Analysis of mechanisms underlying EDS1-PAD4 cooperation in *Arabidopsis* immune signaling

Inaugural–Dissertation

zur

Erlangung des Doktorgrades

der Mathematisch-Naturwissenschaftlichen Fakultät

der Universität zu Köln

vorgelegt von

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Köln, April 2007

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

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Tag der Disputation:	14. Juni 2007

Publications

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

Abstract

Plants have evolved a multilayered immune system to counter pathogen attacks. EDS1 (Enhanced Disease Susceptibility 1) and PAD4 (Phytoalexin Deficient 4) are two plant-specific lipase-like proteins that function as essential regulators of plant innate immunity. They are crucial for basal defence that restricts growth of virulent pathogens and for race-specific resistance to avirulent pathogens triggered by TIR (Toll-Interleukin 1) type NBS-LRR (Nucleotide Binding Site – Leucine Rich Repeats) immune receptors. Moreover, EDS1 and PAD4 generate and perceive (a) signal(s) needed to induce systemic immunity. These regulators stimulate accumulation of the phenolic defence signaling molecule salicylic acid (SA) and SA, in turn, induces their expression creating a positive feedback loop in defence potentiation. *EDS1* and *PAD4* transcript and correspondent protein levels increase upon pathogen challenge. However, earlier changes in expression of a set of distinct genes which are EDS1- and PAD4-dependent imply the activation of pre-existing EDS1/PAD4 complexes through post-translational mechanism(s). In this work I investigated the relative importance of transcriptional regulation and post-transcriptional processes for EDS1 and PAD4 protein functions. I characterized *Arabidopsis thaliana* transgenic lines overexpressing either EDS1, PAD4 or both. Only lines cooverexpressing EDS1 and PAD exhibited growth retardation associated with constitutive activation of the SA pathway and increased resistance to virulent pathogens resulting from a faster SA pathway activation. These lines exhibit also increased tolerance to chemically induced oxidative stress consistent with a known role of EDS1 and PAD4 in processing reactive oxygen species (ROS) - derived signals. The insufficiency of EDS1-PAD4 cooverexpression to fully recapitulate defence activation implies the existence of post-translational mechanisms of regulation. The existence of regulatory post-translational modifications of the EDS1 protein was investigated and lines expressing constitutively or conditionally activated functional epitope-tagged EDS1 were generated. The data presented here demonstrate that EDS1 and PAD4 operate as a signaling unit. The basis of the observed dramatic biotic and abiotic stress phenotypes will be further investigated as it should provide important insight into EDS1 and PAD4 functions.

Zusammenfassung

Pflanzen haben ein mehrschichtiges Immunsystem entwickelt um Pathogene abzuwehren. EDS1 (Enhanced Disease Suseptibility 1) und PAD4 (Phytoalexin deficient 4) sind zwei pflanzenspezifische Lipase-artige Proteine die als essentielle Regulatoren des angeborenen pflanzlichen Immunsystems fungieren. Beide Regulatoren werden sowohl für die basale Abwehr, die das Wachstum von virulenten Pathogenen begrenzt, als auch für die durch Immunrezeptoren der Klasse TIR(Toll-Interleukin 1) NBS-LRR (Nucleotide Binding Site – Leucine Rich Repeats) kontrollierte rassen-spezifische Abwehr gegen avirulente Pathogene, benötigt. Darüber hinaus sind EDS1 und PAD4 für die Ausbildung der systemischen Resistenz essentiell, die die Pflanze nach erstmaliger Infektion vor weiteren Infektionen schützt. EDS1 und PAD4 stimulieren des Weiteren die Akkumulierung des Abwehrsignals Salizylsäure (SA), welches wiederum die Transkription von *EDS1* und *PAD4* aktiviert, wodurch eine Amplifizierung der Abwehrreaktion hervorgerufen wird. Frühre Arbeiten haben gezeigt, dass EDS1-PAD4 Proteinkomplexe bereits in unbehandelten, gesunden Pflanzenzellen existieren. *EDS1* und *PAD4* Transkript sowie korrespondierende Proteinlevel steigen nach Pathogeninokulation an. Die Akkumulation der EDS1-PAD4 Komplexe tritt aber zeitlich nach einer EDS1/PAD4-abhängigen transkriptionellen Reprogrammierung anderen Genen auf, so dass man eine post-translationale Aktivierung von EDS1 und PAD4 postulieren kann. In dieser Arbeit wurde die Bedeutung der transkriptionellen Aktivierung und der post-transkriptionellen Prozessen für die Funktion von EDS1 und PAD4 untersucht. Dazu wurden transgene *Arabidopsis thaliana* Linien untersucht, die entweder EDS1 oder PAD4 alleine oder beide zusammen überexprimieren. Nur Linien, die EDS1 und PAD4 gemeisam überexprimieren, zeigen eine Wachstumshemmung, eine konstitutive Aktivierung des SA-abhängigen Signalweges und eine erhöhte Resistenz gegenüber virulenten Pathogen. Die EDS1/PAD4-Überexpressör-linien wiesen zudem eine erhöhte Toleranz gegenüber Chemikalien die oxidativen Stress verursachen auf, was konsistent ist mit der bekannten Rolle von EDS1/PAD4 als Modulator von Redoxsignalen. Da die Co-Überexpression von EDS1/PAD4 nicht zu einer vollständigen Abwehrreaktion (z.B. fehlender hypersensitiver Zelltod) führt, kann daraus geschlossen werden, dass EDS1 und

PAD4 auch post-translational reguliert werden. Die mögliche Existenz post-translationaler Modifikation(en) von EDS1 wurde untersucht. Dazu wurden verschiedene Linien generiert, die eine konstitutive oder eine induzierbare Aktivierung von EDS1 aufweisen. Die Daten in dieser Arbeit demonstrieren, dass EDS1 und PAD4 zusammen als Signaleinheit operieren. Die Ursache der erhöhten Resistenz gegenüber biotischen und abiotischen Stress in den EDS1/PAD4-Überexpressör-Linien wird weiter untersucht und sollte wichtige Hinweise auf die Funktion von EDS1 und PAD4 geben.

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Table of abbreviations

::	fused to (in the context of gene fusion constructs)
° C	degree Celsius
avr	avirulence
bp	base pair(s)
BTH	benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester
C	carboxy-terminal
CaMV	cauliflower mosaic virus
CC	coiled-coil
cDNA	complementary DNA
cfu	colony forming unit
d	day(s)
dATP	deoxyadenosinetriphosphate
dCTP	deoxycytidinetriphosphate
dGTP	deoxyguanosinetriphosphate
dH_2O	deionised water
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleosidetriphosphate
dsRNAi	double-stranded RNA interference
DTT	dithiothreitol
dTTP	deoxythymidinetriphosphate
EDS1	Enhanced Disease Susceptibility 1
EDTA	ethylenediaminetetraacetic acid
Emwa1	*Hyaloperonospora parasitica* isolate Emwa1
ET	ethylene
EtOH	ethanol
Fig.	Figure
FMO	flavin-dependent monooxygenase
g	gram
g	gravity constant (9.81 ms^{-1})
h	hour(s)
HR	hypersensitive response
HRP	horseradish peroxidase
kb	kilobase(s)
kDa	kiloDalton(s)
l	litre
LRR	leucine-rich repeats
m	milli
M	molar (mol/l)
µ	micro
min	minute(s)
mM	millimolar
mRNA	messenger ribonucleic acid

N	amino-terminal
NBS	nucleotide binding site
ng	nanogram
nm	nanometer
Noco2	*Hyaloperonospora parasitica* isolate Noco2
NUDIX	nucleoside diphosphates linked to some other moiety x
OD	optical density
P_{35SS}	double 35S promoter of CaMV
PAA	polyacrylamide
PAD4	Phytoalexin Deficient 4
PAMP	pathogen-associated molecular pattern
PCR	polymerase chain reaction
PAGE	polyacrylamide gel-electrophoresis
pH	negative decimal logarithm of the H^+ concentration
PR	pathogenesis related
Pst	*Pseudomonas syringae* pv. *tomato*
pv.	pathovar
R	resistance
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	rounds per minute
RPM	resistance to *Pseudomonas syringae* pv. *maculicola*
RPP	resistance to *Peronospora parasitica*
RPS	resistance to *Pseudomonas syringae*
RT	room temperature
RT-PCR	reverse transcription-polymerase chain reaction
SA	salicylic acid
SAG101	Senescence Associated Gene 101
SAR	systemic acquired resistance
SDS	sodium dodecyl sulphate
sec	second(s)
TBS	Tris buffered saline
T-DNA	transfer DNA
TIR	*Drosophila* Toll and mammalian interleukin-1 receptor
TLR	Toll-like receptor
Tris	Tris-(hydroxymethyl)-aminomethane
U	unit
UV	ultraviolet
V	Volt
v/v	volume per volume
w/v	weight per volume

1. INTRODUCTION

In the natural environment plants are continuously under attack by microbial, animal and viral pathogens with diverse life styles and infection strategies [1]. Exposure to potentially lethal assaults has shaped modern plants through evolution and resulted in the development of a multilayered innate immune system. The robustness and effectiveness of plant immunity is illustrated by the fact that most plants remain healthy [2, 3]. Understanding how interactions at the interface between plants and pathogens are regulated is an essential to enhance plant survival as one primary source of food, materials and energy.

1.1 *Arabidopsis thaliana* as model system for studying plant-microbe interactions

The small flowering plant, *Arabidopsis thaliana*, a member of the mustard family (*Brassicaceae*), has emerged as the model plant species in biology. *Arabidopsis* was chosen because it offers many advantages: a short life cycle (about 6 weeks), high fertility, small size, a relatively small genome and efficient transformation by *Agrobacterium tumefaciens* [4]. In the last decade extensive genetic and physical maps of all its five chromosomes were made available, large collections of *Arabidopsis* mutants were generated and approximately 115 Mb of its 125 Mb total genome have been sequenced [5, 6]. Also, analyses of *Arabidopsis* accessions from different geographical locations reveals a high degree of natural genetic variation that can be used to gain insight into fundamental biological processes [7].

Pathogens of *Arabidopsis* that belong to the major classes of plant disease agents have been described and the dissection of these interactions by genetic and biochemical means has enormously improved our understanding of mechanisms underlying plant responses to pathogen attack [1-3].

1.1 The plant immune response

While animals also have an adaptive immune system based on specialized cell types and creation of antigen-specific receptors by somatic recombination [8], plant defense against pathogen attacks relies on the innate immune system (the only exception being antiviral RNA silencing which exhibits features of adaptive recognition) [9]. The plant immune system consists of both pre-formed barriers (such as waxy cuticle, cell wall and antimicrobial compounds accumulating before pathogen challenge) and induced defences [1]. Recent evidence shows that the inducible component of the plant immune system can be divided into two main layers [3] : pathogen associated molecular patterns triggered immunity (PTI) and effector triggered immunity (ETI).

1.1.1 Pathogen associated molecular patterns (PAMP) triggered immunity (PTI)

Microbial or pathogen associated molecular patterns (known as MAMPs or PAMPs) are highly conserved microbial molecules that have essential functions. They are present in entire classes of both pathogenic and non pathogenic microbes but are generally not found in the host [10]. These features make PAMPs ideal “non self” molecules which are recognized by pattern recognition receptors (PRRs) in both plants and animals [10]. Examples of PAMPs perceived by plants are flagellin, Elongation Factor Tu (EF-Tu) and lipopolisaccharides from bacteria, chitin and ergosterol from true fungi, and heptaglucoside and transglutamminase from oomycetes [11]. The most well characterized PAMP in plants is a portion of the flagellin protein. Flagellin builds up the flagellar filaments that are indispensable for bacterial motility [11]. Exposure of *Arabidopsis* plants, protoplasts or cell cultures to purified flagellin or to its N-terminal 22 amino acid peptide flg22 leads to a series of downstream events including an oxidative burst, mitogen associated protein kinase (MAPK) cascade activation, callose deposition at the cell wall, ethylene production, and the rapid transcriptional reprogramming and growth inhibition of seedlings [12, 13]. The flagellin receptor *FLS2* (*Flagellin Sensing 2*) was identified in a genetic screen for mutants insensitive to flg22 and encodes a receptor-like

kinase (RLK) which is internalized upon flagellin perception by receptor-mediated endocytosis [14]. Structurally, FLS2 contains an extracellular LRR (Leucine Rich Repeat) domain and an intracellular serine/ threonine kinase domain [15]. In analyses using spray inoculated pathogenic bacteria, *Arabidopsis fls2* mutant plants exhibited increased susceptibility while pre-treatments with flg22 on wild type plants induced increased resistance. More recently another *Arabidopsis* PRR, the EF-Tu receptor EFR (EF-Tu receptor), was shown to play a major role in restricting colonization by *Agrobacterium tumefaciens* [16]. Thus, PAMP recognition plays an important role in priming defences against pathogens [17]. These results are consistent with the general concept of PAMP triggered immunity (PTI) in which the recognition of PAMPs triggers downstream responses that in many cases are sufficient to halt microbes from progressing in their colonization attempts [2]. Downstream responses to EF-Tu recognition overlap with those observed upon flagellin perception [16] indicating that different PRRs converge to common signaling pathways and defence outputs [3]. *EFR* also codes for an RLK containing an extracellular LRR and internal serine/threonine kinase domain [16]. Within the *Arabidopsis* genome 200 RLKs were identified. Twenty eight of them are up regulated after PAMP perception [16, 18, 19]. These genes represent a potential PRR arsenal for perception of yet further PAMPs that have not yet been molecularly characterized [16].

1.1.2 Pathogen effector triggered susceptibility (ETS)

An efficient way through which pathogens appear to overcome PTI is by secretion of effector proteins into the plant cell [20-23]. This phenomenon was recently termed effector triggered susceptibility (ETS) and evidence for the existence of effector proteins interfering with the signaling cascade downstream of PAMP perception has accumulated in the last few years [1, 2]. The best characterized pathosystem in this respect is the interaction between *Arabidopsis* and strains of pathogenic Gram-negative bacteria, *Pseudomonas syringae*. All known *P. syringae* strains contain a *hypersensitive response and pathogenicity* (*hrp*) – locus encoded type III secretion system (TTSS) [24, 25]. The TTSS generates a molecular syringe upon contact with the host through which effector

proteins and toxins are secreted into the host cell [26]. Some *Pseudomonas syringae* TTSS effectors have been described that interfere with PTI by mimicking or inhibiting eukaryotic cellular functions. For example HopM and AvrE effectors target host vesicle transport [27]. AvrPto and the E3 Ubiquitin Ligase AvrPtoB block PTI at an early stage before MAPK cascade activation [28], while AvrRpm1 and AvrRpt2 target RIN4 (Rpm1 Interacting Protein 4) a negative regulator of PTI [29]. Other type III effectors from phytopathogenic bacteria belonging to the genera *Pseudomonas*, *Xanthomonas*, *Ralstonia*, *Erwinia* and *Pantoea* have been identified and for some of them a biochemical function was experimentally assigned [20, 21]. Only for a few of them has the corresponding host target been identified [20, 21].

Effector proteins were isolated also from fungi and oomycetes [22, 23]. Phytopathogenic fungi and oomycetes do not possess TTSS. However, they form a specialized infection structure called the haustorium that invaginates the host cell membrane with minimal disruption [30]. The precise mechanism(s) through which fungal effectors are delivered from the haustorium into the host cell are unclear [23, 31]. A large collection of candidate secreted effector proteins was identified for *Phytophthora* species [32]. These effectors share a signal peptide for secretion and an RxLR motif followed by a glutamate/aspartate rich domain which is hypothesized to act as a host-targeting signal [23, 32-34]. These structural features are absent in identified effectors from true fungi suggesting the existence of different delivery mechanisms between fungi and oomycetes [23]. An example of a fungal effector protein secreted into the host and interfering with PTI is given by Avr3a from *Phytophthora infestans* which can suppress cell death in *Nicotiana benthamiana* induced by the elicitin INF1, also from *Phytophthora infestans* [35].

1.1.3 Effector triggered immunity (ETI)

In order to counter microbial attempts to subvert PAMP recognition, plants have evolved receptors capable of recognizing pathogenic effectors [1]. Recognition is followed by a series of downstream events such as a massive oxidative burst, accumulation of phenolic compounds including salycilic Acid (SA) and transcriptional reprogramming in both local and systemic tissues. There is also accumulation of antimicrobial compounds at the

site of attempted penetration, activation of a phosphorylation cascade and most commonly a form of localized programmed cell death termed hypersensitive response (HR) [1]. This series of responses is normally sufficient to block the pathogen and being induced by effector recognition has been termed Effector Triggered Immunity (ETI) [2, 3]. Effectors that are specifically recognized are, in this context, called Avirulence (Avr) proteins.

Genes encoding ETI receptors are called Resistance (R) proteins and have been cloned from various plant species. Comparative analyses led to identification of a limited number of R proteins structure motifs [1]. The most abundant class of R protein in *Arabidopsis* has a central NBS (Nucleotide Binding Site) domain and C-terminal LRRs (Leucine Rich Repeats), so called NBS-LRR proteins [36]. A further subdivision within this class can be made according to the type of N-terminus. Some NBS-LRR have similarity to the intracellular domains of Toll and Interleukin-1 receptors from *Drosophila* and humans respectively (TIR-NBS-LRR proteins). Others have a predicted coiled-coil domain (known as CC-NBS-LRR proteins) [1]. The type of N-terminus correlates with the R protein requirement for particular downstream signaling components upon recognition ([37], see below). In *Arabidopsis*, examples of TIR-NBS-LRR are RPP1, RPP4, involved in *Hyaloperonospora parasitica* race specific recognition [38, 39], and RPS4 recognizing *Pseudomonas syringae* pv. *tomato* DC3000 (hereafter *Pst* DC3000) expressing *AvrRps4* [40]. Examples of CC-NBS-LRR are RPS2, RPM1 and RPS5 involved in the recognition of DC3000 expressing respectively AvrRpt2, AvrRpm1 or AvrB, and AvrPphB [41-43] and RPP8 and RPP13 also involved in race specific recognition of downy mildew [44, 45].

Initially, the simple genetic relationship between plant *R* and pathogen *Avr* genes suggested a direct receptor – ligand binding model for their biochemical interaction. Some examples of direct recognition have been described [46-48]. However, evidence emerging in the last decade points towards a wider engagement of indirect recognition strategies as described by the "guard" model [1]. According to this model pathogen effectors target and modify host proteins in order to subvert defence responses or gain nutrients. An R protein guards particular host proteins and perceives modifications induced by the effector (Avr), thereby triggering defense activation [1]. The two best

characterized examples of indirect recognition are provided by AvrRpm1 and AvrPphB [49, 50]. AvrRpm1 targets RIN4 and other so far unidentified host protein(s) [29, 49]. Upon interaction AvrRpm1 causes a hyperphosphorylation of RIN4 which correlates with the activation of RPM1 [49]. AvrPphB is a cysteine protease that targets the host protein kinase PBS1 (AvrPphB Susceptible1). PBS1 cleavage leads to the activation of RPS5 which triggers resistance signaling [50]. According to this model the relatively limited number of identified receptors in plants could account for interception of many pathogen effectors [51]. Thus, few receptors guarding key host proteins would in fact be sufficient to monitor the presence of multiple effectors having the same target [1]. Experimental evidence supports this hypothesis. For example, in addition to AvrRpm1 the bacterial effector AvrB targets RIN4 leading to a similar RPM1 activation [49].

The existence of different recognition modes is supported by phylogenetic studies. In the case of direct Avr-R interaction signatures for diversifying selection in corresponding *Avr* and *R* genes were observed [47, 52]. In evolutionary terms, this can be explained as the result of selective pressure to escape recognition by diversification from the pathogen side and to evolve new recognition specificities from the plant side. In the indirect recognition scenario, mutations in *Avr* genes affecting recognition may also affect virulence functions being recognized. Accordingly, no clear sign of diversifying selection in *R* or *Avr* genes involved in indirect recognition events could be observed [53, 54].

Not surprisingly pathogens have evolved further effectors to interfere with ETI and plants, in turn, new receptors to detect them. A clear example is given by the DC3000 AvrRpt2 effector and the *Arabidopsis* receptor RPS2 [49]. AvrRpt2 encodes a cysteine protease which targets RIN4. Cleavage of RIN4 by AvrRpt2 impairs AvrRpm1 induced RPM1 activation. In turn Rps2 is capable of recognizing RIN4 cleavage and activates HR [49].

How R proteins are activated and how their activation leads to resistance is not fully understood but recent results shed some light on this phenomenon. A negative intra-molecular regulatory function was shown for the LRR domain [36, 55-57] which was previously shown to be important in determining recognition specificity [58-60]. Receptor activation is thought to involve intra-molecular rearrangements to expose the NBS domain, allowing cleavage and cycling of bound ATP [55, 56, 61-63]. This

presumably allows the amino-terminal domain of the receptor to interact with downstream signaling molecules that trigger the defense response. At least in one case homomeric oligomerization was observed as a very early event upon recognition [64] reminiscent of what is observed for animal Nod-Like Receptors (NLR) which are related to plant NBS-LRR proteins in their domain structure [65].

Two recent pieces of data showed how two different receptors, MLA10, a CC-NBS-LRR receptor from barley, and N, a TIR-NBS-LRR receptor from tobacco, require nuclear localization to trigger downstream responses upon perception of the correspondent effectors (AvrMla 10 from the fungus *Blumeria graminis* fs *hordei* for MLA10 and the p50 replicase protein from Tobacco Mosaic Virus for N) [66, 67]. After AvrMla10 recognition, MLA10 was shown to interact specifically with the transcription factors WRKY1 and WRKY2, negative regulators of plant basal defense, drawing a molecular link between recognition and activation of downstream defence [66]. Unpublished results also reveal that a nuclear pool of the *Arabidopsis* TIR-NBS-LRR receptor RPS4 is important for defense activation (L. Wirthmueller and J. Parker, unpublished)

1.1.4 General terminology

Interactions between plants and microbes are classified according to their outputs: when a specific pathogen race is recognized and stopped through ETI the interaction is defined as incompatible and the pathogen race avirulent. When the pathogen can successfully colonize the plant and cause disease, the interaction is defined as compatible and the pathogen virulent [1]. If all members of a microbial species are not capable to infect a particular plant species the interaction is defined non-host or species level resistance [68]. Non-host defense consists of both constitutive and inducible mechanisms. Two layers of inducible responses have been shown to be involved in blocking a host non-adapted pathogen. Pre-invasive mechanisms act before the pathogen gains access to the plant interior and are compromised in so called *pen* (*penetration*) mutants [69-71]. Post-invasive mechanisms are instead activated after penetration [70].

Even in a compatible interaction, a so called basal defence is activated in susceptible plants. The existence of basal defence mechanisms can be demonstrated by the fact that

plants lacking functional basal defense signaling components support higher growth of virulent pathogens compared to wild type [1]. Genetic overlap between ETI and basal resistance responses suggests that one function of R-mediated signaling is to more rapidly and effectively activate defence mechanisms that are shared by both pathways [1, 72].

1.1.5 Systemic Acquired Resistance

Defense activation in systemic tissues follows the localized HR, resulting in heightened resistance to subsequent pathogen attacks. This phenomenon is known as systemic acquired resistance (SAR) and its establishment is dependent on SA accumulation [73]. The generation, translocation and perception of a non species-specific SAR signal moving from the infected leaves systemically are necessary for SAR induction [73, 74]. The nature of this signal(s) is still unclear. The fact that in early grafting experiments in tobacco, root-stocks expressing a bacterial SA degrading enzyme were still capable of inducing SAR in wild type scions, indicated that SA is not the mobile signal [75]. Possible connections with lipid metabolism and SAR signal generation emerged from studies of *Arabidopsis* mutant lines carrying mutations in genes involved in fatty acid metabolism and associated altered SAR responses [74]. Also, *Arabidopsis* plants carrying mutations in the *DIR1* (*Defective in induced resistance 1*) gene, coding for a putative lipid transfer protein, are impaired in the generation or translocation of the SAR signal [76]. Most recently, the involvement of the fatty acid-derived signal molecule jasmonic acid (JA) was implicated in the establishment of SAR [77].

NPR1 is a central positive regulator of SAR signaling that functions downstream of SA [78]. Accumulation of SA induces a change in cellular redox potential triggering the reduction of NPR1 from cytosolic, disulphide-bound oligomers to active monomers [79]. Monomers translocate to the nucleus where they can interact with TGA transcription factors. These interactions may stimulate the binding of TGA factors to SA-responsive elements in the promoters of *PR* genes. The consequent transcriptional reprogramming likely contributes to the establishment of SAR [80]. Transcriptional data indicated that NPR1 co-ordinates up regulation of the secretory apparatus to ensure proper folding and localization of PR proteins [81].

MPK4 (mitogen activated protein kinase 4) instead encodes for a negative regulator of SAR establishment and *mpk4* plants show constitutive SAR response [82]. Such negative regulation is dependent on the MPK4 kinase activity since that stable inactive MPK4 variants were unable to complement the *mpk4* phenotype [82].

SNI1 (*suppressor of npr1-1, inducible 1*) is also a negative regulator of SAR, encoding a leucine-rich nuclear protein with similarity to Armadillo repeats proteins [83, 84]. SNI1 specifically represses *NPR1*-dependent SA responsive genes, probably by serving as a scaffold for formation of a chromatin remodeling complex [83, 84]. Pathogen infection triggers an increase in somatic DNA recombination, which results in transmission of changes to the offspring of infected plants [85]. *SNI1* also negative regulates this phenomenon suggesting a possible mechanistic link between short-term defense response and a long-term survival strategy [86].

1.1.6 Salicylic acid and jasmonic acid / ethylene pathways

ETI (effector triggered immunity) is effective against pathogens that feed on living plant tissues throughout their life cycle (obligate biotrophs, such as *Hyaloperonospora* species) or in the first phase of colonization (hemibiotrophs, such as *Phytophtora*, *Colletotrichum* and *Pseudomonas* species). ETI is not effective against pathogens that feed on dead plant tissues and that can induce host cell death by releasing toxins (necrotrophs, such as *Botrytis* and *Alternaria* species) [87]. This distinction is reflected by a differential engagement of downstream pathways in response to pathogens with different lifestyles. While the salicylic acid (SA) pathway plays a major role in response to biotrophs and hemibiotrophs, the jasmonic acid (JA) and ethylene (ET) pathways are essential for activating responses to necrotrophs [87].

Although SA, JA and ET pathways overlap partially in terms of gene activation, analyses of specific SA and JA marker genes induction (such as *Pathogenesis Related 1* (*PR1*) and *Pathogenesis Related 2* (*PR2*) for SA and *Plant Defensin 1.2* (*PDF1.2*) for JA respectively) upon different treatments and within different mutant backgrounds revealed mutual antagonism between the SA and JA/ET pathway [87, 88]. This is reflected by the increased resistance to biotrophs in plants impaired in JA signaling and to necrotrophs in

plants impaired in SA signaling. Reciprocally, mutations constitutively activating the SA pathway or JA/ET pathway resulted in increased susceptibility against necrotrophs and biotrophs, respectively [87, 88]. However, cases of additivity between the two pathways have been however also observed as well as cases of reciprocal inhibition between the. activation of the JA end ET pathway indicating a much more complex cross-talk between pathways activations whose spatial and temporal aspects are not fully appreciated yet [88].

Two genes that are involved in the antagonism between SA and JA/ET pathways are *NPR1* and *MPK4*. JA pathway repression by SA pathway activation requires NPR1, but not its nuclear localization, suggesting a specific NPR1 cytosolic function [89]. On the other hand, MPK4 activity is essential for the repression of the SA pathway and the activation of the JA/ET pathway [82, 90].

1.2 The disease resistance signalling proteins EDS1 and PAD4

Arabidopsis EDS1 (*Enhanced Disease Susceptibility 1*) and *PAD4* (*Phytoalexin Deficient 4*) are two key components of the plant innate immune system. *EDS1* was originally identified in a mutational screen for defects in *RPP1* and *RPP5* mediated resistance to avirulent isolates of *Hyaloperonospora parasitica*[38], whereas *PAD4* was isolated in a screen for enhanced disease susceptibility to *Pseudomonas syringae* pv. m*aculicola* strain ES4326 [91]. Further genetic analyses in *Arabidopsis* demonstrated that both EDS1 and PAD4 are required for resistance triggered by the same spectrum of R proteins belonging to the TIR-NBS-LRR class [91-93]. This requirement was also observed in other plant systems [94-96]. By contrast, most CC-NBS-LRR proteins trigger local responses independently of EDS1 and PAD4, suggesting that the NBS-LRR N-terminal domain may specify requirements for downstream signaling components [37]. However, the identification of receptors containing a CC domain and showing dependency on EDS1 and PAD4 indicates that this distinction is probably an over simplification [97].

While *Arabidopsis eds1* plants exhibited a complete loss of TIR-NBS-LRR mediated resistance, *pad4* mutants still retained the capability to develop a delayed HR. Upon infection by *Hyaloperonospora parasitica* avirulent isolates this results in no HR and

hyphal growth in *eds1* plants while in *pad4* plants hyphal growth is accompanied by trailing necrosis, a delayed HR which doesn´t stop the pathogen but follows its spreading [93]. For this reason a probable engagement of EDS1 at earlier stages during R mediated responses was initially hypothesized [93]. More recent findings demonstrated however that the different impact on R-mediated defence by *EDS1* and *PAD4* is due to partial genetic redundancy between *PAD4* and *SAG101*, another component of the *EDS1*/*PAD4* node ([98], see below).

The contribution of EDS1 and PAD4 to basal defense seems to be equivalent: *eds1* and *pad4* plants infected by virulent isolates of *H. parasitica* or virulent strains of *P. syringae* have similar levels of enhanced susceptibility compared to wild type plants [92, 93]. EDS1 and PAD4 are also required for the accumulation of the signaling molecule SA upon pathogen challenge [93, 99, 100]. SA in turn induces EDS1 and PAD4 expression creating a positive feedback loop which leads to defense signal potentiation [93].

Structurally, EDS1 and PAD4 are related, possessing two conserved domains: a conserved lipase-like domain encompassing a putative catalytic triad (Ser - Asp – His), and the so called EP (EDS1/PAD4) domain [93], which is unique to higher plants and shared only with one other plant protein, SAG101 (Senescence Associated Gene 101) [98, 101]. The lipase-like domain is less conserved and putative catalytic triad missing in SAG101 although this protein was originally described as an acyl hydrolase involved in senescence regulation [98, 101]. Despite these structural features and the previous reported activity for SAG101, pathogen defense complementation assays using mutated *EDS1* and *PAD4* versions together with biochemical assays performed in our laboratory indicate that EDS1, PAD4 and SAG101 are not lipases (S. Rietz and J. Parker, unpublished). Different approaches are currently being followed to identify the so far elusive biochemical function of these proteins.

EDS1 and PAD4 localize to the nucleus and to the cytoplasm while SAG101 localizes only to the nucleus [98]. The importance of EDS1, PAD4 and SAG101 compartmentalization and the possibility that EDS1 and PAD4 might be shuttled between nuclear and cytoplasmic compartments are currently being investigated. By different means (Yeast two-hybrid, co-immunoprecipitations from plant soluble extracts and Fluorescence Resonance Energy Transfer (FRET) experiments) EDS1 was shown to

homodimerize in the cytoplasm, to associate with PAD4 and to interact directly with SAG101 in the nucleus [37, 98]. The evidence obtained so far suggests the existence of distinct EDS1-PAD4 and EDS1-SAG101 complexes. Furthermore, analyses of EDS1, PAD4 and SAG101 protein levels in the corresponding *Arabidopsis* mutant backgrounds indicate that EDS1, PAD4, and SAG101 are stabilized by their interacting partners [98].

Arabidopsis *sag101* plants do not have an obvious plant defence phenotype. However, analyses of *pad4/sag101* double mutant combinations indicated a partial genetic redundancy between *SAG101* and *PAD4* in both basal defence and ETI [98]. Redundancy was also observed for the described function of SAG101 and PAD4 in non-host resistance [70]. EDS1, PAD4 and SAG101 are in fact required for the activation of post-invasive non-host resistance mechanisms as demonstrated by the analysis of double and triple mutant combinations with the penetration mutant *pen2* [70].

More recently EDS1 was reported to be necessary for the establishment of SAR. *Eds1* mutant plants are impaired in mounting systemic immunity upon challenge with avirulent bacterial strains that induce an EDS1 independent localized HR [77]. Unpublished results from our laboratory demonstrate also that PAD4 plays a role of similar importance for SAR establishment as EDS1. In contrast to *dir1*, *eds1* and *pad4* mutants are impaired both in the SAR signal generation and perception (L. Jorda and J. Parker, unpublished). The requirement for EDS1 and PAD4 in SAR is observed most clearly when SAR establishment ensues from an HR triggered by recognition mediated by CC-NBS-LRR ([77]; L. Jorda and J. Parker, unpublished).

Microarray analyses led to the discovery of new genetic components of the EDS1 defence signaling node by the identification of genes whose expression changed in an *EDS1* or *PAD4* dependent fashion upon infection with *Pst* DC3000 expressing either AvrRpm1 or AvrRps4. Among them, *FMO* (*Flavin dependent Mono Oxygenase*) was shown to be a positive defense regulator with an important function also in SAR establishment [102, 103]. *NUDT7*, a member of the Nudix Hydrolase family, is a negative regulator of plant defense activation [102]. A third gene displaying EDS1 and PAD4 dependent up regulation upon pathogen challenge was *At5g55450*, a *Lipid Transport Protein like* gene related to *DIR1* [102].

During last few years, a broader function of EDS1 and PAD4 has been implied by a number of genetic epistasis analyses. Consistent with their function as signaling components downstream to the TIR-NBS-LRR activation, EDS1 and PAD4 are required for the constitutive defense activation phenotype observed in *snc1* (*suppressor of npr1-1 constitutive 1*) mutant plants [104, 105]. *SNC1* encodes a TIR-NBS-LRR and a recessive point mutation in the portion between its NBS and LRR domains leads to defense activation associated with constitutive high SA levels, *PR* gene transcriptional up-regulation and dwarfism [104, 105]. All these phenotypes are suppressed in the *eds1/snc1* and *snc1/pad4* double mutants [104, 105]. A genetic screen for suppressors of the *snc1* phenotype, led to the identification of the so called *mos* (*modifier of snc1*) mutants [106, 107]. Among them *MOS3* and *MOS6*, coding for a nucleoporin and an importin respectively, appears once more to connect NBS-LRR signaling and the nuclear import-export machinery [106, 107]. Currently, analyses are being performed in our laboratory to determine whether the compartmentalization of EDS1 and PAD4 is altered in *mos3*, *mos6*, *mos3*/*snc1* and *mos6*/*snc1* mutant backgrounds (A. Garcia and J. Parker, unpublished).

MPK4 kinase activity is necessary for both SA pathway repression and ET/JA pathway activation. *Arabidopsis mpk4* plants are severely dwarfed, accumulate high levels of SA, show constitutive SAR activation and constitutive *PR* gene up-regulation [82, 90]. This activation results in increased resistance against *H. parasitica* and *Pst* DC3000, and to increased susceptibility to *Alternaria brassicicola* [90]. The *mpk4* phenotype is entirely dependent on EDS1 and PAD4, since mutations in these genes suppress the de-repression of the SA pathway and suppress the block of the ET/JA pathway in *mpk4/eds1* and *mpk4/pad4* plants [82, 90]. These data therefore place EDS1 and PAD4 as regulators not only of the SAR induction but also of the antagonism between the SA- and ET/JA-mediated defense systems.

The existence of a potentiating signal loop activated by ROS and SA and requiring EDS1 and PAD4 was shown for the *lsd1* (*lesion simulating disease 1*) conditioned runaway cell death (RCD) [108]. EDS1 and PAD4 are not required for the oxidative burst and HR following RPM1 mediated recognition but are needed for generation of RCD in *lsd1* after triggering the RPM1 pathway or provision of ROS [92, 108]. Furthermore, *lsd1* mutants fail to acclimate to excess excitation energy in high light, causing ROS overload and cell

death due to photooxidative stress [109]. The *lsd1* mutant plants display lower catalase activity and reduced stomatal conductance which contributes to a lowering of the internal CO_2 concentration, consequent reduced electron consumption by CO_2 fixation and ultimately ROS over accumulation [109]. Stomatal conductance, reduced catalase activity and ROS accumulation are all restored to wild-type levels in *pad4/lsd1* and *eds1/lsd1* plants [109].

A potential role of EDS1 and PAD4 in processing ROS signals was further supported by the work of K. Apel and colleagues [110]. The conditional *Arabidopsis flu* mutant has been used to determine biological events triggered by singlet oxygen release [111]. Immediately after a dark/light shift of the *flu* mutant, singlet oxygen ($^1O_2$) is generated within the plastids, activating several stress responses that include growth inhibition of mature plants and seedling lethality [111]. These stress responses do not result from physico-chemical damage caused by singlet oxygen, but are attributable to the activation of a genetically determined stress response program triggered by the *EXECUTER1* gene [112]. One of the genes that is rapidly up regulated in *flu* upon dark/light shift is *EDS1* [113]. The release of singlet oxygen in the *flu* mutant triggers a drastic increase in the concentration of free SA and activates the expression of *PR1* and *PR5* genes [113]. These changes depend on the activity of *EDS1* and are suppressed in *flu/eds1* double mutants [113]. Soon after the start of singlet oxygen production, the synthesis of JA and 12-oxophytodienoic acid (OPDA) also start and plants stop growing and induce a cell-death response [113]. The inactivation of *EDS1* does not affect oxylipin synthesis, growth inhibition or the initiation of cell death, but it allows plants to recover faster from singlet oxygen-mediated growth inhibition and it suppresses the spread of necrotic lesions in leaves [113]. Hence, singlet oxygen activates a complex stress-response program and *EDS1* plays a key role in initiating and modulating several steps of it.

1.3 Thesis aims

EDS1 and PAD4 protein levels are up-regulated upon pathogen challenge by virulent and avirulent races of both *Pst* DC3000 or *H. parasitica* and by treatments with BTH (benzo-1,2,3-thiadiazole-7-carbothioic acid S-methyl ester) a functional analogue of SA [38, 93, 99, 100]. It was further shown that the transcriptional induction of *EDS1* upon generation of singlet oxygen anticipates SA accumulation, pointing to a direct capability of ROS to induce *EDS1* expression [113]. *PAD4* transcriptional up- regulation is strongly dependent on the expression of functional EDS1 protein while the up regulation of *EDS1* transcript is only partially compromised in *pad4* mutant plants [93].

Already 3 h after pathogen challenge with avirulent bacterial strains transcriptional changes dependent on EDS1 and PAD4 have been described indicating early activation of the EDS1/PAD4 pathway [102]. EDS1 and PAD4 proteins are present and interact with each other already before pathogen challenge and so far no protein up-regulation at these early time points after infection has been reported [93]. This points to an involvement of previously existent EDS1 and PAD4 complexes. However, it can not be ruled out that a very early general protein up-regulation might occur only at the site of infection which might be overlooked in analyses of input protein levels from total plant tissues.

With this work I aimed to determine the regulatory role of EDS1 and PAD4 protein up regulation by generating and characterizing *Arabidopsis* transgenic lines over expressing EDS1, PAD4 or both.

Furthermore, by comparisons between unchallenged and pathogen challenged wild type and over expressors, I investigated the existence of post translational mechanisms of regulation involved in EDS1 and PAD4 signaling activation.

2. MATERIAL AND METHODS

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

2.1 Materials

2.1.1 Plant materials

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

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

Accession	Abbreviation	Original source
Columbia	Col-0	J. Dangl[a]
Wassilewskija	Ws-0	K. Feldmann[b]

[a]University of North Carolina, Chapel Hill, NC, USA
[b]University of Arizona, Tucson, AZ, USA

Table 2.2. Mutant and transgenic *Arabidopsis* lines used in this study

Gene	Accession	Description	Reference/Source
eds1-1	Ws-0	EMS	[38]
pad4-5	Ws-0	T-DNA	[114]
snc1/npr1-1/eds1-2	Col-0/L*er*	EMS/EMS/FN	[115]
mpk4	L*er*	T-DNA	[82]
nudt7-1	Col-0	T-DNA	[102]
mpk4/MPK4HA	L*er*	Floral dipping of *mpk4*	[90]
mpk4/MPK4^{Y124G}HA	L*er*	Floral dipping of *mpk4*	[90]
CaMV35S::gEDS1-strepII	Ws-0	Floral dipping of *eds1-1*	[116]
promEDS1::gEDS1-strepII	Ws-0	Floral dipping of *eds1-1*	[116]
CaMV35S::cPAD4-strepII	Ws-0	Floral dipping of *pad4-5*	J. Bautor[a]
promPAD4::cPAD4-strepII	Ws-0	Floral dipping of *pad4-5*	J. Bautor[a]

EMS: ethylmathane sulfonate; FN: fast neutron; d*Spm*: defectice *Suppressor-mutator*; T-DNA: transfer-DNA

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

2.1.2 Pathogens

2.1.2.1 *Hyaloperonospora parasitica*

Table 2.3 *Hyaloperonospora parasitica* isolates used in this study

Isolate	Original source	Reference
Emwa1	Oospore infection of a single seedling	[39]
Noco2	Conidia isolated from a single seedling	[117]

Peronospora parasitica isolates and their interaction with *Arabidopsis* ecotypes

***Arabidopsis* ecotype**	***Peronospora parasitica* isolate**	
	Emwa1	Noco2
Col-0	incompatible (*RPP4*)	compatible
Ws-0	compatible	incompatible (*RPP1*)

2.1.2.2 *Pseudomonas syringae pv. tomato*

Pseudomonas syringae pv. *tomato* (*Pst*) strain DC3000 expressing the avirulence determinant *avrRps4* [40] from the broad host range plasmid pVSP61 [118] or DC3000 containing empty pVSP61 were used throughout this study. The *Pst* isolates were originally obtained from R. Innes (Indiana University, Bloomington Indiana, USA).

2.1.3 Oligonucleotides

Listed below are primers used in this study that were synthesised by Invitrogen or Sigma. Lyophilised primers were resuspended in nuclease-free water to a final concentration of 100 pmol/µl (= 100 µM). Working stocks were diluted to 10 pmol/µl (=10 µM).

Table 2.4 List of primers used in this study

Primer	**Sequence (5′ → 3′)**	**Purpose**
KLJ26	GGCGATGAAGCTCAATCCAAACG	RT-PCR Actine For
KLJ27	GGTCACGACCAGCAAGATCAAGACG	RT-PCR Actine Rev
KLJ1	GTAGGTGCTCTTGTTCTTCCC	RT-PCR *PR1* For
KLJ2	CACATAATTCCCACGAGGATC	RT-PCR *PR1* Rev
JK7	AATGAGCTCTCATGGCTAAGTTTGCTTCC	RT-PCR *PDF1.2* For
JK8	AATCCATGGAATACACACGATTTAGCACC	RT-PCR *PDF1.2* Rev

Primer	Sequence (5′ → 3′)	Purpose
MB70	TCATACGCAATCCAAATGTTTAC	RT-PCR *EDS1* For
MB71	AAAAACCTCTCTTGCTCGATCAC	RT-PCR *EDS1* Rev
EG34	TGGTCGACGCTGGCATACT	RT-PCR *PAD4* For
EG35	GGTTGAATGGCCGGTTATCA	RT-PCR *PAD4* Rev
MB16	GACAACACCAGAATCCTCATGCAA	RT-PCR *At5g55450* For
MB17	ATGGATACGAACAATACCAGAAC	RT-PCR *At5g55450* Rev
EDS3	GGATAGAAGATGAATACAAGCC	*eds1-1* genotyping For
EDS1r	ACCTAAGGTTCAGGTATCTGT	*eds1-1* genotyping Rev
MW23	CAAACGTCAAGAGAGCTGAG	*EDS1-strep* genotyping For
LW52	TCATTTTTCAAATTGAGGATGAGACCA	*EDS1-strep* genotyping Rev
EG24	GTCTGTCGGTTGTATACTCGG	MPK4/MPK4^{Y124G}HA genotyping For
EG25	AGGGATAGCCCGCATAGTCA	MPK4/MPK4^{Y124G}HA genotyping Rev
MW31	CTTCAATGGCGGTGTTTTC	*snc1* genotyping For
MW32	GGCATGCGTAATCTGCAATATCTAA	*snc1* genotyping Rev

For.: forward; Rev.: reverse

2.1.4 Enzymes

2.1.4.1 Restriction endonucleases

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

2.1.4.2 Nucleic acid modifying enzymes

Standard PCR reactions were performed using home made *Taq* DNA polymerase. Modifying enzymes and their suppliers are listed below:

Taq DNA polymerase	home made
SuperScript™ II RNase H$^-$ Reverse Transcriptase	Invitrogen™ (Karlsruhe, Germany)

2.1.5 Chemicals

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

2.1.6 Antibiotics

Kanamycin (Kan)	50 mg/ml in H_2O
Rifampicin (Rif)	100 mg/ml in DMSO

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

2.1.7 Media

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

Pseudomonas syringae media

NYG broth

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

pH 7.0

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

Arabidopsis thaliana media

MS (Murashige and Skoog) solid medium (MS plates)

MS powder including vitamins and MES buffer	4.8	g/l
Sucrose	10.0	g/l
Plant agar	9.0	g/l

pH 5.8

For selection of transgenic *Arabidopsis* plants carrying the phosphinothricin acetyltransferase (*PAT*) gene that confers Basta® (glufosinate-ammonium) resistance, DL-Phosphinothricin (PPT) was added to the agar plates:

DL-Phosphinothricin (100 mg/ml)	1:10000

For selection of transgenic *Arabidopsis* plants carrying the *nptII* (*neomycin phosphotransferase*) gene that confers Kanamycin resistance, Kanamycin was added to the agar plates:

Kanamycin (50 mg/ml in H_2O)	1:500

MS (Murashige and Skoog) liquid medium

MS powder including vitamins and MES buffer	4.8	g/l
Sucrose	10.0	g/l

For oxidative stress response analyses Methyl Viologen (MV) was added. A stock of 100mM MV was prepared and diluted in the MS liquid medium to reach a final concentration of 1 or 2 μM.

DL-Phosphinothricin, plant agar and MS powder including vitamins and MES buffer was purchased from Duchefa (Haarlem, The Netherlands). Kanamycin solution and Methyl Viologen powder were purchased from Sigma-Aldrich (Deisenhofen, Germany).

2.1.8 Antibodies

Listed below are primary and secondary antibodies used for immunoblot detection

Table2.5 Primary antibodies

Antibody	Source	Dilution	Reference
α-EDS1	rabbit polyclonal	1:500	S. Rietz[a]
α-PAD4	rabbit polyclonal	1:500	S. Rietz[a]
α-strepII HRP conjugated	Mouse monoclonal	1:5000	IBA (Göttingen, Germany)

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

Table 2.6 Secondary antibodies

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

HRP: horseradish peroxidase

2.1.9 Buffers and solutions

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

DNA extraction buffer (Quick prep)

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

PCR reaction buffer (10x)

Tris	100 mM
KCl	500 mM
$MgCl_2$	15 mM
Triton X-100	1 %
pH 9.0	

Stock solution was sterilised by autoclaving and used for homemade *Taq* DNA polymerase.

DNA gel loading dye (6x)

Sucrose	4 g
EDTA (0.5 M)	2 ml
Bromophenol blue	25 mg
H_2O to 10 ml	

TAE buffer (50x)

Tris	242 g
EDTA	18.6 g
Glacial acetic acid	57.1 ml
H_2O to 1000 ml	
pH 8.5	

Lactophenol trypan blue

Lactic acid	10 ml
Glycerol	10 ml
H_2O	10 ml
Phenol	10 g
Trypan blue	10 mg
Before use dilute 1:1 in ethanol.	

Ethidium bromide stock solution	Ethidium bromide10mg/mlH_2O
	Dilute 1:40000 in agarose solution

BTH solution	BTH (commercial product BION®, Syngenta) was resuspended in dH_20 to a final concentration of 300µM

SDS-PAGE:

Resolving gel (10%): for 20 ml gel mold volume

1,5M Tris (pH 8.8)	5 ml
H_2O	7,9 ml
10 % SDS	0,2 ml
30 % Acrylamide/Bis solution, 29:1 (BioRad)	6,7 ml
TEMED (BioRad)	0,008 ml
10 % APS	0,2 ml

Resolving gel (5%): for 20 ml gel mold volume

0,5 M Tris (pH 6.8)	1 ml
H_2O	2,2 ml
10 % SDS	0,04 ml
30 % Acrylamide/Bis solution, 29:1 (BioRad)	0,67 ml
TEMED (BioRad)	0,004 ml
10 % APS	0,04 ml

Running buffer (10x)

Tris	30.28 g
Glycine	144.13 g
SDS	10 g
H_2O to 1000 ml	

Sample buffer (2x)

Tris	0.125	M
SDS	4	%
Glycerol	20	% (v/v)
Bromphenol blue	0.02	%
Dithiothreitol (DTT)	0.2	M
pH 6.8		

Western blotting:

Transfer buffer (10x)

Tris	58.2	g
Glycine	29.3	g
SDS (10 %)	12.5	ml
H_2O to 1000 ml		
pH 9.2		

Before use dilute 80 ml 10 x buffer with 720 ml H_2O and add 200 ml methanol.

TBS-T buffer

Tris	10	mM
NaCl	150	mM
Tween®20	0.1	%
pH 7.5 (HCl)		

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

2.2 Methods

2.2.1 Sequence Analyses

Allignements were generated using the software ClustalX [119]. Sequences were edited by using the software GeneDoc version 2.6.002 (www.psc.edu/biomed/genedoc). Prediction of phosphorylation sites were performed using the software NetPhos version 2.0 (http://www.cbs.dtu.dk/services/NetPhos/ , [120]).

2.2.2 Maintenance and cultivation of Arabidopsis plant material

Arabidopsis seeds were germinated by sowing directly onto moist compost (Stender AG, Schermbeck, Germany) containing insecticide (10 mg l^{-1} Confidor WG 70 (Bayer, Germany)). Seeds were cold treated by placing sawn pots on a tray with a lid and incubating them in the dark at 4° C for three days. Pots were subsequently transferred to a controlled environment growth chamber, covered with a propagator lid and maintained under short day conditions (10 hour photoperiod, light intensity of approximately 200 µEinsteins m^{-2} sec^{-1}, 23° C day, 22° C night, and 65 % humidity). Propagator lids were removed when seeds had germinated. If required for setting seed, plants were transferred to long day conditions (16 hour photoperiod) to allow early bolting and setting of seed. To collect seed, aerial tissue was enveloped with a paper bag and sealed with tape at its base until siliques shattered.

2.2.3 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.

2.2.4 *Arabidopsis* seed sterilization

For *in vitro* growth of *Arabidopsis*, seeds were sterilised. Approximately 50 - 100 *Arabidopsis* seeds were put into a 1.5 ml closable microcentrifuge tube. Open microcentrifuge tubes were put in a plastic rack. 100 ml of 12 % Sodium-hypochloride solution (chlorine bleach) were poured into a beaker and put together with the seed into an exsiccator. The exsiccator was connected to a vacuum pump. 10 ml of 37 % HCl was directly added into the hypochloride solution so that yellow-grenish vapours were forming and the solution was bubbling heavily. The lid of the exsiccator was closed immediately and vacuum was generated, just enough to get an air tight seal. This was left for 4 – 8 h. After the sterilisation period, the exsiccator was slightly opened under a fume hood for 5 min to let out the gas. The lid was closed again, brought to a sterile bench and sterilised seeds were taken out of the exsiccator. Seeds were left for 15 min in opened vessel under the sterile workbench. Sterilised seed were stored for several days at 4° C or directly plated out on suitable culture media.

2.2.5 Glufosinate selection of *Arabidopsis* transformants on soil

Seed collected from floral-dipped plants (see 2.2.4) were densely sown on soil and germinated as described before. Once cotyledons were fully opened but before true leaves appeared, 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.

2.2.6 Inoculation and maintenance of *Hyaloperonospora parasitica*

H. parasitica isolates were maintained as mass conidiosporangia cultures on leaves of their genetically susceptible *Arabidopsis* ecotypes over a 7 day cycle (see 2.1.2.1). Leaf tissue from infected seedlings was harvested into a 50 ml Falcon tube 7 d after inoculation. Conidiospores were collected by vigorously vortexing harvested leaf material in sterile dH_2O for 15 sec and after the leaf material was removed by filtering through miracloth (Calbiochem) the spore suspension was adjusted to a concentration of 4×10^4 spores/ml dH_2O using a Neubauer counting cell chamber. Plants to be inoculated had been grown under short day conditions as described above. *H. parasitica* conidiospores were applied onto 2-week-old seedlings by spraying until imminent run-off using an aerosol-spray-gun. Inoculated seedlings were kept under a propagator lid to create a high humidity atmosphere and incubated in a growth chamber at 18° C and a 10 h light period. For long term storage *P. parasitica* isolate stocks were kept as mass conidiosporangia cultures on plant leaves at -80° C.

2.2.7 Quantification of *H. parasitica* sporulation

To determine sporulation levels, seedlings were harvested 5 d after inoculation in a 50 ml Falcon tube and vortexed vigorously in 5 – 10 ml water for 15 sec. Whilst the conidiospores were still in suspension 10 µl were removed twice and spores were counted under a light microscope using a Neubauer counting cell chamber. For each tested *Arabidopsis* genotype, three pots containing approximately 30 seedlings were infected per experiment and harvested spores from all seedlings of each pot were counted with sporulation levels expressed as the number of conidiospores per gram fresh weight.

2.2.8 Lactophenol trypan blue staining

Lactophenol trypan blue staining was used to visualize *P. parasitica* mycelium and necrotic plant tissue [121]. Leaf material was placed in a 15 ml Sarstedt tube (Nümbrecht, Germany) and immersed in lactophenol trypan blue. The tube was placed into a boiling

water bath for 2 min followed by destaining in 5 ml chloral hydrate solution (2.5 g/ml water) for 2 h and a second time overnight on an orbital shaker. After leaf material was left for several hours in 70 % glycerol, samples were mounted onto glass microscope slides in 70 % glycerol and examined using a light microscope (Axiovert 135 TV, Zeiss, Germany) connected to a Nikon DXM1200 Digital Camera.

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

Pseudomonas syringae pv. *tomato* strains described in 2.1.2.2 were streaked onto selective NYG agar plates containing rifampicin (100 µg/ml) and kanamycin (50 µg/ml) from -80° C DMSO stocks. Streaked plates were incubated at 28° C for 48 h before storing at 4° C and refreshed weekly.

2.2.10 *P. syringae* pv. *tomato* DC3000 growth assay

Pst DC3000 cultures were grown for two days on NYG broth agar plates containing rifampicin (100 µg/ml) and kanamycin (50 µg/ml) at 28°C. Bacteria were then scratched from the plates and directly transferred into a solution of 10 mM $MgCl_2$ with 0,02% Silwet L-77 (Lehle Seeds, USA) until reaching an optical density of OD600 = 0,1 equal to 5 X 10^7 cfu/ml. Four-week-old plants were surface sprayed with the bacterial suspension. Leaves were harvested 3 and 72 h after infection and surface sterilized (30 s in 70% ethanol, followed by 30 s in sterile distilled water). Four leaf discs from four different leaves were taken by using a coak borer (∅ 0.55 cm) for excision, and ground in 10mM $MgCl_2$ with a microfuge tube plastic pestle. After grinding of the tissue, the samples were thoroughly vortex-mixed and diluted 1:10 serially. Samples were finally plated on NYG broth agar plates containing rifampicin (100 µg/ml) and kanamycin (50 µg/ml). Plates were placed at 28 ° C for 2 days, after which the colony-forming units were counted.

For each line three replicates were performed and for each replicate counts were performed twice.

2.2.11 Sterile growth

Magenta boxes (Sigma-Aldrich Deisenhofen, Germany), were autoclaved. Under laminar flow hood 50 ml of autoclaved MS solid medium was poured in all Magentas and the medium was let to solidify. Upon solidification, sterilized *Arabidopsis* seeds were sown on the medium surface and the Magentas were sealed. For stratification the Magentas were kept for two days at 4° C in the dark and then transferred in a short day (8 h light/day) growth chamber. After five weeks Magentas were open and samples taken.

2.2.12 Cell size measurements

Plants were grown for four weeks at standard growth conditions (12 h/day light). From five individuals for each line the seventh true leaf was collected and cleared over night in a solution of Ethanol : Acetic Acid (2 : 1). The day after leaves were rehydrated by incubation in an ethanol dilution series of 50%, 33% and 25%. Leaves were incubated for twenty minutes in each dilution. Afterwards samples were transferred in a solution of chloral hydrate, ethanol and glycerol (8:1:1) and incubated over night at room temperature. The day after leaves were mounted onto glass microscope slides and examined using a light microscope (Axioplan 2, Zeiss, Germany) connected to a digital camera (Axiocam MR 5, Zeiss, Germany). Pictures at magnification 20X of adaxial epidermal cells in the most central part of the leaf lamina were taken and the borders of 15-18 cells, excluding stomata and trichomes base cells, were drawn manually. The surface of the drawn area was then measured using the Axiovision version 4.4 software (Zeiss, Germany) and the surface of a single epidermal cell estimated.

2.2.13 Oxidative stress analyses

Seeds were sterilized as described in section 2.2.4 and sown on MS plates without antibiotics. For stratification plates were incubated for two days at 4°C in the dark. Afterwards they were transferred in a growth chamber with standard growth condition (12 h/day light). After 7 days, seedlings were transferred in 96 well microtiter plates (Nunc, Denmark) containing in each well 300 µl of autoclaved MS liquid medium without or with methyl viologen (1 or 2 µM). Plates were closed an their lids sealed with hypoallergenic non-woven tape (Leukopor, Germany). Plates were then placed on shakers in growth chamber with standard growth conditions (12 h/day light). Three days after, three samples of three plants each were weighed and the weight of a single plant was estimated. Weight of plants grown in presence of methyl viologen was expressed as percentage of the average value measured for the same line in the absence of methyl viologen.

2.2.14 Molecular biological methods

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

This procedure yields a small quantity of poor quality DNA. However, the DNA is of sufficient quality for PCR amplification. The aliquots were stored at -20° C. The cap of a 1.5 ml microcentrifuge tube was closed onto a leaf to cut out a section of tissue and 400 µl of DNA extraction buffer were added. A micropestle was used to grind the tissue in the tube until the tissue was well mashed. The solution was centrifuged at maximum speed for 5 minutes in a bench top microcentrifuge and 300 µl supernatant were transferred to a fresh tube. One volume of isopropanol was added to precipitate DNA and centrifuged at maximum speed for 5 minutes in a bench top microcentrifuge. The supernatant was discarded carefully. The pellet was washed with 70 % ethanol and dried. Finally the pellet was dissolved in 100 µl 10 mM Tris-HCl pH 8.0 and 1 µl of the DNA solution was used for a 20 µl PCR reaction mixture.

2.2.14.2 Isolation of total RNA from *Arabidopsis*

Total RNA was prepared from 3- to 6-week-old plant materials. Liquid nitrogen frozen samples (approximately 50 mg) were homogenized 2 x 15 sec to a fine powder using a Mini-Bead-Beater-8™ (Biospec Products) and 1.2 mm stainless steel beads (Roth) in 2 ml centrifuge tubes. After the first 15 sec of homogenisation samples were transferred back to liquid nitrogen and the procedure was repeated. 1 ml of TRI® Reagent (Sigma) was added and samples were homogenised by vortexing for 1 min. Samples were centrifuged for 10 min. at 4° C at 12000 g and supernatants incubated for 5 min at room temperature to dissociate nucleoprotein complexes. 0.2 ml of chloroform was added and samples were shaken vigorously for 15 sec. After incubation for 3 min at room temperature samples were centrifuged for 15 min at 12000 *g* and 4° C. 0.5 ml of the upper aqueous, RNA containing phase were transferred to a new microcentrifuge tube and RNA was precipitated by adding 0.5 volumes of isopropanol and incubation for 10 min at room temperature. Subsequently, samples were centrifuged for 10 min at 12000 *g* and 4° C. The supernatant was removed and the pellet was washed by vortexing in 1 ml of 70 % ethanol. Samples were again centrifuged for 10 min at 12000 *g* and 4° C, pellets were air dried for 10 min and dissolved in 20 µl H_2O. Samples were incubated for 5 minutes at 55°C and then immediately stored at -80° C.

2.2.14.3 Polymerase chain reaction (PCR)

Standard PCR reactions were performed using home made *Taq* DNA polymerase. All PCRs were carried out using a PTC-225 Peltier thermal cycler (MJ Research). A typical PCR reaction mix and thermal profile is shown below.

Table 2.7 PCR reaction mix (20 µl total volume):

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

Table 2.8 Thermal profile

Stage	Temperature (°C)	Time period	No. of cycle
Initial denaturation	94	3 min	1 x
Denaturation	94	30 sec	
Annealing	50 - 60	30 sec	25 - 40
Extension	72	1 min per kb	
Final extension	72	3 min	1 x

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

RT-PCR was carried out in two steps. SuperScript™ II RNase H^- Reverse Transcriptase (Invitrogen) was used for first strand cDNA synthesis by combining 1 - 1.5 µg template total RNA, 1 µl oligo $dT_{18}V$ (0.5 µg/µl, V standing for an variable nucleotide), 5 µl dNTP mix (each dNTP 2.5 mM) in a volume of 13.5 µl (deficit made up with H_2O). The sample was incubated at 65° C for 10 min to destroy secondary structures before cooling on ice for oneminute. Subsequently the reaction was filled up to a total volume of 20 µl by adding 4 µl of 5x reaction buffer (supplied with the enzyme), 2 µl of 0.1 M DTT and 0.5 µl reverse transcriptase. The reaction was incubated at 42° C for 60 min before the enzyme was heat inactivated at 70° C for 10 min. For subsequent normal PCR, 1 µl of the above RT-reaction was used as cDNA template.

2.2.14.5 Restriction endonuclease digestion of DNA

Restriction digests were carried out using the manufacturer´s recommended conditions. Typically, reactions were carried out in 0.5 ml tubes, using 1 µl of restriction enzyme per 10 µl reaction. All digests were carried out at the appropriate temperature for a minimum of three hours.

2.2.14.6 Agarose gel electrophoresis of DNA

DNA fragments were separated by agarose gel electrophoresis in gels consisting of 1 – 3 % (w/v) SeaKem® LE agarose (Cambrex, USA) in TAE buffer. Agarose was dissolved in TAE buffer by heating in a microwave. Molten agarose was cooled to 50° C before 2.5 µl of ethidium bromide solution (10 mg/ml) was added. The agarose was pored and allowed to solidify before being placed in TAE in an electrophoresis tank. DNA samples were loaded onto an agarose gel after addition of 2 µl 6x DNA loading buffer to 10 µl PCR- or restriction-reaction. Separated DNA fragments were visualised by placing the gel on a 312 nm UV transilluminator and photographed.

2.2.15 Biochemical methods

2.2.15.1 *Arabidopsis* total protein extraction for immunoblot analysis

Total protein extracts were prepared from 3- to 5-week-old plant materials. Liquid nitrogen frozen samples were homogenized 2 x 15 sec to a fine powder using a Mini-Bead-Beater-8™ (Biospec Products) and 1.2 mm stainless steel beads (Roth) in 2 ml centrifuge tubes. After the first 15 sec of homogenisation samples were transferred back to liquid nitrogen and the procedure was repeated. 150 µl of 2x SDS-PAGE sample buffer was added to 50 mg sample on ice. Subsequently, samples were briefly vortexed, boiled for 5 min at 96 ° C and centrifuged at 20000 *g* and 4° C for 20 min in a bench top

centrifuge. Supernatants were transferred to clean centrifuge tubes and stored at -20° C if not directly loaded onto SDS-PAGE gels.

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

Denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the Mini-PROREAN® 3 system (BioRad) and discontinuous polyacrylamide (PAA) gels. Gels were made fresh on the day of use according to the manufacturer instructions. Resolving gels were poured between to glass plates and overlaid with 500 µl of water-saturated 2 - isopropanol. After gels were polymerised for 30 – 45 min the alcohol overlay was removed and the gel surface was rinsed with dH_2O. Excess water was removed with filter paper. A stacking gel was poured onto the top of the resolving gel, a comb was inserted and the gel was allowed to polymerise for 30 - 45 min. In this study, 10 % resolving gels were used, overlaid by 5 % stacking gels. Gels were 0.75 mm or 1.5 mm in thickness.

If protein samples were not directly extracted in 2x SDS-PAGE sample buffer (see 2.1.11) proteins were denatured by adding 1 volume of 2x SDS-PAGE sample buffer to the protein sample followed by boiling for 5 min.
After removing the combs under running water, each PAA gel was placed into the electrophoresis tank and submerged in 1x running buffer. A pre-stained molecular weight marker (Precision plus protein standard dual colour, BioRad) and denatured protein samples were loaded onto the gel and run at 80 - 100 V (stacking gel) and 100 – 150 V (resolving gel) until the marker line suggested the samples had resolved sufficiently.

2.2.15.3 Immunoblot analysis

Proteins that had been resolved on acrylamide gels were transferred to Hybond™-ECL™ nitrocellulose membrane (Amersham Biosciences) after gels were released from the glass plates and stacking gels were removed with a scalpel. PAA gels and membranes were

pre-equilibrated in 1 x transfer buffers for 10 min on a rotary shaker and the blotting apparatus (Mini Trans-Blot® Cell, BioRad) was assembled according to the manufacturer instructions. Transfer was carried out at 100 V for 70 min or over night at 30 V at 4° C. The transfer cassette was dismantled and membranes were checked for equal loading by staining with Ponceau S for 5 min before rinsing in copious volumes of deionised water. Ponceau S stained membranes were scanned and thereafter washed for 5 min in TBS-T before membranes were blocked for 1 h at room temperature in TBS-T containing 5 % blotting grade milk powder (Roth). The blocking solution was removed and membranes were washed briefly with TBS-T. Incubation with primary antibodies was carried out overnight by slowly shaking on a rotary shaker at 4° C in the following conditions: α-EDS1 1:500 in TBS-T + 2 % milk powder, α-PAD4 1:500 in TBS-T + 0,9 % milk powder. Next morning the primary antibody solution was removed and membranes were washed 3 x 15 min with TBS-T at room temperature on a rotary shaker. Primary antibody-antigen conjugates were detected using a horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody diluted 1:5000 in TBS-T + 2% milk powder. Membranes were incubated in the secondary antibody solution for 1 h at room temperature by slowly rotating. The antibody solution was removed and membranes were washed as described above. In the case of the α-strepII antibodies, already HRP conjugated, the membrane were incubated for six h an a rotary shaker at 4° C in with an antibody dilution of 1:5000 in TBST + 1% milk powder. After being washed as described above, detection immediately followed. Detection was performed by chemiluminescence using the SuperSignal® West Pico Chemimuminescent kit or a 9:1 - 3:1 mixture of the SuperSignal® West Pico Chemimuminescent- and SuperSignal® West Femto Maximum Sensitivity-kits (Pierce) according to the manufacturer instructions. Luminescence was detected by exposing the membrane to photographic film (BioMax light film, Kodak).

2.2.15.4 Protein purification using StrepII affinity purification

2.2.15.4.1 Standard purification from OE_E1s plant material

StrepII affinity protein purification was performed according to the protocol described by Witte et al., with modifications described below [122]. For one purification, 1 g of *Arabidopsis* leaf material was ground in liquid nitrogen and thawed in 1,5 ml StrepII EX buffer listed below. The slurry was aliquoted in 2 ml micro centrifuge tubes and then centrifuged for 15 min at 4ºC (14000 rpm). The supernatant was ultra centrifuged for 20 min at 4ºC (100000 rpm). The supernatant was transferred to a new micro centrifuge tube, sampled (Input), and 240 µl slurry of StrepTactin Sepharose (IBA GmbH, Göttingen, Germany) was added. The Sepharose matrix is based on Sepharose 4FF with a bead size of 45–165 µm. Binding was performed by incubation in an end-over-end rotation wheel for 60 min at 4ºC. The slurry was transferred into a micro spin column (BioRad 732-6204, Hercules, CA) and the unbound fraction let flow through. The resin was washed twice with 1 ml and four times with 0.5 ml StrepII W buffer. For elution, 90 µl of Elution buffer representing the void volume of the system were carefully applied to the resin but not recovered.

Table 2.9 StrepII affinity purification buffers:

StrepII EX:		**StrepII W:**		**Elution:**	
Tris-HCl[a]	100 mM	Tris-HCl[a]	50mM	Tris-HCl[a]	10 mM
EDTA	1 mM	EDTA	0.5 mM	Desthiobiotin	10mM
NaCl	150 mM	NaCl	150 mM	NaCl	150 mM
DTT	10 mM	DTT	2 mM	DTT	2 mM
AEBSF[b]	0.5 mM	Triton X-100	0.05%	Triton X-100	0.05%
Aprotinin	5 µg/ml				
Leupeptin	5 µg/ml				
PI[c]	1:100 dilution				
Triton X-100	0.5%				
avidin	100 µg/ml				

[a]Tris-HCl: pH 8.0

[b]AEBSF: 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride

[c]PI: Proteinase Inhibitor cocktail (Sigma p9599)

Four times 100 µl Elution buffer were passed through and collected in two pools of 200 µl. From each pool, 20 µl were sampled for SDS-PAGE analysis (Elution). All samples taken for electrophoresis analysis were mixed with a 2 x SDS-loading buffer and heated for 5 min at 96º C prior to loading.

2.2.15.4.2 Purification from OE_E1s plant material for LC-MS

For OE_E1s LC-MS analyses StrepII affinity purification was performed following the standard protocol with the following modifications.
While the first two washes of the resin were performed with the StrepII W buffer indicated above, the last four were performed using StrepII W without Triton X - 100.
The elution buffer composition was also modified by removing both NaCl and Triton. Only two times 100µl of elution buffer were employed for the elution and the two eluates pooled. To 50 µl of eluate were added 0,4 µg of sequencing grade modified trypsin (Promega) and the samples were incubated over night at room temperature. The following day the samples were analyzed by LC-MS/MS (Micromass Q-Tof-2, Waters), at the Mass Spectrometry facility of the Max-Planck-Institute for Plant Breeding Research (Cologne, Germany), following their standard protocol.

2.2.15.4.3 StrepII affinity purification from OE_E1s plant material for *in vivo* phosphorylation analyses

For OE_E1s phosphorylation analyses StrepII affinity protein purification was performed following the standard protocol with the following modifications. The phosphatase inhibitors indicated below (purchased from Sigma-Aldrich, Deisenhofen, Germany) were added to the StrepII EX buffer until reaching the indicated final concentration:

NaF	50 mM
$NaVO_4$	10 mM
PPIC[a]	dilution 1 : 100 from the stock
B-Gly-P[b]	10 mM

[a] Protein Phosphatase Inhibitor Cocktail (Sigma P2850)

[b] Glycerol-2-phosphate

After elution, samples were concentrated from starting 400 µl to final 20 µl using Vivaspin500 columns (VIVASCIENCE, Hannover, Germany). To all concentrated samples were added 1.1 µl λ Protein Phosphatase buffer 10X (New England Biolabs, Frankfurt, Germany) and, as indicated in figures, 80 – 100 ng β-Casein (Sigma-Aldrich, Deisenhofen, Germany) and/or 25 units of λ Protein Phosphatase (New England Biolabs, Frankfurt, Germany). All the samples were incubated for 1 h at 37° C. 10 µl of each sample were mixed with 10 µl of sample buffer (2X) and loaded onto SDS-PAGE without any boiling.

For detection the gel was stained with Pro-Q® Diamond phosphoprotein gel stain (Molecular Probes, Invitrogen), following the manufacturers instruction. The detection was performed at the fluorescence scanner Typhoon 8600 (Amersham Biosciences) with an excitation wave length of 532 nm and an emission filter 580 nm BP30. Exposures were set between 600 and 650 V PMT (photomultiplier tube) voltage.

The gel was subsequently rinsed in distilled water and then stained with SYPRO ® Ruby (Molecular Probes, Invitrogen) following the manufacturer´s instruction. Protein signals were visualised by placing the gel on a 312 nm UV transilluminator and photographed.

2.2.15.4.4 Standard purification from NP_E1s plant material

The affinity purification was performed as for OE-E1s plants with some modifications. For one purification, 2 g of *Arabidopsis* leaf material was ground in liquid nitrogen and thawed in 3 ml StrepII EX buffer. The slurry was aliquoted in 2 ml micro centrifuge tubes and then centrifuged for 15 min at 4ºC (14000 rpm). The supernatant was ultra centrifuged for 20 min at 4ºC (100000 rpm). The supernatant was aliquoted in to two new

micro centrifuge tube, sampled (Input fraction), and 240 µl slurry of StrepTactin Sepharose (IBA GmbH, Göttingen, Germany) was added to both tubes. Binding was performed by incubation in an end-over-end rotation wheel for 60 min at 4ºC. The slurry was transferred into a micro spin column (BioRad 732-6204, Hercules, CA) and the unbound fraction let flow through. The resins were washed twice with 1 ml and four times with 0.5 ml StrepII W buffer. For elution, 90 µl of Elution buffer representing the void volume of the system were carefully applied to the resin but not recovered. Four times 100 µl Elution buffer were passed through and collected in two pools of 200 µl. The eluates were pooled and concentrated using Vivaspin500 (VIVASCIENCE, Hannover, Germany) up to 20 µl. The concentrated eluates mixed with sample buffer (2X) and heated for 5 min at 96ºC prior to SDS-PAGE analysis.

2.2.15.4.5 Purification from OP_E1s plant material for *in vivo* phosphorylation analyses

For OP_E1s phosphorylation analyses StrepII affinity protein purification was performed following the OP_E1s standard protocol with the same modifications indicated in section 2.2.13.3

2.2.15.4.6 *In vitro* phosphorylation analyses

Standard purification from OE_E1s plant material was performed. After elution the samples were concentrated from the starting 400 µl up to final 30µl. Each sample was divided into two identical aliquot of 15 µl and to each were added 1.6µl of PKA reaction buffer 10 X (New England Biolabs) and ATP (Sigma Aldrich) to reach a final concentration of 200 µM. Subsequently to the samples were added 250 units of cAMP dependent protein kinase) PKA catalytic subunit (New England Biolabs), and/or 50 ng histone (New England Biolabs) as indicated in figures. All samples were incubated at 30°C for 1 h. Subsequently they were separated by SDS-PAGE and the gel stained with Pro-Q® Diamond phosphoprotein gel stain (Molecular Probes, Invitrogen), following the manufacturers instruction. The gel was subsequently rinsed in distilled water and then

stained with SYPRO ® Ruby (Molecular Probes, Invitrogen) following the manufacturer´s instruction.
Detection was performed as described in section 2.2.13.3

2.2.15.5 Determination of free and total salicylic acid in leaves

Extraction and quantification of total and free salicylic acid (SA) were performed as described previously with modifications [123]. Samples (approximately 200 mg of liquid nitrogen frozen leaf tissue) were homogenised in 0.6 ml of 80 % methanol using a Mini-Bead-Beater-8^{TM} (Biospec Products) and 1.2 mm stainless steel beads (Roth) in 2 ml Eppendorf tubes. Samples were centrifuged at 12000 *g* at 4° C for 10 min. The supernatants were collected into fresh 2 ml Eppendorf tubes. The extraction procedure was repeated once more with the residues and supernatants were combined. Under vacuum at 30° C methanol was evaporated and samples subsequently dissolved in 0.5 ml 0.1M sodium acetate (NaOAc) pH 5.0 by 15 min shaking and 5 min incubation in an ultrasonic bath. Each sample was divided into two aliquots of 0,25 ml and to each aliquot were added 0.25 ml of 0.1 M NaOAc pH 5.0 containing beta-glucosidase (20 U/ml; EC 3.2.1.21; almond, Sigma) to determine total SA, or 0.25 ml of 0.1 M NaOAc pH 5.0 without beta-glucosidase, to determine free SA. Samples to determine total SA incubated at 37° C for 3 h. Subsequently to all samples were added 25 µl TFA (Trifluoroacetic acid) and 600 µl EtOAc (Ethyl acetate) the tubes were mixed for 1 min on a shaker. Samples were then centrifuged at 12000 *g* for 5 min at room temperature. The upper EtOAc phase was collected in a fresh 2 ml Eppendorf tubes. The EtOAc extraction was repeated twice and all three EtOAc fractions pooled and subsequently evaporated under vacuum at 30° C. The pellet was resuspended in 80 % methanol (100 µl / 200 mg initial fresh weight) for 15 min on a shaker and 5 min in the ultrasonic bath. To remove undissolved debris, samples were centrifuged for 5 min at 12000 *g* and 4° C and the clear supernatants transferred to HPLC vials. The quantification procedure by HPLC was performed by P. Bednarek (MPIZ, Cologne). Analyses of processed leaf samples were performed on an Agilent (Palo Alto, CA) 1100 HPLC system equipped with DAD and FLD detectors. Samples were analyzed on a Xterra C-18 column (150/3, 3.5; Waters, Milford, MA)

using 0.1% trifluoroacetic acid as solvent A and 98% acetonitrile/0.1% trifluoroacetic acid as solvent B at a flow rate of 0.4 ml/min at 40°C (gradient of solvent A: 96% at 0, 80% at 5, 70% at 23, 0% at 25 min). The salicylic acid peak was identified based on its retention time as well as absorbance and emission UV spectra. Salicylic acid was quantified by comparing its peak area on the FLD chromatograms (ex. 304 nm; em. 415 nm) with respective calibration curve prepared for authentic standard.

2.2.15.6 Determination of camalexin and scopoletin in leaves

Camalexin levels determinations were performed as previously described [124]. Samples (approximately 200 mg of liquid nitrogen frozen leaf tissue) were homogenised in 0.6 ml of 80 % methanol using a Mini-Bead-Beater-8^{TM} (Biospec Products) and 1.2 mm stainless steel beads (Roth) in 2 ml Eppendorf tubes. Samples were centrifuged at 12000 *g* at 4° C for 10 min. The supernatants were collected into fresh 2 ml Eppendorf tubes. The extraction procedure was repeated once more with the residues and supernatants were combined. Under vacuum at 30° C methanol was evaporated and samples subsequently redissolved in 60 % methanol (150 µl / 100 mg initial fresh weight) by mixing for 15 min on a shaker and 5 min in the ultrasonic bath. To remove undissolved debris, samples were centrifuged for 5 min at 12000 *g* and 4° C and the clear supernatants transferred to HPLC vials. The quantification procedure by HPLC was performed by P. Bednarek (MPIZ, Cologne). Camalexin content was determined using a DiodeArray (DAD) at 330 nm and with a fluorescence detector at emission 318 nm/excitation 385 nm. Actual camalexin amounts were determined by comparisons with respective calibration curve prepared for authentic standard.

To determine scopoletin leaf content the same procedure described for total SA determination was followed (see section 2.2.14). No scopoletin standard was available at the time of the measurements. For this reason data were expressed as peak areas.

3. RESULTS

3.1 Summary

An increase of EDS1 and PAD4 protein abundances is observed upon challenge by a number of different pathogens [125]. To assess the importance of such protein up regulation in relation to the signaling activities of EDS1 and PAD4, multiple independent *Arabidopsis thaliana* transgenic lines over expressing either AtEDS1 or AtPAD4 were selected and characterized. The absence of any obvious phenotype together with the knowledge of the strong intimate connection between the signaling functions of EDS1 and PAD4 prompted me to generate double EDS1 and PAD4 over expressor lines (OE_E1/P4, see below). Compared to wild type plants these lines exhibited growth retardation that was correlated with a reduction in cell size, likely resulting from the interference with or perturbation of an intrinsic developmental program. Also, OE_E1/P4 plants exhibited increased resistance to virulent pathogens associated with a form of localized cell death specifically induced after challenge. Despite a slight constitutive activation of the Salicylic Acid (SA) pathway in the unchallenged state, OE_E1/P4 plants displayed a stronger and quicker defence response than wild type or individual EDS1 and PAD4 over expressors to virulent pathogens. This indicated a requirement for posttranslational changes downstream or independent of EDS1 and PAD4 protein up regulation in full defence activation. Therefore, the capability of OE_E1/P4 plants to respond to chemically induced changes in the cellular redox status that could be qualitatively reminiscent to those observed during the early stages of the pathogen response, was assessed. Also, the existence of potential post translational regulation mechanisms, such as protein modifications or protein-protein interactions in response to pathogen triggers, was investigated. Finally, to improve the extent and synchronicity of EDS1 pathway induction for analysis of EDS1 pathway activation steps, *Arabidopsis* lines were generated that had constitutive or conditional activation of the EDS1 pathway coupled with expression of functional strepII tagged EDS1 proteins.

3.2 Generation of Arabidopsis thaliana lines expressing EDS1 or PAD4 strepII fusion proteins

Both EDS1 and PAD4 proteins are up regulated upon pathogen challenge or chemical induction of the SAR response [125]. To investigate the importance of such up regulation in the signal relay during pathogen response and SAR establishment, the behavior of *Arabidopsis* lines over expressing either EDS1 or PAD4 was investigated.

Arabidopsis eds1-1 and *pad4-5* null mutants (accession Wassilewskija; WS-0) had been previously transformed with constructs for the expression respectively of AtEDS1 or AtPAD4 strepII affinity tag fusion proteins under either the *CaMV 35S* promoter (CaMV35S::gEDS1 - strepII and CaMV35S::cPAD4 - strepII) or the corresponding natives promoters (promEDS1::gEDS1 - strepII and promPAD4-cPAD4 - strepII, the latter not used in this study) ([116, 122]; J. Bautor and J. Parker, unpublished). A schematic representation of the constructs employed is shown in **Fig. 3.1**. As native *AtEDS1* promoter the previously characterized 1,4 kb 5´ region from the *Arabidopsis* accession L-*er* was utilized [98]. Multiple independent homozygous CaMV35S::cPAD4 - strepII lines (hereafter OE_P4s) carrying a single transgene insertion were made available (J. Bautor and J. Parker, unpublished). The nomenclature used for the OE_P4s lines hereafter is indicated in **Table 3.1**. CaMV35S::gEDS1 – strepII and promEDS1::gEDS1 - strepII T_1 seeds were germinated on soil and transgenic plants expressing the marker gene *bar* (*bialaphos resistance*) carried by both the constructs used for transformation (see **Figure 3.1**) and conferring resistance to PPT (phosphinothricin

Table 3.1 *Arabidopsis thaliana* transgenic lines expressing the PAD4 strepII fusion protein

Construct	Previous nomenclature (J. Bautor)	Nomenclature hereafter
CaMV35S::cPAD4 - strepII	AC12 2/4	OE_P4s.1
	AC12 15/6	OE_P4s.2
	AC12 23/5	OE_P4s.3

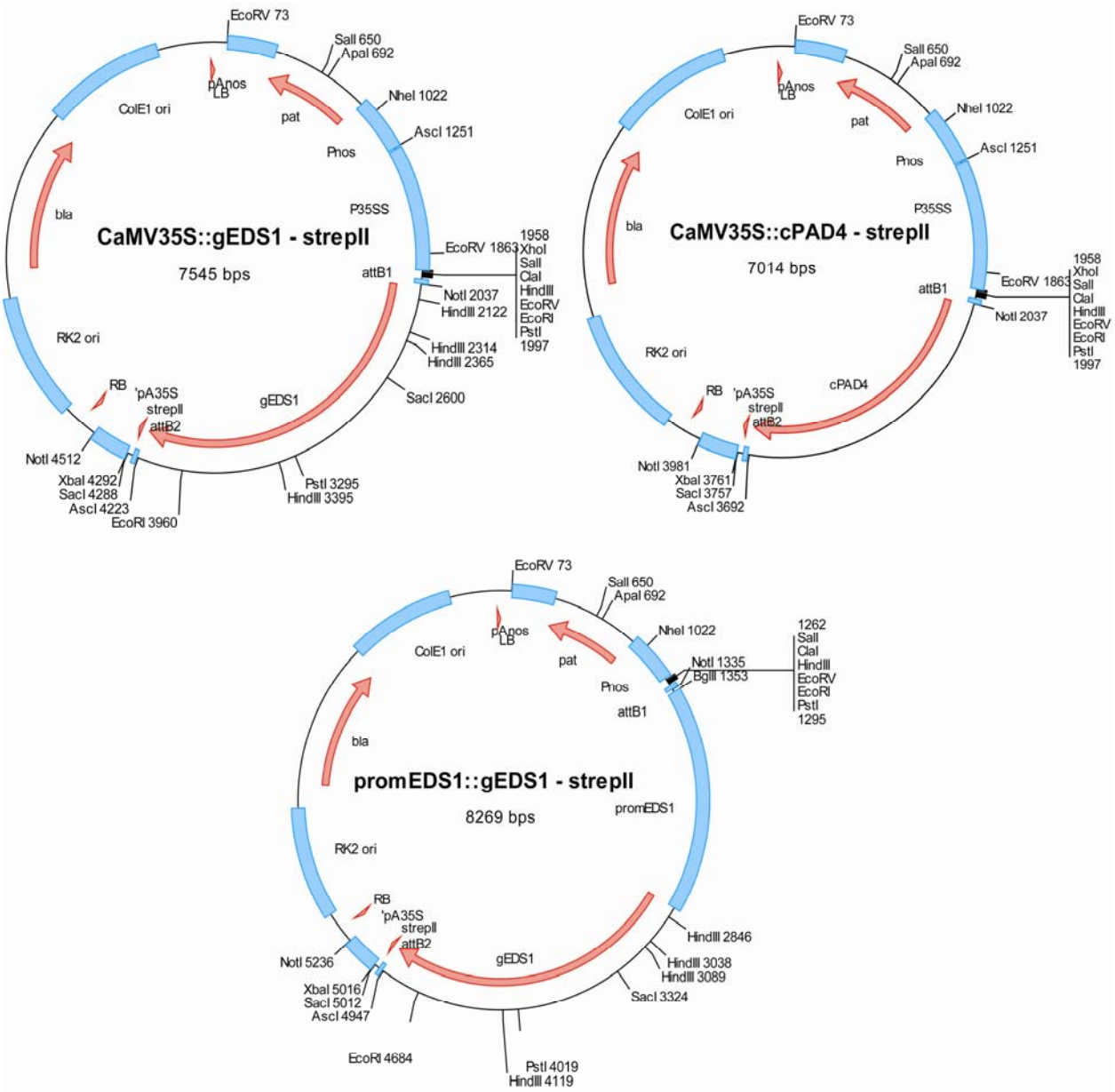


Fig. 3.1 Constructs employed for the generation of *Arabidopsis thaliana* lines

Essential features and restriction sites are depicted in the maps. The CaMV35S::gEDS1-strepII and CaMV35S::cPAD4-strepII vectors allow expression of respectively EDS1 and PAD4 N-terminal strepII tagged fusion proteins under control of the double 35S promoter of cauliflower mosaic virus (P35SS), whereas the promEDS1::gEDS1-strepII vector allows expression of the EDS1 N-terminal strepII tagged fusion protein under control of the EDS1 native promoter. Genomic *EDS1* and *PAD4* cDNA were employed. (attB1) attachment site B1; (attB2) attachment site B2; (LB) left border; (RB) right border; (pat) phosphinothricin acetyltransferase gene conferring PPT-resistance; (bla) β-lactamase gene conferring ampicillin resistance.

[126]) were selected by BASTA® spraying. T_2 seeds were collected from surviving plants and germinated on PPT-containing MS plates to monitor the segregation of the *bar* gene. In **Table 3.2** are reported the observed segregation ratios of Basta resistant to susceptible plants are reported and the nomenclature used for the transgenic lines hereafter.

Multiple transgenics containing a single transgene insertion were selected for each

Table 3.2 *Arabidopsis* transgenic lines expressing the EDS1strepII fusion protein

T2 seeds from each line were germinated on PPT containing MS plates and scored for PPT resistance: (Res) resistant, (Sus) susceptible. χ^2(3:1) is the χ^2 calculated with expected values of 3 resistant individuals to 1 susceptible. χ^2 (1 degree of freedom, P=0,05) = 3,84.

Single insertion transgenic lines selected for further analyses are indicated in bold font.

Construct	Line	Res	Sus.	Ratio (Res/Sus)	χ^2(3:1)
CaMV35S::gEDS1 - strepII	OE_E1s.1	83	2	42,5	17,44
	OE_E1s.2	**151**	**43**	**4,51**	**0,62**
	OE_E1s.3	**80**	**23**	**4,48**	**0,29**
	OE_E1s.4	**79**	**22**	**4,59**	**0,42**
	OE_E1s.5	31	5	7,20	1,78
	OE_E1s.6	**57**	**18**	**4,17**	**0,03**
	OE_E1s.7	**83**	**35**	**3,37**	**1,03**
	OE_E1s.8	**62**	**30**	**3,07**	**2,13**
promEDS1::gEDS1 - strepII	OP_E1s.1	51	5	11,2	5,79
	OP_E1s.2	60	4	16	9
	OP_E1s.3	**214**	**82**	**3,61**	**0,86**
	OP_E1s.4	**151**	**58**	**3,6**	**0,63**
	OP_E1s.5	**151**	**41**	**4,68**	**1,02**
	OP_E1s.6	**99**	**31**	**4,19**	**0,07**
	OP_E1s.7	**94**	**46**	**3,04**	**3,46**

construct (**see Table 3.2**). For each selected line 6 T_2 PPT resistant individuals were transferred on soil and T_3 seeds were collected and germinated on PPT containing MS plates. For each selected line (except OE_E1s.3) it was possible to identify at least one individual whose entire progeny was PPT resistant indicative of transgene homozygosity. Homozygous individuals isolated in this way were used in the following experiments.

To determine whether the strepII tag addition might interfere with the normal EDS1 and PAD4 function, complementation experiments were performed. Both the *eds1-1* and *pad4-5* mutants show compromised resistance to avirulent *Hyaloperonospora parasitica* isolate Noco2 (hereafter Noco2) which in the *Arabidopsis* WS-0 accession is recognized by the TIR-NBS-LRR receptor RPP1 [38, 93]. This impairment determines in *eds1-1* heavy sporulation and complete absence of host cell death (HR). By contrast *pad4-5* plants sustain heavy sporulation but develop a delayed HR that doesn´t stop the pathogen growth but follows it resulting in trailing necrosis [93]. Homozygous T_3 individuals of each selected transgenic line were infected with Noco2 (4 X 10^4 spores/ml) and scored at 5 dpi by both lactophenol trypan blue staining to observe dead cells and mycelium, and inspection under UV light to see trailing plant cell necrosis. For each of the three constructs results obtained from one representative line and *pad4-5*, *eds1-1* and wild type (WS-0) control plants are shown in **Figure 3.2** and **Figure 3.3**. In contrast to the respective mutant backgrounds which showed strong sporulation (*eds1-1*) and sporulation accompanied by trailing necrosis (*pad4-5*), all the selected transgenic lines (represented by OP_E1s.5 in **Figure 3.2** and OE_E1s.6 and OE_P4s.1 in **Figure 3.3**) exhibited normal HR development comparable to wild type plants (WS-0). Similarly, transgenic lines expressing the PAD4 strepII fusion protein under the *PAD4* native promoter in the *pad4-5* mutant background exhibited normal incompatible response to Noco2 (J. Bautor and J. Parker, data not shown). I concluded from these data that both EDS1 and PAD4 strepII fusion proteins were fully functional and decided to further characterize the selected transgenic lines.

Protein expression levels in the selected lines were subsequently tested by Western blot analyses using polyclonal anti-EDS1 or anti-PAD4 antibodies for detection. As shown in **Figure 3.4A** and **B**, the unchallenged OE_E1s lines had variable EDS1 expression levels but in all cases EDS1 amounts were much higher than either unchallenged or challenged

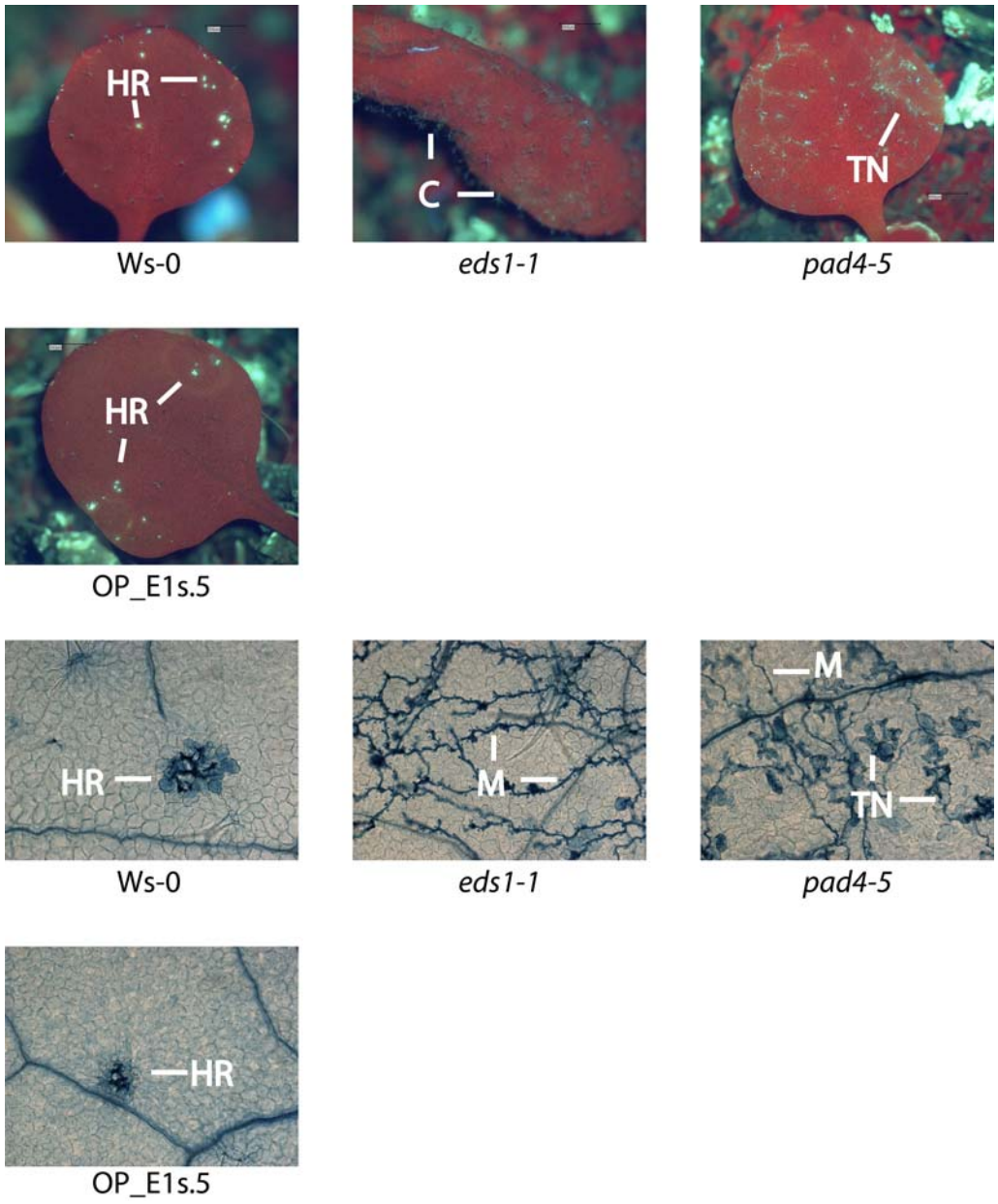


Fig. 3.2 Complementation of *RPP1* mediated resistance to Noco2 in OP_E1s lines

2-week-old OP_E1s.5 plants were spray inoculated with Noco2 (4 X 10^4 spores/ml). As controls wild type (WS-0), *eds1-1* and *pad4-5* plants were included. At 5 dpi plants were screened under UV light to detect cell death associated fluorescence (upper panels) and leaf samples from each line were collected and trypan blue stained to visualize pathogen structures and plant cell death (lower panels). HR: hypersensitive response; TN: trailing necrosis; M: mycelium; C: conidiophores. Two independent experiments gave similar results.

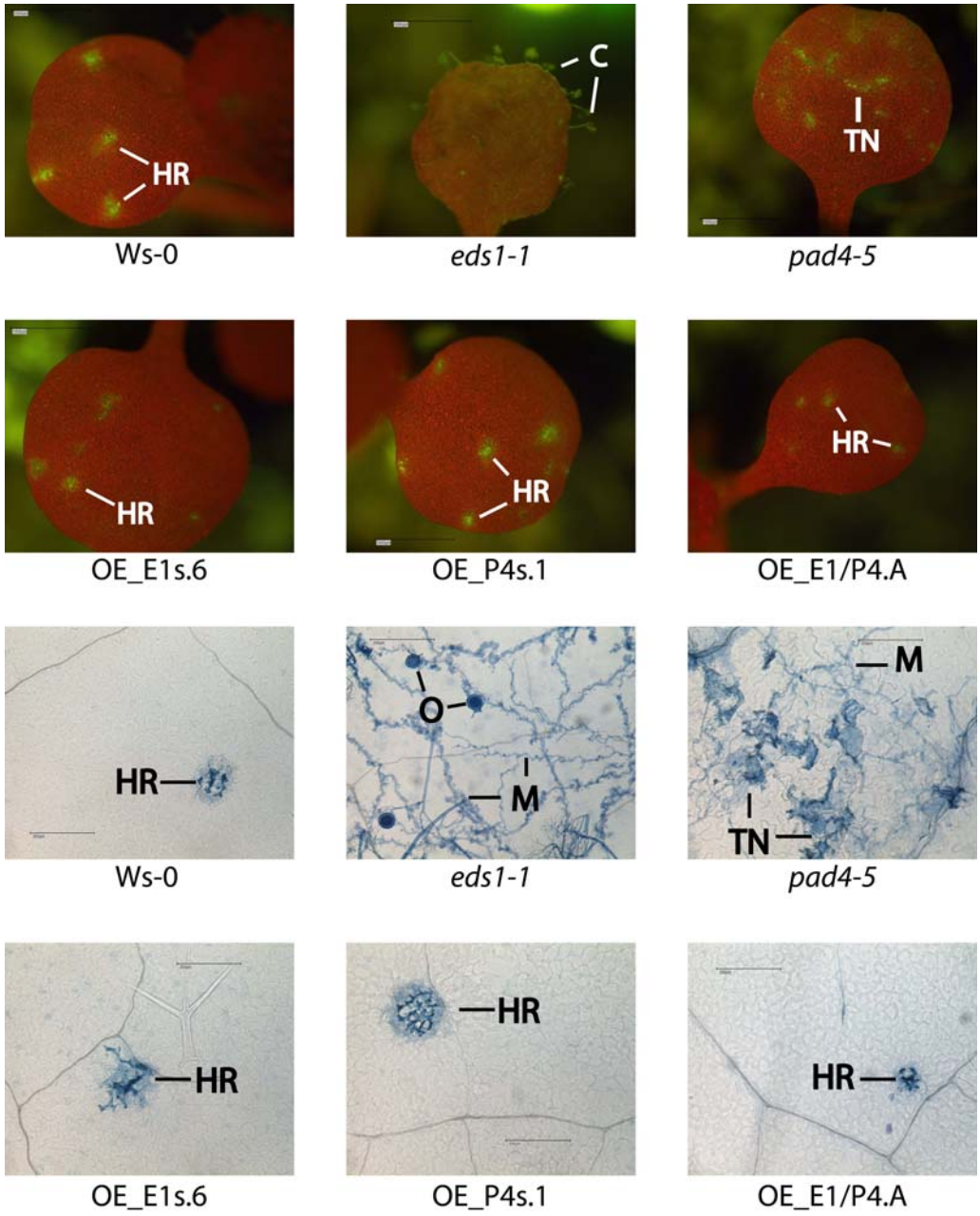
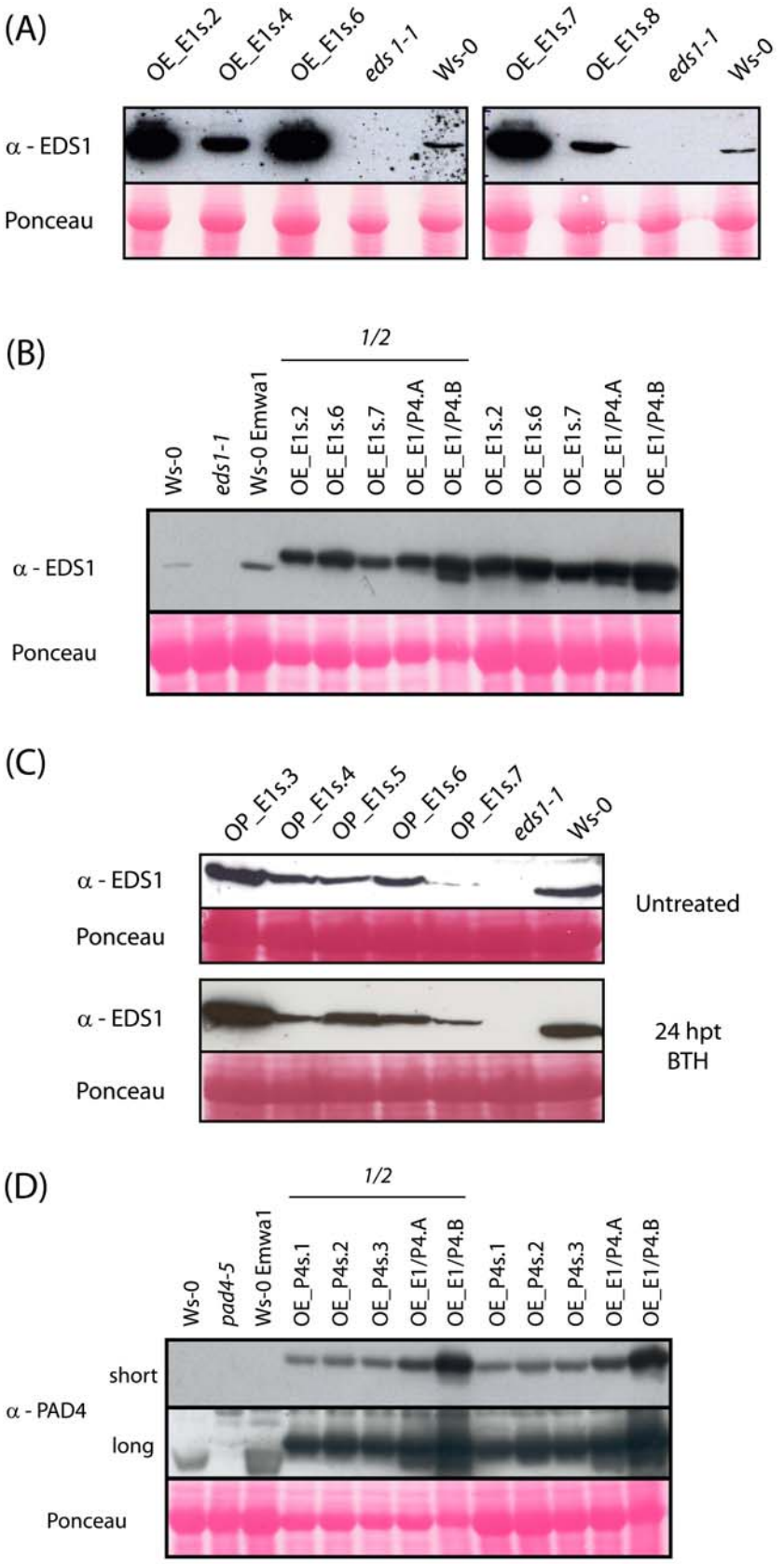


Figure 3.3 Complementation of *RPP1* mediated resistance to Noco2 in OE_E1s, OE_P4s and OE_E1/P4.A lines

2-week-old 35SE1s.15, 35SP4.1 and 35SE1/P4.A (see section 3.2) plants were spray inoculated with Noco2 (4 X 10^4 spores/ml). As controls wild type (WS-0), *eds1-1* and *pad4-5* plants were included. At 5 dpi plants were screened under UV light to detect cell death-associated fluorescence (upper panels) and leaf samples from each line were collected and trypan blue stained to visualize pathogen structures and plant cell death (lower panels). HR: hypersensitive response; TN: trailing necrosis; O: oospores; M: mycelium; C: conidiophores. Two independent experiments gave similar results.


(A)
OE_E1s.2
OE_E1s.4
OE_E1s.6
eds1-1
Ws-0
OE_E1s.7
OE_E1s.8
eds1-1
Ws-0
α - EDS1
Ponceau
(B)
1/2
Ws-0
eds1-1
Ws-0 Emwa1
OE_E1s.2
OE_E1s.6
OE_E1s.7
OE_E1/P4.A
OE_E1/P4.B
OE_E1s.2
OE_E1s.6
OE_E1s.7
OE_E1/P4.A
OE_E1/P4.B
α - EDS1
Ponceau
(C)
OP_E1s.3
OP_E1s.4
OP_E1s.5
OP_E1s.6
OP_E1s.7
eds1-1
Ws-0
α - EDS1
Ponceau
Untreated
α - EDS1
Ponceau
24 hpt
BTH
(D)
1/2
Ws-0
pad4-5
Ws-0 Emwa1
OE_P4s.1
OE_P4s.2
OE_P4s.3
OE_E1/P4.A
OE_E1/P4.B
OE_P4s.1
OE_P4s.2
OE_P4s.3
OE_E1/P4.A
OE_E1/P4.B
short
α - PAD4
long
Ponceau

(Previous page) **Figure 3.4** EDS1 and PAD4 protein levels in selected transgenic lines

Polyclonal anti-EDS1 western blot analyses of 4-week-old plants total protein extracts **(A)** Unchallenged OE_E1s (OE_E1s.2, OE_E1s.4, OE_E1s.6, OE_E1s.7 and OE_E1s.8), *eds1-1* (*eds1*-1) and wild type (Ws-0) plants. **(B)** Unchallenged OE_E1s (OE_E1s.2, OE_E1s.6 and OE_E1s.7), OE_E1/P4.A F1, OE_E1/P4.B F1 (see section 3.5) and wild type plants (WS-0), and Emwa1 challenged (4 X 10^4 spores/ml) *eds1-1* (*eds1-1*) and wild type plants (Ws-Emwa1) 6 dpi. **(C)** OP_E1s (OP_E1s.3, OP_E1s.4, OP_E1s.5, OP_E1s.6 and OP_E1s.7), *eds1-1* (*eds1-1*) and wild type plants (Ws0) untreated (upper panel) and 24 hours after BTH treatment (lower panel).

(D) Polyclonal anti-PAD4 western blot analysis of 4-week-old plants total protein extracts. Unchallenged OE_P4s (OE_P4s.1, OE_P4s.2 and OE_P4s.3), OE_E1/P4.A F1, OE_E1/P4.B F1 (see section 3.5) and wild type plants (WS-0) and Emwa1 challenged (4 X 10^4 spores/ml) *pad4-5* (*pad4-5*) and wild type plants (Ws-Emwa1) 6 dpi. Two different exposure times are shown (short and long).

For semi quantitative comparisons in **(B)** and **(D)** protein extracts were loaded twice: once with the same volume and once with half volume (1/2) of the correspondent control lines.

Relative ponceau stainings below each lane indicate comparable loadings.

wild type plants. In **Figure 3.4B** it is possible to observe that the EDS1 signal obtained from all the OE_E1s lines was larger than that of the wild type plants due to the presence of the strepII tag. This is indicative that the full length fusion protein is expressed and no truncated form can account for the observed *eds1-1* defence phenotype complementation. A signal at the same size was also observed in Western blots using monoclonal anti-StrepII antibody for detection (data not shown). Among the selected OP_E1s lines it was possible to identify lines with EDS1 expression levels lower, similar and higher than wild type plants (WS-0) as shown in **Figure 3.4C** (upper panel). The same trend was also observed 24 h after treatment with BTH (benzol (1,2,3) thiadiazole-7-cabothionic acid S-methyl ester) a SA analogue that induces EDS1 protein levels in wild type plants [93] (**Figure 3.4C**, lower panel). This result indicates a similar behavior between the OP_E1s lines and wild type plants. It further rules out the possibility that over expression in the OE_E1s lines compensates for reduced functionality of the EDS1 strepII fusion protein. Also in this case only a signal corresponding to the size of strepII tagged EDS1 was observed indicating absence of truncated versions.

The line indicated in **Figure 3.4C** as OP_E1s.5 showing an EDS1 expression level very similar to wild type was selected for further analyses.

Unchallenged OE_P4s plants also displayed PAD4 protein levels that were much higher than either unchallenged or pathogen challenged wild type plants (**Figure 3.4D**). Only a signal corresponding to the full length fusion protein could be observed.

Both *eds1-1* and *pad4-5* mutants support higher growth of the virulent *H. parasitica* isolate Emwa1 than wild type plants due to compromised basal defence response [93]. The selected EDS1 and PAD4 transgenic lines were tested to assess whether the over expression of EDS1 or PAD4 complemented the loss of resistance or even could lead to increased resistance against virulent pathogens. Three independent OE_E1s (OE_E1s.2, OE_E1s.6 and OE_E1s.7) and three independent OE_P4s transgenic lines (OE_P4s.1, OE_P4s.2 and OE_P4s.3) were infected with virulent isolate Emwa1 ($4X10^4$ spores/ml). At 5 dpi spores were counted and infected leaves stained with trypan blue to assess the extent of infection. Wild type (WS-0), *eds1-1* and *pad4-5* plants were included. The results are shown in **Figure 3.5**. All the selected transgenic lines exhibited full complementation of the corresponding *eds1-1* or *pad4-5* phenotype in that they supported lower Emwa1 growth similar to wild type plants. This confirms the functionality of the fusion proteins also for basal resistance. None of the selected OE_E1s or OE_P4s lines reduced pathogen growth significantly below that in wild type plants indicating that the over expression of either EDS1 or PAD4 alone does not confer enhanced basal resistance to virulent *H. parasitica*.

(Next Page) **Figure 3.5** The extent of basal resistance in selected transgenic lines against the virulent *H. parasitica* isolate Emwa1

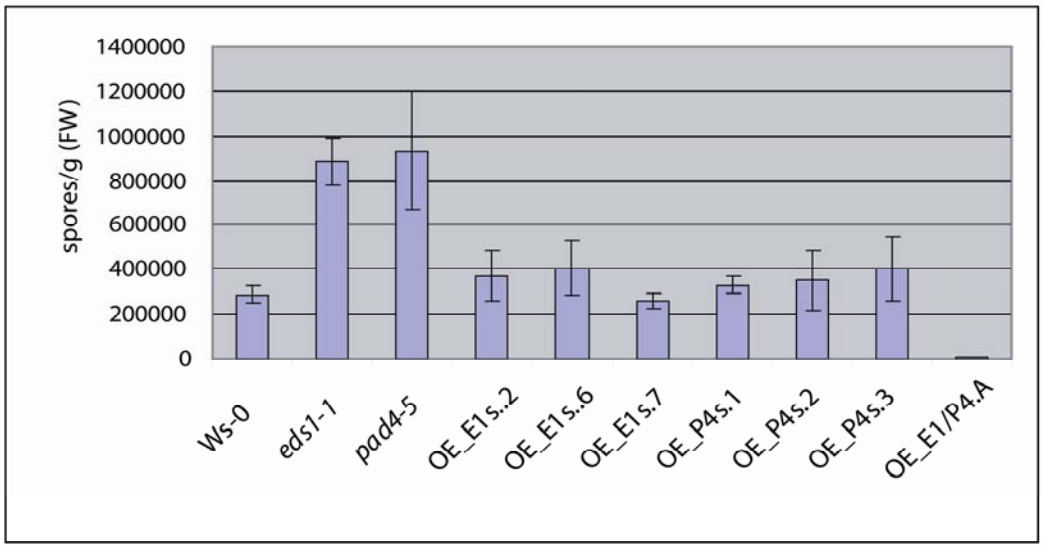
Three independent OE_E1s lines (OE_E1s.2, OE_E1s.6 and OE_E1s.7), three independent OE_P4s lines (OE_P4s.1, OE_P4s.2 and OE_P4s.3) and OE_E1/P4.A plants (see below) were spray inoculated with Emwa1 ($4 X 10^4$ spores / ml). As controls wild type Wassilewskjia (WS-0), Columbia (Col-0) and mutant *eds1-1* and *pad4-5* plants were included.

(A) 5dpi spore counts experiments were performed. Error bars represent standard deviations.

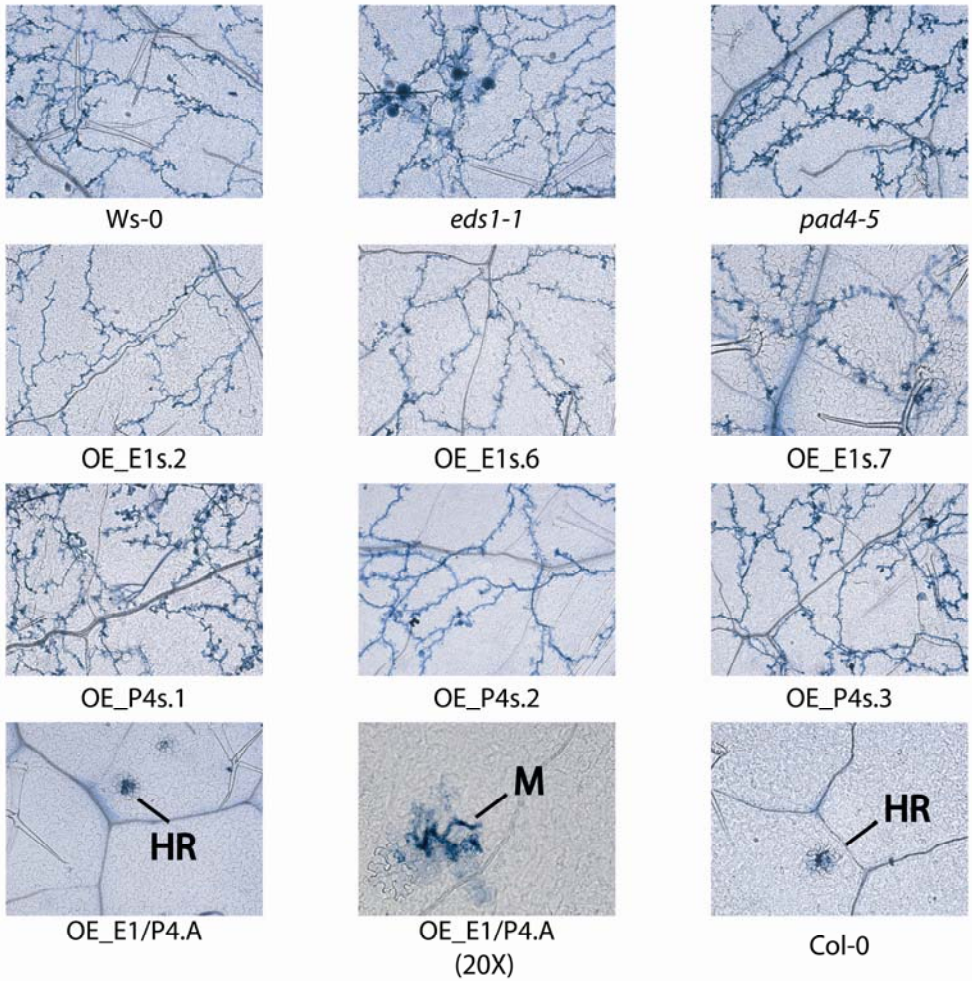
(B) 5dpi leaf samples were collected and trypan blue stained to visualize pathogen structures and plant cell death. All the pictures were taken at the same magnification. Only for the OE_E1/P4.A line a second picture at higher magnification (20X) was taken for displaying greater detail. HR: hypersensitive response; M: mycelium.

Three independent experiments gave similar results.

(A)


1400000
1200000
1000000
800000
600000
400000
200000
0
spores/g (FW)
Ws-0
eds1-1
pad4-5
OE_E1s.2
OE_E1s.6
OE_E1s.7
OE_P4s.1
OE_P4s.2
OE_P4s.3
OE_E1/P4.A

(B)


Ws-0
eds1-1
pad4-5
OE_E1s.2
OE_E1s.6
OE_E1s.7
OE_P4s.1
OE_P4s.2
OE_P4s.3
HR
OE_E1/P4.A
M
OE_E1/P4.A
(20X)
HR
Col-0

3.3 Generation of AtEDS1/AtPAD4 double over expressor lines

Considering the strong genetic and molecular connection between EDS1 and PAD4 I decided to combine single EDS1 and PAD4 over expression lines. This would allow me to test whether the number of EDS1-PAD4 complexes are the limiting factor in triggering defence.

Pollen from OE_P4s.1 plants was used to pollinate OE_E1s.6 emasculated flowers. The OE_P4s.1 transgenic plants generated in the *pad4-5* background are resistant to Kanamycin for the expression of the *nptII* (*neomycin phosphotransferase*) marker gene carried by the T-DNA inserted within endogenous *AtPAD4* gene [93, 127]. F1 seeds were germinated on Kanamycin containing MS plates to verify their identity. Surviving seedlings were transferred onto soil and F2 seeds collected. Since both the constructs for the over expression of EDS1 and PAD4 contain the same *bar* resistance gene to PPT, western blot analyses with commercial monoclonal anti-strepII antibody were performed to identify plants carrying both OE_E1s and the OE_P4s constructs (data not shown). Plants that had a signal for both EDS1 and PAD4 strepII fusion proteins were selected and genotyped for the *eds1-1* and *pad4-5* mutations. F3 seeds were collected and the segregation of the OE_E1s and OE_P4s constructs was monitored by PCR based genotyping. In this way it was possible to identify plants homozygous for both constructs and for the *eds1-1* and *pad4-5* mutations. Hereafter these plants are referred to as OE_E1/P4.A.

3.4 AtEDS1/AtPAD4 dual over expression causes growth abnormalities

During the selection process described above I observed that some of the plants in the OE_E1/P4.A segregating population were obviously smaller in size compared to either the parental lines or to wild type. This growth defect was quantified by measuring the fresh weight of 5-week-old wild type (WS-0), *eds1-1*, *pad4-5*, OE_E1s.6, OE_P4s.1 and OE_E1/P4.A plants. The results are shown in **Figure 3.6**. While the single over expressor lines OE_E1s.6 and OE_P4s.1 had fresh weights comparable to wild type plants (WS-0), the OE_E1/P4.A line had significantly reduced biomass compared to the parental lines

and wild type plants. While growth retardation in the EDS1-PAD4 dual over expressors was consistent between independent experiments, extent varied suggesting it is influenced by environmental conditions. It is notable that *eds1-1* mutants exhibited a higher fresh weight increase compared to wild type, as shown in **Figure 3.6**. Thus, the lack of EDS1 protein, which normally stabilizes PAD4 [98] and the heightened availability of EDS1 and PAD4 strongly influenced plant growth.

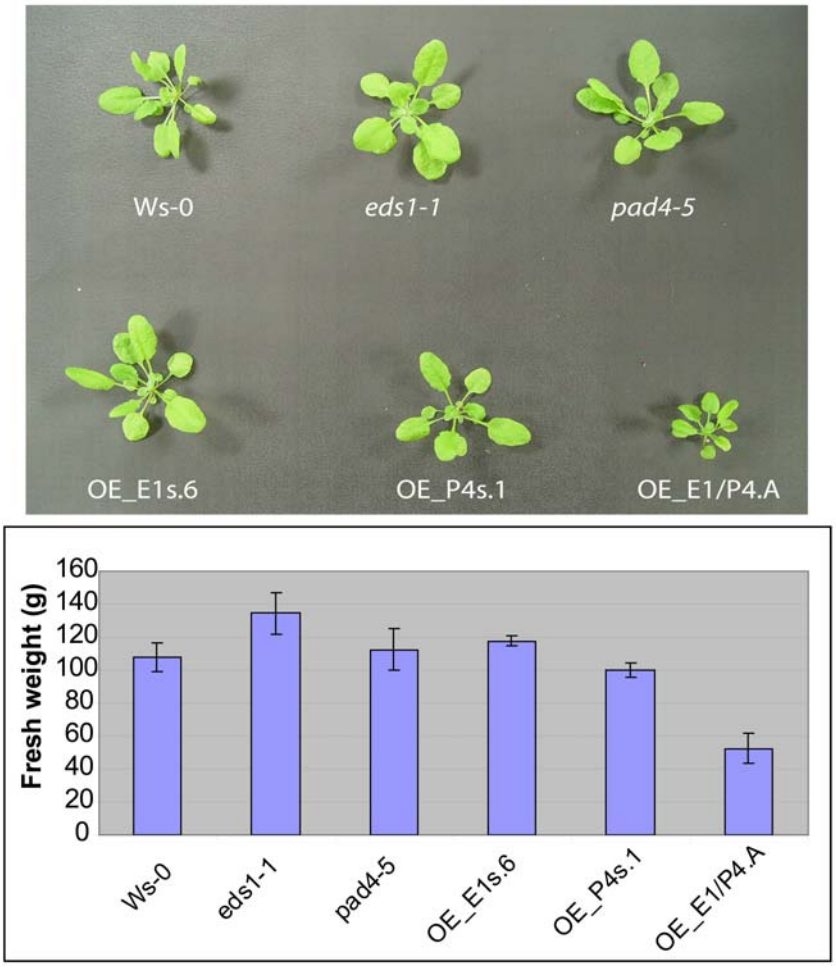
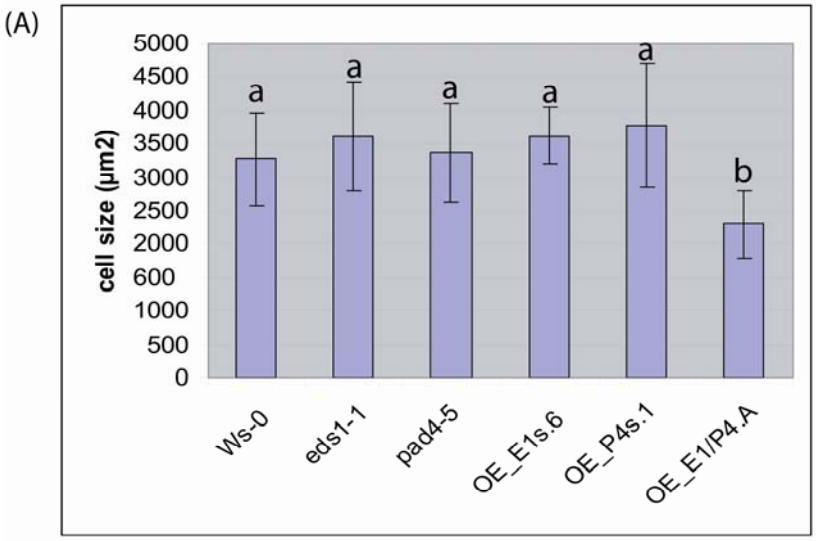


Figure 3.6 Reduced growth of OE_E1/P4.A double over expressor lines

Five-week-old individuals of wild type (WS-0), *eds1-1*, *pad4-5*, OE_E1s.6, OE_P4s.1 and OE_E1/P4.A lines were weighed and the average weight of a single plant was estimated from three samples of three plants (lower panel). Error bars represent sample standard deviations. Pictures of the aerial part of one representative individual from each line are shown in the upper panel. An independent experiment gave similar results.



(B)

	Ws-0	*eds1-1*	*pad4-5*	OE_E1s.6	OE_P4s.1
eds1-1	*0,5*				
pad4-5	*0,84*	*0,64*			
OE_E1s.6	*0,37*	*0,99*	*0,52*		
OE_P4s.1	*0,36*	*0,78*	*0,46*	*0,736*	
OE_E1/P4.A	*0,03*	*0,017*	*0,027*	*0,002*	*0,013*

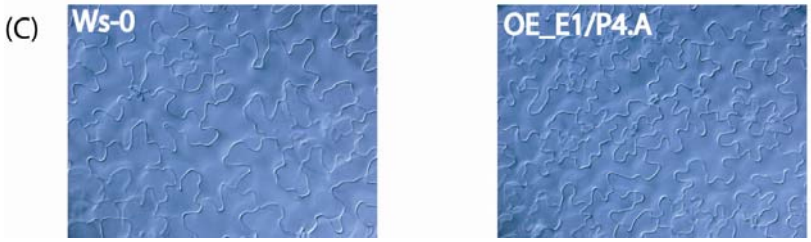


Figure 3.7 Epidermal cell size is reduced in the OE_E1/P4.A plants

The 7th true leaves from five 5-week-old individuals from wild type (WS-0), *eds1-1*, *pad4-5*, OE_E1s.6, OE_P4s.1 and OE_E1/P4.A lines were collected, cleared and mounted on a microscope slide. **(A)** The cell surface of 12-18 contiguous adaxial cells located in the most central portion of the leaf lamina was measured avoiding stomata and trichome base cells. The average single cell size was estimated. Error bars represent sample standard deviation. **(B)** To determine whether the observed differences in average values were statistically significant T test pairwise comparisons were performed. In the central table the calculated null hypothesis probabilities are indicated. Accordingly letters were assigned in (A). Different letters indicate significant differences (P<0,05). **(C)** Two representative pictures taken at the same magnitude for wild type (WS-0) and OE_E1/P4.A plants are shown.

Plants growth is a complex and highly regulated biological process which is mainly determined by cell division and cell elongation events [128]. In order to understand better the nature of the observed developmental phenotype a cell size estimation experiment was performed. The 7th true leaves from five 5-week-old plants were collected, cleared with ethanol and acetic acid and the cell area measured for 12-18 contiguous adaxial epidermal cells in the most central portion of the leaf lamina. Guard cells and trichome base cells were excluded from the estimation. The analysis was performed on OE_E1/P4.A, OE_E1s.6, OE_P4s.1, WS-0, *eds1-1* and *pad4-5* plants. The results are shown in **Figure 3.7**. No significant difference was observed between wild type plants (WS-0) and OE_E1s.6 or OE_P4s.1 single over expressor lines. Mutant *eds1-1* plants did not show enhanced cell size despite the slightly higher whole plant biomass accumulation. OE_E1/P4.A cells were significantly smaller than the control lines suggesting that a substantial contribution to the growth retardation is due to alteration of cell expansion processes, as previously shown for other defence related mutants [82, 129, 130]. However, I can not rule out that alteration in cell division might also be involved in the observed growth phenotype since no cell division analyses were performed so far. The same holds true for the increased biomass of *eds1-1*. Further analyses are needed to elucidate fully the basis to altered growth and biomass production in the lines tested.

3.5 AtEDS1/AtPAD4 dual over expression leads to SA pathway activation

In a number of *Arabidopsis* defence mutants reduced plant size is the consequence of constitutive activation of defence responses that is often associated with accumulation of the phenolic compound salicylic acid (SA) [82, 104, 129, 131-133]. To test whether the dual EDS1 and PAD4 over expression leads to defence activation, expression of defence marker genes was analyzed by semi-quantitative RT-PCR. The SA and jasmonic acid (JA) pathways are two important plant defence signaling systems and their activation is finely tuned during infections [87, 88]. Therefore, the expression of the two marker genes *PR1* (*Pathogenesisi Related 1*) and *PDF1.2* (*Plant Defensin 1.2*) respectively for the SA and JA pathways was analyzed in pathogen unchallenged (healthy) OE_E1/P4.A plants.

Unchallenged OE_E1/P4.A, OE_E1s.6, OE_P4s.1, *eds1-1*, *pad4-5*, wild type plants and pathogen challenged wild type plants were analyzed. The results are shown in **Figure 3.8**. In the unchallenged state a slight *PR1* up regulation was observed in OE_E1/P4.A plants while none of the other lines showed any *PR1* expression. The *PR1* transcript level observed in unchallenged OE_E1/P4.A was, however, lower than in wild type plants after *H. parasitica* infection indicating that simultaneous over expression of EDS1 and PAD4 is not sufficient to fully activate the SA pathway. *PDF1.2* transcript levels, as a marker of JA pathway stimulation, were not increased in any of the unchallenged lines including OE_E1/P4.A, indicating that the SA pathway activation is specifically deregulated.

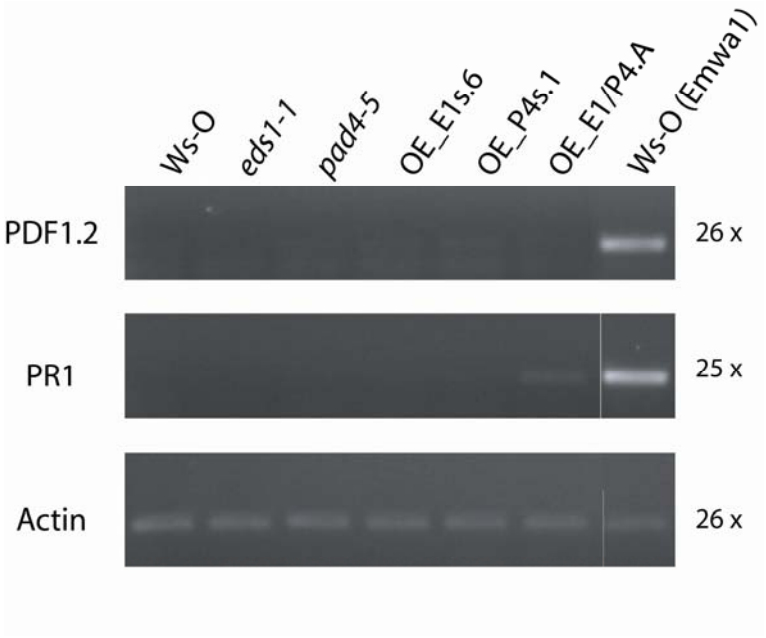


Figure 3.8 Constitutive SA pathway activation in the OE_E1/P4.A plants

Leaf material from 3-week-old unchallenged OE_E1/P4.A, OE_E1s.6, OE_P4s.1, *eds1-1*. *pad4-5* and wild type plants (WS-0) and from *H. parasitica* Emwa1 challenged (4 x 10^4 spores/ml) wild type plants 3dpi (WS-0(Emwa1)) was collected. Total RNA was extracted and the expression of the marker genes *PR1* and *PDF1.2* was assessed by semi quantitative RT-PCR. Equal application of template RNA for reverse transcription is shown by a control PCR reaction detecting *Actin* first strand cDNA. Numbers of cycles used in each PCR reaction are indicated on the right. In all cases additional three cycles showed detectable differences in the observed signal indicating that the assay was performed within the linear range of amplification. Three independent experiments gave similar results.

Together, these data show that the dual over expression of EDS1 and PAD4 leads to slight activation of the SA pathway that is not observed when either one of the two proteins is over expressed. To characterize further SA pathway activation the levels of free and total SA were measured in unchallenged wild type (WS-0), *eds1-1*, *pad4-5*, OE_E1s.6, OE_P4s.1 and OE_E1/P4.A plants. The results are shown in **Figure 3.9**. Compared to parental lines (OE_E1s.6 and OE_P4s.1) and control lines (WS-0, *eds1-1*, *pad4-5*) OE_E1/P4.A plants had increased levels of both free and total SA in the unchallenged state consistent with the observed constitutive up regulation of the SA marker gene *PR1*.

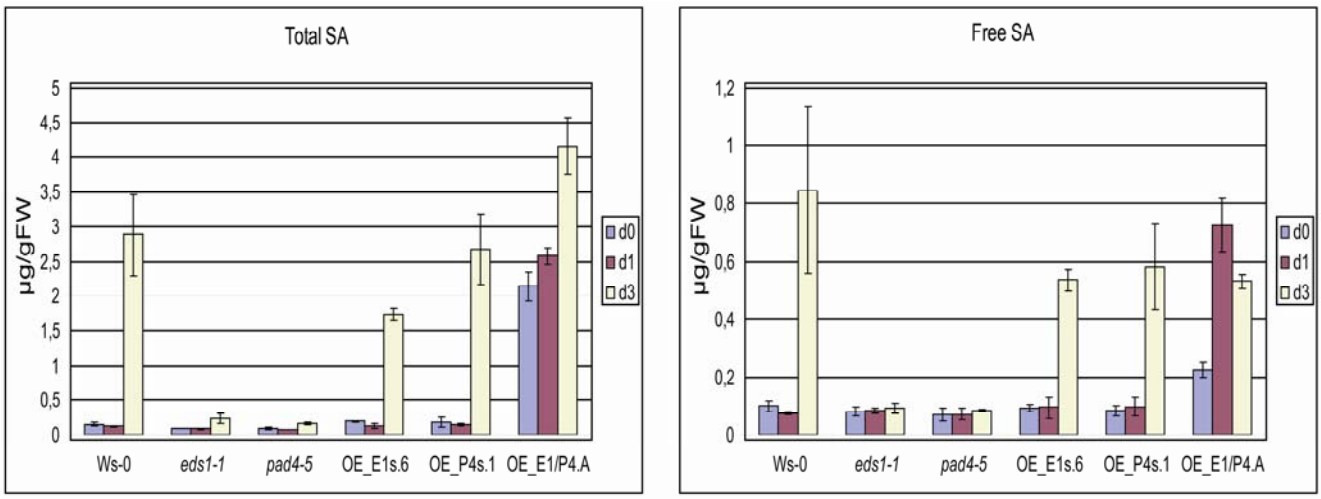


Figure 3.9 Total and Free SA levels in the selected transgenic lines before and after pathogen challenge

Plant material from unchallenged and Emwa1 challenged (4 X 10^4 spores /ml) wild type (WS-0), *eds1-1*, *pad4-5*, OE_E1s.6, OE_P4.1 and OE_E1/P4.A plants 1 and 3 dpi was collected. Extraction and quantification of total and free salicylic acid by HPLC was performed as described in Materials and Methods. Data represent the average from three replicate samples. Error bars represents standard deviations calculated on the three replicates.

In previously characterized defence mutants constitutive activation of the SA pathway was associated with development of spontaneous lesions in the absence of pathogen [102, 132, 134, 135]. In order to assess whether this was also the case in the OE_E1/P4.A plants 3-week-old unchallenged OE_E1/P4.A plants were analyzed by trypan blue

staining together with wild type (WS-0), OE_E1s.6, OE_P4s.1 and the spontaneous lesioning *nudt 7-1* mutant plants [102]. The results are shown in **Figure 3.10**. With the exception of the *nud7-1* plants, none of the analyzed lines exhibited spontaneous lesion development. Thus, I concluded that deregulated SA signaling in the OE_E1/P4.A plants is not associated with lesion development.

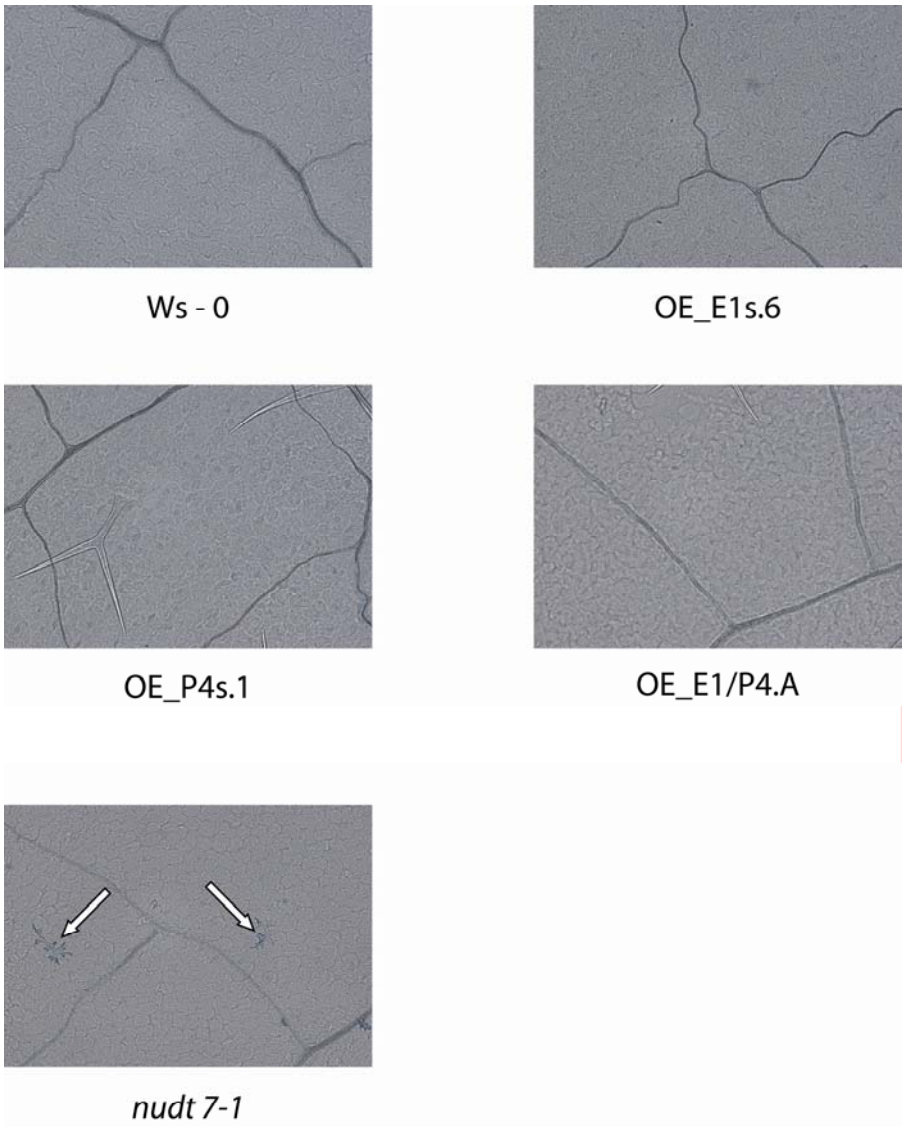


Figure 3.10 Lack of spontaneous lesioning in the OE_E1/P4.A line

Leaf samples from 3-week-old unchallenged wild type (WS-0), OE_E1s.6, OE_P4s.1, OE_E1/P4.A and *nudt 7-1* plants were collected and stained with trypan blue to visualize spontaneous cell death. Only in *nudt 7-1* plants were spontaneous lesions observed (white arrows). Three independent experiments gave similar results.

3.6 AtEDS1/AtPAD4 dual over expressor lines have increased resistance to bacterial and oomycete virulent pathogens

Arabidopsis mutants that have constitutive activation of the SA pathway also have increased resistance against pathogen attack [82, 104, 132, 133, 135]. In some cases increased resistance was shown to be uncoupled from cell death development [104, 133, 136]. To test the capability of the OE_E1/P4.A plants to mount a normal HR in response to avirulent pathogens, OE_E1/P4.A plants were infected with *H. parasitica* Noco2, trypan blue stained and inspected under UV. The results are shown in **Figure 3.2** (page 52) . OE_E1/P4.A plants exhibited normal HR development although the area of tissue undergoing cell death appeared to be less extensive than in wild type plants. Currently, I can not determine whether this is due to the smaller cell size in OE_E1/P4.A plants or to a reduction in the number of cells involved in the response. Some OE_E1.P4.A individuals occasionally produced very high *H. parasitica* sporulation that was comparable to *eds1-1* plants. PCR based genotyping of these individuals, combined with Western blot analyses using monoclonal anti-strepII antibody for detection demonstrated that silencing of both OE_E1s and OE_P4s construct was taking place in these individuals, very likely as consequence of being both constructs driven by the CaMV 35S promoter (data not shown).

I investigated whether the dual over expression of EDS1 and PAD4 and the associated SA pathway activation results in increased basal resistance to virulent pathogens. OE_E1/P4.A plants were infected with virulent *H. parasitica* Emwa1 and spore count and trypan blue staining experiments performed. As shown in **Figure 3.5** (page 57), in comparison to the OE_E1s or OE_P4s lines, the OE_E1/P4.A plants exhibited a strongly enhanced basal resistance to Emwa1 and permitted only very low levels of pathogen sporulation. Trypan blue staining revealed that in contrast to the corresponding parental lines OE_E1s.6 and OE_P4s.1, OE_E1/P4.A plants, produced HR lesions similar to those observed in genetically resistant Col-0 plants despite not carrying any resistance gene capable to recognize the Emwa1 strain. The absence of spontaneous lesions together with the visualization of germinated spores within the area undergoing cell death (see close up at 20 fold magnitude in **Figure 3.5,** page 57) suggest that the cell death phenotype is

directly triggered upon pathogen challenge and not general stress response. Also, since increased resistance against virulent Emwa1 was observed only when both EDS1 and PAD4 are over expressed the notion that EDS1-PAD4 complexes or cooperation is needed to deregulate the plant defence response is reinforced.

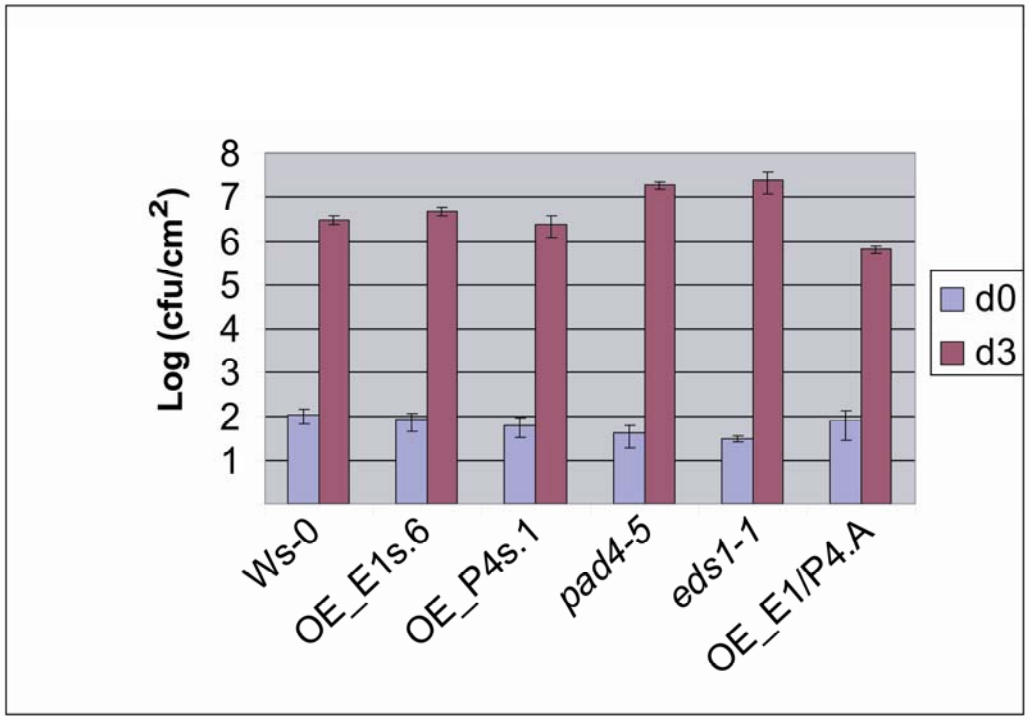


Figure 3.11 Growth of *Pseudomonas syringae* pv *tomato* DC3000 on selected transgenic lines

Wild type (WS-0), *eds1-1*, *pad4-5*, OE_E1s.6, OE_P4s.1 and OE_E1/P4.A 4-week-old plants were infected by surface spraying with DC3000 bacterial suspension of $5X10^7$ cfu/ml. Bacterial titers were measured shortly after inoculation (d0) and 3dpi (d3). Error bars represent sample standard deviations. Three independent experiments of three replicant samples per line gave similar results.

I tested whether increased resistance in the OE_E1/P4.A plants was specific to oomycetes or more generally effective against other pathogens. Therefore, bacterial growth experiments using the virulent bacterial strain *Pseudomonas syringae* pv. *tomato* DC3000 (hereafter *Pst* DC3000) were performed. The inoculation was performed by spraying

bacteria (5 X 10^7 cfu/ml) on to the leaf surface, a method that was shown most recently to best resemble the natural infection mode [137]. OE_E1/P4.A plants together with OE_E1s.6, OE_P4s.1, *eds1-1*, *pad4-5* and wild type (Ws-0) controls were spray inoculated and bacterial growth was measured at 3 dpi. The results are shown in **Figure 3.11.** As expected [38, 93], the basal defence mutant *eds1-1* and *pad4-5* supported higher bacterial growth than wild type plants (WS-0). The single EDS1 and PAD4 over expressors OE_E1s.6 and OE_P4s.1 plants supported bacterial titers at 3dpi that were not statistically different from wild type. Thus, enhanced expression of EDS1 or PAD4 alone did not alter the plant basal defense. OE_E1/P4.A plants had enhanced resistance to *Pst* DC3000, manifested as lower bacterial growth. This result indicates that the increased resistance observed against virulent oomycetes is likely to be a more general phenomenon effective with other pathogens.

3.7 SA pathway activation in OE_E1/P4.A plants is not due to increased sensitivity to pathogen elicitors

Plant basal defence is triggered by recognition of so called Pathogen or Microbe Associated Molecular Patterns (PAMPs or MAMPs [138]) that are essential highly conserved molecules in microorganisms. Plants recognize these non-self components by extracellular receptors belonging to the Receptor Like Kinase (RLKs) class [2, 11]. PAMP recognition leads to the activation of defence responses such as an oxidative burst, up regulation of defence related genes such as *PR1* and to seedling growth inhibition [12]. The increased resistance against virulent pathogens, *PR1* upregulation and growth inhibition of EDS1-PAD4 dual over expressors prompted me to investigate whether the OE_E1/P4.A phenotypes might be explained by a super sensitivity to PAMPs. In this scenario the reduced growth would be the result of hyper responsiveness to non pathogenic microbes normally present in the growing chamber where plants are grown. To test this possibility OE_E1s.6, OE_P4s.1, OE_E1/P4.A, *eds1-1*, *pad4-5* and wild type plants (WS-0) were then grown in sterile MS medium for 5 weeks and their fresh weight measured to assess whether absence of PAMPs would negate the observed developmental

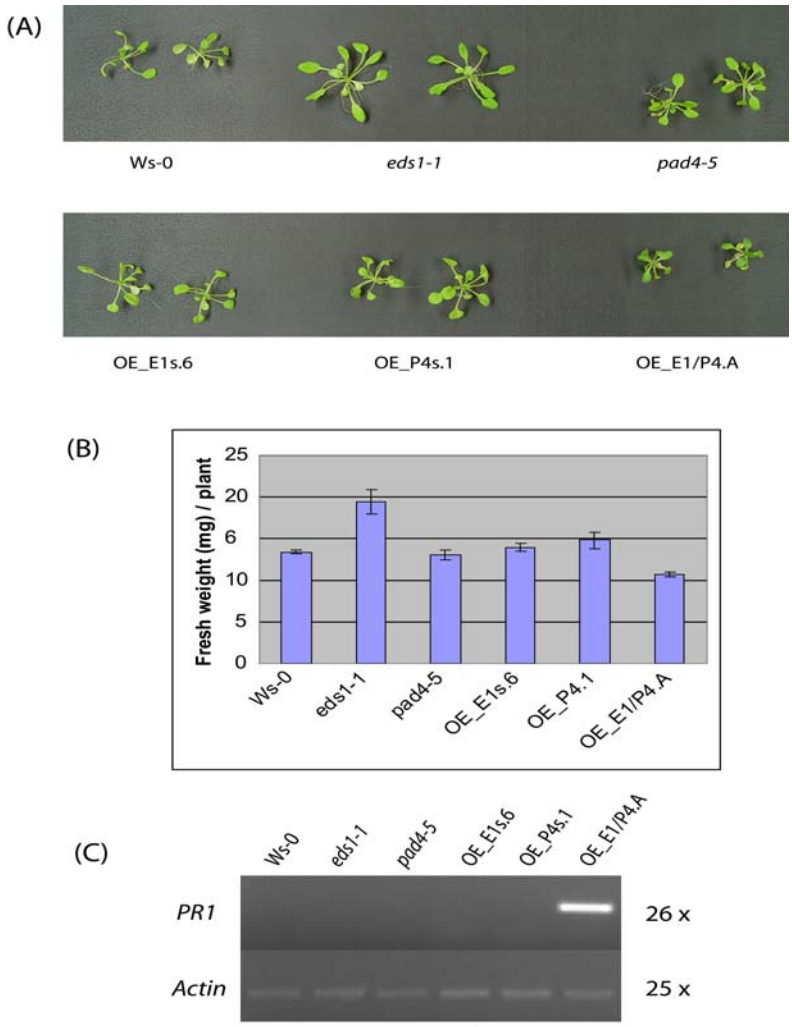


Figure 3.11 Growth retardation and SA pathway activation in sterile grown OE_E1/P4.A plants

Wild type, *eds1-1*, *pad4-5*, OE_E1s.6, OE_P4s.1 and OE_E1/P4.A seeds were surface sterilized and sown in closed Magenta pots containing autoclaved MS solid medium. **(A)** Two representative 5-week-old individuals from each lines are shown. **(B)** After 5 weeks three independent samples each of three seedlings from each line were weighed and the average weight of a single plant was estimated (lower panel). Error bars represent standard deviations. **(C)** Tissues from sterile grown 5 week old OE_E1/P4.A, OE_E1s.6, OE_P4s.1, *eds1-1*. *pad4-5* and wild type plants (WS-0) were collected, total RNA was extracted and the expression of the SA marker gene *PR1* was assessed by semi quantitative RT-PCR. Equal application of template RNA for reverse transcription is shown by a control PCR reaction detecting *Actin* first strand cDNA. Numbers of cycles used in each PCR reaction are indicated on the right. In all cases additional three cycles showed detectable differences in the observed signal indicating that the assay was performed within the linear range of amplification. Two independent experiments gave similar results

phenotype. The results are shown in **Figure 3.11**. Under sterile conditions *eds1-1* plants accumulated significantly more biomass than wild type plants as previously observed in soil-grown plants (see **Figure 3.6** page 59). The extent of the biomass increase over wild type plants was indeed larger than observed in soil-grown plants, reaching differential of ≈30%. This is similar to what reported for other plant defence impaired mutants that showed a general increased fitness when grown in more sterile conditions [139]. The OE_E1/P4.A plants had reduced biomass accumulation also in sterile conditions compared to parental lines OE_E1s.6, OE_P4s.1 and to wild type (Ws-0) plants. I tested whether the growth retardation was also associated with constitutive SA pathway activation in these conditions, by measuring the expression of *PR1* in sterile grown plants. As shown in **Figure 3.11** only sterile grown OE_E1/P4.A plants exhibited *PR1* expression similar to that observed in soil-grown plants (see **Figure 3.8** page 62). From these data I concluded that the reduced growth and the *PR1* activation in the OE_E1/P4.A plants is not the result of higher sensitivity to PAMPs since both defects were retained in sterile growth conditions. These phenotypes are more likely to be the result of perturbation of an intrinsic genetic program through joint EDS1 and PAD4 over expression.

3.8 Reduced growth is observed in independent EDS1/PAD4 dual over expressor lines

All of the phenotypes described so far were tested on a single combination between the two single over expressor lines OE_E1s.6 and OE_P4s.1. To verify that growth retardation, SA pathway activation and increased resistance to virulent pathogens are actually due to over expression of both EDS1 and PAD4 proteins and not a peculiarity of this specific transgenic line combination, a cross between two further independent over expressor lines of EDS1 and PAD4 (denoted OE_E1s.2 and OE_P4s.2 respectively) was performed. Pollen from OE_P4s.2 plants was used to pollinate emasculated OE_E1s.2 flowers to give F1 progeny. Hereafter the resulting line will be referred to as OE_E1/P4.B. A cross between the original OE_E1s.6 and OE_P4s.1 lines (OE_E1/P4.A)

was repeated as control. F1 seeds from each cross were collected and sown on soil together with the corresponding parental lines. At 4 weeks under 10 hours light/day F1 OE_E1/P4.B plants showed obvious growth retardation compared to the parental lines OE_E1s.2 and OE_P4s.2 as shown in **Figure 3.12**. By contrast only some leaf curling could be observed in OE_E1/P4.A compared to the parental OE_E1s.6 and OE_P4.1 plants. EDS1 and PAD4 protein levels were measured in the F1s by Western blot analysis and results are shown in **Figure 3.5** (page 54) . Both OE_E1/P4.A and OE_E1/P4.B F1 plants had higher levels of EDS1 and PAD4 than the corresponding parental lines (OE_E1s.6 and OE_P4s.1, and OE_E1s.2 and OE_P4s.2 respectively). In both F1 over expressor combinations, up regulation was observed for EDS1 and PAD4 endogenous proteins, distinguished from their corresponding tagged versions due to their smaller size (**Figure 3.5** page 54). EDS1 and PAD4 protein accumulation was higher

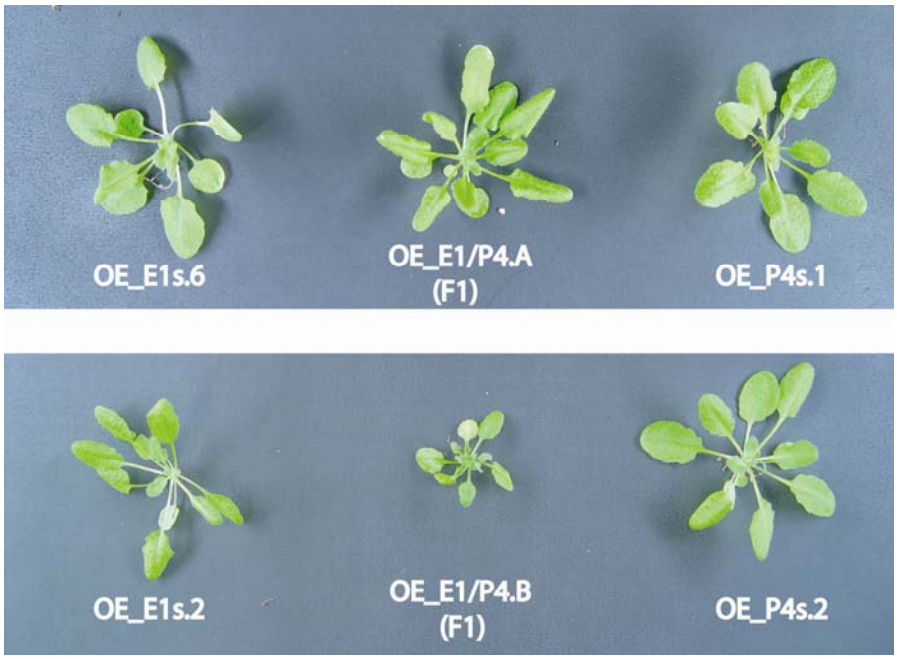


Figure 3.12 Growth phenotype in the F1 of independent EDS1 and PAD4 over expressors combinations

OE_E1/P4.A and OE_E1/P4.B F1 seeds and the seeds from the corresponding parental lines (OE_E1s.15 and OE_P4.1 for OE_E1/P4.A, and OE_E1s.5 and OE_P4s.2 for OE_E1/P4.B) were sown on soil and after 4 weeks the aerial part of one representative individual from each line was photographed. Pictures in the two panels are taken at the same magnification.

in OE_E1/P4.B than in OE_E1/P4.A plants, suggesting a positive correlation between EDS1 and PAD4 protein abundance and growth retardation. Currently, experiments are being performed to assess whether the growth retardation phenotype in the OE_E1/P4.B plants is also associated with SA pathway activation and increased basal defence. From these results I concluded that the growth retardation, and very likely also the other phenotypes observed in the OE_E1/P4.A plants, are not a peculiarity of this particular transgenic line but consequence of the dual EDS1 and PAD4 over expression.

3.9 OE_E1/P4.A plants show accelerated responses upon virulent pathogen attack

In OE_E1/P4.A plants *PR1* levels are lower than in pathogen challenged wild type plants, indicating that the SA pathway is not full activation of by over expression of EDS1 and PAD4 (See **Figure 3.8** page 62). I then decided to characterize the pathogen response timing in the dual EDS1-PAD4 over expressors to test whether the increased resistance is due to an accelerated SA signaling. OE_E1/P4.A plants together with OE_E1s.6, OE_P4s.1, *eds1-1*, *pad4-5* and wild type (Ws-0) plants were infected with virulent *H. parasitica* Emwa1 pathogen (4 x 10^4 spores / ml) and tissue samples collected at 0, 1 and 3 dpi. Mock (water) inoculated wild type plants 3 dpi were also included as control. Total RNA was extracted from all samples and the expression levels of marker genes were analyzed by semi quantitative RT-PCR. The results are shown in **Figure 3.14**. In parallel, the same plant tissues were used to extract total proteins and assess EDS1 and PAD4 protein levels on Western blots by probing with polyclonal anti-EDS1 and anti-PAD4 respectively. The results are shown in **Figure 3.13**. In unchallenged plants PAD4 protein accumulated at higher levels in OE_E1/P4.A than in OE_P4s.1 plants, as previously observed in the OE_E1/P4.A F1 individuals (See **Figure 3.5** page 57). No significant difference was observed between unchallenged OE_E1/P4.A and OE_P4s.1 At the transcriptional level pointing to stabilization of PAD4 protein at the posttranscriptional level by increased EDS1 in the dual over expressors. The observed RT-PCR *PAD4* signals were however, relatively strong for OE_E1/P4.A and OE_P4s.1 unchallenged plants and I can therefore not rule out that they may be near to saturation levels

Quantitative RT-PCR analyses will be performed to measure more precisely quantitative differences in *PAD4* expression among different transgenic lines. In the unchallenged *eds1-1* mutants *PAD4* transcripts accumulated to lower levels than in wild type, confirming the previously reported requirement of functional EDS1 protein for basal *PAD4* transcript accumulation [93]. EDS1 protein levels were slightly higher in unchallenged OE_E1/P4.A compared to the unchallenged parental line OE_E1s.6. Also in this case, *EDS1* transcript levels in OE_E1/P4.A and

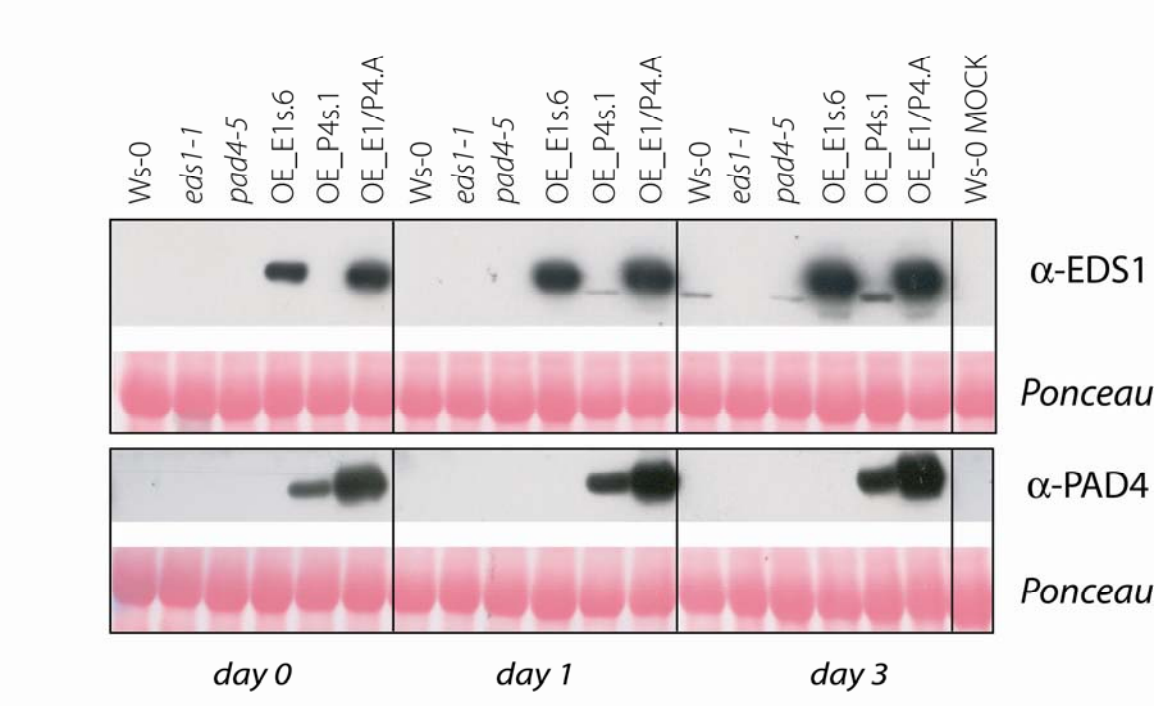


Figure 3.13 EDS1 and PAD4 protein accumulation after virulent pathogen challenge

Wild type (WS-0), *eds1-1*, *pad4-5*, OE_E1s.6, OE_P4s.1 and OE_E1/P4.A plants were spray inoculated with *H. parasitica* Emwa1 (4 x 10^4 spores / ml) and samples from each line collected before (day 0) inoculation and at 1 (day 1) and 3 dpi (day 3). As a control, tissue from water sprayed wild type plants was collected at 3 dpi (Ws-0 MOCK). Total proteins were extracted and analyzed on a Western blot analyses using anti – EDS1 (upper panel) or anti - PAD4 (lower panel). Ponceau staining of the blot indicates comparable loadings of each lane. An independent experiment was performed with similar results.

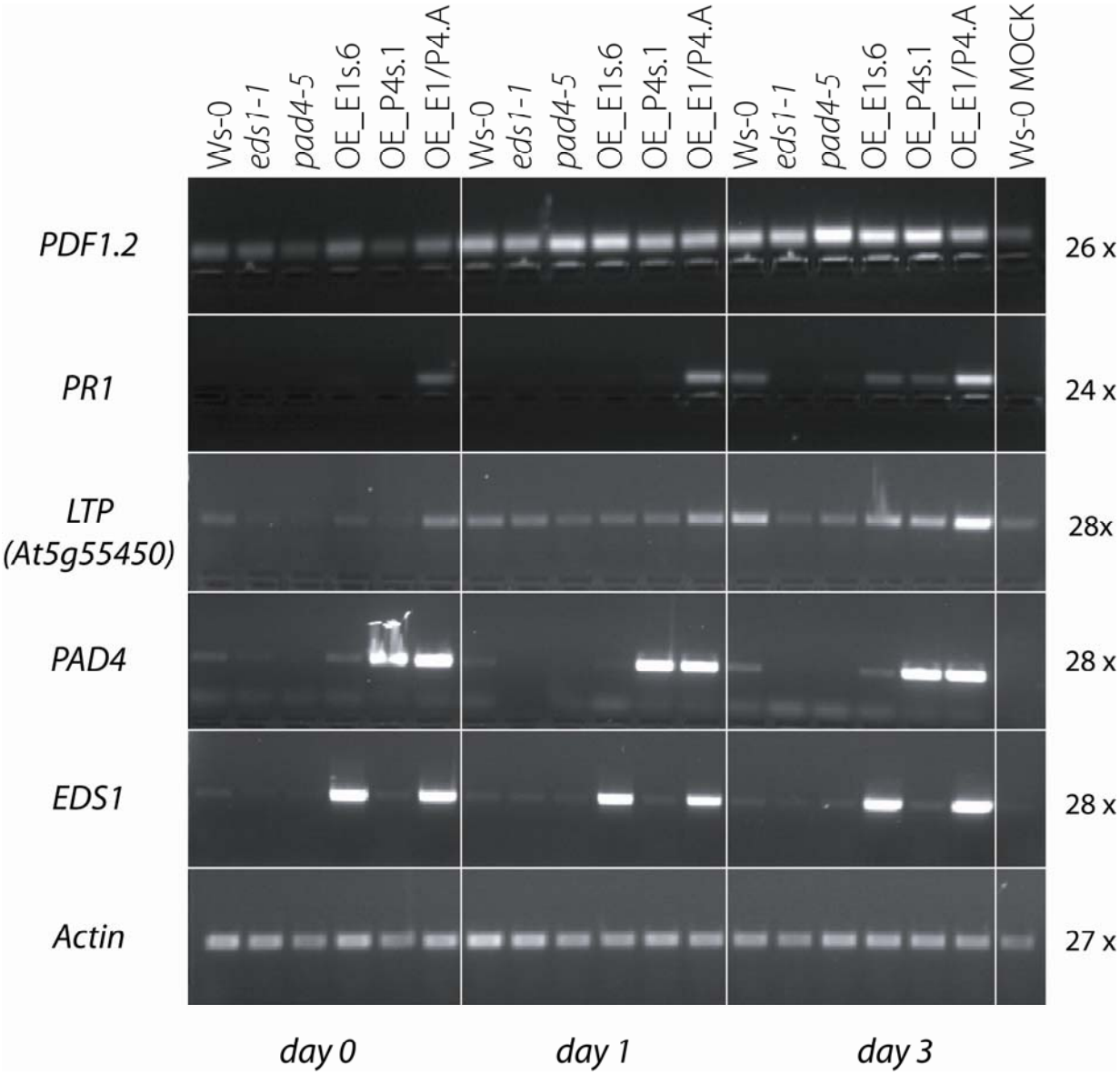


Figure 3.14 Analysis of gene expression after virulent pathogen challenge

From the same series of samples indicated in **Figure 3.13** total RNA was extracted the expression of the indicated genes assessed by semi quantitative RT-PCR. Equal amounts of template RNA for reverse transcription are shown by a control PCR reaction detecting *Actin* first strand cDNA. Numbers of cycles used in each PCR reaction are indicated on the right. In all cases additional three cycles showed detectable differences in the observed signal indicating that the observed signals were not saturated.

OE_E1s.6 plants were similar, consistent with a mutual posttranscriptional stabilization by EDS1 and PAD4 of its partner in the dual EDS1-PAD4 over expressors. It is necessary, however, to confirm the post-transcriptional stabilization by quantitative RT PCR of the samples. As seen above in the unchallenged state, a slight up regulation of the SA pathway marker *PR1* was observed only in OE_E1/P4.A plants. By contrast, the JA pathway marker gene *PDF1.2* was not up regulated in the unchallenged state in any line.

At5g55450, a gene encoding for a putative lipid transfer protein (hereafter *LTP*), was previously shown to be up regulated after bacterial pathogen challenge in an EDS1- and PAD4-dependent fashion [102]. *LTP* transcript levels were also slightly higher in unchallenged OE_E1/P4.A plants compared to unchallenged wild type (Ws-0). At 1dpi of *H. parasitica* Emwa1 infection, there was an increase in EDS1 protein levels in the OE_E1s.6, OE_P4s.1 and OE_E1/P4.A transgenic plants compared to unchallenged. No obvious increase in *EDS1* mRNA was observed in these lines, suggesting a further posttranscriptional stabilization of EDS1 upon pathogen challenge. The fact that the semi quantitative RT-PCR *EDS1* signal for OE_P4s.1 was within the linear amplification range strongly supports this hypothesis. Similarly, evidence for post transcriptional stabilization of PAD4 protein was observed at 1 dpi in both the OE_E1/P4.A and OE_P4s.1 lines (**Figure 3.13**). *PR1* mRNAs were further increased over the unchallenged state of the OE_E1/P4.A 1dpi.

PDF1.2 was up regulated similarly in all lines at both 1dpi and 3 dpi, irrespective of the absence or over expression of either functional EDS1 or PAD4 protein. Also *LTP* was up regulated at both 1 and 3dpi, but up regulation sustainment was dependent on EDS1 and PAD4. Furthermore both *PR1* and *LTP* levels at 3dpi were higher in OE_E1/P4.A plants as compared to wild type or single over expressor lines.

At 3dpi both EDS1 and PAD4 protein had a further up regulation compared to 1 dpi, probably due to post translational stabilization. Consistent with this hypothesis, in comparison to 1dpi, at 3dpi OE_P4.1 plants displayed clear EDS1 protein up regulation while *EDS1* transcripts, whose RT-PCR signal levels were far from the saturation, didn´t change.

Total SA and Free SA were measured in a time course after pathogen challenge with *H. parasitica* Emwa1 (4 X 10^4 spores/ml) in OE_E1/P4.A, OE_E1s.6, OE_P4s.1, *eds1-1*, *pad4-5* and wild type (WS-0) plants. The results are shown in **Figure 3.9** (page 63). At 1dpi only the OE_E1/P4.A plants displayed higher levels of SA, consistent with the observed slight up regulation of *PR1* expression (**Figure 3.14**). A clear increase in SA was observed at 3dpi also in wild type (WS-0), OE_E1s.6 and OE_P4.1 plants. The level of total SA kept rising until 3dpi in OE_E1/P4.A plants and remained significantly higher than in wild type plants. Free SA rapidly became conjugated as recorded (**Figure 3.9** page63). No increase in SA was observed in *eds1-1* or *pad4-5* plants, confirming the previously reported requirement of EDS1 and PAD4 in SA accumulation after pathogen challenge [100, 140].

A typical plant response in both compatible and incompatible interactions is the accumulation of antimicrobial compounds at the site of infection [141]. Compounds that accumulate after pathogen challenge are termed phytoalexins [142]. *PAD4* was originally isolated in a screen to identify *Arabidopsis* mutants impaired in the accumulation of the indole phytoalexin, camalexin [91]. I therefore measured camalexin levels in a time course after infection with *H. parasitica* Emwa1. As shown in **Figure 3.15** very low levels of camalexin were observed before pathogen challenge in all lines. At 1dpi all lines had increased camalexin accumulation. However, OE_E1/P4.A accumulated camalexin to significantly higher levels than all the other lines. At 3dpi camalexin amounts rose in all lines but OE_E1/P4.A remained the highest accumulator. *Eds1-1* and *pad4-5* mutants accumulated significantly lower camalexin levels than wild type plants confirming the requirement of EDS1 and PAD4. Unexpectedly OE_E1s.6 plants displayed lower camalexin levels than wild type plants at 3dpi in this experiment. Further repetitions will be necessary to determine whether this trend in OE_E1s.6 line is reproducible.

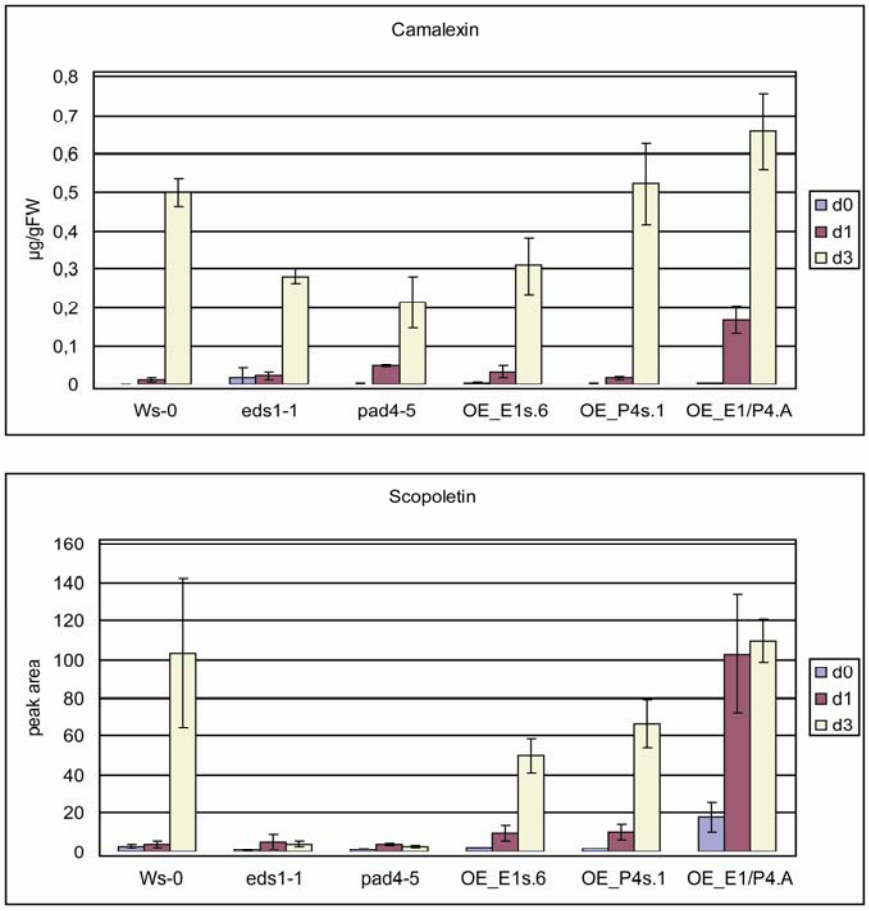


Figure 3.15 Phytoalexin accumulation upon pathogen challenge indifferent plant lines

Wild type (WS-0), *eds1-1*, *pad4-5*, OE_E1s.6, OE_P4s.1 and OE_E1/P4.A plants were spray infected with Emwa1 (4 x 10^4 spores / ml) and samples from each line were collected before (d0), 1 (d1) and 3 (d3) dpi. Extraction and quantification of camalexin and scopoletin by HPLC were performed as described in Materials and Methods. Data represent the average from three replicate samples. Error bars represents sample standard deviations. For scopoletin no chemically pure standard sample was available when the experiment was performed. For this reason scopoletin data are expressed as measured HPLC peak areas.

scopoletin is another phytoalexin which was previously shown to accumulate in *Arabidopsis* in response to applications of phytoprostanes, prostaglandins like molecules which are products of non enzymatic lipids peroxidation [143, 144]. Already in the unchallenged state scopoletin accumulated to significantly higher levels in OE_E1/P4.A plants compared to all the other lines. Reminiscent of what was seen for free and total SA, a further strong increase in scopoletin was observed at 1dpi. In comparison, slight increases in scopoletin were measured in OE_E1s.6 and OE_P4s.1 at 1 dpi and there was

no increase in wild type (**Figure 3.15**). At 3dpi a strong increment in scopoletin content was observed in wild type plants reaching levels comparable to the OE_E1/P4.A double over expressor line that remained high. OE_e1s.6 and OE_P4.1 plants showed a further accumulation of scopoletin but to a lesser extent than what observed in wild type plants. Again, repetitions must be performed to verify the consistency of such behavior of the double and single PAD4 or EDS1 over expressing plants compared to wild type.

All these data taken together indicate that the double over expression of EDS1 and PAD4 leads to a faster activation of the SA pathway as compared to single over expressor lines or wild type.

3.10 OE_E1/P4.A plants exhibit increased tolerance to oxidative stress induced by paraquat treatment

Taken together, the above data show that co over expression of EDS1 and PAD4, even if sufficient to induce some constitutive activation of the SA pathway in unchallenged tissue, does not recapitulate the full extent of the plant response to pathogen attack. Instead, it appears to prime the plant allowing it to respond more quickly to the invading pathogen. This result implies that other signaling components or regulators downstream or independent of the EDS1 and PAD4 protein up regulation are involved in further signal relay leading to specific gene induction and phytoalexins accumulation. On the other hand the co over expression of EDS1 and PAD4 brings the plant to a sort of primed condition which renders faster responses observed also in wild type plants during compatible interaction. I then decided to investigate which possible mechanisms could be involved in such signal relay.

One of the very early cellular events after plant exposure to pathogens is an oxidative burst. This burst is monophasic during compatible interactions and biphasic during incompatible interactions [145, 146]. The defence regulators EDS1 and PAD4 have been previously implicated in the transduction of Reactive Oxygen Species (ROS) derived signals [108, 110]. For example, in *Arabidopsis lsd1* (*lesions simulating disease* 1) plants application of chemicals leading to superoxide production results in a form of spreading

necrosis termed Runaway Cell Death (RCD) that is completely suppressed in *lsd1/eds1* or *lsd1/pad4* double mutants [108, 147]. Furthermore, EDS1 is required in downstream signaling events following the generation of singlet oxygen in the photosensitized mutant *flu* [110]. I tested whether EDS1-PAD4 over expressors displayed altered responsiveness to oxidative stress. The most abundant ROS produced after pathogen challenge are anion super oxide (O^{2-}) and hydrogen peroxide (H_2O_2) [145, 146]. The herbicide methyl

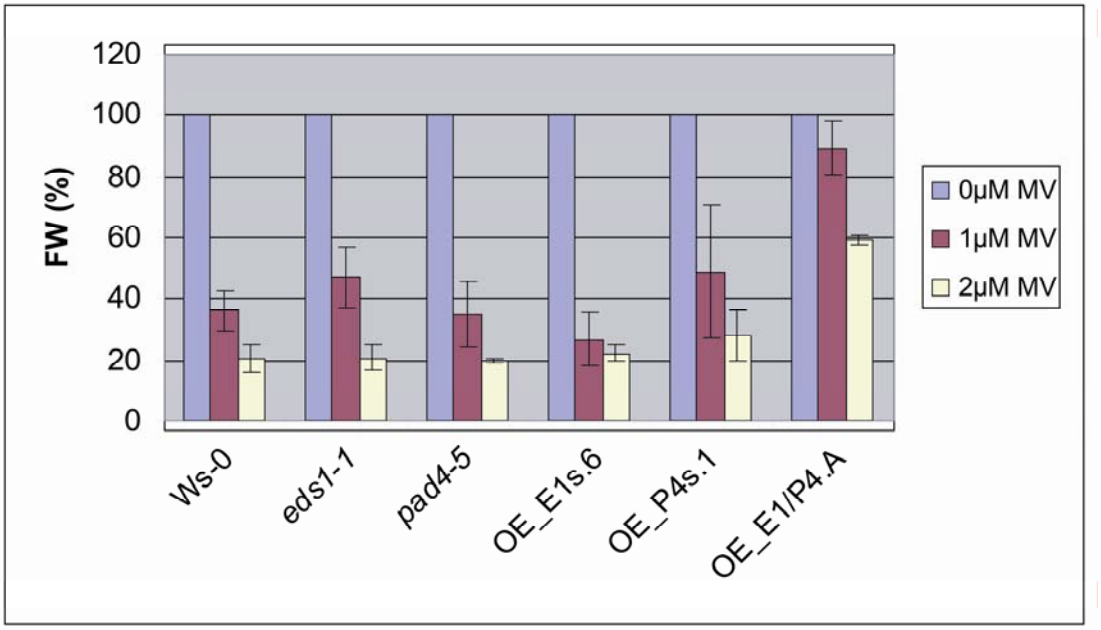


Figure 3.16 Growth response of different plant lines to Methyl Viologen (Paraquat)

Wild type, *eds1-1*, *pad4-5*, OE_E1s.6, OE_P4s.1 and OE_E1/P4.A seeds were surface sterilized and germinated on MS plates. After 7 days seedlings from each line were transferred in liquid MS medium containing 0 (violet bars), 1 (red bars) or 2 µM (yellow bars) Methyl Viologen. After three days the fresh weight from three samples each of three individuals was measured and the average weight of a single plant was estimated. For each line the values were then expressed as percentage of the average single plant fresh weight measured in the absence of MV. Error bars represent sample standard deviation. Three independent experiments gave similar results.

viologen (MV), also known with the commercial name paraquat, induces the production of superoxide and hydrogen peroxide in plants exposed to light, by oxidizing the photosystem I [113, 148]. I tested the sensitivity of OE_E1/P4.A plants to MV compared to OE_E1s.6, OE_P4.1, *eds1-1*, *pad4-5* and wild type (WS-0) plants. Seeds were germinated on solid MS plates and after one week transferred to MS liquid medium with

different concentrations of MV (0, 1 and 2 μM). After 3 days of growth under standard conditions (12 h/day light) the fresh weight of plants was measured. For each line, the growth reduction due to MV application was expressed as the percentage fresh weight measured in the absence of MV. The results are shown in **Figure 3.16**. In the presence of 1μM MV, growth reduction of wild type, OE_E1s.6, OE_P4s.1, *eds1-1* and *pad4-5* plants was ≈ 60%. However, in OE_E1/P4.A plants it was much less and maximally ≈ 5-10%. A further reduction of up to 20-30% for OE_E1/P4.A and 80% for all the other lines was measured in presence of 2μM MV, indicating dosage dependency in the MV induced growth retardation. Different hypotheses can be formulated to explain the observed increased tolerance of the OE_E1/P4.A plants to O^{2-}/H_2O_2 stress. First, I can not rule out that the increased apparent tolerance of EDS1/PAD4 over expressors to MV is due to reduced uptake of MV compared to other lines. Second, the OE_E1/P4.A line could be more tolerant due to heightened activation of the scavenging machinery involved in the detoxification of ROS produced upon MV treatment.
Further experiments are being performed to test these hypotheses.

3.11 An EDS1 pool is phosphorylated

I wished to ascertain how EDS1 and its partner change post-transcriptionally in response to pathogens or oxidative stress in order to trigger downstream changes. Another possibility that could account for the insufficiency of dual EDS1/PAD4 over expression to fully activate defence is that EDS1 and/or PAD4 are regulated post-translationally in response to pathogen stress. The constitutive SA pathway activation (see **Figure 3.8** page 62) might then reflect the inability of a post translational regulatory system to cope with large amounts of EDS1 and/or PAD4 protein accumulating in the OE_E1/P4.A plants. Possible mechanisms of regulation could be EDS1 and/or PAD4 post translational modification(s), EDS1 and/or PAD4 re localization or redistribution between cytoplasmic and nuclear compartment after pathogen challenge [98], or, as observed in many other examples, a combination of the two. An additional post translational regulatory mechanism could be directly related with an activity of EDS1 and PAD4 complexes.

A specific substrate might be released in significant amounts only after pathogen challenge. Basal substrate levels available in the unchallenged condition in combination with the large amounts of the two proteins would then determine the slight constitutive SA pathway activation in unchallenged 35SE1/P4.A plants. While other members of our lab are currently investigating localization dynamics (A. Garcia and J. Parker, unpublished) and developing assays to intrinsic EDS1 and PAD4 biochemical activities (S. Rietz and J. Parker, unpublished), I aimed to assess potential regulation through protein modifications of EDS1 and PAD4 proteins.

A common and well characterized reversible, regulatory modification is phosphorylation [149-151]. *In silico* analysis of the *Arabidopsis* L*er* EDS1 primary amino acid sequence performed with the NetPhos 2.0 software (http://www.cbs.dtu.dk/services/NetPhos [120]) showed the existence of 16 potential phosphorylation sites (score > 0.9). In accordance to what previously reported [152], I hypothesized a probable conservation of critical residues involved in the regulation of EDS1 signaling activity. An alignment between EDS1 amino acid sequences from different plant species was generated and is shown in **Figure 3.17**. Four conserved residues predicted to be potential phosphorylation sites were identified [152]. This prompted me to test whether EDS1 protein signaling activity might be regulated through phosphorylation. So far no EDS1 band shift was observed in one dimensional SDS-PAGE or Western Blot analysis utilizing total protein extracts from challenged or unchallenged plants (data not shown). However, this does not preclude phosphorylation, as reported for other verified phosphorylated proteins [153]. An alternative approach to assess the existence of regulatory phosphorylation sites in EDS1 was followed. In a first step, the strepII affinity purification efficiency [122] in the OE_E1s and OP_E1s lines was assessed. The results are shown in **Figure 3.18**. It was possible to purify coomassie stainable amounts of EDS1 protein, suitable for further

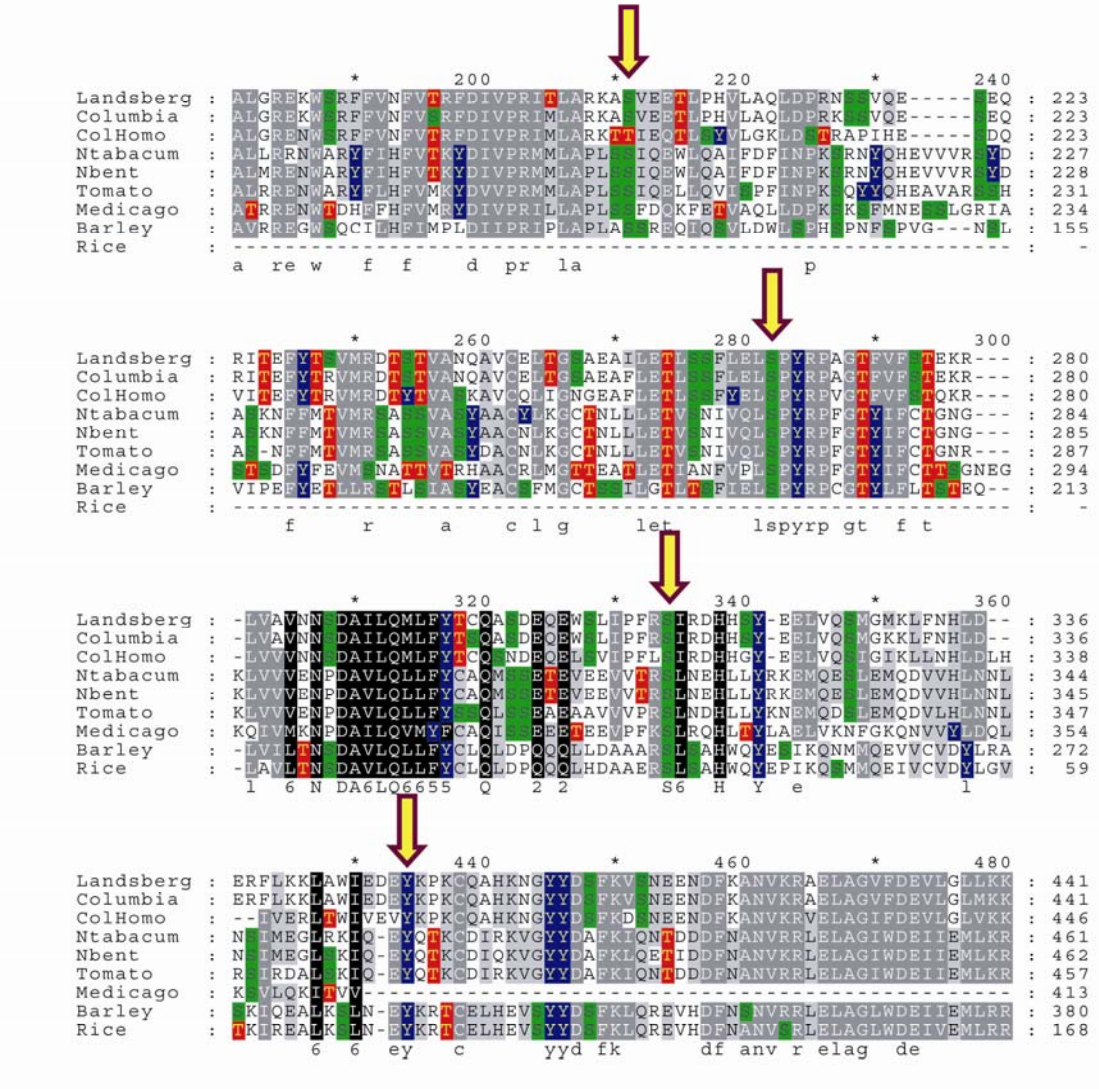


Figure 3.17 Conserved potential phosphorylated residues plant EDS1 amino acid sequence

EDS1 protein sequences from *Arabidopsis thaliana* ecotypes Landsberg and Columbia (containing two EDS1 copies), *Nicotiana tabacum*, *Nicotiana benthamiana*, *Lycopersicon esculentum*, *Hordeum vulgare* and *Oryza sativa* (shown as Landsberg, Columbia and ColHomo, Ntabacum, Nbent, Tomato, Medicago, Barley and Rice respectively) were aligned and conserved residues showing scores higher than 0,9 for predicted phosphorylation by NetPhos 2.0 are indicated with yellow arrows. Overall 16 residues in the Arabidopsis thaliana Landsberg amino acid EDS1 sequence were predicted to be phosphorylated with a score higher than 0.9.

analyses, from OE_E1s.6 and NPE1s.8 plants but not from wild type. The identity of the putative EDS1 band was confirmed by Western blot and LC-MS analyses (data not shown).

An *in vitro* approach was then followed to test whether EDS1 protein can be phosphorylated. Two equal aliquots of strepII purified EDS1 protein from unchallenged OE_E1s.15 plant tissues were incubated under the same conditions with or without a deregulated constitutively active form of cAMP (cyclic adenosine 3',5'-cyclicmonophosphate) dependent protein kinase (PKA) minus its regulatory subunit [154]. To test whether the assay conditions would allow PKA activity, histone protein was incubated with or without PKA, and alone or together with plant extracts from wild type plants as a positive control. For detection of changes in the EDS1 phosphorylation

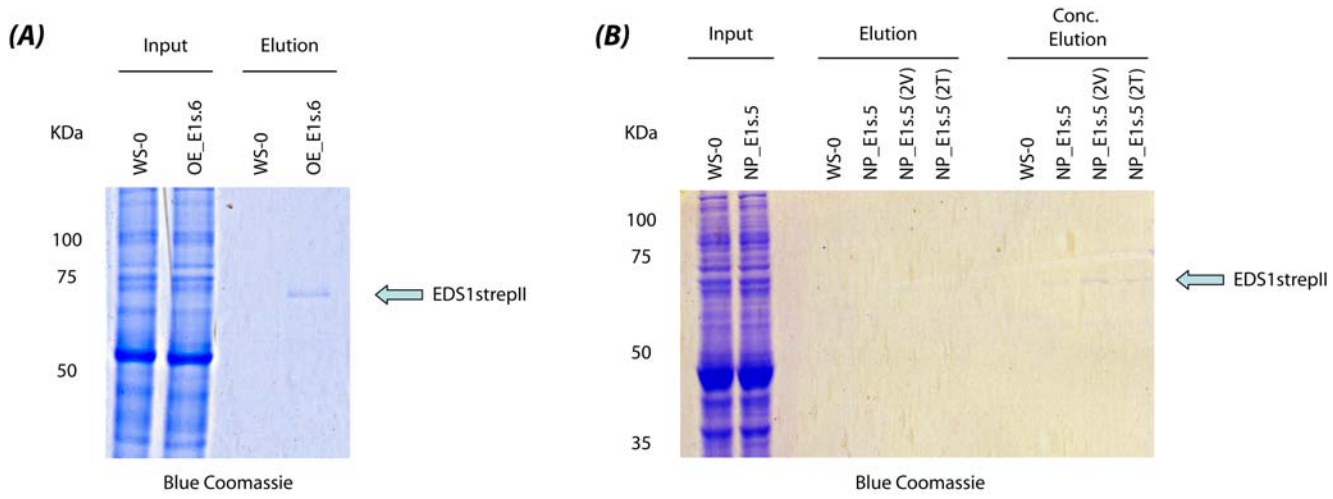


Figure 3.18 EDS1 strepII affinity purification from plant extracts

(A) A strepII affinity purification was performed from unchallenged wild type (WS-0) and OE_E1s.6 plants. Equal volumes of input fractions and eluted fractions were separated by SDS-PAGE and the gel coomassie blue stained. A purified EDS1 protein band of the expected size is indicated by the light blue arrow. **(B)** A strepII purification was performed from unchallenged wild type (WS-0) and OP_E1s.5 plants at different conditions to optimize the purification procedure: standard conditions (see Material and Methods) from wild type and OP_E1s.5 (WS-0 and OP_E1s.5), double tissue amounts of OP_E1s.5 (2V), double time of incubation with the resin from OP_E1s.5 (2T). Equal volumes of input fractions (Input), eluted fractions (Elution) and concentrated eluted fractions (Conc. Elution) were separated by SDS-PAGE and the gel stained with blue coomassie. Purified EDS1 protein bands of the expected size are indicated by the light blue arrow.

state the specific ProQ Diamond phosphostaining (Molecular Probes) followed by total protein assessment by SyproRuby staining was performed as previously described [155]. The results are shown in **Figure 3.19**. The observed differential phospho signal after PKA treatment of comparable amounts of histone and EDS1 protein indicates *in vitro* phosphorylation events. To test whether the phosphorylation happens also *in vivo* and whether it has a role in EDS1 activation, I compared phospho signal from strepII - purified EDS1 derived from unchallenged and pathogen challenged plant tissues. The bacterial strain DC3000 expressing the avirulence gene *AvrRps4* is recognized

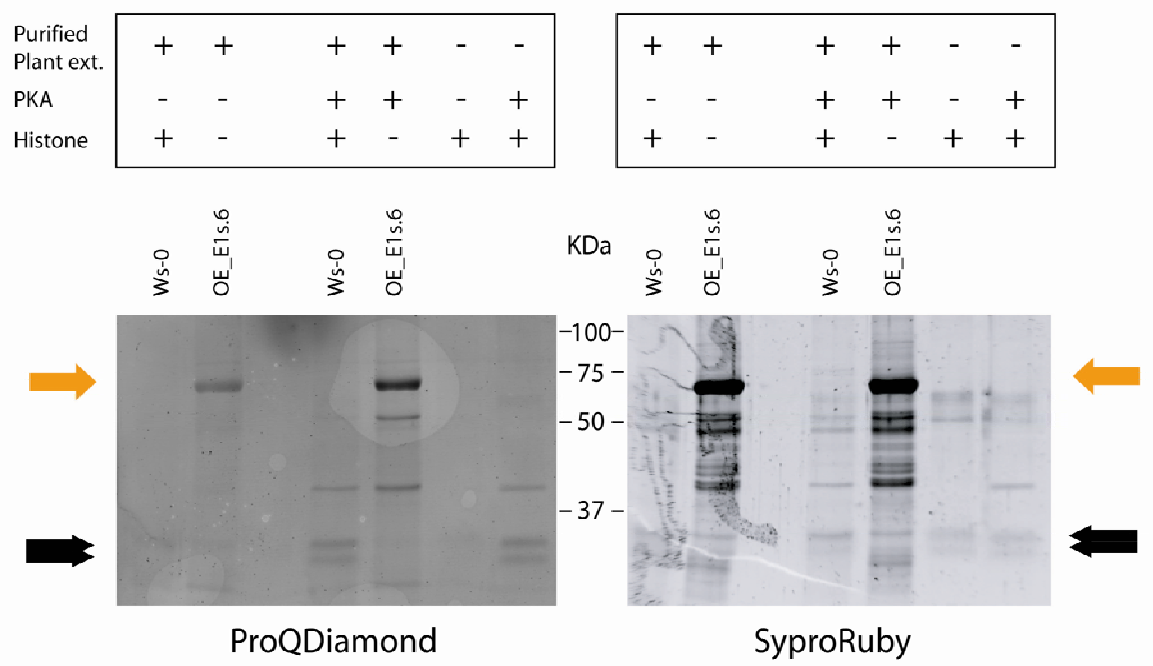


Figure 3.19 *In vitro* EDS1 phosphorylation assay

A strepII affinity purification was performed from unchallenged wild type and OE_E1s.6 plant tissues. The purified fractions were concentrated and divided into two aliquots. To both the wild type purified aliquots 50 ng histone was added as an internal control and they were incubated either in the absence or presence of PKA at 30°C 1 h. Of the two aliquots from OE_E1s.6 one was incubated in the absence of PKA and one in the presence of PKA at 30°C 1 h. Histone alone was also incubated in the absence or presence of PKA at 30°C 1 h as a control. After incubation, all samples were separated by SDS-PAGE and the gel was stained by ProQDiamond phosphostaining (left panel) and subsequently by Sypro Ruby staining to assess total protein amounts (right panel). EDS1 protein bands are indicated by the yellow arrows; Histone bands are indicated by black arrows.

in the *Arabidopsis* ecotype WS-0 by the cognate TIR-NBS-LRR receptor RPS4 leading to EDS1 dependent defence response [40, 92]. I reasoned that in plants challenged by DC3000 *AvrRps4* EDS1 protein should be in its signaling active form. Wild type and OE_E1s.6 plants were inoculated with pathogen by vacuum infiltration of leaves with a bacterial suspension of DC3000 *AvrRps4* (10^7cfu/ml). Leaf tissues were collected at 0 h, 2h and 4 h after bacterial infiltration. The collected tissues were subjected to strepII affinity purification in presence of phosphatase inhibitors in order to maintain potential phosphorylated sites throughout the purification procedure. The purified fractions were concentrated, separated by SDS-PAGE and the gel stained with ProQ Diamond phosphostaining and subsequently with Sypro Ruby. To distinguish a specific phospho signal from the background protein signal [155], phosphatase λ treatments were included. The results are shown in **Figure 3.20A**. The existence of an EDS1 phosphorylated pool was indicated by the observed differential phosphosignal from phoshpatase λ treated and untreated protein. No obvious change in the intensity of this differential signal was observed at different time points after bacterial inoculation, suggesting an unlikely involvement of phosphorylation events in the early activation of EDS1 signaling activity. In order to assess whether phosphorylation events could account for an activation of EDS1 at later stages such as during establishment of SAR the same experiment was performed and later time points (16 h and 24 h after infiltration) analyzed. This produced similar results (**Figure 3.20B**).

Taken together these data suggest that the observed phosphorylation is not correlated with EDS1 signaling activation. An alternative interpretation is that as EDS1 was purified from an over expressor line with much higher EDS1 protein levels than wild type, the phosphorylated pool may represent the actually signaling active EDS1 while the not phosphorylated form would represent an inactive pool that is in excess. In this scenario one would then predict that strepII-purified EDS1 from OP_E1s lines should show enrichment in the phosphorylation signal for the total amount of protein, compared to the OE_E1s lines. To assess this possibility, a strepII purification in the presence of

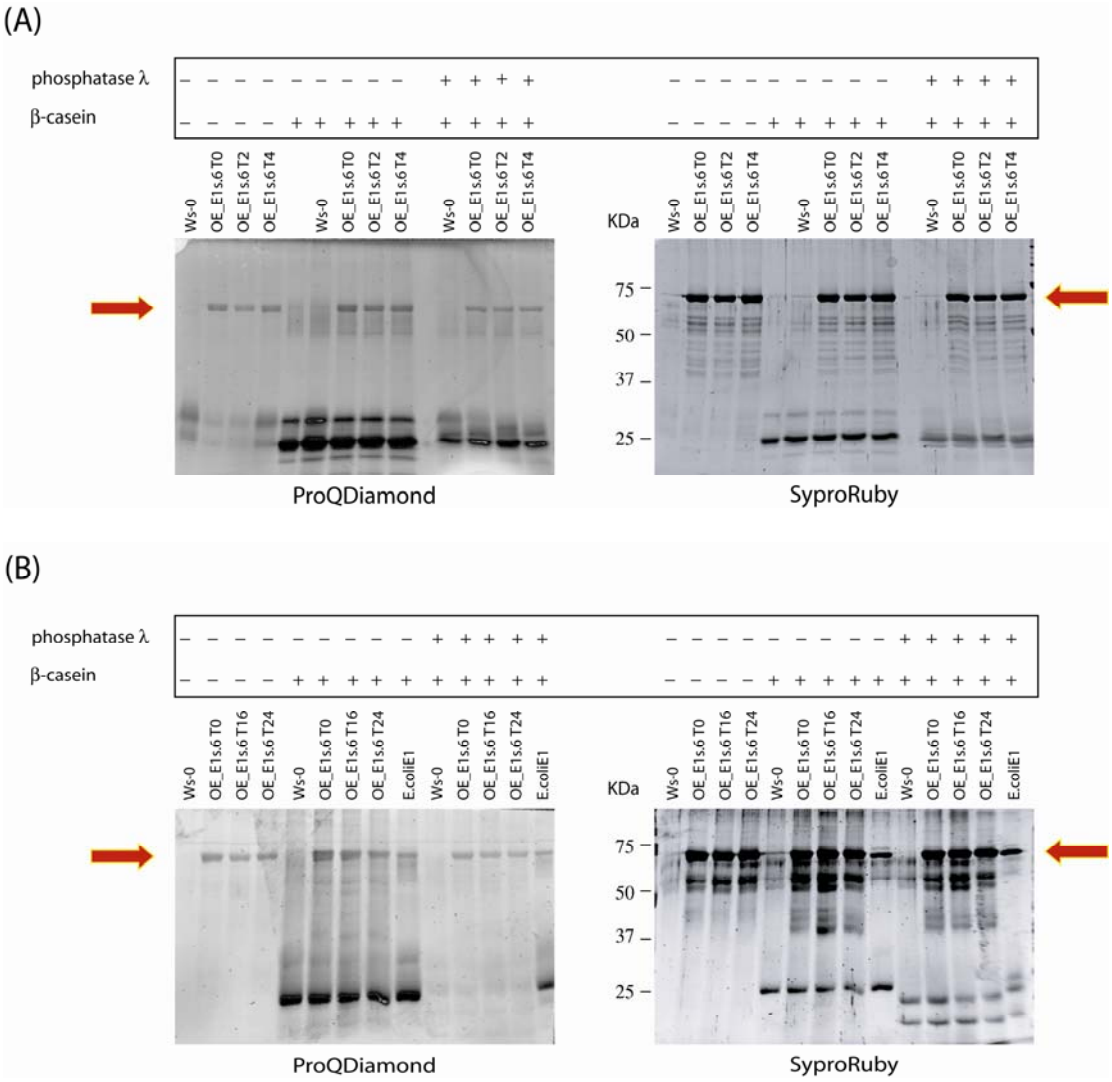


Figure 3.20 *In vivo* phosphorylation analyses of EDS1protein in line OE_E1s.6

(A) StrepII affinity purifications in presence of phosphatase inhibitors was performed from unchallenged wild type (WS-0) and OE_E1s.6 (OE_E1s.6 T0) plant tissue and from challenged OE_E1s.6 plant tissues collected 2h (OE_E1s.6 T2) and 4h (OE_E1s.6 T4) after vacuum infiltration with DC3000 *AvrRps4* (10^7 cfu/ml). The purified fractions were concentrated and aliquoted. β casein (100 ng) and λ phosphatase were added as indicated . All aliquots were incubated at 37°C for 1 hr and then loaded onto an SDS-PAGE. The gel was stained by ProQDiamond phospho staining (Left) and subsequently by SyproRuby staining to assess total protein amounts (Right). **(B)** The same experiment was performed from unchallenged wild type (WS-0) and OE_E1s.6 (OE_E1s.6 T0) plant tissues and from challenged OE_E1s.6 plant tissues collected 16h (OE_E1s.6 T16) and 24h (OE_E1s.6 T24) after DC3000 *AvrRps4* infection. As an additional control *E. coli* expressed purified recombinant EDS1 was also included

EDS1 protein bands are indicated for all gels by the red arrows.

phosphatase inhibitors was performed from OP_E1s.5 and wild type (WS-0) unchallenged and pathogen challenged plant tissues. In this purification phosphatase λ treatments were also included. The result of this experiment is shown in **Figure 3.21**. No significative enrichment in the phosphosignal was observed for the OP-E1s lines compared to OE_E1s. In the case of the OP_E1s.5 there was no increase in the phosphorylated pool compared between unchallenged and challenged plants 2h and 4 h after DC3000 *AvrRps4* vacuum infiltration (data not shown).

Thus, I concluded that the identified phosphorylation is not associated with an active form of EDS1 and unlikely it is involved in EDS1 signalling activation.

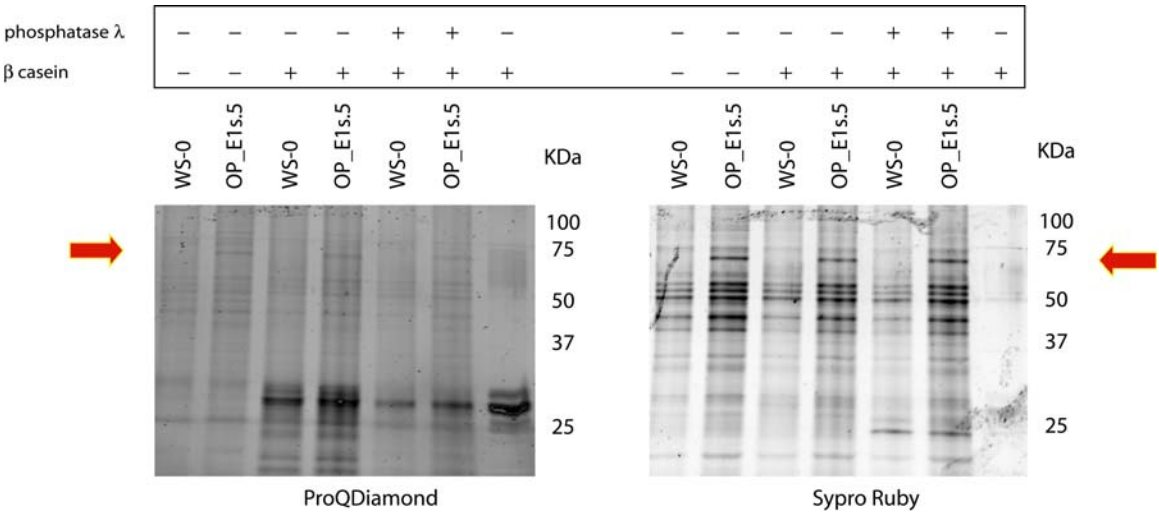


Figure 3.21 OP_E1s in vivo phosphorylation analyses

A StrepII affinity purification was performed from wild type and OP_E1s.5 unchallenged plant tissues in the presence of phosphatase inhibitors. The purified fractions were concentrated and β-casein (80 ng) and λ phosphatase added as indicated. All aliquots were then incubated at 37°C for 1 h and separated by SDS-PAGE. The gel was stained by ProQDiamond (Left) and subsequently by SyproRuby to assess total protein amounts (Right).

EDS1 protein bands are indicated by red arrows.

3.12 EDS1 is N-acetylated

Another broader approach was followed to identify other potential modifications that may be involved in EDS1 activation. StrepII affinity purification was performed from tissues

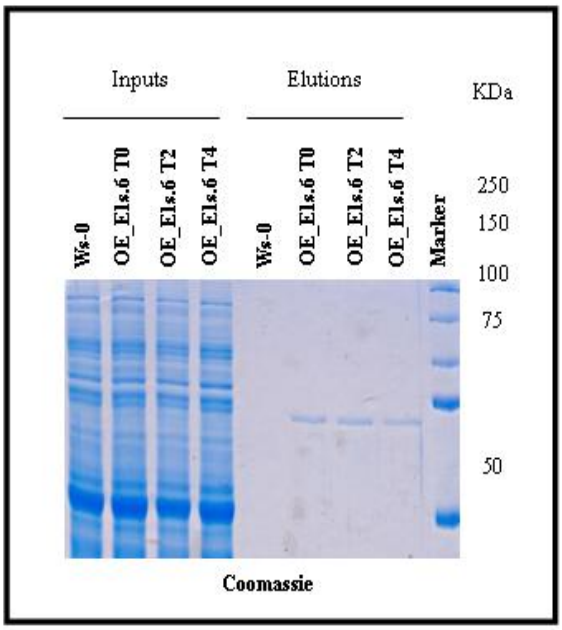
of unchallenged wild type plants, unchallenged OE_E1s.6 plants and challenged OE_E1s.6 plants 2 h and 4 h after vacuum infiltration with *Pst* DC3000 *AvrRps4*. Equal amounts of purified EDS1 from each sample were assessed by coomassie blue staining of SDS-PAGE gels (**Figure 3.22A**). Equal volumes of each purification were digested with trypsin and analyzed by LC/MS without fragmentation in order to identify differential peaks corresponding to peptides with altered flight capabilities related to differential presence of protein modifications. The results are shown in **Figure 3.22B**. No differential peak was identified between the different MS spectra corresponding to different time points. Differences were instead observed with spectra from *E. coli* expressed purified recombinant EDS1 and displayed the presence of an N-acetylation in all the EDS1 samples purified from plant tissues (**Figure 3.22C**). As the N - acetylation was not differential in samples before and after pathogen challenge, involvement of such a modification in the activation of EDS1 at early time points is unlikely. Two kinds of N - acetylation are known: an irreversible form which is estimated to occur on 80 - 90% of the eukaryotic proteins and which would be an unlikely candidate as a regulatory modification [156], and a second reversible one that was shown to be involved in the regulation of histone and transcriptional factors [156, 157]. The fact that no peak corresponding to an unmodified N terminus of EDS1 was observed in the analyzed spectra points to an irreversible modification rather than equilibrium between two different forms of EDS1.

3. 13 EDS1 protein associations *in vivo*

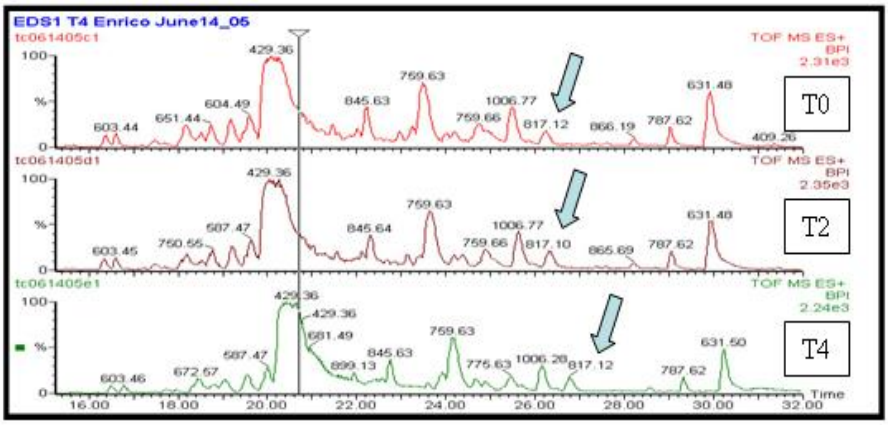
A further possible mechanism through which the EDS1 complex might be regulated post translationally is through physical interaction with so far unidentified protein partner(s) that could specifically associate with the EDS1 complex and modulate its activity upon perceiving a pathogen signal.

I first verified the strepII system as a means to identify protein associations by testing whether PAD4, a known interactor of EDS1, could be affinity copurified together with EDS1 by strepII purification from OP_E1s plant tissues.

(A)



(B)



(C)

Monoisotopic mass of neutral peptide Mr(calc): 1631.85
Variable modifications:
N-term N-Acety (Protein)
Ions Score: 82 Expect: 3.1e-06
Matches (Bold Red): 33/146 fragment ions using 60 most intense peaks

#	b	b^{++}	b*	b^{*++}	b^{0}	b^{0++}	Seq.	y	y^{++}	y*
1	114.05	57.53					A			
2	**261.12**	131.07					F	1519.81	760.41	1502.78
3	**390.17**	195.59			372.16	186.58	E	**1372.74**	686.88	**1355.72**
4	**461.20**	231.11			443.19	222.10	A	**1243.70**	622.35	**1226.67**
5	**574.29**	287.65			556.28	278.64	L	**1172.66**	586.84	**1155.64**
6	**675.33**	338.17			**657.32**	329.17	T	**1059.58**	530.29	**1042.55**
7	732.36	366.68			**714.35**	357.68	G	**958.53**	479.77	**941.51**
8	**845.44**	423.22			827.43	414.22	I	**901.51**	451.26	884.48
9	**959.48**	480.25	942.46	471.73	**941.47**	471.24	N	**788.43**	394.72	771.40
10	1016.50	508.76	999.48	500.24	998.49	499.75	G	**674.38**	337.70	**657.36**
11	1131.53	566.27	1114.51	557.76	1113.52	557.26	D	**617.36**	309.18	600.34
12	**1244.62**	622.81	1227.59	614.30	**1226.61**	613.81	L	**502.33**	251.67	485.31
13	1357.70	679.35	1340.67	670.84	1339.69	670.35	I	**389.25**	195.13	372.22
14	1458.75	729.88	1441.72	721.36	1440.74	720.87	T	**276.17**	138.59	259.14
15							R	175.12	88.06	158.09

(Previous page) **Figure 3.22** EDS1 N – acetylation identification

(A) A StrepII purification from unchallenged wild type plants (WS-0), unchallenged OE_E1s.15 plants (OE_E1s T0) and challenged OE_E1s.6 plants 2h (OE_E1s T2) and 4 h (OE_E1s T4) after infiltration with DC3000 *AvrRps4* (10^7 cfu/ml) was performed. Input fractions and correspondent purified fractions were loaded onto an SDS-PAGE and the gel was blue coomassie stained. **(B)** The strepII purified EDS1 fractions described in (A) were digested with trypsin and analyzed by LC/MS without fragmentation. In figure are shown the resulting spectra: from unchallenged OE_E1s.6 plants (T0), from challenged OE_E1s.6 plants 2 h and 4 h after Pst DC3000 *AvrRps4* infiltration (T2 and T4 respectively). Peaks corresponding to the acetylated EDS1 N-terminus are shown by grey arrows. **(C)** Ion series produced from the N terminal peptide of EDS1 after fragmentation. In the table are reported the expected ion masses in the presence of an N – acetylation. The masses actually observed after the fragmentation of the N terminus of EDS1 are shown in red.

Since EDS1-PAD4 association exists in healthy plants [93], unchallenged OP_E1s.5 and wild type tissues (WS-0) as negative control were used. Results are shown in **Figure 3.23**. I found that it was possible co-purify PAD4 with EDS1-strepII, as indicated by a specific band on a Western blot identified with polyclonal anti-PAD4 antibodies. No PAD4 band was observed in the fraction purified from wild type tissues ruling out non-specific interaction between PAD4 and the affinity matrix in the absence of EDS1-strepII. Experiments using either pathogen-challenged or unchallenged plant material didn´t identify further EDS1 interactors. Similarly PAD4 strepII affinity purification from OE_P4s lines led only to the co purification of endogenous EDS1 and not SAG101 consistent with a previous study [98]. Other tags, for example the TAP (tandem affinity purification) attached to EDS1 or PAD4 also failed to identify new component besides the known interactors (J. Bautor, B. Feys an J.Parker, unpublished; [158]).

I therefore concluded that either no further protein-protein interactions are involved in EDS1-PAD4 signaling regulation or transient interactions taking place in the living cells are too short timed or weak to be captured by the affinity purification systems used so far.

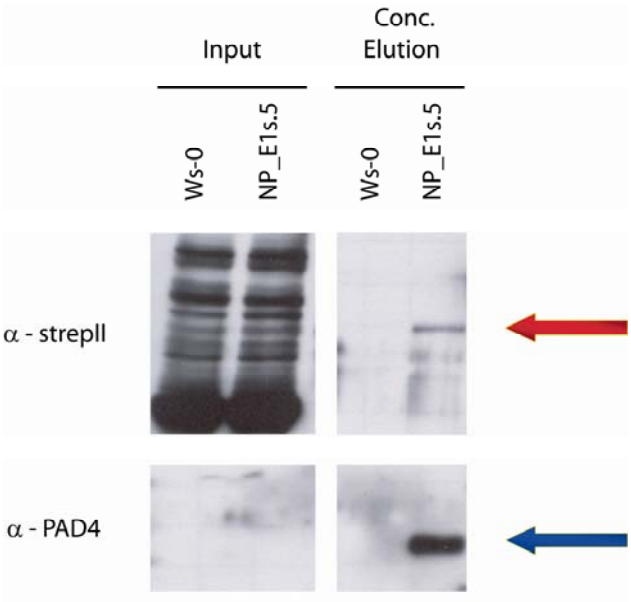


Figure 3.23 Co purification of PAD4 by EDS1 strepII affinity purification

A strepII purification from unchallenged NPE1s.8 and wild type (WS-0) plant tissues was performed. Equal volumes of input fractions and concentrated eluted fractions respectively were analyzed in western blot analyses using monoclonal anti-strepII (upper panels) and polyclonal anti-PAD4 antibodies (lower panels). In the input fractions aspecific bands cross reacting with the strepII antibody indicate equal starting protein amounts. EDS1 and PAD4 protein bands are indicated by the red and blue arrow respectively.

3.14 Strategies to constitutively or conditionally activate the EDS1 pathway

I reasoned that protein modifications or interactions may occur at time points different to those analyzed after pathogen challenge. It is also likely that only a subset of cells under direct exposure to the pathogen were responding in the above experiments. This would dilute any specific change by background "noise". In order to test these possibilities I followed a genetic approach to constitutively or conditionally activate the EDS1 pathway in lines expressing EDS1-strepII protein.

3.14.1 Genetic constitutive activation of the EDS1 pathway

The *Arabidopsis snc1* mutant carries a recessive point mutation in a TIR-NB-LRR *R* gene that leads to constitutive defence activation which is EDS1- and PAD4-dependent [104, 105]. Reasoning that within this background EDS1 is constantly signaling I crossed the selected transgenic OE_E1s.4 and OP_E1s.5 lines with *snc1/eds1-2* mutant plants (hereafter OE_E1s/*eds1*/*snc1* and OP_E1s/*eds1*/*snc1*, respectively). Pollen from OE_E1s.4 and OP_E1s.5 plants, both Basta® resistant, was used to pollinate emasculated *snc1/eds1-2* plants. The resulting F_1 seeds were grown on soil and their identity verified by Basta® spraying. F_2 seeds were collected from surviving plants and grown on soil. Homozygous plants for the *snc1* mutation carrying at least one copy of the OE_E1s or OP_E1s construct were identified because of their typically reduced size [104, 105]. Seeds were collected from these plants and the segregation of the dwarf phenotype was checked in the next generation. F_3 plants from both crosses in comparison to their correspondent parental lines and *snc1* mutant plants are shown in **Figure 3.24**. Even if still segregating, the OE_E1s/*eds1*/*snc1*, and OP_E1s/*eds1*/*snc1* plants had a severely reduced size compared to the parental lines *snc1/eds1-2*, OE_E1s.4 or OP_E1s.5. OE_E1s/*eds1*/*snc1* and OP_E1s/*eds1*/*snc1* had bigger size and strong attenuation of leaf curliness in comparison to *snc1* mutant plants. Occasionally, the generated crosses displayed an additional phenotype, the yellowing of the younger rosette leaves. These differences may be due to the combination of *Arabidopsis* backgrounds created in generating these crosses: the paternal lines are in WS-0 while the maternal line is a cross between *snc1* plants (ecotype Col-0) and *eds1-2* plants (L-*er*). In the OE_E1s/*eds1*/*snc1* and OP_E1s/*eds1/snc1* plants this was particularly evident from the shape of the leaves that were similar to L-*er*. Nonetheless, an obvious requirement for both *snc1* mutation homozygosity and at least one copy of the constructs expressing EDS1 (both confirmed by PCR analyses with specific primers; data not shown) was necessary to observe dwarf phenotype. Thus constitutive activation of the EDS1 pathway was evident in these crosses. Currently OE_E1s/*eds1*/*snc1* and OP_E1s/*eds1*/*snc1* are under selection and propagation to get suitable tissues amounts for biochemical analyses.

Figure 3.24 Genetic constitutive activation of the EDS1 pathway

F3 seeds from the OE_E1s/*eds1*/*snc1*a nd OP_E1s/*eds1*/*snc1*lines (see text for details) were sown on soil together with the parental lines (*snc1/eds1-2*, OE_E1s.4 and OP_E1s.5 respectively) and *snc1* plants for phenotypel comparisons. Four-week-old plants are displayed.

3.14.2 Genetic conditional activation of the EDS1 pathway

AtMPK4 encodes a negative regulator of SAR and *mpk4* plants have a constitutive SAR response which is EDS1- and PAD4- dependent [82]. Such negative regulation is dependent on the MPK4 kinase activity since stable inactive MPK4 variants were unable to complement the *mpk4* defect [82]. As in the case of *snc1* I reasoned that EDS1 would be constitutively activated in the *mpk4* background. Since *mpk4* plants are like the *snc1* plants dwarf and since the MPK4 activity is essential for its role in negative regulating SAR a specific approach was developed to conditionally inactivate MPK4 in the plant. In a recent publication J. Mundy and colleagues generated *mpk4* mutant plants expressing

a conditional loss-of-function HA tagged MPK4 mutated allele (hereafter MPK4^{Y124G}HA) [90]. This allele carries a mutation in its ATP-binding-pocket which leads to a sensitization to the bulky C3-1´-naphtyl (NaPP1) Src tyrosine kinase inhibitor [90, 159]. The specificity of the inhibition was demonstrated by comparisons to *mpk4* mutants expressing HA tagged wild type MPK4 (hereafter MPK4HA) [90]. I then decided to use both these lines, MPK4^{Y124G}HA and MPK4HA, for crosses with the OP_E1s.5 and OE_E1s.4 lines described above. These crosses offer a tool to conditionally trigger the EDS1 pathway in a more homogeneous and synchronized way while allowing first the growth of normal plants, important to avoid side effects deriving from development perturbations. In this case pollen from the MPK4HA and MPK4^{Y124G}HA plants was used to pollinate emasculated flowers from both OP_E1s.5 and OE_E1s.4 plants. Both the MPK4HA and MPK4^{Y124G}HA lines were generated in the *mpk4* background which carries the Kanamycin (Kan) resistance gene *nptII* in the Ds element used to disrupt the endogenous *MPK4* gene [82]. The constructs for the over expression of both MPK4 versions and both the OE_E1s and OP_E1s constructs carry the same PPT resistance. The identity of F_1 individuals was therefore checked by growing F_1 seedlings in Kan/PPT containing MS plates. Resistant plants were transferred to soil and F_2 seeds collected. Aliquots of F_2 seeds were sown on MS plates containing either PPT or Kan to check independently the segregation of the *mpk4* mutation and the segregation of two constructs carrying the PPT resistance. Individuals from lines showing no

Table 3.3 Current situation of the MPK4HA and MPK4^{Y124G}HA crosses

Line	*mpk4*	*eds1-1*	MPK4HA Or MPK4^{Y124G}HA	NPE1s or OE_E1s
NPE1s.8/MPK4HA	Hom	Hom	Het	Hom
NPE1s.8/MPK4^{Y124G}HA	Hom	Hom	Het	Hom
OE_E1s.8/MPK4HA	Hom	Hom	Hom	Het
OE_E1s.8/MPK4^{Y124G}HA	Hom	Hom	Het	Hom

Hom, homozygous; Het, heterozygous

segregation for both the *nptII* and the *bar* marker genes were transferred on soil and genotyped. Dominant PCR based markers for the detection of the MPK4HA, MPK4^{Y124G}HA, OE_E1s and OP_E1s constructs were developed (See Materials and Methods). Plants containing at least one copy of the MPK4 and EDS1 construct were genotyped for the *eds1-1* mutation. F_3 seeds were collected from the selected plants and the status of the MPK4HA, MPK4^{Y124G}HA, OE_E1s and OP_E1s constructs was determined by genotyping 10 – 18 F_3 individuals. The current situation is shown in **Table 3.3**. During the selection procedure it was possible to identify plants homozygous for both the *mpk4* and *eds1-1* mutation, carrying at least one copy of the corresponding construct for the expression of the strepII EDS1 fusion protein and no copy of the correspondent MPK4HA or MPK4^{Y124G}HA construct. An example of such situation is shown in **Figure 3.25**. These plants showed full complementation of the *mpk4* dwarf phenotype confirming once more the full functionality of the EDS1 strepII fusion proteins. Also for these lines the selection is under completion. Once propagated to get suitable tissue amounts for biochemical studies, these lines should provide a useful tool to check kinetics of the EDS1 complex activation upon conditional inhibition of the MPK4 activity.

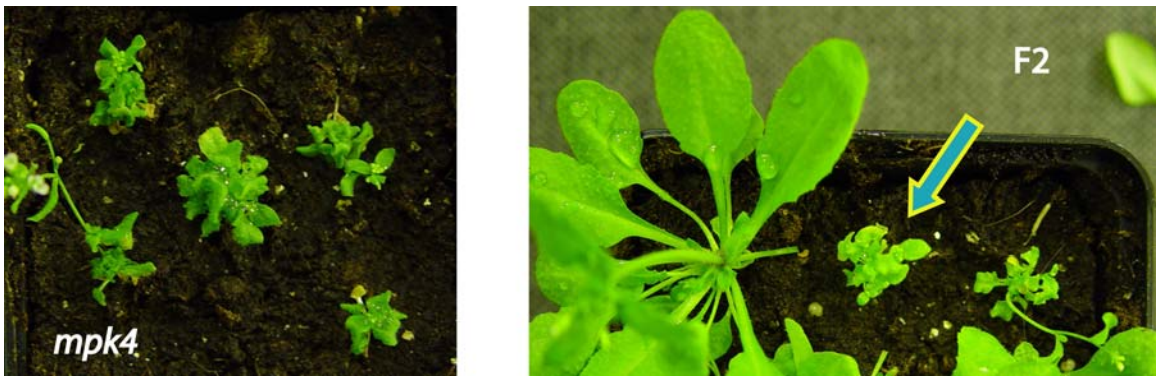


Figure 3.25 Observed phenotypical segregation in the MPK4HA and MPK4^{Y124G}HA crosses

OP_E1s.5/MPK4HA F2 plants were grown on soil alongside *mpk4* plants for phenotypical comparisons. The blue arrow indicate a plant homozygous for both *mpk4* and *eds1-1* mutation, containing at least one copy of the NPE1s transgene and no copy of the MPK4HA construct. Similar phenotypes were observed in OP_E1s.5/MPK4^{Y124G}HA, OE_E1s.4/MPK4HA and OE_E1s.4/MPK4^{Y124G}HA F2 segregating populations.

4. DISCUSSION

During the last years a key function of EDS1 and PAD4 in plant immunity has been demonstrated [125]. Inducible defences against biotrophic and hemibiotrophic potential pathogens have been shown to require EDS1 and PAD4 signaling activity: ETI mediated by TIR-NBS-LRR as well as basal defense against virulent pathogens, post-invasive non-host resistance and SAR are all compromised in *eds1* and *pad4* mutant plants [70, 125]. Accurate placement of EDS1 and PAD4 signaling functions within the series of events following pathogen challenge is complicated by their involvement in a positive feedback loop with SA and ROS, so that their immediate signaling activity is very difficult to be discriminated from actions in signal potentiation [125].

The biochemical activity(ies) of EDS1 and PAD4 is still not known. However, a potential lipase activity, hypothesized on the basis of conserved motifs in both EDS1 and PAD4 amino acid sequence, has been ruled out (S. Rietz and J. Parker, unpublished). Accordingly attempts to obtain structural information by crystallographic analyses together with assays to measure potential alternative activities, hypothesized on the basis of the biological context in which EDS1 and PAD4 operate, are being performed (S. Rietz and J. Parker, unpublished).

A better understanding of how EDS1 and PAD4 proteins are regulated in relation to their activation of downstream defense responses could provide an important insight to their biological role in the plant. The localization of EDS1 and PAD4 in nuclear and

cytoplasmatic compartments [98, 116] needs to be considered in formulating a EDS1/PAD4 activity. It also suggests that signaling through relocalization may be central to EDS1/PAD4 functions. Also, protein interaction studies, together with targeted gene expression analyses, led to the identification of three furher components, SAG101, FMO1 and NUDT7, of the EDS1 and PAD4 regulatory node [98, 102].

EDS1 and *PAD4* appear to be regulated at two different levels: transcriptionally and post transcriptionally. *EDS1* and *PAD4* both transcripts and proteins are up regulated after pathogen challenge or BTH treatment [93]. In several *Arabidopsis* mutant backgrounds that have EDS1 and PAD4 dependent constitutive defense activation, such as *mpk4* and *snc1*, up regulation of *EDS1* and *PAD4* was observed ([90] A. Garcia and J. Parker, unpublished). These data suggest a potential link between *EDS1* and *PAD4* transcriptional control and their signaling activation. However, in these deregulated mutant plants high levels of SA and pleiotropic effects due to the mutations have been described making it very difficult to specifically pin down the relative importance of *EDS1* or *PAD4* up regulation in defence activation [82, 104]. Furthermore, EDS1 and PAD4 proteins are already present in unchallenged tissues [93], and after pathogen challenge gene expression changes dependent on EDS1 and PAD4 take place at early time points [102] before any reported protein up regulation [93, 102]. This implies the activation of pre-existing EDS1 and PAD4 protein complexes and the existence of post translational regulatory mechanisms.

In this study I investigated the relative importance of transcriptional regulation and post transcriptional processes in EDS1 and PAD4 protein signaling. *Arabidopsis* lines over expressing either EDS1 or PAD4 or both were characterized. Growth retardation and enhanced basal resistance was observed only for the dual EDS1-PAD4 over expressors. From these data I conclude that EDS1 and PAD4 do not function separately but within a unique signaling unit, consistent with previous genetic and protein interactions data [37, 98, 125].

The dual EDS1-PAD4 over expression led in unchallenged plants to de-regulation and in pathogen challenged plants to the faster activation of the SA pathway. It was however not sufficient to fully recapitulate EDS1/PAD4 dependent defence activation. This proves the

existence of not yet identified post transcriptional mechanisms contributing to the regulation of EDS1 and PAD4 signaling functions.

Finally, increased tolerance to chemically induced oxidative stress observed in the OE_E1/P4 lines strengthens a connection between the transduction of ROS generated signals and EDS1 and PAD4 protein functions. Consistently, new potential functions of EDS1 and PAD4 and mechanisms of activation of these regulators can now be hypothesized.

4.1 EDS1 and PAD4 single over expressor lines do not exhibit obvious defense phenotypes

To test the importance of *EDS1* and *PAD4* up regulation in relation to their signaling activity, I generated *Arabidopsis thaliana* lines over expressing either EDS1 or PAD4 strepII fusion proteins. The strepII tag was selected because of its very small size (7 amino acid), unlikely to interfere with protein function, and because its addition could allow purification of EDS1 or PAD4 from plant tissues after a series of treatments [122]. Also, transcriptional and protein up regulation of SAG101 had been observed upon pathogen challenge [116]. Since SAG101 activity is redundant with PAD4, I restricted my analysis to EDS1 and PAD4 over expression.

Multiple independent transgenic lines over expressing fully functional EDS1 or PAD4 strepII fusion proteins at much higher levels than either unchallenged or pathogen challenged wild type plants were selected (Figure 3.2, 3.3 and 3.4).

Over expression of other plant defense signaling components has been previously reported to result in increased resistance against virulent pathogens. In *Arabidopsis* over expression of *NPR1* or its interacting partner *TGA5*, both involved in SAR regulation, leads to increased resistance to virulent downy mildew isolates [160, 161]. Increased resistance to *Pst* DC3000 (hereafter DC3000) was observed in *Arabidopsis* transgenic plants over expressing either *NPR1* or *NDR1* (*Non race specific Disease Resistance 1*), encoding a protein required by most CC-NBS-LRR receptors [160, 162]. Also, over expression in *Arabidopsis* plants of *FMO1*, a positive component of EDS1/PAD4 resistance, led to increased resistance to virulent races of *P. syringae* and *H. parasitica*

[121]. In contrast to these examples, no increased resistance to a virulent isolate of *H. parasitica* was observed in EDS1 or PAD4 over expressing transgenic plants (Figure 3.5), indicating that the increasing of EDS1 or PAD4 proteins alone is not sufficient to enhance plant basal defence.

4.2 EDS1/PAD4 dual over expressor lines have retarded growth

Available genetic data point towards an intimate interaction between EDS1 and PAD4 signaling activities [125]. The only example of a function for PAD4 that is independent of EDS1, emerged from analyses of the interaction between *Arabidopsis thaliana* and green peach aphids ([163], V. Pedagaraju et al., unpublished). Also, physical association between EDS1 and PAD4 together with their mutual stabilization suggests that EDS1 and PAD4 operate as a signaling unit [93, 98]. This is also consistent with the finding that both proteins are up regulated in wild type plants upon pathogen challenge or BTH treatment [93, 99, 140]. I hypothesized that over expression of EDS1 or PAD4 alone does not lead to defense activation due to limited availability of the corresponding protein partner. To test this hypothesis crosses between single EDS1 and PAD4 over expressor lines were made and *Arabidopsis* transgenics over expressing simultaneously EDS1 and PAD4 selected.

Dual over expression of EDS1 and PAD4 resulted in growth attenuation compared to the single over expressors or wild type plants (Figure 3.6). Compromised growth has been described for a number of mutants showing constitutive activation of defense responses. The *snc1*, *mpk4* and *cpr1* (*constitutive expression of PR 1*) *Arabidopsis* mutants all display dwarfism associated with high SA content, constitutive activation of defense genes such as *PR1* and *PR2* and increased resistance [90, 104, 105, 164]. Similarly, reduced growth and defense activation were recently been described for plants carrying a mutation in the *NUDT7* gene, whose expression is dependent on *EDS1* and *PAD4* [102]. Complete suppression of the growth an defence phenotypes was observed in *eds1*/*nudt7* plants [102] and preliminary results show increased EDS1 protein accumulation in the *nudt7* background, (M. Straus and J. Parker, unpublished). In general, stunted growth in constitutive defense mutants has been interpreted as the consequence of the metabolic

cost of defense activation [139]. This cost has most likely determined the evolution of complex regulatory networks to limit activation of otherwise detrimental defense pathways and to tailor the response to the attacking pathogen in order to avoid the deployment of ineffective defence.

To understand whether the growth retardation due to EDS1/PAD4 dual over expression might be a consequence of defense pathway activation, marker gene expression analyses by semi-quantitative RT-PCR were performed. A specific activation of the SA pathway (monitored as deregulated expression of the SA marker gene *PR1*) and not of the JA pathway was observed (Figure 3.8). Furthermore, an increased accumulation of both free and total SA in OE_E1/P4 lines compared to the single over expressors and wild type was observed, in accordance with the gene expression data (Figure 3.9).

Plant growth is a complex and highly regulated process in which cell division and cell elongation events are essential factors [128]. In *mpk4* mutants dwarfism was shown to be associated with reduced cell size [82]. However no abnormal response to hormones due to the *mpk4* mutation was observed [82]. A more general role for SA in interfering with plant development emerged by analyses of other mutants with constitutive defense activation. In *acd6* (*accelerated cell death 6*), *agd2* (*aberrant growth and death 2*), *lsd6* (*lesion simulating disease 6*) and *ssi1*(*suppressor of SA-insensitivity* 1) plants, defense activation interfered with cell growth by affecting cell enlargement, endoreduplication and/or cell division [129]. In all of these mutants high levels of SA were measured. An SA contribution to cell morphological changes in these mutants was shown by suppression of cell development alterations in crosses between *acd6*, *agd2*, *lsd6* or *ssi1* mutants with transgenic *Arabidopsis* plants expressing the SA-degrading enzyme NahG, a bacterial salicylate hydroxylase [129]. However, catechol the NahG product, was demonstrated to have pleiotropic effects and the results of these analyses should be interpreted carefully [165, 166]. Observed cell developmental phenotypes of *acd6*/*npr1*, *agd2*/*npr1*, *lsd6*/*npr1* and *ssi1*/*npr1* double mutant combinations indicated also a potential function of NPR1 in promoting cell division or suppressing endoreduplication, confirmed by analyses of single *npr1* mutant plants [129]. In another study, *Arabidopsis cpr5* (*constitutive expressor of PR genes 5*) plants, showing defense activation dependent on SA but only partially dependent on EDS1, PAD4 and NPR1, displayed alterations of

cell size, endoreduplication processes and cell division in both trichomes and epidermal cells [167]. EDS1 and PAD4 are two key components of the SA pathway and their over expression leads to increased SA levels. The availability of experimental evidence indicating a function of SA in affecting cell development, prompted me to test whether the plant growth phenotype in the dual EDS1 and PAD4 over expressors was determined by cell size alterations. A smaller cell size was estimated in OE_E1/P4 lines compared to wild type and single EDS1 or PAD4 over expressors (Figure 3.7). In contrast to what observed in *acd6* or *agd2* plants [129, 168, 169], abnormally enlarged cells in the mesophyll were not seen (data not shown). At present I cannot rule out that alterations in cell division could contribute to the decrease in fresh weight of the OE_E1/P4 lines. Such analyses need to be performed. To determine the specific contribution of SA or NPR1 to this developmental phenotype crosses with *eds16* (*enhanced disease susceptibility 16*), an isochorismate synthase shown to be the major SA source after pathogen challenge, and *npr1* mutant plants are being done. The observed silencing induced by the presence of two constructs driven by the CaMV 35S promoter would on the other hand render the generation of these lines problematic. This problem could be solved by the fact that substantial growth retardation was observed in the F1 progeny of two EDS1 and PAD4 single over expressor lines (Figure 3.12). Crosses between single EDS1 and PAD4 over expressors and *eds16* or *npr1* mutants will be performed and the resulting lines used to test the effects of *eds16* and *npr1* mutations on the growth phenotype in corresponding F1 progenies.

4.3 EDS1/PAD4 dual over expressor display increased resistance and inappropriate HR development in response to virulent pathogens

A common feature described for many defense mutants is the spontaneous development of lesions in the absence of the pathogen: *nudt7*, *cpr5* (*constitutive expression of PRs 5*), *ssi2* (*suppressor of salicylate insensitivity of npr1-5*), *acd6* and *agd2*, among others, display spontaneous cell death [102, 135, 168-170]. In many cases this was shown to be dependent on SA [168-171]. In contrast to these mutants OE_E1/P4 did not exhibit lesion

development in the absence of the pathogen (Figure 3.10). Thus, growth retardation is not a consequence of cell death initiation.

In another group of mutants defense activation resulted in increased resistance to virulent pathogens but also in impairment in HR development against avirulent pathogens. For example, *Arabidopsis dnd1* and *dnd2* (*defense no death1* and *2*) mutants are not capable of developing a wild type HR but have enhanced resistance to avirulent *P. syringae* strains [172, 173]. Both genes encode a predicted cyclic nucleotide-gated ion channel [133, 173] and *DND1* was shown to be involved in calcium fluxes, one of the earliest events following pathogen challenge [174]. Similarly, the *Arabidopsis hrl1* (*hypersensitive response like lesions1*) mutant is characterized by increased resistance to virulent pathogens [136] and suppression of HR induced by *Pst* DC3000 expressing *AvrRpm1*, probably as result of constitutive SAR activation [136]. No obvious suppression of HR following *RPP1* mediated recognition was instead observed in OE_E1/P4 plants upon *H. parasitica* isolate Noco2 challenge.

All the constitutive defense mutants described exhibit higher resistance to virulent pathogens than wild type plants. The same was observed for the OE_E1/P4 line (Figure 3.5 A and B and 3.11). Most strikingly, despite the lack of *R* genes involved in the recognition of the *H. parasitica* virulent isolate Emwa1, the dual over expressor lines developed an HR (Figure 3.5B). The strict pathogen inducibility of the cell death response is demonstrated by the fact that no spontaneous lesions formation is primed in the unchallenged state. *Arabidopsis edr1 edr2* and *edr3* (*enhanced disease resistance1*, *2* and *3*) mutants, carrying mutations in genes coding for a conserved MAPKK kinase, a PH-START domain containing protein and a Dinamin related protein 1E respectively, exhibit increased resistance only towards the fungal pathogen *Erysiphe cichoracearum* but a normal response against the virulent bacterial strain *Pst* DC3000 [175-178]. The double over expression of EDS1 and PAD4 led instead to a condition of heightened resistance with broader effectiveness against downy mildew and bacterial pathogens (Figure 3.5B).

In addition to defenses deployed after pathogen entry, plants can restrict bacterial pathogen entry through stomatal openings regulation [137]. The activation of this defence is dependent on FLS2 activity and requires SA accumulation [137]. This phenomenon

explained the failure to observe increased susceptibility in *fls2* plants to bacteria infiltrated directly into the plant tissue [17]. No test was performed so far to verify whether *eds1* or *pad4* are involved in the stomatal response to bacteria but the reported involvement of *eds16* creates however a link between SA pathway regulation and stomatal control [137, 179]. Experiments using bacteria directly infiltrated into the plant tissue will be performed to test whether a contribution to the observed increased resistance to bacteria derives from the activation of the stomatal response. However, increased resistance observed in OE_E1/P4 plants to downy mildew, that has a different entry strategy, and the association of this resistance with HR development suggests the involvement of additional mechanisms in the deregulated resistance response.

4.4 Growth inhibition in the EDS1/PAD4 dual over expressors is not due to hyper sensitivity to PAMPs

Exposure of Arabidopsis seedlings to flg22, the active 22mer from the N-terminal portion of flagellin, induces growth inhibition, and transcriptional activation of defence related genes such as *PR1* [12]. Recently flagellin from *P. syringae* pv. *tomato* was shown to trigger cell death in the non-host species *Nicotiana benthamiana* in an *NbFLS2* dependent fashion [180]. Furthermore, pre-treatments with flg22 induced resistance to spray inoculated DC3000 in *Arabidopsis* [17]. *Eds1* and *pad4* mutant plants retained the flg22 induced resistance and growth inhibition, indicating that PTI operates independently of EDS1 and PAD4 signaling activities [17]. However *EDS1* and *PAD4* were induced upon flg22 perception and none of the analyzed mutations in JA, ET and SA pathways genes compromised defense activation by flg22 [17]. This led to the hypothesis of pathway activation, that together which would result in high robustness of the response [17]. In this scenario the activation of complementary pathways would mask the SA pathway contribution to PTI.

The double EDS1 and PAD4 over expressor lines showed growth retardation, constitutive *PR1* activation, increased resistance to multiple virulent pathogens and an HR-like response upon attack by a virulent downy mildew isolate. I tested whether these responses were the consequence of an increased responsiveness to PAMPs. The growth

phenotype could then be interpreted as the result of PTI activation by exposure to non pathogenic microbes normally present in the environment. Increased responsiveness to PAMPs carried by virulent pathogens could also explain the observed increased resistance and potentially the development of cell death during compatible interactions. Disruption of another *Arabidopsis* gene, *PDR8/PEN3* (*Pleiotropic Drug Resistance 8/ PENETRATION RESISTANCE 3*), coding for a plasma membrane ABC transporter, has been reported to determine spontaneous lesions development, defence genes activation and increased resistance to virulent pathogens [181]. Gene induction and lesions development were attenuated when plants were grown in sterile conditions, indicating potential defense activation by microbes present in the environment [181]. The behavior of *pdr8* plants in non-sterile conditions at high humidity has not been characterized leaving open the possibility that the phenotype is suppressed by high humidity in the sterile environment.

Under sterile conditions the OE_E1/P4 lines retained both reduced growth and constitutive *PR1* activation, indicating that the observed phenotypes are not the result of PAMP hyper-responsiveness but more likely a consequence of an intrinsic genetic program (Figure 3.11 A, B and C). This was consistent with the fact that *Arabidopsis nudt7* plants, in which the EDS1 and PAD4 pathway is constitutively activated, showed normal responsiveness to flg22 (M. Straus and J. Parker, unpublished). Also, *eds1* mutants had increased biomass compared to wild type in both non-sterile and sterile conditions (Figure 3.6 and Figure 3.11 B). The difference was however much more pronounced in sterile conditions. This once more is probably a consequence of the metabolic costness of default basal defence activation present also in wild type plants and suppressed in *eds1-1* mutants.

4.5 EDS1/PAD4 dual over expression leads to an accelerated response to virulent pathogens

SA pathway activation in unchallenged OE_E1/P4 plants led to low *PR1* transcript accumulation well below the amplitude reached after pathogen challenge (Figure 3.8). Also, cell death in OE_E1/P4 plants was triggered only upon pathogen challenge

indicating that high levels of EDS1 and PAD4 proteins are not sufficient to recapitulate the full pathogen response (Figure 3.5). To better characterize defense induction, time course experiments after infection with a virulent *Hyaloperonospora parasitica* isolate were performed and candidate gene expression, EDS1 and PAD4 protein levels and phenolic compound accumulation monitored (Figures 3.9, 3.13, 3.14 and 3.15). The results of these experiments indicate a quicker activation of the SA pathway in response to virulent pathogens induced by dual over expression of EDS1 and PAD4.

The concept of "priming", originally elaborated to describe a phenomenon observed in mammalian monocytes and macrophages apply also to plants. It describe a "sensitized" condition leading to more rapid responses to subsequent attacks [182, 183]. Induced Systemic Resistance (ISR) is induced by nonpathogenic root-colonizing bacterium *P. fluorescens* WCS417 and also represents a "priming" mechanism. It´s independent of SA and of *PR*-gene activation but requires JA and ET [184]. Analyses of local and systemic levels of JA and ET revealed that ISR induction is not associated with changes in the production of these signal molecules but rather with an enhanced sensitivity in their perception [185]. Consistent with this idea microarray analyses demonstrated changes in expression of virtually no gene in the systemic tissues upon ISR induction, while upon subsequent pathogen challenge ISR induced plants showed more rapid induction of ≈ 80 genes compared to naïve plants [186]. Priming was also described for plants treated with the chemical compound BABA (β-Aminobutyric acid) a non-protein amino acid that potentiates plant responses and confers resistance to biotic and abiotic stresses in a SA, JA and ET independent fashion [182, 183]. The state of plants treated with BABA or in which SAR or ISR have been induced has thus be considered as "primed", to indicate the increased velocity of response to following attacks [182, 183]. The data presented here suggest that OE_E1/P4 plants are also in a "primed" condition. The observed up regulation of *PR1* in the unchallenged state represents a significant difference with ISR induced or BABA treated plants and reflects more similar SAR-induced plants. Constitutive SAR expression was previously believed to suppress cell death [136]. This was not observed in OE_E1/P4 plants. A key regulator of SAR induction is *NPR1* [187]. Determining the *NPR1* contribution to the observed defense phenotype would allow a

better characterization of the primed state of the double EDS1/PAD4 over expressor plants.

The observation that a series of responses were induced only upon pathogen challenge in the OE_E1/P4 lines, points to post translational control of EDS1/PAD4 signalling. Probable post translational stabilization of each protein was observed upon pathogen challenge but must be confirmed by quantitative analyses of *EDS1* and *PAD4* transcript and protein levels (Figure 3.13 and 3.14). However stabilization is unlikely to account fully for defense activation, since the EDS1 and PAD4 protein levels obtained in unchallenged OE_E1/P4 plants are considerably higher than in pathogen challenged wild type plants (Figure 3.4 C and D). To assess whether the defence activation is mediated by endogenous SAG101, *sag101* mutant plants over expressing EDS1 and PAD4 will be generated and characterized. Furthermore the proportion of cytosolic nuclear and cytosolic EDS1 and PAD4 before and after pathogen challenge will be monitored in OE_E1/P4 to assess whether activation occurs through protein relocalization.

4.6 EDS1/PAD4 double over expression leads to increased tolerance to paraquat

EDS1 and PAD4 signaling activity was previously shown to be necessary to process ROS-derived signals since *eds1* and *pad4* mutations lead to partial and total suppression of the ROS induced *flu* and *lsd1* phenotypes, respectively [108, 110]. I tested whether the increased resistance to virulent pathogens could be due to a higher sensitivity to early ROS production combined with a potential higher ROS signal transmission in the EDS1 and PAD4 over expressor lines. The major form of ROS produced during pathogen response is superoxide (O^{2-}) which is quickly converted into hydrogen peroxide (H_2O_2) [146, 188]. The herbicide paraquat or methyl viologen (MV) stimulates production of O^{2-} and H_2O_2 from chloroplasts in plants exposed to light [113, 148]. Also, paraquat induced damages are alleviated by expression of the mammalian anti-apoptotic protein Bcl-2 [189]. This indicates that also for MV treatments, cell death is induced through activation of a genetic program rather than by direct damage. When grown in liquid medium containing low concentrations of MV, OE_E1/P4 plants had less severe growth retardation than either wild type plants and single over expressors (Figure 3.16).

Several hypotheses can be formulated to explain the observed increased tolerance of the double over expressor plants to MV.

The plant response to ROS production is a consequence of aerobic life [146, 188, 190]. Sources of ROS are present in all plant compartments, in the mithocondria during respiration, in chloroplasts during photosynthesis, in peroxisomes during photorespiration, in glyoxisomes during fatty acid oxidation and most importantly in the apoplast during HR response, but also during cell growth and developmental cell death [146, 188, 190]. Superoxide (O_2^-) is produced by reduction of dioxygen which can inactivate enzymes containing Fe-S clusters [188]. In acidic environments O_2^- is converted into hydroperoxide radical ($HO_2^{\bullet}$) which can also cause membrane oxidation [188]. Normally O_2^- is enzymatically converted to H_2O_2 by super oxide dismutase (SOD). H_2O_2 can inactivate enzymes by oxidizing their thiol groups and being relatively more stable than O_2^- can migrate to different cell compartments or to neighboring cells [188]. Also, in presence of metallic ions H_2O_2 can be converted by Haber-Weiss reaction to the much more reactive hydroxyl radical $OH^{\bullet}$ which damages a wide range of bio-molecules [188]. Given the potential ROS toxicity, in all compartments ROS accumulation is regulated by scavenging machineries consisting of enzymatic and non enzymatic components involved for ROS removal. SOD is the only plant enzyme known to scavenge superoxide, while multiple enzymes are involved in H_2O_2 scavenging [190]. Among them, catalases scavenge H_2O_2 without requiring reducing potential but only when high concentrations of this ROS are reached. At lower concentrations ascorbate peroxidases (APXs) and glutathione peroxidases (GPXs) convert H_2O_2 into water utilizing ascorbate and glutathione as reducing agents, respectively. No enzyme is known that scavenges hydroxyl radicals, so that the only strategy plants seem to have adopted is to prevent their formation by removing H_2O_2 and O^{2-} and by sequestrating metal ions with metal binding proteins such as ferritin or methallothioneins [188]. ROS are no longer considered simply as toxic by-products of essential biological processes, but as important signaling molecules whose specificity is determined by their identity, their concentration and the timing or localization of their production [188]. Importantly, low doses of O^{2-} and H_2O_2 have been shown to induce protective mechanisms and acclimation responses against oxidative and abiotic stress, while high doses trigger cell death [188, 189].

There are several possible causes of the increased tolerance of OE_E1/P4 plants to paraquat treatments.

A quicker activation of the scavenging machinery upon MV treatment could take place in in the double over expressors. Gene expression experiments are now being performed to test this possibility. The gene *Fer1* *Arabidopsis* plants over expressing a thylakoidal isoform of *APX* show increased oxidative tolerance upon MV treatment but still retain *Fer1* normal induction [191]. Expression analysis upon MV treatment of *Fer1* (*Ferritin1*), a specific molecular marker for H_2O_2 generation [113, 192], and of other genes codifying for ROS scavenging enzymes will be performed. In this way I will monitor on one hand the MV induced H_2O_2 production, gaining an indirect estimation of the MV up take, and on the other I will assess whether a quicker activation of the scavenging machinery is taking place. However, complementary biochemical approaches will also be followed. In relation to what observed during pathogen challenge and especially in relation with the observed HR response against a virulent pathogen, this increased oxidative tolerance is quite surprising. Another possible explanation is the contrasting action of ROS at different concentrations. *Arabidopsis eds1-1* plants hand infiltrated with high concentrations of MV (25 μM) show 24 hpt reduced induced cell death compared to wild type plants (M. Bartsch and J. Parker, unpublished results). Assuming a role of the EDS1 signaling pathway in both oxidative acclimation and cell death induction by H_2O_2, the double over expressor lines should also exhibit increased sensitivity to higher concentrations of MV and develop cell death at a quicker rate than wild type. Experiments to assess this hypothesis are being performed.

Finally, the demonstrated importance of ROS metabolism in growth regulation and specifically in cell elongation and division could represent a further connection between the observed growth phenotype and ROS signals transduction [193, 194].

4.7 Post translational regulation of EDS1 and PAD4

I have demonstrated that over expression of EDS1 and PAD4 together leads to a partial deregulation of defences. Therefore, there has to be a post translational component that contributes to defence activation upon pathogen challenge.

I focused my analyses on the potential existence of protein modifications and protein interactions which could be triggered upon pathogen challenge representing a potential switch between EDS1 and PAD4 signaling inactive and active forms. An alternative, but not exclusive mechanism of regulation, could be EDS1 or PAD4 re-localization. A third possibility would be an intrinsic biochemical activity of EDS1 and PAD4, such as the processing of a substrate(s) upon pathogen challenge. Also these possibilities are now being explored.

Two different modifications of EDS1 were identified in this study: N-acetylation and phosphorylation (Figures 3.20, 3.21 and 3.22). The identified N-acetylation had the hallmarks of an irreversible protein modification, as only the modified EDS1 version was identified, and as such is unlikely to be a candidate for regulatory modification (Figure 3.22).

Phosphorylation of EDS1 appeared to be invariable between unchallenged and challenged plants at different time points (Figure 3.20). Also, there was no relation between the strength of phospho-signal and the relative amounts of protein likely to be active (Figure 3.21). Different interpretations of these findings can be made. First, it is possible that changes in the phosphorylation status of EDS1 happen transiently and were missed at the time points analyzed. Second, changes in the phosphorylation status might be restricted to a subset of cells undergoing direct attack. Therefore, crosses were performed to analyze the status of EDS1 in backgrounds in which the EDS1 pathway is constitutively or conditionally activated (See section 3.11).

The function of EDS1 and PAD4 in transducing ROS related signals suggests that redox related protein modification may determine their signaling activity. Redox related reversible protein modifications such as S-nitrosylation and thiol-disulphide conversion, mainly involve cysteine residues [195-199]. In EDS1 and PAD4 multiple cysteines are conserved among different plant species (**Figure 4.1 and 4.2)** were identified, consistent with potential conserved redox regulation. Sequence comparison of known S-nitrosylated proteins, has defined a S-nitrosylation motif: (His ,Lys,Arg) / (Cys) / (hydrophobic) / (X) / (Asp,Glu), where X is any amino acid [195]. This motif was not identified in the EDS1 or PAD4 amino acid primary sequences. There are, however, several examples of

validated S-nitrosylated proteins in which the acid-base motif is revealed only in the tertiary or quaternary structure of the protein [200].

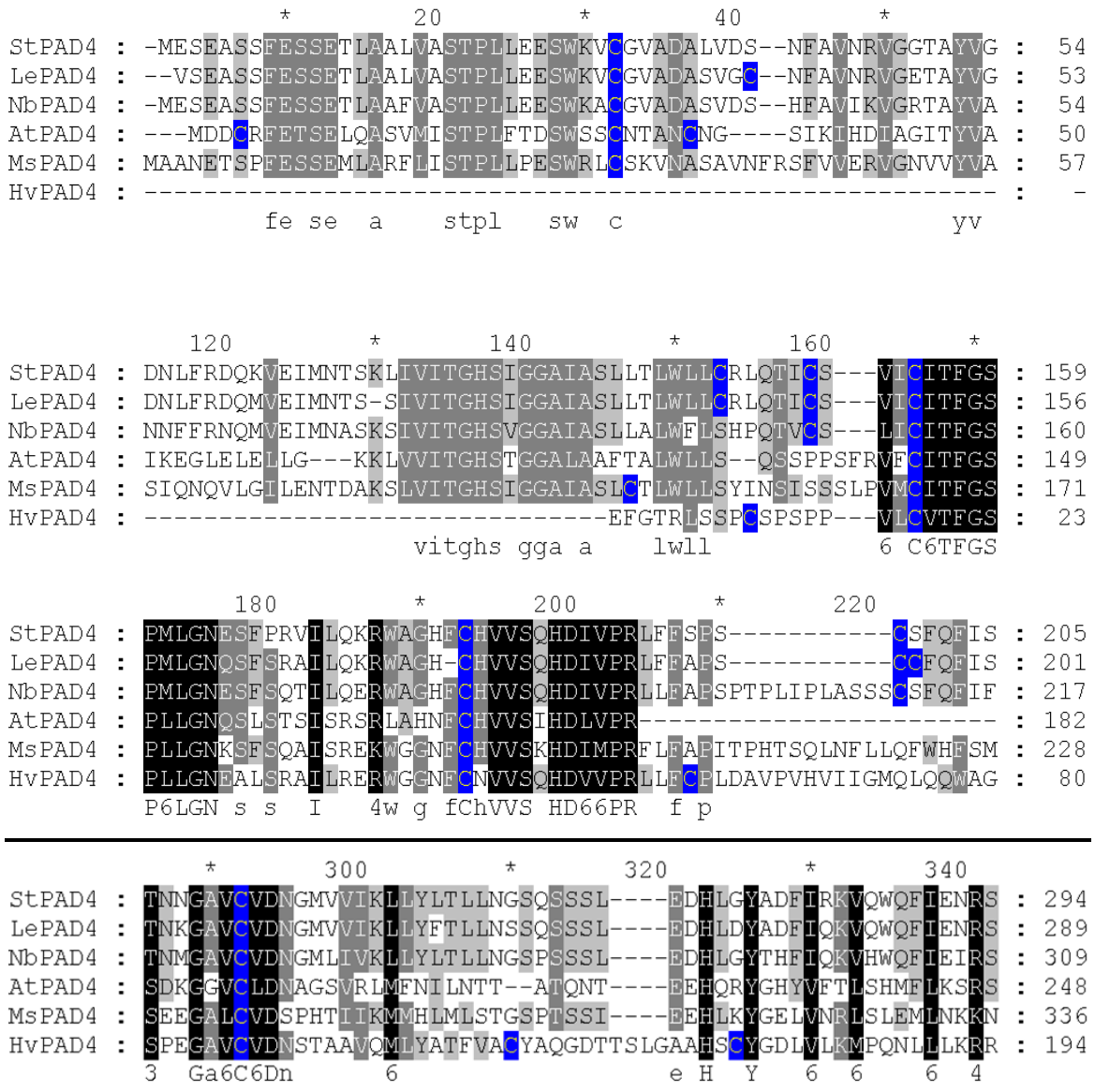


Figure 4.1 Conserved cysteines in the PAD4 amino acid sequence from different plant species

Three portions of an alignment between PAD4 amino acid sequences from different plant species: *Solanum tuberosum* (StPAD4), *Lycopersicon aesculentum* (LePAD4), *Nicotiana benthamiana* (NbPAD4), *Arabidopsis thaliana* (AtPAD4), *Medicago sativa* (MsPAD4), *Hordeum vulgare* (HvPAD4) are shown. Cysteines are highlighted by the blue background.

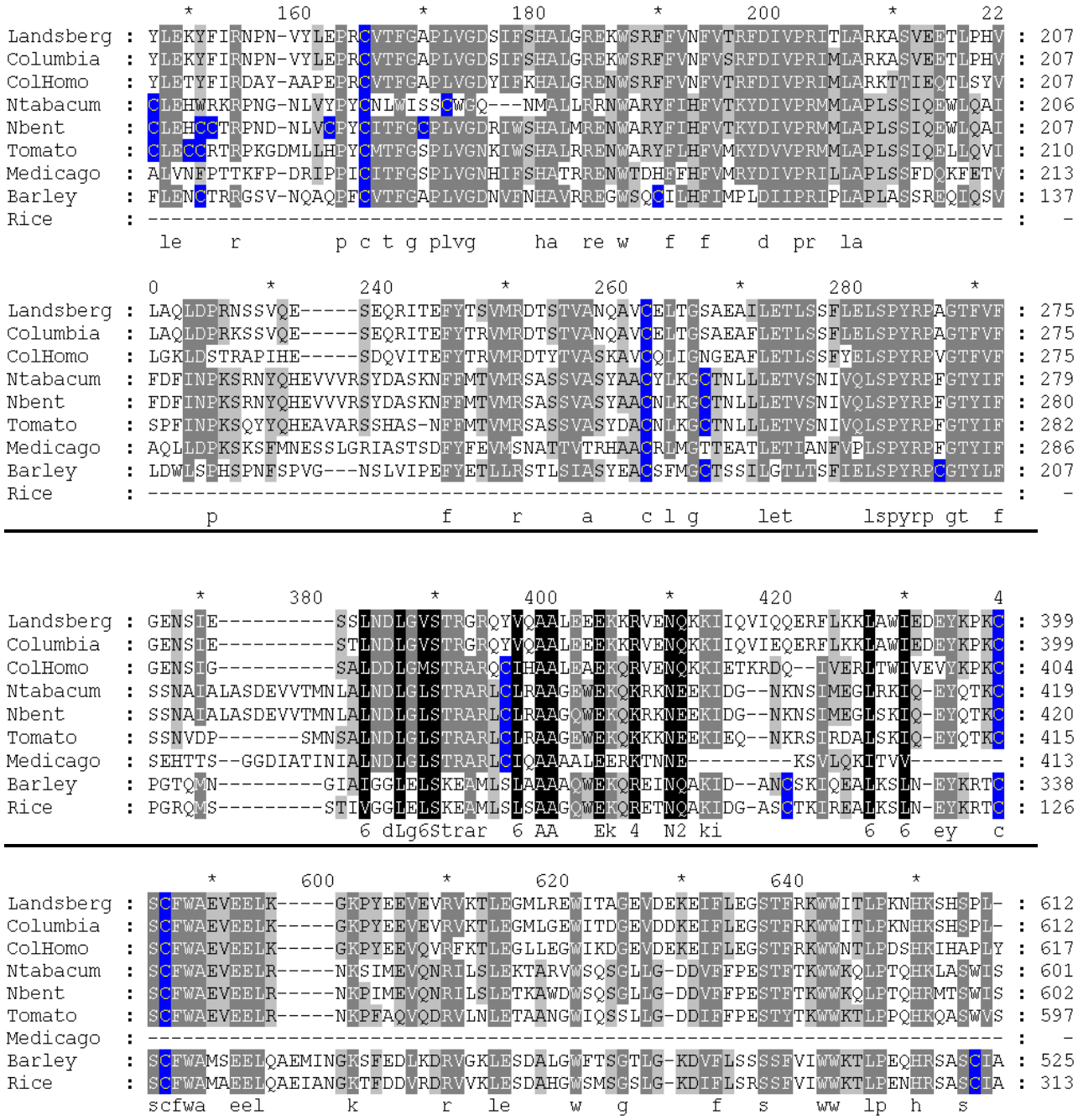


Figure 4.2 Conserved cysteines in EDS1 amino acid sequence from different plant species

Three portions of an allignement between EDS1 amino acid sequences from different plant species: Rice, barley, *Medicago sativa* (Medicago), Tomato, *Nicotiana benthamiana* (Nbent), Tobacco (Ntabacum), *Arabidopsis thaliana* accession Col-0 (ColHomo and Columbia to indicate the two EDS1 copies present in this accession) and Landsberg.

Cysteins are highlighted by the blue background.

Redox sensors involved in responses to oxidative stress in bacteria and yeast are activated through redox-dependent modifications. Some well characterized examples are the transcription factors OXYR in *E.coli* and YAP1 in yeast. In response to peroxide treatment, OXYR activates the expression of the *oxyR* regulon that includes several detoxifying enzymes [198, 199]. The molecular mechanism by which OXYR is activated is unclear. On one hand, OXYR regulation could be achieved through thiol-disulphide bond conversion. On the other hand different redox-dependent modifications (among which S-nitrosylation) of different single cysteines, leading to discrete changes in DNA binding activity could occur [198, 199]. YAP1 is a bZIP DNA-binding protein of the AP-1 family, also involved in oxidative stress response in yeast [198]. Normally YAP1 is continuosly shuttled between nucleus and cytoplasm, but only low levels of protein accumulate inside the nucleus [198]. Exposure to the disulphide stress-inducing oxidant diamide leads to the formation of a disulfide bond in the YAP1 C-terminal cysteine-rich domain [198]. The disulphide bond causes a protein rearrangement that inhibits nuclear export, promoting the transcriptional activation of YAP1 target genes [198]. In response to H_2O_2, a thiol-disulphide relay switch involving another yeast protein, ORP1, leads to the formation of a intramolecular disulphide bond between two cysteines in YAP1, again leading to nuclear accumulation and activation of YAP1 target genes [198].

Regulation through thiol-disulphide conversion was also reported for the SUMO E1 subunit Uba2 and the E2-conjugating enzyme Ubc9, components of the SUMOylation machinery in humans [201]. Oxidative stress or macrophages activation leads to formation of a reversible inter molecular disulphide bridge between catalytic cysteines of Uba2 and Ubc9, resulting in repression of the SUMOylation machinery [201].

Thus, redox regulation of EDS1 and PAD4 might be a mechanism through which ROS modulate EDS1 and PAD4 activities. OE_E1/P4 plants exhibited increased levels of scopoletin in the unchallenged state as compared to wild type plants. Non enzymatic oxidation of fatty acids by free radicals leads in plants to the formation of phytoprostanes, prostaglandin like molecules structurally similar to mammalian isoprostanes [202]. The fact that application of phytoprostanes induced accumulation of both scopoletin and camalexin in *Arabidopsis*, could indicate a link between ROS induced non enzymatic fatty acid peroxidation and EDS1 and PAD4 signalling activation [143, 144].

Furthermore, the lipophylic nature of these compounds could explain the presence in both EDS1 and PAD4 of a conserved lipase-like domain which could be involved in lipid binding for activation, rather than in lipid processing.

4.8 Working Hypotheses

In **Figure 4.3** is depicted a model of EDS1 and PAD4 activation that can be tested experimentally. The production of ROS upon pathogen challenge or paraquat treatment

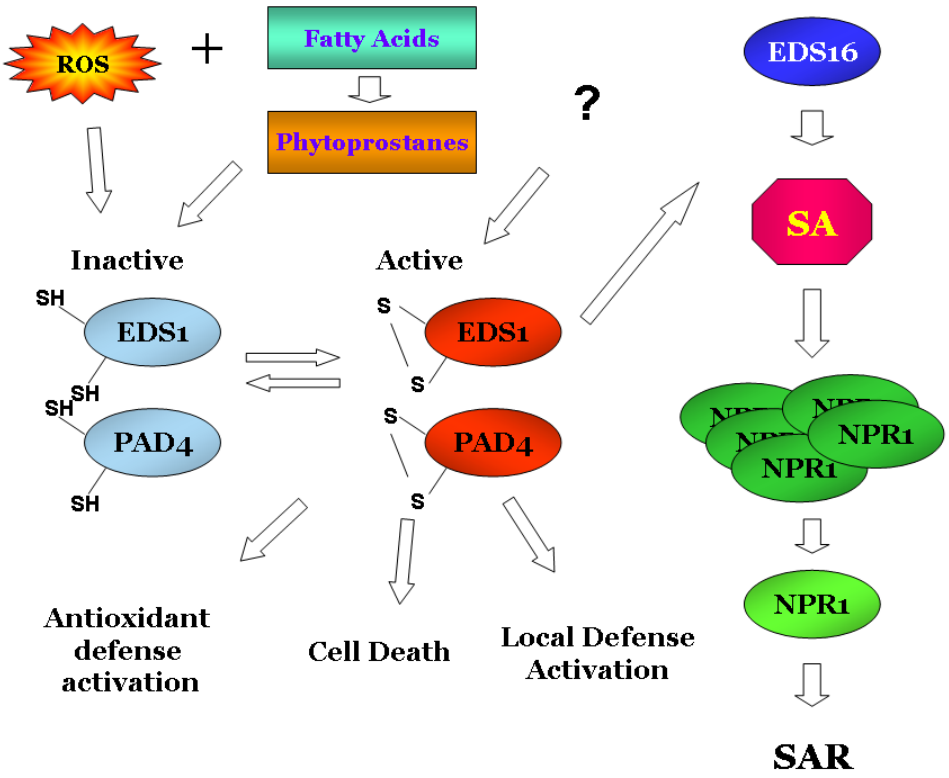


Figure 4.3 Working model for the post translational activation of EDS1 and PAD4

ROS generation upon paraquat treatment or pathogen challenge leads, directly or indirectly (through phytoprostanes generation) to intramolecular thiol-disulphide conversion, leading to signaling activation of EDS1 and PAD4 by conformational changes potentially resulting in alteration of their localization. EDS1 and PAD4 activate antioxidant responses or cell death depending on the ROS concentration perceived. EDS1 and PAD4 activation results in increase in SA levels by EDS16 activation and consequently in SAR induction by SA induced monomerization and nuclear translocation of NPR1.

results directly or indirectly (passively through phytoprostanes generation) into the conversion of EDS1 and PAD4 from a signaling-inactive to a signaling-active form. This conversion happens through redox-based protein modifications. Since EDS1 and PAD4 interact before and after pathogen challenge and the two proteins can be co-purified in reducing conditions ([93], **Figure 3.23** page 90) intra-molecular disulphide bridges are more likely to be involved in EDS1 and PAD4 activation. Upon activation, EDS1 and PAD4 in turn activate different responses dependent on the ROS concentration perceived. Persistent exposure to low ROS concentrations would lead to activation of the antioxidant machinery while acute oxidative stress would lead to cell death response. Activation of EDS1 and PAD4 leads to increased production of SA, consequent monomerization of NPR1 and activation of SAR. The observed slight constitutive activation of the SA pathway in the double EDS1/PAD4 over expressor lines could result from the availability at low abundance of ROS (or phytoprostanes) already in the unchallenged status combined with large amounts of EDS1 and PAD4 proteins. Constitutive activation of the SAR response would result in the observed primed status and determine enhanced disease resistance in the OE_E1/P4 line. Constitutive activation of SAR would result in plant growth retardation through alterations of normal cell development. In the OE_E1/P4 line development of HR upon virulent downy mildew challenge results by an increased sensitivity to ROS.

To assess the contribution of *NPR1* and *EDS16* to the observed developmental and plant defense phenotypes by genetic analyses will allow a better characterization of the source of increased resistance and developmental alteration. Application of different concentrations of paraquat, in combination with gene expression analyses, will determine whether EDS1 and PAD4 drive an antioxidant response or a ROS induced cell death program. Also, biochemical analyses to determine whether EDS1 or PAD4 redox protein modifications occur after pathogen challenge or during oxidative stress will clarify whether a molecular link exists between cell redox alterations and activation of EDS1/PAD4 signaling activities. Finally, the generation by crosses of lines expressing EDS1 strepII functional fusion proteins in genetic backgrounds in which the EDS1 and PAD4 pathway is constitutively or conditionally activated, will facilitate the identification of potential transient regulatory events.

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ERKLÄRUNG

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Acknowledgments

I would like to thank Dr. Jane Parker for all the support she provided me throughout my PhD period at the MPIZ and for proficuous discussion and suggestions.
Thanks: I really learned a lot from my PhD experience.

Thanks to Professor Martin Hülskamp, Professor Ulf-Ingo Flügge and especially to Professor Paul Schulze-Lefert for accepting to be members of my PhD examining committee.
Special thanks to Dr. Sabine Zachgo for giving her disponibility to act as "Beisitzer" at my exam.

A big thank to all the members of the JP lab, past and present, for helping me to get started and for the nice working environment. I will not forget you. "In fact, Indeed".

Special thanks to my always evolving "lunch group": Klaus-Peter, Nieves, Jagreet, Ana, Doris, Jaqueline, Dorit and Aurelie for keeping patiently listening to my daily complaining.

Thanks to all my friends inside and outside the PSL department who always encouraged me to give my best: Cristina, Sandra, Chiara, Mariam, Simone, Fabio, Vittoria, Moritz, Maria, Hugues, Matthieu and Diana.

A tribute to all the members of the notorious TATA band I had the honour to belong to. That was real fun.

Sorry to all of those who supported me and I am now forgetting. There are so many people that I would need a second volume. It´s now 7:00 and I have just finished to prepare my thesis for printing after an over night job fighting with pc, figure formats and evil printers. I am really not in the right shape to remember names. I hope you will understand.

The biggest thank of all to my family, my parents and my sister, because I always know I can truly count on them, because they believe in me and because they are always close to me despite the physical distance.
This PhD thesis is dedicated to them.