Dissection of *EDS1*-dependent and salicylic acid-independent defence signalling pathways in *Arabidopsis thaliana*: a chemical biology approach

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Abbreviations

-	fused to (in the context of gene/protein fusion constructs)
◦C	Degree Celsius
µ	Micro
µM	Micromolar
Aad	α-aminoadipic acid
ABA	Abscisic acid
Ala	alanine
ALD1	AGD2-like defense response protein1
At	*Arabidopsis thaliana*
Avr	avirulence
CC	Coiled-coil
cDNA	Complementary DNA
d	Day(s)
ddH2O	Demineralized, deionized water
Dex	Dexamethasone
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleosidetriphosphate
DTT	Dithiothreitol
EDS1	Enhanced Disease Susceptibility1
EDTA	Ethylenediaminetetraacetic acid
ETI	Effector-triggered immunity
EtOH	Ethanol
FW	Fresh weight
g	Gram
GUS	Glucuronidase
h /H	Hour(s)
Hpa	Hyaloperonospora arabidopsidis
HR	Hypersensitive response

HTS	High-throughput-screening
IAA	Indole-3-acetic acid
IC50	Half maximum inhibitory concentration
ICS1	Isochorismate Synthase1
JA	Jasmonic acid
kDa	kiloDalton(s)
l / L	Litre
LOX	13-Lipoxygenase
LRR	Leucine-rich repeats
LUC	Luciferase
Lys	lysine
m	Milli
M	Molar(mol/L)
MAMP	Microbe-associated molecular patterns
MAPK	Mitogen-activated protein kinase
MeJA	Methyl jasmonate
min	Minute(s)
mM	millimolar
mRNA	Messenger ribonucleic acid
MW	Molecular weight
n	Nano
NB	Nucleotide binding site
ng	Nanogram
NLR	NOD-like receptor
nM	nanomolar
NPR1	Nonexpresser of PR genes1
N-terminal	amino-terminal
p35S	35S promoter of CaMV
PAD4	Phytoalexin Defient4
PAGE	Polyacrylamide gel-electrophoresis
PCR	Polymerase chain reaction

pEDS1	Native EDS1 promoter
PEPC	Phosphoenolpyruvate carboxylase
pH	Negative decimal logarithm of H+ concentration
PR	Pathogenesis related
PRR	PAMP/ pattern recognition receptor
Pst	*Pseudomonas syringae* pv tomato
PTI	PAMP-triggered immunity
pv.	Pathovar
qRT-PCR	Quantitative real time polymerase chain reaction
R	Resistance
RB	Rose bengal
RFU	Relative fluorescence units
RLU	Relative light units
RNA	Ribonucleic acid
rpm	Revolutions per minute
RPM	Resistance to *Pseudomonas syringae* pv. *maculicola*
RPP	Resistance to *Peronospora parasitica*
RPS	Resistance to *Pseudomonas syringae*
RT	Room temperature
SA	Salicylic acid
SAG101	Senescence Associated Gene 101
SAR	Systemic acquired resistance
SAR	Structure-activity relationship
SDS	Sodium dodecyl sulphate
Sec/s	Second(s)
SID2	Salicylic Acid Induction Deficient2
TBS	Tris buffered saline
TBTA	Tris(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine
TIR	Drosophila Toll and mammalian interleukin-1 receptor
Tris	Tris-(hydroxymethyl)-aminomethane
Trp	tryptophan

Tyr	tyrosine
UV	Ultraviolet
V	Volt(s)
v/v	Volume per volume
VSP	Vegetative storage protein
w/v	Weight per volume
wt	Wild-type
YFP	Yellow fluorescent protein

Summary

Plants have to survive in an environment where they are constantly under attack from a wide range of pathogens, and they utilise sophisticated, multi-layered defence mechanisms to resist infection. The *Arabidopsis thaliana* protein Enhanced disease susceptibility1 (EDS1), together with its signalling partners, Phytoalexin deficient4 (PAD4) and Senescence associated gene101 (SAG101), constitutes a central regulatory hub in plant immunity. EDS1 is essential for basal defence and for effector-triggered immune responses mediated via TIR-NB-LRR (Toll-Interleukin1 receptor-Nucleotide Binding site-Leucine Rich Repeat) receptors. EDS1-dependent signalling can be divided into two branches: a salicylic acid (SA)-dependent branch and a SA-independent branch. *Flavin-dependent mono-oxygenase 1* (*FMO1*) is a marker gene for the EDS1-dependent, SA-independent signalling pathway. *FMO1* is dependent on *EDS1* and *PAD4*, and plays a crucial role in basal defence and TIR-NB-LRR-mediated immune responses. However, the mechanistic details of *FMO1* signalling and other components involved modulation of *EDS1*-dependent, SA-independent defence signalling are unknown. I aimed at identifying some of these unknown components, using a chemical biology approach and screening for selective compounds that can be used as tools in applications complementing genetic approaches. The advantage of this method lies in its potential to circumvent problems generally encountered in genetic approaches, such as redundancy of gene function, lethality of mutants and pleiotropic effects.

I developed a bi-directional screening procedure for chemicals activating or inhibiting FMO1 accumulation, using a transgenic *A. thaliana* line expressing a YFP-tagged FMO1 protein under control of the native *FMO1* promoter in a *fmo1-1* mutant background. Three compounds selectively activating *FMO1* expression were identified after screening and verification, from amongst a library of 1488 compounds. The selected chemicals; merbromin, monensin sodium salt, and thaxtomin A, could induce expression of *FMO1*, independent of SA accumulation, thus confirming the SA-independent nature of *FMO1* defence regulation. All three chemicals were extensively tested for their role in modulation of plant immune responses. Merbromin leads to the upregulation of several defence genes known to be linked to *FMO1*, such as *EDS1*, *PAD4* and *Isochorismate synthase1* (*ICS1)*. However, it does not induce accumulation of SA and expression of *Pathogenesis related1* (*PR1*), suggesting that it causes perturbations at several points in the *FMO1*-related signalling pathway. Monensin sodium salt also leads to an increase in expression of *EDS1*, *PAD4*, *ICS1* and *PR1*, along with SA accumulation. Monensin sodium salt activates mitogen-activated protein kinases (MAPKs), which could play a role in induction of *FMO1* expression.

Thaxtomin A was the most specific modulator of FMO1 signalling, and does not affect early defence responses such as MAPK activation and production of reactive oxygen species. It causes SA accumulation and enhances expression of *EDS1*, *PAD4*, *ICS1*, and *PR1*. Thaxtomin A-induced *FMO1* expression is dependent on *EDS1* and *PAD4*. I found thaxtomin A to be a specific activator of *PAD4* expression, with enhanced *PAD4* mRNA accumulation, independent of *EDS1*. There are also indications that *PAD4* may have an *EDS1*-independent role in thaxtomin A-induced *FMO1* upregulation. However, the presence of both, *EDS1* and *PAD4*, is essential for maximum upregulation of *FMO1* by thaxtomin A. Identification of the molecular target of thaxtomin A may lead to unknown components involved in *EDS1*-dependent, SA-independent signalling and greater mechanistic knowledge of the signalling pathway.

Zusammenfassung

Pflanzen leben in einer Umgebung in der sie ständigen Angriffen durch Unmengen von Pathogenen ausgesetzt sind. Dabei benutzen Pflanzen hochentwickelte, mehrschichtige Verteidigungsmechanismen um einer Infektion zu widerstehen. Das Protein „Enhanced disease susceptibility1“ (EDS1) bildet zusammen mit seinen Interaktionspartnern „Phytoalexin deficient4“ (PAD4) und „Senescence associated gene101“ (SAG101) eine zentrale regulatorische Einheit in der Signalweitergabe des pflanzlichen Immunsystems von *Arabidopsis thaliana*. EDS1 ist sowohl unverzichtbar für basale Immunantworten, als auch für Effektor-induzierte Immunantworten, welche durch „Toll-interleukin1 receptor-nucleotide binding site-leucine rich repeat“ (TIR-NB-LRR) Rezeptoren ermöglicht werden. EDS1-vermittelte Signalwege können dabei salicylsäure(SA)-abhängig oder SA-unabhängig sein. Das Gen *Flavin-dependent mono-oxygenase1* (*FMO1*) gilt als Repräsentant des EDS1-abhängigen, SA-unabhängigen Signalwegs. *FMO1* benötigt EDS1 und PAD4 und spielt ebenfalls eine entscheidende Rolle für die basale Resistenz und während TIR-NB-LRR-vermittelter Immunantworten. Details der FMO1-übermittelten Signalwege, sowie andere Komponenten die einen Einfluss auf EDS1-abhängige, SA-unabhängige Immunsignalwege haben, sind bis jetzt noch nicht bekannt. Das Ziel meiner Arbeit war diese unbekannten zusätzlichen Komponenten zu identifizieren. Dafür wurde ein chemisch-biologischer Ansatz gewählt der auf einer Selektion chemischer Komponenten basiert, welche eine genetische Komplementation hervorrufen. Der Vorteil dieser Methode besteht in der Vermeidung von häufig auftretenden Problemen genetischer Selektionsansätze, z.B. Redundanz der Genfunktion, hohe Sterblichkeitsrate der Mutanten oder pleiotropische Effekte.

Ich habe ein bidirektionales Selektionsverfahren entwickelt, wodurch chemische Substanzen die eine Akkumulation von FMO1 anregen bzw. verhindern mit Hilfe einer transgenen *A. thaliana* Linie, welche ein YFP-markiertes FMO1 Protein unter seinem nativen Promoter im *fmo1-1* Mutantenhintergrund exprimiert, identifiziert werden konnten. Aus einer Sammlung von 1488 chemischen Substanzen konnten drei Chemikalien identifiziert und verifiziert werden, welche die Aktivierung der *FMO1* Expression hervorriefen. Alle drei Komponenten: Merbromin, Monensin-Natriumsalz und Thaxtomin A, induzierten die *FMO1* Expression unabhängig von einer SA Akkumulation, was ebenfalls eine SA-unabhängige Regulation von *FMO1* bestätigt. Daraufhin wurde der Effekt der drei Chemikalien auf pflanzliche Immunantworten untersucht. Merbromin führte zur Induktion von verschiedenen *FMO1* verbundenen Abwehrgenen, wie beispielsweise *EDS1*, *PAD4* und „*Isochorismate synthase1“* (*ICS1*). Jedoch führte es nicht zu einer Erhöhung des SA Gehalts oder der Genexpression von „*Pathogenesis related1“* (*PR1*), was darauf deutet, dass

es verschiedenen Stellen in FMO1-regulierten Signalwegen stören kann. Monensin-Natriumsalz bewirkte neben der Expressionsinduktion von *EDS1, PAD4, ICS1* und *PR1* auch eine Anreicherung von SA. Monensin-Natriumsalz aktiviert die Mitogen-aktivierten Proteinkinasen (MAPKs), welche eine Rolle in der Induktion der *FMO1* Expression spielen könnten.

Thaxtomin A wirkte am spezifischsten auf FMO1-vermittelte Signalwege und hatte keinen Einfluss auf frühe Immunantworten, wie beispielsweise die Aktivierung der MAPKs oder die Produktion von reaktiven Sauerstoffspezies. Es bewirkt die Anreicherung von SA und eine erhöhte Expression von *EDS1, PAD4, ICS1* und *PR1*. Die von Thaxtomin A-induzierte *FMO1* Expression ist abhängig von *EDS1* und *PAD4*. Dabei habe ich ermittelt, dass Thaxtomin A auch ein spezifischer Aktivator der *PAD4* Expression ist, wodurch es zur erhöhten Anreicherung von *PAD4* mRNA unabhängig von *EDS1* kommt. Weitere Hinweise deuten auf eine EDS1-unabhängige Funktion von PAD4 während der Thaxtomin A-induzierten *FMO1* Expression. Eine maximale Induktion der *FMO1* Expression durch Thaxtomin A ist jedoch nur möglich, wenn sowohl EDS1 als auch PAD4 vorhanden sind. Die Identifikation des molekularen Angriffsziels von Thaxtomin A könnte die bis jetzt unbekannten Komponenten des EDS1-abhängigen, SA-unabhängigen Signalweges aufzeigen und unser Verständnis über Prozesse dieser Signalweiterleitung erweitern.

1 Introduction

Plants have to survive in an environment where they are constantly under attack from a wide range of pathogens. They utilise a sophisticated, multi-layered defence strategy comprising preformed and inducible defence mechanisms to combat pathogens (Jones and Dangl, 2006; Spoel and Dong, 2012). Preformed anti-microbial compounds, and surface structures such as waxy cuticles, act as the primary barrier for the entry and colonisation by non-specialised pathogens. The primary mechanism of inducible defence involves recognition of certain microbe associated molecular patterns (MAMPs) to trigger a basal resistance response known as pattern-triggered immunity (PTI). The second layer of defence is a result of plant-pathogen co-evolution, wherein pathogen-secreted effector molecules are recognised in the plant, leading to a stronger defence response known as effector triggered immunity (ETI). Systemic acquired resistance is an event when local infection by a pathogen immunises the whole plant against subsequent infections (Shah and Zeier, 2013)

1.1 Initial response: MAMP-triggered immunity

Microbial organisms express highly conserved molecular signatures, generally termed as microbe-associated molecular patterns (MAMPs). Chitin and ergosterol from fungi, and flagellin and lipopolysaccharides from bacteria are some of the MAMPs that act as general elicitors in plants (Zipfel et al., 2004). Plant cells have surface pattern recognition receptors (PRRs) which can perceive MAMPS. This MAMP-recognition triggers a number of defence responses such as reactive oxygen species (ROS) and nitrogen oxide (NO) production, altered cytoplasmic Ca^{2+} levels, callose deposition at the site of infection, induction of mitogen-activated protein kinases (MAPKs), and transcriptional activation of defence genes (Nürnberger and Lipka, 2005). These immune responses are together termed as MAMP-triggered immunity and help to arrest pathogen growth (Jones and Dangl, 2006).

Currently described plant PRRs belong to a family of receptor-like kinases (RLKs). The recognition of bacterial flagellin by FLS2 (FLAGELLIN SENSING 2), and of bacterial

elongation factor Tu by EFR (EF-TU RECEPTOR), and the subsequent defence responses in plants, are two well characterised examples of MAMP-triggered immunity (Gómez-Gómez and Boller, 2002; Zipfel et al., 2006). Fungal oligosaccharide perception by the LysM-RLK CERK1 (CHITIN ELICITOR RECEPTOR KINASE 1) receptor has also been studied in detail (Miya et al., 2007).

Pathogens suppress MTI by injecting effector proteins into the plant cell. Effectors can disrupt MTI responses by various means, leading to successful colonisation by the pathogen. This includes disruption of defence signalling or alteration of MAMP perception by the plant. Such pathogens are termed virulent and the plant-microbe interaction is said to be compatible (Chisholm et al., 2006). The resistance activated by virulent pathogens on susceptible hosts is defined as basal disease resistance (Jones and Dangl, 2006).

1.2 Secondary response: Effector-triggered immunity

Plants, unlike mammals, do not possess an adaptive immune system, with highly specific circulating antibodies for pathogen recognition (Stuart et al., 2013). Instead, diverse pathogen virulence factors, called effectors, are recognised in plants using genetically encoded receptor proteins (Dangl et al., 2013). A 'gene for gene' model for ETI in plants was first proposed in the 1940s, and was based on the inherent complementary resistance (R) gene pairs in the host plant and the pathogen avirulence (Avr) genes, determining disease resistance (Flor, 1971). R proteins comprise a central nucleotide binding (NB) and a C-terminal leucine-rich repeat (LRR) domain. Depending on their N-terminal domain, R proteins can be separated into two subclasses (Meyers et al., 2003). One subclass of NB-LRRs contains a N-terminal coiled-coil (CC) domain, whereas the N-terminal of the other subclass resembles the *Drosophila* and mammalian toll-interleukin1 receptor (TIR) (Maekawa et al., 2011). These TIR/CC- NB-LRR receptors can perceive effector molecules secreted by pathogens, thus leading to an immune response, known as R gene-mediated resistance or effector-triggered immunity (ETI). Activation of the NB-LRR proteins triggers transcriptional reprogramming of pathogen-responsive genes and accumulation of salicylic acid (SA) and ROS within the cell. A hypersensitive response (HR)

associated with the programmed cell death (PCD) of infected cells, is a characteristic of ETI.

R proteins can recognize effectors molecules either through direct physical interaction, or indirectly, through host target-perturbation by effectors. Direct effector-R gene interaction is compatible with the gene-for-gene hypothesis (Flor, 1971), but is rare, since plant pathogen effectors far outnumber the more than 150 NB-LRR proteins found in *A. thaliana*. One model of indirect effector recognition involves modification of a host factor that is bound to and monitored (or guarded) by a NLR, leading to activation of that NLR (Deslandes and Rivas, 2012). Another indirect method of effector recognition is based on a 'decoy' strategy, wherein the monitored host protein has no role in resistance, but is merely a bait to trap effectors which target structurally related components of basal defence, thus triggering ETI (van der Hoorn and Kamoun, 2008; Collier and Moffett, 2009).

Since both, MTI and ETI, utilise a highly overlapping signalling network, ETI can be termed as a rapid and heightened MTI response (Tsuda et al., 2009). Immune responses evoked by MTI and ETI in infected plants are similar, such as accumulation of free SA and enhanced expression of pathogenesis-related (PR) genes (Glazebrook, 2005; Vlot et al., 2009), but the differences in the timing and amplitude of these responses is what distinguishes ETI from MTI (Katagiri and Tsuda, 2010).

1.3 Global response: systemic acquired resistance

Furthermore, alongside local defence responses, MTI and ETI also induce defence in the systemic (uninfected) regions of the plant, including *PR* gene expression, SA accumulation and a long-lasting, broad-spectrum resistance to pathogen infection, termed systemic acquired resistance (SAR) (Ryals et al., 1996; Durrant and Dong, 2004; Shah, 2009; Vlot et al., 2009). SAR leads to 'priming' of systemic tissue to evoke faster and stronger defence responses against subsequent infections (Conrath, 2011), analogous to vaccination in humans. Long-distance communication between the primary pathogen infected tissue, and the uninfected systemic tissue, is crucial for SAR activation (Dempsey & Klessig, 2012). Several small molecule plant metabolites, such as methyl salicylate

(MeSA), SA, pipecolic acid (Pip), azelaic acid (Aza), dehydroabietinal (DA) and glycerol-3-phosphate, have been proposed to function in long-distance transport and/or signal amplification during SAR (Shah and Zeier, 2013).

1.4 The EDS1/PAD4 regulatory node

The enhanced disease susceptibility1 protein (EDS1) is a central regulatory hub in plant immunity. It was first identified in a genetic screen for new factors involved in the RPP *resistance to P. parasitica* (*RPP*) gene-mediated resistance against the obligate biotrophic oomycete, *Hyaloperonospora arabidopsidis* (Hpa) (Parker et al., 1996). EDS1 was found to play an essential role in TIR-NB-LRR mediated immunity, and also contribute to basal immunity (Parker et al., 1996; Aarts et al., 1998). Resistant to pseudomonas syringae 4 (RPS4) triggers a TIR-NB-LRR mediated resistance upon recognition of the bacterial effector AvrRPS4 (Hinsch and Staskawicz, 1996). This signalling pathway has been extensively studied and was shown to be EDS1-dependent (Wirthmueller et al., 2007). EDS1 activity was determined to be downstream of TIR-NB-LRR activation, but upstream of defence gene activation, cell death induction and SA production (Feys, et al., 2001; Wirthmueller et al., 2007; Zhang et al., 2003). In contrast to TIR-NB-LRR mediated resistance, the CC-NB-LRR protein resistance to *Pseudomonas syringae* pv *maculicola*1 (RPM1) influences local programmed cell death, SA production and resistance independent of EDS1. However, the EDS1 pathway is still essential for the transduction of defence and death-promoting signals to cells surrounding the infection hub (Rustérucci et al., 2001; Wiermer et al., 2005).

EDS1 interacts directly with two sequence-related signalling partners, phytoalexin deficient 4 (PAD4) (Feys et al., 2001; Feys et al., 2005; Jirage et al., 1999) and senescence-associated gene 101 (SAG101) (Feys et al., 2005), to form heteromeric complexes (Wagner et al., 2013). *PAD4* was initially identified from a genetic screen for plants with enhanced susceptibility to *Pseudomonas syringae* pv. *maculicola* ES4326 (Psm ES4326) (Glazebrook et al., 1996). Genetically, *PAD4* and *SAG101* are partially redundant. *PAD4* can compensate for the lack of *SAG101* and regulate basal and R gene-mediated immunity. However, *SAG101* cannot completely compensate for the loss of *PAD4*, thus hinting at a

unique role for *PAD4* in plant defence. Both *PAD4*, and *EDS1*, have important intrinsic signalling functions in immunity, since *pad4/sag101* double mutants do not have functional basal and TIR-NB-LRR-mediated resistance, similar to the *eds1* mutant (Feys et al., 2005). Interestingly, monocotyledonous plants lack TIR-NB-LRR proteins, but possess EDS1 and PAD4 orthologs, thus indicating an ancestral function of *EDS1* and *PAD4* in basal immunity (Meyers et al., 2003).

Activation of *EDS1* and *PAD* stimulates synthesis of the defence hormone SA via upregulation of *Isochorismate synthase1* (*ICS1*) (Zhou et al., 1998; Feys et al., 2001; Dempsey et al., 2011). SA plays a central role in resistance against biotrophic and hemibiotrophic pathogens (Vlot et al., 2009). Additionally, *EDS1* and *PAD4* expression can also be stimulated by SA, as a part of a positive feedback loop that amplifies resistance locally, and systemically (Jirage et al., 1999; Feys et al., 2001; Vlot et al., 2009). EDS1 and PAD4 are crucial for SAR due to their role in regulation of SA activity (Malamy et al., 1990; Métraux et al., 1990; Gruner et al., 2013).

EDS1, when bound in a complex with PAD4 or dissociated from it, performs different roles in pathogen resistance. EDS1-PAD4 complex formation is necessary for effective basal resistance (Rietz et al., 2011). In transgenic plants with a single amino acid exchange in *EDS1* (eds1^{L262P}), the mutated EDS1 cannot bind to PAD4, but retains interaction with SAG101. Infection by *P. syringae* DC3000 failed to induce SA accumulation and PR1 expression in the eds1^{L262P} mutants plants, and hence SAR in these plants was compromised. These mutants were found to have reduced resistance to virulent *H. arabidopsidis* isolates and *P. syringae* bacteria (Rietz et al., 2011). Conversely, EDS1 dissociated from PAD4 could activate TIR-NB-LRR-triggered cell death upon infection by *H. arabidopsidis*. Reports also suggest that SAG101 cannot compensate for PAD4-dependent induction of cell death (Rietz et al., 2011).

PAD4 is not only important for effective basal and R gene-mediated resitance, but is also essential for resistance to green peach aphid (*Myzus persicae*) (Pegadaraju et al., 2007). This PAD4 activity in plant defence against *M. persicae* was proven to be independent of EDS1 and pathogen-induced SA accumulation (Louis et al., 2012). Resistance to microbial pathogens was not compromised when a conserved serine in the predicted lipase catalytic triad of PAD4 was mutated. However, this mutation partially

compromised resitance to *M. persicae.* Hence, PAD4 was demonstrated to have different molecular functions in defence responses against pathogens and *M. persicae* (Louis et al., 2012). A chemical biology study discovered interaction between PAD4 and a newly identified TIR-NB-LRR protein, VICTR, thus providing the first evidence of association between TIR-NB-LRR proteins and PAD4.

Thus, both EDS1 and PAD4 play crucial regulatory roles in plant defence signalling. There has been some evidence for an EDS1-independent role of PAD4 in resitance reactions. However, both EDS1 and PAD4 are necessary for a full resistance response to pathogens.

1.5 Flavin-dependent mono-oxygenase 1

Flavin-containing monooxygenases (FMOs) were first discovered in 1960s. Since then, mammalian FMOs have been extensively studied (Krueger and Williams, 2005). FMOs can catalyse oxygenation of nucleophilic, heteroatom-containing substrates (i.e. compounds containing nitrogen, sulphur, selenium or iodine), thus rendering them more polar and readily excreted (Cashman, 2002; Krueger and Williams, 2005). There are five functionally expressed *FMO* genes in humans, i.e., human *FMO1-FMO5*. (Lawton et al., 1994). *FMO*s have also been found in bacteria and yeast (Suh et al., 1996; Chen et al., 2011).

FMO diversity in plants is much greater than in other organisms, with 29 genes annotated as *FMO*-like in Arabidopsis thaliana (Schlaich, 2007). The first *FMO*-like gene to be characterised in plants was termed *YUCCA (YUC1*), whose overexpression led to elevated auxin levels (Zhao et al., 2001). In petunia, overexpression of the *YUCCA* ortholog *FLOOZY* also resulted in endogenous accumulation of indole-3-acetic acid (IAA) (Tobeña-Santamaria et al., 2002). *FMO*-like genes involved in auxin signalling have also been found in strawberry, tomato and rice (Expósito-Rodríguez et al., 2011; Liu et al., 2012; Yi et al., 2013). The possible involvement of plant *FMO*-like genes in auxin biosynthesis implicates some plant FMOs in a substrate-specific manner of compound oxidation, distinct from the

non-specific detoxifying mechanisms suggested for the mammalian FMOs (Olszak et al., 2006).

Phylogenetic analysis of *Arabidopsis thaliana FMO*s separates them into three distinct clusters, with group 1 *FMO*s involved in auxin synthesis, group 2 *FMO*s involved in sulphur-oxygenation (Olszak et al., 2006; Schlaich, 2007). Group 3 consists of a pseudogene *AtFMOp,* and its close homolog *AtFMO1*, which has important functions in plant defence signalling (Bartsch et al., 2006; Koch et al., 2006; Mishina and Zeier, 2006; Olszak et al., 2006).

1.5.1 FMO1 in EDS1/PAD4 dependent defence

AtFMO1 (FMO1) was identified as a gene upregulated in *A. thaliana* wild-type plants infected with *Pst* DC3000 strains expressing the effectors *avrRPM1* or *avrRPS4* (Bartsch et al., 2006). This *FMO1* expression was dependent on *EDS1* and *PAD4* and was suppressed in *eds1-1* and *pad4-5* mutants. Arabidopsis thaliana plants constitutively overexpressing FMO1, displayed enhanced resistance to *P. syringae* (Koch et al., 2006). The role of *FMO1* in defence against pathogens was additionally analysed using a T-DNA insertion mutant, fmo1-1 (Bartsch et al., 2006).

Infection of the *A. thaliana fmo1-1* mutant with the oomycete pathogen *H. arabidopsidis* (Cala2), that is recognized by the TIR-NB-LRR–type *R* gene *RPP2*, displays a loss-of resistance phenotype with trailing necrosis. Defects in *FMO1* also partially disabled TIR-NB-LRR mediated resistance and basal defence against Pst DC3000 expressing *avrRPS4*. However, resistance mediated by the CC-NB-LRR proteins RPP8 and RPM1 was unaffected in the *fmo1-1* mutant plants. SA accumulates to wild type levels in the fmo1-1 mutant, during *RPM1* and *RPS4* responses.

Thus, SA production in CC and TIR-NB-LRR triggered resistance is independent of *FMO1* (Bartsch et al., 2006). However, *FMO1* is required for basal resistance to invasive virulent pathogens and for TIR-NB-LRR conditioned resistance and HR lesion formation due to avirulent pathogens (Bartsch et al., 2006; Olszak et al., 2006). The resistance response evoked by RPP8 and RPM1 recognition in the *A. thaliana fmo1-1* mutants was similar to that in plants with defects in EDS1, PAD4 or SAG101. All the above factors lead

to the conclusion that, FMO1 is involved in an SA-independent branch of EDS1/PAD4 defence signalling.

1.5.2 FMO1 in systemic acquired resistance

Analysis of publicly available microarray experiment data led to the identification of uncharacterized *Arabidopsis thaliana* genes involved in SAR establishment. *FMO1* expression was found to be upregulated after infection with the virulent *Pst* DC3000 and avirulent *Pst avrRpm1* bacterial strains, with the avirulent bacterial strains inducing a faster transcriptional upregulation as compared to virulent strains (Mishina and Zeier, 2006). *FMO1* expression at the site of pathogen inoculation was independent of SA production and signalling, however, an intact SA-signalling pathway was essential for systemic *FMO1* expression. SAR establishment was also closely correlated to enhanced *FMO1* expression in systemic tissue (Mishina and Zeier, 2006). A high-throughput genetic screen for SAR-deficient mutants in *A. thaliana* also isolated plants with hitherto uncharacterised mutations in *FMO1* (Zhang et al., 2011). Thus, FMO1 was proven to be an essential component of SAR development.

A study by the Zeier group has delved deeper into the role of FMO1 in SAR induction and had identified the lysine catabolite pipecolic acid (Pip) as a critical regulator of inducible plant immunity (Navarova et al., 2012). Their data suggests that Pip is the mobile signal which is transported from the primary infected leaves to the distal uninfected leaves of the plant during SAR. Within the distal leaf tissue, Pip upregulates expression of *AGD2-like defense response protein1 (ALD1)* and *FMO1*. This in turn potentiates SA production in the systemic leaves, via upregulation of *ICS1*. It was also shown that *FMO1* is required for dehydroabietinal (DA) and azelaic acid (Aza) induced resistance (Chaturvedi et al., 2012; Jung et al., 2009). Moreover, Aza enhances resistance in an *ALD1*-dependent manner (Jung et al., 2009). These findings suggest that a Pip-/*FMO1*-dependent amplification loop is an essential mediator for SAR signal transduction of at least a section of mobile SAR signals. Based on current data, and adapted from a recent publication (Gruner et al., 2013) a model of the position and role of *FMO1* in plant defence signalling (Fig. 1) was created.

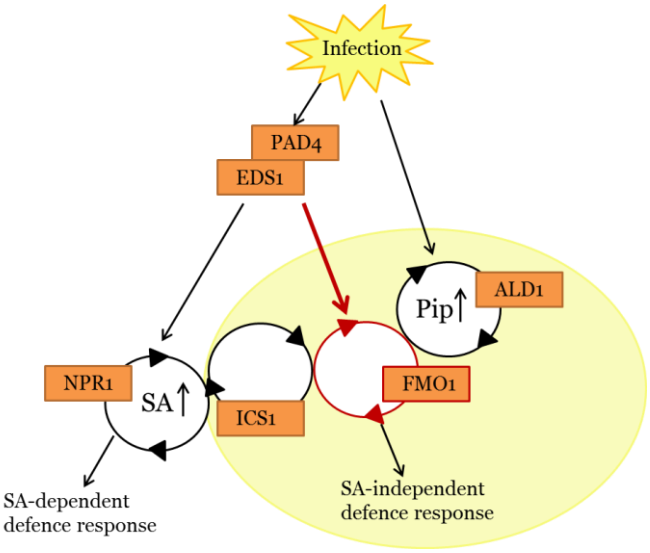

Figure 1: Current model of *FMO1* involvement in plant defence. Infection by pathogens causes upregulation of the *EDS1/PAD4* signalling node, which in turn, promotes production of SA via *ICS1*, causing upregulation of *NPR1* and other SA-dependent defence responses. Infection and activation of *EDS1/PAD4* expression also leads to enhanced production of the SAR signalling metabolite, Pip and expression of *ALD1* in systemic tissues. Enhanced *EDS1/PAD*4 expression, as well as Pip production/*ALD1* expression upregulate expression of *FMO1*. *FMO1* expression also feeds back into the SA-loop by enhancing *ICS1* expression. *FMO1* is a marker gene for the *EDS1/PAD4* pathway and promotes SA-independent defence responses. Adapted from (Gruner et al., 2013)

1.5.3 FMO1 and cell death

One of the first studies on *AtFMO1* dealt with its role in programmed cell death. *AtFMO1* was initially identified as a gene upregulated in the *Arabidopsis thaliana acd11* mutant, which constitutively activates *EDS1*, *PAD4* and SA-dependent PCD and defence (Brodersen et al., 2002; Olszak et al., 2006). Expression was also increased in the *lsd1* mutant, which causes run-away cell death (Dietrich et al., 1997). However, analysis of *acd11/fmo1* mutant plants indicated that *FMO1* is not essential for the activation and progression of run-away cell death in *acd11*. *FMO1* was also found to be expressed during developmental senescence, avirulent, and virulent bacterial infections (Olszak et al., 2006). *FMO1* was also expressed in response to the cell-death inducing chemicals fumonisin B1 (FB1) and rose bengal (RB). Although *FMO1* expression in all the above cases occurred in the region of senescence/HR-like lesions, it does not resolve whether *FMO1* expression has a role in cell death initiation or execution, or whether it is a symptom of cell death.

1.5.4 FMO1 function

Current knowledge about *FMO1* prompts speculation about its role in plant defence. *FMO1* expression is upregulated upon application of FB1, a toxin produced by the necrotrophic fungal plant pathogen *Fusarium moniliforme* (Stone et al., 2000). This could indicate a potential function of *FMO1* in detoxification of pathogen-derived compounds, which would be analogous to the function of FMOs in animals.

Another interesting possibility is that FMO1 can influence the redox state of plant cells. Pathogen-defence responses, especially *EDS1/PAD4*-mediated defence responses, can be regulated by changes in the redox state (Mou et al., 2003; Wiermer et al., 2005). This modulation of the redox state by FMO1 would also be in accordance with the role of FMOs in yeast and animals. The redox changes could be a result of ROS production, or influence on the intracellular glutathione levels (Rauckman et al., 1979; Tynes et al., 1986; Suh et al., 1996). *FMO1* was induced by the extracellular superoxide generating system xanthine/xanthine oxidase (X/XO) and the xanthine dye rose bengal (Olszak et al., 2006). However, this superoxide-induced *FMO1* expression occurred independently of the ROS signal transducing protein kinase OXI1. Hence, the precise relationship between ROS production, *FMO1* activity and defence signalling remains unresolved.

Functional FMO enzymes are characterised by the presence of FAD binding sites, FMO-identifying motifs, and NADPH binding domains. Multiple sequence alignments of FMO1 with functionally characterized FMOs from Arabidopsis, bacteria, yeast, insect, and humans indicated that *A. thaliana* FMO1 also possesses these domains (Bartsch et al., 2006). The conserved Gly residues in the FAD and NADPH binding sites of FMO1 are important for its enzymatic activity and for cofactor binding (Rescigno and Perham, 1994; Kubo et al., 1997). Transgenic *fmo1-1* plants expressing fmo1 with a site–directed mutation converting the conserved Gly residues of the FAD- and NADPH-binding motifs to Ala had impaired pathogen resistance. However, the *fmo1-1* lines could be complemented for pathogen resistance by constitutively expressing wild-type FMO1 (Bartsch et al., 2006). Thus, flavin-dependent monooxygenase activity is essential for *FMO1* defense function.

1.6 Chemical biology and screening strategies

Direct or indirect alteration of protein function via gene mutations to create detectable phenotypes is the key to discovery of new biological functions (Hicks and Raikhel, 2012). The most common approach involves random or directed mutagenesis, e.g. EMS treatment, and gene mapping by breeding. However, such genetic approaches entail several limitations such as lethality due to essential gene functions, genetic redundancy, and pleiotropic effects due to multi-functionality of a gene product.

Chemical genetics is defined as the use of small molecules to alter protein function and thus, analyse the biological function of the target protein (Zheng et al., 2004). Small, cell-permeable molecules can complement classic mutational approaches to analyse biological mechanisms, since interference with small molecules can create swift, conditional and reversible alterations of biological functions (Smukste and Stockwell, 2005). Additionally, chemical activity can be specific for different isoforms of a gene product, e.g. due to formation of a protein complex or post-translational modifications (Tóth and van der Hoorn, 2010). Chemical genetic approaches have been utilised in animal models for cell death and cancer research, and drug development since several years (Schreiber, 1998; Stockwell, 2000; Mayer, 2003; Gangadhar and Stockwell, 2007). In the past decade, use of chemical biology to identify molecules altering plant phenotype and application of such molecules in genetic screens has found broader applications in plant biology as well (McCourt and Desveaux, 2010; Hicks and Raikhel, 2012).

Since the model plant *A. thaliana* can be cultivated in microplates, it is eminently suitable for adoption in high throughput chemical biology assays to identify cellular targets that are inaccessible or recalcitrant to conventional mutational analysis. The first step of a chemical biology study involves development of a robust assay to screen for phenotypic changes (e.g. reporter gene activity or growth inhibition) upon chemical treatment. The next phase involves primary screening with a library of chemical compounds to identify chemicals modulating activity of the targeted gene/protein. Identification of candidate compounds, or 'hits' is only one part of a successful chemical biology screen. The selected compounds need to be critically validated to confirm their activity in the targeted biological process. This validation involves verification of their

activity in secondary screens (e.g. using a different biological readout related to the primary screen), determination of half minimum inhibitory or activating concentration (IC_{50}/EC_{50}) values, and determination of selectivity by comparing their effects on a variety of biological responses. Counterscreening is also necessary to eliminate false positive hits, and isolation of biologically relevant chemicals. These chemicals are useful tools for biological studies involving their target signalling pathway.

However, the ultimate aim of a chemical biology screen is to identify the target of a selected chemical, often a protein, which is essential to analyse the precise mode-of-action of the chemical. The classical and predominant approach for target identification is screening of EMS-mutagenised plants for resistance to the selected chemical. This combination of a chemical screen with a genetic approach is defined as 'chemical genetics'. This approach has been successfully applied for identification of the target of gravicin, and the ABA agonist pyrabactin (Rojas-Pierce et al., 2007; Park et al., 2009). Nevertheless, the step from initial mutant identification to target identification in this approach, is time consuming and labour intensive. Recently, a successful chemical biology screen has identified the target of the chemical jarin, based on its activity within known signalling pathways (Meesters et al., 2014). In addition to genetic approaches, biochemistry techniques such as affinity purification are also widely used for target identification of selective chemicals (Dejonghe and Russinova, 2014). Affinity purification typically involves binding of a tag (e.g. biotin) to the selective small molecule to enable pulldown of the target from amongst a complex mixture. Determination of the structure-activity relationship of the selective compound is crucial before derivatization of the compound for affinity tagging. Analogs of the selected chemical are tested for their activity in the target pathway, to determine the functional groups and moieties vital for its activity. Structure activity relationship is not just useful for compound derivatization, but also provides important clues about the mode-of-action of the candidate chemical. Additional target identification techniques include molecular biology approaches such as the yeast-three-hybrid technology (Cottier et al., 2011) and protein microarrays (Huang et al., 2004). Overall, plant chemical biology has opened new avenues for research into gene function, where traditional genetic techniques have been hitherto ineffective or time consuming.

1.7 Aims of this thesis

The initial discovery of *A. thaliana* FMO1 was followed by several important breakthroughs concerning the role of *FMO1* in plant defence within the span of a year (Bartsch et al., 2006; Koch et al., 2006; Mishina and Zeier, 2006; Olszak et al., 2006). The role of *FMO1* in SAR has also been analysed (Navarova et al., 2012; Gruner et al., 2013). However, genetic approaches have failed to unravel the mechanism of *FMO1* function, and additional components involved in mediating *EDS1*-dependent, SA-independent signalling. Therefore, novel research techniques need to be applied to dissect the intriguing role and function of *FMO1* in plant defence signalling.

Chemical biology has the potential to resolve unsolved research questions, where traditional genetic approaches have failed. The aim of this thesis is to establish a high throughput chemical screen to identify compounds modulating expression of *FMO1* in *A. thaliana*. The candidate chemicals would then undergo secondary and counter-screening to eliminate compounds that affect the reporter readout, leaving only chemicals with activity specific to the *FMO1*-related defence pathway.

Such selective chemicals would then be used as probes to analyse the *EDS1*-dependent and SA-independent defence signalling mechanism in plants. The compound could also help dissect the complex interplay between *FMO1* and cell death activation in plants. Ultimately, biochemical methods and genetic screens could be used to identify the mode-of-action of the chemical and might help identify new *FMO1*-regulatory factors, and provide greater mechanistic knowledge about the *EDS1*-dependent and SA-independent signalling pathway.

2 Screening of natural and synthetic compound libraries uncovers several putative activators

I aimed at identifying small molecules that selectively modulate *FMO1* signalling in *Arabidopsis thaliana*. For this challenging task, I developed a chemical screen using *Arabidopsis thaliana* seedlings expressing a YFP tagged FMO1 fusion protein, under control of the native *FMO1* promoter in a *fmo1* mutant background. Fumonisin B1 (FB1), which is a chemical inducer of *FMO1* (Olszak et al., 2006) was used as a positive control. Initially, I treated *Arabidopsis thaliana pFMO1::FMO1:YFP* seedlings of different ages, with varying concentrations of FB1 and monitored the YFP fluorescence emitted from the plants, over a course of several days.

Eighteen day old seedlings emitted higher fluorescence than thirteen day old seedlings. Hence, 18-day old seedlings were selected for the screening, since the higher fluorescence made YFP detection in a fluorometer easier, and more consistent. I could observe a significant increase in fluorescence in the FB1 treated seedlings, over the mock (DMSO) treated seedlings, starting from 4 days post treatment (Fig. 1). Six days after treatment, there was a three-fold increase in fluorescence relative to the negative control treatment. The standard deviation of the relative fluorescence values at this time point was also lower than that at 7 days post treatment (Fig 1).

Treatment with 10µM FB1 also resulted in a more consistent increase in fluorescence, with lesser variation, in contrast to the treatment with 5µM and 10µM FB1 (data not shown). The negative control treatment with the chemical solvent DMSO (1%), had no effect on the YFP levels of the seedlings, and the fluorescence was constant like non-treated seedlings (data not shown). Hence, the assay conditions were set as treatment of 18-day old *Arabidopsis thaliana pFMO1::*FMO1:YFP seedlings for 6 days with 10µM FB1 as the positive control and 1% DMSO as the negative control (Fig. 3). The effect of FB1 on the *pFMO1::*FMO1:YFP seedlings was also confirmed by fluorescence visualisation using confocal microscopy (Fig. 2).

I performed two chemical screens in this study: (1) a screen for inhibitors of FB1-induced FMO1-YFP expression and (2) a screen for activators of FMO1-YFP expression.

The inhibitor screen utilised the positive control FB1 to induce FMO1-YFP expression in chemical pre-treated seedlings. The chemicals inhibiting FB1-induced YFP fluorescence in the seedlings were considered as inhibitors of FMO1-YFP expression. In the activator screen, *A. thaliana pFMO1::*FMO1:YFP seedlings were treated only with chemicals and the fluorescence emitted was compared to the FB1 (positive) and DMSO (negative) control treatments. Chemicals enhancing YFP fluorescence in the seedlings were considered as activators of FMO1-YFP expression.

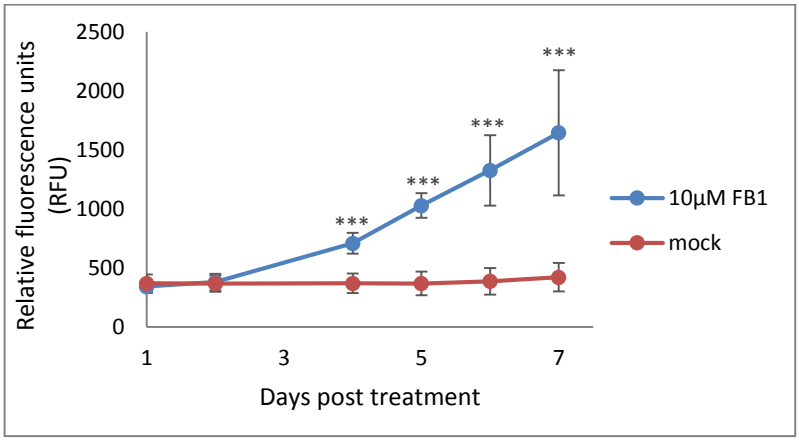

Figure 1: Time course of the effect of fumonisin B1 on *Arabidopsis thaliana pFMO1::FMO1:YFP* seedlings grown in liquid culture. 18 day old seedlings were treated with 10µM FB1 or 1% DMSO (mock). The fluorescence emitted from the seedlings was monitored daily for 7 days after initial treatment. Asterisks indicate significant differences compared to the mock control (Student's t-test ***p<0.001).

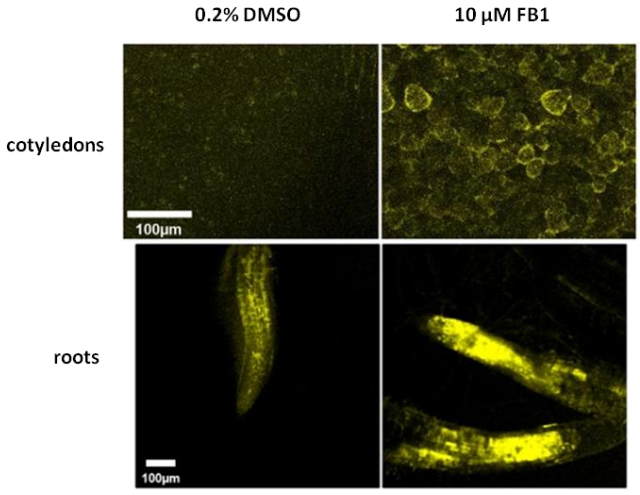

Figure 2: Effect of fumonisin B1 on fluorescence in *Arabidopsis thaliana pFMO1::*FMO1-YFP seedlings. 18 day old *Arabidopsis thaliana pFMO1::*FMO1:YFP seedlings were treated with DMSO and FB1 at the indicated concentrations. Four days after the treatment, the seedlings were examined under a confocal microscope for presence of YFP fluorescence.

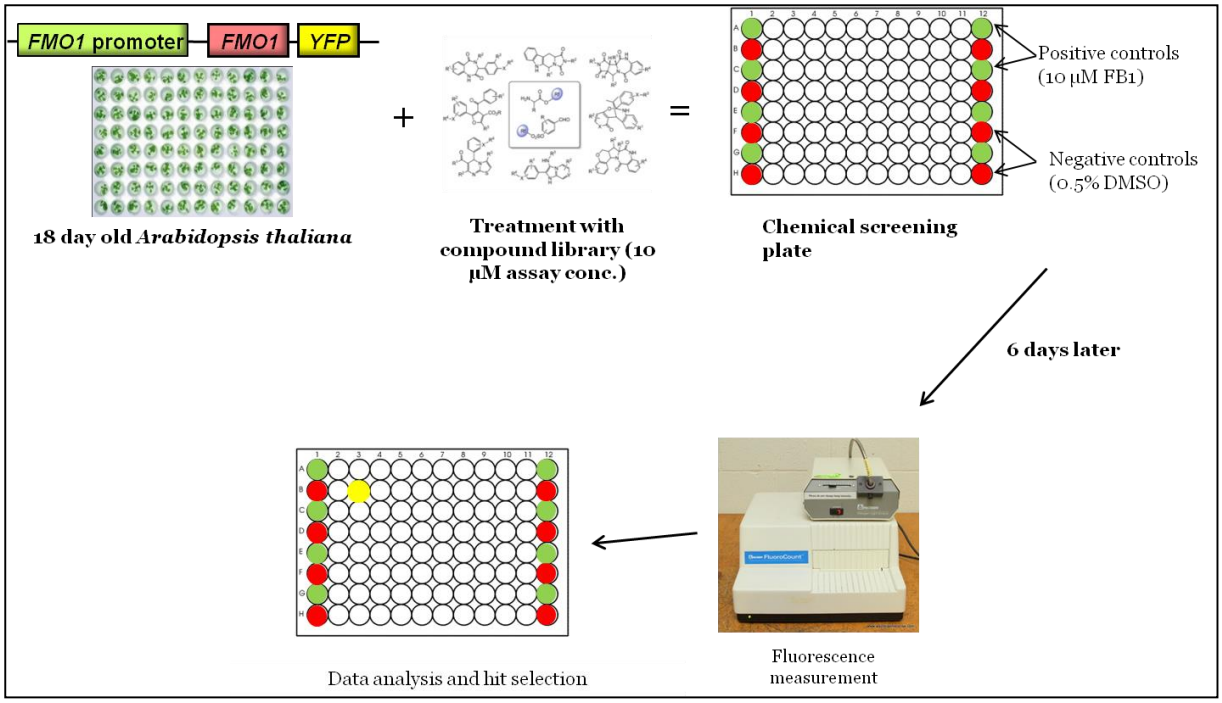

Figure 3: Graphic representation of the chemical screening protocol for activators of FMO1:YFP. 18 day old *Arabidopsis thaliana pFMO1::*FMO1:YFP seedlings were treated for 6 days with 1488 chemical compounds (each at 10-20µM) in duplicate/triplicate. 10µM FB1 and 1% DMSO were the positive and negative controls, respectively. The fluorescence emitted from the seedlings was measured 6 days post treatment. A similar protocol was followed for the inhibitor screen, but the seedlings were treated with 10µM fumonisin B1 one hour after chemical treatment with 1287 chemicals (each at 10-20µM).

2.1 Screen for inhibitors

The inhibitor screen did not yield any chemical inhibitor of FMO1-YFP. The primary reason for this was that FB1, the chemical inducer used for the screen, was a relatively weak activator of FMO1-YFP. The low YFP fluorescence of the treated samples, coupled with their relatively high variance, made it difficult to distinguish any inhibitor hits. The difference between the positive and negative controls was too low to identify a chemical hit inducing YFP fluorescence to an intermediate value.

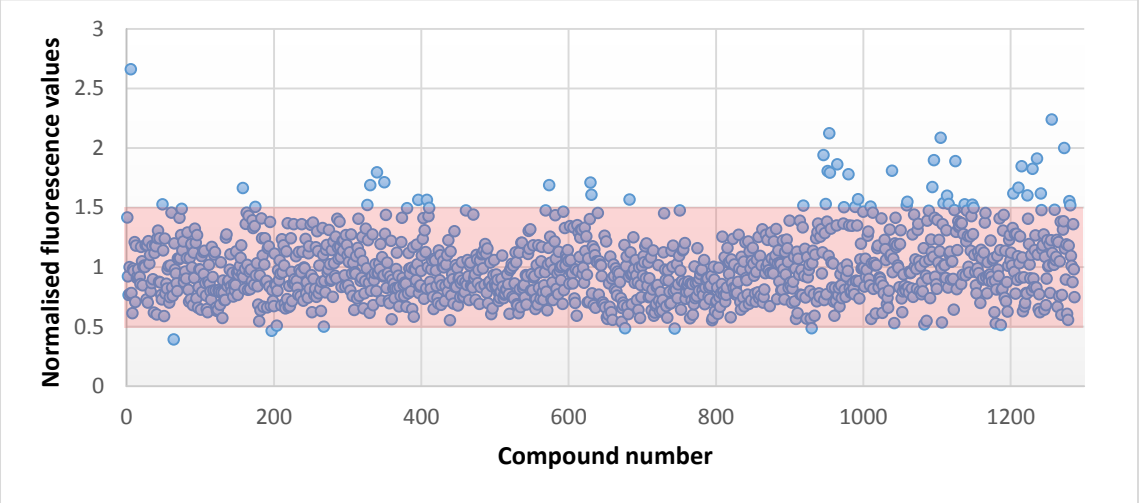

Figure 4: Chemical screen for inhibitors of *pFMO1::*FMO1:YFP expression. 18 day old *A. thaliana pFMO1::*FMO1:YFP seedlings were pre-incubated for 1 h with 1287 chemical compounds (10-20µM each) and expression of the reporter gene was induced by 10µM fumonisin B1. YFP activity was determined after 6 days in duplicate/triplicate samples and the average fluorescence value per compound was normalised to the average activity of the whole plate (96 samples). The shaded area represents the threshold determined for selection of inhibitors.

2.2 Screen for activators

*Arabidopsis thaliana pFMO1::*FMO1:YFP seedlings were screened for chemicals increasing YFP fluorescence using a library of 1488 compounds (Fig. 5). The screening revealed that the vast majority of compounds had no effect on YFP levels in the plants. Around 200 compounds increased the fluorescence to more than 1.5 times the fluorescence value of the plate average/negative DMSO control. The principle behind using the plate average or the negative control for normalisation of the fluorescence, is that since, most chemicals have no effect on YFP levels in the plants, the average fluorescence values of the entire screening plate resemble the negative control. All of these compounds were then rescreened to confirm their role in increasing FMO1-YFP activity. For the majority of these 200 compounds, activity could not be confirmed. However, this was not unexpected, since several of these already showed large variation in activity in the primary screen. Additionally, I had included weak activators of FMO1-YFP expression in the rescreening to avoid missing on false negatives. Hence, I applied a stringent criteria for rescreening putative hits found in the primary activator screen. Thirteen compounds (Fig. 5) could be confirmed as activators of FMO1:YFP expression through the rescreening, giving me a hit rate of 0.87%.

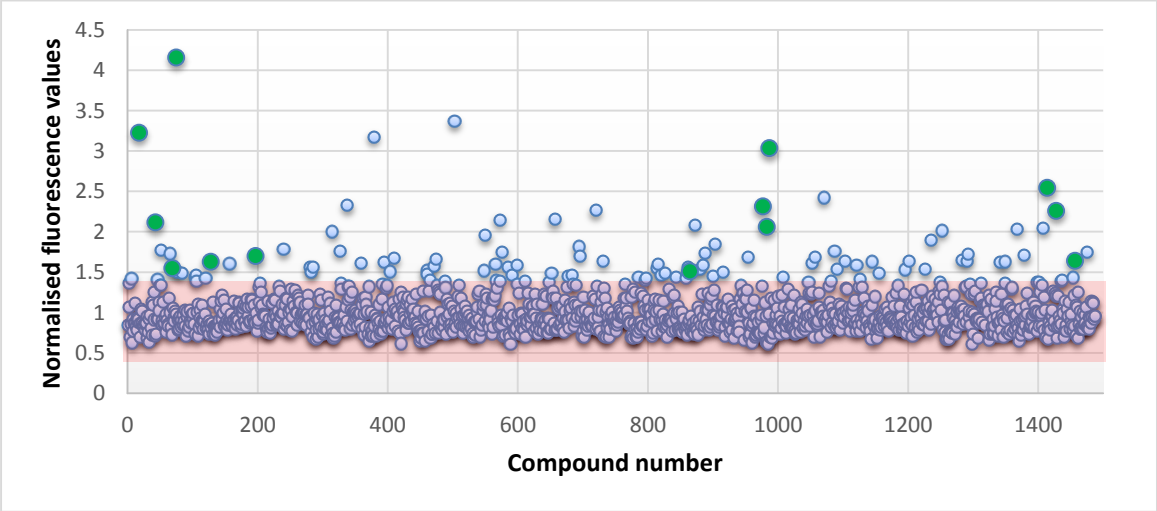

Figure 5: Chemical screen for activators of *pFMO1::*FMO1:YFP expression. 18 day old *pFMO1::FMO1:YFP* seedlings were treated with 1488 chemical compounds (10-20µM each). YFP activity was determined after 6 days in duplicate/triplicate samples and the average fluorescence value per compound was normalised to the average activity of the whole plate (96 samples). The shaded area represents the threshold determined for selection of activators. Rescreening of the candidates above this threshold identified 13 compounds (indicated in green) increasing YFP activity.

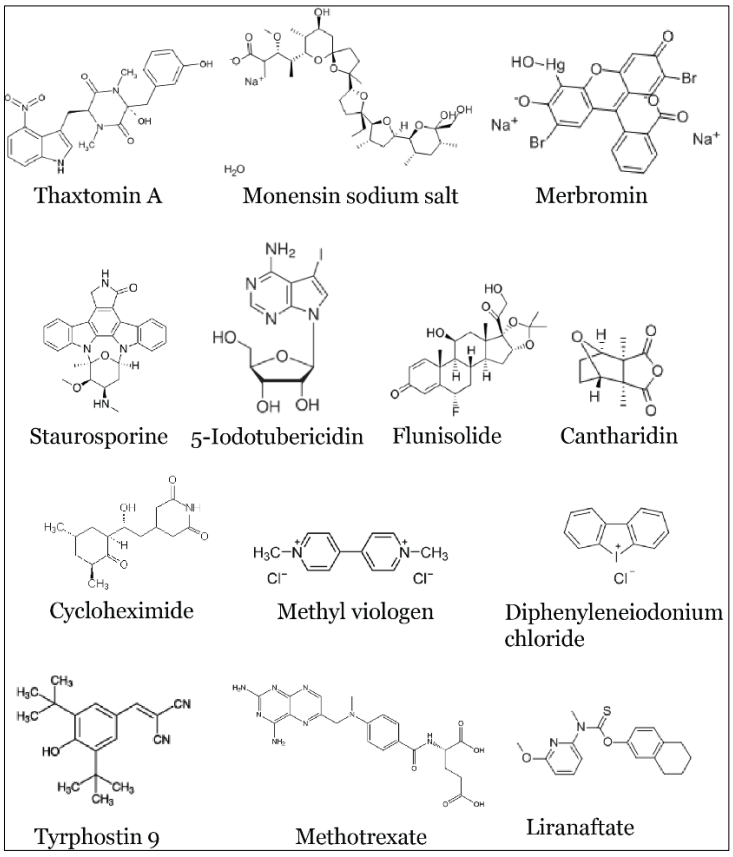

Figure 5: Names and strucutres of selected chemical activators of *pFMO1::*FMO1:YFP expression

2.2.1 Verification of activator chemicals

It was necessary to independently verify that the thirteen selected activator compounds indeed lead to accumulation of FMO1-YFP protein and subsequent increase in YFP fluorescence. Several of the chemicals used in the screening, including the positive control FB1, are known to induce programmed cell death in *Arabidopsis thaliana* (Stone et al., 2000). Auto-fluorescence due to cell death in the plants has a wavelength similar to YFP, which might lead to selection of phytotoxic compounds in the primary screen. Hence, it was important to distinguish between chemicals affecting YFP fluorescence in the original screen, from those modulating FMO1-YFP protein levels, to exclude false positives from the screening hits (Fig. 5).

In order to exclude interference due to autofluorescence, I monitored expression of a different reporter, ß-glucuronidase (GUS) under control of the *FMO1* promoter (Olszak

et al., 2006). However, the *pFMO1*::GUS reporter line proved to be unstable, with high variation of GUS activity even in the untreated samples and after DMSO control treatment (data not shown). Hence, for confirmation of the activator candidates, a Western Blot experiment was performed to check for the accumulation of FMO1-YFP protein after chemical treatment.

Western blot analysis was performed with protein samples extracted from p*FMO1::*FMO1:YFP seedlings treated with the thirteen primary hit compounds (10 µM each) for 1 day and 4 days. The blot was probed with an anti-GFP antibody to detect the presence of FMO1-YFP in the samples. The analysis indicated, that out of the thirteen chemical candidates tested, five led to an increase in accumulation of the FMO1-YFP protein (Fig. 6). FMO1-YFP protein accumulation was strongly induced in seedlings treated for 1 day with thaxtomin A, 5-iodotubercidin and monensin sodium salt. A weaker induction was observed in the merbromin and staurosporine treated samples. No signal was recorded for 4 day-treatment (data not shown).

A time course experiment conducted on similar lines revealed that maximum protein accumulation could be detected 24 hours post treatment, and thereafter declined again (data not shown). Thus, the ideal treatment time for FMO1 activity was 24 hours, and not 4-6 days as observed under primary screening conditions. The five confirmed chemical candidates do not have any obvious structural similarity (Fig. 5, Fig. 6).

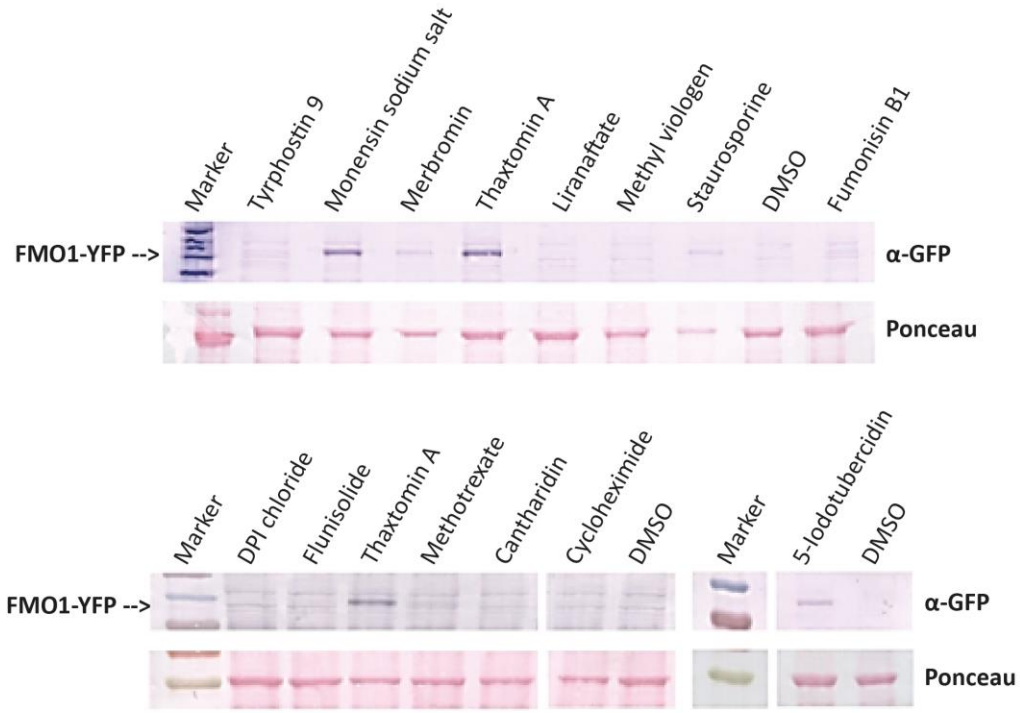

Figure 6: Accumulation of FMO1-YFP protein in response to treatment with primary hit compounds using a Western blot. 18 day old *Arabidopsis thaliana pFMO1*::FMO1:YFP seedlings were treated with 10µM chemical compounds for 24 h. Western blotting was performed with protein extracts from these seedlings using an α-GFP antibody, which binds to YFP. Five of the initial 13 compounds were confirmed as activators of FMO1-YFP. These are listed above along with their chemical structures. At least two independent experiments revealed comparable results. Ponceau staining confirmed loading of equal amounts of protein.

2.2.2 Confirmation of *FMO1* induction in wild-type seedlings by qRT-PCR

The primary and secondary screen had identified five chemicals which increased FMO1-YFP accumulation in *A. thaliana* seedlings. However, I also wanted to know whether treatment with activator candidates could promote *FMO1* transcript accumulation in wild-type Columbia (Col-0) seedlings. It was important to identify whether the effect on *FMO1* in *A. thaliana* seedlings was a general response, or was restricted to the *fmo1-1* mutant background of the seedlings used for the primary screen. Therefore, I analysed the changes in *FMO1* gene expression over time by qRT-PCR, after treatment with the candidate activators. Two week old *A. thaliana* Col-0 seedlings were treated with 10µM merbromin, monensin sodium salt, thaxtomin A, staurosporine and 5-iodotubercidin. The negative control was treatment with 1% DMSO. Samples were collected, up until 48h post-treatment. All five selected chemicals significantly increasing

FMO1 expression 24h post treatment (Fig. 7). Hence, 24h was selected as the ideal treatment time for all future experiments.

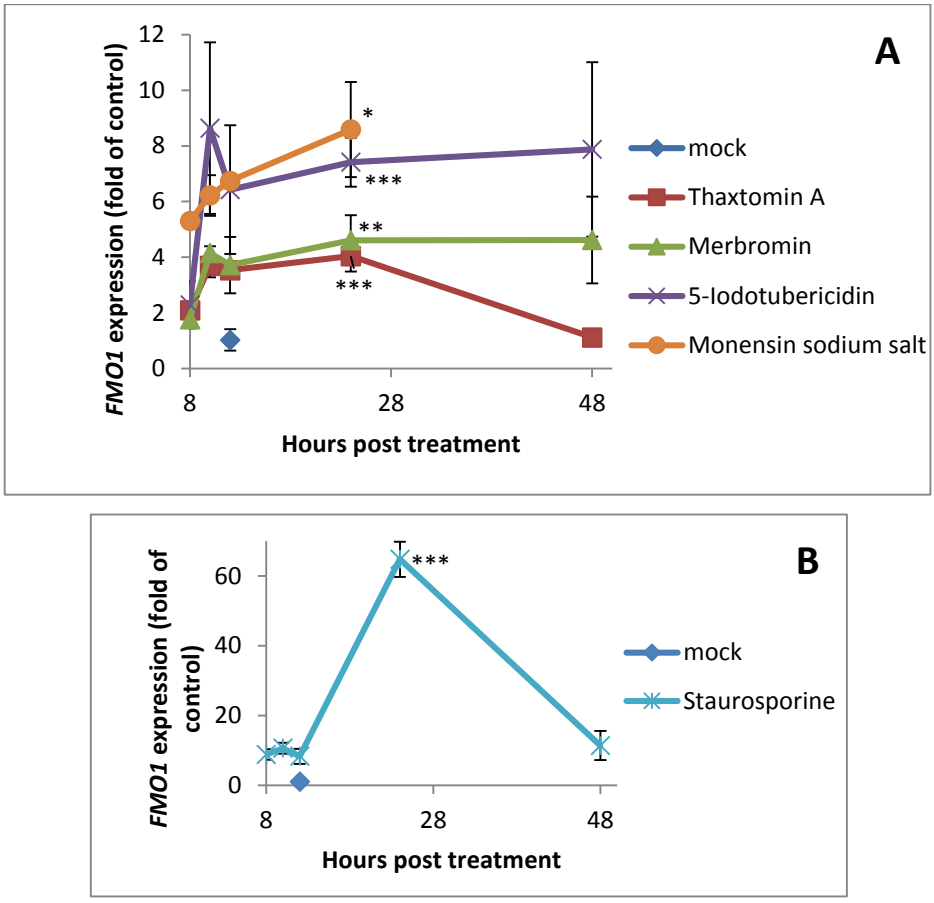

Figure 7: Changes in transcription of *FMO1* over time after treatment with 10µM activator compounds. 14 day old *Arabidopsis thaliana* Col-0 seedlings were treated with 10µM each of **A**: thaxtomin A, merbromin, monensin sodium salt, 5-iodotubercidin and **B**: staurosporine. Plants were harvested at the indicated time points and expression was monitored by qRT-PCR. All five compounds induced *FMO1* expression. Expression was normalized to the untreated control at time 0h and is shown as average of 3 biological replicates ± standard deviation. Asterisks indicate significant differences compared to the mock control (Student's t-test ***p<0.001, **p<0.01, *p<0.05).

The aim of this study was to identify chemicals which selectively activate the *EDS1*-dependent and salicylic acid-independent signalling mechanism in plants. However, staurosporine and 5-iodotubercidin are general kinase inhibitors, and thus are likely to perturb several plant signalling pathways (Massillon et al., 1994; Meggio et al., 1995). Previous screens in our laboratory indicated that staurosporine and 5-iodotubercidin inhibit jasmonic acid and calcium signalling, and also impair expression of PR1-GUS, which

is a marker of salicylic acid signalling (Christian Meesters, Jens Maintz, Vivek Halder and Mohamed Suliman; personal communication). Hence, these two chemicals were excluded from further analysis, and I focused on investigating the activity of thaxtomin A, merbromin and monensin sodium salt.

2.3 Thaxtomin A is a potent *FMO1* upregulator

Thaxtomin A was the strongest activator of FMO1-YFP accumulation (Fig. 6). *FMO1* expression was also enhanced by thaxtomin A (Fig. 7). Thaxtomin A is a phytotoxic compound produced by several *Streptomyces scabies* (King et al., 1989), which is a pathogenic bacterium, responsible for scab disease in potatoes (Lambert and Loria, 1989). Thaxtomin A production is associated with the pathogenicity of the bacteria, and causes formation of scab-like lesions on the potato tuber, which are characteristic of the disease (King et al., 1991; Goyer et al., 1998; Kinkel et al., 1998). Thaxtomin A inhibits cellulose synthesis in plants (Scheible et al., 2003). The strong activity in *FMO1* upregulation, coupled with the known role in plant disease induction, made thaxtomin A an interesting candidate for further analysis.

2.3.1 Timeline and dose-dependence of thaxtomin A-induced *FMO1*

I wanted to determine whether the concentration of thaxtomin A used in previous experiments was appropriate, or whether it needed to be adjusted for an optimal output. *A. thaliana* Col-0 seedlings were treated for 24h with increasing concentrations of thaxtomin A. qRT-PCR analysis was used to determine *FMO1* expression in the seedlings. *FMO1* expression increased with an increase in the concentration of thaxtomin A used for the treatment, upto 50nM thaxtomin A. Beyond this concentration, there was no major and consistent increase in *FMO1* expression, and expression was high, albeit variable. Thus, treatment with 50nM or higher concentration of thaxtomin A significantly increases expression of *FMO1* in *A. thaliana* seedlings. Thaxtomin A had been used before in studies on *A. thaliana* seedlings at a concentration of 100nM (Scheible et al., 2003). 100nM thaxtomin A also had a consistently robust effect on *FMO1* in all my experiments, and hence, this concentration was selected for use in all further experiments, unless otherwise stated.

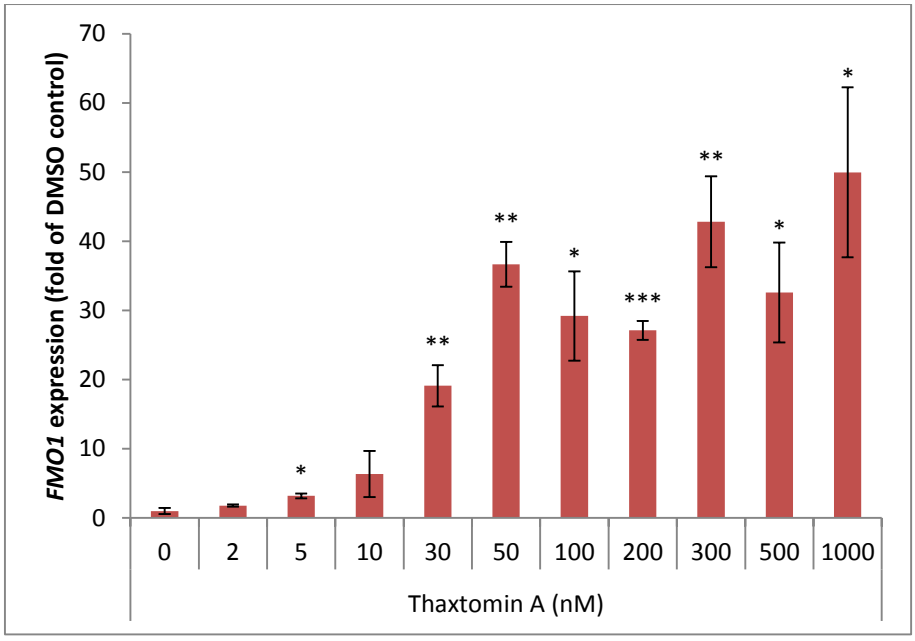

Figure 8: Dose-response of thaxtomin A-induced *FMO1* expression. 14 day old *Arabidopsis thaliana* Col-0 seedlings were treated for 24h with 1%DMSO or with thaxtomin A at the indicated concentrations. *FMO1* activity was quantified using qRT-PCR. Expression was normalized to the DMSO control and is shown as an average of 3 biological replicates ± standard deviation. This experiment was repeated twice with similar results. Asterisks indicate significant differences compared to the DMSO control (Student's t-test ***p<0.001, **p<0.01, *p<0.05). At least two independent experiments revealed comparable results.

The effect of thaxtomin A on *FMO1* over time had been analysed (Fig 7). However, I wanted to ensure that the expression pattern held true for the concentration selected through the thaxtomin A dose dependency test. Thaxtomin A at a concentration of 100nM was used to analyse expression of *FMO1* at different time points. *A. thaliana* seedlings were treated with 100nM thaxtomin A and samples were collected at various time points up until 48h post treatment. There is a low expression of *FMO1* at early time points, but it increases rapidly after 8h post treatment (Fig 9). *FMO1* expression reaches a maximum at 24h, and declines thereafter.

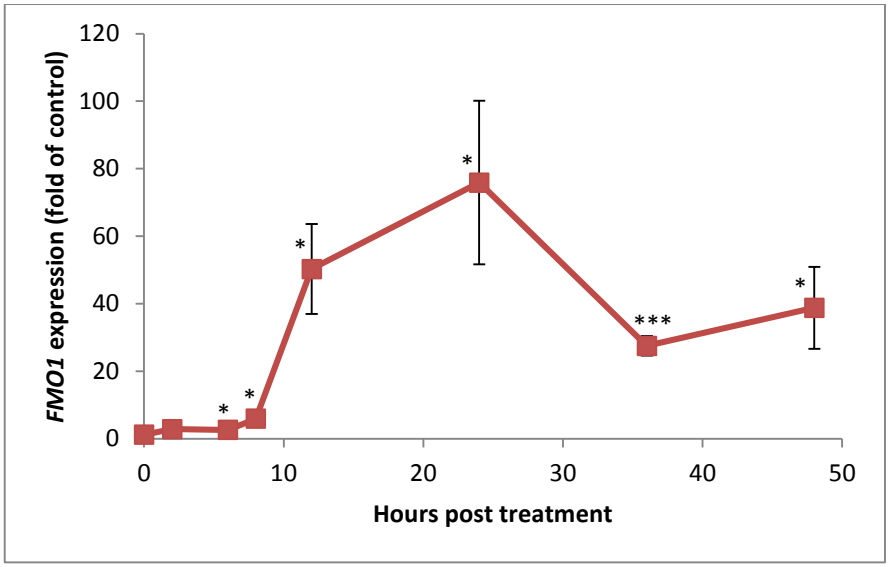

Figure 9: Changes in *FMO1* expression over time, after treatment with 100nM thaxtomin A. 13 day old *Arabidopsis thaliana* Col-0 seedlings were treated for 24h with 1% DMSO or 100nM thaxtomin A. Plants were harvested at the indicated time points, and expression was monitored by qRT-PCR. Expression was normalized to the DMSO control and is shown as an average of 3 biological replicates ± standard deviation. Asterisks indicate significant differences compared to the DMSO control (Student's t-test ***p<0.001, *p<0.05)

2.3.2 Thaxtomin A affects seedling growth, but not germination

Since thaxtomin A is such a potent activator of *FMO1* expression even at low concentrations, the question was whether it also affects plant germination and growth. Wild-type *Arabidopsis thaliana* Col-0 seeds were grown liquid medium containing increasing concentrations of thaxtomin A. All the seeds in the plate germinated. Growth was normal in 2nM thaxtomin A. However, in 5-10nM and higher concentrations of thaxtomin A, the seedlings failed to develop and their growth was arrested (Fig. 10). The seedlings grew normally in the untreated and the 1% DMSO control solutions. Thus, thaxtomin A affects seedling growth, but not germination.

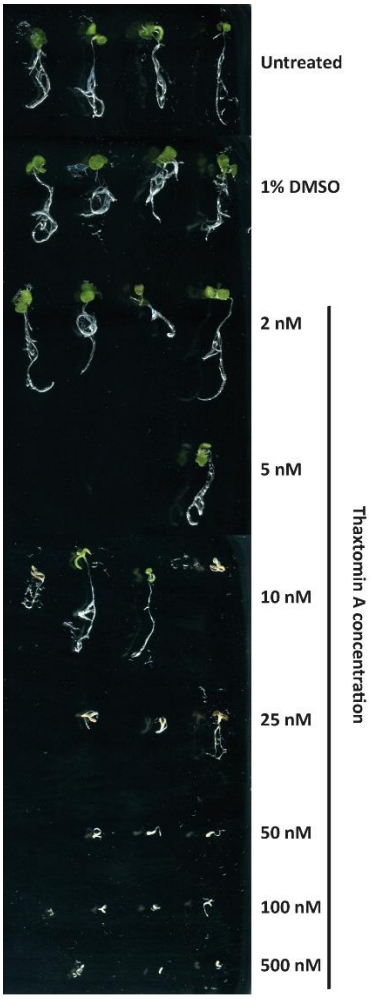

Figure 10: Effect of increasing concentrations of thaxtomin A on germination and growth. *Arabidopsis thaliana* Col-0 seedlings were sown in media containing thaxtomin A at the indicated concentrations. After 2 days of vernalization, the seeds were left to germinate and grow for two weeks. The picture depicts representative plants. Three independent experiments gave similar results.

2.3.3 Thaxtomin A does not trigger MAPK and ROS signalling

Reactive oxygen species (ROS) can act directly as antimicrobial agents (Peng and Kuc, 1992) or indirectly as messengers involved in activation of defence genes in plants (Jabs et al., 1997). I wanted to determine whether thaxtomin A produces ROS signals within the plant, thus leading to activation of defence responses. *A. thaliana* Col-0 seedlings were treated with 100nM thaxtomin A, and the rate of ROS production was compared to treatment with the ROS-inducer flg22 (1µM). ROS production was measured in relative luminescence units produced after injection of a luminol and hydrogen peroxide containing solution. As previously reported (Shi et al., 2013), the flg22-treated

seedlings produced a strong, transient increase in luminescence, over a time period of about 30 minutes (Fig 11). By contrast, thaxtomin A did not affect luminescence and values remained low, similar to the DMSO treatment. Thus, thaxtomin A does not lead to ROS production within the plant.

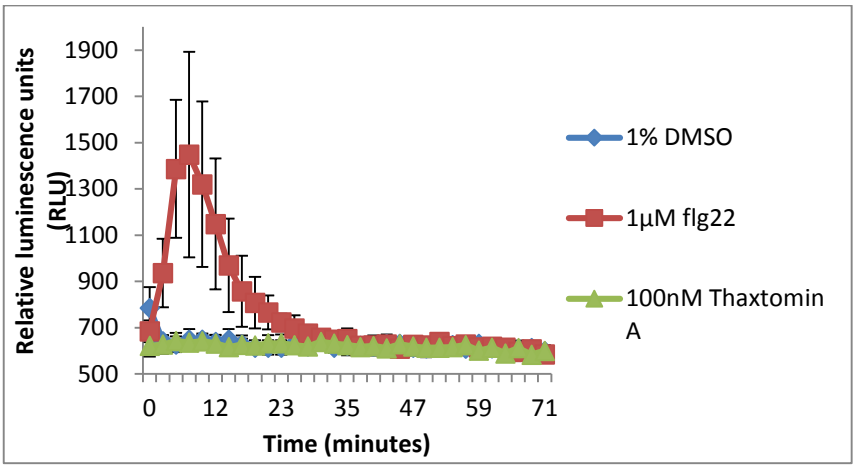

Figure 11: Effect of thaxtomin A and flg22 application on production of reactive oxygen species. 14 day old *Arabidopsis thaliana* Col-0 seedlings in liquid medium were treated with either 100nM thaxtomin A or 1µM flg22 to induce ROS production. 1% DMSO treatment served as a negative control. A solution containing luminol and peroxidase was immediately injected and luminescence was monitored over the indicated time course. Luminescence is depicted in relative light units (RLU). The graph depicts 12 biological replicates ± standard deviation. Two independent experiments gave similar results.

Prolonged activation of MAPK cascades has been known to induce *FMO1* expression (Tsuda et al., 2013). Hence, I wanted to determine whether thaxtomin A triggers MAPK responses, leading to an increase in *FMO1* expression. Seedlings were treated with 100nM thaxtomin A, and DMSO and flg22 treatment were used as negative and positive controls respectively. As expected, there was an accumulation of the active, phosphorylated MPK6 and MPK3 after flg22 application. However, thaxtomin A was unable to trigger accumulation of phosphorylated MAPK proteins (Fig. 12). Thus, thaxtomin A probably acts downstream of MAPK phosphorylation, to induce *FMO1* expression.

Therefore, thaxtomin A is a potent activator of *FMO1* expression, but does not perturb jasmonic acid signalling, and does not trigger early defence signalling responses such as MAPK activation, intracellular calcium spiking and ROS production (Table 1).

Hence, going further, I analysed the role of thaxtomin A in late defence signalling, and activity in regulation of FMO1-related defence responses.

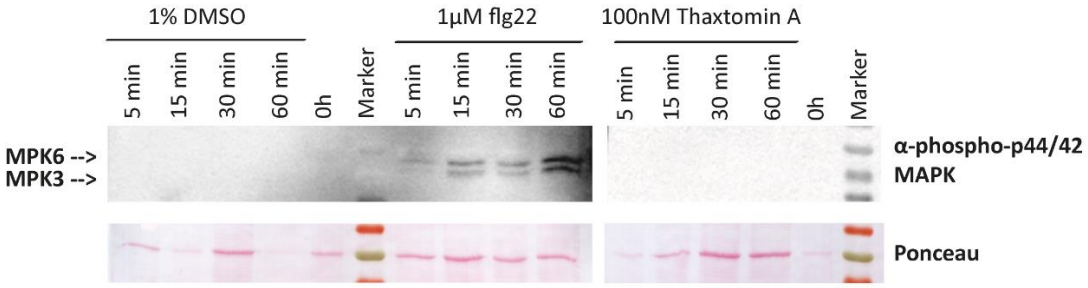

Figure 12: Role of thaxtomin A in MAPK activation. 14 day old *Arabidopsis thaliana* Col-0 seedlings were treated for the indicated time with either 1% DMSO, 100nM thaxtomin A or 1µM flg22 to activate mitogen-activated protein kinases (MAPKs). Accumulation of phosphorylated MAPKs upon application of chemicals was analysed by α-phospho-p44/42-MAPK immunoblot. At least three independent experiments revealed comparable results. Ponceau staining confirmed loading of equal amounts of protein.

Table 1: Overview of the effect of thaxtomin A on plant defence markers. The observed role of thaxtomin A in plant defence in the current screen was compared to its activity in screens for calcium signalling, salicylic acid-dependent *PR1* signalling and in inhibition of the jasmonic acid marker gene *VSP1*. Data compiled from screens by Jens Maintz, Vivek Halder and Mohamed Suliman.

Name	FMO1:YFP	PR1:GUS	Calcium signalling	VSP1:GUS	ROS production	MAPK activation
Thaxtomin A	Activator	Activator	No effect	No effect	No effect	No effect

2.3.4 Analysis of perturbations in amino acid levels

FMO1 is an essential component of systemic acquired resistance (SAR) development in plants and establishment of SAR is closely correlated to enhanced *FMO1* expression in systemic tissue (Mishina and Zeier, 2006). Upon activation of SAR in plants, there is a change in the amino acid profile of the plant (Navarova et al., 2012). There is a significant increase in levels of the defence-inducible amino acids, tyrosine and tryptophan, and a massive accumulation of α-aminoadipic acid (AAD) and the lysine derivative, pipecolic acid. Pipecolic acid is hypothesised to be a mobile signal which is transmitted across the plant, mediating SAR, and leading to upregulation of *AGD2-like defense response protein1*

(ALD1) (Navarova et al., 2012). *ALD1* has been suggested to be important for *FMO1* activation in SAR reactions. Hence, I wanted to know if thaxtomin treatment also perturbs AAD and pipecolic acid accumulation in *A. thaliana* seedlings, thus leading to enhanced *FMO1* expression. Additionally, I also wanted to find out if treatment with thaxtomin A affects the levels of defence-inducible amino acids such as tyrosine and tryptophan. *A. thaliana* Col-0 seedlings were treated with either 1% DMSO or 100nM thaxtomin A and their amino acid profile was analysed.

There was a high alanine content in both, the DMSO and the thaxtomin A-treated seedlings. However, there was no major difference between the treatments. By contrast, levels of pipecolic acid and α-aminoadipic acid were very low (hardly detectable) and did not change upon thaxtomin A treatment (Fig. 13). The levels of the defence-inducible amino acids were also unchanged. Thus, there does not seem to be a role of amino acid signalling in thaxtomin A- induced *FMO1* upregulation.

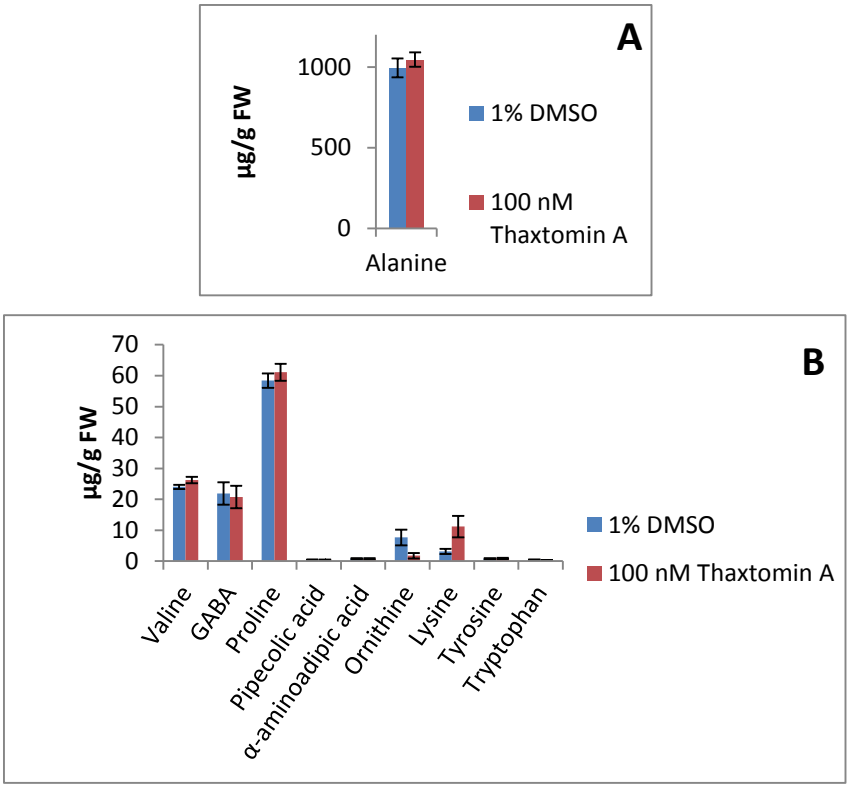

Figure 13: Thaxtomin A induced changes in free amino acid content of *Arabidopsis thaliana* seedlings. 14 day old *Arabidopsis thaliana* Col-0 seedlings were treated for 24h with 1% DMSO or 100nM thaxtomin A. The content of **A**: alanine and **B:** other amino acids in the seedlings was quantified using GC/MS and is shown as an average of 3 biological replicates ± standard deviation.

2.3.5 Thaxtomin A leads to an increase in salicylic acid levels

FMO1 activation triggers a downstream defence responses, leading to upregulation of the salicylic acid biosynthesis gene *Isochorismate Synthase1* (*ICS1)* and production of the defence metabolite, salicylic acid (Mishina and Zeier, 2006). Hence, I wanted to probe whether thaxtomin A, together with induced *FMO1* expression, leads to changes in the salicylic acid content in the plant. The total and free salicylic acid levels in the plant were quantified 24h after treatment with 100nM thaxtomin A, and were compared to the untreated and DMSO controls.

There was a highly significant increase (4-5-fold) in both, the total and the free salicylic acid content of the seedlings after treatment with thaxtomin A (Fig. 14). Thus, thaxtomin A not only induced *FMO1* expression (Fig. 6, Fig. 9), but also led to an increase in salicylic acid production.

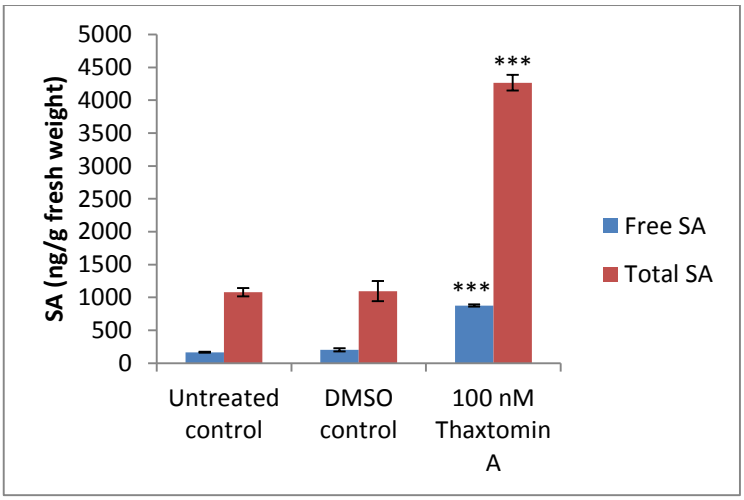

Figure 14: Changes in free and total salicylic acid content of *Arabidopsis thaliana* seedlings due to thaxtomin A treatment. 14 day old *Arabidopsis thaliana* Col-0 seedlings were treated for 24h with 100nM thaxtomin A. The negative controls were treatment with 1% DMSO or no treatment. The free and total salicylic acid content of the seedlings was quantified using GC/MS and is shown as an average of 3 biological replicates ± standard deviation. Asterisks indicate significant differences compared to the DMSO control (Student's t-test ***p<0.001). Two independent experiments gave similar results.

2.3.6 Structure-activity relationship of thaxtomin A

Thaxtomin C is a precursor of thaxtomin A, lacking a methyl group and a phenolic hydroxyl group, and was also isolated from *Streptomyces* species (King et. a., 1994). It is

responsible for induction of soil rot in sweet potatoes and causes necrosis in root tissue (Guan et al., 2012). *A. thaliana* Col-0 seedlings were treated with varying concentrations of thaxtomin C to determine whether it can also induce *FMO1* expression. My analysis indicates that thaxtomin C could also upregulate *FMO1*, although to a lower extent than thaxtomin A (Fig. 15).

Protein target identification and cellular localisation of binding proteins are crucial steps following of a successful chemical screen. Both require covalent modification of the identified chemical. Two such modified thaxtomin A derivatives were available. Thaxtomin 1378 was modified to replace the phenolic –OH group of thaxtomin A with an alkyne group, and thaxtomin 1379 had a rhodamine tag substituting the phenolic –OH group. Thaxtomin 1379, was able to significantly increase *FMO1* expression, albeit to a lower extent, and at higher concentrations, as compared to thaxtomin A. Thaxtomin 1378 was unable to cause significant changes in *FMO1* expression (Fig. 15).

Comparison between activities of thaxtomin A and thaxtomin C indicates that the phenolic –OH group of thaxtomin A, which is absent in thaxtomin C is not essential for its role in upregulation of *FMO1*. The substitution of this –OH group by the rhodamine did not affect activity in thaxtomin 1379, while the alkyne substitution in 1378 greatly reduced activity. This result could be related to differences in charge distribution between the chemicals, leading to differences in uptake by the plant. Alternatively, thaxtomin 1378 could have undergone degradation, since it was synthesized several years ago. It needs to be resynthesized and tested before drawing conclusions from the current results. Thus, changes in thaxtomin A structure can affect its activity in *FMO1* upregulation.

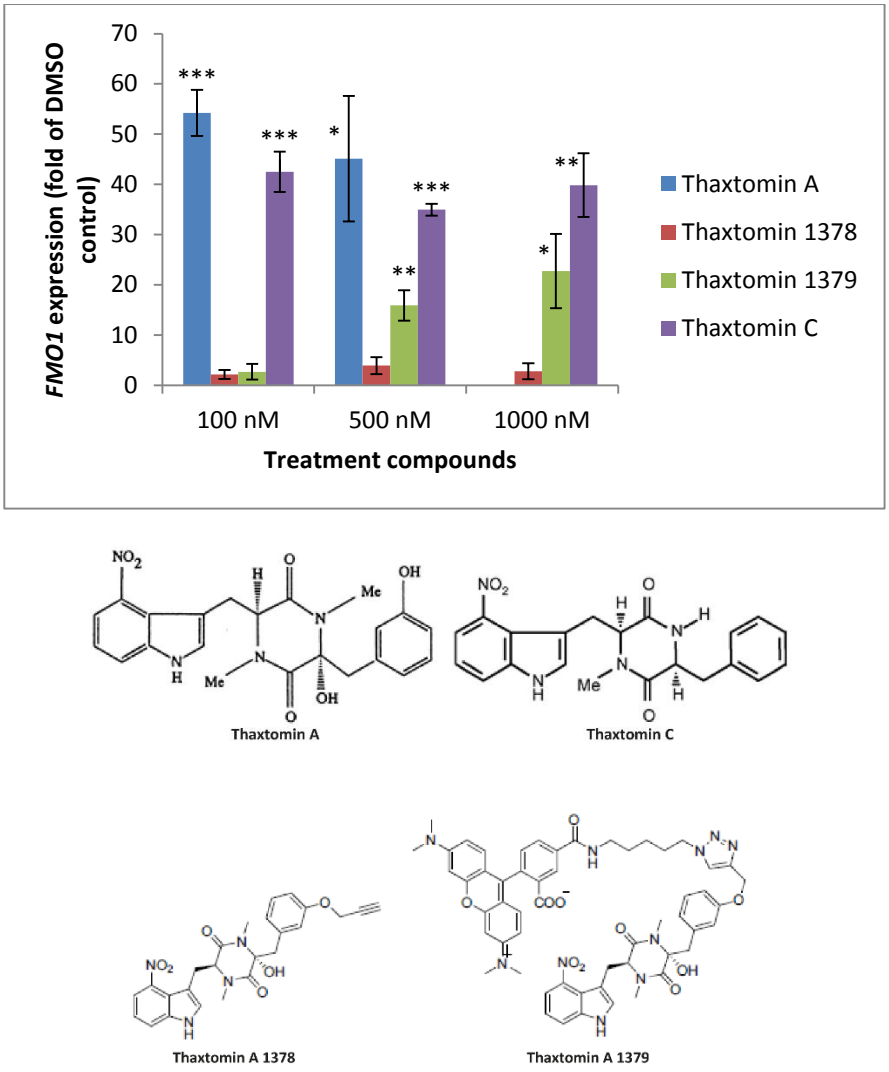

Figure 15: Activation of *FMO1* expression by different thaxtomin A derivatives. 13 day old *Arabidopsis thaliana* Col-0 seedlings were treated for 24h with thaxtomin A, alkyne-tagged thaxtomin A 1378, rhodamine-tagged thaxtomin A 1379 and the thaxtomin A precursor, thaxtomin C, at the indicated concentrations. The negative control was treatment with 1% DMSO. Expression of *FMO1* in the chemical treated samples was normalized to that of the DMSO control samples and is shown as an average of 3 biological replicates ± standard deviation. Asterisks indicate significant differences compared to the DMSO control (Student's t-test ***p<0.001, **p<0.01, *p<0.05). Two independent experiments had similar results. Structures of the thaxtomin A derivatives are depicted below the graph of their activity.

2.3.7 Role of thaxtomin A in other signalling pathways

An ideal hit compound from a chemical screen should act specifically on the pathway of interest, and not create perturbations in other signalling pathways. This increases the likelihood that a single target within the pathway of interest is affected.

Through screens conducted by colleagues in our laboratory, I knew that thaxtomin A has no effect on calcium signalling, and does not inhibit VSP1, which is a marker gene for jasmonic acid signalling. I wanted to identify whether thaxtomin A has any role in the activation of jasmonic acid, abscisic acid or auxin signalling. *A. thaliana* Col-0 seedlings treated for 24h with thaxtomin A were analysed for expression of *VSP1*, *PIN1* and *RAB18*, which are marker genes for the jasmonic acid, auxin and the abscisic acid pathways respectively. Negligible changes (<10-fold increase) in the expression of these genes were observed, when compared to the highly significant changes in *FMO1* expression (150-fold increase) (Fig. 16). Hence, thaxtomin A appears to act specifically on the *EDS1*-dependent defence signalling pathway.

Thaxtomin A is an inhibitor of cellulose synthesis (Scheible et al., 2003). I wanted to ascertain whether activation of *FMO1* is a consequence of the inhibition of cellulose synthesis, or whether *FMO1* upregulation it is independent of cellulose synthesis inhibition. Hence, I measured the changes in *FMO1* expression after treatment with isoxaben, another cellulose synthase inhibitor.

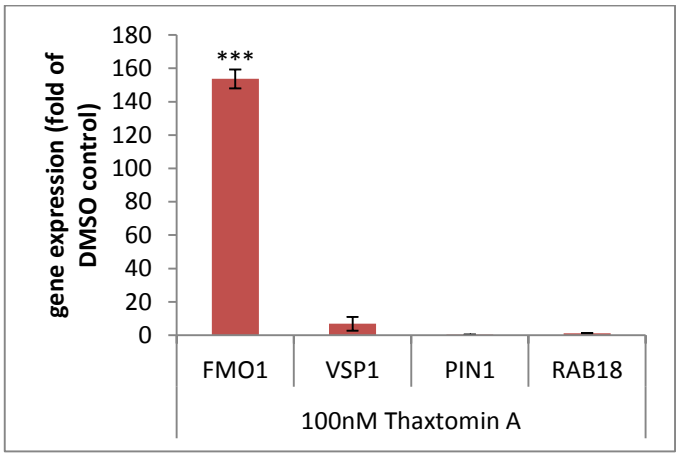

Figure 16: Effect of thaxtomin A treatment on expression of marker genes of major plant signalling pathways. 13 day old *Arabidopsis thaliana* Col-0 seedlings were treated for 24h with 1% DMSO or 100nM thaxtomin A. Activity of *FMO1*, *VSP1*, *PIN1*, and *RAB18* in thaxtomin A treated samples was normalized to that of the DMSO control samples. Gene expression is shown as an average of 3 biological replicates ± standard deviation. Asterisks indicate significant differences compared to the DMSO control (Student's t-test ***p<0.001). Two independent experiments had similar results.

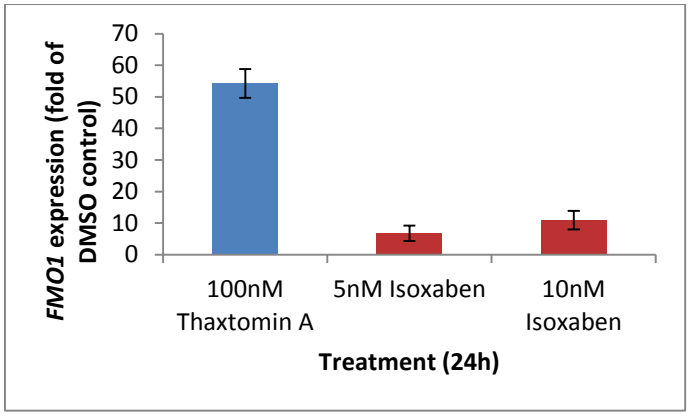

Figure 17: Impact of different cellulose synthesis inhibitors on *FMO1* expression. 13 day old *Arabidopsis thaliana* Col-0 seedlings were treated for with 24h with 1% DMSO, 100nM thaxtomin A, and 5nM or 10nM isoxaben. Expression of *FMO1* in the chemical treated samples was normalized to that of the DMSO control samples and is shown as an average of 3 biological replicates ± standard deviation.

There was a 6-10-fold increase in *FMO1* transcription due to Isoxaben, however, it was far less than the change in *FMO1* transcription (54-fold) due to thaxtomin A (Fig. 17). Thus, there does seem to be some role of cellulose synthase inhibition in *FMO1* activation. But, there is an indication that thaxtomin A also has another mechanism of *FMO1* upregulation, which is independent of its role in inhibition of cellulose synthesis.

2.3.8 *FMO1* expression due to thaxtomin A is dependent on *EDS1* and *PAD4*

During pathogen infection, *FMO1* expression is fully dependent on *EDS1* and *PAD4,* and independent of SA signalling (Bartsch et al., 2006; Mishina and Zeier, 2006). *ALD1* is also important for systemic *FMO1* upregulation during SAR (Navarova et al., 2012). This prompted me to test whether these genes are also essential for thaxtomin A-induced *FMO1* expression. I chose key genes known or hypothesised to be directly upstream or downstream of *FMO1* in the *EDS1*-dependent defence pathway. *FMO1* expression after treatment with thaxtomin A was determined in null mutants of these genes, to help pinpoint the target node/pathway for the chemical. *FMO1* expression was quantified relative to expression of the control gene *EXPR*, since different mutant backgrounds had different basal *FMO1* levels, and led to skewed results when thaxtomin A-induced *FMO1*

expression in mutant seedlings was measured as the fold value of expression in DMSO-treated mutant seedlings.

My analysis indicated that thaxtomin A- induced *FMO1* expression was dependent on *EDS1* and *PAD4*, but not on *SAG101* (Fig. 18). It was also independent of *ICS1/SID2*. This fits with previous published data that *FMO1* activity is *EDS1*- and *PAD4*-dependent, but *ICS1* independent (Bartsch et al., 2006). The data indicates that thaxtomin A-induced *FMO1* is also independent of *ALD1* (Fig. 18). This corresponds to the lack of increase in pipecolic acid levels upon thaxtomin A treatment (Fig. 13) since *ALD1* expression is tightly linked to an increase in pipecolic acid (Navarova et. al., 2012).

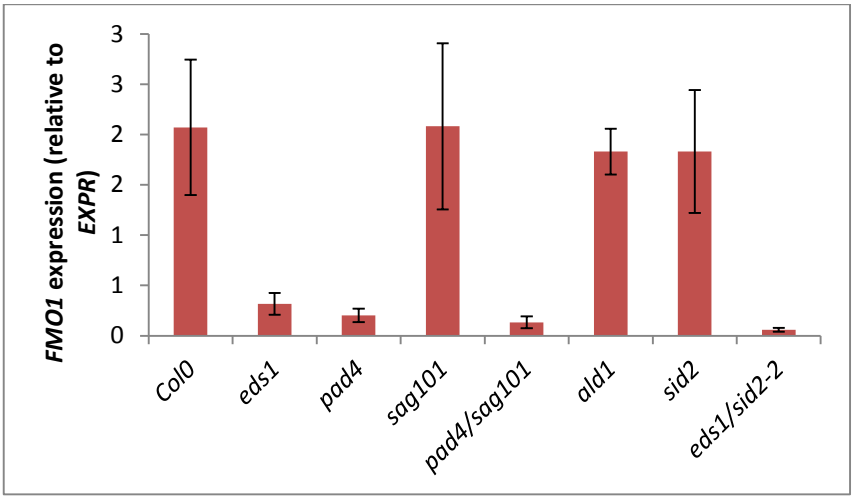

Figure 18: Thaxtomin A induced *FMO1* expression in wild-type and mutant *Arabidopsis thaliana* seedlings. 13 day old *Arabidopsis thaliana* wild-type Col-0 and the indicated mutant seedlings were treated for with 24h with 100nM thaxtomin A. Expression of *FMO1,* relative to *EXPR* expression, is shown as an average of 3 biological replicates ± standard deviation. Two independent experiments showed similar results.

2.3.9 Thaxtomin A is a *PAD4* transcription activator

I had determined that thaxtomin A increased expression of *FMO1* (Fig. 9). However, it was also important to find out which other genes have a change in expression due to thaxtomin A treatment. As thaxtomin A specifically affects *FMO1* expression dependent on *EDS1*-family signalling (Fig. 18), I tested if other *EDS1*-dependent genes are transcriptionally induced upon the chemical application.

Thaxtomin A led to the upregulation of all the genes tested, 24h post treatment. *EDS1* and its signalling partners, *PAD4* and *SAG101* had a significant (>4-fold) increase in expression (Fig. 19). There was a major increase (100-fold) in expression of *PBS3*, which is linked to *EDS1* activation. There was also massive upregulation (140-fold) of the salicylic acid marker gene *PR1*, while the salicylic acid synthesis gene *ICS1* was also upregulated (24-fold) (Fig. 19). This corresponds to the increase in production of salicylic acid observed after thaxtomin A treatment (Fig. 14). Transcription of *ALD1* is also increased (35-fold), although there was no change in pipecolic acid accumulation (Fig. 13). Thus, there is an overall increase in *EDS1*-dependent gene activity.

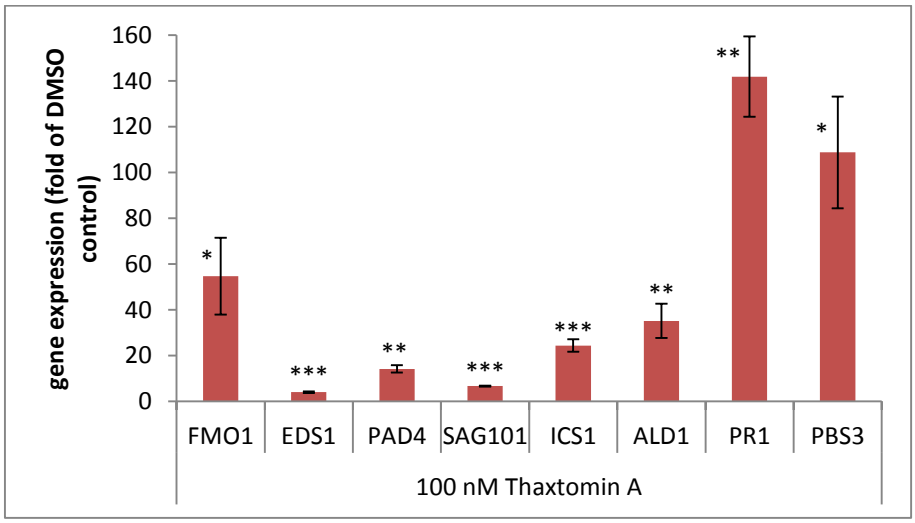

Figure 19: Role of thaxtomin A in *EDS1*-dependent gene upregulation. 13 day old *Arabidopsis thaliana* Col-0 seedlings were treated for 24h with 1% DMSO or 100nM thaxtomin A. Activity of indicated genes in the thaxomin A treated samples was normalized to that in the DMSO control samples. Gene expression is shown as an average of 3 biological replicates ± standard deviation. Asterisks indicate significant differences compared to the DMSO control (Student's t-test ***p<0.001, **p<0.01, *p<0.05). Two independent experiments had similar results.

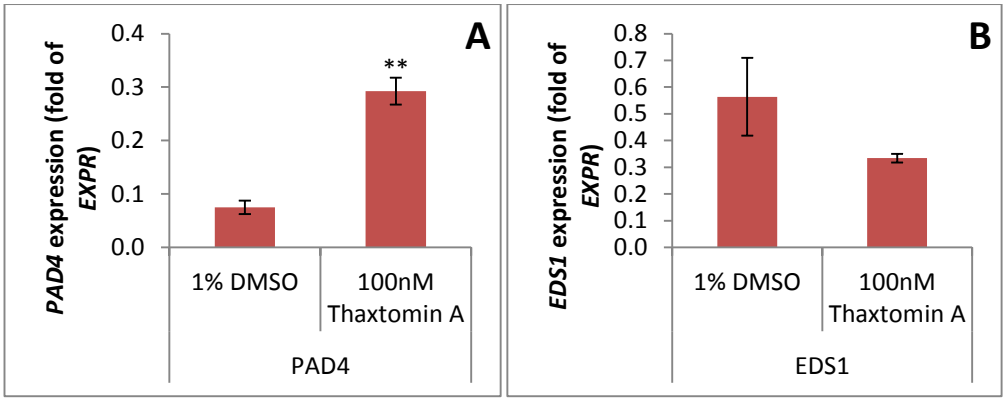

Figure 20: Mutant background influences thaxtomin A induction of *EDS1* and *PAD4*. 13 day old *Arabidopsis thaliana eds1* and *pad4* mutant seedlings were treated for 24h with 1% DMSO or 100nM thaxtomin A. **A:** *PAD4* expression in *eds1* mutant background and **B:** *EDS1* expression in *pad4* mutant background relative to *EXPR* expression, is shown as an average of 3 biological replicates ± standard deviation. Asterisks indicate significant differences compared to the DMSO control (Student's t-test **p<0.01). The results were confirmed in two independent experiments.

EDS1 and *PAD4* were found to be crucial for thaxtomin A-induced *FMO1* upregulation (Fig. 18). Thaxtomin A also leads to an increase in transcription of *EDS1* and *PAD4* (Fig. 19). EDS1 is important for stabilising PAD4 protein structure and they form a heterodimeric complex, which is necessary for basal resistance involving up-regulation of *PAD4* transcription (Rietz et. al., 2011). Hence, the question was, how does thaxtomin A affect *PAD4* transcription in the absence of *EDS1*, and vice versa? *EDS1* expression in a *pad4* null mutant background and *PAD4* expression in an *eds1* null mutant background was analysed after treatment with thaxtomin A. Surprisingly, there was a significant (4-fold) increase in *PAD4* expression in the *eds1-2* background (Fig. 20). This is interesting, since even in the absence of PAD4 protein stabilisation by EDS1, thaxtomin A still drives *PAD4* expression.

However, *EDS1* is not upregulated in the *pad4* mutant background (Fig. 20). Instead, there is a small, but not statistically significant, decrease in *EDS1* expression after thaxtomin A treatment, as compared to the DMSO control. Thus, it appears as if thaxtomin A actively drives *PAD4* expression, while the increase in *EDS1* expression in the wild-type Col-0 seedlings is a feedback effect of *PAD4*-downstream signalling.

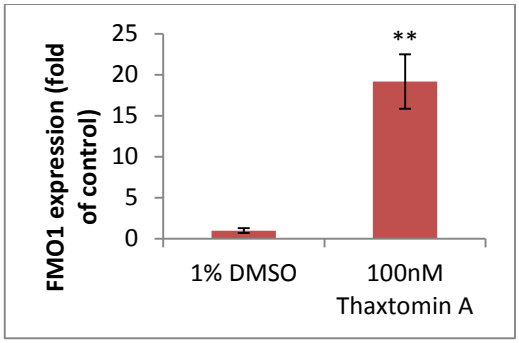

Figure 21: Thaxtomin A induced *FMO1* expression in an eds1^{L262P} mutant seedlings. 13 day old *Arabidopsis thaliana* eds1^{L262P} seedlings were treated for 24h with 1% DMSO or 100nM thaxtomin A. *FMO1* expression in thaxtomin A treated plants, relative to DMSO treated plants, is shown as an average of 3 biological replicates ± standard deviation. Asterisks indicate significant differences compared to the DMSO control (Student's t-test **p<0.01).

I further explored the interaction between EDS1 and PAD4, and the influence of PAD4 stability on *FMO1* expression, by treating *A. thaliana eds1^{L262P}*seedlings with 100nM thaxtomin A. The *eds1^{L262P}* mutant plants have a mutation in EDS1, which prevents interaction with PAD4 and formation of an EDS1-PAD4 protein heterodimer, while retaining interaction with SAG101 (Rietz et al., 2011). It was hypothesised that PAD4 needs EDS1 for protein stabilisation and driving downstream signalling, and thus thaxtomin A would not increase *FMO1* expression in the *eds1^{L262P}* mutant. However, there was a significant (20-fold) induction of *FMO1* after thaxtomin A treatment in the *eds1^{L262P}* plants (Fig. 21). Thus, *PAD4* may have a role in *FMO1* induction, which is independent of its interaction with *EDS1*. However, a functional *EDS1* is still required for complete wild-type-like induction of the *FMO1* gene (Fig. 18).

2.4 Monensin sodium salt and merbromin increase *FMO1* expression

Monensin sodium salt and merbromin were identified as activators of FMO1-YFP in the primary screen (Fig. 5). This was confirmed through a Western blot (Fig. 6). Secondary screening also revealed that monensin sodium salt and merbromin positively affect *FMO1* transcript levels (Fig. 7). They seem to be specific for defence against microbial pathogens, since other screens conducted in our laboratory revealed that they do not act as inhibitors of the jasmonic acid pathway and also do not interfere with calcium signalling (Christian Meesters, Mohamed Suliman and Jens Maintz; personal communication) (Table 2).

2.4.1 EC_{50} determination

The term 'half maximal effective concentration' (EC50) refers to the concentration of a drug, antibody or toxicant which induces a response halfway between the baseline and the maximum. It is commonly used as a measure of a drug's potency. I performed a concentration dependence test of chemical-induced *FMO1* expression by treating *A. thaliana* seedlings for 24h with increasing concentrations of monensin sodium salt and merbromin.

The *FMO1* expression increased with an increase in concentration of chemicals used for the treatment, reaching a maxima at 1 µM for monensin sodium salt treatment (Fig. 22A), and at 20 µM for merbromin treatment (Fig. 22B), before decreasing. The EC_{50} value was calculated as 1 µM for monensin sodium salt and 1µM for merbromin. However, since 10 µM monensin sodium salt and 20 µM merbromin resulted in a consistently high expression of *FMO1*, these concentrations was chosen for most of my subsequent experiments.

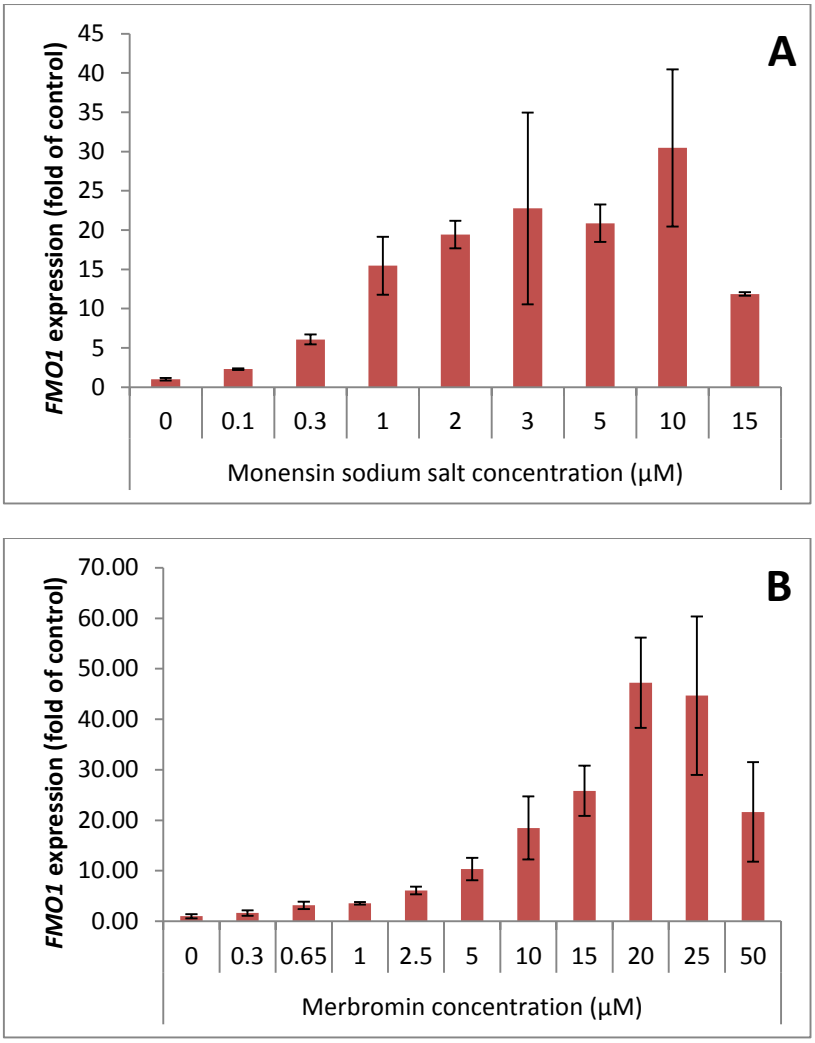

Figure 22: Dose-response of monensin sodium salt and merbromin-induced *FMO1* expression. 14 day old *Arabidopsis thaliana* Col-0 seedlings were treated for 24h with **A:** monensin sodium salt or **B:** merbromin at the indicated concentrations. *FMO1* expression was quantified using qRT-PCR. Expression was normalized to the DMSO control and is shown as an average of 3 biological replicates ± standard deviation. This experiment was repeated twice with similar results.

2.4.2 Monensin sodium salt and merbromin affect germination and growth in a concentration-dependent manner

Monensin sodium salt and merbromin are activators of *FMO1* expression (Fig. 7) and protein accumulation (Fig. 6). *FMO1* upregulation in plants has been linked with an increase in programmed cell death (Olszak et. al., 2006). Hence, I wanted to know whether increasing concentrations of monensin sodium salt and merbromin would hamper germination and growth in the *A. thaliana* seedlings.

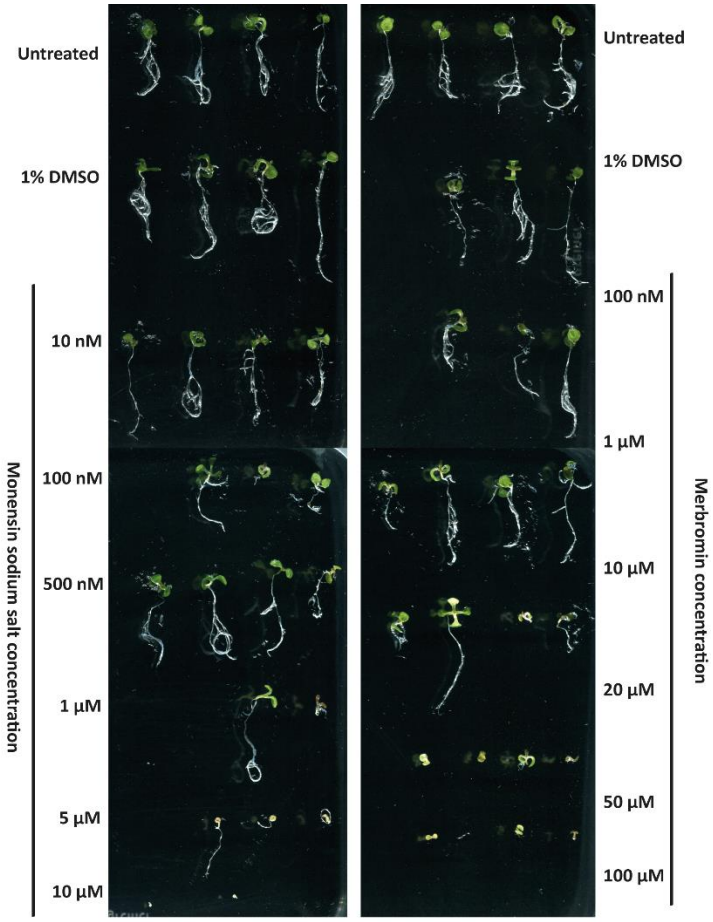

Figure 23: Effect of increasing concentrations of monensin sodium salt on germination and growth. *Arabidopsis thaliana* Col-0 seedlings were sown in media containing monensin sodium salt at the indicated concentrations. After 2 days of vernalization, the seeds were left to germinate and grow for two weeks. The picture depicts representative plants. Two independent experiments gave the same results.

Wild-type *Arabidopsis thaliana* Col-0 seeds were sown into liquid medium containing increasing concentrations of monensin sodium salt and merbromin. The seedlings germinated and grew well in the untreated and the 1% DMSO control treatments (Fig. 23). However, germination was reduced and growth was stunted in plants grown in monensin sodium salt above a concentration of 500 nM and merbromin above a concentration of 10 µM. There was a gradual reduction in size of the seedlings, due to an increase in the concentration of the chemical treatment. Thus, monensin sodium salt and merbromin have a concentration dependent effect on germination and growth of *A. thaliana* seedlings, and a chemical concentration which induces high *FMO1* expression (Fig. 22), also causes severe developmental defects.

2.4.3 Interaction between early defence signalling and *FMO1* upregulation

Monensin sodium salt and merbromin were demonstrated to affect *FMO1* expression (Fig. 7), which is a part of late defence signalling (Bartsch et. al., 2006; Mishina and Zeier, 2006). The above chemicals did not trigger early calcium spikes in plants (Jens Maintz, personal communication). *Arabidopsis thaliana* react to stress or PAMPs (pathogen associated molecular patterns) by production of an oxidative burst, which is a very fast generated signal leading to upregulation of several defence responses (Zipfel et. al., 2004; Mersmann et. al., 2010). Prolonged activation of MAPK signalling can lead to enhanced *FMO1* expression (Tsuda et. al., 2013) However, it was not known whether monensin sodium salt and merbromin triggered these early defence signalling events, which could lead to long term changes in defence gene expression in the plants (Jabs et al., 1997; Tsuda et al., 2013). Therefore, I analysed *A. thaliana* seedlings treated with monensin sodium salt (10 µM) and merbromin (20 µM) for accumulation of active, phosphorylated MAPKs, and production of reactive oxygen species (ROS).

Similar to thaxtomin A treatment, application of monensin sodium salt and merbromin did not generate ROS production in *A. thaliana* seedlings, as compared to the transient increase in ROS after treatment with flg22 (Fig. 24). Thus, ROS production does not mediate upregulation of *FMO1* expression by monensin sodium salt and merbromin.

The active, phosphorylated form of MAPKs was detected in protein extracts of the treated seedlings using an α-phospho-p44/42 antibody. As expected, there was an accumulation of MAPK after flg22 activation (Fig. 25). Merbromin did not influence MAPK phosphorylation. However, monensin sodium salt-treatment increased accumulation of phosphorylated MPK6 and MPK3 protein. Therefore, monensin sodium salt-induced *FMO1* expression could be potentially mediated by MAPK signalling.

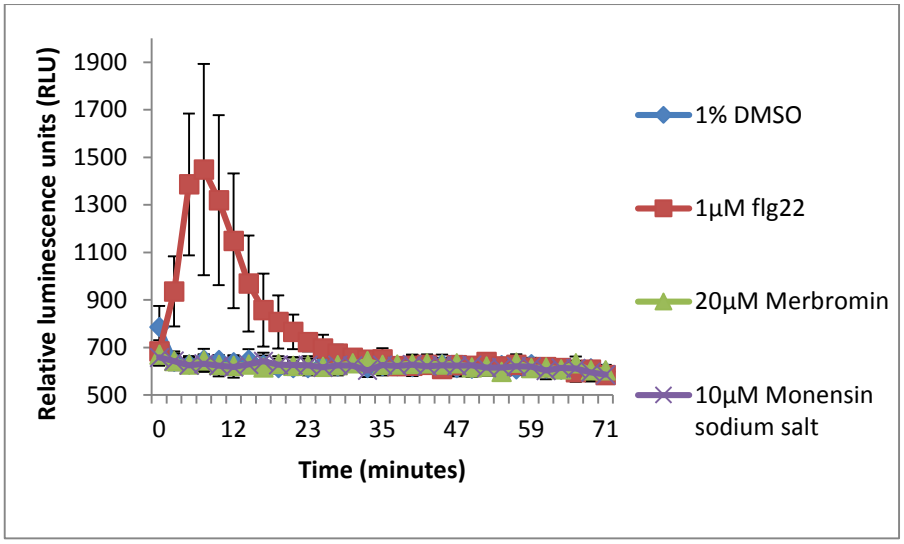

Figure 24: Effect of chemical application on production of reactive oxygen species. 14 day old *Arabidopsis thaliana* Col-0 seedlings in ½ MS medium were treated with either 1% DMSO, 10µM monensin sodium salt, 20µM merbromin or 1µM flg22 to induce ROS production. A solution containing luminol and peroxidase was immediately injected and luminescence was monitored over the indicated time course. Luminescence is depicted in relative light units (RLU). The graph depicts 12 biological replicates ± standard deviation. Two independent experiments gave similar results.

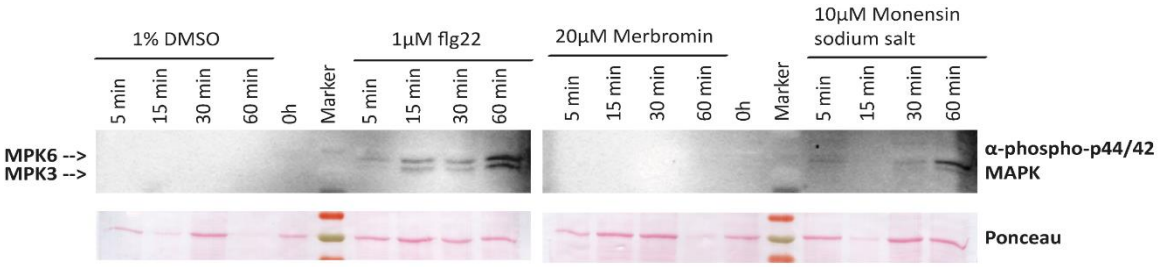

Figure 25: Role of monensin sodium salt in MAPK activation. 14 day old *Arabidopsis thaliana* Col-0 seedlings were treated for the indicated time with either 1% DMSO, 10µM monensin sodium salt, 20µM merbromin or 1µM flg22 to activate mitogen-activated protein kinases (MAPKs). Accumulation of phosphorylated MAPKs upon application of chemicals was analysed by α-phospho-p44/42-MAPK immunoblot. At least two independent experiments revealed comparable results. Ponceau staining was used to compare loading amounts of protein.

Table 2: Overview of the effect of monensin sodium salt and merbromin on plant defence markers. The observed role of monensin sodium salt and merbromin in plant defence in the current screen was compared to its reported activity in screens for calcium signalling, salicylic acid-dependent *PR1* signalling and in inhibition of the jasmonic acid marker gene *VSP1*. Data compiled from screens by Jens Maintz, Christian Meesters, Vivek Halder and Mohamed Suliman.

Name	FMO1:YFP	PR1:GUS	Calcium signalling	LOX2:LUC	VSP1:GUS	ROS production	MAPK activation
Merbromin	Activator	Inhibitor	No effect	Inhibitor	Inhibitor	No effect	No effect
Monensin sodium salt	Activator	Inhibitor	No effect	Inhibitor	Inhibitor	No effect	Activator

2.4.4 Role of monensin sodium salt in other signalling pathways

Monensin sodium salt and merbromin were demonstrated to be inducers of *FMO1* expression (Fig. 7). They were also found to be inhibitors of the jasmonic acid pathway (Christian Meesters and Mohamed Suliman; personal communication) (Table 2). Salicylic acid and jasmonic acid pathways in plants, have antagonistic functions (Spoel et. al., 2003; Mur et. al., 2006). Thus, monensin sodium salt and merbromin could possibly cause an inhibition of the jasmonic acid marker genes *VSP1* and *LOX2*, due to their activity on *FMO1*. However, it was important to identify whether the compounds had any effects on other major plant signalling pathways.

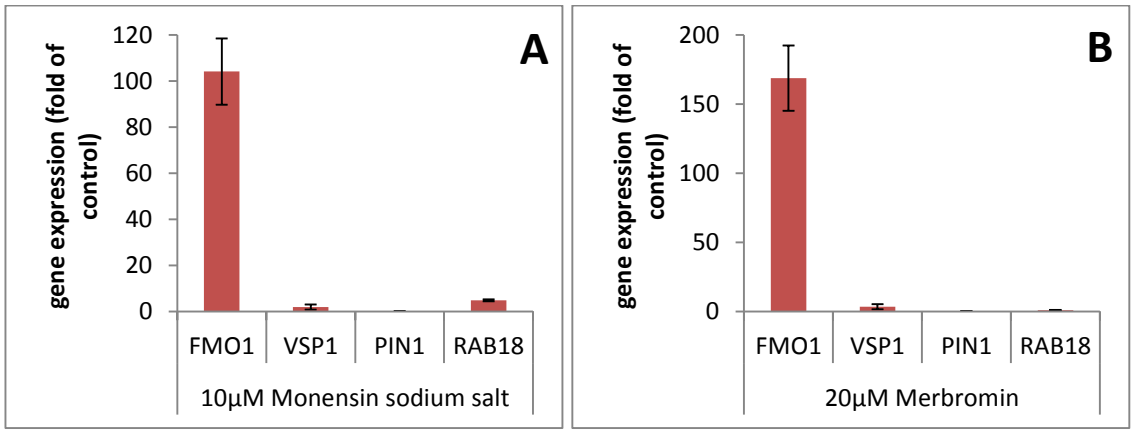

Figure 26: Effect of Monensin sodium salt and merbromin treatment on expression of marker genes of major plant signalling pathways. 13 day old *Arabidopsis thaliana* Col-0 seedlings were treated for 24h with 1% DMSO or **A:** 10µM monensin sodium salt and **B:** 20µM merbromin. Activity of *FMO1*, *VSP1*, *PIN1*, and *RAB18* in chemical treated samples was normalized to that of the DMSO control samples. Gene expression is shown as an average of 3 biological replicates ± standard deviation. Two independent experiments had similar results.

I tested the effect of treatment with monensin sodium salt and merbromin on the activation of the key marker genes in major plant signalling pathways. Compared to the increase in *FMO1* expression (>100-fold), there was little change (<5-fold increase) in the expression of *VSP1*, *PIN1* and *RAB18* which are marker genes for the jasmonic acid, auxin and abscisic acid signalling pathways, respectively. Thus, monensin sodium salt and merbromin seem to be selective activators of *FMO1* expression.

2.4.5 Analysis of changes in amino acid concentration

FMO1 expression is enhanced in systemic tissues during SAR in plants, which is characterised by perturbations in the amino acid content of the plants (Mishina and Zeier, 2006; Navarova et al., 2012). *A. thaliana* seedlings were treated with monensin sodium salt and merbromin for 24h and the seedlings were processed to extract amino acids. The amino acid content of the chemical and the control (1% DMSO), treated seedlings was compared. *A. thaliana* seedlings contained high levels of alanine, whereas all other amino acids were low. However, this is not specific to the treatment, but rather seems to be an artefact of the developmental stage of the plant (Limami et. al., 2008). Importantly, treatment with monensin sodium salt and merbromin did not result in an increase in amino acid content of *A. thaliana* seedlings, including that of the defence inducible amino acids like tyrosine, tryptophan, Pip and α-aminoadipic acid (Fig. 27). Thus, there does not seem to be a role of amino acid signalling in monensin sodium salt and merbromin-induced *FMO1* upregulation.

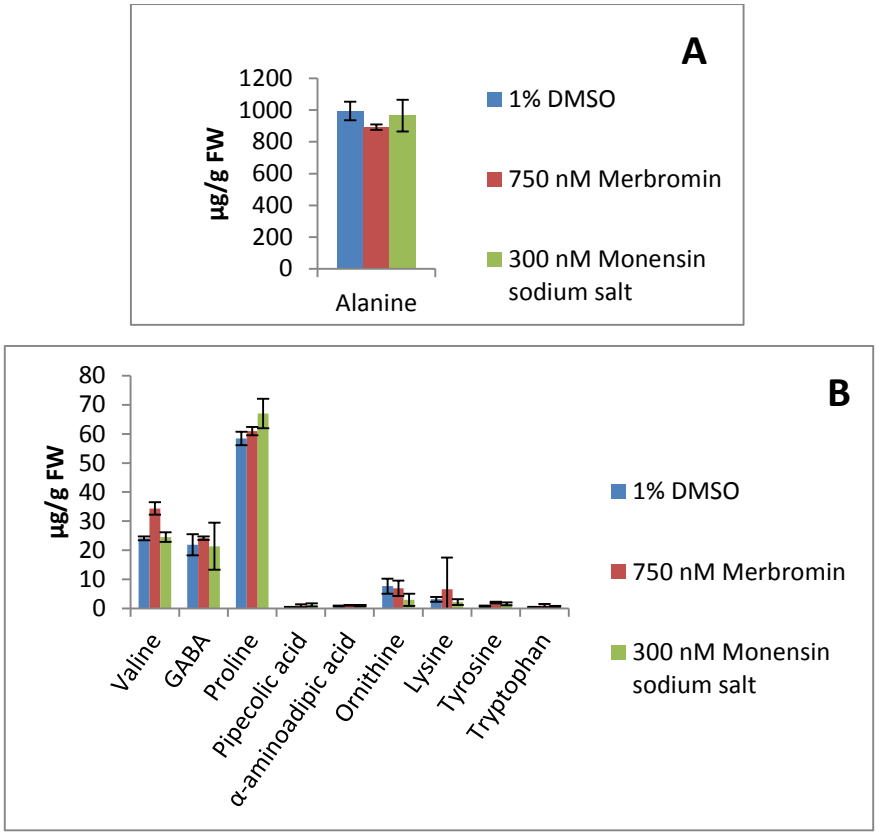

Figure 27: Effect of merbromin and monensin sodium salt treatment on free amino acid content of *Arabidopsis thaliana* seedlings. 14 day old *Arabidopsis thaliana* Col-0 seedlings were treated for 24h with 1% DMSO or 300nM monensin sodium salt. The amino acids content of the seedlings was quantified using GC/MS and is shown as an average of 3 biological replicates ± standard deviation.

2.4.6 Effect of monensin sodium salt and merbromin on defence gene expression

I had established that monensin sodium salt and merbromin act as an inducers of *FMO1* (Fig. 7). However, I also wanted to identify whether they act on *FMO1* alone, or also on other genes known to be dependent on *EDS1*/PAD4. I treated seedlings with monensin sodium salt and analysed the expression of genes known to be upstream or downstream of *FMO1* in the *EDS1/PAD4* signalling pathway (Gruner et al., 2013).

qRT-PCR analysis of the treated seedlings revealed that, not only did the chemical treatments significantly increase expression of *EDS1* and its associated genes *PAD4* and *SAG101*, but they also led to upregulation (>20-fold) of other *EDS1* and *PAD4* dependent genes such as *ICS1* and *ALD1* (Fig. 28). Monensin sodium salt also caused a massive increase (>300-fold) in transcription of *PR1*, and thus, causes a strong transcriptional

upregulation of genes involved in resistance to pathogens. On the contrary, merbromin significantly induces expression of *EDS1*-dependent genes such as *PBS3*, and the SA-biosynthesis gene *ICS1*, but does not significantly increase expression of the SA-marker gene *PR1*. Therefore, merbromin activates *EDS1/PAD4* dependent signalling responses, but may cause blocking of signalling downstream of *ICS1*.

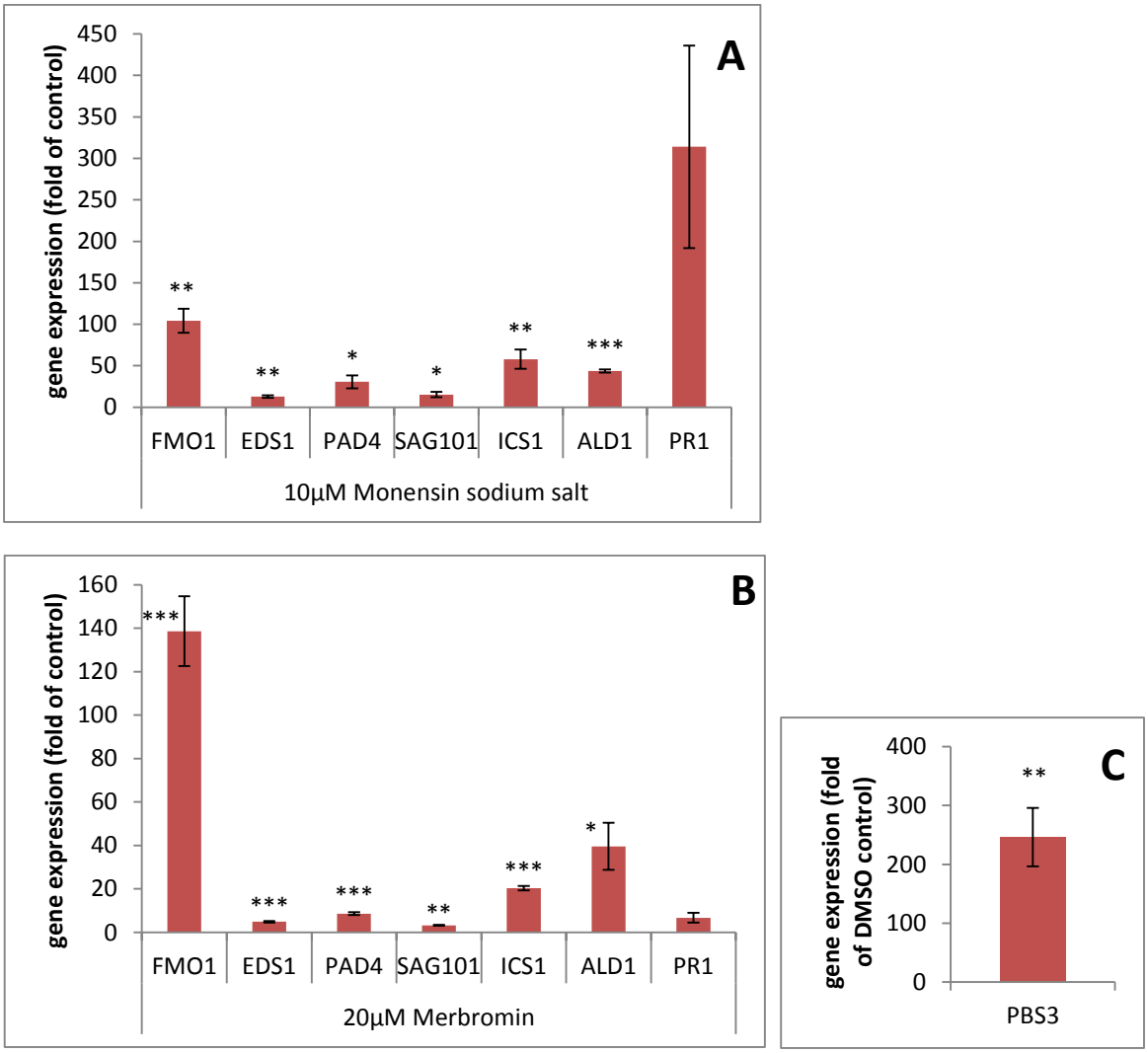

Figure 28: Effect of Monensin sodium salt and Merbromin on defence gene expression. 13 day old *Arabidopsis thaliana* Col-0 seedlings were treated for 24h with 1% DMSO, **A:** 10µM monensin sodium salt or **B:** and **C:** 20 µM merbromin. Activity of indicated genes in the chemical-treated seedlings was normalized to that in the DMSO-treated seedlings. Gene expression is shown as an average of 3 biological replicates ± standard deviation. Asterisks indicate significant differences compared to the DMSO control (Student's t-test ***p<0.001, **p<0.01, *p<0.05). Two independent experiments had similar results.

2.4.7 Monensin sodium salt, but not merbromin leads to an increase in salicylic acid

Monensin sodium salt and merbromin were proven to increase transcription of the SA synthesis gene, *ICS1* (Fig.28). Thus, it was hypothesised that the production of SA within the plant should increase after the chemical treatment.

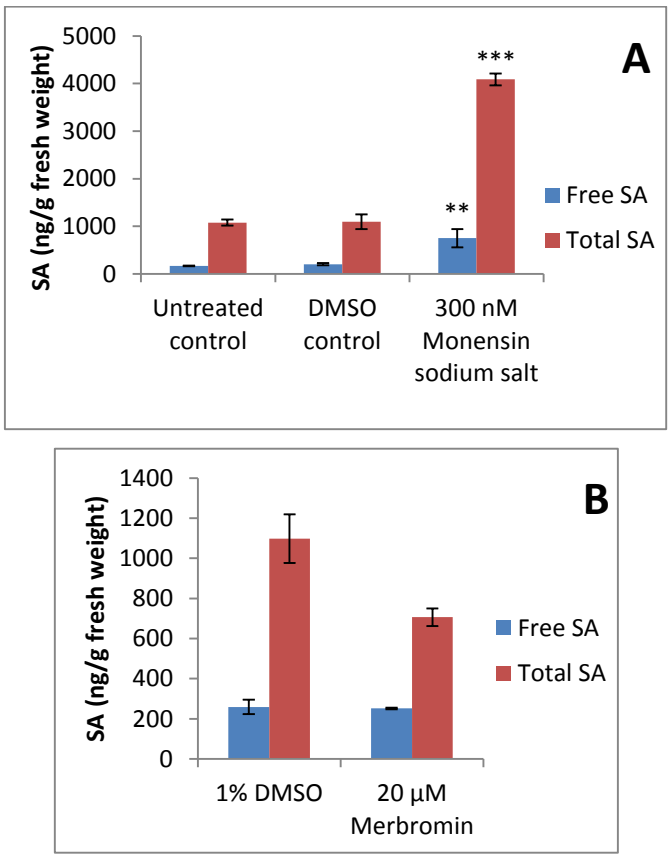

Figure 29: Changes in free and total salicylic acid content of *Arabidopsis thaliana* seedlings due to monensin sodium salt and merbromin treatment. 14 day old *Arabidopsis thaliana* Col-0 seedlings were treated for 24h with **A:** 300nM monensin sodium salt and **B:** 20 µM merbromin. The negative controls were treatment with 1% DMSO or no treatment. The free and total salicylic acid content of the seedlings was quantified using GC/MS and is shown as an average of 3 biological replicates ± standard deviation. Asterisks indicate significant differences compared to the DMSO control (Student's t-test ***p<0.001, **p<0.01). Two independent experiments gave similar results.

I treated *A. thaliana* seedlings with monensin sodium salt (300 nM) and merbromin (20 µM) for 24h. The SA content of the seedlings was analysed after extraction according to a previously published protocol (Straus et. al., 2010). Treatment with monensin sodium salt caused a significant (4-fold) increase in both, the total and the free SA levels, as compared to the untreated and the DMSO control treatments (Fig. 29A). This increase in

the SA content of the plant also corresponds to the massive increase in transcription of *PR1* after treatment with monensin sodium salt. On the contrary, merbromin which enhances expression of the SA-biosynthesis gene *ICS1*, did not increase SA content in the treated seedlings. The absence of an increase in SA could account for the lack of upregulation of *PR1* expression by merbromin. This bolsters my hypothesis that merbromin could potentially cause a block in defence signalling, downstream of *ICS1*.

2.4.8 Role of monensin sodium salt and merbromin in other signalling pathways

Several genes which have a role in modulating *FMO1* expression, have been identified previously (Bartsch et. al., 2006; Mishina and Zeier, 2006; Navarova et. al., 2012). I wanted to identify whether activation of *FMO1* expression by monensin sodium salt and merbromin also involves these genes. Hence, I treated seedlings of different mutants in key genes related to *FMO1* and analysed the *FMO1* expression in these mutants. As in the case of thaxtomin A treatment, changes in *FMO1* expression was quantified as fold of *EXPR* expression.

Monensin sodium salt-induced expression of *FMO1* was independent of SA synthesis and signalling, with an increase in *FMO1* expression in the SA-mutants *sid2* and *sid2/npr1* after chemical treatment (Fig. 30A). The overall expression level of *FMO1* was low in the wild-type and mutant seedlings, even after chemical treatment (Fig. 30A). Hence, this experiment needs to be repeated for greater clarity about additional genes mediating monensin sodium salt-induced *FMO1* expression.

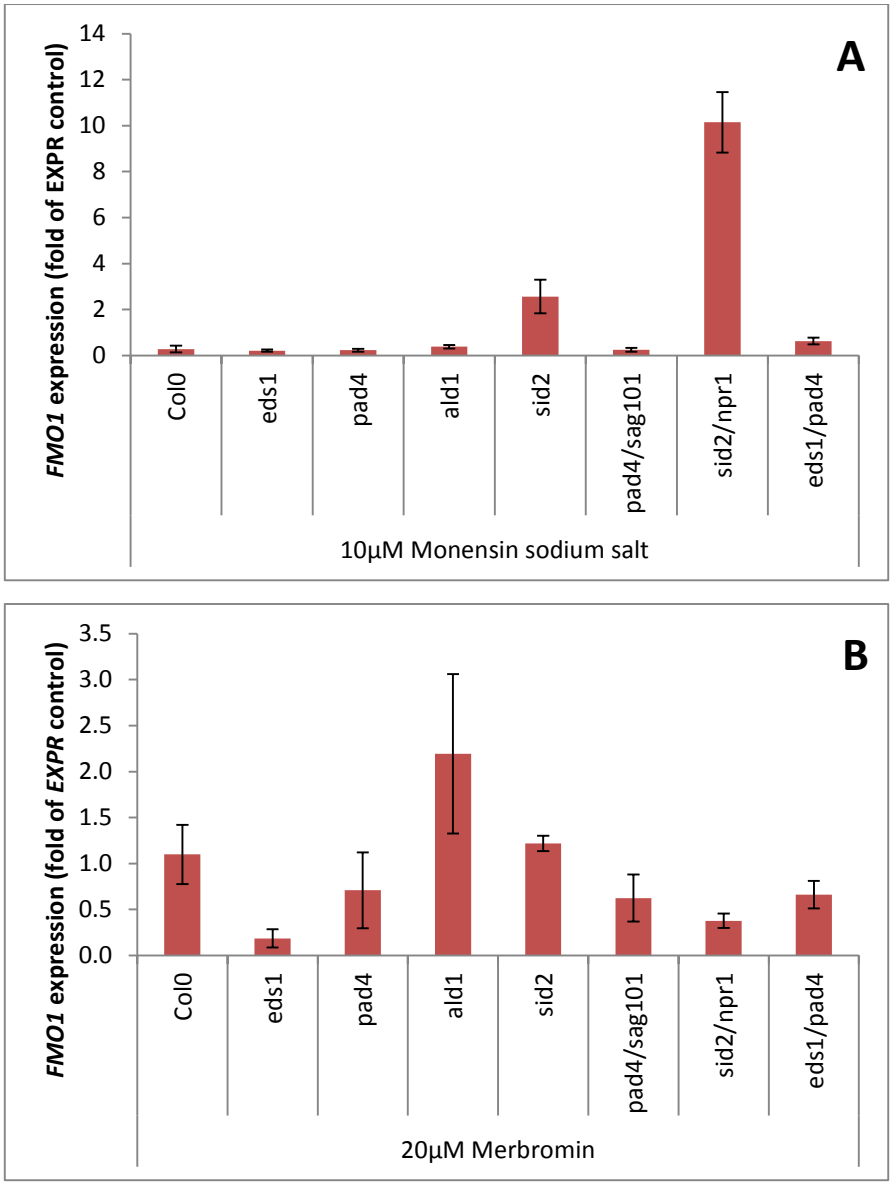

Figure 30: Monensin sodium salt and merbromin-induced *FMO1* expression in wild-type and mutant *Arabidopsis thaliana* seedlings. 13 day old *Arabidopsis thaliana* wild-type Col-0 and the indicated mutant seedlings were treated for with 24h with 1% DMSO, **A:** 10 µM monensin sodium salt, or **B:** 20 µM merbromin. Expression of *FMO1,* relative to *EXPR* expression, is shown as an average of 3 biological replicates ± standard deviation. Two independent experiments showed similar results.

Merbromin-induced *FMO1* expression was dependent on *EDS1*, and was independent of *SID2* and *ALD1* (Fig. 30B). The lack of dependence on *ALD1*, together with unchanged Pip content in merbromin-treated *A. thaliana* seedlings suggests that merbromin-induced *FMO1* expression, unlike *FMO1* expression during SAR is not mediated by Pip and *ALD1*. Thus, merbromin- and monensin sodium salt-induced *FMO1* expression is independent of SA, confirming previously published reports about *FMO1* (Bartsch et al., 2006; Mishina and Zeier, 2006).

3 Discussion

3.1 Primary screening and hit selection

For a good inhibitor screen, one needs a high difference between the negative and positive controls. The higher the difference between the absolute values and lower the variation (standard deviation), the easier it is to clearly distinguish inhibitors from amongst a whole library of chemicals. The high difference between the controls also makes it easier to identify intermediate hits, which mildly reduce activity of the positive control. However, in the screen for inhibitors of FMO1-YFP, the positive control could only increase YFP fluorescence to about 3-fold of the negative control activity. The inhibitor screen revealed that the chemicals tested did not clearly reduce YFP fluorescence, and it remained within the zone of standard deviation of the positive control (Fig. 4). Hence, I could not successfully identify any inhibitors of FMO1-YFP.

The hunt for activators also faced problems due to the low difference (3-fold) in FMO1-YFP fluorescence values between the controls (Fig. 1). The time point selected for the screen was later than the reported timeline for *FMO1* expression (Bartsch et al., 2006; Mishina and Zeier, 2006). However, chemical induced *FMO1* expression was demonstrated to increase from 2 days post treatment, with a maximum at 4 days post treatment (Olszak et al., 2006). Since I was using a chemical inducer, fumonisin B1 (FB1), reported in the same publication, the timeline was thought to be appropriate. As an additional proof, the YFP fluorescence emitted from the seedlings was also visualised at the same time point, 4 days post treatment, under a confocal microscope. Fluorescence microscopy confirmed that FB1 treatment enhanced YFP fluorescence in *A. thaliana pFMO1*::FMO1:YFP seedlings.

Cantharidin and methotrexat were identified twice from different libraries, which confirmed their stable activity under the screening conditions. The fact that known activators of *FMO1*, such as methyl viologen and cycloheximide (Arabidopsis EfP browser-Winter et al., 2007) were identified, was proof of principle for the activator screen. However, there is another aspect to the detection of cycloheximide as an activator of *FMO1*. Cycloheximide inhibits protein synthesis (Lüttge et al., 1974) and thus, leads to cell

death. FMO1 is tightly linked to cell death in plants (Olszak et al., 2006). A large number of compounds used for screening, including the positive control FB1, were phytotoxic, and induced cell death in the plants after 6 days of incubation (Stone et al., 2000). Cell death produces auto-fluorescence in the plants, which can be detected at the same wavelength as YFP fluorescence. Hence, this created the possibility of selecting several false hits, which would cause cell death in the plants, but not induce FMO-YFP. This made the stringent rescreening I used essential for confirmation of activators. Hence, every compound which increased activity to more than the standard deviation of the negative control was rescreened. This low threshold helped to identify several activators which might have been missed otherwise.

Programmed cell death reduces the pH inside the plant cells, which quenches YFP fluorescence (Young et al., 2010). This could possibly be a reason behind the low YFP activity observed in our screen. Since *FMO1* is linked to cell death, an activator of *FMO1* could potentially induce cell death after 6 days of treatment, and thus quench the YFP signal, making it harder to identify hits.

In retrospect, it might have been faster and more convenient to perform the screen with another reporter tagged to FMO1, but the current screen also was successful at identifying chemicals with a hitherto unknown activity on *FMO1* expression. In this sense, the original aim of this project was achieved. However, perhaps a modified screen would have been successful at identifying inhibitors of FMO1 and increasing the hit rate of the activator screen from the current 0.87%. However, the hit rate may also be linked to the composition of the library used for screening. In this project, small hand-selected libraries had a higher hit rate (2.8%) than the large Prestwick chemical library (0.5%), which mainly comprised of human drugs selected for high chemical and pharmacological diversity. A small kinase-inhibitor library also had a high hit rate (3.75%), possibly due to the ubiquity of kinase involvement in several signalling pathways.

The data from the initial activator screen was scrutinized, and several analysis methods were applied, to prevent intermediate hits from falling between the cracks. A graph was made for every screening plate, inclusive of the positive and negative control values for that plate. Each data point was considered in relation to the controls, and the standard deviation of the control values. Every chemical with an average data value equal

to or more than twice the standard deviation of the negative control was selected for rescreening.

I also utilized another analysis method, wherein the individual compound values were normalised to the average value of their screening plate. A threshold of 1.5 times the average plate values was set and all compounds with a mean value at or above this threshold were rescreened. A graph utilising this normalisation technique is depicted in Fig. 3 and Fig. 4.

The third method employed for selection of candidate activators utilised the so-called Z score (Malo et al., 2006). The Z score considers the variation in the measurements of the readout while normalizing the data and thus adds a higher degree of confidence to candidate selection. The average of the whole screening plate is compared to individual compound measurements, since a majority of compounds are expected to be inactive, and thus can function as controls.

The candidates selected via all three statistical methods were pooled together to make a list of compounds for rescreening. Although most of the candidates could be picked from any of the three methods employed, a few were unique to only one of these techniques. Thus, I utilised three different methods for analysis of the primary screen data, to prevent missing out on false negatives in the screen.

The western blot after a time-course assay (data not shown) revealed that FMO1-YFP accumulation was maximum at 24h post treatment. This time point also coincided with the observed maxima of *FMO1* expression. However, FMO1-YFP accumulation could not be detected in plants, after 4 days of treatment with chemicals. This observation contradicts the time line of maximum FMO1-YFP fluorescence activity in the primary screen. This data again points to the role of cell-death induced auto-fluorescence interfering with the actual YFP fluorescence in the primary screen. However, this very screen was successful in the goal of identifying chemical activators of FMO1-YFP.

One hypothesis to explain this time of accumulation is that, activators of FMO1-YFP produce an increase in fluorescence, one day post treatment, but this fluorescence may be too low to detect in our experimental setup. The chemical upregulation of *FMO1* expression, which has been linked to programmed cell death (Olszak et al., 2006) might

increase auto–fluorescence in the seedlings, thus boosting the total fluorescence readout after 6 days of treatment. Hence, activators of *FMO1* expression, along with cell-death inducers such as cycloheximide and methotrexate were selected from the primary screen.

Ultimately, the screen for activators of FMO1-YFP was successful, and I could identify 5 chemicals increasing expression of FMO1. Since staurosporine and 5-iodotubercidin were general kinase inhibitors, and are likely to be involved in perturbations of several plant signalling pathways (Massillon et al., 1994; Meggio et al., 1995), I only focused on three chemicals for subsequent analysis of their role in *FMO1* upregulation. The three selected chemicals are: thaxtomin A, merbromin and monensin sodium salt.

Determination of the concentration dependence of these chemicals in various bioassays, revealed that activator concentrations which greatly increase FMO1 expression (Figs. 8, 22), also cause severe retardation of germination and growth in the seedlings (Figs. 10, 23). While all three selected activator chemicals have a known cytotoxic effect (Duval et al., 2005; Gores et al., 1988; Puerner & Siegel, 1972), the loss of seedling viability may also be a contribution of sustained high expression of *FMO1*, since *FMO1* has been linked to programmed cell death (Olszak et al., 2006). Additionally, since thaxtomin A is a cellulose synthesis inhibitor (Scheible et al., 2003), and thaxtomin A treatment also increases cell death in *A. thaliana* over time (Duval et al., 2005), it can explain the stunted growth and death observed in thaxtomin A-treated seedlings in the germination and growth assay (Fig. 10).

3.2 Origin and activity of thaxtomin A: a potent *FMO1* inducer

In this study, thaxtomin A was found to be a potent activator of *FMO1*, also causing significant increases in the expression of several other defence-related genes. Determining the concentration dependence revealed that *FMO1* expression can be significantly induced by thaxtomin A, even at nanomolar concentrations (Fig. 8), with maximum expression at 24h post treatment (Fig. 9). For ease of use and to evoke a consistent response, treatment for 24h with 100nM thaxtomin A was used for all subsequent experiments.

Thaxtomin A is a phytotoxin secreted by several species of the gram-positive filamentous bacteria in the genus *Streptomyces*, including *S. scabies*, *S. acidiscabies*, and *S. turgidiscabies* (King et al., 1989; Loria et al., 1995). Thaxtomin A causes scab disease in potatoes and other taproot crops like turnip and radish (Lawrence et al., 1990; King et al., 1991; King et al., 1992; Loria et al., 1997). Scab disease has a major economic impact on worldwide potato production (Lambert and Loria, 1989; Loria et al., 2006). The pathogenicity of various *S. scabies* isolates is positively correlated to their ability to produce thaxtomin A (King et al., 1991; Loria et al., 1995; Goyer et al., 1998; Kinkel et al., 1998), and thaxtomin A is responsible for the formation of scab-like lesions on the potato tuber, which are characteristic of the disease (King et al., 1991; Goyer et al., 1998; Kinkel et al., 1998).

Thaxtomin A causes reduced seedling growth, dramatic cell swelling, and inhibition of cellulose synthesis in *A. thaliana*, even at nanomolar concentrations (Scheible et al., 2003). The same group later discovered that thaxtomin A reduces crystalline cellulose and increases pectins and hemicellulose in the cell wall. Like the cellulose-synthase inhibitor isoxaben, thaxtomin A also changes the expression of genes involved in primary and secondary cellulose synthesis as well as genes associated with pectin metabolism and cell wall remodelling (Bischoff et al., 2009). Defects in cell wall integrity and callose deposition have been shown to trigger defence responses in plants (Cano-Delgado et al., 2003; Hernández-Blanco et al., 2007).

Hence, plants were treated with the cellulose synthesis inhibitor isoxaben, to determine whether thaxtomin A-induced *FMO1* expression is a consequence of its action on the cell wall. Comparison of *FMO1* expression in thaxtomin A-treated plants versus isoxaben-treated plants revealed that while isoxaben does lead to mild (10-fold) accumulation of *FMO1* mRNA, thaxtomin A has a far more potent effect on *FMO1* with a 50-fold increase in expression (Fig. 17). Previous studies have also confirmed that thaxtomin A has a greater effect on defence gene expression, as compared to isoxaben (Bischoff et al., 2009). The concentration of isoxaben and thaxtomin A used for the treatment was comparable for their effect on cellulose synthesis inhibition (Desprez et al., 2002; Scheible et al., 2003; Bischoff et al., 2009). Therefore, *FMO1* expression is not merely a result of perturbations in the cell wall structure and integrity, but thaxtomin A

seems to have a role in plant defence signalling that is unique from the cellulose synthesis inhibitor isoxaben.

Previous reports indicated that application of 200nM thaxtomin to *A. thaliana* seedlings for 48h can induce expression of the JA marker genes *VSP1* and *VSP2* (Bischoff et al., 2009). However, the thaxtomin A producing *S. scabies* did not alter JA-related gene expression in potato plants (Arseneault et al., 2014). Additionally, in my experiments, 100nM thaxtomin A did not have a significant effect on expression of the JA-marker gene *VSP1*. Expression of the abscisic acid marker gene *RAB18*, and the auxin marker gene *PIN1* was also not enhanced (Fig. 16). Therefore, thaxtomin A seems to have a specific effect on activation of *FMO1*-related defence signalling.

3.2.1 Structure-activity relationship of thaxtomin A bioactivity

Thaxtomin A production by *S. scabies* is correlated to its pathogenicity on potato plants in scab disease development, and is responsible for development of necrotic scab-like lesions on the potato tubers (King et al., 1991; Loria et al., 1995; Goyer et al., 1998; Kinkel et al., 1998). The 4-nitrotryptophan and phenylalanine groups in thaxtomin A were deemed important for its phytotoxicity (King et al., 1992). Glucosylation of thaxtomin A by *Bacillus mycoides* ameliorated its toxicity, and potato tuber necrosis was reduced by more than 80% (King et al., 2000). Glucosylation of thaxtomin A occurred at the C-14 hydroxyl group, and not at the more reactive C-20 phenolic group, as might have been expected (King et al., 2000). Thaxtomin A (Fig. 3.1 A) also undergoes in vitro glucosylation in *S. scabies* to form thaxtomin A-di-glucoside (TAG) (Acuna et al., 2001). TAG (Fig. 3.1 B) fails to produce thaxtomin A-like necrosis symptoms on potato tubers. Inoculation of potato tubers with ^{14}C-radiolabelled thaxtomin A revealed that scab-resistant potato cultivars have the ability to glucosylate thaxtomin A, while scab-susceptible cultivars do not. Thus, glucose conjugation is a method of thaxtomin A detoxification in potato plants and plays an important role in resistance to scab disease.

Thaxtomin C (Fig. 3.1 C) is a precursor of thaxtomin A, and was also isolated from *Streptomyces* species (King et al., 1994). Thaxtomin C induces soil rot in sweet potatoes and causes necrosis in root tissue (Guan et al., 2012). It is structurally similar to thaxtomin

A, and the structural domains deemed important for bioactivity are intact (King et al., 1992). Application of thaxtomin C to *A. thaliana* seedlings also significantly increases FMO1 expression, albeit to a lower extent than thaxtomin A.

Identification of a direct target of a compound, and its mode of action, is the ultimate goal of a chemical biology approach. A popular technique to identify direct protein targets of a chemical is to attach an alkyne group to the chemical, which enables subsequent labelling of the protein-compound complex via the so-called 'click-chemistry' (Speers et al., 2003). Fluorescent labelling of a compound enables localisation of the compound and potentially of the compound-protein complex within an organism. Hence, thaxtomin A with an alkyne or a rhodamine label was applied to *A. thaliana* seedlings and *FMO1* expression was quantified. The labels were attached by substituting the phenolic –OH group, which was shown to be dispensable by the comparison of *FMO1* expression between thaxtomin A, and thaxtomin C, which lacks the aforementioned –OH moiety. Rhodamine-labelled thaxtomin A, but not alkyne-labelled thaxtomin A, could significantly induce *FMO1* (Fig. 15). This difference in activity between the two thaxtomin A derivatives could possibly be due to a difference in charge distribution. However, this data needs to be confirmed with freshly synthesised thaxtomin A derivatives before drawing conclusions. Microscopic analysis of treated *A. thaliana* seedlings indicated that rhodamine-labelled thaxtomin A could be easily taken up, because it was clearly detectable within the cells of all tissues (data not shown).

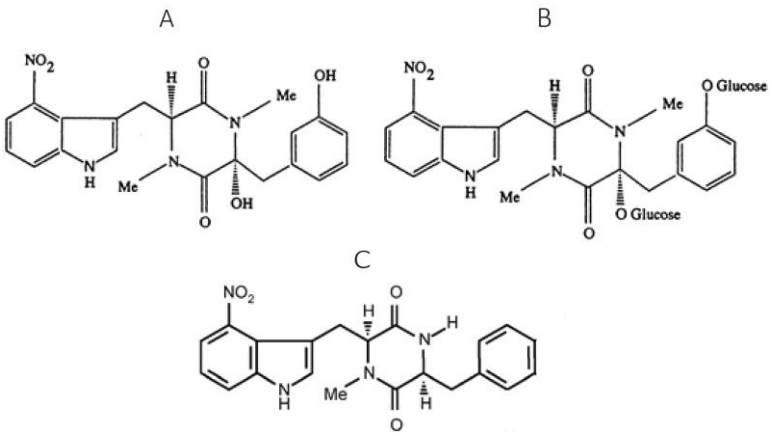

Fig 31: Structure of thaxtomin A (A), thaxtomin A-di-glucoside (TAG) (B) (Acuna et al., 2001) **and thaxtomin C (C)**

3.2.2 Auxin inhibits thaxtomin A-induced *FMO1*

Several greenhouse and field trials established that foliar treatment of potato plants with the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) reduced common scab on the harvested tubers (McIntosh et al., 1982; McIntosh et al., 1985; McIntosh et al., 1988; Thompson et al., 2014). 2,4-D treatment reduced scab disease symptoms and the harvested potato tubers had decreased sensitivity to thaxtomin A, with a reduction in thaxtomin A-induced necrosis. In *Arabidopsis thaliana*, thaxtomin A–induced chlorosis and seedling death was also reduced by the 2,4-D treatment (Tegg et al., 2008). Monitoring of *FMO1* expression after thaxtomin A treatment with or without 2,4-D, revealed that 2,4-D significantly inhibits thaxtomin A-induced *FMO1* expression (Supp. Fig. 1). This result is intriguing because it raises the question whether reduction in necrotic cell death due to 2,4-D application leads to inhibition of *FMO1*, or whether the reduced *FMO1* expression causes inhibition of cell death and necrotic scabs.

It is also possible that the reduced *FMO1* expression is due to crosstalk between auxin and *EDS1*/*PAD4* dependent signalling pathways. Overexpression of the *FMO*-like *YUCCA* genes leads to overproduction of auxin in several plant species (Zhao et al., 2001; Tobeña-Santamaria et al., 2002; Expósito-Rodríguez et al., 2011; Liu et al., 2012). The YUCCA protein YUC6 has been implicated in auxin biosynthesis by converting indole-3-pyruvate (IPA) to indole-3-acetate (IAA) (Dai et al., 2013). Salicylic acid (SA) causes stabilization of the Aux/IAA repressor proteins due to global repression of auxin-related genes, including the gene encoding the TIR1 receptor. This inhibitory effect of SA on auxin responses is a part of the SA-mediated disease resistance mechanism (Wang et al., 2007). Application of 100nM thaxtomin A reduced expression of the auxin marker gene *PIN1* about three-fold. However, treatment with IAA increases *EDS1* expression (Arabidopsis eFP Browser-Winter et al., 2007). This data raised intriguing questions about the involvement of auxin signalling in modification of *FMO1* activity. However, they were beyond the scope of this project, and hence, were not addressed and explored further.

3.2.3 *FMO1* expression in plants: at death's door?

One of the first studies on *FMO1* dealt with its role in programmed cell death. *FMO1* was initially identified as a gene upregulated in the *Arabidopsis thaliana acd11* mutant, which constitutively activates *EDS1*, *PAD4* and SA-dependent PCD and defence (Olszak et al., 2006). *FMO1* was also found to be expressed during developmental senescence, infection by avirulent and virulent bacteria, and in response to the cell-death inducing chemicals FB1 and rose bengal (RB) (Olszak et al., 2006). Although in all the above cases *FMO1* expression occurred in the region of senescence/HR-like lesions, it does not resolve whether *FMO1* expression has a role in cell death initiation or execution or whether it is a symptom of cell death.

In the current study, several cell death inducers were also identified as activators of *FMO1* expression in the activator screen. One rationale for this could be that the primary screening conditions were such that they cell death-inducers could be selected as putative hits, since cell death-induced auto-fluorescence overlapped with the targeted YFP signals. However, secondary screens eliminated these false hits and only compounds activating *FMO1* expression were selected. These confirmed inducers of *FMO1* expression such as thaxtomin A, merbromin and monensin sodium salt have also been linked to cell death induction (Puerner and Siegel, 1972; Gores et al., 1988; Duval et al., 2005). These results, therefore hint at underlying ties between cell death and *FMO1* expression.

Thaxtomin A has been reported to induce PCD in *A. thaliana* cell culture. The lowest concentration of thaxtomin A tested, 500nM, lead to a 25% cell death rate after 24h, as compared to a 12% cell death rate in the control at the same time point (Duval et al., 2005). In the current study, *A. thaliana* seedlings were treated with a maximum concentration of 100nM thaxtomin A for 24h, which is 5-fold lower than that used by Duval et al (2005). Therefore, I hypothesise that there would only be a minor increase in PCD due to thaxtomin A treatment in this study. However, this needs to be confirmed with a cell death assay under my experimental conditions.

One rationale behind identifying a chemical inducer of *FMO1* was that it would help us dissect the complex interplay between *FMO1* and cell death activation in plants and identify components mediating *FMO1* expression. Induction of *FMO1* by chemical

treatment would identify whether *FMO1* alone could trigger plant cell death. However, none of the *FMO1* activators identified in this study have been proven to act exclusively on *FMO1* expression. Additionally, other studies have found them to be involved in cell death induction (Puerner and Siegel, 1972; Gores et al., 1988; Duval et al., 2005). Hence they cannot, at present, be used to investigate *FMO1*-cell death interactions. One way to resolve this question is to control *FMO1* expression with an inducible promoter, such as promoters responsive to dexamethasone, ethanol, tetracycline, etc. (Schena et al., 1991; Moore et al., 2006). This would create a clean system wherein all parameters except *FMO1* expression would be unchanged, which can then be used to monitor cell death induction as compared to that in a dexamethasone-treated wild-type plants.

3.2.4 Positioning thaxtomin A activity within the signalling network

Thaxtomin A was apparently not involved in early defence responses such as ROS production, MAPK activation, and intracellular calcium spiking (Fig. 11, 12, 13). Hence, it was hypothesised that thaxtomin A acts on defence gene transcription without the involvement of the aforementioned signalling cascades. Previous reports suggest that thaxtomin A can induce defence responses in *A. thaliana* (Errakhi et al., 2008; Bischoff et al., 2009; Duval and Beaudoin, 2009). Multiple studies on this subject produced conflicting results (Duval et al., 2005; Errakhi et al., 2008; Bischoff et al., 2009). Thaxtomin A was found to increase expression of the SA-marker gene *PR1* (Bischoff et al., 2009), whereas other studies (Duval et al., 2005; Errakhi et al., 2008) found no evidence of thaxtomin A-induced increase in *PR1* expression. In the current work, Thaxtomin A (100nM) was found to strongly activate (140-fold) *PR1* expression (Fig. 19). Application of the thaxtomin A-producing *S. scabies* to potato plants was also proven to upregulate expression of the SA-related genes *PR-1b*, *PR-2* and *PR-5* (Arseneault et al., 2014). Duval et al(2005) and Errakhi et al(2008) had used 2µM and 10µM thaxtomin A respectively, for their experiments, which is 20-100 times more concentrated than the thaxtomin A used in this study. Thus, thaxtomin A-induced *PR1* expression could be concentration dependent. 2µM and 10µM thaxtomin A were also found to produce 40-50% cell death in *A. thaliana* cell culture

(Duval et al., 2005), and this could also contribute to the lack of *PR1* induction by thaxtomin A.

Several independent studies have established that during local defence responses in plants, *FMO1* expression is independent of SA production and signalling (Bartsch et al., 2006; Gruner et al., 2013; Koch et al., 2006; Mishina & Zeier, 2006; Olszak et al., 2006). Thaxtomin A-induced *FMO1* was proven to be independent from SA signalling by monitoring expression in the *A. thaliana salicylic acid deficient2* (*sid2)* mutant seedlings. Pathogen-induced *FMO1* expression does not trigger SA accumulation in basal defence, but is crucial for SA production and signalling in systemic tissue during SAR (Bartsch et al., 2006; Mishina and Zeier, 2006). Thaxtomin A triggered a 4-5-fold increase in free and total SA levels in wild-type *Arabidopsis thaliana*. This also corresponds to the increase in expression of the SA-marker gene *PR1* after thaxtomin A application.

Since pipecolic acid (Pip) has been shown to be an important defence signal leading to *FMO1* activation during SAR (Navarova et al., 2012), I measured Pip levels by gas chromatography-mass spectrometry (GC/MS) in activator-treated plants. However, there was no obvious change in the Pip content of the treated plants as compared to the control plants. This data corresponds to the wild type-like thaxtomin A-induced increase in *FMO1* expression in the A. thaliana *ald1* mutant, which is defective in Pip accumulation. Thus, thaxtomin A treatment does not trigger Pip production in plants, and thaxtomin A-induced *FMO1* upregulation is not dependent on *ALD1*. However, thaxtomin A treatment leads to an increase in *PAD4* expression, and *P. syringae*-induced *ALD1* expression and Pip production in systemic tissues is dependent on *PAD4* (Mishina and Zeier, 2006). Thus, the increase in *ALD1* expression after thaxtomin A treatment can be linked to enhanced *PAD4* activity in the treated plants.

Pathogen-induced *FMO1* expression is controlled by *EDS1* and *PAD4* (Bartsch et al., 2006). Notably, thaxtomin A-induced *FMO1* is also completely dependent on *EDS1* and *PAD4*, but independent of *SAG101* (Fig. 18). *EDS1* is known to form structurally similar heterodimers with *PAD4* and *SAG101*, leading to downstream defence signalling cascades (Wagner et al., 2013). However, if both the heterodimers use similar or different pathways is currently unknown. In the case of thaxtomin A treatment, the *EDS1-PAD4* heterodimer, but not the *EDS1-SAG101* heterodimer, might be crucial for *FMO1* activity. Hence, the

dimers formed between *EDS1* and its interaction partners *PAD4* and *SAG101* may have distinct roles in plant defence signalling. Thaxtomin A can thus be a useful tool to functionally investigate the dimers formed between *EDS1* and its interaction partners, *PAD4* and *SAG101*.

EDS1 and *PAD4*, amongst other genes, are significantly upregulated in response to thaxtomin A (Fig. 19). This shifted the focus of thaxtomin A activity to a potential target upstream of *FMO1*, and it gave rise to the hypothesis that thaxtomin A targets the signalling pathway upstream of, or directly at *EDS1-PAD4*. This makes thaxtomin A a potential tool to probe the dynamics of the *EDS1-PAD4* signalling mechanism. Additional analyses with mutants in *EDS1* and *PAD4* revealed that thaxtomin A-induced *PAD4* expression is partially *EDS1*-independent (Fig. 20). However, *EDS1* cannot be upregulated by thaxtomin A in the absence of *PAD4*. Therefore, thaxtomin A actively drives PAD4 expression. The enhanced *EDS1* expression in *A. thaliana* wild-type seedlings upon thaxtomin A treatment might be due to a feedback mechanism of *PAD4*-controlled defence signalling. In the *A. thaliana eds1*^{L262P} mutant, the interaction between EDS1 and PAD4 is impaired and formation of an EDS1-PAD4 protein heterodimer is prevented, while interaction with SAG101 is retained. Surprisingly, thaxtomin A treatment significantly enhanced *FMO1* expression in the *A. thaliana eds1*^{L262P} mutants (Fig. 21). This observation indicates that PAD4 may act independently of *EDS1* to drive *FMO1* expression, similar to previous reports describing the independence of *PAD4* from *EDS1* in certain defence processes (Pegadaraju et al., 2007; Rietz et al., 2011; Louis et al., 2012). However, the presence of both, *EDS1* and *PAD4*, is essential for maximum possible upregulation of *FMO1* expression by thaxtomin A.

3.3 Merbromin and monensin sodium salt induce *FMO1* expression

Two additional chemicals, merbromin and monensin sodium salt, were also identified as activators of *FMO1* expression during the library screening. Merbromin is produced and used globally as an antiseptic under the trademark ‘mercurochrome’. It is a xanthene dye and acts as a photosensitizer for the generation of singlet oxygen (Gollnick et al., 1992;

Martínez et al., 1993). Interestingly, another xanthene dye, rose bengal (RB), has also been proven to upregulate *FMO1* in *A. thaliana*. Shikimate dehydrogenase is a conserved and essential enzyme in plants, fungi, and bacteria, and hence, is an attractive target for herbicides and antimicrobial agents. A recent study discovered that merbromin is an inhibitor of the shikimate dehydrogenase enzyme from the bacterium, *Pseudomonas putida* (Peek et al., 2014).

The third chemical activator of *FMO1* expression, monensin sodium salt, is derived from *Streptomyces cinnamonensis* (Westley, 1982) and is a monovalent cation ionophore which facilitates the exchange of Na+, K+ and protons across membranes and thus affects the acidification of cellular compartments (Pressman and Fahim, 1982). It has been used extensively to analyse the function of the Golgi apparatus in a variety of animal and plant systems. Ultrastructure studies indicate that monensin causes accumulation of Golgi-derived swollen vesicles in the cytoplasm of treated plant cells (Morré et al., 1983; Mollenhauer et al., 1988; Zhang et al., 1993).

However, there has not been any substantial research into the interactions between plants and merbromin and monensin sodium salt in the past decade. To my knowledge, this is the first report about the involvement of merbromin and monensin sodium salt in plant defence signalling.

Merbromin and monensin sodium salt were found to activate *FMO1* expression in a chemical screen for activators of FMO1-YFP. Both chemicals showed a concentration dependent effect on *FMO1* expression. The EC_{50} value was calculated to be 1µM for monensin sodium salt and 10µM for merbromin. Interestingly, monensin sodium salt drastically affects growth and germination at concentrations exceeding EC_{50} value (1µM), and growth of *A. thaliana* seedlings was completely blocked at 10µM. This inhibition could be linked to cytotoxic effects of the induced *FMO1*-expression in these seedlings (Olszak et al., 2006). Likewise, 10µM monensin was also shown to inhibit the germination and growth of ryegrass seedlings (Mollenhauer et al., 1986). Merbromin, in a concentration dependent manner, also impaired germination and growth of *A. thaliana* seedlings, and was previously shown to inhibit growth of cucumber seedlings (Puerner and Siegel, 1972). In the current experiments, seedlings grew normally in 1µM merbromin, but at higher concentrations, growth was inhibited, and germination and growth were completely

blocked at 100µM merbromin. Thus, the growth of *A. thaliana* seedlings is repressed at a chemical concentration proven to induce strong *FMO1* expression.

Reactive oxygen species (ROS) are signalling molecules that accumulate rapidly in response to pathogen infection. The superoxide producer RB has previously been found to induce *FMO1* expression (Olszak et al., 2006). Prolonged activation of MAPK signalling cascades has also been shown to upregulate *FMO1* (Tsuda et al., 2013). Hence, I tested ROS production and MAPK activation in seedlings after merbromin and monensin sodium salt application. Like in the case of thaxtomin A, merbromin did not activate MAPK and ROS production (Fig. 24, 25), and thus, these processes do not mediate merbromin-induced *FMO1* expression. However, monensin sodium salt had no effect on ROS production, but induced accumulation of phosphorylated MPK6 and MPK3. Thus, monensin sodium salt-induced MAPK activation could possibly mediate enhanced expression of FMO1 in *A. thaliana* seedlings.

Evaluation of the specificity of merbromin and monensin sodium salt activity on *EDS1*/*PAD4* dependent signalling revealed that while both chemicals strongly induced *FMO1* expression (>100-fold), they have a negligible effect on the JA-marker gene *VSP1* and the abscisic acid marker gene *RAB18* (<5-fold). Previous experiments in our laboratory indicate that merbromin and monensin sodium salt act as inhibitors of JA signalling, while they have no effect on intracellular calcium signalling (Christian Meesters, Mohamed Suliman and Jens Maintz; personal communication). Inhibition of JA-mediated processes by an activator of *EDS1*/*PAD4*-dependent signalling is possible due to the antagonistic nature of SA and JA signalling pathways (Spoel et al., 2003; Koornneef and Pieterse, 2008).

Since Pip was deemed to be an important signal in SAR reactions, responsible for triggering *FMO1* expression (Návarová et al., 2012), I also analysed the amino acid content of merbromin and monensin sodium salt treated plants. However, there was no effect of the chemical treatment on the levels of defence-inducible amino acids such as Pip and α-aminoadipic acid (Fig. 27). Thus, induced expression of *FMO1* by selected chemical activators is not mediated by perturbations of amino acid content of the plants.

The effect of chemical treatment on genes known to be involved in the *EDS1*/*PAD4* pathway was analysed to identify the potential target site/location of chemical activity

within the signalling pathway. Both monensin sodium salt and merbromin significantly upregulated expression of *EDS1*, and its signalling partners, *PAD4* and *SAG101*. The activity of genes known to be dependent on *EDS1*/*PAD4* such as *ICS1*, *ALD1* and *PBS3* was also significantly enhanced (Fig. 28). Thus, both these chemicals act upstream of *FMO1* and drive its expression. Application of monensin sodium salt increased *FMO1* expression in the SA-defective A*. thaliana* mutants, *sid2* and *sid2/npr1* (Fig. 30A). This provides additional proof for previously published reports about the SA-independence of *FMO1* expression (Bartsch et al., 2006; Gruner et al., 2013; Koch et al., 2006; Mishina & Zeier, 2006; Olszak et al., 2006). However, treatment of other signalling mutants with monensin did not modulate FMO1 expression. Merbromin treatment of mutant and wild-type *A. thaliana* seedlings revealed that merbromin-induced *FMO1* expression is independent of *ICS1* and *ALD1*, and is partially dependent on *EDS1* (Fig. 30B). Therefore, I unfortunately cannot draw any conclusions about the dependence of merbromin and monensin sodium salt-induced *FMO1* expression on other genes in the *EDS1/PAD4* pathway.

Pathogen-induced *FMO1* expression is crucial for SA production and signalling in systemic tissue during SAR (Mishina & Zeier, 2006). Monensin sodium salt also promotes SA accumulation and leads to a 4-5-fold increase in free and total SA levels in treated plants. This also corresponds to the increase in expression of the SA synthesis gene *ICS1*, and the SA-marker gene *PR1* in monensin sodium salt-treated plants. Monensin sodium salt was found to be a mild inhibitor of PR1 in a screen for PR1-GUS inhibitors (Vivek Halder; personal communication). However, that data was only semi-quantitative, and the current study has proved on several fronts that monensin sodium salt is involved in upregulating SA signalling. Merbromin, on the other hand, has quite an intriguing role in SA signalling. It can significantly upregulate expression of the SA-biosynthesis gene *ICS1*, but not of the SA-marker gene *PR1* (Fig. 28B). There was also no increase in free and total SA content of the plant after treatment with merbromin (Fig. 29B). Hence, merbromin could activate expression of all genes in the *EDS1/PAD4* pathway, but potentially block signalling downstream of *ICS1*, causing a lack of increase in SA accumulation and SA-dependent signalling.

Thus, merbromin and monensin sodium salt have been proven to be chemical inducers of the EDS1/PAD4 defence signalling pathway, and can be used as tools to analyse the activity of genes in this pathway.

3.4 Concluding Remarks

I established and performed a chemical screen for activators and inhibitors of *FMO1* expression. The primary screen and stringent secondary screening yielded five chemicals significantly increasing *FMO1* expression. Three candidate compounds, namely, thaxtomin A, merbromin and monensin sodium salt, were selected for additional analyses. All three candidate compounds did not enhance expression of marker genes of other major plant signalling pathways, and are selective activators of *EDS1/PAD4* dependent signalling. Merbromin is a strong activator of *EDS1*-dependent gene expression, but possibly blocks signalling downstream of *ICS1*. Monensin sodium salt triggers MAPK activation, which could play a role in upregulation of *FMO1* expression in monensin sodium salt-treated *A. thaliana* seedlings. Thaxtomin A is a potent activator of *FMO1* expression, and selectively acts on *PAD4* expression, thus driving defence signalling downstream of *EDS1*/*PAD4*. Interesting parallels can be drawn between pathogen- and chemical-induction of EDS1/PAD4 dependent defence responses in plants. Thaxtomin A treatment could potentially mimic pathogen infection in plants, thus triggering significant transcriptional changes in defence gene expression. However, all the experiments in this study used *A. thaliana* seedlings in liquid culture enhanced with activator chemicals. Additional experiments are needed to determine whether application of thaxtomin A on soil-grown *A. thaliana* plants can trigger similar defence responses. If soil-grown plants respond to thaxtomin A treatment in in a similar manner to the seedlings, further experiments can be developed to test microbial interactions with thaxtomin A-treated plants. A potential decrease in pathogen activity and disease symptoms after infection of thaxtomin A-treated plants would be proof of the biological role of thaxtomin A in plant immunity.

Overall, the screen was successful in its main goal of identifying chemical modulators of *FMO1* expression. These candidate chemicals can be useful tools for dissection of *FMO1*-related defence responses, for identification of new components involved in modulation of *EDS1*-dependent, SA-independent signalling.

5 Materials & methods

5.1 Materials

5.1.1 Plant materials

Arabidopsis thaliana ecotype, mutant lines and transgenic lines used in this study are listed in Table 5.1, 5.2 and 5.3.

Table 5.1: Wild type Arabidopsis lines used in this study

Accession	Abbreviation	Original Source
Columbia	Col-0	J. Dangl[a]

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

Table 5.2: Mutant Arabidopsis lines used in this study

Mutant allele	Accession	Reference/Source
eds1-2	Col-0/ Ler0[a]	Bartsch et al., 2006
pad4-1	Col-0	Glazebrook et al., 1996
ald1	Col-0	Návarová et al., 2012
sid2-1	Col-0	Wildermuth et al., 2001
sag101-1	Col-0	Feys et al., 2005
pad4-1/sag101-1	Col-0	Feys et al., 2005

[a] Ler *eds1-2* allele introgressed into Col-0 genetic background, 8th backcross generation

Table 5.3: Transgenic Arabidopsis lines used in this study

Line	Construct	Reference/Source
FMO1-YFP	*pFMO1*::FMO1-YFP	Michael Bartsch[a]
FMO1-GUS	*pFMO1*::GUS	Olszak et al., 2006
$EDS1^{L262P}$	HA:eds1L262P	Rietz et al., 2011

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

5.1.2 Chemicals

Laboratory grade chemicals and reagents were purchased from Sigma-Aldrich (Deisenhofen, Germany), Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany), Invitrogen™ (Karlsruhe, Germany), Bio-Rad (Hercules, USA), SYNCHEM OHG (Felsberg/Altenburg, Germany).

The following chemical libraries were used:

- Hand-selected libraries (204 compounds, 10mM)

- Prestwick chemical library (1,280 compounds, 10 mM) (Prestwick Chemical, Illkirch, France)

- Kinase inhibitor library (84 compounds, 2 mM) (Biomol GmbH, Hamburg, Germany)

5.1.3 Media

Media were sterilized by autoclaving for 20 min at 121◦C. Before addition of heat labile compounds the media or solutions were cooled to approx. 60°C.

MS medium: 1/2× Murashige and Skoog Basal Salt Mixture (Sigma-Aldrich)

0.5% (w/v) Sucrose

4 g/L Phytagel (Sigma-Aldrich) for solid medium

pH 5.8

5.1.4 Buffers and solutions

Buffers were sterilized by autoclaving for 20 min at 121°C if needed sterile. Before addition of heat labile compounds, the media were cooled to approx. 60°C. Stock solutions of compounds for plant treatment were dissolved in DMSO at 10 mM concentration, if not stated otherwise.

5.2 Methods

5.2.1 Maintenance and cultivation of Arabidopsis plants

Arabidopsis thaliana seeds were germinated by directly sowing them on moist compost (Stender AG, Schermbeck, Germany) supplemented with Wuxal fertilizer (Nitzsch; Kreuztal, Germany). Pots were transferred to a controlled environment growth chamber, covered with a propagator lid (10 hour photoperiod, light intensity of approximately 200 µEinsteins/m/sec, 23◦C day, 22◦C night and 65% humidity). Propagator lids were removed when seeds had germinated. To allow early bolting and setting of seeds, 4 week old plants were transferred to long day conditions (16 hour photoperiod). Seeds were collected by enveloping aerial tissue with a sealed paper bag until siliques were shattered.

5.2.2 Chemical treatment of Arabidopsis plants

Arabidopsis thaliana seeds were sown in liquid medium, vernalised for 2 nights at 4°C and then transferred to a controlled environment growth chamber with 12h photoperiod. The seedlings were treated with different chemicals as indicated in micro well plates. Double treatments were performed such as that the compound of interest was incubated 1 h before stimulus with fumonisin B1.

5.2.3 Confocal laser scanning microscopy (CLSM)

Detailed analysis of intracellular fluorescence war performed by confocal laser scanning microscopy using a Leica TCS SPS AOBS (Leica, Wetzlar, Germany) based Axiovert microscope equipped with an Argon ion laser as an excitation source. YFP tagged proteins were excited by a 514 nm laser line. YFP fluorescence was selectively detected by using an HFT 514 dichroic mirror and BP 535-590 band pass emission filter. Images were analyzed with Leica Lite software.

5.2.4 Biochemical methods

5.2.4.1 Isolation of total protein extract from Arabidopsis

Proteins from 1 *Arabidopsis* seedling were extracted by grinding frozen material and subsequent addition of 80 µL protein extraction buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 10mM NaF, 25 mM _-Glycerophosphate, 2 mM Na3VO4, 10% (v/v) Glycerol, 0.1% (v/v) Tween 20, 1 mM DTT (added fresh from 100 mM stock solution), 1 mM PMSF (added fresh from 200 mM stock solution in ethanol)).

The lysate was mixed thoroughly and chilled on ice. Cell debris was spun down by centrifugation at 4◦C for 1 min at 15,000 g. The supernatant was transferred to a fresh tube. Protein amounts were determined using the method of Bradford (1976) with the Bio-Rad Protein Assay (Bio-Rad Munchen, Germany) and BSA solutions between 0 and 1.6 mg/ml as internal standard.

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

Protein samples boiled for 5 min in Laemmli Sample Buffer (Bio-Rad, Munchen, Germany) were subjected to SDS-PAGE on a gel containing 12% polyacrylamide. The whole amount of proteins was visualized by coomassie blue R250 (Thermo Scientific, Rockford, USA).

5.2.4.3 Immunublot analysis

On a SDS-PAGE separated proteins were electroblotted onto a Hybond ECL nitrocellulose membrane (Amersham, GE Healthcare) in Towbin buffer (25 mM Tris, 195 mM Glycin, 20% (v/v) methanol). To monitor protein transfer and loading, the membrane was stained with 0.1% Ponceau S (Sigma-Aldrich) in 5% (v/v) acetic acid, followed by extensive washes in water. The membrane was blocked for at least 1 hour with milk powder in TBST (10 mM Tris, 1.5 mM NaCl, pH 7.5, 0.05% (v/v) Tween20). The α-Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), Cell Signaling Technology / New England Biolabs GmbH, Frankfurt am Main, Germany) has been used already for detection of *Arabidopsis* MPK3, MPK4 and MPK6 (Ranf et al., 2011). The α-GFP mouse monoclonal antibody (Roche, Mannheim, Germany) was used for detection of FMO1-YFP. The primary antibody

was diluted 1:5000 (α-Phospho-p44/42) or 1:2000 (α-GFP) in TBST and hybridized with the membrane over night at 4◦C. After washing the membrane 3 times in TBST, the second anti-body (α-rabbit-HRP, Amersham, GE Healthcare) or goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, USA) was hybridized (1:5000 in TBST) with the membrane. After 1 hour incubation with 3 subsequent washing steps, the secondary antibody was detected using the Pierce ECL Western Blotting Substrate (Thermo Scientific, Rockford, USA) and Kodak biomax light autoradiographic film (Sigma-Aldrich).

5.2.4.4 Salicylic acid measurement

SA measurements was obtained of leaf material (100 to 200 mg fresh weight) according to Straus et al. (2010), using a chloroform/methanol extraction and analyzed by gas chromatography coupled to a mass spectrometer (GC-MS, Agilent, Santa Clare, USA).

5.2.5 Molecular biological methods

5.2.5.1 Isolation of total RNA from Arabidopsis

Total RNA was extracted from 2 week old plant material. Liquid nitrogen frozen samples (approximately 20 mg) were homogenized using a bead shaker in 1.5 ml centrifuge tubes. Further processing was performed using the RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) and the Bio-Budget Plant RNA kit following the manufacturer's instructions. The RNA was eluted with 20 µl RNase-free water.

5.2.5.2 cDNA synthesis

Synthesis of cDNA was performed using SuperScript^{TM}II Reverse Transcriptase (RT) (Invitrogen) following the suppliers instructions. Briefly, 1 µl Oligo(dT) primers, 1 µg RNA and 1 µl dNTP Mix was incubated for 5 min at 65◦C. 1× Reaction buffer, 10 µM DTT and 1 µl RT were added to the samples. The synthesis was performed over 60 min at 42°C, with heat inactivation of the enzyme for 15 min at 70°C.

5.2.5.3 Quantitative real-time PCR (qRT-PCR)

Transcript analysis in time course experiments and before microarray hybridization were performed by qRT-PCR. cDNA corresponding to 500 ng RNA from three biological replicates was diluted 1:20 before continuing the protocol. SYBR green assays were developed using iQ™SYBR®Green Supermix (Bio-Rad, Hercules, USA) with gene-specific primers and an adapted protocol. The reaction set up was adjusted to a total volume of 25 µl with 12.5 µL iQ SYBR® Green Supermix, 0.1 µl of each primer (100 µM), 2.3 µl of distilled water and 10 µl diluted cDNA template. PCR was performed on a 'iQ5 multicolor real-time PCR detection system' (Bio-Rad). Expressions were calculated using the CT method (Schmittgen and Livak, 2008). To simplify data interpretation, expression levels in DMSO treated control were fixed to 1 and relative values were calculated. The gene of an expressed protein (At4g26410) served as internal control. This was previously proposed as reference gene (Czechowski et al., 2005) and RefGenes analysis (Hruz et al., 2011) revealed this gene to be the most stable expressed gene in *A. thaliana* seedlings.

qRT-PCR program:

Step	**Temperature**	**Time**	**Cycles**
Initial denaturing	95°C	2 min	
Denaturing	95°C	20 sec	40X
Annealing	55°C	30 sec	
Elongation	72°C	25 sec	
Denaturing	95°C	1 min	
Melting curve	55-95°C	1 min	Gradual step-wise increase of temperature by 0.5°C per step

The following primers (listed as 5'-3') have been used:

- FMO1-qRT-fw: GTTCGTGGTTGTGTGTACCG

- FMO1-qRT-rw: TGTGCAAGCTTTTCCTCCTT

- EDS1-qRT-fw: CGAAGACACAGGGCCGTA

- EDS1-qRT-rw: AAGCATGATCCGCACTCG

- PAD4-qRT-fw: GGTTCTGTTCGTCTGATGTTT

- PAD4-qRT-rw: GTTCCTCGGTGTTTTGAGTT

- SAG101-qRT-fw: CATTCCTCTGCTCCGAGAAC

- SAG101-qRT-rw: CGTTTTAACGTCGGTTCGAT

- PR1-qRT-fw: TTCTTCCCTCGAAAGCTCAA

- PR1-qRT-rw: AAGGCCCACCAGAGTGTATG

- ALD1-qRT-fw: ACTTGGTGGCAGCACAAAAC

- ALD1-qRT-rw: ATCACCAGTCCCAAGGCTTATC

- ICS1-qRT-fw: TTCTGGGCTCAAACACTAAAAC

- ICS1-qRT-rw: GGCGTCTTGAAATCTCCATC

- PBS3-qRT-fw: ACACCAGCCCTGATGAAGTC

- PBS3-qRT-rw: CCCAAGTCTGTGACCCAGTT

- VSP1-qRT-fw: TCATACTCAAGCCAAACGG

- VSP1-qRT-rw: ATCCTCAACCAAATCAGC

-PIN1-qRT-fw: ACA AAA CGA CGC AGG CTA AG

-PIN1-qRT-rw: AGC TGG CAT TTC AAT GTT CC

-RAB18-qRT-fw: ATCCAGCAGCAGTATGACGA

-RAB18-qRT-rw: CCAGTTCCGTATCCTCCTCC

- Expr-qRT-fw: GAGCTGAAGTGGCTTCCATGAC

- Expr-qRT-rw: GGTCCGACATACCCATGATCC

5.2.6 Reporter assays

5.2.6.1 Quantitative GUS assay

For quantitative GUS analysis, two to four *Arabidopsis* seeds were germinated in each well of a 48-well microplate and grown in MS medium. Chemical treatment was performed at the indicated concentration and incubated for five to 24 hours as indicated. Seedling extracts were prepared and fluorimetric GUS activities relative to protein concentration were determined as described by Kienow et al. (2008). Briefly, seedlings were frozen in liquid nitrogen and homogenized in 100 µL Lysis-buffer (50 mM Na(PO4), 1 mM EDTA, 0.1% (v/v) Triton X-100, 10 mM _-mercaptoethanol (fresh), pH 7.0). Debris was spun down at 15,000 g and the supernatant was transferred to a fresh tube. To 50 µL sample were added 50 µL of MUG-substrate (2 mM 4-methylumbelliferyl-_- D-glucoronid in lysis buffer). These samples were incubated at 37◦C and aliquots were taken after 0, 30 and 60 minutes. The reaction was stopped by an excess of 0.2 M Na(CO)3 and fluorescence was measured in a fluorimeter. The GUS activity was calculated in relation to a internal standard and normalized to the protein concentration. Latter was determined using the method of Bradford (1976) with the Bio-Rad Protein Assay (Bio-Rad Munchen, Germany) and BSA solutions between 0 and 1.6 mg/ml as standard.

5.2.7 Chemical screen

Chemical screens were performed using the transgenic *Arabidopsis* FMO1p::FMO1:YFP reporter line. *Arabidopsis* seedlings were grown in hydroponic culture under sterile conditions directly in 96-well microplates. Each well contained one seedling in 160 µl ½ MS medium. 17 day old seedlings were treated for the activator screen in duplicate or triplicate with chemicals in ½ MS medium (10 µM: Kinase inhibitor library and Prestwick library; 10-20 µM: Hand-selected library). 10µM FB1 was added as a positive control, and DMSO served as a negative control. The seedlings were pre-treated with the chemicals for 1 h before adding 10µM FB1 for the inhibitor screen. 6 days after chemical treatment the semi quantitative fluorescence measurement assay was performed using a fluorometer. Fluorescence values were measured thrice per well and averaged for

analysis. Each screening plate had alternating positive and negative controls on the columns at the left and right edges.

5.2.8 Monitoring the oxidative burst

Oxidative burst analysis assay in *Arabidopsis* seedlings was adapted from a previously published protocol (Mersmann et. al., 2010). The assay measures active oxygen species released by seedlings by H_2O_2 dependent luminescence of luminol (Keppler et al.,1989). Briefly, *Arabidopsis thaliana* seedlings were grown in ½ MS media in 96-well microplates for 2 weeks. The seedlings were washed with 300 µL H_2O and then relaxed in 98 µl H_2O under light for 2 hours. The chemicals to be tested were added to the seedlings, immediately prior to triggering the reaction with flg22. This was performed automatically in a luminometer by injecting 100 µl of a solution containing flg22 (2 µM), luminol (400 µM) and horseradish peroxidase (0.02 mg/ml, Sigma-Aldrich, P6782). Luminescence from each single well was detected for 2 seconds and each well was measured every 2 minutes. Luminescence was measured in 31 cycles over a total period of 72 minutes. Each experiment contained 12 biological replicates.

5.2.9 Probe synthesis

Synthesis of molecular probes containing a rhodamine and an alkyne tag attached to thaxtomin A was performed by the group of Prof. Markus Kaiser (ZMB, Universiat Duisburg Essen).

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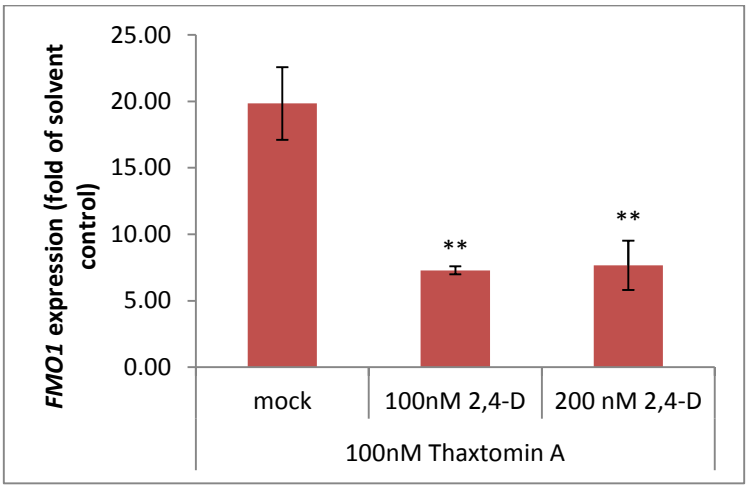
Supplementary data



Supplementary figure 1: Effect of thaxtomin A treatment, with or without 2,4-D, on *FMO1* expression. 13 day old *Arabidopsis thaliana* Col-0 seedlings were treated with 1% DMSO+1% ethanol and 100nM thaxtomin A+ 1% ethanol, 100nM 2,4-D or 200nM 2,4-D for 24h. *FMO1* expression in treated seedlings, relative to that in DMSO and ethanol (mock) treated seedlings is depicted as an average of 3 biological replicates ± standard deviation. Asterisks indicate significant differences compared to the mock treated thaxtomin A sample (Student's t-test **p<0.01).