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Identification of systemic acquired resistance-inducing molecules in plants by a new metabolomic approach

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II. ABBREVIATIONS

AOC	Allene oxide cyclase
AOS	Allene oxide synthase
AzA	Azelaic acid
cfu	Colony forming units
Col-0	Columbia-0
DC3000	*Pseudomonas syringae* pv. *tomato* DC3000 – virulent
DES	Divinyl ether synthase
dNTPs	Deoxynucleoside triphosphates
eds1-2	*Enhanced disease susceptibility 2-1*
ET	Ethylene
HPL	Hydroperoxide lyase
h	Hour(s)
9/13-HPOD/T	9/13-hydroperoxy-octadecadi-/trienoic acid
hpt	Hour(s) post treatment
JA	Jasmonic acid
jar1	*Jasmonic acid resistant 1*
LOX	Lipoxygenase
med4-2	*Methyl esterase deficient 4-2*
min	Minute(s)
ml	Milliliter(s)
mM	Millimolar
MS	Mass spectrometry
nm	Nanometers

npr1	*Nonexpresser of pathogenesis-related proteins 1*
O/N	Overnight
OD	Optical density
ONA	9-oxo-nonanoic acid
qRT-PCR	Quantitative real time polymerase chain reaction
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase polymerase chain reaction
SA	Salicylic acid
sid2	*Salicylic acid induction-deficient 2*
WT	Wild type
µl	Microliter(s)
µM	Micromolar

III. SUMMARY

Systemic acquired resistance (SAR) is a resistance response against a broad spectrum of microorganisms. This resistance is activated by a local pathogen attack. Infected leaves release signals, which are translocated via the phloem to the systemic leaves where they induce resistance. SAR can be mimicked by the conditional over expression of the bacterial effector *AvrRpm1* from a dexamethasone-inducible transgene. Local expression of *AvrRpm1* induces enhanced resistance in the distal tissue against *P. syringae*.

ENHANCED DISEASE SUSCEPTIBILITY1 (*EDS1*) is essential for SAR signal generation and/or transmission (Jorda, Vlot, and Parker, unpublished) and one of the main regulators of SA signalling. By comparing the metabolomic profile of *AvrRpm1*-expressing WT as compared to *eds1-2* mutant plants, this thesis aimed to identify signalling molecules involved in SAR. To this end, we established a new extraction protocol and verified new potential SAR-inducing signals by HPLC (high-performance liquid chromatography), FTICR-MS (fourier transform ion cyclotron resonance mass spectrometry), and LC-MS (liquid chromatography coupled with mass spectrometry). The new potential SAR-signals Neopterin, HPOD (hydroperoxy-octadecadienoic acid), ONA (9-oxo-nonanoic acid), and AzA (azelaic acid) accumulated in plants extracts in a time-dependent and in an *EDS1*-dependent manner. SAR-inducing activity was confirmed by experiments performed in WT *Arabidopsis* plants. Tests in JA-insensitive, SA signalling, and SA-deficient mutants showed that SA production and signalling are required for SAR downstream from Neopterin, ONA, and AzA. Furthermore, a mixture of ONA and AzA appears to require SA, but not *NPR1*-mediated (SA) signalling to trigger SAR. Taken together, this work presents new potentially SAR-inducing compounds, which are still under investigation.

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1. Chapter – INTRODUCTION

Plants are able to protect themselves against attacks of diverse microbial pathogens. On the one hand, pathogens have evolved strategies to secure that they manage to enter and proliferate. On the other hand, plants also have developed a number of strategies to counteract these attacks.

1.1 Basal resistance

In general, there are two types of defences induced in plants interacting with pathogens, local and systemic defence responses (Hammond-Kosack 2000, Schenk, Choo et al. 2010). Both defences will be introduced in this chapter to get an overview of how plants protect themselves against pathogenic microorganisms.

There are many ways for pathogens to enter plants, for instance by gaining access to the intercellular spaces and internal tissue of the leaf. To this end, pathogens and microbes have to cross a first border of defence, including the cuticle and epidermis of the plant as well as the cell wall. They must penetrate the leaf epidermis and enter e.g. via stomata (natural opening), wounds, pores, or via gas exchange routes (Underwood, Melotto et al. 2007). Pathogens that are not able to overcome that first border of defence are termed non-host pathogens in line with the nomenclature of the corresponding form of defence: non-host resistance (NHR) (Thordal-Christensen 2003, Mysore and Ryu 2004). NHR confers immunity to plants against the majority of microbial pathogens and represents the most robust and durable form of plant resistance in nature (Cheng, Zhang et al. 2012).

Host pathogens that are not fended off by NHR can be divided into two types. According to their lifestyles they are divided into biotrophs and necrotrophs. Biotrophs feed on living host tissue, whereas necrotrophs kill the host tissue and feed on the remains (Glazebrook 2005). The plant´s ability to conquer attack by biotrophic pathogens and withstand the concomitant biotic stress depends on how effectively the plant recognises incoming signals (primary immune response) (Maffei, Mithofer et al. 2007). Plant cells harbour on the extracellular surface special pattern recognition receptors (**PRR**s) which recognize so-called elicitors delivered by pathogens (Zipfel 2008). These, in turn, initiate downstream signalling events that result in the activation of basal resistance (Chisholm, Coaker et al. 2006). These evolutionarily conserved elicitors are termed microbial- or pathogen-associated molecular patterns (MAMPS or **PAMPs**) and the activated immune response is called PAMP-triggered immunity (**PTI**) (Jones and Dangl 2006, Bittel and

Robatzek 2007, Boller and He 2009). As part of PTI perception of divers pathogen-derived signals facilitates transcriptional reprogramming through mitogen-activated protein kinase (MAPK) cascades (Ishihama and Yoshioka 2012) and activation of WRKY transcription factors (Asai, Tena et al. 2002, Nurnberger, Brunner et al. 2004) as early defence responses.

For most of the known PAMPs, the corresponding PRRs have been identified (Bittel and Robatzek 2007). One of the best known PAMPs is flg22, a peptide of the bacterial flagellin protein. It is recognized by FLS2 (FLAGGELIN SENSING 2) (Felix, Duran et al. 1999), a leucine-rich repeat receptor-like kinase (LRR-RLK) (Chinchilla, Bauer et al. 2006). LRR-RLKs are single-pass transmembrane proteins composed of an LRR ectodomain (eLRR), a transmembrane domain and a Ser/Thr protein kinase domain related to *Drosophila Pelle* (Shiu and Bleecker 2001).

Another important PAMP is the *N*-acylated peptide of the bacterial elongation factor-Tu (EF-Tu, elongation factor thermo unstable) (Kunze, Zipfel et al. 2004), which is recognized by the EF-receptor (EFR) (Zipfel, Kunze et al. 2006) from *Arabidopsis thaliana* (*Arabidopsis*). In addition, lipopolysaccharides (LPS), chitins and glucans serve as PAMPs (Nurnberger, Brunner et al. 2004, Ron and Avni 2004, Schwessinger and Zipfel 2008, Zipfel 2008).

On the one hand, PTI can suppress colonization of plants by pathogens (Figure 1). On the other hand, some successfully entered pathogens are able to suppress PTI and redirect host metabolism to support their own nutrition and growth. To this end, bacterial pathogens use their type III secretion system (T3SS) to deliver into plant cells up to 30-40 type III effector proteins (T3Es; effectors) and other virulence factors such as the phytotoxin coronatine (jasmonic acid mimic, see below) (Nomura, Melotto et al. 2005). Effectors interfere with PTI, resulting in effector-triggered susceptibility (**ETS**) (Figure 1). In turn, specific pathogen effectors are recognized by R proteins of plants. Such a recognition event results in effector-triggered immunity (**ETI**) or *R* gene-mediated resistance (Figure 1).
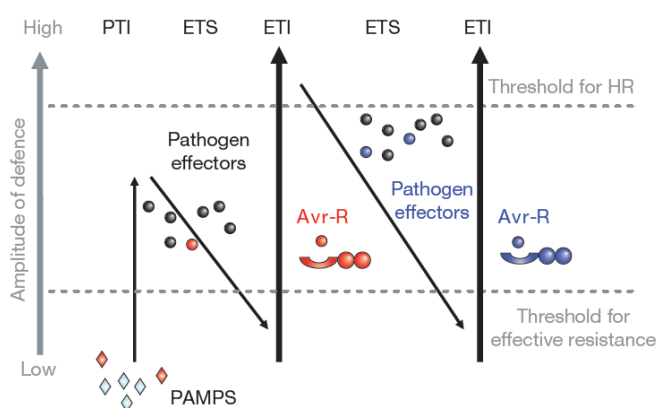
Figure 1: The „zigzag model" of plant defence. This model reflects defence and counter defence of plants versus host pathogens for immunity and susceptibility on both sides. PAMP: pathogen-associated molecular pattern, PTI: PAMP-triggered immunity, ETS: effector-triggered susceptibility, ETI: effector-triggered immunity, HR: hypersensitive response, Avr: effector, -R: resistance proteins. Illustration taken from (Jones and Dangl 2006).

There are different classes of *R*-genes. The first major class encodes a cytoplasmic protein with a nucleotide binding site (NBS), a C-terminal LRR and a putative coiled coil domain (CC) at the N-terminus (CC-NBS-LRRs). A second class of resistance proteins consists of cytoplasmic proteins which possess LRR and NBS motifs and an N-terminal domain with homology to the mammalian toll-interleukin-1-receptor (TIR-NBS-LRRs) (Staswick, Tiryaki et al.). The majority of R proteins from *Arabidopsis* belong to either one of these two classes. The rice genome lacks TIR-NB-LRRs, but contains several NB-LRRs with N-terminal domains of unknown function (X-NB-LRRs) emphasizing the complexity of NB-LRR evolution and function in plants (Monosi, Wisser et al. 2004).

PTI and ETI are effective against biotrophic and hemibiotrophic pathogens, but not against necrotrophic pathogens (Glazebrook 2005). ETI is an amplified version of PTI and results in a hypersensitive response (**HR**), a form of programmed cell death, at the infected site to keep the pathogen isolated from the remaining healthy tissue (Jones and Dangl 2006, Mur, Kenton et al. 2008). Besides an HR, ETI (and to a lesser extent PTI) is accompanied by other responses such as extracellular pH increase, oxidative burst, nitric (NO) generation, secondary metabolite synthesis, cell wall strengthening, and the expression of pathogenesis-related (**PR**) proteins (Silipo, Erbs et al. 2010). Some avirulent (**avr**), ETI/HR-inducing pathogens are well known to induce SAR (see below). However, an HR is not a causative signal for SAR induction. Some pathogens cause necrotic disease symptoms instead of HR and also trigger SAR (Cameron, Dixon et al. 1994, Mishina and Zeier 2006).

To sum this part up PTI is an ancient form of innate immunity (Chisholm, Coaker et al. 2006, Jones and Dangl 2006), during which PRRs recognize PAMPs. Pathogens adapted to their host plants are able to inhibit PTI by delivering virulence effectors into host cells (Abramovitch, Janjusevic et al. 2006, Zhou and Chai 2008, Boller and He 2009, Cui, Xiang et al. 2009). R-protein-mediated recognition of the same effectors in turn leads to a strongly amplified defence in the form of ETI. Although PTI and ETI employ distinct immune receptors, they seem to use a similar signalling network (Tsuda, Sato et al. 2009) by activating a largely overlapping set of genes (Navarro, Zipfel et al. 2004, Zipfel, Kunze et al. 2006).

1.2 Salicylic acid – signalling molecule involved in systemic plant defence

Downstream from PTI or ETI diverse plant hormones act as central players in triggering the plant immune signalling network (Bari and Jones 2009, Pieterse, Leon-Reyes et al. 2009, Katagiri and Tsuda 2010). Plant hormones were originally recognized as regulators of growth and development (Santner and Estelle 2009). Three hormones appear to be

mainly involved in defence. These are **SA** (salicylic acid), **JA** (jasmonic acid) and **ET** (ethylene).

The phytohormone SA accumulates in plants in response to biotrophic and hemi-biotrophic pathogen (Grant and Lamb 2006) during PTI and ETI responses. Phytohormones induce similar sets of genes in tobacco and *Arabidopsis* (Vlot, Dempsey et al. 2009). These include *PR* (*PATHOGENESIS-RELATED PROTEIN1*) genes such as the SA marker gene *PR1*. As mentioned above, PTI and ETI are usually associated with the induction of SAR (Durrant and Dong 2004, Mishina and Zeier 2007, Vlot, Klessig et al. 2008). Mutants that are either impaired in SA accumulation or insensitive to SA display an enhanced susceptibility to pathogens and compromised SAR (Bari and Jones 2009). Thus, SA is essential for PTI, ETI, and SAR.

SA controls the nuclear translocation of NPR1 (NONEXPRESSOR OF *PR* GENES1) through cellular redox changes (Spoel and Dong 2012). In the absence of pathogens, NPR1 is retained in the cytoplasm as an oligomer. After pathogen infection NPR1 is released into the nucleus as a monomer. It acts as a co-activator for transcription factors to induce defence-related genes. Therefore, NPR1 acts downstream of SA (Cao, Bowling et al. 1994). In addition, NPR1 suppresses the expression of *ICS1 (ISOCHORISMATE SYNTHASE1)*. Since this leads to SA biosynthesis inhibition (see below), NPR1 appears to be part of a feedback loop regulating SA signalling.

NPR1 is degraded by the proteasome (Spoel, Mou et al. 2009). Zheng et al. (Fu, Yan et al. 2012) described NPR3 and NPR4, paralogues of NPR1, as SA receptors that bind SA to different affinities. Both function as ubiquitin ligase to mediate NPR1 degradation in an SA-regulated manner. A double *npr3/npr4* mutant contains elevated levels of NPR1, but is not able to mount a SAR response. In addition, ETI-triggered HR cell death and local immunity are impaired (Fu, Yan et al. 2012).

How SA is synthesized is not fully defined until now, but it is published that it can be synthesized from phenylalanine (PAL pathway) and then converted to SA either through free benzoic acid, benzoyl glucose or through *o*-coumaric acid as a precursor depending on the plant species (Figure 2) (Garcion, Lohmann et al. 2008). Further analysis showed that an SA-deficient *Arabidopsis* mutant *sid2* is impaired in ICS (Wildermuth, Dewdney et al. 2001). This provides strong evidence that another pathway exists to synthesise SA (via isochorismate (IC); IC pathway). The IC pathway also is used by bacteria to produce SA (Leistner 1999). Although the IC pathway appears to be the major route to synthesize SA in plants upon pathogen infection (Wildermuth, Dewdney et al. 2001, Garcion, Lohmann et al. 2008), the enzyme necessary to convert IC into SA has not yet been found in plants (Fragniere, Serrano et al. 2011).
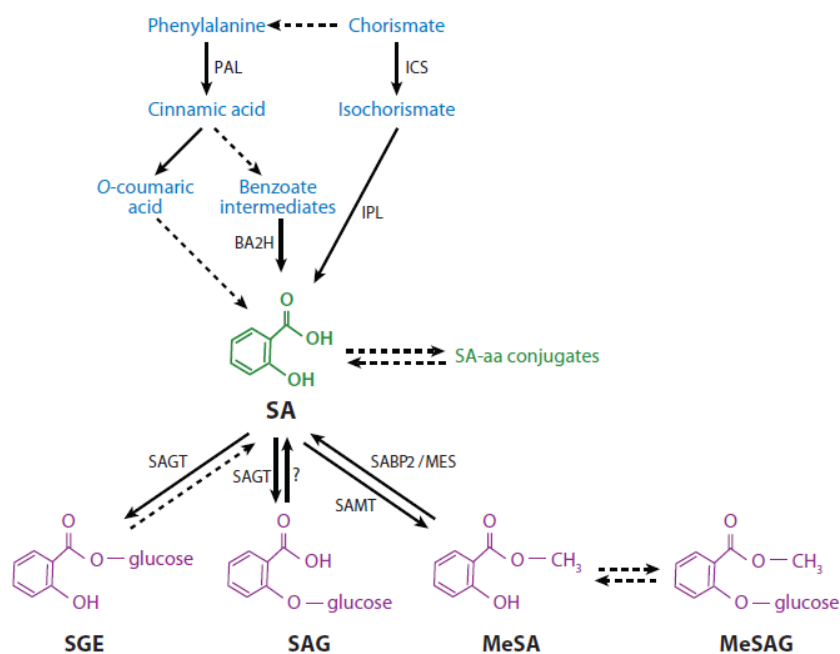
Figure 2: Simplified schematic of SA biosynthesis and metabolism. SA can be synthesized via two different pathways starting from phenylalanine or chorismate. PAL: phenylalanine ammonium lyase, ICS: isochorismatete synthase, IPL: isochorismate pyruvate lyase, BA2H: benzoic acid-2-hydroxylase, SA: salicylic acid, SAGT: SA glucosyltransferase, SABP2: SA-binding protein 2, MES: methyl salicylate esterase, SAMT: SA methyltransferase, SGE: salicyloyl glucose ester, SAG: SA *O-ß*-glucoside, MeSA: methyl salicylate, MeSAG: methyl salicylate *O-ß*-glucoside. Illustration taken from (Vlot, Dempsey et al. 2009).

As it is shown in Figure 2, SA can be further converted into different derivates, including SA *O-ß*-glucoside (SAG) as the major glycosylated conjugate or into the SA glucose ester (SGE) as a minor one (Enyedi, Yalpani et al. 1992, Edwards 1994, Lee and Raskin 1998, Dean, Mohammed et al. 2005). SAG is actively transported from the cytosol into the vacuole, where it may function as an inactive storage form of SA (Vlot, Dempsey et al. 2009). Alternatively, SA can be converted into the volatile methyl salicylate (MeSA) (Shulaev, Silverman et al. 1997, Huang, Cardoza et al. 2003). In 2007, it has been shown that MeSA acts as a mobile inducer of SAR in tobacco (Park, Kaimoyo et al. 2007). Moreover, as a mobile precursor for distal SA accumulation (Forouhar, Yang et al. 2005, Kumar and Klessig 2008). During further processing MeSA can be converted into methyl salicylate *O-ß*-glucoside (Figure 2) (MeSAG). However, the enzyme responsible for the formation of MeSAG has not yet been identified (Song, Koo et al. 2008).

1.3 EDS1 – a key player in SA signalling

In this thesis, I work with the *eds1-2* (*enhanced disease susceptibility1-2*) mutant of *Arabidopsis thaliana*. This mutant may help to identify signals involved in bacterially induced SAR. EDS1 is a lipase-like protein that controls PTI (Schurink, Aarts et al. 1998,

Feys, Wiermer et al. 2005, Wiermer, Feys et al. 2005, Ochsenbein, Przybyla et al. 2006). In addition, EDS1 also is essential for ETI in response to TIR-NB-LRR R proteins and for SAR.

EDS1 interacts with PAD4 (PHYTOLAXIN DEFICIENT4) and SAG101 (SENESCENCE ASSOSIATED GENE101). The three corresponding genes are closely related to each other and are required for PTI as well as ETI mediated by resistance proteins belonging to TIR-NB-LRR class (Aarts, Metz et al. 1998, Falk, Feys et al. 1999, Feys, Moisan et al. 2001, Wiermer, Feys et al. 2005). EDS1 together with PAD4 and SAG101 stimulates the accumulation of SA during PTI, ETI, and SAR (Figure 3) (Feys, Moisan et al. 2001, Feys, Wiermer et al. 2005, Tsuda, Sato et al. 2009, Vlot, Dempsey et al. 2009). This action of EDS1 and partners upstream from SA was concluded from experiments, in which the enhanced disease susceptibility phenotypes of the *eds1* and *pad4* mutants could be rescued by treatment of the plants with SA (Feys, Moisan et al. 2001, Rusterucci, Aviv et al. 2001). SA, in turn, enhances the expression of *EDS1* and *PAD4* indicating that EDS1 and PAD4 are part of an SA-dependent positive feedback loop (Figure 4) (Wiermer, Feys et al. 2005).

EDS1 forms homodimers in the cytoplasm. In addition, EDS1 forms heteromeric complexes with PAD4 in the nucleus and cytoplasm. EDS1- SAG101 heteromeric complexes localise strictly to the nucleus (Feys, Wiermer et al. 2005, Zhu, Jeong et al. 2011). The coordination of nuclear and cytoplasmic trafficking of EDS1 is important for the full plant disease resistance response (Figure 3) (Cheng, Germain et al. 2009, Garcia, Blanvillain-Baufume et al. 2010). Cytoplasm-localised EDS1 leads to pathogen-induced cell death, whereas localisation of EDS1 in the nucleus triggers transcriptional reprogramming leading to downstream defence responses (Figure 3) (Garcia, Blanvillain-Baufume et al. 2010).

The *Arabidopsis* TIR-NB-LRR receptor RPS4 recognises the bacterial effector AvrRps4 from *P. syringae* (Gassmann, Hinsch et al. 1999). In 2011 two groups showed that EDS1 is intimately involved in this recognition process. Both groups showed that EDS1 interacts with AvrRps4 as well as with RPS4 (Bhattacharjee, Halane et al. 2011, Heidrich, Wirthmueller et al. 2011). These interactions occur in the nucleus and are essential for EDS1-mediated transcriptional reprogramming towards defence. Furthermore, these interactions gave evidence that an RPS4-EDS1 receptor signalling complex can accumulate in the cytoplasm, indicating the existence of compartment-specific RPS4-EDS1 defence branches (Figure 3) (Heidrich, Wirthmueller et al. 2011). The cytosolic branch leads to PCD (Figure 3).
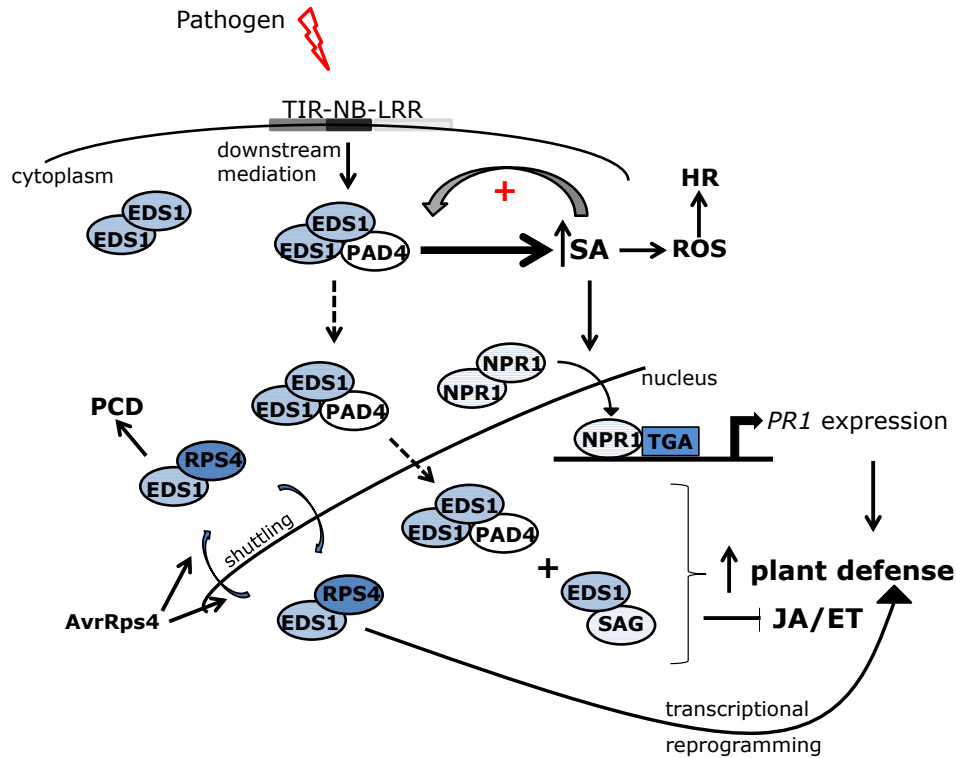
Figure 3: Model for different EDS1-dependent processes. Activation of different plant defence responses, including programmed cell death, is coordinated by EDS1 with different interaction partners. These processes are triggered in the nucleus and the cytoplasm of Arabidopsis. TIR: Toll-interleukin-1-receptor domain, NB-LRR: nucleotide binding-leucin-rich-repeat, EDS1: enhanced disease susceptibility1, PAD4: phytolalexin deficient 4, SAG: senescence associated gene 101, RPS4: receptor for the bacterial effector AvrRps4, SA: salicylic acid, TGA: transcription factor, *PR1*: pathogenesis related protein1 gene, ROS: reactive oxygen burst, HR: hypersensitive response, JA: jasmonic acid, ET: ethylene.

1.4 Phytohormones - crosstalk between SA, JA and ET

SA, JA, including JA derivatives, and ET are well established as primary signals in immune responses in plants (Browse 2009, Pieterse, Leon-Reyes et al. 2009, Vlot, Dempsey et al. 2009). As described above, SA is mainly associated with defence against biotrophic pathogens during PTI and ETI, whereas JA (and ET) are more closely associated with defence against necrotrophic pathogens and insects (Bostock 2005, Glazebrook 2005). SA and JA can act antagonistically or synergistically depending of the relative concentrations of SA and/or JA. ET is an important modulator of SA-JA crosstalk. Cross talk among these defence-related hormones as well as with other hormone-dependent signalling pathways is a rapidly developing theme in plant immune signalling research (Spoel and Dong 2008, Pieterse, Leon-Reyes et al. 2009, Robert-Seilaniantz, Grant et al. 2011).

The SA and JA-signalling pathways are thought to act mainly antagonistically, i.e. activation of one pathway suppresses the activation of the other (Feys and Parker 2000, Kunkel and Brooks 2002). For example, the SA signalling pathway is activated in *Arabidopsis* by *P. syringae*, whereas the JA signalling pathway is suppressed by the same pathogen. In that case, the SA pathway induces resistance against *P. syringae*, but the infected plant is at the same time rendered more susceptible to e.g. the necrotrophic fungus *Alternaria brassicicola* (Spoel, Johnson et al. 2007). Under certain conditions, however, it was shown that SA and JA may function synergistically (Pena-Cortés, Albrecht et al. 1993, Doares, Narvaez-Vasquez et al. 1995, Schenk, Kazan et al. 2000, van Wees, de Swart et al. 2000, Spoel, Koornneef et al. 2003, Mur, Kenton et al. 2006). Low concentrations of JA and SA resulted in a synergistic effect, whereas higher concentrations act antagonistically. This provides evidence for a dependency of the SA-JA interaction on the relative concentration of each hormone (Mur, Kenton et al. 2006). In 2008, Koornneef et al. demonstrated that also the timing and sequence of the hormone treatments/inductions may be important for the outcome of the SA-JA interaction (Koornneef, Leon-Reyes et al. 2008).

Different proteins regulating SA-JA crosstalk have been identified in *Arabidopsis* *(*Petersen, Brodersen et al. 2000, Kachroo, Shanklin et al. 2001, Li, Brader et al. 2004, Brodersen, Petersen et al. 2006, Ndamukong, Abdallat et al. 2007)*. These include EDS1 (Brodersen, Petersen et al. 2006) and NPR1 (Spoel, Koornneef et al. 2003). For example interacts EDS1 with PAD4 to control antagonism between SA and JA/ET defence response in the nucleus (Figure 3) (Gupta, Willits et al. 2000).

Also during SA-JA signal interaction, ET can play a critical role. ET may shape the SA-JA signal interaction during plant defence because it is required for processes in both pathways (Ndamukong, Abdallat et al. 2007, Leon-Reyes, Spoel et al. 2009, Zander, La Camera et al. 2010). The *Arabidopsis npr1* mutant is impaired in SA signalling and blocked in SA -mediated suppression of JA-regulated genes (Spoel, Koornneef et al. 2003, Leon-Reyes, Spoel et al. 2009). Nuclear localisation of NPR1 is essential for SA-responsive defence gene expression, but not for SA -mediated suppression of the JA-pathway (Spoel, Koornneef et al. 2003). By contrast, cytoplasmic NPR1 may be involved in SA-JA crosstalk. In the nucleus NPR1 interacts with TGA transcription factors to activate transcription of SA-responsive *PR* genes (Dong 2004, Lindermayr, Sell et al. 2010). Tested, *tga* mutants that are impaired in SA-responsive gene expression also display compromised SA-mediated suppression of the JA pathway. This indicates that TGAs also are important for SA-JA cross talk (Ndamukong, Abdallat et al. 2007, Leon-Reyes, Spoel et al. 2009, Zander, La Camera et al. 2010). In 2009, it was shown that exogenous applied ET bypass the need of NPR1 by the suppression of the JA response by SA (Leon-Reyes, Spoel et al. 2009). A model was suggested in which ET modulate the

NPR1 dependency of SA/JA antagonism. However, further molecular mechanisms underlying the crosstalk remains elusive (Zhang, Wang et al. 2012).

From a study with ET-insensitive (Tetr) tobacco plants, it was concluded that ET is essential for the onset of SA-dependent SAR, triggered upon infection of the plants with tobacco mosaic virus (TMV) (Verberne, Hoekstra et al. 2003). Moreover, the synergistic effect of ET on SA-induced *PR1* expression is blocked in the ET-insensitive *Arabidopsis* mutant *ein2* (*ET-insensitive protein 2*) (Lawton, Potter et al. 1994, De Vos, Van Zaanen et al. 2006). This indicates that the SA pathway is modulated by ET through EIN2 (Pieterse, Leon-Reyes et al. 2009). Moreover, it was shown in tobacco that ET is essential for the onset of SAR (Verberne, Hoekstra et al. 2003).

ET interacts mainly synergistically with JA. The best example is the regulation of the *Arabidopsis* plant defensin gene *PDF1.2* (*plant defensin1.2*), which requires concomitant activation of the JA and ET response pathway (Penninckx, Thomma et al. 1998). Additionally, activation of the JA and ET response pathway has been demonstrated in ISR (induced systemic resistance), which is triggered after colonization of plant roots by beneficial pathogen (Van der Ent, Verhagen et al. 2008, Van Wees, Van der Ent et al. 2008). This is also highlighting the importance of ET in plant defence response.

1.5 The secrets of SAR

Systemic acquired resistance (SAR) is a long-lasting disease resistance in the systemic tissues of local pre-infected plants. This mechanism provides protection against a broad spectrum of harmful microbes, e.g. bacteria, fungi, or viruses (Shah 2009, Vlot, Dempsey et al. 2009). During SAR establishment, accumulation of SA leading to local defence is followed by systemic, SA-dependent immunity (Vlot, Dempsey et al. 2009). Once the plant defence response is activated in the infected tissue, long distance signals move through the phloem from the infected site to protect the systemic healthy tissue from subsequent infections (Durrant and Dong 2004, Vlot, Klessig et al. 2008). Since systemic *PR1* expression tightly correlates with SAR development it is often used as a marker to indicate SAR (Liu, von Dahl et al. 2011).

SA itself was in discussion for a long time to serve as the long-distance, mobile signal for SAR. However, grafting experiments with tobacco plants expressing the bacterial *NahG* gene (encoding the SA-degrading enzyme salicylate hydroxylase) proved that SA is not the mobile signal (Vernooij, Friedrich et al. 1994). However, after pathogen attack SA accumulates and is converted into MeSA by BSMT1 (BENZOIC ACID/SALICYLIC ACID CORBOXYLMETHYLTRANSFERASE1). The rising SA levels inhibit the MeSA esterase (MSE)

(Figure 4; depicted in red; lower leaf). MeSA travels via the phloem into the systemic tissue and is hydrolysed back to SA by MSE (Figure 4; upper leaf) (Dempsey and Klessig 2012). Accumulation of SA in the systemic tissue is also required to support SAR (Vernooij, Friedrich et al. 1994), most likely by activating NPR-dependent transcriptional reprogramming (Figure 4).

MeSA has been shown to function as a mobile SAR signal in tobacco, Arabidopsis, and potato (Park, Kaimoyo et al. 2007, Vlot, Klessig et al. 2008, Manosalva, Park et al. 2010, Liu, von Dahl et al. 2011). There are controversial opinions about MeSA and its requirement for SAR establishment. Attaran et al. (2009) showed that *Arabidopsis* knockout mutants that lack BSMT1 and fail to accumulate MeSA are still able to produce SA and activate SAR (Attaran, Zeier et al. 2009). The Klessig lab, however, showed the opposite: in their hands SAR was abolished in the same mutants (Liu, Yang et al. 2010). In 2011 the same lab suggested that the necessity for MeSA to trigger SAR might be dependent on the time of infection during the day (Liu, von Dahl et al. 2011). It was shown that the extent of light exposure after the first inoculation plays a critical role in determining the extent to which SAR development depends on MeSA. If plants receive a short light exposure (<3.5 hours) after the first inoculation, MeSA and its metabolising enzymes are required for SAR (Figure 4; yellow flashs). By contrast, when plants were exposed to light for a longer period (>3.5 hours) immediately following the first inoculation, the resulting SAR development is independent of MeSA (Liu, von Dahl et al. 2011).

In addition to MeSA, different SAR signals that are related to lipids or lipid metabolism have been described (Maldonado, Doerner et al. 2002, Truman, Bennett et al. 2007, Chaturvedi, Krothapalli et al. 2008, Jung, Tschaplinski et al. 2009, Chanda, Xia et al. 2011). One of these proposed signals is JA. SAR is compromised in JA mutants, but the link between SAR and JA seems to be unclear since SAR is not altered in all JA signalling mutants (Cui, Bahrami et al. 2005, Mishina and Zeier 2007). As described above, JA and SA signalling are in a complex relationship with antagonistic and synergistic effects (Mur, Kenton et al. 2006). Both hormones increase rapidly after pathogen infection (Mishina and Zeier 2006, Attaran, Zeier et al. 2009). Analysis of JA pathway mutants indicated that pathogen-induced MeSA production is dependent on JA biosynthesis/signalling (Attaran, Zeier et al. 2009). Furthermore, it was seen that exogenous application of MeSA promotes *AtBSMT1* expression suggesting that JA promotes SAR by up-regulating *AtBSMT1* expression and MeSA production in the inoculated leaf (Figure 4, green arrow) (Koo, Kim et al. 2007, Attaran, Zeier et al. 2009).

Another SAR signal related to lipid metabolism is dependent on the *Arabidopsis SFD1* (*SUPPRESSOR OF FATTY ACID DESATURASE DEFICIENCY1*; also known as *GLY1*) gene.

The corresponding mutant fails to develop SAR following a local inoculation with avirulent *P. syringae* pv. *tomato* (*Pst*). Systemic SA accumulation is also impaired in *sfd1* mutant plants (Nandi, Welti et al. 2004). Interestingly, the *sfd1* mutant can respond to SAR signals present in petiole exudates from leaves inoculated with avirulent *Pst* (such petiol exudates will further be referred to as AvrPex). By contrast, AvrPex isolated from *sfd1* mutant plants fail to induce SAR in naïve, wild type (WT) plants, suggesting a defect in *sfd1* to induce or emit the long distance SAR signal (Chaturvedi, Krothapalli et al. 2008). Mutations in several genes, which similarly to *SFD1* encode enzymes involved in chloroplast galactolipid metabolism (*FAD7*, *SFD2*, *MGD1*), also abolish SAR. Since basal resistance is not affected by any of the corresponding mutations, the phenotype appears to be strictly related to SAR (Nandi, Welti et al. 2004, Chaturvedi, Krothapalli et al. 2008).

Efforts to identify the *SFD1*-dependent SAR signal showed that the immediate product of the SFD1-supported enzymatic reaction in the chloroplast, G3P (glycerol-3-phosphate), may be a long distance signal triggering SAR (Chanda, Xia et al. 2011) (Figure 4). G3P is an obligatory precursor for the synthesis of all glycerolipids in plants. Interestingly, it interacts with **DIR1** (DEFECTIVE IN INDUCED RESISTANCE 1), a lipid transfer protein that is essential for SAR signal production or its emission from the primary infected leaf (Maldonado, Doerner et al. 2002). Together, G3P and DIR1 may be sufficient to trigger SAR (Figure 4) (Chaturvedi, Krothapalli et al. 2008).

Additional analyses of AvrPex from *Arabidopsis* identified DA (dehydroabietinal) as a putative mobile SAR signal. DA is not only a potent resistance inducer; it is also rapidly translocated throughout the plant (Figure 4). Local treatment with DA induces SAR to virulent pathogens in *Arabidopsis*, tobacco and tomato, but does not enhance SAR to avirulent *Pst* in *Arabidopsis*. DA-induced resistance requires SA biosynthesis and signalling. Additional experiments with trypsin indicated that one or more proteins are required for DA-dependent SAR induction (Chaturvedi, Venables et al. 2012).

A newly identified putative SAR signal is Pip (pipecolic acid) (Figure 4). Pip is a product of lysine degradation in plants and animals. Elevated levels of Pip were found in *Psm (P. syringae* pv. *maculicola*)-inoculated leaves, in leaves systemic to the inoculated leaves, and in petiole exudates from the inoculated leaves of *Arabidopsis*. Defects in SAR as well as PTI and ETI are associated with a lack of Pip production in *ald1* (*AGD2-like defence response protein1*) mutant plants. Exogenously applied Pip complements these resistance defects of the *ald1* mutant and increases pathogen resistance in WT plants (Navarova, Bernsdorff et al. 2012).

For further explanation of different interactions, Figure 4 illustrates more SAR-related processes activated after pathogen attack. With regards to defence, SFD1, G3P, DA and

AzA (azelaic acid) are expected to act synergistically (double-headed green arrows ⊕; lower leaf). After signals are transported via the phloem to the un-inoculated systemic leaf, DIR1 and G3P inhibit expression of *BSMT1* (depicted in red). At the same time, G3P and DA are thought to support gene expression towards conversion of MeSA into SA (Dempsey and Klessig 2012) (Figure 4, depicted in red; upper leaf). Both these actions shift the SA-MeSA equilibrium in the direction of SA, thus favouring the development of SAR. In addition, FMO1 (FLAVIN DPENDENT MONOOXYGENASE 1) is proposed to positively regulate SA accumulation in the systemic tissue via ICS1 (loop; upper leaf). Alternatively, FMO1 may activate an alternative pathway for SAR induction (dashed blue line). AzA-induced resistance requires FMO and ALD1 (depicted with dashed lines) (Dempsey and Klessig 2012). The signal AzA will be discussed in more detail in chapter 1.7.
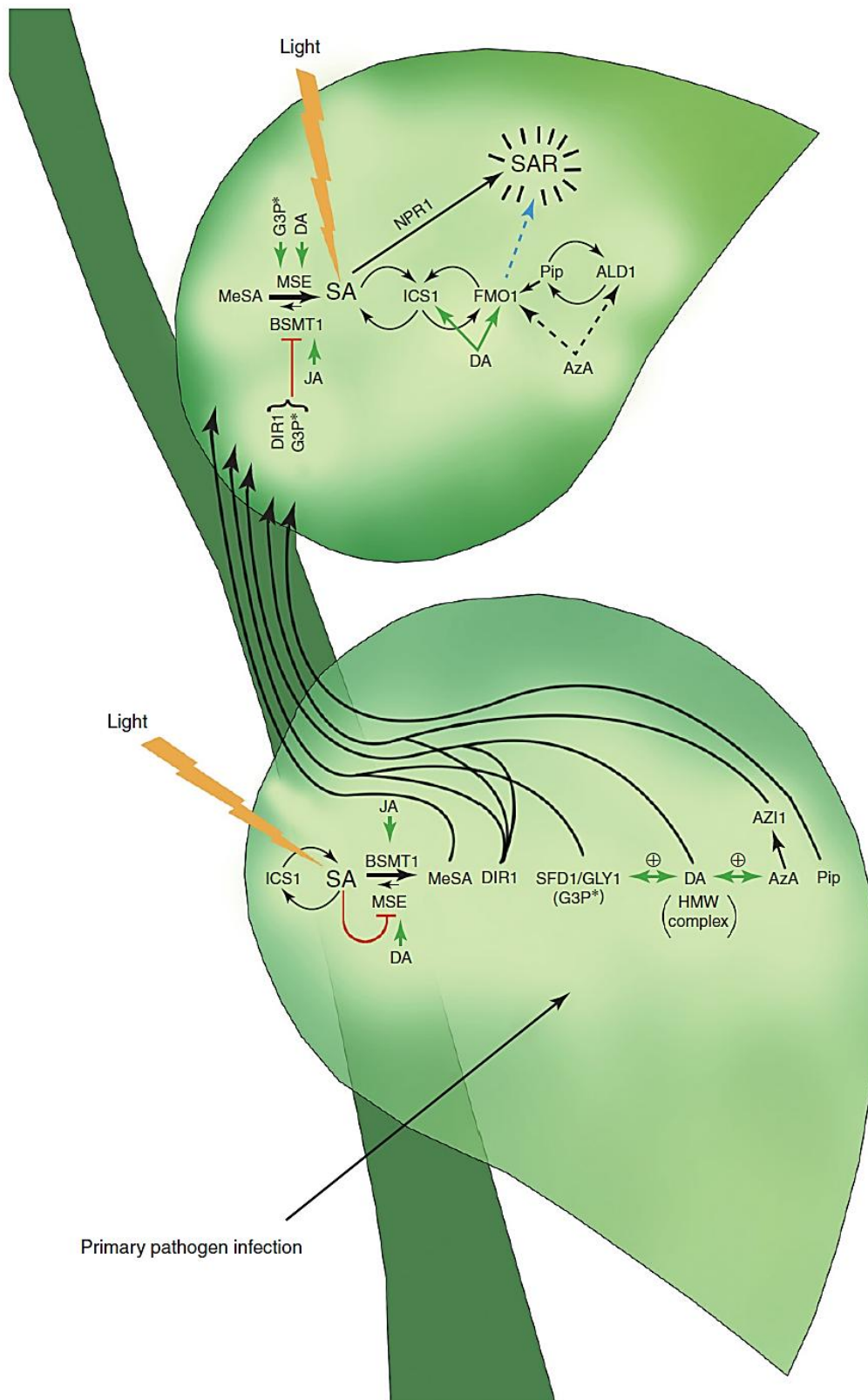
Figure 4: Putative long-distance SAR signals. <u>Local/inoculated leaf:</u> SA: salicylic acid, MeSA: methyl salicylate, DIR1: DEFECTIVE IN INDUCED RESISTANCE1, Pip: pipecolic acid, SFD1: SUPPRESSOR OF FATTY ACID DESATURASE DEFICIENCY1, G3P*: Glycerol-3-Phosphate, DA: dehydroabietinal, AzA: azelaic acid, ICS1: ISOCHORISMATE SYNTHASE1, MSE: MeSA esterase (depict in red), BSMT1: BENZOIC ACID/SA CARBOXYL METHYLTRANSFERASE 1, BSMT1: BENZOIC ACID/SALICYLIC ACID CARBOXYL METHYLTRANSFERASE 1, JA: jasmonic acid, AZI1: AzA induced 1.

<u>Systemic leaf:</u> NPR1: NON-EXPRESSOR OF, ALD1: AGD2-LIKE DEFENCE RESPSONSE PROTEIN, FMO1: FLAVIN DEPENDENT MONOOXYGENASE.

<u>Symbols:</u> synergistic interaction: double-headed green arrow ⊕, red mark: enhancement, SAR signal-induced gene expression: green arrows, amplification loop: black circle, bold and longer arrows: primary direction of MeSA/SA, requirement for induced resistance: dashed lines, alternative pathway: dashed blue line, light: yellow arrows. For further explanations see the text. Illustration taken from (Dempsey and Klessig 2012).

Many other mutants have been described that are incapable of triggering SAR. These include *npr1* (Cao, Bowling et al. 1994, Delaney, Friedrich et al. 1995, Wang, Amornsiripanitch et al. 2006), *sid2* (ICS mutant) (Wildermuth, Dewdney et al. 2001), *eds1* (*enhanced disease susceptibility 1*) (Truman, Bennett et al. 2007), and *pad4* (*phytoalexin deficient 4*) (Attaran, Zeier et al. 2009). All of these mutants are also deficient in accumulation of SA after infection. Not only lipids and hormones are involved in SAR, but also proteins and peptides are potential SAR signalling factors. For example, the *Arabidopsis* apoplastic aspartic protease CDR1 (CONSTITUTIV DISEASE RESISTANCE 1) cleaves an unknown protein, subsequently inducing a systemic defence response (Xia, Suzuki et al. 2004, Simoes, Faro et al. 2007, Lascombe, Bakan et al. 2008). Overexpression of rice CDR1 (*Os*CDR) leads to constitutive activation of defence responses in rice and *Arabidopsis* (Prasad, Creissen et al. 2009, Prasad, Creissen et al. 2010).

1.6 Azelaic acid

In addition to the putative SAR signalling molecules introduced above azelaic acid (AzA; also called nonanoic acid) has been coined as a potential SAR signal in pathogen-induced resistance in *Arabidopsis* (Jung, Tschaplinski et al. 2009). Since it is an important subject of this work, AzA will be introduced in more detail here.

Gas chromatography (**GC**) coupled with mass spectrometry (Kirsch, Takamiya-Wik et al.) of active (defence-inducing) AvrPex revealed an average of 6.2-fold higher levels of the saturated dicarboxylic acid AzA ($C_9H_{16}O_4$) as compared to inactive exudates (Jung, Tschaplinski et al. 2009). Several experiments were performed to further characterise the metabolite. Locally applied AzA is able to reduce bacterial growth from *P. syringae pathovar maculicola* (*Psm*) in *Arabidopsis*. Furthermore, locally applied deuterium-labelled AzA was found in systemic tissue, confirming that AzA can function as a mobile signal. The AzA-induced resistance requires both SA synthesis and several components of the SA signalling pathway. In line with this, AzA-treated plants exhibit elevated SA accumulation and *PR1* expression. As a conclusion, AzA was hypothesized to prime plant cells for SA production upon infection (Jung, Tschaplinski et al. 2009). Since SAR is a form of priming and AzA can move in plants, AzA may be a mobile signal for SAR.

Priming means that plants are more sensitised (primed) to more quickly and effectively activate defence responses as compared to unprimed plants (Conrath 2006). Priming is beginning to be elucidated and described in several plant species (Conrath 2006, Beckers, Jaskiewicz et al. 2009, Jaskiewicz, Conrath et al. 2011). It can be activated as SAR, but also by other molecules such as ß-amino-butyric acid (BABA) (Ton and Mauch-Mani 2004).

The *Arabidopsis* gene *AZI1* (*AZELAIC ACID INDUCED1*, *At*4g12470) is induced by AzA treatment. *AZI1* encodes a predicted secreted protease inhibitor/seed storage/lipid transfer family protein and has no significant homology to *DIR1*. Analyses of two different *azi1* mutant lines revealed normal susceptibility to local infection with several strains of *P. syringae*, but a defect in SAR (Jung, Tschaplinski et al. 2009). Pathogen growth was not restricted in the systemic tissue of pre-infected plants, while the mutants showed reduced accumulation of SA and *PR1* transcripts upon secondary infection of- the systemic tissue. Therefore, the *azi1* mutants were less capable of SAR-inducing priming of the systemic tissue. Further experiments indicated that *azi1* mutants can recognize defence/priming signal(s) because AzA or AvrPex triggered resistance in treated leaves of the mutant plants against *Pseudomonas*. Controversely, AvrPex from *azi1* mutants did not trigger resistance in WT plants, indicating that AZI1 may modulate production and/or translocation of a mobile signal(s) during SAR (Jung, Tschaplinski et al. 2009).

1.7 Another defence factor – oxylipins and general synthesis

As introduced above different defence mechanisms have evolved in plants to prevent disease. In the following an important process related to defence will be described which is relevant for this work. Lipid peroxidation refers to the oxidative degradation of lipids (Mosblech, Feussner et al. 2009, Zoeller, Stingl et al. 2012), which results in the accumulation of compounds like JA (belongs to oxylipins) and AzA (not an oxylipin), which we have seen from the previous sections to play an important role in plant immunity.

Oxylipins have antimicrobial effects, stimulate plant defence gene expression, and regulate plant cell death (Croft, Juttner et al. 1993, Weber, Chetelat et al. 1999, Prost, Dhondt et al. 2005). Alteration in oxylipins synthesis is thought to modify the plant response to pathogen infection (Rance, Fournier et al. 1998, Vijayan, Shockey et al. 1998, De Leon, Sanz et al. 2002, Farmer, Almeras et al. 2003). For example, wounding causes the accumulation of ROS and the degradation of linolenic acid, the precursor of JAs, from membrane lipids (Turner, Ellis et al. 2002). Upon wounding the oxylipin JA is synthesised. It activates transcription factors leading to the activation JA-responsive

genes resulting in defence against necrotrophic pathogen and herbivores (Glazebrook 2005).

Lipid peroxidation is dependent on the **9-LOX** (9-Lipoxygenase) and **13-LOX** (13-Lipoxygenase) pathways (Figure 5) and the CYP74 enzyme families. LOXs add molecular oxygen to either position 9 (9-LOX) or position 13 (13-LOX) of the C18 chain of linoleic and linolenic acids (Howe and Schilmiller 2002). Starting with linolenic acid (Figure 5 left side, green box) the 9-LOX pathway results in the production of colnelenic acid (yellow circle), 9-oxo-nonanoic acid (ONA) and nonadienal (NDE) (blue circle), 10-OPDA (10-oxo-phytodienoic acid, red circle), and α and γ ketol (green circle). By contrast, the 13-LOX pathway (Figure 5 right side, red box) results in the production of etherolenic acid (yellow circle), 12-oxo-dodecenoic acid and 3-hexenal (blue circle), α and γ ketol (green circle), as well as JA which is converted into various derivatives, including MeJA (**8**). Biological functions were assigned to each of the indicated (Blee 2002, Howe and Schilmiller 2002, Liavonchanka and Feussner 2006).

9-LOX (**1**) and 13-LOX (**1**) catalyse the oxygenation of predominantly linolenic (Figure 5, 18:3) and linoleic (18:2, **LA**) acids into reactive hydroperoxides (**HPO**) (Figure 5). 9-LOX produce 9-HPOT (**2**) ((*S*)hydroperoxy-(10*E*,12*Z*,15*Z*)-octa-decatrienoic acid) and 13-LOX produce 13-HPOT (**2**) (see also Figure 5). 9-/13 **HPOT** are processed further by allene oxide synthase (**AOS, 3, 3**), hydroperoxide lyase (**HPL, 4, 4**), divinyl ether synthase (**DES, 5, 5**), peroxygenase and epoxy alcohol synthase (both not shown). AOS, HPL, and DES belong to the Cyp74 family.

Under normal environmental conditions the level of oxylipins is low, but this rapidly increases in response to mechanical wounding, herbivores, or pathogen attack, and other environmental or developmental inputs (Hughes, De Domenico et al. 2009), such as osmotic shock, drought or UV light (Howe and Schilmiller 2002).
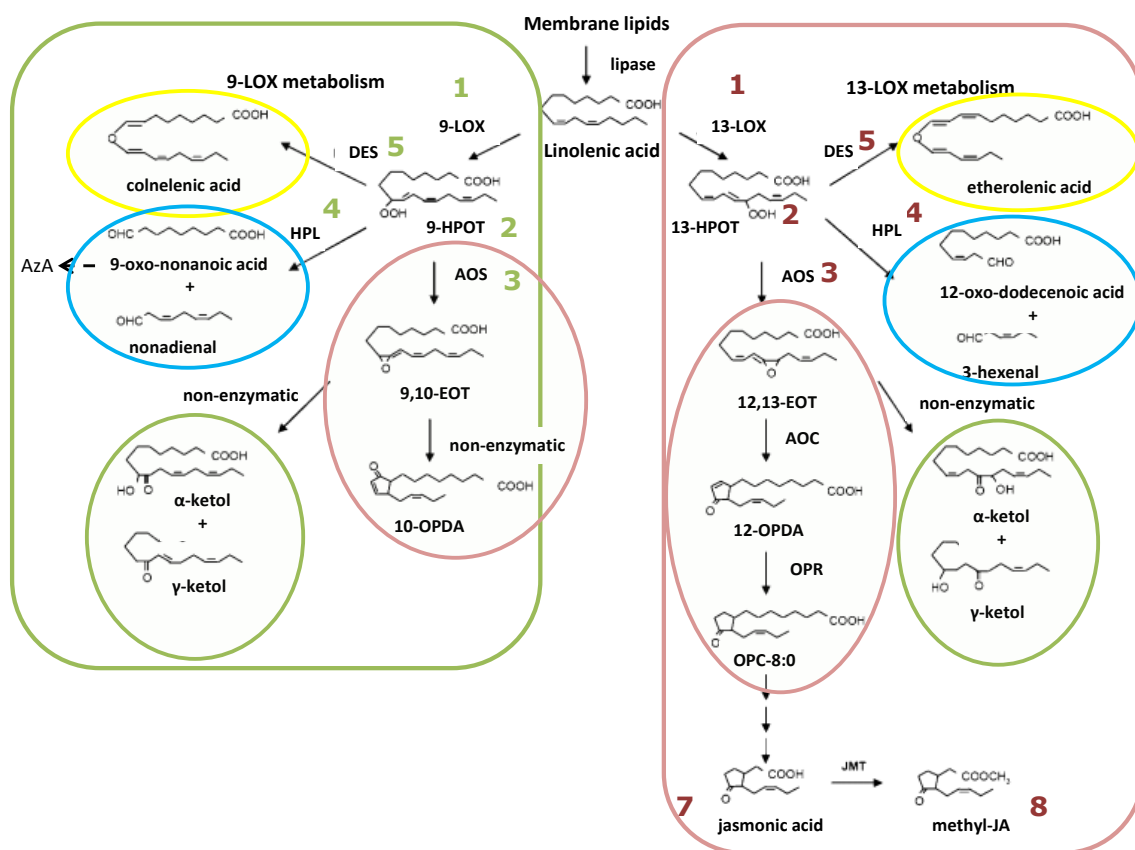
Figure 5: Metabolism of α−linolenic acid. LOX: lipoxygenase. HPOT: (*S*) hydroperoxy-(10*E*,12*Z*,15*Z*)-octa-decatrienoic acid. AOS: allene oxide synthase. HPL: hydroperoxide lyase. DES: divinyl ether synthase. OPR: oxophytodienoate reductase. AOC: allene oxide cyclase. OPDA: oxo-phytodienoic acid. EOT: epoxy octadecatrienoic acid. OPC: 8-[3-oxo-2-*cis*-{(*Z*)-pent-2-enyl} cyclopentyl] octa-noic acid (OPC-8:0). Illustration taken from (Hughes, De Domenico et al. 2009).

The following paragraphs will describe different enzymes/pathways in more detail starting from the different LOXs followed by members of the CYP74 enzyme family. Finally, the differences between the 9-LOX and 13-LOX localization will be discussed in more detail.

1.8 Plant lipoxygenases

LOXs are monomeric, non-heme containing and non-sulphur containing, but iron-containing dioxygenases widely expressed in fungi, animal and plant cells (Hughes, De Domenico et al. 2009). They are absent from prokaryotes and archaea, although a LOX-related genomic sequence was found in bacteria (Ivanov, Heydeck et al. 2010).

As mentioned above, starting with linolenic acid LOXs can produce 9-HPO<u>T</u> (oxidation at C9) or 13-HPO<u>T</u> (oxidation at C13) (Figure 6 A). Starting with linoleic acid, 9-LOX and 13-LOX can produce 9-HPO<u>D</u> (hydroperoxy-octadeca<u>di</u>enoic acid) or 13-HPO<u>D</u> (Figure 6

B) (Feussner and Wasternack 2002). This means that 9-LOX specifically form 9-hydroxyperoxy fatty acids, whereas 13-LOX pre-dominantly catalyses 13-hydroxyperoxy fatty acids (Huang and Schwab 2011). Some LOX, e.g. LOX2 of soybeans exert dual 9- and 13-lipoxygenase activity (Hughes, West et al. 2001, Fukushige, Wang et al. 2005).

A   linolenic acid (18:3)   **B**   linoleic acid (18:2)

9-LOX   13-LOX   9-LOX   13-LOX

9-HPOT   13-HPOT   9-HPOD   13-HPOD

…   …   …   …
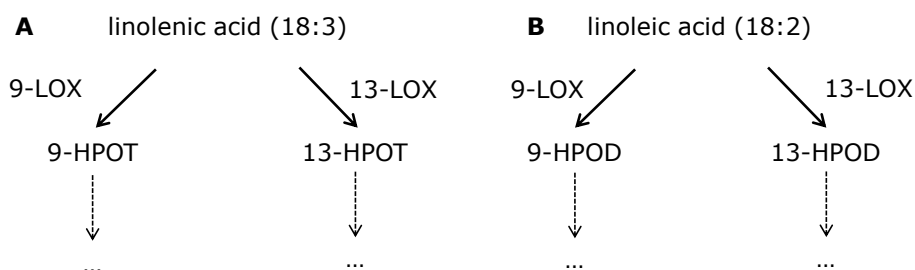
Figure 6: Two degradation pathway starting from linolenic acid (A) or linoleic acid (B). (A) 9-HPOT and 13-HPOT are synthesized through 9-LOX and 13-LOX from linolenic acid (18:3). (B) Starting from linoleic acid (18:2), 9-LOX or 13-LOX produce 9-HPOD or 13-HPOD. LOX: lipoxygenase, HPOT: hydroperoxy-oktadeca<u>t</u>rienoic, HPOD: hydroperoxy-octadeca<u>d</u>ienoic acid.

In *Arabidopsis* 6 different LOX (*At*LOX) proteins were identified (Bannenberg, Martinez et al. 2009). The *At*LOX-1 (*At*1g55020) and *At*LOX-5 (*At*3g22400) are 9-LOXs and oxygenate linolenic and linoleic acid (Bannenberg, Martinez et al. 2009). Both are involved in root development (Vellosillo, Martinez et al. 2007). *At*LOX-1 is involved in response to bacteria (Madoka Ayano 2010). *At*LOX1 is probably locates to the cytosol. Expression of the corresponding gene is activated in roots as well as in leaves upon leaf senescence, pathogen infection, and exposure to MeJA (Melan, Dong et al. 1993, Melan, Enriquez et al. 1994, He, Fukushige et al. 2002). Genetic studies identified a role of the pathogen-induced 9-LOX pathway in different plant species e.g. in tobacco after infection with *P. parasitica* var*. nicotianae* (Rance, Fournier et al. 1998), in *Arabidopsis* infected with *P. syringae* pv *tomato* (Vellosillo, Martinez et al. 2007, Vicente, Cascon et al. 2012), in potato infected with *P. infestans* (Gobel, Feussner et al. 2002), in almond infected with *A. carbonarius* (Mita, Fasano et al. 2007), and in pepper infected with *X. campestris* pv *vesicatoria* (Hwang and Hwang 2010).

*At*LOX2 (*At*3g45140), *At*LOX3 (*At*1g17420), *At*LOX4 (*At*1g67560), and *At*LOX6 (*At*1g72520) are 13-LOX (Bannenberg, Martinez et al. 2009). They display a selective oxygenation of linolenic acid. *At*LOX2 is localized in the chloroplast thylakoid and envelope membrane (Peltier, Ytterberg et al. 2004) and the corresponding gene is expressed in leaves and flowers (Bell, Creelman et al. 1995). Gene transcription is activated after exposure of plants to MeJA (Bell and Mullet 1993) and GLV (green leafy volatiles) (Kishimoto, Matsui et al. 2005) and is down-regulated during senescence (He, Fukushige et al. 2002). Upon wounding, *At*LOX2 is essential for JA formation (Bell, Creelman et al. 1995). The *AtLOX3* gene is expressed in roots, whereas gene

transcription of *AtLOX3* and *AtLOX4* in leaves is activated during senescence (He, Fukushige et al. 2002) as well as by jasmonates (Chung, Koo et al. 2008). In conclusion, less is known about the localization, activity as well as function of the Arabidopsis LOXs *At*LOX3, *At*LOX4, *At*LOX5, and *At*LOX6 (Bannenberg, Martinez et al. 2009).

The rice genome encodes 14 LOX proteins (Umate 2011). Four of the LOXs are annotated to contain a chloroplast precursor. Furthermore, all of the rice LOXs display high level protein similarity with the LOXs from *Arabidopsis*. To this end, LOXs with dual 9- and 13- LOX activity have been identified in potato and soybeans (Hughes, West et al. 2001, Fukushige, Wang et al. 2005).

1.9 CYP74 – key enzymes AOS, HPL, and DES

After the oxidation of linoleic and linolenic acid by 9-/13-LOX, three different branches can be followed to produce oxylipins or other fatty acid fragments. In this chapter, the key enzymes of these three branches will be described. Hydroperoxide lyase (**HPL**) will be described in most detail because of its relevance for this work.

The key enzymes that use 9-/13-HPOD and 9-/13-HPOT as substrates are **HPL**, **AOS** (allene oxide synthase), and **DES** (divinyl ether synthase), which belong to the cytochrome P450 (P450 or CYP) superfamily (Song and Brash 1991, Stumpe, Gobel et al. 2006). All P450 enzymes are thiolate-coordinated heme proteins and most of them function as monooxygenases (Guengerich 2002). CYP74 enzymes are not confined to plants, but also occur in bacteria and animals. Enzymes belonging to the CYP74 family have related protein primary structure and similar enzymatic functions. They share common substrate and reaction. From 9-/13-HPOD and 9-/13-HPOT epoxy allelic carbocation intermediate are chemically synthesized and are common substrates of AOS, HPL, and DES (Figure 7) (Kuroda, Oshima et al. 2005).

The AOS (Figure 5, **3**) branch of the 13-LOX pathway is involved in the synthesis of JA. AOS is known as CYP74A and converts 13-HPOT into an unstable allene oxide [(12,13-EOT (epoxy octadecatrienoic acid)]. This unstable oxide is in turn converted by AOC (allene oxide cyclase) into the JA precursor **12-OPDA** (12-oxo-phytodienoic acid) (Agrawal, Rakwal et al. 2002). All the enzymes required for 12-OPDA biosynthesis appear to be closely connected (Vidi, Kanwischer et al. 2006). After reduction of 12-OPDA and addition of three steps of β–oxidation, (+)-7-iso-JA is formed (Figure 5, red box) (Turner, Ellis et al. 2002). In the absence of AOC a non-enzymatic pathway (hydrolysis) leads to the accumulation of α- and γ-ketols instead of 12-OPDA (Figure 5) (Grechkin and Hamberg 2000).

In vitro studies indicated that the AOS branch (Figure 5, **3**) of the 9-LOX pathway also leads to the production of α- and γ-ketols or to the accumulation of the JA-like metabolite 10-OPDA. (Grechkin, Mukhtarova et al. 2000, Hamberg 2000). To our knowledge, the physiological significance of α- and γ-ketols and their quantity inplanta have not been examined to date (Stumpe, Gobel et al. 2006).

HPLs, also called CYP74B and C, produce aldehydes and oxo-acids related to defence. As illustrated in Figure 5 (green box, No. 4, blue circle) and Figure 7, HPL is needed to produce ONA (9-oxo nonanoic acid) and AzA (Kuroda, Oshima et al. 2005, Jung, Tschaplinski et al. 2009). HPLs are widely found in the plant kingdom and are well characterized in dicotyledonae as producing stress-inducible compounds (Feussner and Wasternack 2002). HPLs are grouped into two groups by their substrate specificities: 13-HPL (CYP74B) and 9-/13-HPL (CYP74C) (Figure 5 and Figure 7) (Kuroda, Oshima et al. 2005). 13-HPL preferentially cleaves 13-HPOD/T into C_6-aldehyes [hexenal, (3*Z*)-hexenal] and (9*Z*)-12-oxo-9-dodecenoic acid (Figure 7) (Matsui, Shibutani et al. 1996, Koeduka, Stumpe et al. 2003). The produced C_6-aldehydes are involved in plant signalling, defence, and production of aroma of fruits, vegetables, and green leaves (Gardner, Weisleder et al. 1991, Hatanaka 1993). 9-/13-HPL (CYP74C) can act on both 13- and 9-hydroperoxides, resulting in the formation of C_9-aldehydes [(3*Z*)-nonenal, (3*Z*,6*Z*)-nonadienal] and C_9-oxo-nonanoic acids (ONA, Figure 5, left side, blue circle) (Figure 7) (Matsui 2006). ONA can be converted into AzA which serve as a defensive compound. (3*Z*)-Hexenal, (3*Z*)-nonenal and (3*Z*,6*Z*)-nonadienal are enzymatically (CYB74B, C) or spontaneously isomerized into (2*E*)-hexenal and (2*E*)-nonadienal, respectively. Aldehydes produced by HPL have functions as defensive compounds against some herbivores (Vancanneyt, Sanz et al. 2001, Kessler, Halitschke et al. 2004).
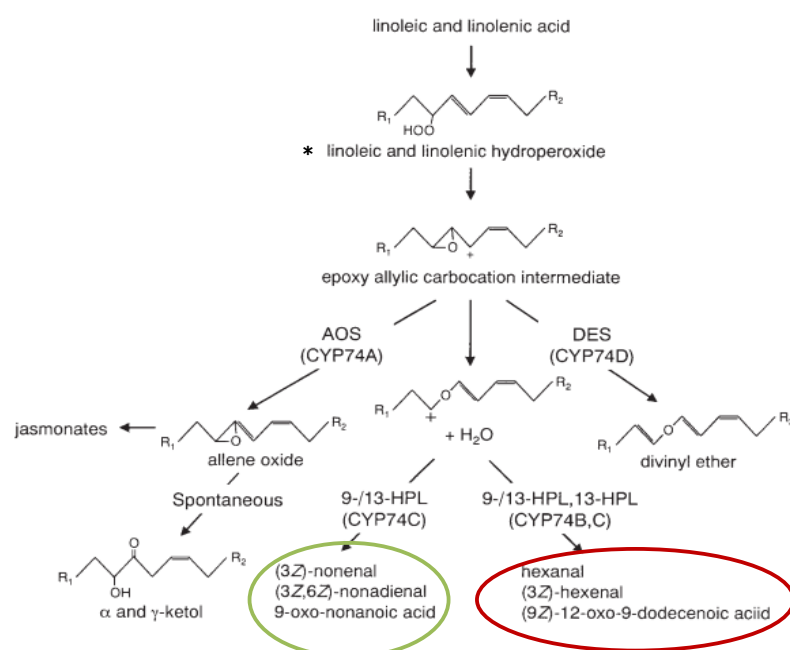
Figure 7: Reaction mechanism for the CYP74-mediated production of allene oxide, aldehydes and oxo-acids, as well as divinyl ether. The hydroperoxide (9-/13-HPOD/T, *) is converted into the epoxy allylic carbocation intermediate. From this intermediate AOS produces JA and α- and γ-ketol. DES produces divinyl ether. HPL catalyzes rearrangement into C_6-aldehydes (red circle) and C9-aldehydes (green circle). AOS: allene oxide, DES: divinyl ether synthase, HPL: hydroperoxide lyase, 13-HPL: 13-hydroperoxide lyase, 9-/13-hydroperoxide lyase, R_1 and R_2: represent 9-/13-HPOD/T. Illustration taken from (Kuroda, Oshima et al. 2005).

HPLs have been found in many plant species, including alfalfa, almond, barley, and rice (Noordermeer, Veldink et al. 1999, Koeduka, Stumpe et al. 2003, Kuroda, Kojima et al. 2005, Kuroda, Oshima et al. 2005, Mita, Quarta et al. 2005).

Interestingly, the presence of HPLs varies between the different ecotypes of *Arabidopsis*. In 2008, Kishimoto et al. reported a 9-HPL (*At*4g15440) activity against fungi in ecotype No-0 (Kishimoto, Matsui et al. 2008). In 2006 and 2008 two different groups presented that the *Arabidopsis* ecotype Col-0 lacks the 9-HPL gene, while the 13-HPL gene has been shown to be mutated and non-functional (Matsui 2006, Chehab, Kaspi et al. 2008). Therefore, Zoeller et al. concluded that Col-0 must use a non-enzymatic pathway to produce products of fatty acid fragments, including ONA and AzA (Zoeller, Stingl et al. 2012).

The rice (*Oryza sativa*) genome encodes three different HPLs (*Os*HPL1 to *Os*HPL3) that metabolize different hydroperoxides (Chehab, Raman et al. 2006). *Os*HPL1 and *Os*HPL2 are the first CYP74C enzymes found in monocots (Kuroda, Oshima et al. 2005). Tong et al. reported in 2012 that *Os*HPL3 is critical for herbivore-induced plant defence responses (Tong, Qi et al. 2012). It can act as a direct and indirect defence response by affecting the level of JA and GLVs against different invaders.

DES (Cyp74D) is the last key enzyme that converts LOX-derived fatty acid hydroperoxides into divinyl ether (**DVE**) fatty acids (Grechkin 2002). 9-HPOT is converted into colneleic and colnelenic acids (Figure 5, green box - yellow circle). Both are plant defence compounds (Weber, Chetelat et al. 1999, Itoh and Howe 2001) with antimicrobial activity against pathogenic bacteria, fungi, and oomycetes (Prost, Dhondt et al. 2005). Also, DVEs inhibit mycelial growth and especially spore germination of several *Phytophthorae* (Weber, Chetelat et al. 1999, Prost, Dhondt et al. 2005). 13-HPOT is converted into etheroleic acid and etherolenic acid (Figure 5, red box – yellow circle) (Grechkin, Fazliev et al. 1995, Hamberg, Ponce de Leon et al. 2002).

In tobacco (*Nicotiana tabacum*) *Nt*DES1 forms DVEs in response to pathogen attack. DES is thought to participate in local rather than in systemic defence responses, because expression of *Nt*DES1 is elicited in the immediate vicinity of infected tobacco sites with *P. parasitica* var. *nicotianae* (Fammartino, Cardinale et al. 2007).

1.10 13-LOX, 9-LOX metabolism–endocellular localisation

In general oxylipins are not pre-formed. They are synthesised in response to mechanical injury, herbivore attack, and other environmental inputs like touch response or drought (Howe and Schilmiller 2002). In response to wounding, for instance, ROS are activated that triggers LOXs (Bhattacharjee 2012). In addition, senescence triggers the activation LOX (Spiteller 2003). As discussed above, LOX oxidize free fatty acids in the cytosol or chloroplast and activate the oxylipin pathway including the jasmonate and HPL pathway (Mosblech, Feussner et al. 2009). In plants two different fatty acid fragmentation pathways takes place (Zoeller, Stingl et al. 2012). The enzymatic oxidative fragmentation pathway, described above, the non-enzymatic fragmentation pathway triggered by singlet oxygen ($^1O_2$) and other ROS (Morris, MacKerness et al. 2000, Mueller, Mene-Saffrane et al. 2006).

The key enzymes of the 9-LOX and 13-LOX pathway are located in different cell compartments. In the 13-LOX signalling pathway the enzymes LOX, AOS, and AOC, leading to OPDA synthesis, are located in the plastids (Bell, Creelman et al. 1995, Leon, Royo et al. 2002, Chen, Hackett et al. 2004). Subsequently, OPDA is transferred from the plastid to the peroxisome where the production of the active form of JA and MeJA takes place (Figure 8) (Feussner and Wasternack 2002, Liavonchanka and Feussner 2006, Wasternack 2007, Balbi and Devoto 2008).

There exists little information about the subcellular localisation of enzymes belonging to the 9-LOX pathway (Hughes, De Domenico et al. 2009). A cytosolic localisation appears to be clearly established for 9-LOX themselves (Figure 8) (Stumpe, Gobel et al. 2006, Fammartino, Cardinale et al. 2007). For the other enzymes, which are downstream in this pathway, other locations were described. Tobacco DES is located in the cytosol (Stumpe, Gobel et al. 2006, Fammartino, Cardinale et al. 2007). 9-AOS is detected in the amyloplast and leucoplast of cells from roots of potato. The protein 9-HPL is associated with lipid bodies when transiently expressed in tobacco protoplasts (Mita, Quarta et al. 2005). Also, *Medicago truncatula* 9/13-HPL is associated with lipid bodies with a cytosolic distribution (Heil and Kost 2006). A similar localisation was found for the cucumber 9-/13-HPL (Chehab, Raman et al. 2006).
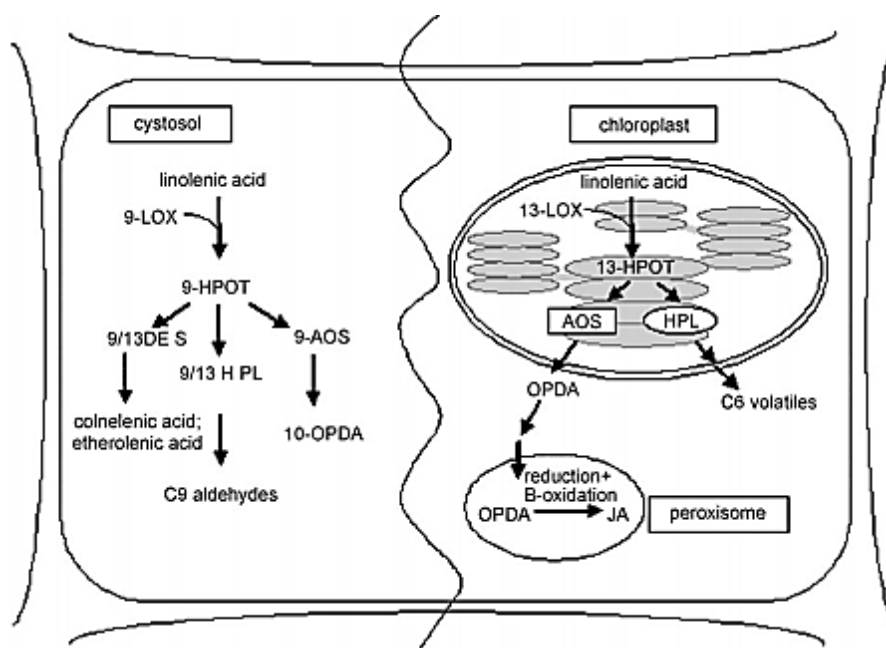
Figure 8: Schematic representation of endocellular compartmentation of 9- and 13-LOX metabolism. LOX: lipoxygenase, HPOT: (*S*)hydroperoxy-(10*E*,12*Z*,15*Z*)-octadecatrienoic acid, AOS: allene oxide synthase, HPL: hydroperoxide lyase, DES: divinyl ether synthase, OPR: oxo-phytodienoate reductase, OPDA: oxo-phytodienoic acid. Illustration taken from (Hughes, De Domenico et al. 2009).

1.11 Finding new signals in bacterially induced SAR

As introduced above, the *EDS1* gene is required for ETI downstream from TIR-NBS-LRR-type R proteins. By contrast, the same gene is not involved in ETI downstream from CC-NBS-LRR-type R proteins (Aarts, Metz et al. 1998). Nevertheless, SAR downstream from the CC-NBS-LRR protein RPM1 was abolished in *eds1* mutant Arabidopsis (Truman, Bennett et al. 2007). The *P. syringae* effector AvrRpm1 activates RPM1. Therefore, a comparison of the molecular response to AvrRpm1 of WT versus *eds1* mutant plants provides a unique opportunity to distinguish between local, ETI-related and true systemic signalling events during SAR. For a correct interpretation of the results of such approach it is essential to know if EDS1 affects SAR in the local, infected or in the systemic tissue. In experiments prior to this work, it was tested if EDS1 is required in the local, infected tissue for SAR signal generation and/or transmission or in the systemic tissue for signal perception and/or propagation. To this end, AvrPex were collected from *Arabidopsis* wild type (WT) and *eds1* mutant plants. Subsequently, WT and *eds1* mutant *Arabidopsis* plants were infiltrated with these AvrPex and *PR1* expression was analysed in the treated tissue. AvrPex from WT plants induced the expression of *PR1* in WT plants, but not in the *eds1* mutant (Jorda, Vlot, and Parker, unpublished). This indicates that EDS1 is required for SAR signal recognition or amplification. In addition, AvrPex from *eds1* mutants were incapable of inducing *PR1* gene expression in healthy WT *Arabidopsis*. These results give

evidence that EDS1 is required for SAR signal generation or transmission from the primary infected tissue (Jorda, Vlot, and Parker unpublished). Therefore, extracts from WT plants that are induced with AvrRpm1 are likely to contain SAR signals that are absent from similar extracts from the *eds1* mutant.

This work set out to identify SAR-related defects in the *eds1* mutant at the metabolite level by using a new experimental set up, in which a bacterial infection is not necessary. To this end, SAR is induced by the conditional over expression of the effector protein AvrRpm1 from a dexamethasone (DEX)-inducible transgene. The local HR is intact in WT and mutant, whereas the *eds1* mutant is not able to generate or emit SAR signals (Figure 9).
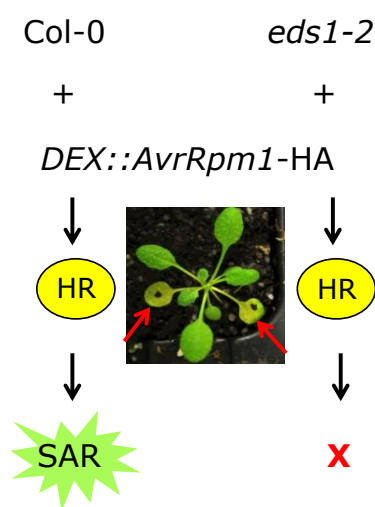
Figure 9: Experimental set-up. SAR is induced by the bacterial effector *AvrRpm1* expressed from a dexamethasone (DEX)-inducible transgene. Col-0 WT and *eds1* mutant *Arabidopsis* plants are capable of mounting an HR (red arrow), whereas only the WT is capable of mounting a SAR response.

1.12 Metabolomic approach

Metabolomics plays a key role in understanding the function of genes and the cellular system of an organism (Fiehn 2002, Bino, Hall et al. 2004, Oksman-Caldentey and Saito 2005, Hall 2006, Saito, Dixon et al. 2006, Schauer and Fernie 2006, Hagel and Facchini 2007). Understanding the metabolic pathways and networks of plants is more difficult as compared to animals. Plants for instance produce orders of magnitude more metabolites than animals.

Different approaches can be used to detect chemical compounds in plants, e.g. chromatography-mass spectrometry (Kirsch, Takamiya-Wik et al.), mass spectrometry (MS), nuclear magnetic resonance (NMR), or fourier transformed-infrared spectrometry (FT-IR) (Saito and Matsuda 2010). MS is usually preceded by metabolite separation using analytical methods including gas chromatography (GC), liquid chromatography (LC), and capillary electrophoresis (Krishnan, Kruger et al. 2005, Dettmer, Aronov et al. 2007, Eisenreich and Bacher 2007, Dunn 2008). Following MS, there are two popular platforms

to identify metabolites by their mass: ChemSpider (http://www.chemspider.com/) and PubChem (http://pubchem.ncbi.nlm.nih.gov/). Other interesting platforms are more dependent on the analytical method used and/or combine different databases and reveal pathways, e.g. the GC-MS-derived Golm Metabolome Database (GMD; http://gmd.mpimp-golm.mpg.de/; (Kopka, Schauer et al. 2005)) and MassTRIX (www.masstrix.org; (Suhre and Schmitt-Kopplin 2008)).

Different preparations of samples are necessary for a successful analysis of metabolites. Satisfactory results may be achieved under specific chromatographic conditions and after pre-purification steps (Guerard, Petriacq et al. 2011) of the plant extracts to avoid clogging of membranes or columns. Pre-purification may include separation of compounds, for example polar versus hydrophobic or positively versus negatively charged (Last, Jones et al. 2007). Pre-purification is also important to avoid excessive ion overlap and ion suppression of minor metabolites by major compounds (Novak, Tarkowski et al. 2003, Ek, Kartimo et al. 2006).

MS is mainly used for measurement of ion abundance and mass-to-charge ratios (m/z) after ionization of molecules (Last, Jones et al. 2007). MS can detect and resolve a broad range of metabolites with speed, sensitivity and accuracy. A small number of metabolites were identified but many remain unidentified (Villas-Boas, Mas et al. 2005, Glinski and Weckwerth 2006). New insights are gained through rapid improvements in mass spectrometry–based methods (Kirsch, Takamiya-Wik et al.) and computer hardware as well as software that is capable of interpreting large datasets.

1.13 Goals of this PhD work

The aim of this thesis was to identify new SAR signalling compound(s) by comparing the molecular profile of extracts from AvrRpm1-expressing WT *Arabidopsis* plants against similar extracts from mutant that are incapable of generating SAR signals (*eds1*). The first step was to narrow down the active compound(s) in MeOH extracts from induced WT vs *eds1-2* plants. Identification of metabolites will be done by using HPLC, FTICR-MS and LC-MS. Furthermore, it was tested if SAR induction is regulated by the identified compound/metabolite(s). New SAR signalling compounds should be integrated in the existing network of known defence signals. Therefore, a third step was to characterise the active compound(s) by elucidating its activity in existing signalling pathways (SA-pathway and JA-pathway) and by using different *Arabidopsis* mutants (*eds1*, *npr1*, *jar1,* and *sid2*). This work was done in collaboration with Prof. Dr. Wilfried Schwab and Dr. Thomas Hoffmann from the Technical University of Munich (TUM, Biotechnology of natural products) and Dr. Schmitt Kopplin from the Helmholtz Zentrum Muenchen.

2. Chapter – MATERIAL AND METHODS

2.1 MATERIAL

2.1.1 Plant material

Arabidopsis thaliana ecotype Col-0 (Columbia-0) was used in this study and is referred to as wild type (WT). The *eds1-2* mutant allele and the *DEX::AvrRpm1-HA* transgenic line were described (Bartsch, Gobbato et al. 2006) (Mackey, Holt et al. 2002). These two lines were crossed to yield *eds1-2 DEX::AvrRpm1-HA* (Dr. Corina Vlot-Schuster and Dr. Jane Parker, unpublished). The mutant lines *jar1* (Staswick, Yuen et al. 1998)(gift from Dr. Antonie Schaeffner), *med4-2* (Vlot, Liu et al. 2008), *npr1* (Cao, Glazebrook et al. 1997), and *sid2* (Wildermuth, Dewdney et al. 2001) were used.

2.1.2 Soil for *Arabidopsis*

The breeding soil (Floraton 1, Floragard, Oldenburg, Germany) was mixed with silica sand in a ratio of 5:1 and poured in 4-well pots. Soil was well watered and seeds were placed with a toothpick on the soil. Pots were covered with wrapping film and stored for 2 days at 4 °C (to synchronize germination) before being transferred into the plant growth chamber.

2.1.3 Bacterial strains

For different experiments the bacterial strains *P. syringae* pv *tomato* (DC3000, virulent strain) and DC300 carrying the bacterial effector AvrRpm1 (DC3000 *AvrRpm1*, avirulent strain) were used.

2.1.4 Chemicals

All chemicals were used in a high purity grade and purchased either from Sigma Aldrich GmbH (Taufkirchen, Germany, consecutively named Sigma), Carl Roth GmbH (Karlsruhe, Germany) or from other sources as indicated in the tables.

2.1.5 DNA-Polymerases

SensiMix™ SYBR Low-ROX Kit (Bioline, Luckenwalde, Germany) was used for qPCR analysis.

2.1.6 Enzymes used for cDNA-synthesis

The enzymes mentioned in Table 1 were used to produce cDNA from isolated plant RNA of *Arabidopsis*.

Table 1: Enzymes used for cDNA-synthesis

Name	Source
SuperScript™II Transcriptase	Invitrogen, Karlsruhe, Germany
SuperScript™III Transcriptase	Invitrogen, Karlsruhe, Germany
RNaseOut™	Invitrogen, Karlsruhe, Germany

2.1.7 Primers

The primers used in this study are outlined in Table 2. The primers were used at a concentration of 10 µM for qPCR. The identified fragment sizes of the products are specified for every primer pair. The internal reference or house-keeping gene *TUBULIN* (Truman, Bennett et al.) is referred to as *TUB.* The target genes quantified via qPCR against the *TUB* control are *Avr* (DC3000 effector *AvrRpm1*) as well as *PR1*, *LOX1,* and *LOX5*. All primers were used at the same annealing temperature of 60 °C.

Table 2: Primer used for qRT-PCR. Bp: base pairs, F: forward, R: reverse.

Acronym	Name	Sequ. 5´-3´	fragment size	Target gene
HB-65	*PR1* F	CTACGCAGAACAACTAAGAGGCAAC		*Arabidopsis PR1*
HB-66	*PR1* R	TTGGCACATCCGAGTCTCACTG	220 bp	
HB-69	*TUB* F	GTACCTTGAAGCTTGCTAATCCTA		*Arabidopsis Tubulin*
HB-70	*TUB* R	GTCAAAGGTGCAAAACCAAC	186 bp	
FW-1	*qAvr* F	CGAACTCAGCCCCTACAGAC		*DC3000 AvrRpm1*
FW-2	*qAvr* R	GTCGTTCTGCAGCTGAATTG	217 bp	
FW-3	*qLOX1* F	GGCTCTGGAGAAGGAGAGGT		*Arabidopsis Lipoxygenase 1*
FW-4	*qLOX1* R	GACACCTTCACCAGGCGTAT	226 bp	

FW-5	*qLOX5* F	AACCGTCCAAACGGATACAG		*Arabidopsis*
FW-6	*qLOX5* R	GGACGGTTAGGGAGAAAACC	213 bp	*Lipoxygenase*
				5

2.1.8 Antibiotics

Table 3 shows the different antibiotics used in selective media with their final working concentrations.

Table 3: Antibiotics used and their working concentration

Name	Working concentration [µg/ml]	Source
Kanamycin	50	Roth, Karslruhe, Germany
Rifampicin	50	Duchefa Biochemie, Germany

Kanamycin was dissolved in water (ddest. water) and rifampicin was dissolved in 100% Dimethylsulfoxide (DMSO). Stock solutions (1000x working concentration) were sterilized through 0.22 µm sterile filters (Millipore, Billerica, MA, United States), aliquoted, and stored at -20 °C.

2.1.9 Media and solutions

Table 4 outlines the buffer compositions and the chemicals required for phenol-chloroform-based RNA extraction (Logemann, Schell et al. 1987). Tables 5 until 9 outline media, buffers and solutions used for various other applications as indicated in the titles of the Tables in bold.

Table 4: Solutions used for plant RNA extraction

Name	Composition	Source
TriReagent, per 100ml (adjusted to pH 5.0 before adding phenol)	3.05 g Ammoniumrhodanide (Ammoniumthiocyanate)	Roth, Karlsruhe, Germany
	9.44 g Guanidinthiocyanat	Merck, Darmstadt, Germany
	5 ml Glycerol	Roth, Karlsruhe, Germany
	3 M Na-Acetate pH 5.2	Merck, Darmstadt, Germany
	40 ml H_2O	
Solution used	38 ml Phenol	Roth, Karlsruhe, Germany
	Chloroform	Merck, Darmstadt, Germany
	2-Propanol	Roth, Karlsruhe, Germany
	Ethanol	Merck, Darmstadt, Germany

Table 5: Media composition for bacteria cultivation

Name	Composition	Source
NYGA, per liter, pH 7.0	5 g Bacto-proteose Peptone	Roth, Karlsruhe, Germany
	3 g yeast extract	Roth, Karlsruhe, Germany
	20 ml Glycerol	Roth, Karlsruhe, Germany

For agar plates 18 g of Agar-Agar (Merck, Darmstadt, Germany) was added to the media after pH adjustment. Media, with our without Agar-Agar, were autoclaved for 20 minutes at 120 °C. After autoclaving, the media were cooled down to ca. 55 °C for addition of selective antibiotics (Table 3).

Table 6: Composition of apoplast buffer

Name/Application	Composition (per liter)	Source
Apo-Buffer	2.5 mM Tris; pH 7.4	Roth, Karlsruhe, Germany
	1 mM EDTA	Sigma, Taufkirchen, Germany
	30 mM MgCl$_2$	Roth, Karlsruhe, Germany

The apoplast buffer was autoclaved.

Table 7: Different chemicals used for metabolite isolation

Name/Application	Working concentration	Source
Processing of	30 µM Dexamethasone	Sigma, Taufkirchen, Germany
DEX::AvrRPM1-HA plants for	0.01% Tween-20	Bio Rad, Munich, Germany
metabolite isolation	100% Methanol	Merck, Darmstadt, Germany
	100% Petroleumether	Merck, Darmstadt, Germany
	100% Dimethyl ether	Merck, Darmstadt, Germany

Filter	Application	Source
Bond Elute – C18, 100MG, 1M	Concentrating of petroleum-ether phase prior to HPCL	Agilent Technologies, Oberhaching, Germany

A 30 mM DEX stock solution (in 100% Ethanol) was filter-sterilized (see section 2.1.8), aliquoted, and stored at -20 °C. For promoter activation of DEX-plants, DEX was diluted to a concentration of 30 µM in 0.01% Tween20.

Table 8: Chemicals used to perform HPLC and LC-MS

Name/Application	Working concentration	Source
Processing for HPLC and LC-MS	100% Methanol	Roth, Karlsruhe, Germany
	100% Acetonitrile	J.T. Baker, Deventer, The Netherland
	100% formic acid	Roth, Karlsruhe, Germany

Table 9: Solutions used in SAR experiments

Name	Composition	Source
MOCK solution, pH 7.0	10mM $MgCl_2$	Merck, Darmstadt, Germany
Bacteria isolation solution	10mM $MgCl_2$	Merck, Darmstadt, Germany
	0.01% Silwet	Lehle Seeds, Texas, USA
Azelaic acid	Stock: 1 M in 100% Ethanol	Sigma, Taufkirchen, Germany
9-oxononanoic acid	Stock: 0.1 M in bidest. H_2O	Chiron AS, Trondheim, Norway
D-(+)-Neopterin	Stock: 1 M in bidest. H_2O	Sigma, Taufkirchen, Germany
Seduheptulose	Stock: 1 M in bidest. H_2O	Sigma, Taufkirchen, Germany
7,8-Dihydroneopterin	Stock: 1 M in bidest. H_2O	Sigma, Taufkirchen, Germany

Azelaic acid was dissolved in 5 mM MES (2-(*N*-morpholino) ethansulfonic acid, Roth, Karlsruhe, Germany, pH 5.6). All the other chemicals were dissolved in water (see section 2.2.16).

2.2 METHODS

2.2.1 Plant growth conditions

Arabidopsis plants were grown in plant growth chambers with 10 hour light 14 hour dark cycles at 70% relative humidity and 22 °C. The light intensity during the 'day' was kept at 100 µE m^{-2} sec^{-1}.

2.2.2 Seed storage

Seeds were harvested and stored in a paper bag, which was permeable to air. Seeds were stored in a dark and dry place at room temperature.

2.2.3 Bacterial strains and culture conditions

DC3000 or DC3000 carrying AvrRpm1 were maintained on NYGA medium. Selective antibiotics were rifampicin and kanamycin (both at 50 µg/ml). Bacteria were grown at 28 °C (MMM-Friocell 111, Munich, Germany).

2.2.4 Leaf sample preparation for gene expression analysis

Leaves of 5-week-old plants (Col-0 *Dex::AvrRpm1* and *eds1-2 Dex::AvrRpm1*) were treated with 0.01% Tween-20 or with 1µM DEX solved in 0.01% Tween-20. Treatments were performed with a painting brush. Treated and systemic (untreated) tissue was harvested before and 2, 4, 8, 24, 48 and 72 hours after treatment. Harvested material was frozen in liquid nitrogen and kept at -80 °C until RNA was isolated as described below.

2.2.5 Phenol-RNA extraction and cDNA-synthesis

Frozen plant material was ground under liquid nitrogen and RNA was isolated by the phenol-extraction method (Logemann, Schell et al. 1987). Quality and concentration of the RNA samples were determined by measuring the absorption at 260 nm and 280 nm

using the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). An absorption of 1.0 at 260 nm equals a concentration of 40 µg/ml of RNA. The A260/A280 ratio was used to assess the purity of total RNA and to detect the presence of protein, phenolics or other contaminants that absorb at or near 280nm. A ratio of approximately 1.8 to 2.0 is generally accepted for pure RNA. The A260/A230 ratio is a second purity measure, which should commonly be in the range of 2.0-2.2. An appreciably lower ratio may indicate the presence of contaminants absorbing at 230 nm. RNA integrity was analysed by using 1% agarose gel electrophoresis.

Synthesis of cDNA was performed by using Superscript^{TM}II or Superscript^{TM}III, Reverse Transcriptase following the manufacturer's instructions (Invitrogen, California, USA).

2.2.6 qRT-PCR (quantitative real-time-PCR) analysis

qRT-PCR is a form of PCR in which data are collected in real-time as the reaction proceeds. Continuous data collection enables the most important application of real-time PCR, target quantification. cDNA was used as a template, in order to quantify the expression of genes-of-interest. Expression of genes was normalized against the reference or house-keeping genes *TUB* (Table 2). To conduct qRT-PCR, the 7500 Real Time PCR System from Applied Biosystems (Darmstadt, Germany) and SYBR Green mixes (2.1.5) were used following the manufacturer´s instructions.

2.2.7 Apoplast extraction

Col-0 *DEX::AvrRpm1-HA* and *eds1-2 DEX::AvrRpm1-HA* plants were sown as a lawn. Four-week-old plants were sprayed until drop-off with 30 µM dexamethasone (DEX, Sigma, Taufkirchen, Germany) in 0.01% Tween-20. 4.5 hours later all above-ground tissue was harvested by cutting the plants right above the soil. The apoplast was extracted as follows. APO-buffer was infiltrated into the tissue by applying and carefully releasing a mild vacuum. The vacuum was kept for 10 minutes and afterwards slowly released. The vacuum-infiltrated tissue was collected in 30 ml syringes and buffer (with apoplast compounds) was spun out of the tissue in 50 ml tubes (900 x g, for 20 min at 4°C). The resulting apoplast extracts were concentrated and purified by solid phase extraction (Laudert, Pfannschmidt et al.): C18 columns were loaded with the samples and eluted five times with methanol (MeOH). The eluates were dried by evaporation and dissolved in 300 µl of 100% DMSO. To this end, 2 µl of the fractions in 100% DMSO were diluted in 998 µl water and tested in the SAR bio-assay (see 2.2.17 for details). In

parallel, the eluates from the C18 column were dried by evaporation dissolved in 100% MeOH and handed to Dr. Thomas Hoffmann (TUM, in collaboration with Prof. Dr. Wilfried Schwab, Biotechnology of Natural Products) to perform analytical LC-MS (see 2.2.13).

2.2.8 Collection of MeOH extracts

Three-week-old plants (Col-0 *Dex::AvrRpm1*-HA and *eds1-2 Dex::AvrRmp1*-HA) were sprayed with 30 µM DEX in 0.01% Tween-20 to induce expression of *AvrRpm1*. After 4.5 hours plants were harvested (all above-ground parts) and powdered with a mortar and pestle in liquid nitrogen. 1 ml of 100% MeOH was added per 100 mg of grained material. Sample were mixed, and incubated for 1 h in the dark while rotating at 28 rpm at room temperature. Subsequently, samples were centrifuged at 4000 rpm at 4 °C and for 10 min. The clear and green supernatants were transferred to falcon tubes. The samples were dried by evaporation and dissolved in 100% DMSO. To this end, 2 µl of the fractions in 100% DMSO were diluted in 998 µl water and tested in the SAR bio-assay (see 2.2.17 for details).

2.2.9   Separation of MeOH extracts into different phases

Per MeOH extract, thirty ml (equals 3 g of ground plant material, see 2.2.8) was dried by evaporation and the pellet was dissolved in a mixture of MeOH and water (v/v 1:9). An equal volume of petroleum ether (PE) was added and properly shaken. After the separation of two phases the lower one was isolated and transferred into a new flask. The remaining upper PE-phase was dried by evaporation and the pellet was dissolved in 300 µl of 100% DMSO. The isolated (lower) phase was mixed with the same volume of dimethyl ether (DME) and the resulting lower phase was again transferred to a new flask. Both phases were dried by evaporation and the pellet of the ether-phase was dissolved in 300 µl of 100% DMSO. Additionally, the water phase was evaporated to get rid of possible PE and DME contaminations. To this end, 2 µl of the fractions in 100% DMSO (PE and DME) were diluted in 998 µl water and tested in the SAR bio-assay (see 2.2.17 for details).

2.2.10 Dividing Petrolether phase by using different MeOH concentrations

100 µl of PE-phase in DMSO (2.2.9) was taken and dissolved in a mixture of MeOH and water (v/v 1:1). Subsequently, 1 ml of each mixed sample (WT and *eds1* mutant) was loaded onto a C18 column to concentrate the metabolites. The columns were consecutively washed with 5 ml of 25%, 50%, 75% and 100% of MeOH followed by an additional wash with PE to elute all remaining metabolites. Each flow through was separately collected and dried by evaporation. Each sample was dissolved in 300 µl of 100% DMSO. To this end, 2 µl of the fractions in 100% DMSO were diluted in 998 µl water and tested in the SAR bio-assay (see 2.2.17 for details).

2.2.11 Sample preparation for preparative HPLC

The samples of 75%, 100% MeOH and the additional wash with PE were pooled (PE-phase). This process was performed for 5, 10 or 20 different PE-phases (independent biological replicates) each of WT and *eds1* mutant. The pooled samples were dried by evaporation and handed to Dr. Thomas Hoffmann (TUM) to perform HPLC. For the following experiment dried pellets were dissolved in 100% of MeOH:

5 samples = 3 ml; 10 samples = 6 ml; 20 samples = 12 ml

Optionally, a short ultrasound pulse was used to make sure all dry matter was dissolved. Per experiment, the processed samples were centrifuged at the maximum speed (depending on the rotor) for 15 min at 4 °C. The supernatant was transferred into a new tube. Subsequently, the whole volume was fractionated by HPLC as described in 2.2.12 by using a MeOH-gradient. Additionally, the UV absorption signal of the HPLC eluate (chromatogram) was recorded at 370 nm. Fractionated samples were used to test for SAR-inducing activity in *Arabidopsis* WT and *eds1-2* mutants as described in 2.2.17. Bio-active (SAR-inducing) fractions were correlated with the UV absorption pattern of the HPLC chromatogram.

2.2.12 Preparative HPLC

HPLC was performed in collaboration with Dr. Thomas Hoffmann in the laboratory of Prof. Dr. Wilfried Schwab (TUM). The Jasco HPLC system (Jasco GmbH, Groß-Umstadt, Germany) consisted of two Jasco PU-2087 Plus pumps connected to a Jasco UV-2075 Plus variable wavelength detector set at 260 nm, an Advantec CHF122SC fraction collector (Tokyo Seisakusho Kaisha Ldt., Japan) and an Agilent LC/MSD Trap XCT mass spectrometer (Agilent, Waldbronn, Germany). The (HP)LC column was a Synergi 4u

Fusion-RP 80, 25 cm x 21.5 mm (Phenomenex, Aschaffenburg, Germany). The HPLC solvents were 0.1 % formic acid in water (A), 0.1% formic acid in methanol (B) and 0.1 % formic acid in acetonitrile (C). For separation of compounds dissolved in 100% MeOH a gradient was used from 100% A to 100% B and back to 100% A as described below (MeOH-gradient). The injection volume was 950 µl per sample. A flow rate of 10 mL/min was used. About 2% of the eluting volume was diverted to the mass spectrometer. In parallel 9.3 ml fractions were collected at one fraction per minute from 5 to 45 minutes (fraction 5-45). For MS analysis, ionization parameters were as follows: the voltage of the capillary was -4000 V and the end plate was set to −500 V. The temperature of the dry gas (N_2) was 330 °C at a flow of 9 L/min. The full scan mass spectra of the metabolites were measured from *m/z* 50 to 800 until the ICC target reached 30000 or 200 ms, whichever was reached first. Tandem mass spectrometry was performed using helium as the collision gas, and the collision energy was set at 1.0 V. Mass spectra were acquired in negative and positive ionization mode. Data analysis was performed using the Jasco ChromPass Version 1.9.302.1124 software (Jasco GmbH, Groß-Umstadt, Germany) and the 6300 Series Trap Control Version 6.2 software (Bruker Daltonics GmbH, Bremen, Germany).

Optionally, fractions from the MeOH-gradient were dried by evaporation and solved in 100% of MeOH. Subsequently, fractions were further fractionated across an acetonitrile (ACN) gradient as described below. Injection volume, flow rate, fractionation and MS were performed as outlined for the MeOH gradient.

MeOH-gradient (A: H_2O + 0.1% HCOOH; B: MeOH + 0.1% HCOOH):
0 – 2 min   100% A
2 – 30 min   0 – 100% B (gradient)
30 – 35 min   100% B
35 – 60 min   100% - 0 A (gradient)

ACN-gradient (A: H_2O + 0.1% HCOOH; C: Acetonitrile + 0.1% HCOOH):
0 – 2 min   50% A
2 – 30 min   50 – 100% C (gradient)
30 – 35 min   100% C
35 – 60 min   50% - 0 C (gradient)

Fractions from the MeOH-gradient and ACN-gradient were dried by evaporation, dissolved in 100% DMSO. To this end, 2 µl of the fractions in 100% DMSO were diluted in 998 µl water and tested in the SAR bio-assay (2.2.17).

2.2.13 Analytical LC-MS

Analytical LC-MS was performed with samples from section 2.2.7 and 2.2.12.

A Bruker Daltonics esquire 3000^{plus} ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) connected to an Agilent 1100 HPLC system (Agilent Technologies) equipped with a quaternary pump and a diode array detector was utilized for quantification. Components were separated with a Phenomenex Luna C-18 column (150 mm long × 2.0 mm, particle size 5 μm; Phenomenex, Aschaffenburg, Germany) that was held at 28 °C. Injection volume was 5 µl. The full-scan mass spectra were measured in a scan range from 50 to 800 *m/z* with a scan resolution of 13,000 *m/z*/s until the ICC target reached 20,000 or 200 ms, whichever was achieved first. The ionization parameters were as follows: the voltage of the capillary was 4000 V and the end plate was set to -500 V. The capillary exit was 121 V and the Octopole RF amplitude 150 Vpp. The temperature of the dry gas (N$_2$) was 330 °C at a flow of 9 l min^{-1}. Tandem MS was carried out using helium as the collision gas (3.56 × 10^{-6} mbar) with 1-V collision voltage. Spectra were acquired in the positive and negative ionization mode. Auto-tandem mass spectrometry was used to break down the most abundant [M+H]$^+$, [M-H]$^-$, or [M+HCOO]$^-$ ions of the different compounds. Data analysis was performed using the DataAnalysis 3.1 software (Bruker Daltonics).

2.2.14 Fourier transform ion cyclotron resonance-MS (FTICR-MS)

This method was applied and evaluated in cooperation with the group of Dr. Schmitt-Kopplin at the Helmholtz Zentrum Muenchen. Masses were analysed in the negative ionization mode with a maximum error of 2.0 ppm (parts per million). The maximum error is given as a relative measure in units of ppm (Huber, Batchelor et al.). The maximum error between the measured mass and the nominal mass depends on the instrument you are using. It also controls the number of false positive calls (increases with larger max. error) and the number of false negative calls (increases with smaller max. error) (http://metabolomics.helmholtz- muenchen.de/masstrix2/doc. html#max_error).

2.2.15 Assessment of SAR in *Arabidopsis*

To perform SAR experiments fresh bacterial strains were used. Bacteria were grown O/N on NYGA medium (Table 5) with antibiotics (Table 3). On the day of the inoculation cells were removed from the plates and resuspended in 10 mM $MgCl_2$, pH 7. DC3000 *AvrRpm1* was diluted to yield a suspension containing 1 x 10^6 colony forming units (CFU/ml). The bacterial concentration was reached by dilution of bacteria in 10 mM $MgCl_2$, pH 7. The optical density of the undiluted suspension was measured at a wavelength of 600 nm by using a photometer (Ultrospec 3100 pro, GE Healthcare, Munich, Germany). The required dilution was calculated by assuming the suspension held 10^8 CFU/ml of bacteria when OD600 = 0.2.

The first two true leaves (Figure 10, red arrows) of 5-week-old *Arabidopsis* plants were infiltrated with 1 x 10^6 CFU/ml of DC3000 *AvrRpm1* or with 10 mM $MgCl_2$ (MOCK) as a control. Infiltration was performed from the abaxial side of the leaves by using a syringe without needle. Alternatively, SAR was induced in plants carrying a *DEX::AvrRpm1-HA* transgene by brushing the first two true leaves per plant with 1 µM dexamethasone (DEX) solved in 0.01% Tween-20 or with 0.01% Tween-20 as a control.

Three days after the primary treatment, the next two "upper" or systemic (2°) leaves (Figure 10, blue arrows) were infiltrated with 1 x 10^5 CFU/ml of DC3000. After another four days, systemic leaves were harvested and growth of bacteria was analysed.

Figure 10: SAR induction. Four genotypes were used: Col-0, *eds1-2*, Col-0 *Dex::AvrRpm1-HA* and *eds1-2 Dex::AvrRpm1-HA*. 1° leaves (red arrows) for the first two genotypes were infiltrated with DC300 *AvrRpm1* or with 10 mM $MgCl_2$ as a control. The other two genotypes were treated with a brush with 1 µM dexamethasone or with 0.01% Tween-20 as a control. After 3 days 2° leaves (systemic, blue arrows) were infiltrated with DC3000. After another 4 days the 2° infected leaves were harvested and bacteria growth was analysed.

For analysis of pathogen growth in the systemic leaves, bacteria were extracted from three 6 mm leaf discs per sample (in triplicate per genotype and 1° treatment). The discs were shaken at 600 rpm in 500 µL of 10 mM $MgCl_2$ with 0.01% Silwet for 1 hour at 25 °C. The resulting bacterial suspension was diluted in 10 mM $MgCl_2$ in five serial 10x steps. Subsequently, 20 µl of each dilution was pipetted (or spotted) onto NYGA plates with antibiotics and grown for two days at 28 °C. Colonies were counted in spots containing between 10 and 100 colonies. Subsequently, the bacterial titer in the leaf was calculated as CFU/cm^2.

2.2.16 Assessment of SAR induced by different metabolites and metabolite fractions from *Arabidopsis*

The first two true leaves of 5-week-old *Arabidopsis* WT and mutant plants were treated either with DC3000 *AvrRpm1*, MOCK, 5 mM MES, solvent (water treated with use chemicals: PE, MeOH, ACN), and 0.2% DMSO. Furthermore, all collected fractions were dried by evaporation, dissolved in 100% DMSO, and dissolved to 0.2% DMSO. 100 µM, 500 µM, 1 mM, 5 mM, and 10 mM AzA (Azelaic acid) were dissolved in 5 mM MES because 5 mM MES was the negative control of AzA. 0.25 mM and 0.5 mM of ONA (9-oxo-nonanoic acid) were solved in water. 0.5 mM and 1 mM of D-(+)-neopterin were solved in water. To this end, 1 mM seduheptulose, and 7,8-dihydroneopterin were solved in water. After first inoculation (with different chemicals, fractions as mentioned above) systemic leaves were infected as described in 2.2.15 and the growth of the bacteria was analysed (see 2.2.15).

3. Chapter – RESULTS

3.1 Overview

In previous work 2D gel analysis was used to compare apoplast extracts from WT and *eds1* mutant plants expressing the bacterial effector AvrRpm1 from a DEX-inducible transgene. Different gel spots were identified that accumulate in the apoplast of *Arabidopsis* in an *EDS1*-dependent manner (Vlot, Parker conducted the analysis at the Max Planck Institute for Plant Breeding Research, Cologne, Germany, unpublished). By using mass spectrometry seven apoplastic proteins were identified. The seven corresponding genes were named *AtAED1-7* (*Arabidopsis thaliana APOPLASTIC EDS1-DEPENDENT 1-7*). In this thesis, experiments were conducted to analyse if metabolites can be found in apoplast extracts that differentially accumulate in WT as compared to *eds1* mutant plants.

First, it was proven that the experimental set-up using DEX-inducible expression of AvrRpm1 to trigger SAR results in a "real" SAR response that is not due to DEX movement. The metabolic patterns of apoplast extracts from *AvrRpm1*-expressing WT and *eds1-2* mutant were then analysed by using LC-MS (in cooperation with Prof. Dr. Wilfried Schwab and Dr. Thomas Hoffmann from the Technical University of Munich (TUM). We found one mass peak that was highly present in WT and not in mutant plants. Therefore, the method was changed from apoplast extraction to MeOH extraction from WT and mutant plants. In MeOH extracts, more metabolites were discovered that were highly present in *Arabidopsis* WT, but not *eds1-2* mutant plants. Subsequently, a new approach was established and further improved to narrow down the active compounds able to induce SAR in the WT plants. By using HPLC, FTICR-MS, and LC-MS SAR-inducing metabolites were identified. HPOD (hydroperoxy-octadecadienoic acid), ONA, AzA, and neopterin induced systemic immunity in WT plants. ONA, AzA and neopterin were also tested in SA-deficient and JA-insensitive *Arabidopsis* mutants to characterize if the corresponding defence signalling pathway are involved in SAR triggered by these metabolites.

3.2 SAR induction by *AvrRpm1* expression in dexamethasone-treated plants

SAR can be induced by infection of plants with a pathogen. We implemented an alternative approach to induce SAR by activating a dexamethasone-inducible transgene encoding the bacterial effector *AvrRpm1*. AvrRpm1 induces ETI in *Arabidopsis*, but we had yet to prove that this in turn induces true SAR.

Biological SAR was induced by a local infection of Col-0 and *eds1-2* with DC3000 *AvrRpm1* in 10 mM MgCl$_2$. MOCK (10 mM MgCl$_2$ without bacteria) acted as control. Secondary treatment followed three days after the first inoculation in the systemic tissue and was done with DC3000. After another four days bacteria were isolated out of systemic, treated tissue and diluted to determine growth.

DEX-induced SAR was triggered by a primary treatment of Col-0 *DEX::AvrRpm1-HA* (Col-0 *DEX*) and *eds1-2 DEX::AvrRpm1-HA* (*eds1-2 DEX*) with 1 µM dexamethasone (1 µM DEX) solved in 0.01% Tween-20. Tween-20 acted as control (Tween). Secondary treatment followed three days later with DC3000 in the systemic tissue. As in the first SAR-experiment described above, analysis of bacteria in the systemic tissue followed four days after the secondary infection.

After a primary treatment with one of the control solutions (MOCK, Tween) the treated leaves looked green and healthy (Figure 11 A, arrow). By contrast, leaves treated with DC3000 *AvrRpm1* (Figure 11 B, arrow) or with DEX displayed yellow-spotted lesions (Figure 11 C, arrow).
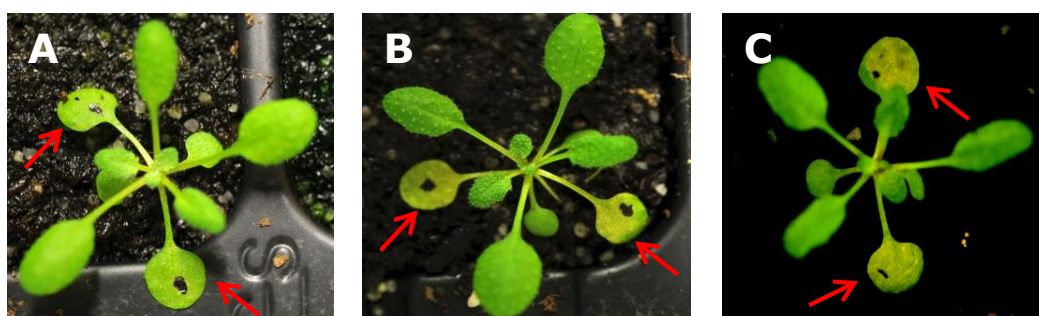
Figure 11: 1° treatment of WT and *eds1-2* mutant *Arabidopsi*s plants. (A) MOCK and Tween treated plants (exemplarily MOCK-treated WT). (B) Local treatment with bacteria in WT plants. (C) Treatment with dexamethasone for DEX-induced SAR in Col-0 *DEX::AvrRpm1-HA*. Comparable observations were made in *eds1-2* mutant and *eds1-2 DEX::AvrRpm1-HA* plants. Pictures exemplarily show WT (A and B) and Col-0 *DEX::AvrRpm1-HA* (C) plants.

After 2° inoculation with DC3000, the systemically infected leaves of uninduced plants (MOCK and Tween, 1° treatment) looked yellow spotted. In comparison, the systemically infected tissue of induced plants (DC3000 *AvrRpm1* or 1 µM DEX, 1° treatment) looked more or less green. Isolation and quantification of bacteria in the 2° infiltrated tissue indicated a SAR induction in WT plants in response to 1° bacterial infection (Figure 12 A). The mutant did not support SAR as indicated by similar bacterial growth in MOCK or DC3000 *AvrRpm1* pre-treated plants. Figure 12 B shows a significant DEX-induced SAR in WT plants in response to 1° DEX treatment. By contrast, there is no detectable DEX-induced SAR in the *eds1-2 DEX* mutant (Figure 12 B).
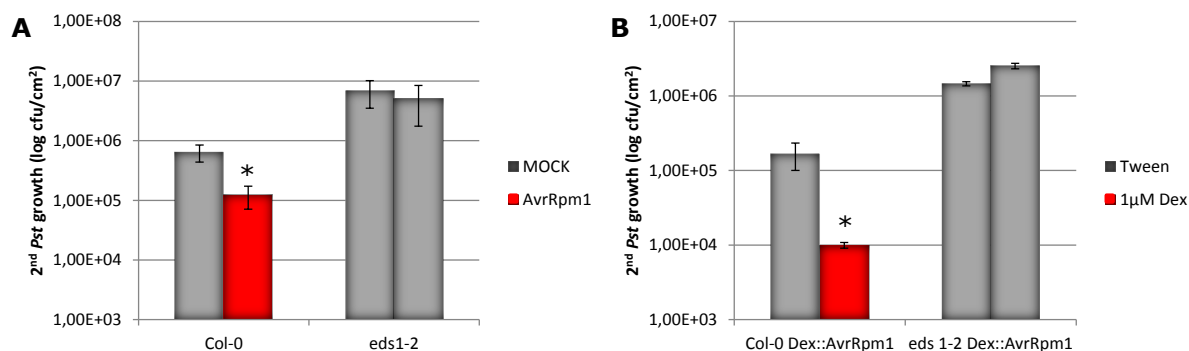
Figure 12: Analysis of bacteria growth in the systemic tissue after 2° infection. (A) 2° DC3000 growth in uninduced (MOCK-treated) plants and in bacterially induced (DC3000 *AvrRpm1*) WT and *eds1-2* mutant plants. (B) 2° DC3000 growth in Tween-treated and DEX-treated Col-0 *DEX::AvrRpm1-HA* and *eds1-2 DEX::AvrRpm1-HA* plants. Asterisks above of bars indicate statistically significant differences as compared to control treatment (* P < 0.05, Student´s t test). Experiments were repeated three times with comparable results.

When WT and *eds1-2* mutant plants were treated locally by brushing with the 0.01% Tween-20 control as well as with 0.1 mM DEX and 1 µM DEX, each solved in 0.01% Tween-20, no SAR was detectable as compared to treatment with DC3000 *AvrRpm1* (Supplement Figure 39). This indicates that different concentrations of DEX solved in 0.01% Tween-20 and the solvent itself do not have SAR-inducing activity in the WT *Arabidopsis*.

3.3 Molecular characterization of SAR in *DEX::AvrRpm1-HA* plants

When considering the results of bacterial - and DEX-induced-SAR (Figure 12) it is impossible to conclude if the DEX-inducible-SAR is a "real" SAR response and not due to movement of DEX and consequent systemic expression of *AvrRpm1*. To investigate if the SAR response may be due to DEX-movement we performed qRT-PCR analysis of tissue of treated and systemic the Col-0 *DEX* and *eds1-2 DEX* plants collected at different time points after Tween or DEX treatment.

Leaves of both plant genotypes were treated either with 0.01% Tween-20 (control) or with 1 µM DEX solved in 0.01% Tween-20. Treated and systemic, untreated tissues were harvested before and 2, 4, 8, 24, 48 and 72 hpt (hours post treatment). Gene expression was analysed for *AvrRpm1* and *PR1* normalised to *Tubulin*.

AvrRpm1 gene expression is induced via DEX treatment in local, treated (Figure 13 A), but not in the systemic, untreated tissue of Col-*DEX* plants (Figure 13 B). The expression level of *AvrRpm1* after DEX treatment is enhanced almost 100-fold in the treated tissue as compared to Tween treated tissue (Figure 13 A).

In the *eds1-2* mutant plants *AvrRpm1* expression is activated via DEX treatment in the local, treated tissue over time (Figure 13 C). The gene expression pattern in local treated tissue was comparable to the expression pattern of *AvrRpm1* in the WT background (Figure 13, compare C to A). Furthermore, there was almost no difference in expression of *AvrRpm1* in the systemic tissue of DEX pre-treated as compared to Tween pre-treated plants (Figure 13 D).

Figure 13: Expression of *AvrRpm1* in the Col-0 *DEX* and *eds1-2 DEX* mutant plants. Local tissue of WT and *eds1-2* DEX plants was treated either with Tween or DEX and expression of *AvrRpm1* was analysed in the treated and systemic tissues before and 2, 4, 8, 24, 48 and 72 hpt (hours post treatment). Gene expression was normalised to Tubulin. (A) Col-0 *DEX,* locally treated tissue with Tween-20 or DEX. (B) Col-0 *DEX,* systemic untreated tissue. (C) *eds1-2 DEX,* locally treated tissue with Tween-20 or DEX. (D) *eds1-2 DEX*, systemic untreated tissue. This experiment was done in three independent biological replicates.

PR1 expression is similarly induced by Tween or DEX treatment of Col-0 *DEX* plants (Figure 14 A). A similar trend is observed in the *eds1-2 DEX* mutant background with *PR1* expression increasing to higher levels in the mutant as compared to WT plants (Figure 14 A and C). By contrast, the *PR1* expression level in the systemic tissue was elevated faster and to a higher extent in Col-0 *DEX* as compared to *eds1-2 DEX* mutant plants (Figure 14 B and D). An early and transient boost of *PR1* transcripts was detectable only in the systemic tissue of locally DEX-treated Col-0 DEX plants (Figure 14 B, in 2 out of 3 experiments).
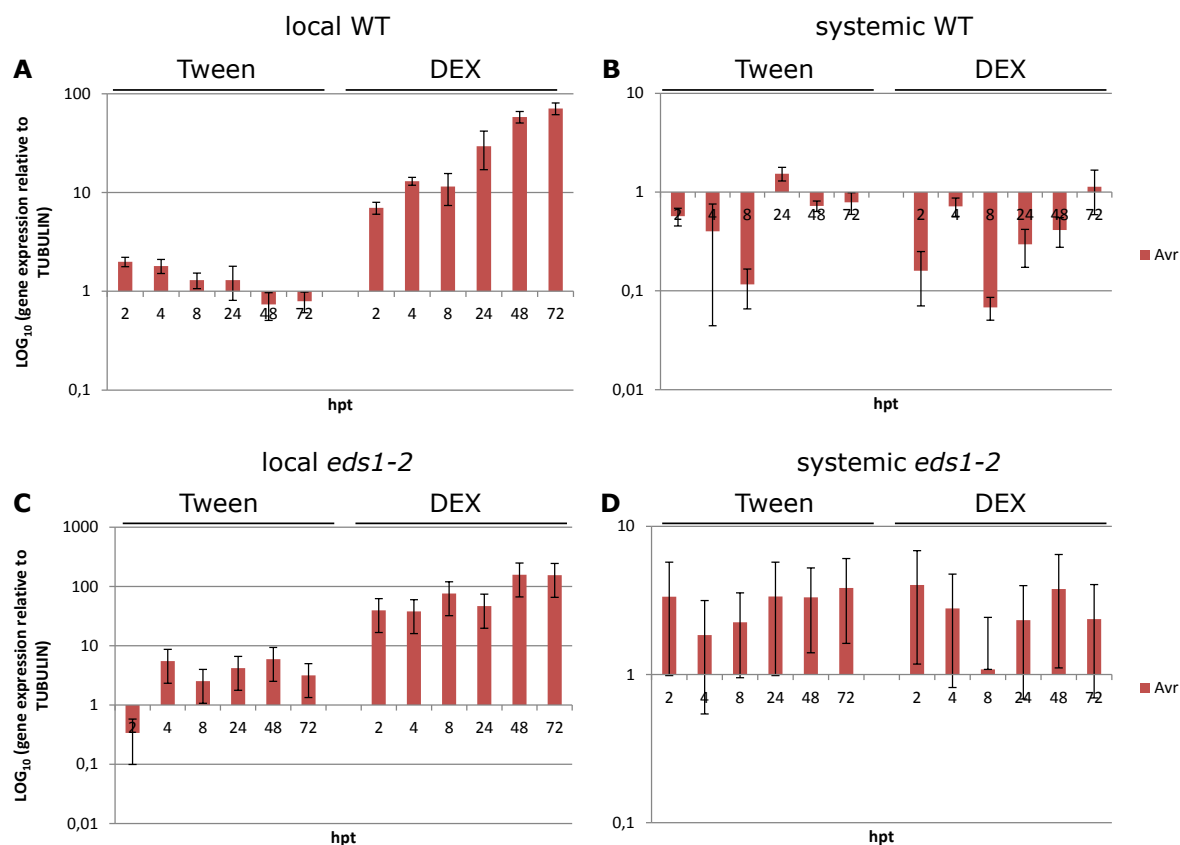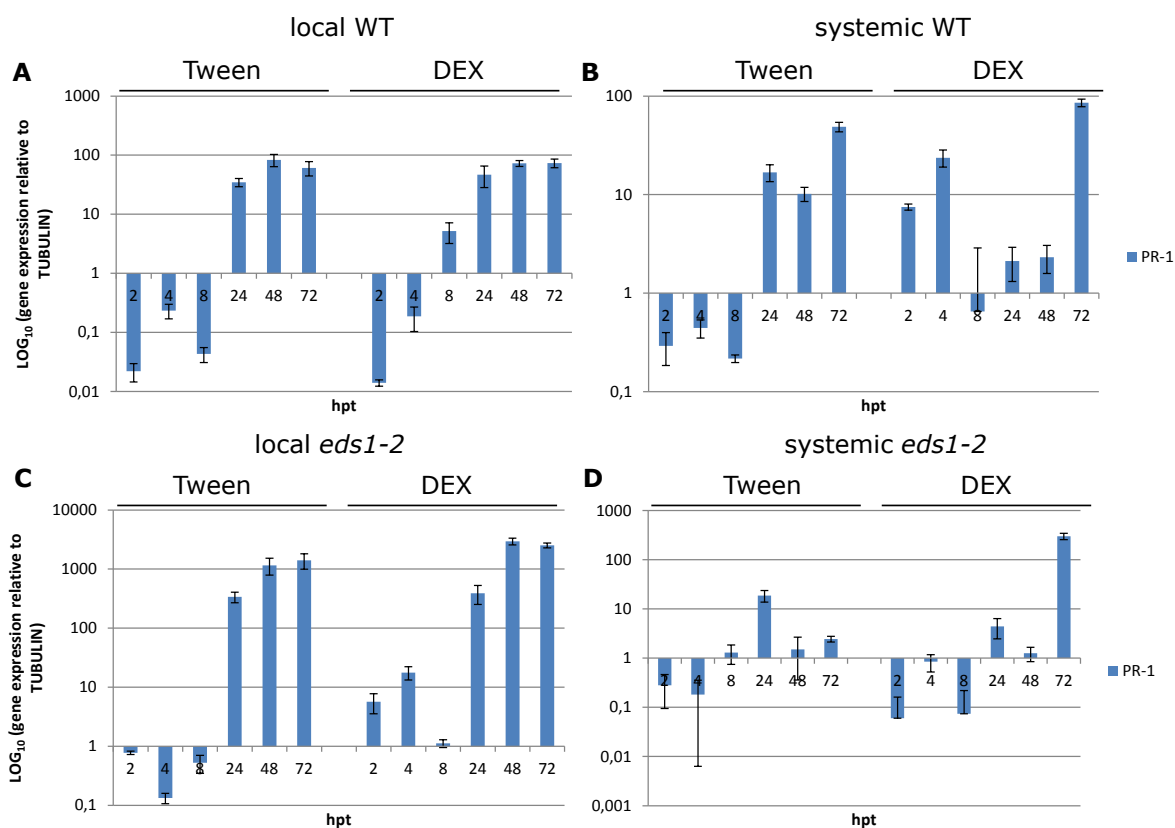
Figure 14: Expression of *PR1* in Col-0 *DEX* and *eds1-2 DEX* mutant plants. Local tissue of Col-0 *DEX* and *eds1-2 DEX* plants was treated either with Tween or DEX and expression of *PR1* was analysed in local and systemic tissue before and 2, 4, 8, 24, 48 and 72 hpt (hours post treatment). (A) Col-0 *DEX,* locally treated tissue with Tween-20 or DEX. (B) Col-0 *DEX,* systemic untreated tissue. (C) *eds1-2 DEX,* locally treated tissue with Tween-20 or DEX. (D) *eds1-2 DEX*, systemic untreated tissue. This experiment was done in three independent biological replicates with similar results.

Taken together, we conclude that the DEX-induced SAR response in the Col-0 *DEX* plants (Figure 12 B) is a "real" SAR that is not due to DEX movement. This was confirmed by the fact that induction of *AvrRpm1* expression was not observed in the systemic tissue of DEX-treated WT and mutant plants (Figure 13 B and D). In addition, a boost of *PR1* expression was observed only in the systemic tissue of DEX-treated WT, but not *eds1-2* mutants plants (Figure 14 B). Furthermore, we point out that the slight expression of *AvrRpm1* in the local, treated tissue in response to Tween treatment likely caused by some basal expression due to leakiness of the DEX-inducible promoter (Figure 13 A, C, and D). Also, *PR1* expression can be induced in response to Tween or DEX treatment in both plant types (Figure 14 A and C). This indicates that the plants are highly sensitive to application (brush) and/or to the solutions used.

3.4 Hunting for metabolites by using two different methods

With the aim of identifying metabolites occurring only in the WT and not in the *eds1-2* mutant, apoplast extracts of *AvrRpm1*-expressing plants were analyzed using LC-MS. Five biologically independent sample sets were analysed in cooperation with Dr. Thomas Hoffmann and Prof. Dr. Wilfried Schwab (TUM).

The DEX-inducible promoter of Col-0 *DEX::AvrRpm1-HA* and eds1-2 *DEX::AvrRpm1-HA* was activated by spray inoculation with 30 µM of DEX. Four and a half hours later, APO buffer was vacuum-infiltrated into the leaves and apoplast extracts were isolated by centrifugation. Samples were concentrated by using a C18 column. Columns were washed with MeOH five times and eluates were analysed by LC-MS. With this method only one unidentified metabolite was found that differentially accumulated in WT as compared to *eds1-2* mutant plants. The metabolite has a mass of 400.69 to 400.82 m/z and a retention time on the HPLC column of ca. 2412 seconds. It was found in the fourth MeOH eluate when negative ions were analysed (MS 'negative mode', Supplement Figure 40).

When plants were locally treated with apoplast extracts from Col-0 *DEX* plants, SAR responses were not detectable in the systemic leaves of WT or *eds1*-2 mutant plants (Supplement Figure 41). This shows that the metabolites in the apoplast extracts may not have SAR-inducing activity.

Because apoplast extracts did not trigger SAR and because the apoplast extracts contained only a limited number of compounds, of which only one differentially accumulated between Col-0 *DEX* and *eds1-2 DEX*, we adapted the approach and – for the purpose of our metabolomics study – used whole tissue MeOH extracts for following analyses. By using MeOH extraction followed by LC-MS it was possible to detect more metabolites (negative ion mode) that were enhanced in Col-0 *DEX* plants as compared to the mutant. The differentially accumulating metabolite found in the apoplast extracts was confirmed in the MeOH extracts. When the positive ionization mode of the MS was used, two additional metabolites were found. One was enhanced in WT and the other one was enhanced in the *eds1-2* mutant background.

When MeOH-extracts of Col *DEX* and *eds1-2 DEX* plants were tested for their SAR-inducing activity in naïve, non-transgenic plants, systemic immunity was not detectable in WT or in the *eds1-2* mutant (Supplement Figure 42). Thus, similar to the apoplast extracts, the MeOH extracts from *AvrRpm1*-expressing plants did not trigger SAR.

3.5 Liquid-liquid phase separation of MeOH extracts

The last observation indicated that metabolites isolated by the MeOH extraction approach, do not induce SAR. The MS analysis of MeOH extracts revealed more metabolites as compared to MS analysis of apoplast extracts. This lead us to further work on this approach.

The MeOH-extracts were separated into different fractions by liquid-liquid extraction using petroleum ether (PE, 1) followed by dimethyl ether (DME, 2) (Figure 15 A). The PE, DME, and remaining aqueous phase (3) were dried, dissolved in 100% DMSO (Figure 15 B), and diluted to a final concentration of 0.2% DMSO. These fractions were infiltrated into lower leaves of Col-0 and *eds1-2* plants to test if they trigger SAR.
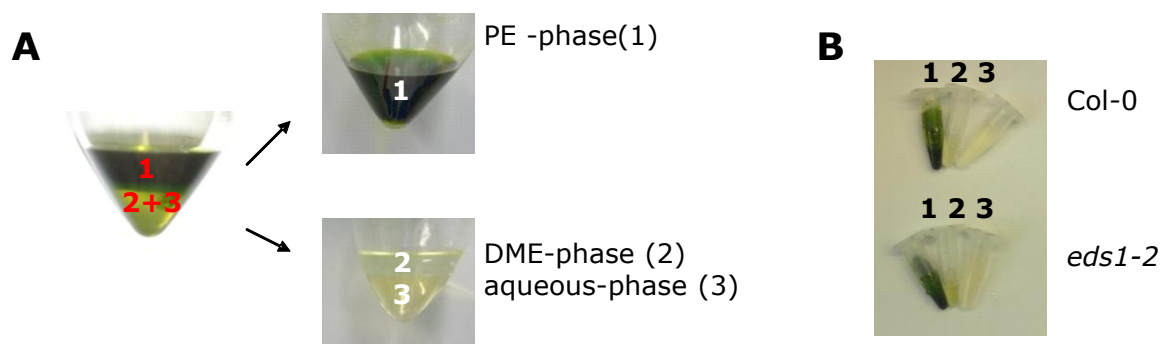
Figure 15: Separation of MeOH-extracts into different phases. (A) The MeOH extracts of Col-0 *DEX* and *eds1-2 DEX* were separated into different phases by using petroleum ether (PE, 1) and dimethyl ether (DME, 2). After separation an aqueous-phase was left (3). (B) After drying by evaporation, the metabolites from the different fractions were solved in 100% of DMSO. No visible difference was observed between the fractions (1, 2, 3) from Col-0 *DEX* (upper picture of B) as compared to those from *eds1-2 DEX* (lower picture of B).

After 3 days a secondary infection with DC3000 followed in the systemic tissue. Bacterial growth in the systemic leaves was determined after 4 additional days (Figure 16). Figure 17 presents the control (primary treatment with 10 mM $MgCl_2$ (MOCK (**1**), uninfected)), which is not able to induce SAR. By contrast, a primary infection with DC3000 *AvrRpm1* initiated SAR (**2**). Other negative controls were 0.2% DMSO (**3**) and the solvent-treated water (solvents, **4**). The negative controls did not induce a SAR response. The PE-fraction of Col-0 *DEX* (**5**) was capable of initiating SAR in the systemic leaves. The DME-fraction (**6**) and aqueous-fraction (**7**) did not trigger SAR. The PE-phase (**8**) of *eds1-2 DEX* also was infiltrated into Col-0 plants and triggered an insignificant SAR. No SAR response was detectable in WT plants neither in response to the DME-phase (**9**) nor to the aqueous-phase (**10**) of extracts from *eds1-2 DEX* (Figure 16).
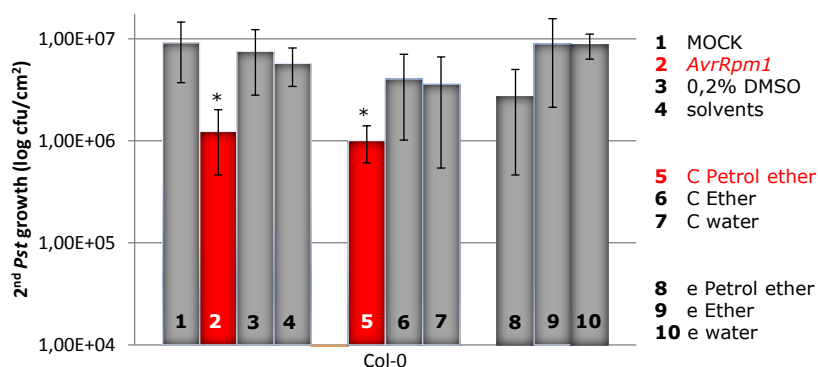
Figure 16: SAR experiment with phases from the liquid-liquid extraction. WT plants were locally treated with MOCK (1), DC3000 *AvrRpm1* (2), 0.2% DMSO (3), chemical treated water (solvents, 4) and with the petrol ether (5, 8), ether (6, 9) and aqueous rest (water, 7, 10) phases from the Col-0 *DEX* (C) and *eds1-2 DEX* (e) extracts, respectively. Growth of secondary DC3000 inoculum was scored four days after infection of the systemic tissue. Asterisks above bars indicate statistically significant differences as compared to the MOCK control (* $P < 0.05$, Student´s t test). Experiments were repeated three times with comparable results.

SAR was not detectable when the active phase (PE) of Col-0 *DEX* was tested in *eds1-2* mutant plants (Supplement Figure 43).

Together, the data show that the SAR-active compounds in the MeOH extracts are soluble in PE and are thus very apolar.

3.6 Solvent exchange: PE to MeOH

For further experiments, including HPLC and LC-MS it was important to have the metabolites solved in MeOH. This solvent (and not PE) is compatible with the different analytical methods. Therefore, we mixed 100 µl each of the PE-phase from Col-0 *DEX* and *eds1-2 DEX* mutant plants with 900 µl of a mixture of water and MeOH (v/v 1:1) and loaded the samples on C18 columns to concentrate the phases. The first flow through was discarded and the column was washed with 5 ml of 25%, 50%, 75% and 100% of MeOH followed by a final wash with PE. The eluates were dried by evaporation, dissolved in 100% of DMSO and diluted down to 0.2% of DMSO with water. Lower leaves of 4- to 5-week-old *Arabidopsis* plants were treated with the different samples and a classical SAR experiment was performed. To this end, the different MeOH eluates of WT (C) and mutant (e) were used for a first treatment as compared to the positive control, DC3000 *AvrRpm1*, and the negative controls, MOCK, 0.2% DMSO, and solvents. A few days later, the systemic tissue of all pre-treated plants was infected with DC3000 and bacterial growth was analysed. Figure 17 illustrates that samples 7-9 from Col-0 *DEX* induced defence in the systemic tissue to a similar degree as the positive control (**2**). Samples 7-9 are the 75% and 100% MeOH eluates and the final PE column wash of Col-0 *DEX*

extracts. The negative controls (1, 3-4) as well as the corresponding MeOH eluates from the mutant extract (12-14) did not induce systemic resistance in WT plants.

Figure 17: SAR induced by different MeOH eluates from C18 columns. WT plants were locally treated with MOCK (1), DC3000 *AvrRpm1* (2), 0.2% DMSO (3), solvents (4), and with the C18 column eluates in MeOH concentrations of 25% (5, 10), 50% (6, 11), 75% (7, 12), and 100% (8, 13), as well as in a final PE wash (9, 14). Extracts were from Col-0 *DEX* (C) and *eds1-2 DEX* (e). Growth of secondary DC3000 inoculum was scored four days after infection of the systemic tissue. Asterisks above bars indicate statistically significant differences as compared to the MOCK control (** $P < 0.01$, Student´s t test). Experiments were repeated three times with comparable results.

SAR was not detectable when *eds1-2* mutants were pre-treated with the SAR-inducing samples 7-9 of Col-0 *DEX* or with the corresponding samples from *eds1-2 DEX* plants (Supplement Figure 44).

The elution of SAR bio-active compounds by higher MeOH concentrations confirms that the active metabolites are very apolar.

3.7 HPLC divided PE phases from WT and mutant into different fractions

To narrow down the SAR-inducing compound(s) in the MeOH extracts the PE phases in MeOH (from 3.5) were further fractionated by using HPLC. Five to 20 (depending on the experiment) biologically independent MeOH extracts from Col-0 *DEX* and *eds1-2 DEX* were extracted with PE and concentrated on C18 columns. The columns were consecutively eluted with 5 ml of 75% MeOH, 100% MeOH, and PE. The eluates were collected, consolidated per genotype (WT and mutant), dried by evaporation, and dissolved in 100% of MeOH. Subsequently, the HPLC was performed across a MeOH gradient to fractionate the samples. One fraction was collected per minute. Fractions were eluted from the HPLC column across a concentration gradient of 0% to 100% MeOH and back to 0% MeOH (water, section 2.2.12). The fractions 21 until 40 (corresponding to MeOH concentration of 75% to 100%) were dried by evaporation and separated into lipid and non-lipid accordingly to 3.5. The lipid phase was dried by evaporation and

dissolved in 100% DMSO. Processed fractions were tested in 4-to-5-week old WT *Arabidopsis* plants for SAR-inducing activity.

Figure 18 shows that HPLC fractions no. 23, 24, 26, 29, and 34 from Col-0 *DEX* (C) trigger SAR to a similar extent as the positive control (DC3000 *AvrRpm1*, 2). By contrast, the corresponding HPLC fractions from the mutant extracts did not induce SAR in WT *Arabidopsis* plants. Also, SAR was not detectable when bio-active HPLC fractions from Col-0 *DEX* and the corresponding fractions from the mutant were tested in the *eds1-2* mutant (Supplement Figure 45).

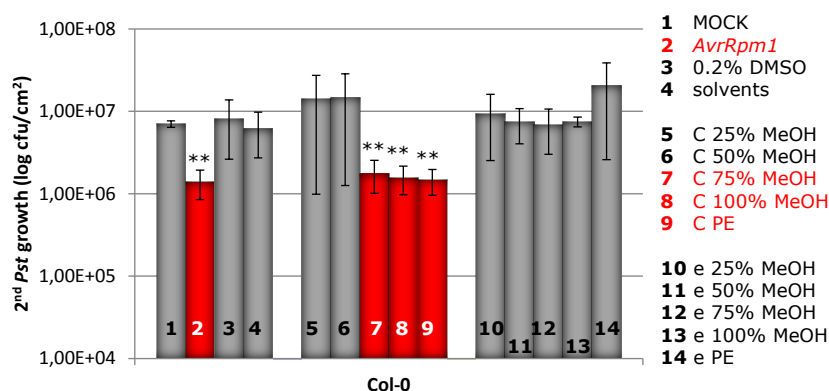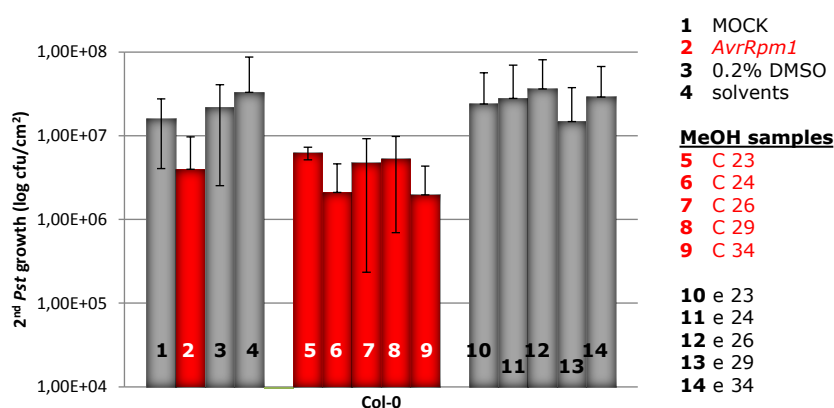**Figure 18: SAR induced by different HPLC fractions.** WT plants were locally treated with MOCK (1), DC3000 *AvrRpm1* (2), 0.2% DMSO (3), solvents (4), and with the HPLC fractions 23 (5, 10), 24 (6, 11), 26 (7, 12), 29 (8, 13), and 34 (9, 14) from Col-0 *DEX* (C) and *eds1-2 DEX* (e). Growth of secondary DC3000 inoculum was scored four days after infection of the systemic tissue. This experiment was repeated three times with similar results.

The UV absorption signal at 260 nm of the HPLC chromatogram of Col-0 *DEX* and *eds1-2 DEX* looked similar (Figure 19). In three repetitions of this experiment, we could distinguish three separate areas in the chromatogram that contained SAR-inducing compounds. These three areas were always in a similar range of the chromatogram as judged by the UV absorption signal of the SAR bio-active fractions (Figure 19, bio-active ranges marked in grey). It should be noted that the number of SAR-inducing fractions varied slightly per repetition. For instance, the SAR-inducing activity in the first bio-active range (Fractions 23, 24, and 26 in Figures 19 and 20) sometimes was concentrated in one fraction. In addition, fraction numbering varies per repetition due to some variability between individual HPLC runs as well as in the starting point of fraction collection. Therefore, fractions are always related to the UV absorption signals of the individual chromatograms (see below).
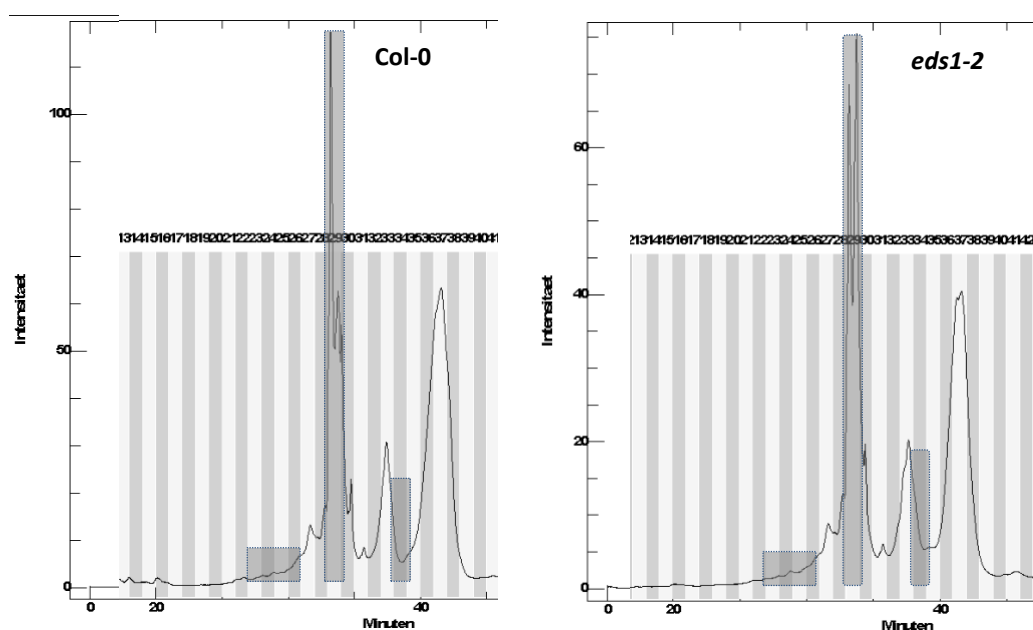
Figure 19: UV absorption signal of the HPLC chromatograms of extracts from Col-0 *DEX* and *eds1-2* *DEX* plants. SAR-inducing fractions were found in three ranges of the chromatogram. These ranges are marked in grey.

3.8 FTICR-MS reveals 38 masses present in SAR-inducing samples

To identify SAR-inducing metabolites present in the HPLC fractions from Col-0 *DEX* extracts we performed FTICR-MS (Fourir transform ion cyclotron resonance mass spectrometry) in cooperation with Dr. Philippe Schmitt-Kopplin and Dr. Basem Kanawati (Helmholtz Zentrum Muenchen, Research Unit BioGeoChemistry). FTICR-MS is high resolution mass analysis that results in the determination of the chemical formula of the compounds detected.

MeOH extracts from Col-0 *DEX* and *eds1-2 DEX* were processed as described above. The resulting HPLC fractions were tested for their SAR-inducing activity in WT *Arabidopsis*. Figure 20 (A) shows that HPLC fractions 5, 8, 11, 14, and 15 displayed SAR-inducing activity. The fractions 5, 8, and 11 from Col-0 *DEX* and *eds1-2 DEX* plants were tested in an additional experiment with similar results (Supplement Figure 46). By contrast, SAR-inducing fractions from Col-0 *DEX* and the corresponding fractions from the mutant did not trigger SAR activity in the *eds1-2* mutant plants (Supplement Figure 47).
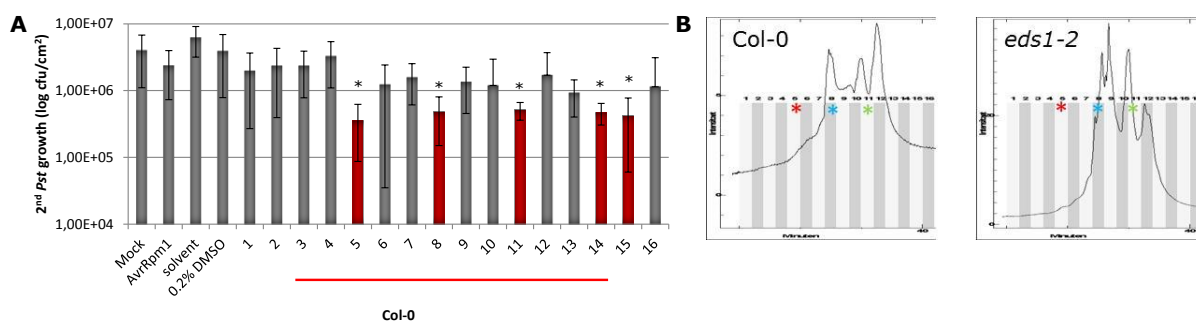
Figure 20: SAR experiment with HPLC fractions that were used for FTICR-MS. (A) Local leaves of WT *Arabidopsis* were treated with MOCK, DC3000 *AvrRpm1*, solvent, 0.2% DMSO, and different HPLC fractions from Col-0 *DEX*. Growth of secondary DC3000 inoculum was scored four days after infection of the systemic tissue. (B) SAR-inducing fractions were found in three ranges of the chromatogram (coloured stars). Asterisks above bars indicate statistically significant differences as compared to the MOCK control (* P < 0.05, Student´s t test). Experiment was repeated twice with comparable results (Supplement Figure 46).

According to the UV absorption signal of the HPLC chromatogram (Figure 20 B), the SAR-inducing fractions 5, 8, and 11 from Col-0 *DEX* represent the same three SAR bio-active ranges of the HPLC chromatogram detected above (compare Figure 19 to Figure 20 B).

The fractions 3 to 14 of Col-0 *DEX* and *eds1-2 DEX* were analysed by FTICR-MS. On the one hand, masses detected in the SAR-inducing fractions from Col-0 *DEX* (5, 8, and 11) were compared to masses detected in the other non-SAR-inducing fractions from Col-0 *DEX* (3, 6 ,7 ,9, 10, 12, 13, and 14) as well as with the corresponding fractions from *eds1-2 DEX* (3-14). The SAR-inducing fraction 14 was not considered because it contained residual tensides, probably from washing of glass bottles. The tensides were detected by the FTICR-MS analysis. To this end, the HCE3 (Hierachical Clustering Explorer) monodimensional clustering software was used. In this comparison, 38 masses were detected that were present only in SAR-inducing fractions (Supplement Table 10). On the other hand, when SAR-inducing fractions from Col-0 *DEX* were compared only to the corresponding fractions from *eds1-2 DEX* the program detected 320 masses that were present in Col-0 *DEX* and not or to a significant lower intensity in the mutant.

Subsequently, the 320 differentially accumulating masses from the final comparison were analysed with the MassTRIX program. This program annotates metabolites in high precision MS data. It for instance marks identified chemical compounds on KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways by using the KEGG/API. Out of 320 masses the program identified four metabolites (Figure 21) present in the Col-0 *DEX* fractions and not in the corresponding *eds1-2 DEX* fractions.

id	mass	formula	name
C11472	370.0066138254	C7H16O13P2	D-glycero-D-manno-Heptose 1,7-bisphosphate
C00447	370.0066138254	C7H16O13P2	Sedoheptulose 1,7-bisphosphate
C06148	513.006310162	C9H18N5O14P3	2,5-Diamino-6-(5'-triphosphoryl-3',4'-trihydroxy-2'-oxop...
HMDB06823	513.006310162	C9H18N5O14P3	2,5-Diamino-6-(5'-triphosphoryl-3',4'-trihydroxy-2'-oxop...
C05923	513.006310162	C9H18N5O14P3	2,5-Diaminopyrimidine nucleoside triphosphate

carbon fixation

folate biosynthesis

Figure 21: Potentially SAR-related metabolites identified in Col-0 *DEX* samples by the Mass TRIX program. The program identified sugar compounds belonging to the carbon fixation pathway and two metabolites of the folate biosynthesis pathway.

The identified compounds D-glycero-D-manno-heptose 1.7-bisphosphate and seduheptulose 1,7-bisphosphate are components of the carbon fixation pathway. Another two metabolites were identified which are mapped to the folate biosynthesis pathway. These metabolites are 2,5-diamino-6-(5´-triphosphoryl-3´,4´-trihydroxy-2´-oxopentyl)-amino-4-oxopyrimidine and 2,5-diaminopyrimidine nucleoside triphosphate.

In summary, FTICR-MS led to the identification of four metabolites that accumulate to a higher concentration in the Col-0 *DEX* extracts as compared to *eds1-2 DEX*. These compounds may thus be related to SAR.

3.9 D-(+)-Neopterin induces SAR in *Arabidopsis*

In the following experiment, we aimed to characterize if the metabolites or pathway identified by the FTICR-MS are involved in SAR. Because the four identified metabolites are not commercially available, close relatives/derivatives of these metabolites in the respective biochemical pathway were purchased.

Metabolites tested in plants were seduheptulose from the carbon fixation pathway as well as D-(+)-neopterin and 7,8-dihydroneopterin from the folate biosynthesis pathway. These components were dissolved in water and diluted to 1 mM. Two lover leaves from 5-week-old WT *Arabidopsis* plants were treated and a classical SAR experiment was performed.

Pre-treatment with D-(+)-neopterin (1 mM) triggered a tendency towards SAR (Figure 22 A). Pre-treatment with seduheptulose or 7,8-dihydroneopterin did not trigger SAR. A second experiment was performed to confirm these results. To this end, the same concentration of D-(+)-neopterin was used. In addition, a lower concentration was tried to induce systemic defence. Figure 22 B indicates clearly that the SAR-inducing tendency in response to 1 mM neopterin seen in Figure 22 A is not reproducible. By contrast, by

using a lower concentration of D-(+)-neopterin (500 µM) a clear and significant SAR was observed (Figure 22 B).
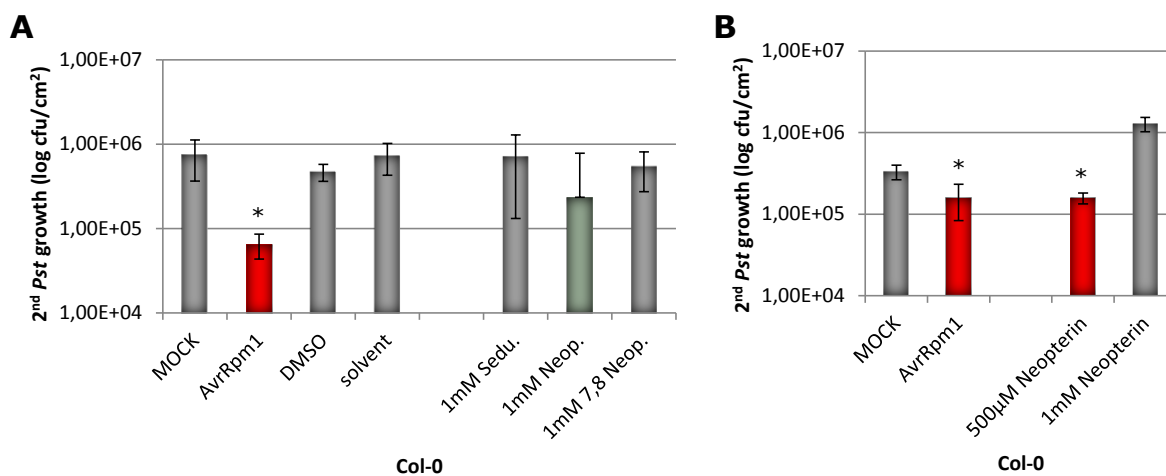
Figure 22: SAR induced by D-(+)-Neopterin. WT plants were locally treated with MOCK, DC3000 *AvrRpm1,* seduheptulose (Sedu.), D-(+)-neopterin (Neop.), and 7,8-dihydroneopterin (7,8 Neop.). Growth of secondary DC3000 inoculum was scored four days after infection of the systemic tissue. (A) Pre-treatment with 1 mM of the compounds used. (B) SAR response to different concentrations of D-(+)-neopterin. Asterisks above of bars indicate statistically significant differences as compared to the MOCK control (* P < 0.05, Student´s t test).

These data indicate that the folate biosynthesis product D-(+)-neopterin can trigger SAR in WT *Arabidopsis* plants. By contrast, additional test are required to establish if 7,8-dihydroneopterin (an intermediate of the folate biosynthesis pathway, Figure 22 A) and seduheptulose, might be involved in SAR, for instance by using lower concentration than 1 mM.

3.10   HPLC across ACN gradient further separates SAR-inducing metabolites

The SAR-related pathways and metabolites identified by FTICR-MS were more polar in nature than expected from the solubility of the active compounds in PE and higher MeOH concentrations. Therefore, another approach was taken to identify apolar SAR-inducing metabolites in the three SAR bio-active ranges of the MeOH gradient chromatogram presented in Figure 19. To this end, new samples of Col-0 *DEX* were processed as described above (3.5). The UV absorption signal of the HPLC chromatogram was used to predict which fractions could contain SAR-inducing compounds. By this approach, seven fractions were selected. These were numbered 22, 25, 28, 29, 30, 31, and 34. Each of these fractions was further separated by HPLC across an ACN gradient. The HPLC was linked to a mass analyser for immediate determination of mass spectra of the HPLC eluates. In parallel, fractions were collected. Fractions were eluted from the HPLC column

across a concentration gradient of 50% to 100% ACN and back to 50% of ACN. Each of the seven fractions from MeOH gradient was fractionated into 40 new ACN fractions.

The mass spectra of the seven ACN gradient HPLC-MS runs were compared to find differences in the MS patterns. Exemplarily the MS pattern for MeOH fraction 22, 25, and 28 is shown in Figure 23. These were compared, because overlap in mass accumulation can be expected between (neighbouring) fractions from the original MeOH gradient fractionation. Such overlaps would result in similar LC-MS spectra of the respective ACN HPLC-MS runs, but would – in a SAR bio-assay - represent the same bio-active molecules activating SAR. To avoid excessive overlap between ACN fractions from different original MeOH fractions, ACN fractions from the seven different HPLC runs were selected that display varying mass spectra. For instance, from the ACN gradient of MeOH fraction 22, 34 fractions were selected (Figure 23 A). These were termed 22.1 – 22.34 with the first number corresponding to the MeOH fraction and the second number referring to the ACN fraction. The ACN fractions 25.1-25.16 from MeOH fraction 25 were taken (B), because this part of the MS pattern looked different as compared to those of MeOH fractions 22 and 28. The ACN fractions 28.16-28.34 from the MeOH fraction 28 (C) were taken, because this part of the MS pattern looked different as compared to the other MeOH fractions.
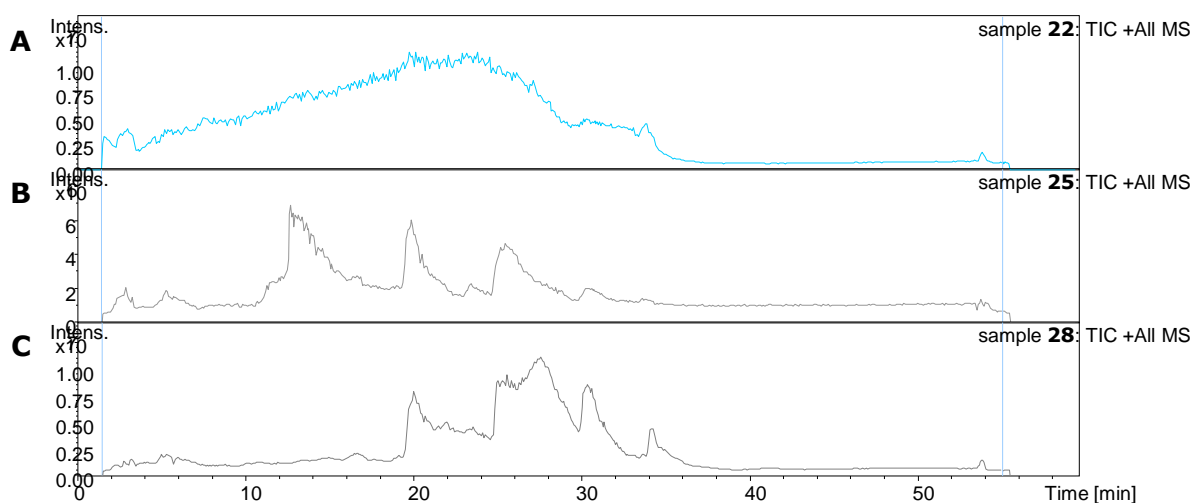
Figure 23: Mass spectra of MeOH fractions after separation of masses across an ACN gradient. (A) MeOH fraction 22. (B) MeOH fraction 25. (C) MeOH fraction 28.

In total, 138 fractions were selected, dried by evaporation. Leftover was separated into a lipid and non-lipid phase by adding PE. Additionally, the lipid phase was dried by evaporation and the pellet was solved in 100% DMSO. WT *Arabidopsis* plants were locally treated with the different fractions and a classical SAR experiment was performed.

The result of the performed SAR experiments is summarized in Figure 24, which illustrates that some fractions are able to trigger a more efficient SAR (SAR ++) than

DC3000 *AvrRpm1*. Figure 24 also illustrates that some samples trigger SAR to a similar extend as DC3000 *AvrRpm1* (SAR +). By contrast, other ACN fractions did not trigger SAR (SAR -) in WT *Arabidopsis* plants.
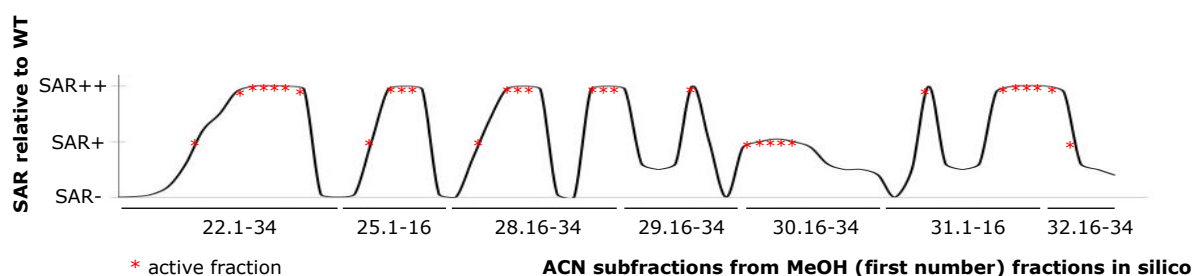
Figure 24: Summary of SAR experiment with 138 different HPLC ACN samples. First number refers to MeOH fraction. Second number refers to ACN sub-fraction. SAR ++: SAR-inducing activity stronger than with DC3000 *AvrRpm1* pre-treatment, SAR +: similar SAR-inducing activity as compared to DC3000 *AvrRpm1* pre-treatment, SAR -: no SAR-inducing activity. Red Asterisks indicate bio-active SAR inducing fractions.

3.11 MS in the positive ion mode reveals metabolites highly present in the mutant as compared to WT *Arabidopsis*

To confirm previous data (Figure 24) a 2nd experiment was performed. Therefore, new MeOH extracts from Col-0 DEX and *eds1-2 DEX* plants were processed as described above and a HPLC MeOH gradient was performed. 16 different MeOH fractions from Col-0 *DEX* and e*ds1-2 DEX* mutants were analysed. The UV absorption signal of the MeOH gradient HPLC was used to estimate which fractions could contain SAR-inducing activity (Figure 25). Fractions 5 and 6 were pooled and expected to cover the first SAR bio-active range of the HPLC chromatogram identified above (Figure 19). Fraction 8 represents the middle SAR bio-active range and fractions 11, 12, and 13 were pooled and expected to include that third SAR bio-active range from Figure 19.
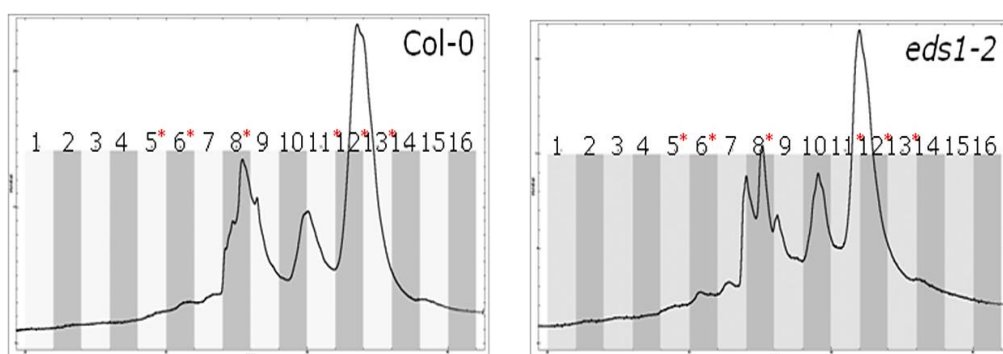
Figure 25: UV absorption signal of the HPCL chromatogram from Col-0 *DEX* and *eds1-2 DEX* extracts. (*) predicted SAR-inducing fractions.

The MeOH fractions 5/6, and 8 from Col-0 *DEX* (C) and mutant (e) extracts were dried by evaporation and dissolved in 100% of MeOH. Subsequently, HPLC across an ACN gradient was performed as in 3.10. Predicted SAR bio-active ACN fractions were dried by evaporation and dissolved in 500 μl of 100% MeOH. These include ACN fraction 8-11 from the pooled MeOH fraction 5/6 (5/6.8 to 5/6.11) and ACN fractions 8-12 from MeOH fraction 8 (8.8 to 8.12). The pooled MeOH fraction 11, 12, and 13 was not analysed due to time constraints.

60 μl of the fractions 5/6.8-11 and the fractions 8.8-12 from Col-0 *DEX* and *eds1-2 DEX* plants were analysed by analytical LC-MS in the positive and negative ion mode. The positive ion mode of the LC-MS revealed 49 masses that accumulated in the mutant extracts to a higher intensity as compared to WT (Supplement Table 11). By contrast, by using the negative ion mode masses were detected that were highly present in the Col-0 *DEX* extracts as compared to the mutant extract.

3.12 ONA, AzA, HPOD and an unknown compound are identified in Col-0 *DEX* and be related to SAR

The MS analysis (negative ion mode) of the fraction C5/6.8 from Col-0 *DEX* revealed four significant mass peaks with a higher intensity as compared to the corresponding peaks in the mutant extract. The first peak with a mass of 171 (m/z) was identified as ONA (9-oxo-nonanoic acid, Figure 26, turquoise) by using a standard (supplement Figure 50 A). The peak with a mass of 187 (m/z) was identified as AzA (azelaic acid, Figure 26, red) and the peak with a mass of 311 was identified as 9-HPOD (9-hydroperoxy octadecadienoic acid, Figure 26, grey). Both were compared to standards shown in Figure 48 B and C in the supplemental material of this thesis. The fourth and last peak-of-interest could not be identified and will be referred to as an unknown compound (Figure 26, royal blue, 255 m/z). ONA, AzA, 9-HPOD and the unknown compound were detectable in the corresponding ACN fraction of the *eds1-2 DEX* extract, but at a significant lower intensity (Figure 26, lower panel). When the detected metabolites were analysed in C5/6.9-11 and e5/6.9-11, the observed peak intensities were similar as in e5/6.8.
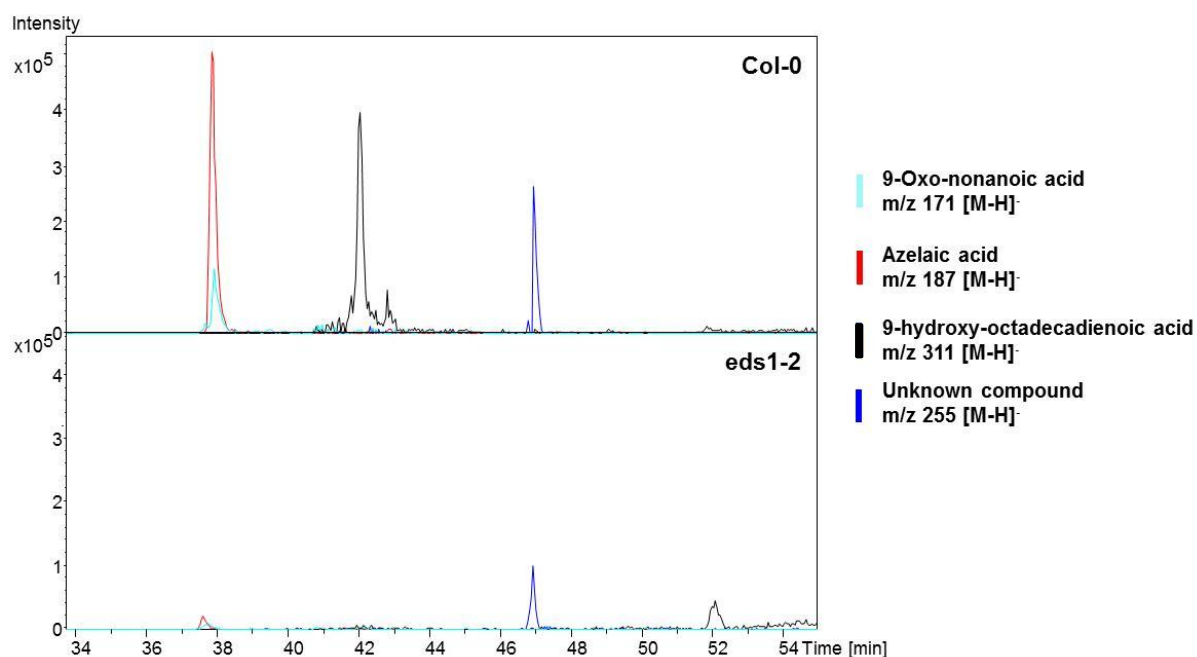
Figure 26: MS analysis of fraction 5/6.8. (upper panel) Col-0 *DEX*. (lower panel) *eds1-2 DEX*, turquoise ONA: 9-oxononanoic acid, red AzA: Azelaic acid, dark blue HPOD: 9-hydroxy-octadecadienoic acid, royal blue: unknown compound (preliminary results).

It should be noted that this experiment could be repeated only once due to time constraints; the data should therefore be viewed as preliminary. The same compounds were not detected in fractions 8.8-12 from Col-0 *DEX* and *eds1-2 DEX*.

175 µl of each of the analysed fractions were dried by evaporation and solved in 17 µl of 100% DMSO. Subsequently, a classical SAR experiment was performed to test the SAR-inducing activity of each fraction.

Figure 27 shows that the fractions C5/6.8 (Figure 27 A) and C8.8 (Figure 27 B) from Col-0 *DEX* triggered systemic defence to a similar degree as the positive control. Furthermore, Figure 27 shows that the fractions C5/6.9 (A) and C8.9 (B) did induce a slight SAR response in WT plants. By contrast, the tested fractions 5/6.10-11 and 8.10-12 did not trigger SAR in WT *Arabidopsis*.

Figure 27: SAR experiment with different ACN fractions. WT plants were locally treated with MOCK, DC3000 *AvrRpm1*, 0.2% DMSO, solvent and different fractions from the HPLC. (A) Fractions 5/6.8-11 from Col-0 *DEX* (C) and *eds1-2 DEX* (e) plants. (B) Fractions 8.8-12 from Col-0 *DEX* and *eds1-2 DEX* plants. Growth of secondary DC3000 inoculum was scored four days after infection of the systemic tissue. Asterisks above bars indicate statistically significant differences as compared to MOCK control (* P < 0.05, Student´s t test).

By contrast, SAR-inducing fractions from Col-0 *DEX* and corresponding fractions from *eds1-2 DEX* did not trigger systemic resistance in the *eds1-2* mutant from Arabidopsis (Supplement Figure 50). Also, none of the fractions isolated from the *eds1-2 DEX* system triggered SAR in WT plants (Figure 27).

Together, the data indicate that ONA, AzA, HPOD, and an unknown compound may be related to SAR in WT *Arabidopsis*. Additionally, we suggest that the accumulation of these compounds during defence signalling is regulated in an *EDS1*-dependent manner.

3.13 The SAR-related compounds ONA, AzA, and HPOD are unstable and may accumulate during the extraction procedure

To confirm the results from Figure 27, a new SAR experiment was performed as described above. Surprisingly, Figure 28 indicates a shift of the SAR-inducing activity from the ACN fractions 8 to 9 (compare Figure 28 to Figure 27). Now, the fraction C5/6.9 triggered systemic immunity and not the fraction C5/6.8 (Figure 28 A). Similarly, fraction C8.9 triggered SAR rather than fraction C8.8 (Figure 28 B).
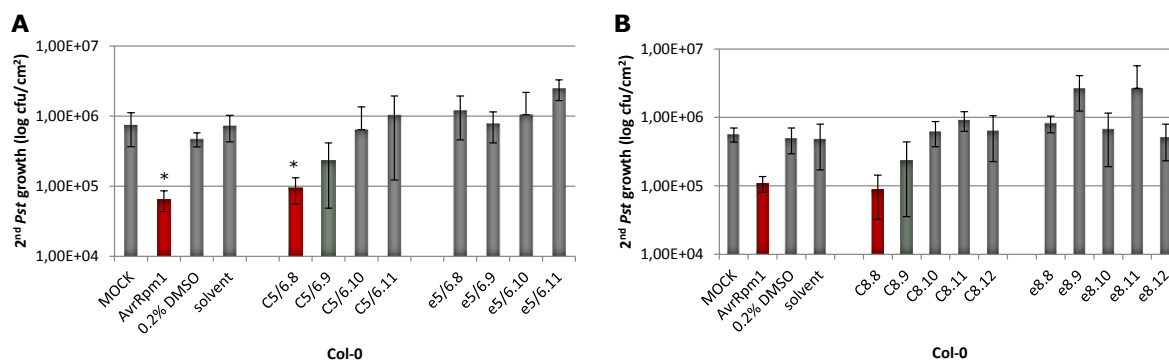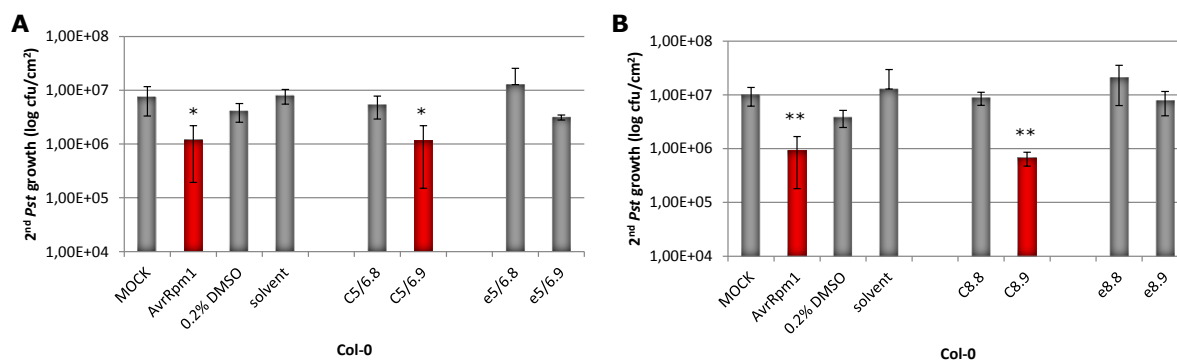
Figure 28: SAR experiment with ACN fractions. WT plants were locally treated with MOCK, DC3000 *AvrRpm1*, 0.2% DMSO, solvent and different fractions from the HPLC. (A) Fractions 5/6.8-11 from Col-0 *DEX* (C) and *eds1-2 DEX* (e) plants. (B) Fractions 8.8-12 from Col-0 *DEX* and *eds1-2 DEX* plants. Growth of secondary DC3000 inoculum was scored four days after infection of the systemic tissue. Asterisks above bars indicate statistically significant differences as compared to MOCK control (* $P < 0.05$, ** $P < 0.01$, Student´s t test). This experiment was conducted with the same ACN fractions as the experiment of Figure 27, but at a later time point.

Subsequent MS analysis of the fraction C5/6.8 revealed a sharp reduction in the levels of ONA, AzA, HPOD, and the unknown compound. After two months of storage of the fraction at minus 80 °C, only 9-HPOD could be detected, but at a much reduced level as compared to the original measurement (Figure 29). The other compounds could not be detected at all. This finding confirms that ONA, AzA, 9-HPOD, and the unknown compound may be relevant for SAR with their degradation in the samples occurring in parallel with loss of SAR-inducing activity. However, ONA, AzA, 9-HPOD, and the unknown compound could not be detected in fractions C5/6.9 and C8.8-9, indicating that other metabolites may be present in these fractions that are responsible for triggering SAR.
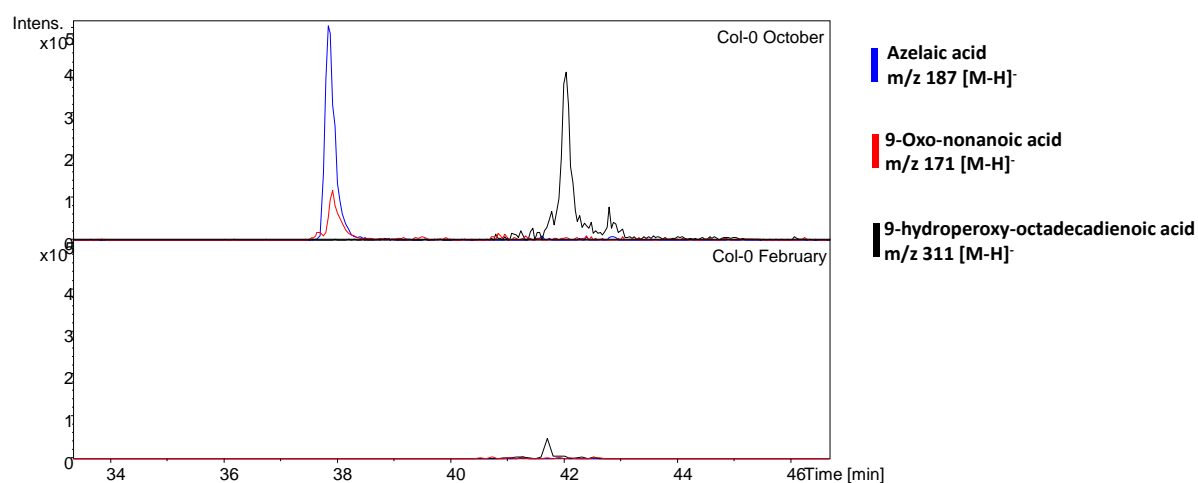
Figure 29: Second MS analysis of fraction 5/6.8 HPLC. (upper panel) Col-0 *DEX* measured in October. (lower panel) Col-0 *DEX* fraction in February, red ONA: 9-oxo-nonanoic acid, royal blue AzA: Azelaic acid, black HPOD: 9-hydroperoxy-octadecadienoic acid (preliminary results).

It should be noted that the whole experiment could be repeated only once due to time constraints; the data should therefore be viewed as preliminary.

Notably, the SAR-inducing activity of the fractions had also accumulated over time or rather during the extraction procedure.

Supplement Figure 42 shows that SAR induction was not triggered by pure MeOH extracts. It was only after the liquid-liquid extraction of the extracts that SAR was triggered by material extracted from Col-0 DEX. To confirm a dependency of SAR on ONA, AzA, HPOD, and the unknown compound the accumulation of these compounds was measured in pure MeOH extracts from Col-0 *DEX* and *eds1-2 DEX* plants.

Figure 30 shows that an unknown compound (C) was significantly increased in MeOH extracts from *eds1-2 DEX* plants as compared to Col-0 *DEX*. Interestingly, the unknown compound (C, unknown I) has the same mass as ONA. By using a standard we could show that this metabolite is not ONA, because the unknown compound has a different retention time on the HPLC column. Possible, it is a derivative or isomer of ONA. By contrast, AzA (A), 9-HPOD (B) and the unknown compound (D, unknown II) accumulated to similar levels in the MeOH extracts from both plant types.
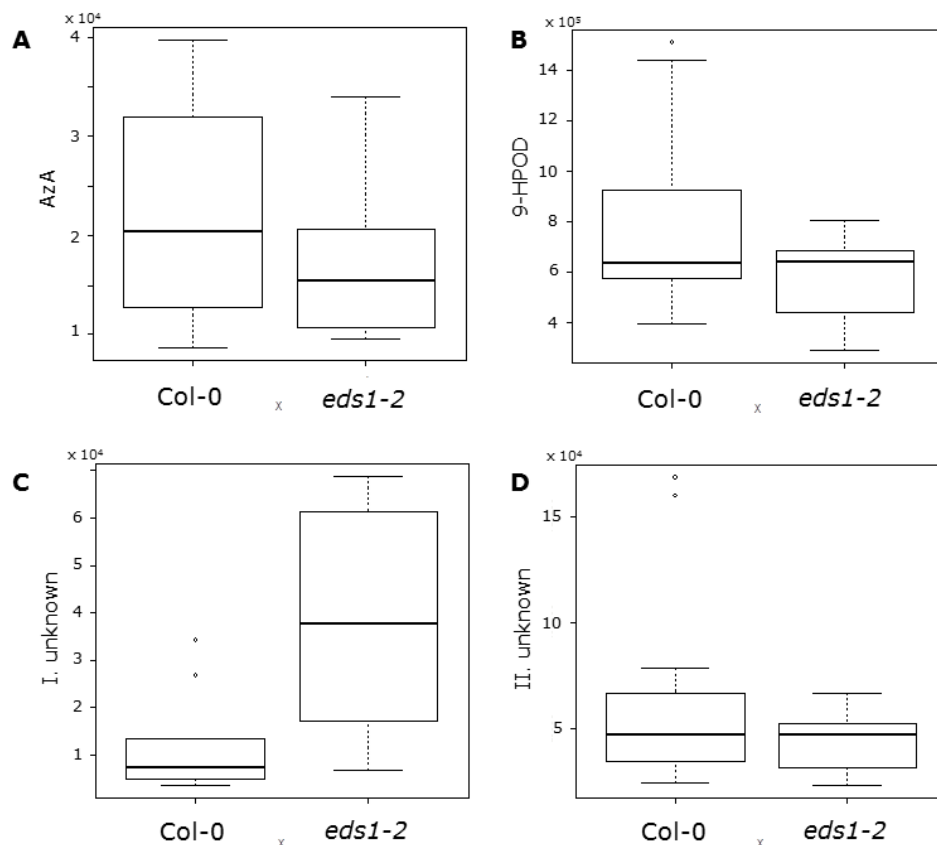
Figure 30: MS measurement of pure MeOH extracts from Col-0 *DEX* and *eds1-2 DEX*. (A) AzA. (B) 9-HPOD. (C) possibly a derivative or isomer of ONA. (D) the unknown compound. The box plots include data from 10 independent biological replicates.

In summary, the differential accumulation of ONA, AzA, 9-HPOD, and the unknown compound in extracts from Col-0 *DEX* as compared to *eds1-2 DEX* is required for SAR. MeOH extracts do not display such differential accumulation. At the same time, these extracts do not trigger SAR. It can be hypothesized that ONA, AzA, 9-HPOD, and the unknown compound accumulate by enzymatic or non-enzymatic (e.g. oxygenation) reactions in the SAR-inducing extracts during the extraction procedure. Importantly, the prerequisites for their accumulation in the extracts are given only in the extracts from Col-0 *DEX* plants and not in extracts from the mutant, confirming a strong correlation between SAR and ONA, AzA, 9-HPOD, and the unknown compound.

3.14   AzA and ONA induce SAR in Arabidopsis

AzA was previously described as a SAR-inducing compound (Jung, Tschaplinski et al. 2009). Jung et al. used 1 mM of AzA to induce systemic defence in *Arabidopsis*. Together with our data, different concentrations of AzA (solved in 5 mM MES) were tested in classical SAR experiments WT *Arabidopsis*. Figure 31 shows that 1 mM of AzA, but also higher concentrations (5 mM and 10 mM) trigger SAR. By contrast, lower concentrations of AzA (100 µM and 500 µM) do not trigger SAR.

Figure 31: SAR experiment with different concentrations of AzA. WT plants were locally treated with MOCK, DC3000 *AvrRpm1*, 5 mM MES and different concentration of AzA. Growth of secondary DC3000 inoculum was scored four days after infection of the systemic tissue. Asterisks above bars indicate statistically significant differences as compared to MOCK or MES controls (* P < 0.05, Student´s t test). Experiments were repeated two times with similar results.

The concentration of AzA in SAR-inducing HPLC fractions was estimated to be in the range of 500 µM. This means that the concentration of AzA in the bio-active fractions is in a biologically active range even if a 500 µM AzA control did not reproducibly trigger SAR. However, AzA was not the only metabolite identified in the bio-active ACN fraction from Col-0 *DEX*. Therefore, different concentrations of ONA and mixtures of ONA with AzA were tested for SAR-inducing activity.

Figure 32 shows that a concentration of 500 µM of ONA triggers systemic immunity. SAR was not triggered by 100 µM of ONA. In addition, mixture of 250 µM AzA with lower concentrations of ONA also did not trigger SAR. By contrast, a mixture of 250 µM AzA with 250 µM ONA did trigger SAR in WT *Arabidopsis*. The latter SAR induction may be caused by ONA, because experiments below will reveal that 250 µM of ONA alone can trigger SAR (Figure 33 A). Together, the data indicate that AzA and ONA can induce SAR in Arabidopsis. ONA is active at lower concentrations than AzA.
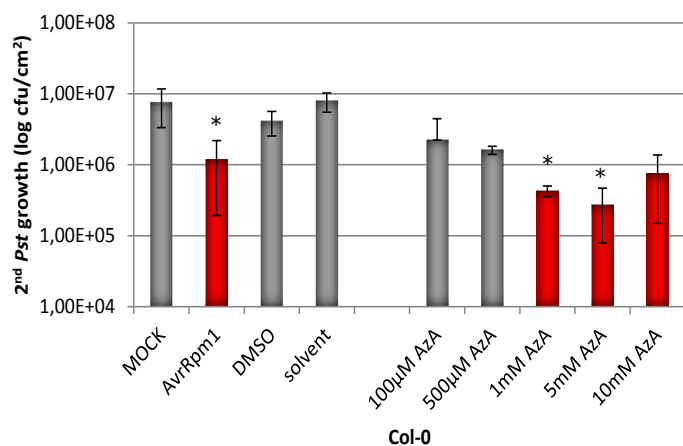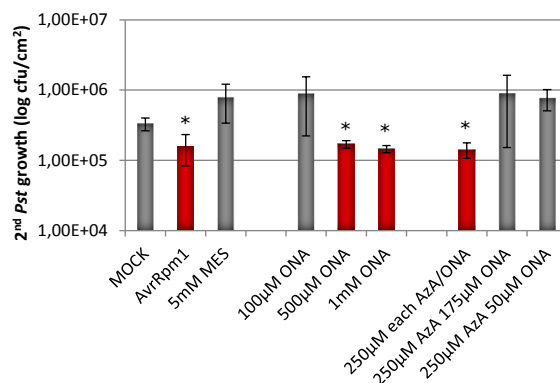
Figure 32: SAR experiment with different concentrations of ONA and AzA. WT plants were locally treated with MOCK, DC3000 *AvrRpm1*, 5 mM MES, different concentrations of ONA, mixtures of ONA and AzA in different concentrations. Growth of secondary DC3000 inoculum was scored four days after infection of the systemic tissue. Asterisks above bars indicate statistically significant differences as compared to MOCK and MES control (* P < 0.05, Student´s t test). Experiment was done twice with similar results.

3.15 ONA, AzA and neopterin trigger SAR in SA-signalling and JA-insensitive mutants

The SAR-inducing activity of AzA, ONA and neopterin was tested in SA-deficient (*sid2*), SA signalling (*med4-2*, *npr1*), and JA-insensitive (*jar1*) mutants as well as in *eds1-2*. This was done to establish which defence signalling pathways are required for SAR induction

By using DC3000 *AvrRpm1* or AzA SAR is triggered in the WT only (Figure 33 A). By contrast, 0.25 mM of ONA triggers SAR in WT (A), *jar1* (B) and *med4-2* (C) plants. This concentration of ONA was not tested in *npr1* (D), *eds1-2* (E) and *sid2* (F) plants. By using a higher concentration of ONA (0.5 mM), it conferred systemic resistance to WT (A) and *npr1* (D) plants only. In addition, SAR was triggered in WT (A) and *npr1* (D) plants by a mixture of ONA and AzA. neopterin was able to trigger SAR in WT (A), *jar1* (B), and *med4-2* (C) plants, but not in the *npr1* (D) mutant. By contrast, *eds1-2* (E) and *sid2* (F) mutant plants were insensitive to any of the different compounds used. It should be noted that this experiment has not yet been repeated. Therefore, the data are considered preliminary, also in light of their poor statistical significance.
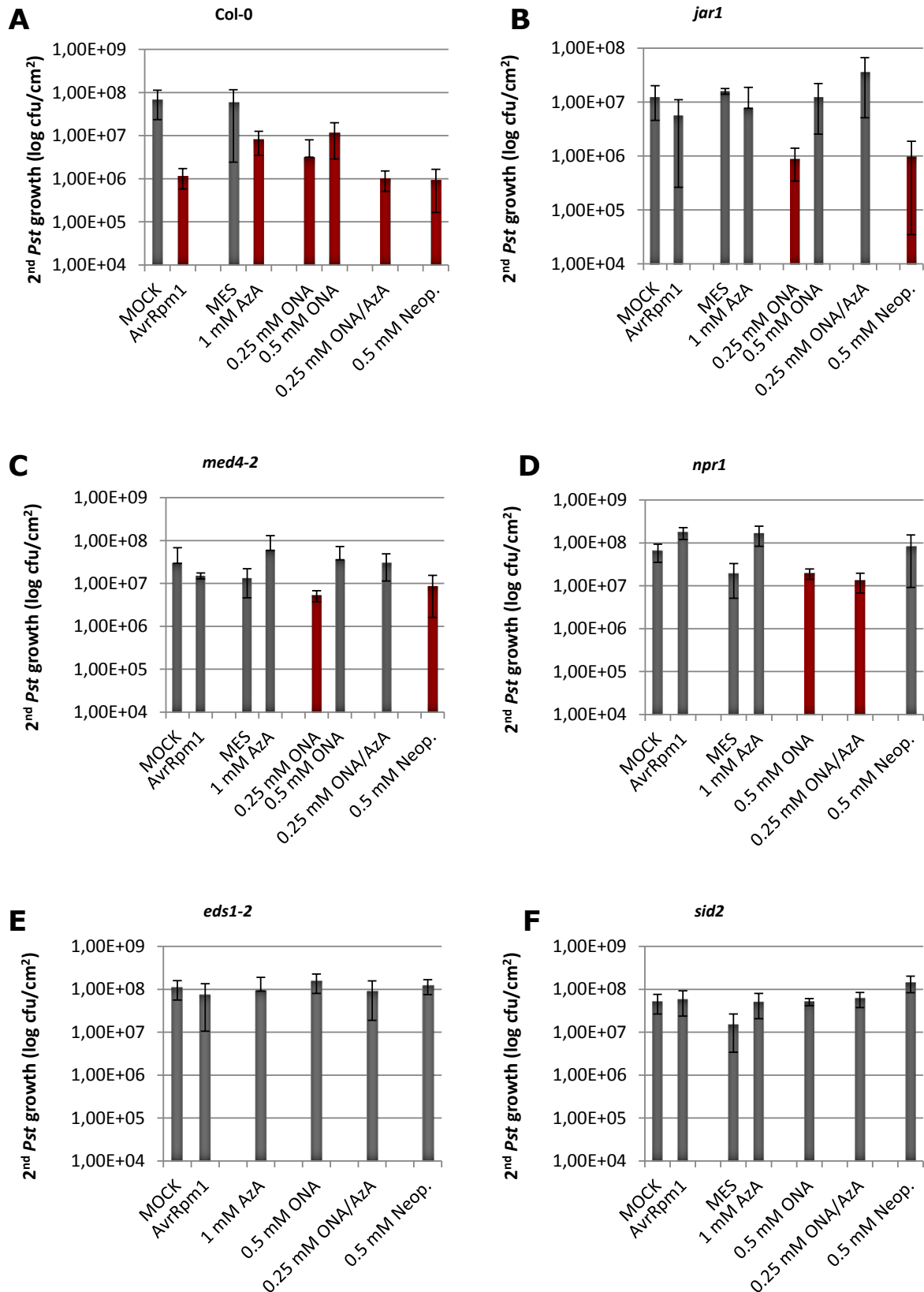
Figure 33: SAR experiment in defence signalling mutants. WT and mutant plants were locally treated with MOCK, DC3000 *AvrRpm1*, MES, 1 mM AzA, 0.25 mM ONA, 0.5 mM ONA, a mixture of 0.25 mM ONA and 0.25 µM AzA, and 0.5 mM neopterin. Growth of secondary DC3000 inoculum was scored four days after infection of the systemic tissue. Asterisks above bars indicate statistically significant differences as compared to

MOCK and MES control (* P < 0.05, Student´s t test). This experiment was done once and is considered preliminary.

Nevertheless, these results indicate that AzA-induced resistance is *EDS1*-dependent and requires SA and JA signalling for full activity. ONA-induced resistance is also dependent of *EDS1* and SA, but may be independent of *NPR1* and of JA signalling. Neopterin-induced resistance is dependent on *EDS*1 and SA, possibly also on *NPR1*, but not on JA signalling.

3.16 *LOX1* and *LOX5* expression are regulated in a time-dependent manner

LOX1 of *Arabidopsis* encodes a 9-LOX and was shown to be involved in response to pathogen infection (Melan, Dong et al. 1993, Melan, Enriquez et al. 1994, He, Fukushige et al. 2002). *LOX5*, also encoding a 9-LOX, appears to be involved in response to insects and wounding (Bannenberg, Martinez et al. 2009, Kilaru, Herrfurth et al. 2011). Because HPOD, ONA, and AzA accumulate downstream from 9-LOX, the gene expression of both *LOX1* and *LOX5* was monitored in the experimental set-up used in this thesis. Leaves of Col-0 *DEX* and *eds1-2 DEX* were treated with either Tween or DEX and harvested before and 2, 4, 8, 24, 48 and 72 hpt. The RNA was isolated and cDNA was generated.

pPCR with gene-specific primers, showed the expression level of *LOX1* increased to much higher levels after DEX treatment as compared to Tween treatment (Figure 34 A) in Col-0 *DEX* and *eds1-2 DEX* plants. There was little difference in *LOX1* induction detectable between Col-0 *DEX* and *eds1-2 DEX* mutant plants. In the experiment shown in Figure 34, WT transiently supported more *LOX1* expression as compared to the mutant at 48 hpt. In a second experiment, a similar difference was observed at 72 hpt. If relevant, this difference in *LOX1* induction between Col-0 DEX and mutant is small and transient.

The expression level of *LOX5* slightly decreased to a similar extent upon Tween or DEX treatment of leaves of Col-0 *DE*X and *eds1-2 DEX* plants (Figure 34 B).
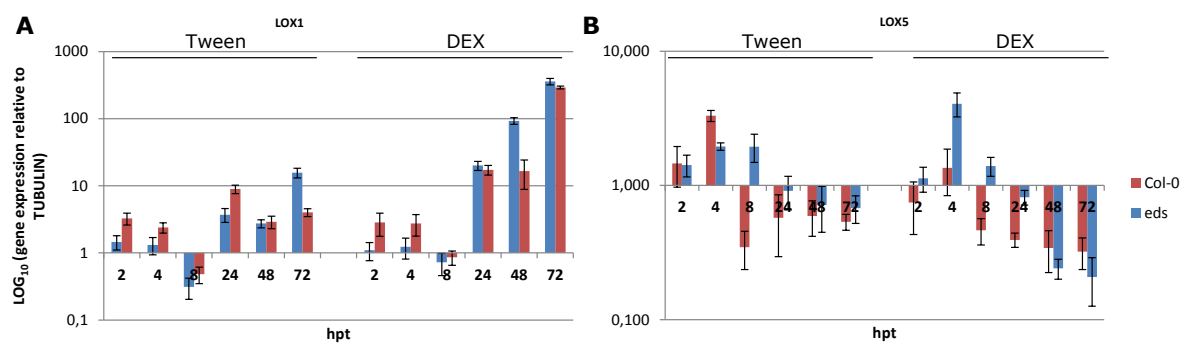
Figure 34: Expression of *LOX1* and *LOX5* in Col-0 *DEX* and *eds1-2 DEX* plants. Leaves were treated with Tween or DEX and harvested before and 2, 4, 8, 24, 48, and 72 hpt (hours post treatment). Gene expression was monitored by using qRT-PCR and normalized to TUBULIN. (A) Relative expression of *LOX1*. (B) Relative expression of *LOX5*. Experiment was repeated twice with similar results.

4. Chapter – DISCUSSION

Various signalling pathways are activated after microbial attack of plants. The main goal of the plant is to keep the intruder isolated from healthy tissue by establishing a fast and strong immune response. In 1961, Frank Ross provided the first evidence for long-distance regulation of induced resistance. After local treatment of tobacco leaves with TMV, plants showed necrotic lesions. This HR reaction of lower leaves led to enhanced resistance in the upper leaves in response to a second infection (Ross 1961). This phenomenon was introduced as systemic acquired resistance (SAR), which is considered a long-lasting and broad spectrum disease resistance. In 1970, Van Loon et al. described that locally and systemically induced resistance were associated with the induction of PR proteins (Van Loon and Van Kammen 1970). Different factors are described that are able to trigger SAR. SA undoubtedly plays an important role. However, an increasing number of other metabolites potentially involved in SAR are being uncovered by research groups worldwide. In 2012, Dempsey and Klessig reviewed the state-of-the-art of different SAR-inducing signals and how these interact with each other in an emerging SAR signalling network (Dempsey and Klessig 2012).

The aim of this thesis was to add new signalling partners to this network by using the *Arabidopsis* WT Col-0 as compared to the *eds1-2* mutant, which is incapable of emitting SAR signals. The metabolite profile was compared of WT and *eds1-2* mutant plants upon expression of the bacterial effector AvrRpm1 from a DEX-inducible transgene. Moreover, a new extraction method was employed. First, metabolites from plant MeOH extracts were fractionated into highly lipophilic, lipophilic and remaining aqueous phases. Subsequently, the bio-active highly lipophilic phase was transferred back to MeOH via solid phase extraction and metabolites were separated by HPLC across a MeOH gradient. FTICR-MS of SAR-inducing HPLC fractions from Col-0 *DEX* as compared to mutant plants revealed neopterin as a new potential SAR signalling molecule. *In planta* tests confirmed that neopterin can trigger SAR. According to literature, it is used as a marker for immune system activation in humans (Murr, Widner et al. 2002).In plants, neopterin is part of the folate biosynthesis pathway (see below).

Additionally, by performing HPLC across an ACN gradient followed by LC-MS on SAR-inducing fractions from the previous MeOH gradient, HPOD, ONA, AzA, and an unknown compound were identified in bio-active fractions from Col-0 *DEX* plants. Finally, *in planta* tests confirmed that AzA and ONA as well as a mixture of AzA and ONA can induce SAR. According to literature, 9-HPOD and 13-HPOD play a role in plant-fungus interactions (Burow, Gardner et al. 2000) and display antimicrobial activity (Sucharitha and Devi 2010). AzA is involved in systemic immunity in *Arabidopsis* (Jung, Tschaplinski et al.

2009). ONA, the precursor of AzA, is part of the lipid peroxidation/degradation pathway (Zoeller, Stingl et al. 2012). By contrast, little is known about its role in plant immunity.

4.1 SAR induction by *AvrRpm1* expression in dexamethasone-treated plants

In this work, SAR is triggered by bacterial infection or is mimicked by expression of the bacterial effector AvrRpm1 from a DEX-inducible transgene. By using the latter system for the metabolomics studies, it is ensured that only metabolites are detected that accumulate in the plant, but not metabolites derived from bacteria. After the promoter is activated the DEX plants show HR-like symptoms (Figure 11 C) that are similar to a bacteria-induced HR in Col-0 and *eds1-2* plants (Figure 11 B). This indicates that defence signalling is activated upon expression of *AvrRpm1*. On the one hand, resistance was confirmed by elevated expression of *PR1*, a SAR-marker, in the DEX-treated tissue (Figure 14 B). On the other hand, it was not yet clear if DEX-induced SAR was a "true" SAR or caused by movement of DEX into the systemic tissue. The latter would trigger expression of *AvrRpm1* in the systemic tissue, which would lead to a resistance that is similar to SAR. In the experiment shown in Figure 12 B, local DEX treatment of Col-0 *DEX* plants reduced growth of a secondary DC3000 inoculum in the systemic tissue by approximately 10-fold as compared to its growth in uninduced plants. By contrast, the *eds1-2* mutant did not show SAR (Figure 12). This phenomena fits to the described *eds1-2* phenotype (Truman, Bennett et al. 2007).

Tween treatment led to a slight expression of *AvrRpm1* in the treated leaves of Col-0 *DEX* and mutant (Figure 13 A and C). We conclude that this expression level is due to leakiness of the DEX-inducible promoter (Figure 14 A and B). As expected, DEX treatment induced *AvrRpm1* expression in the treated leaves (Figure 13 A and C) (Geng and Mackey 2011). By contrast, local DEX treatment did not affect expression of *AvrRpm1* in the systemic tissue of Col-0 *DEX* and mutant, confirming that DEX-induced SAR is not due to mobility of DEX and subsequent systemic expression of *AvrRpm1* (Figure 13 B).

PR1 is used as a marker for SAR induction (Van Loon and Van Strien 1999). Therefore, *PR1* gene expression was monitored in treated and systemic leaves of both plant types in response to both treatments (DEX and Tween) (Figure 14). Both treatments trigger similar levels of *PR1* induction in the treated leaves. In general, these results indicate that the plants are very sensitive. It appears that the plants respond to handling and to the solutions used. DEX treatment led to an early and transient boost of *PR1* transcripts in the systemic leaves of WT *DEX* (Figure 14 B). By contrast, the mutant showed higher

PR1 expression levels after DEX treatment in the treated leaves (Figure 14 C), but lower expression levels as compared to Col-0 *DEX* in the systemic tissue (Figure 14 C and D).

Taken together, these data confirm known phenotypes of the *eds1-2* mutant, which responds normally to *AvrRpm1* in treated/infected tissues. By contrast, *AvrRpm1*-induced SAR, including systemic induction of *PR1* expression, is compromised in the *eds1-2* mutant (Truman, Bennett et al. 2007).

In summary, these experiments confirmed that initiation of SAR or SAR-like disease resistance is possible by a bacterial effector in the absence of other pathogen-derived elicitors.

4.2 Outcome of the different extraction methods

In collaboration with Dr. Thomas Hoffmann and Prof. Dr. Wilfried Schwab (TUM, Biotechnology of Natural Products) the metabolite profiles of extracts from *AvrRpm1*-expressing WT and *eds1* mutant plants were analysed. First, we focused on the apoplast. In previous work, Vlot et al. found proteins in Col-0 *DEX* which accumulated in the apoplast in an *EDS1*-dependent manner (Vlot, Parker conducted the analysis at the Max Planck Institute for Plant Breeding Research, Cologne, Germany, unpublished). Moreover, the apoplast presents the first site of contact with pathogens and plays a crucial role in triggering and coordinating many defence responses (Wojtaszek 2000). It is also known that the apoplast is the route for diffusible signals orchestrating the defence response (Chappell, Levine et al. 1997). By performing apoplast extraction followed by LC-MS analysis, one metabolite was found in the negative ion mode that accumulated to higher levels in the apoplast of Col-0 *DEX* as compared to *eds1-2 DEX* plants (Supplement Figure 41).

In general, relatively few masses were detected in the apoplast extracts. This might be improved by optimizing the composition of the apoplast extraction buffer to increase the detection of metabolites (Witzel, Shahzad et al. 2011). However, additional problems were faced in this approach. By using the vacuum pump during the apoplast extraction procedure, there was no secure way to avoid damaging the plant material, in particular the cell membranes. Therefore, changes of the metabolic state of the apoplast may appear due to differential cytosolic leakage during different extractions. Furthermore, apoplast extracts from *AvrRpm1*-expressing WT did not trigger SAR. Isolation of the apoplast provides a relatively small subset of metabolites from the plant. This might not be enough to trigger SAR. Along the same line, the amount of plant material used to

isolate apoplast extract might not have been sufficient. Finally, essential metabolites could have been lost by usage of C18 columns to concentrate the apoplast extracts.

In conference with Dr. C. Vlot-Schuster as well as with Dr. Thomas Hoffman the extraction method was changed to MeOH-extraction of whole plant tissue. This should guarantee that more metabolites can be found by using LC-MS. Indeed, upon performing this extraction method, the MS detected more masses that accumulated differentially in Col-0 *DEX* versus *eds1-2 DEX* plants as compared to the apoplast extraction method. It is important to note that the previously found, potentially SAR-related metabolite in the apoplast was also detectable at higher levels in the MeOH extracts from Col-0 *DEX* as compared to *eds1-2 DEX* plants. We were not able to identify any metabolites based on their masses by different searching tools and databases (i.e. KEGG). By contrast, the analyses made clear that the scope of the search had to be narrowed. We were not sure what kind of metabolites we were looking for and faced a wide variety of possibilities. There is a huge discrepancy between the number of genes in e.g. *Arabidopsis* and the number of known reactions catalysed by different types of enzymes in the same plant. Moreover, very often MS alone is insufficient to identify the exact position of oxidation on molecules, to differentiate between isomers, or to provide the precise structure of unusual and/or unstable metabolites (Prakash, Shaffer et al. 2007). This lead to the conclusion that certain parameters of the SAR-active metabolites in the extracts should first be determined before further analysis of masses.

To further evaluate the success of the MeOH extraction method, we performed a SAR experiment with the extracts. Unfortunately, local treatment with MeOH extracts did not trigger SAR in WT or mutant *Arabidopsis* (Supplement Figure 42). Preliminary experiments showed that DEX-treated leaves must be attached to the plant for at least four hours to detect SAR upon infection of systemic leaves two days later (Vlot and Parker, unpublished). However, it is not yet clear if SAR signals have fully matured in that limited period of time. In view of the results of SAR experiments with apoplast and MeOH extracts, which were harvested 4.5 hours after DEX treatment of Col-0 *DEX* or *eds1-2 DEX* plants, we assume that in time SAR signals are not yet fully developed/matured.

Since more masses were detected in the MeOH extracts as compared to the apoplast extracts, the MeOH extracts were used for further experiments.

4.3 Extraction method – bio-assay-assisted separation of SAR signalling molecules

The metabolites in the MeOH extracts were separated into hydrophobic and hydrophilic phases by using liquid-liquid extraction. This was one of the most important steps of this work and provided essential information about the nature of the SAR-inducing compound(s) in the extracts. Liquid-liquid extractions were performed with PE and DME. PE is a mixture of pentane and hexane. PE is more apolar than DME and yields a more specific extract for neutral lipids (Dowgiallo 1975), i.e., energy reserve and phospholipids. The PE phase contained highly lipophilic molecules, whereas the DME phase contained lipophilic molecules. Both phases as well as the aqueous rest were processed and applied to plants in a SAR bio-assay. Figure 16 shows that the PE phase from *Col-0 DEX* plants triggers SAR in WT *Arabidopsis*. By contrast, the same PE phase was not able to induce defence in the *eds1-2* mutant (Supplement Figure 43). Moreover, the PE phase from *eds1-2 DEX* did not trigger SAR in WT plants. Together, these results show that the SAR-inducing activity in Col-0 *DEX* is highly lipophilic and dependent of *EDS1*. Most likely, proteins are not associated with this SAR-inducing activity, because these should be fully denaturated during the extraction procedure using 100% MeOH.

The fact that the PE phase from Col-0 *DEX* plants triggers SAR in naïve WT plants (Figure 16) indicates that the extraction method is working. At least three independent repetitions of this experiment confirmed that metabolites isolated by PE can trigger SAR. The PE phase was not analysed by LC-MS. Therefore, no statement can made about its exact lipid composition. However, further bio-assay-assisted purification steps continued to indicate that the SAR-inducing metabolites are highly lipophilic nature. An advantage of this extraction method is simplicity and low cost.

For further experiments it was important to have the SAR-inducing compound(s) in MeOH. Figure 17 reveals that SAR-inducing metabolites from the PE phase from Col-0 *DEX* eluted from a C18 column with 75% and 100% MeOH and with PE. Each of these eluates triggered SAR in WT *Arabidopsis*; they were combined into one sample (PE-MeOH phase) for further processing. By contrast, the three constituents of the PE-MeOH phase isolated from *eds1-2 DEX* did not trigger SAR in WT plants. Finally, SAR could not be triggered in the *eds1-2 Arabidopsis* mutant with the bio-active constituents of the PE-MeOH phase from Col-0 *DEX* (Supplement Figure 44).

Taken together, it can be hypothesized that a certain process takes place in the extracts that is involved in developing or maturing SAR-inducing compounds. As discussed above, the MeOH extracts from Col-0 *DEX* plants did not trigger SAR. After liquid-liquid extraction, however, the PE and PE-MeOH phases are able to trigger SAR. Perhaps the

MeOH extract contains precursors of SAR-inducing molecules that mature during liquid-liquid extraction. During such extractions, the metabolites are exposed to oxygen, which can support e.g. non-enzymatic lipid peroxidation. Alternatively, the MeOH extract might contain inhibitors of SAR and/or specific inhibitors of the SAR-inducing metabolites isolated by PE. Such inhibitors would mask the presence of SAR-inducing metabolites in the bio-assay used, but may have been lost during the liquid-liquid extraction.

Further fractionation of the PE-MeOH phase was performed to narrow down the number of potentially active compound(s). By using HPLC across a MeOH gradient, SAR-inducing activities from the PE-MeOH phase could be separated into three bio-active ranges of the HPLC chromatogram (Figure 19). By contrast, fractions from the corresponding ranges of the chromatogram of extracts from *eds1-2 DEX* did not trigger SAR (Figure 19). Similar to the PE and PE-MeOH phases, SAR-inducing HPLC fractions from Col-0 *DEX* did not induce systemic immunity in *eds1-2* mutant plants (Supplement Figure 45). Furthermore, related to the HPLC chromatogram the SAR-inducing fractions were eluted with ca. 90% of MeOH (first bio-active range), 100% MeOH (second bio-active range), and with 75% of MeOH (third bio-active range). This is consistent with the elution of SAR-inducing metabolites from the PE phase eluting from C18 columns in MeOH concentrations of 75% and more (Figure 17).

SAR induction is regulated by diverse signalling pathways and compounds (see the introduction of this thesis). In this work, we mainly focused on lipids involved in SAR in *Arabidopsis*. There are many examples of lipid-related processes that are linked to SAR. For instance, SAR is abolished in different mutants affected in chloroplast glycerolipid metabolism (Chaturvedi, Krothapalli et al. 2008). *EDS1* codes for a lipase-like protein with unknown function. According to different experiments we showed that SAR-inducing fractions from Col-0 *DEX* were not able to trigger systemic immunity in the *eds1-2* mutant. Moreover, the highly lipophilic SAR signalling metabolites from Col-0 *DEX* extracts are missing from extracts from the *eds1-2 DEX* mutant. Although a direct effect of the mutation on SAR signal accumulation is unlikely, one could hypothesize that the mutant lacks an important enzyme involved in the lipid signalling pathway leading to SAR-inducing activity. Alternatively, *EDS1* may affect SAR signal accumulation more indirectly due to its pronounced effect on SA signalling.

4.4 FTICR-MS

Fourier transform ion cyclotron resonance (FTICR)-MS provides a very high resolution mass analysis. This method allows the measurement of the empirical formula for thousands of metabolites. Therefore, this method was applied to identify metabolites in

SAR-inducing versus non-active HPLC fractions. The limiting aspect of this MS method is that it does not include chromatographic separation prior to mass analysis. Therefore, isomers cannot be distinguished, which, given the functional importance of isomers in biology, is an important hindrance (Aharoni, Ric de Vos et al. 2002). Before FTICR-MS was conducted new MeOH gradient HPLC fractions were tested in a SAR experiment. The three bio-active ranges of the chromatogram were confirmed (Figure 20 A and B) and assigned to fractions 5, 8, and 11 of the Col-0 *DEX* extract (Figure 20). The fractions 3 until 14 from extracts of each genotype were analysed by FTICR-MS in an effort to identify metabolites involved in SAR. From the UV chromatogram it was not expected that the fractions 14 and 15 from the Col-0 *DEX* extract would induce SAR. After mass analysis these SAR-inducing fractions were excluded, because they contained soap leftovers the glass flasks used.

When the HCE (Hierachical Clustering Explorer) software compared masses detected in SAR-inducing fractions from Col-0 *DEX* (C5, C8, and C11) to masses detected in all other fractions from Col-0 DEX and mutant plants, 38 different masses were identified that were present in the SAR-inducing fractions only (Supplement Table 10). Subsequently, masses in the SAR-inducing fractions from Col-0 *DEX* were compared to masses in the corresponding fractions from mutant extracts. In this comparison, 320 different masses were found that were specific for WT and thus potentially related to SAR. Of these 320 masses, four could be identified (Figure 21). D-glycero-D-manno-heptose 1,7-bisphosphate and seduheptulose 1,7-bisphosphate belong to the carbon fixation pathway and have the same mass. Therefore, these are either isoforms or one and the same compound. In addition, two metabolites of the folate biosynthesis pathway were identified. These are 2.5-diamino-6-(5´-triphosphoryl-3´,4´-trihydroxy-2´-oxopentyl)-amino-*4*-oxopyrimidine and 2.5-diaminopyrimidine nucleoside triphosphate. None of these four metabolites could be purchased in a pure form. Therefore, close relatives/derivatives were purchased. Figure 35 A shows that sedoheptulose 1,7-diphospate phosphatase (3.1.3.37) hydrolyses seduheptulose-1,7.bisphosphate into sedoheptulose-7P. Both of these compounds were not commercially available. Subsequently, sedoheptulosekinase (2.7.1.14) dephosphorylates sedoheptulose-7P into sedoheptulose, which was available and purchased (Figure 35 A, blue star). Furthermore, Figure 35 B shows that the identified metabolite 2.5-diaminopyrimidine nucleoside triphosphate is converted in plants into 2.5-diamino-6-(5´-triphosphoryl-3´,4´-trihydroxy-2´-oxopentyl)-amino-4-oxopyrimidine. When additional metabolites in the metabolic pathway were checked for their availability, the 7,8-dihydroneopterin (Figure 35 B, left blue star) and the D-(+)-neopterin were the next available compounds and were purchased. The illustrated pathway indicates that 7,8-dihydronepopterin is converted into neopterin (Figure 35 B, right blue star).

A
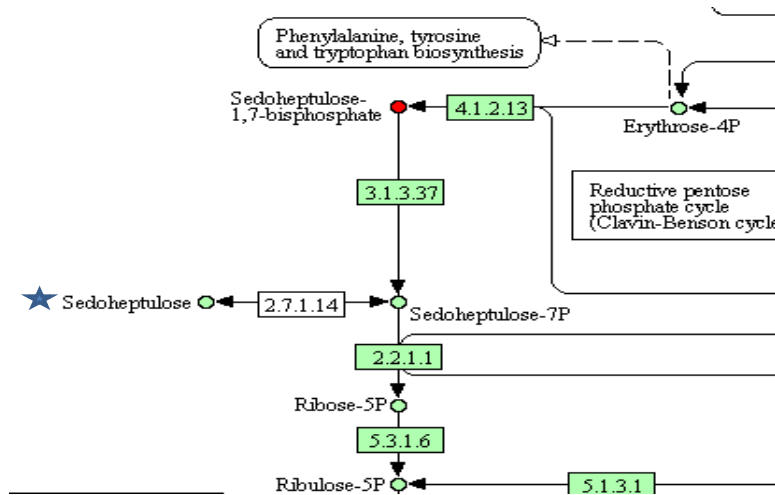
B
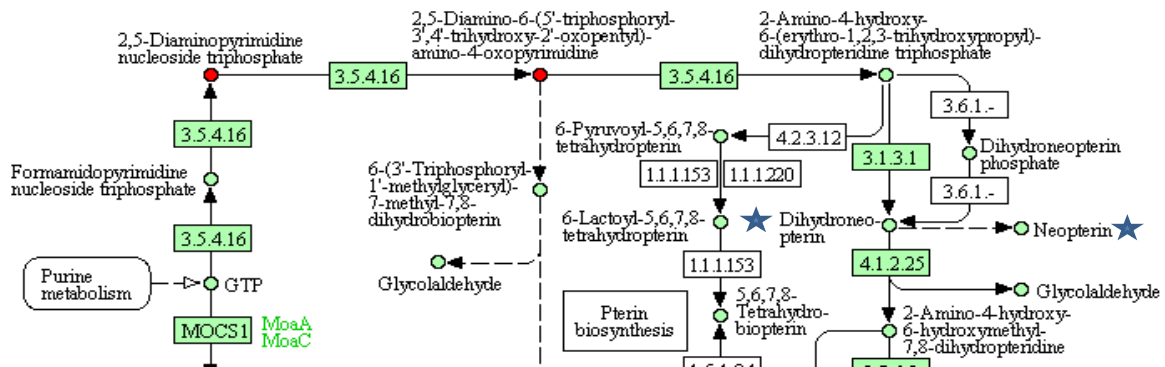
Figure 35: Potentially SAR-inducing metabolites in their biochemical pathways. (A) Identified metabolite seduheptulose-1,7-bisphosphate from the carbon fixation pathway (red dot). The compound sedoheptulose (blue star) was purchased. (B) Identified metabolites 2,5-diamino-6-(5´-triphosphoryl-3´,4´-trihydroxy-2´-oxopentyl)-amino-4-oxopyrimidine and 2,5-diaminopyrimidine nucleoside triphosphate in the folate biosynthesis pathway (red dots). The derivatives D-(+)-neopterin and 7,8-dihydroneopterin (blue stars left and right) were purchased.

The FTICR-MS data were analysed by the MassTRIX program, which compares masses detected in different runs within compound and pathway annotations. The four identified metabolites were found in the negative ion mode. They were part of annotated pathways in *Arabidopsis*. However, most of the detected masses did not lead to an identification of specific metabolites or pathways. There are different problems to face when considering why few metabolites were identified. One reason might be the ion mode used for MS analysis. The four metabolites were identified in the negative ion mode. In principle it is expected that most of the metabolites are identified in the positive ion mode (personal communication with Dr. Thomas Hoffmann). Another factor is that the MassTRIX program takes masses into consideration that were identified with high intensity (10^7) by FTICR-MS. It is predicted that only masses with high intensity are significant. By contrast, we suggest that also masses detected with lower intensity might be of interest and SAR-inducing. Another reason for the relatively low identification efficiency might be

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that plant extracts in general contain many unidentified metabolites, unidentified derivatives, precursors, and metabolites with attached substrates or chemical groups. Therefore, results have to be confirmed by at least another measurement. This is currently in progress.

4.5 Neopterin in plant immunity

Treatment of *Arabidopsis* with 0.5 mM of D-(+)-neopterin (253.215 g/mol, $C_9H_{11}N_5O_4$) triggered resistance in the systemic, untreated tissue against DC3000 (Figure 22 B). However, a higher concentration of 1 mM did not induce a significant SAR response (Figure 22 A). Similarly, 1 mM of the other compounds tested seduheptulose and 7,8-dihydroneopterin (Figure 22 A) did not induce SAR. Increasing evidence suggests an essential role for sugars in plant innate immunity (reviewed in (Bolouri Moghaddam and Van den Ende 2012)). Although seduheptulose is a sugar, it might not itself trigger defence or SAR. Alternatively, the concentration used might have been too high. Therefore, future experiments should include SAR tests using lower concentrations of seduheptulose.

This is the first work establisheding a link between neopterin and SAR. Neopterin is part of the plant folate biosynthesis pathway, which is not yet fully understood, but is probably similar to that in bacteria (Scott, Rébeillé et al. 2000, Hanson and Gregory Iii 2002). Neopterin is most likely synthesised from guanosine triphosphate (GTP). With the help of the enzyme GTP-cyclohydrolase I (GCHI), GTP is converted to dihydroneopterin triphosphate (www.neopterin.net, (Hossain, Rosenberg et al. 2004)). Next steps lead to production of 7,8-dihydroneopterin as well as its conversion to neopterin (dephosphorylation/oxidation) (Sucher, Schroecksnadel et al. 2010). Since 7,8-dihydroneopterin is the immediate precursor for neopterin, it would be surprising if it does not trigger SAR while neopterin does. Therefore, it was concluded that the concentration used for the bio-assay (1 mM) might be too high. Higher concentrations of 7,8-dihydroneopterin as well as of neopterin might for instance lead to toxicity in plants. Consequently, a lower concentration of 7,8-dihydroneopterin should be tested for SAR-inducing activity. In addition, it has yet to be established if the enzyme required for the conversion of 7,8-dihydroneopterin to neopterin is expressed in uninfected plants.

Increased amounts of neopterin are produced in human-macrophages upon stimulation with the cytokine interferon-y (Huber, Batchelor et al. 1984). Increased neopterin production is used as a marker to detect acute infections, including infections by viruses (e.g. HIV), intracellular bacteria or parasites as well as autoimmune diseases, malignant tumor disease and neurological and cardiovascular disease (Murr, Widner et al. 2002).

Interestingly, an increased concentration of neopterin is associated with ROS production in humans (Murr, Fuith et al. 1999). ROS play important roles in innate immunity both in mammals and in plants.

In plants, treatment with neopterin might also lead to ROS production and concomitant activation of defence signalling pathways. Such events would then lead to SAR activation as detected in our experiments. The *AtFOLT1* gene has the closest homology with mammalian mitochondria folate transporters (MFTs). However, disruption of the *AtFOLT1* gene in *Arabidopsis* does not lead to phenotypic alteration in folate-sufficient or folate-deficient plants. The *atfolt1* null mutant contains WT levels of folates in chloroplasts and preserves the enzymatic capacity to catalyse folate-dependent reactions in this compartment (Bedhomme, Hoffmann et al. 2005).

As shown in this thesis, the identified metabolite neopterin can trigger SAR. Our data provide evidence that neopterin is a strong signal to induce SAR. We would expect that also other metabolites or precursor of neopterin might trigger SAR. As described above we also want to test a lower concentration of 7,8-dihydroneopterin in *Arabidopsis*. Another interesting point might be, if metabolites accumulating further downstream in this pathway are involved in SAR. As described above, neopterin is used as a marker for different diseases in humans. Therefore, it would be interesting if neopterin accumulates after pathogen infection in the local tissue and serves as a marker in response to disease in plants as well.

Tetrahydrofolate and its derivatives are essential cofactors for the one-carbon transfer reaction in all organisms. Plants make folates *de novo* from pterin, p-aminobenzoate (PABA), and glutamate (Scott, Rébeillé et al. 2000, Hanson and Gregory Iii 2002). Humans and other mammals lack a complete folate-synthesis pathway and thus need dietary folate. Therefore, plant foods are a major folate source (DellaPenna 1999, Scott, Rébeillé et al. 2000). The richest sources are leafy vegetables, legumes, and certain fruits. By contrast, folate levels are extremely low in cereals and in root and tuber crops. Since the past 10 years, efforts have been ongoing to develop bio-fortified food to reduce folate-deficiency in humans (Hossain, Rosenberg et al. 2004). We hypothesize that such bio-fortified crop plants also might display elevated disease resistance in the field.

4.6 LC-MS acetonitrile gradient

To get a better separation of the SAR-inducing lipid (derived) metabolites in the extracts, the SAR-inducing HPLC fractions from the MeOH gradient were fractionated further by HPLC across an ACN gradient. By comparing mass spectra recorded of the different HPLC

chromatograms an attempt was made to limit the number of overlaps in SAR-inducing activities between ACN fractions from the different MeOH fractions. HPLC-assisted separation of compounds is never absolute and a partial overlap of compounds in neighbouring fractions must be expected. Such overlapping compounds in neighbouring MeOH fractions would display an identical fractionation/elution pattern when fractionated across an ACN gradient.

Subsequently, masses were analysed in SAR-inducing ACN fractions from the first SAR bio-active range of the MeOH gradient of WT extracts. These mass peaks were compared to those in corresponding fractions from the *eds1-2 DEX* mutant. By using the positive ion mode of the MS, 49 masses were identified that were highly present in the *eds1-2* mutant as compared to WT plants (Supplement Table 11). These masses might correspond to accumulating compounds, which cannot be converted further in the mutant due to loss of the EDS1 lipase-like protein. Moreover, the levels of other enzymes may also be altered due to compromised defence signalling in the mutant. The accumulation of intermediates of abrogated pathways in the mutant might lead to toxicity and therefore, high susceptibility of the plant to pathogen attack. Identification of these accumulating compounds can help to characterize the *eds1-2* mutant further and expose altered signalling pathway.

When the negative ion mode of the MS was used, 9-HPOD, ONA, AzA and an unknown metabolite were identified as accumulating to a higher level in SAR-inducing fractions from *AvrRpm1*-expressing WT as compared to mutant plants (Figure 26). The identified compounds are products of the 9-LOX pathway and at least two of them trigger systemic protection against DC3000 in WT *Arabidopsis* plants (Figure 31 and 32). The latter experiment thus confirmed the SAR-inducing activities of ONA and AzA. However, the SAR-inducing activity of the ACN fraction containing 9-HPOD, ONA, and AzA lasted only a short period of storage at minus 80 ºC (Figure 28 A and 29). In fact, when the (previously) SAR-inducing fraction from Col-0 *DEX* plants was re-analysed by MS, AzA, ONA and the unknown compound were not detected (Figure 29), confirming alteration of the SAR-inducing compounds during storage at minus 80 ºC. By contrast, 9-HPOD could still be detected, but at a 30-fold lower level as compared to the first measurement (Figure 29). This result indicates that this level of 9-HPOD cannot trigger SAR. Although it cannot be excluded that higher levels of 9-HPOD can induce SAR, 9-HPOD might 'only' be required to support the SAR-inducing activities of AzA and ONA. In future, it is essential to test the SAR-inducing activity of different concentrations of 9-HPOD to distinguish between these two possibilities. If local application of 9-HPOD triggers SAR, it is of high interest to test if it is converted in plants to ONA and AzA and if this is required for SAR (see below).

Taken together, the results reveal a strong correlation between ONA, AzA, and SAR. Both SAR experiments with ACN fractions indicate that additional SAR-active metabolites might be present in the first and middle bio-active range of the MeOH gradient. One or more of these appear to elute from the HPLC column exactly one fraction after the fraction containing HPOD, ONA, and AzA and is/are more stable at minus 80 °C.

4.7 ONA and AzA in plant immunity

Lipid peroxidation is an essential process to induce plant pathogen defence. Lipids can be oxidized (fragmented) by LOXs (lipoxygenases) and/or ROS (reactive oxygen species) (Mosblech, Feussner et al. 2009). Degradation of lipids is leading to different compounds by two different pathways (Figure 5). The signalling compounds produced are jasmonates (e.g. JA), antimicrobial and antifungal compounds such as aldehydes or divinyl ethers, and volatiles (Feussner and Wasternack 2002). ONA and AzA also can be produced by the 9-LOX pathway.

As introduced above, lipid degradation can be enzymatic or non-enzymatic (Figure 36). The enzymatic LOX pathway was described in detail in the introduction of this thesis. This fragmentation pathway was described in plants only, involves LOX and HPL (hydroperoxide lyase) acts on free fatty acids (Figure 36 A) (Matsui 2006, Zoeller, Stingl et al. 2012). Until recently, a ROS-induced pathway was described only *in vitro* and in animals *in vivo* (Figure 36 B) (Schneider, Porter et al. 2008). The non-enzymatic pathway has been investigated in detail by Schneider et al (2008) and include spontaneous fragmentation of lipid peroxide dimers (Schneider, Porter et al. 2008, Zoeller, Stingl et al. 2012). In mammalian systems, this pathway leads to fragmented glycerolipids (Hazen 2008). A similar ROS-dependent fragmentation of glycerolipids in plants has only recently been proposed (Zoeller, Stingl et al. 2012).
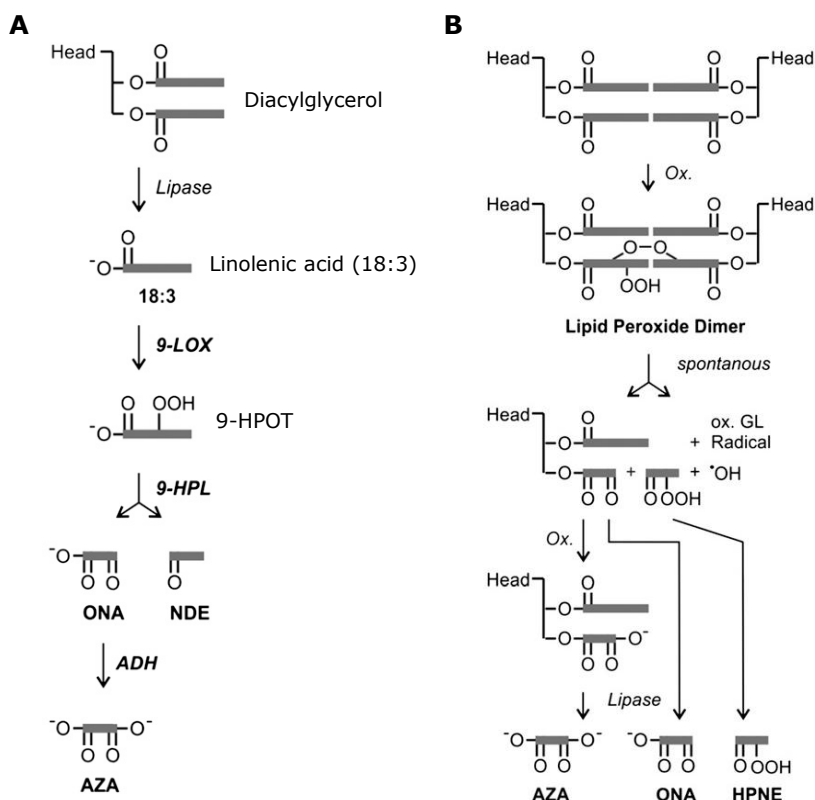
Figure 36: Fatty acid fragmentation pathways. (A) Enzymatic oxidative fragmentation pathway. (B) Radical-catalyzed oxidative fragmentation pathway. LOX: lipoxygenase, HPOT: hydroperoxy-octadecatrienoic acid, HPL: hydroperoxide lyase, NDE: nonadienal, ONA: 9-oxononanoic acid, ADH: aldehyde dehydrogenase, Ox.: oxidation, GL: glycerolipid, HPNE: hydroperoxy nonenal. Transformed illustration taken from (Zoeller, Stingl et al. 2012).

As described in the previous section 9-HPOD, ONA, AZA, and an unknown compound were found in SAR-inducing HPLC fractions from extracts from WT plants and not in the corresponding fractions from the *eds1-2* mutant. ONA and AzA indeed triggered SAR in the bio-assay used in this thesis. According to literature, AzA is involved in local and systemic immunity in *Arabidopsis* against *P. syringae* (Jung, Tschaplinski et al. 2009, Chaturvedi, Venables et al. 2012). AzA was proposed to prime plants for SA accumulation and was suggested to be a long-distance SAR signal (Jung, Tschaplinski et al. 2009). Interestingly, results of Zoeller et al. led to a converse hypothesis about AzA and its relation to SAR (Zoeller, Stingl et al. 2012). This group was neither able to measure AzA in the systemic tissue after infection with an avirulent *Pst* strain nor have they found reduced bacteria growth after AzA treatment of plants. Furthermore, they showed that AzA levels accumulating during a primary infection do not correlate with the strenght of the cocomitant SAR response. Therefore, Zoeller et al. (2012) claim that AzA does not play a role in SAR. This was, in turn, not the case in this thesis. Similar to results of Jung et al. (Jung, Tschaplinski et al. 2009) bacteria growth was reduced upon systemic infection of plants that had been treated locally with 1 mM of AzA. To reconcile the different results, one could argue that AzA is not the end product of this pathway: it is

further converted into PIM (pimelic acid) by ß-oxidation (Zoeller, Stingl et al. 2012). PIM is essential for the biosynthesis of biotin. It serves as a vitamin and also as a cofactor for different enzymes that catalyse carboxylation, decarboxylation, and transcarboxylation (Knowles 1989). So far, PIM was not detected in our extracts, but it will be tested for its SAR-inducing activity in a bio-assay. Perhaps the dependency of SAR on AzA (or PIM) varied between research groups due to differential conversion of AzA into PIM. This could be due to e.g. differences in experimental conditions, including in plant growth conditions.

Zoeller et al. (2012) analysed pathogenesis-associated lipid peroxidation in plants and proposed that AzA is a marker for lipid peroxidation rather than a SAR signal (Zoeller, Stingl et al. 2012). Figure 36 (presented by Zoeller et al.) shows that AzA and ONA accumulate in both enzymatic and non-enzymatic lipid degradation pathways. In the enzymatic pathway, free fatty acids are oxidized by 9-LOX into 9-HPOT, which is transformed by 9-HPL into NDE (nonadienal, volatile) and ONA. ADH (aldehyde dehydrogenase) then converts ONA into AzA. The non-enzymatic pathway can take place by the presence of singlet oxygen or other free radicals (Durand, Bultel-Ponce et al. 2009, Hamberg 2011). With regards to a possible enzymatic generation of ONA in plants, it is of interest, that Col-0 lacks HPLs (Chehab, 2008, Matsui, 2006). Therefore, Zoeller et al. suggest that AzA must be accumulated by the non-enzymatic pathway. This can be a pathway that is similar to that in animal systems (Figure 36) (Zoeller, Stingl et al. 2012). In light of the findings in this thesis, one could speculate that there is another enzyme in Col-0, which takes over the function of HPL to produce ONA from 9-HPOD/T. Alternatively, ONA and AzA detected in this work could be generated by the non-enzymatic pathway.

With respect to the discussion about enzymatic and non-enzymatic pathways to produce AzA, we cannot fully exclude either. During the first 4.5 hours after DEX treatment of the plants, compounds can have accumulated as a result of either one or both pathways. Therefore, gene expression was analysed of the 9-LOX-encoding genes *LOX1* and *LOX5*. Expression of *LOX1* was clearly induced upon expression of AvrRpm1, but to similar levels in WT and *eds1-2* mutant plants. Any difference in the level or timing of *LOX1* expression in WT as compared to *eds1-2* mutant plants was either negligible and/or not reproducible. Therefore, it appears that expression of the gene encoding the first enzyme in the (LOX-mediated) biosynthetic pathway towards AzA is not dependent on *EDS1.* Interestingly, a *lox1* mutant was previously shown to be SAR defective (Vicente, Cascon et al. 2012). This SAR defect was linked to a compromised accumulation of 9-KOT (9-Keto-10(*E*),12(*Z*)-octadecadienoic acid). One could ask the question if AzA accumulation correlates with 9-KOT production. Figure 37 illustrates that both metabolites are produced from hydroperoxides, but in different pathways. Hydroperoxide (9-HPOD and 9-

HPOT) is oxidized to 9-KOT or catalysed to 9-Oxo-C$_9$ (ONA) followed by transformation into AzA.
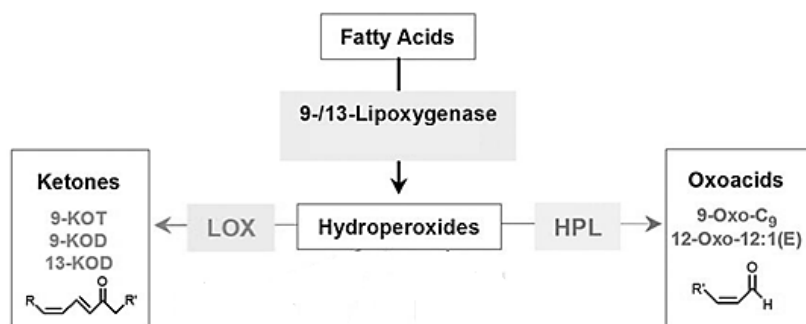

Figure 37: Pathway of fatty acid oxygenation. LOXs oxidize fatty acid into hydroperoxides and further by LOX into 9-KOT, 9-KOD, and 13-KOD. Hydroperoxides can be also catalyzed by HPL into 9-Oxo-C$_9$ or 12-Oxo-12:1(E). LOX: lipoxygenase, 9-KOT: 9-Keto-10(*E*),12(*Z*), 15(*Z*)-octadeca-trienoic acid, 9-KOD: 9-Keto-10(*E*),12(*Z*)-octadecadienoic acid, 13-KOD: 13-Keto-9(*Z*),11(*E*),15(*Z*)-octadecadienoic acid, HPL: hydroperoxide lyase, 9-Oxo-C$_9$: 9-Oxo-nonanoic acid, 12-Oxo-12:1(E): 12-Oxo-10(*E*)-dodecenoic acid. Transformed illustration taken from (Vellosillo, Martinez et al. 2007).

Importantly, Zoeller et al. (2012) showed that AzA accumulates to normal levels in a *lox1lox5* double mutant (Zoeller, Stingl et al. 2012). Thus, AzA does not appear to be involved in *LOX1*-mediated SAR. Since the accumulation of ONA was not analysed in the *lox1* mutant background, it cannot be excluded that this compound is involved in *LOX1*-dependent SAR signalling. Therefore, in future experiments we will analyse if ONA and/or AzA rescue the SAR-deficient phenotype of the *lox1* mutant. Taken together, enzymatic generation of ONA and AzA cannot be excluded by the experiments shown in this thesis, but the *EDS1*-independent expression of *LOX1* does not provide support for the plant's use of the enzymatic pathway to produce ONA and AzA.

MeOH extracts harvested 4.5 hours after DEX treatment of Col-0 DEX plants did not trigger SAR in WT *Arabidopsis*. Since SAR-inducing activity was isolated from the same extracts after further purification steps, something happened to the samples establishing a change from non-active MeOH extracts into active PE-MeOH phases and active MeOH and ACN HPLC fractions. This something must be non-enzymatic, because all enzyme activity was lost in the MeOH extract due to protein denaturation. If we compare signal intensities of the 9-HPOD and AzA mass peaks between MeOH extracts and ACN fractions, it appears that the level of 9-HPOD decreases during sample purification by roughly 10-fold (data not shown). The level of AzA increased during purification and reached a roughly 10-fold higher level in the ACN fraction as compared to the MeOH extract (data not shown, see below). Although this is suggestive for a non-enzymatic conversion of 9-HPOD to AzA during purification of the extracts, it is unknown if such a conversion can occur in plants or *in vitro*. Possibly, accumulation of 9-HPOD was enzymatic, but unrelated to the accumulation of AzA, which was likely formed at least in part during extract purification and thus via the non-enzymatic pathway.

It should be noted that we cannot 1:1 compare signal intensities of mass peaks between MeOH extracts and ACN fractions (Figure 30 and Figure 26). First, the solvent and metabolite compositions vary between these samples and may affect signal intensities measured for specific compounds. Second, single MeOH extracts were measured and compared to ACN fractions from 20X concentrated extracts. Finally, loss of compounds during the purification procedure from MeOH extract to ACN fraction cannot be judged. Therefore, the comparisons used in the paragraph above are crude estimations that require additional experiments for confirmation. For instance, spiking of the MeOH extracts with deuterium-labelled 9-HPOD and AzA would allow quantification of how much of these metabolites is lost during the extraction. If the number of extracts analysed per LC-MS run is then kept constant, such an experiment would provide information on the kinetics of the different metabolites (appearance/disappearance) in the extracts. Moreover, spiking extracts with deuterium-labelled 9-HPOD only should allow us to directly analyse if 9-HPOD in the extracts is converted to ONA and/or AzA.

Of the metabolites identified, ONA shows the best correlation with SAR. ONA is absent from the non-SAR-inducing MeOH extracts, but accumulates to relatively high levels in bio active (SAR-inducing) ACN fractions from WT, but not in the corresponding non-SAR-inducing fractions from *eds1-2* mutant plants. Strikingly, the MeOH extracts contain a metabolite with the same mass as ONA, but with a different retention time on the HPLC column. This metabolite might be a derivative or – more likely – isomer of ONA. Theoretically, it is possible that other metabolites in the MeOH extract affect the retention time of ONA. This can be tested by spiking of the MeOH extracts with an ONA standard. If such an experiment confirms that the ONA-like substance in the MeOH extracts is not ONA, the SAR-inducing activity of extracts/fractions would fully correlate with the accumulation of ONA. Since ONA would be absent from MeOH extracts, it must form during extract purification via the non-enzymatic lipid degradation pathway. Activation of this pathway is expected, since the extract is exposed to oxygen during purification. Alternatively, the ONA-like substance in the MeOH extracts could act as the ONA precursor. Its conversion to ONA also would have to be non-enzymatic.

One could argue that ONA was found in this thesis as an artifact of the extraction method used to isolate metabolites from plants. However, it still represents a striking difference between WT and *eds1-2* mutant plants. Although ONA was formed in the extract from WT plants, most likely by non-enzymatic lipid degradation, one or more prerequisites for its generation were missing from extracts from the mutant. One of these prerequisites might be certain ROS species. Normally, ROS accumulation is triggered downstream from AvrRpm1 recognition. However, *EDS1* affects ROS signalling during the plant defence response, including the accumulation of certain ROS species (Straus, Rietz et al. 2010). Alternatively, the *eds1-2* mutant might simply lack the precursor of ONA. In either case,

the dependency of ONA accumulation on *EDS1* is evident and might indicate functional relevance of ONA and/or the pathway leading to ONA during establishment of SAR *in vivo*. Therefore, we hypothesize that ONA in plants pays a relevant contribution to SAR signalling.

In planta, ONA is either relatively quickly converted into PIM (via AzA, see above) or occurs in its membrane-bound (esterified) form. Since lower concentrations of ONA as compared to AzA are sufficient to trigger SAR, it can be hypothesized that ONA itself rather than e.g. AzA or PIM, is relevant for SAR. This might require a bound form of ONA. During defence signalling and concomitant SAR establishment, ONA may be bound by a lipid-transfer protein. Candidate lipid-transfer proteins include DIR1 and AZI1, which are both required for SAR (Jung et al., 2009; Maldonado et al., 2002). Although additional experiments are required to support these hypotheses, the experiments described in this thesis provide strong support for a role of ONA in SAR signalling.

4.8 Neopterin, ONA, and AzA in SA and JA signalling pathways

To place the SAR signalling molecules identified in this work in known defence signalling pathways, their SAR-inducing capacity was tested in various *Arabidopsis* mutants. It should be noted that this experiment was in part performed only once and that the statistical significance of SAR leaves room for improvement. Therefore, the data should be considered preliminary. For the purpose of this discussion, a statistically not significant, but clear trend towards SAR is considered a positive SAR response.

In the performed experiments the mutants *eds1-2* and *sid2* never responded to pre-treatment with Neopterin, ONA, AzA or the mixture of AzA and ONA (Figure 33 E and F). *SID2* is responsible for up to 90% of SA accumulating in response to pathogen attack (Wildermuth, Dewdney et al. 2001) and the corresponding mutant does not support SAR in response to a primary infection. The controls in this work confirmed the SAR-deficiency of the *sid2* mutant. *EDS1* also acts upstream of SA accumulation during defence responses and is SAR-defective (see the introduction of this thesis). Since both mutants do not mount a SAR response upon a local treatment with neopterin, AzA, ONA, or a mixture of AzA and ONA, all SAR signalling molecules found in this thesis require intact SA synthesis and/or SA signalling via *EDS1* for downstream signalling to establish SAR (Figure 38).

One of the key signalling regulators downstream from SA is NPR1. Of the signalling molecules tested, neopterin and AzA, but not ONA or a combination of AzA and ONA, required *NPR1* for downstream signalling towards SAR. Thus, a clear dependency on SA

and SA signalling was established for neopterin and AzA. In the case of AzA, this confirms previously described findings (Jung, Tschaplinski et al. 2009). ONA or the combination of AzA and ONA require SA, but not *NPR1*, to trigger SAR. Most likely, ONA induces SA-dependent, *NPR1*-independent signalling routes towards defence. In that case, the combination of AzA and ONA would trigger SAR in the *npr1* mutant via the ONA-induced route (Figure 38).

A dependency of the newly identified SAR signalling molecules on JA signalling was tested only in the *jar1* mutant. *JAR1* is involved in signalling downstream from JA (Van Poecke and Dicke 2003). There is some controversy around the role of JA signalling and *JAR1* in SAR in response to pathogen attack with diverse *P. syringae* strains (Shah 2009). At least one previous study indicated that JA is upregulated in response to DC3000 (*AvrRpm1*) (Truman, Bennett et al. 2007). Furthermore, this JA-accumulation was accompanied by expression of JA-responsive genes. Some JA mutant are SAR-defective, others support normal SAR. The control experiment in this thesis using DC3000 *AvrRpm1* support the hypothesis that *JAR1* participates in biologically induced SAR. Of the new signalling molecules tested, it can only be concluded that neopterin does not require JA signalling to trigger SAR. In response to ONA and/or AzA, the dependency on *JAR1* varied within or between experiments. For instance, in the experiment shown in Figure 33 B, two different concentrations of ONA that normally each trigger SAR, show a differential dependency on *JAR1.* Additional experiments, possibly including more JA signalling mutants, are required to unravel the dependency on JA signalling of SAR downstream from ONA and/or AzA.

Finally, interplay of neopterin, ONA and/or AzA with a previously established SAR signal was tested by using the *med4-2* mutant. In this mutant, expression of several *AtMES* genes is reduced, which leads to reduced conversion of the SAR signal MeSA to SA in the uninoculated systemic leaves of *Arabidopsis* (Vlot, Liu et al. 2008). SAR can be activated in this mutant if the primary infection takes place in the morning, but not if the primary infection takes place in the afternoon (Liu, von Dahl et al. 2011). In this thesis, the primary treatments were started in the morning but during long infiltration time some plants were treated in the afternoon and a dependency of SAR on MeSA hydrolysis could be observed in the control, in which the primary treatment was performed with DC3000 *AvrRpm1* (Figure 33 C). From Figure 33 C it is difficult to conclude if there is an interplay between MeSA and neopterin, AzA and/or ONA. Considering data from an experiment using the *med4-1* mutant (not shown), it can be concluded that neopterin triggers SAR independently of MeSA. Similar to *jar1*, the *med4* mutants respond differentially to ONA and/or AzA.
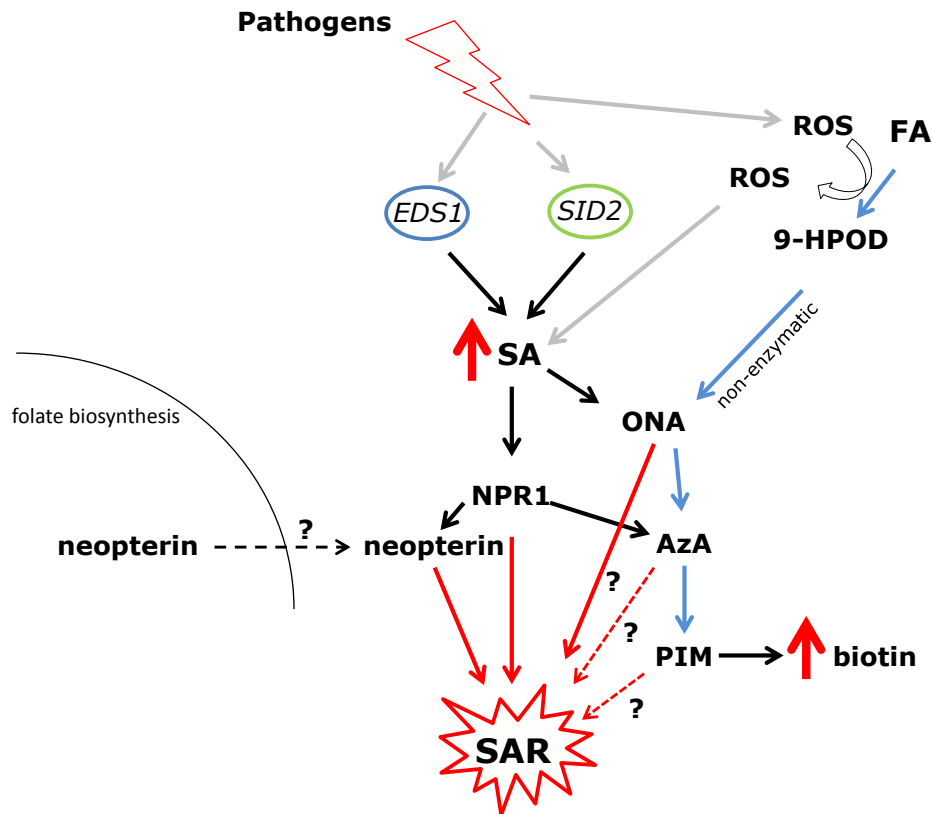
Figure 38: Predicted working model for new potential SAR-inducing signals. The identified metabolites as well as results of this thesis were included in a SAR model for *Arabidopsis*. Some of the regulatory pathways are still under investigation (question marks). For further explanation see the text above. *EDS1*: ENHANCED DISEASE SUSCEPTIBILITY, *SID2*: *SALICYLIC ACID INDUCTION DEFICIENT 2*, SA: salicylic acid, ROS: reactive oxygen species, FA: fatty acid, 9-HPOD: 9-hydroperoxy-octadecadienoic acid, ONA: 9-oxo-nonanoic acid, NPR1: NONEXPRESSOR OF PATHOGENESIS-RELATED PROTEINS1, AzA: azelaic acid, PIM: pimelic acid, SAR: SYSTEMIC ACQUIRED RESISTANCE, grey arrows: stumulus, blue arrows: lipid degradation, black arrows: requirement, red arrow: induction of SAR, thick red arrow: accumulation.

In summary, neopterin, AzA, and ONA trigger SAR via SA and *EDS1.* Neopterin and AzA trigger *NPR1*-dependent SA signalling mechanisms, whereas ONA induces SA-dependent, but *NPR1*-independent signalling routes (Figure 38).

4.9 Conclusion and Outlook

In the first part of this thesis we confirmed that SAR can be induced by the expression of the bacterial effector AvrRpm1 from a DEX-inducible transgene. Subsequently, extracts from *AvrRpm1*-expressing plants were used to isolate and compare the metabolite pattern from WT versus *eds1-2* mutant plants. We established a new extraction method and found new SAR-inducing metabolites that accumulated in plants or extracts in an *EDS1*-dependent and time-dependent manner. In addition to neopterin and ONA, also the previously established SAR signal AzA was confirmed as an *EDS1*-dependent SAR-

inducing signal in *Arabidopsis*. Neopterin, ONA and/or AzA all appear to require SA and/or functional SA signalling to trigger SAR (Figure 38). ONA and AzA are products of the 9-LOX pathway, but we hypothesize that in this work these were non-enzymatically produced during the extraction procedure of metabolites from plants. Finally, this work identified neopterin and ONA as new SAR-inducing signals.

For future plans we have to first confirm the results in at least one more biologically independent repetition of large-scale LC-MS experiments in this work. Moreover, SAR-inducing ACN fractions should be measured also by FTICR-MS (positive and negative ion mode) to increase the chance of additional compound identification (e.g. of the unknown compounds differentially accumulating between WT and mutant).

It would be of interest to analyse masses from *eds1-2* fractions that accumulate to higher levels in the mutant as compared to WT plants. This also should be done by LC-MS (focusing on apolar molecules) and by FTICR-MS (focusing on polar molecules) to get an idea about the unknown compounds (by formula). Similar analyses can be done on pure MeOH extracts. To get a deeper insight into the lipid profile of the *eds1-2* mutant, application of TLC (thin layer chromatography) or GC-FID (flame ionization detector) for fractionation of organic compounds might provide an answer.

ONA and AzA are products of the 9-LOX pathway. It would be interesting to further analyse this pathway in relation to defence and/or SAR. First, 9-HPOD must be analysed to test if this compound itself induces SAR or if it supports the SAR-inducing activity of ONA and AzA. Also, by using deuterium-labelled 9-HPOD it could be tested if this compound is converted into ONA and/or AzA prior to SAR establishment. Subsequently, by using LC-MS it could be measured how the accumulation of the SAR-inducing molecules 9-HPOD, ONA and/or AzA is regulated in plants over time (time-course experiment). To this end, Col-0 *DEX* and *eds1-2 DEX* plants can be used. Similar to the measurement of *PR1* and *AvrRpm1* transcripts, samples should be collected at different time points after DEX treatment. Oxidative processes occurring in the extracts should be inhibited (Zoeller, Stingl et al. 2012) to ensure that plant-derived and not extract-derived compounds are measured. In case ONA and AzA in plants are derived, if only in part, from the LOX pathway, other 9-LOX products should be analysed. 9-HPOD/T is converted into ONA and the volatile compound nonadienal. To this time, the measurement of nonadienal is in progress in the head space of Col-0 *DEX* and *eds1-2 DEX* plants (Bichlmeier, Ghirado, and Vlot, Helholtz Zentrum Muenchen).

The experiments in this thesis show that neopterin, ONA, and AzA trigger systemic resistance. It is currently not clear if these compounds are mobile in plants and thus trigger SAR or if they activate local disease resistance signalling leading to the emission of (other) SAR signals. First, local immunity in response to treatment with neopterin,

ONA and AzA should be tested with e.g. bacterial and fungal pathogens. Furthermore, by using deuterium-labelled compounds their potential translocation through the vascular system can be investigated.

Genetic modification (mutants, silencing lines, and over-expressors) of *Arabidopsis* can be used to get a deeper insight into (signalling) pathways involved in SAR dependent on neopterin, ONA, and/or AzA. With regards to ONA and AzA, underexpression of the gene coding for ADH could for example be used to analyse if ONA itself triggers SAR or if it has to be converted to AzA. In addition, expression of *AZI1* is induced by AzA and *azi1* mutants do not display SAR in response to pathogens or AzA (Jung, Tschaplinski et al. 2009). It would be interesting to test how this mutant reacts to treatment with the AzA precursor ONA. SAR-related signalling downstream from ONA and/or AzA can be investigated in more detail by using microarray analysis of treated and systemic tissues. The same goes for neopterin. With regards to this compound, this is the first work linking folate biosynthesis to SAR. In future work, we will test if the end product of this pathway, folic acid, also triggers SAR.

Taken together, this thesis describes the identification and initial characterization of two new SAR-inducing metabolites, neopterin and ONA. These metabolites are able to trigger SAR in the dicotyledonous plant *Arabidopsis thaliana*. Recent work by others has for the first time shown that SAR-like disease resistance can be activated in the monocotyledonous crop plants maize (Balmer, de Papajewski et al. 2013) and barley (Dey, Wenig, Knappe, and Vlot, unpublished). In terms of potential applications towards crop protection, it will be of interest to test the SAR-inducing metabolites found in this study in these crops.

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6. Chapter - Supplement
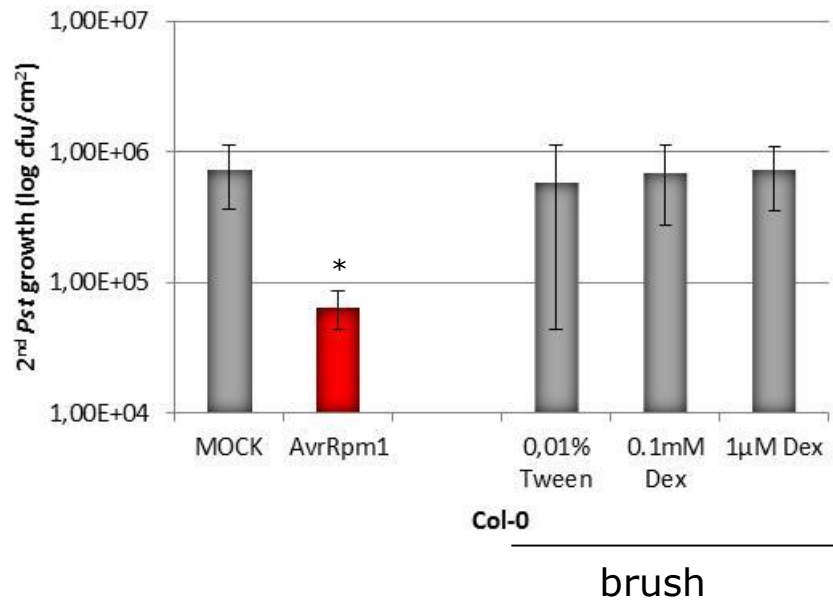
Figure 39: Treatment of WT Col-0 plants with 0.01% Tween, 100 µM DEX and 1 µM DEX. WT plants were locally treated with MOCK, DC3000 *AvrRpm1*, and different samples of Tween and DEX. Treatment was performed by infiltration (MOCK and DC3000 *AvrRpm1*) and by using a brush. Growth of secondary DC3000 inoculum was scored four days after infection of the systemic tissue. Asterisk above bars indicate statistically significant differences as compared to MOCK control (* $P < 0.05$, Student´s t test). Experiment was repeated three times with comparable results.
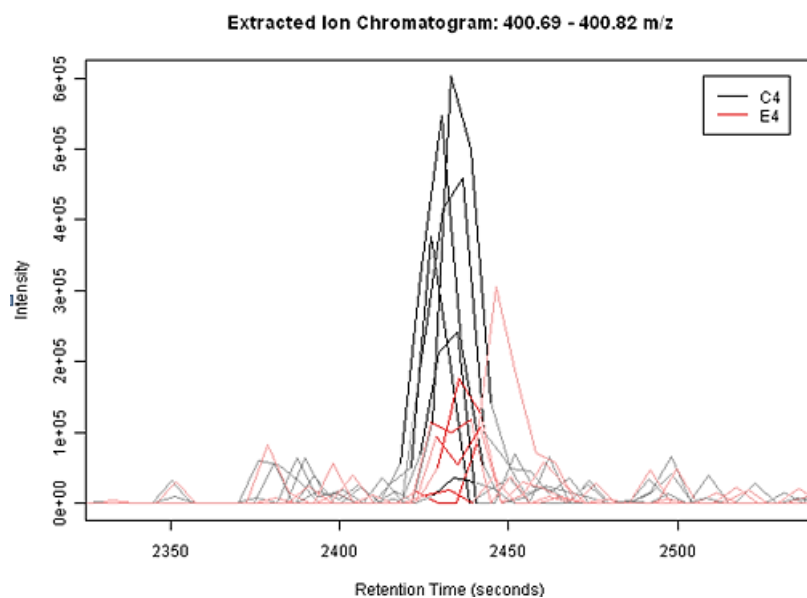
Figure 40: LC-MS data. In the fourth MeOH-wash-sample from Col-0 *DEX* (C4) as compared to *eds1-2 DEX* (E4) a metabolite was identified. Black lines (C4) indicate 5 biological repetitions for Col-0 (C) and red lines (E4) indicate 5 biological repetitions for *eds1-2*.
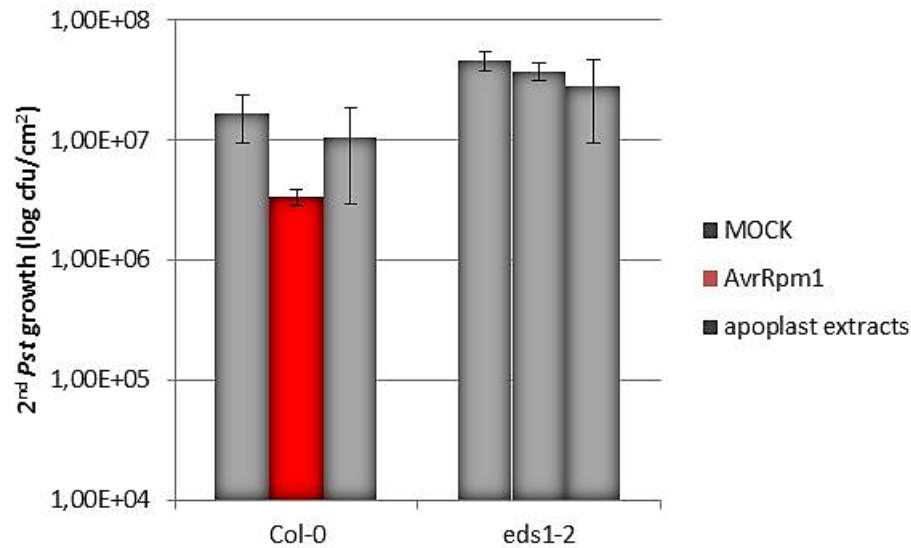
Figure 41: SAR experiment to test apoplast extracts. WT and *eds1-2* mutants were local treated with MOCK, DC3000 *AvrRpm1* apoplast sample from Col-0 *DEX* and *eds1-2 DEX.* Growth of secondary DC3000 inoculum was scored four days after infection of the systemic tissue. Asterisk above bars indicate statistically significant differences compared to MOCK control (* P < 0.05, Student´s t test). Experiment was repeated twice with similar results.
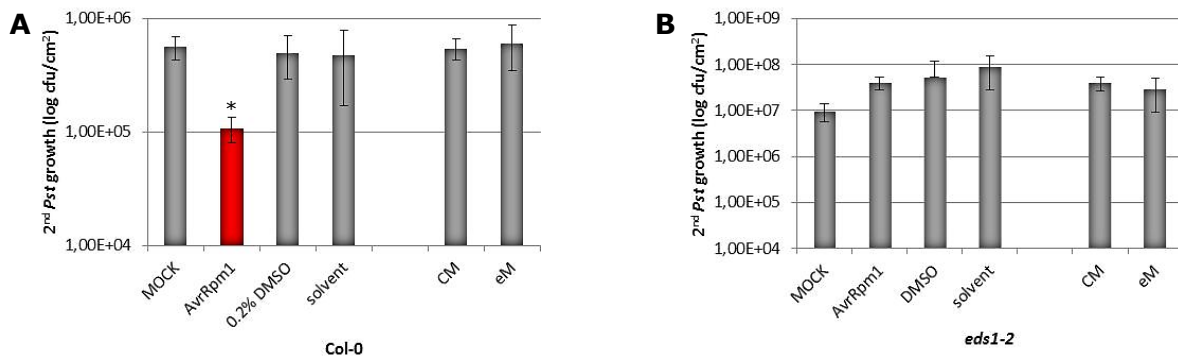
Figure 42: SAR experiment to test MeOH extracts. WT (Col-0) and mutants (eds1-2) were locally treated with MOCK, DC3000 *AvrRpm1*, 0.2% DMSO, solvent, and MeOH extracts (M) from Col-0 *DEX* (C) and *eds1-2 DEX* (e). Growth of secondary DC3000 inoculum was scored four days after infection of the systemic tissue. Asterisk above bars indicates statistically significant differences compared to MOCK control (* P < 0.05, Student´s t test). Experiment was repeated twice with similar results.
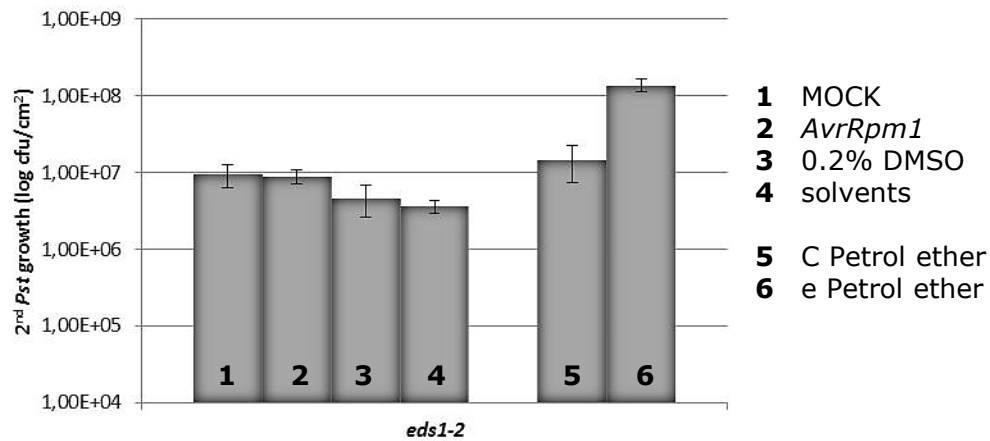
Figure 43: SAR experiment to test SAR inducible PE-phase from Col-0 *DEX* in *eds1-2* mutant. Mutant plant were locally treated with MOCK, DC3000 *AvrRpm1*, 0.2% DMSO, solvent, and PE-fraction Col-0 *DEX* (C) and *eds1-2 DEX* (e). Experiment was repeated twice with similar results.
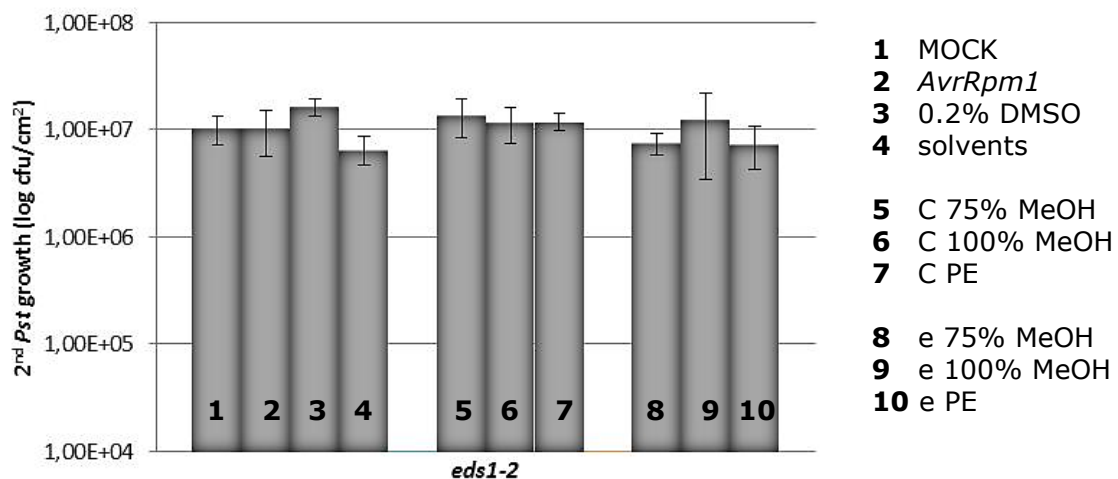
Figure 44: SAR experiment to test SAR inducible MeOH-fractions as compared to negative fraction of mutant in *eds1-2* mutant *Arabidopsis*. *Eds1-2* mutant plants were locally treated with MOCK, DC3000 *AvrRpm1*, 0.2% DMSO, solvent, and different samples from Col-0 *DEX* (C) and *eds1-2 DEX* (e). Growth of secondary DC3000 inoculum was scored four days after infection of the systemic tissue. Experiment was repeated twice with similar results.

1 MOCK
2 *AvrRpm1*
3 0.2% DMSO
4 solvents

MeOH samples
5 C 23
6 C 24
7 C 26
8 C 29
9 C 34

10 e 23
11 e 24
12 e 26
13 e 29
14 e 34
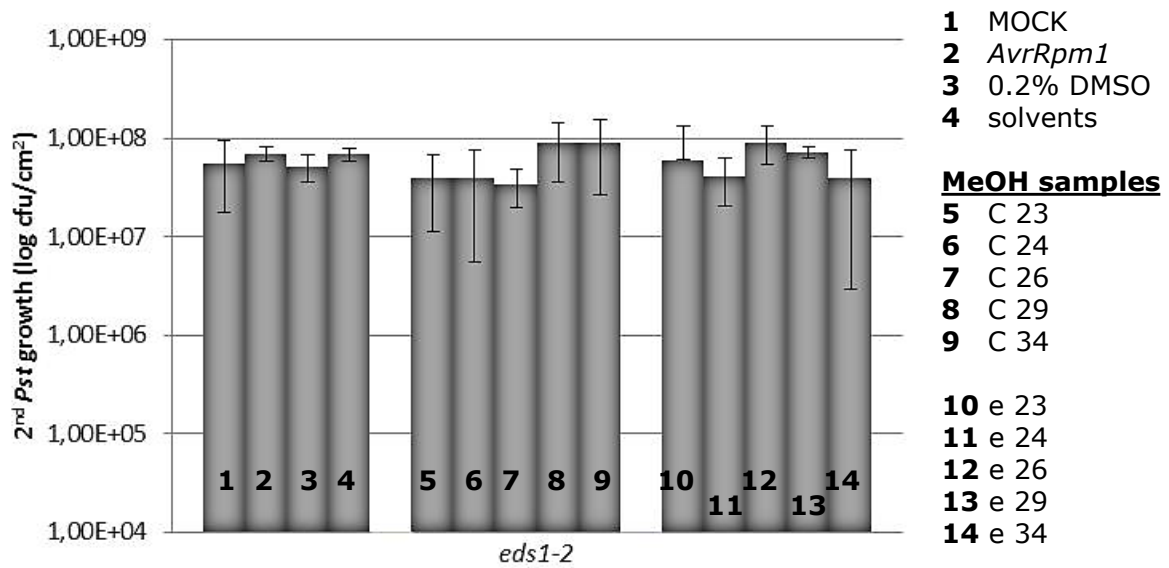
Figure 45: SAR experiment to test SAR inducible HPLC samples from Col-0 *DEX* as compared to negative fraction of mutant in *eds1-2* mutant *Arabidopsis*. Mutant plant were locally treated with MOCK, DC3000 *AvrRpm1*, 0.2% DMSO, solvent, and different samples from Col-0 *DEX* (C) and *eds1-2 DEX* (e). Growth of secondary DC3000 inoculum was scored four days after infection of the systemic tissue. Experiment was repeated twice with similar results.
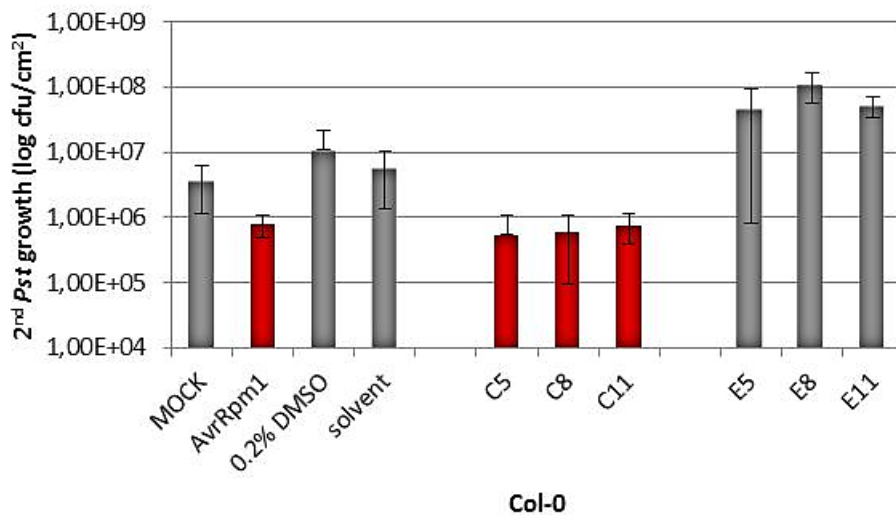
Figure 46: SAR experiment to test HPLC fractions in WT *Arabidopsis* before FTICR-MS. WT Arabidopsis plant were locally treated with MOCK, DC3000 *AvrRpm1*, 0.2% DMSO, solvent, and different samples from Col-0 *DEX* (C5, C8, C11) and *eds1-2 DEX* (e5, e8, e11). Growth of secondary DC3000 inoculum was scored four days after infection of the systemic tissue. Experiment was repeated twice with similar results.

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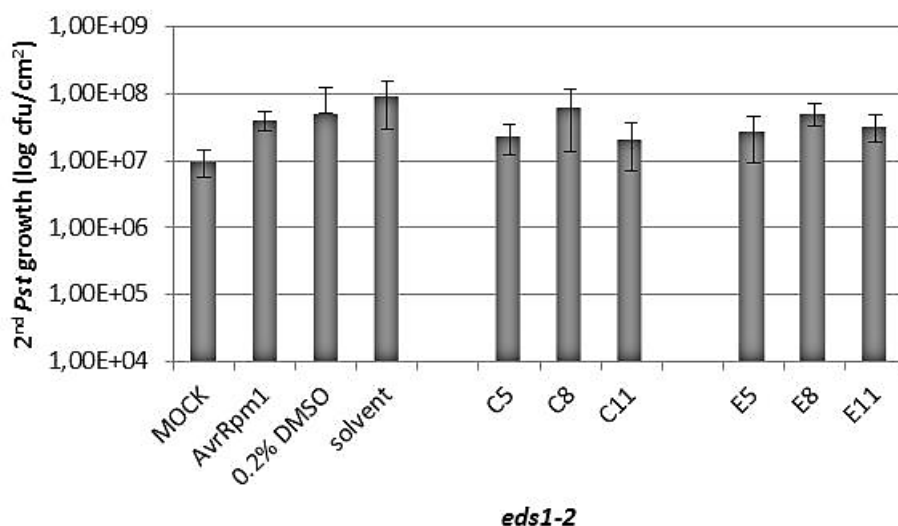
Figure 47: HPLC MeOH samples from Col-0 *DEX* and *eds1-2 DEX* were tested in eds1-2 mutant from *Arabidopsis*. Mutant plant were locally treated with MOCK, DC3000 *AvrRpm1*, 0.2% DMSO, solvent, and different samples from Col-0 *DEX* (C) and *eds1-2 DEX* (E). Growth of secondary DC3000 inoculum was scored four days after infection of the systemic tissue. Experiment was repeated twice with comparable results.

Table 10: Identified metabolites by FTICR-MS present in positives of Col-0 *DEX* (5, 8, and 11) as compared to corresponding samples from mutant.

mass m/z	intensity		mass m/z	intensity
183,10268	6198194		378,9185	31210906
226,966	13086593		384,9354	49357820
242,94355	36639376		409,1695	0
248,9605	1,48E+08		425,1474	0
272,95894	0		432,1686	0
288,93393	0		440,889	0
288,93649	5424133		446,9059	14845990
289,93727	0		452,9229	59558292
292,98674	57540232		465,232	0
294,95344	15380357		475,1898	0
295,18913	25574552		487,3795	36819424
304,91403	9914859		497,3638	81797112
305,91738	0		503,3744	3,18E+08
306,91915	94432360		504,3777	1,06E+08
310,93098	40991928		507,3654	9599902
316,94795	1,65E+08		508,8764	0
345,20483	43288788		514,8934	14886986
362,94089	13854260		561,38	70253472
363,17662	0		577,3749	1,26E+08

Table 11: Identified metabolites from LC-MS in positive ion mode highly present in mutant as compared to Col-0 *DEX*.

mass m/z	retention time (sec.)		mass m/z	retention time (sec.)
151.19-151.45	2185		385.48-385.65	2668
152.18-152.44	155		386.43-386.64	2668
173.04-173.45	747		419.45-419.65	2631
194.16-194.35	1579		438.64-438.91	2512
207.27-207.53	2185		463.47-463.74	2630
216.16-218.44	1758		474.44-474.65	2609
219.09-219.34	1879		507.46-507.64	2628
229.1-229.41	2182		508.44-508.66	2628
229.98-230.32	2182		546.51-546.83	2759
234.97-235.97	747		551.41-551.63	2628
250.16-250.43	2629		552.36-552.65	2627
256.23-265.64	2847		561.46-561.69	2607
256.31-256.55	2715		562.4-562.72	2606
278.27-278.54	2716		595.41-595.62	2626
283.26-283.62	2850		596.37-596.67	2626
291.04-291.23	2181		598.86-599.39	2959
305.36-305.74	2844		620.49-620.93	2961
321.31-321.54	2603		635.36-635.54	2618
322.29-322.53	2603		639.43-639.64	2625
326.38-326.65	2433		649.35-649.65	2658
327.37-327.55	2433		664.53-664.78	2945
339.17-339.55	2553		683.39-683.63	2624
340.36-340.56	2553		684.36-684.65	2624
375.38-375.65	2631		728.35-728.61	2622
			730.49-730.73	2935
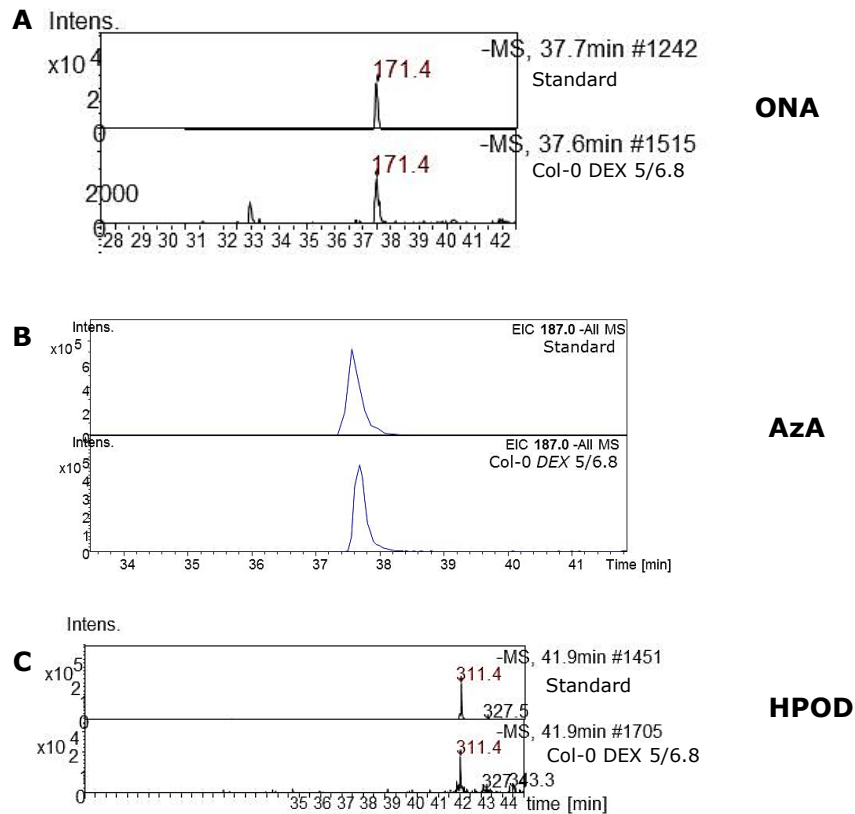
Figure 48: Measurement of Col-0 _DEX_ sample 5/6.8 compared to standards. Peaks of C5/6.8 were analysed with different standards synthesised from linoleic acid by LOX and HPL. (A) Identified as ONA 171.4 m/z. (B) Identified as AzA with 187 m/z. (C) Identified as HPOD 311.4 m/z. LOX: Lipoxygenase, HPL: hydroperoxide lyase, HPOD: hydroperoxy-octadecadienoic acid. Preliminary results.
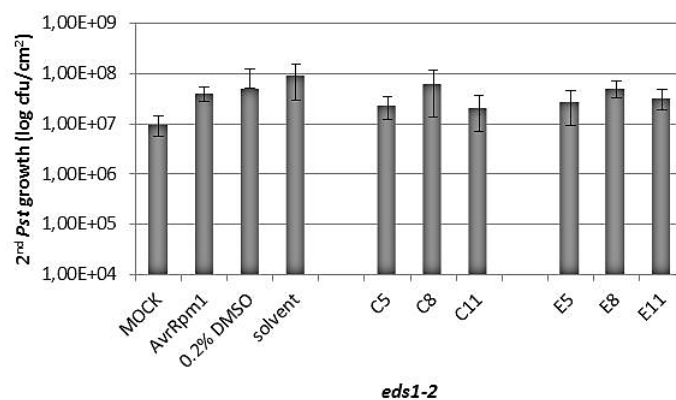
Figure 49: SAR experiment of new HPLC ACN samples from Col-0 _DEX_ and _eds1-2 DEX_ in mutant from _Arabidopsis._ Mutant plant were locally treated with MOCK, DC3000 _AvrRpm1_, 0.2% DMSO, solvent, and different samples from Col-0 _DEX_ (C5/6.8-9, C8.8-9) and _eds DEX_ (e5/6.8-9, e8.8-9). Growth of secondary DC3000 inoculum was scored four days after infection of the systemic tissue. Preliminary results.

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"Identification of mobile defence-inducing signals in plants, an experimental approach", International Conference on Arabidopsis Research (ICAR) 2012, Vienna, Austria, **Poster**

Finni Wittek, Thomas Hoffmann, David Mackey, Jane E. Parker, Wilfried Schwab, A. Corina Vlot "Identification of mobile defense-inducing signals in plants, an experimental approach", International Conference on Arabidopsis Research (ICAR) 2011, Madison, Wisconsin, USA, **Poster**

Finni Wittek, Heiko Breitenbach, Marion Wenig, Thomas Hoffmann, Wilfried Schwab, Jane E. Parker, A. Corina Vlot "Characterization of defence reactions in *Arabidopsis thaliana* and analysis of apoplastic, defence-related proteins in two plant species", Tri-National *Arabidopsis* Meeting (2010), Salzburg, Austria, **Poster**

Finni Wittek, Steffen Rietz, Thomas Hoffmann, Wilfried Schwab, Jane E. Parker, A. Corina Vlot "Identification of mobile defense-inducing signals in plants, an experimental approach", 1st International Metabolomics & More Symposium 2010, Freising, Germany, **Poster**