Analysis of fatty acid hydroxylating cytochrome P450 enzymes

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Abbreviations

½ MS	Murashige & Skoog medium half strength
A	Adenine
A. thaliana	Arabidopsis thaliana
aa	Amino acids
Abs	Optical absorbance
ADE 2d	Selection marker for adenine auxotrophy
ALA	5-aminolevulinic acid
ampR	Ampicillin resistance gene
amu	Atomic mass units
ATR1	A. thaliana cytochrome P450 reductase 1
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BSA	Bovine serum albumin
C	Cytosine
cDNA	Complementary DNA
CE	Collision energy
CFP	Cyan-fluorescent protein
CO	Carbon monoxide
CPR	Cytochrome P450 reductase
CYP	Cytochrome P450
d	Day (s)
dde	Delayed dehiscence
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Dinucleotide triphosphate
DP	Declustering Potential
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra-acetic acid
ER	Endoplasmic reticulum
ESI	Electrospray ionization
et al.	et alii
EtOH	Ethanol
f1 ori	f1 phage origin of replication
FA	Fatty acid
FAA	Formaldehyde-acetic acid-alcohol solution for tissue fixation
FAD	Flavin adenine dinucleotide
FW	Fresh weight
His	Histidine
HPLC	High performance liquid chromatography
hpw	Hours post wounding
hr	Hour
h	hours
JA	Jasmonic acid
JA-Ile	Jasmonoyl-isoleucine
L/R reaction	Ligation and restriction in one step
LB	Luria-bertani

min	Minute
MS	Mass spectrometry
NADP+	Nicotinamide adenine dinucleotide phosphate, oxidized form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
NEB	New England Biolabs
OD600	Optical density at 600 nm wavelength of light
oLAC	Lac operator
OPDA	Oxo-phyto-dienoic acid
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Poly-ethylene glycol
pGAL	Galactose promoter
PMSF	Phenylmethanesulfonylfluorife
pT7	T7 promoter
QTOF	Quadrupole-time-of-flight
RBS	Ribosome binding site
RNA	Ribose nucleic acid
rpm	Revolutions per minute
RT	Reverse transcriptase
SDS	Sodium dodecyl sulfate
sec	Second (s)
SGI	Synthetic media for S. cerevisiae
T	Thymine
TCA	Trichloroacetic acid
TE	Tris/EDTA
Temed	N, N, N', N'-Tetramethylethylendiamine
TFA	Trifluoroacetic acid
TOF	Time-of-flight
Tris	2-amino-2-hydroxymethyl-1,3-propanediol
Triton X-100	4-octylphenol polyethoxylate
tT7	T7 terminator
UHPLC	Ultra high performance liquid chromatography
URA3	Selection marker for uracil auxotrophy
UV	Ultra violet (light)
v/v	Volume to volume ratio
Vmax	Maximal reaction velocity
w/v	Weight to volume ratio
x g	Gravitation
X-Gluc	5-Bromo-4-chloro-1H-indol-3-yl β-D-glucopyranosiduronic acid
YFP	Yellow fluorescent protein
YPGA	S. cerevisiae medium: yeast extract, bactopeptone, glucose and adenine
YPGE	S. cerevisiae medium: yeast extract, bactopeptone, glucose and ethanol

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1 Introduction

"In nature, plants are subjected to frequent, if not constant, harassment and bombardment from the environment by wind, hail, large herbivores, insects, fungi and other pathogens, being attacked both above and below the ground" (Davies 1987). Plants possess an innate immune system which recognizes the attacking organism on a molecular basis. The responses to such molecules are often regulated by plant hormones and happen in an antagonistic or synergistic manner (Pieterse et al. 2009). Furthermore, plants react also to mechanical wounding by a lipoxygenase- (LOX) based pathway by which polyunsaturated fatty acids are oxidized, forming primary and secondary oxidation products (Hildebrand 1989). These oxygenated fatty acid derivatives, referred to as oxylipins, are not only present in plants but also in animal kingdom, where structural analogs to plant oxylipins may be prostaglandins which are derived from eicosatetraenoic acid (arachidonic acid) (Bergey et al. 1996). In both kingdoms the oxylipins derive from membrane lipids and have a similar biosynthetic pathway, since lipoxygenases and cytochrome P450 enzymes are employed to oxidize fatty acids (Bergey et al. 1996; Blée 2002; Lee et al. 2008). Oxylipins are also present in fungi. They serve as developmental triggers and can play a role in fungal-host interaction (Tsitsigiannis and Keller 2007). Oxylipins perform essential functions in plant life cycle (Feussner and Wasternack 2002). In the defense signaling pathways oxylipins play a major role. Upon tissue injury, it comes to perturbation of cellular membranes and membrane lipids are released. Lipases, such as DEFECVTVIE IN ANTHER DEHISCENCE1 (DAD1) and DONGLE (DGL), can specifically cleave the unsaturated fatty acid at the *sn1* position (Ellinger et al. 2010; Hyun et al. 2008). Thus unsaturated fatty acids, such as (9Z,12Z,15Z)-octadecatrienoic acid (α-linolenic acid, α-LeA) and hexadecatrienoic acid, are provided to represent the beginning of the jasmonic acid biosynthesis (Vick and Zimmerman 1983).

1.1 Jasmonates

Jasmonic acid and its derivatives, collectively referred to as jasmonates, belong to a family of oxylipins that originate from the enzymatic oxygenation of α-LeA or hexadecatrienoic acid (Wasternack and Kombrink 2010). Jasmonates are mainly synthesized by plants and may also be produced by some fungi (Miersch et al. 1991).

1.1.1 Biosynthesis of Jasmonic acid

The biosynthesis of jasmonic acid (JA) takes place in two cellular compartments. The first part is located in the chloroplast and begins with α-LeA, which may be released from the *sn1* position of galactolipids of the plastidial membrane. This reaction may be pathway- and stimuli-specific regulated by the phospholipases *DAD1* and *DGL*. *DGL* is expressed in the leaves and may maintain the basal level of JA under normal conditions. Therewith *DGL* regulates vegetative growth and may be involved in forming the JA burst upon wounding. While *DGL* may serve in the early phase of wound response, *DAD1* may take over at later phases of wound-induced JA production (Hyun et al. 2008). The 13-lipoxygenase (13-LOX) enzymes introduce an oxygen at the position 13 of α-LeA (Figure 1), forming the (13S)-hydroperoxyoctadecatrienoic acid (13-HPOT) (Feussner and Wasternack 2002). Among the six LOXs of *A. thaliana*, four of them (LOX2, LOX3, LOX4, LOX6) have been identified as 13-LOXs (Bannenberg et al. 2009). Studies revealed that the bulk of JA formation in the first hour (h) upon wounding is mediated by LOX2 (Glauser et al. 2009; Schommer et al. 2008). By analysis of the *lox2-1* mutant JA and JA-Ile are still synthesized upon wounding (Glauser et al., 2009). This suggests the participation of other 13-LOXs in the JA formation. A proteomic study among-JA-induced proteins assigned LOX3 as increased and thus JA-pathway related as well (Gfeller et al. 2011). Furthermore, LOX-6 showed a favored role in contribution to JA formation at least in the wound response (Caldelari et al. 2011; Chauvin et al. 2012). Activities of LOX3 and LOX4 were described in vascular tissues (Vellosillo et al. 2007). Catalyzed by the ALLENE OXIDE SYNTHASE (AOS), a CYP74A enzyme, an allene oxide is formed from (13*S*)-hydroperoxyoctadecatrienoic acid (13-HPOT) (Vick and Zimmerman 1987), as shown in Figure 1. The highly unstable allene oxide is converted to a cylopentenone by the ALLENE OXIDE CYCLASE (AOC) (Ziegler et al. 2000) and thus forming cis(+)-12-oxophytodienoic acid (OPDA). Another source of OPDA in *A. thaliana* are the Arabidopsides. In *A. thaliana* the class of Arabidopsides comprises oxidized galactolipids that contain cyclopentenones (Hisamatsu et al. 2003; Hisamatsu et al. 2005). Esterified to the glycerol backbone, cyclopentenones like 12-*oxo*-phytodienoic acid (OPDA) and/or dinor-12-*oxo*-phytodienoic acid (dnOPDA) can be present in such structures. One or more cyclopentenones can be esterified to the backbone or sugar moiety of the lipid backbone (Anderson 2006). OPDA is exported from the chloroplast by a so far unknown mechanism. Next, the peroxisomal ABC transporter (PXA1) imports OPDS into the peroxisome (Theodoulou et al. 2005). The peroxisomal OPDA reductase (OPR3) specifically converts *cis*-(+)-OPDA to 3-oxo-2-(2-pentenyl)-cyclopentane-1-octanoic acid (OPC-8) (Schaller et al. 2000; Strassner et al. 2002). OPC-8 is then activated as Co-A-ester by OPC-8:CoA ligase 1 (OPCL1) to form OPDA-CoA which then enters three cycles

of ß-oxidation leading to shortening of the carboxylic acid side chain. Three enzymes are mainly involved in the ß-oxidation process, acyl-CoA oxidase (ACX), multifunctional protein (MFP) and 3-ketoacyl-CoA thiolase (KAT) (Li et al. 2005; Schaller and Stintzi 2009; Vick and Zimmerman 1984), leading to jasmonoyl-CoA (JA-CoA). In the last step of biosynthesis, a hydrolysation of JA-CoA takes place, releasing the free acid. The hydrolyzation reaction might be mediated by peroxisomal acyl-thioesterases (*At*ACH1 and *At*ACH2), forming the (+)-7-*iso*-jasmonic acid, which is further modified in the cytosol (Li et al. 2005; Tilton et al. 2004). JAR1 mediates the conjugation reaction of isoleucine (Ile) to JA forming (+)-7-*iso*-jasmonoyl-isoleucine (JA-Ile), the biologically active substance (Staswick and Tiryaki 2004).

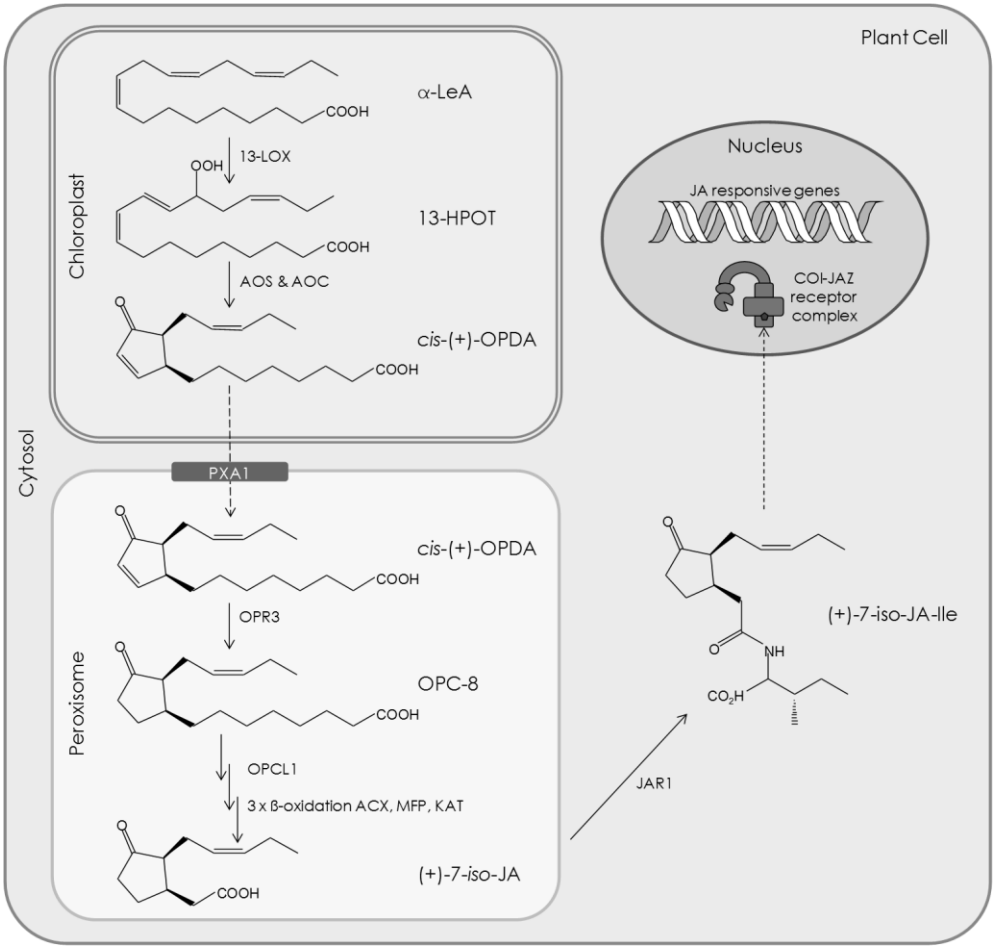

Figure 1: Synthesis of Jasmonic acid (JA) and conjugation to the biologically active jasmonoyl-isoleucine (JA-Ile).
Alpha-linolenic acid (α-LeA), 13-lipoxygenase (13-LOX), (13S)-hydroperoxyoctadecatrienoic acid (13-HPOT), allene oxide cyclase (AOC); allene oxide synthase (AOS), cis-(+)-12-oxophytodienoic acid (cis-(+)-OPDA), peroxisomal ABC transporter (PXA1), OPDA reductase3 (OPR3), 3-oxo-2-(2-pentenyl)-cyclopentane-1-octanoic acid (OPC-8), OPC-8:CoA ligase 1 (OPCL1), acyl-CoA oxidase (ACX), multifunctional protein (MFP), 3-ketoacyl-CoA thiolase (KAT), JA-amino acid synthetase (JAR), coronatine insensitive (COI), jasmonate zim domain (JAZ).

An assay for quantification of (+)-7-*iso*-JA-Ile from tomato-extracts indicates that this compound is chemically more stable than it was assumed before (Fonseca et al. 2009). Moreover, by use of a recombinant JAR1, it was shown that (+)-7-*iso*-JA-Ile is exclusively formed, since JAR1 has a strong preference for Ile over other amino acids (Suza et al. 2010). JA-Ile is assumed to diffuse from the cytosol into the nucleus, where it can bind to the COI-JAZ receptor complex (Koo and Howe 2012), in more detail shown in Figure 2. The biosynthesis of JA-Ile is regulated and controlled by development and environment (Creelman and Mullet 1997; Koo et al. 2009; Wasternack 2007). Both JA and JA-Ile play a crucial role in regulation of defense induction in vegetative tissue. JA and JA-Ile accumulate upon various biotic and abiotic stresses. Mechanical wounding and herbivory of *A. thaliana* leaves effectively triggers the *de novo* synthesis and accumulation of JA/JA-Ile in the damaged tissue. The level of JA and JA-Ile can increase about 25-fold within 5 min after wounding (Chung et al. 2008; Glauser et al. 2008; Koo et al. 2009). The nippiness of this response indicates the presence of JA/JA-Ile biosynthetic enzymes in the unwounded tissue prior to stimulation. In agreement with this view is the fact that the rate-limiting step in JA and JA-Ile synthesis most likely is the release of fatty acyl substrates from plastidial gylcerolipids by lipases (Ishiguro et al. 2001; Koo and Howe 2012; Stenzel et al. 2003b). In *A. thaliana* the release of OPDA from Arabidopsides may be an alternative mechanism leading to an even more rapid formation of JA-Ile. An remaining question is the mechanism by which the extracellular signal activates the plastidial lipases that provide the substrate for JA-Ile production (Koo and Howe 2012). The production of the wound-hormone JA/JA-Ile is a response to an environmental signal, like wounding. It was shown, that wounding can provoke an electrical signal (Herde et al. 1999). This signal is transmitted from wounded leaves by membrane depolarization. Upon such a signal, many genes, *AOS* and *LOX2*, for instance, are up-regulated of which the corresponding proteins are part of the JA biosynthesis (Reymond et al. 2000). Putative cation channels, encoded by the glutamate-receptor-like (GLR) genes, control the distal expression of wound-regulated genes (Mousavi et al. 2013). The transcriptional response goes beyond the study of Reymond et al. (2000), all genes of the JA-biosynthesis are wound-inducible (Wasternack et al. 2006). Taken together, these findings account for the regulation of JA metabolism by positive feedback loop, substrate availability, and tissue specificity (Koo et al. 2006; Wasternack 2007).

1.1.1.1 Signal perception by the SCF^{COI}-complex

In plant hormone sensing and signaling the ubiquitin-proteasome system plays a central role (Santner and Estelle 2010). A main part of this system is

the Skp1/Cullin/F-box (SCF) complex, functioning as an E3 ubiquitin ligase (Figure 2). Accordingly, the F-box protein can recognize a target protein and ubiquitinylate it, so that the ubiquitinylated protein is subjected to degradation in the proteasome. In case of JA signaling and perception, the protein CORONATINE INSENSITIVE 1 (COI1) works as the F-box protein (Xie et al. 1998). Thus, perception of the JA signal happens via SCF^{COI}-complex. Other proteins that are participating in JA-Ile perception are the JASMONATE ZIM DOMAIN (JAZ) proteins (Thines et al. 2007). In *A. thaliana* the family comprises 12 JAZ proteins. JAZ proteins can bind to COI1 upon presence of JA-Ile. The interaction of JAZ and COI is mediated by JA-Ile, a fact that has been used for screenings to determine the biological activity of a JA-derivative of interest. JA and MeJA are for example unable to promote the binding between COI and JAZ (Thines et al. 2007). The MYC2 transcription factor (TF) is another part of the JA-Ile perception. Additionally to MYC2, MYC3 and MYC4 were also found to participate in the JA response in *A. thaliana* (Cheng et al. 2011; Fernandez-Calvo et al. 2011). Jasmonate insensitive 1 (JIN1) encodes this MYC-related transcriptional activator with a typical DNA binding domain of a basic helix-loop-helix leucine zipper motif (Dombrecht et al. 2007). MYC2 binds to an extended G-Box promoter motif and interacts with JAZ proteins and acts as both activator and repressor of distinct JA-responsive gene expression in *A. thaliana* (Lorenzo et al. 2004). In the unstressed state, MYC2 binds to the G-Box of a JA-responsive gene but cannot activate the transcription because of the JAZ protein that is binding to MYC2. Thus, JAZ proteins are working as repressors of the JA-responsive genes (Wasternack and Hause 2014). Other co-repressors of the JA-responsive genes are the two interacting proteins TOPLESS (TPL) and Novel Interactor of JAZ (NINJA) (Pauwels et al. 2010). TPL cannot directly bind to DNA but can interact with two histone deacetylases (HDAs) that can perform chromatin modifications. HDAs are part of basic mechanism that cause the suppression of gene expression and are involved in *A. thaliana's* defense (Berr et al. 2012). In case of JA signaling HDA6 and HDA19 are genetically linked to TPL. Potentially, the JAZ-mediated repression of the JA-responsive genes is an effect of HDA6 and HDA19 that are linked to the JAZ via TPL and NINJA (Wasternack and Hause 2013). After crystallization of the COI-JAZ receptor complex (Sheard et al. 2010) a mechanism for JA-Ile perception was established. If JA-Ile as ligand is present, the Jas domain (domain of JAZ for binding to COI and other TFs) of the JAZ proteins can interact with COI. The binding might be increased by IP_5 (Mosblech et al. 2011; Sheard et al. 2010). Upon wounding, the level of JA-Ile rises within 5 minutes (min) (Glauser et al. 2008), which leads to binding of the JAZ proteins to the COI complex. Accordingly, the JAZ is ubiquitinylated by the E3 ubiquitin ligase and thus targeted for degradation by the 26S-

proteasome. Upon JAZ degradation the MEDIATOR25 (MED25) subunit of the eukaryotic Mediator complex could bind to MYC2. MED25 was lately described as an integrative hub in JA-mediated gene expression (Cevik et al. 2012). The JA-responsive gene can now be transcribed. Basically the fundamental concept of JA-Ile perception is established. Open questions remain regarding the ubiquitination of the JAZs, the exact interactions of proteins in the SCF^{COI}-complex at high and low JA-Ile concentrations as well as their stability (Wasternack and Hause 2013).

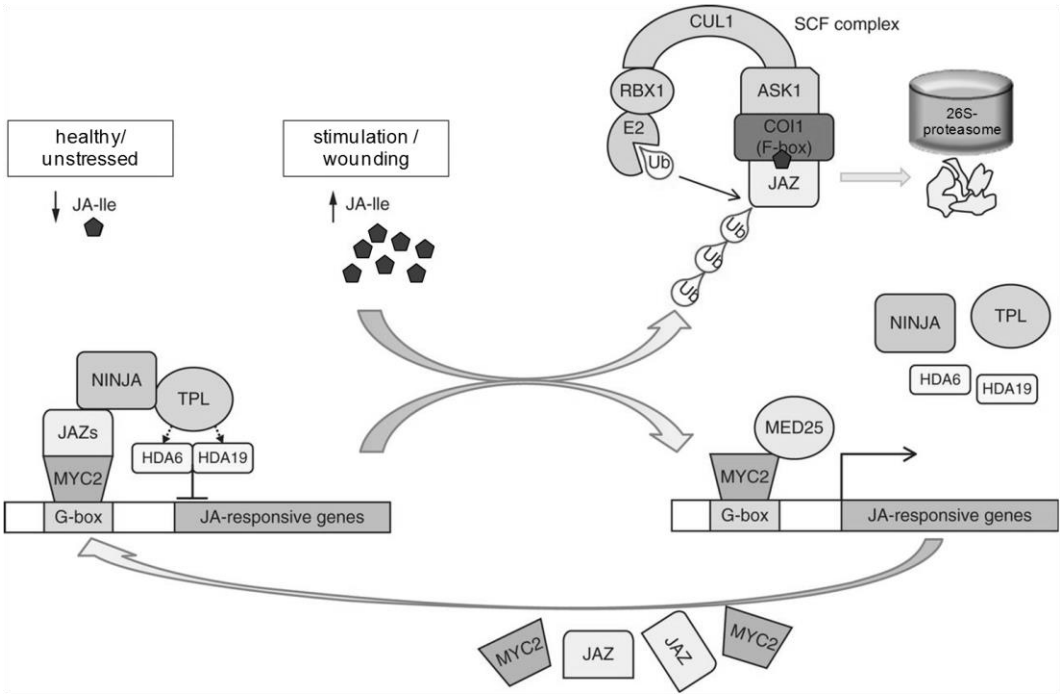

Figure 2: Jasmonoyl-isoleucine (JA-Ile) perception via the COI1–JAZ co-receptor complex.
In the unstressed state (left), the level of JA-Ile is low. The binding of MYC2 to a G-box of the promoter of a JA-responsive gene cannot activate transcription. The repressor Jasmonate ZIM domain protein (JAZs) is bound to MYC2 and thus prevents transcription. The co-repressors Novel Interactor of JAZ (NINJA) bound to JAZs, and TOPLESS (TPL) suppress transcription by the use of HISTONE DEACETYLASE 6 (HDA6) and HDA19. Wounding (right, high JA-Ile level) leads to a recruitment of JAZs by COI1. JAZ is thus ubiquitinylated and subsequently degraded by the 26S proteasome. Accordingly, MYC2 can activate transcription of early JA-responsive genes. Among them are also those encoding JAZ and MYC2. The transcription is mediated by the subunit 25 of Mediator complex (MED25). Further abbreviations: Arabidopsis SKP1 (S-phase kinase-associated protein 1) homologue (ASK1), Cullin (CUL), ubiquitin-conjugating enzyme (E2), bHLHzip transcription factor (MYC2), RING-H2 protein (RBX), complex consisting of Skp1, Cullin-1 and F-box protein (SCF-complex), ubiquitin (Ub), Modified from (Wasternack and Hause 2013).

1.1.1.2 JA-signal amplification and propagation *in planta*

The patterns of leaf primordia origination, on the flanks of the shoot apical meristem (SAM), results in one of the most noticeable characters of shoot morphology, phyllotaxis. Generally phyllotactic patterns are regular and thus

define whole taxonomic groups (Dengler 2006). The phyllotaxis can change during development, such as changes from vegetative to reproductive shoot phase (Poethig 1990). The most common pattern among dicotolydons is a helical phyllotaxis (Mitchison 1977). In such species leaf primordia are initiated at a more or less constant angle of 137.5 ° along the ontogenetic helix. Additional helices that are steeper on the ontogenetic helix are termed parastichies (Kirchoff 1984). The parastichies in an adult *A. thaliana* rosette (Figure 3) lead to a direct vascular connection of leaves n±5 and n±8. Thus the leaf 8 is connected to leaves 5, 13 and 16 (Dengler 2006). Communication of environmental harassment (Davies 1987), like wounding, can be mediated in the plant through the parastichies. By the use of non-invasive surface electrodes it was shown, that wounding can provoke an electrical signal (Herde et al. 1999). This signal was transmitted from wounded leaves to those with a vascular connection (leaves n±5 and n±8) by membrane depolarization. Putative cation channels, encoded by the glutamate-receptor-like (GLR) genes, control the distal expression of wound-regulated genes. Among them are the JAZ genes (Mousavi et al. 2013). The GLR genes are involved in propagation of the wound stimulus by an electrical signal and thus can activate the JA biosynthesis in distal leaves (Mousavi et al. 2013). By earlier studies it was found out that JA rapidly accumulates in tissues both proximal and distal to the site of wounding (Glauser et al. 2009). Within 30 seconds (sec) JA accumulates near to the site of wounding. In distal leaves JA increases significantly within 120 sec, especially in leaves with direct vascular connections the accumulation of JA is very quick. The average velocity of signal propagation that leads to JA accumulation in distal leaves is 3.4-4.5 cm min^{-1} (Glauser et al. 2009).

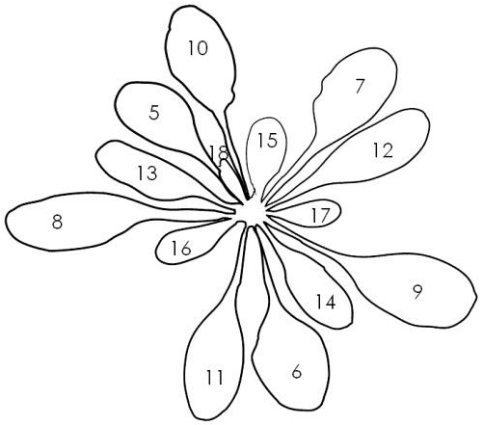

Figure 3: Schematic *A. thaliana* rosette about 5 weeks after sowing.
The leaves are numbered from youngest to oldest. *A. thaliana* leaves grow in a developmental pattern: in adult-phase rosettes, leaf n shares direct vascular connections to leaves n±5 and n±8. Thus the wounded leaf 8 is connected to leaves 5, 13 and 16, these connections are termed parastichies (Dengler 2006). The figure is modified from (Mousavi et al. 2013)

1.1.2 Metabolic fate of jasmonate

As a odoriferous compound methyl jasmonate (MeJA) has been isolated from oil of *Jasminum grandiflorum* (Demole et al. 1962). Since then, jasmonates have been described in many plants species. The JA carboxy methyl-transferase (JMT) mediates the methylation of JA and forms MeJA (Figure 4), which has been assumed also to be the active compound of JA-action (Seo et al. 2001). When JMT was expressed in wild tobacco, it was found to negatively affect the JA-Ile formation. Thus, the biological activity of MeJA is only observable when MeJA is de-methylated and further conjugated to form JA-Ile (Stitz et al. 2011). The discovery of a JA-Ile mediated binding of JAZ proteins to the jasmonate receptor COI complex provides the proof that MeJA itself is not the biologically active molecule. MeJA, however, is widely used in application on plants, either to overcome a JA deficiency or to enhance the JA response.

Further JA derivatives are the sulfated JA species. Among the 18 sulfotransferases of *A. thaliana* the sulfotransferase 2a (ST2a) was identified as the protein that is able to specifically convert 11-hydroxy-JA (11-OH-JA) and 12-hydroxy-JA (12-OH-JA) to the corresponding sulfated derivatives, referred to as HSO_4-JA (Gidda et al. 2003), as shown in Figure 4. The expression of ST2a is mediated by OPDA, JA, JA-Ile and 12-OH-JA. The sulfotransferase reaction is mediated by a family of four Adenosin-5'-phosphosulfate-kinases (APK). For the double mutant *apk1apk2* a 5-fold reduction in 12-OH-JA and 12-HSO_4-JA was shown. At the same time an increase of 12-gylcosylester-JA (12-O-Glc-JA) was monitored (Mugford et al. 2009). These findings indicate that 12-OH-JA is derivatized to either 12-O-Glc-JA or 12-HSO_4-JA and these reactions happen simultaneously. Another indication for a link between sulfur metabolism and 12-OH-JA was found upon the analysis of the *fou8* mutant. The *fou8* loss of function allele of adenosine bisphosphate phosphatase FIERY1 leads to many different metabolic phenotypes including an increased enzymatic oxygenation of fatty acids and increased jasmonate synthesis. An elevated LOX2 level leads to the increase in oxidized fatty acids and the constitutive activation of the JA synthesis. Thus, the *fou8* plants exhibit a JA-phenotype of shorter roots and smaller growth (Rodriguez et al. 2010). The conversion of 3'phosphoadenosine-5'-phosphate (PAP) to AMP, a side-product of the reaction of sulfotransferase, is affected as well. As a result, the sulfur-metabolism and thus the sulfatation of glucosinolates and 12-OH-JA is dramatically changed (Lee et al. 2012). The triple mutant fou8apk1apk2 leads to a suppression of the fou8 phenotype. This fact indicates that the sulfur futile cycle affects the LOX activity, which is necessary for JA biosynthesis. This, however,

is a convincing evidence for a crosstalk between sulfur metabolism and JA biosynthesis (Rodriguez et al. 2010).

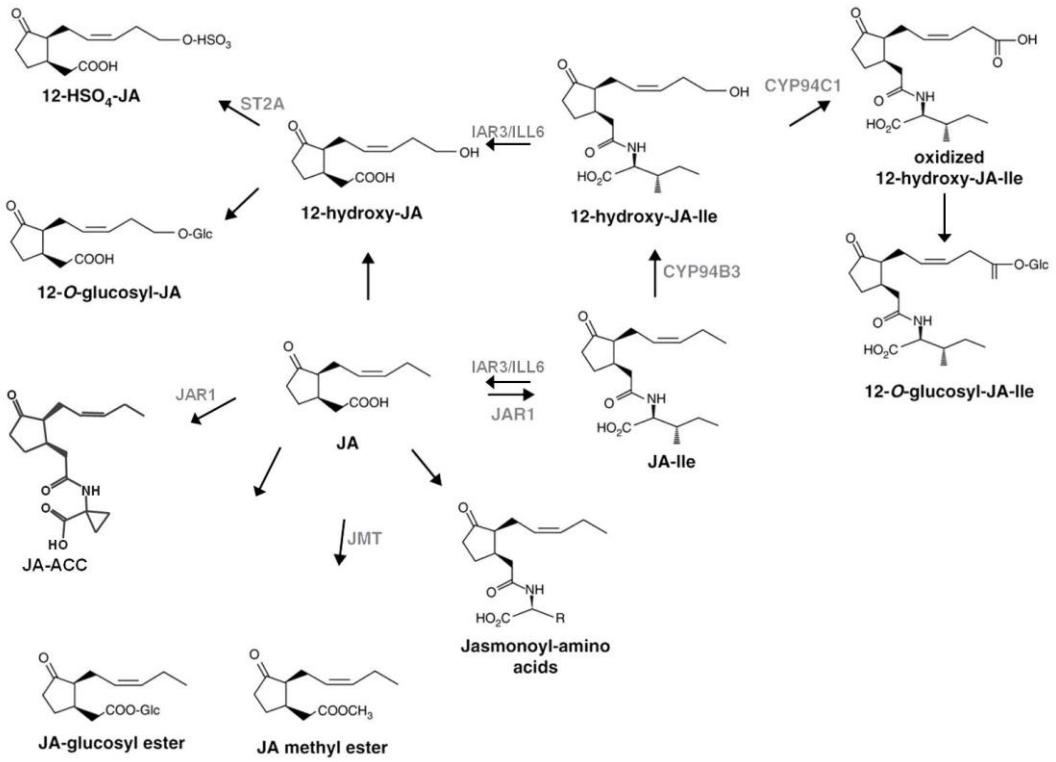

Figure 4: Metabolic fate of jasmonic acid (JA) and jasmonoyl-isoleucine (JA-Ile).
Enzymes which have been cloned and characterized are given in grey, JA-amino acid synthetase (JAR1), JA carboxy-methyl-transferase (JMT), sulfotransferase 2A (ST2A), indole acetic acid-alanine (IAA-Ala) resistant 3 (IAR3), IAA-leucine resistant (ILR)-like gene 6 (ILL6), Cytochrome P450B3 (CYP94B3) and Cytochrome P450C1 (CYP94C1). Jasmonoyl-1-amino-cyclopropane-1-carboxylic acid conjugate (JA-ACC). Modified from Wasternack 2013.

The glycosylated derivatives are another group of jasmonate derivatives. They exist as glycosyl-esters, which are assumed to be inactive. The glycosylation at the COOH-group of the JA molecule blocks the position for further derivatization by JAR1. Other glycosylated derivatives are O-glycosylated at C11 or C12, they are known to accumulate rapidly upon wounding of leaves (Glauser et al. 2008; Miersch et al. 2008). For unwounded soybean leaves it has been shown, that the accumulation of 12-O-Glc-JA can be up to threefold higher than JA. In wounded tomato leaves the accumulation of 12-O-Glc-JA happens subsequently to the accumulation of JA and OH-JA (Miersch et al. 2008). The exact biological role of 12-O-Glc-JA has not been described. It is hypothesized as a transport form according to glycosides of salicylic acid (SA) and benzoic acid (Wasternack and Hause 2013). In the Mimosoideae-plants like *Albizzia* and *Samanea saman* 12-O-Glc-JA was identified as a COI/JAZ independent leaf closing factor (LCF) (Nakamura et

al. 2011). The involvement of JA-derivatives in nyctinastic leaf movement has been confirmed by another group. Gene expression data of a *Medicago truncatula* mutant with a defective pulvinus revealed down-regulation of genes involved in JA synthesis and metabolism (Zhou et al. 2012).

1.1.2.1 JA-Ile hydroxylation –JA signal attenuation?

Plant hormones are orchestrated in spatial and temporal manner. Thus, the homeostasis of an active compound has to be tightly regulated. These regulations have to be present in both, biosynthetic and catabolic branches (Heitz et al. 2012). Plants have mechanism available to attenuate the JA signal. One example are the JAZ genes, that are co-induced with the JA biosynthetic pathway to rapidly restore the repression at the promoter of JA responsive genes (Chung et al. 2008). For other plant hormones, the involvement of cytochrome P450 (CYP) enzymes in activation and deactivation is well known (Mizutani and Ohta 2010). The over-expression of CYP707, for example, depletes the ABA pools upon germination, resulting in hyper-dormancy. CYP707 was thus identified as ABA-8'-hydroxylase (Saito et al. 2004). For over-expression of CYPs that are involved in brassinolide- and gibberellin-oxidation also severe growth defects are known (Turk et al. 2005; Zhu et al. 2006).

The hydroxylation reaction of JA-Ile at the terminal carbon atom of the pentenyl side chain has been recently ascribed to the three enzymes, CYP94B1, CYP94B3 and CYP94C1 (Heitz et al. 2012; Kitaoka et al. 2011; Koo et al. 2011; Koo et al. 2014), see Figure 4. Originally described as fatty acid hydroxylases (Benveniste et al. 2006), they fulfill their function at the fatty acid-derived phytohormone JA-Ile. Another way of deactivation of the JA-Ile signal is described on a different route. In case of auxin, for instance, the conjugation to amino acids serves as inactivation or storage of the active compound (Piotrowska and Bajguz 2011). For JA-Ile the counter reaction might be a key to signal inactivation, thus a de-conjugation reaction is likely (Widemann et al. 2013). A gene family of auxin amidohydrolases came into the focus of research. For several of the 7 family members a function in de-conjugation of auxin is known (Rampey et al. 2004). The gene indole acetic acid resistant 3 (IAR3) encodes a indole-acetic acid-alanine (IAA-Ala) hydrolase (Davies et al. 1999) that was initially described as a wound and JA-induced gene and formerly named Jasmonate Responsive 3 (JR) (Titarenko et al. 1997). For tobacco a herbivory-induced IAR3-related JA-Ile hydrolase (JIH1) was described, which attenuates the JA-Ile signal by de-conjugating the JA-Ile (Woldemariam et al. 2012). For *A. thaliana* two amidohydrolases, IAR3 and