Functional analysis of small bioactive molecules in jasmonate signaling

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Abstract

Plants exhibit multitudes of defense mechanisms against different kinds of stress. Jasmonic acid (JA) is one of the identified signaling compounds mediating plant’s response to wounding, attack by herbivores or necrotrophic pathogens. Central parts of the JA signaling pathway have recently been unraveled by demonstrating that (+)-7-iso-JA-L-Ile is the most bioactive form of JA and that the SCF<sup>COI1</sup>-complex functions as its receptor. However, many other components of the JA signaling pathway remain unknown. This includes how and where protein kinases may be involved in JA signaling. Likewise, the mechanistic details of cross-talk between different hormone signaling pathways are unknown. Using a chemical biology approach, screening for selective compounds that can be used as tools in applications complementing genetic approaches, I aimed at identifying some of these unknown components. The advantage of this method is that it has the potential to circumvent redundancy of gene function, lethality of mutants and pleiotropic effects, problems generally encountered in genetic approaches.

I developed a screening procedure for bioactive compounds that uses a transgenic <i>Arabidopsis thaliana</i> line, harboring the JA-responsive reporter gene LOX2p::LUC. This procedure allowed bidirectional screening for activators or inhibitors of reporter expression. Sifting through approx. 1,700 natural compounds, I identified one activator of reporter gene expression and 16 inhibitors of methyl jasmonate induced reporter expression. Critical validation of these primary hits revealed that the putative activator in fact interfered with the activity of the luciferase reporter. It presumably binds and stabilizes luciferase, thereby enhancing its apparent activity, whereas reporter gene expression was not affected. After validation and characterization of the inhibitors, one compound (12) was identified as selective inhibitor of JA signaling. Structure-activity relationship studies, using derivatives of the compound, defined parts of the molecule that where indispensable for its bioactivity. Based on this analysis, a derivatized probe was designed that harbors a ‘photoreactive’ benzophenone for establishing covalent binding and an alkyne residue to attach a detectable fluorophore using ‘click chemistry’. Importantly, this probe retained activity and was used in first affinity-based target identification experiments.

In a second screen using a small, targeted library of 84 known protein kinase inhibitors, I identified three compounds that impaired JA signaling. This finding suggests the involvement of protein kinases in the JA signaling pathway that has been previously reported. Among the identified inhibitors was 5-iodotubercidin, a nucleoside antibiotic. A derivative of this compound, toyocamycin, was previously described to selectively impair auxin signaling, which is mechanistically related to JA signaling. Several structural analogs were investigated with respect to their effect on JA-dependent
reporter expression or JA-independent readouts. Toyocamycin was considered to be the most specific derivative. To elucidate the role of toyocamycin in the *Arabidopsis* hormonal signaling network, I performed a microarray analysis after treatment with toyocamycin. The expression data showed that this compound modulates expression of *JAZ* genes, which are repressors of JA induced gene expression. Toyocamycin also modulated genes, which may be involved in hormonal crosstalk between e.g. auxin or salicylic acid signaling. The fact that toyocamycin caused a root growth phenotype, which is dependent on allene oxide synthase (AOS) and jasmonoyl-isoleucine synthetase (JAR1), indicated that multiple targets may exist, because inhibition of the *LOX2* marker was independent of JAR1.

Identification of the protein targets of toyocamycin and compound 12 may eventually lead to identification of yet unknown components in JA signaling or hormonal crosstalk.
Zusammenfassung


In einem zweiten Screen mit einer kleinen zielgerichteten Chemikalienbibliothek, die aus 84 beschriebenen Proteinkinaseninhibitoren besteht, habe ich drei Proteinkinaseninhibitoren identifiziert, welche den JA Signalweg beeinflussten. Dies deutet auf eine Rolle von Proteinkinasen im JA Signalweg hin. Unter den identifizierten Inhibitoren war 5-Iodotubercidin, ein Nukleosidantibiotikum. Ein Derivat der Verbindung, Toyo-

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<tr>
<td>µ</td>
<td>Micro</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>12-O-Glc-JA</td>
<td>12-0-β-D-glucopyranosyljasminic acid</td>
</tr>
<tr>
<td>ABA</td>
<td>Abscisic acid</td>
</tr>
<tr>
<td>ACC</td>
<td>Aminocyclopropane carboxylic acid</td>
</tr>
<tr>
<td>ACX</td>
<td>Acyl-CoA oxidase</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine-5'-diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine-5'-monophosphate</td>
</tr>
<tr>
<td>AOC</td>
<td>Allene oxide cyclase</td>
</tr>
<tr>
<td>AOS</td>
<td>Allene oxide synthase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>bHLHzip</td>
<td>Basic helix-loop-helix leucine zipper</td>
</tr>
<tr>
<td>cm</td>
<td>Centimeter</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>COI1</td>
<td>Coronatine insensitive 1</td>
</tr>
<tr>
<td>COR</td>
<td>Coronatine</td>
</tr>
<tr>
<td>cps</td>
<td>Counts per second</td>
</tr>
<tr>
<td>CTS1</td>
<td>COMATOSE1</td>
</tr>
<tr>
<td>DAD1</td>
<td>DEFECTIVE ANTHER DEHISCENCE 1</td>
</tr>
<tr>
<td>DARTS</td>
<td>Drug affinity responsive target stability</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>Demineralized, deionized water</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Demineralized water</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dnOPDA</td>
<td>Dinor-OPDA</td>
</tr>
<tr>
<td>dpi</td>
<td>Dots per inch</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EAR</td>
<td>ERF-associated amphiphilic repression domain</td>
</tr>
<tr>
<td>EOT</td>
<td>Epoxy-octadecatrienoic acid</td>
</tr>
<tr>
<td>ERF</td>
<td>Ethylene response factor</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>g</td>
<td>Gravity constant (9.81 ms⁻¹)</td>
</tr>
<tr>
<td>GDL</td>
<td>DONGLE</td>
</tr>
<tr>
<td>GO</td>
<td>Gene Ontology</td>
</tr>
<tr>
<td>GUS</td>
<td>β-Glucuronidase</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
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</tbody>
</table>
HPOT | Hydroperoxy-octadecatrienoic acid
HTS | High-throughput-screening
IAA | Indole-3-acetic acid
IC$_{50}$ | Half maximum inhibitory concentration
InsP$_5$ | Inositol pentakisphosphate
InsP$_6$ | Inositol hexakisphosphate
IP3K | Inositol-3-kinase
IP4K | Inositol-4-kinase
JA | Jasmonic acid
JA-Ile | Jasmonoyl-isoleucine
JA-Leu | JA-leucine
JAR1 | Jasmonate resistant 1
JA-Trp | JA-tryptophan
JA-Val | JA-valine
JAZ | Jasmonate-ZIM-domain
JID | JAZ-interacting domain
JMT | JA methyltransferase
KAT | L-3-ketoacyl CoA thiolase
L | Liter
LCF | Leaf-closing factor
LOX | 13-Lipoxygenase
LUC | Luciferase
m | Milli
M | Molar (mol/L)
MAPK | Mitogen-activated protein kinase
MeJA | Methyl jasmonate
MFP | Multifunctional protein
min | Minute(s)
MYC2 / 3 / 4 | Myogenic regulatory factor 2 / 3 / 4
NINJA | Novel Interactor of JAZ
OPC-8 | 3-Oxo-2-(2’Z)-pentenyl)-cyclopentan-1-octanoic acid
OPCL1 | OPC-8:CoA ligase
OPDA | 12-Oxophytodienoic acid
OPR3 | OPDA reductase 3
PACOR | Photoaffinity tagged COR
PAGE | Polyacrylamide gel electrophoresis
PCR | Polymerase chain reaction
PDF1.2 | PLANT DEFENSIN 1.2
PED3 | PEROXISOME DEFECTIVE 3
pH | Negative decimal logarithm of the H$^+$ concentration
PL | Phospholipase
PR1 / 4 | PATHOGENESIS RELATED 1 / 4
PXA1 | PEROXISOMAL ABC TRANSPORTER 1
qRT-PCR | Quantitative real time polymerase chain reaction
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>RLU</td>
<td>Relative light units</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase PCR</td>
</tr>
<tr>
<td>s</td>
<td>Seconds</td>
</tr>
<tr>
<td>SA</td>
<td>Salicylic acid</td>
</tr>
<tr>
<td>SAR</td>
<td>Structure-activity relationship</td>
</tr>
<tr>
<td>SCF-complex</td>
<td>Skp, Cullin, F-box containing complex</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>SPRi</td>
<td>Surface plasmon resonance imaging</td>
</tr>
<tr>
<td>ST2a</td>
<td>Sulfitotransferase 2a</td>
</tr>
<tr>
<td>TAP</td>
<td>Tandem affinity purification</td>
</tr>
<tr>
<td>TAT</td>
<td>TYROSINE AMINOTRANSFERASE</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBTA</td>
<td>Tris(1-benzyl-1H-1,2,3-triazol-4-yl)methylamine</td>
</tr>
<tr>
<td>TCEP</td>
<td>Tris(2-chlorethyl)phosphate</td>
</tr>
<tr>
<td>TE</td>
<td>Thioesterase</td>
</tr>
<tr>
<td>TIR</td>
<td>TRANSPORT INHIBITOR RESPONSE</td>
</tr>
<tr>
<td>TPL</td>
<td>TOPLESS</td>
</tr>
<tr>
<td>Ub</td>
<td>Ubiquitin</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VSP</td>
<td>VEGETATIVE STORAGE PROTEIN</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per volume</td>
</tr>
<tr>
<td>w/w</td>
<td>Weight per weight</td>
</tr>
<tr>
<td>X-Gluc</td>
<td>5-Bromo-4-chloro-3-indolyl-β-D-glucuronide</td>
</tr>
<tr>
<td>ZIM</td>
<td>Zinc-finger protein expressed in Inflorescence Meristem</td>
</tr>
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</table>
1 Introduction

1.1 The past, present and future of jasmonic acid signaling

Jasmonic acid (JA) and its derivatives, collectively referred to as jasmonates, comprise a group of oxylipin signaling molecules in plants that share a high degree of structural and functional similarity to prostaglandins found in animals. Both types of compound are derived from fatty acid oxidation pathways that are activated in response to stress. Correspondingly, jasmonate levels increase drastically and rapidly upon wounding, UV irradiation, ozone treatment and other abiotic stresses (Wasternack, 2007). In addition, responses to biotic stress, such as herbivore attack or infection by microbial pathogens also depend on jasmonates. In healthy, non-stressed plants jasmonates mediate developmental processes, such as root growth seed germination, tuber formation, tendril coiling, trichome initiation, flower development and senescence (Wasternack, 2007; Browse and Howe, 2008). Jasmonates operate via changing genome wide gene expression, which is in part mediated by the transcription factor MYC2 (Lorenzo et al., 2004; Dombrecht et al., 2007; Fonseca et al., 2009a). Elucidation of their mode of action has long been hampered by the fact that plants contain many JA derivatives, biosynthetic precursors and JA metabolites, which differ considerably in their biological activities (Krumm et al., 1995; Kramell et al., 1997; Stintzi et al., 2001; Staswick and Tiryaki, 2004; Taki et al., 2005; Miersch et al., 2008).

Most of our current knowledge about synthesis and function of jasmonates has been derived from mutants that are defective in JA biosynthesis or the physiological response to JA treatment (Berger et al., 2002; Wasternack, 2006; Browse, 2009b). However in addition to such biological approaches, chemical strategies have also always been integral part of jasmonate research. During the first years after discovery of jasmonates as plant constituents that are important for plant growth and development (Ueda and Kato, 1980; Dathe et al., 1981), extensive studies on structure-activity relationships were carried out. These included the synthesis of numerous JA derivatives and determining their impact on various plant responses (Wasternack, 2007; Wasternack and Kombrink, 2010). This type of work provided first insights into structural requirements for jasmonate bioactivity and aimed at development of tools for target identification. This approach led to the synthesis of the highly active jasmonate analog coronal, which was inspired by the extremely high bioactivity of the bacterial phytotoxin coronatine (COR) (Mithöfer et al., 2004).

Because of its exceptional structure and bioactivity, COR has been essential for two important discoveries in jasmonate research: (1) Application of COR in a genetic screen yielded the Arabidopsis thaliana mutant coronatine insensitive 1 (coi1), which is defective in a central regulatory unit of jasmonate signaling (Feys et al., 1994), and (2) its defined
stereochemical structure provided the blueprint for identification and synthesis of the plant endogenous most bioactive jasmonate, (+)-7-iso-jasmonoyl-L-isoleucine (Fonseca et al., 2009b).

1.1.1 The jasmonate biosynthesis pathway

The plant-specific pathway of JA biosynthesis has been unraveled. It comprises at least ten mostly well-characterized enzymatic reactions that are outlined in Figure 1.1 (Wasternack, 2007; Schaller and Stintzi, 2009). After the initial release of α-linolenic acid (C18:3) from plastidial galactolipids by phospholipases such as DAD1 or GDL (Ishiguro et al., 2001; Hyun et al., 2008), it is oxidized by a 13-lipoxygenase (LOX). The product 13(S)-hydroperoxy-octadecatrienoic acid [13(S)-HPOT] is converted to 12,13(S)-epoxy-octadecatrienoic acid [12,13(S)-EOT] by allene oxide synthase (AOS), which represents the first committed step of JA biosynthesis. In Arabidopsis AOS is a single copy gene and the loss-of-function mutant is deficient in JA (Park et al., 2002; von Malek et al., 2002). Allene oxide cyclase (AOC) converts the allylic epoxide product of AOS into optically pure (9S,13S)-12-oxo-phytodienoic acid [cis-(+)-OPDA], which is the endproduct of the plastid-localized part of the JA biosynthesis pathway and represents the first cyclic compound with pronounced bioactivity (Stenzel et al., 2003; Wasternack, 2007). Note that the same set of enzymes acting on hexadecatrienoic acid (C16:3) generate dinor-OPDA (Weber, 1997). Interestingly, in Arabidopsis chloroplasts OPDA and dnOPDA have also been identified as constituents of membrane galactolipids and upon selective release by lipases may act as signals or contribute to JA biosynthesis (Stelmach et al., 2001; Andersson et al., 2006).

For further conversion OPDA is translocated from chloroplasts to peroxisomes, which in part is mediated by the ATP-binding cassette transporter CTS1 (identical to PXA1/PED3) (Theodoulou et al., 2005). OPDA reductase (OPR3) catalyzes the reduction of OPDA to OPC-8 (Fig. 1.1). In Arabidopsis and tomato only this isoform is found in the peroxisome and the JA-deficient phenotype of the opr3 mutant indicates that other isoforms do not contribute to JA biosynthesis (Sanders et al., 2000; Stintzi and Browse, 2000). The following β-oxidation is initiated by activation of the carboxylic acid moiety to the corresponding CoA ester by OPC-8:CoA ligase 1 (OPCL1), one member of a large superfamily of acyl-coenzyme A synthetase in Arabidopsis (Schneider et al., 2005; Koo et al., 2006; Kienow et al., 2008). After three rounds of β-oxidation, jasmonoyl-CoA is cleaved by a yet unknown thioesterase (TE) giving rise to (+)-7-iso-JA that equilibrates to the more stable (-)-JA. An overview of all steps of JA biosynthesis is shown in Figure 1.1 Arabidopsis mutants that have been important for understanding JA biosynthesis are highlighted in blue.

1.1.2 Metabolic conversion and structure-activity relationship (SAR) of jasmonates

JA is subject to enzymatic modifications as evident from numerous JA metabolites that were shown to be constituents in various plant species and tissues or to accumu-
Figure 1.1: Pathway of jasmonic acid biosynthesis. Upon release of α-linolenic acid (C18:3) from galactolipids by phospholipases (PL) in the chloroplast, the hydroperoxy compound 13(S)-HPOT is formed by 13-lipoxygenase (LOX). The unstable allene oxide 12,13(S)-EOT is generated by allene oxide synthase (AOS) and further converted to (9S,13S)-OPDA by allene oxide cyclase (AOC). In the peroxisome OPDA is reduced to OPC-8 by OPDA reductase 3 (OPR3). Subsequent activation to the corresponding CoA ester by OPC-8:CoA ligase (OPCL1) allows shortening of the carboxylic acid side chain via the fatty acid β-oxidation machinery, comprising acyl-CoA oxidase (ACX), multifunctional protein (MFP) and L-3-ketoacyl CoA thiolase (KAT). The endproduct, jasmonoyl-CoA, is cleaved by a putative thioesterase (TE) yielding (+)-7-iso-JA, which equilibrates with the more stable (-)-JA. *Arabidopsis* mutants that have been important for understanding JA biosynthesis are indicated in blue. Reaction scheme modified after Westernack and Kombrink (2010).
late in response to stress (Wasternack, 2007; Göbel and Feussner, 2009). Among these metabolites are the methyl ester (MeJA), amino acid conjugates of JA, e.g. JA-Ile, JA-Leu, JA-Val or the hydroxylation products 12-OH-JA (Fig. 1.2 includes more JA metabolites). The abundance and bioactivity of different metabolites may vary considerably (Miersch et al., 2008), which immediately raises two important questions: (1) What is the biological significance of such large number of JA derivatives? (2) What are the structural requirements for bioactivity in different bioassays?

After the first reported JA-induced plant responses, e.g. growth inhibition (Ueda and Kato, 1980; Dathe et al., 1981), the increasing structural and functional diversity of identified, natural jasmonates stimulated a strong interest in structure-activity relationships (SAR) and extensive synthesis of novel JA derivatives, standard approaches executed in drug research that precede target identification steps. From early chemical-biological exercises, distinct structural requirements for JA-activity were defined based on various biological responses, such as tuber formation, root growth inhibition, tendril coiling, alkaloid formation or expression of JA responsive genes (Koda et al., 1992; Staswick et al., 1992; Blechert et al., 1995, 1999; Miersch et al., 1999). These requirements include that a cyclopentanone ring carrying a keto group at C-6 is essential, that an intact pentenyl side chain is required for activity, that formation of amino acid conjugates generally increases bioactivity or that hydroxylation at C-11 or C-12 impairs biological activity.

Data about SAR provided only first hints about the functional significance of particular derivatives or distinct structural units. Some of the JA metabolites, such as 12-OH-JA, 12-HSO₄⁻-JA, 12-O-Glc-JA, may even occur in distinct tissues or organs of some plants (e.g. Zea mays, Glycine max) at concentrations that are orders of magnitude higher than those of JA, suggesting a function as inactivated storage form of JA (Miersch et al., 2008). Indeed, formation of 12-OH-JA and 12-HSO₄⁻-JA is accompanied with a partial suppression of JA signaling (Gidda et al., 2003; Miersch et al., 2008). Likewise, accumulation of several glucosides of JA and JA derivatives has been observed upon wound-induced JA biosynthesis, and collectively, these data indicate a role of JA metabolism in turning off JA signaling by conversion of active jasmonates into metabolites that are at least partially inactive (Glauser et al., 2008; Miersch et al., 2008; Glauser et al., 2010).

Still, the question about what is the most bioactive jasmonate in planta remained unsolved. The presence of two chiral centers in jasmonic acid (see Fig. 1.2) allows four possible stereo isomers of jasmonates; however, only the (3R,7S) and (3R,7R) configurations occur in natural compounds, e.g. JA or JA-Ile, since isomerization at C-3 apparently does not occur (Koda et al., 1992; Holbrook et al., 1997). Based on the facts that the ratio of the stereo isomers, (+)-7-iso-JA vs. (-)-JA, in plant tissues increases drastically in response to wounding (Schulze et al., 2006) and that the jasmonate mimic COR, in which epimerization is not possible, shows extremely high bioactivity, a search for stereo specific compounds among 40 different JA derivatives was performed. This uncovered only (+)-7-iso-JA-L-Ile to be exceptionally active (as well as the non-natural (+)-JA-L-Ile), whereas all other tested compounds showed only weak or no activity. Those included JA, MeJA, OPDA and other JA amino acid conju-
Figure 1.2: Metabolism of jasmonic acid. Conjugation of (+)-7-isoJA to isoleucine by jasmonoyl-isoleucine synthetase (JAR1) generates the natural bioactive jasmonate (+)-7-iso-JA-L-Ile, which is converted to inactive 12-OH-(+)-7-iso-JA-L-Ile by JA-Ile hydroxylase (CYP94B3). JAR1 presumably also catalyzes the conjugation of JA to other amino acids such as valine, leucine, phenylalanine, tryptophan or the ethylene precursor aminocyclopropane carboxylic acid (not shown). Other common JA transformations include hydroxylation to 12-OH-JA, which can be sulfated by a sulfotransferase (ST2a) to 12-HSO₄-JA or glucosylated to 12-O-glucosyl-JA or conjugated with isoleucine to 12-OH-(+)-7-iso-JA-L-Ile (presumably by JAR1), hydroxylation to 11-OH-JA and methylation at the carboxyl group by JA methyltransferase (JMT) to MeJA. Enzymes catalyzing formation of JA-1-glucoside or reduction to 9,10-dehydro-JA, cis-jasmone or cucurbitic acid are not yet known. Note that only a selection of JA metabolites is shown and their absolute stereoconfiguration is mostly not established or represented by racemic mixtures. Chiral centers in (+)-7-iso-JA are highlighted in red.

For the biosynthetic enzyme jasmonoyl-isoleucine synthetase (JAR1) from tomato a strong preference for (+)-7-iso-JA over (-)-JA and isoleucine over other amino acids has recently been demonstrated [Suza and Staswick, 2008; Suza et al., 2010], suggesting that JAR1 activity is tightly coupled to (+)-7-iso-JA formation before its epimerization can occur. Inactivation of jasmonate signaling originating from (+)-7-iso-JA-L-Ile was recently shown to involve hydroxylation of the jasmonate residue by a cytochrome P450 monooxygenase (CYP94B3) leading to 12-hydroxy-JA-Ile and attenuation of jasmonate responses [Koo et al., 2011; Kitaoka et al., 2011]. In addition, jasmonate signaling may also be turned off by other mechanisms, such as epimerization at C7 converting active (+)-7-iso-JA-L-Ile to inactive (-)-JA-L-Ile or methyl esterification to (+)-7-iso-JA-L-Ile-Me, which was also shown to be inactive [Fonseca et al., 2009b]. The natural occurrence of JA-L-Ile methyl esters indicates that this mechanism of hormone inactivation may indeed operate in planta [Hause et al., 2000].
1.1.3 Searching for signaling components - dissecting the JA signaling pathway

Genetic and molecular analyses have been pivotal for the current understanding of JA biosynthesis and function in signaling processes. In contrast to many other alternative approaches, mutant analysis offers the potential to provide clear, unequivocal information about how gene products affect plant function. Some JA-related mutants were initially identified based on recognizable phenotypic characteristics, such as defective flower development in the mutants delayed dehiscence 1 (dde1/opr3) and delayed dehiscence 2 (dde2/aos), defective in anther dehiscence 1 (dad1), or deficiency in unsaturated fatty acids in the fatty acid desaturase triple mutant fad3 fad7 fad8, but subsequently turned out to be affected in JA biosynthesis (McConn and Browse, 1996; McConn et al., 1997; Sanders et al., 2000; Ishiguro et al., 2001; von Malek et al., 2002). Other JA-related mutants were identified in screens by applying jasmonates (MeJA, COR, etc.) to chemically enforce a phenotype and by selecting resistant plants (Berger et al., 2002; Westernack, 2006; Browse, 2009b). Among the large number of these mutants, three have turned out to be of particular importance: jasmonate resistant 1 (jar1), coronatine insensitive 1 (coi1), and jasmonate insensitive 1 (jai1/jin1/myc2).

MeJA and other jasmonates strongly impair root growth and the jar1 mutant of Arabidopsis was found to be insensitive to this inhibition (Staswick et al., 1992). Subsequent biochemical analyses showed that the enzyme encoded by JAR1 catalyzes the conjugation of JA with isoleucine to JA-Ile (Staswick et al., 2002; Staswick and Tiryaki, 2004; Suza and Staswick, 2008). In the jar1 mutant JA-Ile levels are drastically reduced and most, but not all, JA responses impaired (e.g. fertility is retained), indicating the important and central signaling function of JA-Ile, as outlined above (Staswick and Tiryaki, 2004; Staswick, 2008; Browse, 2009a).

The coi1 mutant of Arabidopsis was isolated in a screen for plants that were resistant to root growth inhibition by COR (Feys et al., 1994). Subsequent work showed that coi1 is also insensitive to MeJA, male sterile and impaired in almost all jasmonate responses (Westernack, 2007; Browse, 2009b). The identification of COI1 as F-box protein was seminal for subsequent work on JA signaling (Xie et al., 1998), but the suggested function of COI1 acting as an E3 ubiquitin ligase in a Skp/Cullin/F-box complex (SCF\textsubscript{COI1}) and as such being involved in marking other regulators for degradation was only recently confirmed by discovery of the so-called jasmonate-ZIM-domain (JAZ) proteins (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007). Although extensive genetic screens provided numerous mutants affected in JA signaling, the genetic approach failed to identify SCF\textsuperscript{COI1} targets (Browse, 2009b).

Another informative mutant originating from a screen for jasmonate insensitive root growth is the Arabidopsis mutant jai1/jin1, which is defective in the basic helix-loop-helix leucine zipper (bHLHzip)-type transcription factor MYC2 (Lorenzo et al., 2004). Importantly, MYC2 is involved in differential regulation of two branches of the JA signaling pathway. One of these branches, positively regulated by MYC2, activates expression of genes involved in the wound response, e.g. VSP2, LOX3, TAT; the other branch, negatively regulated by MYC2, is required for expression of pathogen defense
genes, e.g. PDF1.2, PR1, PR4. In comparison to coi1, the jai1/jin1/myc2 mutant is defective in fewer JA responses and shows a weaker phenotype, suggesting that MYC2 acts downstream of COI1 and may be involved in mediating crosstalk between different signaling pathways (Lorenzo et al., 2004; Dombrecht et al., 2007).

Since genetic screens did not provide additional signaling candidate components acting downstream of COI1 or targets of SCF^COI1, alternative experimental approaches were exploited, including yeast-two-hybrid screening, transcriptional profiling, and biochemical purification. An important step towards understanding JA signaling was the discovery of the JAZ proteins, which function in repression of JA responsive genes and are encoded by a gene family of 12 members in Arabidopsis (Chung et al., 2009). They were identified by three different experimental approaches: (1) Their rapid JA-induced expression in stamens of Arabidopsis flowers (Thines et al., 2007), (2) by cloning of the dominant JA-resistant Arabidopsis mutant jai3-1 (Chini et al., 2007), (3) identification and cloning of a gene acting as repressor of JA-inhibited root growth in Arabidopsis, which turned out to represent a splice variant of JAZ10 (Yan et al., 2007). All JAZ proteins contain a ZIM domain of 27 amino acids in their central part and a Jas domain near the C-terminus. They are translocated into the nucleus via a nuclear localization signal present in the Jas domain (Browse, 2009a; Chung et al., 2009; Grunewald et al., 2009). Beside the sheer number of different JAZ proteins, their functional diversity may be further increased by the occurrence of different splice variants, e.g. as demonstrated for JAZ10, which may contribute to the sensitivity and specificity of JA signaling (Yan et al., 2007; Chung et al., 2009). Another regulatory role seems to be provided by homo- and heterodimerization of particular JAZ proteins, and the demonstration of 38 combinatorial interactions among the 66 possible heterodimeric combinations clearly emphasizes the potential regulatory flexibility in JA signaling (Chini et al., 2009b; Chung et al., 2009). The output of JAZ protein action is dependent on the specific transcription factors affected by JAZ proteins (Lorenzo et al., 2004; Fernández-Calvo et al., 2011; Qi et al., 2011; Song et al., 2011).

Recently, new pieces for understanding the JA signaling puzzle were supplied by biochemical analysis (Pauwels et al., 2010). Tandem affinity purification (TAP) was applied in order to identify new JAZ interactors and to monitor the dynamics of JAZ complex assembly. First, the half-life of a JAZ1-firefly luciferase fusion protein upon treatment of cultured cells with JA was determined, and subsequently this time-frame (1 min after JA treatment) was used for purifying proteins interacting with the JAZ-TAP-tagged bait. In addition to several known JAZ-interactors, such as JAZ12 reflecting the reported dimerization of JAZ proteins, MYC3 as close relative to MYC2, and COI1, a novel protein called NINJA (Novel Interactor of JAZ) was identified as an adaptor protein that interacts with the ZIM domain of most JAZ proteins, with exception of JAZ7 and JAZ8 (Pauwels et al., 2010). In addition, NINJA interacts via its EAR motif with the co-repressor TOLLESS (TPL), which was previously identified to mediate auxin-dependent transcriptional repression in Arabidopsis (Szemenyei et al., 2008; Pauwels et al., 2010). This exciting finding highlights the mechanistic similarity between auxin and jasmonate signaling beyond the common SCF-ubiquitin-ligase-catalyzed degradation of repressor proteins Aux/IAA and JAZ, respectively (Chini
et al., 2009a; Santner and Estelle, 2009; Wasternack and Kombrink, 2010; Pauwels and Goossens, 2011).

With all the identified components, the scenario of JA signaling via the SCF\(^{COI1}\) complex can be summarized as shown in Figure 1.3. In cells containing low levels of the bioactive jasmonate, \((-)-7\text{-iso-JA-L-Ile}\), JAZ proteins bind to the positive regulators of JA signaling, MYC2 and related transcription factors, which reside on promoters of JA-responsive genes (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007). By additional interaction with the adaptor protein NINJA, the general co-repressor TPL is recruited to the MYC2–JAZ–NINJA complex, which is thought to effectively prevent downstream signaling (Pauwels et al., 2010). This repression is relieved under conditions that activate JA biosynthesis and leads to an increase of endogenous \((-)-7\text{-iso-JA-L-Ile}\) via JAR1 activity, which may occur very rapidly, e.g. 5 min after wounding (Chung et al., 2008; Glauser et al., 2008; Koo et al., 2009, Koo and Howe, 2009). \((-)-7\text{-iso-JA-L-Ile}\) levels will promote physical interaction between COI1 and JAZ proteins, which allows ubiquitination of JAZ by the SCF\(^{COI1}\) complex (Chini et al., 2007; Thines et al., 2007; Katsir et al., 2008). JAZ repressor(s) are then degraded via the 26S proteasome, which leads to de-repression of MYC2 and thereby allowing expression of JA-responsive genes, and release of NINJA and TPL, which may get engaged in interactions with other proteins (Causier et al., 2012).

This model is supported by a wealth of experimental evidence that was generated by yeast-two-hybrid protein interaction studies, expression analyses, binding assays, pull-down experiments, analyses employing mutants and overexpression lines for various signaling components presented (for reviews see Kazan and Manners, 2008; Staswick, 2008; Browse, 2009a; Chini et al., 2009a; Chung et al., 2009; Fonseca et al., 2009b; Wasternack and Kombrink, 2010; Pauwels and Goossens, 2011). The mechanism of JA signaling via SCF complex-dependent proteasomal degradation exhibits striking similarities to the signaling mechanisms by auxin, ethylene and gibberelin, where similar components participate (Chini et al., 2009a; Santner and Estelle, 2009; Pauwels and Goossens, 2011).

1.1.4 Target identification of the bioactive jasmonates, JA-Ile and COR

The results obtained so far demonstrate that the COI1–JAZ–JA-Ile complex is an essential unit in jasmonate perception and signaling, but the participation of additional components could not be excluded because bioassays were performed with crude extracts or partially purified COI1 and JAZ proteins. It also remained unknown in which order the COI1–JAZ–JA-Ile complex assembled. Unraveling these molecular details required purified components and structural information of the complex, which was established by \textit{in silico} homology modeling of COI1 and crystal structure analysis of the COI1–JAZ co-receptor (Yan et al., 2009; Sheard et al., 2010).

Experimental evidence for direct binding of JA-Ile or COR by COI1 was provided by three independent approaches (Yan et al., 2009). (1) Retention of COI1 from crude plant extracts was achieved by affinity chromatography, using a matrix with JA coupled to the amino group of a 1,6-diaminohexane spacer on sepharose beads, generating
Figure 1.3: Model of jasmonate action in regulation of gene expression. In the resting state, at low level of JA-Ile, transcription factors (e.g., MYC2) are bound to their target sequence at the promoter of jasmonate-responsive gene (e.g., G-box), but their activity is repressed by interaction with JAZ proteins, the adaptor protein NINJA and the co-repressor TOPLESS (TPL). Upon stimulation by stress (e.g., wounding or infection by necrotrophic pathogens) or developmental cues, (+)-7-iso-JA-L-Ile rapidly accumulates and binds to the jasmonate receptor COI1 (F-box protein), which is part of the SCF$^{COI1}$ complex, comprising the protein components ASK1, Cullin, Rbx and the E2 ubiquitin-conjugating enzyme. JAZ proteins are recruited from their initial binding site to the SCF$^{COI1}$ complex, which acts as an E3 ubiquitin ligase transferring ubiquitin (Ub) from the E2 ubiquitin-conjugating enzyme to the COI1-interacting JAZ proteins. Upon polyubiquitination, JAZ proteins are degraded by the 26S proteasome thereby releasing MYC2 and activating expression of JA responsive genes, including those encoding JAZ proteins, while NINJA and TPL could engage in other interactions. Synthesis of new JAZ repressors results in termination of JA gene expression. Modified after Pauwels et al. (2010).

a jasmonoylamide (Fig. 1.4). Although COI1 was selectively eluted from the column by JA-Ile (and not JA), this approach does not exclude the possibility that other proteins contribute to this interaction. (2) Surface plasmon resonance (SPR) measurements revealed that the three purified components, COI1, JA-Ile and JAZ1, are sufficient to form a complex, thereby ruling out the possibility that other COI1 co-purified proteins are involved in JA-Ile perception. The SPR technology records changes in light refraction on sensor chip surfaces that occur upon interaction between two (or more) binding partners, one of which is covalently linked to the sensor chip surface. This approach also verified that JAZ1 alone (immobilized on the sensor chip) could neither bind JA-Ile/COR nor COI1. Unfortunately, the inverse experimental set-up, COI1 immobilized on the chip surface, could not be realized. (3) To gain direct evidence for COI1 binding JA-Ile/COR, a photoaffinity probe, PACOR, was designed and synthesized that contained three functional groups, COR, biotin, and the photoreactive phenyldiazirine group (Fig. 1.4). Upon incubation with affinity-purified His-tagged COI1 and exposure to UV irradiation, the PACOR probe was covalently linked to the protein through photolysis, which allowed simultaneous detection of the probe-labeled receptor/COI1 after SDS-PAGE separation by immuno-blotting using anti-His and anti-biotin antibodies. Importantly, PACOR retained reasonable biological activity and binding to COI1 was
competed out by COR, suggesting that COI1 directly binds JA-Ile/COR and functions as a receptor for JA-Ile/COR (Yan et al., 2009).

Further insight into the mechanism of jasmonate perception was eventually provided by the crystal structure of COI1 in complex with JA-Ile and a JAZ peptide (Sheard et al., 2010). First, binding assays using $^3$H-labeled COR and purified proteins confirmed that the jasmonate receptor is a co-receptor consisting of COI1 and JAZ proteins (Sheard et al., 2010). High-affinity, saturable binding of $^3$H-COR to COI1 occurred only in the presence of full-length JAZ protein and active (+)-7-iso-JA-L-Ile effectively competed for binding, whereas the inactive stereoisomer (-)-JA-L-Ile was less effective. By contrast, $^3$H-COR showed no binding to JAZ proteins and only marginal binding to COI1, accounting for less than 2% in comparison to the COI1–JAZ complex. Mapping of the COI1-binding region in the JAZ proteins revealed that a minimal sequence of 21 amino acids (Glu200-Val220 defining the JAZ degron peptide) is sufficient for establishing the COI1–JAZ–JA-Ile complex (Sheard et al., 2010). The crystal structure of COI1 in complex with the JAZ1 degron and JA-Ile/COR revealed that the ligand is buried deeply in the binding site and only the keto and the carboxyl groups of COR/IA-Ile remain exposed. This provides the surface for interaction with the JAZ degron peptide and upon its binding the COR/IA-Ile ligand is completely covered by the two components, explaining high-affinity binding of the hormone to the COI1–JAZ co-receptor (Sheard et al., 2010). Inositol pentakisphosphate (InsP$_5$) was identified as third critical component of the jasmonate co-receptor complex COI1–JAZ, extending the similarity to the auxin receptor complex TIR–Aux/IAA, which contains an inositol hexakisphosphate (InsP$_6$) molecule bound underneath the auxin-binding site (Tan et al., 2007). For the COI1–JAZ1 co-receptor it was demonstrated that InsP$_5$ is crucial for high affinity ligand binding, suggesting that interaction of its phosphate residues with basic amino acids of COI1 is involved in shaping the ligand-binding pocket properly (Sheard et al., 2010).

Although the observed binding mode of COR/IA-Ile by the COI1–JAZ1 co-receptor is markedly differed from that predicted by computational modeling, it does not preclude the previously proposed sequential order for assembling the receptor complex (Yan et al., 2009). Eventually, the bioactive jasmonate is in direct contact with both COI1 and JAZ1 supporting the molecular glue mechanism previously proposed for the auxin system (Tan et al., 2007). Despite differences in structural and mechanistic details, jasmonate perception follows the same common theme as previously established for other plant hormones, including auxin, gibberellin, ethylene and abscisic acid (Santner and Estelle, 2009).

### 1.1.5 The hunt is not yet over: Open questions and searching for additional JA signaling pathways and components

Despite the enormous progress in understanding JA perception and signaling, many important questions remain to be answered. Perhaps the most captivating is to explain the multitude of biological jasmonate responses and the pronounced differential bioactivity of jasmonate derivatives, which are difficult to reconcile with only one com-
Figure 1.4: Structures of synthetic probes used for jasmonate target identification. Jasmonic acid immobilized on sepharose beads served as affinity matrix for purification of JA interacting proteins. The photoaffinity- and biotin-tagged coronatine (PACOR), a bioactive jasmonate mimic, was used to demonstrate that COI1 has the capacity to bind COR and functions as jasmonate receptor. Similarly, photoaffinity- and biotin-tagged 12-O-β-D-glucopyranosyl-(-)-jasmonic acid (12-O-Glc-JA) labeled the (-)-LCF-binding membrane protein. The bioactive jasmonate residues are labeled in blue and the photoreactive trifluoromethyl diazirine and benzophenone residues in dark red.

mon perception and signaling mechanism as outlined above for the COI1–JAZ–JA-Ile module. Thus, the search for missing signaling components is not yet over.

For example, OPDA, the bioactive precursor of JA biosynthesis, has signaling properties that are markedly different from JA, MeJA or JA-Ile (Weiler et al., 1994; Stintzi et al., 2001; Taki et al., 2005; Wasternack, 2007; Westernack, 2007; Böttcher and Pollmann, 2009). In Eschscholtzia californica OPDA induced alkaloid biosynthesis. In Bryonia dioica as well as in Phaseolus vulgaris it was identified as endogenous signaling molecule mediating tendril coiling (Weiler et al., 1994; Blechert et al., 1995; Stelmach et al., 1998). In cultured tobacco cells OPDA, as well as JA, induced a transient increase in cytoplasmic Ca^{2+} concentrations, whereas JA-Ile did not (Walter et al., 2007), and in the conditional flu mutant of Arabidopsis OPDA was suggested to antagonize JA-promoted cell death (Danon et al., 2005; Reinbothe et al., 2009). In the Arabidopsis opr3 mutant, which is defective in JA biosynthesis, the activation of the complete set of wound-inducible gene required the application of both OPDA and JA suggesting that two independent signaling pathways exist, which is further supported by the observation that opr3, in comparison to coil, showed increased resistance to fungal and insect attack and concomitant activation of COI1-dependent genes that are also regulated by JA, and COI1-independent genes that are not induced by JA (Stintzi et al., 2001). Likewise, global expression profiling experiments using Arabidopsis and different jasmonates revealed that OPDA triggers expression of distinct set of genes
that are related to the wound response (Taki et al., 2005). Many plant species contain high levels of OPDA, both as free acid and bound in chloroplast membranes as galactolipids that further increase upon wounding or infection. The mechanisms that initiate distinct signaling cascades from the SCF$^{COI1}$ complex depending on either OPDA or JA/JA-Ile are currently unknown (Miersch et al., 2008; Ribot et al., 2008).

Similarly, COI1-independent defense responses (to wounding or infection) are only poorly characterized. Distinct and informative mutants have not yet emerged from genetic analyses (Reymond, 2000; Reymond et al., 2004; Devoto et al., 2005; Ribot et al., 2008; Stotz et al., 2011).

Other examples of JA derivatives with distinct bioactivities are cis-jasmone, which induced expression of a set of genes in Arabidopsis that is different from MeJA treatment (Bruce et al., 2008), and 12-0-β-D-glucopyranosyljasmonic acid (12-O-Glc-JA), which has been identified as specific regulator of nycnastic leaf movement, leaf-closing factor (LCF), in the Leguminosae Samanea saman and Albizia julibrissin (Ueda et al., 2000; Ueda and Nakamura, 2007; Nakamura et al., 2008a).

For the latter, chemical synthesis and studies of structure-function relationships revealed that only the (3R,7R) epimer of 12-O-Glc-JA, (-)-LCF, displayed high leaf-closing activity. The corresponding aglycon, (-)-12-OH-JA (also known as tuberonic acid), showed low activity, whereas all other compounds tested were inactive, including (+)-ent-LCF, JA, JA-Ile, and COR. This suggested that the mechanism of leaf closing operates independent of the COI1–JAZ module (Nakamura et al., 2008a, 2011b).

To unravel this signaling pathway, the direct target of (-)-LCF was identified as plasma membrane resident protein using a biotin-tagged affinity probe containing a photoreactive benzophenone residue (Nakamura et al., 2008b, 2011a). Although the identity of the protein target has not yet been uncovered, its successful and efficient labeling has been made possible by first establishing structure-function relationships and subsequent design and optimization of the affinity probe for photoaffinity cross-linking. This highlights the importance of alternative experimental strategies for dissecting signaling pathways in systems that are not tractable by genetic approaches.

Jasmonate action is integrated into a network with other signaling processes, including biotic and abiotic responses as well as the action of other hormones (Glazebrook, 2005; Lorenzo and Solano, 2005; Pieterse et al., 2009; Santner and Estelle, 2009). While a synergistic and antagonistic interaction between jasmonate, salicylic acid, abscisic acid and auxin is well documented, the molecular mechanisms and components participating in these processes are not yet identified. However, the recent identification of the general repressor TPL, which also acts in auxin signaling, may provide a possible link (Szemenyi et al., 2008; Pauwels et al., 2010; Causier et al., 2012). Likewise, the participation of mitogen-activated protein kinases (MAPK), as well as other protein kinases and phosphatases in jasmonate signaling has been established, some of which operate in a COI1-independent manner (Jensen et al., 2002; Brodersen et al., 2006; Ortiz-Masia et al., 2007; Takahashi et al., 2007). However, how these regulatory steps are integrated into the current model of JA signaling remains unclear.

The identification of signaling components or their molecular characterization often involves the application of biochemical or other combined chemical and biological
methods. This approach is used, when genetic approaches fail and mutants cannot be isolated because of redundant or indispensable gene functions. Identification of selective chemicals, acting as agonist or antagonists of a response, and their application in genetic screens has recently found broader application in plant biology (Blackwell and Zhao, 2003; Walsh, 2007; Hicks and Raikhel, 2009; McCourt and Desveaux, 2010).

However, in contrast to other plant hormone responses (Armstrong et al., 2004; Gendron et al., 2008; De Rybel et al., 2009; Park et al., 2009; Lin et al., 2010), jasmonate signaling has not been subject to such alternative chemical genetic interrogation in search for additional signaling components.

The only exception documented so far is bestatin, an inhibitor of aminopeptidases and leukotriene A4 hydrolase in plants and animals (Zheng et al., 2006; Harbut et al., 2011). Bestatin was shown to specifically activate expression of JA-responsive genes in tomato and Arabidopsis in a COI1-dependent manner, but without strict requirement for JA biosynthesis. This lead to the hypothesis that bestatin exerts its function through modulation of key regulators in JA signaling (Zheng et al., 2006). To identify these (novel) regulators, bestatin was applied in a genetic screen for mutants resistant to root growth inhibition, which bestatin exerted as efficiently as MeJA. Although numerous bestatin resistant (ber) mutants were isolated by this approach, novel JA signaling components have not been uncovered (Zheng et al., 2006). However, the rationale for such screen and its feasibility is documented by the finding that one of the ber mutants turned out to be a new jin1/myc2 allele (Zheng et al., 2006).

### 1.2 Chemical genetics and screening strategies

Traditional forward genetic approaches have been widely used to identify genes or sets of genes that are responsible for a particular phenotype. In model organisms this strategy often involves random or directed mutagenesis and gene mapping by breeding. Major limitations of such mutational approaches are lethal mutations because of essential gene functions, pleiotropic effects caused by multi-functionality of a gene product, and redundant gene functions.

Small cell-permeable molecules with activating or inhibitory bioactivity have the potential to complement mutational approaches for dissection of biological processes because chemical interference can be performed in a conditional, dose-dependent and reversible manner (Smukste and Stockwell, 2005). Chemical genetic techniques have long been applied in animal systems in areas such as cancer research, cell death and drug development (Schreiber, 1998; Stockwell, 2000; Mayer, 2003; Gangadhar and Stockwell, 2007).

More recently these approaches have also found application in plant biology (Blackwell and Zhao, 2003; Armstrong et al., 2004; Walsh, 2007; Serrano et al., 2007; Hicks and Raikhel, 2009; Tóth and van der Hoorn, 2010; McCourt and Desveaux, 2010). In particular, the model plant A. thaliana provides excellent prerequisites for scanning small molecule libraries for compounds acting on cellular targets that are inaccessible or recalcitrant to conventional mutational analysis. It is suitable for cultivation
in microplates and thus allows high-throughput screening (HTS) using miniaturized bioassays (Walsh 2007; Armstrong et al. 2004; Tóth and van der Hoorn 2010; Lin et al. 2010). Studies that have systematically explored the potential of small molecules to interfere with plant-specific processes include the gravitropic response, auxin-, abscisic acid- or brassinosteroid-mediated signaling, plant cell morphogenesis, and innate immunity (Zhao et al. 2003; Armstrong et al. 2004; Zouhar et al. 2004; Surpin et al. 2005; Serrano et al. 2007; Yoneda et al. 2007; DeBolt et al. 2007; Gendron et al. 2008; Robert et al. 2008; Schreiber et al. 2008; Knoth et al. 2009; Lin et al. 2010; Serrano et al. 2010; He et al. 2011). The methods applied to screen for bioactivity of small molecules differ greatly, ranging from visual evaluation of phenotypic alterations such as seed germination or hypocotyl length (Zhao et al. 2007; Robert et al. 2008; Savaldi-Goldstein et al. 2008; Bassel et al. 2008; De Rybel et al. 2009; Serrano et al. 2010; He et al. 2011) to quantifying biometric traits such as enzyme activities or reporter gene expression (Zhao et al. 2003; Armstrong et al. 2004; Serrano et al. 2007; Kim et al. 2011). All these screens use miniaturized assay formats in microplates.

For such chemical screening procedures a large number of chemical libraries is commercially available. Combinatorial libraries contain simple compounds with a limited variety of building blocks (Smukste and Stockwell 2005). Natural compound libraries on the contrary typically comprise compounds of high chemical diversity, biochemical specificity and other molecular properties that make them favorable as lead structures for drug discovery (Koehn and Carter 2005). However, natural products are limited by the building blocks of living organisms, which can be increased by designing libraries with compounds of semi-synthetic origin. One challenge in chemical biological research is to choose a library that fits best the own interest. Suppliers often offer preselected collections of pharmacophore libraries, which comprise ‘drug-like molecules’ (Muegge 2002). Typically, compounds in chemical libraries fulfill the following criteria: they are able to cross the cell membrane, they contain substructures similar to known bioactive molecules and they do not contain high reactive functional groups that are likely to cause cytotoxic effects (Mayer 2003).

It is important to note that identification of candidate compounds in a chemical screen is only the first step of a successful chemical genetic strategy. Bioactive compounds originating from the primary screen need to be critically validated. This includes verification of their activity in secondary screens (e.g. using a biological readout that is related but not identical to the primary screen), determination of IC50 values, and evaluation of their selectivity by comparing their effects on a variety of biological responses. The latter assays should include dedicated counter screens to identify false positive hits, leaving only biological relevant chemicals.

Ultimately, the identification of the protein target of a bioactive small molecule is of fundamental importance for understanding its mode of action. To this end, various experimental strategies can be applied. For instance following traceable derivates, such as radioactive labeled or otherwise tagged molecules, can lead to successful identification of the target via fractionation in chromatography- or affinity-based purification. Such techniques have contributed fundamentally to our recent understanding of the COI1–JAZ co-receptor (compare section 1.1.4). Other methods for target identifica-
tion are the yeast-three-hybrid technology (Kley, 2004; Cottier et al., 2011), phage display (Crameri et al., 1994), quartz crystal microbalances (Cooper and Singleton, 2007) or protein microarrays (Huang et al., 2004). Techniques requiring modified derivatives reach their limits, because the modified derivatives may lose their bioactivity. The classical approach without ligand derivatization is application of a selective compound in a genetic screen to identify mutants with a discriminative effect to chemical treatment. Recent achievements in elucidating hormone-resistant Arabidopsis mutants exemplify the power of this chemical genetic strategy (Chini et al., 2007; Thines et al., 2007; Murase et al., 2008; Shimada et al., 2008; Park et al., 2009; Santner and Estelle, 2009).

However, the step from initial mutant identification to target identification is labor intensive and time consuming. In combination with the described biochemical methods, genome wide microarray studies can also basically contribute to understanding the mode of action of a compound (Bassel et al., 2008; Park et al., 2009; Lin et al., 2010). A general strategy for chemical biological research is depicted in Figure 1.5.

**Figure 1.5: From high-throughput screening to target identification.** A robust biological readout can be applied in a primary screen for chemical compounds with bioactivity on the desired pathway. Such primary hits are verified in secondary screens and evaluated for their biological relevance in counter screens. Confirmed hits can be used as tool in genetic screening procedures or more generally in chemical biological or bioinformatic approaches to identify their cellular target and mode of action.
1.3 Aims of this thesis

Over the past few years the field of jasmonate research has seen exciting developments and many details of JA biosynthesis, metabolism, perception and signal transduction have been unraveled. And yet, many questions remain open. They include, but are not restricted to the following: (1) How is selectivity of diverse biological JA responses established? (2) What is the mode of action and perception mechanism for different bioactive jasmonates? (3) What are the molecular mechanisms of cross-talk between different hormonal signaling pathways and what are the missing components?

The aim of this thesis is to answer these questions, which will likely provide an increased mechanistic understanding of plant hormone perception and signaling beyond the specific action of jasmonates. Since chemical biological approaches in jasmonate signaling remain unused, I aim at identification of compounds that specifically impair or modulate jasmonate signaling.

Screening a natural compound library may lead to identification of compounds that modulate jasmonate signaling via unknown mechanisms, while application of a kinase inhibitor library may uncover if kinases are involved in jasmonate signaling and how they exert their function in the signaling pathway.

To identify compounds with such a specific effect on JA signaling, a robust chemical screening procedure will be developed. As such, a reporter-based chemical screen in *Arabidopsis thaliana* will afford at least semi-quantitative data that allow discrimination between compounds with high and low bioactivity. After establishment of screening conditions and identification of compounds that affect the reporter readout, secondary and counter screens will be applied. These will verify, if the identified compounds affect JA signaling in a selective and unique way. Selective compounds can be applied in subsequent genetic screens and/or other chemical biological approaches to uncover new components in the JA signaling network.
2 Screening of a natural compound library uncovers one putative activator and several putative inhibitors

For the challenge to identify new bioactive molecules that selectively impair JA signaling in Arabidopsis, I designed a chemical screening procedure in Arabidopsis seedlings harboring an inducible firefly luciferase (LUC) reporter construct under the control of the jasmonate-inducible promoter of the Lipoxygenase 2 (LOX2) gene (Jensen et al., 2002). Such a reporter provides excellent prerequisites for scanning small molecule libraries for compounds acting on cellular targets that are inaccessible or intractable to conventional genetic screening (McCourt and Desveaux, 2010). Treatment of Arabidopsis seedlings, which were directly grown in 96-well microplates, with 100 µM MeJA lead to accumulation of LUC. MeJA induces the LOX2 promoter already one hour after application. The over 24 hours accumulated LUC reporter can be detected as flash LUC activity after triggering the LUC reaction with D-luciferin (Fig. 2.1). Initial luciferase activity is proportional to the amount of protein expressed from the activated reporter gene (and the turnover of the protein). However, since luciferase is inhibited by its product oxyluciferin, maximum enzyme activity declines over time and reaches a steady state after a certain time. For data analysis in the chemical screen, I decided to use the average of the initial triggered phase (Fig. 2.1), because averaging the luminescence of the ‘triggered phase’ would increase differences in LUC activity between control and compound treatment that modulates reporter expression.

Sifting through a library comprising about 1,700 small molecules of natural and semi-synthetic origin (AnalytiCon Discovery), I identified a single compound, 1-propyl-2-carboxy-3,8-dihydroxy-anthraquinone (766), that apparently activated expression of the reporter gene (see section 2.1, Fig. 2.3). Conversely, screening the same library for inhibitors uncovered several small molecules that strongly impaired MeJA-induced expression of the LOX2p::LUC reporter gene. Three of the identified compounds, cycloheximide 8, and the two trichothecene mycotoxins diacetoxyscirpenol 15 and neosolaniol 16, inhibit protein synthesis (Serrano et al., 2010). An example of one microplate in a screen for inhibitors including the translational inhibitors is shown in Figure 2.2. The whole screen for inhibitors is shown in section 2.2 (Fig. 2.8).

Note that the MeJA-stimulated LOX2p::LUC expression varies considerably across all samples of this primary screen. This is largely attributed to variable seedling size
Figure 2.1: Time course of LUC activity. 12-day-old Arabidopsis LOX2p::LUC seedlings were treated with 100 µM methyl jasmonate (MeJA) to induce reporter gene expression. After 24 h in vivo LUC activity measurement was initiated by addition of luciferin (t = 0 h) and recorded for the indicated time. Values represent average activity (± standard deviation, n = 33) in counts per second (cps). The red line represents activities of induced samples. The blue line represents activities of samples treated with the DMSO control. Activities in the chemical screen and subsequent LUC assays are based on the average of each value in the triggered/flash phase (green shaded area). Values of the steady state phase (blue shaded area) were not considered for comparisons.

Figure 2.2: Chemical screen for inhibitors of reporter gene expression. 12-day-old LOX2p::LUC seedlings were preincubated for 1 h with the indicated compounds (each at 25 µM) and expression of the reporter gene was induced by 100 µM methyl jasmonate. LUC activity was determined after 24 h in duplicate samples, values (± standard deviation) are normalized to the average activity of the whole plate (96 samples). The shaded area shows a two-fold upper and lower threshold of the plate average. Three candidate compounds (8, 15, 16; dark red diamonds) were confirmed in a secondary screen (Fig. 2.9 A) and identified as translational inhibitors, cycloheximide (8), diacetoxyscirpenol (15), and neosolaniol (16). Other candidates outside the indicated thresholds have not been confirmed (gray diamonds).
and orientation within individual microplate wells, thus leading to variable luminescence detection, which effectively renders the assay only semi-quantitative. However, stringent selection criteria for primary hits and their confirmation with increased samples numbers reduces the false discovery rate.

2.1 Screen for activators

The screening procedure of the chemical library of natural and semi-synthetic origin uncovered only one chemical compound that apparently activated expression of the reporter gene (Fig. 2.3, structure see Fig. 2.5). I confirmed the higher LUC activity in an independent experiment using the same reporter (Fig. 2.4 A). To verify the effect on jasmonate signaling, I used a second MeJA inducible marker, VSP1p::LUC (VSP1: VEGETATIVE STORAGE PROTEIN 1) as secondary readout. The induction by 766 alone was much weaker than the induction by MeJA (Fig. 2.4 B). However, application of 766 together with MeJA caused an almost six-fold hyperactivation in VSP1p::LUC (Fig. 2.4 C).

![Figure 2.3: Chemical screen for activators of reporter gene expression. 12-day-old Arabidopsis seedlings harboring the LOX2p::LUC reporter gene were treated for 24 h with 1,728 different compounds (each at 25 µM). One of these compounds (766) caused an increase in LUC activity, which is presented as average ± standard deviation from duplicate samples. cps: counts per second.]

2.1.1 Derivatives of 766 modulate LOX2:LUC

Compound 766 (1-propyl-2-carboxy-3,8-dihydroxy-anthraquinone) is a derivative of 9,10-anthracenedione (anthraquinone) (see table 2.1) and derivatives are commercially available. To investigate if other anthraquinones were able to modulate the expression of the reporter, I tested the structure-function relationship (SAR) by application of 31 other anthraquinone derivatives to the LOX2 reporter line (For structures see Table 2.1 and supplemental Fig. S.2).
Figure 2.4: Activation of reporter expression upon chemical treatment.
A: 12-day-old Arabidopsis seedlings harboring the LOX2p::LUC reporter gene were treated for 24 h with 766 or MeJA (25 µM). 766 induces LOX2 similar to MeJA. B: 12-day-old Arabidopsis seedlings harboring the VSP1p::LUC reporter gene were treated with 766 or MeJA (25 µM) for 24 h. DMSO served as negative control. 766 causes weak induction of VSP1 compared to MeJA. C: 12-day-old Arabidopsis seedlings harboring the VSP1p::LUC reporter gene were pretreated with 766 or MeJA (25 µM) for one hour. Then reporter expression was induced with MeJA (100 µM) for 24 h. DMSO served as negative control. Double application of 766 and MeJA causes strong hyperactivation. LUC activity is presented as average (± standard deviation, n = 2-4) from one representative experiment out of two. Asterisks indicate significant differences compared to the respective control (Student’s t-test **p<0.05, *p<0.1). cps: counts per second.

Of all derivatives tested, only 47 and 48 showed inducing activity similar to 766 (Fig. 2.5 A, green bars). However, strong hyperactivation compared to MeJA alone, as in the VSPp::LUC reporter, could only be observed for 766 together with MeJA (Fig. 2.5 A, blue bars). Interestingly, two compounds, 18 and 39, inhibited the MeJA-induced expression of the reporter (Fig. 2.5 A & B, blue bars).

The derivatives 47, 48 and 766 share the carboxylic acid at carbon atom 2 and differently substituted carbon chains at carbon atom 1 (see Fig. 2.5). The derivatives 18 and 39 only share the carboxylic acid at carbon atom 2. Together, all bioactive derivatives contain a carboxylic acid group at C-2, whereas only the activating compounds contain a substituted carbon chain at C-1. This indicates that a structure-activity relationship at carbon atoms 1 and 2 exists, which may have different impact on their activity.

2.1.2 766 modulates LUC activity, rather than JA signaling

The use of reporter lines in chemical screenings provides an indirect readout for monitoring gene expression and depends (1) on the size of the promoter fragment and its insertion site and (2) on the activity of the reporter gene product. To verify the effect of 766 on the LOX2p::LUC line, I tested its effect by monitoring the activity of the β-glucuronidase (GUS) under the control of the LOX2 promoter instead of LUC in a secondary assay. Neither 766 nor 18 had an effect on the expression of the GUS reporter line (Fig. 2.6 A, green bars). After application of MeJA compound 18 prevented the induction of LOX2p::GUS efficiently (Fig. 2.6 A, blue bars). Interestingly, 766 did also inhibit the induction of LOX2p::GUS by MeJA in contrast to hyperactivating the LUC reporter.
Figure 2.5: Activation of LOX2p::LUC after application of anthraquinones.

12-day-old seedlings harboring LOX2p::LUC were pretreated for one hour with anthraquinones as indicated (25 µM). JA-specific expression was induced with MeJA (100 µM) for 24 h. DMSO served as negative control. A: Effect of 23-48, 47, 48 and 766 activate the reporter. 766 and MeJA together cause strong hyperactivation. 39 inhibits MeJA-induced reporter expression. B: Effect of 18-22. MeJA or 766 activate the reporter. 766 and MeJA together cause hyperactivation. 18 inhibits MeJA-induced reporter expression. LUC activity is presented as average of two independent experiments with at least four replicates ± standard deviation. Asterisks indicate significant differences compared to the respective control (Student’s t-test **p<0.02, *p<0.05). cps: counts per second.

Monitoring an alternative reporter is still an indirect method of testing gene expression. Therefore I tested the effect of 766 on endogenous mRNA accumulation at different time points after chemical application to the LOX2p::LUC reporter line. 766 did neither induce the expression of LUC nor of the endogenous JA-signaling markers LOX2 or VSP1. It rather inhibited the expression of LOX2 (Fig. 2.6 B).

This result indicated that 766 may rather modulate LUC activity than gene expression. For verification of this hypothesis I applied 766 to Arabidopsis seedlings harboring a construct, which expresses LUC under the control of the CaMV35S promoter. The monitored activities after application of 766 were almost three-fold higher compared to the DMSO control (Fig. 2.7 A). The derivative 18 caused a two-fold reduction in LUC activity. To verify the effect on LUC activity I also performed an in vitro LUC assay using recombinant luciferase (Roche). DMSO, 766 or 18 were incubated in reaction buffer containing ATP. Upon application of a mixture with luciferin and LUC the luminescence was repeatedly measured at five different time points. Intriguingly, 766 inhibited the reaction at time point zero (Fig. 2.7 B) and even stronger at later time points (Fig. 2.7 C). Compound 18 did not cause any significant effects although the activity was constantly lower than the control with higher variation between samples.
Figure 2.6: Effect of selected anthraquinones on LOX2p::GUS activity and gene expression.
12-day-old seedlings harboring different reporters were pretreated for one hour with anthraquinones as indicated (25 µM). JA-specific expression was induced with MeJA (100 µM) for 24 h. DMSO served as control. A: Chemical intervention on LOX2p::GUS activity. 18 and 766 both inhibit MeJA-induced LOX2p::GUS and do not induce the reporter itself. GUS activity was quantified in plant extracts using 4-methylumbelliferyl-β-D-glucuronide as substrate in a fluorimetric assay and is related to the protein concentration. Activities are normalized to the control. The data represent the average of four biological replicates ± standard deviation. Asterisks indicate significant differences compared to the respective control (Student’s t-test **p<0.02, *p<0.05). B: Chemical intervention on gene expression. LUC expression is induced, LOX2 expression is inhibited and VSP1 expression is unchanged after application of 766. The picture depicts RT-PCR on RNA of Arabidopsis LOX2p::LUC. Plants were treated as indicated and RNA harvested at the indicated time points.

Figure 2.7: Effect of selected anthraquinones on LUC in vivo and in vitro.
A: 12-day-old seedlings harboring CaMV35S::LUC were treated for 24 h with anthraquinones as indicated (25 µM). 766 enhances LUC activity, whereas 18 inhibits. LUC activity is presented as average of six replicates ± standard deviation. cps: counts per second. B, C: 766 inhibits in vitro LUC activity. Compounds were mixed with ATP. One second after injection of a mixture of luciferin and luciferase luminescence was detected over 10 s with a luminometer (B). Measurement was repeated at different time points (C). LUC activity is presented as absolute luminescence or is normalized to the control. One sample without ATP served as negative control. The data represent the average of five replicates ± standard deviation. Asterisks indicate significant differences compared to the DMSO control (Student’s t-test **p<0.02, *p<0.05). RLU: relative light units.
These results suggest that probably 766 binds to LUC. *In vivo* 766 enhances activity, since it may stabilize the protein by binding and preventing it from degradation. *In vitro* the compound impairs enzyme activity, because it may bind to the enzyme. Proving the hypothesis of stabilizing function may be done via immunodetection of LUC in extracts but was not of further interest for the overall project.

Table 2.1: Anthraquinone derivatives of 766.
The structures of 766 and anthraquinone are shown. The numbering of the carbon atoms is depicted next to anthraquinone. The table names the functional groups of each derivative at its respective carbon atom. For full structures see supplemental Fig. S.2.
2.2 Screen for inhibitors

The chemical screen for inhibitors of MeJA-induced LOX2p::LUC expression showed big variations in LUC activity. Each plate consisting of 96 different chemicals was analyzed for itself and candidates were obtained by comparing activities of the chemical plate to one separate control plate. Screening the natural compound library yielded 16 candidates that apparently prevented the induction of LOX2p::LUC gene expression (Fig. 2.8).

![Chemical screen for inhibitors of reporter gene expression.](image)

**Figure 2.8:** Chemical screen for inhibitors of reporter gene expression. 12-day-old *Arabidopsis* seedlings harboring the LOX2p::LUC reporter gene were pretreated for one hour with 1,728 different compounds (each at 25 µM) before addition of MeJA (100 µM). The overall screen was performed in 13 separate steps with each containing one separate control plate. Due to their relative activity compared to the respective control plate, 16 compounds were chosen for further analysis (red diamonds). The plot presents relative average LUC activities (normalized to the respective control plate) after treatment with the chemical library (n = 2).

In a secondary assay for confirmation of the 16 candidates, only 10 of those inhibited the LOX2p::LUC expression significantly (Fig. 2.9 A). One candidate is cycloheximide (8), a compound which is known to inhibit protein synthesis. Two other candidates, the mycotoxins diacetoxyscirpenol (15) and neosolaniol (16), are also translational inhibitors (Serrano et al. 2010). Candidate 17, piericidin, is a potent inhibitor of the mitochondrial type I NADH-ubiquinone oxidoreductase in the respiratory chain and is considered to bind to the ubiquinone binding site of the enzyme (Darrouzet et al. 1998). Because 17 generally may interfere with energy dependent metabolism or signal transduction pathways it was excluded from further studies. The translational inhibitors 8, 15 and 16 were also not considered for secondary and counter screens.

For the other candidate compounds (2, 3, 4, 5, 6, 7, 9, 10, 11, 12, 13, 14) no additional information was available in the suppliers database (For structures of confirmed candidates see Fig. 2.11).
Figure 2.9: Activity of selected compounds on various LUC and GUS reporters. 12-day-old *Arabidopsis* seedlings harboring the indicated reporter gene were pretreated with compounds (25 µM). Reporter expression was induced by MeJA (100 µM) or flg22 (1 µM). A: Confirmation of the candidate-modulated LOX2p::LUC activity is shown as average of two replicates ± standard deviation. B: Only confirmed and selected candidates were tested for their effect on VSP1p::LUC activity. LUC activity is shown as average of two independent experiments with two replicates each ± standard deviation. Asterisks indicate significant differences compared to the control (Student’s t-test **p<0.02, *p<0.05). C: LOX2p::GUS and VSP1p::GUS reporter activities were induced by MeJA (blue staining). Pretreatment with selected compounds interferes with reporter activation (reduced staining). D: WRKY29p::GUS reporter activity was induced by flg22 (blue staining). Only 10 and 12 do not interfere with reporter activation. Histochemical GUS staining shows one representative out of at least two independent experiments.

2.2.1 Compound 12 is the most selective compound

To ensure that the inhibition of gene expression is specific for JA signaling, I performed an analysis of the candidate’s effect using different *Arabidopsis* reporter lines and different readouts. In a secondary screen I tested expression of *VSP1* by *in vivo* detection of luminescence in *Arabidopsis* VSP1p::LUC seedlings after application of MeJA and the respective candidate compounds (Fig. 2.9 B). The reporter expression was not affected by compound 4. MeJA-induced LUC expression was completely suppressed by 6, 7 and 12. Compounds 5 and 10 were less effective but still provoked significantly reduced reporter expression.

To confirm the results obtained with the *Arabidopsis* lines expressing the LUC reporters I repeated the experiment with two lines harboring the β-Glucuronidase (GUS) fused to the LOX2 and VSP1 promoter, respectively (Fig. 2.9 C). Compared to the
LOX2p::LUC reporter 4 and 10 did behave different. Both compounds did not or only slightly inhibit the GUS reporter in both GUS reporter lines. 5, 6, 7 and 12 inhibited GUS accumulation completely.

As counter screen for verification of the compound’s selectivity the effect on the JA independent Arabidopsis line WRKY29p::GUS was tested. This line harbors the WRKY29 promoter fused to GUS. WRKY29 expression is inducible by the elicitor epitope flg22, which originates from bacterial flagellin (Asai et al., 2002). JA-specific candidate compounds should not affect this readout. Compounds 6 and 7 showed strong inhibition of the reporter (Fig. 2.9 D). 4 and 5 where just slightly able to inhibit the reporter expression whereas 10 and 12 did not have any effect.

Since the observed effect of the chemicals on the reporter lines is only indirect and dependent on a functional transcription and translation machinery, a reporter-independent readout was applied as other counter screen. The oxidative burst reaction in Arabidopsis is a very fast generated signal as response to stress or pathogen-associated molecular patterns such as flg22 (Rentel et al., 2004; Zipfel et al., 2004; Nühse et al., 2007; Galletti et al., 2008; Mersmann et al., 2010). This production of reactive oxygen species (ROS) is independent of JA. Arabidopsis leaf discs were incubated with the respective candidate compounds and then elicited with flg22. Increasing ROS levels were monitored by their reaction with luminol and a peroxidase, which results in luminescence emission.

The only compounds without any effect on ROS production are 10 and 12 (Fig. 2.10 A). Whereas 4 slightly and 6 almost completely inhibited the oxidative burst, 5 promoted ROS production. After treatment with 7, ROS accumulated slower and to a lesser extent and after 15 minutes ROS levels stayed at the same level instead of decreasing.

In conclusion 10 and 12 modulate JA-dependent responses but do not interfere
with flg22-mediated responses. To verify the effect on the LOX2 and VSP1 reporters I monitored endogenous mRNA accumulation of LOX2 and VSP1. Compounds 10 and 12 did both cause inhibition of endogenous LUC expression in the LOX2p::LUC reporter line as well as inhibition of endogenous LOX2 at least 3 and 6 hours after MeJA induction. Consistent with the results using LOX2p::GUS, only 12 inhibited endogenous VSP1 expression 3, 6 and 24 hours after MeJA induction. Due to the selective effect of 12 on all JA specific readouts, this compound was considered most interesting to continue with for further analysis (see Table 2.2 for an overview of the effects). For structures of the tested compounds see Fig. 2.11.

Table 2.2: Overview of effects caused by different compounds. The table summarizes the effects of the selected candidate compounds on different readouts. Asterisks indicate significant inhibition compared to the control (Student’s t-test **p<0.02 [red], *p<0.05 [light red]). Minus or plus signs indicate the strength of inhibiting or enhancing effects, estimated from the qualitative readout; red: strong inhibition; light red: weaker inhibition; green: activation. Note that 7 initially causes inhibition of ROS, but causes higher ROS levels at later stages [orange]. Note as well, that 12 is the only compound with an effect on all JA specific readouts and does not affect flg22 dependent readouts.

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<th>Compound</th>
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Figure 2.11: Structure of selected candidate compounds. The picture depicts structures of candidate compounds that were selected after the secondary screen for analysis in counter screens. No further information was available in the suppliers database. For structures of all confirmed candidates see supplemental Fig. S.1.
2.2.2 IC$_{50}$ of compound 12 and gene expression

To see whether the previously used concentration is appropriate for further experiments, I tested the response of Arabidopsis LOX2p::GUS seedlings to different concentrations of 12. This experiment confirmed the strong inhibition at 25 µM and indicates that the half maximum inhibitory concentration (IC$_{50}$) of 12 is around 10 µM (Fig. 2.12 A).

The concentration of 10 µM was then used to analyze expression of LOX2 at different time points. Seedlings were pretreated with 12 and subsequently LOX2 expression was induced by addition of MeJA. LOX2 is induced already one hour after induction and reaches a peak after eight hours (Fig. 2.12 B). In plants pretreated with 12, LOX2 expression is not prevented but reduced at every time point. Although this indicates a rather weak effect, I decided to analyze genome wide gene expression using the AGRONOMICS1 microarray [Rehran et al., 2010]. Samples were collected eight hours after treatment and before hybridization with the microarray, LOX2 expression was tested via quantitative real-time PCR. Despite the significant differences between the different treatments (Fig. 2.12 C), the microarray data did only yield a few significant differentially expressed genes, which even did not include the JA-downstream markers LOX2 and VSP1 (data not shown).

Figure 2.12: Dose response of 12 and LOX2 expression.
A: 12-day-old Arabidopsis seedlings harboring the LOX2p::GUS reporter gene were pretreated with 12 for one hour at the indicated concentrations. MeJA (100 µM) was added and incubated for 24 h. GUS activity was quantified in plant extracts using 4-methylumbelliferyl-β-D-glucuronide as substrate in a fluorimetric assay and related to the protein concentration. Activities are normalized to the control and are shown as average of 4 replicates ± standard deviation. B: 14-day-old Arabidopsis seedlings (Col-0) were pretreated with DMSO or 12 (10 µM) and LOX2 expression was induced by application of MeJA (100 µM). Plants were harvested at the indicated time points and expression was monitored by qRT-PCR. C: 14-day-old Arabidopsis seedlings (Col-0) were treated as in B and harvested after 8 hours. After confirming the inhibitory effect of 12, these RNA samples were taken for microarray hybridization. Expression was normalized to the untreated control and is shown as average of 3 biological replicates ± standard error. Asterisks indicate significant differences compared to the MeJA control (Student’s t-test **p<0.02, *p<0.05).
2.2.3 12 inhibits root growth and degradation of JAZ

Since 12 interferes with JA-specific gene expression, an obvious question is, whether the compound also affects other JA-related phenotypes or not. If grown on plates containing solid medium supplemented with different concentrations of 12, *Arabidopsis* root growth elongation was impaired with increasing concentrations, whereas the aerial part of the plant was not impaired (Fig. 2.13 A & B). The IC₅₀ for the root growth phenotype is between 10 and 30 µM, which is higher than for the LOX2p::GUS reporter. If subsequently transferred to soil all plants did recover and developed normally as did untreated plants (data not shown).

JA biosynthesis or signaling mutants do not respond to JA induced inhibition of primary root growth. To see whether such mutants would be resistant to 12 as well, I tested *Arabidopsis* mutants for their response to 12. Neither the JA-biosynthesis mutant *aos* nor *jar1-1*, which cannot produce the bioactive jasmonate (+)-7-iso-JA-L-Ile, were resistant to inhibition of root growth elongation (Fig. 2.13 A).

One central component in JA signaling is the proteosomal degradation of JAZ proteins. To investigate if this part of JA signaling is inhibited by 12 I tested the JAZ degradation in an *Arabidopsis* line (CaMV35Sp::JAZ-GUS, Thines et al. (2007)), that constantly overproduces a JAZ1-GUS fusion protein. The degradation of JAZ protein can be detected 30 minutes after application of MeJA in roots. After incubation with 12 the JAZ1 fusion protein was not degraded, while it was fully degraded after treatment with the DMSO control (Fig. 2.14). This effect was already visible at concentrations of 3 µM. The proteasome inhibitor MG132 served as positive control for inhibition of this degradation.

Thus 12 may interfere with proteasome activity or exert its function via other ways to interfere with JA signaling.

![Figure 2.13: Root growth phenotype after treatment with compound 12. A: *Arabidopsis* seeds were germinated on solid medium containing 12 and grown vertically under day/night conditions. After 9 days pictures were taken and root length was measured using ImageJ software. Root length is shown as average of at least 19 plants ± standard deviation. B: The picture depicts representative *Arabidopsis* Col-0 plants. Two independent experiments gave the same results.](image)
2.2.4 Structure-activity relationship of 12

Analysis of the structure of 12 revealed that the core structure consisted out of aminocytisine. Cytisine is a plant alkaloid from Faboidae (Izaddoost et al., 1976). This core structure is connected to two different functional groups (biphenyl-carboxylic acid and 3-methoxy-propionic acid) via two amide bonds. The commercial availability of numerous derivatives of aminocytisine opened the opportunity to study the SAR of 12. I tested cytisine derivatives carrying different substituents for their ability to modulate reporter expression of Arabidopsis LOX2p::LUC in response to MeJA. Some of the derivatives are depicted in Figure 2.15; for all structures tested see supplemental Figure S.3.

Only 49 and 52 prevented expression of LOX2p::LUC to the same extent as 12 (Fig. 2.15 A). Both compounds share with 12 the core structure of aminocytisine and the biphenyl-carboxylic acid as substituents at the same position. All other tested derivatives did not cause any effect. Concluding from this, the position of the biphenyl-carboxylic acid, as well as the connection to aminocytisine is important for the ability to inhibit MeJA-induced LOX2p::LUC reporter expression. The alternative position can be varied with different substituents, defining the site for modification of 12 without impairing its activity.

The ultimate goal in chemical biology is to identify a compound’s direct target and its mode of action. For target identification I used a photoaffinity cross-linking and protein purification approach with a modified version of the original compound. Since it appears to be possible to modify 12 at one position of the molecule, I went for targeted modifications including a photoreactive benzophenone group and an alkyne. Modified derivatives were synthesized and provided by a collaborating group (Prof. Markus Kaiser, ZMB, Universität Duisburg Essen). Benzophenones are chemically very stable,
A 12-day-old *Arabidopsis* LOX2p::LUC were pretreated with derivatives of 12 (25 µM) and induced with MeJA (100 µM). 12, 49 and 52 inhibit MeJA-induced LOX2p::LUC activation. LUC activity is presented as average of two independent experiments ± standard deviation with 6 replicates each.

B A modified derivative (65) inhibits MeJA-induced LOX2p::LUC activation. LUC activity is presented as average of six replicates ± standard deviation. At least two independent experiments gave similar results. Asterisks indicate significant differences compared to the control (Student's t-test **p<0.02, *p<0.05). Structures of selected compounds are depicted including the core structure cytisine (56) and biphenyl-carboxylic acid (57).

2.2.5 First steps towards target identification

Since 65 was able to modulate the JA response in the LOX2p::LUC reporter line, I initiated protein labeling experiments to identify its direct protein target. The labeling was performed on *Arabidopsis* cell cultures to first establish experimental conditions. The advantage of in dark conditions grown cell culture is that it contains less light absorbing pigments than seedlings, which would complicate photoaffinity cross-linking with UV light.
Arabidopsis cell cultures were treated for one hour with 65 and irradiated at the same time with UV-light to establish covalent cross-linking with C-H bonds of the protein target. Since the probe does not contain a group, which can be purified with affinity-based chromatography or detected on a gel, it was necessary to label the probe after cross-linking. This was performed using copper-catalyzed 1,3-dipolar cycloaddition (‘click’ reaction) between the alkyne of the probe and an fluorescent probe containing an azide.

After separation of the labeled protein extracts on a polyacrylamide gel, it could be observed, that 65 binds several different proteins (Fig. 2.16 A, lane 3). This binding was competed out by addition of 12 (Fig. 2.16 A, lane 4), which indicated specific binding of 65 to a similar protein target of 12.

Since the band pattern of proteins reflects the whole-protein-stain of the gel (Fig. 2.16 B), an obvious question was, if the UV-cross-link just established general unspecific bonds to several proteins and that the observed competition may in fact be competition for uptake. Thus I performed the UV-cross-linking on protein extracts, in which no competition for uptake would occur. Intriguingly, the result reflects completely the result of the previous experiment (Fig. 2.16 C and D). Consequently, the labeled proteins are bound by 65 in a specific manner. A control for unspecific UV-cross-linking (66), which contained a benzylamine fused to a benzophenone and an alkyne (see supplemental Fig. S.3), did not label any proteins. This indicates as well the specific labeling by 65. If one target would exist, that is bound at lower concentrations of the ligand than the other proteins, lower concentrations of the probe would maybe result in a more specific band pattern. Unfortunately lowering the concentrations of 65 resulted just in a decrease in overall signal instead of appearance of more specific protein bands (data not shown).

However, these findings encourage to continue the efforts to pull down the protein target. Modifying the experimental conditions in a targeted approach and increasing the spatial resolution by 2D-SDS-PAGE may eventually lead to identification of specific protein target(s). Unfortunately these additional experiments could not be performed under the time constraints of this PhD thesis.
Figure 2.16: Photoaffinity labeling of *Arabidopsis* proteins with a photoreactive derivative of 12. *Arabidopsis* cell cultures or *Arabidopsis* cell culture protein extracts were treated for one hour with 100 µM of 65. This high concentration was used, since the compound was less efficient at inhibiting reporter activity compared to 12. UV-cross-linking was performed using a hand-held UV lamp at 254 and 366 nm wavelength. Protein extracts were labeled with a fluorophore (Alexa Fluor® 555) and labeled proteins were visualized on a fluorescence scanner. Subsequently the gel was stained with coomassie blue to reveal unlabeled and labeled proteins. A: Fluorescence of gel after pulldown on cell culture. B: Coomassie stain of gel after pulldown on cell culture. C: Fluorescence of gel after pulldown on cell culture extracts. D: Coomassie stain of gel after pulldown on cell culture extracts. (1): DMSO, (2): UV-cross-link control (66, 100 µM, Fig. S3), (3): 65 (100 µM), (4): 65 (100 µM) + 12 (400 µM)
3 Screening of a kinase inhibitor library identifies toyocamycin as modulator of JA signaling

In addition to the screen of a natural compound library, I performed a second screen using the MeJA inducible LUC reporter construct LOX2p::LUC in A. thaliana. The second chemical library contained 84 known kinase inhibitors (Biomol GmbH). Phosphorylation and dephosphorylation of proteins via specific or non-specific kinases are common modifications in metabolism and signaling pathways. One advantage of this rather small library is that it contains a variety of specific and non-specific kinase inhibitors with already described target(s). The probability to cause any effects in planta is rather high compared to libraries selected for chemical diversity.

3.1 Three kinase inhibitors disturb JA signaling

The screen for kinase inhibitors, which modulate the expression of LOX2 did not uncover any activator of LOX2p::LUC. Out of 84 kinase inhibitors only three compounds caused robust inhibition of MeJA-induced LOX2p::LUC expression (Fig. 3.1). Compound 6, staurosporine, is a general kinase inhibitor, which was shown to inhibit more than 80 percent out of 290 tested human kinases. In contrast to 6 compound 16, tyrphostin AG-879, is more specific. It is an inhibitor of protein tyrosine kinases and mitogen-activated protein kinases, which are involved in human breast or prostate cancer. The third compound, 5-iodotubercidin, is a nucleoside analog and also a general kinase inhibitor. As adenosine analog it was shown to inhibit human adenosine kinase. Several structural derivatives of 5-iodotubercidin are described to specifically inhibit auxin signaling, which is mediated via SCFTIR1, which is mechanistically related to JA signaling. Because of the functional similarity between auxin and JA signaling pathways, 5-iodotubercidin was selected for further studies. As inhibitor of both pathways it could enhance the understanding of how hormonal crosstalk between auxin and JA may be mediated. Thus, different derivatives of 5-iodotubercidin were compared for their ability to interfere with MeJA-induced reporter expression (for structures see supplemental Fig. S.4). Sangivamycin is the strongest inhibitor of LOX2p::GUS expression with an IC50 of 0.3 and 1 µM (Fig. 3.2 A). Toyocamycin is less effective and inhibits half maximum at 1 µM. The weakest effect is caused by tubercidin and 5-iodotubercidin, which both exhibit an IC50 of 3 µM. For IC50 values see Table 3.1.
12-day-old *Arabidopsis* seedlings harboring the LOX2p::LUC reporter gene were pretreated for one hour with 84 different kinase inhibitors (each at 10 \( \mu \)M). The reporter was induced with MeJA (100 \( \mu \)M) for 24 h. In two independent experiments only three compounds were able to inhibit expression of the LOX2p::LUC reporter (red). Activities were normalized to the control. The plot presents relative average LUC activities of two independent experiments.

### 3.1.1 Toyocamycin is the most selective derivative

For all nucleosides I did a secondary assay with two MeJA-inducible reporter constructs, VSP1p::GUS and OPCL1p::GUS. The expression of VSP1p::GUS was most efficiently inhibited by sangivamycin, although the IC\(_{50}\) (1 \( \mu \)M) is lower than in the LOX2 reporter line (Fig. 3.2 B, Table 3.1). The IC\(_{50}\) of toyocamycin on VSP1p::GUS expression is between 1 and 3 \( \mu \)M and thus close to the IC\(_{50}\) on the LOX2 reporter line. Tubercidin and 5-iodotubercidin have IC\(_{50}\) values around 30 \( \mu \)M. All together, the tendency to inhibit LOX2 and VSP1 is the same. *OPCL1* is as well inducible but its gene product acts in the biosynthesis of JA downstream of LOX2. Intriguingly, the effect of the nucleosides on OPCL1p::GUS expression is very different to expression of LOX2p::GUS (Fig. 3.2 C). Sangivamycin and 5-iodotubercidin are causing an inhibitory effect at concentrations higher than 10 \( \mu \)M, whereas tubercidin and toyocamycin inhibit OPCL1p::GUS expression only at 30 \( \mu \)M. Overall, toyocamycin is the least effective at 30 \( \mu \)M, where the other analogs cause already complete inhibition.

As counter assay for testing JA independent readouts I analyzed expression of the *Arabidopsis* WRKY29p::GUS line, which is inducible by flg22. All nucleoside analogs inhibited the expression of WRKY29p::GUS to the same extent (IC\(_{50}\) 1 \( \mu \)M), although tubercidin appeared to be less effective than the other analogs at 10 \( \mu \)M (Fig. 3.2 D).

To sort out, if kinases are more generally affected or if there may be inhibition of specific kinases, I tested, whether the compounds are able to inhibit the activation of the flg22-induced MAPK cascade. *Arabidopsis* seedlings treated with the respective nucleoside were triggered with flg22 to induce activation of the MAPK cascade. The
Arabidopsis plants harboring the respective promoter fused to GUS were grown in liquid culture for 2 weeks and subsequently treated with the indicated compounds for 24 h (LOX2, VSP, OPCL1) or 5 h (WRKY29). Control treatment was MeJA (LOX2, VSP, OPCL1) or flg22 (WRKY29) only. GUS activity was quantified in plant extracts using 4-methylum bending-glucuronide as substrate in a fluorimetric assay and related to the protein concentration. Activities are normalized to the control. The data represent the average of at least three biological replicates ± standard deviation.

**A**: LOX2p::GUS activity is inhibited by all nucleosides.

**B**: VSP1p::GUS activity is inhibited by all nucleosides.

**C**: OPCL1p::GUS activity is less inhibited than the other reporters and least affected by toyocamycin.

**D**: WRKY29p::GUS activity is inhibited by all nucleosides.

Active, phosphorylated form of three MAPKs (MPK3, MPK4 and MPK6) was detected via a commercial antibody (Fig. 3.3). Sangivamycin and 5-iodotubercidin prevented the activation of MPK3 and MPK4 almost completely and prevented the activation of MPK6 slightly at 10 µM. The general kinase inhibitor staurosporine inhibited the activation of all three MAP kinases. Even at 1 µM sangivamycin and 5-iodotubercidin prevented the phosphorylation (data not shown), whereas toyocamycin and tubercidin did not inhibit at even 10 µM (Fig. 3.3). Since the MPK6 is also activated by JA (Takahashi et al., 2007), it would be interesting to see, whether the same effect of the kinase inhibitors could be observed. Unfortunately, the activation could not be detected by the commercial antibody (data not shown).

### Table 3.1: IC<sub>50</sub> values of nucleoside analogs on various *Arabidopsis* GUS reporter.

The table shows IC<sub>50</sub> values of the nucleoside analogs on the activity of the depicted GUS reporter. IC<sub>50</sub> values have been estimated from the GUS activities shown in Fig. 3.2.

<table>
<thead>
<tr>
<th>5-Iodotubercidin (µM)</th>
<th>Tubercidin (µM)</th>
<th>Toyocamycin (µM)</th>
<th>Sangivamycin (µM)</th>
</tr>
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<tr>
<td>3</td>
<td>3</td>
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<td>0.3 - 1</td>
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<td>30</td>
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<thead>
<tr>
<th>IC&lt;sub&gt;50&lt;/sub&gt; on reporter activity of:</th>
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<tbody>
<tr>
<td>LOX2p::GUS</td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>3 - 10</td>
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<tr>
<td>10 - 30</td>
</tr>
</tbody>
</table>
Due to the described effect on mechanistically related SCF$^{TIR1}$ mediated auxin signaling (Hayashi et al., 2009) and the less general effect of toyocamycin (no inhibition of MAPKs, less inhibition of OPCL1p::GUS), I focused on toyocamycin for the remainder of this study.

### 3.1.2 Toyocamycin inhibits root growth in a JAR1 and AOS dependent manner

To investigate if application of toyocamycin in addition to impairing LOX2 reporter expression also caused other phenotype in Arabidopsis, I grew plants in the presence of this compound. Arabidopsis plants grown on solid MS medium supplemented with different concentrations of toyocamycin showed severe inhibition of primary root elongation (Fig. 3.4 A). To see whether JA signaling is involved in this root phenotype, I tested Arabidopsis mutants impaired in JA biosynthesis or signaling for their response to toyocamycin. Since root growth was efficiently inhibited at 0.3 μM, I chose this concentration for further analysis. Root growth elongation in coi1-16 and jin1-1 was as efficiently inhibited as in wild type (Fig. 3.4 B), indicating a COI1 independent regulation of root growth inhibition. However the JA-biosynthesis mutant aos and the JA-Ile-synthesis mutant jar1-1 were less susceptible to root growth inhibition by toyocamycin (Fig. 3.4 B). It has been shown that kinases are involved in root growth elongation, since several kinase inhibitors can prevent root growth elongation in Arabidopsis seedlings (Baskin and Wilson, 1997). To see if the effect of toyocamycin on root growth elongation and the resistant phenotype of jar1-1 is based on inhibition of kinase activity, I tested the response of Arabidopsis wild type and jar1-1 to the general kinase inhibitor staurosporine. This compound also inhibits root growth and jar1-1 is fully responsive as the wild type (Fig. 3.4 C).
Figure 3.4: Effect of Toyocamycin on Arabidopsis root growth and gene expression in Arabidopsis mutants. Arabidopsis seeds were germinated on solid medium containing chemicals as indicated and grown vertically under day/night conditions. After seven to nine days pictures were taken and root length was measured using ImageJ software. Root length is shown as average of at least 15 plants ± standard deviation and normalized to the untreated control if not stated otherwise. 

A: Arabidopsis wild type roots are strongly affected by 0.3 µM toyocamycin. Absolute root length is shown in cm. 
B: Root growth is less inhibited by toyocamycin in jar1-1 (blue bars) and aos (yellow bars). 
C: The general kinase inhibitor staurosporine inhibits root growth in jar1-1 as strong as in Arabidopsis wild type. 
D: MeJA has an additive effect to root growth inhibition by toyocamycin (0.3 µM), since root growth is even stronger impaired by the double treatment. jar1-1 roots are not inhibited by MeJA. 
E: COR has an additive effect to root growth inhibition by toyocamycin (0.3 µM), since root growth is even stronger impaired by the double treatment. aos and jar1-1 root growth is as strong inhibited by COR as in Arabidopsis wild type. 
F: 14-day-old Arabidopsis seedlings were pretreated with DMSO or toyocamycin (10 µM) and LOX2 expression was induced by application of MeJA (100 µM). Plants were harvested at the indicated time points and LOX2 expression was monitored by semiquantitative RT-PCR. Actin served as control for equal amounts of RNA. Experiments with comparable results were repeated at least two times.
To verify that this phenotype is independent of the root phenotype induced by JA, solid MS medium was supplemented with MeJA or the JA-Ile analog COR together with toyocamycin. Root growth of wild type and aos was inhibited by MeJA, whereas roots of jar1-1 were resistant (Fig. 3.4 D), since jar1-1 cannot convert JA to bioactive JA-Ile. The effects of MeJA and toyocamycin together were additive, since roots were shorter after double treatment compared to treatment with the single compounds. After application of COR, wild type, aos and jar1-1 are inhibited in their root growth (Fig. 3.4 E), as they are all able to perceive COR. The effect caused by COR was slightly stronger than the one caused by MeJA at the respective concentrations, but root growth was still stronger impaired, if toyocamycin and COR were applied together. Roots of aos and jar1-1 are still slightly longer than in wild type after COR and toyocamycin treatment. Together these data show that toyocamycin induces a root phenotype different from JA, which may not be mediated via COI1 and MYC2.

Whereas the root phenotype caused by toyocamycin depends on JAR1, LOX2 gene expression surprisingly does not. Expression of endogenous LOX2 was inducible by COR in wild type and jar1-1 (Fig. 3.4 F). Simultaneous application of toyocamycin prevents this induction in wild type. This inhibitory effect could also be observed in jar1-1, suggesting that two independent effects of toyocamycin on root growth and gene expression exist.

3.1.3 Toyocamycin does not inhibit SCF-complex mediated processes

Toyocamycin was described to prevent auxin-dependent degradation of Aux/IAA repressors without impairing proteasome activity, suggesting a general role in the SCF-ubiquitination pathway (Hayashi et al., 2009). To confirm a potential role in SCF-mediated processes I conducted similar experiments by monitoring JA dependent degradation of JAZ1-GUS fusion protein in Arabidopsis seedlings expressing JAZ1-GUS. MeJA initiates the degradation of the JAZ1-GUS fusion protein via the SCF COI1 proteasome pathway. The presence of the fusion protein can be detected via histochemical GUS staining. After 30 minutes incubation with MeJA degradation of JAZ1-GUS could be observed in comparison to mock treated plants (Fig. 3.5 A). This degradation was prevented in the presence of the potent proteasome inhibitor MG132. Even at high concentrations of 50 µM, toyocamycin was not able to prevent the degradation of the fusion protein and toyocamycin treatment itself did not induce degradation of the protein. This indicates that toyocamycin does not have any effect on the SCF COI1 mediated degradation of JAZ repressors.

Another SCF-mediated process in Arabidopsis is early petal development, which depends on UNUSUAL FLORAL ORGANS (UFO), which encodes a F-box protein and was shown to be a transcriptional co-factor to regulate flower development (Durfee et al., 2003; Chae et al., 2008). Arabidopsis plants impaired in different components of the SCF UFO complex exhibit severe floral morphological defects (Ni et al., 2004). If toyocamycin generally would interfere with SCF-mediated processes, application of the compound during flower development could probably reduce fertility of such plants. Thus, I sprayed Arabidopsis flowers on five consecutive days with toyocamycin and
Figure 3.5: MeJA-dependent degradation of the JAZ protein and flower development after toyocamycin treatment. A: Seven-day-old *Arabidopsis* CaMV35Sp::JAZ1-GUS seedlings were incubated for one hour with the indicated chemical. The seedlings were then treated with or without MeJA (10 µM) to induce degradation of the fusion protein. The presence of JAZ1-GUS was visualized by histochemical GUS staining. Toyocamycin does not prevent MeJA-induced degradation of the fusion protein. The proteasome inhibitor MG132 was included as control for prevention of JAZ1-GUS degradation. Three independent experiments showed comparable results. B: Shoots of flowering *Arabidopsis* wild type plants were sprayed with DMSO (0.05%) or Toyocamycin (50 µM) in Silwet L-77 (0.05%) on five consecutive days. Toyocamycin does not have any effect on flower development, since siliques developed normally. Pictures were taken after flowers developed siliques. Three independent experiments showed comparable results.

Toyocamycin inhibits jasmonate downstream markers in a JAR1 independent manner while inhibition of the elongation of the primary root is dependent on functional JAR1 and AOS. Although JA signaling seems to be modulated by toyocamycin the SCF-complex mediated degradation of JAZ proteins, one central part in the JA signaling pathway, is unaffected.
Figure 3.6: Expression of \textit{LOX2} after MeJA and toyocamycin stimulus. 14-day-old \textit{Arabidopsis} seedlings (Col-0) were pretreated with DMSO or toyocamycin (10 \textmu M) and \textit{LOX2} expression was induced by application of MeJA (100 \textmu M). Plants were harvested at the indicated time points and expression was monitored by qRT-PCR. Toyocamycin reduced \textit{LOX2} expression after MeJA treatment. B: 14-day-old \textit{Arabidopsis} seedlings (Col-0) were treated as in A and harvested after 8 hours. Toyocamycin causes reduced \textit{LOX2} expression after MeJA treatment, but did not affect the untreated control. Expression was normalized to the untreated control and is shown as average of 3 biological replicates ± standard error. Asterisks indicate significant differences compared to the control (Student’s t-test **p<0.02, *p<0.05).

3.1.4 Analysis of global gene expression

To gain a better understanding of the particular phenotype caused by toyocamycin, I decided to analyze the impact on global gene expression after incubation with the compound. As first step, I tested how expression is changed over time after application of toyocamycin and MeJA. Already one hour after MeJA stimulus on \textit{Arabidopsis} seedlings, an increase of \textit{LOX2} transcript could be observed (Fig. 3.6 A). Over time \textit{LOX2} expression increased further and reached its maximum between eight and twelve hours. If seedlings were additionally treated with toyocamycin this increase was strongly inhibited (Fig. 3.6 A). The biggest differences between control and toyocamycin treatment were observed eight and twelve hours after the MeJA stimulus.

Based on these observed differences, an eight hour treatment was chosen for the microarray setup. \textit{Arabidopsis} seedlings were incubated with toyocamycin one hour before the MeJA stimulus was given. After eight hours samples were harvested and \textit{LOX2} expression was verified via quantitative real-time PCR. \textit{LOX2} transcript is highly induced by MeJA (40-fold compared to untreated control), whereas toyocamycin and MeJA together cause only a 12-fold induction (Fig. 3.6 B). The single treatment of toyocamycin did not cause any effect at this time point. This indicated a successful treatment and therefore the samples were hybridized with the AGRONOMICS1 microarray (Rehrauer et al., 2010).

Comparing the microarray expressions after toyocamycin treatment with the respective DMSO control showed that toyocamycin alone induced the expression of 1,441 genes (Fig. 3.7 A). In combination with MeJA 1,040 genes were upregulated in comparison with MeJA alone. 701 genes were coregulated in both comparisons.

In comparison to the DMSO control, toyocamycin inhibited the expression of 1,373 genes (Fig. 3.7 A). By the double treatment of toyocamycin/MeJA a total of 1,057
Figure 3.7: Misregulated gene expression and Gene Ontology (GO) of genes affected by toyocamycin. 14-day-old Arabidopsis plants were pretreated with DMSO or toyocamycin (10 µM) and then incubated for eight hours with DMSO or MeJA (100 µM). A: Microarray expression values were compared to the respective control (DMSO or MeJA) and two-fold misexpression was plotted into a venn diagram. 1,441 (740 + 701) genes were upregulated by toyocamycin and 1,040 (339 + 701) genes were upregulated by toyocamycin and MeJA. 1,373 (653 + 720) genes were downregulated by toyocamycin and 1,057 (337 + 720) genes were downregulated by toyocamycin and MeJA. B: Genes that were misregulated by toyocamycin alone were subject ed to GO analysis. Enrichment of GO terms for cellular compartment is shown as percentage of total misregulated genes.

was inhibited compared to MeJA alone. 720 genes were coregulated with the single treatment. Thus, there are more genes misregulated by toyocamycin alone than by the combination of toyocamycin with MeJA with an even smaller number being misregulated specifically only together with MeJA.

To get an impression about which processes are affected by toyocamycin, I performed an analysis of Gene Ontology (GO) terms (TAIR, Berardini et al. (2004)) that are affected by toyocamycin. Interestingly, the GO term for cellular compartment was enriched in genes, misregulated by toyocamycin, whose gene products are localized to the chloroplast (Fig. 3.7 B). Genes misregulated by toyocamycin in combination with MeJA showed the same enrichment and analysis via a different tool (AmiGO, Ashburner et al. (2000)) confirmed this enrichment (data not shown).

To verify that JA downstream signaling is inhibited by toyocamycin, I compared the expression of previously tested LOX2 and VSP1/2 (Fig. 3.8). Both marker genes were strongly induced by MeJA, and this was prevented by the pretreatment with toyocamycin. Jasmonate biosynthesis occurs in chloroplast and peroxisome (see introduction Fig. 1.1). Based on the fact that the GO term chloroplastic localization was enriched in genes misregulated by toyocamycin, I analyzed microarray expressions of several

Figure 3.8 (following page): Microarray expression levels of selected genes. 14-day-old Arabidopsis seedlings (Col-0) were pretreated with DMSO or toyocamycin (10 µM) and JA-specific expression was induced by application of MeJA (100 µM). Plants were harvested eight hours after MeJA stimulus. Expression of three independent replicates was analyzed on an AGRONOMICS1 microarray (Rehrauer et al. 2010). Absolute expression values are shown as average ± standard deviation. LOX2, the main lipoxygenase of JA biosynthesis, AOS and AOC2, the major allene oxide cyclase, are localized to the chloroplast as well as LOX3, LOX4, LOX6, AOC1, AOC3 and AOC4. The committed step of JA synthesis in the peroxisome is catalyzed by OPR3. OPCL1, which activates OPC-8 for β-oxidation is also localized to the peroxisome. VSP1 and VSP2 are common downstream markers for JA signaling.
Figure 3.9: Microarray expression levels of JAZ, Aux/IAA and DELLA. 14-day-old Arabidopsis seedlings (Col-0) were pretreated with DMSO or toyocamycin (10 µM) and JA-specific expression was induced by application of MeJA (100 µM). Plants were harvested eight hours after MeJA stimulus. Expression of three independent replicates was analyzed on an AGRONOMICS1 microarray [Rehrauer et al., 2010]. Expression was normalized to the respective control and is shown as average ± standard deviation. A: Mainly JAZ are upregulated by toyocamycin compared to the DMSO control. B: JAZ expression was analyzed after treatment with toyocamycin in combination with MeJA and normalized to expression after MeJA single treatment. Expression of JAZ5, 7 and 8 is strongly induced, while expression of other JAZ is rather unchanged.

selected genes of JA biosynthesis. Beside LOX2, the major lipoxygenase participating in JA biosynthesis, the gene family comprises five additional members, of which only LOX3, 4 and 6 have the chloroplast target sequence [Stenzel et al., 2003]. All of them exhibit lower expression levels and do not behave as LOX2. The other two chloroplastic enzymes participating in JA biosynthesis, AOS and AOC2, behave similar to LOX2. Whereas AOC1 and AOC4 show just a similar trend, expression of AOC3 behaves different and is upregulated after toyocamycin treatment.

Expression of the committing single-copy gene OPR3 and of the OPC-8 activating acyl-coenzyme A synthethase OPCL1 in the peroxisome shows a complete different pattern. It is not inhibited by toyocamycin but rather induced or unchanged. These observations coincide with the results of GO term analysis, which was enriched in the GO term for chloroplastic localization.

Because of the described effect of toyocamycin on auxin signaling, I wanted to see, which effect it has on the transcriptional regulated repressors of auxin signaling. I compared the microarray expression of all repressors of the mechanistically related signaling pathways auxin, gibberellin and jasmonic acid. Interestingly, predominantly JAZ expression was induced eight hours after toyocamycin stimulus (Fig. 3.9 A), whereas expression of Aux/IAA and DELLA was not significantly changed. Briefly, expression of JAZ5 was affected strongest with almost eight-fold higher expression than in the DMSO control. JAZ1, JAZ6, JAZ9 and JAZ10 transcripts were less affected by toyocamycin but exhibited still a more than two-fold upregulation. This means that toyocamycin mostly affects JA signaling concerning the transcriptional regulation of the repressors. Because JAZ expression is known to be induced by MeJA alone, I wanted to see, if after application of MeJA and toyocamycin any additional or reducing effects on JAZ expression exist. Indeed, most JAZ were upregulated by MeJA with the exception of JAZ4, JAZ11 and JAZ12 (See supplemental Fig. S.6).
None of the Aux/IAA or DELLA was modulated by MeJA. Intriguingly, JAZ5, JAZ7 and JAZ8 exhibited even higher expression after application of toyocamycin together with MeJA (Fig. 3.9 B). Since JAZ5 expression is already more than seven-fold higher with toyocamycin alone, this may explain the higher expression in combination with MeJA.

3.1.5 Structure-activity relationship of toyocamycin

Toyocamycin is a structural analog of adenosine. Adenosine is phosphorylated into its nucleotides (mono-, di- or triphosphate), as such providing energy for numerous biological processes. To rule out the possibility that toyocamycin acts as adenosine analog, I tested the activity of adenosine and its corresponding nucleotides on the LOX2p::LUC reporter. None of the derivatives was able to induce reporter expression itself (data not shown) and all derivatives did not have any effect on MeJA-induced expression (Fig. 3.10 A, blue bars). If toyocamycin would compete with adenosine or its nucleotides for a protein target, the inhibitory effect would vanish upon excess application of the adenosine derivatives. However, the effect of toyocamycin on the expression of LOX2p::LUC was not significantly affected, neither at equimolar concentrations nor at concentrations ten-fold higher than toyocamycin (Fig. 3.10 A, rose bars). This means that the effect on this reporter is not based on replacement of adenosine by toyocamycin.

Adenosine can be phosphorylated to AMP by the adenosine kinase. Toyocamycin was previously shown to inhibit recombinant human adenosine kinase, but with only moderate activity (Ugarkar et al., 2000). Thus, binding to Arabidopsis adenosine...
kinase and phosphorylation of toyocamycin in planta could be possible. To see if a phosphorylated form is the active form, I tested two different derivatives for their effect on LOX2p::LUC. Toyocamycin-BOC cannot be phosphorylated, whereas toyocamycin-sulfamate (toyocamycin-H$_3$NSO$_3$) mimics the 5'-phosphate and cannot be phosphorylated either (Fig. 3.11). Even at higher concentrations of 30 µM, toyocamycin-BOC was not able to inhibit the reporter expression (Fig. 3.10 B). Toyocamycin-sulfamate caused even strong inhibition at low concentrations. This means that apparently the phosphorylated form of toyocamycin is the active form. For identification of a direct protein target, we obtained toyocamycin-sulfamate containing an alkyne as linker for click chemistry. This derivative showed the same activity as toyocamycin-sulfamate (data not shown) and may be used in affinity-based target identification experiments, which have been initiated. Unfortunately, further experiments have not been performed due to time restrictions for this PhD thesis.

Figure 3.11: Structure of toyocamycin and modified derivatives. The structures of toyocamycin and modified derivatives is depicted. Targeted modification of toyocamycin yielded the inactive toyocamycin-BOC and the highly bioactive toyocamycin-sulfamate (H$_3$SNO$_3$). Toyocamycin-click was as active as toyocamycin-sulfamate, contains an alkyne and may be used in target identification experiments using affinity-based labeling methods in combination with click chemistry.
4 Discussion

Many components of jasmonate biosynthesis or signaling have been unraveled, such as the perception of the most bioactive jasmonate (+)-7-iso-JA-L-Ile by the COI1–JAZ co-receptor module. However, some components remain unknown, such as mode of action and perception of other bioactive jasmonates or the molecular mechanism of cross-talk between different hormonal signaling pathways. Genetic approaches in jasmonate research have reached their limits and the usage of chemical genetics in plant sciences is rapidly expanding. Despite the successful achievements by chemical genetic approaches in plant biology, such as in auxin, ABA or ethylene signaling (Zhao et al., 2003; Armstrong et al., 2004; Park et al., 2009), this methodology remains unexploited in jasmonate research.

This was the reason to design a chemical screen in Arabidopsis, which should be the basis for uncovering chemical compounds, that selectively modulate JA signaling. Unveiling the cellular target of such compounds may answer open questions in JA research, which may not have been answered due to redundancy of gene function or lethality of mutants. The here described chemical screening procedure was developed to enable simultaneous handling of high numbers of chemicals to allow screening of bigger chemical libraries comprising molecules of high chemical diversity. This requirement was fulfilled by using Arabidopsis seedlings, which could be grown directly in 96-well microplates. As readout, the MeJA inducible firefly luciferase (LUC) reporter LOX2p::LUC was chosen. The advantage of such reporter-based screen is that it affords semi-quantitative data that allow discrimination between compounds with high and low bioactivity. Additionally, it allows bidirectional screening for either activators of reporter expression or for compounds that prevent reporter activation after MeJA stimulus. The LUC activities were based on the average of the first four hours after initiation of LUC measurement, which approximates the integrated activity over time and yielded most stable and reliable results. Unfortunately the LUC activities showed considerable variations between different samples, which can be attributed to size (biomass) and orientation of seedlings in the microplates. Because of this finding, deep analysis of the data and stringent threshold values, which dictate the selection of candidate compounds from the analyzed population, were necessary.

The small kinase inhibitor library (chapter 3), containing only 80 compounds, was screened in one day. Due to the small number of chemicals, controls were included on the same plate and the whole screen was repeated once completely. This assured a stringent selection of candidates. Conversely, large scale screening is time consuming and cannot be repeated as a whole. The natural compound library (chapter 2) comprised over 1,700 different compounds, which were supplied in a 96-well microplate format. The screening procedure was performed in small steps, each including a maxi-
maximum of three different chemical plates at the same time. Instead of including controls on each of the screening plates, a separate control plate was included. This was considered for practical reasons, because in this case, the 96-well format of the chemical library could be directly transferred to the 96-well formatted screening plates. Candidate compounds were selected by comparison of LUC activities to the average LUC activity of the control plate.

It could be observed that the average activities during the different steps of the screening procedure differed considerably (compare supplemental Fig. S.5). Averages of whole plates sometimes differed up to 50% compared to the average of the control plate. This plate-to-plate variation indicates that minor changes in growth conditions may have major effects on LUC activity and that it may not be sufficient to rely on controls on a separate microplate. Such datasets need critical statistic evaluation to gain confident selection of candidates. The evaluation using comparison to a control plate generated a set of candidates, of which one was selected for further characterization (compound 12). The selectivity of this compound proves the principle of this selection method but maybe different rather statistic based approaches could be useful for future screenings.

One confident selection method of candidates in high-throughput-screening (HTS) is based on using the so called Z-score (Malo et al., 2006). The Z-score is a simple normalizing method, which considers the variation of the readout, thus adding a higher degree of confidence to candidate generation. It includes the average of the whole plate as control, since most compounds are expected to have no effect, thus rendering the effect of single compounds negligible (\( Z = \frac{x_i - \bar{x}}{s_x} \) where \( x_i \) is the raw measurement on the \( i^{th} \) compound, \( \bar{x} \) and \( s_x \) are the mean and the standard deviation, respectively, of all measurements within the plate) (Malo et al., 2006). Although candidates of the natural compound library have been selected already, I applied the Z-score to the dataset of this library. This analysis revealed that all of the confirmed candidates would have met the selection criteria, while some of the not confirmed candidates would have been discarded in the first place. Intriguingly, this method would have led to some other candidate compounds, but their relevance remains to be shown.

Based on these findings, I performed the same screen on another chemical library (Prestwick Chemical Library\textsuperscript{®}, 1,200 compounds). For first candidate selection a more relaxed threshold was used (data not shown) and final candidate generation may be done including the Z-score. The true value of such analysis on this screen remains to be shown by subsequent analysis of the selectivity of such candidates but in other screens which used the Z-score it proved to be applicable (Lee et al., 2010; Thorne et al., 2011; Saydmohammed et al., 2011).

However, the fact that the screen on the natural compound library uncovered one apparent activator and several inhibitors, including cycloheximide (8) and the two trichothecone mycotoxins diacetoxyscirpenol (15) and neosolaniol (16), proves the concept of the screening procedure. Since 8, 15 and 16 have in common that they inhibit protein synthesis in general (Serrano et al., 2010), any of these compounds may serve as suitable positive control in reporter-based screens for inhibitors.
4.1 766 can be lead structure for designing novel luciferase ligands

The screen for activators of LOX2p::LUC uncovered compound 766, as apparent activator. 766 is a derivative of 9,10-anthracenedione (anthraquinone) (see Table 2.1). It was first isolated from Streptomyces griseorubiginosus and showed inhibitory activity on the binding of the transcription factor AP-1 to its recognition site in rat cell cultures (Naruse et al., 1998; Goto et al., 1998). The same compound and related derivatives were shown to have antifungal and cytotoxic activity (Tang et al., 2004; Poumale et al., 2006; Lee et al., 2007). Other anthraquinones like physcion, rhein and emodin are described to have antifungal activity. They can protect plants against phytopathogens such as Botrytis cineria, Erysiphe graminis, Plasmopara viticola or induce expression of defense genes (Kim et al., 2004; Godard et al., 2009; Vinale et al., 2008). Additionally, higher anthraquinone levels were observed in damaged buckthorn (Rhamnus alpinus) leaves (Bañuelos et al., 2004). This findings initially emphasized the possibility of 766 to be able to modulate defense response pathways.

SAR studies indicated the importance of the functional groups at carbon atoms 1 and 2 to activate the LUC reporter. Of all anthraquinones tested, the compounds 44, 47 and 48 are the only compounds, which have carbon chains of the same or longer length at carbon atom one compared to 766. All of these compounds also have a carboxylic acid group at the second carbon atom (see structures in Fig. 2.5). The chains of 47 and 48 are longer and both compounds cause higher reporter expression than the negative control. Note in this experiment the low activity of 766, which is based on general problems with expression of the reporter. This is potentially caused by successive silencing during propagation of the plants (Nocarova et al., 2010). The activity of 47 and 48 indicates that a carbon chain at the first carbon atom may be of importance for effective binding to a protein target. Unfortunately, compound 44, which also contains a carbon chain at C-1, did not modulate the reporter response. Based on the missing activity, no clear conclusion about SAR can be drawn. By contrast, compound 18 and 39 caused inhibition of the reporter (see structures in Fig. 2.5). They contain also a carboxylic acid group at C-2. Since both compounds also modulated the response in the same reporter line, this indicated, that the group at C-2 may be involved in binding to a protein target but that the carbon chain at C-1 may be important for activating LOX2 reporter expression.

In secondary assays, 766 did not induce endogenous LOX2 expression or expression of the LOX2p::GUS reporter. This were first indications that this compound does not modulate JA signaling. In a LUC overexpression line I observed higher LUC activities caused by 766. Intriguingly, the compound inhibited LUC activity in vitro. The apparent in vivo activation may in fact reflect increased enzyme stability. LUC is inhibited by its product oxyluciferin and it has been reported that the LUC protein is sensitive to ligand-based stabilization, leading to accumulation of the enzyme (Thompson et al., 1991; Chiou and Ueda, 1994; Leitão and Esteves da Silva, 2010). In luminescence based detection of LUC, an increased enzyme level will be reflected in
higher luminescence emission. Thus ligand-based stabilization of LUC in vivo may appear as reporter activation, if the ligand binds competitively to LUC, still allowing its reaction with D-luciferin. Such binding could decrease LUC activity in vitro, because less enzyme would react with D-luciferin, while some of it is occupied by the other ligand (Thorne et al., 2010b,a).

By showing the inhibitory activity of 766 in vitro on recombinant LUC, I provide strong evidence that this compound acts on LUC. Adding an excess of luciferase to the in vitro assay after LUC inhibition could strengthen the hypothesis that 766 inhibits the enzyme. This experiment could verify that ATP is still available in the system and that in fact LUC is inhibited. If the compound binds competitively could be resolved by enzyme kinetics.

If 766 binds LUC, thus increasing enzyme levels in planta, how does 18 cause inhibition of the reporter? If this compound would be a non-competitive ligand of LUC, it would prevent the LUC reaction in planta as well as in vitro. However, 18 did not affect in vitro LUC activity, although activity seems to be slightly reduced and more variable. This may be ascribed to just weak binding to LUC. Thus, 18 is not a true ligand of LUC and the inhibitory effect in planta could be attributed to other effects, such as toxicity of the compound. If the functional group at carbon two may be involved in LUC binding remains to be shown. Stronger evidences for SAR need a more targeted approach by modifying the molecule at single carbon atoms and testing their effectiveness on LUC inhibition.

Finally, docking simulations suggest as well that 766 binds to the luciferase. The crystal structure of firefly luciferase (Photinus pyralis) has been resolved (Conti et al., 1996). I used this crystal structure as basis for docking simulations of the binding of D-luciferin and 766 to the enzyme using an online tool (DockingServer, Bikadi and Hazai, 2009). Both molecules were calculated to bind at the same site of the enzyme although in different orientations. The estimated binding energy of luciferin binding was -6.31 kcal/mol, whereas 766 released -4.42 kcal/mol upon binding. Both molecules established π-π-bonds with His245 but 766 additionally established π-π-bonds with Phe247. Since the original crystal structure established for LUC does not include the substrate bound to the enzyme, I compared the results with the crystal structure of luciferase from the japanese firefly (Luciola cruciata), which is very similar to Photinus pyralis luciferase and includes oxyluciferin and AMP into the structure (Kajiyama et al., 1992; Nakatsu et al., 2006). Indeed the respective His is bound to oxyluciferin via π-π-bonds, whereas AMP interacts with the respective Phe, supporting the hypothesis of competitive inhibition of LUC by 766. An example of the binding pocket with D-luciferin and 766 is shown in Figure 4.1.

Since 766 appears to bind LUC, this raised the question if there are any possible applications of this ligand. Increased LUC stability via the addition of competitive inhibitors could improve the sensitivity of weak promoters as already proposed by Thompson et al. (1991). Although detection limits for LUC are very low (Stanley, 2000) uninduced or weak promoters may not yield enough enzyme for detection. This problem can be approached by application of competitive LUC inhibitors, which improve protein stability and lead to detectable enzyme concentrations. Thus, 766 could be a lead structure for such a ligand and extensive SAR studies and more detailed
Docking simulation was achieved using the free version of DockingServer (Bikadi and Hazai, 2009) based on the crystal structure of firefly luciferase (Conti et al., 1996) and the structures of D-luciferin and 766. The model shows one of several possibilities, how the molecules hypothetically both bind to the same part of the enzyme. Each possible conformation potentially released energy.

**A:** D-luciferin bound to luciferase, **B:** 766 bound to luciferase.

*in silico* modeling could improve the effectiveness of a ligand. I did not perform any additional experiments, since proving the mechanism of 766–LUC interaction was not the main interest of this project.

However, the discovery of 766 as putative ligand of the reporter enzyme demonstrates the importance of secondary and counter screens for verification of a candidate compound. This is a general problem of using reporters in chemical screens, which highlights advantages of reporter independent readouts.

### 4.2 Uncovering selective inhibitors

An alternative way of screening is to look for inhibitors of reporter expression. To approach this, *Arabidopsis* seedlings were preincubated for one hour with different chemicals before LOX2p::LUC expression was induced by MeJA. The corresponding LUC activities were detected 24 hours after induction.

After uncovering the compounds 4, 5, 6, 7, 8, 10, 12, 15, 16, 17 (see structures in supplemental Fig. S.1) as inhibitors of MeJA-induced LOX2p::LUC reporter expression in *Arabidopsis*, I verified the specificity for JA signaling only for 12. The translational inhibitors 8, 15 and 16 were excluded from this study. Candidate 17, piericidin inhibits the mitochondrial type I NADH-ubiquinone oxidoreductase in the respiratory chain (Darrouzet et al., 1998). Because it may generally interfere with energy dependent pathways it was as well excluded. It is worth noting, that piericidine was also identified in other chemical screens performed in the lab (flg22-repressed anthocyanin accumulation, Serrano et al., (2012); flg22-induced Ca$^{2+}$ spike, Jens Maintz, unpublished results). The compounds 4, 5, 6 and 7 inhibited the JA independent induction of the reporter WRKY29p::GUS and interfered with flg22 induced ROS production, whereas 10 and 12 did not. This only left 10 and 12 as JA selective compounds, of which 12 inhibited all MeJA-induced readouts, whereas 10 did not interfere with the
GUS reporters and exhibited a less pronounced effect on the VSP1p::LUC reporter.

In parallel, the screen on the kinase inhibitor library yielded three different inhibitors of MeJA-induced LOX2p::LUC expression. Two are non-specific kinase inhibitors (staurosporine and 5-iodotubercidin) and one is an inhibitor of protein tyrosine kinases and mitogen-activated protein kinases (MAPK), tyrphostin AG-879. Thus, tyrphostin AG-879 has the potential to cause more specific effects than the other two inhibitors. Nevertheless, I focused on the analysis of 5-iodotubercidin and three of its derivatives (tubercidin, sangivamycin and toyocamycin), based on the published effect on SCF\(^{TIR1}\) mediated auxin signaling (Hayashi et al., 2009), which is mechanistically similar to JA signaling. Tyrphostin AG-879 still will be included in secondary and counter screens, which aim at confirming candidates from the Prestwick Chemical Library\(^{R}\), which has not been included in this study. Likewise, staurosporine will be included into these verifications, although its effects on numerous human kinases (Karaman et al., 2008) may indicate that it is less specific. This suggests that its inhibition of JA signaling requires critical evaluation.

Testing the four different nucleoside analogs (5-iodotubercidin, tubercidin, toyocamycin and sangivamycin) for their ability to interfere with the expression of different reporters revealed, that all inhibited JA independent expression of \(WRKY29\) to the same extent, whereas ten-fold differences in their IC\(_{50}\) for the JA dependent reporters could be observed. This indicates that all four compounds may have one or more common targets, that are involved in the flg22 dependent activation of \(WRKY29\), but that they have a different specificity for JA dependent expressions. Of all nucleosides tested, sangivamycin exhibited the strongest effect on each reporter and also prevented the flg22 dependent activation of MAPKs. This indicates that this compound is less specific than the others, because it inhibited all responses tested. 5-Iodotubercidin was less effective on the expression of the \(LOX2\) or \(VSP1\) reporters, but also prevented the activation of MAPKs. This only left tubercidin and toyocamycin, of which toyocamycin caused stronger effects on the expression of the \(LOX2\) and \(VSP1\) reporter, whereas it was less effective on the expression of the \(OPCL1\) reporter. Both compounds did not interfere with flg22-dependent activation of MAPKs.

In conclusion, based on critical evaluation of all biological reporters tested, I decided to focus on compound \(12\) and toyocamycin as selective inhibitors of JA signaling. The main question emerging was, where to put the effect of the compounds into the \(Arabidopsis\) hormonal signaling network, which I tried to answer for each compound by different experimental strategies.

4.3 Analysis of compound 12 and its effect on hormone signaling

To figure out the mode of action of compound \(12\) one important point was to determine how fast it affects (JA)-responses. The degradation of JAZ proteins is a rapid response and depends on a JA stimulus (Chini et al., 2007; Thines et al., 2007). The prevention of JAZ1-GUS degradation mediated by \(12\) indicates that this compound is taken up efficiently by the seedlings and that it is a potent inhibitor of specific processes already
one hour after application.

Compound 12 not only affects rapid responses in Arabidopsis, but it also affects long term responses. Root growth was inhibited by 12 and this effect appears to be independent of JA or JA-Ile synthesis since the biosynthesis mutants aos and jar1 both are as sensitive as the wild type. This is a strong indication that root growth inhibition is independent of JA signaling. Further proof of this hypothesis may be delivered after testing the JA-Ile receptor mutant coi1 and the MYC2 transcription factor deficient mutant jin1 for their responsiveness to 12. Additionally, testing mutants of auxin, ethylene or ABA signaling may shed light on the question whether 12 might inhibit root growth by a different mechanism, since some Arabidopsis mutants of these phytohormone signaling pathways are resistant to root growth inhibition by the respective hormone [Roman et al., 1995; Ghassemian et al., 2000; Lorenzo et al., 2004; Růžička et al., 2007].

One way to get more insight into the question what is affected by 12 would be application of the compound in a genome wide microarray expression analysis. This approach could show, which signaling pathways are involved in the Arabidopsis response to 12. I performed such a microarray experiment in parallel to the microarray with toyocamycin. Because of time and budget constraints, I decided to test only one time point and only Arabidopsis wild type plants for their response to 12 and toyocamycin. Eight hours treatment appeared to be a suitable time point to observe robust effects with both compounds. Selected genes may later be analyzed via quantitative real-time PCR (qRT-PCR) at different time points and in selected Arabidopsis mutants to see the impact on fast responses as well as on defined signaling pathways.

Due to the lower effect of 12 on the LOX2 inhibition (IC50 10 µM) compared to the more potent inhibitor toyocamycin (IC50 1 µM), I performed careful analysis of the samples concerning RNA integrity and significant LOX2 inhibition in qRT-PCR. Interestingly, the trend of gene expression of genes such as LOX2 or VSP1/VSP2 in the microarray dataset was as expected, but unfortunately the differences between control and application of 12 were not significant. Even with lowered significance levels (p ≤ 0.1) only a total of 129 genes was misregulated by 12 alone and 50 genes in combination with MeJA. The low differences in gene expression cannot be attributed to the new AGRONOMICS1 tilling array, since it was shown that AGRONOMICS1 and the previously commonly used ATH1 [Redman et al., 2004] yield nearly identical expression fold changes [Rehrauer et al., 2010]. The big differences between expression fold changes of qRT-PCR and the AGRONOMICS1 tiling array are a commonly observed phenomenon (Annegret Rok, Moritz Schön, MPI for Plant Breeding Research, personal communication). The resulting low hit rate for the array with 12 makes fast and easy data analysis practically impossible. However, those data may still be used to support upcoming experiments, since trends in gene expression are still valid. In retrospect, a higher concentration of 12 could have improved the microarray but the decision for the finally used, less effective concentration was made, to ensure that at least one treatment would yield usable data, in the case that the effect of toyocamycin would be too strong. Ironically, exactly the other way came true.
4.4 Target identification

Root growth elongation was inhibited by 12 but it was not possible to link this phenotype to JA signaling by testing different JA biosynthesis/signaling mutants. This missing link indicates that the compound may be either involved in an unknown JA signaling pathway or act on another hormone signaling pathway. Beside the approach to integrate it into the signaling network via analysis of known mutants or via microarray analysis, identification of the direct protein target would be an elegant way to arrange the compound’s effect into the network with the possibility to unravel its mode of action. In similar approaches chemical probes have been successfully developed to label e.g. members of a lectin protein family or to identify a target membrane protein of the jasmonate glycoside that controls leaf movement in *Albizia saman* (Ballell et al., 2005; Nakamura et al., 2008b).

Prerequisite for this approach is a specific compound, that is taken up efficiently, exhibits strong binding affinity to its target and is detectable. The first steps towards target identification via affinity based labeling usually comprise SAR studies. These aim at identification of indispensable parts of the molecule. Identification of sites that allow modifications without losing bioactivity enable linking of fluorophores or biotin. Such a modified compound can then be used as probe for purification or detection of bound proteins.

The established SAR for 12 allowed modification on the side of the propanoic acid-methyl ether. The weak effect of 12 (IC$_{50}$ 10 µM) compared to a stronger inhibitor like toyocamycin (IC$_{50}$ 1 µM) indicates the possibility of weak binding to the target. To approach this problem, the probe 65, which contains a photoreactive benzophenone group was synthesized. This probe was provided by Prof. Markus Kaiser (ZMB, Universität Duisburg Essen). The photoreactive group can be used to establish covalent cross-linking to the target protein. A benzophenone was chosen, since it can be worked with in ambient light, does hardly react with solvents and modifies C-H bonds within 3 Å of the carbonyl oxygen (Dormán and Prestwich, 1994, 2000). Benzophenone-containing molecules can be synthesized relative easily via peptide chemistry using p-benzoyl-L-phenylalanine (Kauer et al., 1986). In favor of a small alkylene group, the direct incorporation of a bulky fluorescent or biotin tag was abandoned, since the probe may be easier taken up by the cell and may be more likely to distribute within living cells in an unbiased manner. Using ‘click chemistry’, a copper-catalyzed 1,3-dipolar cycloaddition between azide and alkyn, a commercial fluorophore can be added after UV-cross-linking (Speers et al., 2003). In the literature several examples exist in which specific proteins or protein families have been characterized employing photoreactive and/or ‘click chemistry’ probes (for reviews see Puri and Bogyo (2009); Kalesh et al. (2010); Kolodziejek and van der Hoorn (2010); Heal et al. (2011)). Indeed, the probe was able to prevent activation of the LOX2p::LUC reporter, although the concentrations were higher than with compound 12. This lower efficiency may be attributed to insolubility (the solutions with 65 appeared more turbid than with 12) or less efficient uptake of the probe.

The fact that 65 was able to label proteins in a concentration dependent and by 12
competable manner suggests that the probe indeed targets proteins, but unfortunately not as specific as expected. The UV-cross-linking control did not label any proteins, which indicates that labeling by 65 is not based on unspecific UV-cross-linking. If among these labeled proteins a main target may be masked remains unclear. This question may be answered by increasing the spatial resolution via separation of proteins on two-dimensional gels. In the case of that no main protein target can be identified, MS-analysis of single bands will show, if the proteins belong to a family of proteins, which all may be labeled at a highly conserved domain. It has been shown that e.g. proteases or kinases are involved in plant defense and that single probes can label phosphatases of the same family [van der Hoorn and Jones 2004].

Alternative methods for target identification

If photoaffinity cross-linking and purification with modified versions of the ligand does not prove to be useful, other methods could be applied to identify a protein target. Such label-free methods can also be used to verify target binding after successful pulldown via an independent method.

Binding of ligands may stabilize proteins and make them less prone to degradation as hypothesized for the interaction of 766 and the luciferase. This fact can be exploited by applying a probe to protein extracts with subsequent digestion of proteins. Stabilized proteins will still be detectable in the digested extract. This so called drug affinity responsive target stability (DARTS) has been successfully applied [Lomenick et al. 2009; Aghajan et al. 2010]. DARTS does not require derivatized compounds for affinity binding, which has the advantage that the original bioactive compound can be used. The method is restricted to abundant proteins but due to missing washing steps, as in classical affinity purification, no signal is lost. This increases the chance to identify lower abundant proteins.

Another very sensitive technique for proving direct interaction is surface plasmon resonance (SPR). The SPR technology records changes in light refraction on sensor chip surfaces that occur upon interaction between two (or more) binding partners, one of which is covalently linked to the sensor chip surface. SPR is the current leading technology for label-free detection of protein interactions [Hall et al. 2007]. Together with modern imaging techniques surface plasmon resonance imaging (SPRi) forms a powerful tool for affinity-based biosensors in high throughput screens [Ray et al. 2010]. SPRi has been utilized as SPR-microscopy based protein arrays for analysis of affinity-tagged proteins during the expression and purification process or to improve drug research or food applications [Ro et al. 2005; Jung et al. 2005; Ro et al. 2006; Shankaran et al. 2007; Ray et al. 2010]. An Arabidopsis protein array using SPR has been described already but the study was interested in ligands of a single protein, thus screening 150,000 chemical compounds for interaction with one protein hybridized on the chip [Yoshitani et al. 2005]. Instead of linking only one protein to the sensor chip, proteins from cDNA libraries could be spotted onto the sensor chip and the ligand would be injected over the surface to detect the interaction partner. The recent development of nanohole arrays increases spatial resolution, facilitating the development of
protein arrays (De Leebeeck et al., 2007). To my knowledge such a study has not been performed in Arabidopsis and depends on creating own cDNA libraries or on complete cDNA collections as from the ORFeome project (Yamada et al., 2003; Gong et al., 2004). The advantages of SPRi protein arrays are that even natural low abundant proteins are detectable and that it enables kinetic characterization of the protein–ligand interaction (Rich et al., 2002).

Innovative methods such as DARTS and SPRi protein arrays are two applicable independent methods to identify the direct target and do not need derivatized compounds. Thus, both techniques could be used with either 12 or toyocamycin. However, other approaches for target identification exist. For a survey of such methods see Tóth and van der Hoorn (2010), Cottier et al. (2011) or Tashiro and Imoto (2012). The identification of a direct protein target will provide more insights into the mode of action of 12 or toyocamycin, which in turn may provide another point of view onto JA signaling or the Arabidopsis hormone signaling network.

4.5 Analysis of toyocamycin and its effect on Arabidopsis hormone signaling

Potential targets of toyocamycin

Toyocamycin, which was initially isolated as anti-Candida antibiotic from Streptomyces toyocaensis (Nishimura et al., 1956), modulates auxin signaling potentially via the SCF-ubiquitination pathway (Hayashi et al., 2009). It would be conceivable that it may have a general effect on SCF-ubiquitination regulated processes. The Arabidopsis genome contains more than 600 F-box genes, which suggests that plants make extensive use of SCF complexes to regulate multiple biological processes (Gagne et al., 2002; Risseeuw et al., 2003).

Auxin signaling is mediated via the SCF$^{TIR1}$ complex, that is necessary for ubiquitination of Aux/IAA repressors and their subsequent proteosomal degradation (Gray et al., 2001; Dharmasiri et al., 2005; Kepinski and Leyser, 2005; Tan et al., 2007). Toyocamycin was shown to effectively prevent degradation of these Aux/IAA repressors (Hayashi et al., 2009). This mechanism is similar to the JAZ-degradation in JA-Ile signaling, that is mediated by SCF$^{COI}$ (Chini et al., 2007; Thines et al., 2007; Yan et al., 2009; Fonseca et al., 2009b; Sheard et al., 2010). Intriguingly, toyocamycin did not prevent JAZ1 from degradation. Likewise, the floral development, which is dependent on SCF$^{UFO}$ (Durfee et al., 2003; Ni et al., 2004; Chae et al., 2008) was not disturbed after application of toyocamycin. This indicates that toyocamycin does not target SCF-ubiquitination pathways in general but rather acts specifically on auxin and JA signaling or hormonal crosstalk.

Based on the similarity to adenosine it would be conceivable that toyocamycin substitutes adenosine and adenosine nucleotides, thus disturbing energy driven processes in general. That such a replacement is the cause for toyocamycin’s effect on JA signaling is rather unlikely, since neither adenosine nor its corresponding nucleotides were able to outcompete the reporter inhibition. However, it may still act as adenosine analog.
on JA independent processes. The question, in which form toyocamycin is active was answered by application of a sulfonated form, which mimics a phosphate group, and a protected form that cannot be phosphorylated. The protected form was inactive, whereas the sulfonated form exhibited stronger bioactivity concerning LOX2p::LUC inhibition than toyocamycin. This suggests that phosphorylated toyocamycin is the bioactive form. In this scenario, toyocamycin first has to be phosphorylated, which may be the reason for the lower bioactivity of toyocamycin. It has been shown that 5'-deoxytoyocamycin retained activity on inhibition of the auxin responsive DR5p::GUS reporter (Hayashi et al., 2009). This indicates, that unphosphorylated toyocamycin inhibits auxin signaling, whereas it has to be phosphorylated for its effect on JA signaling. Phosphorylation of toyocamycin could be proven by conducting radiolabeling experiments in which radiolabeled ATP serves as donor for phosphorylation of toyocamycin in vivo with subsequent purification and detection of (phosphorylated) toyocamycin. Such phosphorylation could be catalyzed by a adenosine kinase in planta, which is bound to and maybe inhibited by toyocamycin, since toyocamycin was shown to inhibit human adenosine kinase [Bergstrom et al., 1984; Ugarkar et al., 2000; González and Moldes, 2004].

Toyocamycin not only inhibits human adenosine kinase, it also was shown to inhibit phosphatidylinositol-4-kinase (PI4K) of human epithelial cell cultures (Nishioka et al., 1990). PI4K and phosphatidylinositol-3-kinase (PI3K) both have been shown to be required for circadian rhythm or white light induced stomatal opening and ABA induced stomatal closing in Arabidopsis (Jung et al., 2002). Additionally, PI4K is activated upon SA stimulus in Arabidopsis suspension cells (Krinke et al., 2007). PI3K is required for auxin induced ROS generation in guard cells, for root gravitropism and for endosomal trafficking (Joo et al., 2005; Jaillais et al., 2006). Wortmannin, an inhibitor of PI3K, affects auxin distribution, because inhibition of PI3K impairs the polar distribution of PIN1, an auxin efflux carrier (Jaillais et al., 2006). If toyocamycin also affects the PI3K or PI4K activity, thus interfering with auxin transport remains to be shown. Indeed, an effect on auxin distribution potentially explains toyocamycin’s effect on the auxin signaling pathway. Whether toyocamycin impairs PI3K or PI4K activity can be tested by comparing its effect with the effect of the PI3K inhibitors wortmannin or LY294002 on stomatal movement, ROS generation in guard cells or endosomal trafficking. The latter can be tested by application of FM 4-64, which is a dye for following membrane internalization and transport (Vida, 1995).

Since several possible targets of toyocamycin exist, it would be interesting to identify its real target(s) in planta. Based on the fact that toyocamycin appears to be more active as monophosphate, I obtained a sulfonated probe, which mimics the 5’-phosphate and which contains an alkyne tag for ‘click chemistry’ (structure in supplemental Fig. S3). This probe retained its activity, but preliminary experiments failed to label any proteins. This may be the result of (1) not fully optimized conditions for performing such experiments, (2) weak binding to the protein or (3) a low abundant protein target. Optimal conditions for protein labeling will be established using the modified version of compound 12 (65), but those conditions may differ from optimal conditions for labeling with toyocamycin. Strong (covalent) binding of toyocamycin,
which is necessary for employing the ‘click-reaction’ without dissociation of protein and ligand, may occur at the CN-group of the molecule. Tubercidin, the derivative with no reactive group at C-5 causes similar effects on JA-signaling, as well as no inhibition of flagellin mediated MAPK activation. This fact makes it rather unlikely that toyocamycin binds covalently via the CN-group. However, toyocamycin and tubercidin may have different targets and a JA-specific target may not be shared between those compounds.

How does toyocamycin mediate root growth inhibition?
The effect of toyocamycin on root growth elongation was already described in the early 1970s. Root growth was inhibited in rice, barnyard grass, crab grass, lucerne, tomato and turnip at rather high concentrations (> 20 µM) (Yamada et al., 1972). This study aimed just at identification of compounds with growth regulating properties and did not continue with analyzing the effect of toyocamycin. I showed that the root phenotype in *Arabidopsis* was less pronounced in *aos* and *jar1-1*. Both mutants have in common that they contain no or only low levels of JA-Ile, because *aos* does not produce JA and *jar1-1* cannot conjugate JA to Ile. Thus, root growth inhibition by toyocamycin appears to depend on JA-Ile. Interestingly, the JA-Ile receptor mutant *coi1* did fully respond to treatment with toyocamycin. The mutant *jin1*, which lacks the transcription factor MYC2, did behave identical to the wild type. MYC2 is predominantly expressed in the root and contributes mainly to root growth inhibition by JA (Fernández-Calvo et al., 2011). Thus, functional JA-Ile signaling is not necessary for root growth inhibition by toyocamycin. The fact that toyocamycin and MeJA/COR contributed additively to root growth inhibition indicates that toyocamycin modulates root growth via a different mechanism than via COI1 and MYC2. That JA-Ile is necessary for this phenotype implies, that toyocamycin may modulate jasmonate levels. If JA-Ile is the necessary jasmonate it may be perceived via an additionally, yet unknown mechanism. Another possibility would be that JAR1 forms a novel bioactive jasmonate, which is not involved in the classical signal transduction JA–COI1–MYC2.

JAR1 belongs to the GH3 gene family of adenylating enzymes, that conjugates amino acids to IAA, SA or JA (Staswick et al., 2002, 2005). Indeed, it was shown that JAR1 is able to conjugate Ile, Val, Leu, Phe and the ethylene precursor ACC to jasmonic acid. However, the enzyme has a strong preference for (+)-JA and Ile (Staswick and Tiryaki, 2004, Guranowski et al., 2007, Suza and Staswick, 2008). Commonly, auxin is deactivated by conjugation of amino acids to IAA, since it was shown e.g. that IAA-Trp and also JA-Trp inhibit auxin responses in *Arabidopsis* roots (Staswick, 2009). Despite the fact that *jar1* is functionally a null allele, this mutant still exhibits very low amounts of JA-Ie and increased levels of JA-Phe and JA-ACC, inferring that another enzyme is able to form these derivatives (Staswick and Tiryaki, 2004, Suza and Staswick, 2008). If such an enzyme is also involved in either activation or inhibition of JA/auxin signals remains to be shown. The fact that the root phenotype caused by toyocamycin depends on functional JA synthesis (resistant *aos* mutant), but does not need high amounts of JA-Ile, could mean that the signal is a different JA-derivative than JA-Ile as e.g. JA-Phe or JA-ACC.
Measurements of jasmonate levels in COR and toyocamycin treated seedlings have been initiated to verify the hypothesis that toyocamycin modulates jasmonate levels. However, data on this are not yet available. In addition, I will collect published mutants for testing their responsiveness to toyocamycin (see supplemental Table S.1). This may shed light on which known mechanisms are involved in toyocamycin’s root growth inhibition. Additionally, a genetic screen for suppressor mutants of toyocamycin induced root growth inhibition may uncover mutants that are impaired in JA-(amino acid conjugate) levels and may even lead to identification of the gene, that is responsible for residual JA-Ile levels in *jar1-1*.

Understanding toyocamycin’s effect using microarray data?

Since the mode of action of toyocamycin remains unclear, the analysis of global gene expression could help explaining, how toyocamycin exerts its function on the hormonal signaling network. Seedlings were pretreated with toyocamycin or DMSO as control. One half of each pretreatment was then treated with MeJA or DMSO, respectively. The samples were collected eight hours after chemical treatment and analyzed on the AGRONOMICS1 microarray.

After toyocamycin treatment roughly 2,800 genes were misregulated compared to the DMSO control. Around 2,000 genes were misregulated after double treatment together with MeJA compared to MeJA treatment alone. This confirmed a strong effect of toyocamycin. The high numbers of misregulated genes demand careful selection criteria, if one wants to generate candidate genes, that are involved in JA signaling/crosstalk.

After subjecting the misregulated genes to analysis of GO terms, I observed an enrichment for genes, of which the encoded products are localized to the chloroplast. MeJA-induced expression of the chloroplastic enzymes of JA biosynthesis, *LOX2*, *AOS* and *AOC2*, was inhibited by toyocamycin. *AOC1* and *AOC4* are also localized to the chloroplast and showed similar trends in gene expression. The isoforms *LOX3*, *LOX4* and *AOC3* were upregulated by toyocamycin, which may be due to feed-back regulations of low *LOX2* or *AOC2* expression. Thus, those two isoforms may in part compensate for missing expression of the major isoforms. In contrast to the chloroplastic isoforms of JA biosynthesis, the isoforms acting in the peroxisome were transcriptionally regulated differently. Since these genes were not inhibited by toyocamycin, this explains the low effect of toyocamycin and also of the other derivatives on the OPCL1p::GUS reporter (IC$_{50}$ 30 µM). That OPCL1p::GUS expression was inhibited at these high concentrations may be explained by toxic effects of the nucleoside derivatives.

These observations lead to the question, whether or not toyocamycin modulates JA levels. Expression of the chloroplast localized part of JA biosynthesis appears to be impaired. In addition, expression of the JA sulfotransferase (*ST2a*) shows a similar pattern as *LOX2*, *AOS* or *AOC2*, while the JA methyltransferase (*JMT*) and the JA-Ile hydroxylase (*CYP94B3*, see introduction Fig. 1.2) are strongly upregulated by the double treatment of toyocamycin and MeJA compared to MeJA alone (see Fig. 1.2A-C). If this expression is reflected on protein level, this may indicate, that the bioactive jasmonate may be converted to an inactive form, thereby inhibiting JA signaling.
Nevertheless, this would not explain the induced JAZ expression after toyocamycin treatment in the microarray. In this case, JAZ expression would be induced via another mechanism than by bioactive jasmonate.

Expression of JAZ was shown to be linked to degradation of JAZ proteins (Thines et al., 2007). A similar regulation exists for the Aux/IAA repressors in auxin signaling (Kepinski and Leyser, 2005). JA dependent degradation of JAZ or auxin dependent degradation of Aux/IAA proteins rapidly induces their own expression after hormone stimulus. Whereas toyocamycin prevents the degradation of Aux/IAA proteins (Hayashi et al., 2009), I showed that toyocamycin does not prevent the degradation of JAZ1. Intriguingly, the expression of JAZ1, 5, 6, 9 and 10 was induced by toyocamycin, which could explain inhibition of JA signaling, if this higher expression is translated into higher JAZ protein levels. However, the microarray was only performed at a rather late time point, leaving the question unanswered, how JAZ and/or Aux/IAA are regulated shortly after toyocamycin treatment. Two scenarios are conceivable: (1) Toyocamycin rapidly induces JAZ expression, which in turn would lead to higher protein levels or (2) toyocamycin induces JAZ expression only at later stages, which fits better with the observation, that protein levels at early time points do not change (see JAZ1-GUS degradation in Fig. 4.3). This hypothesis can be verified by monitoring protein levels in the JAZ1-GUS overproducing line at later time points and by analyzing JAZ transcript levels at different time points. These experiments could show, if JAZ1 protein levels are elevated eight hours after toyocamycin treatment or if JAZ are differently expressed at early and late time points.

After double treatment with MeJA and toyocamycin, expression of JAZ7 and JAZ8 were more induced than the other JAZ transcripts compared to MeJA treatment. JAZ7 and JAZ8 both lack two arginine residues, which are important for interaction with COI1 (Chung et al., 2010, Pauwels et al., 2010, Pauwels and Goossens, 2011). Based on the findings that the root phenotype was independent of COI1 and that no change in the COI1-dependent degradation of JAZ1 could be observed, the upregulation of JAZ7 and JAZ8 may indicate their involvement in COI1 independent signaling. To prove this hypothesis, experiments have been initiated that will analyze if JAZ expression is inducible in Arabidopsis wild type and the coi1 mutant at different time points.

The microarray data yielded more indications that toyocamycin is involved in hormonal crosstalk. Not only expression of the JA inactivating enzymes was upregulated, also two genes belonging to the same family as JAR1, which are involved in auxin metabolism exhibited an high expression after treatment of toyocamycin and MeJA (see Fig. 4.12 A-E). IAA-LEUCINE RESISTANT-LIKE 5 (ILL5) and GH3.3 belong to the group of IAA-amino acid conjugating or hydrolyzing enzymes (Davies et al., 1999, Staswick et al., 2005). Intriguingly, ILL5 was shown to be upregulated during systemic response to P. syringae in an Arabidopsis auxin transporter mutant (Truman et al., 2010). This even opens the possibility, that toyocamycin also may be involved in SA signaling. The transcription factor WRKY70 participates in a convergent node of SA and JA signaling. Overexpression of WRKY70 activates SA-induced genes and repression of WRKY70 induces JA responsive genes (Li et al., 2004). In the microarray data I observed a strong activation of WRKY70 expression after toyocamycin treat-
Figure 4.2: Microarray expression of selected genes. 14-day-old Arabidopsis seedlings (Col-0) were pretreated with DMSO or toyocamycin (10 µM) and JA-specific expression was induced by application of MeJA (100 µM). Plants were harvested eight hours after MeJA stimulus. Expression of three independent replicates was analyzed on an AGRONOMICS1 microarray (Rehrauer et al., 2010). Absolute expression values are shown as average ± standard deviation. A: Expression of the JA methyltransferase (JMT) is induced by double treatment of toyocamycin and MeJA. B: Expression of a sulfotransferase (ST2a) is induced by MeJA, which partially is inhibited by toyocamycin. C: Expression of JA-Ile hydroxylase (CYP94B3) is induced by MeJA, but even stronger induced in combination with toyocamycin. D: IAA-LEUCINE RESISTANT-LIKE GENE 5 (ILL5) is strongly induced by double treatment of toyocamycin and MeJA. E: GH3.3 is slightly induced by toyocamycin, but even more by the double treatment with MeJA and toyocamycin. F: WRKY70 is strongly induced by toyocamycin. MeJA prevents this induction.

ment, which is negatively regulated by addition of MeJA (see Fig. 4.2 F). This (and also some generated candidate genes, see below) emphasizes that toyocamycin may be involved in modulating the crosstalk between different hormone signaling pathways.

For truly understanding the mode of action of toyocamycin in JA signaling, it is crucial to either identify a direct protein target or to establish genetic evidence. For the latter I analyzed the microarray data and generated candidate genes, of which available mutants will be tested for their response to toyocamycin. Candidates were selected for genes, that are induced after toyocamycin treatment but not induced after double treatment of toyocamycin and MeJA. They are also not significantly misregulated by MeJA. These candidates would represent genes, that are negatively regulated by MeJA, which only can be observed due to the inducing effect of toyocamycin (see supplemental Fig. S.7 and Table S.2).

Finally, mutants of these respective genes will be obtained and tested for their responsiveness to toyocamycin but it is worth noting that among these candidates some genes are, that are already known to be involved in plant defense. These genes include e.g. AIG1, NIMIN-2, SARD1 or several genes from the WRKY transcription factor
family (Reuber and Ausubel, 1996; Glocova et al., 2005; Weigel et al., 2005; Wang et al., 2008; Zhang et al., 2010; Wang et al., 2011). Also unknown genes with proposed function in plant defense are in this candidate set (AT3G04210 and AT5G48567). If any of these candidate genes is important in toyocamycin mediated crosstalk remains to be shown.

4.6 Concluding remarks

I wanted to identify unknown components of the JA signaling pathway, which I tried to achieve by using a chemical biology approach. Thus, I designed a bidirectional chemical screen for compounds that are able to modulate JA signaling in *Arabidopsis*. In this reporter based screen I identified several compounds, of which I analyzed three in more detail. Compound 766 was found by screening a natural compound library of about 1,700 chemicals for activators of LOX2p::LUC reporter expression. This compound appeared to activate the LUC reporter, but secondary and counter assays indicated that it may bind directly to or influence LUC enzyme, thereby presumably stabilizing the protein. By application of derivatives of this compound to LOX2p::LUC seedlings, I was able to establish SAR, which indicated that the substituents at C-1 and C-2 are important for activity. Based on these findings, 766 could be used as lead structure for development of new LUC ligands. Such ligands could be either new substrates for the enzyme itself or could be used as enzyme stabilizing compounds, that increase enzyme levels in reporter lines with weak promoter activity, by preventing protein degradation.

Screening the natural compound library for inhibitors of MeJA-induced LOX2p::LUC expression, I identified 16 compounds that inhibited reporter expression. After validation in secondary and counter assays, compound 12 was shown to affected only JA-dependent responses, whereas it did not have any effect on JA-independent readouts. Compound 12 not only inhibited downstream markers of JA signaling, it also prevented degradation of JAZ1 protein. Thus, its mode of action can be integrated upstream of JAZ into the JA signaling pathway. Establishing SAR showed that a molecular core of aminocytisine and the substituent biphenylcarboxylic acid both are important for the bioactivity of 12. A derivatized probe containing a photoreactive group and an alkyne for ‘click chemistry’ retained bioactivity and was used in preliminary affinity-based target identification experiments. These experiments showed that this probe labels proteins, which could be competed out by 12. The identification of direct protein targets is not completed, but experiments to continue this effort are underway.

The screen using a small, targeted library of known protein kinase inhibitors identified three inhibitors of MeJA-induced LOX2p::LUC expression (staurosporine, 5-iodotubercidin and tyrphostin AG-879). This indicates the involvement of protein kinases in the JA signaling pathway. However, I cannot rule out the possibility, that these inhibitors may target a non-kinase protein, which might be the reason for their effect on JA signaling. A derivative of 5-iodotubercidin, toyocamycin, was already described to specifically inhibit auxin signaling, which is mechanistically similar to JA
signaling (Hayashi et al., 2009). After testing four derivatives of 5-iodotubercidin for their effect on JA-specific reporter expression and JA-independent readouts, toyocamycin appeared to affect JA-signaling most selectively. Based on this finding and based on the described effect of toyocamycin on auxin signaling, I focused on the analysis of this compound. Toyocamycin inhibits not only JA signaling. Microarray data and published results (Hayashi et al., 2009) showed that it may also affect auxin or SA signaling. How this compound exerts its function remains unknown, but I showed that it acts upstream of JAZ expression. Whether modulated JAZ expression is dependent on COI1 will be shown by expression analysis in the coi1 mutant, but the distinct expression of JAZ7 and JAZ8 indicate the possibility of a COI1-independent mechanism, since their gene product lacks the functional Jas domain for COI1-interaction. The fact that JAZ1 degradation was not prevented by toyocamycin indicates that the compound may act somewhere within the hormonal signaling network, rather than specifically modulating COI1-dependent JA signaling or that it has multiple targets. Additional indications that toyocamycin exerts its function in COI1 independent manner were provided by the fact that the pronounced root growth phenotype caused by toyocamycin was independent of COI1. Surprisingly, this was dependent on JAR1 and AOS, while the inhibition of LOX2 by toyocamycin was independent of JAR1, which also suggests the possibility of multiple targets. Identification of the direct target(s) in similar approaches as the protein labeling with 12/65 will be crucial for the understanding of the mode of action. In addition, genetic evidences by analysis of Arabidopsis mutants or by genetic screening using the distinct root phenotype may contribute to understanding the effect of toyocamycin.

The identification of compound 12 and toyocamycin as modulators of the JA-signaling pathway is only the first step to discover new components of the hormone signaling network. While identification of a direct protein target of 12 may already yield a new component that exerts its function upstream of JAZ, the analysis of the effect of toyocamycin may enhance our understanding of how protein kinases are involved in JA signaling or how JA signaling is integrated into the hormone signaling network.
5 Materials & methods

5.1 Materials

5.1.1 Plant materials

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

<table>
<thead>
<tr>
<th>Accession</th>
<th>Abbreviation</th>
<th>Original Source</th>
</tr>
</thead>
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<tr>
<td>Columbia</td>
<td>Col-0</td>
<td>J. Dangl $^a$</td>
</tr>
<tr>
<td>Columbia (gl1-1)</td>
<td>Col-5</td>
<td>Nottingham <em>Arabidopsis</em> Stock Centre $^b$</td>
</tr>
<tr>
<td>Columbia (gl1-1)</td>
<td>Col-6</td>
<td>Nottingham <em>Arabidopsis</em> Stock Centre $^b$</td>
</tr>
</tbody>
</table>

$^a$ University of North Carolina, Chapel Hill, NC, USA

$^b$ Nottingham, UK

<table>
<thead>
<tr>
<th>Mutant allele</th>
<th>Accession</th>
<th>Reference/Source</th>
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<tr>
<td>coi1-16</td>
<td>Col-5</td>
<td>Ellis and Turner (2002)</td>
</tr>
<tr>
<td>jar1-1</td>
<td>Col-0</td>
<td>Staswick et al. (1992)</td>
</tr>
<tr>
<td>jin1-1</td>
<td>Col-0</td>
<td>Berger et al. (2002)</td>
</tr>
<tr>
<td>aos</td>
<td>Col-6</td>
<td>Park et al. (2002)</td>
</tr>
</tbody>
</table>

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:
- Natural compound library (1,728 compounds, 10 mM) (AnalytiCon Discovery GmbH, Potsdam, Germany)
- Kinase inhibitor library (84 compounds, 2 mM) (Biomol GmbH, Hamburg, Germany)
Table 5.3: Transgenic *Arabidopsis* lines used in this study

<table>
<thead>
<tr>
<th>Line</th>
<th>Accession</th>
<th>Construct</th>
<th>Reference/Source</th>
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<tbody>
<tr>
<td>LOX2:GUS/LUC</td>
<td>Col-0</td>
<td><em>LOX2p::GUS/LUC</em></td>
<td>Jensen et al. (2002)</td>
</tr>
<tr>
<td>VSP1:GUS</td>
<td>Col-5</td>
<td><em>VSPp::GUS</em></td>
<td>Ellis and Turner (2001)</td>
</tr>
<tr>
<td>VSP1:LUC</td>
<td>Col-5</td>
<td><em>VSPp::LUC</em></td>
<td>Ellis and Turner (2001)</td>
</tr>
<tr>
<td>OPCL1:GUS</td>
<td>Col-0</td>
<td><em>OPCL1p::GUS</em></td>
<td>Kienow et al. (2008)</td>
</tr>
<tr>
<td>35Sp:GUS</td>
<td>Col-0</td>
<td><em>CaMV35Sp::GUS</em></td>
<td>Imre Somssich$^a$</td>
</tr>
<tr>
<td>35Sp:LUC</td>
<td>Ler</td>
<td><em>CaMV35Sp::LUC</em></td>
<td>Ferenc Nagy$^b$</td>
</tr>
<tr>
<td>JAZ1-GUS</td>
<td>Col-0</td>
<td><em>CaMV35Sp::JAZ1-GUS</em></td>
<td>Thines et al. (2007)</td>
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<tr>
<td>WRKY29:GUS</td>
<td>Col-0</td>
<td><em>WRKY29p::GUS</em></td>
<td>Imre Somssich$^a$</td>
</tr>
</tbody>
</table>

$^a$ Max-Planck-Institute for Plant Breeding Research, Cologne, Germany  
$^b$ Institute of Plant Biology, Szeged, Hungary

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.

Cell culture medium:  
1× Murashige and Skoog Basal Salt Mixture (Sigma-Aldrich)  
3% (w/v) Sucrose  
1% (v/v) 2,4-Dichlorophenoxyacetic acid solution  
(stock solution 0.1% (w/v)  
2% (v/v) Vitamins solution (1% myo-Inositol, 0.05% nicotinic acid, 0.05% pyridoxin HCl, 0.05% thiamin HCl)  
$pH$ 5.7

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 seeds were germinated by directly sowing them on moist compost (Stender AG, Schermbeck, Germany) or jiffy-9 pots (Jiffy International AS, Ryomgaard, Denmark) 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 Maintenance and cultivation of Arabidopsis cell culture

Arabidopsis cell cultures were maintained shaking (100 rpm) at 25°C at dark conditions. Inoculum of main cultures was transferred every 7 days to fresh cell culture medium. Maintenance of Arabidopsis cell cultures was conducted by Lydia Bollenbach. Experimental cell cultures were taken 5 days after inoculation.

5.2.3 Chemical treatment of Arabidopsis plants

Arabidopsis plants in liquid medium were treated with different chemicals as indicated in microwell plates. Double treatments were performed such as that the compound of interest was incubated 1 h before stimulus with MeJA, COR or flg22. In adenosine/AMP/ADP/ATP competition assays a mixture of toyocamycin and the respective competitor was added as first treatment 1 h before MeJA stimulus. Adenosine, AMP, ADP and ATP stock solutions were prepared in potassium phosphate buffer (5 mM, pH 7.0).

Spray application of compounds was conducted in aqueous solution supplemented with 0.05% (v/v) Silwet L-77. Final DMSO concentration was 0.05% (v/v).

Treatment of Arabidopsis cell culture or protein extracts was performed for one hour in 48-well microplates on ice. For UV-cross-linking, a maximum of 300 µL culture/extract was added per well. The culture/extract was irradiated from the top with a hand-held UV lamp at 254 and 366 nm wavelength by placing the lamp directly on the microplate during chemical treatment.

5.2.4 Biochemical methods

5.2.4.1 Isolation of total protein extract from Arabidopsis

Proteins from 2-4 Arabidopsis seedlings 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 β-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 München, Germany) and BSA solutions between 0 and 1.6 mg/mL as internal standard.

Proteins where precipitated using the method of Wessel and Flügge (1984). Briefly, samples were set to a volume of 200 µL. 250 µL Chloroform:Methanol (1:4) was added, mixed and spun down for 10 min at 4°C and 15,000 g. The supernatant was discarded and the bottom phase was mixed with 200 µL methanol. After a second centrifugation step the pellet was dried for 30 min and then dissolved in Laemmli Sample Buffer (Bio-Rad, München, Germany) (Laemmli, 1970).

Proteins from *Arabidopsis* cell culture were extracted in 1× PBS (1.6 mM KH₂PO₄, 1552 mM NaCl, 30 mM Na₂HPO₄, pH 7.4) + 1% SDS. For chemical treatment of protein extracts, the extraction was conducted without SDS. Cells were collected by short centrifugation and lysated with sonication in PBS. Debris was collected by centrifugation at 4°C for 15 min at 15,000 g and the supernatant was transferred to fresh tubes.

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

Protein samples boiled for 5 min in Laemmlı Sample Buffer (Bio-Rad, München, Germany) were subjected to SDS-PAGE on a gel containing 12% polyacrylamide. SDS-PAGE gels containing fluorescent probe-labeled proteins were washed 3 times with ddH₂O and labeled proteins were visualized by in-gel fluorescence scanning using a FLA-7000 scanner (Fujiﬁlm, Düsseldorf, Germany) with excitation and emission at 532 and 580 nm, respectively. The whole amount of proteins was visualized by coomassie blue R250 (Thermo Scientific, Rockford, USA). Note that samples after ‘click chemistry’ contained Cu(I) and β-mercaptoethanol and were not boiled before SDS-PAGE to avoid chemical protein degradation.

### 5.2.4.3 Immunoblot 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 2 hours with milk powder in TBST (10 mM Tris, 1.5 mM NaCl, pH 7.5, 0.05% (v/v) Tween20). A commercial antibody (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). This antibody was diluted 1:1000 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) 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).

5.2.5 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 an overhead stirrer (RW-20, IKA®-Werke GmbH & Co. KG, Staufen, Germany) in 1.5 mL centrifuge tubes. Further processing was performed using the RNeasy®Plant Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer’s instructions.

To yield micro RNAs ≥ 21 nt the protocol was adapted following the manufacturer’s instructions. Briefly the tissue was lysed in the provided buffer and purified in the QIAshredder spin column. The lysate was mixed with 0.75 volumes of ethanol and bound to the RNeasy spin column. Two times washing was performed with a following washing step of 80% ethanol. The RNA was eluted with RNase-free water.

5.2.5.2 cDNA synthesis

Synthesis of cDNA was performed using SuperScript™II Reverse Transcriptase (RT) (Invitrogen) following the suppliers instructions. Briefly, 1 µL Oligo(dT) primers, 500 ng RNA and 1 µL dNTP Mix was incubated for 5 min at 65°C. 1× Reaction buffer and 10 µM DTT were added. Shortly samples were incubated at 42°C before addition of the RT. The synthesis was performed over 60 min with heat inactivation of the enzyme for 15 min at 70°C.

5.2.5.3 Reverse-Transcriptase PCR

Reverse-Transcriptase PCR (RT-PCR) was performed using the Superscript®One-Step RT-PCR system (Invitrogen, Darmstadt, Germany) following the manufacturers instructions. 100 ng RNA template and 1 µL of each primer (10 µM) were used in a 50 µL reaction volume. The PCR program was repeated 30 times.

<table>
<thead>
<tr>
<th>RT-PCR program:</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
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<tr>
<td>Initial denaturing</td>
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</table>
The following primers have been used:
- Actin: Actinfw 5’ CCTTGTACGCAGTGGCTGCTACAACCCTGTATTTG 3’
- Actin: Actinrw 5’ ATAGTGGTCACCACACTGACCAATATGACAC 3’
- LOX2: LOX2fw 5’ TACTTTCCAACCGGCAAC 3’
- LOX2: LOX2rw 5’ AGAGATACGGCCCTGCTTCTCCTATCCCGGC 3’
- LUC: LUCfw 5’ GTCGAGTCTCCAGACTTTGAACG 3’
- LUC: LUCrw 5’ CTGGCCTTCTGAGATGGAAG 3’
- VSP1/2: VSPfw 5’ AGCCGAACCTTGGAGAAAGG 3’
- VSP1/2: VSPrw 5’ GCTTAAAACCCCTCCAGAGGA 3’

After RT-PCR, samples were subjected to agarose gel electrophoresis for visualization of transcripts. Actin served as internal control for equal loading of RNA.

5.2.5.4 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 1:100 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, 1 µL of each primer (10 µM) 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 Arabidopsis seedlings.

qRT-PCR program:

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<th>Cycles</th>
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<td>temperature increase</td>
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The following primers have been used:
- Expr-qRT-fw: 5’ GAGCTGAAGTGGCTTCCA TGAC 3’
- Expr-qRT-rw: 5’ GGTCCGACATACCCATGTAC 3’
- LOX2-qRT-fw: 5’ TACTTTCCAACCGGCAAC 3’
- LOX2-qRT-rw: 5’ CCTGTCTCTGGATCGTGAT 3’
5.2.5.5 Microarray hybridization

Arabidopsis seedlings were pretreated for one hour with DMSO, \textit{12} (10 \(\mu\)M) or toyocamycin (10 \(\mu\)M). One half of the samples was then treated with DMSO, the other half was treated with MeJA (100 \(\mu\)M). RNA was extracted eight hours after treatment. Analysis of RNA integrity was performed by Dr. Bruno Hüttel (Max Planck Genome Centre Cologne).

RNA was hybridized with the AGRONOMICS1 microarray as described by Rehrauer et al. (2010). Microarray hybridization was performed by Bruno Hüttel.

5.2.6 Reporter assays

5.2.6.1 Histochemical GUS staining

For histochemical staining, seedlings were placed in GUS substrate solution (50 mM sodium phosphate buffer, pH 7.0, 0.1\% (v/v) Triton X-100, 3 mM \(\text{K}_3\text{Fe(CN)}_6\), 1 mM 5-bromo-4-chloro-3-indolyl-\(\beta\)-D-glucuronide (X-Gluc)), vacuum infiltrated and incubated over night at 37\(^\circ\)C. Stained samples were destained by a series of washes in 80\% ethanol (Jefferson, 1987).

5.2.6.2 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 \(\mu\)L Lysis-buffer (50 mM Na(PO\(_4\)), 1 mM EDTA, 0.1\% (v/v) Triton X-100, 10 mM \(\beta\)-mercaptoethanol (fresh), pH 7.0). Debris was spun down at 15,000 \(g\) and the supernatant was transferred to a fresh tube. To 50 \(\mu\)L sample were added 50 \(\mu\)L of MUG-substrate (2 mM 4-methylumbelliferyl-\(\beta\)-D-glucoronid in lysis buffer). These samples were incubated at 37\(^\circ\)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 München, Germany) and BSA solutions between 0 and 1.6 mg/mL as standard.

5.2.6.3 Monitoring JAZ1 degradation

Transgenic Arabidopsis expressing CaMV35Sp::JAZ1-GUS (Thines et al., 2007) were cultivated in liquid MS medium for 7 days. Degradation of the JAZ-GUS construct was monitored as in Thines et al. (2007). Briefly, roots of transgenic 35::JAZ1-GUS were pretreated for 1h as indicated. Degradation of the fusion protein was induced by addition of 10\(\mu\)M MeJA for 30 min. Histochemical GUS staining using GUS substrate
solution was performed over night at 37°C. Results were observed under a optical microscope.

5.2.6.4 Luciferase assay (in vivo)

Transgenic *Arabidopsis* plants harboring the LUC reporter were assayed in multiwell plates (OptiPlate-96, Perkin Elmer, USA) containing 200 µL MS medium. The medium was supplemented with 0.25 mM Luciferin (5 mM potassium phosphate, pH 7.8, 4 mM D-luciferin free acid (SYNCHEM OHG, Germany), 0.01% (v/v) Triton X-100). Over a time of up to 24 h each single well was repeatedly measured for 2 s either using a TopCount NXT™ Microplate Scintillation and Luminescence Counter (Perkin Elmer, USA) or a Luminometer (Centro LB 960, Berthold Technologies, Germany). Plates for TopCount readouts were sealed with TopSeal®-A Microplate Press-On Adhesive Sealing Film (Perkin Elmer, USA). For allowing transpiration holes were pierced into the Sealing Film. Values of the time span until the LUC activity decreased to a stable value (usually 4-6h) were averaged and taken into analysis.

5.2.6.5 Luciferase assay (in vitro)

*In vitro* LUC assays were performed in black 96-well microplates using recombinant luciferase (Roche). Chemicals (25 µM) were incubated with 0.1 µM ATP in 100 µL cold Tris-HCl buffer (100 µM, pH 7.8). 100 µL of a cold mixture of luciferase (5 ng/mL) and luciferin (50 µM) (Luciferase stock: 1 µM in 100 µM Tris-HCl, pH 7.8; Luciferin stock: 4 mM D-luciferin free acid in 5 mM potassium phosphate, pH 7.8, 0.01% (v/v) Triton X-100) were injected, and luminescence measurement was initiated after 1 s delay. Luminescence was detected over 10 s with a luminometer (Centro LB960 XS3, Berthold Technologies, Bad Wildbad, Germany). Data were collected from at least five technical replicates.

5.2.7 Chemical screen

Chemical screens were performed using the transgenic *Arabidopsis* LOX2p::LUC reporter line. *Arabidopsis* seedlings were grown in hydroponic culture under sterile conditions directly in 96-well microplates. Each well contained two seedlings in 200 µL MS medium. 12-14 day old seedlings were pretreated with chemicals for 1 h in MS medium (10 µM: Kinase inhibitory library; 25 µM: Natural compound library). 100µM MeJA was added to induce reporter expression, DMSO served as negative control. 24 h after chemical treatment the semiquantitative LUC assay was performed using a TopCount NXT™ Microplate Scintillation and Luminescence Counter (Perkin Elmer, USA), as described in section 5.2.6.4. A maximum of 288 chemicals was screened at the same time.

Luminescence of the first 4-6 hours was averaged and taken into analysis. One microplate with 24 uninduced and 24 induced replicates served as control. Values of each microplate were compared to the control plate. For the activator screen only values above the average of the negative control were considered as hit. The threshold for the
inhibitor screen was set to the lower value of the control’s standard deviation. Only samples with luminescence values below this threshold were considered as hit.

5.2.8 Root growth elongation assay

Root growth assays were performed on square petri dishes containing approximately 35 mL solid MS medium including the respective compounds for testing. Seeds were sown in 2-3 rows of about 20-30 seeds and plates were kept at 4°C for two days to synchronize germination. Plates were transferred to growth cabinets and plants were grown vertically for 7-10 days. Pictures were taken from the bottom using a conventional image scanner at 600 dpi. Root length was measured using ImageJ software [Abramoff et al., 2004].

5.2.9 Monitoring the oxidative burst

Oxidative burst analysis in *Arabidopsis* leaf discs was performed following standard procedures [Gomez-Gomez et al., 1999]. The assay measures active oxygen species released by leaf tissue by H$_2$O$_2$ dependent luminescence of luminol [Keppler et al., 1989]. Briefly, *Arabidopsis* leaves were cut into leaf discs (diameter 6 mm) and incubated overnight floating on H$_2$O. Leaf discs were transferred into 96-well microplates containing 50 µL H$_2$O and respective chemicals for testing. These were incubated for one hour before triggering the reaction with flg22. This was performed automatically in a luminometer (Centro LB960, Berthold Technologies, Bad Wildbad, Germany) by injecting 50 µL of a solution containing flg22 (2 µM), luminol (400 µM) and horseradish peroxidase (0.1 mg/mL, Sigma-Aldrich, P6782). Luminescence was measured every 2 minutes over a total period of 32 minutes. Each experiment contained 6 biological replicates.

5.2.10 Probe synthesis and click reaction

Synthesis of molecular probes containing a photoreactive benzophenone and an alkyne was performed by the group of Prof. Markus Kaiser (ZMB, Universität Duisburg Essen).

Copper-catalyzed 1,3-dipolar cycloaddition between molecular probes and an azide containing fluorophore (Alexa Fluor® 555, Invitrogen) was performed as described (Speers et al., 2003; Kaschani et al., 2009). Briefly, cleared protein extracts in 1 × PBS (1.06 mM KH$_2$PO$_4$, 155.2 mM NaCl, 3 mM Na$_2$HPO$_4$, pH 7.4) were supplemented with SDS to a final concentration of 1% SDS. The reaction was performed in a total volume of 50 µL. In 4 consecutive steps including mixing of the sample, 1 µL of the following reagents was added: Alexa Fluor 555 (1mM in DMSO), TBTA (1.7 mM, fresh in tert-butanol), CuSO$_4$ (50 mM), TCEP (100 mM, fresh). Samples were shaked for one hour at room temperature before addition of 1× Laemmli Sample Buffer (BioRad, München, Germany) [Laemmli, 1970]. Note that protein samples were not boiled before subjecting them to SDS-PAGE.
5.2.11 Microarray data analysis

Microarray data processing was performed by Emiel Ver Loren van Themaat (MPI for Plant Breeding Research, Cologne). Normalization and statistical analysis of the data was performed as described by [Irizarry et al., 2003; Smyth, 2004]. Comparison of fold-change expression was performed between the following treatments:
- MeJA vs. DMSO
- 12 vs. DMSO
- Toyocamycin vs. DMSO
- 12 + MeJA vs. MeJA
- Toyocamycin + MeJA vs. MeJA

Two-fold misregulated expression was analyzed using GeneVenn [Pirooznia et al., 2007] to see coregulated genes.

Gene Ontology analysis was performed using the online tool of The Arabidopsis Information Resource [Berardini et al., 2004] and the AmiGO online tool [Ashburner et al., 2000].
References


Supplement

Figure S.1: Structure of in a secondary screen confirmed candidates. The picture depicts structures of all candidates, whose activity on LOX2p::LUC could be confirmed. Known compounds are: cycloheximide, 8; diacetoxyscirpenol, 15; neosolaniol, 16; piericidin, 17.

Figure S.3: Structural derivatives of compound 12. The structures of all tested derivatives of compound 12 are shown. Only 12, 49 and 52 exhibited bioactivity. The modified version 65 contains a benzophenone photoaffinity group and an alkyne group for click chemistry. Compound 66 exhibits also a benzophenone and an alkyne but not the part of 12, which was shown to be active.
Figure S.4: Structure of nucleoside analogs and modified derivatives. The structures of the four nucleoside analogs toyocamycin, tubercidin, 5-iodotubercidin and sangivamycin are depicted. Targeted modification of toyocamycin yielded the inactive toyocamycin-BOC and the highly bioactive toyocamycin-sulfamate (H$_3$SNO$_3$). Toyocamycin-click was active as toyocamycin-sulfamate, contains and alkyne and may be used in target identification experiments using affinity based labeling methods in combination with click chemistry.
Figure S.5: Variation of LUC activities in the screen for inhibitors of the natural compound library. 12-day-old Arabidopsis seedlings harboring the LOX2p::LUC reporter gene were pretreated for one hour with 1,728 different compounds (each at 25 µM) before addition of MeJA (100 µM). The overall screen was performed in 13 separate steps with each containing one separate control plate. The plot presents average LUC activities after application of each compound (n = 2). The blue line depicts the average of each screening plate. The red line depicts the average of the respective control plate.

Figure S.6: Microarray expression levels of JAZ, Aux/IAA and DELLA. 14-day-old Arabidopsis seedlings (Col-0) were pretreated with DMSO and JA-specific expression was induced by application of MeJA (100 µM). Plants were harvested eight hours after MeJA stimulus. Expression of three independent replicates was analyzed on an AGRONOMICS1 microarray [Rehrauer et al., 2010]. Expression was normalized to the DMSO control and is shown as average ± standard deviation. Only JAZ are upregulated by MeJA with the exception of JAZ4, JAZ11 and JAZ12.
Table S.1: Arabidopsis mutants for root growth assays. The table depicts Arabidopsis mutants, that will be considered for testing their response to toyocamycin and possibly 12. They are selected based on their known root growth response to hormonal or pathogen stress. This table is not exhaustive and more mutants may be considered.

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Figure S.7 (following page): Microarray expression of selected genes. 14-day-old Arabidopsis seedlings (Col-0) were pretreated with DMSO or toyocamycin (10 µM) and JA-specific expression was induced by application of MeJA (100 µM). Plants were harvested eight hours after MeJA stimulus. Expression of three independent replicates was analyzed on an AGRONOMICS1 microarray (Rehrauer et al., 2010). Expression is shown relative to expression after DMSO control treatment. Genes were selected after following criteria: ≥2-fold induction after toyocamycin treatment; <2-fold misregulation after MeJA treatment; >2-fold difference between double treatment toyocamycin + MeJA and single treatment with MeJA alone; ratio toyocamycin treatment / double treatment with toyocamycin + MeJA is <0.7 or >1.5. Table S.2 shows the same candidates and includes the average expression values.
### Table S.2: Microarray expression of selected genes

14-day-old *Arabidopsis* seedlings (Col-0) were pretreated with DMSO or toyocamycin (10 μM) and JA-specific expression was induced by application of MeJA (100 μM). Plants were harvested eight hours after MeJA stimulus. Expression of three independent replicates was analyzed on an AGRONOMICS1 microarray (Rehrauer et al. [2010]). Genes were selected after following criteria: >2-fold induction after toyocamycin treatment; <2-fold misregulation after MeJA treatment, >2-fold difference between double treatment toyocamycin + MeJA and single treatment with MeJA alone; ratio toyocamycin treatment / double treatment with toyocamycin + MeJA is <0.7 or >1.5.

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**Table S.2 – continued from previous page**
Danksagung

An dieser Stelle möchte ich allen Leuten danken, die mich während der gesamten Arbeit unterstützt und zum Gelingen der Arbeit beigetragen haben.

Ganz besonderer Dank gilt Dir Erich, für Deine große Geduld und die immer offene Tür. Deine Fähigkeit, mir meinen eigenen Finger zu zeigen und ihn in Bild oder Wort zu fassen hat maßgeblich zum Erfolg des ein oder anderen Vortrages, Posters und auch dieser Arbeit beigetragen.

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Dr. Wim Soppe many thanks for accepting the task of being "Beisitzer" in the examination committee.

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An letzter Stelle danke ich meinen Eltern, die mich auf dem gesamten Weg immer unterstützt haben und immer da waren, wenn ich sie brauchte. Ihr habt – ohne es zu merken – erheblich zu diesem Erfolg beigetragen, einfach dadurch, dass Ihr da wart!
Erklärung


Köln, 5. Dezember 2011
Curriculum Vitae

Christian Meesters

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geboren am: 15.05.1983 in Goch
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Nationalität: deutsch

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01/2008 - 09/2008 Diplomarbeit an der Universität Bonn mit dem Titel: ‘Expressionstudien zu Genen des Zuckerstoffwechsels der austrocknungstoleranten Pflanze Craterostigma plantagineum’
Seit 11/2008 Max Planck Institut für Pflanzenzüchtungsforschung, Köln
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Köln, Dezember 2011