Gene expression profiling leading to identification of essential components in EDS1/PAD4-regulated plant defence

Inaugural–Dissertation

zur

Erlangung des Doktorgrades

der Mathematisch-Naturwissenschaftlichen Fakultät

der Universität zu Köln

vorgelegt von

Michael Bartsch

aus Lübeck

Köln, Mai 2005

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

Berichterstatter:	Prof. Dr. Paul Schulze-Lefert
	Prof. Dr. Diethard Tautz
Prüfungsvorsitzender:	Prof. Dr. Martin Hülskamp
Tag der mündlichen Prüfung:	4. Juli 2005

Summary

Plants possess multiple mechanisms to detect pathogen attack and protect themselves against colonisation. The antagonistic interplay of positive and negative regulators allows the plant to spacially and temporarily control defence responses. *EDS1* (*Enhanced Disease Susceptibility1*) and *PAD4* (*Phytoalexin Deficient4*) encode lipase-like proteins that positively regulate plant basal resistance to virulent pathogens. Additionally, *EDS1* and *PAD4* are recruited by resistance (*R*) genes of the TIR-NBS-LRR but not of the CC-NBS-LRR type in *R* gene-mediated resistance. Previous experiments demonstrated that *EDS1* and *PAD4* are required for accumulation of salicylic acid (SA), a phenolic signal in defence to biotrophic pathogens. Recent findings suggest that *EDS1* and *PAD4* promote defence also independently of SA. This as yet uncharacterised *EDS1/PAD4*-controlled pathway is important for full expression of local *R* gene-triggered and basal resistance as well as for systemic immunity.

To identify components involved specifically in *EDS1/PAD4*-controlled signalling, transcriptional profiles of *Arabidopsis thaliana* wild-type, *eds1* and *pad4* mutant plants were examined during early *R* gene-mediated defence using whole-genome oligonucleotide microarrays. In wild-type, the inoculation with strains of the bacterial plant pathogen, *Pseudomonas syringae* pv. *tomato*, expressing either *avrRpm1* (avr1; recognised by a CC-NBS-LRR-type R protein) or *avrRps4* (avr4; recognised by a TIR-NBS-LRR-type R protein) triggered transcriptional changes in a similar set of genes but with different kinetics. Sets of genes with *EDS1- and PAD4*-dependent expression in healthy, avr1- or avr4-challenged leaves were identified. For a subset of these genes, corresponding insertional mutants were isolated and tested for alterations in pathogen resistance. The mutant screen resulted in the identification of a flavin-dependent monooxygenase (FMO) as a positive regulator and two sequence-related NUDIX (nucleoside diphosphates linked to some other moiety x) hydrolases as negative regulators of plant disease resistance. This study demonstrates for the first time that FMOs and NUDIX hydrolases can modulate host defence responses against pathogens in any biological system. The findings presented here support the view that *EDS1* and *PAD4* control the expression of both positive and negative regulators as a mean to fine-tune plant immune responses.

Zusammenfassung

Pflanzen besitzen vielfältige Detektions- und Abwehrmechanismen, die sie gegen einen Pathogenangriff schützen. Dabei erlaubt das antagonistische Zusammenwirken von positiven und negativen Regulatoren der Pflanze ihre Abwehrmaßnahmen zeitlich und räumlich zu steuern. *EDS1* (*Enhanced Disease Susceptibility1*) und *PAD4* (*Phytoalexin Deficient4*) kodieren Proteine mit Homologie zu eukaryotischen Lipasen und sind positive Regulatoren der pflanzlichen basalen Resistenz gegen virulente Pathogene. Ferner erfordert auch die durch Resistenzproteine (R) der TIR-NBS-LRR-Klasse (aber nicht der CC-NBS-LRR-Klasse) vermittelte Abwehrreaktion gegen avirulente Pathogene EDS1 und PAD4. Beide Proteine werden sowohl bei basaler als auch TIR-NBS-LRR vermittelter Resistenz für die Akkumulation der phenolischen Signalsubstanz Salizylat (SA) benötigt. Dennoch gibt es Hinweise auf eine SA-unabhängige Signalfunktion von EDS1 und PAD4, welche für eine effektive lokale als auch systemische Abwehrreaktion essentiell ist.

Um Komponenten dieses *EDS1/PAD4*-abhängigen Signalweges zur identifizieren, wurde während der frühen Phase der *R*-Gen vermittelten Pathogenabwehr eine vergleichende Transkriptionsanalyse mittels Oligonukleotid-Mikroarrays zwischen *Arabidopsis thaliana* Wildtyp und den Mutanten *eds1* und *pad4* durchgeführt. In Wildtyp-Pflanzen führten Inokulationen mit isogenen Stämmen des bakteriellen Pflanzenpathogens *Pseudomonas syringae* pv. *tomato*, welche die Avirulenzproteine avrRpm1 (avr1; detektiert von CC-NBS-LRR R-Protein RPM1) bzw. avrRps4 (avr4; detektiert von TIR-NBS-LRR R-Protein RPS4) exprimieren, zur Induktion bzw. Repression von ähnlichen Gengruppen, allerdings mit unterschiedlicher Kinetik. Weiterhin wurden Gengruppen mit einer *EDS1/PAD4*-abhängigen Expression im unbehandelten Zustand und nach Pathogenbehandlung mit avr1 oder avr4 identifiziert. Für einige dieser Kandidatengene wurden Insertionsmutanten isoliert und auf Veränderung ihrer Pathogenresistenz untersucht. Die Phenotypisierung der Insertionsmutanten führte zur Identifizierung einer Flavin-abhängigen Monooxygenase (FMO) als positiven Regulator und zweier NUDIX- (*nucleoside diphosphates linked to some other moiety x*) Hydrolasen als negative Regulatoren pflanzlicher Abwehrreaktionen. Die vorliegende Arbeit zeigt erstmalig, dass FMOs und NUDIX-

Hydrolasen des Wirtes dessen Abwehrmaßnahmen gegen Pathogene modulieren können. Des Weiteren konnte dargelegt werden, dass *EDS1* und *PAD4* die Expression von positiven sowie von negativen Regulatoren steuern und damit zur Feinregulierung von pflanzlicher Pathogenabwehr beitragen.

Abbreviations

° C	degree Celsius
avr	avirulence
avr1	*Pseudomonas syringae* pv. *tomato* DC3000 expressing *avrRpm1*
avr4	*Pseudomonas syringae* pv. *tomato* DC3000 expressing *avrRps4*
BTH	benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester
CC	coiled-coil
cDNA	complementary DNA
cfu	colony forming unit
dpi	days post inoculation
DEPC	diethylpyrocarbonate
dH_20	deionised water
DNA	deoxyribonucleic acid
EDS1	Enhanced Disease Suseptibility1
FMO	flavin-dependent monooxygenase
f. sp.	forma specialis
g	gravity constant (9.81 ms^{-1})
GUS	beta-glucuronidase
h	hours (post inoculation)
HR	hypersensitive response
LRR	leucine-rich repeats
Mg	(treatment with) magnesium chloride solution
mRNA	messenger ribonucleic acid
NBS	nucleotide binding site
NT	non-treated
NUDIX	nucleoside diphosphates linked to some other moiety x
OD	optical density
PAD4	Phytoalexin Deficient4
PAMP	pathogen-associated molecular pattern

PCR	polymerase chain reaction
pH	negative decimal logarithm of the H^+ concentration
PR	pathogenesis related
Psm	*Pseudomonas syringae* pv. *maculicola*
Pst	*Pseudomonas syringae* pv. *tomato*
pv.	pathovar
qRT-PCR	quantitative real-time polymerase chain reaction
R	resistance
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-PCR	reverse transcription-polymerase chain reaction
SA	salicylic acid
SAR	systemic acquired resistance
T-DNA	transfer DNA
TIR	*Drosophila* Toll and mammalian interleukin-1 receptor
UV	ultraviolet
vir	virulence
WT	wild-type

Table of contents

1. Introduction

Plants are the ultimate source of food for humans and animals. The concentration on a few plant species in agriculture and forestry and their cultivation in monoculture has favoured the out break of plant diseases, sometimes with devastating consequences. A better understanding of plant-pathogen interactions will enable us to develop means that will help to make plant production more predictable.

1.1 *Arabidopsis thaliana* as a model host of plant pathogens

Despite the lack of any important commercial value of this member of the mustard family, *Arabidopsis thaliana* has several traits which make it an ideal model organism for plant genetic research. Its small plant size, the short life cycle (about 6 weeks) and its large seed production make it possible to grow it on limited space and in rapid manner. Because of the named advantages and its relatively small genome (125 Mb), *Arabidopsis* was the first plant whose genome has been fully sequenced in a multinational effort (Initiative, 2000). The availability of the genome sequence was the starting point for the development of genomic tools, making it possible to study transcriptional changes at a whole genome-wide scale (Redman et al., 2004).

Arabidopsis is host to different classes of pathogens including oomycetes, fungi, viruses and bacteria (Mauch-Mani and Slusarenko, 1993). Depending on the mode of the infection, pathogens are classified as necrotrophic (host cell is killed), biotrophic (cell remains alive) or hemibiotrophic (cell is killed later in the infection process). Knowledge about the existence of pathogens that attack *Arabidopsis* was subsequently used to study plant-microbe interactions in this model organism. The obligate biotrophic pathogen oomycete *Peronospora parasitica* (Crute et al., 1992; Parker et al., 1993; McDowell et al., 2000) and biotrophic bacterial strains of *Pseudomonas syringae* (Whalen et al., 1991; Volko et al., 1998) were especially useful to unravel mechanisms of plant disease and host resistance.

1.2 Plant defence against biotrophic pathogens

The observation that most plants appear healthy in an environment full of potential disease causing agents lead to the conclusion that plants have developed extremely effective defence systems that normally protect them from disease.

1.2.1 Non-host resistance

The most prevalent form of disease resistance in the field is called “non-host resistance”. It is defined as resistance expressed by an entire plant species to a pathogen that normally infects another plant species (Heath, 2000). Although non-host resistance is not well understood at the molecular level, it involves preformed defence barriers (e.g. cuticle on the leaf surface) and inhibitory plant metabolites (e.g. alkaloids, cyanogenic glycosides, phenols) as well as inducible defence mechanisms. Induction of non-host defence responses can be triggered by recognition of pathogen-associated molecular patterns (PAMPs) (reviewed in Medzhitov and Janeway, 2002). PAMPs are molecules which are characteristic for an entire pathogen class, e.g. flagellin of bacterial pathogens and are recognized in flies, mammals and plants by Toll-like receptors (TLR) (Lemaitre et al., 1997; Felix et al., 1999; Hayashi et al., 2001; Zipfel et al., 2004). Although broadly structurally similar molecular components mediate PAMP-triggered signal transduction pathways in plants and animals, it is not clear if these similarities are due to convergent evolution or common ancestral origin (Nurnberger et al., 2004).

1.2.2 *R* gene-mediated resistance

Although non-host resistance provides plants with a relatively robust protection from disease, pathogens have developed virulence factors (effectors) that help them to overcome defence mechanisms of certain plant species. As a countermeasure against virulent pathogens, plants have evolved a race-specific resistance that is effective against specific pathogen isolates, thus turning a normally compatible interaction (host develops disease) into an incompatible interaction (host is resistant). The genetic basis for this so-called race-specific resistance is embodied in the gene-for-gene hypothesis (Flor, 1971) which states that pathogen recognition is conferred by products of plant resistance (*R*) and corresponding pathogen avirulence (*Avr*)

genes. The recognition event triggers a rapid defence response that often includes a localised programmed cell death of plant cells at the site of attempted invasion, a phenomenon termed hypersensitive response (HR). The accumulation of the phenolic defence molecule salicylic acid (SA) can contribute to HR but is not essential in all cases (Mur, 1997; Kachroo et al., 2000; Mur et al., 2000; Shapiro and Zhang, 2001; Chandra-Shekara et al., 2004). The function of SA in plant defence was extensively studied on SA depleted plants expressing a bacterial salicylate hydroxylase (NahG) which removes SA by conversion to catechol and on the *Arabidopsis* SA synthesis mutant *sid2* (Salicylic Acid Induction Deficient2) (Wildermuth et al., 2001). *SID2* encodes an isochorismate synthase, suggesting that SA accumulated during pathogen infection is derived from chorismate. Substantial SA accumulation and transcriptional activation of the SA marker gene *PR1* (*Pathogenesis-Related1*) were detected in *Arabidopsis* treated with avirulent strains of *P. syringae* as early as 4 and 10 hours post inoculation (h), respectively (Feys et al., 2001; Zhang et al., 2004).

Several *R* and corresponding *Avr* genes have been cloned. The largest class of *R* genes encode intracellular proteins with a central nucleotide-binding site (NBS) and C-terminal leucine rich repeats (LRRs). Based on the domain at the N-terminus, NBS-LRR proteins are divided into two sub-classes. One class is defined by a domain that has homology to Drosophila Toll and mammalian interleukin (IL)-1 receptors (TIR) and the other class by a coiled-coil domain (CC). Examples of Avr-R protein pairs, identified in genetic studies of the *Arabidopsis-Pseudomonas syringae* system, are avrRpm1 recognised by RPM1 (CC-NBS-LRR type) and avrRps4 recognised by RPS4 (TIR-NBS-LRR type) (Dangl et al., 1992; Hinsch and Staskawicz, 1996). By introducing *avrRpm1* or *avRps4* into the virulent strain *Pseudomonas syringae* pathovar *tomato* DC3000 (*Pst*), both resulting strains (*Pst*-avr1 and *Pst*-avr4) are converted into avirulent pathogens in *Arabidopsis* plants that express the corresponding R protein.

Avr genes are defined by their ability to induce disease resistance in host plants but subsequent studies demonstrated that *Avr* genes in the absence of corresponding *R* genes confer a selective advantage to the pathogen as they act as virulence factors (Kearney and Staskawicz, 1990; Ritter and Dangl, 1995). Bacterial pathogens of animals and plants utilise both a type III secretion pathway to deliver *Avr* gene products into the host cell (Hueck, 1998).

It was assumed that the recognition process between the products of *R* and *Avr* genes is based on a direct interaction. However, molecular characterisation of corresponding R and Avr protein pairs indicate that this event is rather an exception than the rule. Based on these observations the guard hypothesis postulated that Avr proteins (as their function as virulence factors) bind to plant virulence targets (Dangl and Jones, 2001). The role of R proteins is in detection of this Avr protein-virulence target complexes rather than the perception of the Avr protein alone. Thus, R proteins may monitor the binding status or stability of plant virulence targets ("guardees"). The guard hypothesis is supported by various recent findings. In the *Arabidopsis-Pseudomonas* interaction, RIN4 (RPM1-interactor protein4) was identified as guardee targeted by the two sequence unrelated virulence factors avrRpm1 and avrB (Mackey et al., 2002). RIN4 was shown to interact with avrB and avrRpm1 but also with the CC-NBS-LRR protein RPM1 conferring resistance against *Pseudomonas syringae* expressing *avrRpm1* or *avrB*. Reduction of RIN4 protein levels hampers resistance to both bacterial strains, indicating that RIN4 is essential for RPM1 mediated resistance. The guard hypothesis made it plausible how a limited number of approximately 128 NBS-LRR type genes in the *Arabidopsis* genome (Initiative, 2000; Dangl and Jones, 2001) can confer resistance to numerous pathogen races. As R proteins may be guarding a limited number of plant components that are the preferred targets of multiple virulence factors, R proteins should be able to detect invasion of multiple races of pathogens.

1.2.3 Basal resistance

Even when plants are attacked by virulent pathogen (not detected by R proteins), pathogen growth is to some extent restricted. This phenomenon, called basal resistance, became apparent with the identification of "enhanced disease susceptibility" mutants that allowed an even stronger development of disease than wild-type susceptible hosts (Glazebrook et al., 1996). Although, virulent pathogens escape *R* gene-mediated recognition, they trigger a delayed and weak defence response with similarity to *R*-mediated defence (e.g. SA accumulation and host transcriptional reprogramming but no HR). Thus R protein action ensures that defence responses are triggered in a rapid and strong manner, thus preventing host colonisation.

1.2.4 Systemic acquired resistance and the role of salicylic acid

A successful local defence response in *R*-mediated resistance not only leads to local resistance but mediates enhanced resistance to subsequent infections in previously unchallenged parts of the plant. This type of resistance, often referred to as systemic acquired resistance (SAR), is effective against a broad range of biotrophic pathogens (Ryals et al., 1996). The defence signalling molecule SA accumulates locally in *R*-mediated resistance and is then allocated to systemic leaves via the phloem (Metraux et al., 1990; Shulaev et al., 1995). The coincidence of SA accumulation in systemic leaves and the establishment of SAR led many researchers to believe that SA is the SAR mediating signal. Results from grafting experiments between wild-type and transgenic tobacco expressing the bacterial SA degrading enzyme, NahG, led to the conclusion that SA is not the SAR signal as it was found that the NahG rootstock (SA deficient) was still able to produce and translocate the SAR signal to the wild-type rootstock scion (Vernooij et al., 1994). The reciprocal grafting experiment demonstrated that the NahG systemic scion was unable to perceive the SAR signal emitted from the wild-type scion, indicating that SA accumulation in the systemic tissue is essential for the establishment of SAR.

Consistent with the requirement for SA in SAR establishment, spray application of SA or its synthetic analogue BTH (benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester) induces SAR and transcriptional activation of a typical set of *PR* genes. Recent publications revealed that SA-mediated redox changes activate the key SA-response regulator, NPR1 (Non-expresser of Pathogenesis-Related genes1), by shifting NPR1 from its inactive oligomeric to its active monomeric form (Mou et al., 2003). Upon its activation, NPR1 binds to TGA transcription factors and stimulates the DNA-binding activity of these transcription factors to promoter elements of SA-responsive genes, resulting in *PR* gene up-regulation (Despres et al., 2003).

1.3 SA-independent signalling

The findings that SA deficiency in plants only partially compromises local resistance, the relatively late accumulation of SA and the SA-independent nature of the SAR signal demonstrate that SA-independent signalling pathways exist.

1.3.1 Early signalling events

Early cellular re-programming events, preceding SA signalling, are induced upon pathogen recognition. Changes in the ion permeability of the plasma membrane resulting in influxes of calcium (Ca^{2+}), protons (H^{+}) and an efflux of potassium (K+) and chloride (Cl^{-}) ions are one of the earliest signalling events after pathogen exposure. Increased intracellular Ca^{2+} levels are upstream of the production of reactive oxygen species (ROS) (Ligterink et al., 1997; Grant et al., 2000b), a process known as oxidative burst. ROS are produced during the oxidative burst by plasma-membrane-bound NADPH oxidases (Torres et al., 2002), cell wall attached peroxidases (Kawano, 2003) and apoplast-located amine oxidases (Allan and Fluhr, 1997). There are several roles discussed for ROS, including direct pathogen toxicity (Bussink and Oliver, 2001) and the reinforcement of plant cell walls by cross-linking of cell wall polymers (Bradley et al., 1992). The oxidative burst also triggers a change of the cellular redox status, thus connecting it to the SA signalling cascade. There is also evidence for ROS as SA-independent signal that controls early changes in gene expression via a mitogen-activated protein kinase (MAPK) (Grant et al., 2000a). Also, plant protein tyrosine phosphates (PTPs) are discussed to detect redox changes and subsequently regulate MAPKs which then might activate transcription factors (Gupta and Luan, 2003; Laloi et al., 2004). Another early signalling event is mediated by Ca^{2+}-dependent protein kinases (CDPKs). CDPKs are thought to sense the increase of the Ca^{2+} concentration triggered in response to different abiotic and biotic stresses and transduce this information via protein kinase activity to downstream signalling events (Ludwig et al., 2004). The biological relevance of CDPK-signalling was reinforced by the finding that CDPK-silenced *Nicotiana benthamiana* plants showed a weaker and delayed hypersensitive response upon race-specific elicitation in a *R*-mediated resistance response (Romeis et al., 2001).

1.3.2 Jasmonic acid and ethylene signalling

Plants defective in SA signalling are not more susceptible to the necrotrophic fungus *Botrytis cinerea*. Whereas *Arabidopsis* mutant plants with defects in jasmonic acid (JA) signalling (*coi1, coronatine insensitive1*) and ethylene (ET) perception (*ein2*, *ethylene insensitive2*) display an impaired resistance to *Botrytis cinerea.* In contrast to hyper-susceptible SA-deficient mutants, *coi1* plants are more resistant to virulent

stains of *Pseudomonas syringae*. The contrasting actions of SA and JA signalling were also reflected by gene expression analysis in which *coi1* and SA signalling mutants had mainly opposite effects on global gene expression (Glazebrook et al., 2003). Notably, comparative *Arabidopsis* gene expression profiling experiments after JA and SA application found that these signalling molecules also induce a common set of genes (Schenk et al., 2000). These results suggest a complex interplay of SA and JA/ET in modulating gene expression and resistance to plant pathogens.
The observation that in *Arabidopsis* JA/ET-controlled gene expression was induced by non-host but not by host biotrophic powdery mildew pathogens and that ectopic activation of JA/ET signalling conferred resistance to two biotrophic host pathogens suggests that host biotrophic pathogens either fail or actively repress the JA/ET signalling cascade (Zimmerli et al., 2004).

1.3.3 Evidence for lipid-derived signals in regulating plant defences

Besides JA that is derived from linolenic acid, other fatty acid-derived molecules have been implicated as modulators of plant defence signalling. Changes in abundance and composition of oxylipins that are derived from oxidation of fatty acids occurred upon pathogen attack or after wounding (Weber et al., 1997). Alméras et al. (2003) demonstrated that the electrophilic character of many oxylipins makes them potent transcriptional activators of certain marker genes for abiotic and biotic stress.
The identification of several *Arabidopsis* mutants deficient in aspects of lipid metabolism also points to an important role of lipid signalling in plant defence. For example, *Arabidopsis* mutant *ssi2* (*suppressor of SA-insensitivity2*) is deficient in oleate caused by a mutated gene encoding a stearoyl-acyl-carrier-protein desaturase and displays constitutive high levels of SA and a enhanced resistance to various biotrophic pathogens (Kachroo et al., 2001; Shah et al., 2001). Analysis of *ssi2NahG* plants revealed that elevated levels of SA were not essential for the *ssi2* phenotype. Deficiencies in the synthesis of polyunsaturated glycerol lipids in the double mutant of *fatty acid desaturase7* (*fad7*) and *fad8* resulted in a partially defective oxidative burst, reduced cell death and impaired resistance to avirulent strains of *P. syringae* (Yaeno et al., 2004). Another protein, ACD11 (accelerated cell death11) with *in vitro* sphingolipid transfer activity was shown to be a negative regulator of programmed cell death in *Arabidopsis* (Brodersen et al., 2002). The lethal recessive *acd11*

mutation triggered spontaneous cell death and constitutive up-regulation of a subset of defence genes including genes encoding the lipase-like proteins *EDS1* and *PAD4* (*EDS1/PAD4* regulatory role in plant defence is discussed in sections 1.5 and 1.6). Spontaneous cell death was abolished in *acd11NahG* but can be restored by BTH treatment. Notably, BTH induced cell death did not occur in *acd11eds1* and only partially in *acd11pad4*.

Evidence for the role of lipid-derived molecules in SAR signalling comes from the finding that a mutation in *DIR1* (*Defective in induced resistance1*) encoding a lipid transfer-like protein prevents the emission of a yet unidentified SAR signal after an otherwise intact local defence response. Similarly, mutations in the *SFD1* (*Suppressor of fatty acid desaturase deficiency1*) gene, which affects plastidic glycerolipid composition, compromises the SAR response but not basal resistance to *P. syringae* (Nandi et al., 2004).

1.4 Transcriptional reprogramming during plant defence responses

The complexity of signalling events following pathogen recognition with multiple signalling molecules and regulators is so immense that a global view on changes at the level of the metabolome or proteome is not yet technically feasible. However, development of large scale gene expression profiling technologies, in particular the emergence of oligonucleotide arrays, allows monitoring of transcriptional reprogramming during plant defence on a genome-wide scale (Redman et al., 2004).

In the following overview I will focus on studies performed on *Arabidopsis* as high quality, large scale and comparable gene expression profiling data sets are most advanced for this plant species. Examining the transcriptional changes in *Arabidopsis* upon challenge with different pathogens revealed that up to 23% of the total genes had altered transcript levels (Scheideler et al., 2002; Tao et al., 2003).

The earliest transcriptional changes are thought to be triggered by recognition of PAMPs. Zipfel et al. (2004) reports that treatment with the bacteria derived flagellin led to an up-regulation of 966 of approximately 23000 monitored *Arabidopsis* genes within 30 minutes (min) of application. Consistent with the idea that the first transcriptional changes are triggered by PAMP recognition is the finding that the transcriptional profiles of plants treated with *Pst*-hrpA$^-$ (mutant strain unable to deliver

type III effectors), *Pst* or *Pst*-avr1 do not differ considerably within first 2 h but at later time points (de Torres et al., 2003). *Pst*-avr1 triggered changes in gene expression were not observed before 3 h.

Induced non-host, basal and *R* gene-mediated resistance share common signalling events (e.g. Ca^{2+}-fluxes, ROS burst, SA induction). These similarities are reflected by the observation that all three defence systems induce and repress common sets of genes (Maleck et al., 2000; Tao et al., 2003; Zipfel et al., 2004).

Large differences in the host transcriptional profiles after infiltration of virulent and avirulent *P. syringae* strains were only observable at early time points (3, 6 and 9 h) but the profile of the compatible interaction at later time points (30 h) were similar to profile of the incompatible interaction at earlier time points (9 h) (Tao et al., 2003). Thus, differences in the transcriptional profiles between *R*-meditated and basal resistance appear to be quantitative rather than qualitative. The action of R proteins seems to accelerate and amplify transcriptional reprogramming of basal defence responses.

Although R proteins of the TIR- and CC-NBS-LRR class differ in their dependency on some signalling components, their action induces and represses a common set of genes. This was recently demonstrated by comparing large-scale gene expression profiles of RPP4- (Recognition of Peronospora parasitica4; TIR-NBS-LRR type), RPP7- and RPP8- (both CC-NBS-LRR type) mediated resistance responses to *Peronospora parasitica* (Eulgem et al., 2004).

1.5 *EDS1* and *PAD4* are positive regulators in plant defence signalling

Genetic screens in *Arabidopsis* for mutants with altered defences led to the identification of several important resistance signalling components. *EDS1* was first identified as a mutant compromised in *R*-mediated resistance to *Peronospora parasitica* (Parker et al., 1996). *PAD4* was discovered in a screen for mutants with defects in basal resistance to virulent *P. syringae* pathovar *maculicola* (*Psm)* (Glazebrook et al., 1996). Since their discovery nearly a decade ago, a great amount of knowledge accumulated about their important role in defence signalling.

EDS1 and *PAD4* are both required for resistance to various classes of pathogens (oomycetes, bacteria and viruses) recognised by TIR-NBS-LRR type R proteins

(Parker et al., 2000; Peart et al., 2002). Although, CC-NBS-LRR-mediated resistance signalling is normally dependent on NDR1 (Non-race specific Disease Resistance1) and independent of EDS1 and PAD4 at least one exceptions was found with CC-NBS-LRR type R protein HRT mediating viral resistance in an EDS1/PAD4-dependent manner (Chandra-Shekara et al., 2004). Also two CC-domain containing proteins with predicted trans-membrane domain, RPW8.1 and RPW8.2, conferring resistance to powdery mildew pathogens, depend on EDS1 and PAD4 (Xiao et al., 2003; Xiao et al., 2005).

Characteristically, TIR-NBS-LRR type R protein-mediated resistance is totally abolished in *eds1* null mutants but still partially functional in *pad4* mutant lines. This was illustrated well in the different phenotypes of *eds1* and *pad4* to avirulent *P. parasitica* strains. In contrast to strictly delimited HR in wild-type, pathogen growth is unimpeded in *eds1* whereas in *pad4* a delayed HR response allows hyphal growth leading to trailing plant cell necrosis (Feys et al., 2001). Further, SA accumulation in TIR-NBS-LRR-mediated resistance was totally abolished in *eds1* but only partially disabled in *pad4* (Feys et al., 2001). Similarly, the ROS burst was found to be still intact in *pad4* but not in *eds1* plants (Rusterucci et al., 2001).

Constitutive resistance triggered by deregulated TIR-NBS-LRR type R proteins was found to be dependent on EDS1 and PAD4 (Shirano et al., 2002; Zhang et al., 2003), suggesting a signalling role for *EDS1/PAD4* genetically down-stream of R protein action. Further evidence for an EDS1/PAD4 function downstream of R protein action comes from analysis of *BONZAI1* (*BON1*) encoding a calcium-dependent phospholipid-binding protein. *BON1* is a negative regulator of the *R* gene *SNC1*. The *bon1* mutation results in *SNC1*-mediated constitutive defence responses and growth defects which require *EDS1*, *PAD4* and SA accumulation (Yang and Hua, 2004).

Studies on *Arabidopsis* genes that negatively effect the EDS1/PAD4 pathway were valuable to elucidate the role of EDS1/PAD4 in transducing ROS and SA defence signals. The lesion-mimic mutant *lsd1* (*lesion simulating disease resistance response1*) displays a deregulated cell death response (run away cell death) upon various abiotic and biotic stresses (Dietrich et al., 1994). The deregulated cell death in *lsd1* is caused by its inability to restrict ROS-derived signals. Epistatic analysis revealed that both *EDS1* and *PAD4* are necessary for *lsd1* conditioned run away cell death even in response to an artificial provision of ROS or an SA analog (Rusterucci et al., 2001). Cooperation of ROS and SA is known to be important in triggering

resistance to pathogens (Shirasu et al., 1997). Thus, it was proposed that EDS1/PAD4 regulate an ROS/SA signal amplification loop under negative control of LSD1 (Rusterucci et al., 2001).

MPK4 (MAP kinase4) was recently found to negatively regulate SA accumulation (Petersen et al., 2000) and positively regulate JA/ET signalling both in an EDS1- and PAD4-dependent manner (P. Brodersen and colleagues, personal communication). Thus, EDS1 and PAD4 might modulate the previously discussed antagonism between SA and JA/ET signalling.

While expression of local resistance and plant cell death triggered by CC-NBS-LRR R proteins upon pathogen recognition is the same as wild-type in *eds1* and *pad4*, these mutants fail to establish SAR. Experiments with phloem exudates indicate that *eds1* is defective in emitting SAR signals from local tissue but also in its perception in systemic tissue (L. Jorda, unpublished). Although *eds1* compared to *pad4* is more defective in TIR-mediated resistance, their deficiency in basal resistance seems to be equivalent (Feys et al., 2001; Rusterucci et al., 2001).

Jirage et al. (1999) found that PAD4 function is redundant in the defence response to *Psm-avrRpt2* (recognised by CC-NBS-LRR R protein RPS2) but required in response to virulent *Psm*. Thus, it was proposed that PAD4 is required for amplification of weak signals that occur by infection of virulent pathogens. In contrast, RPS2-derived signals are strong enough to trigger defence responses and thus do not require amplification by PAD4 (Jirage et al., 1999).

Disabled SA accumulation can only partially explain the *eds1* and *pad4* mutant phenotypes as *R* gene-mediated resistance in *eds1* and *pad4* is more severely compromised than in the SA-deficient *sid2* or *NahG* plants (Feys et al., 2001 and this study).

EDS1 and PAD4 have pockets of homology to eukaryotic lipases (Falk et al., 1999; Jirage et al., 1999). It was therefore suggested that they might play a role in lipid based signalling by hydrolysing a lipid substrate. Lipase activity has indeed been reported for the EDS1/PAD4-related *Arabidopsis* protein associated with senescence control, SAG101 (Senescence Associated Gene101) (He and Gan, 2002). Despite trying various potential substrates under different reaction conditions, S. Rietz and colleagues (MPIZ, Cologne) did not observe lipase activity for EDS1, PAD4 and SAG101. Thus, the biochemical nature of EDS1/PAD4 derived signal remains elusive. Recent findings demonstrate that EDS1, PAD4 and SAG101 work in concert

to regulate basal and TIR-NBS-LRR resistance (Feys et al., submitted). A common signalling function of the three lipase-like proteins is further supported by the finding that EDS1 forms dimeric and potentially multimeric complexes with PAD4 and SAG101 inside the plant cell (Feys et al., 2001 and Feys et al., submitted).

1.6 EDS1 and PAD4-controlled gene expression

As observed for other signalling components involved in plant defence responses, mRNA and protein levels of EDS1 and PAD4 are induced upon pathogen challenge (Feys et al., 2001). Notably, EDS1 and PAD4 proteins exist in the cell prior to pathogen attack, potentially transducing the early defence promoting signals.

Furthermore, it was shown that *EDS1* and *PAD4* positively influence mutually their mRNA accumulation upon pathogen challenge (Jirage et al., 1999; Feys et al., 2001; Eulgem et al., 2004). Consistent with their signalling role closely downstream of TIR-NBS-LRR function and in basal resistance, EDS1 and PAD4 are required for pathogen-triggered gene induction from early time points on (Zhou et al., 1998; de Torres et al., 2003).

Glazebrook et al. (2003) applied the microarray technology to study transcriptional changes in *Arabidopsis* wild-type and mutant plants upon inoculation with virulent *P. syringae*. Their study revealed that mutations in *SID2* and *PAD4* effected common but also different sets of genes. The common set of *SID2* and *PAD4*-controlled genes includes *PR1* and most likely represents genes that are induced by SA. The set of genes which is effected by *pad4* but not by *sid2* was predicted to function in a yet unknown signalling pathway.

By monitoring transcriptional changes in 8000 genes during RPP4-signalling, Eulgem et al. (2004) recently identified seven *PAD4* co-regulated genes (including *EDS1*) with no requirement for *NDR1*, *NPR1* or SA (*NahG*) but with suppressed mRNA levels in *pad4-1*. The authors predict that these genes are involved in the EDS1/PAD4 signalling process but they did not demonstrate the biological relevance of these *PAD4*-coregulated genes in pathogen resistance.

I was interested in identifying essential components of this EDS1/PAD4-regulated SA-independent defence pathway.

1.7 Thesis aims

As described, several lines of evidence point to the existence of an *EDS1/PAD4*-controlled signalling pathway in *Arabidopsis* that functions independently of SA. This mainly uncharacterised *EDS1/PAD4* pathway is important for full expression of local *R* gene-triggered and basal resistance as well as for systemic defence responses. However, the nature of this signalling pathway or the genetic components involved are largely unknown. I intended to characterise this important *EDS1/PAD4*-conditioned pathway by means of comparative transcriptional profiling of defence responses in wild-type, *eds1* and *pad4*. In particular, I aimed to combine data derived from the transcriptional profiling experiment with the use of *Arabidopsis* insertion mutant resources to identify, in a targeted approach, novel essential regulators in the *EDS1/PAD4*-controlled defence signalling pathway.

2. Materials and Methods

The Materials and Methods section is subdivided into three 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) and microarray-related methods are addressed in the third part (2.3).

2.1 Materials

2.1.1 Plant materials

Arabidopsis thaliana wild-type and mutant lines used in this study are listed in the following two tables.

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

Accession	Abbreviation	Original source
Columbia-0	Col-0	J. Dangl[a]
Landsberg-erecta-0	Ler-0	Nottingham *Arabidopsis* Stock Centre[b]
Wassilewskija-0	Ws-0	K. Feldmann[c]

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

[b]Nottingham, UK

[c]University of Arizona, Tucson, USA

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

Plant line	Accession	Description	Original source
eds1-1	Ws-0	EMS	Parker et al.,1996
eds1-2	Ler-0	FN	Falk, et al., 1999
pad4-1	Col-0	EMS	Glazebrook et al., 1997
pad4-2	Ler-0	FN	Jirage et al., 1999
pad4-5	Ws-0	T-DNA	Feys et al., 2001
sid2-1	Col-0	EMS	Wildermuth et al., 2001
Atfmo-1	Col-0	SALK_026163 (T-DNA)	This study[a]
Atfmo-2	Ler-0	GT_3_108523 (DS)	This study[b]
Atnud2.1	Col-0	GABI_158B10 (T-DNA)	This study[c]
Atnud4.1-1	Col-0	SALK_046441 (T-DNA)	This study[a]
Atnud4.1-2	Col-0	SALK_104293 (T-DNA)	This study[a]
Atmrp7	Col-0	SALK_120950 (T-DNA)	This study[a]
Atprk	Col-0	SAIL_46_E06 (T-DNA)	This study[a]
Atltp	Col-0	SALK_109557 (T-DNA)	This study[a]
Atgh	Col-0	SALK_038957 (T-DNA)	This study[a]
AtFMO::GUS	Col-0	Promoter-GUS	J. Mundy, unpublished

[a] SALK collection (Alonso et al., 2003) distributed by Nottingham Arabidopsis Stock Centre.

[b] Ds-insertion line (Sundaresan et al., 1995) distributed by Nottingham Arabidopsis Stock Centre.

[c] GABI-Kat, Max-Planck-Institute for Plant Breeding Research (Rosso et al., 2003).

2.1.2 Pathogens

2.1.2.1 *Peronospora parasitica*

Table 2.3 *Peronospora parasitica* isolates used in this study.

Isolate	Original source	Reference
Cala2	Oospore infection of a single seedling	(Holub et al., 1994a)
Emco5	Oospore infection of a single seedling	(Holub et al., 1994a)
Noco2	Conidia isolated from a single seedling	(Parker et al., 1993)

Peronospora parasitica isolates and their interaction with *Arabidopsis* ecotypes

***Arabidopsis* ecotype**	***Peronospora parasitica* isolate**		
	Cala2	Emco5	Noco2
Col-0	incompatible (*RPP2*)	intermediate (sporulation on cotyledons)	compatible
Ler-0	compatible	incompatible (*RPP8*)	incompatible (*RPP5*)
Ws-0	incompatible (*RPP1A*)	compatible	incompatible (*RPP1*)

2.1.2.2 *Pseudomonas syringae* pv. *tomato*

Pseudomonas syringae pv. *tomato* strain DC3000 expressing the avirulence determinants *avrRps4* (Hinsch and Staskawicz, 1996) or *avrRpm1* (Grant et al., 1995) from the broad host range plasmid pVSP61 (Innes et al., 1993) or DC3000 containing empty pVSP61 were used throughout this study. The *P. syringae* pv. *tomato* isolates were originally obtained from R. Innes (Indiana University, Bloomington Indiana, USA).

2.1.2.3 *Golovinomyces orontii*

Inoculum of *Golovinomyces orontii* was kindly provided by the group of R. Panstruga (Max-Planck-Institute for Plant Breeding Research).

2.1.3 Oligonucleotides

Listed below are primers used in this study which were synthesised by Operon or Metabion. 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
ActF	TGCGACAATGGAACTGGAATG	*Actin2* RT-PCR
ActR	CTGTCTCGAGTTCCTGCTCG	*Actin2* RT-PCR
LB	ATATTGACCATCATACTCATTGC	LB primer for GABI-KAT
LBa1	TGGTTCACGTAGTGGGCCATCG	LB primer for SALK
M128	CACCATTGTGCAAGCTTTTCCTCCT	GT_3_108523 (*Atfmo-2*)
M129	GTTCATCGGTGATGGCGAAACTCCTC	GT_3_108523 (*Atfmo-2*)
M130	TCGTTTCCGTCCCGCAAGT	GT_3_108523 (*Atfmo-2*) Ds3'3 for detection of DS element (Sundaresan et al., 1995)
M143	TGTTCAGGGTGTGGCTCAGTG	SAIL_46_E06 (*Atprk*)
M144	GAAGAAGATATCGCTTTGGCCT	SAIL_46_E06 (*Atprk*)
M145	TTGATTGTTGTTGTCTTTTGCTTC	SALK_104293 (*Atnud4.1-2*)
M146	CGATGGCAAGTTTTACAGTGG	SALK_104293 (*Atnud4.1-2*)
M147	CGTACGAGAGAATATAAGAGAAAAG	*sid2-1* detection
M152	GCAAATTCACTCTCCTCGCCAC	*sid2-1* detection
MB111	CCAATAAACAAAGGGCACGGA	SALK_046441 (*Atnud4.1-1*)
MB112	CCACTCCTCTCCTGGACAACG	SALK_046441 (*Atnud4.1-1*)
MB27	GATCGTTTCATTTTCAATGACTTG	SALK_109557 (*Atltp*)
MB28	AAGGTGACTGAAAAATCACTGC	SALK_109557 (*Atltp*)
MB42	TTAAGCAGTCATATCTTCTTTTTCTTC	*AtFMO* qRT-PCR
MB46	TGCTGCAAATCATCAAGGCAA	SALK_120950 (*Atmrp7*)
MB47	GGCACTCTTCTTTTCAGTGTGGC	SALK_120950 (*Atmrp7*)
MB53	GGAAGCGGATAAAGGGATGATCC	*AtFMO* qRT-PCR
MB58	TCAATGGATGGATTGTTCCCC	SALK_026163 (*Atfmo-1*)
MB59	GGCAACAATTAAACAGTTACTCGCA	SALK_026163 (*Atfmo-1*)
MB60	TCATGGCTCTCATAACATGCAA	SALK_038957 (*Atgh*)
MB61	CCAAAATTCTCTGGTCATATCCG	SALK_038957 (*Atgh*)
MB97	TCCAAGCTTCCCTTCACGTCTC	GABI_158B10 (*Atnud2.1*)
MB98	AGCCCATCGGCAAGCTTTAAC	GABI_158B10 (*Atnud2.1*)

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 10 x reaction buffer.

2.1.4.2 Nucleic acid modifying enzymes

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

Taq DNA polymerase (home made)
PfuTurbo® DNA polymerase (Stratagene®, Heidelberg, Germany)
T4 DNA ligase (Roche, Mannheim, Germany)
DNaseI (Roche, Mannheim, Germany)
SuperScript™ II RNase H^- Reverse Transcriptase (Invitrogen™ , Karlsruhe,Germany)
Gateway™-Technology
LR Clonase™ Enzyme mix (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

Ampicillin (Amp)	100 mg/ml in H_2O
Carbenicillin (Carb)	50 mg/ml in H_2O
Gentamycin (Gent)	15 mg/ml in H_2O
Kanamycin (Kan)	50 mg/ml in H_2O
Rifampicin (Rif)	100 mg/ml in DMSO
Tetracycline (Tet)	12.5 mg/ml in 70 % ethanol

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

2.1.7 Buffers and solutions

General buffers and solutions are displayed in the following listing. All buffers and solutions were prepared with Milli-Q® water. Buffers and solutions for molecular

biological experiments were autoclaved and sterilised using filter sterilisation units, respectively. Buffers and solutions not displayed in this listing are denoted with the corresponding methods.

DEPC-H_2O	Diethylpyrocarbonate	0.1	% in H_2O
	Well mixed, left O/N and autoclaved for 30 min.		
DNA extraction buffer (Quick prep)	Tris	200	mM
	NaCl	250	mM
	EDTA	25	mM
	SDS	0.5	%
	pH 7.5 (HCl)		
DNA gel loading dye (6 x)	Sucrose	4	g
	EDTA (0.5 M)	2	ml
	Bromphenol blue	25	mg
	H_2O to 10 ml		
Ethidium bromide stock solution	Ethidium bromide	10	mg/ml in H_2O
	Diluted 1:40000 in agarose solution		
GUS staining solution	Na_2HPO_4 (1M)	11.54	ml
	NaH_2PO_4 (1M)	8.46	ml
	$K_3Fe(CN)_6$ (0.05 M)	2	ml
	$K_4Fe(CN)_6$ (0.05 M)	2	ml
	EDTA (0.05 M)	4	ml
	Triton X-100 (10 %)	2	ml
	H_2O	90	ml
	pH 7.0		
	Prior to use add 5 ml methanol and 550 µl X-Gluc stock solution (50 mg/ml DMF) to 50 ml staining solution.		

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.		
PCR reaction buffer (10 x)	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 with *Taq* DNA polymerase.		
BTH solution	BTH (commercial product BION®, Syngenta) was resuspended in dH_20 to the desired concentration prior use.		

2.2 Methods

2.2.1 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.

2.2.2 Generation of *Arabidopsis* F_1 and F_2 progeny

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

2.2.3 Inoculation and maintenance of *P. parasitica*

P. parasitica isolates were maintained as mass conidiosporangia cultures on leaves of their genetically susceptible *Arabidopsis* ecotypes over a 7 day cycle. Leaf tissue from infected seedlings was harvested into a 50 ml Falcon tube 7 days after inoculation. Conidiospores were collected by vigorously vortexing harvested leaf material in dH_2O for 15 seconds and after the leaf material was removed by filtering through miracloth (Calbiochem) the spore suspension was adjusted to the desired concentration using a Neubauer counting cell chamber. Plants to be inoculated were grown under short day conditions as described above. *P. 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 hour light period. For long term storage *P. parasitica* isolate stocks were kept as mass conidiosporangia cultures on plant leaves at -80° C.

2.2.4 Quantification of *P. parasitica* sporulation

To determine sporulation levels, seedlings were harvested 5-7 d after inoculation in a 50 ml Falcon tube and vortexed vigorously in 5 – 10 ml water for 15 seconds. 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, two pots containing approximately 30 seedlings were

infected per experiment and harvested spores from all seedlings of each pot were counted twice with sporulation levels expressed as the number of conidiospores per gram fresh weight.

2.2.5 Lactophenol trypan blue staining

Lactophenol trypan blue staining was used to visualise necrotic plant tissue and *P. parasitica* mycelium (Koch and Slusarenko, 1990a). 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 minutes 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. For Figure 3.13 infected leaves were examined under UV-light to exhibit cell death-associated fluorescence.

2.2.6 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 hours before storing at 4° C and refreshed weekly.

2.2.7 *P. syringae* pv. *tomato* inoculations and growth assay

P. syringae pv. *tomato* cultures were started from a small amount of bacteria grown on NYG agar plates containing rifampicin (100 µg/ml) and kanamycin (50 µg/ml) in 20 ml NYG broth containing rifampicin (100 µg/ml) and kanamycin (50 µg/ml). The 20 ml cultures were incubated overnight at 28° C and 200 rpm in a rotary shaker. For hand infiltrations applied for the microarray samples see section 2.3.1. For growth assays, 2.5 ml of the overnight cultures were used to inoculate 50 ml of NYG broth in 300 ml Erlenmeyer flasks supplemented with antibiotics. The flasks were incubated at 28° C and 200 rpm in a rotary shaker for 3 hours. The required OD_{600} reading at this time point was 0.2. Cultures were transferred to sterile 50 ml Falcon tubes and pelleted at 4500 rpm for 10 minutes at 20° C. Bacteria were washed by resuspending the pellet

in 40 ml of 10 mM sterile $MgCl_2$ and subsequent centrifugation at 4500 rpm for 10 minutes at 20° C. The supernatant was promptly removed and each pellet resuspended in 50 ml of sterile 10 mM $MgCl_2$. For vacuum-infiltration the concentration of bacteria was adjusted to 5 x 10^5 cfu/ml in 600 ml of 10 mM $MgCl_2$ containing 0.002 % Silwet L-77 (Lehle seeds, USA).

Single pots of nine 4- to 5-week old plants, grown under short day conditions were routinely used for bacterial growth assays. Two hours before vacuum-infiltration, plants were watered and kept under a dH_2O-humidified lid. Plants were vacuum-infiltrated with bacteria by inverting the pots and carefully submerging all leaf material in 600 ml of diluted bacterial suspension contained within a plastic exsiccator. Vacuum was applied and maintained within the exsiccator for 3 minutes before being gradually released. Periodic swirling and tapping of the exsiccator helped to dislodge any air bubbles that accumulated at the surface of the leaves. Any non-infiltrated leaves remaining at this stage were removed by hand. Excess of bacterial solution was removed by inverting the pots and gently dipping the plants in water.

Day zero (T0) samples were taken one hour after infiltration by using a cork borer (∅ 0.55 cm) to excise and transfer four leaf discs from four independent plants to a 1.5 ml centrifuge tube, resulting in a total excised area of 1 cm^2. This was repeated with a second batch of four leaf discs from four independent plants. The discs were then macerated with a plastic pestle in 100 µl of sterile 10 mM $MgCl_2$. Subsequently, 900 µl of sterile 10 mM $MgCl_2$ were added (10^{-1} dilution) and 100 ml of each sample were plated onto NYG agar (Rif^{100}, Kan^{50}). Day three (T3) samples were taken in an identical manner to that of T0 except that four leaf discs from four independent plants per genotype were taken in triplicates. For each sample a dilution series ranging between 10^{-1} and 10^{-7} was made and 15 µl aliquots from each dilution were spotted sequentially onto a single NYG agar plate (Rif^{100}, Kan^{50}). All bacteria plates were incubated at 28° C for two days before colony numbers were counted.

2.2.8 *A. thaliana* powdery mildew *Golovinomyces orontii*

Powdery mildew *G. orontii* was propagated on *A. thaliana* ecotype Col-0 plants cultivated at 20° C and 16 h light/ 8 h darkness, 80 % humidity in a growth chamber.

2.2.9 Isolation of *Arabidopsis* genomic DNA (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.

2.2.10 Isolation of total RNA from *Arabidopsis*

Total RNA was prepared from 4-week old plant materials. Liquid nitrogen frozen leaf samples (approximately 80-100 mg) were homogenized 15 seconds 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 homogenisation samples were kept frozen in liquid nitrogen until the next step of the extraction procedure. 1 ml of RNAwiz® Reagent (Ambion) was added and samples were homogenised by vortexing for 1 minute. For dissociation of nucleoprotein complexes the homogenised samples were incubated for 5 minutes at room temperature. 0.2 ml of chloroform was added and samples were shaken vigorously for 20 seconds. After incubation for 10 minutes at room temperature samples were centrifuged for 15 minutes at 12000 *g* and 4° C. The upper aqueous, RNA containing phase was transferred to a fresh 2 ml microcentrifuge tube containing 0.5 ml DEPC-water. The RNA was precipitated by adding 1 ml isopropanol, subsequent mixing and incubation for 10 minutes at room temperature. Samples were centrifuged for 15 minutes at 12000 *g* and 4° C. The supernatant was removed and the pellet was washed by vortexing in 1 ml of ice cold 75 % ethanol. Samples were again centrifuged for 5 minutes at 12000 *g* and 4° C. Supernatant was discarded and pellets were allowed to air-dry for 10 minutes and

dissolved in 25 µl DEPC-water. Samples were immediately transferred to and stored at -80° C.

2.2.11 Polymerase chain reaction (PCR)

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

Reaction mix (20 µl total volume):

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

Thermal profile

Stage	**Temperature (°C)**	**Time period**	**No. of cycle**
Initial denaturation	94	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

2.2.12 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 µg template total RNA, 1 µl primer $dT_{18}V$ (0.5 µg/µl, V standing for an variable nucleotide), 5 µl dNTP mix in a volume of 13.5 µl (deficit made up with DEPC-water). Samples were incubated at 65° C for 10 minutes. Subsequently, the reactions were filled up to a total volume of 20 µl with 4 µl of 5 x reaction buffer, 2 µl of 0.1 M DTT and 0.5 µl reverse transcriptase. The reactions were incubated at 42° C for 60 minutes before the enzyme was heat inactivated at 70° C for 10 minutes. For subsequent normal PCR, 1 µl of the above RT-reaction was used as cDNA template. As template total RNA for the reverse transcription reaction was not DNase treated, a control reaction for each RNA preparation was performed in which the reverse transcription reaction was incubated without reverse transcriptase enzyme (enzyme replaced by equal volume of DEPC-water) to check in the following PCR for contamination by genomic DNA.

2.2.13 Quantitative real-time polymerase chain reaction (qRT-PCR)

A quantitative real-time PCR kit (Brilliant SYBR Green QPCR Core Kit, Stratagene) was used to determine the amount of transcript accumulation of a gene of interest. Reactions were carried out according to the manufacturer's protocol. Primer combinations that specifically amplify the investigated gene and a gene serving as an internal standard were used in independent reactions performed on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, California, USA). Data were analysed by the comparative $\Delta\Delta C_T$ method (ABI PRISM 7700 User Bulletin).

Quantitative real-time PCR reaction mix :

Template cDNA	2 µl
Upstream primer (100 pmol/µl)	50 nM
Downstream primer (100 pmol/µl)	50 nM
dNTP-mix (dATP, dGTP, dCTP, dTTP)	0.2 mM (each)
Taq DNA polymerase (2.5 U)	2.5 U
PCR amplification buffer (10x)	1/10 of reaction volume

Glycerol solution (50%)	8 % (v/v)
DMSO (100%)	3 % (v/v)
Diluted SYBR green (1:3000)	2.5 µl
DEPC-water ad	50.0 µl

Thermal profile Stage:			
Initial denaturation	1x	95°C	10 min
Next three steps	50x		
Denaturation		95 °C	30 sec
Annealing		55 °C	30 sec
Extension		72 °C	1.5 min
Final extension	1x	72 °C	3 min

2.2.14 Plasmid DNA isolation

Standard alkaline cell lysis minipreps of plasmid DNA were carried out using the GFX™ micro plasmid prep kit from Amersham Biosciences according to the manufacturer´s instructions.

2.2.15 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 30 minutes.

2.2.16 Isolation of DNA fragments from agarose gel

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

2.2.17 DNA sequencing

DNA sequences were determined by the "Automatische DNA Isolierung und Sequenzierung" (Gross et al.; Hentrich; Vianello et al.; Lawton et al.; Tully et al.; Terras et al.; TolkerNielsen et al.; Heath; Wan et al.; Kawano) service unit at the MPIZ on Applied Biosystems (Weiterstadt, Germany) Abi Prism 377 and 3700 sequencers using Big Dye-terminator chemistry (Sanger et al., 1977).

2.2.18 Standard DNA sequence analysis

Sequence data were analysed mainly using SeqMan™ II version 5.00 (DNASTAR, Madison, USA), EditSeq™ version 5.00 (DNASTAR, Madison, USA) and Ione Manager 6 version 6.00 (Scientific and Educational software, USA).

2.2.19 Staining for beta-glucuronidase (GUS) activity

Plant material was covered with GUS-staining solution in appropriate reaction tubes. Open tubes were placed in an exsiccator and vacuum was applied for 3 - 5 minutes. Vacuum was released and this procedure was repeated twice. Tubes were closed and incubated at 37° C over night. After incubation of the leaves, the GUS staining solution was discarded. Plant material was rinsed with deionised water and tissues were cleared by placing them into 70 % ethanol. The ethanol was exchanged until tissues were completely cleared and GUS-staining was visible. Tissues were stored in 70 % ethanol until examined.

2.2.20 Determination of total salicylic acid levels in leaves

Extraction and quantification of total salicylic acid were performed in a modified way as described previously (Enyedi et al., 1992). 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™ (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. To each sample 0.5 ml of 0.1 M NaOAc pH 5.0 containing beta-glucosidase (20 U/ml; EC 3.2.1.21; almond, Sigma) was added and incubated at 37° C for 3 h. After incubation, 50 µl TFA (Trifluoroacetic acid) and 600 µl EtOAc (Ethyl acetate) was added and 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.

2.2.21 Identification of *Arabidopsis* insertion mutants

Insertion mutants corresponding to the *EDS1/PAD4*-dependent genes were identified using T-DNA express (http://signal.salk.edu/cgi-bin/tdnaexpress). To detect plants homozygous for the insertion, two separate PCRs were performed as described on the T-DNA express web page. One PCR was performed to detect the insertion and one to detect the wild-type allele. Table 2.6 lists the primer combinations used for the characterisation of the insertion mutants.

Table 2.6 Primer combinations used for the characterisation of the insertion mutants.

Mutant name	Mutant ID	T-DNA detection	Wild-type allele detection
Atfmo-1	SALK_026163 (T-DNA)	MB58/LBa1	MB58/MB59
Atfmo-2	GT_3_108523 (DS)	M128/M130	MB129/MB128
Atnud2.1	GABI_158B10 (T-DNA)	MB98/LB	MB97/MB98
Atnud4.1-1	SALK_046441 (T-DNA)	M111/LBa1	M111/M112
Atnud4.1-2	SALK_104293 (T-DNA)	M146/LBa1	M145/M146
Atmrp7	SALK_120950 (T-DNA)	MB46/LBa1	MB46/MB47
Atprk	SAIL_46_E06 (T-DNA)	-	M143/M144*
Atltp	SALK_109557 (T-DNA)	M27/LBa1	MB27/MB28
Atgh	SALK_038957 (T-DNA)	MB61/LBa1	MB61/MB60

* PCR for wild-type allele detection was repeated twice on independent DNA preparations.

To detect the *sid2-1* point mutation, the region around the point mutation was PCR amplified from genomic DNA (primers M147/M152) and the PCR products purified and sequenced.

2.2.22 Sequence analysis

The annotations of FMO-like protein sequences used for the construction of the phylogentic tree are listed in Table 2.7.

Table 2.7 Annotations of FMO-like amino acid sequences

Name	Annotation	Organism
HsFMO1	NP_002012; gi|4503755	*Homo sapiens*
HsFMO2	NP_001451; gi|4503757	*Homo sapiens*
HsFMO3	NP_001002294; gi|50541961	*Homo sapiens*
HsFMO4	NP_002013; gi|4503759	*Homo sapiens*
HsFMO5	NP_001452; gi|4503761	*Homo sapiens*
HsFMO6	XP_371326; gi|51458831	*Homo sapiens*
HsHomologue	NP_620139; gi|20270325	*Homo sapiens*

OsFMO1	XP_470552.1; gi|50920383	*Oryza sativa*
OsFMO2	NP_917203; gi|34911712	*Oryza sativa*
OsFMO 3	NP_919084; gi|34915254	*Oryza sativa*
OsFMO 4	NP_922668; gi|37536732	*Oryza sativa*
yFMO	NP_012046; gi|41629686	*Saccharomyces cerevisiae*
DmFMO1	NP_611859; gi|19922866	*Drosophila melanogaster*
DmFMO2	NP_610217; gi|19921694	*Drosophila melanogaster*
CeFMO1	NP_492038; gi|25141385	*Caenorhabditis elegans*
CeFMO11	NP_501968; gi|25150462	*Caenorhabditis elegans*
CeFMO12	NP_501972; gi|17541300	*Caenorhabditis elegans*
CeFMO13	NP_499356; gi|17555726	*Caenorhabditis elegans*
CeFMO14	NP_506370; gi|25145785	*Caenorhabditis elegans*
CeFMO15	NP_503352; gi|17561948	*Caenorhabditis elegans*
AtFMO1	gi|25513456	*Arabidopsis thaliana*
At5g45180		*Arabidopsis thaliana*
At5g61290		*Arabidopsis thaliana*
At1g62570		*Arabidopsis thaliana*
At1g12130		*Arabidopsis thaliana*
At4g28720		*Arabidopsis thaliana*
At1g62580		*Arabidopsis thaliana*
At1g04180		*Arabidopsis thaliana*
At1g62620		*Arabidopsis thaliana*
At5g43890		*Arabidopsis thaliana*
At1g12200		*Arabidopsis thaliana*
At5g07800		*Arabidopsis thaliana*
Yucca3	At1g04610	*Arabidopsis thaliana*
At1g48910		*Arabidopsis thaliana*
At1g62600		*Arabidopsis thaliana*
At1g62560		*Arabidopsis thaliana*
At1g65860		*Arabidopsis thaliana*
At2g33230		*Arabidopsis thaliana*
At1g21430		*Arabidopsis thaliana*
At1g62540		*Arabidopsis thaliana*
At1g63370		*Arabidopsis thaliana*
At1g12140		*Arabidopsis thaliana*
Yucca2	At4g13260	*Arabidopsis thaliana*
At5g25620		*Arabidopsis thaliana*
Yucca	At4g32540	*Arabidopsis thaliana*
At1g63340		*Arabidopsis thaliana*

Estimates of sequence divergence (%) were calculated with p-distance algorithm and illustrated using a neighbor-joining tree constructed with MEGA 2.1 software (Kumar et al., 2001).

2.3 Microarray analysis

2.3.1 Sample preparation

Fully expanded leaves of 4-week old plants were hand-infiltrated with a 10 mM $MgCl_2$ suspension of a bacterial strain at a density of 1 X 10^7 colony-forming units (cfu)/ ml using needleless syringes (Katagiri, 2002). The bacterial concentration was adjusted according to its corresponding optical density (OD_{600} 0.02). The leaf tissue was harvested at the times points indicated in Table 3.1 and immediately frozen in liquid nitrogen. The time of inoculation was 10:30 am (1.5 h after the light-period started) in both replicate experiments. Total RNA was prepared from frozen tissue using RNAwiz (Ambion, Austin, USA) as described in section 2.2.10. RNA quality and concentration was measured in a spectrophotometer according to the manufacturer's instructions (BioPhotometer, Eppendorf AG, Hamburg, Germany). The absorption ratio 260/280 of all 21 samples were between 1.9 - 2.1 indicating that good quality RNA was isolated (low protein contamination). All RNA samples were adjusted to a concentration of 1 µg/µl with DEPC-H_2O and aliquots were separated on an agarose gel to evaluate if the RNA is intact. Equal amounts of RNA were pooled from two independent experiments. Each independent experiment used two leaves (about 80-100 µg fresh weight) from two different plants per sample. Thus, the final sample was a pool of four leaves from four different plants derived from two independent experiments. Complementary RNA labelling, hybridisation and data collection from the hybridised GeneChip were performed by S. Debey in the Department “Molekulare Tumorbiologie und Tumorimmunologie” of Prof. Dr. J.L. Schultze at the University of Cologne according to the manufacturer's instructions (Affymetrix, Santa Clara, USA). In brief, total RNA was reverse transcribed using SuperScript™ II RNase H^- Reverse Transcriptase (Invitrogen™) and $T7(dT)_{24}$ primer. The first strand cDNA was used for double-strand cDNA synthesis. Purified double-strand cDNA was used to generate biotin-labelled cRNA by in vitro transcription reactions. cRNA was fragmented and

used for hybridisation to GeneChip Arabidopsis ATH1 Genome Array (Affymetrix, Santa Clara, USA). After the washing and staining procedure the arrays were scanned in an Agilent GeneArray Scanner (Agilent, Palo Alto, USA).

2.3.2 Data analysis

Data from each individual chip were normalised to each other by setting their average signal intensity to 100 (arbitrary units) using Microarray Suite 5.0 software (Affymetrix). This procedure termed “global scaling” was previously described (Zhu and Wang, 2000) and ensures comparability between chips.

The data for all 22746 probe sets (not including spike controls) represented on the GeneChip were extracted into one Excel spreadsheet. When an expression value was < 5, it was set to 5. This procedure prevented high ratio values for probe sets that were hardly expressed above the noise level. The noise value was below a signal intensity of 12 for all 21 arrays. Two classes of expression signal ratios were calculated (Table 2.8) and $\log_2$ transformed for each probe set. To describe the change in expression in wild-type samples upon the different treatments, of each probe set (gene) the signal value in pathogen-treated sample was divided by their signal value in the corresponding mock-treated sample resulting in “wild-type ratios”. To illustrate changes triggered by the infiltration procedure “WT Mg 3 h / WT NT 3 h” ratios were calculated. To illustrate the mutant effects on gene expression, corresponding signal values from mutant and wild-type were divided giving rise to the “mutant ratios”.

Table 2.8 Expressions ratios created and applied in the Data analysis of this study.

	Ratios	**Short term used in Figures**
wild-type ratios	WT avr1 3 h / WT Mg 3 h	WT avr1 3 h / WT Mg
	WT avr1 6 h / WT Mg 6 h	WT avr1 6 h / WT Mg
	WT avr4 3 h / WT Mg 3 h	WT avr4 3 h / WT Mg
	WT avr4 6 h / WT Mg 6 h	WT avr4 6 h / WT Mg
	WT Mg 3 h / WT NT 3 h	WT Mg 3 h / WT NT
	eds1 avr1 3 h / WT avr1 3 h	eds1 avr1 3 h / WT avr1

mutant ratios	eds1 avr1 3 h / WT avr1 3 h	eds1 avr1 3 h / WT avr1
	eds1 avr1 6 h / WT avr1 6 h	eds1 avr1 6 h / WT avr1
	eds1 avr4 3 h / WT avr1 3 h	eds1 avr4 3 h / WT avr4
	eds1 avr4 6 h / WT avr1 6 h	eds1 avr4 6 h / WT avr4
	eds1 NT 3 h / WT NT 3 h	eds1 NT 3 h / WT NT
	pad4 avr1 3 h / WT avr1 3 h	pad4 avr1 3 h / WT avr1
	pad4 avr1 6 h / WT avr1 6 h	pad4 avr1 6 h / WT avr1
	pad4 avr4 3 h / WT avr1 3 h	pad4 avr4 3 h / WT avr4
	pad4 avr4 6 h / WT avr1 6 h	pad4 avr4 6 h / WT avr4
	pad4 NT 3 h / WT NT 3 h	pad4 NT 3 h / WT NT

"Pathogen responsive" probe sets were selected in Excel and had to meet the following criteria that were similar as described in Tao et al., 2003: Two at least $\log_2$ +1 or $\log_2$ -1 expression changes in wild-ratios (pathogen treated versus mock treated). In this ratio calculation probe sets were only included when the larger value of the two expression signals was at least 50 (3839 probe sets met this criteria, Table 3.2). From the pathogen responsive gene set avr1- and avr-4 induced genes were selected. avr1-induced genes had to be up-regulated at both time points (3 and 6 h) with a $\log_2$ ratio of at least 1 with a minimum expression value of 50. From the avr1-induced gene set, genes were selected that were repressed in both *eds1* and *pad4* at both time points with a log2 value of -1 or smaller (Group II) compared to wild-type Thus, the selection of Group II genes was based on four mutant $\log_2$ ratios with a value of -1 or smaller. The avr4-induced and *EDS1/PAD4*-dependent genes (Group III) were selected analogously. For the selection of genes repressed in both *eds1* and *pad4* in non-treated samples (Group I, selection criteria listed in Table 3.4A), more stringent criteria were applied as this selection is based on only two mutant ratio values.

The selected $\log_2$ expression ratios were subjected to complete linkage hierarchical clustering analysis and visualised using Genesis software 1.5.0 (Sturn et al., 2002). The resulting clustergrams were extracted to Adobe Illustrator 10 to improve the legend for the experimental conditions.

3. Results

To identify components involved specifically in *EDS1- and PAD4*-controlled signalling, transcriptional profiles of wild-type plants and mutants during early *R* gene-mediated defence were examined. Sets of genes with strong *EDS1/PAD4*-dependent transcriptional activation were identified and for a subset of these genes corresponding insertion mutants were tested for altered defence responses. The targeted mutant screen resulted in the identification of one positive and two homologous negative regulators of plant defence responses.

3.1 Microarray analysis

3.1.1 Experimental design

In order to determine the impact of *EDS1* and *PAD4* on early pathogen-triggered transcriptional changes on a genomic-wide scale, expression profiling experiments were performed.

The experimental design (Table 3.1) includes 21 samples derived from leaves of wild-type *Arabidopsis* of accession Wassilewskija (WT) and null mutants *eds1-1* and *pad4-5* in the same background. Leaves were untreated (NT), treated with $MgCl_2$ solution (Mg) or with *Pseudomonas syringae* strains expressing either *avrRps4* (abbreviated avr4; triggering *EDS1/PAD4*-dependent resistance) or *avrRpm1* (avr1; conditioning *EDS1/PAD4*-independent resistance and localised plant cell death). These abbreviations for experimental conditions will be used from hereon (see also Table 3.1). Leaves were harvested at 3 and 6 hours post-inoculation (h) and analysed using the Affymetrix GeneChip ATH-1 representing 23734 genes (Redman et al., 2004).

The *Pseudomonas syringae-Arabidopsis* pathosystem was chosen because the response phenotypes of wild-type, *eds1* and *pad4* have been characterised in detail (Aarts et al., 1998; Jirage et al., 1999; Feys et al., 2001; Rusterucci et al., 2001). Moreover, bacterial leaf inoculations at high density provide a synchronised and uniform infection.

There were several reasons for choosing early time points for harvesting material after inoculation. First, early cellular re-programming appears to be crucial for successful defence against pathogens (Grant and Mansfield, 1999; de Torres et al., 2003). Thus, essential defence regulators might be up-regulated at early time points. The massive transcriptional changes at later time points involving re-programming of whole metabolic pathways and detoxification system might mask the important early transcripts. Second, a principle aim was to identify components of SA-independent defence. Therefore I chose one time point prior (3 h) and one at the beginning (6 h) of SA accumulation. Third, *EDS1* and *PAD4* transcripts are themselves induced at early time points, and therefore genes participating in an *EDS1/PAD4*-controlled pathway might be co-regulated with *EDS1/PAD4*.

As the oxidative burst, SA accumulation and HR development is still intact in *eds1* and *pad4* upon avr1-treatment, only genes specifically regulated by EDS1/PAD4-derived signals should be blocked in the mutants. I therefore anticipated that examining *RPM1*-triggered re-programming in wild-type and mutants is an elegant way to discriminate between transcripts controlled by ROS and SA and those specifically depend on an EDS1/PAD4 signal.

Non-treated samples of wild-type and mutants were included in the experimental design to identify a potential regulatory function of EDS1/PAD4 in healthy tissue.

Table 3.1 Experimental samples for gene expression microarray analysis.

Plant[a]	Treatment[b]	Timepoints (h)	HR appearance (h)[C]
Wild-type (WT)	Non-treated (NT)	3	-
	10 mM $MgCl_2$ (Mg)	3, 6	-
	avr1	3, 6	6
	avr4	3, 6	24
eds1-1	Non-treated (NT)	3	-
	10 mM $MgCl_2$ (Mg)	3, 6	-
	avr1	3, 6	6
	avr4	3, 6	-
pad4-5	Non-treated (NT)	3	-
	10 mM $MgCl_2$ (Mg)	3, 6	-
	avr1	3, 6	6
	avr4	3, 6	24

[a] All plants were in Wassilewskija background
[b] *Pseudomonas syringae* pv. *tomato* strain DC3000 expressing either *avrRpm1* (avr1) or *avrRps4* (avr4) were inoculated at 1 X 10^7 cfu/ml resuspended in 10 mM $MgCl_2$ (Mg).
[c] HR appearance was checked by trypan blue staining. - stands for HR not detected within 24 h.

3.1.2 Sample preparation and quality control

To reduce the biological variability in the samples used for transcriptional profiling several strategies were applied. First, all plants of one replicate experiment were grown in parallel under defined conditions in the same climate-controlled chamber. Second, the timing of the HR occurrence was observed and only sample sets that displayed the typical timing of HR (see Table 3.1 and Figure 3.1) were included for microarray analysis. Expression of HR upon avr4-treatment was *EDS1*- but not *PAD4*-dependent, whereas an HR induced by avr1 occurred in an *EDS1*/*PAD4*-independent manner (Figure 3.1). Third, each of the 21 samples was a pool of total RNA from four different plants derived from two different experiments. Forth, prior to sample pooling, the expression of *AtERF13* (At2g44840, marker gene for early defence responses; (Onate-Sanchez and Singh, 2002) and *PR1* (AT2G14610, marker gene for SA-regulated defence responses; Uknes et al., 1992) was determined. Sample sets where these pathogenesis marker genes were induced in non-treated or mock-treated tissue were excluded from analysis. Finally, purity and intactness of the extracted total RNA was evaluated with a spectrophotometer and gel electrophoresis prior to sample pooling (for details refer to Materials and Methods). The procedures of cDNA synthesis, subsequent cRNA synthesis and the hybridization of the biotinylated cRNA to the 21 arrays are described in Materials and Methods. After hybridisation, data from each individual chip were normalised to each other by setting their average signal intensity to 100 (arbitrary units). This procedure termed “global scaling” was previously described (Zhu and Wang, 2000) and ensures comparability between chips.

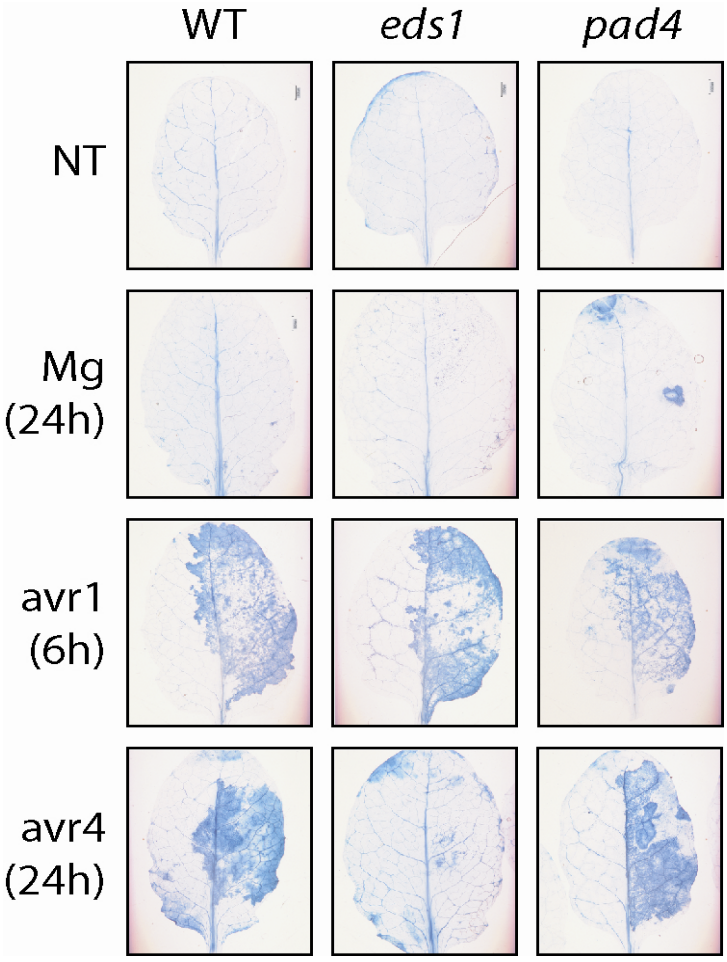


Figure 3.1. Timing of hypersensitive cell death triggered by avirulent *Pseudomonas syringae* inoculation (avr1 or avr4, for abbreviations see Table 3.1).
Leaves of wild-type and mutant plants were treated as described in Table 3.1 except that here only the right half of each leaf was inoculated. Leaves were subsequently harvested and stained with trypan blue at the indicated time points to visualize plant cell death as described in Materials and Methods.

3.1.3 Global transcriptional changes upon inoculation with avirulent *P. syringae* in wild-type plants

From the 22746 probe sets (usually a set of 11 different oligonucleotides representing one or several homologous genes) on the ATH-1 chip, 72.87 % of the probe sets were expressed at least in one of the 21 datasets (representing the 21 experimental conditions listed in Table 3.1).

Table 3.2. Number of probe sets represented and expressed on the ATH-1 array.

	Number of probe sets	**% probe sets**
Probe sets represented on ATH-1 chip	22746	100.00
Probe sets with at least one "present" call in the 21 samples (wild-type and mutant samples)	16576	72.87
Probe sets with at least two calls UP or DOWN in wild-type upon either pathogen treatment (pathogen responsive probe sets)	3839	16.88
Probe sets upregulated at 3 and 6 hpi by avr1 in wild-type samples	961	4.22
Probe sets upregulated at 3 and 6 hpi by avr4 in wild-type samples	207	0.91
Probe sets up-regulated by both bacterial treatments at both time points in wild-type samples	190	0.84

For analysis of transcriptional changes upon pathogen inoculation and the influence of the *eds1-1* or *pad4-5* mutations on these changes, two classes of expression ratios were calculated. First, to identify and to describe pathogen-responsive probe sets in the wild-type data, ratios of the absolute expression values of pathogen treated and mock-treated data were calculated for each probe set (wild-type ratios). These wild-type ratio classes are: WT avr1 3h / WT Mg 3h; WT avr1 6 h / WT Mg 6h; WT avr4 3h / WT Mg 3h; WT avr4 6 h / WT Mg 6h. To identify probe sets already induced by the inoculation procedure, I calculated the ratio WT Mg 3h / WT NT. The second class of ratios compared corresponding expression values from mutant and wild-type (e.g. *eds1* avr1 3h / WT avr1 3h displayed in short: *eds1* avr1 3h / WT avr1; mutant ratios). For illustration in clustergrams all ratios were $\log_2$ transformed resulting in $\log_2$ expression ratios.

For further analysis, 3839 pathogen-responsive probe sets were identified that had at least two log_2 expression ratios of >= +1 (pathogen-induced) or <= -1 (pathogen-repressed) in the wild-type data sets (Table 3.2). The 3839 pathogen-responsive probe sets represent 16.88% of all ATH-1 probe sets. This proportion is similar to the 15% pathogen-responsive genes found previously in *Pst-avrRpt2* treated samples 7 h using cDNA arrays (Scheideler et al., 2002). Probe sets that were either weakly up-regulated/repressed or at only at one time point by one bacterial treatment were missed. The stringent filtering method was applied to ensure robust sampling.

Hierarchical clustering of the wild-type ratios from 3839 pathogen-responsive probe sets in gene and treatment dimensions revealed that the effect of avr1- and avr4-inoculation on gene induction and repression is similar at 6 h, showing a 57.8 % overlap between avr1 and avr4 up-regulated probe sets at 6 h (Figure 3.2). In contrast, avr1 and avr4 transcriptional profiles at 3 h substantially differed as avr4-treatment induced changes in fewer genes compared to avr1-treatment. The greatly reduced impact of avr4-treatment on early transcriptional changes compared to avr1-induced changes was mirrored by the later induction of HR in avr4-inoculated tissues. (Figure 3.1). Later *R* gene-mediated defence responses triggered by avr4 compared to avr1 were also found by Bennett et al. (2005) in *Arabidopsis* ecotype Col-5 using biophoton imagining to record defence responses. However, the reason of differences in timing remains unknown.

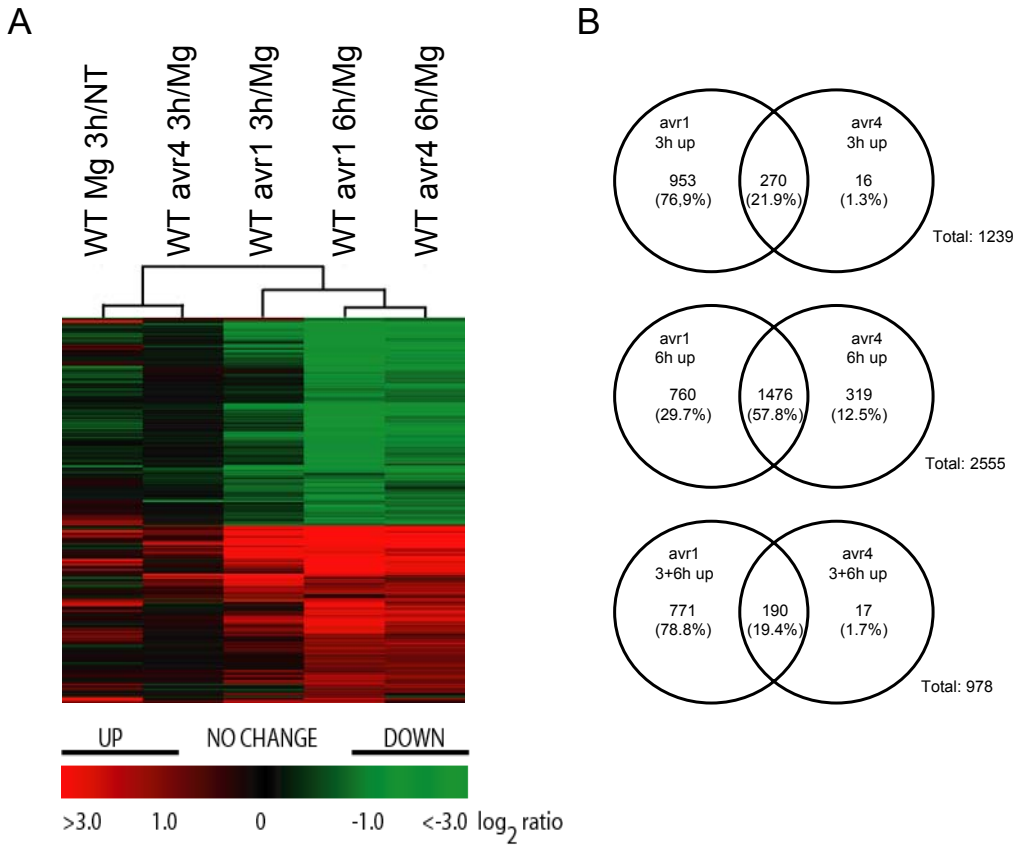


Figure 3.2. Avr1 and avr4 trigger transcriptional changes in a common set of target genes but with different timing.
(**A**) Expression ratios of 3839 pathogen responsive probe sets (see Table 3.2) were used for hierarchical clustering in gene and treatment dimensions (see Material and Methods for details). Expression ratios were $\log_2$ transformed and displayed in colour code. Positive ratios (pathogen induced probe sets) were depicted in red, negative ratios (pathogen repressed) in green and ratios around zero (no change upon pathogen treatment) in black. The term "WT avr4 3h/Mg" is the short form for the ratio "wild-type avr4 3h/ wild-type Mg 3h". The dendogram above the clustergram represents the relatedness of the overall expression pattern between the different experimental conditions. (**B**) Overlap between avr1- and avr4-induced probe sets are illustrated by Venn diagrams for 3h, 6h and both time points. Abbreviations are defined in Table 3.1.

Substantial overlap between avr1- and avr4-induced transcriptional changes at 6 hpi is most likely due to the induction of common downstream signalling events such as the salicylic acid pathway. The number of specifically avr1- or avr4-induced genes, as depicted in Figure 3.2B, is overestimated since many genes which fall in either the avr1 or avr4 specific class are just below the threshold of a two-fold induction. More stringent filtering criteria identified only four probe sets representing six genes that were specifically induced by avr1 (Table 3.3) but no specifically avr4-induced genes using analogous search criteria. The unique avr1-induced gene set might be part of an RPM1- (CC-NBS-LRR) specific signal cascade. Alternatively, they may be

induced just prior and during the onset of the programmed cell death triggered by avr1. In support of the latter idea, the listed *peroxidase* (At1g14540) is reported to be induced upon AAL-toxin treatment leading to cell death (Gechev et al., 2004).

Table 3.3 Genes specifically up-regulated by avr1 (at least 3-fold UP with minimum absolute values of 100 (3h) and 200 (6h)) but not by avr4 (less than 1.5-fold UP or absolute value smaller than 50). For abbreviations see Table 3.1.

			absolute expression values					
Affymetrix Probe ID	**AGI Number**	**Gene Description**	**WT Mg (3h)**	**WT Mg (6h)**	**WT avr1 (3h)**	**WT avr1 (6h)**	**WT avr4 (3h)**	**WT avr4 (6h)**
266455_at	At2G22760	basic helix-loop-helix (bHLH) family protein	17.66	5	106.02	467.74	20.4	43.68
261474_at	At1G14540	anionic peroxidase	9.32	17.18	278.88	309.48	30.48	18.22
256407_at	AT5G43610 AT1G66570 AT2G14670	sucrose transporter-related	27.32	5	126.42	277.22	26.82	32.58
256763_at	At3G16860	phytochelatin synthetase-related	53.48	54.78	215.1	217.14	64.32	74.72

3.1.4 Effect of the *eds1* and *pad4* mutations on avr1- and avr4-triggered transcriptional changes

Before analysing the impact of mutations in *EDS1* and *PAD4* on global gene transcription, the expression levels of *EDS1* and *PAD4* themselves were examined (Figure 3.3). In wild-type *EDS1* and *PAD4* were strongly induced upon pathogen-treatment at both 3 and 6 h but not in non- or mock-treated samples. This early induction of *EDS1* and *PAD4* differed from the induction profile of *PR1* (a marker gene for SA-signalling) that was induced at 6 h but not at 3 h.

Up-regulation of *EDS1* upon avr1- but especially upon avr4-treatment was reduced in *pad4*, and *vice versa*. As *eds1-1* is a point mutant, *eds1* mRNA was still present in the mutant but at lower abundance than in wild-type, indicating that functional EDS1 is required for maximum *EDS1* up-regulation. Transcript levels of *PAD4* were never

observed in *pad4-5* derived samples in accordance with previous findings, demonstrating that *pad4-5* is a mRNA null mutant (Feys et al., 2001).

Taken together, these results confirmed the previous finding that EDS1- and PAD4-derived signals participate in a positive feed back loop on *EDS1* and *PAD4* expression (Feys et al., 2001).

In contrast to previous findings that *PR1* up-regulation in CC-NBS-LRR-triggered signalling was not effected in *pad4* (Zhou et al., 1998; Fellbrich et al., 2002), I found that *PR1* transcript levels were reduced in *eds1-1* and *pad4-5* upon avr1-treatment at 6 hpi when compared to wild-type. As the previous transcript analysis examined later time points (12 or 24 h), my finding suggests a function of EDS1/PAD4 in early *PR1* regulation.

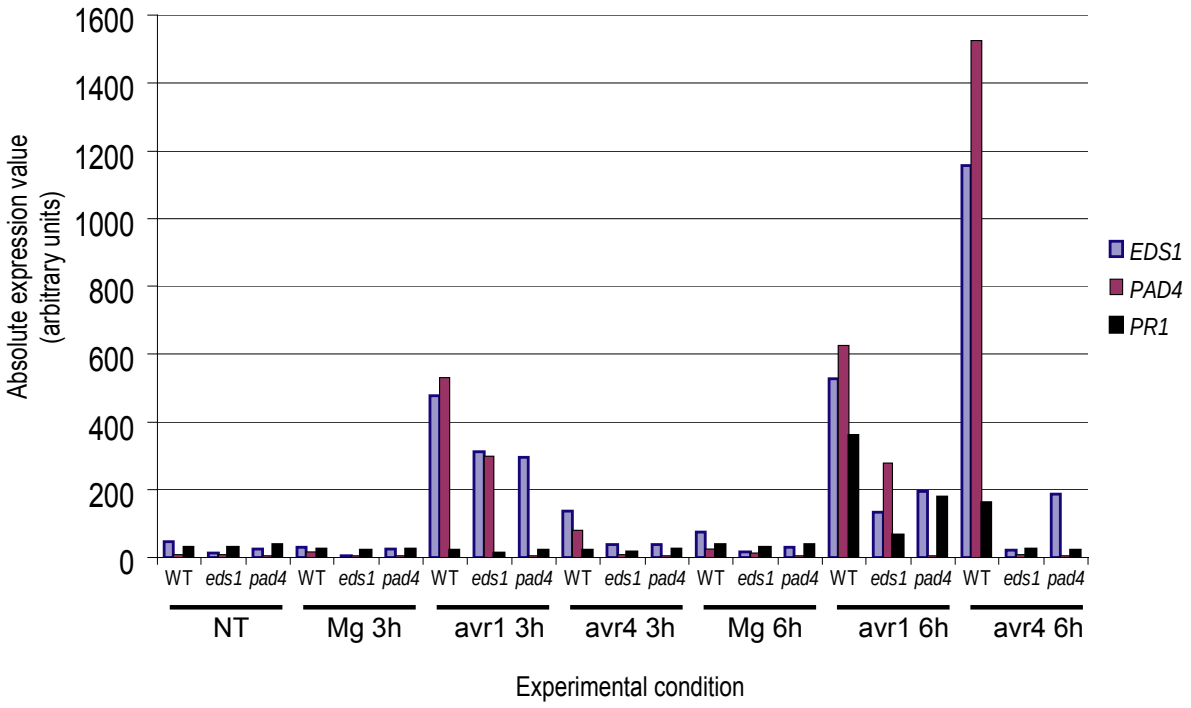


Figure 3.3. Absolute expression levels for *EDS1*, *PAD4* and *PR1* mRNAs in healthy and pathogen-inoculated wild-type and mutant plants.
Expression values were obtained after global scaling of all 21 GeneChips to a target value of 100 to achieve comparability between the data sets (see Materials and Methods). Genes and corresponding probe sets used in this figure: *EDS1* (252373_at; At3g48090), *PAD4* (252060_at; At3g52430) and *PR1* (266385_at; At2g14610).

Figure 3.4A shows $\log_2$-ratios (infected mutant/infected wild-type) for all of the 3839 pathogen responsive probe sets. The column with expression ratios "*eds1* avr4 / WT

avr4" at 6 h reveals the strong impact of *eds1* in blocking avr4-triggered transcriptional changes (Figure 3.4A and 3.4C). In contrast, avr1 transcriptional changes were substantially unaffected by *eds1* (note the predominantly black shading in the columns representing "eds1 avr1 / WT avr1"). Overall the *pad4* expression ratios mirrored the *eds1* ratios with the difference that the *pad4* ratios had a lower magnitude. The finding that *eds1* blocked avr4-triggered gene expression changes to a greater extent compared to *pad4* is in accordance with the finding that *eds1* is defective in early downstream signalling events such as ROS burst, programmed cell death and SA induction whereas *pad4* only partially disables these processes after avr4-inoculation. Upon avr1-inoculation these early defence signalling events are still intact in both *eds1* and *pad4*, reflected by my finding that *eds1* and *pad4* had little effect on avr1-triggered transcriptional changes (Figure 3.4B).

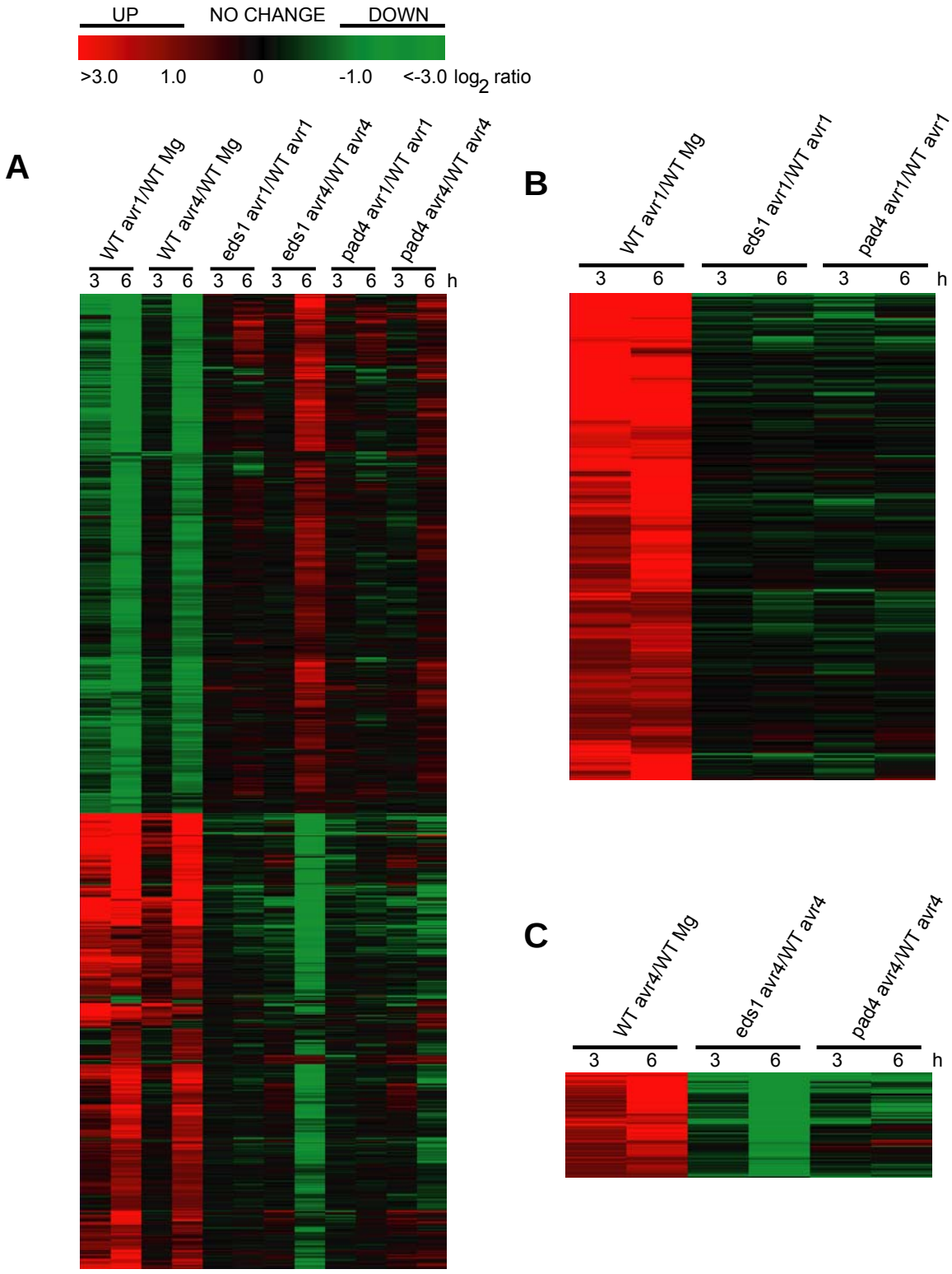


Figure 3.4. Changes in *Arabidopsis* gene expression triggered by avr1 and avr4 and the impact of *eds1* and *pad4* on these changes are visualised in expression ratios after hierarchical clustering in probe set dimension.
(**A**) Expression ratios for 3839 pathogen responsive probe sets in wild-type (first four columns) and in *eds1* and *pad4* (last 8 columns) are shown in this clustergram. Clustergram (**B**) represents 961 avr1-induced probe sets (at both 3 and 6h) and their expression in the mutants relative to wild-type. Clustergram (**C**) represents 207 avr4-induced (both at 3 and 6h in wild-type) probe sets and their expression in the mutants compared to wild-type. Abbreviations are the same as in Table 3.1.

3.1.5 Identification of genes controlled by *EDS1* and *PAD4*

EDS1 and PAD4 share homology to lipases and current genetic and molecular data indicate that they act in the same signalling pathway (Glazebrook, 1999; Rusterucci et al., 2001; Brodersen et al., 2002; Mateo et al., 2004a). I therefore hypothesised that signalling molecules derived from EDS1 and PAD4 control the expression of a common set of downstream signalling components. For that reason, I searched for probe sets that were repressed in both *eds1* and *pad4* (abbreviated *eds1*/*pad4*) compared to wild-type. Querying repression in both *eds1* and *pad4* increased the robustness of the filtering results. However, one disadvantage of this approach was that probe sets that are specially blocked by either mutation would be lost. Since my main focus was on identification of novel EDS1/PAD4 signalling components and evaluation of their biologically relevance, I exploited the increased reliability of this data set.

First, I identified genes whose basal expression in healthy tissue was substantially blocked in *eds1*/*pad4* using Microsoft Excel. Only four probe sets representing four genes (termed Group I) met the strict filter criteria (Table 3.4A). These *EDS1/PAD4*-dependent genes in healthy tissue include two genes that were previously shown to be important regulators of plant defence: *Accelerated cell death* 6 (*ACD6*) and *Pathogen and circadian controlled 1* (*PCC1*) (Lu et al., 2003; Sauerbrunn and Schlaich, 2004). For *ACD6*, *PAD4*-dependent expression in healthy plants was reported previously (Lu et al., 2003). For *PCC1* and At2g14560 a basal expression independent of *PAD4* was reported (Sauerbrunn and Schlaich, 2004; Huang et al., 2005) which is in conflict with my data. The *PAD4*-dependent expression of *PCC1* was confirmed by semi-quantitative RT-PCR on biological independent samples (data not shown).

The other two genes in Group I have not been previously described. Although, AT5G54610 contains an ankyrin repeat motif like *ACD6* that mediates protein-protein interactions, these sequences are otherwise not significantly related. The potential signalling role of genes in Group I will be addressed in the Discussion.

EDS1 and *PAD4* are redundant in *RPM1*-mediated local resistance and plant cell death. However, both genes are required for the production and emission of a subsequent SAR signal (L. Jorda, A.M. Maldonado, C. Lamb and J. Parker,

unpublished data). Also, *EDS1* and *PAD4* are transcriptionally activated upon avr1-treatment. I hypothesised that EDS1/PAD4-derived signals are generated upon triggering of the RPM1 pathway. Thus, genes that are avr1-induced in wild-type but not or significantly less in *eds1/pad4* might represent genes that are under specific EDS1/PAD4 control independently of ROS and SA that are produced to the same extent as in wild-type (Rusterucci et al., 2001; Feys et al., 2001). Also their early up-regulation at 3 h would be indicative of SA-independent regulation.

Six genes with *EDS1/PAD4*-dependent up-regulation upon avr1-challenge were identified (referred to as gene Group II; Table 3.4B). None of the Group II genes have been previously reported to be functionally linked to disease resistance. There are reports for the putative *flavin-containing monooxygenase* (here referred to as *AtFMO*, AT1G19250) to be up-regulated (along with *EDS1* and *PAD4*) in *acd11* (Brodersen et al., 2002). Also, for *AtMRP7* (*Multidrug Resistance-associated Protein 7*) up-regulation in response to ectopic SA application was reported (Kolukisaoglu et al., 2002). BLAST searches revealed that the lipid transfer-like protein (At5g55450) found in Group II is sequence related to the SAR regulator *DIR1* (31% amino acid identity). *DIR1* itself was not pathogen-induced in the microarray experiment presented here (data not shown).

I reasoned that genes belonging to Group II may be defence regulators closely associated in the EDS1/PAD4 pathway. I therefore evaluated corresponding T-DNA insertional mutants for altered local defences (see section 3.2).

From the 207 avr4-induced probe sets, I found 28 probe sets representing 29 genes to be consistently (both at 3 and 6 h) and strongly both *EDS1- and PAD4-* dependent (Group III, Table 3.4c). The result that only 28 of the 207 avr4-induced probe sets were apparently blocked in *eds1/pad4* was due to the stringent filtering criteria which missed genes that were just over the $\log_2$ ratio -1 criterion for *EDS1/PAD4*-dependency or were blocked in only one mutant. A search for avr4-induced genes (at both 3 and 6 h) that were not blocked in either *eds1 or pad4* (genes whose wild-type expression value were not 1.5-fold increased compared to *eds1*/*pad4* at both 3 and 6h) did not return any hit. Thus, all of the 207 avr4-induced genes were at least at one time point blocked by either *eds1* or *pad4*. As mentioned above, most avr4-induced genes were repressed in *eds1*, whereas in *pad4* a significant number of genes were expressed like in wild-type upon avr4-inoculation (see Figure 3.4C). Only three avr4-induced genes were found to be repressed in *pad4* but not in *eds1* at both

time points. All three genes are encoded by the chloroplast genome. Atcg00540 encodes a cytochrome f apoprotein involved in the photosynthetic electron transport (Dinkins et al., 1994). For the two other genes the TAIR data base (http://www.arabidopsis.org/index.jsp) predicts that Atcg00760 encodes a chloroplast ribosomal protein and that Atcg00740 encodes a RNA polymerase subunit. Future qRT-PCR experiments will need to be performed to validate the expression profiles of these three genes before any conclusion can be made.

Group III contains several genes previously associated to plant defence signalling such as *BONZAI1* (Yang and Hua, 2004), *MKK4* (Asai et al., 2002), *PR-5* (Reuber et al., 1998), *AtWRKY46* (Eulgem et al., 2000; Kalde et al., 2003) as well as *EDS1* and *PAD4* themselves. As stated in the introduction, *BONZAI1* is a negative defence regulator of TIR-NBS-LRR resistance with requirement for *EDS1/PAD4*. Interestingly, a close homologue of the NUDIX (nucleoside diphosphates linked to some other moiety x) hydrolase *AtNUD2.1* (Group II) was found in Group III (referred to as *AtNUD4.1*). *AtNUD2.1* stands for "*Arabidopsis* NUDIX on chromosome 2, 1st gene identified on this chromosome", analogous to the annotation of the first cloned *Arabidopsis* NUDIX gene *AtNUDT1* (Dobrzanska et al., 2002). Expression of *AtNUD4.1* (along with seven other genes) was previously found to be blocked in *pad4* but not in *ndr1*, *npr1 or NahG* during *RPP4*-signalling (Eulgem et al., 2004). In that study, the authors used the previous version of the Affymetrix GeneChip (representing approximately 8000 genes) that did not represent any of the six genes in Group II.

Genes of Group II (avr1-induced in *EDS1/PAD4*-dependent manner) were also transcriptionally activated by avr4-treatment in an *EDS1/PAD4*-dependent manner but solely at 6 h their wild-type expression reached high levels. Low wild-type expression levels at 3 h upon treatment with avr4 prevented them to be included in Group III (see supplement Figure for absolute signal values of Group II genes in all 21 experimental conditions). Similarly, mRNAs of Group III genes were also induced after avr1-inoculation but to a comparable extent in wild-type, *eds1-1* and *pad4-5* (data not shown).

Table 3.4A. Genes suppressed in *eds1* and *pad4* in non treated tissue[a] (Group I)

Affymetrix Probe ID	AGI Number	Gene Description	Gene symbol	absolute values			signal log_2 ratio	
				WT NT	eds1 NT	pad4 NT	eds1 NT /WT NT	pad4 NT/WT NT
245265_at	AT4G14400	ankyrin repeat family protein	*ACD6*	548.28	111.08	178.98	-2.30	-1.62
248169_at	AT5G54610	ankyrin repeat family protein		197.10	44.42	50.64	-2.15	-1.96
256766_at	AT3G22231	Pathogen and circadian controlled 1	*PCC1*	497.46	57.24	14.36	-3.12	-5.11
265837_at	AT2G14560	expressed protein		152.40	17.20	13.74	-3.15	-3.47

[a] Genes were selected which were at least 4-fold suppressed in *eds1* and at least 2-fold in *pad4* compared to wild-type with a minium expression value of 100.

Table 3.4B. avr1-induced genes in both *EDS1*- and *PAD4*-dependent manner[a] (6 probe sets/6 genes; Group II)

Affymetrix Probe ID	AGI Number	Gene Description	Gene symbol	Signal log_2 ratio					
				WT avr1 3h/Mg	WT avr1 6h/Mg	eds1 avr1 3h/WT	eds1 avr1 6h/WT	pad4 avr1 3h/WT	pad4 avr1 6h/WT
257185_at	At3g13100	ABC transporter family protein (AtMRP7)	*AtMRP7*	3.86	4.65	-1.84	-1.81	-1.45	-1.31
260179_at	At1g70690	kinase-related	*AtPRK*	3.39	6.77	-1.72	-1.63	-2.47	-1.29
248062_at[b]	At5g55450	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	*AtLTP*	4.04	0.62	-1.32	-4.67	-1.70	-1.87
256012_at	At1g19250	flavin-containing monooxygenase family protein / FMO family protein	*AtFMO*	3.51	8.18	-2.54	-1.97	-3.51	-1.19
263852_at	At2g04450	MutT/nudix family protein	*AtNUD2.1*	4.74	4.81	-2.10	-2.24	-2.23	-1.93
249743_at	At5g24550	glycosyl hydrolase family 1 protein	*AtGH*	4.28	5.35	-2.57	-3.20	-3.72	-1.83

[a] Probe sets were selected which were at least 2-fold induced upon avr1 (both at 3 and 6h) with a minimum expression value of 50.
[b] 248062_at does not meet the criteria for 2-fold up-regulation in ratio "WTavr1 6h/Mg" as the absolute level in "WT Mg 6h"was already high. Nevertheless 248062_at displayed a consistent *eds1/pad4* dependency and was therefore included in this table.

Table 3.4C. avr4-induced genes in both *EDS1*- and *PAD4*-dependent manner[c] (28 probe sets/29 genes; Group III)

				Signal log_2 ratio					
Affymetrix Probe ID	**AGI Number**	**Gene Description**	**Gene symbol**	**WT avr4 3h/Mg**	**WT avr4 6h/Mg**	**eds1 avr4 3h/WT**	**eds1 avr4 6h/WT**	**pad4 avr4 3h/WT**	**pad4 avr4 6h/WT**
246293_at	At3g56710	sigA-binding protein		2.31	2.23	-2.56	-3.73	-2.59	-1.29
246777_at	At5g27420	zinc finger (C3HC4-type RING finger) family protein		4.11	5.03	-1.98	-5.13	-1.38	-1.58
246821_at	At5g26920	calmodulin-binding protein		3.89	6.64	-2.41	-5.03	-1.80	-1.01
247493_at	At5g61900	copine BONZAI1 (BON1)	*BON1*	1.76	5.91	-1.43	-4.90	-1.37	-2.49
247602_at	At5g60900	lectin protein kinase family protein		2.22	1.64	-1.36	-2.34	-1.89	-1.08
248322_at	At5g52760	heavy-metal-associated domain-containing protein		2.95	4.99	-1.96	-7.51	-1.87	-2.15
249417_at	At5g39670	calcium-binding EF hand family protein		2.64	4.45	-2.21	-6.20	-2.34	-1.89
251400_at	At3g60420	expressed protein		2.96	4.67	-2.02	-6.25	-2.65	-1.71
252060_at	At3g52430	phytoalexin-deficient 4 protein	*PAD4*	2.43	5.93	-2.96	-7.44	-4.01	-8.25
252373_at	At3g48090	disease resistance protein 1	*EDS1*	2.31	3.99	-1.89	-5.90	-1.89	-2.62
254243_at	At4g23210	protein kinase family protein		1.69	4.31	-1.04	-4.16	-1.29	-2.63
254271_at	At4g23150	protein kinase family protein		2.51	4.98	-1.60	-5.43	-1.77	-4.41
254784_at	At4g12720	MutT/nudix family protein	*AtNUD4.1*	1.77	3.86	-1.06	-5.12	-1.10	-1.05
255406_at	At4g03450	ankyrin repeat family protein		1.41	4.35	-1.72	-5.06	-1.56	-1.76
256183_at	At1g51660	mitogen-activated protein kinase kinase (MAPKK), putative (MKK4)	*MKK4*	1.97	3.47	-1.62	-4.53	-1.45	-1.39
257623_at	At3g26210	cytochrome P450 71B23, putative (CYP71B23)	*CYP71B23*	1.83	3.13	-1.21	-4.18	-1.55	-2.42
257763_s_at	At3g23110/At3g23120	disease resistance family protein/leucine-rich repeat family protein		3.44	2.17	-2.07	-6.21	-3.29	-2.97
259272_at	At3g01290	band 7 family protein		1.74	4.13	-1.97	-5.35	-1.71	-1.22
259385_at	At1g13470	expressed protein		1.51	2.18	-3.34	-4.93	-2.72	-1.79
259925_at	At1g75040	pathogenesis-related protein 5 (PR-5)	*PR-5*	1.68	2.22	-1.08	-3.89	-1.37	-1.68
260046_at	At1g73800	calmodulin-binding protein		2.75	4.18	-2.88	-5.78	-2.44	-2.06
260068_at	At1g73805	calmodulin-binding protein		2.73	4.65	-2.84	-6.82	-3.26	-2.31
260804_at	At1g78410	VQ motif-containing protein		3.73	5.22	-1.80	-6.79	-3.09	-2.46
261476_at	At1g14480	ankyrin repeat family protein		1.99	1.95	-2.00	-2.40	-1.84	-1.69
263783_at	At2g46400	WRKY family transcription factor	*AtWRKY46*	1.54	4.22	-1.44	-5.20	-1.15	-1.38
264434_at	At1g10340	ankyrin repeat family protein		2.19	3.99	-1.46	-5.45	-1.63	-1.42
264866_at	At1g24140	matrixin family protein		3.38	5.88	-1.60	-5.88	-1.04	-2.24
265597_at	At2g20142	expressed protein		3.80	3.89	-1.64	-5.02	-2.24	-1.15

[c] Probe sets were selected analogous to Table 3.4B.

3.2 Screen for altered resistance phenotypes to the oomycete *Peronospora parasitica* isolate Cala2

For some of the *EDS1/PAD4*-dependent genes identified here an important role in defence signalling has been suggested and in some cases demonstrated. To test the functional relevance of the yet uncharacterised genes in plant defence, homozygous T-DNA insertion mutants of the six genes in Group II and of *AtNUD4.1* (Group III) were identified in accession Col-0 (see Materials and Methods for details) and tested for altered *RPP2*- (TIR-NBS-LRR) mediated resistance to the oomycete *Peronospora parasitica* isolate Cala2 (Sinapidou et al., 2004). Although the genes were identified in data sets derived from bacterial treated tissue, I assumed that these genes might have a conserved role in TIR-NBS-LRR-mediated resistance along with *EDS1* and *PAD4*.

3.2.1 Aberrant defence responses in *Atfmo* and *Atnud4.1* T-DNA insertional mutants

Pathogen growth and the plant response were evaluated 7 dpi with *P. parasitica* isolate Cala2 using trypan blue to stain infection structures of the oomycete and hypersensitive plant cell death on the first two true leaves (Koch and Slusarenko, 1990b). Defence responses in Col-0 wild-type resulted in localised cell death at attempted sites of infection not allowing the pathogen to develop hyphal structures as described before (Holub et al., 1994b). In leaves of *pad4-1* (partially susceptible control in Col-0 background) abundant hyphal growth was observed which was surrounded by dead plant cells (trailing necrosis) as described previously (Glazebrook et al., 1997b; Kalde et al., 2003).

Of the seven homozygous T-DNA insertion lines in Col-0 background, six showed wild-type like HRs, whereas *Atfmo-1* displayed a partial loss of resistance (Figure 3.5A). This partial loss of resistance was manifested as larger areas of cell death (an extended HR) at attempted infection sites, trailing plant cell necrosis, sporadic free hyphae and, in rare cases, the development of sporangiospores and oospores from 8 dpi on. The hyphal growth and sporulation in *Atfmo-1* was consistently weaker than in *pad4-1*. In contrast, free hyphal growth (not associated with plant cell death) was more often observed in *Atfmo-1* compared to *pad4-1*. *Atnud4.1-1* appeared to develop more HR lesions than Col-0 wild-type and displayed a slight dwarf

phenotype with faintly curled leaves in the unchallenged state which is reminiscent of constitutively active defence mutants.

As the initial Cala2 screen revealed aberrant defence responses in *Atfmo-1* and *Atnud4.1-1*, independent insertion mutants were identified for *AtFMO* and *AtNUD4.1* (Figure 3.5B) and included in a detailed investigation for aberrant defence phenotypes.

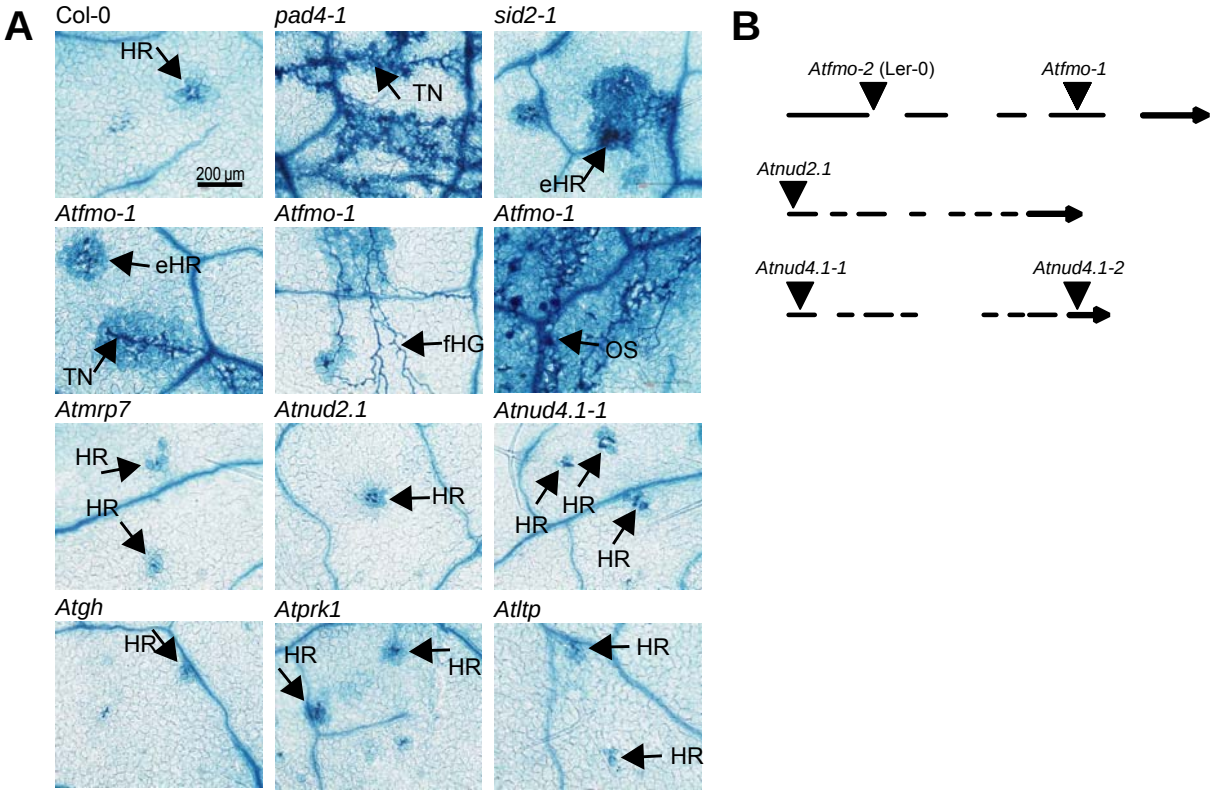


Figure 3.5. Pathogen growth and response phenotypes of avirulent *P. parasitica* Cala2 (*RPP2*) on Col-0 wild-type, *pad4-1, sid2-1* and different insertional lines and schematic insertion mutant representation for *AtFMO*, *AtNUD2.1* and *AtNUD4.1*.

(**A**) The insertional lines correspond to *EDS1/PAD4*-dependent genes in Table 3.4B and 3.4C (*Atnud4.1-1*). For a detailed list of the T-DNA insertion mutants see Materials and Methods. Plants were spray inoculated with conidiospores at $4x10^4$ spores/ml 14 to 18 days after sowing and first true leaves were harvested 7dpi for trypan blue staining. A tightly localised hypersensitive response plant cell death (HR) developed at attempted sites of infection in Col-0 wild-type, *Atmrp7, Atnud2.1, Atnud4.1-1, Atgh, Atprk1* and *Atltp*. A partial loss of *RPP2*-mediated resistance was detected in *pad4-1* as trailing necrosis (TN), in *sid2-1* as extended HR cell death (eHR) and in *Atfmo-1* as eHR, TN and sporadically as free hyphal growth (fHG) and development of oospores (OS). Infected plants of the Cala2-susceptible Ler-0 ecotype grown in parallel showed heavy sporulation 7 dpi (data not shown). (**B**) Schematic representation of the exon-intron structure and mutants for *AtFMO*, *AtNUD2.1* and *AtNUD4.1*: *Atfmo-1* (SALK_026163), *Atfmo-2* (GT_3_108523); *Atnud2.1* (GABI_158B10); *Atnud4.1-1* (SALK_046441) and *Atnud4.1-2* (SALK_104293).

3.3 Defining the role of *AtFMO* in plant defence

3.3.1 *AtFMO* is required for *EDS1/PAD4*-controlled defence responses

To validate further the *Atfmo-1* response phenotype, the homozygous *Ds*-(*Dissociation* element) (Sundaresan et al., 1995) insertion mutant in Ler-0 background (*Atfmo-2*) was tested for *RPP5*-mediated resistance to *P. parasitica* isolate Noco2 (Figure 3.6). As expected, an HR was triggered by *P. parasitica* isolate Noco2 in Ler-0 wild-type leaves due to recognition by RPP5 (Reignault et al., 1996). The *pad4-2* mutant (in accession Ler-0) exhibited trailing necrosis with some sporulation, whereas *Atfmo-2* exhibited trailing necrosis and sporulation to a lesser extent than in *pad4-2*. Thus, two independent defective *AtFMO* alleles in different *Arabidopsis* backgrounds showed similar partial loss of TIR-NBS-LRR-mediated resistance to *P. parasitica*.

Treatment with the SA analogue BTH prior to infection reverted the *Atfmo-2* mutant susceptible phenotype to resistance (Figure 3.6) as previously shown for *eds1* and *pad4* (Parker et al., 1996; Feys et al., 2001). These results demonstrate that *AtFMO* is a necessary component in *R* gene-mediated resistance and that BTH perception is still intact in *Atfmo.* Thus, *AtFMO* might act up-stream or independently of SA signalling.

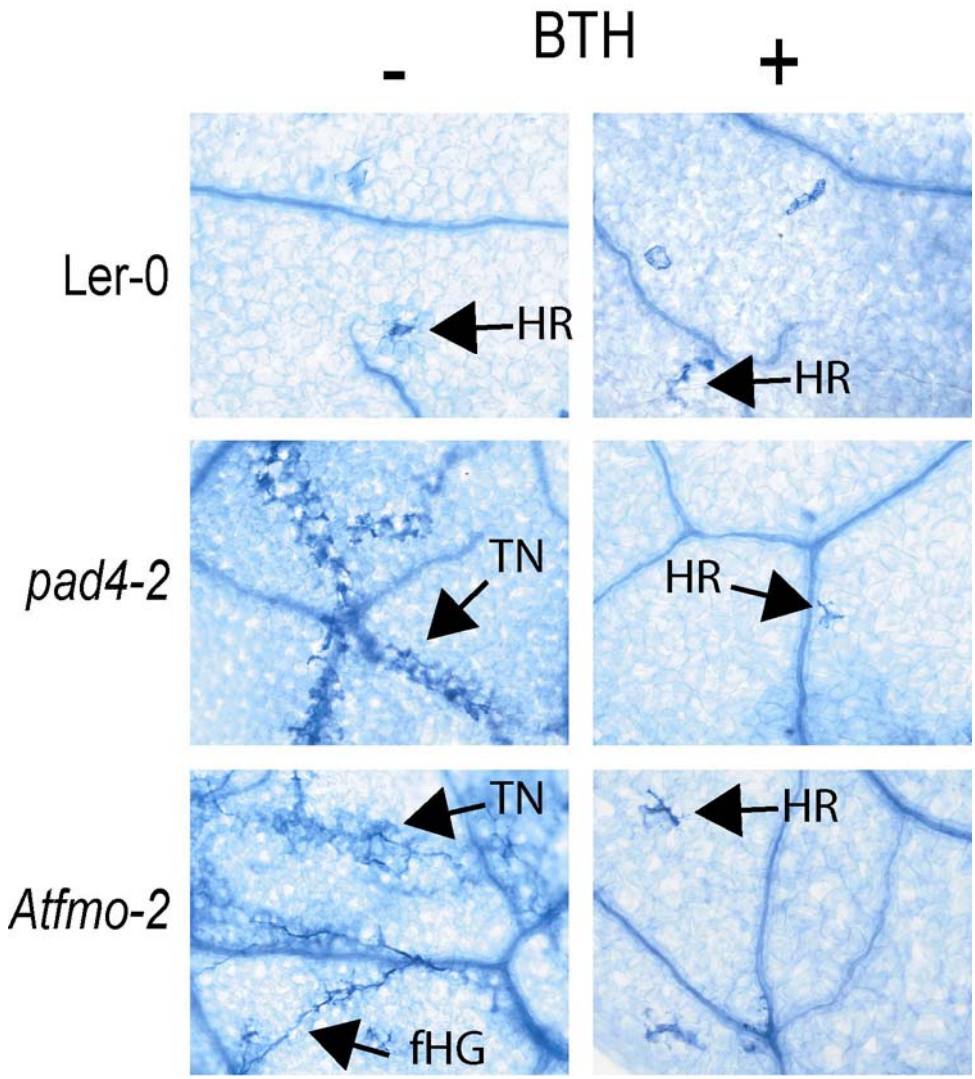


Figure 3.6. BTH perception is intact in *pad4-2* and *Atfmo-2*.
Two days prior to inoculation with avirulent *P. parasitica* isolate Noco2 (recognised by RPP5 in Ler-0 wild-type) plants were sprayed (+) with the SA-analogue BTH (30 µM in water) or treated with water only (-). Leaf tissue was trypan blue stained 7 dpi to visualise pathogen structures and plant cell death. Abbreviations of oomycete structures and plant responses are the same as in Figure 3.5.

Further pathogen assays with the two independent *Atfmo* mutants in Col-0 and Ler-0 were performed alongside wild-type, *eds1* or *pad4* mutants in the corresponding accessions to test whether *AtFMO* functions only in an *EDS1/PAD4*-controlled resistance pathway. I found that *AtFMO* is required for full resistance to *P. syringae* expressing *avrRps4* but not to the isogenic strain expressing *avrRpm1* thus resembling *PAD4*, although the defect in *RPS4*-mediated resistance was less strong in *Atfmo-1* (Figure 3.7). As with *PAD4* (Feys et al., 2001), *AtFMO* was found to be redundant in *RPP8*-mediated resistance to *P. parasitica* isolate Emco5. Restricted

trailing necrosis in *eds1-2,* as also found previously (Aarts et al., 1998; McDowell et al., 2000), suggests that *EDS1* function is needed for development of restricted HRs in *RPP8*-mediated defence.

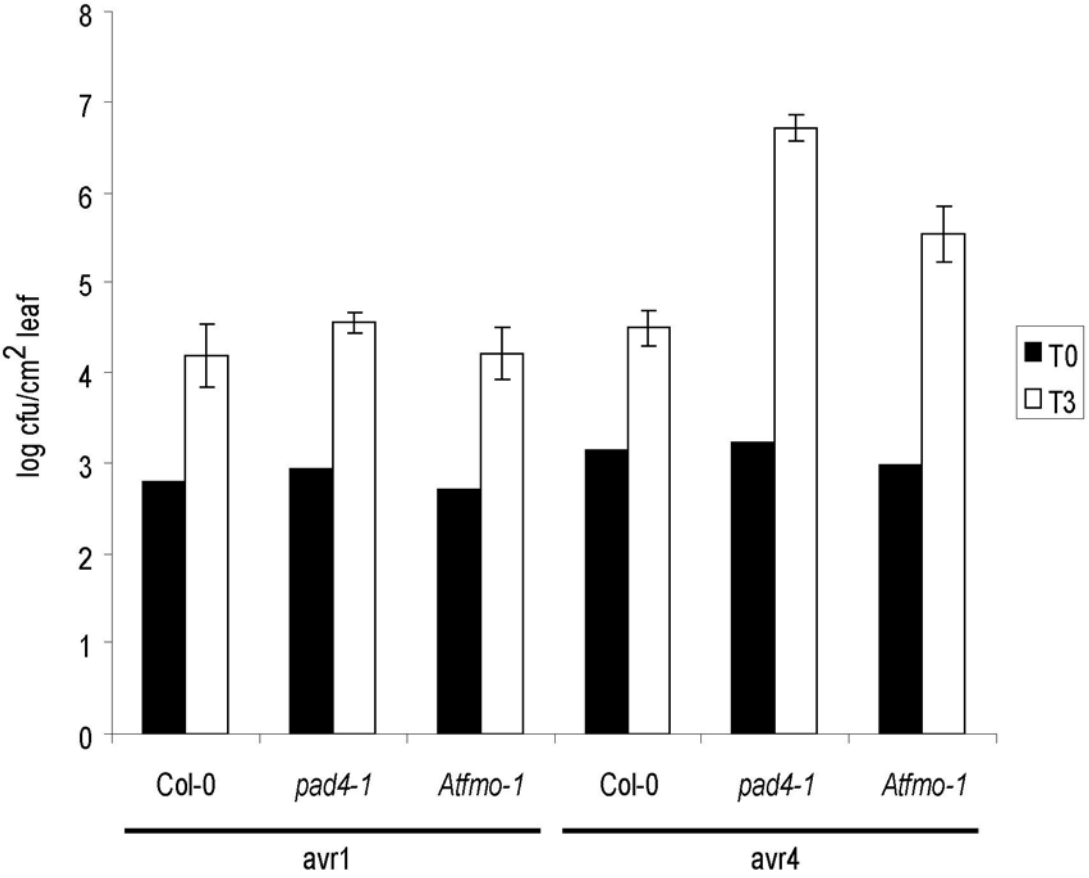


Figure 3.7. Growth of avirulent *P. syringae* strains either expressing *avrRpm1* (avr1) or *avrRps4* (avr4) in leaves of Col-0 wild-type, *pad4-1* and *Atfmo-1*.
Leaves of 4- to 5-week old plants were vacuum infiltrated with bacterial suspensions at $5x10^5$ cfu/ml and bacterial titers were determined in triplicate at 0 (T0) and 3 (T3) dpi as described in Materials and Methods. Data represent the average from three replicate samples (+/- standard deviation). An independent experiment gave similar results.

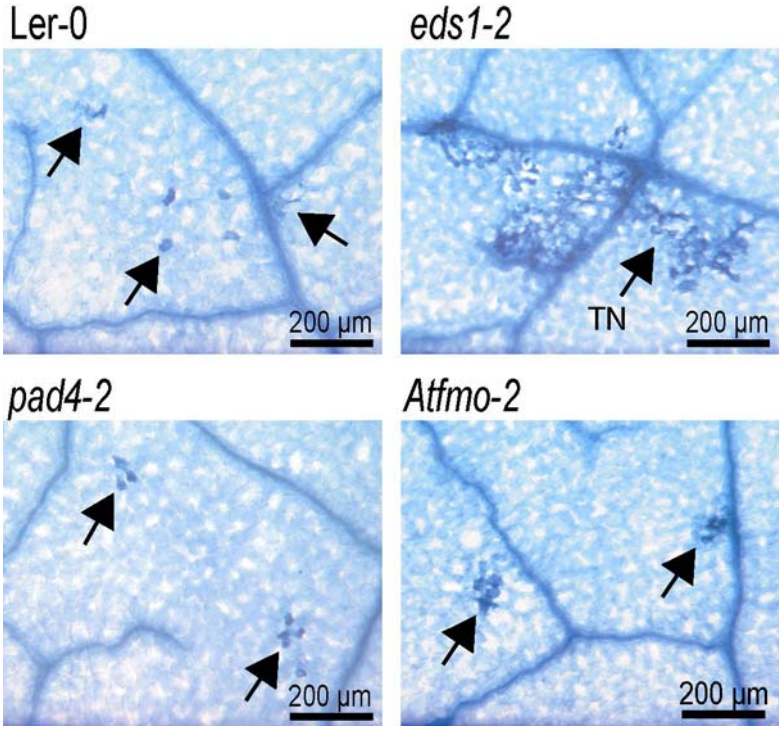


Figure 3.8. Pathogen growth and plant response phenotypes of avirulent *P.parasitica* isolate Emco5 (*RPP8*) on Ler-0 wild-type, *eds1-2, pad4-2* and *Atfmo-2*.
Plants were spray inoculated as described in Figure 3.5. Tightly localised HR cell death developed at attempted sites of infection (arrows) in Ler-0 wild-type, *pad4-2* and *Atfmo-2*, whereas in *eds1-2* extended HRs (not shown) or limited trailing necrosis (TN) occurred. Infected plants of the Emco5-susceptible Ws-0 ecotype grown in parallel showed heavy sporulation 7 dpi (data not shown).

As *eds1* and *pad4* plants are defective in basal resistance, the growth of virulent isolates of *P. parastica* was evaluated in the respective *Atfmo* mutants. These assays revealed a requirement for *AtFMO* in basal resistance (Figure 3.9). In comparison to *eds1* or *pad4*, the deficiency in basal resistance in *Atfmo* mutants was more pronounced than in *R* gene-meditated resistance.

I conclude from the results of the pathogen tests that *AtFMO* mediates resistance controlled by R proteins of the TIR-NBS-LRR class (RPP2, RPP5 and RPS4) but not of the CC-NBS-LRR class (RPM1 and RPP8). Additionally, a defect in basal resistance was found in *Atfmo*. Thus, the defence assays performed so far suggest that AtFMO is a necessary regulator of basal and TIR-NBS-LRR-mediated resistance, acting in the same signalling pathway as EDS1 and PAD4.

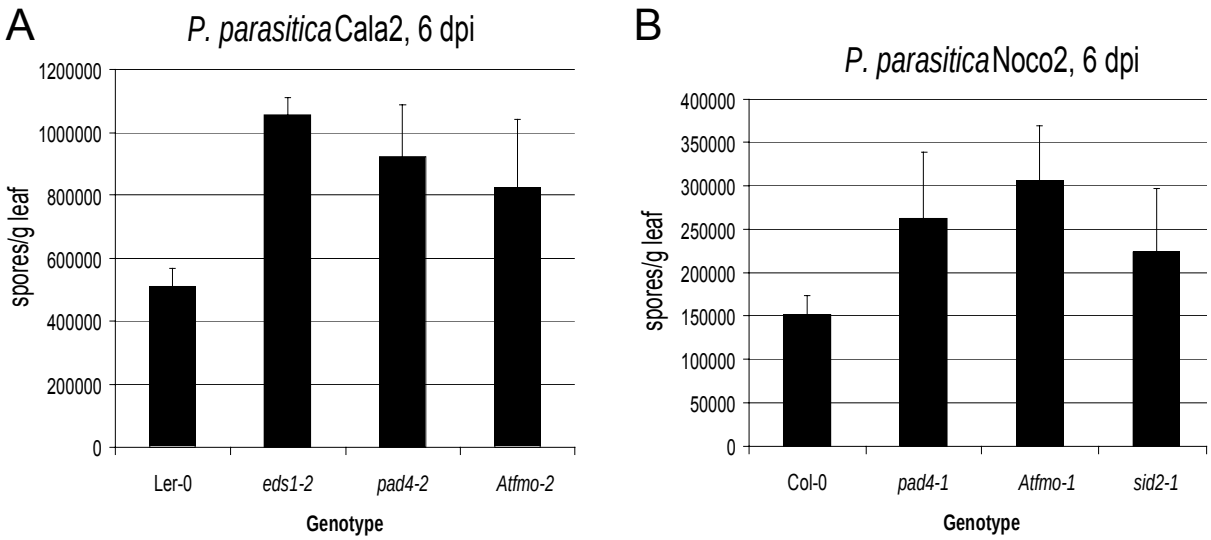


Figure 3.9. Extent of sporulation of virulent *P. parasitica* isolates in wild-type and mutant plants.
Spray inoculations were performed in the same way as described in Figure 3.5. Quantitative evaluations of sporulation strength were performed as described in Materials and Methods. Resistant control plants were used in both assays and did not develop any sporulation (data not shown; Resistant ecotypes were Col-0 containing *RPP2* in the Cala2 assay and Ler-0 containing *RPP5* in the Noco2 assay). Data represent the average from four replicate samples (+/- standard deviation). An independent experiment gave similar results.

3.3.2 Analysis of *AtFMO* expression and SA accumulation in wild-type and mutants

The microarray data in this study demonstrated that *AtFMO* mRNA is up-regulated upon inoculation with avirulent *P. syringae*. To get an idea of the spacial control of *AtFMO* expression we infected *AtFMO*-promoter-GUS lines in Col-0 background (*AtFMO*::GUS) with virulent and avirulent strains of *P. parasitica*. Upon avirulent *Peronospora* infection, GUS staining was observed at sites surrounding the HRs (Figure 3.10). Areas of GUS staining were tightly restricted and usually five mesophyll cells in diameter. In the compatible interaction, weaker GUS staining was observed only in cells penetrated by haustoria but not in further surrounding cell layers.

This data demonstrates that *AtFMO* is transcriptionally activated in compatible and incompatible pathogen interactions, which is in accordance with its positive regulatory role in basal and TIR-NBS-LRR-mediated resistance. The finding that

AtFMO::GUS is activated during the infection with virulent *P. parasitica* (defence response not associated with plant cell death at 4 dpi) demonstrates that AtFMO is not just up-regulated upon plant cell death. The exact timing of *AtFMO* expression upon pathogen infection needs to be addressed in future time course experiments.

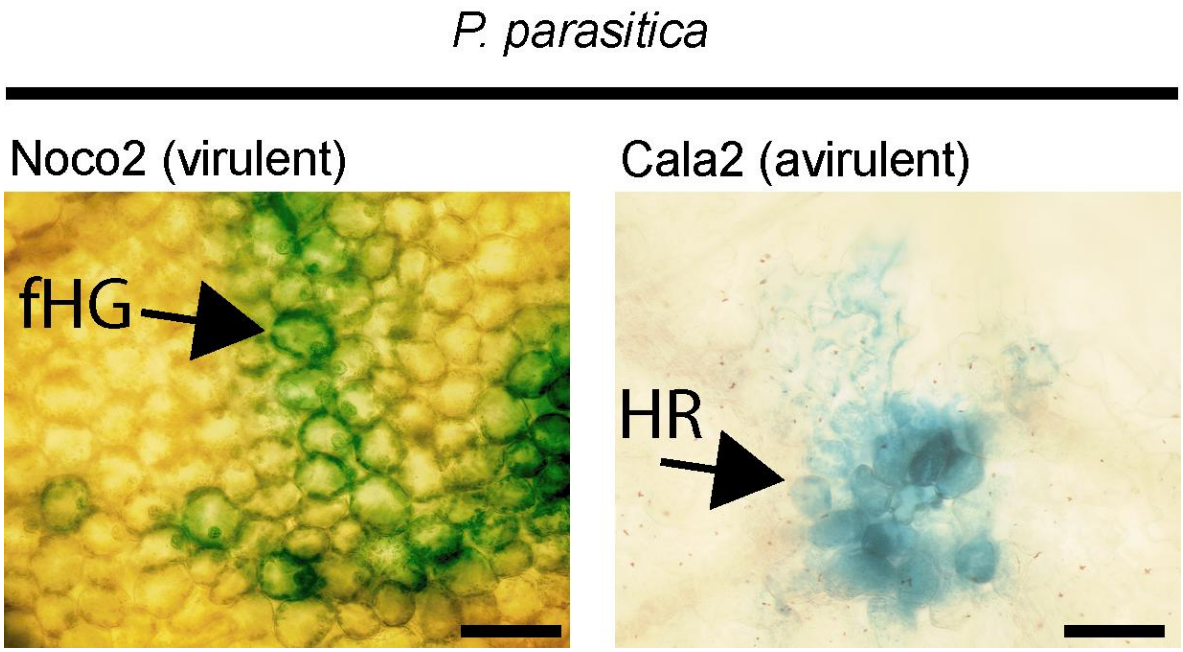


Figure 3.10. Inoculation of *AtFMO*-promoter-GUS lines in accession Col-0 with *P. parasitica* indicate that *AtFMO* is locally induced in compatible and incompatible interactions.
Leaves were processed to visualize beta-glucuronidase activity as described in Materials and Methods. The pictures were taken 4 dpi with *P. parasitica* isolate Noco2 and 7 dpi with isolate Cala2. The contrast in the Noco2 picture was enhanced with Photoshop software for a better display of the free hyphal growth (fHG). In non treated leaves GUS staining was only observed at hydathodes at the leave margins (data not shown and personal communication with John Mundy). *AtFMO*-promoter-GUS lines were kindly provided by John Mundy. The scale bar is 80 µm.

I tested whether *AtFMO* mRNA accumulation is at least partially independent of SA signalling by measuring the effect of *sid2-1* (SA synthesis mutant in Col-0 accession) on *AtFMO* expression by quantitative real-time PCR (qRT-PCR). The results presented in Figure 3.11 confirmed the *PAD4*-dependent up-regulation of *AtFMO* transcript as found in the microarray data (here in Col-0, whereas microarray samples were in Ws-0 background). Surprisingly, *AtFMO* transcript levels were found to be elevated in mock- and avr1-treated *sid2-1* samples, indicative of negative regulation of *AtFMO* by SA. This idea is supported by the finding that ectopic SA application reduces *AtFMO* transcript levels to 62% of mock treated samples (based

on microarray data from the Gene Investigator database, https://www.genevestigator.ethz.ch/). However, the qRT-PCR results are considered preliminary for the following reason. Several weeks after the samples for qRT-PCR analysis were collected, plants from the same growth chamber were found to be contaminated by an as yet uncharacterised pathogen which caused the strongest disease symptoms on *sid2-1*. Thus, high levels of *AtFMO* transcripts in *sid2-1* might have been caused by an undetected infection of *sid2-1* plants. Further independent qRT-PCR experiments will give more certain results.

If the effect of depleted SA levels in *sid2* on *AtFMO* expression is valid, the qRT-PCR results would argue for a negative regulatory function of SA on *AtFMO* mRNA accumulation as seen for some JA-induced genes (Spoel et al., 2003).

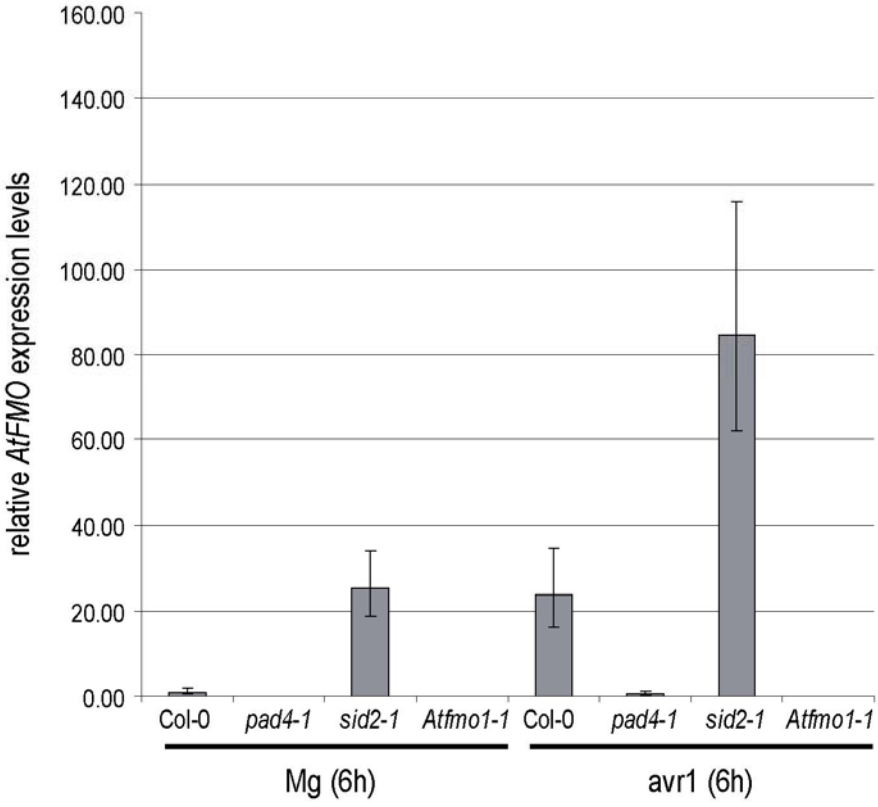


Figure 3.11. Relative expression of *AtFMO* mRNA in mock (Mg) and avr1-inoculated leaves derived from Col-0 wild-type, *pad4-1*, *sid2-1* and *Atfmo1-1*.
Transcript levels of *AtFMO* were determined by quantitative real-time PCR using SYBR green dye as described in Materials and Methods. *AtFMO* mRNA levels were normalised relative to the internal control *Actin2* and calculated relative to expression in mock treated Col-0 at 6h (Col-0 Mg 6 h). The error bars (standard deviation) are derived from three technical replicates from one biological sample set. The experiment has been performed once more on independent biological samples with similar results. Nonetheless these results are considered preliminary as discussed in the text. Abbreviations for treatments are as described in Table 3.1.

Salicylic acid is a well characterised defence signal that promotes resistance. To evaluate if defects in SA accumulation in *Atfmo* could account for the enhanced susceptibility phenotype, SA levels in non-, mock- and pathogen-treated wild-type and mutant plants were determined. SA levels in *Atfmo-1* were not significantly different from wild-type 24 h after avr1- or avr4-inoculation (Figure 3.12). In contrast to previous reports (Feys et al., 2001), a small but significant reduction in SA levels in *pad4-1* upon avr1-treatment compared to the corresponding wild-type sample was found. In non- and mock-treated samples basal SA levels were reduced in *pad4-1* and *Atfmo-1* compared to wild-type. Only trace amounts of SA was detected in *sid2-1* samples.

These data show that the resistance defects in *Atfmo* are not related to a deficiency in SA accumulation. Therefore, I reasoned that *AtFMO* is likely to be important for SA-independent signalling in the EDS1/PAD4 pathway.

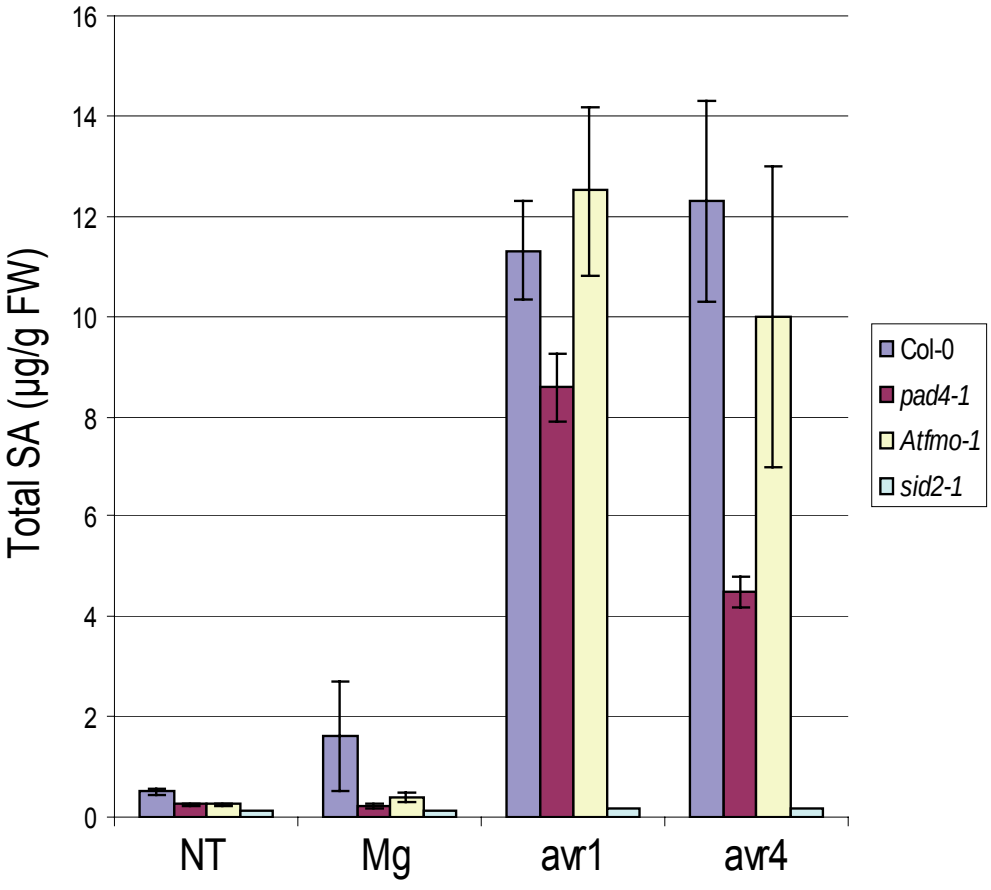


Figure 3.12. Accumulation of total salicylic acid (SA) in Col-0 wild-type, *pad4-1*, *Atfmo-1* and *sid2-1* in untreated (NT), mock treated (Mg) and leaves inoculated with avirulent *P. syringae* expressing either *avrRpm1* (avr1) or *avrRps4* (avr4).
Leaves of 4-week old plants were vacuum infiltrated with the respective bacterial strains at 5 X 10^6 cfu/ml. Extraction and quantification of total salicylic acid by HPLC 24 hpi was performed in triplicate as described in Materials and Methods. In *sid2-1* SA was observed in trace amounts without induction upon pathogen challenge. HPLC analysis was performed by P. Bednarek (MPIZ, Cologne). Data represent the average from three replicate samples (+/- standard deviation).

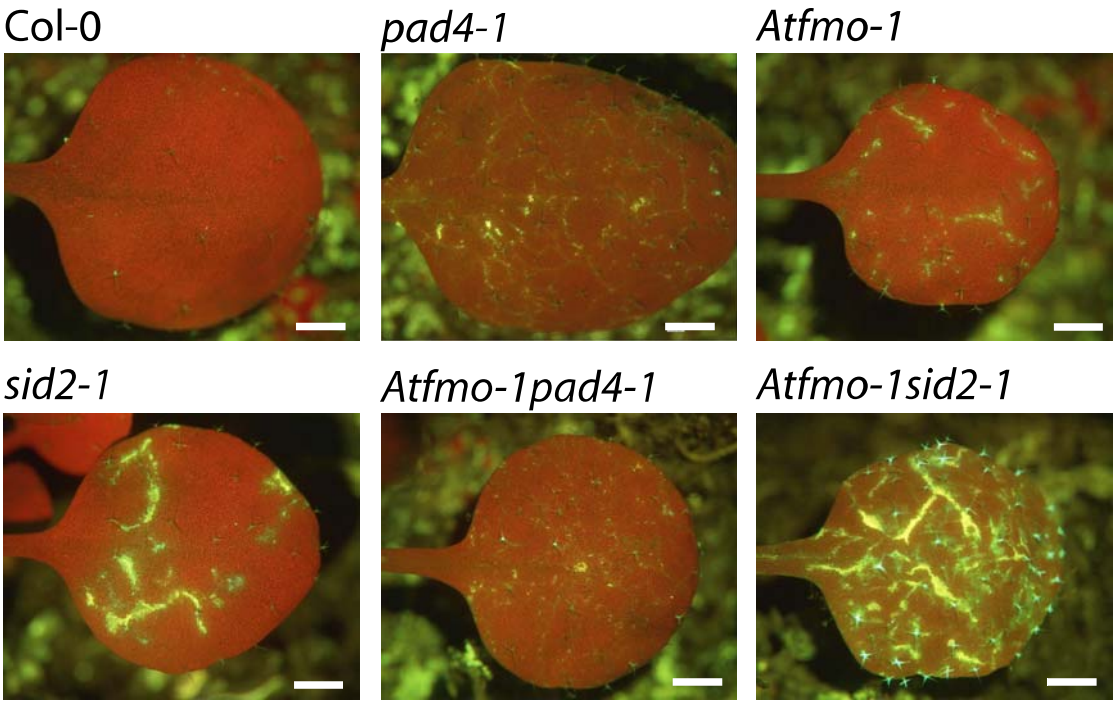
3.3.3 Double mutant analysis supports an SA-independent function of *AtFMO*

The stronger loss of in *R* gene-mediated (Figure 3.5A) and basal resistance (Figure 3.9B) in *Atfmo* over *sid2-1*, demonstrates that reduced SA levels in *Atfmo* were not the main cause for *Atfmo* partial susceptibility. These findings rather suggest that *Atfmo* is defective in a signalling pathway which functions up-stream and partially independently of SA-signalling. If this is the case, I reasoned that combined disruption of the *AtFMO*- and the SA-mediated defence pathways would result in additive effects.

I therefore generated homozygous *Atfmo-1sid2-1* and *Atfmo-1pad4-1* double mutants in accession Col-0 and tested them along with the corresponding single mutants for *RPP2*-mediated resistance (Figure 3.13). The *Atfmo-1pad4-1* double mutant plants supported hyphal growth and a sporulation to levels comparable to *pad4-1* alone. The only difference between *Atfmo-1pad4-1* and *pad4-1* was that more hyphae grew without trailing necrosis in the double mutant (trypan blue analysis, data not shown). In contrast, strong genetic additivity was observed between *Atfmo-1* and *sid2-1*. Whereas none of the single mutant plants (both 0/27) permitted pathogen sporulation at 5 dpi, nearly all of the corresponding double mutant plants supported sporulation (26/27). This semi-quantitative data correlated with the pathogen phenotype (extent of trailing necrosis monitored under UV-light) that showed additive effects between *Atfmo-1* and *sid2-1* but not between *Atfmo-1* and *pad4-1* (Figure 3.13A).

The results of this double mutant analysis support the idea that *AtFMO* functions in an *EDS1/PAD4*-controlled signalling process which is substantially independent of SA signalling.

A



B

Resistance phenotype (*RPP2*) in single and double mutants of *Atfmo-1*.

	Pathogen phenotype			
	total number of plants examined	**HR**	**TN**	**TN + Sporulation**
Col-0	18	18	0	0
pad4-1	27	0	1	26
sid2-1	27	0	27	0
Atfmo-1	27	0	27	0
Atfmo-1sid2-1	27	0	1	26
Atfmo-1pad4-1	27	0	0	27

Figure 3.13. *Atfmo-1* and *sid2-1* display additivity in loss of *RPP2*-mediated resistance.
(**A**) Growth phenotypes of virulent *P. parasitica* Cala2 in wild-type and mutant plants. Spray inoculations were performed in the same way as described in Figure 3.5A but at higher inoculum density (4 X 10^5 spores/ml). Plants were analysed with a binocular at 5 dpi under UV-light to visualize cell death-associated fluorescence. Col-0 developed only small spots of cell death (HR), *pad4-1* and *Atfmo-1pad4-1* displayed sporulation and thin trails of cell death (TN), *Atfmo-1* and *sid2-1* showed broader trails of cell death without sporulation, while *Atfmo-1sid2-1* exhibited severe TN with sporulation. The scale bar is 1000 µm. (**B**) Quantitative evaluations of resistance phenotype by scoring plants 5 dpi with *P. parasitica* Cala2. The three pathogen phenotype classes are hypersensitive cell death with no trailing necrosis or sporulation (HR), trailing necrosis with no occurrence of conidiophores (TN) and trailing necrosis with occurrence of conidiophores (TN+sporulation).

3.3.4 AtFMO has motifs characteristic for flavin-dependent monooxygenases but does not have close homologues in *Arabidopsis*

Sequencing of the *AtFMO* coding sequence (CDS) derived from Col-0 mRNA revealed that the published TAIR sequence for At1g19250 is incomplete as it lacks a stretch of 45 nucleotides (http://www.arabidopsis.org/). The corrected CDS (1593bp) was translated to the amino acid sequence (530 aa) which was found to be identical to protein GI 25513456 from the NCBI database (http://www.ncbi.nlm.nih.gov/). The corrected sequences were used for all further analyses.

Flavin-dependent monooxygenases (FMO) bind the co-factor FAD and catalyse oxygenation of various substrates containing nucleophilic nitrogen, sulphur, phosphorous and selenium atoms at the expense of NADPH (Poulsen and Ziegler, 1995). The extensively studied mammalian FMOs function in detoxification processes of xenobiotics (Lawton et al., 1994a), the single yeast FMO (yFMO) functions as a redox regulator under oxidative stress (Suh et al., 2000) and an insect FMO (SNO) is involved in plant toxin detoxification (Naumann et al., 2002). Little is known about the function of FMOs in plants. Only for one homologous FMO group, consisting of *Arabidopsis* Yucca, its two homologues and Floozy from *Petunia* are functional data available that suggest they are involved in auxin synthesis (Zhao et al., 2001; Tobena-Santamaria et al., 2002).

Amino acid sequence alignments of AtFMO with its rice homologue and with functionally characterised FMOs from yeast, insect and human revealed that three motifs typical for FMOs are conserved in AtFMO (Figure 3.14A). The FAD-binding-, at least partially the NADPH-binding- and the previous described FMO-identifying-motif (Fraaije et al., 2002) are present in AtFMO suggesting that AtFMO is *bona fide* a FMO. The lack of conservation in the second glycine (G) of the NADPH binding motif of AtFMO occurs in 15 out of the 24 related sequences in *A. thaliana* (data not shown) and also in the recently described bacterial FMO (bFMO) for which catalytic FMO activity was demonstrated(Choi et al., 2003).

Phylogenetic analysis revealed that AtFMO is an isolated member of the Arabidopsis FMO-like family and that AtFMO does not have close relatives in other non-plant organisms (Figure 3.14B). This may be indicative of a plant-specific function of AtFMO in pathogen defence. Two related sequences in rice (XP_470552, XP_474948) have been identified (54 %, 34.4% amino acid identity respectively)

suggesting that FMO function in plant defence is conserved between monocot and dicot plants.

It has to be noted that BlastN searches found At5g45180 to be closely related to *AtFMO* but microarray data from this study and from the Nottingham Arabidopsis Stock Centre's microarray database (http://affymetrix.arabidopsis.info/narrays/experimentbrowse.pl) indicated that At5g45180 is not expressed (data not shown). Furthermore, J. Mundy (personal communication) reports that sequence analysis of At5g45180 revealed a stop codon in the beginning of the third exon. Thus, unlike other FMO-like proteins in *Arabidopsis*, AtFMO forms an isolated branch in the phylogenetic tree. The potential signalling function of AtFMO will be discussed in the Discussion.

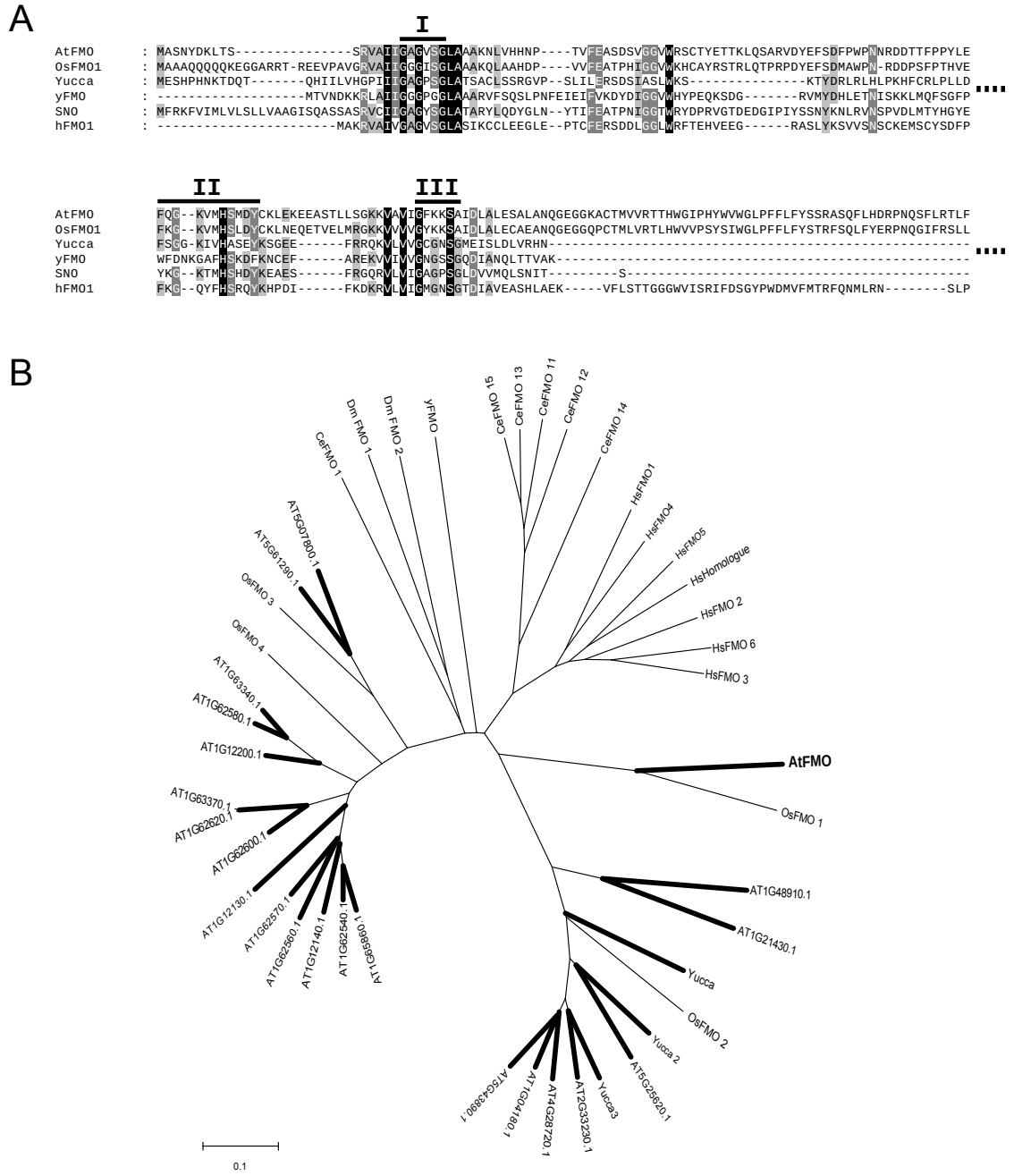


Figure 3.14. AtFMO is an isolated member of the FMO-like protein family.
(**A**) Protein alignment of parts of the predicted amino acid sequence from AtFMO with related sequences from rice OsFMO (XP_470552), Arabidopsis Yucca (At4g32540), insect Tyria jacobaeae SNO (CAD12369) and human hFMO1 (NP_002012). Common FMO-motifs are indicated in the top line: (**I**) FAD-binding motif "GXGXXG", (**II**) FMO-identifying sequence motif "FXGXXXHXXX(Y/F)" and (**III**) NADPH-binding domain "GXGXX(G/A)". (**B**) Phylogenetic tree of AtFMO and related amino acid sequences from *Arabidopsis thaliana* (24 sequences), *Oryza sativa* (4) *Drosophila melanogaster* (2), *Caenorhabditis elegans* (6) and *Homo sapiens* (7). Sequences resulted from BlastP search in the MIPS database (for Arabidopsis sequences; http://mips.gsf.de/) and NCBI (refseq database; http://www.ncbi.nlm.nih.gov/) with an e value of 10^{-10} as the exclusion limit. Sequences for yFMO (did not meet the exclusion limit) and OsFMO (not listed in the refseq database) were added manually. For a detailed list of the protein annotations and the construction of the tree see Materials and Methods. Branches leading to Arabidopsis FMO-like sequences were printed in bold. The scale bar represents the proportional difference between sequences

3.4 Functional characterisation of *AtNUD2.1* and *AtNUD4.1*

As mentioned above, preliminary observations indicated that *Atnud4.1-1* displayed properties of a constitutive defence mutant. The fact that two homologous genes (*AtNUD2.1* and *AtNUD4.1*) were identified as strongly *EDS1/PAD4*-dependent prompted me to characterise them in more detail.

3.4.1 Sequence and transcriptional analysis for *AtNUD2.1* and *AtNUD4.1*

BLASTP searches led to the identification of five additional protein sequences from *Arabidopsis* with homology to AtNUD4.1 and AtNUD2.1. In accordance with the TAIR annotation, AtNUD4.1 and AtNUD2.1 both contained the NUDIX-motif Gx5Ex7REUxEExGU (where U is a hydrophobic and x any amino acid; Figure 3.15A) (Bessman et al., 1996). NUDIX hydrolases are a family of proteins which catalyze the hydrolysis of a wide spectrum of substrates, predominantly nucleoside diphosphates linked to some other moiety x (Bessman et al., 1996). As NUDIX substrates include cell toxic compounds like dinucleoside polyphosphates, ADP-ribose, NADH, nucleotide sugars, or ribo- and deoxyribonucleoside triphosphates, it was suggested that NUDIX hydrolases are “house cleaning enzymes” as they might clear the cell of potentially deleterious endogenous nucleotide metabolites (Bessman et al., 1996; Dunn et al., 1999).

The phylogentic relationship and expression patterns of *AtNUD4.1*, *AtNUD2.1* and their five *Arabidopsis* homologues are depicted in Figure 3.15B. Another NUDIX hydrolase, At2g04430 was found to be pathogen inducible in an *EDS1/PAD4*-dependent manner but solely at 6 h, as can be seen in Figure 3.15B. Interestingly, although At5g47240 mRNA levels did not change upon pathogen challenge in wild-type samples, much higher transcript levels in *eds1* and *pad4* than in wild-type was observed in the avr4-treated samples at 6 h.

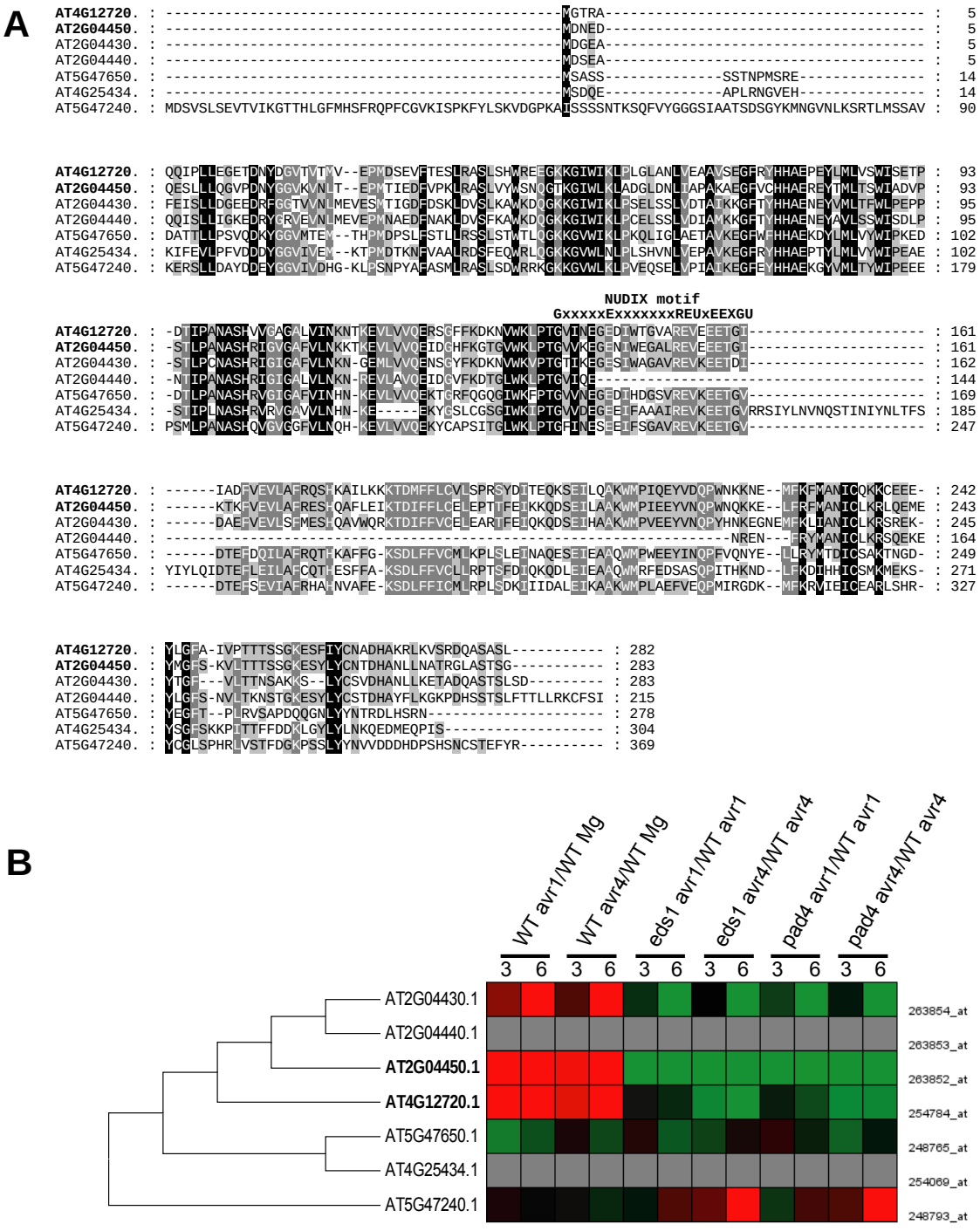


Figure 3.15. AtNUD2.1 (At2g04450) and AtNUD4.1 (At4g12720) are members of a small protein family in Arabidopsis.
(**A**) Protein sequence alignment of AtNUD2-1, AtNUD4.1 reveals that the NUDIX motif is present in all but one protein (At2g04440). U stands for any hydrophobic amino acid. (**B**) A phylogenetic tree based on amino acid sequence similarity of the seven NUDIX-like proteins and the corresponding gene expression ratios. Grey colour in the expression ratio graph means the gene is not expressed in the data set.

3.4.2 T-DNA mutants of two homologous NUDIX hydrolases display constitutive defence responses and enhanced basal resistance

The finding that the two homologues *AtNUD4.1* and *AtNUD2.1* are pathogen-induced in an *EDS1/PAD4*-dependent manner, suggested some redundancy between them. Consequently, *AtNUD2.1AtNUD4.1-1* double mutant plants were derived from crosses and brought to homozygosity. To validate the initially identified dwarf phenotype of *Atnud4.1-1*, an independent T-DNA mutant in Col-0 background was identified (referred to as *Atnud4.1-2*) in the Salk T-DNA selection (Scholl et al., 2000) and included in the further analysis.

First the growth phenotypes of the two independent *Atnud4.1* mutants, *Atnud2.1* and of the double mutant *Atnud2.1Atnud4.1-1* were examined. Germination rate and the early growth appearance of the NUDIX single and double mutants did not differ from wild-type (data not shown) but when the first leaves (from 2 weeks on) expanded cotyledons of *Atnud4.1* and *Atnud2.1Atnud4.1* mutants pointed down to the earth whereas wild-type cotyledons were positioned parallel to the soil (Figure 3.16A: shown for *Atnud4.1-1*). Later in development, both *Atnud4.1* mutants and *Atnud2.1Atnud4.1-1* but not *Atnud2-1* displayed leaf wrinkling and a reduced plant size and weight (Figure 3.16B and 3.17). This phenotype was especially pronounced in *Atnud2.1Atnud4.1*. A first inspection suggests that a reduced leaf size and not the delayed development of leaves are the reason of the dwarf phenotypes (data not shown).

Microscopic inspection of trypan blue stained leaves revealed spontaneous single cell death in *Atnud4.1-1, Atnud4.1-2* and especially strong in *Atnud2.1Atnud4.1-1* but not in Col-0 wild-type and *Atnud2.1* (Figure 3.16C). Dead cells were observed in all three leaf cell layers (epidermis, palisade parenchyma and sponge parenchyma).

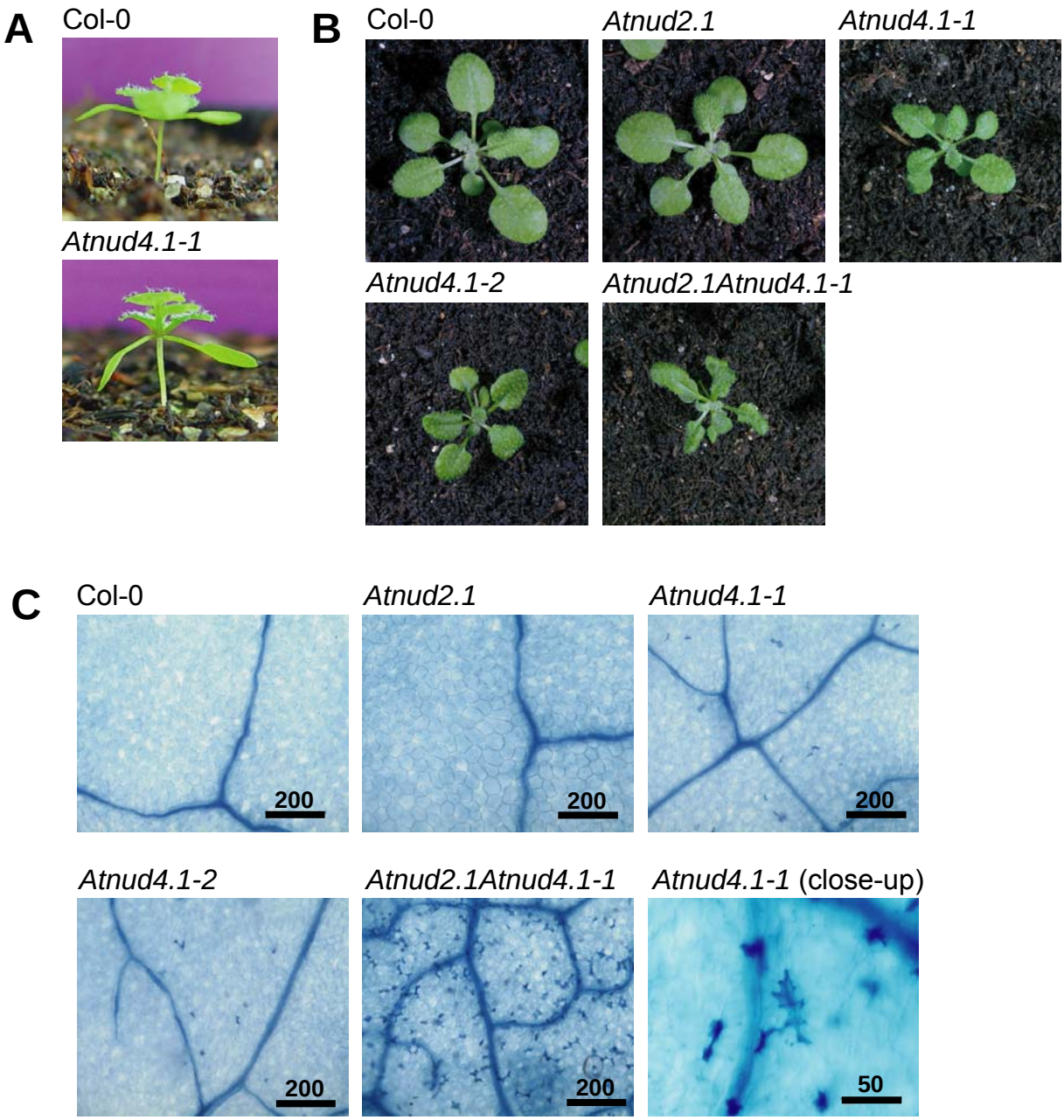


Figure 3.16. Developmental phenotypes of Col-0 wild-type, *Atnud2.1* and *Atnud4.1* single and double mutant plants grown under short day.
(**A**) Side view on 15-day old plants of Col-0 wild-type and *Atnud4.1-1*. (**B**) 25-day old plants of wild-type and mutant plants in top view. (**C**) Fully developed leaves of Col-0 wild-type and mutant plants were harvested 4 weeks after sowing and stained with trypan blue. Spontaneous plant cell death (seen as dark blue spots) was observed in 4-weeks old leaf samples of *Atnud4.1-1*, *Atnud4.1-2* and *Adnud2-1Atnud4.1-1* but not in Col-0 and *Adnud2-1*. Similar results were observed in 3-week old leaves (data not shown). The scale bar unit is µm.

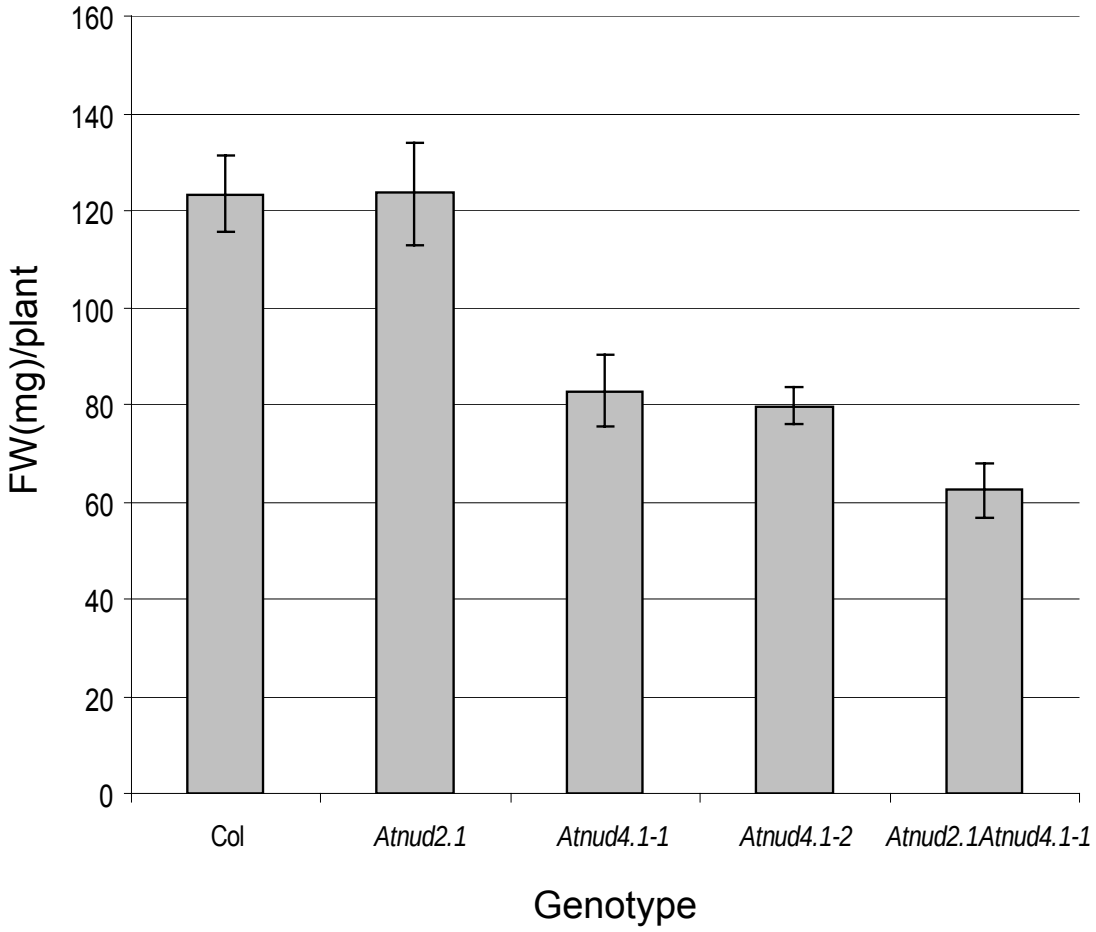


Figure 3.17. Plant fresh weight (FW) of 3-week old Col-0 wild-type, single mutants *Atnud2.1*, *Atnud4.1-2*, *Atnud4.1-2* and double mutant *Atnud2.1Atnud4.1-1*.
The average weight (+/- standard deviation) per plant was calculated from the weight of the aerial tissue of five plants per genotype.

As the growth defects were reminiscent of constitutive defence mutants, the NUDIX related single and double mutants were tested for basal resistance to virulent *P. parasitica* isolate Noco2. Microscopic analysis of the *P. parasitica* infection phenotypes revealed membrane damage in cells around hyphal structures in both the single mutant of *Atnud4.1* and the *Atnud2.1Atnud4.1* double mutant. In contrast, cells from Col-0 wild-type and *Atnud2.1* that were in close proximity to hyphal structures did not show cell damage (no trypan blue staining of plant cells; Figure 3.18A).

Visible sporulation on Col-0 wild-type and *Atnud2.1* occurred 5 dpi and peaked at 7 dpi, whereas first weak sporulation on both single mutants of *Atnud4.1* and the *Atnud2.1Atnud4.1* double mutant was delayed at 7 dpi and never reached the

sporulation intensity of wild-type (Figure 3.18B). Quantitative determination of the degree of sporulation revealed additive effects between *Atnud2.1* and *Atnud4.1*. This result correlates with the quantitative weight data (Figure 3.17).

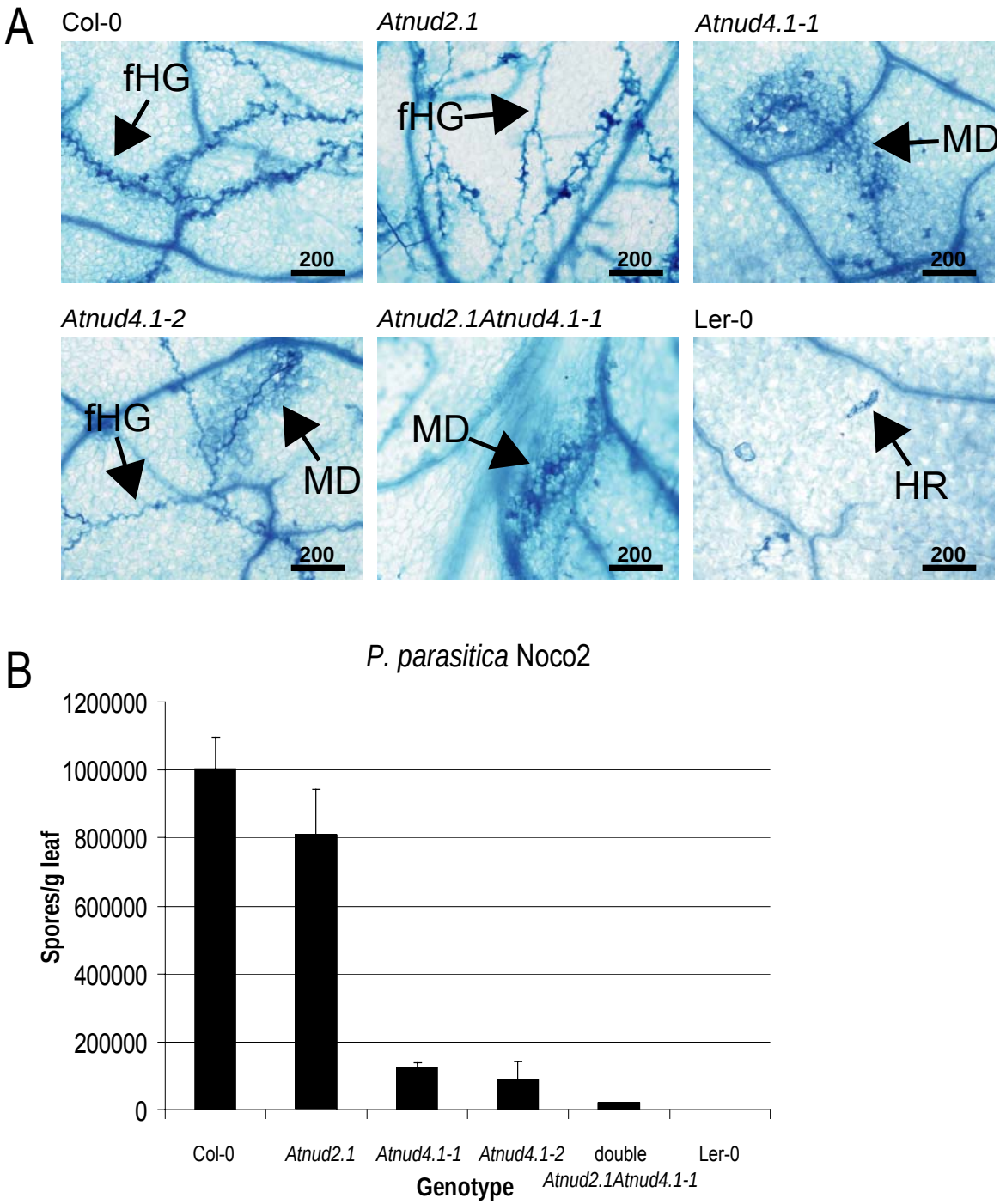


Figure 3.18. Plant response phenotypes and growth of virulent *P. parasitica* isolate Noco2 on Col-0 wild-type, *Atnud2.1*, *Atnud4.1-1*, *Atnud4.1-2* and *Atnud2.1Atnud4.1-1*.
Plants were spray inoculated with conidiospores at $4x10^4$ spores/ml 14 to 18 days after sowing and harvested 7 dpi for trypan blue staining (**A**) or spore counting (**B**). (**A**) Free hyphal growth (fHG) can be detected in Col-0 wild-type and *Atnud2.1* whereas in both *Atnud4.1* mutants and the double mutant

hyphal growth is accompanied at least partially by membrane damage (MD) in the adjacent mesophyll cells seen as dark blue trypan blue stained areas. The scale bar unit is µm. (**B**) Pathogen sporulation was determined in four replicate samples (+/- standard deviation). The level of sporulation of the double mutant was at the detection limit (2/20 of the examined plants displayed weak sporulation). An independent experiment gave similar results.

Few *Arabidopsis* mutants have been described that have enhanced resistance to virulent *P. parasitica*. Of these, *pmr4-1* (*powdery mildew resistance4-1*), first identified as a resistant mutant to *Golovinomyces orontii,* was shown to be more resistant to virulent *P. parasitica* (Vogel and Somerville, 2000). I compared the basal resistance response of the NUDIX mutants with *pmr4-1* to *P. parasitica* and *G. orontii*. Basal resistance to *P. parasitica* Noco2 was similar in *Atnud4.1-1* and *pmr4-1* (Figure 3.19A). In contrast, no enhanced resistance to *G. orontii* was detected by visual inspection in any of the NUDIX mutants whereas the *pmr4-1* was resistant, as previous described (Figure 3.19B).

Measurements of total SA levels revealed that high concentrations of SA accumulate in healthy leaves of *Atnud4.1* and reached levels comparable to wild-type pathogen-treated tissue (Figure 3.20). Taken together these results suggest that *Atnud4.1* and *Atnud2.1Atnud4.1* are in hyper-responsive state in the absence of a pathogen challenge.

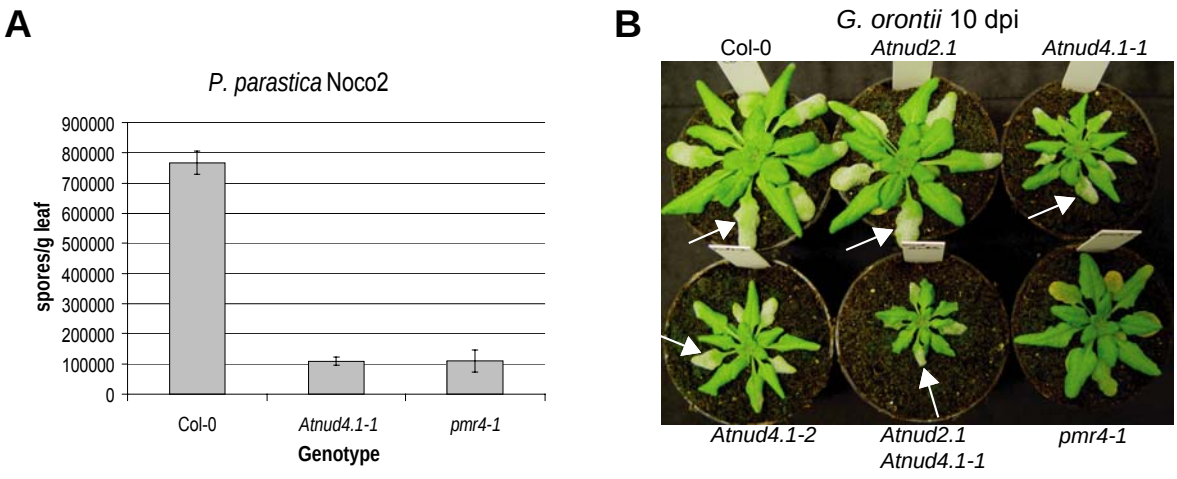


Figure 3.19. *Atnud4.1* and *Atnud2.1Atnud4.1-1* display enhanced basal resistance to *P. parasitica* but not to *Golovinomyces orontii*.
(**A**) Virulent *P. parasitica* sporulation was determined as described for Figure 3.9. (**B**) Spores of *G. orontii* were applied to leaves of 4-week old plants by rubbing them with heavily sporulating leaves. At 10 dpi, pictures of representative plants were taken. No sporulation but yellowing of the infected *pmr4-1* leaves was observed. Arrows indicate leaves with sporulation.

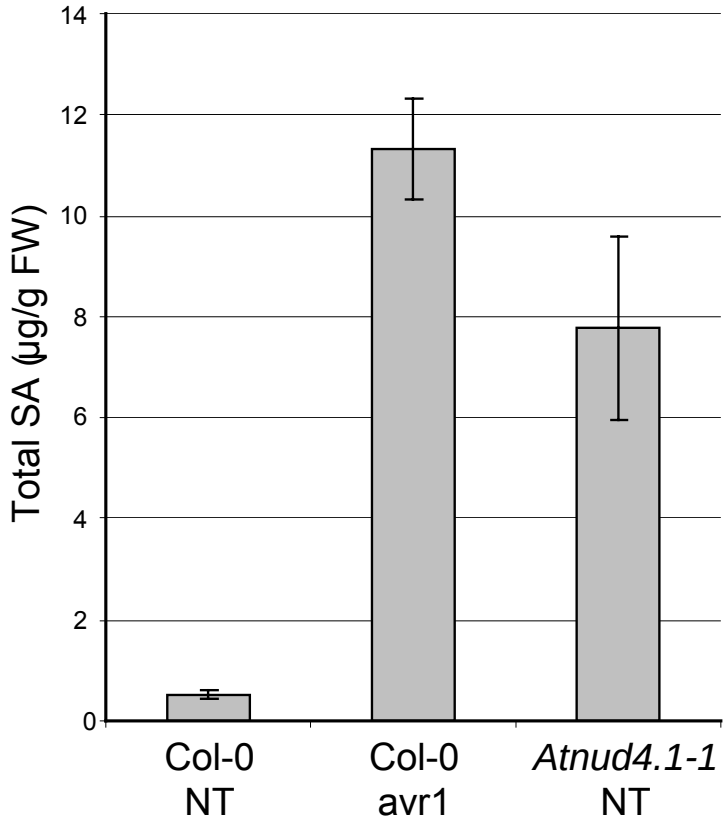


Figure 3.20. Accumulation of total salicylic acid (SA) in non-treated (NT) leaves of *Atnud4.1-1* in comparison to Col-0 wild-type in untreated and pathogen-treated state.
Leaves of 4-week old plants were vacuum infiltrated with a suspension of avirulent *P. syringae* expressing *avrRpm1* (avr1, at 5 X 10^6 cfu/ml). Extraction and quantification of total salicylic acid by HPLC after 24 hpi was performed as described in Materials and Methods. Data represent the average from three replicate samples (+/- standard deviation). The experiment was repeated three times with similar results.

4. Discussion

EDS1 and *PAD4* are required for *RPS4*- but not for *RPM1*-mediated resistance to *Pseudomonas syringae* expressing *avrRps4* or *avrRpm1*, respectively. Transcriptional profiles of wild-type and mutants plants during early *R* gene-mediated defence were examined to identify components involved specifically in *EDS1- and PAD4*-controlled signalling. In this study, I found that infection with bacteria expressing *avrRpm1* (avr1) or *avrRps4* (avr4) triggered transcriptional changes in a similar set of genes but with different kinetics (abbreviations for the experimental conditions are defined in Table 3.1). Further, I identified sets of genes with an *EDS1- and PAD4*-dependent transcriptional expression in healthy tissue (Group I), in avr1- (Group II) and avr4- (Group III) challenged leaves (Table 3.4). For a subset of these genes their biological relevance in modulating resistance was tested in pathogen assays, resulting in the identification of a flavin-dependent monooxygenase as a positive regulator and two sequence-related NUDIX hydrolases as negative regulators of plant disease resistance. Neither FMOs nor NUDIX hydrolases were previously shown to be associated with host defence responses against pathogens in any biological system.

4.1 A quantitative model to explain defence-related global transcriptional reprogramming and corresponding mutant effects

I found that *RPM1*- and *RPS4*-mediated global transcriptional changes differ in timing but result in repression or induction of similar sets of genes at 6 h. This finding is in agreement with previous studies that revealed that different *R* genes and basal resistance responses affect overlapping sets of genes (Tao et al., 2003; Eulgem et al., 2004). The authors concluded that signals derived from different *R* genes and basal resistance must converge up-stream of gene regulation. Common signalling events such as redox changes, Ca^{2+} fluxes and SA accumulation might represent these convergence points. On the other hand, the *RPM1*- and *RPS4*-controlled pathways differ in their requirement for certain genetic loci (e.g. *EDS1/PAD4/AtFMO*

in the *RPS4*-pathway and *NDR1* in the *RPM1*-pathway) (Aarts et al., 1998; Feys et al., 2001 and this study). A quantitative signalling model was originally postulated by Toa et al. (2003) to explain the quantitative rather than qualitative differences between compatible and incompatible plant-pathogen interactions and to illustrate the differential defects of *ndr1* in basal, *RPS2*- and *RPM1*-mediated resistance. I modified this model and applied it to discuss the observations that: (1) on the global-scale, similar genes sets are regulated by *RPM1* and *RPS4*, (2) *EDS1, PAD4 and AtFMO* are required in basal and *RPS4*-mediated resistance but not in *RPM1*-mediated resistance, (3) the extent of loss of basal resistance in *eds1*, *pad4* and *Atfmo* is similar whereas dissimilar strong defects are seen in *RPS4*-mediated resistance for the three mutants (Figure 4.1).

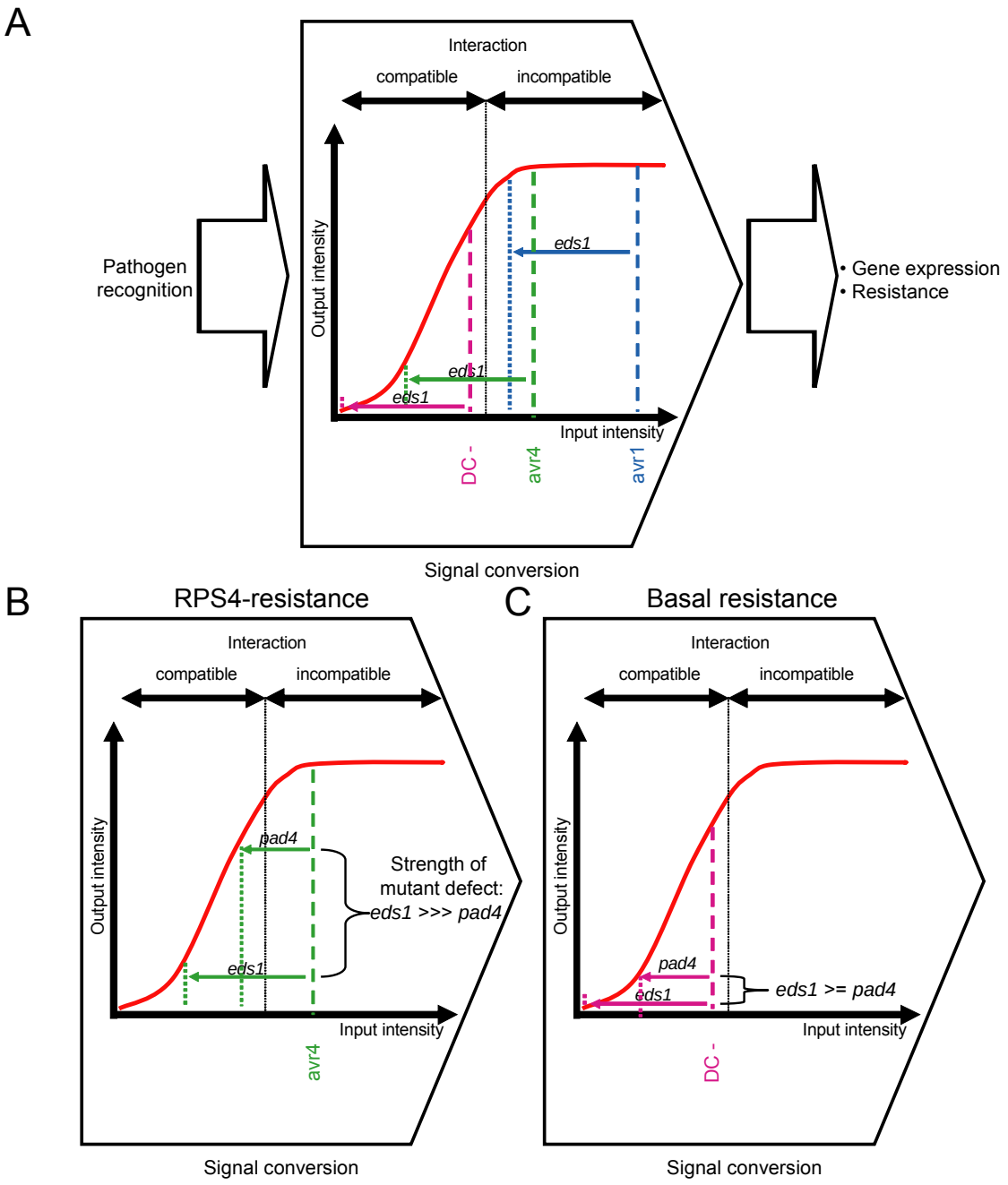


Figure 4.1. Quantitative model to illustrate signalling in compatible and incompatible interactions and the differential requirement for genetic loci in plant defence (modified from Toa, et al., 2003 and Eulgem, 2005).
(**A**) Recognition of pathogens by the basal or R protein defence system generates input signals of different intensities for a common signal conversion mechanism. The recognition process in basal and R protein-mediated resistance differs (reflected by different classes of receptor molecules) but both systems feed into a common signal conversion mechanism. This mechanism converts the different signal input strength to quantitatively different output, reflected in gene expression and resistance strength. The mode of this signal conversion mechanism is illustrated by the saturation curve depicted in red. Recognition of avr1 and avr4 is defined by higher input intensities whereas recognition of virulent bacterial strains (DC-) is defined by low intensity input values. Consequently incompatible interactions are defined by expression changes in many genes with high amplitudes of expression (high output intensity) whereas compatible interactions affect fewer genes with lower expression amplitudes. A mutation in *EDS1* reduces non-specifically the input signal in basal, RPS4- and RPM1-mediated resistance. (**B**) The defect in RPS4-mediated resistance is more severe in *eds1* compared to *pad4* whereas (**C**) similar strong defects in *eds1* and *pad4* are observed in basal resistance.

According to the quantitative model, *eds1* reduces non-specifically the input signal in basal, *RPS4*- and *RPM1*-mediated resistance. The model can also be applied to effects of *pad4* or *Atfmo*. As *RPM1* generates an input signal strength that is in the saturated phase in wild-type plants, an input reduction in *eds1* causes a minor reduced output signal, resulting in no or minor defects in pathogen-induced gene expression or resistance. In contrast, the *RPS4*-emitted input signal lies at the edge of the saturation curve and a further reduction in *eds1* below the compatibility/incompatibility threshold leads to a drastically reduced output signal compared to wild-type. As a consequence, mutations in *EDS1* have a big impact on *RPS4*-mediated gene regulation and resistance, turning an incompatible interaction to a compatible interaction. One experiment to evaluate this model would be to test if partially functional mutant alleles of *RPM1* (as described in Tornero et al., 2002) are *EDS1*-dependent. The experiment would combine a weak *rpm1* allele with the *eds1* mutation by crossing and quantify the growth of *P. syringae* expressing *avrRpm1* in the homozygous *rpm1eds1* double mutants.

The saturation curve suggested by Toa and colleagues did not include a lag-phase but I introduced this modification to explain the similar defects of *eds1*, *pad4* and *Atfmo* in basal resistance and their differential effects in *RPS4*-mediated resistance (Figure 4.1B;C). The different strong effects *eds1* and *pad4* in reducing the input signal are depicted as different long arrows. For the sake of simplicity, *Atfmo* is not represented in Figure 4.1 but the *Atfmo* defect would be illustrated by a shorter arrow compared to *pad4* to mirror its less strong loss of *RPS4*-mediated resistance.

If one considers the SAR signal as a weak input signal in systemic tissue, the prediction would be that, besides *EDS1/PAD4*, *AtFMO* is also required for perception of the SAR signal. Future SAR assays will evaluate the prediction that *AtFMO* is essential for the establishment of SAR.

The notion presented here of a genetic requirement for *EDS1* and *PAD4* in signalling pathways that have weak input signals is consistent with the previously proposed “amplification role” of *EDS1/PAD4*, necessary for potentiating weak signals (Jirage et al., 1999; Feys et al., 2001; Rusterucci et al., 2001).

Although this model is helpful to explain similar gene expression profiles during *RPM1*- and *RPS4*-signalling despite their different genetic requirements, it is oversimplified in several respects. The model cannot explain the expression patterns for all genes. For example, genes in Group I - III were repressed in both *eds1* and

pad4 to a larger extent than a non-specifically reduced input level could account for. Also, a mutation which lowers the input level just below the incompatibility threshold would give the pathogen an opportunity to establish a mechanism which actively lowers the defence response of its host. Manipulation of the host defence response by the pathogen provides an additional level of complexity which is not addressed in this model. Active suppression of the plant host defence response by *P. syringae* has been previously reported (Chen et al., 2000; Abramovitch et al., 2003; Cui et al., 2005).

In summary, according to the model presented here, *EDS1*, *PAD4* and *AtFMO* function in both *RPM1*- (CC-NBS-LRR) and *RPS4*- (TIR-NBS-LRR) mediated signalling. Their mutant defect in local defence is however only observable in plant-pathogen interactions that emit a low to medium intensity input signal.

Such a quantitative effect of another defence modulator has been described at the molecular level. In barley, *RAR1* is genetically required for *MLA6*- but not for *MLA1*-conferred resistance to the powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (Bieri et al., 2004). Analysis of the R protein levels revealed that in both instances RAR1 elevates steady state protein levels of MLA1 and MLA6. Initially lower protein levels of MLA6 and a further reduction in *rar1* resulted in a genetic requirement for *RAR1* in *MLA6*- but not in *MLA1*-mediated resistance despite similar biochemical function of RAR1 in elevating steady state MLA protein levels.

4.2 *EDS1/PAD4*-regulated genes might function either downstream of *EDS1/PAD4* or as part of a positive feed back loop

As mentioned in the introduction, epistatic analyses with constitutively activated *R* genes were indicative for an *EDS1/PAD4* function coincident or directly downstream of *R* genes (Shirano et al., 2002; Zhang et al., 2003). In agreement with the idea of an early function of *EDS1/PAD4* in the genetic defence signalling cascade, I was unable to detect genes with a strong up-regulation upon avr4-treatment independently of both *EDS1* and *PAD4*. This finding suggests that either there are no components up-stream of *EDS1/PAD4* or these components are not transcriptionally regulated. However, positioning of signalling components in a genetic pathway can not be performed solely based on transcriptional data. A good example of the more complex nature of interactions between defence signalling components comes from

the study of the *Arabidopsis* gain-of-function mutant *acd6*. Basal mRNA levels of *ACD6* are repressed in *eds1* and *pad4* (this study and Lu et al., 2003) suggesting that *ACD6* is genetically downstream of *EDS1/PAD4*. But as the resistance phenotype and growth defects of the *acd6* gain-of-function mutant are partially dependent on *PAD4* and *acd6* displays enhanced *EDS1/PAD4* mRNA levels (Lu et al., 2003), it was suggested that *ACD6* functions up-stream of *EDS1/PAD4* in a positive signal amplification loop (Dong, 2004). Consequently, an *EDS1/PAD4*-dependent expression pattern observed in the Group I–III genes suggests their involvement either downstream of *EDS1/PAD4* or as part of a positive feed back loop. Also for the NUDIX hydrolases AtNUD2.1 (Group II) and AtNUD4.1 (Group III), identified as negative regulators of plant defence, and for the flavin-dependent monooxygenase AtFMO, identified as positive defence regulator, the exact position in the signalling pathway has to be determined. Double mutant analyses of *eds1* and *pad4* with *Atnud4.1* will help to position the NUDIX hydrolase *AtNUD4.1* in the EDS1/PAD4-controlled signalling pathway. Recent preliminary results indicate enhanced basal resistance in transgenic *Arabidopsis* lines over-expressing *AtFMO* (data not shown). If this result can be confirmed, the gain-of-function phenotype of the *AtFMO* over-expresser lines will be tested for its dependency on *EDS1/PAD4*, thus helping to position *AtFMO* in relation to *EDS1/PAD4* in the signalling pathway leading to resistance.

I examined transcriptional changes in *R* gene-mediated resistance. Consistent with the quantitative model discussed above stating that *R* gene-meditated and basal resistance target similar sets of genes, *EDS1/PAD4* are also transcriptionally activated in compatible plant-pathogen interactions (Feys et al., 2001). Consistent with a partially *R* gene-independent transcriptional activation, *EDS1* and *PAD4* but also *AtNUD2.1* and *AtNUD4.1* are induced by the PAMP flagellin peptide flg22 (Zipfel et al., 2004). Application of flg22 prior to bacterial challenge restricts pathogen invasion in some cases. *AtFMO* was not listed as a strongly flg22-induced gene in the supplement data of Zipfel et al. (2004). Nevertheless, the activation of *AtFMO*::GUS upon infection with virulent *P. parasitica* (Figure 3.10) is indicative for partially *R* gene-independent transcriptional regulation of *AtFMO* and for an induction not solely associated with plant cell death.

Notably, *EDS1/PAD4* are not required for flg22-induced resistance (Zipfel et al., 2004). As flagellin induces SA, JA and ethylene signalling, Zipfel et al. (2004)

proposed that perturbation of one signalling pathway is not sufficient to abolish flg22-mediated resistance.

4.3 *EDS1/PAD4*-dependent genes – More to discover?

I analysed insertion mutants corresponding to all six members of Group II and to one member of Group III (Table 3.4). For three (*AtFMO*, *AtNUD2.1* and *AtNUD4.1*) out of the seven genes, I was able to demonstrate a regulatory function in plant defence. Although insertion mutants corresponding to *AtMRP7*, *AtLTP*, *AtPRK* and *AtGH* were not deficient in *RPP2*-mediated defence, they may be defence regulators of SAR or basal resistance. Also functional redundancy might have hindered the identification of a knock-out phenotype. For example, the *lipid transfer protein-like* gene *AtLTP* (At5g55450; Group II) has three sequence-related genes in the *Arabidopsis* genome. Two of these are also pathogen inducible (At5g55410 and At5g55460, data not shown) and the third is *DIR1* which is not pathogen responsive but has been shown to be essential for the establishment of SAR (Maldonado et al., 2002). Similarly, the *Atnud2.1* mutant phenotype was only detectable as an additive effect in the *Atnud2.1Atnud4.1* double mutant plants. Thus, future studies will focus on the identification of sequence-related genes of Group II and corresponding double mutants will be examined for altered resistance responses.
Considering that Group I and III contain several previously defined defence regulators, some of the yet uncharacterised genes in these gene group might have a regulatory role in plant resistance. Thus, I anticipate that analysis of corresponding insertion mutant plants may result in the identification of new defence regulators.

Do the gene descriptions of the *EDS1/PAD4*-dependent genes listed in Table 3.4 give any hint to the signalling functions of EDS1/PAD4? In Table 3.4, five genes are predicted to encode proteins of the ankyrin repeat family (including *ACD6*) and five genes are linked to Ca^{2+} signalling or binding (including *BONZAI1*). A joint signalling role of *EDS1/PAD4* with the ankyrin repeat protein encoding *ACD6* was proposed recently (Dong, 2004) although the biochemical nature of cooperation between EDS1/PAD4 and ACD6 is unknown. Also, the potential involvement of EDS1/PAD4 in Ca^{2+} signalling is not investigated yet and still needs to be addressed in future

experiments. Later in the Discussion, I propose a potential role of the *EDS1/PAD4*-controlled NUDIX hydrolases in Ca^{2+} signalling.

Large-scale forward genetic screens were successfully applied to identify genetic components in plant defence signalling (Ausubel et al., 1995; Glazebrook et al., 1997a). A disadvantage inherent to these screens is the need to examine large numbers of plants and thus it is difficult to detect mutants with subtle alterations in the defence response. The success of the present study in identifying as yet uncharacterised regulators of plant resistance suggests that combining transcriptional profiling and classic insertion mutant analysis is a promising approach to refine the plant defence signalling network. The availability of large microarray data sets derived from various experimental conditions allows identification of genes that are strictly co-regulated with previous defined pathogen regulators. The well established insertion mutant resources allow evaluation of the biological relevance of such genes in plant resistance. In future, the integration of genome-wide expression data with other data resources (e.g. protein-protein interaction data) should provide a powerful tool to predict gene functions *in planta*, as successfully already applied in yeast and human (Lee et al., 2004; Basso et al., 2005).

4.4 *AtFMO* - a positive regulator of plant defence responses

4.4.1 *AtFMO* transcript accumulation and defence function is partially independent of SA

I identified *AtFMO* as a gene whose transcript accumulation during *RPM1*-mediated signalling was significantly reduced in both *eds1* and *pad4*. To date, no genes were reported to be transcriptionally *EDS1/PAD4*-dependent in CC-NBS-LRR-mediated defence signalling. However, there are some genes described whose expression is blocked in *eds1/pad4* but not in SA-deficient plants in basal or TIR-NBS-LRR-mediated resistance. Eulgem et al. (2004) identified genes blocked in *pad4* but not in *NahG* during *RPP4*-signalling (including *AtNUD4.1*) but the authors did not demonstrate that these genes are functionally relevant in plant defence. In contrast, for *Agd2-Like Defence Response Protein1* (*ALD1*) an essential function in local and systemic defence responses was established (Song et al., 2004a). Transcriptional

up-regulation of *ALD1* upon virulent *P. syringae* infection was found to be blocked in *pad4* but not in *sid2-1*, *NahG* or *npr1* (Song et al., 2004b). The authors suggested that PAD4, besides controlling SA accumulation, also emits another signal that controls *ALD1* mRNA accumulation. ALD1 has amino-transferase activity *in vitro* and *ald1* mutant plants displayed reduced SA levels. Therefore, it was proposed that ALD1 produces an amino-acid derived signal which stimulates SA synthesis and plant resistance to pathogens. In the microarray data from the present study I found that *ALD1* (At2g13810, 265658_at) transcripts were strongly up-regulated at 6 h by avr1- (log_2 ratio +5.8) and avr4- treatment (+4.2) but barely at 3 h. *ALD1* up-regulation was strongly repressed in *eds1* (compared to wild-type) upon avr1- (-2.8) and avr4- (-5.4) inoculations, whereas *pad4* suppressed *ALD1* mRNA significantly less strongly upon challenge with avr1 (-0.9) compared to avr4 (-4.2). The expression data from this study reveal that *PAD4,* and even more strongly *EDS1*, positively regulate *ALD1* transcript accumulation, possibly by an SA independent mechanism.
As for *ALD1*, the data presented in this study point to AtFMO´s participation in an SA-independent pathway. In preliminary qRT-PCR analysis, I found that *AtFMO* transcript accumulation was not blocked but rather enhanced in SA depleted *sid2-1* plants (Figure 3.11). Further, the strong additive effects in loss of *RPP2*-resistance in an *Atfmo-1sid2-1* double mutant (Figure 3.13) combined with only marginally reduced SA levels in *Atfmo-1* in *RPS4*-mediated resistance (Figure 3.12) demonstrate a role of *AtFMO* in an SA-independent pathway.

4.4.2 AtFMO – possible biochemical activity and substrates

This study is the first report that functionally links genes encoding FMO-like proteins to pathogen defence in any biological system. In the following section I discuss what is known about the activity and the biological functions of FMOs in non-plant and plant organisms. Based on this knowledge, I will speculate about the potential activity of AtFMO and suggest experiments which might help unravel AtFMO function in promoting plant resistance to biotrophic pathogens.
Until recently only five expressed mammalian *FMO* genes were identified and designated, based on their amino acid similarities, to the gene families *FMO1* – *FMO5* (Lawton et al., 1994a). With the identification of three novel expressed *FMO* genes in mouse it became apparent that the mammalian *FMO* gene family is more

complex than previously thought (Hernandez et al., 2004). The physiological role of mammalian FMOs is poorly understood. The only function which seems to be essentially linked to the mammalian FMOs is its role in detoxification of endogenous and exogenous xenobiotics (Lawton et al., 1994b). The detoxification process catalysed by these microsomal proteins is oxygenation of nucleophilic atoms in structurally diverse compounds.

Despite the limited knowledge about the physiological role of mammalian FMOs, some insights to the catalytic cycle of FMOs have been gained (Krueger, 2005). FMOs bind NADPH as cofactor and FAD as prosthetic group. As the first step of the FMO catalytic cycle, FAD is reduced by NADPH to FADH2. Second, FADH2 reacts with molecular oxygen to a stable Flavin-hydroperoxide (FAD-OOH). The FAD-OOH bound enzyme is thought to be the predominant form in the cell that oxidises any nucleophilic substrate which gains access to the active site. As no prior substrate binding is required, substrate specificity is determined if the substrate gains access to the FAD pocket. With purified mammalian FMOs a significant production of ROS was caused by the “substrateless” reaction of FAD-OOH and NADPH bound in the same FMO protein (Rauckman et al., 1979; Tynes et al., 1986). This observation led Krueger and Wiliams (2005) to speculate that ROS production by FMOs could play a role in controlling the cellular redox state and consequently redox dependent gene expression. However, the same authors point out that ROS production by mammalian FMOs may only occur by purified but not necessarily the native enzyme that is located in the endoplasmatic reticulum (ER).

Interestingly, the single yeast FMO (yFMO) was shown to function in cellular redox control (Suh et al., 2000). yFMO is located on the cytoplasmic surface of the ER where it oxidises glutathione (GSH to GSSG) that is then transported inside the ER to maintain an oxidising environment. The oxidising environment in the ER established by yFMO was shown to be essential for proper folding of disulfide-containing proteins (Suh et al., 1999). Further, a FMO-deletion strain was unable to grow under reductive stress. As yeast contains only a single FMO which does not accept a wide range xenobiotic compounds, it was suggested that the yFMO function in maintaining the cellular reducing potential may be the ancestral activity of the FMO protein family (Suh et al., 1996).

In contrast to the single yeast *FMO* and the relatively small gene family of mammalian FMOs, the *Arabidopsis* genome contains a significantly larger number of

FMO-like genes (27 *FMO-like* genes according to Plant Gene Family Evolution Page: http://www.tc.umn.edu/~cann0010/genefamilyevolution/). Previous phylogenetic analysis (Fraaije et al., 2002; Naumann et al., 2002) and this study (Figure 3.14) revealed that the plant FMOs are divided into three separate plant specific clusters. When only considering predicted amino acid sequences from *Arabidopsis*, one finds that the cluster containing AtFMO has only one other predicted protein which is likely to be encoded by a pseudogene (as mentioned in Results). The two other plant clusters include multiple *Arabidopsis* members. Because of the large and diverse FMO family *in planta*, it was previously speculated that FMO in *A. thaliana* might have plant-specific functions (Naumann et al., 2002).

The idea of plant-specific functions of FMO is supported by the finding that an FMO (YUCCA) in *Arabidopsis* catalyzes *in vitro* the N-oxygenation of tryptamine, which is rate-limiting step in tryptophan-dependent auxin biosynthesis (Zhao et al., 2001). A mutant over-expressing *YUCCA* was identified with elongated hypocotyls by an activation tagging approach. The phenotype was caused by *YUCCA* mRNA over-expression resulting in doubling of the free indole-3-acetic acid (IAA, the main plant auxin) concentration compared to wild-type. Although tryptamine was not yet identified *in planta* (Ljung et al., 2002) the finding that *yucca* is more resistant to toxic tryptophan analogues, possibly by their enhanced conversion to the corresponding non-toxic forms, suggests that auxin is produced via a tryptophan-dependent pathway controlled by YUCCA. No loss-of-function mutant is reported for *YUCCA*, possibly due to redundancy with at least nine other sequence-related genes in the *Arabidopsis* genome. In contrast, a *YUCCA* orthologue in petunia *Floozy* (*FZY*) was identified as insertion mutant that has disturbed leaf and flower architecture (Tobena-Santamaria et al., 2002). The *fzy* plants contained wild-type levels of auxin but ectopic *FZY* over-expression caused excessive auxin accumulation suggesting that *FZY* also is involved in auxin synthesis.

Plant FMOs were initially implicated in catalysis of the first step in the biosynthesis of certain glucosinolates based on *in vitro* inhibitor studies in *Brassica* species (Bennett et al., 1993; Bennett et al., 1995; Oldfield et al., 1999). Glucosinolates are a diverse group of secondary metabolites promoting plant resistance against insects and pathogens (Rask et al., 2000). The initial step in glucosinolate synthesis is the oxidation of the amino group from a variety of amino acids and its analogues to an oxime group. Although the initial studies pointed to the involvement of FMOs in

glucosinolates synthesis, to date no plant FMO with a corresponding function has been characterised. Moreover, recent studies in *Arabidopsis* identified a subgroup of cytochrome P450s (P450s) to catalyse the first step in the glucosinolate synthesis (Wittstock and Halkier, 2000; Hansen et al., 2001; Glawischnig et al., 2004). However, there might be redundant functions between P450s and FMOs in glucosinolate synthesis, similar to the overlapping substrate specificity for many human P450s and FMOs (Krueger, 2005).

Does this knowledge about FMOs provide a lead to the potential substrate and the derived signal of AtFMO in plant defence? Neither the *AtFMO* insertion mutants, nor the *AtFMO* over-expresser lines (preliminary examination) have developmental defects. Thus, AtFMO is most likely not involved in auxin synthesis. This notion is further supported by the diverged amino acid sequences of AtFMO and YUCCA (Figure 3.14B). Our current knowledge about plant FMOs does not hint to the biochemical enzymatic activity of AtFMO or its substrate other than it might be a plant specific signal.

It is tempting to speculate that AtFMO, analogous to the yFMO function (Suh et al., 1999, 2000), regulates the redox homeostasis during pathogen defence. Considering the finding that the key regulator of plant immunity NPR1 is redox controlled (Mou et al., 2003) and EDS1/PAD4 are suggested to function in a ROS amplification loop (Rusterucci et al., 2001; Mateo et al., 2004b), the redox link is worth exploring further. I envisage testing the redox link of AtFMO in multiple experiments. First, *Atfmo* plants will be challenged by different biotic and abiotic stresses that cause reductive or oxidising imbalances and then assayed for altered phenotypes and GSH-GSSG ratio compared to wild-type. Additionally, GSH will be offered as substrate in an *in vitro* AtFMO enzymatic activity assay. AtFMO enzymatic activity on potential substrates can be tested *in vitro* by photometric determination of the NADPH concentration at 340nm, since FMO activity and NADPH consumption are linked (Tynes and Hodgson, 1985). Although mammalian FMOs accept a wide range of substrates, mainly amino groups have been oxidised to N-hydroxyl groups in other species (Zhao et al., 2001; Naumann et al., 2002; Choi et al., 2003).

It is intriguing that AtFMO is potentially linked to an amino-group oxidising function since ALD1 displays *in vitro* aminotransferase activity (Song et al., 2004b). As discussed, both AtFMO and ALD1 are likely to be involved in an *EDS1*/*PAD4*-controlled pathway independent of SA. Thus, amino group-containing compounds

might be modified by ALD1 and AtFMO to generate a signal that promotes plant resistance.

Such candidate amino group-containing substrates could be sphingosine and related long-chain sphingoid bases that are the backbone of sphingolipids (Merrill et al., 1997). I reasoned that the transcriptional activation of *AtFMO* in *acd11* (ACD11 has *in vitro* sphingosine transfer activity) and the recently discussed role of sphingolipids in plant signalling (Liang et al., 2003; Worrall et al., 2003) further justifies testing.

The typical FMO motifs are conserved in AtFMO (Figure 3.14A). Nevertheless, it remains unclear whether FMO activity is essential for the function of AtFMO in defence. Thus, I have created site directed mutants with mutations in the FAD and NADPH binding sites of AtFMO. Constructs for *in planta* over-expression of wild-type *AtFMO* and site directed mutants were created and stably transformed in *Atfmo-1* plants. Lines over-expressing wild-type or mutant FMO forms were tested for complementation of *Atfmo-1* in *RPP2*-mediated resistance. First results indicate that over-expression of wild-type *AtFMO* but not the site directed mutant constructs complement the resistance defect (data not shown). These preliminary results, if confirmed, suggest that intact FMO motifs in AtFMO are crucial for its defence function.

As mentioned above, preliminary data suggest that *AtFMO* over-expression causes enhanced basal resistance. If the over-expression phenotype can be confirmed, the over-expression lines and loss-of-function mutants of *AtFMO* would be an ideal platform for metabolic profiling to identify the AtFMO-derived signal.

The biochemical function of AtFMO remains speculative. The finding that AtFMO represents an isolated member of the FMO-like protein family suggests that AtFMO is involved in generation of a plant specific signal important for plant defence. Identification of the biochemical nature of this AtFMO-derived signal should deepen our understanding of the signalling events in plant immunity and the specific activities of EDS1 and PAD4.

4.5 NUDIX hydrolases as negative regulators of plant defence responses

Mutant *Atnud2.1* and *Atnud4.1* plants did not display defects in the initial screen for *RPP2*-mediated resistance. However, the fact that I found two sequence related genes as strongly *EDS1/PAD4*-dependent and the observation of a dwarf phenotype of *Atnud4.1* prompted me to investigate their potential function in regulating plant defence.

4.5.1 Constitutive defence symptoms and enhanced resistance in NUDIX hydrolase knock-out mutants

In the absence of pathogen treatment, the T-DNA insertion mutant *Atnud4.1* and more strongly the double mutant *Atnud2.1Atnud4.1*, but not *AtNud2.1* single mutant, displayed symptoms of constitutively active defence signalling. The mutant phenotypes included spontaneous cell death, dwarfism and wrinkled leaves (Figure 3.16). The elevated SA levels observed in unchallenged leaves of *Atnud4.1* (Figure 3.20) most likely caused the leaf wrinkling as it is a typical characteristic of mutants with enhanced SA levels (Bowling et al., 1997; Li et al., 2001). Microscopic analysis of trypan blue stained leaves revealed that spontaneous cell death in *Atnud4.1* and *Atnud2.1Atnud4.1* occurred in epidermis, palisade parenchyma and spongy parenchyma (Figure 3.16C and data not shown). Spontaneous cell death occurred already in young plants in an uniformly spaced manner in single cells and not in clusters as observed in other cell death mutants such as *acd6* (Rate et al., 1999), *cpr5* (Bowling et al., 1997) or *acd11* (Brodersen et al., 2002). Judged from macroscopic analysis until seven weeks post sowing, the NUDIX mutants never developed necrotic patches nor early leaf senescence. In contrast, *Atnud4.1* and *Atnud2.1Atnud4.1* appeared to have greener leaves (darker green) compared to wild-type. The occurrence of single cell death was also recently discovered in 6-week old *Arabidopsis* plants with mutations in *AtMLO2*, although solely in mesophyll cells (C. Consonni and R. Panstruga, unpublished). AtMLO2 and its functional homologue MLO in barley behave as negative regulators of plant defence responses and corresponding mutants are resistant to normally virulent powdery mildew strains (Büschges et al., 1997 and C. Consonni and R. Panstruga, unpublished).

Atnud4.1 and *Atnud2.1Atnud4.1* but not *Atnud2.1* displayed quantitatively enhanced resistance to a virulent isolate of *P. parasitica* (Figure 3.18). Based on inspections with the naked eye, susceptibility to *G. orontii* remained unaltered in inoculated leaves of both single and double NUDIX hydrolase mutants (Figure 3.19B). The *Arabidopsis* mutant *pmr4* displayed resistance to both pathogens as previous described (Vogel and Somerville, 2000). Nishimura and colleagues (2003) reported that the *NahG* transgene fully and *npr1* partially restored *G. orontii* susceptibility to *pmr4*. Further they showed that *pmr4* causes hyper-activation of SA response genes. Consequently, the authors concluded that *pmr4* resistance signals through the SA pathway. In contrast, I found that *Atnud4.1*, with strongly elevated SA levels, was not or only marginally affected in susceptibility to *G. orontii*. Thus, I propose that hyper-activation of SA signalling is not sufficient itself to confer resistance to *G. orontii*. It is possible that the *NahG* effect in reversing the *pmr4* resistance to wild-type susceptibility is based on the combination of SA depletion and pleiotropic effects caused by catechol accumulation. The compromising influence of catechol to non-host resistance has been described previously (van Wees and Glazebrook, 2003).

The *Atnud2.1* mutant defects became apparent only in the *Atnud2.1Atnud4.1* double mutant combination. The lack of a single mutant phenotype in *Atnud2.1* might be due to partial redundancy with the sequence-related gene At2g04430, which is also pathogen induced at the 6 h timepoint (see Figure 3.15B).

Plant mutants with enhanced basal resistance to virulent pathogens could be caused by the lack of a plant gene that is required for growth and reproduction of the pathogen. Recently, such a plant disease compatibility factor was discovered in *Arabidopsis*: *PRM6* is necessary for powdery mildew susceptibility (Vogel et al., 2002). Constitutive defence expression and high SA levels in the *Atnud4.1* mutant plants provide evidence that NUDIX hydrolases function as negative regulators of defence responses including plant cell death rather than being a plant disease compatibility factor necessary for *P. parasitica* infection.

4.5.2 Potential NUDIX hydrolase function in regulating plant stress responses

I showed that the typical NUDIX domain was conserved in AtNUD2.1 and AtNUD4.1 (Figure 3.15A), thus indicating these proteins might indeed hydrolyse a nucleotide substrate. Recent studies revealed that AtNUD4.1 catalyses the hydrolysis non-cyclic adenosine diphosphate ribose (ADP-ribose) to AMP and ribose-5-phosphate *in vitro* (E. Kraszewska, Polish Academy of Sciences, Warsaw, personal communication). From the battery of substrates tested, AtNUD4.1 was most active on ADP-ribose, although with a high Km value of 1.2 mM. The low enzymatic activity might be due to the fact that ADP-ribose is not the native substrate of AtNUD4.1 or that the test conditions were not optimal. However, these results strongly indicate that AtNUD4.1 is indeed a NUDIX hydrolase.

What potential NUDIX hydrolase activity could lead to its dual role in restricting defence responses and plant cell death? The first NUDIX hydrolase described was the *E. coli* enzyme MutT which catalyses hydrolysis of the oxidized deoxyguanosine nucleotide, 8-oxo-dGTP, to its corresponding monophosphate (8-oxo-dGMP) (Maki and Sekiguchi, 1992). Thus, MutT prevents the incorporation of oxidised nucleotides into DNA, which would otherwise result in mutations. AtNUD4.1 did not complement the *E. coli mutT* mutant or hydrolyse 8-oxo-dGTP (E. Kraszewska, personal communication).

Since the identification of MutT, a large number of enzymes have been discovered which share the NUDIX domain (also known as the MutT domain) with MutT but have a broader enzymatic activity as they hydrolase a variety of nucleotide substrates, some of which are cytotoxic (Bessman et al., 1996). All NUDIX hydrolase substrates have in common that they contain an oligophosphate chain which is esterified on one or both ends.

Besides the “house cleaning” function, NUDIX hydrolases might have a regulatory role in stress signalling by removing nucleoside phosphates that act as stress or pro-apoptotic signals (Bessman et al., 1996; Safrany et al., 1998). Support for the “stress signalling hypothesis” comes from the finding that some mammalian pathogenic viruses and bacteria express NUDIX hydrolases, some of which are essential for infectivity (McLennan, 1999). Interestingly, a mutational screen in the pathogen *Actinobacilus pleuropneumoniae* for genes required for the survival in its host (pig)

identified an ADP-ribose hydrolase encoding gene (Sheehan et al., 2003). Also, the finding that *Mycobacterium tuberculosis* can survive within its host macrophages, despite the oxidative bursts that the host generates to fight infection, led Kang and colleagues (2003) to speculate that the pathogen's ADP-ribose hydrolase provides protection against damaging oxidative stresses and ROS imbalance. Analogous to the NUDIX hydrolase function in pathogens, plants might utilise NUDIX hydrolases to restrict the damaging effects caused by the plant defence response. A role of nucleotides in stress signalling in plants has been implicated previously (Hunt et al., 2004). For example, extracellular ATP is proposed to activate calcium signalling in abiotic stress and wound responses (Jeter et al., 2004).

If one assumes that free ADP-ribose is the biologically relevant substrate of AtNUD2.1 and AtNUD4.1, recent findings demonstrate an interesting link between free ADP-ribose and stress induced cell death. In mammalian cells, levels of free ADP-ribose increased under oxidative stress conditions due to NAD^+ decay catalysed by the enzymes PARP/PARG (poly ADP-ribose polymerase/ poly ADP-ribose glycohydrolase) in the nucleus or by mitochondrial damage and subsequent ADP-ribose leakage (Richter and Schlegel, 1993; Chakraborti et al., 1999; Davidovic et al., 2001; Hara et al., 2002). *In planta*, it was recently shown that if PARP activity was reduced by chemical inhibitors or gene silencing, plants became more resistant to abiotic stresses and stress induced cell death was reduced (De Block et al., 2005). The authors propose that the enhanced stress tolerance by PARP inhibition is caused by the reduction of NAD^+ decay and thus cellular energy homeostasis is maintained even under stress conditions. Although not addressed in this publication, I reason that PARP-silenced plants might have reduced cellular levels of free ADP-ribose caused by reduced NAD^+ decay under stress conditions. This might represent the opposite situation as in the NUDIX mutants where ADP-ribose might accumulate to higher levels and thus lead to cell death due to its direct toxicity or a pro-apoptotic signalling function.

A signalling function of free ADP-ribose is well established in the mammalian system where free ADP-ribose was shown to trigger Ca^{2+} influxes by binding to and activating the plasma membrane cation channel TRPM2 (transient receptor potential melastatin2) (Perraud et al., 2001). TRPM2 contains an enzymatically active NUDIX domain whose binding to ADP-ribose was shown to be essential in oxidative stress-induced Ca^{2+} gating (Perraud et al., 2005). Consistent with the notion that ADP-

ribose acts as a second messenger for Ca^{2+} influx, suppression of ADP-ribose accumulation by ectopic over-expression of an ADP-ribose hydrolase inhibited oxidative triggered gating of TRPM2 (Perraud et al., 2005).

Based on these data, it is tempting to speculate that AtNUD2.1 and AtNUD4.1 may restrict plant defence-associated Ca^{2+} fluxes by the removal of ADP-ribose, thus limiting Ca^{2+} triggered defence responses.

The constitutive defence response in the *Arabidopsis* NUDIX hydrolase mutants would then be a consequence of failure to remove cellular ADP-ribose and its subsequent accumulation. Direct measurement of cellular free ADP-ribose concentrations is technically difficult (Perraud et al., 2005) and few measurements have been made in human erythrocytes (Guida et al., 1992) and to my knowledge none *in planta*. However, if high levels of free ADP-ribose are the cause for the mutant phenotype in *Atnud2.1* and *Atnud4.1*, a prediction would be that ectopic expression of an ADP-ribose hydrolase would reverse the *Atnud* mutant defects.

The conserved NUDIX domain in AtNUD2.1 and AtNUD4.1 and enzymatic activity of AtNUD4.1 on ADP-ribose suggest that both enzymes catalyze the hydrolysis of a nucleoside diphosphate derivative. Although the substrates of the NUDIX hydrolases identified here remain unknown, plants may utilise a NUDIX hydrolase-based system to control and restrict the damaging effects of resistance responses.

4.5.3 Control of NUDIX hydrolases by EDS1 and PAD4

For the plant it is vital to activate its defence machinery upon pathogen recognition in a rapid manner in order to prevent colonisation. As the successful defence response often includes the energy consuming synthesis of defence compounds and the activation of a host cell death program at the site of infection, it is apparent that these responses have to be restricted in a spatially and timely manner. Another important reason why plants must restrict and balance the defence machinery is based on the finding that antagonism exists between defence signalling pathways. For example, activation of SA signalling confers enhanced resistance to many biotrophs but represses JA-controlled defences against insects (Felton et al., 1999; Cipollini et al., 2004).

EDS1 and *PAD4* were previously shown to promote the expression of genes with important positive regulatory function in plant defence, e.g. *ACD6* (Lu et al., 2003)

and *ALD1* (Song et al., 2004b). The transcriptional dependence of *AtNUD2.1* and *AtNUD4.1* on *EDS1*/*PAD4* and the finding of Eulgem et al. (2004) that *AtNUD4.1* is not repressed in *NahG* plants suggest that an EDS1/PAD4-derived signal promotes the expression of the NUDIX hydrolases independently of SA. Further, it suggests that EDS1/PAD4 as positive regulators control the expression of NUDIX hydrolases which have a negative regulatory function in plant defence. Such a regulatory mechanism was previous included in a theoretically model to explain the spacing patterns of trichomes on the leave surface (Hulskamp, 2004). It stated that the activator of trichome development controls the production of its own inhibitor. Further, the model requires an auto-catalytic loop for the activator. Interestingly, this model predicts that the activator and its inhibitor show the highest expression at the same locations. If one applies this model to the EDS1/PAD4-NUDIX hydrolase regulatory function, striking similarities can be found. First, EDS1 and PAD4 have an auto-regulatory function that is also shown in this study on the transcriptional level. Second, EDS1/PAD4 and the NUDIX hydrolases are transcriptionally co-regulated. Third, EDS1/PAD4 might control the transcript levels of the NUDIX hydrolases.

However, if *eds1* and *pad4* display reduced levels of *NUDIX hydrolase* transcripts why are *eds1* and *pad4* not more resistant as the NUDIX hydrolase mutants? I assume that the reduced mRNA expression levels of NUDIX hydrolases in *eds1/pad4* are sufficient to prevent a NUDIX hydrolase defective gain-of-resistance phenotype. Only strong inhibition of NUDIX hydrolase function in the corresponding insertion mutants might results in enhanced resistance.

It is not clear what causes elevated levels of SA in the *Atnud4.1* mutant. NUDIX hydrolases might negatively regulate the SA pathway or alternatively the EDS1/PAD4 pathway up-stream of SA signalling. To address this question and to establish whether NUDIX mutant-conferred resistance and cell death is dependent on SA and/or EDS1/PAD4/FMO signalling, a study of the double mutants *Atnud4.1eds1*, *Atnud4.1pad4*, *Atnud4.1Atfmo* and *Atnud4.1sid2* will be performed.

The *EDS1/PAD4*-dependency for the NUDIX hydrolases transcript accumulation points to a close genetic relationship between *EDS1/PAD4* and the NUDIX hydrolases. Strikingly, G. Li identified the NUDIX hydrolase At5g47240 in a yeast-two-hybrid (Y2H) screen for interactors of SAG101 (G. Li, personal communication). From this microarray study I found that At5g47240 transcripts were not induced by avr1 or avr4 in wild-type but highly elevated in *eds1* and *pad4* compared to wild-type

in the “avr4 6 h” sample sets (see Figure 3.15B). Future interaction studies will test potential direct interactions between NUDIX hydrolases and EDS1, PAD4 or SAG101.

Taken together these findings suggest that EDS1/PAD4 (potentially with SAG101) and NUDIX hydrolases regulate the extent of defence responses and cell death. The strong double mutant phenotype of *Atnud2.1Atnud4.1* including growth defects and enhanced resistance to *P. parasitica* provides an ideal basis for suppressor mutagenesis. The characterisation of these mutations will help to identify components of the NUDIX pathway involved in restricting cell death and defence responses.

Outlook

I identified a flavin-dependent monooxygenase as positive and two sequence related NUDIX hydrolases as negative regulators of defence. Future experiments will further characterise these essential regulators and evaluate the initial results pointing to their involvement in an EDS1/PAD4-specific signalling pathway. The initial findings support the view that EDS1 and PAD4 control the expression of positive and negative resistance regulators in a way that balances defence responses. The challenge now is to identify the signals derived from EDS1, PAD4, AtFMO, AtNUD2.1 and AtNUD4.1 and to understand the mechanisms by which these signals are perceived and transduced.

References

Aarts, N., Metz, M., Holub, E., Staskawicz, B.J., Daniels, M.J., and Parker, J.E. (1998). Different requirements for EDS1 and NDR1 by disease resistance genes define at least two R gene-mediated signaling pathways in Arabidopsis. Proc. Natl. Acad. Sci. U. S. A. **95,** 10306-10311.

Abramovitch, R.B., Kim, Y.J., Chen, S., Dickman, M.B., and Martin, G.B. (2003). Pseudomonas type III effector AvrPtoB induces plant disease susceptibility by inhibition of host programmed cell death. Embo J **22,** 60-69.

Allan, A.C., and Fluhr, R. (1997). Two distinct sources of elicited reactive oxygen species in tobacco epidermal cells. Plant Cell **9,** 1559-1572.

Almeras, E., Stolz, S., Vollenweider, S., Reymond, P., Mene-Saffrane, L., and Farmer, E.E. (2003). Reactive electrophile species activate defense gene expression in Arabidopsis. Plant J **34,** 205-216.

Alonso, J.M., Stepanova, A.N., Leisse, T.J., Kim, C.J., Chen, H., Shinn, P., Stevenson, D.K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C.C., Parker, H., Prednis, L., Ansari, Y., Choy, N., Deen, H., Geralt, M., Hazari, N., Hom, E., Karnes, M., Mulholland, C., Ndubaku, R., Schmidt, I., Guzman, P., Aguilar-Henonin, L., Schmid, M., Weigel, D., Carter, D.E., Marchand, T., Risseeuw, E., Brogden, D., Zeko, A., Crosby, W.L., Berry, C.C., and Ecker, J.R. (2003). Genome-wide insertional mutagenesis of Arabidopsis thaliana. Science **301,** 653-657.

Asai, T., Tena, G., Plotnikova, J., Willmann, M.R., Chiu, W.L., Gomez-Gomez, L., Boller, T., Ausubel, F.M., and Sheen, J. (2002). MAP kinase signalling cascade in *Arabidopsis* innate immunity. Nature **415,** 977-983.

Ausubel, F.M., Katagiri, F., Mindrinos, M., and Glazebrook, J. (1995). Use of Arabidopsis-Thaliana Defense-Related Mutants to Dissect the Plant-Response to Pathogens. Proc. Natl. Acad. Sci. U. S. A. **92,** 4189-4196.

Basso, K., Margolin, A.A., Stolovitzky, G., Klein, U., Dalla-Favera, R., and Califano, A. (2005). Reverse engineering of regulatory networks in human B cells. Nat Genet **37,** 382-390.

Bennett, M., Mehta, M., and Grant, M. (2005). Biophoton imaging: A nondestructive method for assaying R gene responses. Molecular Plant-Microbe Interactions **18,** 95-102.

Bennett, R., Donald, A., Dawson, G., Hick, A., and Wallsgrove, R. (1993). Aldoxime-Forming Microsomal-Enzyme Systems Involved in the Biosynthesis of Glucosinolates in Oilseed Rape (Brassica-Napus) Leaves. Plant Physiology **102,** 1307-1312.

Bennett, R., Ludwigmuller, J., Kiddle, G., Hilgenberg, W., and Wallsgrove, R. (1995). Developmental Regulation Gf Aldoxime Formation in Seedlings and Mature Plants of Chinese-Cabbage (Brassica-Campestris Ssp Pekinensis) and Oilseed Rape (Brassica-Napus) - Glucosinolate and Iaa Biosynthetic-Enzymes. Planta **196,** 239-244.

Bessman, M.J., Frick, D.N., and O'Handley, S.F. (1996). The MutT Proteins or ``Nudix'' Hydrolases, a Family of Versatile, Widely Distributed, ``Housecleaning'' Enzymes. J. Biol. Chem. **271,** 25059-25062.

Bieri, S., Mauch, S., Shen, Q.H., Peart, J., Devoto, A., Casais, C., Ceron, F., Schulze, S., Steinbiss, H.H., Shirasu, K., and Schulze-Lefert, P. (2004). RAR1 positively controls steady state levels of barley MLA resistance proteins and enables sufficient MLA6 accumulation for effective resistance. Plant Cell **16,** 3480-3495.

Bowling, S.A., Clarke, J.D., Liu, Y.D., Klessig, D.F., and Dong, X.N. (1997). The *cpr5* mutant of Arabidopsis expresses both *NPR1*-dependent and *NPR1*-independent resistance. Plant Cell **9,** 1573-1584.

Bradley, D.J., Kjellbom, P., and Lamb, C.J. (1992). Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defense response. Cell **70,** 21-30.

Brodersen, P., Petersen, M., Pike, H.M., Olszak, B., Skov, S., Odum, N., Jorgensen, L.B., Brown, R.E., and Mundy, J. (2002). Knockout of Arabidopsis accelerated-cell-death11 encoding a sphingosine transfer protein causes activation of programmed cell death and defense. Genes Dev **16,** 490-502.

Büschges, R., Hollricher, K., Panstruga, R., Simons, G., Wolter, M., Frijters, A., van Daelen, R., van der Lee, T., Diergaarde, P., Groenendijk, J., Töpsch, S., Vos, P., Salamini, F., and Schulze-Lefert, P. (1997). The barley *Mlo* gene: A novel control element of plant pathogen resistance. Cell **88,** 695-705.

Bussink, H.J., and Oliver, R. (2001). Identification of two highly divergent catalase genes in the fungal tomato pathogen, Cladosporium fulvum. European Journal of Biochemistry **268,** 15-24.

Chakraborti, T., Das, S., Mondal, M., Roychoudhury, S., and Chakraborti, S. (1999). Oxidant, mitochondria and calcium: an overview. Cell Signal **11,** 77-85.

Chandra-Shekara, A.C., Navarre, D., Kachroo, A., Kang, H.-G., Klessig, D., and Kachroo, P. (2004). Signaling requirements and role of salicylic acid in HRT- and rrt-mediated resistance to turnip crinkle virus in Arabidopsis. Plant J **40,** 647-659.

Chen, Z., Kloek, A.P., Boch, J., Katagiri, F., and Kunkel, B.N. (2000). The Pseudomonas syringae *avrRpt2* gene product promotes pathogen virulence from inside plant cells. Mol Plant Microbe Interact **13,** 1312-1321.

Choi, H.S., Kim, J.K., Cho, E.H., Kim, Y.C., Kim, J.I., and Kim, S.W. (2003). A novel flavin-containing monooxygenase from Methylophaga sp strain SK1 and its indigo synthesis in Escherichia coli. Biochemical and Biophysical Research Communications **306,** 930-936.

Cipollini, D., Enright, S., Traw, M.B., and Bergelson, J. (2004). Salicylic acid inhibits jasmonic acid-induced resistance of Arabidopsis thaliana to Spodoptera exigua. Mol Ecol **13,** 1643-1653.

Crute, I.R., Holub, E.B., Tor, M., Brose, E., and Beynon, J.L. (1992). The identification and mapping of loci in *Arabidopsis thaliana* for recognition of the fungal pathogens: *Peronospora parasitica* (downy mildew) and *Albugo candi*da (white blister). In Advances in Molecular Genetics of Plant-Microbe Interactions. Vol. 2, E.W. Nester and D.P.S. Verma, eds (Dordrecht: Kluwer Academic Publ.), pp. 437-444.

Cui, J., Bahrami, A.K., Pringle, E.G., Hernandez-Guzman, G., Bender, C.L., Pierce, N.E., and Ausubel, F.M. (2005). Pseudomonas syringae manipulates systemic plant defenses against pathogens and herbivores. PNAS **102,** 1791-1796.

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

Dangl, J.L., Ritter, C., Gibbon, M.J., Mur, L.A.J., Wood, J.R., Goss, S., Mansfield, J., Taylor, J.D., and Vivian, A. (1992). Functional homologs of the *Arabidopsis RPM1* disease resistance gene in bean and pea. The Plant Cell **4,** 1359-1369.

Davidovic, L., Vodenicharov, M., Affar, E.B., and Poirier, G.G. (2001). Importance of poly(ADP-ribose) glycohydrolase in the control of poly(ADP-ribose) metabolism. Exp Cell Res **268,** 7-13.

De Block, M., Verduyn, C., De Brouwer, D., and Cornelissen, M. (2005). Poly(ADP-ribose) polymerase in plants affects energy homeostasis, cell death and stress tolerance. Plant J **41,** 95-106.

de Torres, M., Sanchez, P., Fernandez-Delmond, I., and Grant, M. (2003). Expression profiling of the host response to bacterial infection: the transition from basal to induced defence responses in RPM1-mediated resistance. Plant Journal **33,** 665-676.

Despres, C., Chubak, C., Rochon, A., Clark, R., Bethune, T., Desveaux, D., and Fobert, P.R. (2003). The Arabidopsis NPR1 disease resistance protein is a novel cofactor that confers redox regulation of DNA binding activity to the basic domain/leucine zipper transcription factor TGA1. Plant Cell **15,** 2181-2191.

Dietrich, R.A., Delaney, T.P., Ukness, S.J., Ward, E.R., Ryals, J.A., and Dangl, J.L. (1994). Arabidopsis mutants simulating disease resistance response. Cell **77,** 565-577.

Dinkins, R.D., Bandaranayake, H., Green, B.R., and Griffiths, A.J.F. (1994). A Nuclear Photosynthetic Electron-Transport Mutant of Arabidopsis-Thaliana with Altered Expression of the Chloroplast, Peta Gene. Current Genetics **25,** 282-288.

Dobrzanska, M., Szurmak, B., Wyslouch-Cieszynska, A., and Kraszewska, E. (2002). Cloning and characterization of the first member of the Nudix family from Arabidopsis thaliana. J Biol Chem **277,** 50482-50486.

Dong, X. (2004). The role of membrane-bound ankyrin-repeat protein ACD6 in programmed cell death and plant defense. Sci STKE **2004,** pe6.

Dunn, C.A., O'Handley, S.F., Frick, D.N., and Bessman, M.J. (1999). Studies on the ADP-ribose pyrophosphatase subfamily of the nudix hydrolases and tentative identification of trgB, a gene associated with tellurite resistance. J Biol Chem **274,** 32318-32324.

Enyedi, A.J., Yalpani, N., Silverman, P., and Raskin, I. (1992). Localization, conjugation and function of salicylic acid in tobacco during the hypersensitive reaction to tobacco mosaic virus. Proc.Natl.Acad.Sci.USA **89,** 2480-2484.

Eulgem, T., Rushton, P.J., Robatzek, S., and Somssich, I.E. (2000). The WRKY superfamily of plant transcription factors. Trends Plant Sci **5,** 199-206.

Eulgem, T., Weigman, V.J., Chang, H.S., McDowell, J.M., Holub, E.B., Glazebrook, J., Zhu, T., and Dangl, J.L. (2004). Gene expression signatures from three genetically separable resistance gene signaling pathways for downy mildew resistance. Plant Physiol **135,** 1129-1144.

Falk, A., Feys, B.J., Frost, L.N., Jones, J.D.G., Daniels, M.J., and Parker, J.E. (1999). *EDS1*, an essential component of *R* gene-mediated disease resistance in *Arabidopsis* has homology to eukaryotic lipases. Proc. Natl. Acad. Sci. U. S. A. **96,** 3292-3297.

Felix, G., Duran, J.D., Volko, S., and Boller, T. (1999). Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. Plant J **18,** 265-276.

Fellbrich, G., Romanski, A., Varet, A., Blume, B., Brunner, F., Engelhardt, S., Felix, G., Kemmerling, B., Krzymowska, M., and Nurnberger, T. (2002). NPP1, a Phytophthora-associated trigger of plant defense in parsley and Arabidopsis. Plant J **32,** 375-390.

Felton, G.W., Korth, K.L., Bi, J.L., Wesley, S.V., Huhman, D.V., Mathews, M.C., Murphy, J.B., Lamb, C., and Dixon, R.A. (1999). Inverse relationship between systemic resistance of plants to microorganisms and to insect herbivory. Curr Biol **9,** 317-320.

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

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

Fraaije, M.W., Kamerbeek, N.M., van Berkel, W.J., and Janssen, D.B. (2002). Identification of a Baeyer-Villiger monooxygenase sequence motif. FEBS Lett **518,** 43-47.

Gechev, T.S., Gadjev, I.Z., and Hille, J. (2004). An extensive microarray analysis of AAL-toxin-induced cell death in Arabidopsis thaliana brings new insights into the complexity of programmed cell death in plants. Cell Mol Life Sci **61,** 1185-1197.

Glawischnig, E., Hansen, B.G., Olsen, C.E., and Halkier, B.A. (2004). Camalexin is synthesized from indole-3-acetaidoxime, a key branching point between primary and secondary metabolism in Arabidopsis. Proc. Natl. Acad. Sci. U. S. A. **101,** 8245-8250.

Glazebrook, J. (1999). Genes controlling expression of defense responses in *Arabidopsis*. Current Opinion in Plant Biology **2,** 280-286.

Glazebrook, J., Rogers, E.E., and Ausubel, F.M. (1996). Isolation of *Arabidopsis* mutants with enhanced disease susceptibility by direct screening. Genetics **143,** 973-982.

Glazebrook, J., Rogers, E.E., and Ausubel, F.M. (1997a). Use of Arabidopsis for genetic dissection of plant defense responses. Annual Review of Genetics **31,** 547-569.

Glazebrook, J., Chen, W., Estes, B., Chang, H.S., Nawrath, C., Metraux, J.P., Zhu, T., and Katagiri, F. (2003). Topology of the network integrating salicylate and jasmonate signal transduction derived from global expression phenotyping. Plant J **34,** 217-228.

Glazebrook, J., Zook, M., Mert, F., Kagan, I., Rogers, E.E., Crute, I.R., Holub, E.B., Hammerschmidt, R., and Ausubel, F.M. (1997b). Phytoalexin-deficient mutants of *Arabidopsis* reveal that *PAD4* encodes a regulatory factor and that four *PAD* genes contribute to downy mildew resistance. Genetics **146,** 381-392.

Grant, J.J., Yun, B.W., and Loake, G.J. (2000a). Oxidative burst and cognate redox signalling reported by luciferase imaging: identification of a signal network that functions independently of ethylene, SA and Me-JA but is dependent on MAPKK activity. Plant Journal **24,** 569-582.

Grant, M., Brown, I., Adams, S., Knight, M., Ainslie, A., and Mansfield, J. (2000b). The RPM1 Plant disease resistance gene facilitates a rapid and

sustained increase in cytosolic calcium that is necessary for the oxidative burst and hypersensistivecell death. The Plant Journal **23 (4),** 441-450.

Grant, M.R., and Mansfield, J. (1999). Early events in host-pathogen interactions. Current Opinion in Plant Biology **2,** 312-319.

Grant, M.R., Godiard, L., Straube, E., Ashfield, T., Lewald, J., Sattler, A., Innes, R.W., and Dangl, J.L. (1995). Structure of the *Arabidopsis RPM1* gene enabling dual specificity disease resistance. Science **269,** 843-846.

Gross, G.G., Janse, C., and Elstner, E.F. (1977). Involvement of malate, monphenols, and the superoxide radical in hydrogen peroxide formation by isolated cell walls from horseradish (*Armoracia lapathifolia* Gilib.). Planta **136,** 271-276.

Guida, L., Zocchi, E., Franco, L., Benatti, U., and De Flora, A. (1992). Presence and turnover of adenosine diphosphate ribose in human erythrocytes. Biochem Biophys Res Commun **188,** 402-408.

Gupta, R., and Luan, S. (2003). Redox control of protein tyrosine phosphatases and mitogen-activated protein kinases in plants. Plant Physiology **132,** 1149-1152.

Hansen, C.H., Wittstock, U., Olsen, C.E., Hick, A.J., Pickett, J.A., and Halkier, B.A. (2001). Cytochrome P450 CYP79F1 from Arabidopsis Catalyzes the Conversion of Dihomomethionine and Trihomomethionine to the Corresponding Aldoximes in the Biosynthesis of Aliphatic Glucosinolates. J. Biol. Chem. **276,** 11078-11085.

Hara, Y., Wakamori, M., Ishii, M., Maeno, E., Nishida, M., Yoshida, T., Yamada, H., Shimizu, S., Mori, E., Kudoh, J., Shimizu, N., Kurose, H., Okada, Y., Imoto, K., and Mori, Y. (2002). LTRPC2 Ca2+-permeable channel activated by changes in redox status confers susceptibility to cell death. Mol Cell **9,** 163-173.

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

He, Y., and Gan, S. (2002). A gene encoding an acyl hydrolase is involved in leaf senescence in Arabidopsis. Plant Cell **14,** 805-815.

Heath, M.C. (1998). Involvement of reactive oxygen species in the response of resistant (hypersensitive) or susceptible cowpeas to the cowpea rust fungus. New Phytol **138,** 251-263.

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

Hentrich, W. (1979). Allelwirkung und pleiotropie mehltauresistenter mutanten des ml-o locus der gerste. Arch. Züchtungsvorsch., Berlin **9,** S. 283-291.

Hernandez, D., Janmohamed, A., Chandan, P., Phillips, I.R., and Shephard, E.A. (2004). Organization and evolution of the flavin-containing monooxygenase genes of human and mouse: identification of novel gene and pseudogene clusters. Pharmacogenetics **14,** 117-130.

Hinsch, M., and Staskawicz, B. (1996). Identification of a new *Arabidopsis* disease resistance locus, *RPS4*, and cloning of the corresponding avirulence gene, *avrRps4*, from *Pseudomonas syringae* pv. *pisi*. Molecular Plant-Microbe Interactions **9,** 55-61.

Holub, E.B., Beynon, J.L., and Crute, I.R. (1994a). Phenotypic and genotypic characterization of interactions between isolates of *Peronospora parasitica*

and accessions of *Arabidopsis thaliana*. Molecular Plant-Microbe Interactions **7,** 223-239.

Holub, E.B., Beynon, L.J., and Crute, I.R. (1994b). Phenotypic and Genotypic Characterization of Interactions between Isolates of Peronospora-Parasitica and Accessions of Arabidopsis-Thaliana. Molecular Plant-Microbe Interactions **7,** 223-239.

Huang, Z., Yeakley, J.M., Garcia, E.W., Holdridge, J.D., Fan, J.B., and Whitham, S.A. (2005). Salicylic acid-dependent expression of host genes in compatible Arabidopsis-virus interactions. Plant Physiol **137,** 1147-1159.

Hueck, C.J. (1998). Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiol Mol Biol R **62,** 379-+.

Hulskamp, M. (2004). Plant trichomes: a model for cell differentiation. Nat Rev Mol Cell Biol **5,** 471-480.

Hunt, L., Lerner, F., and Ziegler, M. (2004). NAD - new roles in signalling and gene regulation in plants. New Phytol **163,** 31-44.

Initiative, T.A.G. (2000). Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. Nature **408,** 796-815.

Innes, R.W., Bisgrove, S.R., Smith, N.M., Bent, A.F., Staskawicz, B.J., and Liu, Y.-C. (1993). Identification of a disease resistance locus in *Arabidopsis* that is functionally homologous to the *RPG1* locus of soybean. The Plant Journal **4,** 813-820.

Jeter, C.R., Tang, W., Henaff, E., Butterfield, T., and Roux, S.J. (2004). Evidence of a novel cell signaling role for extracellular adenosine triphosphates and diphosphates in Arabidopsis. Plant Cell **16,** 2652-2664.

Jirage, D., Tootle, T.L., Reuber, T.L., Frost, L.N., Feys, B.J., Parker, J.E., Ausubel, F.M., and Glazebrook, J. (1999). Arabidopsis thaliana *PAD4* encodes a lipase-like gene that is important for salicylic acid signaling. Proc. Natl. Acad. Sci. U. S. A. **96,** 13583-13588.

Kachroo, P., Yoshioka, K., Shah, J., Dooner, H.K., and Klessig, D.F. (2000). Resistance to turnip crinkle virus in Arabidopsis is regulated by two host genes and is salicylic acid dependent but NPR1, ethylene, and jasmonate independent. Plant Cell **12,** 677-690.

Kachroo, P., Shanklin, J., Shah, J., Whittle, E.J., and Klessig, D.F. (2001). A fatty acid desaturase modulates the activation of defense signaling pathways in plants. Proc Natl Acad Sci U S A **98,** 9448-9453.

Kalde, M., Barth, M., Somssich, I.E., and Lippok, B. (2003). Members of the Arabidopsis WRKY group III transcription factors are part of different plant defense signaling pathways. Mol Plant Microbe Interact **16,** 295-305.

Kang, L.W., Gabelli, S.B., Cunningham, J.E., O'Handley, S.F., and Amzel, L.M. (2003). Structure and mechanism of MT-ADPRase, a nudix hydrolase from Mycobacterium tuberculosis. Structure (Camb) **11,** 1015-1023.

Katagiri, F., Thilmony, R.L., and He, S.Y. (2002). The Arabidopsis thaliana-Pseudomonas syringae interaction. In The Arabidopsis Book, C.R. Somerville and E.M. Meyerowitz, eds (Rockville, MD: American Society of Plant Biologists), doi/10.1199/tab.0039, http://www.aspb.org/publications/arabidopsis.

Kawano, T. (2003). Roles of the reactive oxygen species-generating peroxidase reactions in plant defense and growth induction. Plant Cell Reports **21,** 829-837.

Kearney, B., and Staskawicz, B.J. (1990). Widespread distribution and fitness contribution of *Xanthomonas campestris* avirulence gene *avrBs2*. Nature **346,** 385-386.

Koch, E., and Slusarenko, A. (1990a). *Arabidopsis* is susceptible to infection by a downy mildew fungus. Plant Cell **2,** 437-445.

Koch, E., and Slusarenko, A. (1990b). Arabidopsis Is Susceptible to Infection by a Downy Mildew Fungus. Plant Cell **2,** 437-445.

Kolukisaoglu, H.U., Bovet, L., Klein, M., Eggmann, T., Geisler, M., Wanke, D., Martinoia, E., and Schulz, B. (2002). Family business: the multidrug-resistance related protein (MRP) ABC transporter genes in Arabidopsis thaliana. Planta **216,** 107-119.

Krueger, S.K. (2005). Mammalian flavin-containing monooxygenases: structure/function, genetic polymorphisms and role in drug metabolism. Pharmacol. Therap. **In Press, Corrected Proof**.

Kumar, S., Tamura, K., Jakobsen, I.B., and Nei, M. (2001). MEGA2: molecular evolutionary genetics analysis software. Bioinformatics **17,** 1244-1245.

Laloi, C., Apel, K., and Danon, A. (2004). Reactive oxygen signalling: the latest news. Current Opinion in Plant Biology **7,** 323-328.

Lawton, M.P., Cashman, J.R., Cresteil, T., Dolphin, C.T., Elfarra, A.A., Hines, R.N., Hodgson, E., Kimura, T., Ozols, J., Phillips, I.R., and et al. (1994a). A nomenclature for the mammalian flavin-containing monooxygenase gene family based on amino acid sequence identities. Arch Biochem Biophys **308,** 254-257.

Lawton, M.P., Cashman, J.R., Cresteil, T., Dolphin, C.T., Elfarra, A.A., Hines, R.N., Hodgson, E., Kimura, T., Ozols, J., Phillips, I.R., Philpot, R.M., Poulsen, L.L., Rettie, A.E., Shephard, E.A., Williams, D.E., and Ziegler, D.M. (1994b). A Nomenclature for the Mammalian Flavin-Containing Monooxygenase Gene Family Based on Amino-Acid-Sequence Identities. Archives of Biochemistry and Biophysics **308,** 254-257.

Lee, I., Date, S.V., Adai, A.T., and Marcotte, E.M. (2004). A Probabilistic Functional Network of Yeast Genes. Science **306,** 1555-1558.

Lemaitre, B., Reichhart, J.M., and Hoffmann, J.A. (1997). Drosophila host defense: Differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. Proc. Natl. Acad. Sci. U. S. A. **94,** 14614-14619.

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

Liang, H., Yao, N., Song, J.T., Luo, S., Lu, H., and Greenberg, J.T. (2003). Ceramides modulate programmed cell death in plants. Genes Dev. **17,** 2636-2641.

Ligterink, W., Kroj, T., zurNieden, U., Hirt, H., and Scheel, D. (1997). Receptor-mediated activation of a MAP kinase in pathogen defense of plants. Science **276,** 2054-2057.

Ljung, K., Hull, A.K., Kowalczyk, M., Marchant, A., Celenza, J., Cohen, J.D., and Sandberg, G. (2002). Biosynthesis, conjugation, catabolism and homeostasis of indole-3-acetic acid in Arabidopsis thaliana. Plant Mol Biol **49,** 249-272.

Lu, H., Rate, D.N., Song, J.T., and Greenberg, J.T. (2003). ACD6, a novel ankyrin protein, is a regulator and an effector of salicylic acid signaling in the Arabidopsis defense response. Plant Cell **15,** 2408-2420.

Ludwig, A.A., Romeis, T., and Jones, J.D. (2004). CDPK-mediated signalling pathways: specificity and cross-talk. J Exp Bot **55,** 181-188.

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

Maki, H., and Sekiguchi, M. (1992). MutT protein specifically hydrolyses a potent mutagenic substrate for DNA synthesis. Nature **355,** 273-275.

Maldonado, A.M., Doerner, P., Dixon, R.A., Lamb, C.J., and Cameron, R.K. (2002). A putative lipid transfer protein involved in systemic resistance signalling in Arabidopsis. Nature **419,** 399-403.

Maleck, K., Levine, A., Eulgem, T., Morgan, A., Schmid, J., Lawton, K.A., Dangl, J.L., and Dietrich, R.A. (2000). The transcriptome of Arabidopsis thaliana during systemic acquired resistance. Nature Genetics **26,** 403-410.

Mateo, A., Muhlenbock, P., Rusterucci, C., Chang, C.C., Miszalski, Z., Karpinska, B., Parker, J.E., Mullineaux, P.M., and Karpinski, S. (2004a). LESION SIMULATING DISEASE 1 is required for acclimation to conditions that promote excess excitation energy. Plant Physiol **136,** 2818-2830.

Mateo, A., Muhlenbock, P., Rusterucci, C., Chang, C.C.C., Miszalski, Z., Karpinska, B., Parker, J.E., Mullineaux, P.M., and Karpinski, S. (2004b). Lesion simulating disease 1 - Is required for acclimation to conditions that promote excess excitation energy. Plant Physiology **136,** 2818-2830.

Mauch-Mani, B., and Slusarenko, A.J. (1993). *Arabidopsis* as a model host for studying plant-pathogen interactions. Trends in Microbiology **1,** 265.

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

McLennan, A.G. (1999). The MutT motif family of nucleotide phosphohydrolases in man and human pathogens (review). Int J Mol Med **4,** 79-89.

Medzhitov, R., and Janeway, C.A., Jr. (2002). Decoding the patterns of self and nonself by the innate immune system. Science **296,** 298-300.

Merrill, A.H., Schmelz, E.M., Dillehay, D.L., Spiegel, S., Shayman, J.A., Schroeder, J.J., Riley, R.T., Voss, K.A., and Wang, E. (1997). Sphingolipids - The enigmatic lipid class: Biochemistry, physiology, and pathophysiology. Toxicol Appl Pharm **142,** 208-225.

Metraux, J.P., Signer, H., Ryals, J., Ward, E., Wyss-Benz, M., Gaudin, J., Raschdorf, K., Schmid, E., Blum, W., and Inverardi, B. (1990). Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. Science (Wash.). **250,** 1004-1006.

Mou, Z., Fan, W., and Dong, X. (2003). Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. Cell **113,** 935-944.

Mur, L., Bi, Y-M, Darby, RM, Firek, S and Draper, J. (1997). Compromising early salicylic acid accumulation delays the hypersensitive response and increases viral dispersal during lesion establishment in TMV-infected tobacco. Plant Journal **12,** 1113-1126.

Mur, L.A.J., Brown, I.R., Darby, R.M., Bestwick, C.S., Bi, Y.M., Mansfield, J.W., and Draper, J. (2000). A loss of resistance to avirulent bacterial pathogens in tobacco is associated with the attenuation of a salicylic acid-potentiated oxidative burst. Plant Journal **23,** 609-621.

Nandi, A., Welti, R., and Shah, J. (2004). The Arabidopsis thaliana Dihydroxyacetone Phosphate Reductase Gene SUPPRESSOR OF FATTY ACID DESATURASE DEFICIENCY1 Is Required for Glycerolipid Metabolism and for the Activation of Systemic Acquired Resistance. Plant Cell **16,** 465-477.

Naumann, C., Hartmann, T., and Ober, D. (2002). Evolutionary recruitment of a flavin-dependent monooxygenase for the detoxification of host plant-acquired pyrrolizidine alkaloid-def ended arctiid alkaloids in the moth Tyria jacobaleae. Proc. Natl. Acad. Sci. U. S. A. **99,** 6085-6090.

Nishimura, M.T., Stein, M., Hou, B.H., Vogel, J.P., Edwards, H., and Somerville, S.C. (2003). Loss of a callose synthase results in salicylic acid-dependent disease resistance. Science **301,** 969-972.

Nurnberger, T., Brunner, F., Kemmerling, B., and Piater, L. (2004). Innate immunity in plants and animals: striking similarities and obvious differences. Immunol Rev **198,** 249-266.

Oldfield, M.F., Bennett, R.N., Kiddle, G., Wallsgrove, R.M., and Botting, N.P. (1999). Biochemical characterisation of an aldoxime-forming flavoprotein involved in 2-phenylethylglucosinolate biosynthesis in Brassica species. Plant Physiol. Biochem. **37,** 99-108.

Onate-Sanchez, L., and Singh, K.B. (2002). Identification of Arabidopsis ethylene-responsive element binding factors with distinct induction kinetics after pathogen infection. Plant Physiology **128,** 1313-1322.

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

Parker, J.E., Szabo, V., Staskawicz, B.J., Lister, C., Dean, C., Daniels, M., and Jones, J.D.G. (1993). Phenotypic characterization and molecular mapping of the *Arabidopsis thaliana* locus *RPP5*, determining resistance to *Peronospora parasitica*. Plant Journal **4,** 821-831.

Parker, J.E., Feys, B.J., Van der Biezen, E.A., Noël, N., Aarts, N., Austin, M.J., Botella, M.A., Frost, L.N., Daniels, M.J., and Jones, J.D.G. (2000). Unravelling *R* gene-mediated disease resistance pathways in *Arabidopsis*. Molecular Plant Pathology **1,** 17-24.

Peart, J.R., Cook, G., Feys, B.J., Parker, J.E., and Baulcombe, D.C. (2002). An *EDS1* orthologue is required for *N*-mediated resistance against tobacco mosiac virus. Plant Journal**,** in press.

Perraud, A.L., Fleig, A., Dunn, C.A., Bagley, L.A., Launay, P., Schmitz, C., Stokes, A.J., Zhu, Q., Bessman, M.J., Penner, R., Kinet, J.P., and Scharenberg, A.M. (2001). ADP-ribose gating of the calcium-permeable LTRPC2 channel revealed by Nudix motif homology. Nature **411,** 595-599.

Perraud, A.L., Takanishi, C.L., Shen, B., Kang, S., Smith, M.K., Schmitz, C., Knowles, H.M., Ferraris, D., Li, W., Zhang, J., Stoddard, B.L., and Scharenberg, A.M. (2005). Accumulation of free ADP-ribose from mitochondria mediates oxidative stress-induced gating of TRPM2 cation channels. J Biol Chem **280,** 6138-6148.

Petersen, M., Brodersen, P., Naested, H., Andreasson, E., Lindhart, U., Johansen, B., Nielsen, H.B., Lacy, M., Austin, M.J., Parker, J.E., Sharma, S.B., Klessig, D.F., Martienssen, R., Mattsson, O., Jensen, A.B., and

Mundy, J. (2000). Arabidopsis MAP kinase 4 negatively regulates systemic acquired resistance. Cell **103,** 1111-1120.

Rask, L., Andreasson, E., Ekbom, B., Eriksson, S., Pontoppidan, B., and Meijer, J. (2000). Myrosinase: gene family evolution and herbivore defense in Brassicaceae. Plant Mol Biol **42,** 93-113.

Rate, D.N., Cuenca, J.V., Bowman, G.R., Guttman, D.S., and Greenberg, J.T. (1999). The gain-of-function Arabidopsis acd6 mutant reveals novel regulation and function of the salicylic acid signaling pathway in controlling cell death, defenses, and cell growth. Plant Cell **11,** 1695-1708.

Rauckman, E.J., Rosen, G.M., and Kitchell, B.B. (1979). Superoxide radical as an intermediate in the oxidation of hydroxylamines by mixed function amine oxidase. Mol Pharmacol **15,** 131-137.

Redman, J.C., Haas, B.J., Tanimoto, G., and Town, C.D. (2004). Development and evaluation of an Arabidopsis whole genome Affymetrix probe array. Plant J **38,** 545-561.

Reignault, P., Frost, L.N., Richardson, H., Daniels, M.J., Jones, J.D.G., and Parker, J.E. (1996). Four *Arabidopsis RPP* loci controlling resistance to the *Noco2* isolate of *Peronospora parasitica* map to regions known to contain other *RPP* recognition specificities. MPMI **9,** 464-473.

Reuber, T.L., Plotnikova, J.M., Dewdney, J., Rogers, E.E., Wood, W., and Ausubel, F.M. (1998). Correlation of defense gene induction defects with powdery mildew susceptibility in Arabidopsis enhanced disease susceptibility mutants. Plant J **16,** 473-485.

Richter, C., and Schlegel, J. (1993). Mitochondrial calcium release induced by prooxidants. Toxicol Lett **67,** 119-127.

Ritter, C., and Dangl, J.L. (1995). The *avrRpm1* gene of a *Pseudomonas syringae* pv. *maculicola* is required for virulence on Arabidopsis. MPMI **8,** 444-453.

Romeis, T., Ludwig, A.A., Martin, R., and Jones, J.D.G. (2001). Calcium-dependent protein kinases play an essential role in a plant defence response. Embo Journal **20,** 5556-5567.

Rosso, M.G., Li, Y., Strizhov, N., Reiss, B., Dekker, K., and Weisshaar, B. (2003). An Arabidopsis thaliana T-DNA mutagenized population (GABI-Kat) for flanking sequence tag-based reverse genetics. Plant Mol Biol **53,** 247-259.

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

Ryals, J.L., Neuenschwander, U.H., Willits, M.C., Molina, A., Steiner, H.-Y., and Hunt, M.D. (1996). Systemic acquired resistance. Plant Cell **8**.

Safrany, S.T., Caffrey, J.J., Yang, X., Bembenek, M.E., Moyer, M.B., Burkhart, W.A., and Shears, S.B. (1998). A novel context for the 'MutT' module, a guardian of cell integrity, in a diphosphoinositol polyphosphate phosphohydrolase. Embo J **17,** 6599-6607.

Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. Proceedings of the National Academy of Sciences USA **74,** 5463-5467.

Sauerbrunn, N., and Schlaich, N.L. (2004). PCC1: a merging point for pathogen defence and circadian signalling in Arabidopsis. Planta **218,** 552-561.

Scheideler, M., Schlaich, N.L., Fellenberg, K., Beissbarth, T., Hauser, N.C., Vingron, M., Slusarenko, A.J., and Hoheisel, J.D. (2002). Monitoring the

switch from housekeeping to pathogen defense metabolism in Arabidopsis thaliana using cDNA arrays. J Biol Chem **277,** 10555-10561.

Schenk, P.M., Kazan, K., Wilson, I., Anderson, J.P., Richmond, T., Somerville, S.C., and Manners, J.M. (2000). Coordinated plant defense responses in Arabidopsis revealed by microarray analysis. Proc Natl Acad Sci U S A **97,** 11655-11660.

Schneider, K., Kienow, L., Schmelzer, E., Colby, T., Bartsch, M., Miersch, O., Wasternack, C., Kombrink, E., and Stuible, H.-P. (2005). A New Type of Peroxisomal Acyl-Coenzyme A Synthetase from Arabidopsis thaliana Has the Catalytic Capacity to Activate Biosynthetic Precursors of Jasmonic Acid. J. Biol. Chem. **280,** 13962-13972.

Scholl, R.L., May, S.T., and Ware, D.H. (2000). Seed and molecular resources for Arabidopsis. Plant Physiol **124,** 1477-1480.

Shah, J., Kachroo, P., Nandi, A., and Klessig, D.F. (2001). A recessive mutation in the Arabidopsis SSI2 gene confers SA- and NPR1-independent expression of PR genes and resistance against bacterial and oomycete pathogens. Plant J **25,** 563-574.

Shapiro, A.D., and Zhang, C. (2001). The role of NDR1 in avirulence gene-directed signaling and control of programmed cell death in arabidopsis. Plant Physiology **127,** 1089-1101.

Sheehan, B.J., Bosse, J.T., Beddek, A.J., Rycroft, A.N., Kroll, J.S., and Langford, P.R. (2003). Identification of Actinobacillus pleuropneumoniae genes important for survival during infection in its natural host. Infect Immun **71,** 3960-3970.

Shirano, Y., Kachroo, P., Shah, J., and Klessig, D.F. (2002). A gain-of-function mutation in an Arabidopsis Toll Interleukin1 receptor-nucleotide binding site-leucine-rich repeat type R gene triggers defense responses and results in enhanced disease resistance. Plant Cell **14,** 3149-3162.

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

Shulaev, V., Leon, J., and Raskin, I. (1995). Is salicylic acid a translocated signal of systemic acquired resistance in tobacco? Plant Cell **7,** 1691-1701.

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

Song, J.T., Lu, H., and Greenberg, J.T. (2004a). Divergent roles in Arabidopsis thaliana development and defense of two homologous genes, ABERRANT GROWTH AND DEATH2 and AGD2-LIKE DEFENSE RESPONSE PROTEIN1, encoding novel aminotransferases. Plant Cell **16,** 353-366.

Song, J.T., Lu, H., McDowell, J.M., and Greenberg, J.T. (2004b). A key role for ALD1 in activation of local and systemic defenses in Arabidopsis. Plant J **40,** 200-212.

Spoel, S.H., Koornneef, A., Claessens, S.M., Korzelius, J.P., Van Pelt, J.A., Mueller, M.J., Buchala, A.J., Metraux, J.P., Brown, R., Kazan, K., Van Loon, L.C., Dong, X., and Pieterse, C.M. (2003). NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. Plant Cell **15,** 760-770.

Sturn, A., Quackenbush, J., and Trajanoski, Z. (2002). Genesis: cluster analysis of microarray data. Bioinformatics **18,** 207-208.

Suh, J.K., Poulsen, L.L., Ziegler, D.M., and Robertus, J.D. (1996). Molecular cloning and kinetic characterization of a flavin-containing monooxygenase from Saccharomyces cerevisiae. Arch Biochem Biophys **336,** 268-274.

Suh, J.K., Poulsen, L.L., Ziegler, D.M., and Robertus, J.D. (1999). Yeast flavin-containing monooxygenase generates oxidizing equivalents that control protein folding in the endoplasmic reticulum. Proc Natl Acad Sci U S A **96,** 2687-2691.

Suh, J.K., Poulsen, L.L., Ziegler, D.M., and Robertus, J.D. (2000). Redox regulation of yeast flavin-containing monooxygenase. Arch Biochem Biophys **381,** 317-322.

Sundaresan, V., Springer, P., Volpe, T., Haward, S., Jones, J.D.G., Dean, C., Ma, H., and Martienssen, R. (1995). Patterns of Gene-Action in Plant Development Revealed By Enhancer Trap and Gene Trap Transposable Elements. Genes & Development **9,** 1797-1810.

Tao, Y., Xie, Z.Y., Chen, W.Q., Glazebrook, J., Chang, H.S., Han, B., Zhu, T., Zou, G.Z., and Katagiri, F. (2003). Quantitative nature of Arabidopsis responses during compatible and incompatible interactions with the bacterial pathogen Pseudomonas syringae. Plant Cell **15,** 317-330.

Terras, F.R.G., Broekaert, W.F., and Janssens, F.A. (1995). A Pathogen-Induced Antifungal Plant Defensin from Radish Leaves. J Cell Biochem**,** 490-490.

Tobena-Santamaria, R., Bliek, M., Ljung, K., Sandberg, G., Mol, J.N., Souer, E., and Koes, R. (2002). FLOOZY of petunia is a flavin mono-oxygenase-like protein required for the specification of leaf and flower architecture. Genes Dev **16,** 753-763.

TolkerNielsen, T., Holmstrom, K., and Molin, S. (1997). Visualization of specific gene expression in individual Salmonella typhimurium cells by in situ PCR. Applied and Environmental Microbiology **63,** 4196-4203.

Tornero, P., Chao, R.A., Luthin, W.N., Goff, S.A., and Dangl, J.L. (2002). Large-Scale Structure -Function Analysis of the Arabidopsis RPM1 Disease Resistance Protein. Plant Cell **14,** 435-450.

Torres, M.A., Dangl, J.L., and Jones, J.D.G. (2002). Arabidopsis gp91phox homologues AtrbohD and AtrbohF are required for accumulation of reactive oxygen intermediates in the plant defense response. PNAS **99,** 517-522.

Tully, T., Preat, T., Boynton, S.C., and Del Vecchio, M. (1994). Genetic dissection of consolidated memory in *Drosophila.* Cell **79,** 35-47.

Tynes, R.E., and Hodgson, E. (1985). Catalytic Activity and Substrate-Specificity of the Flavin-Containing Monooxygenase in Microsomal Systems - Characterization of the Hepatic, Pulmonary and Renal Enzymes of the Mouse, Rabbit, and Rat. Archives of Biochemistry and Biophysics **240,** 77-93.

Tynes, R.E., Sabourin, P.J., Hodgson, E., and Philpot, R.M. (1986). Formation of hydrogen peroxide and N-hydroxylated amines catalyzed by pulmonary flavin-containing monooxygenases in the presence of primary alkylamines. Arch Biochem Biophys **251,** 654-664.

Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., Chandler, D., Slusarenko, A., Ward, E., and Ryals, J. (1992). Acquired resistance in *Arabidopsis*. The Plant Cell **4,** 645-656.

van Wees, S.C., and Glazebrook, J. (2003). Loss of non-host resistance of Arabidopsis NahG to Pseudomonas syringae pv. phaseolicola is due to degradation products of salicylic acid. Plant J **33,** 733-742.

Vernooij, B., Friedrich, L., Morse, A., Reist, R., Kolditz-Jawhar, R., Ward, E., Uknes, S., Kessmann, H., and Ryals, J. (1994). Salicylic acid is not the translocated signal responsible for inducing systemic acquired resistance but is required in signal transduction. The Plant Cell **6,** 959-965.

Vianello, A., Zancani, M., and Macri, F. (1990). Hydrogen peroxide formation and iron ion oxidoreduction linked to NADH oxidation in radish plasmalemma vesicles. Biochimica et Biophysica Acta **1023,** 19-24.

Vogel, J., and Somerville, S. (2000). Isolation and characterization of powdery mildew-resistant Arabidopsis mutants. Proceedings National Academy of Sciences USA **97,** 1897-1902.

Vogel, J.P., Raab, T.K., Schiff, C., and Somerville, S.C. (2002). PMR6, a Pectate Lyase-Like Gene Required for Powdery Mildew Susceptibility in Arabidopsis. Plant Cell **14,** 2095-2106.

Volko, S.M., Boller, T., and Ausubel, F.M. (1998). Isolation of new Arabidopsis mutants with enhanced disease susceptibility to Pseudomonas syringae by direct screening. Genetics **149,** 537-548.

Wan, J., Dunning, F.M., and Bent, A.F. (2002). Probing plant-pathogen interactions and downstream defense signaling using DNA microarrays. Funct Integr Genomics **2,** 259-273.

Weber, H., Vick, B.A., and Farmer, E.E. (1997). Dinor-oxo-phytodienoic acid: A new hexadecanoid signal in the jasmonate family. Proc. Natl. Acad. Sci. U. S. A. **94,** 10473-10478.

Whalen, M.C., Innes, R.W., Bent, A.F., and Staskawicz, B.J. (1991). Identification of *Pseudomonas syringae* pathogens of *Arabidopsis* and a bacterial locus determining avirulence on both *Arabidopsis* and soybean. Plant Cell **3,** 49-59.

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

Wittstock, U., and Halkier, B.A. (2000). Cytochrome P450CYP79A2 from Arabidopsis thaliana L. catalyzes the conversion of L-phenylalanine to phenylacetaldoxime in the biosynthesis of benzylglucosinolate. Journal of Biological Chemistry **275,** 14659-14666.

Worrall, D., Ng, C.K.Y., and Hetherington, A.M. (2003). Sphingolipids, new players in plant signaling. Trends in Plant Science **8,** 317-320.

Xiao, S., Brown, S., Patrick, E., Brearley, C., and Turner, J.G. (2003). Enhanced transcription of the Arabidopsis disease resistance genes RPW8.1 and RPW8.2 via a salicylic acid-dependent amplification circuit is required for hypersensitive cell death. Plant Cell **15,** 33-45.

Xiao, S., Calis, O., Patrick, E., Zhang, G., Charoenwattana, P., Muskett, P., Parker, J.E., and Turner, J.G. (2005). The atypical resistance gene, RPW8, recruits components of basal defence for powdery mildew resistance in Arabidopsis. Plant J **42,** 95-110.

Yaeno, T., Matsuda, O., and Iba, K. (2004). Role of chloroplast trienoic fatty acids in plant disease defense responses. Plant J **40,** 931-941.

Yang, S.H., and Hua, J. (2004). A haplotype-specific Resistance gene regulated by BONZAI1 mediates temperature-dependent growth control in Arabidopsis. Plant Cell **16,** 1060-1071.

Zhang, C., Gutsche, A.T., and Shapiro, A.D. (2004). Feedback control of the Arabidopsis hypersensitive response. Molecular Plant-Microbe Interactions **17,** 357-365.

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

Zhao, Y.D., Christensen, S.K., Fankhauser, C., Cashman, J.R., Cohen, J.D., Weigel, D., and Chory, J. (2001). A role for flavin monooxygenase-like enzymes in auxin biosynthesis. Science **291,** 306-309.

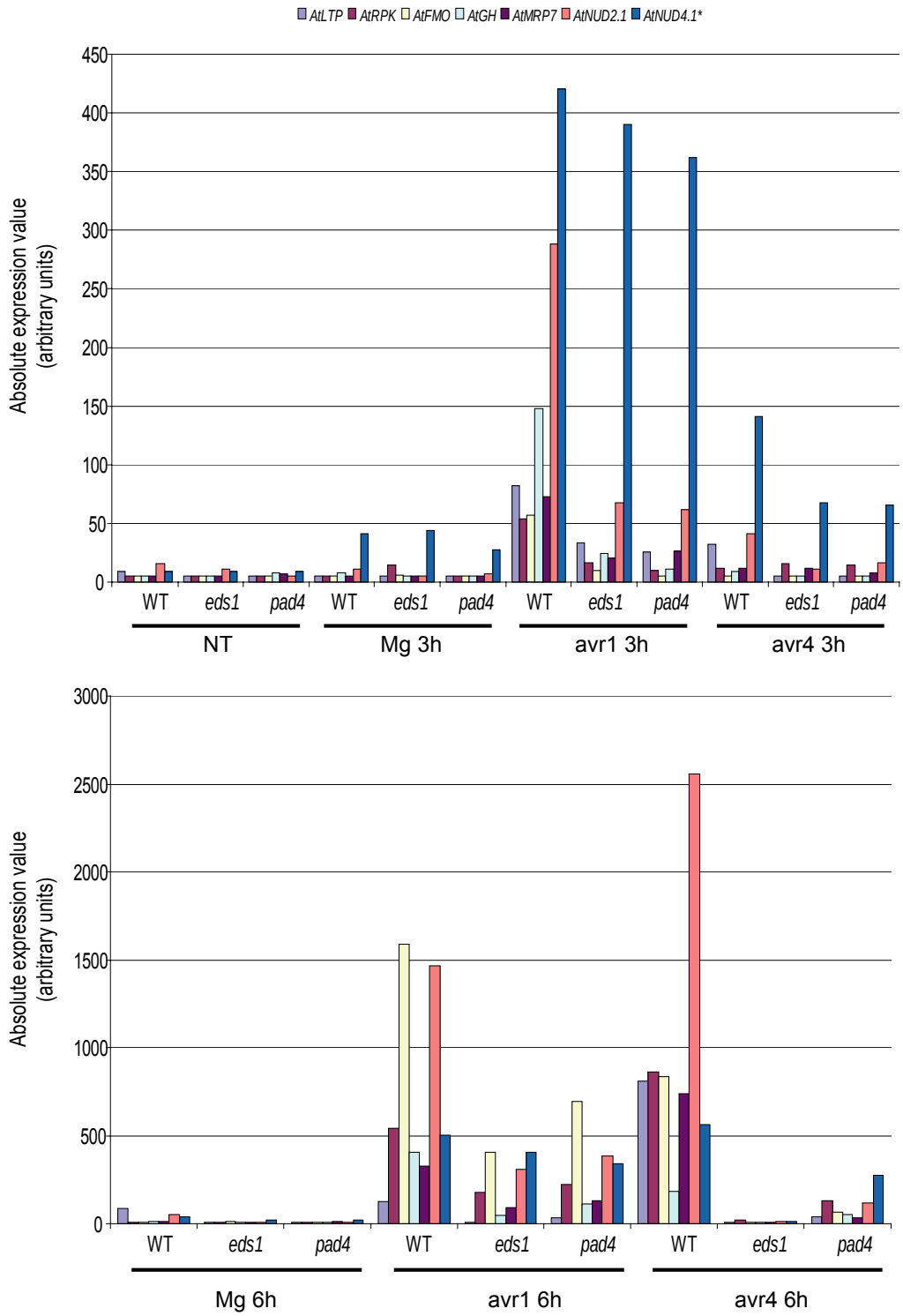
Zhou, N., Tootle, T.L., Tsui, F., Klessig, D.F., and Glazebrook, J. (1998). *PAD4* functions upstream from salicylic acid to control defense responses in Arabidopsis. Plant Cell **10,** 1021-1030.

Zhu, T., and Wang, X. (2000). Large-scale profiling of the Arabidopsis transcriptome. Plant Physiol **124,** 1472-1476.

Zimmerli, L., Stein, M., Lipka, V., Schulze-Lefert, P., and Somerville, S. (2004). Host and non-host pathogens elicit different jasmonate/ethylene responses in Arabidopsis. Plant J **40,** 633-646.

Zipfel, C., Robatzek, S., Navarro, L., Oakeley, E.J., Jones, J.D., Felix, G., and Boller, T. (2004). Bacterial disease resistance in Arabidopsis through flagellin perception. Nature **428,** 764-767.

Supplementary data



Supplement Figure. Absolute expression values for genes of Group II plus *AtNUD4.1* (*values for *AtNUD4.1* were divided by the factor 10 to better fit the scale). For detailed annotations see Table 3.4.

Danksagung

Ein herzliches Dankeschön an…

…meine Betreuerin Frau Dr. Jane Parker, auf die ich mich immer verlassen konnte und die es geschafft hat eine super Atmosphäre in der Arbeitsgruppe zu zaubern. Ich hätte keine bessere Betreuerin finden können!

…Herrn Prof. Dr. Schulze-Lefert danke ich für die Möglichkeit zur Erstellung dieser Dissertation am Max-Planck-Institut und für einige interessante Diskussionen.

…Herrn Prof. Dr. Tautz für die freundliche Übernahme des Korreferats.

…“meinen Post-Doc“ Lucia Jorda für das Einrichten des Parker-Labors nach dem Umzug aus England, ihre stets große Hilfsbereitschaft und ihre fröhliche Art. Muchas, Gracias.

…meine Laborkollegen Shige (siehst Du jetzt stehst Du doch hier ;-), Marcel, Steffen, Stefan, Lennart, Enrico, Jagreet, Jaqueline, Doris, Guangyong, Diether und Koh und an meine Ex-Kollegen Nieves, Pino, Laurent, Adriana und Paul für ihre Hilfsbereitschaft und das tolle Arbeitsklima.

…Jagreet und Stefan für das Korrekturlesen dieser Arbeit.

…Pawel für die Durchführung zahlreicher Salizylsäuremessungen.

…Bill Truman für seine Unterstützung bei der Mikroarrray-Analyse.

…meine Freundin Chiara, die sich besonders liebevoll um mich gekümmert hat und mich darin erinnerte wann es wieder Zeit wurde sich zu rasieren.

…meine Eltern, die in allen Situationen hinter mir stehen und mein Zimmer in Lübeck noch immer für mich reserviert halten, obwohl ich viel zu selten zu Besuch komme…

Publikation

Schneider, K., Kienow, L., Schmelzer, E., Colby, T., Bartsch, M., Miersch, O., Wasternack, C., Kombrink, E., and Stuible, H.-P. (2005). A New Type of Peroxisomal Acyl-Coenzyme A Synthetase from Arabidopsis thaliana Has the Catalytic Capacity to Activate Biosynthetic Precursors of Jasmonic Acid. J. Biol. Chem. **280,** 13962-13972.

Erklärung

"Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von der auf Seite 113 angegebenen Teilpublikation - noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Paul Schulze-Lefert betreut worden."

Köln, im Mai 2005

Lebenslauf

Zur Person

Name: Michael Bartsch Geburtsdatum: 11. Juli 1975

Ausbildung:

seit Februar 2002	Bearbeitung des Dissertationsthemas „Gene expression profiling leading to identification of essential components in EDS1/PAD4-regulated plant defence“ am Max-Planck-Institut für Züchtungsforschung, Köln (Abteilung Molekulare Phytopathologie) unter Leitung von Prof. Dr. P. Schulze-Lefert
April 2001	Studiumabschluß als Diplom Agrarbiologe an der Universität Hohenheim
September 2000 – April 2001	Diplomarbeit angefertigt am Sainsbury Laboratory, John Innes Centre, Norwich, Großbritannien: „Characterisation of GTPases associated with INF1 mediated response to *Phytophthora infestans* in *Nicotiana benthamiana*”. Referent: Dr. D. C. Baulcombe, Koreferent: Prof. Dr. A. Pfitzner
April – Juli 2000	Auslandssemester an der Universität Reading, Großbritannien
Wintersemester 1996	Beginn des Studiums Agrarbiologie in Hohenheim
1986-1995	Carl-Jacob-Burckhardt-Gymnasium, Lübeck
1982-1986	Grundschule Lübeck-Niendorf

Praktische Tätigkeiten

August - Dezember 2001	Wissenschaftlicher Mitarbeiter, Sainsbury Laboratory, John Innes Centre, Norwich, Großbritannien