1-StrepII fusions under 35S promoter (prom35S:EDS1-StrepII lines, E. Gobbato et al, manuscript in preparation). Since in the prom35S:EDS1-StrepII lines the ratio between nuclear and cytoplasmic EDS1 protein levels is not altered when compared to wild-type (Gobbato et al, manuscript in preparation) I speculated that the limited overexpression observed in the EDS1-YFP-GR lines may be due to EDS1 cytosolic retention by the GR fusion. Time course analysis after pathogen infection of prom35S:EDS1-StrepII lines, showed enhanced EDS1 protein levels in pathogen challenged tissue that could not be explained by transcriptional induction of the transgene (Gobbato et al, manuscript in preparation). This evidence points to a post-transcriptional control of EDS1 in which EDS1 protein could be stabilized after pathogen infection. I therefore decided to monitor the accumulation of EDS1-YFP-GR fusion protein after pathogen treatment. Total protein levels of the fusion protein EDS1-YFP-GR were induced 7 d post inoculation with avirulent *H. parasitica* Noco2, but this was stronger in those plants that were pretreated with dexamethasone (Figure 3.11A, bottom panel). Since the EDS1-YFP-GR fusion is under the control of the 35S promoter, is unlikely that the higher protein levels are due to transcriptional upregulation. Hence, these results further hint to a pathogen-triggered stabilization of EDS1 protein. The hypothetical stabilization of EDS1-YFP-GR fusion protein seems greater when coupled with dexamethasone treatment, suggesting that allowing EDS1 nuclear accumulation is important for EDS1 protein stability.

I studied EDS1-YFP-GR fusion protein localization by examining in a confocal microscope the fluorescence in EDS1-YFP-GR lines 6 d after infection with virulent *H. parasitica* Cala2, with and without dexamethasone pre-treatment. Figure 3.11B shows representative confocal laser scanning microscopy images that depict the expected nucleocytoplasmic distribution for the EDS1-YFP-GR constructs in plants pretreated with steroid hormone. By contrast, I did not detect a nuclear signal in leaves of pathogen-challenged seedlings that were not pretreated with dexamethasone (Figure 3.11B). Through these observations I could confirm that GR attachment efficiently withholds EDS1-YFP-GR fusion protein in the cytoplasm and foliar dexamethasone treatment allows fusion protein nuclear accumulation. Consistent with the protein blot results (Figure 3.11A), the fluorescence observed in infected plants not pretreated with dexamethasone was weaker than that of the dexamethasone-pretreated infected plants (Figure 3.11B). This further supports the notion that there is post-translational protein stabilization associated with pathogen inoculation and that protein stabilization is greater when the fusion protein is allowed to enter the nucleus through the dexamethasone treatment. One possible explanation is that normal EDS1 distribution between nuclear and cytosolic compartments is important for maintenance of EDS1 protein steady state levels. Alternatively, the nuclear pool of EDS1 becomes stabilized during the defense response. It should also be considered that it could be due to an artifact of the GR fusion. However, since there was a disproportionate increase in native EDS1 nuclear accumulation within hours of inoculation with *Pst* DC3000 AvrRps4 (Figure 3.3), I favor stabilization of EDS1 protein in the nucleus of pathogen challenged tissue as a physiological and important aspect of EDS1 signaling.

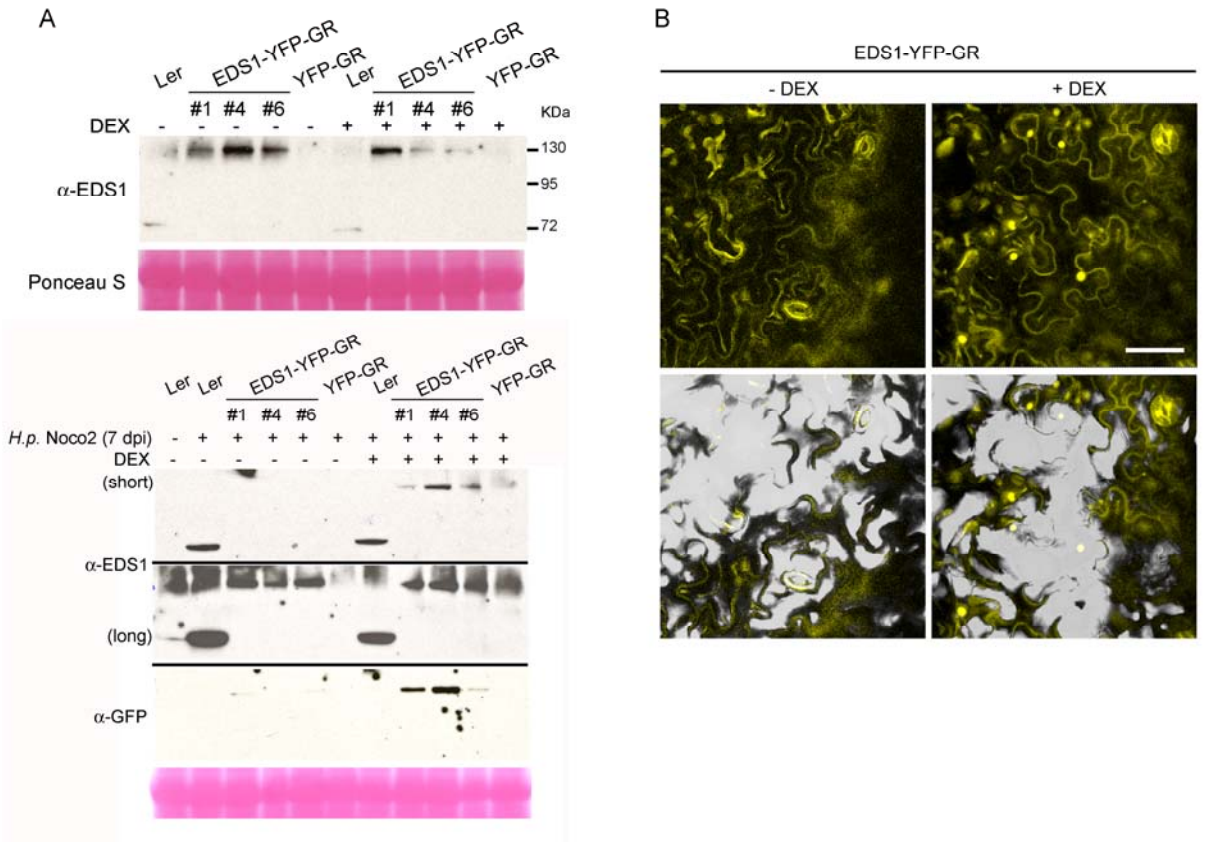


**Figure 3.11. Accumulation and subcellular distribution of EDS1-YFP-GR fusion protein in stable transgenic lines, with or without dexamethasone (DEX) pretreatment.**

(A) Top panel: Protein gel blot of total protein extracts from independent transgenic lines expressing EDS1-YFP-GR or YFP-GR fusion proteins in the L*er eds1-2* mutant background probed with anti-EDS1 antibody. Protein extracts were prepared from 3-week-old plants that were either untreated or after 6 h pretreatment with 30 µM DEX. Bottom panel: Protein extracts of the same transgenic lines were prepared from 3-week-old plants 7 dpi with avirulent *H. parasitica* Noco2. Previous to the infection, plants were either untreated or pretreated with DEX for 6 h. The protein sample loaded in the first lane, corresponding to untreated wild-type L*er*, is the same sample than that of the first lane of the protein blot in the top panel. Its signal was only visible by long exposure of the membrane. Membrane was probed with anti-EDS1 and anti-GFP antibodies. Ponceau S staining of the membrane indicates equal loading and transfer onto the membrane.

(B) Confocal images of YFP fluorescence, and its overlay with bright field, in leaf epidermal tissue of 4-week-old EDS1-YFP-GR (line #1) transgenic plants with or without 6 h pretreatment with 30 µM DEX, 6 dpi with virulent *H. parasitica* Cala2. Bar = 40 µm.

I then measured the ability of EDS1-YFP-GR constructs to complement the *eds1-2* mutation during defense responses, with or without dexamethasone pretreatment. The EDS1-YFP-GR transgenics were first tested for function in TIR-NB-LRR mediated resistance. Trypan blue staining of leaves 7 d after inoculation with avirulent *H. parasitica* Noco2 revealed characteristic RPP5-dependent HR in wild-type L*er* plants and unrestricted growth in YFP-GR expressing transgenics, due to the *eds1-2* mutation (Figure 3.12A) (Aarts et al., 1998).

Importantly, these responses were not affected by dexamethasone pretreatment and I therefore concluded that dexamethasone treatment per se or the expression of the GR containing fusion protein do not affect pathogen colonization. Independent EDS1-YFP-GR expressing lines displayed enlarged HR lesions in response to *H. parasitica* Noco2 in the absence of dexamethasone. The HR lesions were reduced in size in plants pretreated with dexamethasone (Figure 3.12A). The enlarged lesions could be due to a weakened response that fails to contain pathogen growth through rapid death of few cells at infection foci. Another possibility is that the increased lesions reflect inefficiently contained cell death. A mild sporulation on cotyledons of EDS1-YFP-GR transgenic lines without dexamethasone pretreatment was also seen, which would speak in favor of weaker resistance. The absence of spontaneous cell death with or without dexamethasone pretreatment suggests that no absolute deregulation of programmed cell death pathways is present in these transgenic lines (data not shown). I also analyzed competence to express RPS4-triggered resistance to *Pst* DC3000 AvrRps4 in the EDS1-YFP-GR transgenic lines. Similar to observations after avirulent *H. parasitica* inoculation, EDS1-YFP-GR lines exhibited intermediate susceptibility between wild-type and the hypersusceptible YFP-GR line. Strong resistance was observed after dexamethasone pretreatment of EDS1-YFP-GR lines (Figure 3.12B). Dexamethasone treatment is expected to allow fast release of GR fusion proteins into the nucleus. Thus, dexamethasone treatment could allow a similar EDS1-YFP-GR nuclear enrichment to that observed for wild-type EDS1 protein within the first few hours of *Pst* DC3000 AvrRps4 infection (Figures 3.3 and 3.5). These results lead me to conclude that rapid EDS1 nuclear accumulation after infection with the avirulent Pseudomonas is important for RPS4-triggered resistance.

To analyze phenotypes during basal resistance, selected transgenic lines and control plants were spray-inoculated with *H. parasitica* Cala2 which is virulent on L*er*. As seen in Figure 3.12C, the control YFP-GR line was more susceptible than wild-type L*er* plants, with or without dexamethasone pretreatment, indicative of *eds1* enhanced disease susceptibility. Lines expressing the EDS1-YFP-GR fusion construct allowed enhanced pathogen sporulation when compared with wild-type plants in the absence of dexamethasone pretreatment. When plants were pretreated with dexamethasone prior to pathogen infection, EDS1-YFP-GR lines showed similar sporulation than that observable in wild-type plants. The specific enhancement of resistance through dexamethasone pretreatment of EDS1-YFP-GR-expressing transgenic plants suggests that EDS1 nuclear accumulation is also important for basal resistance.

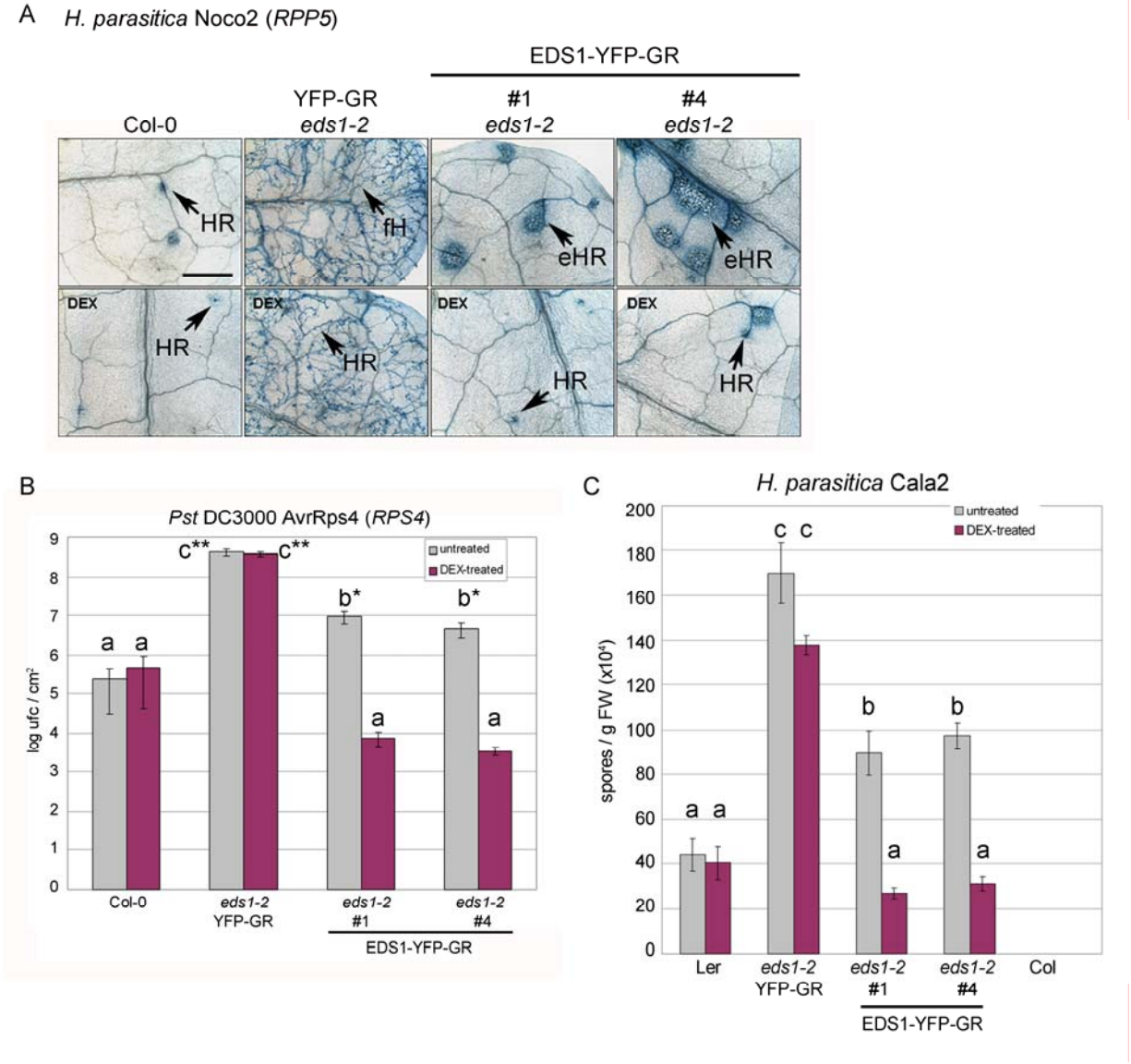


**Figure 3.12. EDS1 nuclear accumulation (through DEX treatment) enhances resistance in GR fusion transgenic lines.**

(A) Visualization of pathogen growth and dead cells in leaves of 3-week-old plants 7 dpi with avirulent *H. parasitica* Noco2 through staining with lactophenol trypan blue. Spray inoculation with conidiospore suspension was performed on plants that were either untreated or pretreated with 30 µM DEX for 6 h. HR: hypersensitive response associated cell death; eHR: enhanced HR; fH: free pathogen hyphae. Bar = 500 µm. Data are representative of two independent experiments.

(B) Leaves of 5-week-old plants of the indicated genotypes were hand-infiltrated with *Pst* DC3000 AvrRps4 and bacterial titers were determined 4 dpi. Spray inoculation with the bacterial suspension was performed on plants that were either untreated plants (grey bars) or 6 h after pretreatment with 30 µM DEX (lilac bars). Values are the average of four samplings from 5 individual plants and error bars represent Standard Error of the mean. Characters a, b and c indicate significant differences (t-test; * p-value <0,05; ** p-value <0,001). Data are representative of two independent experiments.

(C) Pathogen conidiospores were counted on leaves 6 dpi with virulent *H. parasitica* Cala2. Spray inoculation with conidiospore suspension was performed on plants that were either untreated untreated plants (grey bars) or 6 h after pretreatment with 30 μM DEX (lilac bars). Values are the average of 4 replicate samples and error bars represent Standard Error of the mean. Characters a, b and c indicate significant differences (t-test; p-value <0,01). Data are representative of two independent experiments.

## 3.6 Targeting of EDS1 to the nucleus

The data presented so far suggest that nuclear accumulation of EDS1 plays an important role in plant defense activation. To test further whether EDS1 nuclear activity is decisive for defense activation, I generated transgenic plants in which EDS1 would be targeted to the nucleus by attaching the NLS of the simian virus 40 (SV40) large T antigen ($PKK_{128}KRKVGG$) (Kalderon et al., 1984). The NLS was fused to the C-terminus of the gEDS1-mYFP fusion and expressed under its native promoter (promEDS1:gEDS1-mYFP-NLS) in the Col *eds1-2* null mutant background (hereafter referred to as EDS1-YFP-NLS lines). Control transgenic lines expressing EDS1-YFP fused to the corresponding non-functional mutated signal (nls, $PKT_{128}KRKVGG$) were also generated (hereafter referred to as EDS1-YFP-nls lines). Several independent stable transgenic lines were generated and some lines expressing the EDS1-YFP fusion proteins to detectable levels were selected for further analysis. Analysis of intracellular fluorescence by CLSM, confirmed the expected localization of the fusion proteins: exclusive nuclear accumulation in EDS1-YFP-NLS lines and nucleocytoplasmic distribution in EDS1-YFP-nls lines (Figure 3.13A).

An immediate observation of $T_1$ transgenic plants revealed the presence of two independent EDS1-YFP-NLS transgenic plants (NLS #A3 and NLS #A5) that were dwarf, chlorotic and had short stems with asymmetric and curly leaves. Fluorescence analysis in a confocal laser scanning microscope revealed high nuclear YFP signal in both $T_1$ plants, indicating high accumulation of EDS1-YFP-NLS fusion protein (Figure 3.13A). The reduced number of $T_2$ seeds obtained from these two $T_1$ plants, made it difficult to perform a robust segregation analysis in the $T_2$ generation. Nevertheless, the segregation pattern in the $T_2$ families of EDS1-YFP-NLS lines #A3 and #A5, suggested that the morphological defects segregated 3:1 together with the transgene (Basta® resistance). The morphological defects observed in the line NLS #A3 were more pronounced than those of the line NLS #A5, and those plants with stronger phenotype eventually died in normal growth conditions

approximately five weeks after germination (Figure 3.13B). The fact that they represented approximately one fourth of the total $T_2$ grown plants suggest the homozygous plants are not viable. Significantly, $T_2$ plants of the NLS #A3 line with intermediate growth defects (putative heterozygous plants) showed an almost identical phenotype to those NLS #A5 $T_2$ plants with stronger phenotype (putative homozygous) (Figure 3.13B). So far, I have not been able to identify homozygous families in the $T_3$ generation of the NLS #A3 line while I have succeeded in doing so for the NLS #A5 line. This allowed me to confirm that NLS #A5 homozygous plants resemble NLS #A3 heterozygous plants. A thorough fluorescence microscope analysis suggests there is a higher transgene expression in the NLS #A3 line than that observed in the NLS #A5 line, and both lines showed stronger fluorescence signal than other EDS1-YFP-NLS lines without obvious morphological defects. Therefore I hypothesize that the observed growth phenotype may be explained by different levels of EDS1 nuclear accumulation. Interestingly, the fluorescence signal observed in these two EDS1-YFP-NLS lines was within the same range or only slightly higher than that observed in the characterised EDS1-YFP line (Figure 3.13A). Furthermore, the prom35S:EDS1-StrepII transgenic lines previously mentioned accumulate enhanced amounts of EDS1 in both cytosolic and nuclear compartments (Gobbato et al., manuscript in preparation). These results suggest it may be the disproportionate enhanced accumulation of EDS1 protein in the nuclear compartment which is detrimental for plant growth and viability.

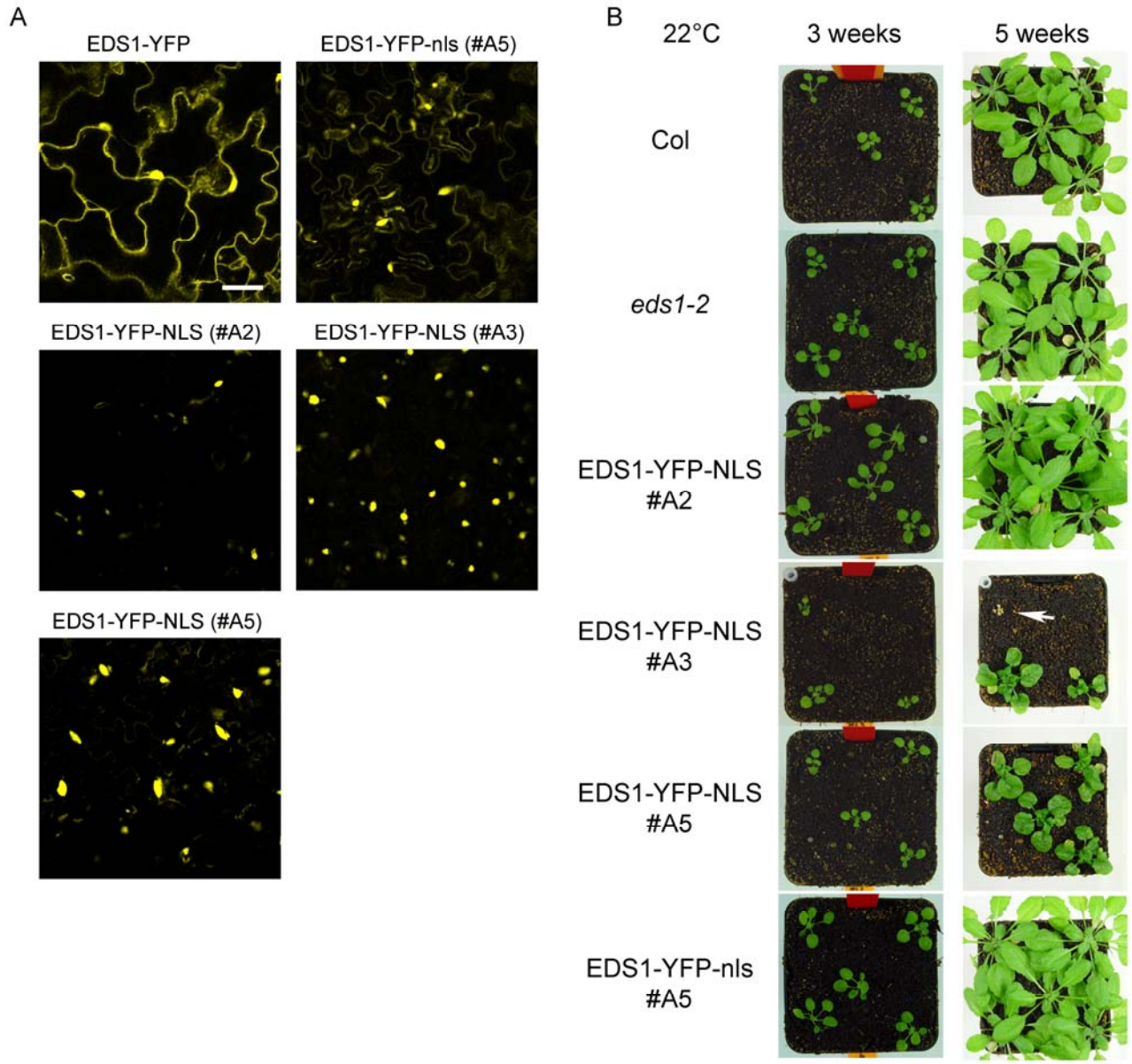


**Figure 3.13. Intracellular fluorescence and growth phenotype of EDS1-YFP-NLS and EDS1-YFP-nls independent transgenic lines.** (A) Confocal images of YFP fluorescence in leaf epidermal tissue of EDS1-YFP-NLS and EDS1-YFP-nls transgenic plants. Images were taken from 3-week-old plants. Bar = 40 µm. (B) Growth phenotype of the generated transgenic lines at 22°C, 3 weeks and 5 weeks after sowing. The white arrow indicates a dead plant among the 5-week-old NLS #A3 plants.

The growth defects observed present some morphological features that resemble the typical phenotypes of plants with high SA levels and constitutively activated defense responses (Bowling et al., 1994; Bowling et al., 1997; Li et al., 2001; Shirano et al., 2002). Many of these phenotypes are suppressed by growing the plants in high temperature or high humidity, and

also by the lack of EDS1 (Clarke et al., 2001; Li et al., 2001; Shirano et al., 2002; Yang and Hua, 2004; Zhou et al., 2004; Wirthmueller et al., 2007). I therefore examined the effect of high temperature on the growth defects of these EDS1-YFP-NLS transgenic lines and found that the morphological defects were suppressed by growing the plants at 28°C (Figure 3.14A). These results suggest that the EDS1-YFP-NLS transgenic lines expressing nuclear localized EDS1 fusion protein could be SA over-accumulators that may also display enhanced resistance. I tested the EDS1-YFP-NLS/nls transgenic lines for race-specific resistance to *H. parasitica* Emwa1 and monitored pathogen growth and cell death by staining the leaves with trypan blue 5 d after inoculation. I found that both fusion proteins complemented the *eds1-2* mutation for RPP4 (TIR-NB-LRR)-mediated resistance and efficiently arrested pathogen colonization (Figure 3.14B). Interestingly, EDS1-YFP-NLS lines #A3 and #A5 exhibited an exacerbated occurrence of cell death which indicates that a cell death program may be deregulated in these plants. It will be important to determine whether these transgenic lines also have spontaneous cell death. With the recent identification of $T_3$ homozygous families it will be possible to evaluate whether EDS1-YFP-NLS plants display enhanced resistance to virulent and avirulent pathogens. Given the nuclear function of RPS4, it will be of particular interest to study their phenotype in resistance to *Pst* DC3000 AvrRps4 conferred by this TIR-NB-LRR receptor. Given the evidence for nuclear enrichment of EDS1 during TIR-NB-LRR-mediated responses (Figure 3.3), the possibility that these plants are "primed" (possess an enhanced capability to mount defense responses (Conrath et al., 2002)) should also be contemplated. To consider this aspect, SA accumulation and transcriptional changes of EDS1-dependent defense genes will be analyzed before and after pathogen challenge to assess whether EDS1-YFP-NLS transgenic lines are able to respond more rapidly to pathogens than wild-type plants.

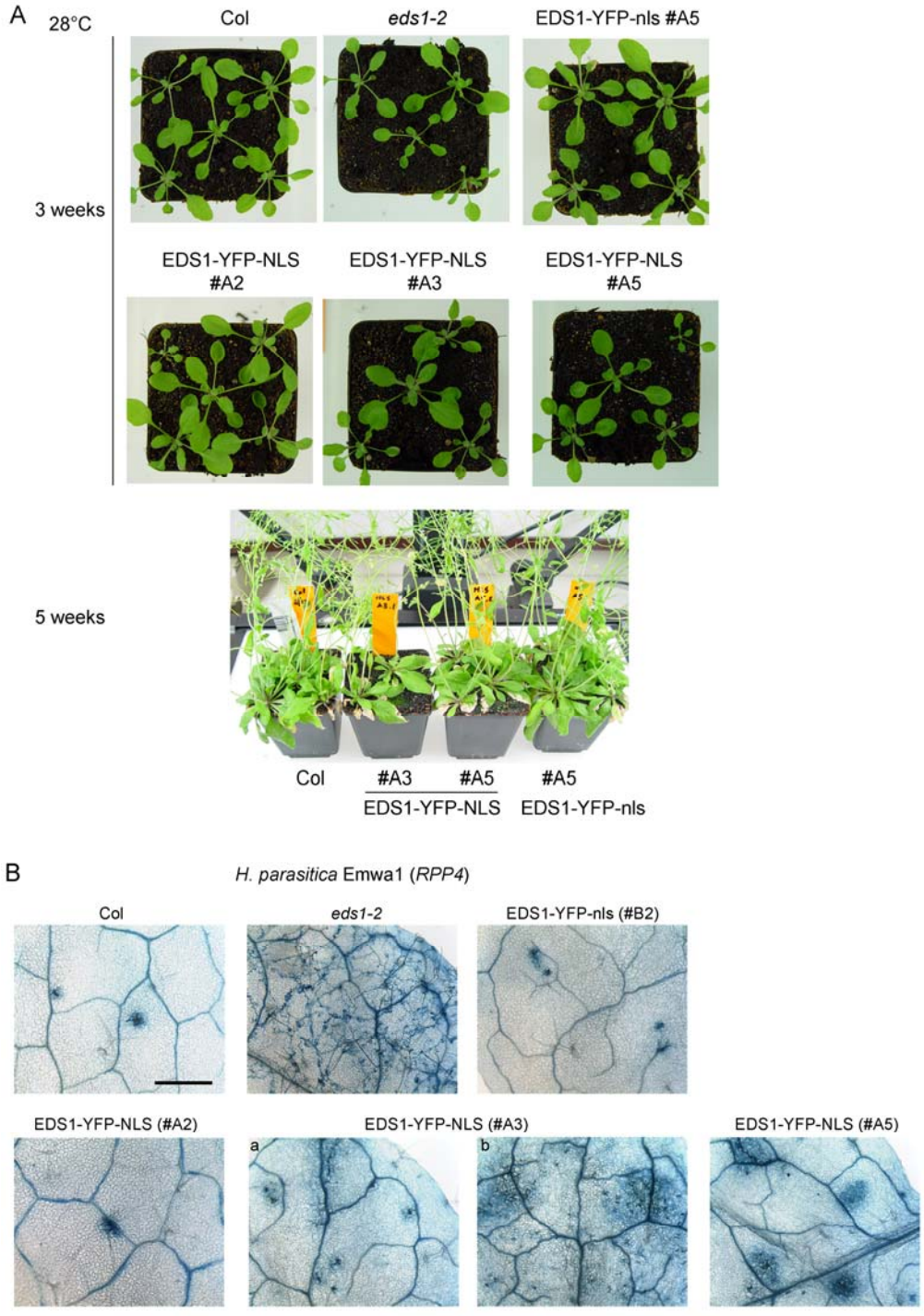


**Figure 3.14.** (A) Growth defects observed in EDS1-YFP-NLS transgenic lines #A3 and #A5 at 22°C were suppressed by growing the plants at 28°C. (B) Visualization of pathogen growth and dead cells in leaves of 3-week-old plants 5 dpi with avirulent *H. parasitica* Emwa1 through staining with lactophenol trypan blue. Bar = 500 µm.

## 3.7 Shuttling of EDS1 complexes?

Proteins or protein complexes carrying functional nuclear export and import signals are generally able to continuously shuttle between the nucleus and the cytoplasm (Xu and Massague, 2004). Using the NetNES 1.1 prediction server (www.cbs.dtu.dk) EDS1 and PAD4 were found to possess a putative NES that fall within a leucine-rich region (Wiermer, 2005). Therefore, EDS1 itself and EDS1 protein complexes have putative NLS (present in EDS1 and SAG101 sequences) and NES (present in EDS1 and PAD4 sequences), which tallies with their subcellular distributions (Falk et al., 1999; Feys et al., 2005). For this reason, we hypothesized that EDS1 might be subject to steady-state shuttling to and from the nucleus, and any shift in this equilibrium would lead to nuclear or cytoplasmic enrichment. Given that steady state accumulation of SAG101 and PAD4 in leaf tissues depend on EDS1 (Feys et al., 2005), I reasoned that EDS1 movement and accumulation would influence distribution and likely functions of its binding partners. To test this hypothesis we made use of the nuclear export inhibitor Ratjadone A (RatA) that specifically inhibits nuclear export mediated by CRM1-type exportins in a similar fashion to the widely used inhibitor Leptomycin B (LMB) (Meissner et al., 2004). After treatment with export inhibitors, constitutively shuttling proteins and complexes accumulate in the nucleus and the cytosolic localization is greatly reduced (Haasen et al., 1999). To test this hypothesis I prepared protoplasts from the stable transgenic line expressing YFP-tagged EDS1 driven by its native promoter (EDS1-YFP line) and monitor its localization on a confocal microscope after treatment with RatA in methanol or mock-treatment with methanol only. I used one EDS1-YFP-NES stable transgenic line (#2-11) as a positive control for the treatment, since we demonstrated that the presence of the NES drives the export of the fusion protein (Figure 3.6), and a stable transgenic line expressing prom35S:mYFP as negative control. As expected, protoplasts of the EDS1-YFP-NES transgenic line expressed fluorescence only in the cytoplasm and this localization was not altered by the control treatment with methanol (Figure 3.15A). However, treatment with 15 ng/ml RatA for 4 h provoked a shift in yellow fluorescence to the nucleus, resulting in a proportion of protoplasts expressing only nuclear fluorescence and some expressing fluorescence in nucleus and cytoplasm (Figure 3.15A). This indicates that RatA treatment was successful in inhibiting NES-driven nuclear export. When I performed a similar RatA

treatment to EDS1-YFP expressing protoplasts, I observed a significant increase in the number of protoplasts expressing exclusively a nuclear signal (82%) compared to protoplasts treated only with methanol (37.5%) (Figure 3.15A and B). RatA or control treatment did not affect the distribution of yellow fluorescence emitted by mYFP which, due to its small size, is able to move passively between cytosol and nucleus (Ishidate et al., 1997). I concluded from this experiment that EDS1 is able to shuttle from the nucleus to the cytoplasm under these conditions. These results also show that in the EDS1-YFP-NES lines, EDS1 fusion protein does enter the nucleus before being shunted out.

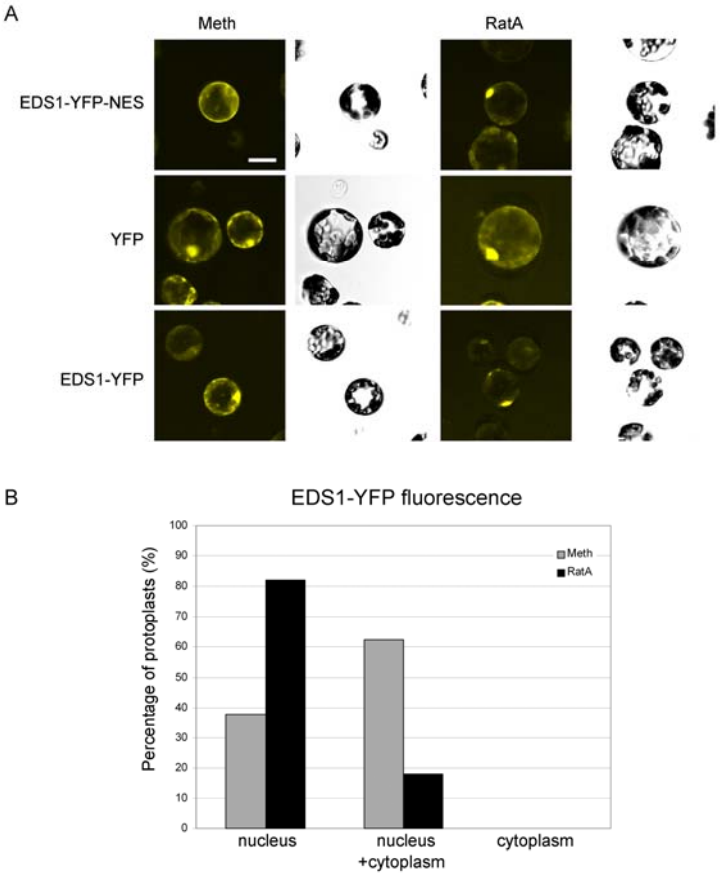


**Figure 3.15. Nucleocytoplasmic shuttling of EDS1 in *Arabidopsis* leaf mesophyll protoplasts.** (A) Confocal images of YFP fluorescence and bright field of *Arabidopsis* protoplasts prepared from leaves of 4-week-old transgenic plants expressing EDS1-YFP-NES (top), YFP (medium), or EDS1-YFP (bottom). Protoplasts were treated for 4 h with 15 ng/ml of the nuclear export inhibitor Ratjadone A (RatA) or mock-treated with methanol. Bar = 10 µm. (B) Quantification of YFP signal in protoplasts of EDS1-YFP transgenic line with respect to its nuclear and cytosolic signal. The graph shows the percentage of protoplasts showing signal in the indicated subcellular fractions, determined from 30 protoplasts. Grey and black bars represent protoplasts treated with methanol (Meth) and RatA respectively.

# 4 DISCUSSION

*Arabidopsis* EDS1 nucleocytoplasmic complexes perform a key role in plant innate immunity against biotrophic and hemi-biotrophic pathogens (Wiermer et al., 2005). EDS1 and PAD4 are required for SA accumulation and for processing ROS signals at the site of infection which allow defense amplification (Feys et al., 2001; Rusterucci et al., 2001). This function is important for containing the growth of virulent pathogens in the absence of specific recognition and for the generation of SAR-inducing signals downstream of immune receptor activation (Wiermer et al., 2005). EDS1 activity was revealed to be engaged early after TIR-NB-LRR-type immune receptor activation and necessary for the generation of reactive oxygen species and HR development (Rusterucci et al., 2001). A recent study of the TIR-NB-LRR receptor RPS4 demonstrated that it signals entirely through EDS1 and that its nuclear pool is essential for defense activation (Wirthmueller et al., 2007). RPS4 requirement for *EDS1* appears not to be at the level of receptor assembly or distribution in the cell prior to or after pathogen sensing. This post-activation signaling activity of EDS1 is essential for RPS4-triggered transcriptional reprogramming and may occur in the nucleus since sub-pools of both RPS4 and EDS1 co-localize in this compartment (Wirthmueller et al., 2007). Up to now the precise EDS1 molecular function is not known. To gain an insight to EDS1 activity and its relationship to subcellular dynamics, I aimed to characterize where in the cell EDS1 complexes are localized during defense responses and in which subcellular compartment(s) EDS1 exerts its function. The results achieved in this analysis, will be summarized, evaluated and discussed with regard to the recent literature. Experiments in progress and future directions are also included in this section. At the end of the section, there is a proposed model indicating the subcellular dynamics and signaling activities of EDS1 and interacting partners during defense responses (Figure 4.2).

## 4.1 Post-transcriptional regulation of EDS1

Transcriptional analyses in of *Arabidopsis* have shown that *EDS1* and *PAD4* genes are upregulated during compatible and incompatible interactions with *Pst* DC3000 and *H.*

*parasitica*. This was believed to be the result of a positive feedback loop activated downstream of EDS1/PAD4 initial signaling since EDS1-PAD4 complexes exist in cells before pathogen inoculation (Feys et al., 2001; Bartsch et al., 2006). Also, *EDS1* and *PAD4* are essential for pathogen-induced SA accumulation triggered during basal and TIR-NB-LRR mediated defenses and hence activation of SA target genes is abolished or reduced in *eds1* and *pad4* mutant plants (Zhou et al., 1998; Feys et al., 2001). The hypothesis that EDS1 and PAD4 proteins function upstream of SA in regulating defense responses is further supported by the finding that application of SA or its analog BTH restored defense gene induction in *pad4* and *eds1* (Zhou et al., 1998; Falk et al., 1999). SA contributes to *EDS1* and *PAD4* transcriptional activation consistent with the participation of EDS1-PAD4 complexes and SA in a positive regulatory loop important for defense potentiation (Falk et al., 1999; Jirage et al., 1999). Enhanced transcript accumulation of *EDS1* and *PAD4* after infiltration with avirulent *Pst* DC3000 expressing AvrRps4 or AvrRpm1 was visible at early time points (3 h after inoculation), anticipating or concomitant with the upregulation of *EDS1*-dependent genes (Bartsch et al., 2006). While EDS1 and PAD4 protein levels increase at 12 h post inoculation with *Pst* DC3000 AvrRps4 (Feys et al., 2001), protein accumulation was not analyzed at earlier time points after pathogen challenge.

However, analyses of stable transgenic lines overexpressing *EDS1* or *PAD4* show that their transcriptional upregulation is important but insufficient in priming defense activation (Gobbato *et al*, manuscript in preparation). Enhanced accumulation of EDS1 or PAD4 proteins in these lines did not accelerate defense responses. Only the combined overexpression of *EDS1* and *PAD4* resulted in increased resistance to biotrophic pathogen. Importantly, activation of downstream events in EDS1/PAD4 double overexpressor lines was greater when coupled with a pathogen trigger, indicating that a post-transcriptional regulatory step is important for EDS1/PAD4 signaling (Gobbato *et al*, manuscript in preparation).

EDS1 and its interacting partners PAD4 and SAG101 form distinct complexes in the cytoplasm and nucleus of healthy plants (Feys et al., 2005). To test whether the spatial dynamics of EDS1 complexes are important for EDS1 pathway activation, protein accumulation and subcellular distribution of EDS1 and PAD4 were examined after pathogen challenge and in the deregulated resistant background *snc1* in which EDS1 pathway is constitutively activated (Li et al., 2001). Combining fluorescence microscopy and cell fractionation analyses, I could show that *snc1* mutation and late stages of infection with

virulent *H. parasitica* lead to increased EDS1 and PAD4 protein steady state levels in both the nucleus and cytoplasm without apparent protein concentration in one particular compartment (Figures 3.1, 3.2 and 3.4). I reasoned that if there were transient changes necessary for defense activation I may not see them in constitutive activated resistant backgrounds or late stages of infection due to compensatory mechanisms vital for growth and development, and therefore decided to study EDS1 localization at early stages of TIR-NB-LRR-mediated defense activation against *P. syringae*.

*Arabidopsis* defense responses to *P. syringae* are triggered within 3 to 6 h post inoculation (Tao et al., 2003) while transcriptional changes associated with *H. parasitica* infection are observed 12 hours to 2 days after inoculation (Eulgem et al., 2004). For this reason, *Arabidopsis – Pseudomonas* interaction provides a shorter time frame and a better system to study transient effects associated with defense activation. Some early responses to virulent *Pst* DC3000 have been previously described, such as the SA-dependent stomatal closure observed 1 h after treatment with concentrated bacteria (Melotto et al., 2006). In *Nicotiana benthamiana*, *EDS1* is necessary for *Pst* AvrPtoB-mediated suppression of early ROS production *in planta* (10 min post elicitation) (Hann and Rathjen, 2007). In this study I assessed EDS1 subcellular localization after triggering the TIR-NB-LRR RPS4 which signals entirely through EDS1 (Wirthmueller et al., 2007). By monitoring early time points after spraying with high doses of *Pst* DC3000 AvrRps4 I could find EDS1 nuclear enrichment within 2 to 4 h after inoculation (Figure 3.3). Since this disproportionate increase of nuclear EDS1 is not accompanied by a corresponding increase in EDS1 amounts in total cellular or nuclei-depleted fractions, the observed nuclear enrichment could be explained by an increase in EDS1 nuclear import, in nuclear retention or by a selective stabilization of the EDS1 nuclear pool. I still need to assess whether the observed EDS1 nuclear enrichment is transient since later it appears to be overridden by an increase in EDS1 whole cellular amounts. It will also be important to study EDS1 localization after virulent *P. syringae* inoculation, to determine if EDS1 nuclear enrichment is specific to the onset of TIR-NB-LRR-mediated immune responses or happening more broadly during compatible and incompatible interactions between *Arabidopsis* and *Pseudomonas.*

In this work I have described the analysis of EDS1-YFP-GR stable transgenic lines in which it is possible to control EDS1 fusion protein nucleocytoplasmic distribution through the use of the steroid hormone dexamethasone (DEX). Untreated plants showed cytoplasmic

localization of the fusion protein whereas DEX-treated plants exhibited protein accumulation in both cytoplasm and nuclei (Figure 3.11B). Accumulation of EDS1-YFP-GR fusion protein increased during TIR-NB-LRR-mediated resistance to *H. parasitica* and the observed increase was maximal when pathogen challenge was coupled with DEX treatment (Figure 3.11A). Since transgene expression in these lines is driven by the constitutive cauliflower mosaic virus 35S promoter, the increased fusion protein accumulation is unlikely to be due to transcriptional upregulation (although this must be tested to be excluded). Therefore, these results suggest that there is pathogen-triggered EDS1 protein stabilization, which is maximal when the protein is allowed to enter the nucleus through DEX-treatment. In view of this data I can build the hypothesis that EDS1 nuclear pool becomes stabilized during TIR-NB-LRR-mediated responses, which would tally with the observed enrichment in nuclear amounts of EDS1 during RPS4-triggered defense. Another possibility is that proper nucleocytoplasmic trafficking and subcellular distribution is important for EDS1 protein function and stability.

In a genetic screen for mutants that suppress the phenotypes of *snc1*, several *mos* mutants were identified that show different degrees of suppression (Palma et al., 2005; Zhang et al., 2005; Zhang and Li, 2005). As mentioned previously, *MOS3* encodes a Nup96, *MOS6* an Importin α3, and *MOS7* is a nucleoporin with homology to Nup88 (Palma et al., 2005; Zhang and Li, 2005); Cheng et al, submitted). EDS1 protein levels are reduced in these *mos* single mutants and in the *snc1 mos* double mutants correlating with the degree of *snc1* phenotype suppression (M. Wiermer, A.V. García and J. Parker, unpublished). Also, while *mos7* mutant plants have reduced EDS1 protein levels compared to wild-type, *EDS1* transcript levels are not significantly changed (Cheng et al, submitted). A plausible explanation for the reduced EDS1 protein levels observed is that a defect in EDS1 movement across the nuclear envelope disturbs proper protein distribution and thus leads to protein destabilization. In this respect, *mos7-1* mutant plants did not show an obvious change in EDS1 nucleocytoplasmic partitioning (Cheng et al, submitted), meaning that if a destabilizing effect exists it becomes equilibrated between both cellular compartments. We hypothesize that the *mos7-1* mutation causes a specific reduction in EDS1 nuclear accumulation which contributes to *snc1* suppression and that EDS1 reduced nuclear levels are compensated by increased EDS1 degradation in the cytoplasm. By the same reasoning, EDS1 nucleocytoplasmic distribution may also be compensated for in *snc1* plants and at late stages of infection, since I observed an overall enhancement of EDS1 accumulation in nuclei-enriched and nuclei-depleted cellular fractions in these tissues (Figures

3.1, 3.2 and 3.4). I hypothesize that EDS1 steady state levels and subcellular distribution are fine tuned to maintain an equilibrated ratio in between the compartments.

The ubiquitin-proteasome system (UPS) controls important outputs of plant hormone signaling (Stone and Callis, 2007). The plant hormone auxin has been shown to bind to the nuclear F-box protein TIR1 and this promotes the interaction between TIR1 and the auxin/indole-3-acetic acid (Aux/IAA) family of transcriptional repressors which are then targeted for degradation (Dharmasiri et al., 2005; Kepinski and Leyser, 2005). Similarly, the interaction between the F-box COI1 with members of the JAZ family of transcriptional repressors was shown to be induced by the JA conjugate JA-Ile, which leads to $SCF^{COI1}$-mediated proteasome degradation of JAZ proteins (Chini et al., 2007; Thines et al., 2007). In the case of gibberellic acid (GA) signaling, the F-box *At*SLY1/*Os*GID2 mediates the degradation of the DELLA protein family of GA repressors in response to GA treatment (Sasaki et al., 2003; Dill et al., 2004). However, GA does not bind directly to the F-box protein but instead to the nuclear receptor GID1, a protein with similarity to hormone sensitive lipases (HSLs) (Ueguchi-Tanaka et al., 2005; Griffiths et al., 2006). Binding of GA to GID1 induces GID1-GA-DELLA complex formation and triggers $SCF^{GID2}$-dependent degradation of DELLA proteins. Therefore, it appears fundamental in plant hormone signaling to induce removal of negative regulators through the SCF-mediated 26S proteasome system in response to changes in hormone levels. It is intriguing whether SA responses are also regulated by the UPS in a similar manner. In this respect, a RING-type ubiquitin E3 ligase was recently identified as a negative regulator of SA accumulation during immune responses (Yaeno and Iba, 2008). Besides, it has been shown that SA signaling pathway is under the regulation of the small ubiquitin-like modifier E3 ligase SIZ1 which negatively regulates *PAD4*- and SA-mediated signaling (Lee et al., 2007a). Identification of *MOS5* as being an important player in *snc1* constitutive resistance that encodes a component of ubiquitin-mediated regulation implicates ubiquitination in R-mediated responses (Goritschnig et al., 2007). Hence, there is some evidence pointing to a role for ubiquitin-mediated protein degradation in modulating the SA pathway but more research is needed to further clarify how this is controlled at the molecular level.

A plausible hypothesis is that in unchallenged cells EDS1 nuclear protein levels may be controlled by the activity of negative regulators of the SA-pathway and defense activation. Increased SA levels may target those negative regulators for proteasome-mediated degradation

and as a consequence, nuclear EDS1 amounts increase. Depending on the extent of similarity with the mentioned hormone pathways, this may happen instead of or in addition to the degradation of transcriptional repressors. If this hypothesis is valid, EDS1 nuclear enrichment should be visible after treatment with SA or its chemical analogue BTH. Another possibility is that nuclear EDS1 is a direct target of an ubiquitin E3 ligase and hence EDS1 nuclear amounts are controlled by proteasome activity. A strategy to unravel these possibilities is to study EDS1 nucleocytoplasmic partitioning and particularly EDS1 nuclear amounts after treatment with a proteasome inhibitor such as epoxomicin. If EDS1 is normally subject to proteasome-mediated degradation, epoxomicin treatment should produce an increase in EDS1 nuclear amounts. On the contrary, if EDS1 negative regulators are controlled by proteasome-mediated degradation, epoxomicin treatment will stabilize them and EDS1 levels will decrease. These experiments are underway. The fact that both EDS1 and GID1 share some homology with HSLs (Falk et al., 1999; Ueguchi-Tanaka et al., 2005) raises the possibility that EDS1 also participates in targeting negative regulators of SA-pathway to proteasome-mediated degradation. Several reports have shown that tight regulation of defense regulators is crucial to achieve the necessary control of immune responses. One such example is the regulation of NB-LRR resistance proteins which have been reported to be subject to constitutive negative regulation either through intramolecular interactions or by interacting proteins (DeYoung and Innes, 2006). Immune regulators with similarity to chaperones have been identified and shown to control R protein accumulation in both a positive and negative manner (Holt et al., 2005). SA overaccumulation and constitutive activation of SA-dependent inducible defenses reduce plant fitness (Heidel et al., 2004; Alcazar et al., 2009), thus it is not surprising that SA pathway components and EDS1 in particular may also be under tight post-transcriptional regulation.

## 4.2 EDS1 nuclear exclusion compromises immune responses

The results showing EDS1 nuclear enrichment during RPS4-mediated resistance to *Pst* DC3000 AvrRps4 (Figure 3.2) indicate that EDS1 nuclear accumulation may have functional significance in activating defense responses. To explore further this possibility and determine in which compartment within the cell EDS1 carries its function, I examined whether manipulation of EDS1 localization affected its function in disease resistance. To obtain plants

with reduced EDS1 nuclear accumulation I generated EDS1-YFP-NES transgenic lines. Attachment of the PKI NES used in this study was shown to be sufficient to trigger rapid nuclear export of heterologous proteins in mammalian cell cultures and in plants (Wen et al., 1995; Matsushita et al., 2003). For a more precise analysis of the effect of EDS1 subcellular distribution on defense activation, I also analyzed EDS1-YFP-GR transgenic lines in which EDS1 nuclear entry should be controlled by addition of DEX. Finally, to gain further insight on the role of nuclear EDS1 I generated stable transgenic lines expressing nuclear localized EDS1 by translational fusion of the NLS of SV40 large T antigen (EDS1-YFP-NLS lines).

The data suggest that shunting EDS1 out of the nucleus through the NES fusion or cytosolic retention of EDS1 by the GR imposes a detrimental effect on defense activation (Figures 3.7 and 3.12), indicating that nuclear EDS1 plays a role in disease resistance. Importantly, the resistance levels observed were significantly higher in the EDS1-YFP-GR lines when EDS1 nuclear accumulation was allowed through DEX-treatment (Figure 3.12). Within the EDS1-YFP-NES lines, the impairment in complementing *eds1-2* enhanced disease susceptibility was greater in the NES #2-10 line with lower level of fusion protein accumulation, suggesting that NES efficiency to drive nuclear export may be reduced by high protein levels. Importantly, fusion protein accumulation in the transgenic line NES #2-10 is similar to that in the line nes #1-1 which show wild-type levels of resistance, indicating that the reduced disease resistance present in line NES #2-10 may be specifically due to lower EDS1 nuclear amounts. The stable transgenic lines expressing mislocalized EDS1 were tested in effector-triggered immunity (R-mediated resistance), effector-triggered susceptibility (basal defenses) and systemic acquired resistance (SAR) and were found to be similarly disabled at all levels of resistance. This data could be explained either by similar requirements for nuclear EDS1 in all types of resistance tested or by the existence of a unique function of EDS1 that affects local and systemic defense responses in the same manner. So far, we are still not able to discriminate whether the EDS1 role in generation of cell death, ROS and SA accumulation, and transcriptional reprogramming are accomplished by the same or distinct molecular functions.

The moderate detrimental effect on resistance provoked by the NES fusion to EDS1 may be caused by the lack of complete nuclear depletion generated by NES and the fact that the fused protein still enters the nuclei before being exported, as shown in the shuttling experiments. Even if the PKI NES used in this study was shown to be one of the most active

signals, nuclear export rate of a specific shuttling protein depends both on the strength and the accessibility of its NES (Henderson and Eleftheriou, 2000). In this respect, a recent report showed that the attachment of PKI NES to a UV-B signaling component succeeded in keeping the fusion protein out of the nucleus only in the absence of UV-B (Kaiserli and Jenkins, 2007). Therefore, certain stimuli and mechanisms for nuclear translocation are strong enough to overcome the presence of an efficient NES. In any case, the incomplete impairment of resistance observed in EDS1-YFP-GR transgenic lines without DEX treatment argues that cytosolic EDS1 plays a role in disease resistance which may differ from EDS1 nuclear activities.

How pathogen perception by immune receptors leads to transcriptional reprogramming is an important gap in understanding the onset of defense responses. Both barley MLA (CC-NB-LRR) and tobacco N (TIR-NB-LRR) proteins interact with plant specific transcription factors (Liu et al., 2004; Shen et al., 2007) which could provide a direct link to transcriptional regulation. Nevertheless, TIR-type receptors still need the presence of EDS1 for downstream gene activation (Liu et al., 2002; Wirthmueller et al., 2007) and yet association between TIR-NB-LRR proteins and EDS1 or partners has never been observed. The molecular mechanism behind TIR-NB-LRR-mediated activation of defense genes is not known and whether this is mediated by an immediate role of EDS1 in transcriptional reprogramming remains to be elucidated.

The lack of obvious DNA-binding domains in the EDS1 sequence suggests that EDS1 function in defense-associated transcriptional reprogramming is unlikely to be through direct association with gene regulatory sequences. Therefore, a hypothesis would be that EDS1 contributes to transcriptional regulation by modulating the activity of transcription factors, for which several scenarios can be imagined. Some possibilities are that EDS1 function somehow increases the interaction between immune receptors and transcription factors, the binding capacity of the complex to target gene regulatory sequences, or causes a post-translational modification of receptor or transcription factor that leads to their activation. Some models with respect to EDS1 function in *Pst* DC3000 AvrRps4 activation of RPS4-mediated transcriptional reprogramming are depicted in Figure 4.1. As mentioned above, one possibility is that EDS1 increases the interaction of RPS4 with transcriptional activators and this activates defense gene expression (Model A). Another options is that *Pst* DC3000 AvrRps4 recognition by RPS4 triggers the recruitment of EDS1 to DNA-bound complexes already containing transcription

factors, either alone (Model B) or together with activated RPS4 (Model C). Alternatively, EDS1 could be constitutively holding transcription factors in a DNA-unbound state and *Pst* DC3000 AvrRps4 recognition may trigger complex dissociation and release of transcriptional activators (Model D). A direct function of EDS1 complexes in transcriptional regulation as a transcriptional repressor should also be considered (Model E). EDS1 association with DNA-bound transcription factors may repress their activity, and defense activation might release the interaction and activate defense gene expression. Data gathered from a microarray analysis after *Pst* DC3000 AvrRps4 inoculation (Bartsch et al., 2006) revealed some genes which were transcriptionally downregulated in an *EDS1*-dependent manner after the pathogen trigger, arguing that EDS1 may participate in transcriptional repression. The models proposed comprise possible roles for EDS1 in gene regulation together with RPS4 and transcriptional activators, but EDS1 may also exert its function through the interaction with transcriptional repressors. All of these EDS1 nuclear activities are likely to be performed through a concerted action with PAD4 and/or SAG101, since double *pad4/sag101* mutants abolished all EDS1-dependent responses (Feys et al., 2005).

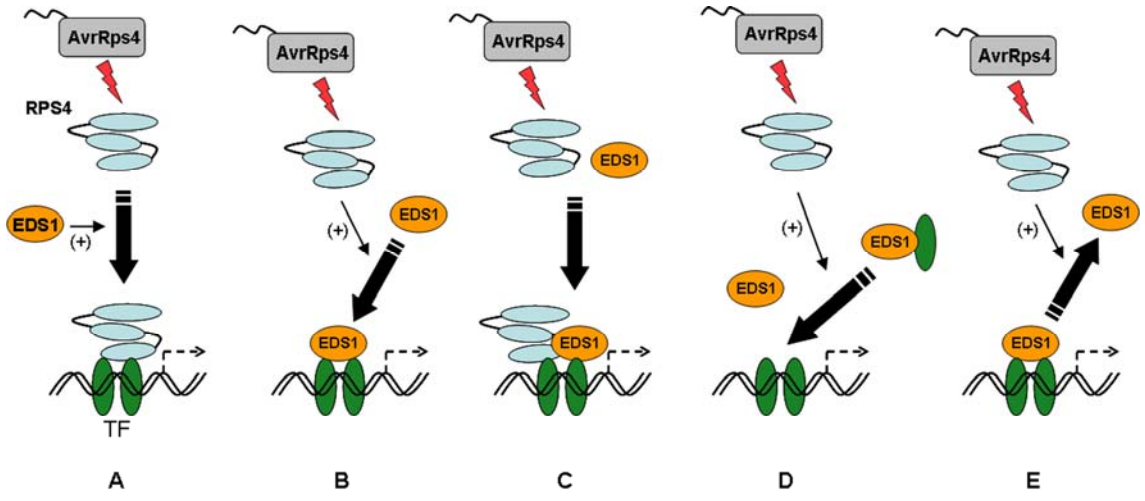


**Figure 4.1. Models for EDS1-dependent RPS4-triggered transcriptional reprogramming depicting possible links between EDS1 and/or RPS4 with transcriptional machinery.** Since EDS1 and RPS4 have no obvious DNA binding domains or known chromatin binding capacity, their role in defense gene regulation is hypothesized to be through the interaction with unidentified positive or negative regulators of transcription. These models represent different scenarios involving transcriptional activators, EDS1 and RPS4 after *Pst* DC3000 AvrRps4 recognition. Arrow with (+) indicate positive effect/impact and dashed arrow indicate movement. TF, transcription factors; AvrRps4, *Pst* DC3000 AvrRps4.

In this direction, the study of fluorochrome-tagged EDS1 mobility in the nuclei and fractionation of nuclear pools could give us information of the compartmentalization of EDS1 within the nuclei, such as whether is bound to some high order complex or chromatin. A more direct approach to study a possible EDS1 association with chromatin would be chromatin immunoprecipitation (Ch-IP), and is currently being explored. Given the chance of a positive result, this type of analysis would allow us to gain insights to the regions of DNA and particular genes targeted by EDS1.

## 4.3 Is there a role for cytosolic EDS1?

Residual disease resistance in EDS1-YFP-NES transgenic lines and EDS1-YFP-GR lines without DEX treatment suggests that cytosolic EDS1 functions in resistance activation (Figures 3.7 and 3.12). Furthermore, the fact that both sets of transgenic lines exhibit some capability to generate programmed cell death in response to pathogen infection (Figures 3.7A and 3.12B) indicates that both fusion proteins are able to function in the development of the hypersensitive response. It should also be considered that there might be leakiness in both systems used for nuclear exclusion and small amounts of EDS1 in the nucleus may be able to fulfill some of its role. Nevertheless, a role in modulating cell death can be envisioned for cytosolic EDS1.

As previously mentioned, *EDS1* is necessary for ROS production and HR generation associated with resistance conferred by various TIR-NB-LRR-type receptors (Rusterucci et al., 2001). Important data regarding EDS1 and PAD4 roles during oxidative stress responses have been provided by analyses of the lesion mimic mutant *lsd1* (Rusterucci et al., 2001; Mateo et al., 2004; Muhlenbock et al., 2008). *LSD1* activity appears to monitor a superoxide-dependent signal and negatively regulate a plant cell death pathway (Jabs et al., 1996). Genetic studies showed that *EDS1* and *PAD4* function in processing ROS is important for *lsd1* conditioned runaway cell death (RCD) and distinct from their functions in local R-mediated resistance (Rusterucci et al., 2001). In *lsd1* mutant plants, superoxide accumulation is detectable in cells surrounding the developing lesions and lesion formation can be triggered by superoxide applications (Jabs et al., 1996). Importantly, *eds1/lsd1* and *pad4/lsd1* double mutants do not initiate spreading lesions in response to superoxide nor during resistance responses conditioned

by TIR- and CC-type NB-LRRs (Rusterucci et al., 2001). Hence, it was proposed that EDS1 and PAD4 activities in *lsd1* RCD operate separately from their function in HR formation and may be related to their roles in defense signal potentiation that are engaged by both types of NB-LRR receptors (Rusterucci et al., 2001). Further work by Karpinski and colleagues, revealed that *lsd1* plants fail to efficiently acclimate to conditions that promote excess excitation energy (EEE) causing chloroplastic ROS overload which leads to photooxidative damage and eventually cell death (Mateo et al., 2004). The defects exhibited by *lsd1* plants included reduced stomatal conductance, reduced peroxisomal catalase activity and enhanced accumulation of foliar ethylene in response to EEE, all of which were suppressed by mutations in *EDS1* and *PAD4* (Mateo et al., 2004; Muhlenbock et al., 2008). Thus it was concluded that LSD1 controls EDS1 and PAD4 activities in order to prevent photooxidative damage. However, the specific molecular activities of EDS1 and PAD4 during oxidative stress remain elusive.

It has been proposed that ROS-triggered plant cell death can occur in two ways: either ROS production can be activated in compartments where antioxidant buffering is low (e.g. apoplast) or antioxidant capacity can be withdrawn from compartments in which ROS production is high (e.g. the chloroplast and peroxisome) (Foyer and Noctor, 2005). An example of the latter is the reduction in peroxisomal catalase activity observed in *lsd1* (Mateo et al., 2004). I hypothesize that EDS1 activity alters either transcriptionally or post-transcriptionally the accumulation of components of the antioxidant machinery which could be accomplished by a participation of EDS1 in retrograde signaling. Chloroplast to nucleus retrograde signaling allows translating plastidial stress into transcriptional activation of nuclear genes encoding organellar proteins (Fernandez and Strand, 2008). Signaling in response to singlet oxygen ($^1O_2$) in plastids was revealed by the identification of a mutant in *Arabidopsis FLU* gene, which encodes a chloroplast protein (Meskauskiene et al., 2001). Transcriptome analysis using *flu* plants showed that singlet oxygen activates drastic transcriptional changes in the nucleus, for which the concerted action of the plastidial proteins EXECUTER1 and EXECUTER2 are required (op den Camp et al., 2003; Lee et al., 2007b). Interestingly, rapid induction of *EDS1* is part of the transcriptional changes induced by the singlet oxygen release in *flu* mutant plants and this upregulation occurred through a signaling pathway operating independently of SA (Ochsenbein et al., 2006). EDS1 function appeared important in singlet oxygen-induced stress responses in *flu* plants for the spread of cell death and recovery after the stress conditions

ceased. However, monitoring marker gene expression in *eds1/flu* double mutants indicated that *EDS1* may only be involved in transcriptional changes mediated by SA-pathway and thus is unlikely that EDS1 transcriptional-related activity could account for its role in cell death and ROS generation.

The N-terminal portion of EDS1, PAD4 and SAG101 protein sequences have homology with eukaryotic lipases (Falk et al., 1999; Jirage et al., 1999; He and Gan, 2002), although no lipase activity has been found to date for any of these proteins and EDS1 predicted lipase active site residues are not required for resistance signaling (S. Rietz and J. Parker, unpublished). Nevertheless, it remains possible that they could be involved in binding a so far unidentified lipid or other molecule that signals in defense activation. A body of evidence points to activities of lipid metabolites and lipid modifications of macromolecules in plant defense responses and programmed cell death (Shah, 2005). *Arabidopsis* mutants *acd5* (*accelerated-cell-death5*) and *acd11* show deregulated programmed cell death observed as spontaneous death of group of cells with some apoptotic features (Brodersen et al., 2002; Liang et al., 2003). *ACD5* encodes a putative ceramide kinase and *ACD11* codes for a sphingosine transfer protein, highlighting the role of sphingolipid metabolism in plant programmed cell death. The phenotypes associated with *acd11* mutation are SA-dependent and are completely suppressed by *eds1* and *pad4* (Brodersen et al., 2002). Interestingly, BTH treatment did not restore lesion formation in *acd11/eds1-2* plants while it resulted in sporadic cell death in *acd11/pad4-2* and initiated runaway cell death in *acd11/nahG* plants. In view of this data, EDS1 role in cell death initiation appears not to be related with its participation in SA pathway. However, as mentioned before, EDS1 and PAD4 role in *lsd1* runaway cell death may be related to their activities in signal potentiation together with SA.

I observed enlarged HR lesions in EDS1-YFP-GR transgenic plants without DEX treatment in response to infection with avirulent *H. parasitica* (Figure 3.12A). The presence of the enhanced lesions could be explained by a weakened resistance response that fails to halt the pathogen within the cells undergoing pathogen attempted penetration. If this is true, it may be due to a defect in defense potentiation which may need nuclear EDS1 activities. Another possibility is that EDS1-YFP-GR lines are defective in containing pathogen-triggered cell death due to an altered ROS metabolism. I have not detected spontaneous cell death in these transgenic lines with or without DEX treatment which implies that programmed cell death is not generally deregulated. One way to tackle this issue would be to study the sensitivity of

EDS1-YFP-GR transgenic plants to treatment with ROS generators, such as paraquat. The herbicide paraquat is a known generator of chloroplastic ROS by acting as a terminal oxidant of the photosystem I (Mehler, 1951). Under light conditions, Photosystem I reduces oxygen to the superoxide radical that subsequently dismutates to hydrogen peroxide. I can assess the effect of paraquat on the initiation of cell death and transcription of stress-related genes in the EDS1-YFP-GR lines with and without DEX, to unravel whether EDS1 function in processing ROS signals and initiation of cell death is mainly cytosolic. It is interesting that the observed enlarged HR lesions resemble those present in *lsd1* plants also after infection with avirulent *H. parasitica* (Rusterucci et al., 2001). True leaves of challenged *lsd1* plants further developed runaway cell death 6 d after infection which I did not observe in EDS1-YFP-GR transgenics. This raises the possibility that, similar to LSD1, EDS1 modulates the localization and/or activity of transcription factors involved in cell death control.

## 4.4 EDS1 is capable of constitutive nucleocytoplasmic shuttling

An alternative to unidirectional relocalization is constitutive nucleocytoplasmic shuttling. Standard microscopic and biochemical methods usually reveal protein localization only in those compartments where the steady state concentration of a protein is above the detection threshold, disregarding the aspect of protein movement and the possibility that a minor fraction of the protein performs an important function in another compartment. During the past years, an increasing number of proteins that shuttle continuously back and forth between the nucleus and the cytoplasm were identified, providing evidence that nucleocytoplasmic shuttling proteins are key factors in relaying information between the two major compartments (Gama-Carvalho and Carmo-Fonseca, 2001; Xu and Massague, 2004). Leptomycin B (LMB) and ratjadones are bacterial toxins that specifically inhibit nuclear export mediated by CRM1-type exportins and have been used to identify constitutively shuttling proteins and complexes, since the inhibition of nuclear export causes their accumulation in the nucleus (Kudo et al., 1998; Carlotti et al., 2000; Huang et al., 2000; Heerklotz et al., 2001; Meissner et al., 2004). Strikingly, many proteins thought to localize exclusively in the cytoplasm were revealed to enter the nucleus through the use of nuclear export inhibitors, e.g. NFkappaB (Carlotti et al., 2000; Huang et al., 2000). LMB and RatA have also been shown to be functional in plants and

to inhibit the *Arabidopsis* homologue, AtCRM1/AtXPO1, in a similar fashion (Haasen et al., 1999; Meissner et al., 2004). Shuttling proteins or complexes typically have both nuclear localization and nuclear export signals, as is the case for EDS1, PAD4 and SAG101 protein complexes (Falk et al., 1999; Feys et al., 2005). Consistent with this, I have found that EDS1 protein is capable of nucleocytoplasmic shuttling in healthy *Arabidopsis* protoplasts. Whether EDS1 is shuttling in a monomeric or dimeric form has not been determined, although EDS1-EDS1 homodimers detected by FRET were only observed in the cytoplasm (Feys et al., 2005). It would be interesting to examine whether PAD4 is as well a nucleocytoplasmic shuttling protein and if EDS1-PAD4 heterodimers are shuttled across the nuclear pores as a complex. This would provide insight on the intracellular dynamics of EDS1 complexes.

The functional significance of EDS1 being a nucleocytoplasmic shuttling protein is still not clear. For this reason it would be useful to determine if there is any change in the protein movement associated with the activation of defense responses, as for example in the speed of EDS1 shuttling which could be revealed by studying the time point after RatA treatment at which maximal nuclear retention is observed. In this regard, the EDS1-YFP line in the *snc1/* Col *eds1-2* background generated in this study could be of use to prepare protoplasts and examine EDS1 movement in a constitutive activated background. Nevertheless, in view of my results it is tempting to speculate with a role for EDS1 in the passaging of signaling molecules between the nucleus and cytoplasm, and hence relaying information between both compartments. Coupled with the previous data suggesting EDS1 degradation in the nucleus, I anticipate that EDS1 ability to move out of the nucleus is important for protein stability. This would also help in maintaining a certain ratio and equilibrium between EDS1 nuclear and cytosolic protein pools.

## 4.5 An EDS1, PAD4, SAG101 signaling triad

The essential role of EDS1 in plant immunity is accomplished through a concerted action with its interacting partners PAD4 and SAG101 (Feys et al., 2001; Feys et al., 2005). PAD4 and SAG101 signal within the EDS1-regulated disease resistance pathway and contribute intrinsic and partially redundant signaling activities in defense activation (Feys et al., 2005). The three proteins share a domain with homology to eukaryotic lipases and a second domain of unknown

function termed EP (for EDS1 and PAD4 defined) (Feys et al., 2001). A combination of cell fractionation, coimmunoprecipitation and FRET experiments have demonstrated that EDS1, PAD4 and SAG101 form molecularly and spatially distinct associations in the nucleus and cytoplasm (Feys et al., 2001; Feys et al., 2005). Furthermore, the presence of the interacting partners is important for EDS1, PAD4 and SAG101 protein stability (Feys et al., 2005), suggesting that they may mututally influence their partners subcellular distributions.

I assessed the role of PAD4 and SAG101 on EDS1 protein accumulation and nucleocytoplasmic partitioning in uninduced conditions and after defense activation through cell fractionation followed by protein gel blot analyses. EDS1 protein levels were found to be reduced in nuclei-depleted and nuclei-enriched extracts of *pad4* and *sag101* healthy plants, with a further reduction in the *pad4-1/sag101-1* double mutant (Figures 3.4 and 3.5). Since a similar ratio to wild-type was present in all fractions I concluded that EDS1 steady-state nucleocytoplasmic distribution is not influenced by its interacting partners, consistent with previous results obtained in transient expression assays (Feys et al 2005). A similar scenario was observed when I examined pathogen-triggered tissues, either with virulent *H. parasitica* or with avirulent *Pst* DC3000 AvrRps4 (Figures 3.4 and 3.5). Notably, EDS1 nuclear enrichment which was visible during RPS4-mediated responses to *Pst* DC3000 AvrRps4 in wild-type, *pad4-1* and *sag101-1* plant extracts was not observed in *pad4-1/sag101-1* double mutant plants (Figure 3.5). At present, it remains unclear whether the observed nuclear enrichment after avirulent *Pseudomonas* infection is determined by an increase in EDS1 nuclear import, nuclear retention or protein stabilization of EDS1 nuclear pool. Thus, it is possible that *pad4-1/sag101-1* double mutant plants are impaired in EDS1 pathogen-inducibility which may happen specifically in the nuclear compartment at early stages of R-mediated responses or in mobilization to the nucleus of pre-existing EDS1 protein. If the absolute resistance suppression present in *pad4/sag101* double mutant plants (Feys et al., 2005; Lipka et al., 2005), is related to the absence of EDS1 nuclear accumulation, expression of the EDS1-YFP-NLS fusion protein in *pad4/sag101* may suppress or attenuate the resistance defects. A cross between one of the selected EDS1-YFP-NLS transgenic lines with the double mutant is in progress. Another possibility to consider is that EDS1 may function as a scaffold that modulates the stability, subcellular distribution and signaling activities of its interacting partners. Hence the effect of withholding EDS1 in the cytosol or pushing it into the nucleus on PAD4 and SAG101 localization should be examined.

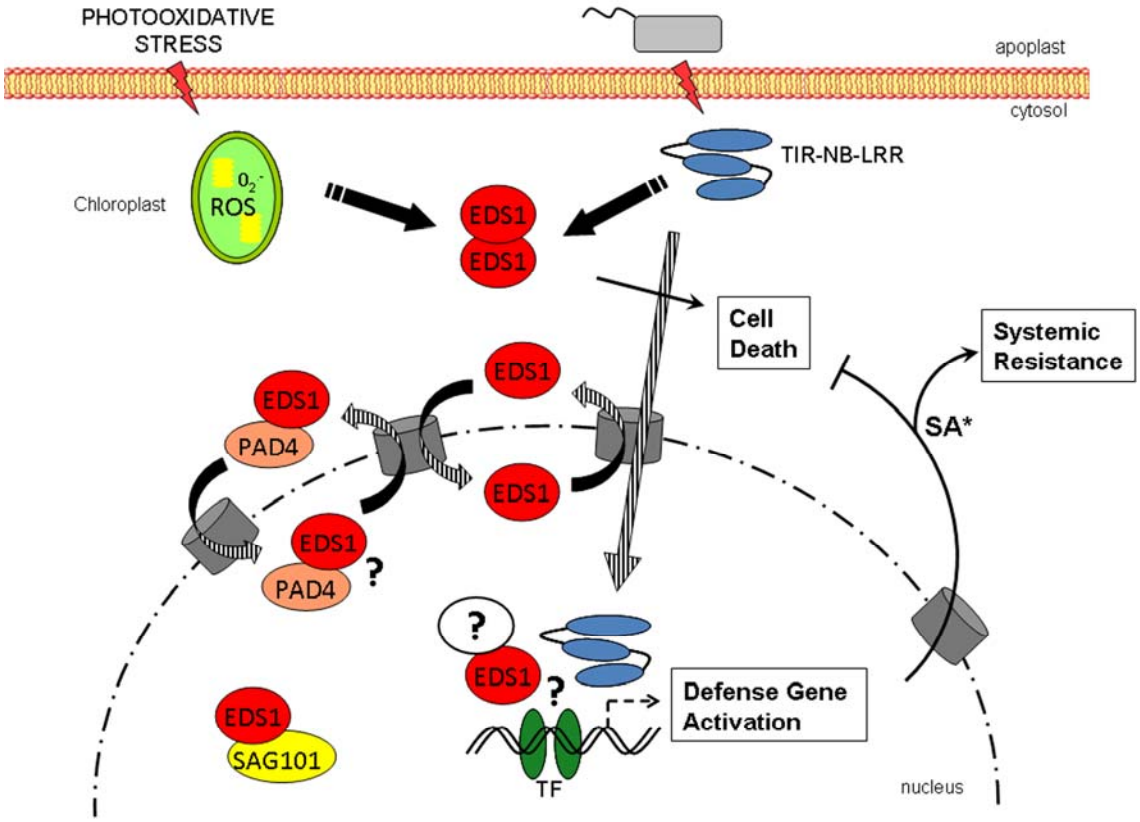


**Figure 4.2. A model of the subcellular dynamics and signaling activities of EDS1 complexes during defense responses and oxidative stress that lead to activation of defense responses such as cell death, defense gene induction and systemic resistance.** EDS1 may be involved in plastid to nucleus retrograde signaling by conveying particular ROS stress signals coming from the chloroplast to the transcriptional machinery in the nucleus. EDS1 processing of ROS signals in the cytoplasm could also participate in triggering of programmed cell death. EDS1 is capable of nucleocytoplasmic shuttling and likely transports PAD4 across the nuclear pore. EDS1 activities in the nucleus may be related to its role in transcriptional reprogramming through the interaction with transcription factors (TFs). This function could be accomplished alone or together with TIR-NB-LRRs and/or its interacting partners PAD4 and SAG101. One output of EDS1 interaction with the transcriptional machinery would be defense gene regulation and the corresponding proteins synthesized could be involved in SA-pathway responses such as modulation of cell death, accommodation of ROS stress and promotion of systemic resistance.

## 4.6 Future perspectives

Some recent reports point to the nucleus as a key compartment for plant immunity (Burch-Smith et al., 2007; Shen et al., 2007; Wirthmueller et al., 2007). The results presented in this study also suggest that EDS1 nuclear activities are important for the activation of plant disease resistance. A function for EDS1 complexes in fine-tuning transcriptional reprogramming could account for its central role in interpreting and transducing various environmental cues such as pathogen and oxidative stress signals, and in modulating the interaction between plant hormone pathways. The specific nuclear activities of EDS1 are not known and due to the absence of obvious DNA binding domains in EDS1 sequence it is tempting to speculate that it functions in concert with yet unidentified transcription factors to regulate gene expression. Studies on the nuclear function of the mammalian NB-LRR CIITA, master regulator of MHC class II gene expression, presents interesting possibilities for the nuclear activities of EDS1 and plant R proteins (Hake et al., 2000; Ting and Davis, 2005). Lacking apparent DNA-binding capacity, CIITA is a potent transcriptional activator that serves as a scaffold for the recruitment of transcription factors and DNA modifying enzymes on MHC class II promoters. Future research could address these possibilities for EDS1 and R proteins by studying potential interaction with transcription factors and chromatin modifying enzymes and by assessing their chromating binding capacity. Techniques including chromatin immunoprecipitation (Ch-IP) should allow us to gain insights on the target DNA regions. The EDS1-YFP-NLS transgenic lines generated in this study will be useful tools to characterize further the nuclear activities of EDS1. Stable transgenic lines expressing a similar contruct driven by an estradiol inducible promoter are being generated for a more controlled and detailed analysis of EDS1 activities upon nuclear entry and to link it with gene expression outputs.

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Köln, im Februar 2009 _____

Ana Victoria García

# Curriculum vitae

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