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Most people say that is the intellect which makes a great scientist.
They are wrong, it is character.

-Albert Einstein-

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Chapter 1

General introduction

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1.1 Plant defense

One of the most evident sets of adaptations in the history of life is plant defense, as plants represent the main source of energy to support other organisms and are the basis of most food webs (Koornneef and Pieterse, 2008). An estimated 300,000 plant species on Earth are attacked by a multitude of organisms including fungi, bacteria, oomycetes, herbivores, nematodes, insects and viruses. Higher plants thus had to evolve sophisticated self-defense mechanisms modulated by the ecological context allowing them to survive and to withstand not only pests and diseases (van Loon, 2015; Yan and Xie, 2015) but also all kinds of abiotic stresses such as drought, flooding, salinity, light intensity and quality, temperature and nutrient shortage (Wasternack and Strnad, 2015). Even if plants are permanently surrounded by a large number of microorganisms, only few are able to attack any given plant species (Bari and Jones, 2009). Plants employ different strategies to defend themselves from pathogens. The first line of defense includes direct defense with constitutive chemical and physical barriers such as trichomes, cuticular waxes or thorns that deter insects and herbivores either physically or in combination with secondary metabolites (Howe, 2004). The second line is an inducible defense that develops after the plant is damaged. This inducible defense has two stages: one is an immediate reaction of constitutive defense compounds known as phytoanticipins (VanEtten *et al.,* 1994). The second includes the *de novo* biosynthesis of phytoalexins with biologically activity against a large number of pathogens (Ahuja *et al.,* 2012). Both types of induction include the production of various secondary metabolites, low molecular weight compounds, that negatively affect pathogens by restricting the infection progress (Piasecka *et al.,* 2015) and affect herbivores by reducing food intake or causing food avoidance (Iason, 2005). The release of volatile organic compounds (VOCs) to attract predators or parasitoids of herbivores is another example of inducible defense response (Svoboda and Boland, 2010). VOCs are considered as secondary metabolites and are usually small size organic compounds such as terpenoids, aldehydes, methanol, acetone, methyl-ethyl-ketone (MEK), methyl-vinyl-ketone (MVK), methyl jasmonate (MeJA), methyl salicylate (MeSA), ethylene (ET) and even sulfur compounds derived from the hydrolysis of glucosinolates in Brassicaceae plants (Vivaldo *et al.,* 2017). Field observations have shown plant-to-plant communication through the emission of VOCs. *Listening* plants induce a defense response making them resistant to most pests and diseases, giving them a selective advantage over plants that did not receive such information. Thus, plants perceive the attacker and translate this perception into an immune response that includes an indirect defense by attracting predators through the production of VOCs (Dicke *et al.,* 2003; Karban *et al.,* 2000; Dolch and Tscharntke, 2000). VOCs emissions can be either constitutively stored in specialized compartments, which are released after wounding, or induced in response to stress resulting in *de novo* biosynthesis involving the activation of a large number of genes (Niinemets *et al.,* 2013).

When the constitutive lines of defense are compromised, plants rely on an inducible defense, a basal mechanism called *innate immune system*, which is triggered upon detection of microbial

signatures such as peptides, lipophilic fatty acids and oligosaccharides, and the so called pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs) such as flagellin and chitin. These inducible defenses induce the production of endogenous damage-associated molecular patterns (DAMPs), among others, cell wall fragments from the host's plant cells, released by lytic enzymes from herbivores or microorganisms (Yu *et al.,* 2017). These cell wall fragments in turn, are recognized by transmembrane pattern recognition receptors (PRRs) and activate responses known as pattern-triggered immunity (PTI) that includes activation of mitogen-activated protein kinase (MAPK) cascades and calcium-dependent protein kinases (CDPK) (Li *et al.,* 2016). The activation of these cascades induce multiple intracellular defense responses that include downstream transcriptional reprogramming such as callose deposition to reinforce the cell wall at the site of infection, and a burst of calcium, an increase in the fluxes in phospholipid production and their turnover, and the production of the phytohormones jasmonic acid (JA), salicylic acid (SA), ET, reactive oxygen species (ROS), nitric oxide (NO) and phytoalexins production. All are the hallmarks of PTI that usually arrests infection and disease progression (Boller and Felix, 2009; Jones and Dangl, 2006; Fürstenberg-Hägg *et al*., 2013). Additional local physiological changes that do not require transcriptional reprogramming include actin filament remodeling (Li *et al.,* 2015). However, successful pathogens are able to suppress PTI leading to an effector-triggered susceptibility (ETS) although plants are able to recognize such effectors and activate a *secondary immune response* called effector-triggered immunity (ETI) leading to disease resistance (Chisholm *et al.,* 2006). ETI is elicited via intracellular nucleotide-binding domain leucine-rich repeat-containing receptors (NLRs) and amplifies PTI basal transcriptional programming that is mostly followed with localized programmed cell death and is referred to as the hypersensitive response (HR) (Zebell and Dong, 2015). The HR is characterized by the development of necrotic lesions at the site of the damage where a quick reaction involves encircling the point of infection making it nearly impossible for the pathogen to spread any further (Dempsey and Klessig, 2012). HR is associated with the resistance response (RR) and triggered when the host possesses a dominant *R* gene that corresponds to a pathogens' dominant *Avr* gene. The HR is highly dependent on the host-pathogen interaction explained by the fact that not all RR are the same due to differences in signaling strength (*R*-to-*Avr* interaction) and activated downstream defenses (Greenberg and Yao, 2004).

In parallel with the activation of the plant responses at the site of infection, also a systemic defense response is induced in distal parts of the plant to protect these undamaged tissues from a subsequent attack. Long-distance stress response signaling can be divided into three major stages. The first stage is the production of a stress signal during the stress response at the damaged site. During the second stage, the stress signal travels from the infested site to distant tissues via the vascular system, cell-to-cell transport or via the gas phase in the leaves or by air from plant to plant. In the third stage, distant tissues perceive and interpret the signal and start an appropriate response by inducing the expression of the resistance related genes (Champigny and Cameron, 2009). This spread of a long-

lasting resistance after a primary attack is known as *systemic acquired resistance* (SAR). SAR is an inducible defense mechanism that provides "immunization" to undamaged distant plant tissues from subsequent pathogen attacks where these tissues are primed to turn on defenses faster (Shah and Zeier, 2013). SAR has been shown to be transferred to the progeny probably by the epigenetic expression of defense-related genes in *Arabidopsis* (Luna *et al.,* 2012). SAR includes the biosynthesis of SA that after reaching non-damaged tissues, induces the expression of the molecular marker of SAR *PATHOGENESIS-RELATED 1* (*PR1*) and *NON-EXPRESSER OF PR GENES1* (*NPR1*) genes after interacting with the TGACG sequence of specific TGA transcription factors (TF) and then inducing PR proteins that confer resistance to a broad spectrum of microorganisms (Durrant and Dong, 2004).

1.2 Phytohormones in plant defense

Wounds induced by feeding, egg deposition or physical injury can trigger a plant defense response that will repel herbivores by releasing toxic secondary metabolites with different biological activities; induce JA production that elicit the synthesis of VOCs that attract parasitoids (War *et al.,* 2012); and/or inhibit microbial growth, to name a few examples. Plant pathogens are generally classified according to their lifestyles as necrotrophs and biotrophs where the former destroys the host cells and feed on contents and the latter derive nutrients from living host tissue through haustoria that invaginate the host cell without disrupting it. Most plant pathogens display both lifestyles and are then called hemibiotrophs (Pieterse *et al.,* 2009). The action and coordination of stress response events require signal transduction, that is, the communication from the site of damage to either distal organs of the plant (long distance), to adjacent cells or same cells. The signaling process is mediated by small signal molecules *i.e.* phytohormones, that work at low concentrations and are mostly transported via the vascular system along with nutrients and water (Lacombe and Achard, 2016). Their biosynthesis and/or activation, transport and binding with receptors to develop a response and their later removal or degradation, are the basic events during the plant stress response. Phytohormones include auxins (AUX), cytokinins (CK), gibberellins (GAs), abscisic acid (ABA), ET, nitric oxide (NO), strigolactones, brassinosteroids (BRs), SA, JA and a number of biologically active hormone-peptides such as systemin, cyclotides, CLAVATA3 (CLV3), S-locus cysteine protein (SCP), phytosulfokine (PSK), ENOD40, PEP1, Rapid Alkalization Factor (RALF), thionines and plant natriuretic peptide (PNP), to name a few. All are integral to plants' biological activities which is shaped by the combined interaction of different phytohormones (Wang and Irving, 2011; Marmiroli and Maestri, 2014). An example in plant defense is the signaling pathways mediated by phytohormones that interact synergistically like JA/ET (Pan *et al.,* 2018) or antagonistically like SA and JA (Thaler *et al.,* 2012) (Fig. 1) in the so-called *crosstalk* determining the outcome of downstream transcriptional responses. The result of this conserved and non-overlapping crosstalk ultimately determines plant survival and fitness and contributes to the epigenetic memory or *priming* for future challenges (Ramirez-Prado *et al.,* 2018; Nemhauser *et al.,*

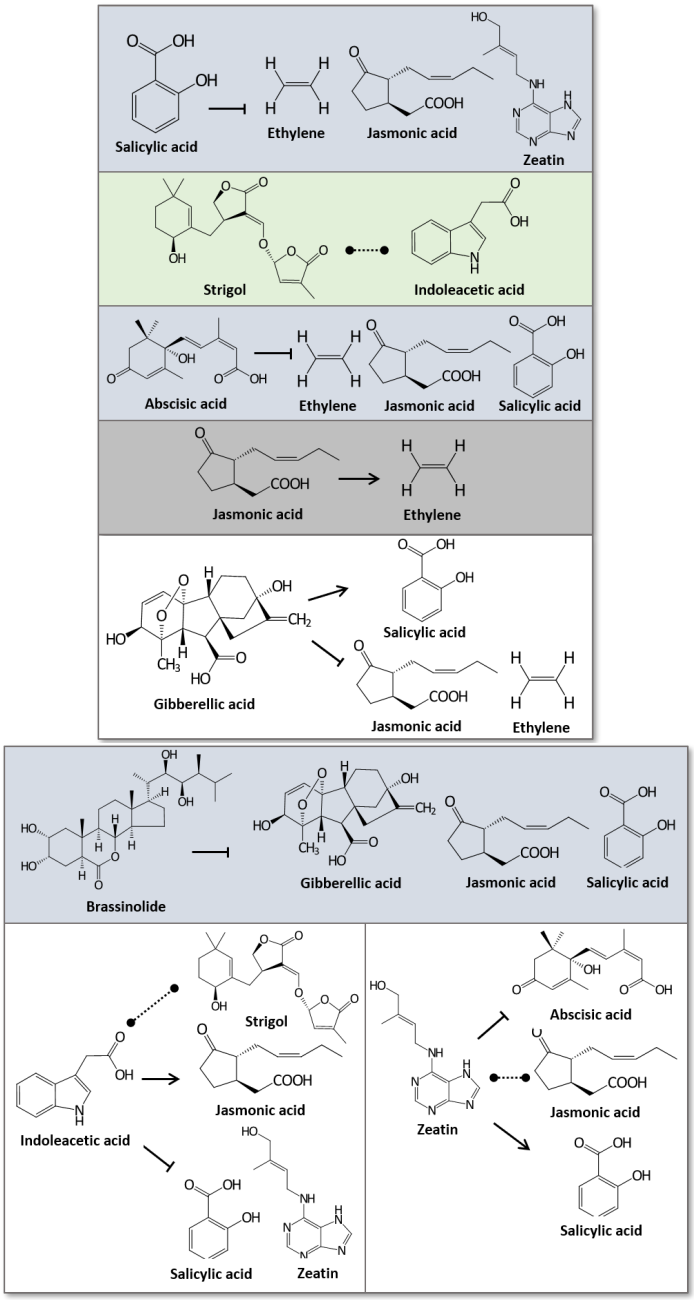

Figure 1. A simplified overview of phytohormones crosstalk and interactions in plants (adapted from Shigenaga *et al*., 2017). Biotrophic pathogens like fungi or oomycetes activate the salicylic acid (SA)-mediated response which in turn represses jasmonic acid/ethylene (JA/ET) whereas necrotrophic pathogens activate the JA/ET response. These phytohormones contribute to the plant's immunity by up- or down-regulating either the SA or JA/ET branches. A synergistic interaction between phytohormones is shown in grey, an antagonistic interaction is shown in blue and phytohormones with multiple and different interactions are shown in white. Either an antagonistic or a synergistic interaction to be confirmed is depicted with a dashed line in green.

2006; Berens *et al.,* 2017).

1.3 Jasmonic acid

Of particular interest are JA and its derivatives, known as jasmonates (JAs), with a central role as signaling molecules, not only in plant growth and development but also in plant responses to biotic and abiotic stress (Devoto and Turner, 2003). The abbreviation JA is used here for the stereoisomer that is biologically active as phytohormone, (3*R*, 7*S*)-jasmonic acid. Early observations in *Arabidopsis* showed that the exogenous application of MeJA but not SA resulted in the production of the defense-related proteins and thionins, normally induced after necrotrophic attack (Penninckx *et al.,* 1996; Epple *et al.,* 1995). Observations in the *Arabidopsis coronatine insensitive-1 (coi1)* mutant defective in the expression of JA-regulated genes, are susceptible to infection by *Alternaria brassicicola* and *Botrytis cinerea* where MeJA application conferred resistance to these necrotrophic pathogens thus confirming that JA is a positive regulator in plant immunity (Thomma *et al.,* 1998).

JAs belong to the family of plant oxylipins which are oxidation products of the polyunsaturated fatty acids (PUFA) linoleic acid (C18:2), α-linolenic acid (C18:3) and hexadecatrienoic acid (C16:3) (Joyard *et al.,* 1998a), the substrates for JA biosynthesis. Plant oxylipins comprise a structurally diverse group of metabolites that can be found as free forms or esterified in galactolipids or phospholipids in thylakoids (Genva *et al.,* 2019; Kombrink, 2012). The major biosynthetic pathways of free and esterified oxylipins start with the hydrolysis of C18:2, C18:3 and C16:3 esterified in the thylakoid glycerolipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) (Griffiths, 2015; Bleé, 2002). Furthermore, upon wounding leaves of *Arabidopsis*, MGDG and DGDG serve as substrate for the *in situ* transformation to the JAs 12-*oxo*-phytodienoic acid (OPDA) and dinor-12-*oxo*-phytodienoic acid (dnOPDA) (Nilsson *et al.,* 2012; Böttcher and Weiler, 2007) by the enzymatic action of 13-lipoxygenase (13-LOX), allene oxide synthase (AOS) and allene oxide cyclase (AOC) on C18:3 or C16:3.

One of the first biosynthetic pathways of plant oxylipins to be characterized was that of JA. Biosynthesis and metabolism of JA merge diverse topics including thylakoid galactolipids, FA biosynthesis and JA metabolism, all integral subject matters in this study. Below, we will briefly discuss FA biosynthesis and oxylipins as the early steps in JA biosynthesis, the formation of JA as the final step in JA biosynthesis and lastly, JA metabolism.

1.3.1 The early steps in jasmonic acid biosynthesis: fatty acids

The *de novo* FA biosynthesis occurs in the chloroplast and is completed in the endoplasmic reticulum (ER) (Ohlrogge and Browse, 1995) although plant mitochondria are capable of limited FA biosynthesis mostly of octanoic acid (C8:0), the precursor of lipoic acid (Wada *et al.,* 1997; Shintani and Ohlrogge, 1994). The major FA found in chloroplasts have chain lengths of 16 or 18 carbons and contain zero to

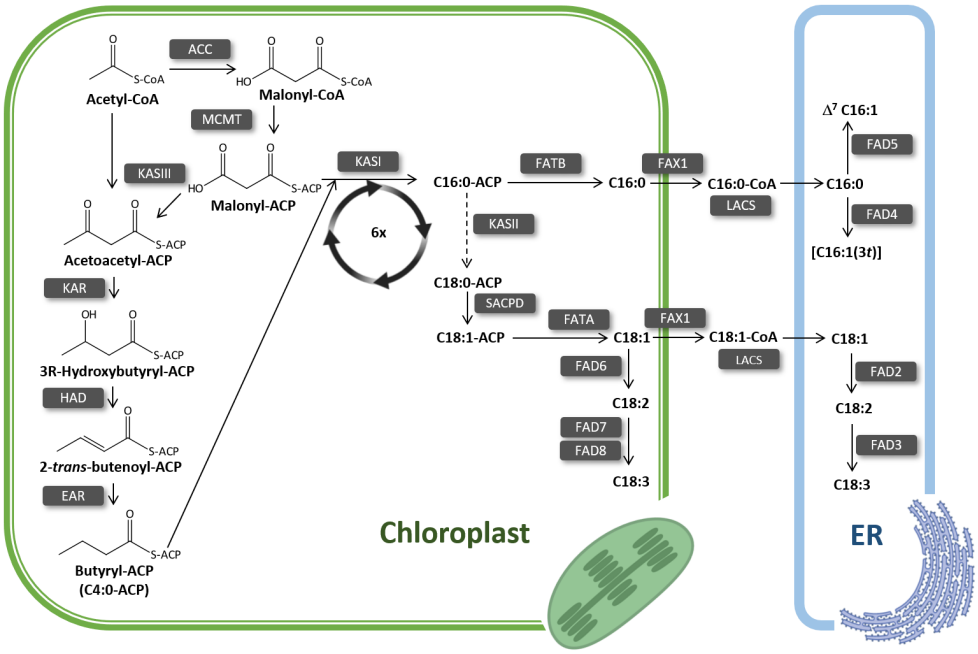

Figure 2. Fatty acid biosynthesis in plants (adapted from Hölzl and Dörmann, 2019). Arabidopsides A-G are present in leaves of *Arabidopsis thaliana* and few other plants and are defined as oxidized monogalactosyldiacylglycerols (MGDG) or digalactosyldiacylglycerols (DGDG) containing at least one residue of 12-*oxo*-phytodienoic acid (OPDA) or dinor-12-*oxo*-phytodienoic acid (dnOPDA). These two jasmonic acid (JA) precursors, are both esterified in different *sn*-positions, thus contributing to their structural diversity. Linolipins A-D occurring in leaves of *Linum usitatissimum*, are oxidized MGDG and DGDG, that can contain one α-linolenic acid chain and one or two (ω5*Z*)-etherolenic acid residues.

three double bonds all in a *cis* configuration and include palmitic acid (C16:0), oleic acid (C18:1), C18:2, C18:3 (Hölzl and Dörmann, 2019). Additionally, MGDG containing C16:3 and phosphatidylglycerol (PG) containing Δ^3-*trans*-hexadecenoic acid [C16:1(3*t*)] can be found in *Arabidopsis* (Mongrand *et al.,* 1998). Membrane glycerolipids have FA attached to the *sn*-1 and *sn*-2 positions of the glycerol backbone and a polar head group attached to the *sn*-3 position leading to a vast structural diversity of glycerolipids (Joyard *et al.,* 1998b).

In plants, FA biosynthesis is catalyzed by acetyl-CoA carboxylase (ACC) and FA synthase (FAS) where all carbon atoms found in FA are derived from the direct product of photosynthesis acetyl-coenzyme A (CoA). The first reaction is catalyzed by the ACC complex that forms malonyl-CoA from the condensation of acetyl-CoA and CO_2. Second, malonyl-CoA is transferred to acyl carrier protein (ACP) by the enzyme malonyl-CoA:ACP malonyltransferase (MCMT). FA are produced by two carbon elongation reactions using malonyl-ACP as the donor. In the first elongation reaction, acetyl-CoA is condensed with malonyl-ACP to produce acetoacetyl-ACP, catalyzed by 3-ketoacyl-ACP synthase 3 (KASIII) in the FAS complex, with the release of CO_2 and the delivery of a ketoacyl-ACP elongated

by two carbon units. Next, acetoacetyl-ACP is reduced in the keto group to a hydroxyl group, this reaction yields 3R-hydroxybutyryl-ACP and is catalyzed by the enzyme 3-ketoacyl-ACP reductase (KAR). Hydroxyacyl-ACP dehydratase (HAD) forms an enoyl group into 2-*trans*-butenoyl-ACP. This product is reduced again to butyryl-ACP by enoyl-ACP reductase (EAR) yielding an elongated acyl-ACP *i.e.* butyryl-ACP (C4:0-ACP) (Brown *et al.,* 2009; Rawsthorne, 2002) (Fig. 2).

Whereas KASIII is the active enzyme in the first cycle of elongation from acetyl-ACP to butyryl-ACP, KASI catalyzes the elongation cycles from C4:0-ACP to C16:0-ACP and KASII performs the step from C16:0-ACP to C18:0-ACP; the latter enzyme is also responsible for the C16:C18 ratio (Hölzl and Dörmann, 2019). The FA thioesterases A (FATA) and FATB catalyze the chain termination by the hydrolysis of the thioester linkage between the acyl group and ACP to release free FA, which is the most common mechanism (Rahman, 2014). The 30 enzymatic reactions that produce C16 and C18 FA take place in the stroma of plastids. Subsequent desaturation reactions can take place in the plastid and in the ER. Desaturation of C18:0-ACP to C18:1-ACP or C16:0-ACP to C16:1-ACP occurs in the plastid via the stromal SSI2-encoded stearoyl-acyl carrier protein (ACP) desaturase (SACPD) that introduces a *cis* double bond into the acyl-ACP in C9 (Lim *et al.,* 2017). The assembly of C16 and C18 saturated and unsaturated FA into glycerolipids occurs either via the *prokaryotic* pathway of lipid biosynthesis that takes place in the chloroplast or via the *eukaryotic* pathway, where C16:0 and C18:1 are exported by fatty acid exporter 1 (FAX1) from the chloroplast to the cytosol as CoA thioesters, catalyzed by long-chain acyl-coenzyme A synthetase (LACS), and are later assembled into glycerolipids in the ER. Desaturation of membrane glycerolipids is catalyzed by membrane bound desaturases of the chloroplast and the ER. Further desaturation of C18:1 to C18:2 is catalyzed by FAD2 in the ER and FAD6 in the plastid while desaturation of C18:2 to C18:3 is catalyzed by FAD3 in the ER and FAD7/FAD8 in the plastid (McConn *et al.,* 1994). Desaturation of C16:0 to [C16:1(3*t*)] is catalyzed by FAD4 that introduces of the Δ^3-*trans* double bond into C16:0 at position *sn*-2 in the carboxylic group of PG (Gao *et al.,* 2009; Haverkate *et al.,* 1964) whereas FAD5 is responsible for the synthesis of Δ^7 C16:1 (Browse *et al.,* 1985) (Fig. 2). The desaturation of the FA chains is an important characteristic for lipid composition. In this way, plants can be classified according to the composition of the (*n*-3) trienoic FA *cis*-7,10,13-hexadecatrieonic acid (C16:3) or *cis*-9,12,15-octadecatrienoic acid (C18:3), esterified in the *sn*-2 position in glycolipids in photosynthetic tissues (Mongrand *et al.,* 1998) as either "C16:3 plants" like the Brassicaceae plant family or "C18:3 plants" like *Spinacia oleracea*, although this classification is species-dependent (Somerville and Browse, 1991).

Oxylipins are signaling molecules generated by the enzymatic oxidation of C18 and C16 PUFA by one, two or four atoms of oxygen catalyzed by the cytochrome P450 CYP74, LOX and cyclo-oxygenase-like enzymes, respectively. Biosynthesis of free and esterified oxylipins occur upon stress (Kourtchenko *et al.,* 2007; Buseman *et al.,* 2006; Vu *et al.,* 2012) or during certain plant developmental processes like flower development (Hause *et al.,* 2000). This oxidative metabolism leads to *hydroxides*,

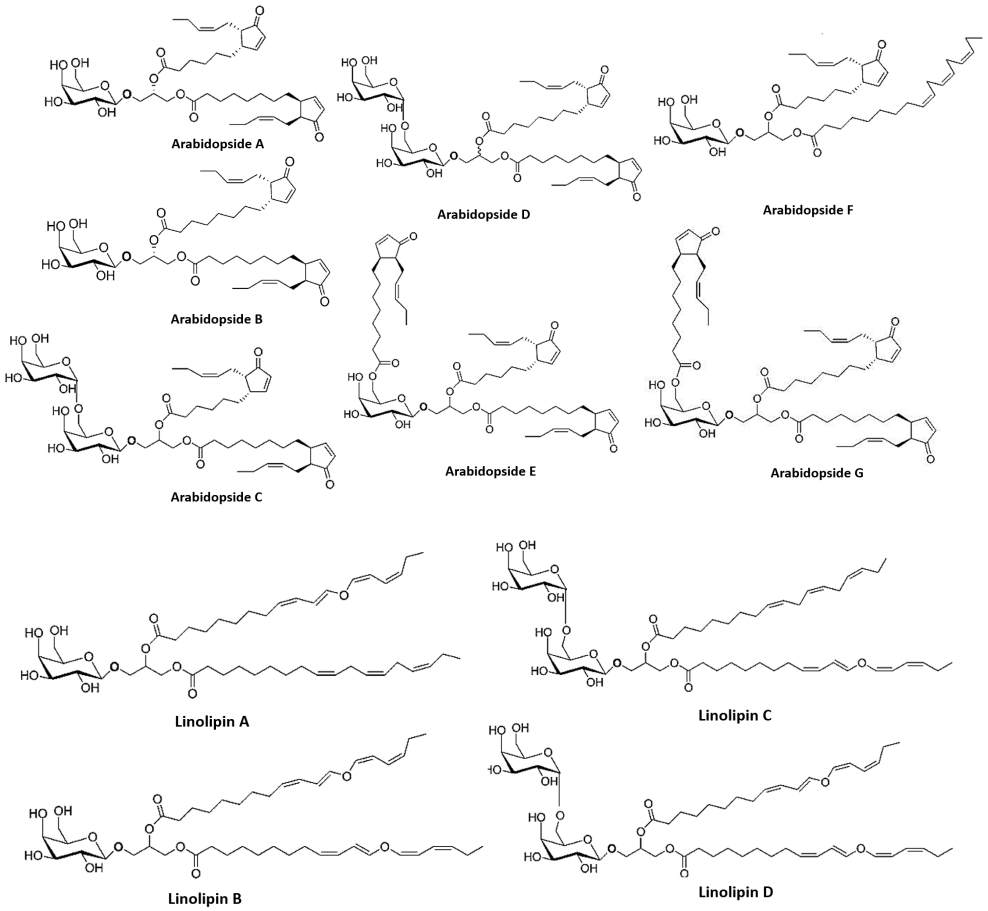

Figure 3. The esterified oxylipins arabidopsides and linolipins (adapted from Genva *et al*., 2019). Arabidopsides A-G, present in leaves of *Arabidopsis thaliana* and few other plants, are defined as oxidized monogalactosyldiacylglycerols (MGDG) or digalactosyldiacylglycerols (DGDG) containing at least one residue of 12-*oxo*-phytodienoic acid (OPDA) or dinor-12-*oxo*-phytodienoic acid (dnOPDA). These two jasmonic acid (JA) precursors, are both esterified in different *sn*-positions, thus contributing to their structural diversity. Linolipins A-D occurring in leaves of *Linum usitatissimum*, are oxidized MGDG and DGDG, that can contain one α-linolenic acid chain and one or two (ω5*Z*)-etherolenic acid residues esterified either in the *sn*-1 or in the *sn*-2 positions.

9- and 13-hydroperoxides FA that include aldehydes, alcohols or *oxo*-acids, divinyl ethers of FA, hydroxy FA, keto FA, epoxyhydroxy FA and JAs and lastly, *α-hydroperoxides aldehydes* (Bleé, 2002; Deboever *et al.,* 2020).

Esterified oxylipins such as the oxidized glycolipids named arabidopsides (A-G), are found exclusively in leaves of *Arabidopsis* where their formation occurs upon biotic and abiotic stress. They contain dnOPDA/OPDA esterified in the *sn*-1 and *sn*-2 position to MGDG and DGDG (Hisamatsu *et al.,* 2003; 2005; Andersson *et al.,* 2006; Kourtchenko *et al.,* 2007; Glauser *et al.,* 2008; Ibrahim *et al.,* 2011). Arabidopsides have so far been found in some *Arabidopsis* species (Nakajyo *et al.,* 2006;

Böttcher and Weiler, 2007), *Olimarabidopsis pumila* (Nilsson *et al.,* 2015), *Ipomoea tricolor* (Ohashi *et al.,* 2005), *Cirsium arvense* (Hartley *et al.,* 2015), *Melissa officinalis* (Zábranská *et al.,* 2012), *Nasturtium officinale* and *Erucastrum canariense* (Pedras and To, 2017). A second family of oxidized galactolipids named linolipins (A-D), are found in leaves of *Linum usitatissimum* and their levels too, increase upon biotic stress. Linolipins are also composed of MGDG and DGDG with at least one divinyl ether residue and a (ω5*Z*)-etherolenic acid chain esterified either in the *sn*-1 or in the *sn*-2 positions. Linolipins A and C have one C18:3 chain and a (ω5*Z*)-etherolenic acid residue whereas linolipins B and D contain two (Chechetkin *et al.,* 2009; 2013) (Fig. 3). Additionally, dnOPDA/OPDA-containing phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and phosphatidylcholine (PC) species have been reported in *Arabidopsis* leaves during the HR or wounding (Buseman *et al.,* 2006; Vu *et al.,* 2012; 2014a; 2014b; 2015). Other lipid classes such as sulfovinyldigalactosylglycerol (SQDG) and phosphatidylinositol (PI) might be targets of enzymatic conversion into OPDA-containing lipids during the HR (Zoeller *et al.,* 2012; Andersson *et al.,* 2006; Kourtchenko *et al.,* 2007).

Oxylipins are widely distributed in fungi, cyanobacteria, algae, mosses, ferns, plants, diatoms and animals in free forms, esterified to phospholipids or galactolipids or in combination with amino acids or methyl groups (Andreou *et al.,* 2009; Stonik and Stonik, 2015). Almost all oxylipins are biologically active against bacteria, fungi and oomycetes (León-Morcillo *et al.,* 2012), can minimize water loss like cutin (Granér *et al.,* 2003) or play a crucial role in plant defense-signaling pathways like the JAs dnOPDA and JA (Creelman and Mullet, 1997; Buseman *et al.,* 2006).

1.3.2 The final steps to jasmonic acid biosynthesis

The biosynthesis of JA includes at least 9 well-characterized enzymatic reactions that occur in the chloroplast and peroxisome. JA biosynthesis was first described in *Vicia faba* (Vick and Zimmerman, 1983) but occurs ubiquitously in monocotyledonous and dicotyledonous plants (Hamberg and Gardner, 1992; Meyer *et al.,* 1984), in corals (Brash *et al.,* 1987) and in *Chlorella* (Ueda *et al.,* 1991). In the first step, the PUFA C18:3 and/or C16:3, both esterified in galactolipids in the thylakoid, are released from their *sn*-1 position by phospholipase 1 (PLA_1), DEFECTIVE IN ANTHER DEHISCENCE 1 (DAD1) and DONGLE (DGL) (Ishiguro *et al.,* 2001). In the next step, 13-LOX inserts oxygen in position C13 to produce 13-(*S*)-hydroxyperoxy-octadecatrienoic acid (13-HPOT). The following enzyme is AOS, which is responsible for the third step that involves dehydration of 13-HPOT into the epoxy-octadecatrienoic acid (12,13-EOT). The conversion of this unstable allene oxide into (9*S*, 13*S*)-OPDA is the fourth step mediated by AOC catalyzing a cyclization reaction (Santino *et al.,* 2013). The parallel pathway of C16:3 also depends on 13-LOX, AOS and AOC to yield 11-(*S*)-hydroperoxy-hexadecatrienoic acid (11-HPHT), 10,11-(*S*)-epoxy-hexadecatrienoic acid (10,11-EHT) and dnOPDA, respectively (Schaller and Stintzi, 2008; Weber *et al.,* 1997). The carrier COMATOSE1/ PEROXISOMAL1/PEROXISOME ABC TRANSPORTER (CTS1/PXA1/PED3) is possibly involved

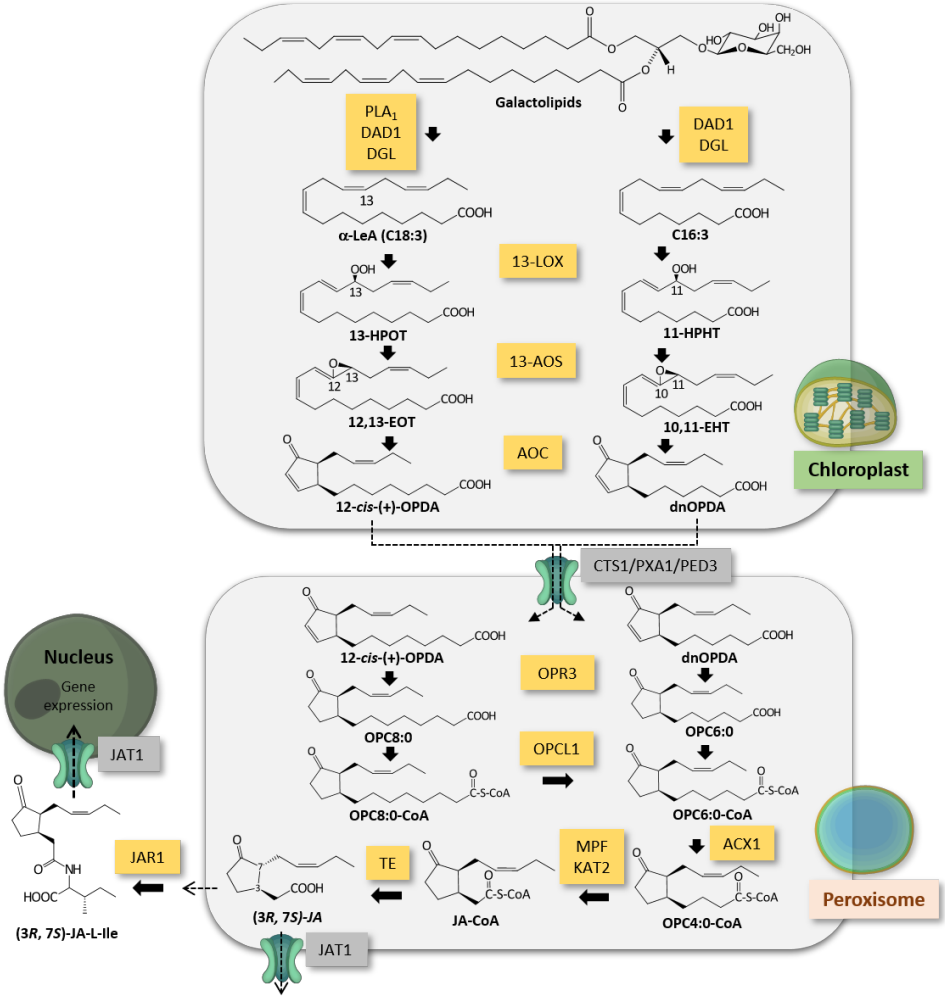

Figure 4. Biosynthesis of jasmonic acid and intracellular transport. Enzymes are indicated with a yellow background. Solid lines indicate an enzymatic reaction and dashed lines indicate intracellular transport. Biosynthesis of JA starts with the release of the precursors C18:3 or C16:3 from galactolipids in the thylakoid by lipases such as PAL_1, DGL and DAD1. Formation of OPDA and dnOPDA is catalyzed by 13-LOX, 13-AOS and AOC where the both JAs are transported to the peroxisome where a chain shortening pathway occurs starting with a reduction catalyzed by OPR3 to yield OPC8:0 or OPC6:0. These are activated to their CoA esters by OPCL1, which then undergoes three rounds of β-oxidation catalyzed by ACX1, MFP and KAT2 to yield (3*R*, 3*S*)-JA. JAR1 catalyzes formation of the amino acid conjugate JA-Ile from JA in the cytosol. JAT1 exports JA across the plasma membrane and imports JA-Ile into the nucleus. Abbreviations for compounds, transporters and enzymes listed in chronological order are: PLA_1, phospholipase 1; DAD1, DEFECTIVE IN ANTHER DEHISCENCE 1; DGL, DONGLE; α-LeA, α-linolenic acid; C16:3, hexadecatrienoic acid; 13-LOX, 13-lipoxygenase; 13-HPOT, 13-(*S*)-hydroxyperoxy-octadecatrienoic acid; 11-HPHT, 11-(*S*)-hydroxyperoxy-hexadecatrienoic acid; 13-AOS, allene oxide synthase; 12,13-EOT, epoxy-octadecatrienoic acid; 10,11-EHT, 10,11-(*S*)-epoxy-hexadecatrienoic acid; AOC, allene oxide cyclase; 12-*cis*-OPDA, *cis*-(+)-*oxo*-phytodienoic acid; dnOPDA, dinor-12-*oxo*-phytodienoic acid; CTS/PXA1/PED3 COMATOSE1/PEROXISOMAL1/PEROXISOME ABC TRANSPORTER; OPR3, OPDA reductase3; OPC8:0, OPCL1, OPC-8:CoA ligase1; 3-*oxo*-2-(2′(*Z*)-pentenyl)-cyclopentane-1-octanoic acid; OPC6:0, 3-*oxo*-2-(2′(*Z*)-pentenyl)-cyclopentane-1-hexanoic-acid; ACX1, acyl CoA

oxidase; MPF, multifunctional protein; KAT2, 3-ketoacyl-CoA thiolase; TE, thioesterase; JAT1, JASMONATE TRANSPORTER1; JAR1, JASMONATE RESISTANT1; JA-L-Ile, (3*R, 7S)*-jasmonoyl-L-isoleucine.

in the export of OPDA and dnOPDA from the chloroplast to the peroxisome (Zolman *et al.,* 2001). The next steps occur in the peroxisome where OPDA and dnOPDA are first reduced by the enzymatic activity of OPDA reductase3 (OPR3) (Wasternack *et al.,* 2006) and subsequently oxidized to 3-*oxo*-2-(2′(*Z*)-pentenyl)-cyclopentane-1-octanoic acid (OPC:8) or to 3-*oxo*-2-(2′(*Z*)-pentenyl)-cyclopentane-1-hexanoic-acid (OPC:6). This is followed by activation of OPC:8 and OPC:6 by esterification to OPC:8-CoA and OPC:6-CoA, respectively, by OPC8:CoA ligase 1 (OPCL1) (Kienow *et al.,* 2008). The sequential β-oxidation steps are catalyzed by an acyl CoA oxidase (ACX), with ACX1 playing the major role (Schilmiller *et al.,* 2007); a multifunctional protein (MFP) involved in the synthesis of OPC:4CoA and a 3-ketoacyl-CoA thiolase (KAT2) that catalyzes the formation of JA-CoA (Cruz-Castillo *et al.,* 2004). Subsequently, (3*R,* 7*S*)-JA is released by thioesterase (TE) where is then exported and converted in the cytosol by JASMONATE RESISTANT1 (JAR1) (Staswick and Tiryaki, 2004) into the biologically active (3*R,* 7*S)*-jasmonoyl-L-isoleucine (JA-Ile) (Fonseca *et al.,* 2009). Both JA and JA-Ile are later transported by JA/JA-ILE JASMONATE TRANSPORTER 1 (JAT1) across the plasma membrane or to the nucleus, respectively (Li *et al.,* 2017) (Fig. 4).

1.3.3 Metabolism of jasmonic acid

The metabolism of JA includes conversion into other compounds, some which have a specific function, whereas others are possibly only degradation products. *Methylation* yields the volatile MeJA (Seo *et al.,* 2001), which is not active on itself, but acts as a fast transport signal through diffusion in the gas phase in the leave tissues and between plants, demethylation is required for activation of the signal. *Conjugation* with amino acids at C-1 yields among others jasmonoyl-L-tryptamine (JA-Trp), jasmonoyl-L-valine (JA-Val), jasmonoyl-L-leucine (JA-Leu), jasmonoyl-L-tyrosine (JA-Tyr), JA-L-methionine (JA-Met), JA-L-alanine (JA-Ala), JA-L-glutamine (JA-Glu) and OPDA-isoleucine (OPDA-Ile) (Kramell *et al.,* 1995; 2005; Yan *et al.,* 2016; Floková *et al.,* 2016), although the biologically active signal compound during the stress response is (3*R,* 7*S)*-jasmonoyl-L-isoleucine (JA-Ile) (Fonseca *et al.,* 2009). The reverse reaction from JA-Ile to JA stops the defense signaling activity (Sánchez-Carranza *et al.,* 2016). *Reduction* of the C6 keto group yielding dihydrojasmonic acid (9,10-DHJA) and 6-HOJA. *Hydroxylation* at C-11 to 11-hydroxyjasmonic acid (11-HOJA) or C12 to 12-hydroxyjasmonic acid (12-HOJA). Hydroxylation seems to be the first step in the degradation of JAs. Hydroxylated JAs have no activity anymore as signal compounds (Kitaoka *et al.,* 2011; Aubert *et al.,* 2015; Heitz *et al.,* 2012; Miersch *et al.,* 2008), however, 12-hydroxyjasmonic acid (12-HOJA) was found to indirectly induce tuberization in potato plants (Simko *et al*., 1996). *Decarboxylation* of JA at position C-1 yields *cis*-jasmone (Koch *et al.,* 1999) and is involved in stress responses (Mueller *et al.,*

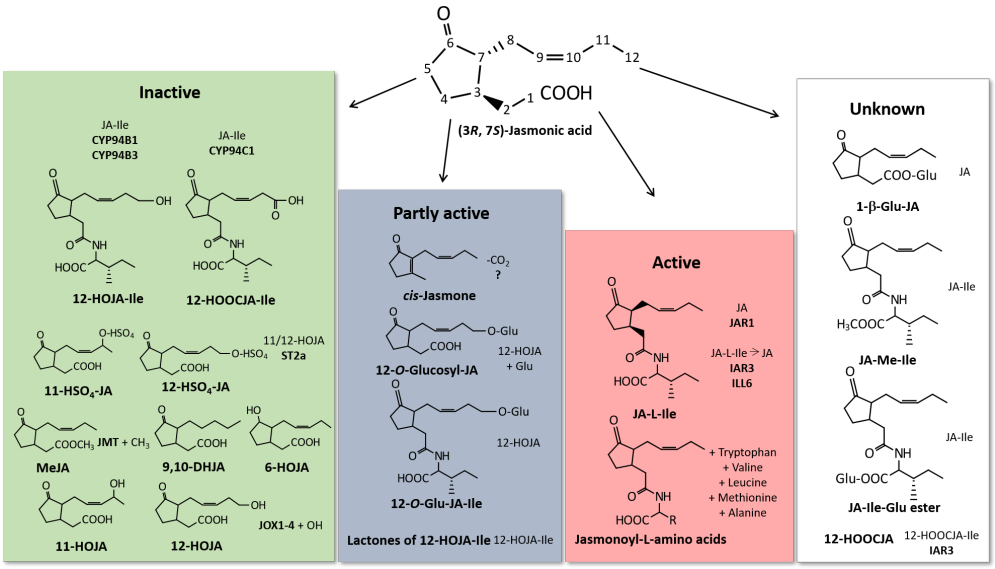

Figure 5. Some examples of the metabolism of jasmonic acid. Enzymes are indicated in bold along with their substrate. Inactive metabolites are shown in green, partly active metabolites are shown in blue, active metabolites are shown in red and unknown activity metabolites are shown in white. Inactive metabolites can undergo hydroxylation, reduction, sulfation and oxidation; partly active metabolites can undergo decarboxylation and glucosylation; active metabolites undergo conjugation to amino acids and metabolites with unknown activity include glucosylation, methylation and esterification. All colored boxes display the precursor in smaller font and on top of the enzyme's name, the enzyme and the product both in bold *e.g.* JA-Ile (precursor) + CYP94B1 (enzyme) = 12-HOJA-Ile (product). Abbreviations for compounds and enzymes are: JA, (3*R,* 7*S)*-jasmonic acid; JA-L-Ile, (3*R,* 7*S)*-jasmonoyl-L-isoleucine, CYP94B3, CYP94B1, CYP94C1, cytochrome P450 enzymes; 12-HOJA-Ile, 12-hydroxy-JA-Ile; 12-HOOCJA-Ile, 12-carboxyjasmonoyl-L-isoleucine; 11/12-HSO_4-JA, hydroxyjasmonic acid sulfate, 11/12-HOJA, 11/12-hydroxyjasmonic acid, ST2a, hydroxyjasmonate sulfotransferase; MeJA, methyljasmonate, JMT, carboxyl methyl transferase; JOX1-4, jasmonate-induced oxygenases; 12-*O*-glucosyl-JA, 12-*O*-glucosyl-jasmonic acid; 12-*O*-Glu-JA-Ile, 12-*O*-glucosyl-jasmonoyl-L-isoleucine; 12-HOJA-Ile, 12-hydoxyjasmonoyl-L-isoleucine; JAR1, JASMONATE RESISTANT1, IAR3, amidohydrolases IAA-ALA-RESISTENT3; ILL6, IAA-LEU RESISTENT-like6; 1-β-Glu-JA, 1-β-glucosyl-jasmonic acid; JA-Me-Ile, jasmonoyl isoleucine methyl ester, JA-Ile-Glu ester, jasmonoyl-L-isoleucine glucosyl ester.

1993) against herbivorous insects (Matthes *et al.,* 2010), it attracts pollinators, florivores (Etl *et al.,* 2016) and parasitic insects and repels some aphids (Birkett *et al.,* 2000). *O-Glucosylation* of JA-Ile and 12-HOJA, *sulfation* of 12-HOJA to 12-HSO_4-JA and formation of JA and JA-Ile glucosyl esters (Miersch *et al.,* 2008) and *esterification* of JA to lasiojasmonates (lasioJAs) (Andolfi *et al.,* 2014) are other metabolic events found in plants. Deactivation of JA/JA-conjugates includes the oxidative inactivation of JA-Ile leading to 12-HOJA-Ile (Koo *et al*., 2011; 2014; Kitaoka *et al.,* 2011; Heitz *et al*., 2012); *deconjugation* of 12-HOOCJA-Ile to 12-HOOC-JA and JA-Ile to JA (Sánchez- Carranza *et al.,* 2016); *hydrolyzation* to JA (Woldemariam *et al*., 2012; 2014); oxidation of JA-Ile to 12-carboxyjasmonoyl-L-isoleucine (12-HOOCJA-Ile) (Heitz *et al*., 2012; Koo and Howe, 2012; 2014); hydroxylation and oxidation of JA-Val, JA-Phe, JA-Leu and JA-Ile (Widemann *et al*., 2015; Kitaoka

et al., 2014; Woldemariam *et al*., 2012; 2014) (Fig. 5). All these reactions can lead to more than 30 different JAs that can be found in gymnosperms, monocots, dicots, bryophytes, lycophytes and algae (Pratiwi *et al.,* 2017; Ponce de León *et al.,* 2015; Jaulneau *et al.,* 2010; Li *et al.,*2009; Rowe *et al.,* 2014). After JA is modified *in planta* into the bioactive JA-Ile, there is no evidence that JA-Ile is transported via the vascular system directing the attention to other JAs that might be better candidates such as MeJA. Supporting evidence comes from the fact that isotopically labeled MeJA was found in the xylem and phloem upon application to tobacco leaves (Thorpe *et al.,* 2007) suggesting that MeJA is immediately converted to JA and JA-Ile. Similarly, isotopically labeled JA was also found in roots after application to leaves of *N. sylvestris* (Zhang and Baldwin, 1997) making both JAs long-distance signaling compounds, thus they can be transported via air (airborne) or vascular system (Heil and Ton, 2008). JA formed from MeJA is capable of inducing the production of VOCs that are an essential part of the plants' defense system (Tamogami *et al.,* 2008; 2012).

1.3.4 Signaling pathway of jasmonates

The core JA signaling module is composed by the Skp1/Cullin/F-box (SCF) E3 ubiquitin ligase complex (SCF^{COI1}) and the F-box protein CORONATINE INSENSITIVE1 (COI1) (Devoto and Turner, 2003). Upon stress, COI1 recognizes JA-Ile and triggers the ubiquitination and 26S proteasomal degradation of the repressor JASMONATE ZIM DOMAIN (JAZ) proteins (Chini *et al.,* 2007; Thines *et al.,* 2007; Yan *et al.,* 2007) (Fig. 6). JAZ proteins control gene expression by either directly inhibiting TF from binding to DNA or through the interaction with the Groucho/Tup1-type co-repressor TOPLESS (TPL) (Pauwels *et al.,* 2010) and the TPL-related proteins (TPR) that can interact directly with JAZ proteins or via the NOVEL INTERACTOR OF JAZ (NINJA) adaptor (Goossens *et al.,* 2017) (Fig. 6). JAZ proteins contain the highly conserved TIFY motif in the ZIM domain (Vanholme *et al.,* 2007) located in the central portion of the protein and the C-terminal JA-associated (Jas) domain, also highly conserved across the JAZ family (Yan *et al.,* 2007). The TIFY motif interacts with the N-terminal JAZ interaction domain (JID) of the basic helix-loop-helix (bHLH) Leu zipper TF MYC2 (Kazan and Manners, 2013). The Jas domain interacts with COI1 and MYC2 (Melotto *et al.,* 2008; Chini *et al.,* 2007; Wager and Browse, 2012). Transcriptional repression mediated by JAZ1, JAZ6 and JAZ9 involves the physical interaction with the chromatin modifying protein HISTONE DEACETYLASE6 (HAD6), an RPD3-type histone deacetylase in *Arabidopsis* that negatively affects the expression of *PLANT DEFENSINE 1.2* (*PDF1.2*)*, VEGETATIVE STORAGE PROTEIN 2* (*VSP2*)*, JASMONATE-INSENSITIVE 1* (*JIN1*) and *ETHYLENE RESPONSE FACTOR1* (*ERF1*) suggesting the involvement of HAD6 in JA-mediated stress response, flowering and senescence (Wu *et al.,* 2008), and HISTONE DEACETYLASE19 (HDA19), which is known to interact with TPL in a repression mechanism during embryogenetic development in *Arabidopsis* (Long *et al.,* 2006) (Fig. 6).

MYC2 is considered as a central regulator not only in plant development, reproduction,

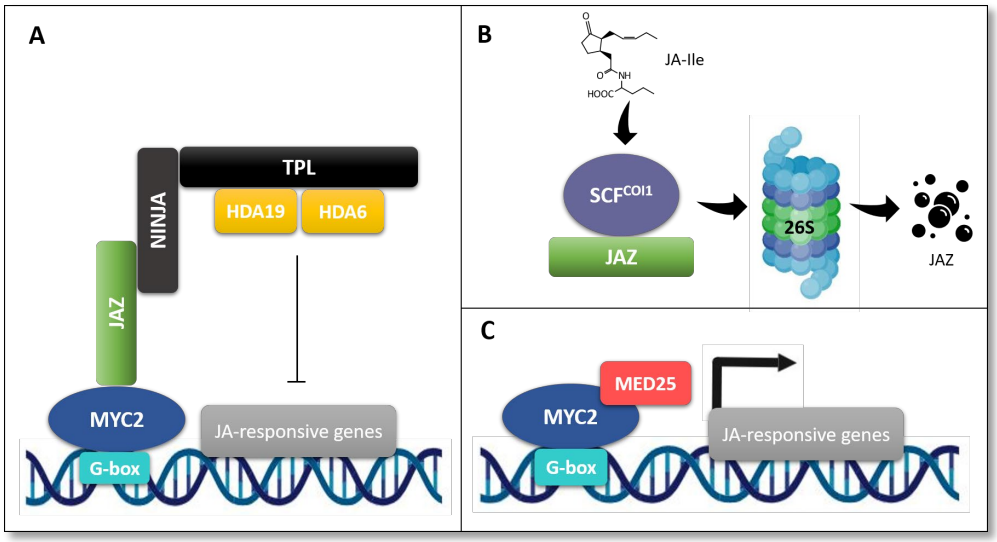

Figure 6. Model of jasmonic acid signaling pathway (adapted from De Geyter *et al.,* 2012). A. In the resting state, JA-responsive gene expression is repressed by JASMONATE ZIM DOMAIN (JAZ) family of proteins that binds to the transcription factor (TF) MYC2. The repressor JAZ interacts either through directly inhibiting the interaction between MYC2 and the MEDIATOR25 (MED25), the coactivator subunit and/or recruiting the corepressor TOPLESS (TPL) directly or through the NINJA adaptor. Repression by JAZ1/6/9 takes place through the interaction with HISTONE DEACETYLASE6 (HDA6) and HDA19 is known to interact with TPL in *Arabidopsis.* B. Upon stress, the F-box protein CORONATINE INSENSITIVE1 (COI1), part of a Skp1/Cullin/F-box (SCF) E3 ubiquitin ligase complex (SCF^{COI1}) recognizes (3*R,* 7*S)*-jasmonoyl-L-isoleucine (JA-L-Ile), the biologically active jasmonate (JAs), that promotes the interaction between JAZ and the COI1, F-box subunit, leads to ubiquitination and the proteasomal degradation of JAZ. C. JAZ-free MYC2 TF forms a homo- or heterodimer and binds to a G-box in the promoter of the JA-responsive genes and the interaction of MYC2 with MED25, finally initiates the transcription of JA-responsive genes.

circadian rhythms (Kazan and Manners, 2013) but also in JA signalling, as it coordinates the JA-mediated defense responses that are specific to different plants and include the biosynthesis of secondary metabolites like flavonoids, glucosinolates and anthocyanins in *Arabidopsis* (Wasternack and Strnad, 2017; Dombrecht *et al.,* 2007), nicotine and phenolamides in *N. attenuata* (Woldemariam *et al.,* 2013), *N. benthamiana* (Todd *et al.,* 2010) and *N. tabacum* (Zhang *et al.,* 2012), artemisinin in *Artemisia annua* (Shen *et al.,* 2016) and terpenoid indole alkaloids (TIA) in *Catharanthus roseus* (Zhang *et al.,* 2011). MYC2 also regulates the crosstalk between JA and ABA and JA and SA (Kazan and Manners, 2013). JA and ABA positively regulate the JA-responsive expression of *VSP1* through the MYC2-regulated NAC-domain, which includes the TF ANAC019 and ANAC0155 both involved in drought tolerance (Hussain *et al.,* 2017). On the other hand, ABA negatively regulates the expression of the JA-responsive *PDF1.2* by activating MYC2, that in turn negatively regulates the TF ORA59 and ERF1, both positive regulators of *PDF1.2* (Vos *et al.,* 2013). MYC2, upon coronatine activation, activates the NAC-domain of the TF ANAC019, ANAC055 and ANAC072 that in turn downregulate

SA levels by activating *S-ADENOSYL-L-METHIONINE: BENZOIC ACID-SALICYLIC ACID CARBOXYMETHYLTRANSFERASE1* (*BSMT1*) and *SALICYLIC ACID GLUCOSYLTRANSFERASE1* (*SAGT1*) thus suppressing the SA biosynthetic gene *ISOCHORISMATE SYNTHASE1* (*ISC1*) (Zheng *et al.,* 2012).

The close homologues MYC3 and MYC4 are functionally redundant to MYC2 although they have a differential gene expression. In *Arabidopsis,* expression of MYC2 is higher in roots whereas expression of MYC3 and MYC4 is higher in aerial parts where they contribute to glucosinolate biosynthesis by interacting with other TF (Schweizer *et al.,* 2013) such as MYB through the JID domain (Ambawat *et al.,* 2013). The trans-activator domain (TAD), adjacent to JID, controls the interaction with MEDIATOR25 (MED25) a coactivator subunit of the complex that enables MYC activity (Çevik *et al.,* 2012). Repression of the *Arabidopsis* MYC3 occurring through the interaction of JAZ9, displays conformational changes that inhibit the interaction of the Jas motif with MED25 (Zhang *et al.,* 2015). Additionally, splice variants of JAZ10 that lack the C-terminal Jas motif but do contain an N-terminal cryptic MYC-interaction domain (CMID), are unable to recruit SCF^{COI1}, leading to the accumulation of these JAZ splice variants in the presence of JA and then re-repress MYC TF, thus balancing back JA homeostasis (Zhang *et al.,* 2017).

1.4 Are jasmonates self-inducible?

As a part of the stress response, signal molecules need to be immediately available either by inducing their biosynthesis or to be released from inactivated forms to modulate the signaling pathway (Ballaré, 2011). The reversible conjugation into inactive derivatives suggests that under the constantly changing physiological conditions, these inactive derivatives can be a source of the (more) active phytohormone while maintaining a steady state concentration of activity in the receptive tissues (Cohen and Bandurski, 1982). Wounding in *Arabidopsis* induces a fast and high accumulation of JA and JA-Ile within 5 min after wounding, followed by the formation of 12-HOJA-Ile after 15 min, 11-HOJA and 12-HOJA after 35 min and 12-carboxyjasmonoyl-L-isoleucine (12-HOOCJA-Ile) after 40 min (Glauser *et al.,* 2008). These fast responses exclude the expression of *LOX2* (Glauser *et al.,* 2009), *OPR3* (Koo *et al.,* 2009), *JAR1* and JAZ proteins (Chung *et al.,* 2008) which raises the question whether this burst of JA accumulation results from a liberation of JA from a yet unknown pool of bound JA or from the conversion of a direct precursor like dnOPDA and/or OPDA. Based on the fact that arabidopsides accumulate quickly and in large amounts following wounding of leaves in *Arabidopsis*, it has been hypothesized that esterified oxylipins like arabidopsides, might be the substrates of dnOPDA/OPDA synthesizing enzymes like LOX, AOS and AOC rather than free FA pools converted to dnOPDA/OPDA and then esterified to galactolipids (Buseman *et al.,* 2006), thus suggesting that these enzymes can act on esterified FA and are constitutively present at least in members of the Brassicaceae (Kourtchenko *et al.,* 2007). This is supported by the observation that leaves of *Arabidopsis* incubated

with ^{18}O-labeled water before wounding, did not accumulate ^{18}O-labeled-arabidopsides, thus proving that arabidopsides were formed *without* free FA formation (Nilsson *et al.,* 2012). Furthermore, esterified dnOPDA/OPDA can be transferred to the galactose moiety of another MGDG by the enzyme acylated galactolipid associated phospholipase 1 (AGAP1), leading to OPDA-acylated MGDG (Nilsson *et al.,* 2015). Nevertheless, the incorporation of the bound dnOPDA and/or OPDA into the JA pathway has not yet been demonstrated.

Biological activities of OPDA include its ability to induce the accumulation of enzymes involved in the biosynthesis of alkaloids in *Eschscholtzia californica* cell cultures (Blechert *et al.,* 1995; Kutchan, 1993) and volatiles in *Phaseolus lunatus* (Koch *et al.,* 1999), tendril coiling in *Bryonia* (Stelmach *et al.,* 1999; Blechert *et al.* 1999; Weiler *et al.,* 1993) and repression of seed germination (Dave *et al.,* 2011). Its accumulation after wounding (Buseman *et al.,* 2006) or osmotic stress (Kramell *et al.,* 2000) suggests a similar and partly overlapping role in the defense mechanisms with that of JA (Taki *et al.,* 2005). The finding of the conjugate OPDA-Ile in wound-induced experiments in *Arabidopsis* flowering plants suggests a possible role in plant defense in a COI1-independent way (Floková *et al.,* 2016). OPDA induces a different set of genes (Taki *et al.,* 2005) that are COI1-independent as it was demonstrated in *Arabidopsis opr3* mutants defective in converting dnOPDA to JA. These wounded mutant plants were still able to accumulate basal levels of dnOPDA, OPDA and JA and upon OPDA treatment, the biosynthetic gene *OPR3* was highly expressed suggesting that there are alternative roles of OPDA during stress response (Stintzi *et al.,* 2001). Signal perception of OPDA during OPDA-related responses is not known yet and much less is known about the biological activity(ies) of dnOPDA.

Regulation of JA biosynthesis can be understood using three basic concepts *i.e.* i) release of C18:3/C16:3 from the membrane upon stimuli, ii) positive feedback loop of the JA-related genes expression by JA (Browse, 2009a; 2009b) and iii) tissue specificity (Wasternack and Song, 2017). Further regulatory factors might include post-translational control like in the case of OPR3 where an equilibrium between its monomer and dimer includes self-inhibition by dimerization (Breithaupt *et al.,* 2006). Other regulatory factors are heteromerization observed in all four AOC in *A. thaliana* (Otto *et al.,* 2016; Stenzel *et al.,* 2012), post-translational modifications (Scholz *et al.,* 2015) and Ca^{2+}-mediated regulation of *LOX2* (Bonaventure *et al.,* 2007). The interaction between AOS and hydroperoxide lyase (HPL) is involved in the formation of volatile and non-volatile oxylipins (Andreou *et al.,* 2009), JAZ proteins, other branches of the LOX pathway (Wasternack and Hause, 2013) and mitogen-activated protein kinase cascade (van Verk *et al.,* 2011).

1.5 *Catharanthus roseus*

1.5.1 Terpenoid indole alkaloids

The genus *Catharanthus* belongs to the Apocynaceae plant family and includes nine species, two subspecies and six varieties endemic to Madagascar *i.e. C. coriaceus*, *C. lanceus*, *C. longifolius*, *C. makayensis, C. ovalis*, *C. ovalis* subsp. *grandiflorus, C. ovalis* subsp. *ovalis, C. ovalis* var. *ovalis, C. ovalis* var. *tomentellus, C. pusillus, C. roseus*, *C. roseus* var. *albus, C. roseus* var. *angustus, C. roseus* var. *nanus, C. roseus* var. *roseus, C. scitulus* and *C. trichophyllus*. The species *C. pusillus* is endemic to India and Sri Lanka (van Bergen, 1996). Nowadays, *C. roseus* is cultivated as an ornamental plant all over the world and is known as Madagascar periwinkle. Its major value comes from its more than 130 different terpenoid indole alkaloids (TIA), a number of TIA that keeps increasing (Wang *et al.,* 2011a; 2011b; 2012a; 2012b). Particularly the dimeric alkaloids α-3',4'-anhydrovinblastine, vincristine and vinblastine are of interest for the production of medicines to treat cancer. Ajmalicine, another commercially important TIA, occurring at high levels in the roots, is used since 1957 to treat hypertension (van der Heijden *et al.,* 2004) and along with vincamine and reserpine also used as peripheral vasodilator. Other TIA not found in *Catharanthus* but also used in medicine are yohimbine for the treatment of erectile dysfunction (Almagro *et al.,* 2015), quinine as antimalarial, quinidine to treat cardiac arrhythmias and strychnine as rodenticide (Seneca, 2007).

TIA belong to a large class of at least 2500 compounds identified so far that are restricted to the order Gentaniales and the plant families Apocynaceae, Loganiaceae, Rubiaceae, Nyssaceae and Icacinaceae (Dugé de Bernonville *et al.,* 2015; Zhang *et al.,* 2018; O'Connor and Maresh, 2006). A drawback for some alkaloids is the low levels in plants, *e.g.* vinblastine and vincristine are around 5 and 0.5 ppm in leaves, respectively, making their production expensive. These low levels are mostly attributed to their complex biosynthesis that takes place in different cells and cellular compartments, especially the spatial separation of vindoline and catharanthine. Due to their commercial importance, great efforts to produce them by biotechnological means have encouraged an intense research for over 40 years to obtain these TIA in *in vitro* cultures (De Luca and St-Pierre, 2000; Zhao and Verpoorte, 2007; Contin *et al.,* 1999; Moreno *et al.,* 1993; Whitmer *et al.,* 2002; El-Sayed and Verpoorte, 2002; Arvy *et al.,* 1994). More recently, *omics* technologies like single-cell metabolomics and imaging mass spectrometry (IMS) (Yamamoto *et al.,* 2016; 2019) have contributed to the creation of metabolome, proteome and transcriptome databases and dedicated libraries of *C. roseus* and other plants such as *Gelsemium sempervirens, Calotropis gigantea* and *Camptotheca acuminate, e.g.* the SmartCell consortium, PhytoMetaSyn, Medicinal Plant Genomic Resources and CathaCyc (Rai *et al.,* 2017).

TIA biosynthesis includes at least 30 enzymatic steps that start from the shikimate and the monoterpenoid (also known as 2-*C*-methyl-D-erythritol 4-phosphate (MEP)) pathways. TIA are formed from the coupling of tryptamine and the monoterpenoid component secologanin (Pan *et al.,* 2016). The first steps in their biosynthesis, leading to loganin, take place in the vascular internal phloem associated parenchyma (IPAP) cells. From these cells an iridoid intermediate, probably loganic acid, is transported to the epidermis (Miettinen *et al.,* 2014). In the epidermis cells the iridoid glycoside secologanin is

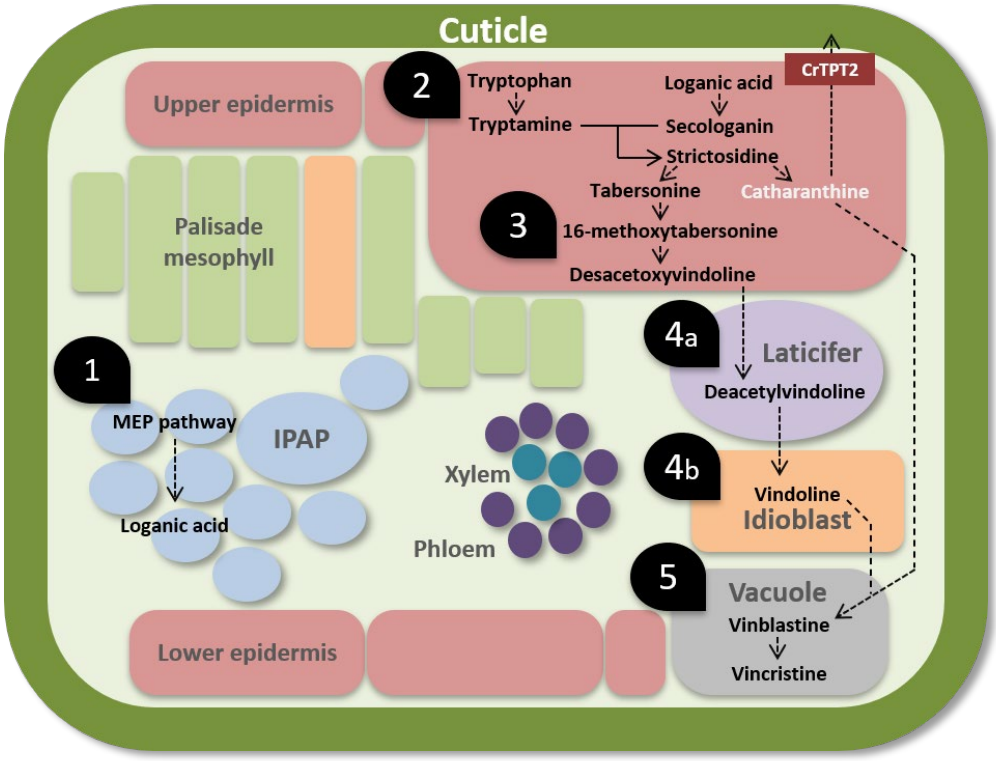

Figure 7. Model of localization for terpenoid indole alkaloid biosynthesis in the leaf epidermis of *Catharanthus roseus* (adapted from Courdavault *et al.,* 2014; Qu *et al.,* 2015). Broken arrows mean multiple enzymatic steps. All biosynthetic steps that lead to TIA take place in the internal phloem associated parenchyma (IPAP) cells, epidermal cells, laticifers and idioblasts of the mesophyll in whole leaves. There are around 30 enzymatic steps involved in vindoline biosynthesis. **1.** The 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway takes place in the IPAP cells and it involves 9 enzymes that lead to the formation of loganic acid. **2.** Loganic acid is exported to the upper epidermis and then converted to secologanin while tryptophan is converted to tryptamine also in the cytosol of IPAP cells. The condensation of secologanin and tryptamine that yields strictosidine occurs in the cytosol of the upper epidermis. Catharanthine and 16-methoxytabersonine are formed in the cytosol of the epidermis cells. Catharanthine is exported via the CrTPT2 transporter to the leaf surface. **3.** The conversion of 16-methoxytabersonine from tabersonine into desacetoxyvindoline is catalyzed by the enzyme 16-methoxy-2,3-dihydro-3-hydroxytabersonine methyl transferase (NMT) and occurs in the epidermis. **4.** Desacetoxyvindoline is exported from the epidermis and hydroxylated either in the laticifers (**4a**) and/or the idioblasts (**4b**) yielding deacetylvindoline. The formation of vindoline comes after acetylation of deacetylvindoline in two enzymatic steps that take place in the nucleocytoplasma of the laticifers (**4a**) and/or idioblasts (**4b**) cells. **5**. Coupling of catharanthine and vindoline occurs in the vacuole of leaves yielding vinblastine and vincristine in approximately 3 enzymatic steps.

synthesized in the cytosol and is coupled to tryptamine in the vacuole to yield strictosidine (Guirimand *et al.,* 2011; Courdavault *et al.,* 2014). The steps starting from strictosidine to catharanthine on one side and to tabersonine on the other side have not been fully elucidated yet. Catharanthine biosynthesis probably occurs in epidermal cells, it is exported via the ATP-binding cassette (ABC) transporter CrTPT2 and is accumulated in the leaf surface (Yu and De Luca, 2013) although IMS and single-cell

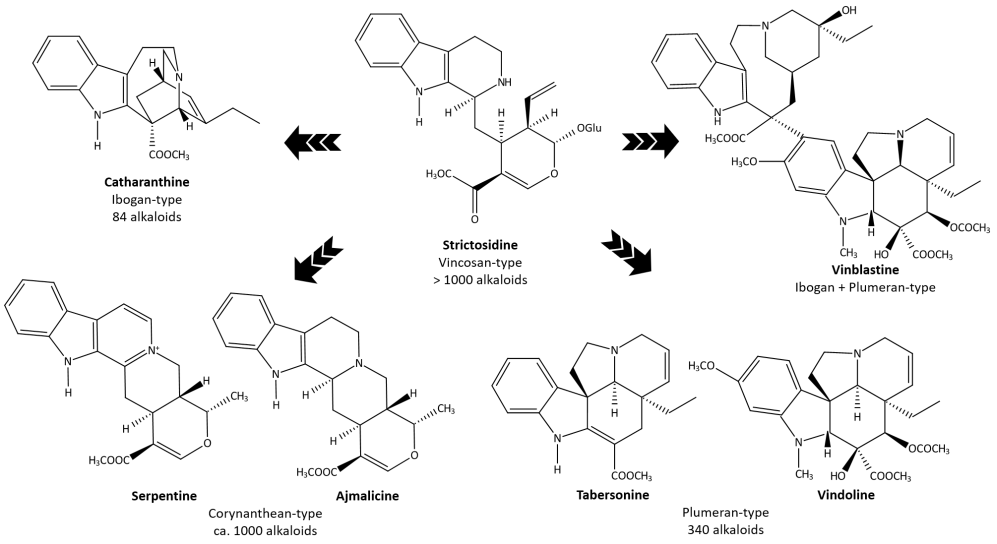

Figure 8. Selected examples of family types of mono and dimeric indole alkaloids occurring in *Catharanthus roseus* (adapted from Szabó, 2008). Broken arrows mean multiple enzymatic steps. Approximate number of alkaloids per family type is shown. The aglycone strictosidine is the common precursor to all family types of terpenoid indole alkaloids. In intact plants of *C. roseus,* catharanthine is found in the leaf epidermis; serpentine and ajmalicine are found in roots, leaves and seedlings; tabersonine in leaves, seedlings and seeds; vindoline is found in laticifers and idioblasts in leaves; same case for the dimeric alkaloid vinblastine.

metabolomics have confirmed the presence of catharanthine in idioblasts and laticifers and in a lower extent in parenchymal cells in a stem longitudinal section of *C. roseus* (Yamamoto *et al.,* 2016; 2019). Biosynthesis of tabersonine probably occurs in epidermal cells as well; it is metabolized into 16-hydroxytabersonine and 16-methoxytabersonine in the cytosol of epidermal cells and subsequently transported to parenchymal cells. In these cells, 16-methoxytabersonine forms 16-methoxy-2,3-dihydro-3-hydroxytabersonine. Desacetoxyvindoline is formed in the epidermis and transported to the idioblasts and laticifers (Courdavault *et al.,* 2014; Roepke *et al.,* 2010) by an unidentified process (Qu *et al.,* 2015). In the nucleocytoplasma of these cells, vindoline is formed which is subsequently transported to the vacuole (Pan *et al.,* 2016). Coupling of catharanthine and vindoline occurs in the vacuole of leaves yielding α-3',4'-anhydrovinblastine, which is further converted to vinblastine and then to vincristine (Courdavault *et al.,* 2014), the two most valuable dimeric alkaloids in *C. roseus* (Fig. 7). Nowadays, these alkaloids are produced industrially by biomimetic coupling of the much more abundant vindoline and catharanthine (Tam *et al.,* 2010). Other TIA derived from tabersonine are 19-hydroxytabersonine,19-*O*-acetyltabersonine and 19-*O*-hörhammericine, only found in roots and cell cultures of *C. roseus* (St-Pierre *et al.,* 2013).

The aglycone of strictosidine is the central biosynthetic intermediate that gives rise to structurally diverse skeletons, based on different intramolecular reactions of one of the two amino

groups (N-1 and N-4) with either of the two aldehyde functions present in strictosidine, which are liberated after glucolysis of secologanin and thus leading to the major skeletons of TIA. By breaking of different C-C bonds and formation of others, further diversity is created by the various plant species. A large number of classes and subclasses of monomers is distinguished such as plumeran-type (*e.g.* tabersonine, vindoline), corynanthean-type (*e.g.* serpentine, ajmalicine), ibogan-type (*e.g.* catharanthine) and vincosan-type (*e.g.* strictosidine) to name a few (Stöckigt and Panjikar, 2007; Seneca, 2007; Szabó, 2008; Farrow *et al.,* 2018) (Fig. 8).

1.5.2 Effect of jasmonic acid on TIA biosynthesis in *Catharanthus roseus*

Plants are constantly exposed to external stimuli that ultimately shape their growth rate, development and defense system. Most of these responses are modulated by phytohormones which form the link between primary and secondary metabolism. JA controls a large number of TF that have a profound effect on plant growth, development and stress responses (Wasternack and Hause, 2013). Precursors of TIA are derived from the MEP and shikimate pathways where at least 6 enzymatic steps out of ca. 30 are JA-responsive, through the expression of the TF OCTADECANOID DERIVATIVE-RESPONSIVE CATHARANTHUS APETALA2-DOMAIN 2 (ORCA2) and ORCA3, members of the ERF subfamily and within the AP2/ERF TF superfamily (van der Heijden *et al.,* 2004; Menke *et al*., 1999; De Geyter *et al.,* 2012; van der Fits and Memelink, 2000). These TF are regulated by the central regulator of JA-responses MYC2, a bHLH TF, by directly binding to the promoter of the ORCA3 gene thus controlling TIA biosynthesis (Zhang *et al.,* 2011). ORCA2 and ORCA3 regulate the expression of at least 13 TIA biosynthetic genes as well as that of *GERANIOL SYNTHASE* (*GES*) and *GERANIOL-8-OXIDASE* (*G8O*, also known as *G10H*) (Miettinen *et al.,* 2014) (Fig. 9). More recently, a cluster of ORCA3, ORCA4 and ORCA5, regulated by MYC2, was identified, where overexpression of ORCA4 and not ORCA3, lead to a higher increase of TIA accumulation in hairy roots of *C. roseus* (Paul *et al.,* 2017). The coordinated interaction of MYC2 and ORCA3 and ORCA4 leads to the activation of *TRYPTOPHAN DECARBOXYLASE* (*TDC*)*, ORCA2* and *ORCA3* interact with their target genes *e.g. STRICTOSIDINE SYNTHASE* (*STR*) via a sequence-specific binding to the GCC element (Memelink, 2009) although it has been recently shown that ORCA3, ORCA4 and ORCA5 coregulate the expression of *STR* (Fig. 9). Moreover, ORCA4 and ORCA5 are JA-inducible although they cannot be regulated by CrMYC2 but possibly by other TF (Paul *et al.,* 2017). Additionally, ORCA4 interacts with the JA-responsive TF bHLH IRIDOID SYNTHESIS1 (BIS1), where overexpression of *BIS1* and *BIS2* resulted in the increased accumulation of methylerythritol-4-phosphate and the expression of the iridoid branch genes *i.e. GES, G8O, 8-HYDROXYGERANIOL OXIDOREDUCTASE* (*8HGO*)*, IRIDOID SYNTHASE* (*IS/P5*β*R5*)*, 7-DEOXYLOGANETIC ACID GLUCOSYLTRANSFERASE* (*7DLGT*) and *7-DEOXYLOGANIC ACID HYDROXYLASE* (*7DLH*), all integral to TIA biosynthesis (van Moerkercke *et al.,* 2015; 2016). The JA-responsive bHLH TF REPRESSOR OF MYC2 TARGETS 1 (RMT1) is

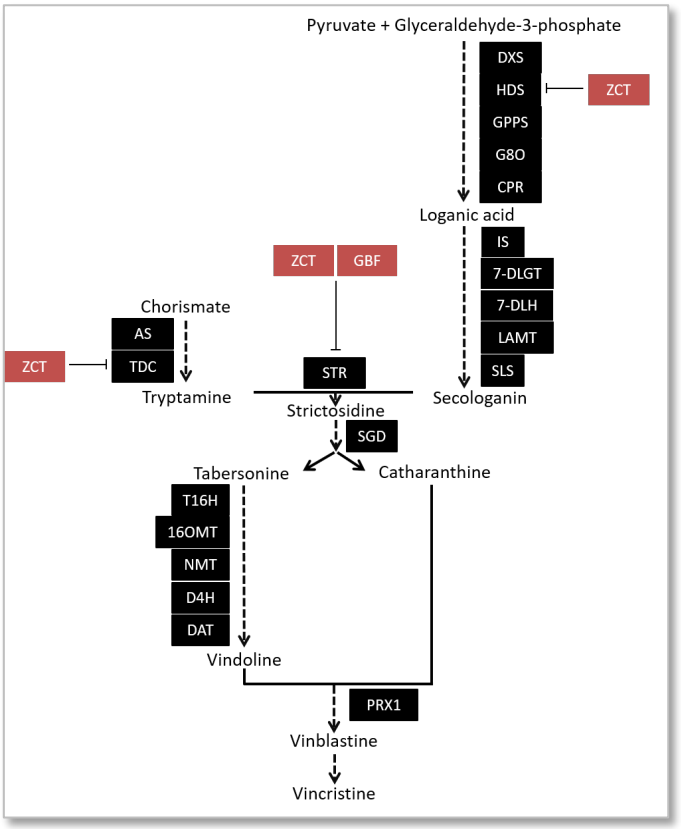

Figure 9. Biosynthetic pathway leading to terpenoid indole alkaloids in *Catharanthus roseus* (adapted from Memelink and Gantet, 2007; Li *et al.,* 2013; Sun and Peebles, 2015; Pan *et al.,* 2018). Broken arrows indicate multiple enzymatic reactions. Genes reported to be regulated by OCTADECANOID DERIVATIVE-RESPONSIVE CATHARANTHUS APETALA2-DOMAIN (ORCA) 3 and ORCA2, both induced by jasmonic acid (JA) or methyljasmonate (MeJA), are indicated in black boxes. Repressors ZINC-FINGER CATHARANTHUS TRANSCRIPTION FACTOR 1 (ZCF1), ZCF1, ZCF2, ZCF3, G-BOX BINDING FACTORS 1 (GFB1) and GFB2 are indicated in red boxes. Abbreviations for enzymes and/or proteins are: AS, anthranilate synthase; TDC, tryptophan decarboxylase; DXS, 1-deoxy-D-xylulose-5-phosphate synthase; HDS, hydroxymethylbutenyl 4-diphosphate synthase; GPPS, geranyldiphosphate synthase; G8O, geraniol 8-oxidase; IS, iridoid synthase; 7-DLGT, 7-deoxyloganetic acid glucosyltransferase; 7-DLH, 7-deoxyloganic acid hydroxylase; LAMT, *S*-adenosyl-L-methionine:loganic acid methyltransferase; SLS, secologanin synthase; STR, strictosidine synthase; SGD, strictosidine β-D-glucosidase; CPR, NADPH cytochrome P450 reductase; T16H, tabersonine 16-hydroxylase; 16OMT, tabersonine 16-*O*-methyltransferase; NMT, 16-methoxy-2,3-dihydro-3-hydroxytabersonine; D4H, desacetoxyvindoline 4-hydrolase; DAT, deacetylvindoline *O*-transferase; PRX1, peroxidase 1.

activated by CrMYC2 and BIS1 and acts as a passive repressor of the targets of CrMYC2. Moreover, CrMYC2 and BIS1 are repressed by JAZ proteins in the absence of JA and de-repressed in the presence of JA via the SCF^{COI1} complex suggesting that JAZ proteins and RMT1 regulate the crosstalk between CrMYC2 and BIS and hence TIA biosynthesis (Patra *et al.,* 2018).

The TF CrWRKY1 preferentially expressed in roots of *C. roseus*, belongs to the group III WRKY superfamily and it is induced by JA, GA and ET. Overexpression of CrWRKY1 upregulates the expression of *TDC* especially, along with that of the TIA biosynthetic genes *ANTHRANILATE SYNTHASE* (*AS*), *1-DEOXY-D-XYLULOSE-5-PHOSPHATE SYNTHASE* (*DXS*), *SECOLOGANIN SYNTHASE* (*SLS*) and *STRICTOSIDINE β-D-GLUCOSIDASE* (*SGD*). CrWRKY1 regulates the expression of the repressors *ZINC-FINGER CATHARANTHUS TRANSCRIPTION FACTOR 1* (*ZCT1*), *ZCT2* and *ZCT3* but downregulates the expression of *CrMYC2*, *ORCA2* and *ORCA3* (Suttipanta *et al.,* 2011). The TF ZCT1, ZCT2 and ZCT3 repress the gene expression of *STR* and *TDC* promoters where all repressors are induced by yeast extract and MeJA (Pauw *et al.,* 2004). However, the *STR* and *TDC* promoters binding sites of ZCT1 and ZCT2 are different from ZCT3, their structures also differ, and their functions are partially different. ZCT1 and ZCT2 but not ZCT3 bind to the promoter region of *HYDROXYMETHYLBUTENYL 4-DIPHOSPHATE SYNTHASE* (*HDS*) and repress its expression (Chebbi *et al.,* 2014). Moreover, silencing ZCT1 in hairy roots of *C. roseus* elicited with MeJA, did not increase TIA accumulation or induced gene expression of *G10H, TDC* and *STR.* Since elevated levels of *ZTC3* were observed, it suggests that all ZTC play overlapping but different functions in TIA biosynthesis (Rizvi *et al.,* 2016). The repressors G-BOX BINDING FACTORS 1 (CrGBF1) and CrGBF2 bind to the NR element in the *STR* promoter indicating a role in the regulation of the expression of *STR* (Sibéril *et al.,* 2001).

1.6 Aim of this thesis

The main goal in this research project was to unravel the basis of the rapid JA-response in elicited cells of *C. roseus*. In order to achieve this goal, an integrated systems biology approach was used with information of the plant metabolic status after induction with JA and dnOPDA using cell suspension cultures of *C. roseus*, as a simplified model. The effect of JA on the accumulation of TIA and the expression of biosynthetic genes in this plant has been extensively documented, although few studies have covered JA's rapid accumulation largely conducted in wound-induced experiments in intact plants such as *Arabidopsis, Solanum lycopersicum* or *N. tabacum.* Additionally, there are currently no studies reporting the effect of JA on primary metabolites such as FA or the putative feedback mechanism of JA in cell suspension cultures and/or in *C. roseus*. Most of the knowledge in the plant JA-mediated stress response field has been studied *in planta*. In order to elaborate a timeline of metabolic events involving both primary and secondary metabolism in a cell suspension model during and after the plant's stress response, we formulated the following questions:

1. What is the effect of time on FA profiles of cell suspension cultures of *C. roseus*? Is there a trend in their FA profiles? How different are the FA profiles in seeds, roots, flower buds, flowers, stems and leaves of *C. roseus* plants? (**Chapter 2**). This chapter describes the FA profiles of cell suspension cultures of *C. roseus* grown over a period

of 21 days and intact organs of *C. roseus* plants, analyzed by gas chromatography-mass spectrometry (GC-MS) where differences, similarities and relationships among FA were assessed using multivariate data analysis (MVDA), univariate data analysis (UVDA) and regression models.

2. Does the stress response in cell suspension cultures of *C. roseus* start with the *de novo* biosynthesis of JA by releasing its FA precursor linolenic acid? Are any of the other FA levels affected by JA? Do these responses fit into the early or late stress response in cell suspension cultures of *C. roseus*? (**Chapter 3**). This chapter describes the effect of JA over a 24 h period on FA of cell suspension cultures of *C. roseus* analyzed by GC-MS. Differences among and between treatments and groups were explored and assessed with MVDA and UVDA.
3. How does JA affect TIA accumulation? Which TIA are affected by JA? Is the TIA accumulation a late stress response in *C. roseus*? (**Chapter 4**). This chapter describes the effect of JA on 11 TIA and loganic acid in cell suspension cultures of *C. roseus* over a 24 h period, all analyzed by high-performance liquid chromatography-diode array detector (HPLC-DAD) and ultra high-performance liquid chromatography-high resolution mass spectrometry (UHPLC-HRMS).
4. Is exogenous dnOPDA converted to JA? Can JA induce its own biosynthesis by a putative feedback loop mechanism? How do results fit into the early stress response in cell suspension cultures of *C. roseus*? (**Chapter 5**). This chapter describes the results concerning a putative self-induction feedback loop using *d*5-JA and *d*5-dnOPDA fed to cell suspension cultures of *C. roseus*. Different analytical platforms were used to monitor the fate of both JAs and their isotopologue profiles and stability in cells and liquid growth medium.
5. Which JAs are present in cell suspension cultures of *C. roseus* after feeding with JA? Which JA conversion catabolite is most abundant? Which JAs are the main turnover catabolites of JA? (**Chapter 6**)**.** This chapter describes the metabolic fate of exogenously added JA in cell suspension cultures of *C. roseus* and liquid growth medium over a 24 h period. Oxidized, hydroxylated, glucosylated, conjugated with isoleucine and reduced JAs' profiles were analyzed by UHPLC-HRMS.

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Chapter 2

Fatty acid profiles of cell suspensions and intact organs of *Catharanthus roseus* using a gas chromatography-mass spectrometry targeted approach

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ABSTRACT

The fatty acid (FA) profiles of cell suspension cultures of *Catharanthus roseus* grown over a period of 21 days and that of intact organs such as flowers, flower buds, stems, leaves, roots and seeds of *C. roseus* plants were compared. The major FA in cell suspension cultures was C16:0 followed by C18:2 and C18:3. Hierarchical cluster analysis (HCA) of gas chromatography-mass spectrometry (GC-MS) of source materials distinguished three major clusters of cell suspension samples according to their age in "older" cells (0, 6, 13 and 17 days), "younger" cells (2, 4, 8, and 10 days) and 21-day-old cells as a single group. At day 21, significantly higher contents of C10:0, C15:0 and especially C20:0 and C22:0 were observed if compared to day 0. C10:0, C16:0, C18:2, C20:0 and C21:0 showed a linear increase in their contents over time. In fact, "younger" cells at exactly day 8 had the highest number of significant changes in all FA. In plant materials, the major FA was C16:0, followed by C18:0 and C18:1 as major compounds in seeds. Roots and stems had similar levels of FA as cell suspension cultures; seeds were several orders of magnitude higher in FA than the rest of the plant organs. Leaves, flowers and flower buds were lower in FA than cell suspension cultures. The ratio of saturated (SFA) and unsaturated FA (UFA) was higher in plant organs than in cell suspension cultures, *i.e.* UFA levels were relatively higher in cell suspension cultures. Analysis of plant materials of *C. roseus* by HCA showed that seeds and roots clustered as two single groups; flower buds and leaves clustered together as a third group and stems and flowers as a fourth one. Differences between seeds and roots might be attributed to the large amounts of FA in seeds such as C16:0, C18-series and C20:0. Both short and long chain FA were relatively more abundant in cell suspension cultures than in plant organs with the exception of roots, rich in both types of FA.

2.1 INTRODUCTION

Fatty acids (FA) are essential molecules present in all living organisms. They are usually bound to glycerol to form lipids or part of the cellular membranes. Saturated FA (SFA) are those in which hydrogen molecules occupy all free bonding positions of the carbon chain, whereas unsaturated FA (UFA) carry one or more double bonds between the carbons of the chain. Thus, various FA can be identified by the length of their carbon chain, the number of double bonds and their positions (Kachroo and Kachroo, 2009). Systematic names are formed by appending the suffix "-oic acid" to the stem of the name of the parent hydrocarbon; the carboxyl carbon is assigned as number 1. According to this system, two numbers separated by a colon designate a FA; the first number is the total number of carbon atoms in the FA and the second is the number of double bonds. Hence, 18:2 refers to a C18 acid with two double bonds *e.g.* octadecadienoic acid (Rezanka and Sigler, 2009). The positions of the remaining double bonds in polyunsaturated FA (PUFA) are deducted easily as they follow the methylene-interrupted pattern and are given additional number(s). The position(s) of a double bond is counted from the carboxylic group and *cis* (*Z*) configuration is assumed in natural compounds (López-Alonso

and García-Maroto, 2000).

FA are one of the most abundant form of reduced carbon chains available in nature along with plant oils where plant FA represent a large pool of diversity with at least 200 different types that occur mainly in plants. The composition in plants consists almost exclusively of 16 and 18-carbon FA, being palmitic acid (C16:0) the major saturated FA followed by the unsaturated linolenic (C18:3), linoleic (C18:2) and oleic acid (C18:1), and are often referred to as the common FA (Millar *et al.,* 2000). SFA and UFA with carbon chains shorter than 12 and longer than 22 are less abundant (Rezanka and Sigler, 2009) and those with chemical structures that differ significantly from the common ones are called unusual FA, which include very long chain FA (VLCFA; C20-C36 *e.g.* erucic acid; C22:1), medium chain FA (MCFA; C8-C14 *e.g.* lauric acid; C12:0), hydroxylated FA (*e.g.* ricinoleic acid; 12OH-C18:1) and FA with different positions of the double bond (*e.g.* petroselinic acid; C18:1 Δ6) (Millar *et al.,* 1998; Ellenbracht *et al.,* 1980; Cassagne *et al.,* 1994) (Fig. 1).

Biosynthesis of the major FA *i.e.* C16 and C18 series, starts by *de novo* biosynthesis of long chain SFA through the combined activity of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS). MCFA biosynthesis occurs in the plastid where acetate (C2) is elongated by sequential addition of further C2 units while attached to a soluble acyl-carrier protein (ACP) that is terminated when the chain reaches the 16 or 18 carbons length. Plants that synthesize C8-C14 FA have an additional acyl-ACP thioesterase that prematurely cleaves the acyl-chain from ACP redirecting FA synthesis from (C16-18) to medium chains (C8-C14) (Millar *et al.,* 2000). VLCFA are synthesized by successive rounds of elongation by an endoplasmic reticulum-localized FA elongation complex of four core enzymes starting with a C18 fatty acyl precursor by two carbons originating from a malonyl-CoA. Each elongation step requires four enzymatic reactions: condensation between an acyl precursor and malonyl-CoA, followed by a reduction, dehydration and another reduction (Millar and Kunst, 1997; Haslam and Kunst, 2013). VLCFA are found esterified to various hydroxyl groups *e.g.* to glycerol as erucic acid in rape seed and particularly to wax alcohols in jojoba in developing seeds where they account for almost two thirds of the total FA (Cassagne *et al.,* 1994) as well as in cuticular waxes as cutin or suberin as a vital barrier (Schreiber, 2010).

Common plant FA are structural components in lipid membranes and their differential occurrence suggests that structure and composition is important for the membrane function (Ohlrogge and Browse, 1995). The proportion and composition of FA in various organisms is genus-, species- and organ-specific and is highly dependent on the environment as well as on the developmental status. Cells undergoing development and/or stress have to adapt their membrane properties like fluidity, permeability and transport. Possibly, by affecting membrane fluidity, it might induce changes in the FA composition or the modification in their length and unsaturation (Harwood, 1996). Changes in lipid composition are not only seen during plant development or when plants are challenged with stress conditions (Du Granrut and Cacas, 2016) but also in *in vitro* cultures undergoing developmental

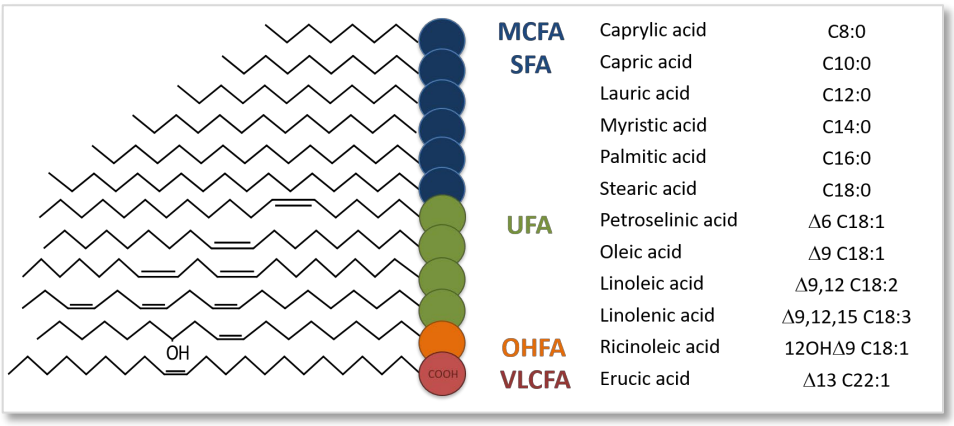

Figure 1. Common FA with their chemical formulae, common names and abbreviations (modified from Töpfer *et al.,* 1995). MCFA: Medium chain fatty acids; SFA: saturated fatty acids; UFA: unsaturated fatty acids; OHFA: hydroxy fatty acids; VLCFA: very long chain fatty acids.

processes and when compared to parent plants the differences might be a reflection of the nutrition as well as the degree of differentiation of photosynthetic tissues (Williams *et al.,* 1991) which are mostly characterized by a high proportion of monogalactosyldiacylglycerols (MGDG) and digalactosyldiacylglycerols (DGDG).

The cultivation of undifferentiated plant cells grown under controlled environmental conditions is a useful tool to study lipid and FA metabolism. Cell suspension cultures represent an excellent and stable system since they have a high growth rate that can be connected with membrane development, since in principle, all lipids and FA found in green tissues of higher plants occur in tissue cultures derived from such plants. In our investigation, we comparatively studied the FA composition and content of cell suspension cultures and intact organs of *Catharanthus roseus* (L.) G. Don. Until now, all the attention on this plant has been focused on the characterization of terpenoid indole alkaloids (TIA) and the metabolic engineering of their biosynthesis (Hughes *et al.,* 2004; Miettinen *et al.,* 2014; Ritala *et al.,* 2014; Asada *et al.,* 2013) and little is known about the FA profile in cell cultures and intact organs of *C. roseus.* Only a few investigations report on the lipid and/or FA profiles *i.e.* senescent leaves (Mishra *et al.,* 2006; Mishra and Sangwan, 2008), stress-induced suspension cultures (Toivonen *et al.,* 1992a; MacCarthy and Stumpf, 1980a; 1980b; 1981) and hairy roots (Toivonen *et al.,* 1992b), seeds (Satyan *et al.,* 2009) and aerial parts (Pandey-Rai, 2006; Brun *et al.,* 2001; Guedes De Pinho *et al.,* 2009) illustrating how little attention has been paid to the integration of these groups of compounds to other aspects of plant development and/or metabolism.

The main goal of the present work was to characterize the FA profiles in cell suspension cultures and plant materials of *C. roseus.* The FA profiles will be the basis for further studies on the effect of various forms of stress, among others, in relation to jasmonates (JAs) biosynthesis and JAs

signaling under stress conditions. After hydrolysis of the plant material *i.e.* liberating the bound FA from the cellular membrane with a base, the obtained free FA were methylated and analyzed with a validated GC-MS-based methodology. Results were assessed by using multivariate data analysis (MVDA).

2.2 EXPERIMENTAL

2.2.1 Cell suspension cultures

Flasks of 250 mL of volume containing 50 mL of Gamborg B5 (Gamborg *et al.,* 1968) medium supplemented with 30 g/L sucrose and 1.86 mg/L of 1-naphthalene acetic acid (NAA) and adjusted to pH 5.8 with 0.1 N KOH were inoculated individually with 20 mL of the CRPP cell line of *C. roseus* and propagated on a rotary shaker (110 rpm) at 25 °C under continuous light (500-1500 lux) and were subcultured every three weeks. A series of 27 Erlenmeyer flasks of the suspended cells were prepared and cells were harvested in triplicates on 0, 2, 4, 6, 8, 10, 13, 17 and 21 days after subculture which corresponds to one growth cycle from lag phase extending into stationary phase. Cells were filtered on Whatman filter paper under partial vacuum. Biomass and media samples were immediately frozen in liquid nitrogen and kept at -80 °C until further analysis.

2.2.2 Plant materials

Approximately 6-month-old plants of *C. roseus* were purchased in June 2013 from Jan van Paridon Bloemen BV (Rijnsburg, The Netherlands) and authenticated by Rogier van Vugt (Hortus Botanicus, Leiden University). Plants were separated into flower buds, flowers, leaves, stems and roots. Seeds from the cultivar Pacifica Red were obtained from Rijnsburg Zaadhandel BV (Rijnsburg, The Netherlands).

2.2.3 Chemicals used for cell suspension cultures

The chemicals used for macro salts were $CaCl_2$ (min. 99%), KH_2PO_4 (min. 99.5%), KNO_3 (min. 99%) and NH_4NO_3 (min. 99%) all purchased from Merck (Darmstadt, Germany), $MgSO_4$ was obtained from OPG Farma (BUVA BV, Uitgeest, The Netherlands). The chemicals used for micro salts H_3BO_3, $MnSO_4.H_2O$, $ZnSO_4.7H_2O$, Na_2EDTA (Merck) and $FeSO_4.7H_2O$ (Brocades-ACF Groothandel NV, Maarssen, The Netherlands) were dissolved into one solution and KI, $NaMoO_4.2H_2O$, $CuSO_4.5H_2O$ and $CoCl_2.6H_2O$ (Merck; Darmstadt, Germany) were dissolved into another solution to avoid precipitations. Thiamine-di-HCl was from Janssen Chimica (Geel, Belgium), pyridoxine-HCl was from Sigma-Aldrich Chemie (Steinheim, Germany), nicotinic acid (99.5%), glycine (99.7%) and NAA were from Merck (Darmstadt, Germany), sucrose (99.7%) and *myo*-inositol (99.7%) were from Duchefa Biochemie (Haarlem, The Netherlands).

2.2.4 Chemicals used for fatty acid determination

The 37 Components of fatty acid methyl esters (FAME) mix were obtained from Supelco (Sigma-Aldrich; Bellefonte, PA, USA) and was used to identify the FAME in the samples by comparison of their retention times (RT) and MS-fragmentation patterns. All other chemicals and solvents were of analytical grade and purchased from common sources. Water was from a Milli-Q water purification system (TGI Pure Water Systems; Brea, CA, USA).

2.2.5 Fatty acid extraction

Fatty acid extraction was carried out according to a protocol developed by PRISNA B.V. (Leiden, The Netherlands, unpublished data). Briefly, samples of 10 mg of lyophilized plant organs or 10 mg of lyophilized cells were spiked with 50 µg of C17:0 as internal standard (IS) and then subjected to hydrolysis by adding 1 mL of 1 M of KOH in 95% ethanol (v/v) to the test tube. All materials were ultrasonicated for 10 min followed by heating in a closed test tube at 80 °C for 30 min. After cooling at room temperature, 1 mL of distilled water was added followed by two times extraction with 1 mL of *n*-hexane containing 0.01% butylated hydroxytoluene (BHT; Sigma-Aldrich, St Louis, MO, USA); after vigorously vortexing, samples were centrifuged for 10 min at 3,500 rpm and the upper hexane layer was removed and discarded. The aqueous layer containing free FA was acidified by adding 200 µL of 6 M HCl and extracted twice with 1 mL *n*-hexane (0.01% w/v BHT) and after centrifugation for 10 min at 3,500 rpm upper layers were pooled and completely dried under a gentle flow of N_2 gas. To the residues 1 mL of boron trifluoride (BF_3; 10 % w/w) in methanol (Sigma-Aldrich, St Louis, MO, USA) was added and the closed tubes were heated for 15 min at 80 °C and then cooled down at room temperature and 1 mL of *n*-hexane was added, after centrifugation the upper layer (100 µL for plant organs and 300 µL for cell suspensions) was subjected to GC-MS analysis.

2.2.6 Gas chromatography-mass spectrometry

Fatty acid methyl esters (FAME) analysis was performed on an Agilent 7890A gas chromatograph equipped with an Agilent 7693 auto sampler and an Agilent 5775C Triple-Axis MSD detector (all from Agilent Technologies Inc., Santa Clara, CA, USA). FAME were separated on a 30 m x 0.25 mm x 0.25 µm film thickness DB-Wax column (J&W; Agilent Technologies Inc., Santa Clara, CA, USA), with a constant flow of 20 mL/min of He as a carrier gas. The injection port was heated to 50 °C. The injection volume was 1 µL with a split ratio of 20:1. The oven temperature was 50 °C for 1 min, then 25 °C/min to 200 °C and then 3 °C/min to 250 °C for 18 min. All mass spectra were acquired in the electron impact (EI) mode for full scan in total ion current (TIC) and selected ion monitoring (SIM) modes. Ions selected for quantification are listed in Table 1. FAME reference standard Mix 37 (Supelco) was used as a control for possible retention time shifts and mass spectra ion identification.

Table 1. Name, formula, *m/z*, retention time for each FAME and ions for identification in SIM mode

Systematic name	Common name	Formula	R.T. (min)	*m/z*	SIM Ions for identification
Decanoic acid	Capric acid	C10:0	5.65	186	74,55,87,143,186
Dodecanoic acid	Lauric acid	C12:0	6.65	214	74,41,43,55,87,143,171,214
Tetradecanoic acid	Myristic acid	C14:0	7.61	242	74,87,143,199,242
Δ-9-Tetradecanoic acid	Myristoleic acid	C14:1	7.81	240	41,55,69,74,87,240,241
Pentadecanoic acid	Pentadecanoic acid	C15:0	8.17	256	57,74,87,143,256
Hexadecanoic acid	Palmitic acid	C16:0	8.82	270	74,87,143,227,270
Δ-9-Hexadecanoic acid	Palmitoleic acid	C16:1	9.02	268	55,69,74,41,83,81,237,269
Heptadecanoic acid	Heptadecanoic acid	C17:0	9.6	284	74,87,143,284
Octadecanoic acid	Stearic acid	C18:0	10.52	298	74,87,143,255,299
Δ-9-Octadecanoic acid	Oleic acid	C18:1*n*9c	10.74	296	55,69,81,96,109,264,297
Δ-9,12-Octadecadienoic acid	Linoleic acid	C18:2	11.24	294	67,81,95,245,263,294
Δ-9,12,15-Octadecatrienoic acid	α-Linolenic acid	C18:3*n*3	11.97	292	67,79,95,108,292
Eicosanoic acid	Arachidic acid	C20:0	12.89	326	43,55,74,87,326
Heneicosanoic acid	Heneicosanoic acid	C21:0	14.32	340	43,55,57,74,87,340
Docosanoic acid	Behenic acid	C22:0	15.9	354	43,55,74,87,143,354
Tetracosanoic acid	Lignoceric acid	C24:0	19.73	382	57,74,87,143,382

2.2.7 Method validation

The GC method for determining FAME was subjected to validation following recommendations of the International Conference on Harmonization (ICH, 2006). Quantification of individual FAME was based on obtained peak areas, which were normalized to that of the internal standard, no correction factors were used.

2.2.7.a. Response factor, linearity, precision and quantification of FA

In order to quantify FA and to check the precision of the method through its repeatability tested as interday precision for three consecutive days, working solutions of 1 mg (weighed in an analytical balance, ENTRIS224-1S Sartorius Lab Instruments GmbH & Co. KG, Göttingen, Germany) of 7 FA standards chosen according to their chain length and degree of unsaturation *i.e.* C12:0, C14:0, C16:0, C18:0 and C18:2 were prepared in 1 mL of *n*-hexane for each individual FA and were treated as a sample starting with basic hydrolysis as described in the Experimental section 2.2.5 of this chapter to obtain the FAME of each FA species. Two independent sets of calibration curves were prepared and analyzed each in two consecutive days by dilution of each working solution of these FA where C17:0 (50 µg/mL, prepared from a working solution of 1 mg/mL of *n*-hexane) was added to each solution as IS, to achieve seven concentration levels (0.1, 0.5, 10, 20, 50, 75 and 100 µg/mL of *n*-hexane) for the first set of solutions and three concentration levels (100, 50 and 10 µg/mL of *n*-hexane) for the second set of solutions. Each sample was analyzed three times and data from both calibration curves was

combined. Quantification was based on the peak area of each FAME, which was identified with the help of the National Institute of Standards and Technology (NIST) library. Peak areas of all FAME species were normalized with that of the IS as reported elsewhere (Mengesha and Bummer, 2010). According to Wychen et al. (2015) and Carvalho et al. (2012), quantification of FAME not included in the calibration curves is achieved by using the regression equations of FAME with similar carbon chain lengths as it is assumed that they have similar response factors and volatility allowing a direct comparison of peak areas. In this way we made five groups of FA: **1**) C10:0 and C12:0 were quantified with the regression equation of C12:0; **2**) C14:0 and C14:1 were quantified with the regression equation of C14:0; **3**) C15:0 and C16:0 were quantified with the regression equation of C16:0; **4**) C18:0, C19:0, C20:0, C21:0, C22:0, C24:0 and C26:0 were quantified with the regression equation of C18:0; and **5**) C18:1, C18:2 and C18:3 were quantified with the regression equation of C18:2. Validation results are expressed in nmol/µL with the relative standard error (RSE %) (Table 2).

2.2.7.b. Limit of quantitation and detection

Limit of quantitation (LOQ) was determined as QL=10 σ/s and limit of detection (LOD) was determined as DL=3.3 σ/s where σ is the standard deviation of the response and s is the slope of the calibration curve (Table 2).

2.2.8 Data analysis

Before statistical analysis, distributions of datasets of cell suspension cultures and plant organs were tested for normality using the Shapiro-Wilk test ($p < 0.05$). A nested one-way ANOVA corrected for multiple comparisons with Dunnett's post hoc test was used to assess significant differences of each FA compared with day 0. Associations between the absolute values of each FA (observed variables) and days (sampling times) were assessed by performing linear or quadratic regression models. For plant organs, significant differences in FA contents among all plant organs was assessed with a nested one-way ANOVA corrected for multiple comparisons with Tukey's post hoc test. Significant differences in C15:0 between flower buds and roots and in C26:0 between flower buds and leaves were tested using a two-tailed nested *t*-test. All statistical tests were performed using GraphPad Prism software (v. 8.4.3.686, La Jolla, CA, USA). Differences with $p < 0.05$ were considered statistically significant. To examine data of cell suspension cultures, a Hierarchical Cluster Analysis (HCA) was performed using averaged absolute values of technical and biological replicates. Euclidean distances and Ward's clustering algorithm were used in SIMCA-P software (v. 15.0.2, Sartorius Stedim Data Analytics AB, Umetrics, Umeå, Sweden).

2.3 RESULTS AND DISCUSSION

2.3.1 Method validation

Calibration plots were run for the major plant FA using C17:0 (50 µg/mL) as IS. The repeatability of the method was evaluated in triplicate technical samples analyzed once with seven different concentrations of an artificial mix of FA of C12:0, C14:0, C16:0, C18:0 and C18:2. Dilutions were made from a working solution of 1 mg prepared in 1 mL of *n*-hexane of each FA where each solution was prepared and analyzed as described in the Experimental section 2.2.5 of this chapter. Repeatability (interday precision) results are expressed as relative standard error (RSE %) (Table 2).

Table 2. Validation data for fatty acid analysis with GC-MS

FA	Regression equation	Linear correlation coefficient	LOD (nmol/µL)	LOQ (nmol/µL)	Repeatability (interday precision) (RSE%)
C12:0	y = 0.3274x - 0.0071	0.9332	1.90	5.75	29.24
C14:0	y = 0.4815x - 0.0201	0.9973	0.32	0.98	11.13
C16:0	y = 0.5044x - 0.0098	0.9986	0.20	0.63	6.25
C18:0	y = 0.4671x - 0.0035	0.9994	0.12	0.38	5.65
C18:2	y = 1.4864x - 0.037	0.9948	0.36	1.10	5.92

LOD: Limit of detection; LOQ: Limit of quantification; RSE: Relative standard error (RSE %).

2.3.2 Fatty acid composition of cell suspension cultures of *C. roseus*

The most abundant FA present in cell suspension cultures of *C. roseus* were C16:0, C18:2 and C18:3 as previously reported in *C. roseus* (Toivonen *et al.,* 1992a; MacCarthy and Stumpf, 1980a; 1980b; 1981; Elkahoui *et al.,* 2004; Radwan *et al.,* 1974; Leathers and Scragg, 1989) whereas C21:0 and C24:0 were detected in lower amounts (Table 3). Even numbered species were predominant over the odd numbered ones *e.g.* C15:0 and C21:0 as previously observed in *C. roseus* (Pandey-Rai *et al.,* 2006; Brun *et al.,* 2001; Guedes De Pinho *et al.,* 2009). Significantly higher contents of C10:0, C15:0 and particularly C20:0 and C22:0 were observed at day 21 in comparison to day 0 which also agrees with the highest amounts of total FA and SFA being observed the same day (Table 3). FA such as C14:0 and C18:1 showed significant differences in their contents in most observation points when compared to day 0. The highest number of significant differences for FA was observed in 8-day-old cells, followed by 10-day-old cells. Considering the growth characteristics of the suspension cultured cells, the growth cycle can be divided in 3 events. Fresh weight results revealed no increase in biomass in the first 2 days, which corresponds to the lag-phase. Subsequently, the biomass increased gradually until the last harvesting point. Evaluation of the dry weight increase showed that the biomass increase, halted at day 13 and declined beyond this point, indicating the onset of the stationary phase. Following the timing of these phases, the cultured cells were in exponential phase from day 2 until day 13 (Supplement 2.7.1). Subsequently, the cells with most significant changes in FA composition were in mid-exponential phase. VLCFA *e.g.* C20:0 and higher, are rarely found in suspension cultures, PUFA with chain lengths up to C26 have been reported for calli and cell suspension cultures of different plant species such as *Hydnocarpus anthelminthica*, *Phaseolus aureus, Cicer arietinum, Petroselinum hortense, Daucus*

Table 3. Contents of fatty acids in cell suspension cultures of *Catharanthus roseus* over a growth period of 21 days (µmol/g DW)

	Day										
	0	**2**	**4**	**6**	**8**	**10**	**13**	**17**	**21**	**F**	***p***
C10:0	2.37±0.04	2.46±0.03	2.33±0.05	1.65±0.62	2.53±0.06	2.58±0.05	2.43±0.05	2.48±0.02	3.55±1.66*	$F_{(8,66)} = 11.47$	<0.0001
C12:0	4.53±0.52	3.47±0.42	3.22±0.29	4.01±0.48	3.21±0.33	3.28±0.21	3.54±0.31	4.25±0.53	2.6±1.37	$F_{(8,18)} = 1.744$	0.1556
C14:0	10.94±0.08	7.95±0.18*	7.49±0.09*	8.02±0.08*	7.09±0.06*	7.47±0.16*	9.17±0.22*	10.23±0.28	10.9±0.68	$F_{(8,18)} = 42.53$	<0.0001
C15:0	4.75±0.05	3.51±0.08	3.29±0.04	3.42±0.04	4.44±0.18	4.8±0.21	3.99±0.04	4.62±0.13	6.99±2.53*	$F_{(8,18)} = 4.643$	0.0033
C16:0	199.05±3.62	173.63±8.78	187.8±6.5	217.4±3.42	181±4.06	187.69±0.45	245.83±4.4*	234.23±5.99*	225.41±5.57	$F_{(8,18)} = 14.42$	<0.0001
C18:0	3.4±0.07	2.91±0.1	2.88±0.14	3.28±0.36	2.63±0.04*	2.79±0.12*	3.1±0.04	3.39±0.09	2.68±0.76	$F_{(8,18)} = 4.265$	0.0051
C18:1	4.19±0.03	7.21±0.24*	8.23±0.33*	7.78±0.09*	5.35±0.06*	5.38±0.04*	5.94±0.13*	4.92±0.23	4.99±0.15	$F_{(8,18)} = 43.66$	<0.0001
C18:2	33.66±0.54	31.56±1.62	38.08±6.68	52.8±0.38	25.34±8.26	37.71±0.27	50.36±5.73	42.42±2.09	32.73±0.82	$F_{(8,18)} = 3.509$	0.0128
C18:3	33.06±0.31	23.35±1.09	28.45±1.09	31.24±0.25	18.44±0.39*	21.3±0.8*	24.5±5.38	30.24±3.76	34.15±1.42	$F_{(8,18)} = 4.911$	0.006
C20:0	5.99±1.1	4.24±0.42	5.29±0.46	5.91±0.64	1.71±0.02	1.75±0.01	7.92±1.05	7.58±1.36	72.19±47.34*	$F_{(8,72)} = 4.56$	0.0002
C21:0	3.49±0.96	2.8±0.4	3.5±0.3	4.79±0.48	6.53±0.86*	3.91±0.17	5.49±1.26*	6.31±0.84*	trace	$F_{(7,64)} = 7.737$	<0.0001
C22:0	14.62±1.73	8.87±1.15	9.27±1.39	9.95±1.39	5.02±0.09	5.57±0.23	12.8±1.61	13.37±1.29	104.67±68.31*	$F_{(8,72)} = 4.362$	0.0002
C24:0	6.25±0.2	4.49±0.13	5.16±0.81	4.48±0.42	4.12±0.08*	4.29±0.21*	6.32±0.39	6.59±0.58	trace	$F_{(7,16)} = 4.921$	0.0040
Total FA	326.3	276.45	304.99	354.73	267.41	288.52	381.39	370.63	500.86		
SFA	255.39	214.33	230.23	262.91	218.28	224.13	300.59	293.05	428.99		
UFA	70.91	62.12	74.76	91.82	49.13	64.39	80.8	77.58	71.87		
SFA/UFA	3.6	3.45	3.08	2.86	4.44	3.48	3.72	3.78	5.97		
Σ C18-series	74.31	65.03	77.64	95.1	51.76	67.18	83.9	80.97	74.55		
Σ C18:3+C18:2 / Σ C18-series	89.79	84.44	85.69	88.37	84.58	87.84	89.23	89.74	89.71		
C18:3/C18:2	0.98	0.74	0.75	0.59	0.73	0.56	0.49	0.71	1.04		
Σ VLCFA	30.35	20.4	23.22	25.13	17.38	15.52	32.53	33.85	176.86		

Values are the mean ± standard error of mean (SEM) of three biological replicates each analyzed three times measured by GC-MS. Significantly differences to day 0 are marked with an asterisk (nested one-way ANOVA with Dunnett's post hoc test, $p < 0.05$).
FA: fatty acid(s); UFA: unsaturated fatty acids; SFA: saturated fatty acids; VLCFA: very long chain fatty acids.
Total FA was calculated by the sum of all FA/day.
SFA was calculated by the sum of all FA/day except the unsaturated C18-series.
UFA was calculated by the sum of only the unsaturated C18-series/day.
SFA-to-UFA ratio was calculated by the ratio of SFA to UFA.
Sum of C18-series was calculated by the sum of C18:0, C18:1, C18:2 and C18:3.
Sum of C18:3+C18:2/sum of C18-series is shown in %.
C18:3-to-C18:2 was calculated by the ratio of C18:3 to C18:2.
Sum of VLCFA was calculated by the sum of C20:0, C21:0, C22:0 and C24:0.

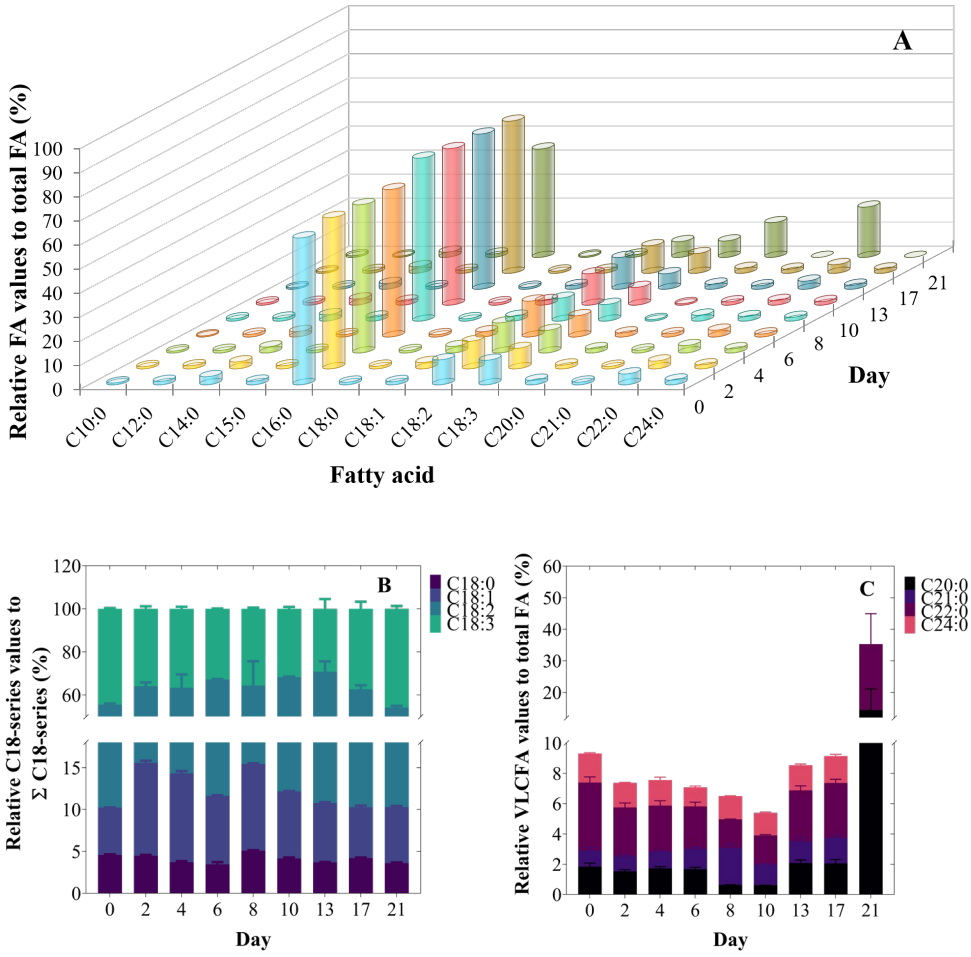

Figure 2. Relative values of fatty acid contents in percentage throughout one growth cycle in cell suspension cultures of *Catharanthus roseus* measured by GC-MS. Averaged data ± relative standard error of mean (SEM) of three biological replicates of each harvesting point analyzed three times is shown. **A.** Relative values of fatty acid to total amount of fatty acids. **B.** Relative C18-series values to the sum of all C18-series FA. **C.** Relative VLCFA to total amount of fatty acids.

carota (Radwan *et al.,* 1974), *Euonymus europaeus* (Gemmrich and Schraudolf, 1980) and the bryophytes *Rhytidiadelphus squurrosus, Eurhynchium striatum* (Hansen and Rossi, 1991) and *Marchantia polymorpha* (Chiou *et al.,* 2001). In cell suspension cultures of *C. roseus*, the only VLCFA found were C20:0, C21:0, C22:0 and C24:0 (Table 3) and their individual relative values to total FA were lower than 5 % except at day 21 where C20:0 and C22:0 showed an increase to 14.4 % and 20.9 % of the total FA, respectively (Fig. 2C). When comparing the summed values of relative VLCFA to total FA, they contributed with approximately 5-10 % during the lag and exponential phases of growth curve and increased up to 35 % of total FA at the last time point in (late) stationary phase (Fig. 2C).

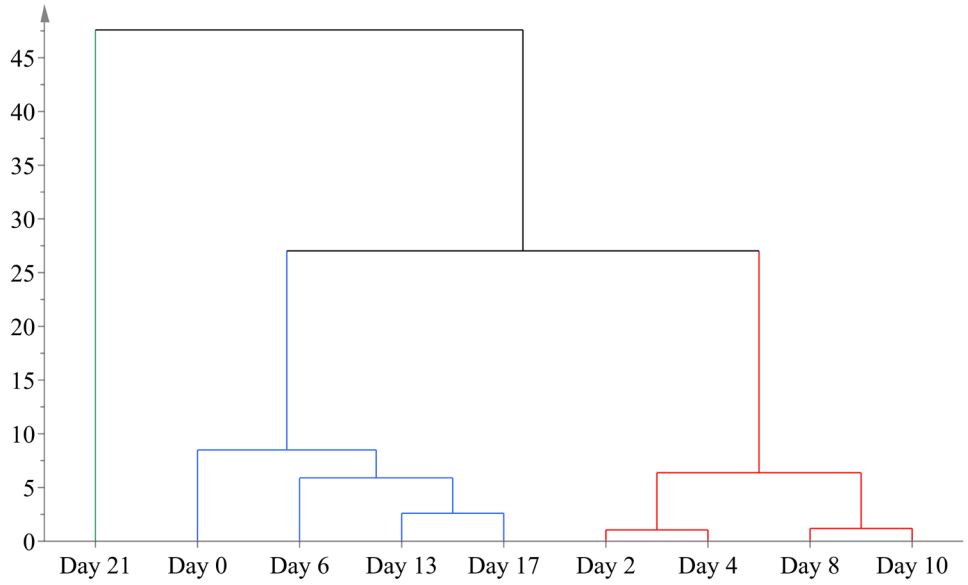

Figure 3. Dendrogram obtained from hierarchical cluster analysis of the fatty acid contents in cell suspension cultures of *Catharanthus roseus* measured by GC-MS and based on four components. Three main groups can be distinguished according to their different fatty acid profiles into "older cells" (days 0, 6, 13 and 17) colored in blue, "younger cells" (2, 4, 8 and 10) colored in red and day 21 stands as a unique group colored in green. Averaged data of three technical and three biological replicates of each harvesting point contained in Table 3 was used. Euclidean distances and Ward's clustering algorithm were used.

Relative VLCFA values to total FA in *C. roseus* were higher if compared to cell suspension cultures of *C. arietinum* and *P. hortense*, where VLCFA individual values for C20:0, C21:0, C22:0 and C24:0 ranged from 0.3 to 3.7 (weight %) (Radwan *et al.,* 1974). It is noteworthy that the amounts of odd and even numbered saturated VLCFA in cell suspension cultures of *C. roseus, C. arietinum* and *P. hortense* occurred in almost similar percentages except in 21-day-old *C. roseus* cells (Fig. 2C), suggesting that growth conditions like low oxygen supply and age of the culture might favor FA elongation and saturation (Radwan *et al.,* 1975b; Radwan and Mangold, 1976), since FAD enzymes involved in the desaturation of FA require molecular oxygen as an electron acceptor and fatty acid elongases require reduced pyridine nucleotides and molecular oxygen (Zhukov, 2018; Shanklin and Cahoon, 1998).

In the case of relative C18 values to the sum of total C18-series (Fig. 2B), C18:2 is the most abundant C18 FA peaking at day 13 with 60 % and coinciding with the maximum biomass increase. It is followed by C18:3 peaking at days 0 (44.5 %) and 21 (45.8 %), showing that cells on these days have similar contents of this FA. Moreover, we observed an inverse time-dependent behavior between relative values of C18:2 and C18:3 to the sum of total C18-series, whereas the summed relative values for C18:2 and C18:3 were very stable, between 84.6 % and 89 %, an increase in C18:3 is connected with a concomitant decrease in C18:2 which is expressed by the variation in the ratio between these

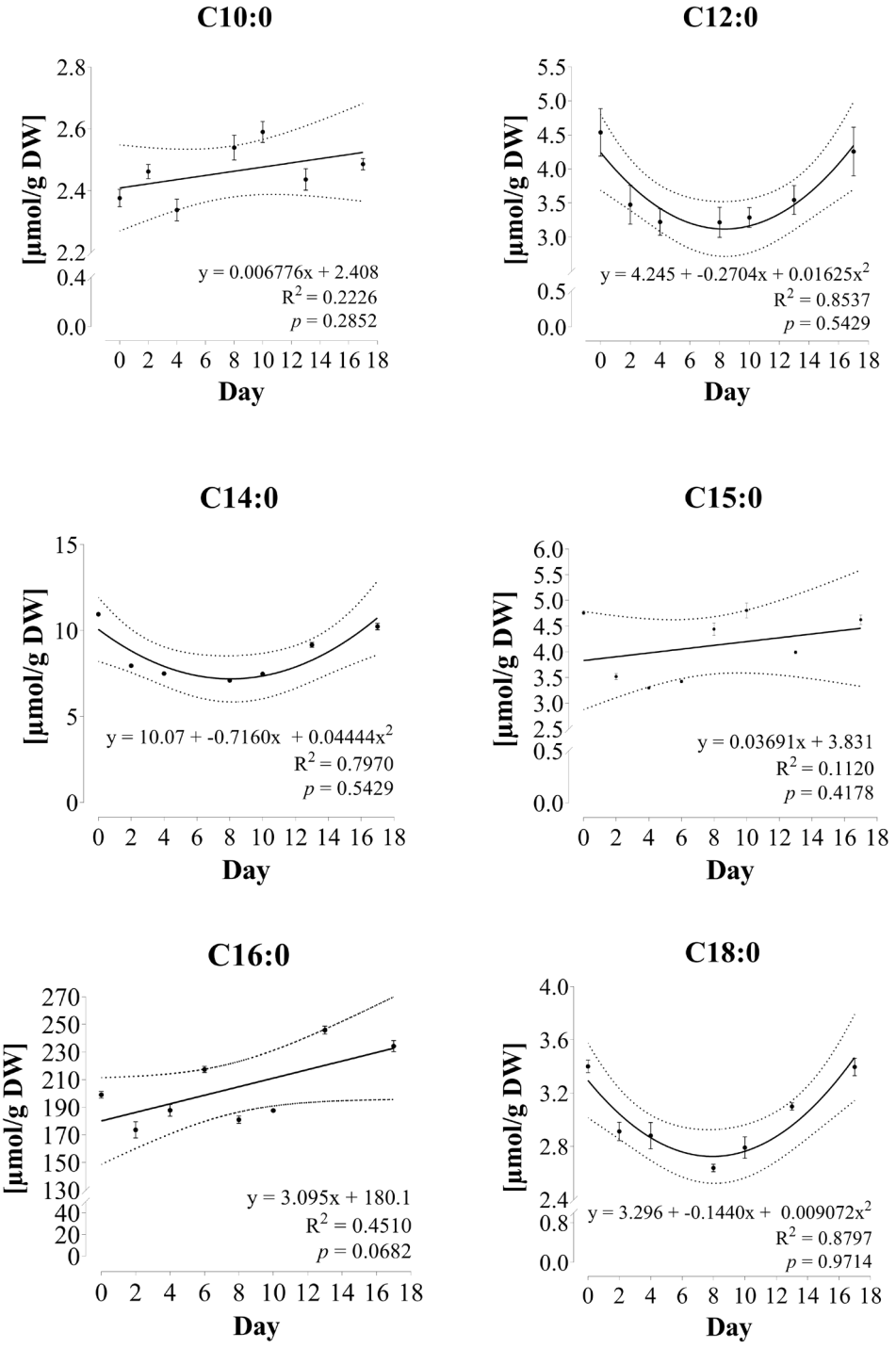

Figure 4. Continued

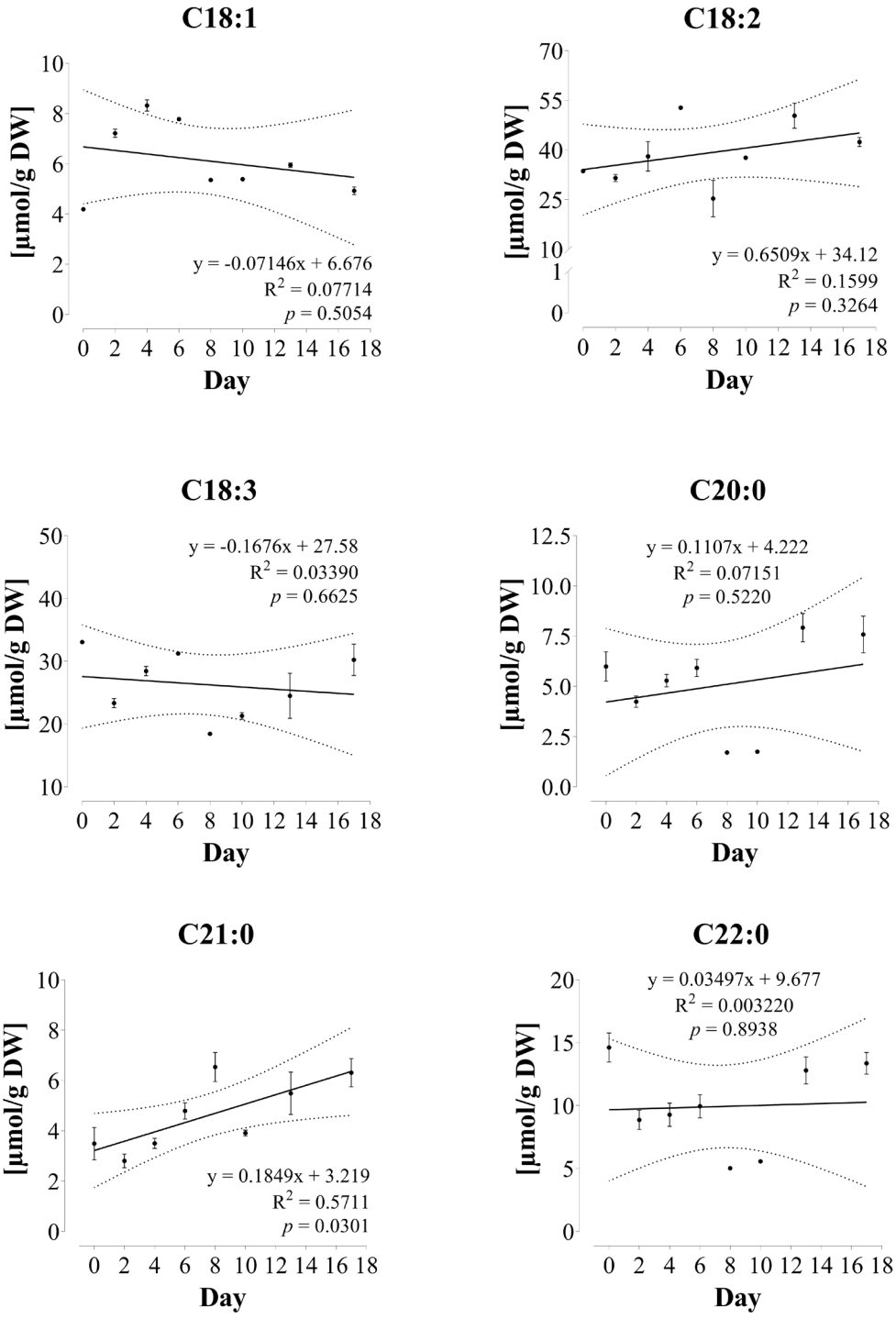

Figure 4. Continued

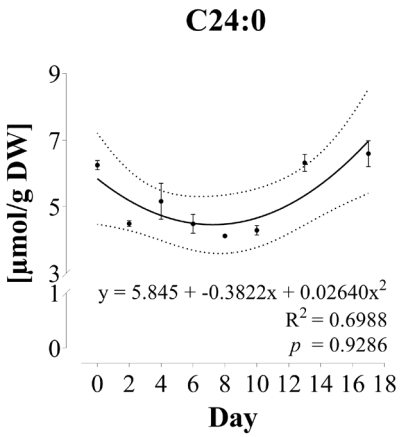

Figure 4. Linear or quadratic relationships between fatty acids amounts and the days after subculture in cell suspension cultures of *Catharanthus roseus* measured by GC-MS. Day is on the *x*-axis and absolute amounts in [µmol/g DW] on the *y*-axis. Averaged data of three technical and three biological replicates for each time point contained in Table 3 was used. Data from day 6 was excluded for C10:0, C12:0, C14:0 and C18:0. Data from day 21 was excluded for all fatty acids. Equations are presented for each fatty acid, the goodness of fit is represented by the coefficient of determination, $p < 0.05$ was considered statistically significant and dotted lines represent the 95% Confidence Intervals (CI).

two FA, varying between 0.49 at day 13 and 1.04 at day 21 (Table 2). In the case of C18:0 and C18:1, their relative values to the sum of total C18-series were low and had similar relative values at days 0, 17 and 21 (Fig. 2B). In order to obtain information about the relationships among data, a hierarchical cluster analysis (HCA) yielding a dendrogram was applied to all samples showing a clear separation into three main groups according to their age as "older cells" harvested at 0, 6, 13 and 17 days, "younger cells" harvested at 2, 4, 8 and 10 days and day 21 as a unique group (Fig. 3). Cells harvested at day 0 are in fact 21-day-old cells as they were measured immediately after transfer to fresh medium and thus are expected to be similar. Levels of C12:0 and C14:0 were higher on day 0 (old cells immediately after subculture) and 21-day-old cells.

Our results partly agree with previous observations in cell suspensions of *C. roseus* where C14:0, C16:0, C18:0, C18:2 and C18:3 were amongst the FA with the largest variations as a function of age (MacCarthy and Stumpf, 1980b), whereas in our results C20:0 and C22:0 were by far the most affected FA but only in 21-day-old cells. Differences found could be attributed to differences in origin of plant material and/or growth conditions such as pH, light, growth regimes, carbon sources and temperature. To further establish a relationship between FA amounts and days after subculture, linear or quadratic fitting models using regression analysis were used to describe for each FA, the change in FA levels over days after subculture *i.e.* the model that fits best a FA's change over time. The goodness of fit for each model is represented by the coefficient of determination (R^2) where a *p* value lower than 0.05 was considered statistically significant (Fig. 4). After visual examination of each FA model, for

C10:0, C12:0, C14:0 and C18:0, day 6 was removed from the regression analysis and not included in the calculations as those points were regarded as outliers and the same applied for day 21 in all FA, as most FA contents in this day showed too much variation that might negatively impact the best-fit values in the model. Nevertheless, in Table 3 values for each day are shown. A linear regression model was fitted for C10:0, C15:0, C16:0, C18:1, C18:2, C18:3, C20:0, C21:0 and C22:0 in order to find the line that best predicts *y* from each unit of *x* where the slope (*m*) says how much the amount of each FA is changing every day and the intercept (*b*) is the *y* values when *x* equals zero. Taking C10:0 as an example in Fig. 4 and according to the linear model, the intercept in the equation equals to 2.408 μmol/g DW at day 0, the slope explains that for every day there was an increase of 0.006776 μmol/g DW and the slope deviation from zero is not significant (p=0.2852). Additionally, the R^2 value tells the fraction of the variation that is shared between *x* and *y*, in other words, it is the percentage of the variation that is explained by a linear model so for C10:0, the model explains 22.26 % of the variability of the data around its mean. Because only the model for C21:0 was significant, interpretation of data in Fig. 4 is inconclusive. The models of C10:0, C15:0, C:16:0, C18:2, C20:0 and C21:0 showed a small daily rate increase. C12:0, C14:0, C18:0 and C24:0 showed a U-shape over the growth cycle, in which the high level of FA at the end of the growth cycle is similar to the level of day 0, *i.e.* just after subculture to fresh medium the levels of these FA went down, although halfway the growth cycle, their levels started to increase again. The major FA in cell suspension cultures belong to the C16- and C18-series FA. Obviously, there are a number of significant changes, but in most cases the changes are rather limited, with significant changes of about 50-100% or even less if compared to day 0. However, the most remarkable changes are for the VLCFA C20:0 and C22:0 at the end of the growth cycle.

Spener et al. (1974) observed that FA concentrations of C12:0 and C14:0 and the unsaturated C18-series showed pronounced differences that were age-dependent in tissue cultures of *Hydnocarpus anthelminthica.* Observations in *in vitro* cultures of *Arabidopsis thaliana* and *Acer pseudoplatanus* showed that levels of the C18-series had significant changes during the exponential phase where C18:3 and C18:2 decreased while C18:1 had a sharp increase. It was suggested that the activities of the FATTY ACID DESATURASES 2/3 (FAD 2, FAD3), respectively responsible of C18:2 and C18:3 formation in phospholipids, have limited rates in rapidly dividing cells such as cell suspension cultures and calli to desaturate C18:1 which ends up in its accumulation in membrane lipids, thus concluding that there is a negative correlation between growth rate and the accumulation of C18:2 and C18:3 (Meï *et al.,* 2015). The same authors also noticed less variations in C18:2 than in C18:3 and C18:1 concluding that C18:2 had no significant correlation with growth rate which is in disagreement with our results, as in *C. roseus* cells, we observed that relative values of C18:2 and C18:3 to the sum of total C18-series changed in conjunction, while C18:2 increased over time, C18:3 decreased and reaching similar levels at days 0 and 21. From the experiments, it is clear that the FA profiles of cell suspension cultures of *C. roseus* are subject to significant changes during one growth cycle.

2.3.3 Fatty acid profile of organs of *C. roseus* plants

The FA profiles of intact organs of *C. roseus* showed a different composition from those of cell suspension cultures. When looking at the averages of total FA, seeds had the highest content of total FA (µmol/g DW) if compared to the other plant parts (Table 4). Additionally, seeds and roots shared the highest values for the summed contents of VLCFA and C18-series FA followed by stems. Nevertheless, stems and roots had the highest ratio of SFA/UFA. Since seeds had the highest composition of FA of all plant organs studied and statistically different to all plant materials in contents of C16, all C18-series and C20:0, we compared the total FA values of all other plant organs using the ratio of seed-to-plant organ; this showed that flower buds had a factor of 181.5, followed by leaves (81.9) and flowers (40.7) (Table 4). The FA composition of each plant organ in terms of relative values to total FA showed that C16:0 was the most abundant FA for all plant organs especially for stems and C18:1 for seeds, followed by C22:0 in roots, C20:0 in stems, C24:0 in roots and C18:0 in seeds (Fig. 5A). In the case of relative values to total C18-series, again seeds stood out for their highest contents of C18:1 (Fig. 5B). Stems and flowers had similar relative values to total C18-series of C18:0 whereas flower buds were rich in C18:2 and C18:3 (Fig. 5B). Relative values of C18:3 to total C18-series were the lowest in most plant organs especially in seeds and roots. Oilseeds are known to accumulate C16:0, C18:0, C18:1, C18:2 and C18:3 (Bach and Faure, 2010; Villalobos *et al.,* 2013; Baud, 2018), like seeds of *Jatropha curcas* with low relative values of C18:3 to β-sitosterol but rich in C18:1, C18:2, C16:0 and C18:0 (Thi *et al.,* 2018). Similarly, seeds of *Helianthus annus* and green beans of *Coffea arabica* are abundant in C18:2, C18:1, C16:0, C18:0, C20:0 C22:0 (Chernova *et al.,* 2019; Mehari *et al.,* 2019). In contrast, seeds of *Brassica napus* are more abundant in C18:1, C18:2 and C18:3 (Chernova *et al.,* 2019). Total amount of VLCFA was higher in seeds and roots (Table 4). Seeds and stems showed similar relative values of C20:0 and C22:0 to total FA, being the only VLCFA present (Fig. 5C). Despite the fact that seeds alone accounted for 70.9 % and 29.1 % of relative values of C20:0 and C22:0 to total VLCFA respectively, when comparing their individual relative values to total FA, they actually account for less than 5 % of total FA in seeds (Fig. 5A). In contrast, flower buds and leaves showed the presence of C20:0, C22:0, C24:0 and C26:0 although with different individual relative values to total VLCFA (Fig. 5C). Similarly, in roots and flowers, only individual relative values to total VLCFA of C20:0, C22:0 and C24:0 were present (Fig. 5C).

Hierarchical cluster analysis was applied to data from plant organs and yielded a dendrogram that allowed the discrimination of samples into 4 groups based on their FA composition: one formed by seeds; the second formed by roots and the third subdivided above-ground organs between flower buds and leaves *versus* stems and flowers (Fig. 6). Of these, flower buds and leaves shared that they are the only plant parts in which C26:0 was detected. The seeds distinguished themselves from the other groups by relatively low levels of the VLCFA and relatively high levels C18:1. Roots are among others characterized by high levels of both C22:0 and C24:0 (Table 4).

Table 4. Contents of fatty acids profiles of different plant organs of *Catharanthus roseus* (µmol/g DW)

	Flowers	Flower buds	Leaves	Roots	Stems	Seeds	F	*p*
C12:0	nd	nd	nd	nd	nd	58.28±0.98	-	-
C14:0	nd	4.28±0.02	nd	22.49±15.95	13.86±2.97	26.37±7.22	$F_{(3,7)} = 1.797$	0.2353
C15:0	nd	2.4±0.11	nd	28.69±14.53	nd	nd	-	-
C16:0	67.71±27.59^{b}	8.94±1.63^{b}	28.05±27.65^{b}	142.49±59.24^{b}	160.53±85.57^{b}	2784.02±354.3^{a}	$F_{(5,12)} = 65.55$	<0.0001
C18:0	20.54±8.31^{b}	1.85±0.21^{b}	9.04±8.96^{b}	34.94±17.44^{b}	29.74±6.36^{b}	1066.66±117.64^{a}	$F_{(5,12)} = 144$	<0.0001
C18:1	4.4±0.82^{b}	2.71±0.06^{b}	3.34±0.84^{b}	25.72±23.26^{b}	8.79±0.79^{b}	2165.78±298.94^{a}	$F_{(5,12)} = 54.13$	<0.0001
C18:2	9.33±3.06^{b}	3.23±0.26^{b}	5.96±3.83^{b}	17.53±13.73^{b}	11.89±1.79^{b}	390.39±62.01^{a}	$F_{(5,12)} = 32.22$	<0.0001
C18:3	3.9±0.63^{b}	2.83±0.12^{b}	3.38±0.7^{b}	3.13±0.13^{b}	5.35±0.64^{b}	10.87±1.49^{a}	$F_{(5,12)} = 13.81$	<0.0001
C19:0	3.24±0.81	2.14±0.25^{b}	2.26±0.81^{b}	5.53±0.9	5.68±0.43^{a}	nd	$F_{(4,10)} = 5.706$	0.0118
C20:0	18.05±7.52^{b}	1.68±0.14^{b}	8.95±9.03^{b}	26.96±6.56^{b}	66.88±68.45^{b}	157.04±20.28^{a}	$F_{(5,12)} = 10.2$	0.0005
C22:0	28.34±11.68	2.31±0.39^{b}	13.72±14.13^{b}	103.73±30.66^{a}	48.76±30.26	64.61±9.11	$F_{(5,12)} = 4.692$	0.0132
C24:0	9.84±3.68	2.96±0.94^{b}	4.96±4.01	96.94±34.7^{a}	nd	nd	$F_{(3,8)} = 4.87$	0.0326
C26:0	nd	1.71±0.47	2.4±1.45	nd	nd	nd	-	-
Total FA	165.35	37.04	82.06	508.15	351.48	6724.02		
SFA	147.72	28.27	69.38	461.77	325.45	4156.98		
UFA	17.63	8.77	12.68	46.38	26.03	2567.04		
SFA/UFA	8.4	3.2	5.5	10	12.5	1.6		
Σ C18-series	38.17	10.62	21.72	81.32	55.77	3633.7		
Σ VLCFA	56.23	8.66	30.03	227.63	115.64	221.65		
Seed/Plant organ	40.7	181.5	81.9	13.2	19.1	1		

Values are the mean ± standard error of mean (SEM) of three biological replicates each analyzed three times measured by GC-MS. Different letters show significant differences among plant organs (nested one-way ANOVA with Tukey's post hoc test, $p < 0.05$). Not enough data for C12:0 allowed ANOVA calculations: (-). No significant differences in C15:0 between flower buds and roots (t=2.517, df=4, p=0.0656) and in C26:0 between flower buds and leaves (t=0.5465, df=4, p= 0.6138) were found (two-tailed nested t-test, $p < 0.05$).

FA: fatty acid(s); UFA: unsaturated fatty acids; SFA: saturated fatty acids; VLCFA: very long chain fatty acids; nd: not detected.

Total FA was calculated by the sum of all FA.

SFA was calculated by the sum of all FA except the unsaturated C18-series.

UFA was calculated by the sum of only the unsaturated C18-series/day.

SFA-to-UFA ratio was calculated by the difference between SFA and UFA.

Sum of C18-series was calculated by the sum of C18:0, C18:1, C18:2 and C18:3.

Sum of VLCFA was calculated by the sum of C20:0, C22:0, C24:0 and C26:0.

Seed-to-Plant organ ratio was calculated by the difference between total FA content of seeds and the total FA content of each plant organ.

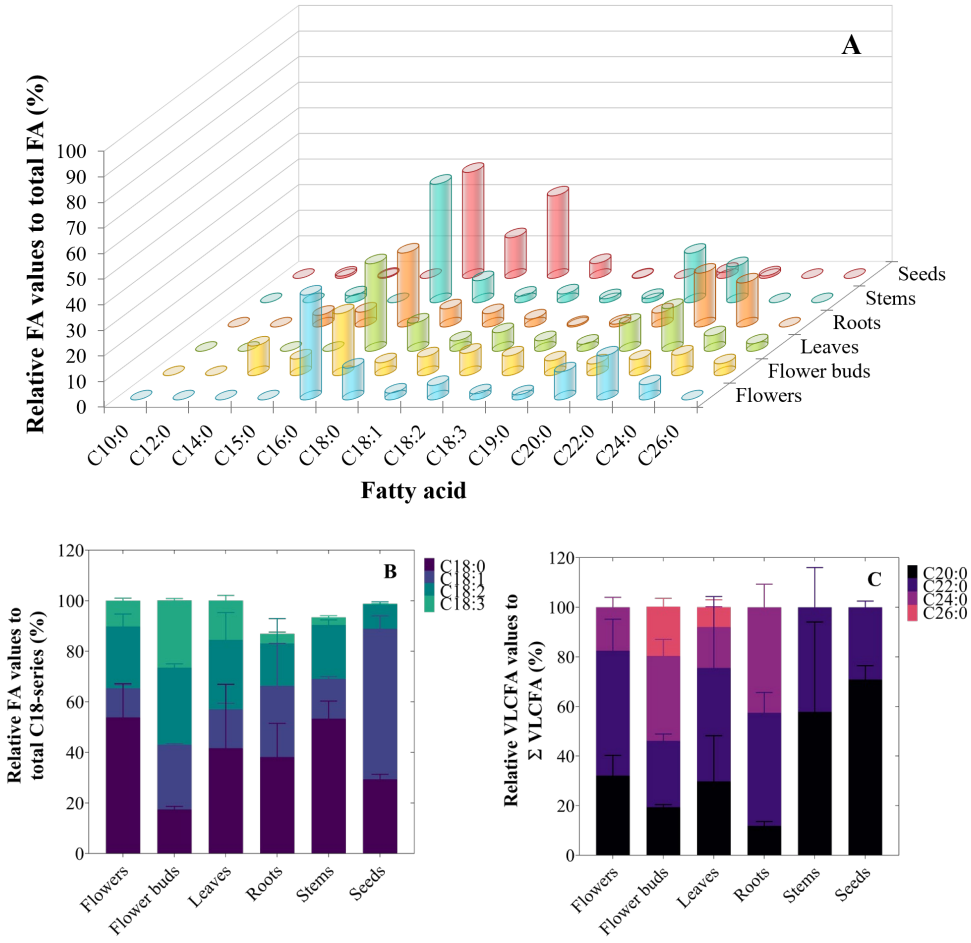

Figure 5. Relative values of fatty acid contents in plant organs of *Catharanthus roseus* measured by GC-MS. **A.** Relative values of fatty acid to total amount of fatty acids. **B.** Relative C18 values to the sum of all C18-series. **C.** Relative VLCFA to the sum of all VLCFA. Averaged data ± relative standard error of mean (SEM) of three technical and three biological replicates of each plant organ is shown and expressed in percentage (%).

There are only a few reports on the FA composition of *C. roseus* plants mostly part of essential oils and extracted by hydrodistillation. Studies of volatiles in leaves and flowers of *C. roseus* reported the presence of C16:0 (4.9 %) and C18:0 (1.5 %) as the major FA in the essential oils of both plant organs whereas C9:0 (3.3 %) and C14:0 (1.3 %) were only present in leaves but not in flowers. This is in partial agreement with our observations since no C14:0 was observed neither in leaves nor in flowers but with high relative values to total FA in flower buds (11.6 %). In contrast, flowers were rich in C16:3 (1.9 %), a FA not observed in our findings, although lower relative levels of C18:0 (0.4 %), C16:0 (0.4 %) and C10:0 (0.6 %) were found if compared to leaves (1.5 %, 4.9 % and 0.8 %, respectively) (Pandey-Rai *et al.,* 2006) which disagrees with our results where C16:0 and C18:0 were

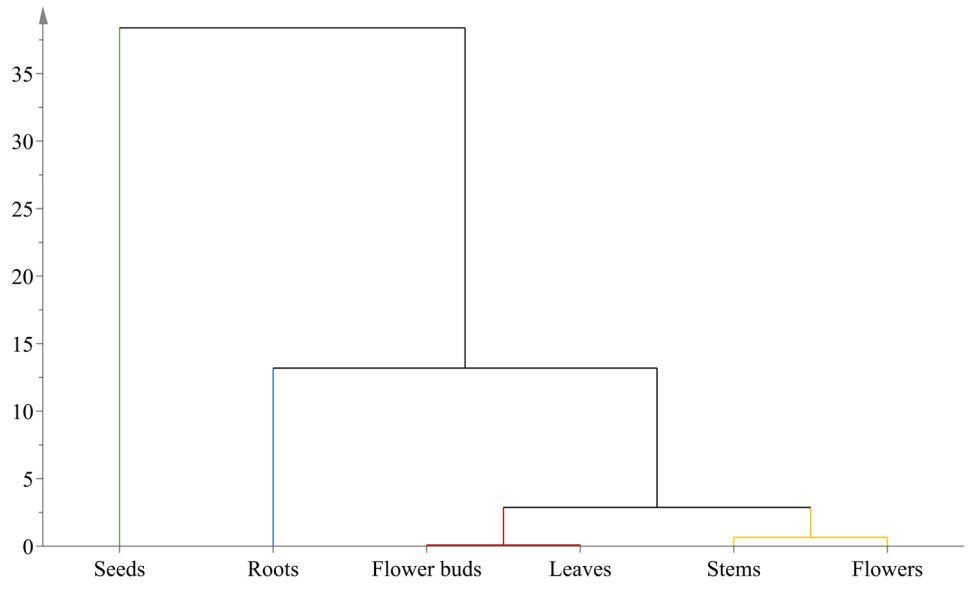

Figure 6. Dendrogram obtained from hierarchical cluster analysis of fatty acid contents in plant organs of *Catharanthus roseus* measured by GC-MS and based on two components. Plant organs were separated into four different groups: seeds as one group (green), roots as a second group (blue), flower buds and leaves as a third group (red) and stems and flowers as a fourth group (yellow). Averaged data of three technical and three biological replicates of each plant organ contained in Table 4 was used. Euclidean distances and Ward's clustering algorithm were used.

among the most abundant FA in flowers. Another study of essential oils of *C. roseus* leaves, reported C16:0 (64.9 %), C14:0 (6.6 %) and C12:0 (2.7 %) as the major FA although low levels (<0.3 %) of FA like C6:0, C7:0, C8:0, C9:0, C11:0, C15:0 were detected (Brun *et al.,* 2001) where none of these SCFA were observed in our experiments. Guedes De Pinho et al. (2009) analyzed by headspace solid-phase microextraction (HS-SPME), leaves, stems and flowers of *C. roseus* and reported for the first time the presence of the ethyl ester of C18:0 in all plant organs although more abundant in leaves (5.1 %), the propyl esters of C12:0, C14:0, C16:0 and C18:0, the ethyl C6:0 mostly in leaves (5.5 %) and flowers (2.2 %), traces of MeJA in stems and flowers and relative low levels of C18:2 (0.2 %) only in leaves. Lawal et al. (2015) reported the presence of the ethyl ester of C18:2 as the major FA in flowers (14 %) and leaves (43.9 %) of *C. roseus*, followed by C18:0 (10.6 %), C16:0 (6.8 %), C18:2 (5.6 %) and C18:3 (1.2 %) all present only in leaves. Other components also reported in these plant materials were carotenoids, 13 carbon compounds, alkanes, alcohols, aldehydes, ketones, terpenoids, phenylpropanoids and sterols (Pandey-Rai *et al.,* 2006; Brun *et al.,* 2001; Guedes De Pinho, 2009; Lawal *et al.,* 2015). The essential oil composition of stems and fruits of *Caralluma europea* consisted mostly of non-aromatic compounds like alkanes and the FA C14:0, C16:0 and C18:2 that accounted for more than 88% of their composition in both plant materials. Similarly, essential oils from branches and flowers of *Periploca laevigata* subsp. *angustifolia* showed the presence of C16:0, C16:1, C14:0 and

C18:1 as well as aldehydes, alkanes, terpenoids and alcohols (Zito *et al.,* 2013). Changes in FA composition in senescent leaves in comparison to young leaves of *C. roseus* were reported. The latter were characterized by the presence of high levels of C16:3 esterified to MGDG and C18:3 to DGDG whereas older leaves showed a higher ratio of sterols-phospholipids, a lower UFA/SFA ratio and alteration in polar lipids indicating the selective degradation of chloroplasts and vacuoles (Mishra and Sangwan, 2008; Mishra *et al.,* 2006). All of these are typical ontogenic events involving C18:3, and in some plant species C16:3, which are important components of the thylakoids where the acyl groups are essential constituents of the photosynthetic apparatus (Mongrand *et al.,* 1998; Griffiths, 2015).

When comparing total FA amounts in cell suspension cultures, they were in the same range as roots and stems. The ratio of SFA/UFA in seeds is quite different from that found in the other plant parts and cell suspension cultures due to the relative high level of UFA (Table 3 and Table 4). The ratio of SFA/UFA was also relatively low in cell cultures with values varying between 2.86 and 5.97, which is in the same range as the ratio in flower buds and leaves. Overall, C16:0 is the major constituent in all plant parts as in cell suspension cultures (Fig. 2A and 5A). Seeds were rich in C18:0 and C18:1 and the only plant organ with C12:0 (Fig. 5B). Furthermore, the C18:0/C18:1 composition in seeds is quite different from cell suspension cultures where these had higher relative values of C18:2. Moreover, C19:0 and C26:0 were only found in plant materials whereas C10:0 and C21:0 were unique to cell suspension cultures. Flower buds were the most abundant in C14:0, C15:0, C18:1, C18:2, C18:3, C19:0 and C26:0 if compared to the rest of the plant organs. Differences in relative values to total FA in cell suspension cultures were moderately higher for C16:0 (1.8-fold), C18:2 (1.6-fold) and C18:3 (1.9-fold) than in leaves although the composition is quite different, C10:0, C12:0, C14:0, C15:0 and C21:0 were absent in leaves. Photoautotrophic cell cultures, like photosynthetic organs, typically have relatively high levels of MGDG and DGDG and esterified FA species such as C18:2, C18:3 and C16:0, whereas in heterotrophic cultures levels of C18:2 and C18:1 are lower (Hüsemann *et al.,* 1980). Also, levels of sterols, sterol esters, sterol glucosides and esterified sterol glucosides are higher, demonstrating that the FA and lipid patterns of photoautotrophic systems resemble those of photosynthetic organs (Hüsemann *et al.,* 1980). Similar patterns were observed in the cell line CRPP of *C. roseus* where the presence of phytosterols, carotenoids and chlorophylls might indicate that this cell line is partially photoautotrophic (Saiman *et al.,* 2015). These observations can also be seen in dedifferentiated tissues such as calli, non-embryogenic calli and somatic embryos where high levels of lipids and FA do not occur (Radwan *et al.,* 1975a; Cunha and Fernandes-Ferreira, 2003; Williams *et al.,* 1991). Similarly, *Petroselinum crispum* (Ellenbracht *et al.,* 1980; López *et al.,* 1999), *B. napus* and *B. campestris* (Staba *et al.,* 1971), *Cucumis melo* (Halder and Gadgil, 1984) and *E. europaeus* (Gemmrich and Schraudolf, 1980) tissue cultures had lower accumulation of particularly C18:1, C18:2, C18:3 and C22:1 when compared to seeds, leaves, roots, stems and cotyledons of those plant species. Yin et al. (2014) observed similar FA contents in cell suspension cultures and seeds from *Capparis spinosa* where levels of C16:0, C18:2 and

C18:1 and C14:0 were the predominant species in both plant materials. These observations are in agreement with the occurrence of PUFA as the major components of most plant tissues *e.g.* during leaf expansion or greening of etiolated tissues (Murphy and Stumpf, 1981). Moreover, the FA composition of stems, fruits and flowers of *Caralluma europaea* and seeds of *Tabernaemontana cymosa* is mostly characterized by the presence of C14:0, C16:0, C18:2 (Zito *et al.,* 2010; Achenbach *et al.,* 1997) as well as traces of SCFA such as C6:0, C8:0, C9:0 and C10:0 (Formisano *et al.,* 2009) where the first three FA species have also been reported to be present in leaves of *C. roseus* (Pandey-Rai *et al.,* 2006; Guedes De Pinho *et al.,* 2009). The observation that levels of C18:3 in cells are higher than in seeds is in agreement with Pollard et al. (2015) who found the FA composition of *in vitro* cultured young embryos of *Camelina sativa* closely matched that of seeds of the same plant. Finally, differences in the biological replicates observed in plant organs and cell suspension cultures of *C. roseus* might be due to differences between the cultivars used (Pacifica Red and Pacifica Punch, respectively), although Dong et al. (2015) demonstrated only significant differences in contents of major FA (C18:2, C16:0, C18:1 and C20:0) of seeds among seven cultivars of *Coffea robusta* but not on their distribution. Additionally, FA contents in seeds of three cultivars of *Chenopodium quinoa* showed that the presence and composition of FA is highly conserved among cultivars (Wood *et al.,* 1993).

2.4 CONCLUSIONS

The application of a GC-MS-based targeted approach proved to be useful to characterize and to distinguish FA profiles between cell suspension cultures grown over a period of 21 days and plant materials of *C. roseus*. After HCA analysis, cell suspensions clustered into three groups *i.e.* 21-day-old cells as a single group, “younger” (2, 4, 8, and 10 days) and “older” cells (0, 6, 13 and 17 days). At day 21 significantly higher contents of C10:0, C15:0, C20:0 and C22:0 than on day 0 were observed. HCA analysis of plant materials showed that flowers and stems, flower buds and leaves clustered into 2 different groups whereas seeds and roots clustered into two separate groups. The ratio SFA/UFA is smaller in cell suspension cultures than in plant organs, with the exception of seeds that had by far the highest FA content and the lowest SFA/UFA ratio. In cell suspension cultures, C18:2 and C:18:3 are the major UFA, whereas in seeds it is C18:1. In all systems, C16:0 is relatively the major compound, in absolute amount it is highest in seeds. Furthermore, differences in contents of C16:0, the C18-series and C20:0 in seeds were statistically different from those in all plant materials. Finally, the chosen statistical approach allowed us to correctly assess differences in FA contents in both experiments in cell suspension cultures and intact plants and seeds taking into consideration the biological and technical replication, as nested nominal variables. Together with the use of a validated GC-MS method, we conclude that our analytical platform is suitable for further studies of the effect of various treatments on total free and bound FA of the cell cultures and plants.

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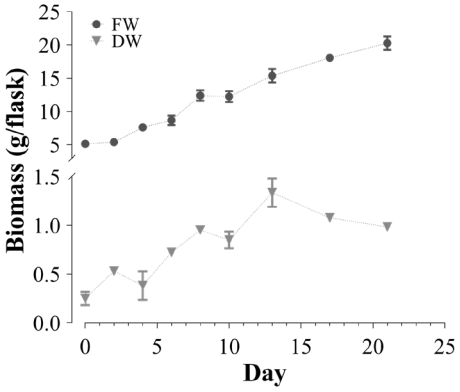
2.7 SUPPLEMENTAL INFORMATION

Supplement 2.7.1. Biomass accumulation in the CRPP line throughout one growth cycle of cell suspension cultures of *Catharanthus roseus*. Day is on the *x*-axis and biomass amounts in [g/flask] on the *y*-axis. Values are the mean ± standard error of mean (SEM) of three biological replicates.



Chapter 3

Effect of jasmonic acid on the fatty acid profiles of cell suspensions of *Catharanthus roseus*

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ABSTRACT

Apart from a structural function in membranes, some fatty acids (FA) are part of the biosynthesis of important derivatives with a signaling function. Of particular interest is the role of linolenic acid (C18:3) as the precursor of jasmonic acid (JA), an important stress hormone and inducer of several defense-related pathways. Earlier tests showed a fast burst of JA upon challenging *Catharanthus roseus* cell suspension cultures with JA; here we aimed to evaluate if this fast burst of JA is due to the induction of the biosynthesis of the precursor C18:3. A targeted profiling was employed to investigate the effect of jasmonic acid (JA) on the fatty acid (FA) profile of cell suspension cultures of *Catharanthus roseus* in a time course experiment (0, 5, 30, 90, 360 and 1440 min after elicitation) using gas chromatography coupled to mass spectrometry (GC-MS) and multivariate data analysis (MVDA) like principal component analysis (PCA). PCA showed some separation of all samples into three main clusters: the first two clusters encompassing the early stage samples (0-30 min and 90-360 min after elicitation) where most changes were associated to mock treatment and the last cluster samples from 1440 min, encompassing samples with changes associated to JA elicitation. Particularly levels of C18:0, C18:1, C18:2 and C18:3 showed an increasing trend after 1440 min of JA treatment compared to untreated cells. However, it appears that the mock treatment with a solution of 40 % ethanol, which is the solvent of JA, has a clear and significant effect on the pattern of FA found in the cells as well and particularly on the C18 series. Under these experimental conditions, no conclusions can be drawn yet about the effect of JA on FA composition, as the effect might be due to an unexpected elicitation-like effect of the solvent ethanol.

3.1 INTRODUCTION

Fatty acids (FA) are essential molecules present in all living organisms as they serve as the major source of complex lipids that are essential components of cellular membranes (Kachroo and Kachroo, 2009). Additionally, they are also key molecules that participate in various biological processes (Li *et al.,* 2015). In plants, composition and turnover of intracellular lipids and FA are frequently altered during development and are among the first targets of environmental signals (Feussner and Wasternack, 2002). For example, the polyunsaturation of FA has proven to be correlated to adaptation when plants are challenged with changes in temperature (MacCarthy and Stumpf, 1980; Kazemi-Shahandashti *et al.,* 2013) and high salinity (Elkahoui *et al.,* 2004). Apparently, they modulate a variety of responses to biotic and abiotic stresses.

Jasmonic acid (JA) and its derivatives, also known as jasmonates (JAs) are derived from FA and are one of the best-studied groups of signal molecules. JAs play a role of master switch in many plant responses to biotic and abiotic factors such as wound response after herbivore attack, ultraviolet light, ozone and drought; but they also control flower, seed and fruit development, seed germination, pollen viability, anthocyanin accumulation, fruit ripening (Creelman and Mullet, 1997), tuberization

in *Solanum* spp., tendril coiling in *Bryonia* (Falkenstein *et al.,* 1991) and promotion of leaf senescence (Wasternack and Hause, 2002; Wasternack *et al*., 2013; De Domenico *et al.,* 2012). The *de novo* biosynthesis of JA occurs through the oxidation of the unsaturated FA linolenic acid (C18:3) and in plants such as *Arabidopsis thaliana* and *Solanum lycopersicum* from 7(*Z*),10(*Z*),13(*Z*)-hexadecatrienoic acid (C16:3) which are released from galactolipids in the thylakoid by phospholipase 1 (PLA_1), DEFECTIVE IN ANTHER DEHISCENCE 1 (DAD1) and DONGLE (DGL) (Ishiguro *et al.,* 2001), although both pathways yield (+)-7-*iso*-JA (Gfeller *et al.,* 2010). The first biosynthetic step is the addition of molecular oxygen to form 13(*S*)-hydroxyperoxydecatrienoic acid (13-HPOT) by a 13-lipoxygenase (13-LOX) with C18:3 as substrate. This FA hydroxyperoxide is dehydrated by allene oxide synthase (AOS) yielding 12,13-epoxy-linolenic acid (12,13-EOT). The cyclization catalyzed by allene oxide cyclase (AOC) leads to the cyclopentenone derivative 12-*oxo*-phytodienoic acid (OPDA) in the C18:3 pathways or dinor-12-*oxo*-phytodienoic acid (dnOPDA) in the C16:3 pathway. Both OPDA and dnOPDA are possibly exported from the chloroplast to the peroxisome by the COMATOSE1/PEROXISOMAL1/PEROXISOME ABC TRANSPORTER (CTS1/PXA1/PED3) (Zolman *et al.,* 2001). Further transformation includes saturation of the Δ^{10}-double bond of OPDA and dnOPDA by OPDA reductase3 (OPR3), followed by three sequential steps of β-oxidation in the peroxisome catalyzed OPC8:CoA ligase 1 (OPCL1), acyl CoA oxidase (ACX1) and the multifunctional protein (MFP) and 3-ketoacyl-CoA thiolase (KAT2), which catalyze the formation of JA-CoA, later liberated by a thioesterase (TE) (Wasternack and Hause, 2013). Conjugation to isoleucine catalyzed by JASMONATE RESISTANT1 (JAR1) in the cytosol yields the biologically active (*3R, 7S*)-jasmonoyl-L-isoleucine (JA-Ile) (Fonseca *et al.,* 2009). Both JA and JA-Ile are transported by JA/JA-ILE JASMONATE TRANSPORTER 1 (JAT1) across the plasma membrane or to the nucleus, respectively (Li *et al.,* 2017) (Fig. 1).

The effects of JA during the stress response are well documented in many plant species where broad changes in gene expression take place. According to Wasternack and Hause (2002), two different responses can be distinguished: (1) the downregulation of housekeeping genes and (2) the upregulation of JA-responsive genes that leads to the synthesis of JA-induced proteins, which starts with the accumulation of enough JA-Ile levels needed to promote CORONATINE INSENSITIVE1-JASMONATE ZIM DOMAIN (COI1-JAZ) interactions (Koo *et al.,* 2009; Koo and Howe, 2009). This in turn promotes the degradation of JAZ proteins that repress the expression of JA-responsive genes. These events will ultimately end up with the accumulation of a wide array of JAs (Koo *et al.,* 2009) and secondary metabolites such as in *Coptis japonica* (Yamada *et al.,* 2011), *Nicotiana benthamiana* (Todd *et al.,* 2010), *Catharanthus roseus* (Lee-Parsons *et al.,* 2004; Moreno *et al.,* 1993; Shukla *et al.,* 2010), *Hypericum perforatum* (Walker *et al.,* 2002), *Taxus cuspidata* (Yang *et al.,* 2008), *Rauvolfia canescens* (Gundlach *et al.,* 1992), *Calendula officinalis* (Wiktorowska *et al.,* 2010), *Eschscholtzia californica* (Mueller *et al.,* 1993), *Panax ginseng* (Dewir *et al.,* 2010), *Lithospermum*

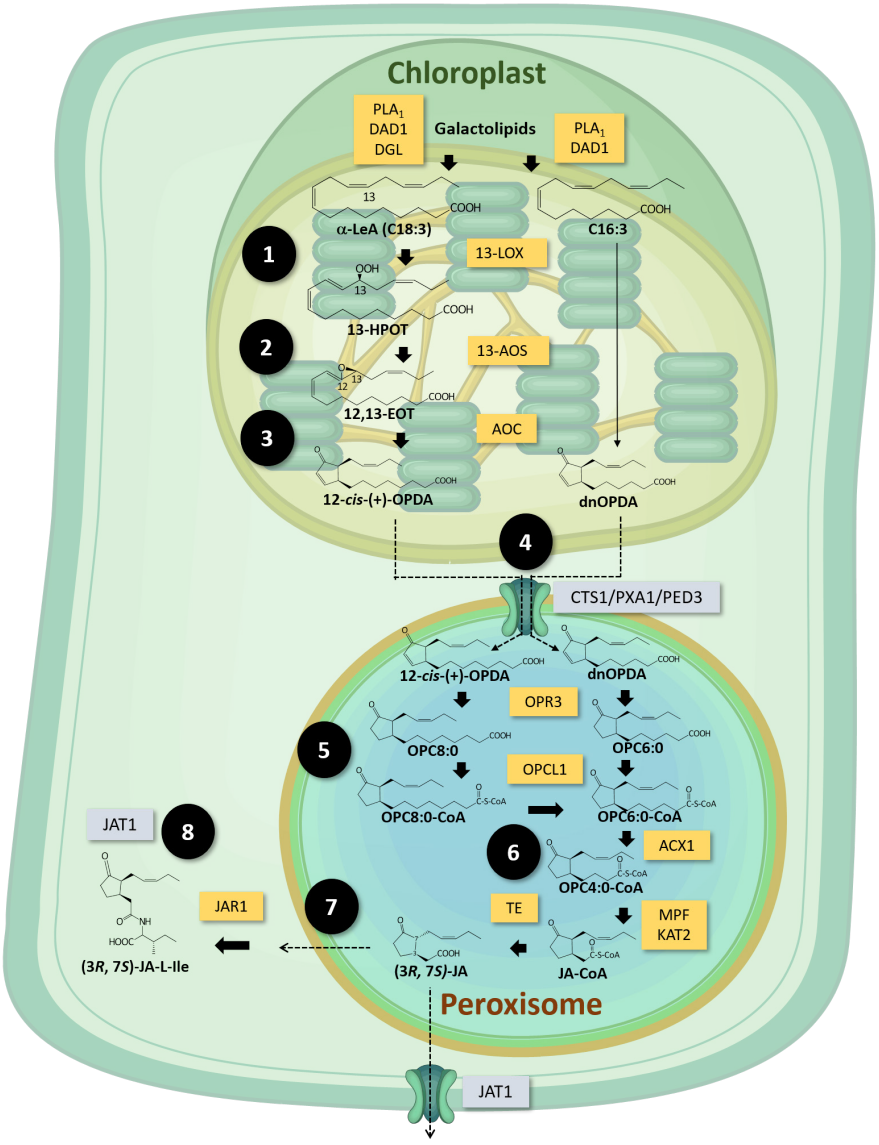

Figure 1. Subcellular compartmentation of JA biosynthesis (modified from Mosblech *et al.*, 2009). The formation of octadecanoid oxylipins including JA starts with the lipase-mediated release of α-linolenic acid (C18:3) from membrane lipids catalyzed by phospholipase 1 (PLA_1), DEFECTIVE IN ANTHER DEHISCENCE 1 (DAD1) and DONGLE (DGL). 13-Lipoxygenase (13-LOX) (**1**) transforms the FA into 13-(*S*)-hydroxyperoxy-octadecatrienoic acid (13-HPOT). Allene oxide synthase (AOS) (**2**) converts 13-HPOT to the unstable intermediate epoxy-octadecatrienoic acid (12,13-EOT), which is the substrate for AOC (**3**), yielding *cis*-(+)-*oxo*-phytodienoic acid (OPDA) and dinor-12-*oxo*-phytodienoic acid (dnOPDA) derived from the hexadecatrienoic acid (C16:3) pathway. OPDA and dnOPDA are exported from the plastid by an unknown mechanism and imported into peroxisomes by the carrier COMATOSE1/PEROXISOMAL1/PEROXISOME ABC TRANSPORTER (CTS1/PXA1/PED3) (**4**). In the peroxisome OPDA is reduced by OPDA-reductase 3 (OPR3) (**5**) to 3-*oxo*-2-(2-(Z)-pentenyl)-cyclopentane-1-octanoic acid (OPC8:0) and dnOPDA to 3-*oxo*-2-(2-(*Z*)-pentenyl)-

cyclopentane-1-hexanoic acid (OPC6:0). Both are activated to their CoA esters by OPC8:CoA ligase1 (OPCL1), which then undergo sequential rounds of β-oxidation catalyzed by acyl CoA oxidase (ACX1), the multifunctional protein (MFP) and 3-ketoacyl-CoA thiolase (KAT2) producing 3-*oxo*-2-(2-(*Z*)-pentenyl)-cyclopentane-1-butyric acid (OPC4:0CoA) and JA-CoA (**6**) which is deactivated by a thioesterase (TE) yielding to (3*R*, 3*S*)-JA. JA is later exported to the cytosol by an unknown mechanism (**7**) where it can be conjugated to isoleucine (Ile) by JASMONATE RESISTANT1 (JAR1) (**8**), forming the biologically active (3*R*, 3*S*)-JA–Ile, that is transported into the nucleus by JA/JA-ILE JASMONATE TRANSPORTER 1 (JAT1).

erythrorhizon (Mizukami *et al.,* 1993) and *Vitis vinifera* (Zhang *et al.,* 2002; Lu *et al.,* 2016) to name a few.

Other defense responses related to JA include an increase of cytoplasmic calcium concentration, protein phosphorylation, production of reactive oxygen species (ROS), ion transport (Zhao *et al.,* 2005), phytoalexin accumulation (Davis and Currier, 1988), and a systemic resistance of *S. tuberosum* against *Phytophthora infestans* (Cohen *et al.,* 1991). The main control point that regulates JA levels is a positive feedback loop observed in some plant species where JAs themselves induce the expression of genes involved in the biosynthesis of their own precursors (Wasternack, 2007). The release of C18:3 from plant membrane lipids by stress activated lipases, provides the substrate for LOX (Wasternack and Hause, 2013) as demonstrated in wound-induced leaves of tomato (Conconi *et al.,* 1996) and exemplified in JAs-treated cell suspension cultures of *E. californica* by increased levels of C18:3 correlating with higher levels of JA (Mueller *et al.,* 1993). Furthermore, using *in vitro* crushed leaves of *Oryza sativa* to mimic the wounding response, proved that JA and JA-Ile biosynthesis occur in both unwounded and wounded tissues as long as the JA biosynthetic pathway in chloroplasts is able to produce OPDA. The exogenous addition of C18:3 to OPDA-inhibited *in vitro* tissues, stimulated OPDA biosynthesis and that of OPDA, JA and JA-Ile in intact tissues, thus demonstrating that the presence of C18:3 is the limiting step following wounding (Christeller and Galis, 2014). Cell suspension cultures represent good model systems to study the effect of JA on both primary and secondary metabolism since they can provide information at the cellular level whereas in studies with complete plants, it is difficult to separate specific cellular mechanisms from those involved in the structure, function and tissue organization in the whole plant (Elkahoui *et al.,* 2004). The main goal of our investigation was to study the effect of JA on the accumulation and behavior of FA present in cell suspension cultures of *C. roseus,* with particular interest in C18:3 in order to determine if the release of this precursor is involved in the fast response of JA after elicitation. A validated GC-MS method in combination with univariate and multivariate data analysis (MVDA) was used to measure and assess FA profiles.

3.2 EXPERIMENTAL

3.2.1 Cell suspension cultures and elicitation with jasmonic acid

Cell suspension cultures of the *C. roseus* cell line CRPP were grown in 250 mL Erlenmeyer flasks containing 50 mL of Gamborg B5 medium (Gamborg *et al.,* 1968) supplemented with 30 g/L sucrose and 1.86 mg/L of 1-naphthalene acetic acid (NAA) and adjusted to pH 5.8 with 0.1 N NaOH. Cell cultures were propagated on a rotary shaker (110 rpm) at 25 °C under continuous light (500-1500 lux) and were subcultured every three weeks by transferring 20 mL of the suspended cells to 50 mL of fresh medium. Four-day-old cell suspension cultures were treated with JA (7.18 µmol/flask; Sigma-Aldrich, St Louis, MO, USA) dissolved in 40% ethanol (v/v) (experimental samples), or 150 µL of 40% ethanol (v/v) as a negative control (mock). Treated and untreated cells were harvested in quadruplicates at 0, 5, 30, 90, 360 and 1440 min after elicitation. Cells were filtered on Whatman filter paper under partial vacuum and biomass and media samples were immediately frozen in liquid nitrogen and kept at -80 °C until further analysis.

3.2.2 Chemicals used for cell suspension cultures

The chemicals used for macro salts, $CaCl_2$ (min. 99%), KH_2PO_4 (min. 99.5%), KNO_3 (min. 99%) and NH_4NO_3 (min. 99%), were purchased from Merck (Darmstadt, Germany) and $MgSO_4$ was obtained from OPG Farma (BUVA BV, Uitgeest, The Netherlands). The chemicals used for micro salts H_3BO_3, $MnSO_4.H_2O$, $ZnSO_4.7H_2O$, Na_2EDTA (Merck) and $FeSO_4.7H_2O$ (Brocades-ACF Groothandel NV, Maarssen, The Netherlands) were dissolved in one solution and KI, $NaMoO_4.2H_2O$, $CuSO_4.5H_2O$ and $CoCl_2.6H_2O$ (Merck) were dissolved into another solution to avoid insolubility problems. Thiamine-di-HCl was from Janssen Chimica (Geel, Belgium), pyridoxine-HCl was from Sigma-Aldrich Chemie (Steinheim, Germany), nicotinic acid (99.5%) and glycine (99.7%) and NAA were from Merck (Schuchardt, Germany), sucrose (99.7%) and *myo*-inositol (99.7%) were from Duchefa Biochemie (Haarlem, The Netherlands).

3.2.3 Chemicals used for fatty acid determination

The mixture of 37 fatty acid methyl esters (37 Components FAME Mix) was obtained from Supelco (Sigma-Aldrich; Bellefonte, PA, USA) and was used to identify the FAME in the samples by comparison of their retention times (RT) and MS-fragmentation patterns. All other chemicals and solvents were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems; Brea, CA, USA).

3.2.4 Fatty acid extraction

Samples of 10 mg of lyophilized cells were spiked with 50 µg of C17:0 as internal standard (IS) and then subjected to hydrolysis by adding 1 mL of 1 M of KOH in 95% ethanol (v/v) to the test tube. The suspension was ultrasonicated for 10 min followed by heating the closed test tube at 80 °C for 30 min. After cooling at room temperature, 1 mL of Milli Q water was added followed by two times

extraction with 1 mL of *n*-hexane containing 0.01% butylated hydroxytoluene (BHT; Sigma-Aldrich, St Louis, MO, USA), by vigorously vortexing and centrifugation for 10 min at 3,500 rpm; the upper hexane layer was removed and discarded. The aqueous layer containing free FA was acidified by adding 200 µL of 6 M HCl and extracted twice with 1 mL *n*-hexane (0.01% BHT) and after centrifugation for 10 min at 3,500 rpm upper layers were pooled and completely dried under a gentle flow of N_2 gas. To the residues 1 mL of boron trifluoride (BF_3; 10 % w/w) in methanol (Sigma-Aldrich, St Louis, MO, USA) was added and the closed tubes were heated for 15 min at 80 °C. After cooling down to room temperature, 1 mL of *n*-hexane (0.01% BHT) was added and after centrifugation, the upper layer (300 µL) was used in GC-MS analysis.

3.2.5 Gas chromatography-mass spectrometry

Fatty acid methyl ester analysis was performed on an Agilent 7890A series gas chromatograph equipped with an Agilent 7693 auto sampler and an Agilent 5775C Triple-Axis MSD detector (all from Agilent Technologies Inc., Santa Clara, CA, USA) and separated on a 30 m x 0.25 mm I.D. x 0.25 µm film thickness DB-Wax column (J&W; Agilent Technologies Inc., Santa Clara, CA, USA), with a constant flow of 20 mL/min of He as a carrier gas. The injection port was heated to 50 °C. The injection volume was 1 µL with a split ratio of 20:1. The oven temperature was 50 °C for 1 min, then 25 °C/min to 200 °C and then 3 °C/min to 250 °C for 18 min. All mass spectra were acquired in the electron impact (EI) mode for full scan in total ion current (TIC) and selected ion monitoring (SIM) modes. GC-MS was controlled by Enhanced Chemstation software version E.02.00.493 (Agilent Technologies Inc., Santa Clara, CA, USA). Ions selected for quantification are listed in Table 1. The 37 Component FAME Mix was used as a control for possible retention time shifts and mass spectra ion identification.

3.2.6 Data analysis

Fatty acids were identified as FAME with the help of the National Institute of Standards and Technology (NIST) library version 2.0f (Agilent Technologies Inc., Santa Clara, CA, USA). Quantification was done by normalization of peak areas of each FAME with that of the IS (C17:0, 50 µg, 1 mg prepared in 1 mL of *n*-hexane) using the same procedure and the same calibration curves described in the Experimental section 2.2.7.a in Chapter 2. Before statistical analysis, distributions were tested for normality using the Shapiro-Wilk test ($p < 0.05$). A nested one-way ANOVA corrected for multiple comparisons with Tukey's post hoc test was used to assess significant differences among untreated cells, mock-treated and JA-treated cells for each FA per time point. Significant differences in C14:1 at 90 min between untreated and mock-treated cells was tested using a two-tailed nested *t*-test. Differences with $p < 0.05$ were considered statistically significant. All statistical tests were performed using GraphPad Prism software (v. 8.4.3.686, La Jolla, CA, USA).

ANOVA results are shown in Table 3.7.1 in the supplemental information section. Principal component analysis (PCA) was performed with the SIMCA-P software (v. 15.0.2, Sartorius Stedim Data Analytics AB, Umetrics, Umeå, Sweden). Absolute value data were mean-centered and scaled using unit variance (UV).

3.3 RESULTS AND DISCUSSION

3.3.1 Multivariate data analysis of GC-MS data

Because of the limited dynamic range of the calibration curves of the FA used for their absolute quantification, some FA species could not be quantified; these are reported as traces or in case of being below the detection limit (LOD) no values are shown in Fig. 3. Multivariate data analysis was applied to follow the relative changes of individual FA between time points, using the normalized peak area of the IS. Thus, principal component analysis (PCA) was applied to the GC-MS absolute averaged data in order to differentiate the samples according to the FAME levels. The PCA score plot of PC1 *vs.* PC2 explained 49.6% and 17.6% of variances, respectively. Three clusters of samples treated with JA can be observed: those of 0, 5 and 30 min; 90 and 360 min and 1440 min after elicitation (Fig. 2). However, PCA did not show a full separation of the clusters of different treatments; moreover, the biological variation of the replicates seems to be larger than between treatments.

The most abundant FA present in non-treated cell suspensions of *C. roseus* were in decreasing amounts: C16:0, C18:2, C18:3, C20:0 and C22:0 (Fig. 3). Some changes are quite large, like for C14:1 and C18:0 where the former was not present after 90 min in JA-treated cells but only in the controls and the latter showed a large increase after 90 min. Over the time of the experiments, we can see that in the JA-treated cells these major FA increase in levels, with the exception of C22:0. After an initial increase, C22:0 decreased together with C10:0, C12:0, C14:1 and C24:0 back to the initial values, with the latter together with C24:0, were significantly different from the control after 360 min. Only C14:0, C15:0, C18:0 and C20:0 showed a significant difference in JA-treated cells versus untreated cells after 90 min. In the mock-treated cells (40% ethanol (v/v)), C14:0 and C20:0 are significantly higher at the end of the experiment when compared to either the control or JA-treated cells; C12:0 showed a similar pattern, although without any significant increase. It is clear that the mock treatment has a significant effect on the pattern of certain FA found in the cells especially after 1440 min of treatment e.g. C10:0, C14:0, C16:0, C18:0, C18:1, C18:2, C18:3, C20:0 and C22:0. Based on these results, the effects of JA and mock treatment after 1440 min are difficult to assess. Even if in the JA-treated cells, C18:0, C18:1, C18:2 and C:18:3 showed a significantly higher content at 1440 min when compared to the control (Fig. 3), these results do not allow a conclusion on the question if elicitation with JA could induce its own biosynthesis through increasing the content of

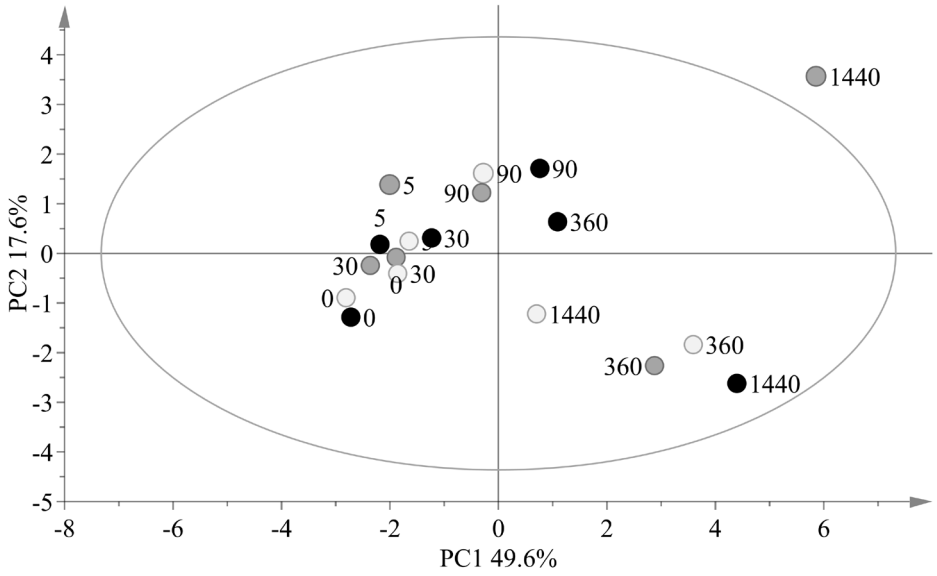

Figure 2. Principal component analysis scores plot (PC1 *vs.* PC2) of FA contents in cell suspension cultures of *Catharanthus roseus* treated with jasmonic acid and measured by GC-MS. Data represent averaged values of four biological replicates each analyzed three times. Black circles: JA-treated cells; white circles: untreated cells; grey circles: mock-treated cells. Numbers refer to 0, 5, 30, 90, 360 and 1440 min after treatment.

C18:3 in cell suspension cultures of *C. roseus*. The effects of stress hormones like JA, on different metabolic pathways have been extensively reported in different plant and cell systems, including *C. roseus*; and yet, the effect of the solvent ethanol was rather unexpected. Ethanol is a natural product that accumulates in plant organs primarily after exposure to anaerobic conditions like flooding or induced anoxia (Davis, 1980; Kimmerer and MacDonald, 1987), during seed deterioration (Woodstock and Taylorson, 1981), seed imbibition (Chen *et al*., 2019), fungal attack on roots (Kelsey *et al*., 2016) and fruit ripening accompanied with an increase of ethylene (ET) (Hyodo *et al*., 1983).

Ethanol along with methanol modulate elicitor-induced defense signaling responses to danger- and microbe-associated molecular patterns (DAMP and MAMP) by synergistically acting with the 22 amino acid bacterial flagellum-derived flg22 peptide, the small peptide systemin and chitosan, a non-acylated polyglucosamine from fungal cell walls (Hann *et al.,* 2014). Moreover, ethanol activates SUMOylation, a post-translational modification that involves small ubiquitin-like modifier proteins that control several cellular processes in response to biotic and abiotic stress, hormone signaling and plant defense among others (Morrell and Sadanandom, 2019). Observations in cell suspension cultures of *Ilex paraguariensis* fed with JA or salicylic acid (SA) dissolved into ethanol, methanol or *n*-propanol showed the accumulation of 1-*O*-ethyl-β- glucopyranoside, methyl-β-glucose and propyl-β-glycoside in both JA- and SA-treated cells, respectively, concluding that

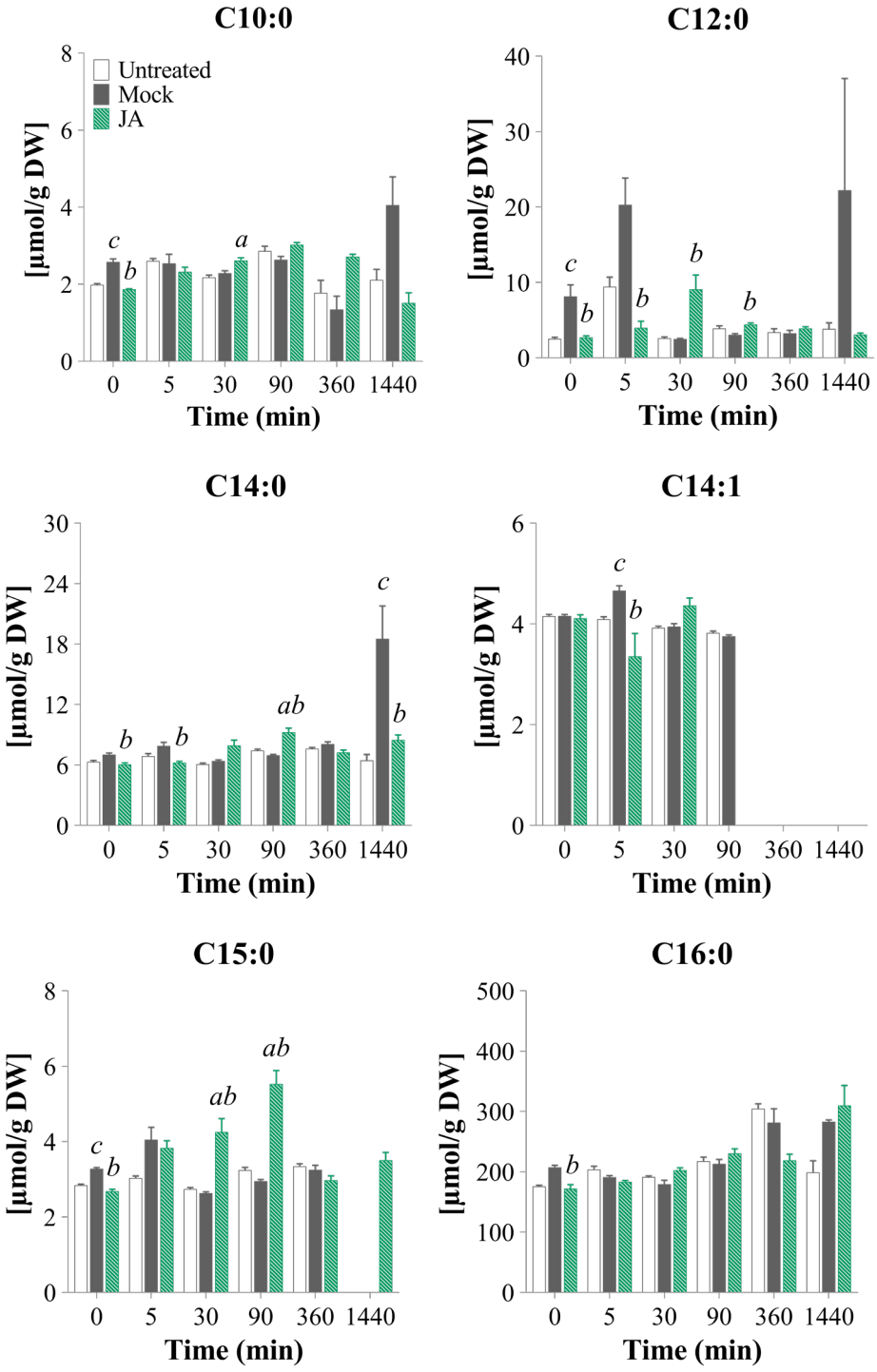

Figure 3. Continued

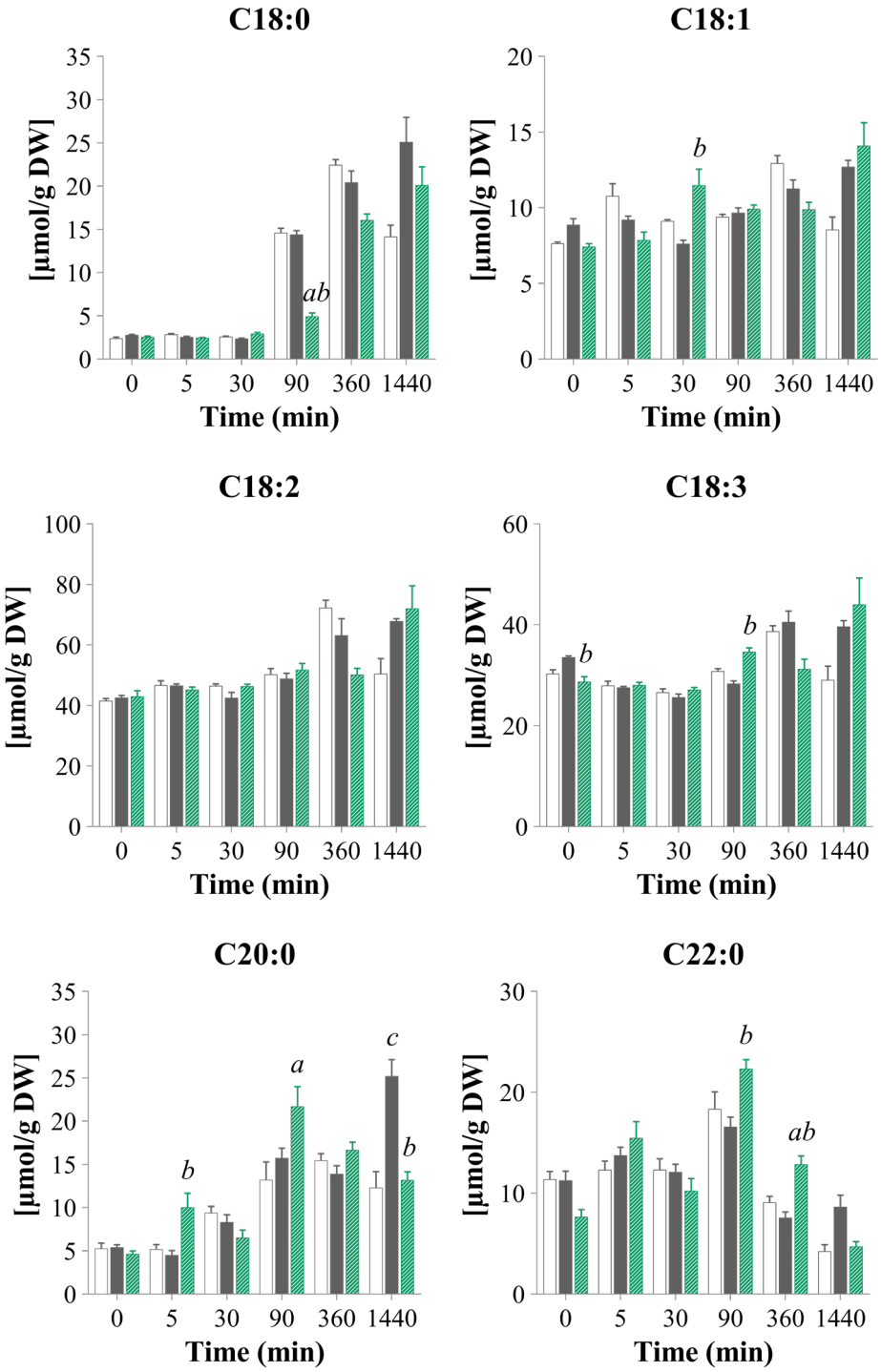

Figure 3. Continued

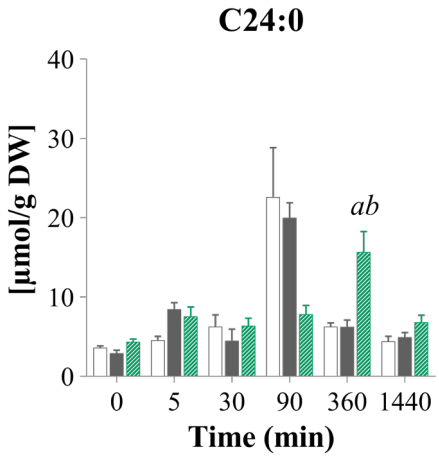

Figure 3. Time course accumulation of fatty acids in cell suspension cultures of *Catharanthus roseus* treated with jasmonic acid and controls measured by GC-MS. Time is on the *x*-axis and absolute amounts in [µmol/g DW] on the *y*-axis. Values are the mean ± standard error of mean (SEM) of four biological replicates each analyzed three times. Significant results are marked with superscripts (nested one-way ANOVA with Tukey's post hoc test, $p < 0.05$). No significant differences for C14:1 at 90 min between untreated and mock-treated cells (t=1.483, df=22, p=0.1524) were found (two-tailed nested *t*-test, $p < 0.05$). C15:0 at 1440 min was not tested because there was not enough data for statistical analysis.
[a] JA-treated significantly different from untreated cells at the same time point of observation.
[b] JA-treated significantly different from mock at the same time point of observation.
[c] Mock significantly different from untreated at the same time point of observation.
Missing values in all FA are due to their levels below the detection limit.

ethanol cannot be considered as an inactive solvent for cell cultures (Kraemer *et al.,* 1999; 2002). Interestingly, in some cases, ethanol can increase contents of secondary metabolites like in hairy root cultures of *Anisodus acutangulus* elicited with ethanol and methyl jasmonate (MeJA) dissolved in dimethyl sulfoxide (DMSO). Results showed that only ethanol and not MeJA significantly increased the contents of the tropane alkaloids anisodamine, anisodine, hyoscyamine and scopolamine mostly after 24 h of treatment when compared to untreated hairy roots (Kai *et al.,* 2012). Additionally, contents of scopolamine in hairy roots of *Atropa baetica* fed with SA dissolved in ethanol were higher in the mock than the SA-treated hairy roots throughout the entire elicitation experiment (4-72 h). A similar effect occurred with hairy roots treated with acetylsalicylic acid (ASA) dissolved in ethanol, in which, after 48 h of treatment, contents of scopolamine were higher in the mock. It is noteworthy that contents of scopolamine were higher upon ASA if compared to the mock, when ASA was dissolved in water and MeJA was used undissolved, confirming the elicitor-like effect of ethanol (el Jaber-Vazdekis *et al.,* 2008). Furthermore, axenic suspension cultures of the endophyte *Fusarium solani* were fed either with an ethanolic leaf extract of *C. roseus* (young leaves, no variety mentioned) or with ethanol in an attempt to increase contents of the anticancer alkaloid camptothecin. Surprisingly, the addition of 5 % (v/v) of ethanol resulted in a 15.5-fold increase of this alkaloid if

compared to the untreated control. Because a reduced glucose uptake was observed combined with ethanol consumption, it was concluded that ethanol has a dual role as an elicitor and as a carbon source resulting in a higher biomass yield and hence camptothecin (Venugopalan and Srivastava, 2015). Previous studies on the CRPP cell line of *C. roseus* treated with JA showed significant variations in secondary metabolism after 24 h to 72 h of treatment compared to the mock, mostly in carotenoids, organic acids, sugars and some amino acids (Saiman, 2014). Similarly, the *C. roseus* cell line A12A2 elicited with SA did show changes in primary metabolites such as sugars and amino acids after 6 to 24 h of treatment, but no FA profiles were made (Mustafa *et al.,* 2009). In these previous studies, no specific effects were detected in solvent control treatments regarding the compound groups investigated; yet these studies did not report on the FA profiles.

3.4 CONCLUSIONS

Using a GC-MS-based targeted profiling in combination with MVDA, we demonstrated the effect of JA and mock treatments on FA contents in a time course experiment in cell suspension cultures of *C. roseus.* Principal component analysis showed three main events: the first taking place from 0-30 min; the second occurring between 90-360 min and the last one at 1440 min after JA-treatment. There is an increase in levels of C16:0, C18:0, C18:1 and C18:3 in JA-treated cells at 1440 min compared to the untreated cells. Only C14:0, C15:0, C18:0, C20:0, C22:0 and C24:0 were significantly induced by JA treatment between 90 and 360 min, if compared to untreated cells. However, the mock-treated cells also showed a significant increase for C14:0 and C20:0 at 1440 min if compared with untreated cells. This means that at this time point, the effect of JA and mock treatment cannot be distinguished. In order to further study the effect of ethanol in cell suspension cultures of *C. roseus*, gene expression of all early JA-biosynthetic genes should be tested. Moreover, the use of ethanol and methanol should be completely discouraged, and lastly, JA should be fed undiluted to cell suspension cultures. Finally, since treatment with JA did not significantly affect levels of its precursor C18:3, with the present methodological approach, we cannot conclude if C18:3 is involved or not in the early stress response in *C. roseus*.

3.5 ACKNOWLEDGMENTS

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3.7 SUPPLEMENTAL INFORMATION

Table 3.7.1. ANOVA results for each test performed for each fatty acid in untreated, mock-treated and JA-treated cells in all studied time points of suspension cultures of *Catharanthus roseus*

			Time (min)			
Fatty acid	**0**	**5**	**30**	**90**	**360**	**1440**
C10:0	$F_{(2,9)} = 48.9$ $p < 0.0001$	$F_{(2,9)} = 2.744$ $p=0.1174$	$F_{(2,9)} = 6.256$ $p=0.0198$	$F_{(2,9)} = 2.178$ $p=0.1692$	$F_{(2,9)} = 3.999$ $p=0.0572$	$F_{(2,8)} =$ 3.723 $p=0.0663$
C12:0	$F_{(2,9)} = 7.091$ $p=0.0142$	$F_{(2,9)} = 5.744$ $p=0.0247$	$F_{(2,9)} = 5.186$ $p=0.0318$	$F_{(2,9)} = 6.158$ $p=0.0207$	$F_{(2,9)} =$ 0.6466 $p=0.5465$	$F_{(2,9)} = 1.27$ $p=0.3267$
C14:0	$F_{(2,9)} = 5.396$ $p=0.0288$	$F_{(2,9)} = 4.501$ $p=0.0442$	$F_{(2,9)} = 4.119$ $p=0.0537$	$F_{(2,33)} = 20.97$ $p < 0.0001$	$F_{(2,9)} = 1.268$ $p=0.3271$	$F_{(2,9)} =$ 7.057 $p=0.0143$
C14:1	$F_{(2,9)} = 0.1042$ $p=0.9021$	$F_{(2,9)} = 12.42$ $p=0.0026$	$F_{(2,9)} = 3.561$ $p=0.0726$	-	-	-
C15:0	$F_{(2,9)} = 19.26$ $p=0.0006$	$F_{(2,9)} = 3.788$ $p=0.064$	$F_{(2,9)} = 7.103$ $p=0.0141$	$F_{(2,33)} = 44.49$ $p < 0.0001$	$F_{(2,9)} = 1.35$ $p=0.3070$	-
C16:0	$F_{(2,9)} = 5.26$ $p=0.0307$	$F_{(2,9)} = 2.25$ $p=0.1613$	$F_{(2,9)} = 1.799$ $p=0.2202$	$F_{(2,9)} =$ 0.4293 $p=0.6636$	$F_{(2,9)} = 2.295$ $p=0.1565$	$F_{(2,8)} =$ 1.586 $p=0.2571$
C18:0	$F_{(2,9)} = 2.192$ $p=0.1677$	$F_{(2,9)} = 2.391$ $p=0.1469$	$F_{(2,9)} = 3.474$ $p=0.0762$	$F_{(2,9)} = 60.48$ $p < 0.0001$	$F_{(2,9)} = 3.311$ $p=0.0836$	$F_{(2,9)} =$ 3.138 $p=0.0925$
C18:1	$F_{(2,9)} = 2.276$ $p=0.1586$	$F_{(2,9)} = 3.476$ $p=0.0767$	$F_{(2,9)} = 4.496$ $p=0.0443$	$F_{(2,9)} =$ 0.3022 $p=0.7464$	$F_{(2,9)} = 2.361$ $p=0.1499$	$F_{(2,9)} =$ 2.637 $p=0.1255$
C18:2	$F_{(2,9)} = 0.09818$ $p=0.9074$	$F_{(2,9)} = 0.1764$ $p=0.8411$	$F_{(2,9)} = 1.084$ $p=0.3785$	$F_{(2,9)} =$ 0.1565 $p=0.8574$	$F_{(2,9)} = 2.514$ $p=0.1357$	$F_{(2,9)} =$ 1.709 $p=0.2349$
C18:3	$F_{(2,9)} = 4.078$ $p=0.0549$	$F_{(2,9)} =$ 0.05363 $p=0.9481$	$F_{(2,9)} =$ 0.4721 $p=0.6383$	$F_{(2,9)} = 8.046$ $p=0.0099$	$F_{(2,9)} = 2.073$ $p=0.1817$	$F_{(2,9)} = 1.06$ $p=0.3861$
C20:0	$F_{(2,9)} = 0.6393$ $p=0.55$	$F_{(2,9)} = 5.034$ $p=0.0341$	$F_{(2,33)} = 3.067$ $p=0.06$	$F_{(2,33)} = 5.344$ $p=0.0098$	$F_{(2,9)} = 1.139$ $p=0.3622$	$F_{(2,9)} = 14$ $p=0.0017$
C22:0	$F_{(2,9)} = 4.931$ $p=0.0358$	$F_{(2,9)} = 1.635$ $p=0.2479$	$F_{(2,33)} = 1.198$ $p=0.3147$	$F_{(2,9)} = 4.226$ $p=0.0508$	$F_{(2,9)} = 9.855$ $p=0.0054$	$F_{(2,9)} =$ 5.029 $p=0.0342$
C24:0	$F_{(2,9)} = 2.299$ $p=0.1561$	$F_{(2,9)} = 3.412$ $p=0.0789$	$F_{(2,33)} = 0.638$ $p=0.5348$	$F_{(2,9)} = 2.553$ $p=0.1324$	$F_{(2,9)} = 6.928$ $p=0.0151$	$F_{(2,9)} =$ 1.093 $p=0.3759$

Not tested due to insufficient data (-).

Chapter 4

Terpenoid indole alkaloid profiles of cell suspension cultures of *Catharanthus roseus*

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ABSTRACT

Cell suspension cultures of the medicinal plant *Catharanthus roseus* (L.) G. Don accumulate a wide variety of terpenoid indole alkaloids (TIA). The cell line (CRPP) used for this study accumulated 10 different TIA *e.g.* catharanthine, tabersonine, serpentine and 7 different α-methylene-indoline alkaloids (α-TIA-1-7) along with the precursors loganic acid and strictosidine upon elicitation with jasmonic acid (JA). The aim of this study was to integrate the knowledge of the fast JA-stress response with later events like the accumulation of TIA in *C. roseus.* Treatment of cells with JA resulted in an increased accumulation for catharanthine, α-TIA-1, serpentine and tabersonine and a decrease of loganic acid, 1440 min after induction when compared to JA-treated cells at 0 min or to untreated cells at the same time point of observation. Strictosidine levels were much higher than all other TIA and were more or less the same during the experiment, only at the last time point it dropped considerably if compared to t=0. Similarly, untreated and mock-treated cells showed a similar drop in strictosidine levels at 1440 min. Catharanthine did not show major differences between the first and the last time point, but at 90 and 360 min the levels were considerably lower for all three samples, with no apparent reason. Levels of loganic acid showed a decrease throughout the experiment for all samples. All these observations are in agreement with our previous results where the accumulation of TIA fit into the late stress response, which depends on the signaling pathway mediated by JA that leads to the expression of genes involved in the biosynthesis of TIA.

4.1 INTRODUCTION

The terpenoid indole alkaloids (TIA) are a group of about 2000 compounds naturally occurring amongst members of the botanical families Apocynaceae, Loganiaceae, Nyssaceae, Rubiaceae and Icacinaceae (O'Connor and Maresh, 2006; Zhang *et al.,* 2018a). Most of these TIA have specific roles in plant defense as antimicrobials, insect deterrents (Luijendijk *et al.,* 1996; Roepke *et al.,* 2010), antifungal (Guéritte *et al.,* 1983) and there is a large number of biologically active agents used in pharmacology such as the antineoplastic dimeric alkaloids vinblastine, vincristine, anhydrovinblastine from *Catharanthus roseus* and camptothecine from *Camptotheca acuminata*, the rat poison strychnine from *Strychnos nux-vomica*, the antihypertensive serpentine from *C. roseus*, the antiarrhythmic ajmalicine from *C. roseus* and *Rauwolfia serpentina*, the stimulant yohimbine from *Pausinystalia yohimbe* and *R. serpentina* and the antimalarial quinine from *Cinchona ledgeriana* to name a few (Kutchan, 1995; van der Heijden *et al.,* 2004; O'Connor and Maresh, 2006). Of particular interest is *C. roseus* (Apocynaceae), which is one of the most studied medicinal plants since it accumulates over 130 different TIA such as vincristine and vinblastine effectively used for some 40 years against different types of cancer (van der Heijden *et al.,* 2004). The amounts of the dimeric TIA in *C. roseus* leaves are very low (around 0.0005 % DW) and consequently difficult to isolate from a mixture of many

compounds with very similar chemical and physical properties (Scott, 1970). Because of these difficulties, tremendous efforts have been focused on their extraction, isolation, separation and structural elucidation using new technologies such as the use of ionic liquids (IL), supercritical fluid extraction (SFE) and molecularly imprinted polymers (MIP) besides the long-known capillary electrophoresis (CE), high-speed counter current chromatography (HSCCC), quantitative nuclear magnetic resonance (qNMR), gas chromatography coupled to mass spectrometry (GC-MS) and ultra high-pressure liquid chromatography coupled to mass spectrometry (UHPLC-MS) (Goldhaber-Pasillas *et al.,* 2013).

Owing to their commercial importance, many efforts to produce them by means of plant cell cultures have been reported (De Luca and St-Pierre, 2000; van der Heijden *et al.*, 2004) such as scale-up in bioreactors (Zhao and Verpoorte, 2007), precursor feeding experiments (Contin *et al.,* 1999; Moreno *et al.,* 1993; Whitmer *et al.,* 2002; El-Sayed and Verpoorte, 2002; Arvy *et al.,* 1994), media optimization (Contin *et al.,* 1998; Smith *et al.,* 1987a) and genetic modifications with biosynthetic genes (Goddijn *et al.,* 1995; Hughes *et al.,* 2004) and transcription factors (Peebles *et al.,* 2009a; 2009b) such as the octadecanoid-responsive *Catharanthus* AP2-domain ORCA1, ORCA2 and ORCA3 (Memelink *et al.,* 2001; Menke *et al.,* 1999a; Li *et al.,* 2013).

Cell suspension cultures of *C. roseus* are able to accumulate catharanthine and tabersonine (Verpoorte *et al.,* 1993) and to transform tabersonine in 16-methoxytabersonine (St-Pierre and De Luca, 1995) but they do not have the enzymatic activity necessary for vindoline biosynthesis (Vázquez-Flota and De Luca, 1998), which only occurs in plastids in photosynthetically active cells. The major problem is the lack of differentiation of cell cultures (Facchini and De Luca, 2008) since the many steps involved are tightly regulated and the expression of biosynthetic genes is cell-, tissue-, development- and environment-specific and also affected by external biotic and abiotic factors (Leonard *et al.,* 2009; De Luca and St-Pierre, 2000).

4.1.1 Biosynthesis of terpenoid indole alkaloids in *Catharanthus roseus*

Terpenoid indole alkaloids are derived from two primary metabolic pathways: the shikimate and the monoterpenoid (also known to be synthesized from 2-*C*-methyl-D-erythritol 4-phosphate (MEP)) pathways that require coordination of the amounts of precursors supplied by both pathways. TIA consist of an indole moiety provided by tryptamine, derived from the amino acid tryptophan and a monoterpenoid component derived from the iridoid glucoside secologanin (Facchini, 2001). The secologanin biosynthetic pathway starts in the vascular cells and/or the epidermal cells with the condensation of glyceraldehyde-3-phosphate and pyruvate to yield 1-deoxy-D-xylulose-5-phosphate catalyzed by 1-deoxy-D-xylulose-5-phosphate synthase (DXS) (Chahed *et al.,* 2000). Next, the condensation between the isomers dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) is catalyzed by geranyl diphosphate synthase (GPPS) leading to geranyl diphosphate

(Chatzivasileiou *et al.,* 2019). Geraniol is formed from geranyl diphosphate by the enzyme geraniol synthase (GES), and through multiple enzymatic reactions that include oxidation, reduction, glycosylation and methylation, the end product is the formation of secologanin (Fig. 1). This iridoid is coupled to tryptamine, yielding strictosidine, the central precursor for all TIA. The first step in the iridoid pathway is the enzyme geraniol 10-hydroxylase/8-oxidase (G8O) which is found in the internal phloem associated parenchyma (IPAP) cells. The encoding gene was first identified by Collu *et al.* (2001). The last two steps, the enzymes loganic acid *O*-methyltransferase (LAMT) and secologanin synthase (SLS) take place in the epidermal cells (Irmler *et al.,* 2000) (Fig. 1). More recently, two additional enzymes were characterized in the secologanin pathway *i.e.* iridoid synthase (IS) (Geu-Flores *et al.,* 2012) and GES (Simkin *et al.,* 2013) (Fig. 1). The full iridoid pathway was eventually elucidated on the level of genome, transcriptome, proteome and metabolome and the complete pathway was successfully expressed in *Nicotiana benthamiana* by Miettinen *et al*. (2014). Some reviews deal with the TIA biosynthesis and regulation (De Luca *et al.,* 2014; Pan *et al.,* 2016), compartmentation and logistics (Courdavault *et al.,* 2014), metabolic engineering (Kellner *et al.,* 2015; Pan *et al.,* 2012; Thamm *et al.,* 2016) and transport (Roytrakul and Verpoorte, 2007). Recently, Qu *et al.* (2018) reported new steps in the catharanthine pathway.

The biosynthesis of all TIA starts with strictosidine which is formed through the condensation of secologanin and tryptamine in the upper and lower epidermis. Tryptamine derives from the shikimate pathway where anthranilate synthase (AS) commits chorismate to anthranilate yielding tryptophan. Decarboxylation of tryptophan to tryptamine is catalyzed by tryptophan decarboxylase (TDC). The condensation of secologanin and tryptamine is catalyzed by strictosidine synthase (STR) and takes place in the cell vacuole. Seven isoforms of STR have been identified in *C. roseus* (de Waal *et al.,* 1995) that are possibly subject to post-translational modifications as they are encoded by a single gene (Pasquali *et al.,* 1999). The next step is catalyzed by strictosidine β-glucosidase (SGD) which yields a highly reactive opened-ring dialdehyde, which through the formation of a carbinolamine between one of the aldehyde groups with the NH_2 group yields cathenamine. Different rearrangements of cathenamine lead to a large structural diversity of TIA skeletons *e.g.* strychnos-, aspidosperma-, corynanthe-, bisindole-, iboga-, quinoline- and ajmalan-type (Geerlings *et al.,* 2000; Leonard, 1999). The reduction of cathenamine by cathenamine reductase (CR) leads to the formation of ajmalicine that can be further oxidized to serpentine in the vacuole by a class III peroxidases (Blom *et al.,* 1991; Sierra *et al.,* 1989; Sottomayor *et al*., 2004). Through epimerization of cathenamine, tetrahydroalstonine is formed by reduction of the enamine/carbinolamine group. The enzyme tetrahydroalstonine synthase (THAS) is thought to be involved in this reaction (Qu *et al.,* 2018; Stavrinides *et al.,* 2015). This compound can be oxidized to yield alstonine. The biosynthetic steps starting from strictosidine leading to tabersonine on one hand and to catharanthine on the other one, are not fully characterized. The biosynthesis of both TIA is believed to occur in the epidermal cells where catharanthine is exported via

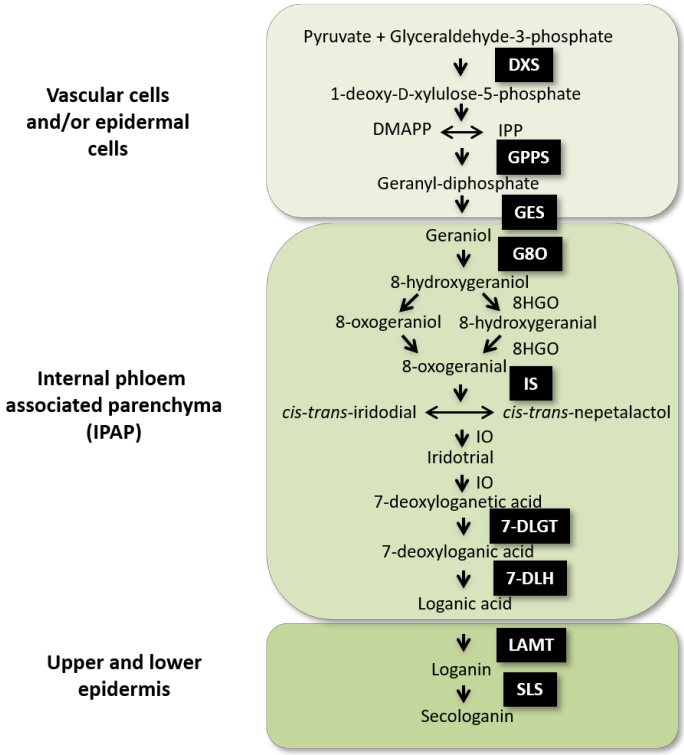

Figure 1. Biosynthetic pathway of TIA in *Catharanthus roseus* (adapted from Murata *et al.,* 2008; Liu *et al.,* 2017; Miettinen *et al.,* 2014). Framed consecutive reactions indicate localization in the plant. Genes previously reported to be regulated by ORCA3 and ORCA2, both induced by jasmonic acid (JA), are indicated in black boxes. Abbreviations for enzymes, proteins and/or products listed in sequential order are: DXS: 1-deoxy-D-xylulose-5-phosphate synthase; DMAPP: dimethylallyl diphosphate; IPP: isopentenyl diphosphate; GPPS: geranyl diphosphate synthase: GES: geraniol synthase occurring in vascular cells and/or epidermal cells; G8O: geraniol 8-oxidase; 8HGO: 8-hydroxygeraniol oxidoreductase; IS: iridoid synthase; IO: iridoid oxidase; 7-DLGT: 7-deoxyloganetic acid glucosyltransferase; 7-DLH: 7-deoxyloganic acid hydroxylase all secoiridoid pathway occurs in the internal phloem associated parenchyma (IPAP) cells and LAMT: *S*-adenosyl-L-methionine:loganic acid methyltransferase and SLS: secologanin synthase occurring in epidermal cells.

the ATP-binding cassette (ABC) transporter CrTPY2 to the leaf surface (Yu and De Luca, 2013) and tabersonine is transported to parenchymal cells. The steps that follow from tabersonine to vindoline are light dependent and need cellular differentiation (Vázquez-Flota *et al.,* 2002; De Luca and St-Pierre, 2000). The biosynthetic steps towards vindoline start with the hydroxylation of tabersonine at the C16 position catalyzed by tabersonine 16-hydroxylase (T16H) that is subsequently converted to 16-methoxytabersonine by 16-hydroxytabersonine-16-*O*-methyltransferase (16OMT) further oxidized to 16-methoxy-2,3-dihydrotabersonine by an unknown hydroxylase (St-Pierre and De Luca, 1995) (Fig. 2). The conversion to desacetoxyvindoline is catalyzed by *N*-methyltransferase 16-methoxy-2,3-dihydro-3-hydroxytabersonine (NMT) and has only been detected in differentiated plants (Dethier and

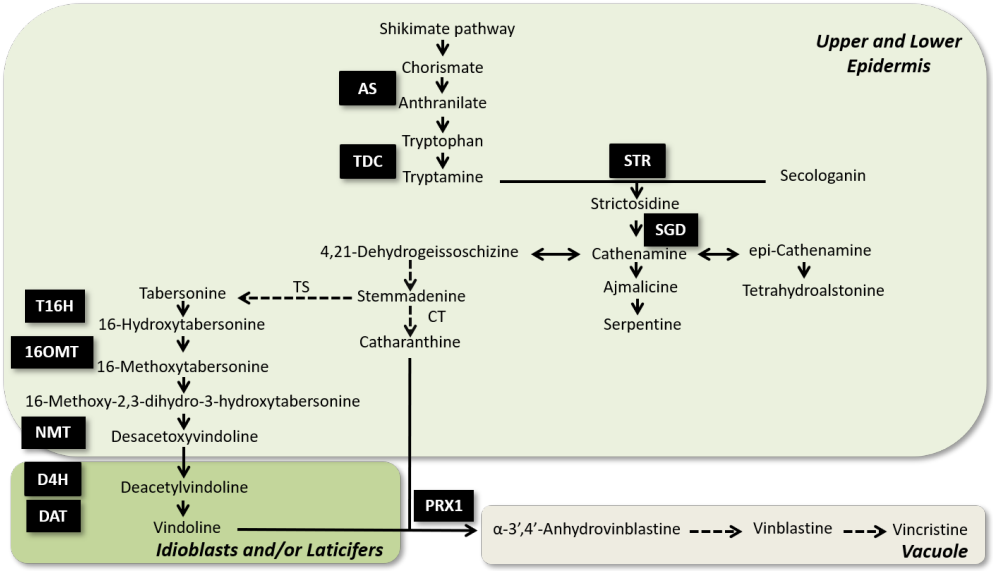

Figure 2. Biosynthetic pathway of TIA in *Catharanthus roseus* (adapted from Liu *et al.,* 2017; De Luca *et al.,* 2014). Broken arrows indicate multiple enzymatic reactions. Genes previously reported to be regulated by ORCA3 and ORCA2, both induced by jasmonic acid (JA), are indicated in black boxes. Abbreviations for enzymes and/or proteins listed in sequential order are: AS: anthranilate synthase; TDC: tryptophan decarboxylase; STR: strictosidine synthase; SGD: strictosidine β-D-glucosidase; TS: tabersonine synthase; CT: catharanthine synthase; T16H: tabersonine 16-hydroxylase; 16OMT: 16-hydroxytabersonine-16-*O*-methyltransferase occurring at the upper and lower epidermis; NMT: 16-methoxy-2,3-dihydro-3-hydroxytabersonine occurs in the epidermis; D4H: desacetoxyvindoline 4-hydrolase; DAT: acetyl-CoA:4-*O*-deacetylvindoline 4-*O*-acetyltransferase and PRX1: peroxidase 1 occurring in the idioblasts and/or laticifers in the mesophyll.

De Luca, 1993). The last two steps towards vindoline biosynthesis are catalyzed by the 2-oxoglutarate-dependent dioxygenase desacetoxyvindoline 4-hydrolase (D4H) (Vázquez-Flota and St- Pierre, 1998) and acetyl-CoA:4-*O*-deacetylvindoline 4-*O*-acetyltransferase (DAT) (St-Pierre *et al.,* 1998) both restricted to the laticifer and idioblasts cells in leaves (Fig. 2). Because NMT, DAT and D4H are organ-specifically distributed in mature plants and thus, absent in plant cell cultures, it shows the extent in the regulation of the biosynthetic steps leading to vindoline (St-Pierre and De Luca, 1995). With the vindoline pathway fully elucidated (De Luca *et al.,* 2014) it was successfully expressed in yeast (Qu *et al.,* 2015). From the intermediate 4,21-dehydrogeissoschizine, stemmadenine might be formed, which then leads to the formation of catharanthine and vindoline (Meijer *et al.,* 1993a; 1993b). Recently, two enzymes in the branching point at stemmadenine were described to isomerize, deacetoxylate and cyclize stemmadenine into catharanthine and tabersonine via catharanthine synthase (CT) and tabersonine synthase (TS) (Caputi *et al.,* 2018) (Fig. 2). Qu *et al.* (2018) reported the elucidation of the full pathway from 19*E*-geissoschizine to tabersonine and catharanthine. The steps leading to the dimeric TIA vinblastine and vincristine involve i) the dimerization of catharanthine and vindoline to α-3',4'-

anhydrovinblastine catalyzed by the class III peroxidase α-3',4'-anhydrovinblastine synthase (PRX1) that ii) hydroxylates the double bond yielding vinblastine and iii) oxidizes the *N*-methyl group yielding vincristine thus marking the end of TIA biosynthesis in *C. roseus*. Expression of *PRX1* occurs only in petals, reproductive organs and leaves, further supporting that the occurrence of TIA downstream of vindoline only occurs in fully developed tissues (Costa *et al.,* 2008) (Fig. 2).

After some 40 years of research on the biosynthesis of TIA in *C. roseus,* most of the pathways have now been characterized and the identified genes have been overexpressed in plants and yeast. Though overexpression of the genes in other plants and yeast has been successful, in terms of yields the production is not of interest. Only by using precursor feeding to transgenic plants or yeast containing a few biosynthetic genes, commercially interesting levels of an alkaloid have been achieved, *e.g.* strictosidine that is produced in *E. coli* or in yeast (Kutchan *et al.,* 1988). A yeast containing strictosidine synthase was able to produce 2 g/L of this alkaloid in 3 days after feeding with tryptamine and berry juice from *Symphoricarpos albus*, which contains about 2% of secologanin and carbohydrates (Geerlings *et al.,* 2001). Apparently, the TIA pathway involves many steps localized in different cells and cellular compartments making the regulation extremely complex, as it involves transport of intermediates between cellular compartments and cells. Transport is in part driven by diffusion, in part by highly selective transporters of different classes that for each single intermediate have different affinity. Signal molecules like jasmonic acid (JA), ethylene (ET) and salicylic acid (SA) may affect various steps in the biosynthesis, changing carbon fluxes through the different parts of the metabolic network. In this chapter, we will describe the effect on TIA biosynthesis after adding JA to *C. roseus* cell cultures.

4.1.2 Effect of jasmonic acid on TIA biosynthesis in *Catharanthus roseus*

The plant stress hormone JA and its methyl ester (MeJA) have an inducing effect on the signal transduction pathway that induces the biosynthesis of TIA in *C. roseus.* Both jasmonates (JAs) induce several of the known TIA biosynthetic pathway genes (Fig. 2) (Collu *et al.,* 2001; Miettinen *et al.,* 2014; Simkin *et al.,* 2013), which results in transiently higher levels of TIA in cell suspension cultures of *C. roseus* (Lee-Parsons *et al.,* 2004). The coordinated expression of biosynthetic genes is mediated through the ORCA transcriptional factors (Menke *et al.,* 1999a), where only ORCA2 and ORCA3 are induced by JA (Li *et al.,* 2013; Memelink *et al.,* 2001; van der Fits and Memelink, 2000) but not via ORCA1. This transcription factor may have a very different function, which is supported by its homology with the *Arabidopsis* dehydration-responsive elements (DREB2A and DREB2B), which are involved in drought-responses (Liu *et al.,* 1998). ORCA2 transactivates the STR promoter, a jasmonate- and elicitor-responsive promoter element (JERE) after induction with MeJA (Menke *et al.,* 1999a). Overexpression of ORCA3 also induced the biosynthetic genes *STR* and *TDC* and is functionally and structurally similar to ORCA2 (Zhou and Memelink, 2016). ORCA3 controls the transcription of

several genes in both the shikimate and mevalonate pathway such as *AS, DXS, D4H, STR* and *TDC* (van der Fits and Memelink, 2000; Pan *et al*., 2012) and ORCA2 controls the expression of *AS, TDC, LAMT, STR, T16H, D4H* and *PRX1* (Li *et al.,* 2013) (Fig. 1 and 2). Expression of *ORCA3* followed by JA treatment has been confirmed in cells of *C. roseus* (Menke *et al.,* 1999b) with a maximal effect after 8 h of treatment with MeJA (Shukla *et al.,* 2010). Moreover, biotic and abiotic elicitors have also been able to induce the gene expression of *TDC, STR* and *SGD* (Ramani and Jayabaskaran, 2008; Ramani and Chelliah, 2007; Smith *et al.,* 1987b; Zhao *et al.,* 2001a) thus demonstrating the vast effect of JA on plant metabolism as it induces both primary and secondary metabolism at the level of gene expression where ORCA3 acts as the central regulator (Memelink *et al.,* 2001). Cell suspension cultures of *C. roseus* overexpressing ORCA3 significantly accumulated more tryptophan and tryptamine but no TIA were found, only feeding of loganin caused an increase in TIA (van der Fits and Memelink, 2000).

The accumulation of TIA can be considered as a late response in the JAs-mediated stress response in *C. roseus,* where the coordinated expression of *TDC, STR* and *SGD* genes takes place after 2 h of induction (Collu *et al.,* 2001; Wei, 2010) and the accumulation of TIA is detected in significant amounts after 4-24 h (Moreno *et al.,* 1996; Vázquez-Flota *et al.,* 2009). The early steps in the JA-mediated response after wounding include a rapid burst of JAs propagating from locally wounded to distally unwounded leaves that takes place within 30 sec (Glauser *et al.,* 2009), preceding any transcriptional activity. As a part of an integrative study on the fast JA-response, we studied TIA accumulation after induction with JA of cell suspension cultures of *C. roseus* to establish a time line of events in the first 24 hours including both primary (Chapter 3) and secondary metabolism (this chapter).

4.2 EXPERIMENTAL

4.2.1 Cell suspension cultures and elicitation with jasmonic acid

Cell suspension cultures of the *C. roseus* cell line CRPP were grown in 250 mL Erlenmeyer flasks containing 50 mL of Gamborg B5 medium (Gamborg *et al.,* 1968) supplemented with 30 g/L sucrose and 1.86 mg/L of 1-naphthalene acetic acid (NAA) and adjusted to pH 5.8 with 0.1 N KOH. Cell cultures were propagated on a rotary shaker (110 rpm) at 25 °C under continuous light (500-1500 lux) and were subcultured every three weeks by transferring 20 mL of the suspended cells to 50 mL of fresh medium. Four-day-old cell suspension cultures were treated with JA (7.18 µmol/flask; Sigma-Aldrich, St Louis, MO, USA) dissolved in 40% ethanol (v/v) or 150 µL of 40% ethanol (v/v) (mock), or nothing (untreated) and were harvested in quadruplicates at 0, 5, 30, 90, 360 and 1440 min after elicitation. Cells were filtered on Whatman filter paper under partial vacuum and biomass and media samples were immediately frozen in liquid nitrogen and kept at -80 °C until further analysis.

4.2.2 Chemicals used for cell suspension cultures

The chemicals used for macro salts, $CaCl_2$ (min. 99%), KH_2PO_4 (min. 99.5%), KNO_3 (min. 99%) and NH_4NO_3 (min. 99%), were purchased from Merck (Darmstadt, Germany) and $MgSO_4$ was obtained from OPG Farma (BUVA BV, Uitgeest, The Netherlands). The chemicals used for micro salts, H_3BO_3, $MnSO_4.H_2O$, $ZnSO_4.7H_2O$, Na_2EDTA (Merck; Darmstadt, Germany) and $FeSO_4.7H_2O$ (Brocades-ACF Groothandel NV, Maarssen, The Netherlands), were dissolved into one solution, and KI, $NaMoO_4.2H_2O$, $CuSO_4.5H_2O$ and $CoCl_2.6H_2O$ (Merck; Darmstadt, Germany) were dissolved into another solution to avoid problems of insolubility. Thiamine-di-HCl was from Janssen Chimica (Geel, Belgium), pyridoxine-HCl was from Sigma-Aldrich (St. Louis, MO, USA); nicotinic acid (99.5%), glycine (99.7%) and NAA were from Merck (Darmstadt, Germany), sucrose (99.7%) and *myo*-inositol (99.7%) were from Duchefa Biochemie (Haarlem, The Netherlands).

4.2.3 Alkaloid standards

Strictosidine and secologanin were provided by Phytoconsult (Leiden, The Netherlands); loganic acid, tabersonine and vindoline were purchased from PhytoLab (Vestenbergsgreuth, Germany); tryptamine was purchased from Sigma-Aldrich Chemical (Steinheim, Milwaukee, WI, USA); tryptophan and ajmalicine were purchased from Sigma-Aldrich (St. Louis, MO, USA); serpentine was purchased from Roth (Karlsruhe, Germany) and catharanthine was a kind gift of Pierre Fabre (Gaillac, France).

4.2.4 Alkaloid and precursor extraction for HPLC

A modified extraction protocol was followed after Moreno *et al.* (1993). Briefly, freeze-dried samples of 50 mg were extracted twice with 5 mL of methanol, vortexed for 1 min, sonicated for 20 min and then centrifuged for 30 min at 3,500 rpm. Pooled samples were reduced to dryness under reduced pressure. To the dried extract 500 µL of 1 M H_3PO_4 was added and the suspension was thoroughly homogenized and then transferred to an Eppendorf tube for centrifugation for 10 min at 13,000 rpm. Extracts were filtered with a 0.2 µm PTFE membrane and then 50 µL were analyzed by HPLC.

4.2.5 HPLC analysis of TIA

Chromatographic separations were carried out on a 250 mm x 4.60 mm, 5 µm Gemini-NX C18 (Phenomenex Inc., Torrance, California, USA) column and a guard column filled with RSil C18 HL (Uetikon) at room temperature. Chromatographic methods were adapted from Tikhomiroff and Jolicoeur (2002). Elutions were performed at a flow rate of 1.5 mL/min. Solvents used for TIA (strictosidine, ajmalicine, serpentine, catharanthine, tabersonine, vindoline, vincristine and vinblastine) analysis were 5 mM Na_2HPO_4 in water adjusted to pH 6 with H_3PO_4; (Solvent A) and acetonitrile (v/v) (Solvent B). Elution was as follows: from 0-20 min, linear gradient from 80:20 to 20:80 (v/v) (A:B); 20-25 min, isocratic elution with 20:80 (v/v) (A:B); 25-30 min, linear gradient from 20:80 to 80:20 (v/v) (A:B); 30-31 min, isocratic elution at 80:20 (v/v) (A:B). Precursors (tryptamine, tryptophan,

loganic acid, loganin and secologanin) were analyzed using an isocratic elution of 85:15 (v/v) of 0.01 M H_3PO_4 in water (Solvent A) and acetonitrile (Solvent B). Elutions were performed at a flow rate of 1.5 mL/min and the injection volume was 50 µL for both methods. The HPLC system was an Agilent 1200 Series consisting of a G1310A binary pump, a G1329A autosampler, a G1322A degasser and a G1315D photo-diode array detector (DAD) controlled by ChemStation software (Agilent v. 03.02; all from Agilent Technologies Inc., Santa Clara, CA, USA). Ultraviolet (UV)-spectra of all peaks were collected (220-320 nm) and chromatograms were recorded at 220, 254, 280, 306 and 320 nm. Detection of target peaks was achieved by comparison of their absorbance with that of reference standards. Every sample was injected once.

4.2.6 Extraction procedures for UHPLC-TOF-HRMS analysis

Approximately 10 mg of lyophilized plant material was extracted twice with 2 mL of isopropanol, vortexed for 1 min and sonicated for 15 min and centrifuged for 15 min at 3,500 rpm at 10 °C. The extracts were dried under a gentle flow of N_2 gas, reconstituted into 1 mL of methanol-water (85:15 v/v) and subjected to SPE (Waters Sep-Pak C18, 1 mL, 100 mg; Milford, MA, USA) purification, previously conditioned with 1 mL methanol and 1 mL methanol-water (85:15 v/v). Samples were loaded and eluted with 1 mL methanol-water (85:15 v/v). The solution (1 mL) was used for UHPLC-TOF-HRMS analyses.

4.2.7 Quantification of TIA

External calibration curves of strictosidine, ajmalicine-HCl, serpentine-HCl, tabersonine-HCl, catharanthine sulphate and loganic acid were constructed over different concentration levels for each TIA and their UV spectra were recorded at 280, 306 and 320 nm. Working solutions were prepared on 0.01 M H_3PO_4 and each sample was analyzed once *i.e.* there are no technical replicates. Peaks were identified based on chromatographic retention times and spectral data compared to their respective reference standards. Detection and quantification were based on peak area of each TIA.

4.2.8 UHPLC-TOF-HRMS analyses

UHPLC-TOF-HRMS analyses were performed on a Micromass-LCT Premier time-of-flight spectrometer (Waters, MA, USA) with an electrospray interface and coupled with an Acquity UPLC system (Milford, MA, USA). ESI conditions: capillary voltage 2400 V, cone voltage 40 V, MCP detector voltage 2400 V, source temperature 120 °C, cone gas flow 20 L/h, desolvation gas flow 800 L/h. Detection was performed in positive and negative ion modes in the *m/z* range 100-1000 Da in a centroid mode with a scan time of 0.5 s. For the dynamic range enhancement (DRE) lock mass, a 2 µg/mL solution of leucin-enkephalin (Sigma-Aldrich, St Louis, MO, USA) was infused through the lock mass probe at a flow rate of 10 µg/min with a Shimadzu LC pump (LC-10ADvp, Duisburg,

Germany). The separation was carried out on a Waters Acquity BEH C18 (Milford, MA, USA) UPLC column 50 mm x 1.0 mm i.d. 1.7 µm with the following solvent system: A= 0.1% (v/v) formic acid-water, B= 0.1% (v/v) formic acid-acetonitrile. The flow rate was 300 µL/min using 2% of B for 4.8 min, 2-98% B in 4.9 min and holding 98% B for 6 min. Every sample was injected once.

4.2.9 Data handling and analysis

Raw LC–MS data were processed using MarkerLynx® software (Waters, Milford, MA, USA). Before statistical analysis, distributions were tested for normality using the Shapiro-Wilk test ($p < 0.05$). A one-way ANOVA with Tukey's multiple comparison test was applied to test: (a) significant differences in contents of TIA between treatments in JA-treated, mock-treated and untreated cells in each time point and, (b) differences of TIA in JA-treated cells compared against to those of JA-treated cells at all time points. All statistical tests were performed in GraphPad Prism software (v. 8.4.3.686, La Jolla, CA, USA). Differences with $p < 0.1$ and $p < 0.05$ were considered statistically significant and are indicated as * and **, respectively. ANOVA results are shown in Table 4.7.1. (a) and Table 4.7.2. (b) in the supplemental information section.

4.3 RESULTS AND DISCUSSION

4.3.1 α-Methylene-indoline alkaloids in cell suspension cultures of *C. roseus*

Alcoholic extractions allowed the detection and quantification of 10 different TIA and 2 precursors by means of their UV spectra and high-resolution (HR) MS data (Table 1 and supplemental Figure 4.7.3.). Seven peaks were noticed and distinguished only by their different retention times (α-TIA-1-7). According to their absorbance under UV light, three maxima were observed at 225, 300 and 325 nm and a deep minimum at 250 mAU (Saxton, 1965; Hisiger and Jolicoeur, 2007) (Fig. 3), which is characteristic of alkaloids possessing an α-methylene indoline chromophore, constituted by a double bond conjugated with a carbonyl group in the α position of the indoline nitrogen atom (Fig. 4A). Moreover, these alkaloids are also present in their *N*-oxide form and thus have a similar UV spectrum (Malikov and Yunusov, 1977; Schripsema *et al.,* 1987). Some examples of these alkaloids are shown in Fig. 4B-E. Several of these, like tubotaiwine, lochnericine (van der Heijden *et al.,* 2004; 1989; Giddings *et al.,* 2011), condylocarpine (El-Sayed *et al.,* 2004) and akuammicine (Scott *et al.,* 1980; Stöckigt and Soll, 1980) have been reported to accumulate in cell suspension and tissue cultures of *C. roseus* (van der Heijden *et al.,* 1989). Among these, based on the exact mass of *m/z* 353.185969 $[M+H]^+$ derived from the molecular formula $C_{21}H_{24}N_2O_3$, the possible α-TIA candidates are 19-hydroxytabersonine, akuammigine and lochnericine, all found in cell suspension and tissue cultures of *C. roseus* (van der Heijden *et al.,* 1989; 2004). However, the aim of this work was not to identify each trace alkaloid present in our cell system but to get a more general overview of the main TIA

Table 1. Detection parameters of TIA in cell suspension cultures of *Catharanthus roseus* by HPLC-DAD and UHPLC-TOF-HRMS

TIA	Wavelength (nm)	HPLC-DAD RT (min)	UHPLC-TOF-MS RT (min)	Dynamic range (µmol/g DW)	$[M+H]^+$	Molecular formula
Strictosidine	225, 275	4.83	1.55	2.91-20.89	530.2264	$C_{27}H_{34}N_2O_9$
Loganic acid	225	5.24	0.78	0.35-3.35	375.1277	$C_{16}H_{24}O_{10}$
Serpentine	250, 306	6.78	1.61	1.07-2.03	349.1549	$C_{21}H_{20}N_2O_3$
α-TIA-1	225, 300, 325	11.43	1.14	0.14-1.32	353.1863	$C_{21}H_{24}N_2O_3$
α-TIA-2	225, 300, 325	11.94	1.27	0.08-0.20	353.1863	$C_{21}H_{24}N_2O_3$
Catharanthine	225, 280	13.27	1.60	0.01-0.20	337.1916	$C_{21}H_{24}N_2O_2$
α-TIA-3	225, 300, 325	17.15	1.36	0.78-1.56	353.1862	$C_{21}H_{24}N_2O_3$
α-TIA-4	225, 300, 325	19.95	1.41	0.009-0.39	353.1862	$C_{21}H_{24}N_2O_3$
Tabersonine	225, 300, 325	21.24	1.66	0.16-0.78	337.1914	$C_{21}H_{24}N_2O_2$
α-TIA-5	225, 300, 325	22	1.55	0.009-0.01	353.1861	$C_{21}H_{24}N_2O_3$
α-TIA-6	225, 300, 325	23	1.62	0.01	353.1877	$C_{21}H_{24}N_2O_3$
α-TIA-7	225, 300, 325	24.65	1.65	0.001-0.03	353.1864	$C_{21}H_{24}N_2O_3$

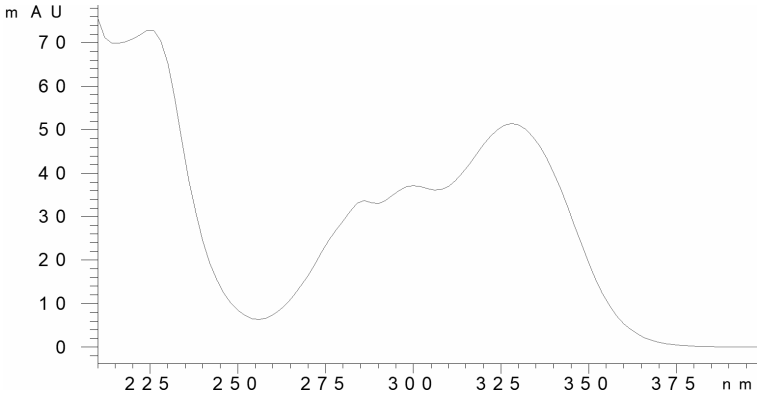

Figure 3. UV spectra of a α-TIA (1-7) detected in cell suspension cultures of *Catharanthus roseus* analyzed by HPLC-DAD.

accumulated after JA treatment. Further studies including their isolation and structural elucidation by NMR- and MS-approaches are needed to fully characterize them.

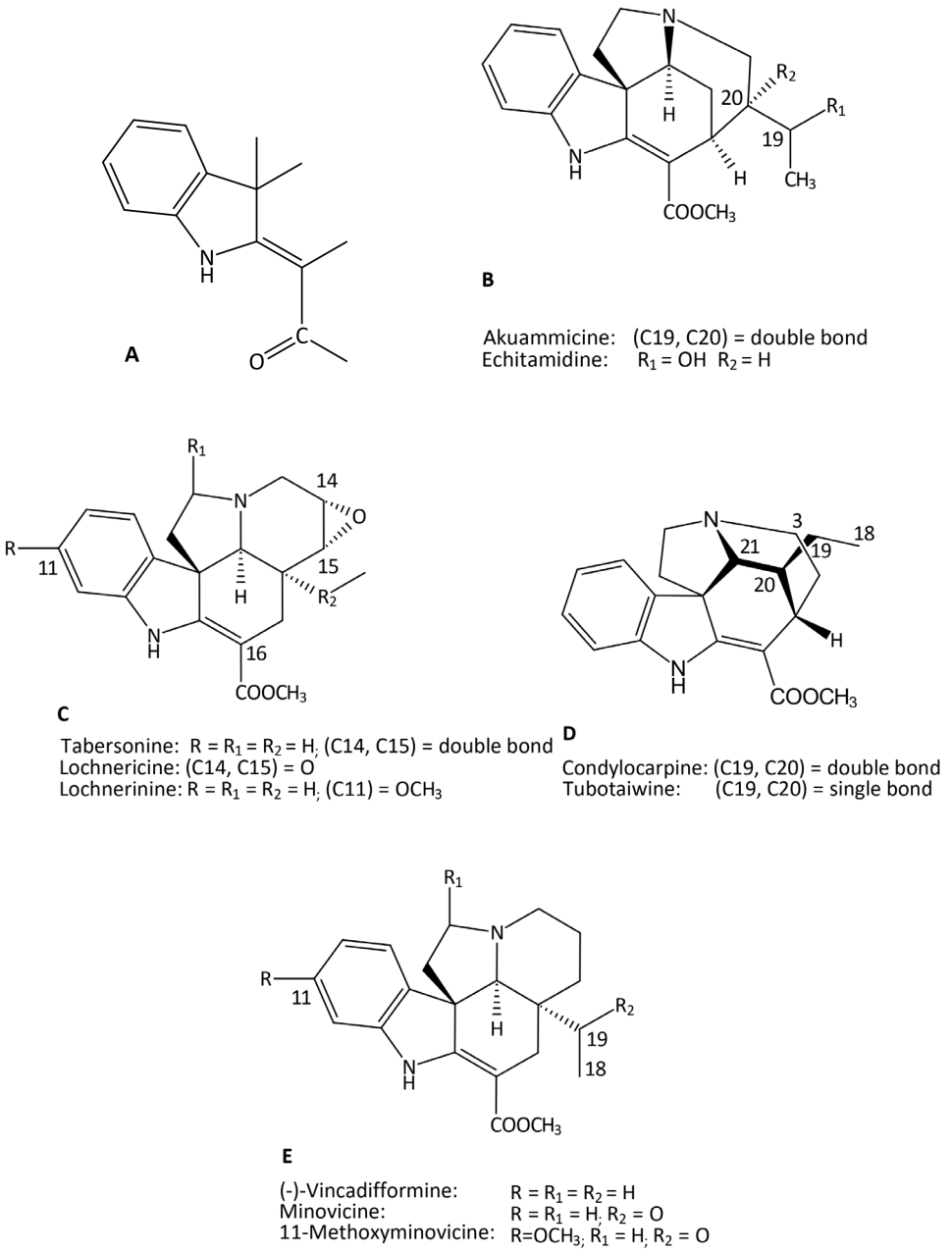

Figure 4. Terpenoid indole alkaloids with a α-methylene indoline chromophore possibly accumulated in cell suspension cultures of *Catharanthus roseus.* **A.** Typical indoline system. **B**. Alkaloids with a C20 skeleton. **C**. Alkaloids with a C21 skeleton (Tabersonine and Lochnericine) and C22 skeleton (Lochnerinine). **D**. Alkaloids with a C20 skeleton. **E**. Alkaloids with a C21 skeleton.

4.3.2 Effect of jasmonic acid on the accumulation of TIA in *C. roseus*

Jasmonic acid treatment was applied to cell suspension cultures of *C. roseus* on the 4th day after subculture. Strictosidine was by far the most abundant TIA in all treatments and controls (1 or 2 orders of magnitude higher than the other TIA found in these experiments) with the highest levels of 20.89 µmol/g DW (at 360 min) and 20.87 µmol/g DW (at 90 min), both in JA-treated cells and followed by mock-treated cells at 360 min (20.69 µmol/g DW) (Fig. 5). Levels of strictosidine in JA-treated cells followed, more or less, the trend of untreated and mock-treated cells at 1440 min. There was a 2.2-fold decrease in strictosidine levels at 1440 min in JA-treated cells if compared to JA-treated cells at t=0 (Fig. 5), although significant differences were observed in JA-treated cells at 1440 min compared to JA-treated cells at 5, 90 and 360 min. At 30 min in JA-treated cells, levels of strictosidine showed a 0.7-fold decrease when compared to mock-treated cells and a 0.9-fold decrease in comparison to untreated cells at 30 min. Levels of strictosidine in JA-treated cells at 90 and 60 min, were significantly higher than JA-treated cells at 30 min. Loganic acid showed lower levels at 30 and 90 min in JA-treated cells in comparison to untreated cells and the mock treatment at these time points. Moreover, levels at 30 min in JA-treated cells were significantly lower than at t=0 and 5 min in JA-treated cells. When compared to JA-treated cells at t=0, a 2.9-fold difference was observed in JA-treated cells at 90 min with a decreasing and significative trend towards the end of the experiment if compared to JA-treated cells at t=0. Neither tryptophan nor tryptamine were detected in this cell line in complete agreement with results of Saiman et al. (2014; 2015). Contents of tabersonine and α-TIA-1 showed similar trends in the JA and mock treatments and in untreated cells over time. JA-treated cells showed for both TIA an increase towards the end of the experiment showing a 1.8-fold increase compared to the mock and a 2-fold increase compared to untreated cells, both at 1440 min (Fig. 5). Levels of α-TIA-2 and α-TIA-3 showed no clear trend, though at 1440 min JA-treated and mock-treated cells did not contain any measurable amount of α-TIA-2. Though accumulation of serpentine seemed to be suppressed in the JA-treated cells (0-360 min), at the end of the experiment it was again about at the same level as untreated and mock-treated cells. Overall, there was a 1.6-fold increase in contents of serpentine in JA-treated cells at 1440 min in comparison to JA-treated cells from the previous time point of observation, and a significant 1.7-fold increase when compared to JA-treated cells to all previous time points. A 0.8-fold decrease in levels of serpentine was seen after 30 min in both JA-treated cells and mock-treated cells in comparison to the control at 30 min. After 90 min, levels of serpentine were lower than untreated and mock-treated cells. However, in the case of catharanthine, higher levels were visible after 1440 min in JA-treated cells when compared to the control and to JA-treated cells at t=0, 90 and 360 min. After 90 and 360 min, levels of catharanthine were significantly lower (more than 10-fold) for untreated, mock-treated and JA-treated cells when compared to earlier time points. There is no clear explanation for this, as other TIA, for as far analyzed, did not show any major changes at these time points. Traces of α-TIA-4-7 were noticed in most treatments though showing a high variation amongst biological replicates. Furthermore, because of their unconfirmed identity and low levels, their quantification was

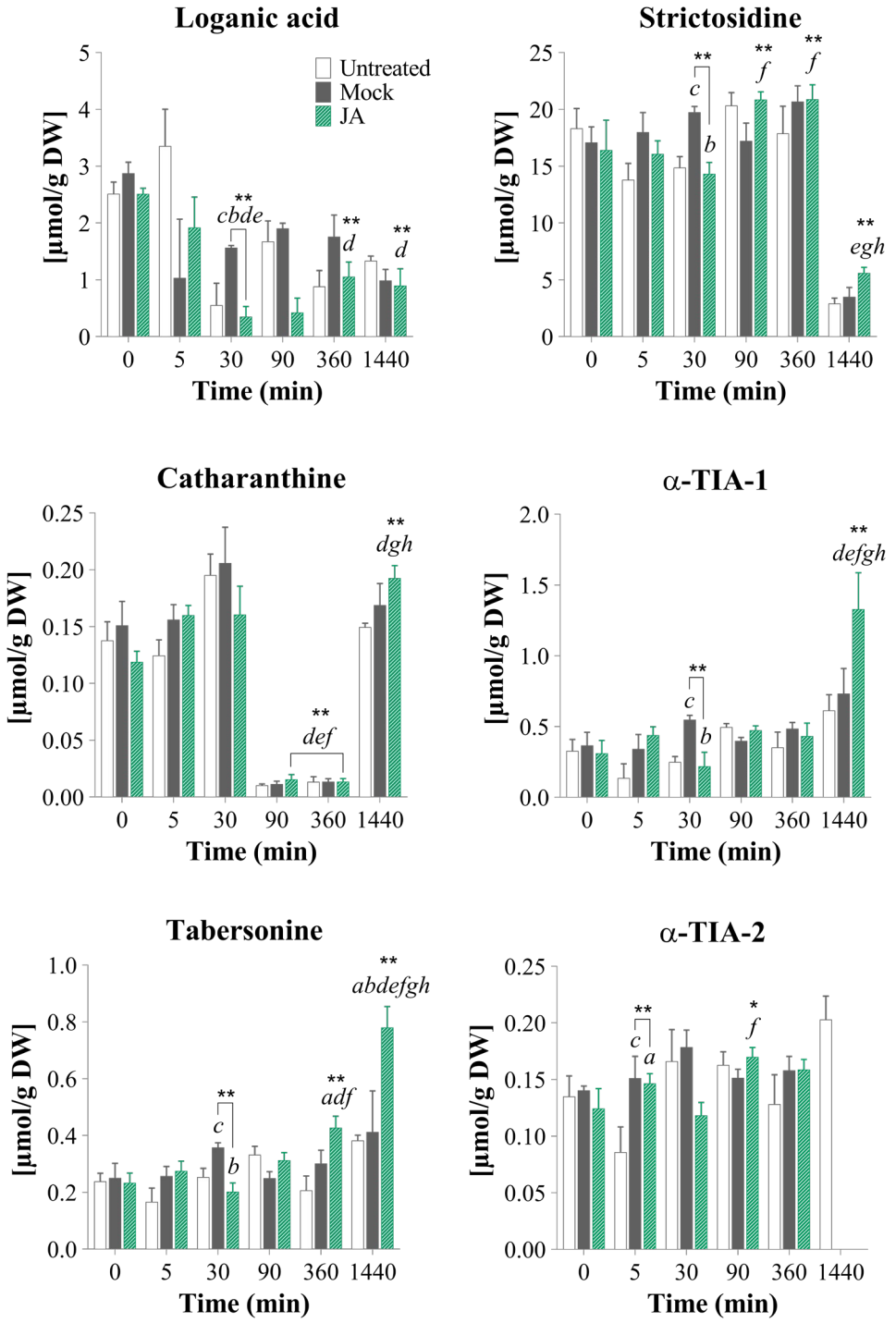

Figure 5. Continued

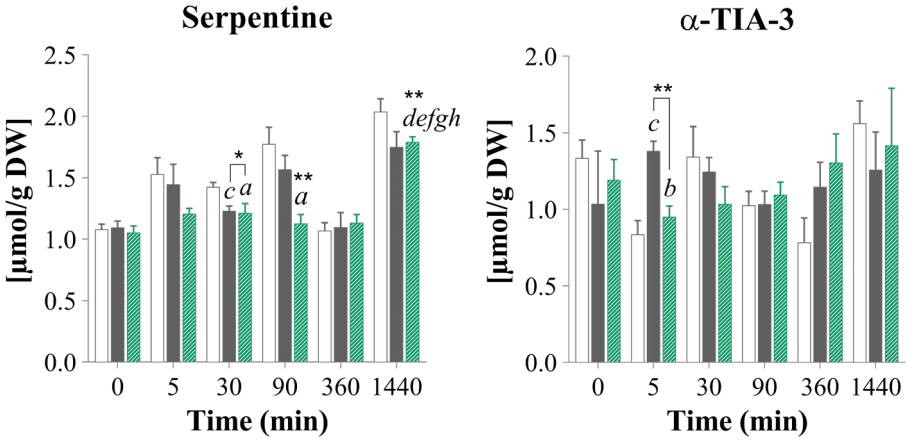

Figure 5. HPLC-DAD analysis of terpenoid indole alkaloids in cell suspension cultures of *Catharanthus roseus* treated with jasmonic acid. Values are the mean ± standard error of mean (SEM) of four biological replicates each analyzed once. Significant results are marked with superscripts (one-way ANOVA with Tukey's post hoc test, $*p < 0.1$, $**p < 0.05$).
[a] JA-treated significantly different from untreated at the same time point of observation.
[b] JA-treated significantly different from mock at the same time point of observation.
[c] Mock significantly different from untreated at the same time point of observation.
[d] JA-treated significantly different from JA-treated at t=0.
[e] JA-treated significantly different from JA-treated at t=5.
[f] JA-treated significantly different from JA-treated at t=30.
[g] JA-treated significantly different from JA-treated at t=90.
[h] JA-treated significantly different from JA-treated at t=360.
[i] JA-treated significantly different from JA-treated at t=1440.
α-TIA 2 in mock and JA treatments at 1440 min was below the detection limit.
Lower levels in catharanthine at 90 and 360 min is due to the absence of this alkaloid in some of the replicates in all treatments.

not possible, although their levels are shown in Figure 6 as areas under the curve (AUC) analyzed by UHPLC-TOF-HRMS. α-TIA-4 increased over time with significant differences in JA-treated cells after 360 min in comparison to JA-treated cells at 5 min. α-TIA-5 showed an increase towards the end of the experiment with significantly higher contents after 360 min when compared to untreated cells at the same time point and to JA-treated cells at 5 and 30 min. A similar trend was observed for α-TIA-6 and α-TIA-7, with peaking levels after 360 min in JA-treated cells. From these four α-TIA, only α-TIA-6 had the lowest levels whereas α-TIA-5 had the highest levels in JA-treated cells at 360 min. Interestingly, these α-TIA showed their highest levels at 360 min in JA-treated cells and none of them seemed to be affected by the mock treatment.

In previous experiments with the same cell suspension cultures (CRPP) of *C. roseus* supplemented with either glucose or sucrose, levels of loganic acid, strictosidine, catharanthine, tabersonine and serpentine were found to be higher in sucrose-supplemented growth medium (Saiman *et al.,* 2014). However, in JA-treated studies in the same cell line but fed with glucose, only loganic acid, strictosidine, serpentine and tabersonine were reported. These precursors and TIA showed

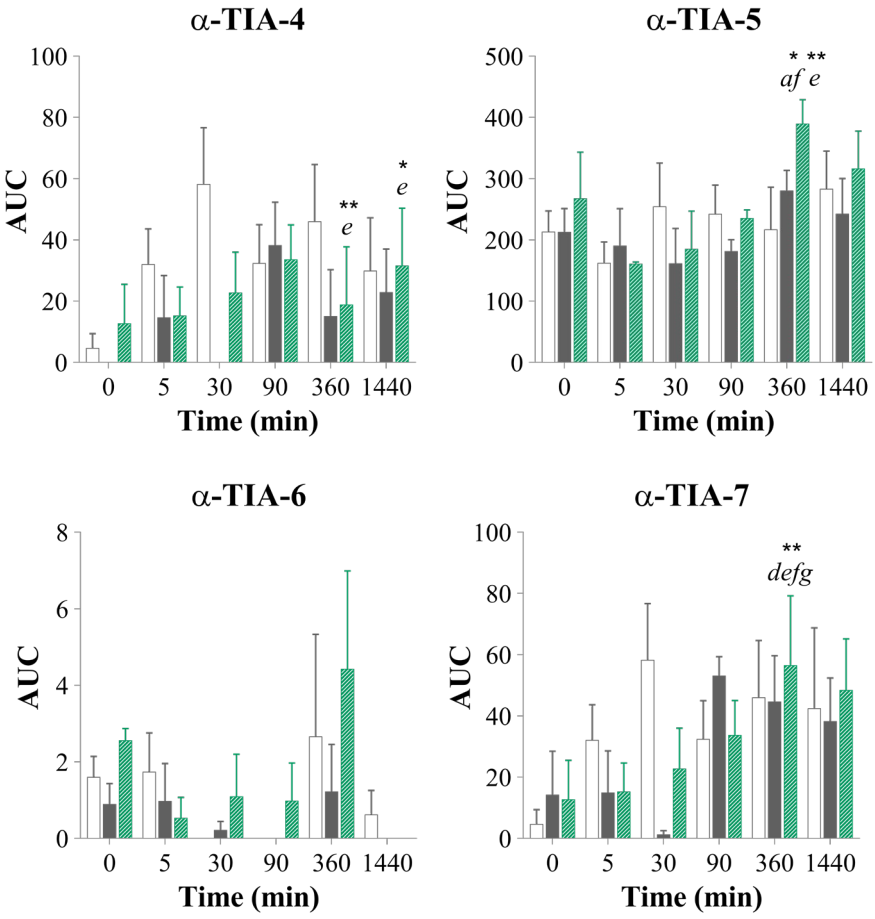

Figure 6. UHPLC-TOF-MS analysis of terpenoid indole alkaloids in cell suspension cultures of *Catharanthus roseus* treated with jasmonic acid. Values are the mean ± standard error of mean (SEM) of four biological replicates each analyzed once. Significant results are marked with superscripts (one-way ANOVA with Tukey's post hoc test, $*p < 0.1$, $**p < 0.05$). α-TIA-6 was not tested due to insufficient data for ANOVA calculations.
[a] JA-treated significantly different from untreated at the same time point of observation.
[b] JA-treated significantly different from mock at the same time point of observation.
[c] Mock significantly different from untreated at the same time point of observation.
[d] JA-treated significantly different from JA-treated at t=0.
[e] JA-treated significantly different from JA-treated at t=5.
[f] JA-treated significantly different from JA-treated at t=30.
[g] JA-treated significantly different from JA-treated at t=90.
[h] JA-treated significantly different from JA-treated at t=360.
[i] JA-treated significantly different from JA-treated at t=1440.

increased levels after 72 h in the JA-treated cells when compared to the untreated (Saiman *et al.,* 2015). Furthermore, experiments with the cell line A12A2 of *C. roseus* showed increased levels of strictosidine and ajmalicine after elicitation with MeJA (El-Sayed and Verpoorte, 2002). Further investigations on

cell suspensions of *C. roseus* treated with 100 µM MeJA showed a 300% increase in serpentine (Lee-Parsons *et al.,* 2004).

According to our previous work and those reported in the literature, 100 µM JA or MeJA is the optimum concentration to elicit secondary metabolism when added in a particular frame of time during cell growth, usually after 4 to 6 days of subculture, *i.e.* in the exponential growth phase when cells are rapidly dividing and growing(Lee-Parsons *et al.,* 2004; Gundlach *et al.,* 1992). Other studies have reported an increased TDC activity after elicitation with 10 µM MeJA or 5 ppm of ET in suspension cultures of *C. roseus,* leading to an increased accumulation of ajmalicine but not of catharanthine within the first 24 h and returning to initial values after 48 h. Even though both treatments lead to the accumulation of TIA, it is noteworthy to observe a differential response in TDC activity with both elicitors. After ethylene exposure, the activity increased transiently after 12 h and peaked at 24 h, whereas exposure to MeJA showed the highest TDC activity after 12 h (Vázquez-Flota *et al.,* 2009). Ajmalicine production is highly inducible by MeJA in cell suspension cultures of *C. roseus* where the highest yield (4.75 mg/L) was observed when cells were treated with 3 mM of $CaCl_2$ and 100 µM of MeJA (Lee-Parsons and Ertürk, 2005). Higher concentrations of this ion showed a negative effect on the accumulation of this alkaloid, which might be explained by the fact that the cytosolic pools of Ca^{2+} may alter signaling processes in which they act as a second messenger, along with protein phosphorylation, in hormone and stress signaling (Arimura and Maffei, 2010; Rudd and Franklin-Tong, 2001; Sun *et al.,* 2010). Another major role of Ca^{2+} in alkaloid biosynthesis induction in *C. roseus* after fungal elicitation is to induce mRNA accumulation of the transcriptional factors *Catharanthus roseus* Box P Binding Factor-like protein 1 (CrBPF-1) and ORCAs that regulate the expression of *STR* in a JA-independent signal transduction pathway (van der Fits *et al.,* 2000).

Besides the effect of JA on TIA accumulation, mock treatment (40% v/v ethanol) transiently induced levels of strictosidine, tabersonine, α-TIA-1, α-TIA-2 and α-TIA-3 when compared to those of JA at the same time point of observation. Most changes were significant due to the treatment and were noticed between 5 and 90 min (Fig. 5) as it was also observed in the fatty acid (FA) profiles of cells (Chapter 3 of this thesis). Both JA- and mock-treatment (40 % v/v ethanol) of the cells induced changes. Similarly, Saiman et al. (2015) found that feeding cell suspension cultures of *C. roseus* with 20% (v/v) of ethanol, increased levels of loganic acid, strictosidine, serpentine and tabersonine at 6 and 72 h compared to the time of treatment. Yet, the addition of JA resulted in an equal or stronger increase compared to the mock, with significantly higher levels for strictosidine, serpentine and tabersonine at 72 h. Furthermore, Saiman (2014) observed a stronger induction of TIA after 72 h of feeding cell suspensions of *C. roseus* with a solution of 50 % (v/v) of ethanol and JA dissolved in 50 % (v/v) of ethanol. Levels of strictosidine, serpentine, catharanthine and tabersonine increased to similar levels as those in JA-treated cells after 72 h. Moreover, levels of these TIA in mock-treated cells were two times higher than those after 24 h of treatment.

Elicitation of suspension cultures of *C. roseus* with mycelial homogenates *i.e. Phytium aphanidermatum, Alternaria zinnae, Verticillium dahlie, Rhodotor ularubra, Eurotium rubrum* to name a few, are able to induce the accumulation of strictosidine lactam, ajmalicine, lochnericine, catharanthine and tabersonine, which includes a rapid and transient increase in the activities of *TDC* and *STR* (Shukla *et al.,* 2010; Eilert *et al.,* 1986; 1987; Tallevi *et al.,* 1986; DiCosmo *et al.,* 1987; Zhao *et al.,* 2001b). Interestingly, the induction of *STR* activity preceded that of *TDC* by 60 h suggesting that endogenous levels of tryptamine might regulate the expression of *TDC* (Eilert *et al.,* 1987; Moreno *et al.,* 1996). All these observations are supported by the fact that fungal elicitors are able to induce endogenous levels of JA in suspension cultures of *C. roseus* (Mueller *et al.*, 1993) which is enough to induce the expression of the *STR* gene (Menke *et al.,* 1999b).

In a large-scale transcriptomic and metabolomic profiling analysis of MeJA-elicited cells of *C. roseus* a gene-to-metabolite network confirmed previous knowledge concerning the coordinated regulation of *TDC* and *STR* by MeJA up to 16-hydroxytabersonine (Rischer *et al.,* 2006). Later steps *e.g. D4H* and *DAT* expression, are not induced by MeJA in cell suspension cultures of *C. roseus* (Vázquez-Flota *et al.,* 2002) explaining the absence of vindoline. Nonetheless, contents of 16-hydroxytabersonine, 16-methoxytabersonine, 16-methoxy-2,3-dihydro-3-hydroxytabersonine and desacetoxyvindoline were significantly induced in *C. roseus* leaves after MeJA treatment (Zhang *et al.,* 2018b*).* It thus seems that the JA effect differs with the type of cell. Rischer *et al.* (2006) reported other metabolic pathways affected by MeJA elicitation. They classed these pathways and reported the following distribution: cell organization and defense (2.4%), metabolism and energy (28%), protein synthesis (3.1%), signal transduction (3.1%), transport (3.6%) and transcription (4.8%).

4.4 CONCLUSIONS

These results extend the knowledge on TIA accumulation in cells of *C. roseus* after JA-induction within the first 24 h. Considering the iridoid precursor loganic acid, results showed a decreasing trend towards 1440 min in all treatments and untreated cells. Strictosidine, as early intermediate, was by far the major TIA with levels up to 20.87-20.89 µmol/g DW at 90 and 360 min in JA-treated cells and almost equally high levels in untreated and mock-treated cells. However, at 1440 min, levels dropped to 2.91, 3.5 and 5.6 µmol/g DW in untreated-, mock- and JA-treated cells, respectively. Significantly increased levels of tabersonine were observed towards the latest time points (360-1440 min), confirming previous observations made after JA elicitation with the same cell line. Additional increases were observed for serpentine (1.5-fold increase), tabersonine (1.8-fold increase), catharanthine (10.5-fold increase) and α-TIA-1 (3-fold increase) in JA-treated cells at 1440 min in comparison to levels at 360 min in JA-treated cells; however, these increases do not add up to the decrease in levels of strictosidine, in this time frame. Apparently, changes are limited, and the *de novo* biosynthesis of TIA does not seem to play a major role in the 1440 min time span. Furthermore, the effect of mock on TIA contents was only

visible between the 5-30 min time points, thus showing that all changes in TIA profiles towards the end of the experiment were the result of treatment with JA. Based on these observations, we support the idea that two independent time-events occur after JA induction. In the early response *i.e.* after 5-30 min of induction, no major effects are observed for TIA levels; only the precursor loganic acid seems to be decreased and the second event, taking place after 1440 min with increased levels of serpentine, tabersonine, catharanthine and α-TIA-1.

4.5 ACKNOWLEDGMENTS

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4.7 SUPPLEMENTAL INFORMATION

Table 4.7.1. ANOVA results for each test performed for each terpenoid indole alkaloid in untreated, mock-treated and JA-treated cells per time point of suspension cultures of *Catharanthus roseus*

			Time (min)			
TIA	**0**	**5**	**30**	**90**	**360**	**1440**
Loganic acid	$F_{(2,9)} = 1.487$ $p=0.2767$	$F_{(2,6)} = 2.157$ $p=0.1969$	$F_{(2,9)} = 6.943$ $p=0.015$	$F_{(2,7)} = 3.077$ $p=0.11$	$F_{(2,9)} = 2.225$ $p=0.164$	$F_{(2,9)} = 1.223$ $p=0.339$
Strictosidine	$F_{(2,9)} = 1.185$ $p=0.3491$	$F_{(2,9)} = 2.067$ $p=0.1825$	$F_{(2,9)} = 12.12$ $p=0.0028$	$F_{(2,9)} = 2.781$ $p=0.1147$	$F_{(2,9)} = 0.9161$ $p=0.4344$	$F_{(2,9)} = 1.656$ $p=0.2440$
Catharanthine	$F_{(2,9)} = 0.9815$ $p=0.4115$	$F_{(2,9)} = 2.61$ $p=0.1277$	$F_{(2,9)} = 0.8652$ $p=0.4532$	$F_{(2,9)} = 0.8978$ $p=0.441$	$F_{(2,9)} = 0.0007463$ $p=0.9993$	$F_{(2,9)} = 2.84$ $p=0.1106$
α-TIA 1	$F_{(2,9)} = 0.1019$ $p=0.9041$	$F_{(2,9)} = 2.979$ $p=0.1017$	$F_{(2,9)} = 7.946$ $p=0.0103$	$F_{(2,9)} = 3.462$ $p=0.0767$	$F_{(2,9)} = 0.6027$ $p=0.568$	$F_{(2,9)} = 4.002$ $p=0.0571$
Tabersonine	$F_{(2,9)} = 0.0524$ $p=0.9492$	$F_{(2,9)} = 2.16$ $p=0.1714$	$F_{(2,9)} = 8.734$ $p=0.0078$	$F_{(2,9)} = 2.588$ $p=0.1294$	$F_{(2,9)} = 5.699$ $p=0.0252$	$F_{(2,9)} = 5.511$ $p=0.0274$
α-TIA 2	$F_{(2,9)} = 0.3054$ $p=0.7441$	$F_{(2,9)} = 4.222$ $p=0.0509$	$F_{(2,9)} = 2.66$ $p=0.1237$	$F_{(2,9)} = 0.9877$ $p=0.4095$	$F_{(2,9)} = 1.005$ $p=0.4038$	-
Serpentine	$F_{(2,9)} = 0.1839$ $p=0.8351$	$F_{(2,9)} = 1.799$ $p=0.2201$	$F_{(2,9)} = 4.762$ $p=0.0388$	$F_{(2,9)} = 8.76$ $p=0.0077$	$F_{(2,9)} = 0.1306$ $p=0.8792$	$F_{(2,9)} = 2.581$ $P=0.1301$
α-TIA 3	$F_{(2,9)} = 0.4451$ $p=0.6541$	$F_{(2,9)} = 14.56$ $p=0.0015$	$F_{(2,9)} = 1.239$ $p=0.3347$	$F_{(2,9)} = 0.1902$ $p=0.8301$	$F_{(2,9)} = 2.463$ $p=0.1403$	$F_{(2,9)} = 0.3123$ $p=0.7393$
α-TIA 4	-	$F_{(2,4)} = 0.3316$ $p=0.7358$	-	$F_{(2,6)} = 0.3404$ $p=0.7244$	$F_{(2,2)} = 0.1253$ $p=0.8887$	$F_{(2,3)} = 1.037$ $p=0.4547$
α-TIA 5	$F_{(2,5)} = 0.4015$ $P=0.6821$	$F_{(2,9)} = 0.1732$ $p=0.8437$	$F_{(2,9)} = 0.5789$ $p=0.5801$	$F_{(2,9)} = 1.221$ $p=0.3394$	$F_{(2,9)} = 3.109$ $p=0.0941$	$F_{(2,9)} = 0.3794$ $p=0.6947$
α-TIA 6	$F_{(2,5)} = 2.013$ $p=0.2285$	-	-	-	-	-
α-TIA 7	-	$F_{(2,4)} = 0.3285$ $p=0.7377$	$F_{(2,4)} = 1.111$ $p=0.4134$	$F_{(2,7)} = 0.7344$ $p=0.5134$	$F_{(2,5)} = 1.902$ $p=0.2431$	$F_{(2,5)} = 2.027$ $p=0.2267$

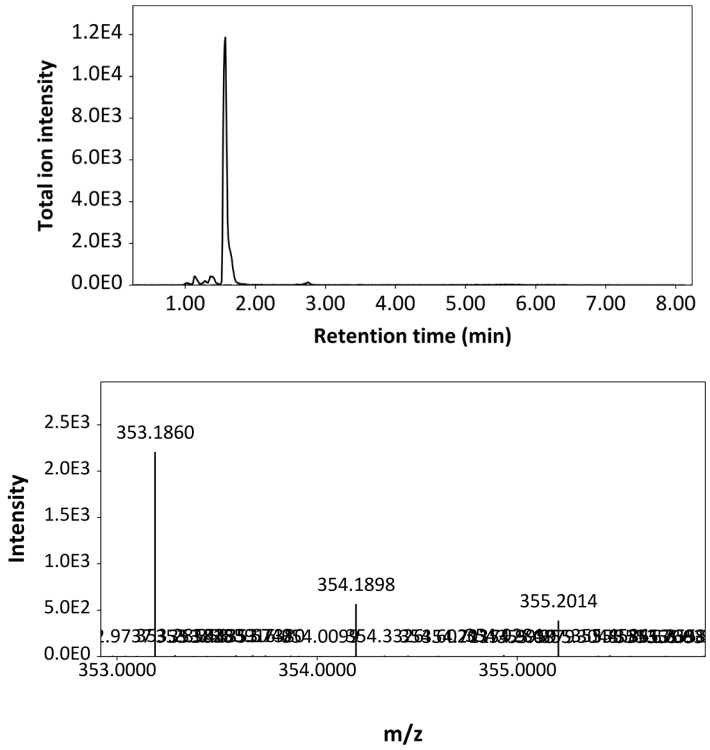
Not tested due to insufficient data for ANOVA calculations: (-).

Table 4.7.2. ANOVA results for tests performed for each terpenoid indole alkaloid against all time points in JA-treated cells of suspension cultures of *Catharanthus roseus*

TIA	**F**	***p***
Loganic acid	$F_{(5,16)} = 6.728$	0.0015
Strictosidine	$F_{(5,17)} = 7.1$	0.0009
Catharanthine	$F_{(5,18)} = 38.96$	<0.0001
α-TIA 1	$F_{(5,18)} = 9.901$	0.0001
Tabersonine	$F_{(5,18)} = 24.82$	<0.0001
α-TIA 2	$F_{(4,15)} = 3.638$	0.029
Serpentine	$F_{(5,18)} = 19.3$	<0.0001
α-TIA 3	$F_{(4,15)} = 0.8712$	0.5038
α-TIA 4	$F_{(5,5)} = 5.631$	0.0405
α-TIA 5	$F_{(5,17)} = 3.381$	0.0266
α-TIA 6	-	-
α-TIA 7	$F_{(5,7)} = 8.065$	0.008

Not tested due to insufficient data for ANOVA calculations: (-).

Figure 4.7.3. UHPLC elution profile of α-TIA-1-7 (top panel) and mass spectrum showing *m/z* 353.1867 (bottom panel) found in cells of suspension cultures of *Catharanthus roseus* analyzed by UHPLC-TOF-MS. Retention times for each α-TIA is as follows: 1.14 min α-TIA-1, 1.27 min α-TIA-2, 1.36 min α-TIA-3, 1.41 min α-TIA-4, 1.55 min α-TIA-5, 1.62 min α-TIA-6, 1.65 min α-TIA-7.



Chapter 5

Effects of dinor-12-*oxo*-phytodienoic acid feeding and study of the early steps of jasmonic acid biosynthesis in cell suspension cultures of *Catharanthus roseus*

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ABSTRACT

In *Catharanthus roseus,* jasmonic acid (JA) plays an important role in regulating terpenoid indole alkaloid (TIA) biosynthesis. We explored the role of JA and its direct precursor dinor-12-*oxo*-phytodienoic acid (dnOPDA) on JA biosynthesis in cell suspension cultures of *C. roseus*. Feeding *d*5-dnOPDA resulted in a fast accumulation of *d*5-JA and *d*5-jasmonoyl-L-isoleucine (*d*5-JA-Ile) in cells demonstrating that cells incorporated exogenous *d*5-dnOPDA. To confirm the production of *d*5-JA-Ile and/or other labeled and unlabeled jasmonates (JAs), cells were fed with *d*5-JA, showing the accumulation of a mass spectrometry (MS) signal corresponding to *unlabeled* JA by observation of the Multiple Reaction Monitoring (MRM) transition *m/z* 209.1→59.0 with the same retention time as the analytical standard of JA. This suggested that *d*5-JA feeding could induce JA biosynthesis. Nevertheless, both targeted and untargeted profiling demonstrated that the putative JA signal was linked to an interference in MRM detection caused by degradation of *d*5-JA into *d*1-JA by hydrogen/deuterium (H/D) exchange in the cell culture conditions. Because no unlabeled JAs were observed in cells or medium, it confirmed that no endogenous JA was produced. The immediate conversion of exogenously fed *d*5-dnOPDA into *d*5-JA and *d*5-JA-Ile indicates that enzymes leading to JA and JA-Ile production are constitutively present and active.

5.1 INTRODUCTION

Plants are sessile organisms that evolved a large number of self-defense mechanisms against biotic and abiotic stresses (Koornneef and Pieterse, 2008; Loon, 2015). The action and coordination of stress response events require signal transduction that is mediated by phytohormones mostly transported via their vascular system along with nutrients and water (Lacombe and Achard, 2016). Of particular interest is jasmonic acid (JA) and its derivatives known as jasmonates (JAs), with a central role as signaling molecule in stress responses (Yan and Xie, 2015).

JAs are plant oxylipins derived from oxidation products of fatty acids (FA), their biosynthesis starts with the release of α-linolenic acid (C18:3) or hexadecatrienoic acid (C16:3) from galactolipids contained in the chloroplast membrane (Joyard *et al.,* 1998) and released from their *sn*-1 position by the phospholipases 1 (PLA_1) DEFECTIVE IN ANTHER DEHISCENCE 1 (DAD1) and DONGLE (DGL) (Ishiguro *et al.,* 2001). Subsequent steps include oxidation by inserting oxygen in position C13 to produce 13-(*S*)-hydroxyperoxy-octadecatrienoic acid (13(*S*)-HPOT), catalyzed by 13-lipoxygenase (13-LOX). Next, 13-LOX produces 13-(*S*)-hydroxyperoxy-octadecatrienoic acid (13(*S*)-HPOT). The enzyme allene oxide synthase (AOS), is responsible for the dehydration of 13-HPOT into the epoxy-octadecatrienoic acid. The conversion of this unstable allene oxide into (9*S*,13*S*)-*cis*-(+)-*oxo*-phytodienoic acid is mediated by allene oxide cyclase (AOC) catalyzing a cyclization reaction (Santino *et al.,* 2013). Export of 12-*oxo*-phytodienoic acid (OPDA) and dinor-12-*oxo*-phytodienoic acid (dnOPDA) from the chloroplast to the peroxisome is possibly carried out by

COMATOSE1/PEROXISOMAL1/PEROXISOME ABC TRANSPORTER (ABC CTS1/PXA1/PED3) (Zolman *et al.,* 2001). Once in the peroxisome, the reduction of dnOPDA/OPDA is catalyzed by OPR3 (OPDA REDUCTASE3) (Wasternack *et al.,* 2006) and followed by the oxidation to 3-*oxo*-2-(2′(*Z*)-pentenyl)-cyclopentane-1-octanoic acid (OPC:8) or to 3-*oxo*-2-(2′(*Z*)-pentenyl)-cyclopentane-1-hexanoic-acid (OPC:6). Activation of OPC:8 and OPC:6 by esterification to CoA yield OPC:8-CoA and OPC:6-CoA, respectively, and the sequential β-oxidation steps are catalyzed by an acyl CoA oxidase (ACX), ACX1 and ACX5 in the case of OPC:6 (Schilmiller *et al.,* 2007). A multifunctional protein (MFP) is involved in the synthesis of OPC:4CoA and, a 3-ketoacyl-CoA thiolase (KAT2) catalyzes the formation of JA-CoA to yield (3*R*, 7*S*)-JA (Weber *et al.,* 1997; Stelmach *et al.,* 2001). The final step is the conjugation with isoleucine that yields the biologically active form (3*R*, 7*S*)-jasmonoyl-L-isoleucine (JA-Ile) (Fonseca *et al.,* 2009) catalyzed by the cytosolic JASMONATE RESISTANT1 (JAR1). JA-Ile is then imported across the nuclear membrane and JA is exported outside the plasma membrane, both by JA/JA-Ile JASMONATE TRANSPORTER 1 (JAT1) (Li *et al.,* 2017) (Fig. 1). It is noteworthy to mention that the expression of all biosynthetic genes of JA is induced by JAs (Sasaki *et al.,* 2001; Wasternack and Feussner, 2018; Chung *et al.,* 2008) suggesting a possible self-induction of JA-biosynthesis.

JA represents the coordinated link between primary and secondary metabolism by activating transcription factors (TF) involved in the JA-mediated defense pathways. In *Catharanthus roseus* (L.) G. Don., these signaling pathways are regulated by the CORONATINE INSENSITIVE1 (COI1)/JASMONATE ZIM DOMAIN (JAZ)/MYC2/ bHLH IRIDOID SYNTHESIS1 (BIS1) signalling module. COI1 is part of a Skp1/Cullin/F-box protein complex acting as an E3 ubiquitin ligase (SCF^{COI1}), which upon recognition of JA-Ile, promotes the interaction between the JAZ repressors and COI1 leading to the ubiquitination and degradation of JAZ proteins and thus activates MYC2 and BIS1 (Chini *et al.,* 2007; 2009; Patra *et al.,* 2018). Activation of these TF, initiates the expression of terpenoid indole alkaloids (TIA) biosynthetic genes through the activation of the TF OCTADECANOID-DERIVATIVE RESPONSIVE CATHARANTHUS AP2-DOMAIN2, (ORCA2) ORCA3 (Menke *et al.,* 1999), ORCA4 and ORCA5 (Li *et al.,* 2013; Memelink *et al.,* 2001; Fits and Memelink, 2000; Paul *et al.* 2017). ORCA2 and ORCA3 regulate the expression of several TIA biosynthetic genes such as *TRYPTOPHAN DECARBOXYLASE* (*TDC*)*, STRICTOSIDINE SYNTHASE* (*STR*) (Memelink, 2009), *GERANIOL SYNTHASE* (*GES*), *GERANIOL-8-OXIDASE* (*G8O* or *G10H*) (Miettinen *et al.,* 2014), *7-DEOXYLOGANETIC ACID GLUCOSYLTRANSFERASE* (*7DLGT*) and *7-DEOXYLOGANIC ACID HYDROXYLASE* (*7DLH*) (Moerkercke *et al.,* 2015; 2016) to name a few.

It is known that exogenous JA and its methyl ester (MeJA) induce several of the TIA biosynthetic pathway genes (Peebles *et al.,* 2009; Lee-Parsons *et al.,* 2004; Kellner *et al.,* 2015; Zhang *et al.,* 2018) but only after 2 h of induction (Collu *et al.,* 2001; Wei, 2010) and the TF ORCA2, ORCA3, ORCA4, ORCA5, BIS1, MYC2 and bHLH REPRESSOR OF MYC2 TARGETS 1 (RMT1) (Patra *et*

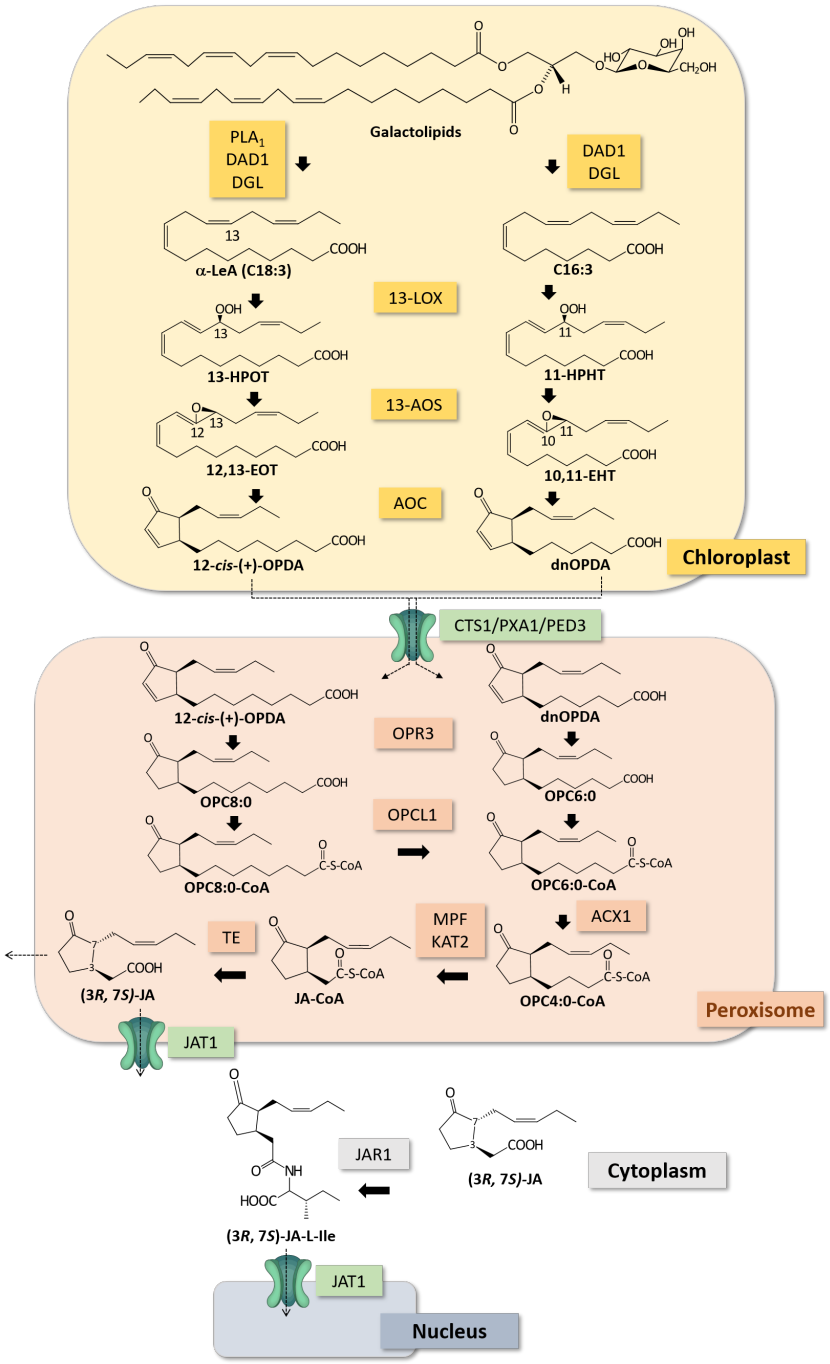

Figure 1. Biosynthesis of jasmonic acid in plant cells starts with the release of a-linolenic acid or hexadecatrienoic acid from their *sn*-1 position from galactolipids in the thylakoid by the lipases PLA_1, DAD1 and DGL. The three consecutive enzymatic reactions including oxidation, dehydration and cyclization are catalyzed by 13-LOX, 13-AOS and AOC, respectively, and take

place in the chloroplast. The final products are OPDA and dnOPDA, which are then exported from the chloroplast and imported into the peroxisome by the COMATOSE1/PEROXISOMAL1/PEROXISOME ABC TRANSPORTER (ABC CTS1/PXA1/PED3). In the peroxisome, OPDA and dnOPDA are reduced by OPR3 and then oxidized to OPC8:0 and OPC6:0. These, are activated to their CoA esters (OPC8:0-CoA and OPC6:0-CoA) and later reduced through three sequential steps catalyzed by ACX1, MPF and KAT2 yielding JA-CoA which is released by TE to form (3*R*, 7*S*)-JA. JA is exported to the cytoplasm where the conjugation with isoleucine by JAR1 renders the biologically active JA-Ile. JA is exported across the plasma membrane and JA-Ile is imported into the nucleus, both by JAT1. Enzymes are indicated with a colored background. Solid lines indicate an enzymatic reaction and dashed lines indicate intracellular transport. Abbreviations for compounds, transporters and enzymes are: PLA_1, phospholipase 1; DAD1, DEFECTIVE IN ANTHER DEHISCENCE 1; DGL, DONGLE; α-LeA, α-linolenic acid; C16:3, hexadecatrienoic acid; 13-LOX, 13-lipoxygenase; 13-HPOT, 13-(*S*)-hydroxyperoxy-octadecatrienoic acid; 11-HPHT, 11-(*S*)-hydroxyperoxy-hexadecatrienoic acid; 13-AOS, allene oxide synthase; 12,13-EOT, epoxy-octadecatrienoic acid; 10,11-EHT, 10,11-(*S*)-epoxy-hexadecatrienoic acid; AOC, allene oxide cyclase; 12-*cis*-OPDA, *cis*-(+)-*oxo*-phytodienoic acid; dnOPDA, dinor-12-*oxo*-phytodienoic acid; CTS/PXA1/PED3 COMATOSE1/PEROXISOMAL1/PEROXISOME ABC TRANSPORTER; OPR3, OPDA reductase3; OPC8:0, OPCL1, OPC-8:CoA ligase1; 3-*oxo*-2-(2´(*Z*)-pentenyl)-cyclopentane-1-octanoic acid; OPC6:0, 3-*oxo*-2-(2´(*Z*)-pentenyl)-cyclopentane-1-hexanoic-acid; ACX1, acyl CoA oxidase; MPF, multifunctional protein; KAT2, 3-ketoacyl-CoA thiolase; TE, thioesterase; JAT1, JASMONATE TRANSPORTER1; JAR1, JASMONATE RESISTANT1; JA-L-Ile, (3*R,* 7*S)*-jasmonoyl-L-isoleucine.

al., 2018; Paul *et al.,* 2017; De Geyter *et al.,* 2012; Moerkercke *et al.,* 2015; 2016) both *in planta* (Pan *et al.,* 2018) and *in vitro* cell cultures (Wei, 2010). Because significant accumulation of TIA, involved in plant defense (Heijden *et al.,* 2004; Zhang *et al.,* 2011), is detected after 4-24 h (Moreno *et al.,* 1996; Vázquez-Flota *et al.,* 2009), their accumulation can be considered as a late response in the JAs-mediated stress response in *C. roseus*.

While most of the knowledge of the early steps in JA biosynthesis is based on *in planta* experiments, notably for the understanding of defense signaling in plant models like *Arabidopsis* or tomato (Glauser *et al.,* 2009; Howe *et al.,* 1996), little is known about the early steps in other plant models and/or in plant cell cultures. Because of the important role of JA in the biosynthesis of the TIA in *C. roseus,* the present work investigates the biosynthesis of the various forms of JA in typical cell cultures experiments especially because such compounds are used for the induction of these secondary metabolites. We formulated the following questions: i) can exogenous dnOPDA be converted in JA and JA-Ile?; ii) can exogenous JA be converted in JA-Ile?; iii) are there other forms of JAs in cells and/or growth medium before and after feeding them with dnOPDA or JA?. To answer these questions, we devised feeding experiments with *d*5-dnOPDA and *d*5-JA. To distinguish between endogenous and exogenous dnOPDA and JA, *d*5-dnOPDA and *d*5-JA were added separately to cell suspension cultures of *C. roseus*. These experiments were intended to provide qualitative (presence of unlabeled dnOPDA/JA after feeding their labeled forms) as well as quantitative data (fate of (labeled) dnOPDA/JA over time).

5.2 EXPERIMENTAL

5.2.1 Cell suspension cultures and elicitation with JA and dnOPDA

Cell suspension cultures of the *C. roseus* cell line CRPP were grown in 250 mL Erlenmeyer flasks containing 50 mL of Gamborg B5 medium (Gamborg *et al.,* 1968; Duchefa Biochemie; Haarlem, The Netherlands) supplemented with 30 g/L sucrose and 1.86 mg/L of 1-naphthalene acetic acid (NAA) and adjusted to pH 5.8 with 0.1 N KOH. Cell cultures were propagated on a rotary shaker (110 rpm) at 25 °C under continuous light (500-1500 lux) and were subcultured every three weeks. Three experiments were performed: **1**) four-day-old cell suspension cultures were treated with 50.65 nmol/flask of *d*5-JA; ((2-((1R,2R)-3-*oxo*-2-((Z)-pent-2-en-1-yl)cyclopentyl-2,4,4-*d*3) acetic-2,2-*d*2 acid; CDN Isotopes Inc. Quebec, Canada) or 54.5 µL of 40% ethanol (mock) or nothing (untreated cells); **2**) five-day-old cell suspension cultures were treated with 7.18 µmol of *d*5-JA ((2-((1R,2R)-3-*oxo*-2-((Z)-pent-2-en-1-yl)cyclopentyl-2,4,4-*d*3) acetic-2,2-*d*2 acid; CDN Isotopes Inc. Quebec, Canada) or 773.7 µL of 40% ethanol (mock) or nothing (untreated cells); **3**) for the last experiment, the JA precursor *d*5-dnOPDA was added (50.65 nmol/flask; 4-*oxo*-5S-(2Z)-2-penten-4,4,5,5,5-*d*5-1-yl-2-cyclopentene-1S-hexanoic acid; Cayman Europe; Tallinn, Estonia). Both labeled compounds were dissolved into 40% ethanol (v/v); 54.5 µL (*d*5-JA **1**); 773.7 µL (*d*5-JA **2**) or 42 µL (*d*5-dnOPDA **3**) were added to the cell culture flasks. The mock treatments in each experiment were made with the same amount of only 40% ethanol (v/v). Treated, mock-treated and control cells were harvested in triplicates at 0, 5, 30 and 1440 min for both *d*5-JA experiments and in duplicates at 5, 30 and 1440 after elicitation (for *d*5-dnOPDA). Cells were filtered on Whatman filter paper under partial vacuum carefully avoiding cross-contamination of cells to the growth medium, and biomass and media samples were immediately frozen in liquid nitrogen and kept at -80 °C; cells and growth media were lyophilized before any further processing.

5.2.2 Extraction procedures for d5-JA and d5-JA-Ile experiments

Approximately 5 mg of lyophilized plant cells and lyophilized growth medium materials were extracted twice in a 2 mL centrifuge tube with 0.8 mL of methanol-water (80:20 v/v), vortexed vigorously for 10 sec and centrifuged for 6 min at 13,000 rpm at 10°C. The combined supernatants (1 mL) were directly transferred into an appropriate vial for both UHPLC-MS/MS and UHPLC-HRMS analyses.

5.2.3 Extraction procedures for d5-dnOPDA experiments

The extraction protocol was according to the method described (Glauser and Wolfender, 2013). Briefly, approximately 10 mg of lyophilized plant cells or growth medium materials were extracted twice with 2 mL of isopropanol, vortexed for 1 min, sonicated for 15 min and centrifuged for 15 min at 3,500 rpm at 10 °C. The supernatants were combined and dried under a gentle flow of N_2 gas, reconstituted into 1 mL of methanol-water (85:15 v/v) and subjected to SPE (Waters Sep-Pak C18, 1 mL, 100 mg; Milford, MA, USA) purification, previously conditioned with 1 mL methanol and 1 mL methanol-water (85:15

v/v). Samples were loaded and eluted with 1 mL methanol-water (85:15 v/v). The solution (1 mL) was filtered (Sartorius RC 0.20 µm; Göttingen, Germany) and used for UHPLC-TOF-MS analyses.

5.2.4 UHPLC-HRMS analysis of JA and JA-Ile

UHPLC-MS/MS analyses were performed on a QTRAP 4000 quadrupole-linear ion trap (Applied Biosystems, Toronto, Canada) with an electrospray interface (ESI) and coupled with an ACQUITY UPLC system (Waters, Milford, MA, USA). Detection and separation method were based and adapted from Glauser et al. 2014. ESI conditions were the following: curtain gas 25 psi, collision gas 5 psi, Ion Spray voltage -4500 V, source temperature 550°C, ion source gas 1: 45 psi, ion source gas 2: 30 psi. Acquisition was performed in negative ion mode. The separation was carried out on a 50 mm x 2.1 mm i.d. 2.1 µm ACQUITY BEH C18 column (Waters, Milford, MA, USA) coupled to an ACQUITY UPLC BEH C18 130 Å, 1.7 µm, 2.1 mm x 5 mm guard column with the following solvent system: A is 0.1% (v/v) formic acid in water, B is 0.1% (v/v) formic acid in acetonitrile. The flow rate was 400 µL/min using 5% of B at 0 min, applying a linear gradient of 5-100% of B in 7 minutes and holding 100% of B for 1 min before returning to 5% of B. The monitoring of targeted analytes was based on appropriate Multiple Reaction Monitoring (MRM) of ion pairs for endogenous JA and *d*5-JA with following mass transitions: JA 209.100→59.000 and *d*5-JA 214.100→62.000. Compound parameters were optimized for the two transitions by Flow Injection Analysis of standard solution: collision energy (CE) was set at -22 eV for JA, -25 eV for *d*5-JA; declustering potential (DP) and entrance potential (EP) were identical for the two JAs, respectively -55 V and -10 V. Dwell time was set at 0.1 sec for JA transition and 0.130 sec for *d*5-JA transition. The injection volume was 5 µL.

UHPLC-HRMS analyses were performed on a Q-Exactive Focus mass spectrometer (Thermo Scientific, Bremen, Germany) with a heated electrospray interface (HESI-II) and coupled with an ACQUITY UPLC system (Waters, Milford, MA, USA). HESI conditions were the following: source voltage 2.5 kV; sheath gas flow rate (N_2) 55; auxiliary gas flow rate 15; spare gas flow rate 3.0; capillary temperature 275 °C; S-Lens RF level 45. Detection was performed in negative ion mode in the *m/z* range 120-1,500 Da. Internal calibration was done by using a mixture of caffeine, methionine-arginine−phenylalanine−alanine−acetate (MRFA), sodium dodecyl sulfate, sodium taurocholate and Ultramark 1621 (Thermo Scientific, Bremen, Germany) in an acetonitrile/methanol/water solution containing 1% formic acid by direct injection. The separation was carried out on a 50 mm x 2.1 mm i.d. 2.1 µm ACQUITY BEH C18 column (Waters, Milford, MA, USA) with the following solvent system: A is 0.1% (v/v) formic acid in water, B is 0.1% (v/v) formic acid in acetonitrile. The flow rate was 600 µL/min using 5% of B at 0 min, a linear gradient of 5-100% of B in 7 minutes and holding 100% of B for 1 min followed by re-equilibration at 5% of B. Analysis of JA and *d*5-JA was based on data-dependent MS/MS events and were performed on the three most intense ions detected in full scan MS (Top3 experiment). The MS/MS isolation window width was 1 Da. Stepped normalized collision

energy (NCE) was set to 15, 30 and 45 units. Full scans were acquired at a resolution of 35,000 FWHM (at *m/z* 200) and MS/MS scans at 17,500 FWHM both with an automatically determined maximum injection time. The injection volume was 6 µL.

5.2.5 Quantification and UHPLC-TOF-MS/MS analysis of d5-dnOPDA, d5-JA and d5-JA-Ile

External calibration curves of *d*5-dnOPDA (Cayman Europe; Tallinn, Estonia), JA (Sigma-Aldrich, St Louis, MO, USA) and JA-Ile (Cayman Chemical, Ann Arbor, MI, USA) were built over different concentration levels. Working solutions were prepared in 1 mL of methanol-water (85:15 v/v). Peaks were identified based on chromatographic retention times and compared to their respective reference standards. Detection and quantification were based on peak area of each JAs. UHPLC-MS/MS analyses were performed on a microTOF-QII spectrometer (Bruker, Bremen, Germany) with an electrospray interface and coupled with an UPLC system (Waters, Milford, MA, USA). ESI conditions: capillary voltage 3100 V, cone voltage 40 V, MCP detector voltage 2400 V, source temperature 120 °C, cone gas flow 20 L/h, desolvation gas flow 800 L/h. Detection was performed in negative ion mode in the *m/z* range 50-600 Da. Internal calibration was done by means of the Lockspray interface (Bruker, Bremen, Germany) by infusing a 2 µg/mL solution of sodium formate (Sigma-Aldrich, St Louis, MO, USA) at a flow rate of 10 µL/min with a Shimadzu LC pump (LC-10ADvp, Duisburg, Germany). Data were averaged over 2 scans for mass correction. The separation was carried out on a 50 mm x 1.0 mm i.d. 1.7 µm Phenomenex C18 UPLC column (Torrance, CA, USA) with the following solvent system: A is 0.1% (v/v) formic acid in water, B is 0.1% (v/v) formic acid in acetonitrile. The flow rate was 300 µL/min using 2% of B at 0 min, a linear gradient of 2-98% of B in 7 min and holding 98% of B for 2 min prior to re-equilibration at 2% of B. Analysis of all JAs was based on published Multiple Reaction Monitoring (MRM) of ion pairs for labeled and endogenous JA, JA-Ile, dnOPDA, OPDA, DHJA, OPC-4 and OPC-6 using the following mass transitions: *d*5-JA 214→170; JA 209→109; JA-Ile 322→130; *d*5-JA-Ile 327→130; dnOPDA 263→156; *d*5-dnOPDA 268→170; OPDA 291→165; *d*5-OPC-4 242→181; *d*5-OPC-6 270→181 and DHJA 211→59 (Balcke *et al.,* 2012; Glauser and Wolfender, 2013). Collision potential (CE) V was set to 18 for every JAs. The injection volume was 5 µL.

5.2.6 Extraction of galactolipids

Modified protocols from Welti et al. 2002 and Kourtchenko et al. 2007 were followed to extract galactolipids in cell suspension cultures of *C. roseus.* Briefly, 100 mg of lyophilized cell material was extracted twice with 3 mL of isopropanol and 100 µL of 0.025% BHT warmed up at 75 ºC. All test tubes were stirred for 5 min and sonicated for 30 min and the combined extracts were transferred to a new test tube. Then, 1.5 mL of chloroform and 600 µL of water were added and vigorously shaken for 1 h. The extraction was repeated three times with a mixture of chloroform-water 2:1 (v/v) and 0.025%

BHT and manually shaken for 30 min. Phase separation of the combined chloroform-water extracts was achieved by adding one volume of a solution of chloroform-potassium sulphate (380 mM) (1:1 v/v), the lower phase was transferred to a new glass test tube and the aqueous phase was reextracted with one volume of chloroform. Both organic phases were combined, dried under gas N_2, dissolved in a small volume of chloroform and subjected to SPE (Waters Sep-Pak silica, 1 mL, 100 mg; Milford, MA, USA) purification, previously conditioned with 1 mL chloroform and 1 mL chloroform-acetone (9:1 v/v). Samples were loaded and neutral lipids were eluted with 1 mL chloroform-acetone (9:1 v/v) and galactolipids eluted with acetone-methanol (9:1 v/v). Furthermore, each fraction was analyzed on a silica thin layer chromatography (TLC) plate developed with chloroform-methanol (85:15 v/v), bands were visualized by staining with anisaldehyde followed by heating. The anisaldehyde stain solution was prepared by adding 135 mL of absolute ethanol, 5 mL of concentrated sulfuric acid, 1.5 mL of glacial acetic acid and 3.7 mL of *p*-anisaldehyde. The solution was stirred vigorously and stored in darkness until further use. Neutral lipids yielded 3 bands at R_f= 0.35-0.45; R_f=0.5-0.6 and R_f=0.7-0.8 and galactolipids yielded 4 bands: at R_f=0.17-0.27; R_f=0.34-0.42; R_f=0.65-0.73 and R_f=0.8-0.85. Each band was scrapped off the plate, washed with methanol-acetone 1:1 (v/v), vortexed for 1 min, centrifuged for 20 min at 13,000 rpm and kept at 4 ºC until further analysis. All fractions were analyzed by UHPLC-HRMS and ^{1}H-NMR.

5.2.7 Extraction of galactose, dnOPDA and OPDA

Analysis of pure galactose, dnOPDA and OPDA in their methyl ester or trimethylsilylated forms was carried on by GC-MS and GC-FID. A solution of 500 ng of either galactose, dnOPDA or OPDA in 1 mL of methanol was evaporated to dryness under a gentle flow of N_2 gas, then each analyte followed two derivatization processes: **1)** methylation was achieved by adding 500 µL of boron trifluoride (BF_3; 10 % w/w) in methanol (Sigma-Aldrich, St Louis, MO, USA), followed by heating the closed tubes at 80 °C for 20 min and then cooling down at room temperature for 5 min. Phase separation was achieved by adding 500 µL of *n*-hexane and was repeated twice; each time, after centrifugation for 5 min at 3,500 rpm, the upper layers were collected, combined and evaporated to dryness under a gentle flow of N_2 gas. Analytes were reconstituted in 100 µL of *n*-hexane and subjected to GC-MS or GC-FID analysis. **2)** trimethylsilylation was achieved by adding 50 µL of pyridine (Sigma-Aldrich, St Louis, MO, USA) and 50 µL of *N,O*-bis-trimethylsilyl-trifluoroacetamide with trimethylchlorosilane (BSTFA+TMCS, Supelco, Sigma-Aldrich, St Louis, MO, USA), the solution was heated at 80 °C for 20 min and left to cool down at room temperature for 5 min. After complete evaporation, analytes were reconstituted in 100 µL of pyridine and subjected to GC-MS or GC-FID analysis. For galactose and JAs analysis in plant materials, approximately 50 mg of lyophilized untreated, mock and JA-treated cells were hydrolyzed with 500 µL of 1 M KOH in methanol at 80 °C for 30 min. After cooling down for 5 min at room temperature, 500 µL of *n*-hexane with 0.01% of BHT and 50 µL of water were added to induce

phase separation, this extraction step was repeated three times. Samples were combined in new test tubes, vortexed and the upper layer was collected into new tubes. Solvents were evaporated to dryness under a gentle flow of N_2 gas. Analytes were derivatized as described above for the pure compounds and reconstituted in 100 µL of *n*-hexane.

5.2.8 Gas chromatographic analyses

Analysis of the silylated galactose and OPDA was performed on an Agilent 7890A gas chromatograph equipped with an Agilent 7693 auto sampler and an Agilent 5775C Triple-Axis MSD detector (all from Agilent Technologies Inc., Santa Clara, CA, USA). Methyl esters or trimethylsilyl derivatized analytes were separated on a 30 m x 0.25 mm x 0.25 µm film thickness DB-5 column (J&W; Agilent Technologies Inc., Santa Clara, CA, USA), with a constant flow of 1 mL/min of He as a carrier gas. The injection port was heated to 60 °C. The injection volume was 5 µL in a splitless mode. The oven temperature was 60 °C for 4 min, then 10 °C/min to 200 °C followed by 20 °C/min to 300 °C, which was maintained for 10 min. All mass spectra were acquired in the electron impact (EI) mode for full scan in total ion current (TIC) mode. Alternatively, an Agilent gas chromatograph 6890 series with an FID detector was used for analysis of galactose, dnOPDA and OPDA (Agilent Technologies Inc., Santa Clara, CA, USA). The instrument was equipped with a 7683 series injector and autosampler, plus a 6890 series integrator. A DB-5 column (J&W; Agilent Technologies Inc., Santa Clara, CA, USA) of 30 m x 0.25 mm x 0.25 µm film thickness was used. The injection port was heated to 60 °C. Injection volume was 5 µL in a splitless mode. The oven temperature was 60 °C for 4 min, then 10 °C/min to 200 °C and then 20 °C/min to 300 °C which was maintained for 10 min. Including a cooldown step of 2.5 min to 60˚C, the total run time was 35.5 min per sample for both MS and FID methods.

5.2.9 ^{1}H-NMR spectroscopy

All samples were evaporated under vacuum until dryness and were reconstituted in 200 µL of methanol-*d*4 (Cambridge Isotope Laboratories, Inc., Andover, MA, USA) and further sonicated to reach complete solubility. To wash the extraction glass tube, 100 µL of methanol-*d*4 were added and then a total of 300 µL were loaded into an NMR tube. ^{1}H-NMR spectra were recorded at 25 °C on a 400 MHz spectrometer (Bruker, Karlsruhe, Germany). MeOD was used as the internal lock. Each ^{1}H NMR spectrum consisted of 128 scans requiring 10 min and 26 sec acquisition time with the following parameters: 0.16 Hz/point, pulse width (PW) = 30° (11.3 µsec) and relaxation delay (RD) = 1.5 sec. A pre-saturation sequence was used to suppress the residual H_2O signal with low power selective irradiation at the H_2O frequency during the recycle delay. FIDs were Fourier transformed with LB = 0.3 Hz.

5.2.10 Data handling and analysis

Raw LC–MS data were processed using Data Analysis® software (Bruker, Bremen, Germany) and Xcalibur 4.3 software (Thermo Fisher Scientific, Waltham, MA, USA). 1H-NMR spectra were manually phased, and baseline corrected and calibrated to methanol-*d*4 at 3.3 ppm using TopSpin software (version 3.0; Bruker, Bremen, Germany). Raw GC-MS and GC-FID data was acquired, integrated and peaks identified using Agilent Chemstation 4.03 (Agilent Technologies, Santa Clara, CA, USA). Before statistical analysis, distributions were tested for normality using the Shapiro-Wilk test ($p < 0.05$). A nested one-way ANOVA corrected for multiple comparisons with Tukey's post hoc test was used to assess significant differences among *d*5-JA, *d*5-JA-Ile and *d*5-dnOPDA in all time points in cells and a nested t-test was used to test significant differences in growth medium between *d*5-JA and *d*5-dnOPDA in all time points. Significant differences among all isotopologues of deuterium labeled JA (high dose and low dose; HD and LD) from 0 min were assessed with a one-way ANOVA. Significant differences between *d*5-JA and "JA-like" for each observation point were assessed with a two-tailed *t*-test with Welch's correction. Significant differences among *d*5-JA and "JA-like" from *d*5-JA at 0 min were assessed with a one-way ANOVA with Dunnett's post hoc test. Differences with $p < 0.05$ were considered statistically significant. All statistical tests were performed using GraphPad Prism software (v. 8.4.3.686, La Jolla, A, USA).

5.3 RESULTS AND DISCUSSION

5.3.1 Use of deuterium labeled jasmonates in feeding experiments

In order to monitor the incorporation in cells and in parallel assessing the levels of endogenous JA-Ile and other JAs in cells and growth medium, the choice to use deuterium labeled JAs such as *d*5-dnOPDA and *d*5-JA for all experiments was based on their ease and suitability to be correctly identified by means of UHPLC-MS/MS along with their isotopologues.

5.3.2 Effects of induction by *d*5-dnOPDA

As a first experiment cell cultures were fed with *d*5-dnOPDA. When analyzing cell contents by targeted MRM detection, already 5 min after feeding cells with *d*5-dnOPDA, levels of *d*5-JA were 1.5-fold higher than those of *d*5-dnOPDA in the cells (Fig. 2A). After 30 min, intracellular levels of *d*5-JA were only half of that at 5 min, whereas the level of *d*5-JA-Ile showed a small increase (Fig. 2A). Levels of *d*5-dnOPDA were about similar at 5 and 30 min, but at 1440 min, it was completely gone (Fig. 2A). Another interesting observation was that in the growth medium, no *d*5-JA-Ile was found although *d*5-JA was present with a 1.5-fold increase at 30 min in comparison to 5 min whereas the opposite was observed for *d*5-dnOPDA with a 1.7-fold decrease at 30 min. Neither *d*5-JA or *d*5-JA-Ile were observed in the medium at 1440 min (Fig. 2B). No unlabeled JAs were observed in the cells or in the growth medium.

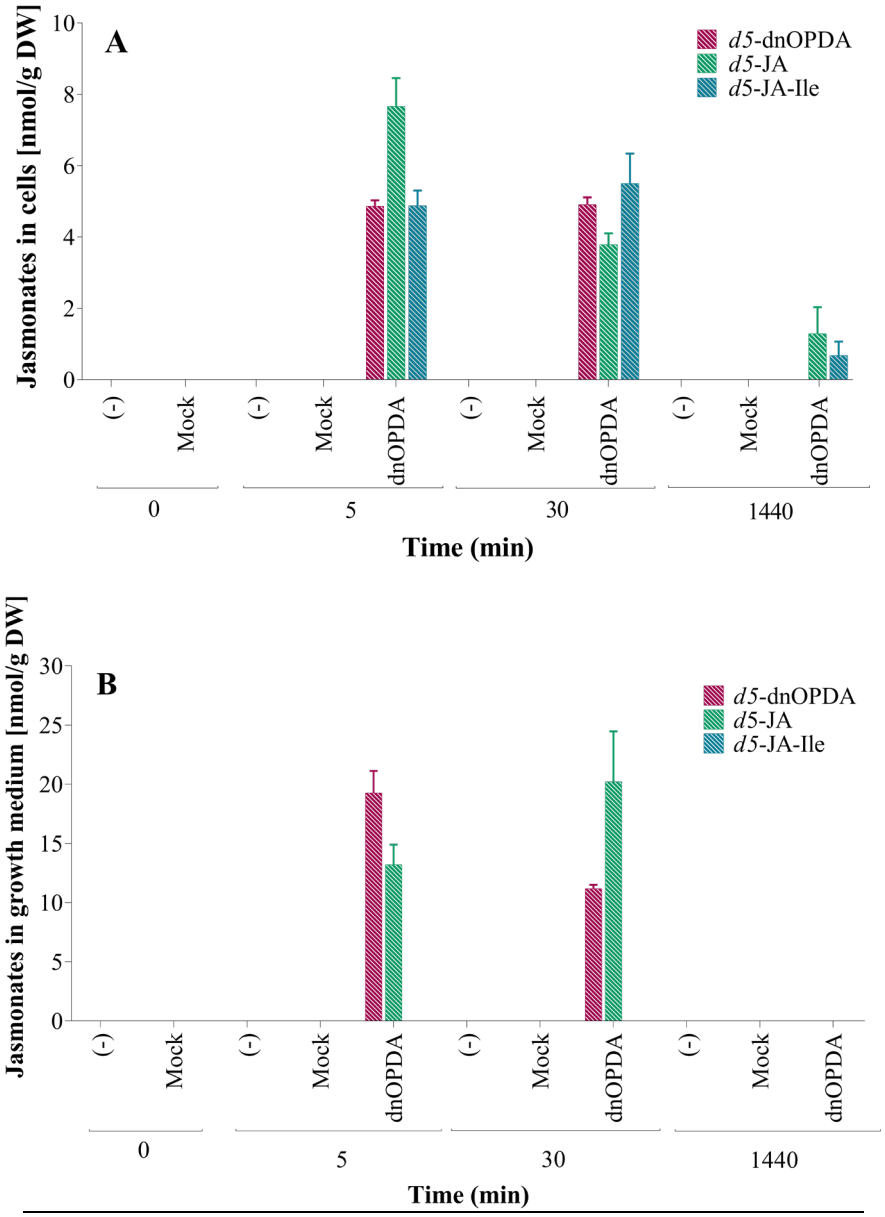

Figure 2. UHPLC-TOF-MS/MS analysis of JAs in lyophilized growth medium and lyophilized cells at different time points (5, 30 min and 24 h) after feeding cell suspension cultures of *Catharanthus roseus* with *d*5-dnOPDA (50.64 nmol/flask). Time is on the *x*-axis and absolute amounts in [nmol/g DW] on the *y*-axis. **A**. Levels of labeled JA, labeled JA-Ile and labeled dnOPDA in cells. **B**. Levels of labeled JA and labeled dnOPDA in growth medium. No unlabeled oxylipins were observed. Values are the mean ± standard error of mean (SEM) of two biological replicates analyzed twice. Significant results are marked with an asterisk (nested one-way ANOVA with Tukey's post hoc test, $p < 0.05$: 5 min: $F_{(2,3)} = 7.854$, p=0.0642; 30 min: $F_{(2,3)} = 1.157$, p=0.4242). No significant differences *d*5-JAs at 5 min (t=0.8431, df=2, p=0.4879) and at 30 min (t=1.795, df=2, p=0.2145) were found (two-tailed nested *t*-test, $p < 0.05$). 1440 min in cells and growth medium was not tested because there was not enough data for statistical analysis.

The experiment of feeding cell suspension cultures of *C. roseus* with *d*5-dnOPDA, delivered a direct proof of the uptake of *d*5-dnOPDA by the cells and its incorporation into *d*5-JA and *d*5-JA-Ile as shown by their induction after 5 and 30 min upon feeding. In addition, the *d*5-JA found in the medium may suggest a signaling function. The relative short timeframe of events indicates that, in our cell suspension system, the machinery to convert dnOPDA into JA is constitutively present and active. Whether this dnOPDA conversion and signaling process, as observed here in the cell culture model, also functions in intact plants requires further studies.

5.3.3 Effects of induction by *d*5-JA, artifactual detection of unlabeled JA

In order to confirm the production of *d*5-JA-Ile, cells were fed with *d*5-JA in an exploratory feeding experiment. A MS/MS MRM signal corresponding to unlabeled JA was observed in cells and growth medium. However, neither the presence of unlabeled JA-Ile nor other unlabeled JAs was observed. These results prompted us to further investigate if unlabeled JA could be induced and if exogenously added *d*5-JA or *d*5-dnOPDA could result in the production of unlabeled JA and JA-Ile. In case of a self-induction of the JA pathway, a fast rise of unlabeled JAs levels is expected to be visible after elicitation with *d*5-JA. In a preliminary qualitative feeding experiment using a targeted method with a MRM experiment in a microTOF-QII spectrometer (unpublished results), we observed the rapid burst of a compound with a mass corresponding to the MRM transition *m/z* 209.1→109.0. The retention time was the same as the JA analytical standard. This metabolite was already present at t=0 after feeding cell suspension cultures of *C. roseus* with *d*5-JA. After 24 h, it decreased to finally reach negligible levels after 48 h. In order to confirm our observations, the feeding experiment was repeated using a low and high dose of *d*5-JA. Cells were analyzed using an untargeted method with a Q-Exactive Focus mass spectrometer in order to screen dnOPDA, OPDA, JA and JAs. To our surprise, JA was not detected, whereas *d5*-JA was found in all experimental samples in both high and low dose *d*5-JA experiments. A qualitative analysis with the Q-Exactive Focus unequivocally confirmed the presence of all deuterium-labeled JA isotopologues *d*1-JA, *d*2-JA, *d*3-JA and *d*4-JA in various proportions, on the basis of their MS^1 and MS^2 spectra over the time course of the feeding experiments (Fig. 3). This indicated a hydrogen/deuterium (H/D) back exchange in the deuterated standard under our experimental conditions. To ensure the possibility that JA was present but undetectable due to a lower sensitivity of the untargeted approach, we performed a MS/MS analysis using a QTRAP 4000 quadrupole-linear ion trap mass spectrometer. The MRM method on the same samples profiled in HRMS displayed again a signal present at t=0 (Fig. 4) corresponding to the MRM transition of JA (*m/z* 209.1→59.0). These results matched our very first experiment but contradicted the observations from the untargeted approach where no JA was observed. The “JA-like” signal followed the same fate as the *d5*-JA signal. An increase was recorded at 5 and 30 min and a drop to a third occurred at 24 h when compared to t=0 (Fig. 4). The presence or absence of endogenous JA over time was unambiguously determined with a

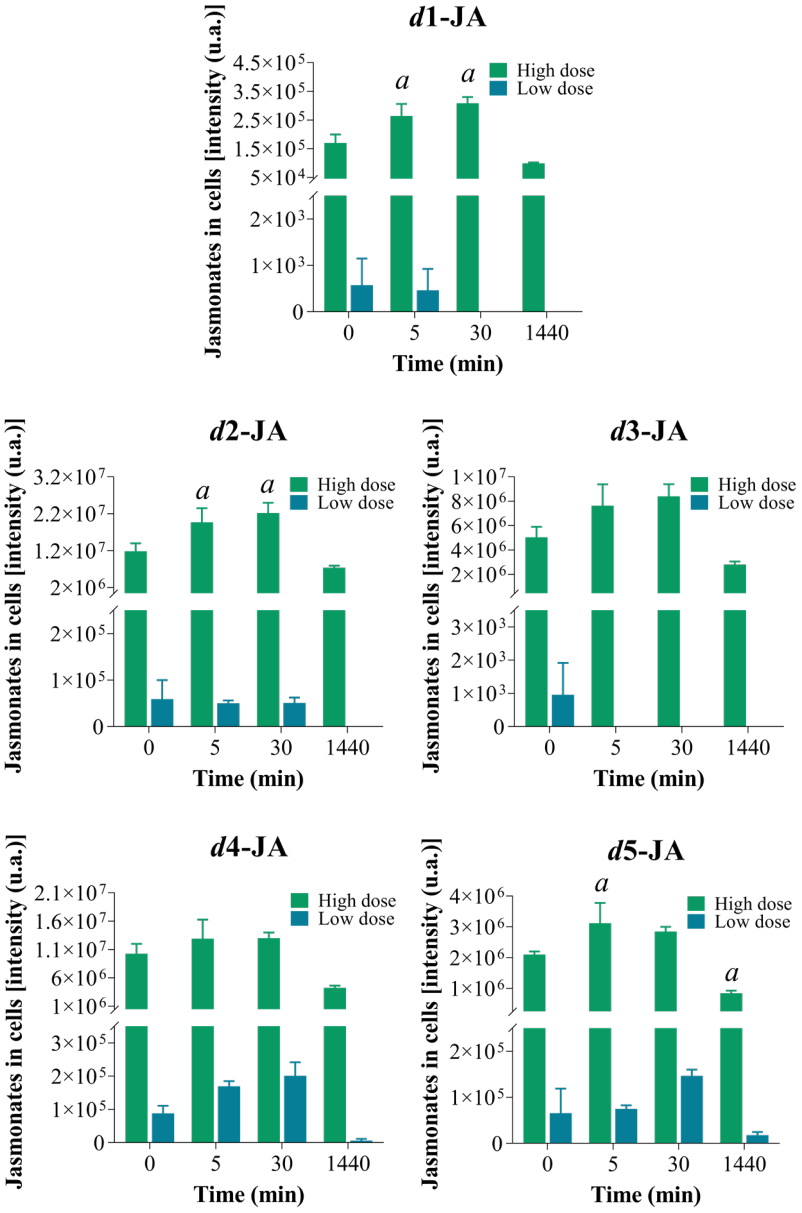

Figure 3. UHPLC-HRMS analysis of all deuterium-labeled JA isotopologues after feeding cell suspensions of *Catharanthus roseus* with *d*5-JA (high and low dose). Time is on the *x*-axis and intensities weighed by extracted mass is on the *y*-axis. Values are the mean ± standard error of mean (SEM) of two biological replicates analyzed once. Significant results from t=0 min are marked with superscripts for high dose (*a*) and low dose (*b*) (one-way ANOVA with Dunnett's post hoc test, $p < 0.05$: *d*1-JA HD: $F_{(3,4)} = 36.21$, $p=0.0023$; *d*2-JA HD: $F_{(3,4)} = 18.92$, $p=0.0079$; *d*2-JA LD: $F_{(2,3)} = 0.09256$, $p=0.9141$; *d*3-JA HD: $F_{(3,4)} = 14.01$, $p=0.0137$; *d*4-JA HD: $F_{(3,4)} = 10.14$, $p=0.0243$; *d*4-JA LD: $F_{(3,4)} = 6.659$, $p=0.0492$; *d*5-JA HD: $F_{(3,4)} = 42.29$, $p=0.0017$; *d*5-JA LD: $F_{(3,4)} = 2.977$, $p=0.1597$). *d*1-JA LD and *d*3-JA LD were not tested due to insufficient data for ANOVA.

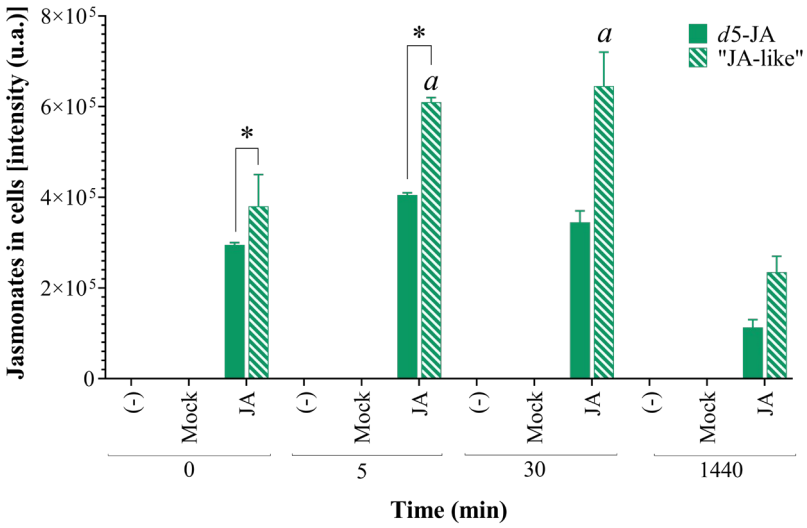

Figure 4. UHPLC-HRMS analysis of *d*5-JA and the "JA-like" signal after induction in cell suspensions of *Catharanthus roseus* with a high dose of *d*5-JA (7.18 µmol/flask). Time is on the *x*-axis and intensities corrected for the extracted biomass on the *y*-axis. Values are the mean ± standard error of mean (SEM) of two biological replicates analyzed twice. Significant differences between *d*5-JA and "JA-like" for each observation point are marked with an asterisk (two-tailed *t*-test with Welch's correction, $p < 0.05$: 0 min: t=1.211, df=1.010, p=0.4378; 5 min t=18.34, df=1.471, p=0.0103; 30 min t=3.795, df=1.220, p=0.1281; 1440 min t=3.135, df=1.447, p=0.1312). Significant differences among *d*5-JA and "JA-like" from *d*5-JA at 0 min are marked with a superscript (*a*) (one-way ANOVA with Dunnett's post hoc test, $p < 0.05$: $F_{(7,8)} = 20.02$, p=0.0002).

second Q-trap mass spectrometer. A final MRM experiment was designed with Q1 and Q3 set at high resolution mode (all other MRM transitions were previously recorded at nominal mass resolution). Even if this setting led to a loss of sensitivity, selectivity increased thus allowing the confirmation that no "JA-like" signal was observed this time. The observations using the untargeted approach were confirmed (supplement 5.7.3). The nature of this artefact signal is most likely due to the presence of *d*1-JA (exchanged from *d*5-JA) as evidenced by the HRMS analysis. In particular, the detection at nominal mass resolution led to a lack of selectivity between JA and *d*1-JA (1 Da). The artefact signal was clearly due to a MRM channel interference since a 3 Da standard window is routinely used with this mode. Additionally, no other unlabeled JAs were observed in the cells. Subsequently, detection set up must be carefully chosen when monitoring deuterated compounds in biological systems. In conclusion, there is no evidence supporting the hypothesis that JA biosynthesis is induced in cells after feeding them with *d*5-JA, the so-called positive feedback loop mechanism, which might operate at the level of gene regulation (Sasaki *et al.,* 2001; Chung *et al.,* 2008; Wasternack and Feussner, 2018) although with the current approach, it does not support a similar mechanism at the (bio)chemical level.

Since it is known *in planta* that dnOPDA and OPDA can be found in bound forms as galactolipid (Kourtchenko *et al.,* 2007; Hisamatsu *et al.,* 2003), MS signals for all known bound forms

in both targeted and untargeted MS experiments were specifically monitored. Furthermore, UHPLC-HRMS analysis of untreated and *d*5-JA-treated cells did not show the presence of galactolipids, dnOPDA and/or OPDA in their bound forms. Their presence was also investigated by means of TLC, GC-MS, GC-FID and ^{1}H-NMR. 2D ^{1}H-NMR data of arabidopsides A and B published by Hisamatsu et al. 2003 were used, choosing the two characteristic signals of the double bond of the cyclopentenone ring to identify (dn)OPDA-containing galactolipids in *C. roseus*, *i.e.* proton positions 8 (dnOPDA) or 10 (OPDA) (δ=7.960 *dd*, *J*=5,9, 1.9 Hz or δ=7.968 *dd*, *J*=5,9, 1.9 Hz), 9 (dnOPDA) or 11 (OPDA) (δ=6.209 *dd*, *J*=5,9, 1.9 Hz or δ=6.210 *dd*, *J*=5,9, 1.9 Hz). None of these signals of arabidopsides were found in the ^{1}H-NMR spectra. In addition, specific GC-FID with derivatization and TLC profiling analyses were performed for their detection (see below and supplements 5.7.1 and 5.7.2), but no traces of arabidopsides were detected neither in treated nor untreated cells.

5.3.4 Stability of *d*5-JA

It is known that a H/D exchange may occur for deuterium atoms located in the alpha position of a keto or a carboxyl functional group (supplement 5.7.4). Thus, to test the stability of labeled JA, three solutions of *d5*-JA (500 ng/mL) were prepared, the first in 40% ethanol (v/v) kept at 4 ºC, the second in ammonium acetate (*d5*-JA at pH 6,500 ng/mL) kept at room temperature and the third one (*d*5-JA, 1.5 mg/mL) prepared in 40% ethanol and kept at room temperature. The first two solutions were analyzed at t=0 h and t=60 h after their preparation. The MS spectra (supplement 5.7.5 and 5.7.6) of the ethanol solution of *d5*-JA kept at 4 ºC showed less than 2-3% of H/D exchange from *m/z* 214.14 into *m/z* 213.15. The aqueous pH 6 solution after 60 h at room temperature showed that the H/D exchange to *m/z* 213.15 and *m/z* 212.15 is about double of that of the 40% ethanol solution kept at 4 ºC. The third solution showed less than 10% of H/D exchange and remained constant over 48 h of analysis. It must be noted that the exchange of these deuterium atoms did not produce unlabeled JA as only four of the five deuterium atoms present are exchangeable. However, when using the MRM mode in a triple quadrupole system with moderate resolution the *d1*-JA form was found to interfere with the transition dedicated to JA. All labeled JA molecules were monitored in both *d*5-JA experiments (low and high dose) to verify their presence in cells fed with *d*5-JA over time.

5.4 CONCLUSION

The hypothesis that JA can induce its own biosynthesis by a feedback loop is based on the observation that in wound-induced experiments in *A. thaliana* the JA biosynthetic precursors dnOPDA and/or OPDA are released from lipid complexes (*e.g.* arabidopside) in the thylakoid membrane (Kourtchenko *et al.,* 2007) and endogenous levels of JA and JA-Ile rapidly increased after wounding leaves of *Arabidopsis* (Browse, 2009a; 2009b). Nevertheless, this could not be confirmed neither in *Solanum lycopersicum, Nicotiana attenuata* nor *P. lunatus* (Pluskota *et al.,* 2007; Miersch and Wasternack, 2000;

Koch *et al.,* 1999). Further experiments with unwounded leaves of *Arabidopsis* sprayed with coronalon, a JA-Ile mimic, alone or in combination with C18:3, showed that endogenous levels of both JAs rapidly accumulated only when treatments were combined with wounding (Scholz *et al.,* 2015). This demonstrated that i) trauma is enough for the fast accumulation of endogenous JAs; ii) their accumulation precedes the expression of the JA biosynthetic genes *LOX2* and *AOS* and iii) exogenously applied coronalon and C18:3 are not taken up by wounded leaves. Additional evidence comes from the rapid burst of JA (within 30 sec) recorded in locally wounded leaves of *Arabidopsis* where this burst is delayed by 1 min in distally unwounded leaves (Glauser *et al.,* 2009). This raises the question whether this burst of JA accumulation results from a liberation of JAs from a yet unknown pool of bound JA or from the conversion of a direct precursor. The presence of direct precursors like C18:3 or OPDA and/or dnOPDA as esters in the *sn-1* and *sn-2* position of galactolipids in the thylakoid, such as mono- or digalactosyldiacylglycerol (MGDG and DGDG like the arabidopsides A-F) (Kourtchenko *et al.,* 2007; Hisamatsu *et al.,* 2005; Andersson *et al.,* 2006; Glauser *et al.,* 2008; Ibrahim *et al.,* 2011), found only in a few plant species so far (Nakajyo *et al.,* 2006; Böttcher and Weiler, 2007; Nilsson *et al.,* 2015; Ohashi *et al.,* 2005; Hartley *et al.,* 2015; Pedras and To, 2017). Apparently, the entire enzymatic machinery involved in the rapid hydrolysis of the arabidopsides and the subsequent formation of OPDA, dnOPDA and JA seems to be constitutively present in *Arabidopsis* leaves (Kourtchenko *et al.,* 2007). Furthermore, Chini et al. (2018) reported a new biosynthetic pathway in *Arabidopsis* where in the absence of OPR3, OPDA can enter β-oxidation to produce dnOPDA, tetranor-OPDA (tnOPDA) and finally 4,5-didehydro-JA, where after leaving the peroxisome, is reduced to JA by the cytoplasmic ORP2. Therefore, 4,5-didehydro-JA could act as a direct precursor of JA in *Arabidopsis.*

By feeding cell suspension cultures of *C. roseus* with *d*5-dnOPDA and *d*5-JA, we investigated both the uptake and biosynthetic fate of these metabolites, and the potential inducing effects on endogenous JA biosynthesis delivering unlabeled JAs. Our observations clearly showed that feeding cell suspension cultures of *C. roseus* with *d*5-dnOPDA, resulted in the immediate accumulation of *d*5-JA and *d*5-JA-Ile already after 5 min in cells, including the accumulation of *d*5-JA in the growth medium, clearly indicating its excretion. The enzymes responsible for the conversion of *d*5-dnOPDA seem to be constitutively present and active. From the dnOPDA feeding experiment it is clear that free dnOPDA is taken up in the cells and immediately converted into JAs. The fast uptake of exogenous dnOPDA and JA into the cells in the suspension cultures plus the excretion of the newly formed (labeled) JA, might mean that any release of dnOPDA or JA from a cell can induce the stress response in other cells, like in cell suspension cultures of *C. roseus* where TIA accumulation has been proven to be induced by JA and MeJA (Collu *et al.,* 2001; Miettinen *et al.,* 2014; Simkin *et al.,* 2013). We propose the hypothesis that dnOPDA might act as a signal compound in our model system where upon feeding with *d*5-dnOPDA, it forms JA and JA-Ile (Fig. 5). The presence of bound (dn)OPDA as a potential source of (dn)OPDA precursors for JA biosynthesis, comparable to the arabidopside complexes, could

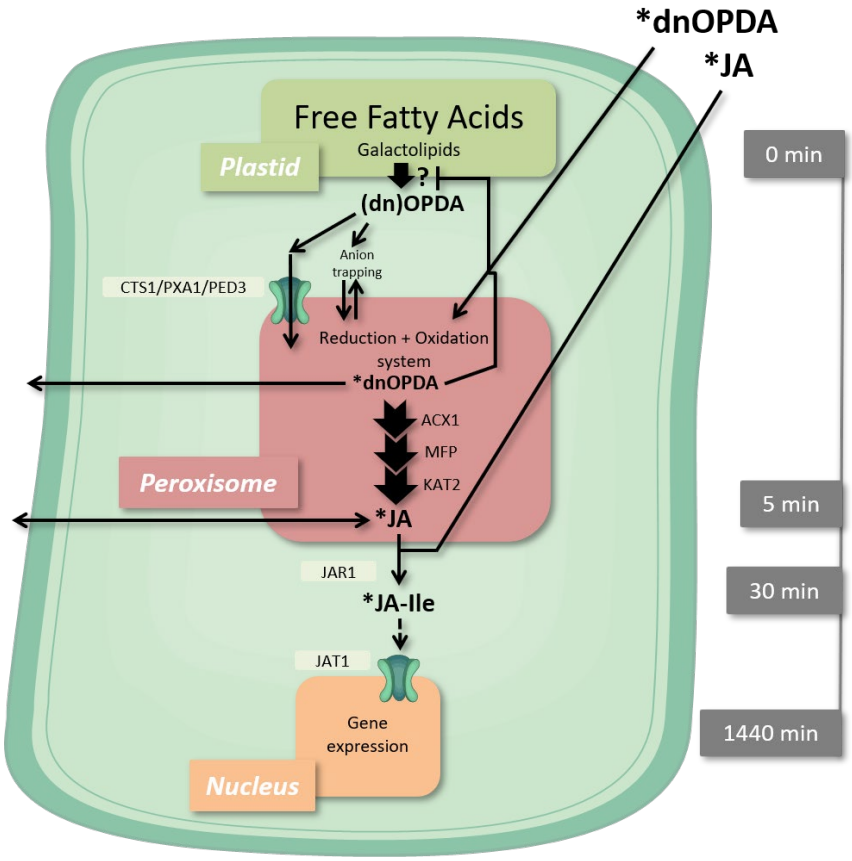

Figure 5. A proposed model for the response in cell suspension cultures of *Catharanthus roseus* after feeding with *d*5-dnOPDA and *d*5-JA. Events in cell compartments such as signaling (dashed arrows), bioconversion (bold arrows) and transport (diffusion and selective transport, black arrows) are depicted. Feeding cells with *d*5-JA (*JA) does not induce the accumulation in cells of *unlabeled* JA or *unlabeled* JA-Ile. Feeding cells with *d*5-dnOPDA (*dnOPDA) does not induce a putative receptor in the plastids leading to the hydrolysis of free fatty acid(s) or their CoA esters from possibly galactolipid-like complexes in the plastids. It is known that dnOPDA is immediately transported by the ABC transporter *COMATOSE1/PEROXISOMAL1/PEROXISOME* (*CTS1/PXA1/PED3*) and in a lesser extent by physical transport by an ion trapping system into the peroxisome where an already present reduction and oxidation system is in place (Zolman *et al.,* 2001). Moreover, feeding cells with *d*5-dnOPDA leads to the accumulation of *d*5-JA in cells, probably from where it is excreted into the growth medium. JA-Ile is imported into the nucleus and JA exported outside the cell by *JA/JA-Ile TRANSPORTER 1* (*JAT1*) (Li *et al.,* 2017) to activate transcription factor (TF) cascades and pathways involved in defense responses in *C. roseus* (Memelink *et al.,* 2001). The three bioconversion arrows after dnOPDA in the peroxisome mean oxidation into OPC6:0, OPC4:0 and JA-CoA catalyzed by an acyl CoA oxidase (ACX1), a multifunctional protein (MFP) and a 3-ketoacyl-CoA thiolase (KAT2) before yielding JA. The cytosolic JASMONATE RESISTANT1 (JAR1) converts JA into JA-Ile.

not be confirmed until now with the current experimental setup in *C. roseus* cells. The most important observation after feeding cells with *d*5-JA, is that it did not induce the accumulation of endogenous unlabeled JA or any other unlabeled JAs in *C. roseus* cells, thus demonstrating that JA was not produced

endogenously and that there is no feedback loop of exogenously added JA in cell suspension cultures of *C. roseus.*

The importance of using more than one single method of analysis is clearly demonstrated by the presence of JA-like transitions *m/z* 209.1→109.0 and *m/z* 209.1→59.0 eluting at the same retention time as the analytical standard of JA, detected by a targeted approach using two independent analytical platforms. Future studies using *d*5-JA should always use analytical methods that prevent the generation of this false JA positive. Moreover, we discourage the use of organic solvents like ethanol for the preparation of deuterium-labeled phytohormone solutions. The choice of more stable deuterium-labeled JA and/or other phytohormones should be carefully studied and it is highly recommended to use carbon-marked phytohormones instead.

5.5 ACKNOWLEDGEMENTS

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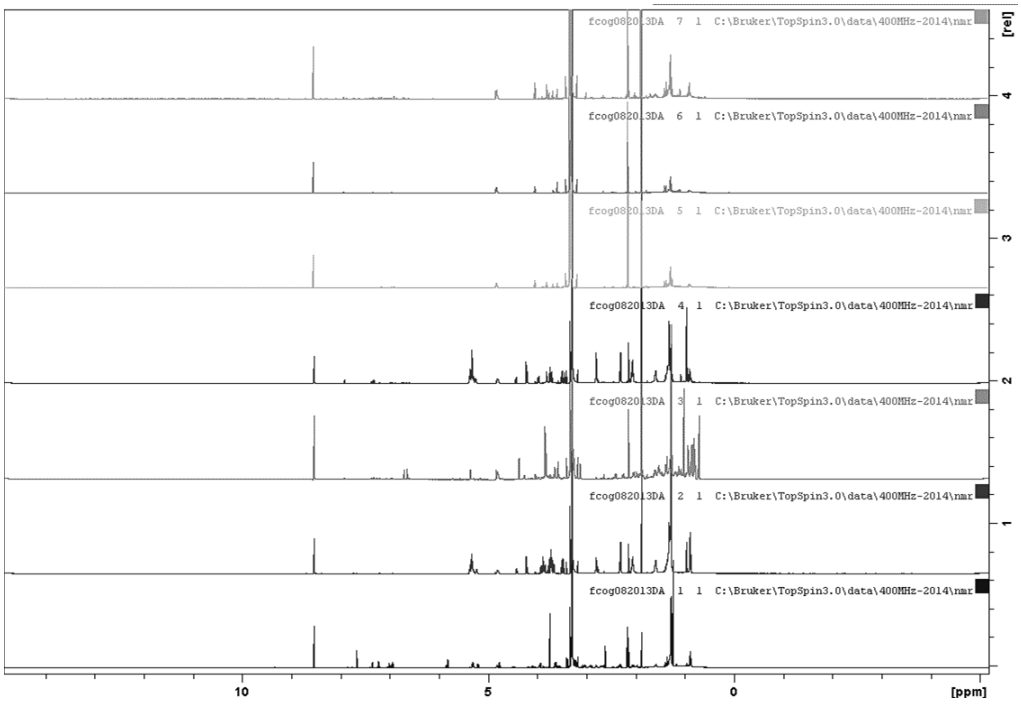
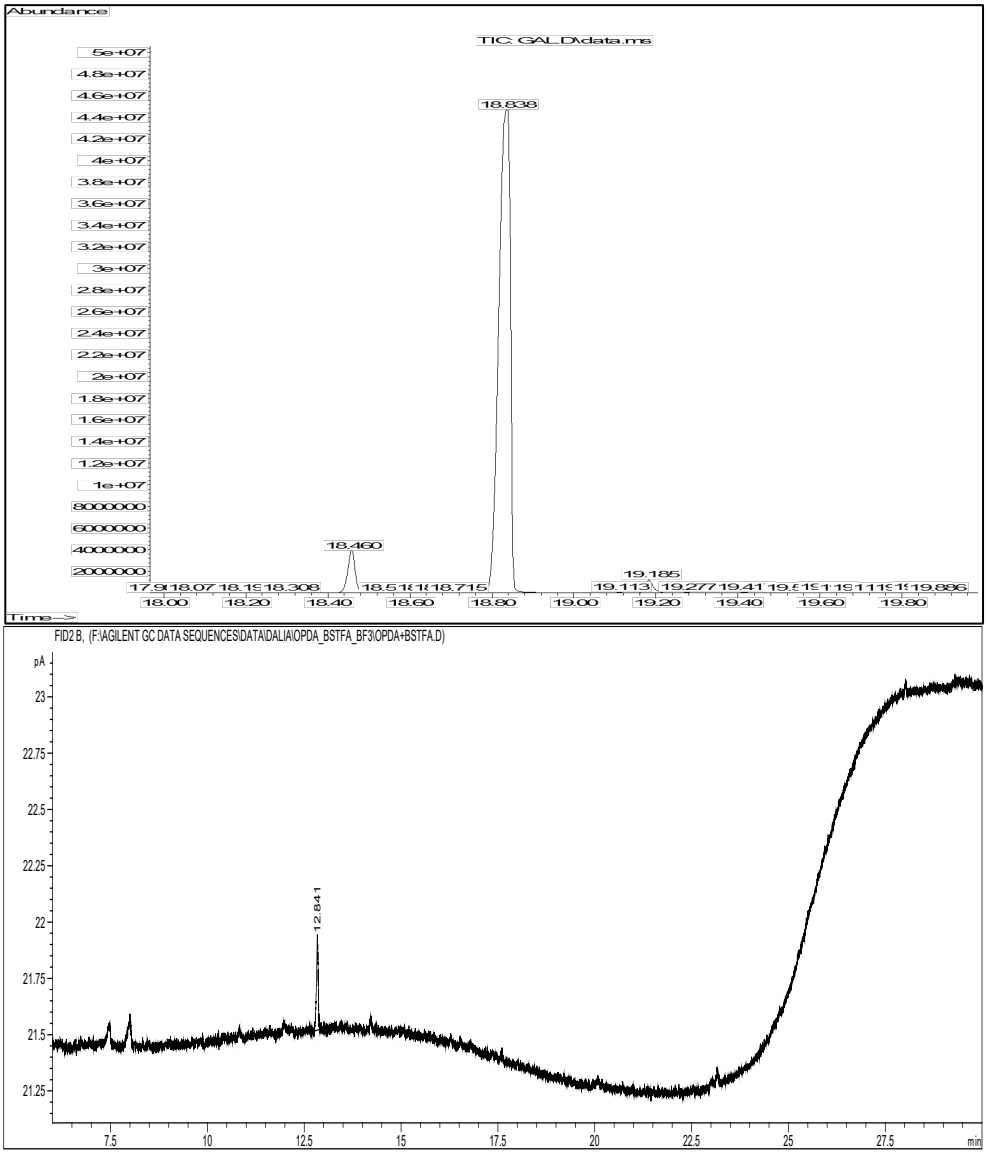
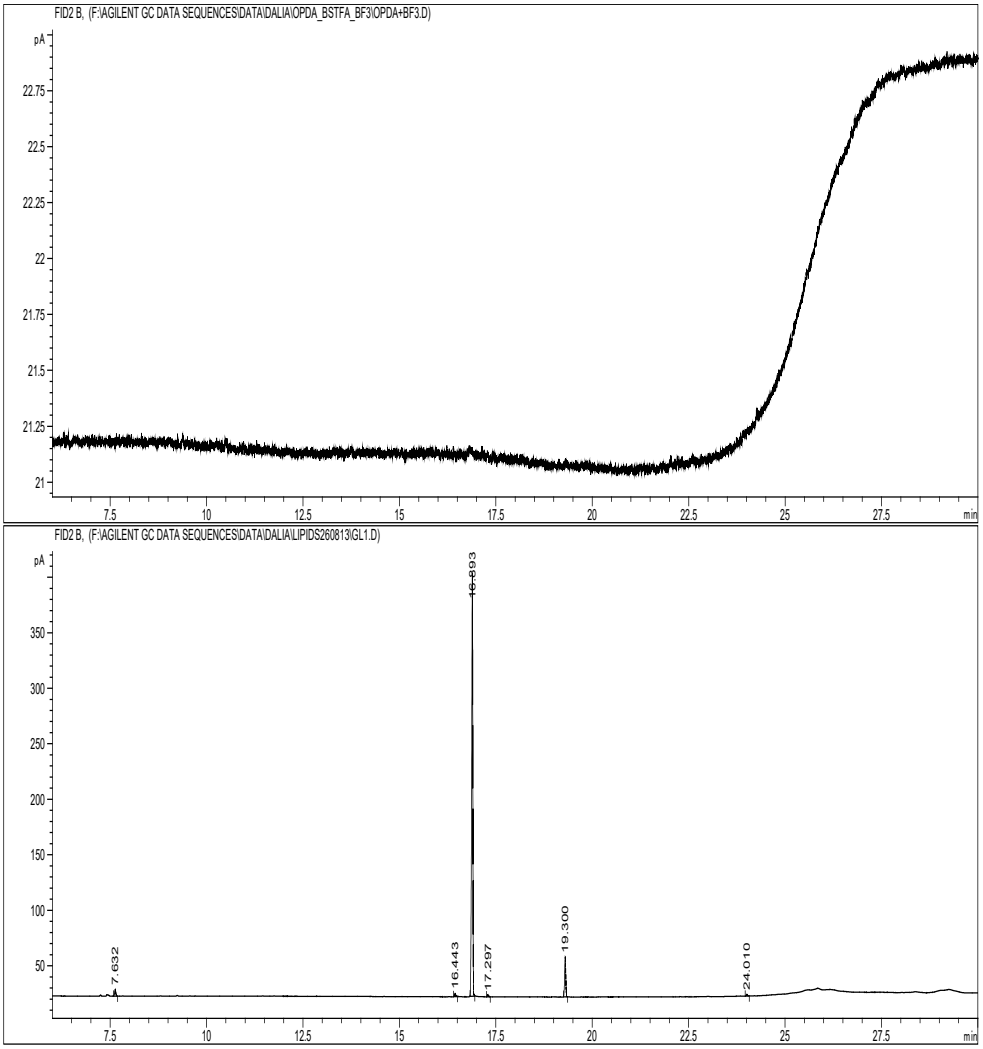
5.7 SUPPLEMENTAL INFORMATION

Supplement 5.7.1. ^{1}H-NMR spectra for the screening of the possible presence of galactolipids (top 4) and neutral lipids in selective extracts (bottom 3) of cell suspension cultures of *Catharanthus roseus.* Lyophilized materials were extracted twice with of isopropanol and 0.025% BHT warmed up at 75 ºC. Phase separation was induced with several steps that included chloroform and water 2:1 (v/v) with 0.025% BHT. Sample enrichment was achieved by a solid phase extraction step (SPE) where galactolipids were eluted with a mixture of acetone-methanol 9:1 (v/v). Each fraction was applied to a silica thin layer chromatography (TLC) plate and bands were visualized with anisaldehyde. All bands were scrapped off the plate, washed with methanol-acetone 1:1 (v/v) and analyzed by ^{1}H-NMR.



Supplement 5.7.2. GC-FID and GC-MS profiles for the screening of OPDA in galactolipids and neutral lipids fraction in JA-treated and untreated cell suspension cultures of *Catharanthus roseus.* A solution of 500 ng of galactose in 100 µL of pyridine derivatized with BSTFA+TMCS (Rt =18.83 min; first panel). OPDA in 100 µL of pyridine derivatized with BSTFA+TMCS (Rt = 12.841 min; second panel). A solution of 500 ng of OPDA in 100 µL of pyridine derivatized with BF_3 (No peak; third panel) An extract of neutral lipids (fourth panel) and galactolipids (fifth and sixth panels) proving the absence of OPDA in these extracts.

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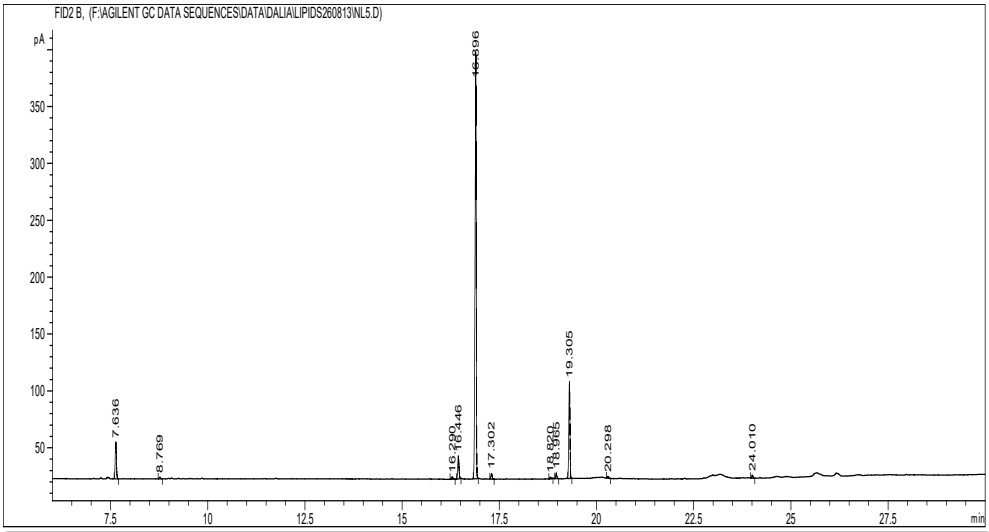
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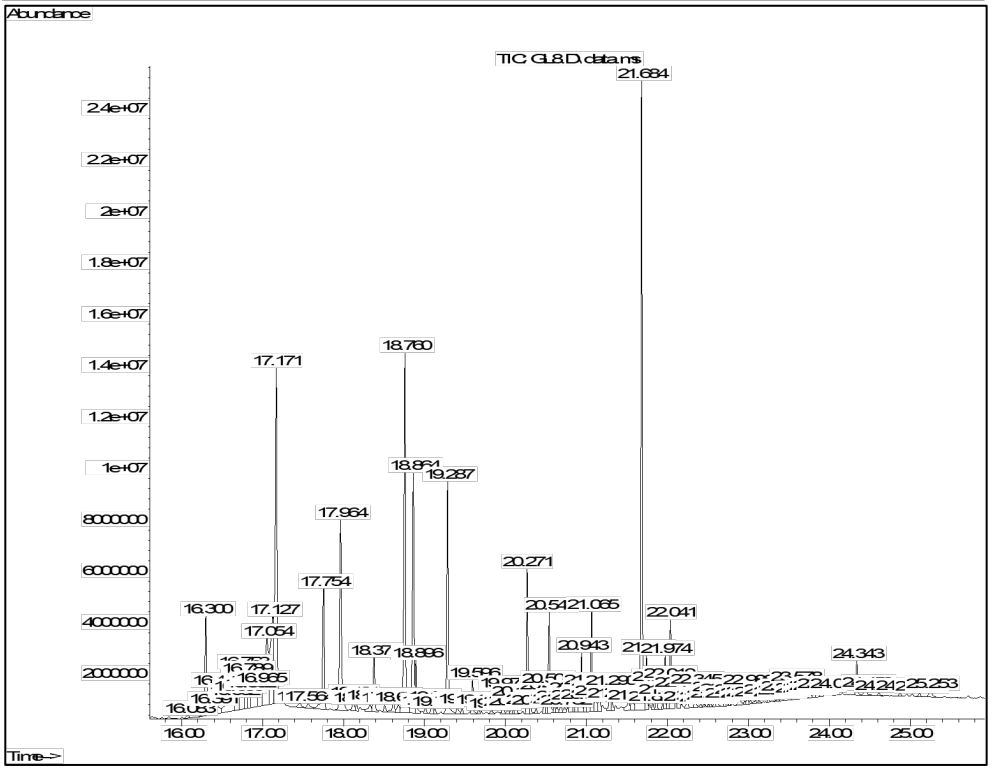
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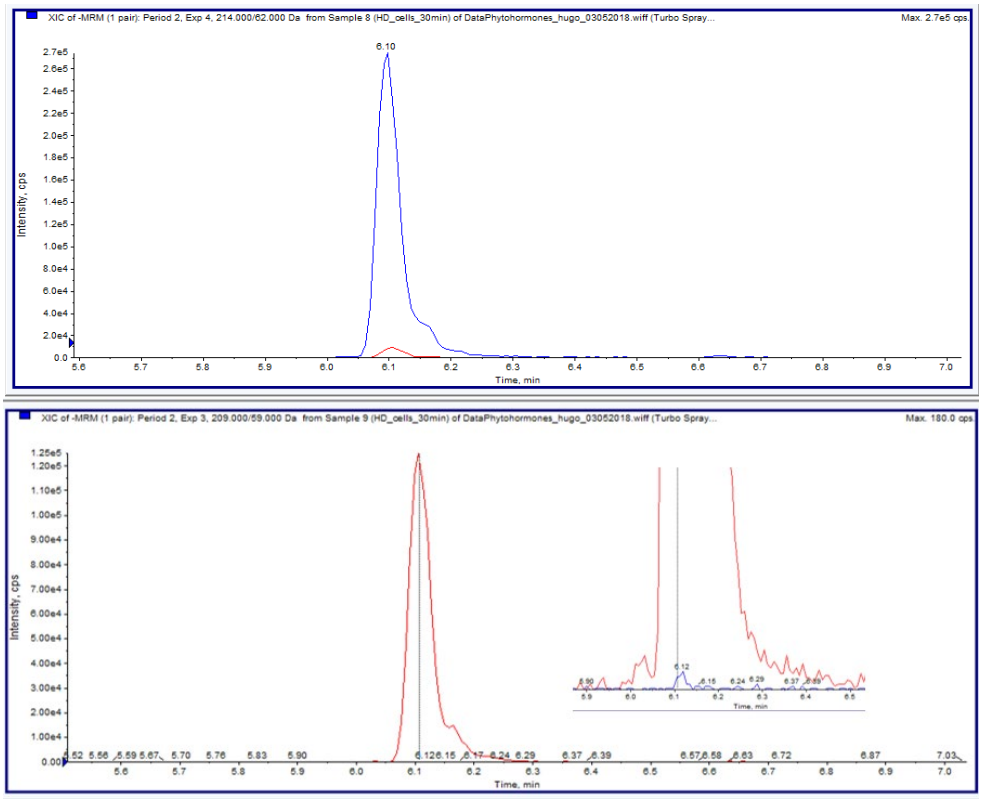
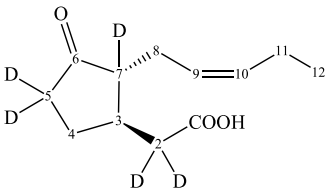
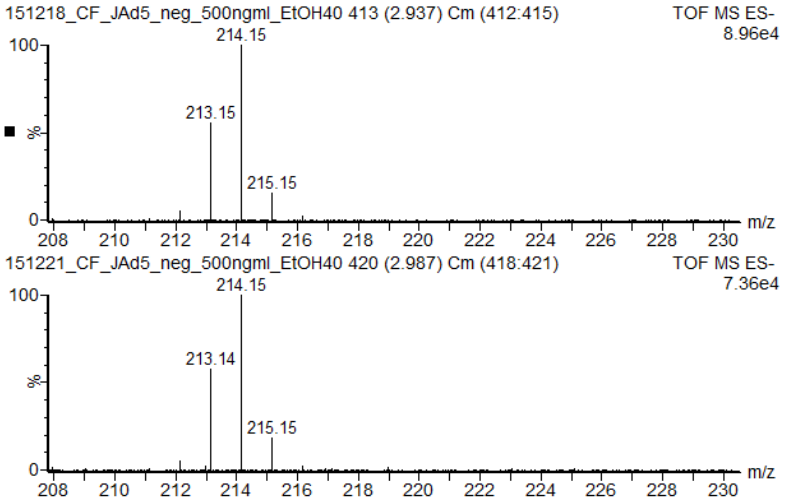
Supplement 5.7.3. Chromatogram of *d*5-JA and JA of cells fed with a high dose of *d*5-JA (7.18 µmol/flask) after 30 min showing the overlapping signals of JA in blue and *d*5-JA in red acquired with a low Q resolution (top panel) and high Q resolution (bottom panel) analyzed with an targeted MS method using a QTRAP 4000 quadrupole-linear ion trap mass spectrometer. An increase in the Q resolution allowed the unambiguous identification of the JA-like compound whose HRMS is *m/z* 210.1246 that corresponds to that of *d*1-JA.



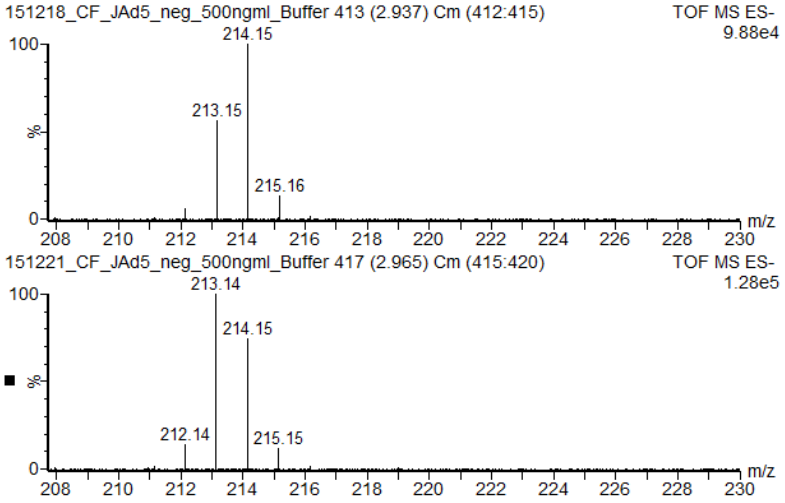
Supplement 5.7.4. Chemical structure of *d*5-JA ((2-((1R,2R)-3-*oxo*-2-((Z)-pent-2-en-1-yl)cyclopentyl-2,4,4-*d*3) acetic-2,2-*d*2 acid) showing the five deuterium atom positions.



Supplement 5.7.5. Mass spectra of *d5*-JA prepared in 40% ethanol (v/v) (500 ng/mL) kept at 4 ºC and analyzed at t=0 h (top) and at t=60 h (bottom) in a targeted approach using a UHPLC-TOF-MS spectrometer. The hydrogen-deuterium exchange (H/D) from *m/z* 214.15 to *m/z* 213.15 is less than 3% thus experimentally proving that *d*5-JA is stable in solution and at low temperature.



Supplement 5.7.6. MS spectra of *d5*-JA prepared in ammonium acetate buffer (pH 6; 500 ng/mL) kept at room temperature and analyzed at t=0 h (top) and at t=60 h (bottom) in a targeted approach using a UHPLC-TOF-MS spectrometer. Ion intensities of *m/z* 213.15 doubled over a period of 60 h followed by *m/z* 212.14 showing that H/D exchange occurred in a solution adjusted to pH 6.



Chapter 6

Metabolism of exogenous jasmonic acid in cell suspension cultures of *Catharanthus roseus*

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ABSTRACT

After jasmonic acid (JA) elicitation of cell suspension cultures of *Catharanthus roseus* (L.) G. Don, derivatives such as jasmonoyl-L-isoleucine (JA-Ile), 12-hydroxyjasmonic acid (12-HOJA), 12-*O*-glucosyl-jasmonic acid (12-*O*-Glc-JA), 12-carboxyjasmonoyl-L-isoleucine (12-HOOCJA-Ile), 12-hydroxyjasmonoyl-L-isoleucine (12-HOJA-Ile) and 9,10-dihydrojasmonic acid (DHJA) accumulated in cells and growth medium shortly after addition of JA. The major products in the growth medium were JA followed by 12-HOJA, DHJA and 12-HOJA-Ile already present at 0 min. At 1440 min contents of all jasmonates (JAs) in cells reached negligible levels. The profiles over time of JA and 12-HOJA showed an inverse correlation in both cells and growth medium. A similar inverse behavior was observed for JA-Ile in cells and growth medium. The major catabolic events in *C. roseus* cells are hydroxylation, oxidation and reduction of JA, JA-Ile and 12-HOJA.

6.1 INTRODUCTION

Jasmonates (JAs) are lipid-derived messengers that regulate plant defense responses and many developmental processes. After the biosynthesis of jasmonic acid (JA) is finished in the peroxisome, the molecule epimerizes into the more stable *trans* configuration (3*R*, 7*S*)-JA (Wasternack *et al.,* 2006). Subsequently, JA may be metabolized into a number of derivatives through several known conversion pathways that may render either active, inactive or partially active metabolites (Wasternack and Song, 2017; Wasternack and Feussner, 2018) (Fig. 1). These metabolic conversions include: (1) *Hydroxylation* at C11 or C12 to 11-hydroxyjasmonic acid (11-HOJA) and 12-hydroxyjasmonic acid (12-HOJA), catalyzed by JASMONATE-INDUCED OXYGENASE 1-4 (JOX) (Caarls *et al.,* 2017); (2) *Conjugation* at C1 with the amino acids tryptamine, tryptophan, valine, leucine, tyrosine, methionine, alanine, glutamine and isoleucine. Conjugation to the biologically active (3*R,* 7*S)*-jasmonoyl-L-isoleucine (JA- Ile) (Fonseca *et al.,* 2009) is catalyzed by JASMONATE RESISTANT1 (JAR1) (Staswick *et al.,* 1992; Staswick and Tiryaki, 2004). Conjugation of *oxo*-phytodienoic acid (OPDA) to isoleucine (OPDA-Ile) was detected in *Arabidopsis* (Floková *et al.,* 2016); (3) *Reduction* of the C6 keto group yields dihydrojasmonic acid (9,10-DHJA) (Miersch *et al.,* 1989; Meyer *et al.,* 1989) and 6-HOJA; (4) *Methylation* to methyl jasmonate (MeJA) is catalyzed by JASMONIC ACID CARBOXYL METHYL TRANSFERASE (JMT) (Seo *et al.,* 2001); (5) *Glucosylation* to 1-β-glucosyl-JA (1-β-Glu-JA) and 12-*O*-β–D-glucopyranosyl-JA (JAG); (6) *Decarboxylation* at C1 yields the highly volatile *cis*-jasmone (Koch *et al.,* 1997); (7) *Conjugation* to 1-aminocyclopropane-1-carboxylic acid (JA-ACC); (8) *O-Glucosylation* of 12-HOJA to 12-*O*-glucosyl-JA (12-*O*-Glc-JA); (9) *Sulfation* of 11/12-HOJA to 11/12-*O*-sulfonyl-JA (11/12-HSO_4-JA), catalyzed by the SULFOTRANSFERASE 2a (ST2a) (Gidda *et al.,* 2003); (10) *esterification* to lasiojasmonates A-C (lasioJAs) (Andolfi *et al.,* 2014). Further modifications of JA-Ile include: (11) *Hydroxylation* to 12-hydroxyjasmonoyl-L-isoleucine (12-

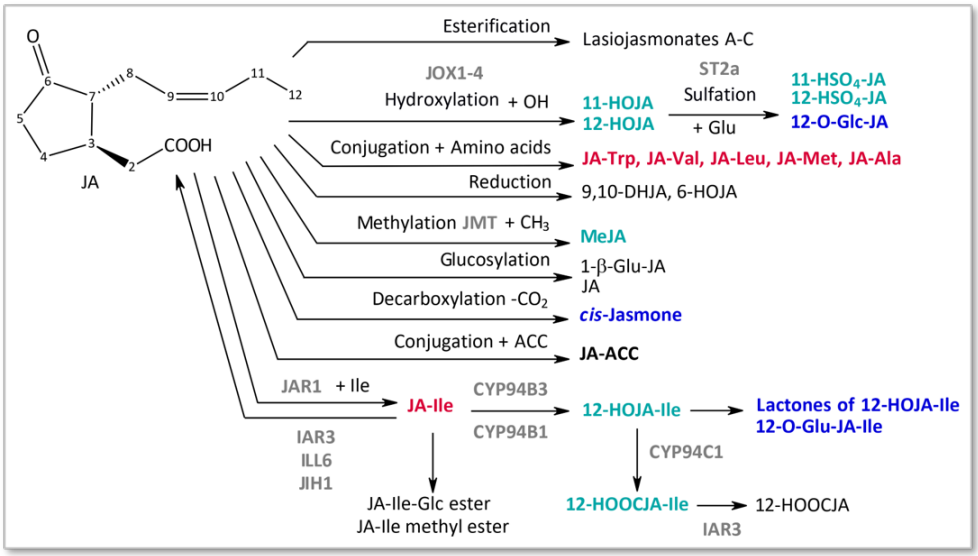

Figure 1. Metabolism of jasmonic acid (adapted from Wasternack and Feussner, 2018 and Wasternack and Strnad, 2015). Enzymes are in gray. Inactive metabolites are in green; partly active metabolites are in blue; active metabolites are in red and metabolites with unknown biological activity are in black. Metabolic conversions shown are: *esterification* to lasiojasmonates A-C; *hydroxylation* to 11-hydroxyjasmonic acid (11-HOJA) and 12-hydroxyjasmonic acid (12-HOJA), catalyzed by JASMONATE-INDUCED OXYGENASES (JOX1-4); *conjugation* to amino acids like isoleucine to JA-Ile is catalyzed by JASMONATE RESISTANT1 (JAR1) and the reverse reaction from JA-Ile to JA by the amidohydrolases IAA-ALA-RESISTENT3 (IAR3) and IAA-LEU RESISTENT-like6 (ILL6); *reduction* to 9,10-dihydrojasmonic acid (DHJA) and 6-HOJA; *methylation* to methyl jasmonate (MeJA), catalyzed by JASMONIC ACID CARBOXYL METHYL TRANSFERASE (JMT); *glucosylation* to 1-β-glucosyl-JA (1-β-Glu-JA) and 12-*O*-β–D-glucopyranosyl-JA acid (JAG); *decarboxylation* to *cis*-jasmone; conjugation to 1-aminocyclopropane-1-carboxylic acid (JA-ACC); *O*-glucosylation of 12-HOJA to 12-*O*-glucosyl-jasmonic acid (12-*O*-glucosyl-JA); *sulfation* of 11/12-HOJA to 11/12-HSO_4-JA is catalyzed by SULFOTRANSFERASE 2a (ST2a). The cytochrome P450 enzymes CYP94B3 and CYP94B1 catalyze the *hydroxylation* of JA-Ile to 12-hydroxyjasmonoyl-L-isoleucine (12-HOJA-Ile). JASMONOYL-L-ISOLEUCINE HYDROLASE 1 (JIH1) *hydrolyzes* JA-Ile to JA. CYP94C1 catalyzes the *oxidation* to 12-HOOCJA-Ile, further *deconjugated* to 12-HOOCJA by IAR3. Lactones of 12-HOJA-Ile are shown as derivatives expected to be formed although not detected yet in plants. JA-Ile can be further converted to JA-Ile methyl ester and JA-Ile-glucosyl ester (JA-Ile-Glc ester).

HOJA-Ile), catalyzed by the cytochrome P450 enzymes CYP94B3 and CYP94B1; (12) *Oxidation* of 12-HOJA-Ile to 12-carboxyjasmonoyl-L-isoleucine (12-HOOCJA-Ile), catalyzed by CYP94C1; (13) *Glucosylation* to JA-Ile-glucosyl ester (JA-Ile-Glc ester); (14) *Methylation* to JA-Ile methyl ester; (15) *Deconjugation* of 12-HOOCJA-Ile and JA-Ile, catalyzed by IAA-ALA-RESISTENT3 (IAR3) and IAA-LEU RESISTENT-like6 (ILL6) (Sánchez-Carranza *et al.,* 2016) and (16) *Hydrolyzation* to JA catalyzed by JASMONOYL-L-ISOLEUCINE HYDROLASE 1 (JIH1) (Woldemariam *et al*., 2012; 2014) (Fig. 2).

Katsir et al. (2008a) defined bioactive JAs as JA derivatives that directly promote CORONATINE INSENSITIVE 1 (COI1) interaction with one or more JASMONATE ZIM DOMAIN

(JAZ) proteins to activate gene expression. Non-bioactive JAs are either precursors or deactivated forms of bioactive JAs like 12-HOJA (Miersch *et al.,* 2008). Katsir et al. (2008b) described the following criteria to determine whether a particular compound is a bioactive signal: (i) the compound is synthesized in plant cells; (ii) the exogenous (added) compound elicits a physiological response in wild type but not in the null mutant insensitive to JAs *coi1* plants; (iii) depletion of the compound in plant tissues by genetic or pharmacological means impairs physiological responses that depend on JA; and (iv) the compound directly promotes COI1-JAZ interaction. According to Katsir et al. (2008b) of all JAs known, only JA-Ile has been shown to fulfill all four criteria. 12-*oxo*-Phytodienoic acid (OPDA), JA and MeJA only fulfill the first three criteria. The strong correlation between JA and JA-Ile suggests a reversible equilibrium of both compounds since high levels of JA are a prerequisite for JA-Ile formation and the reverse reaction from JA-Ile to JA halts the signaling pathway, thus JA availability is a controlling factor (Sánchez-Carranza *et al.,* 2016).

The biological activity of 12-HOJA and its derivatives *i.e.* 12-*O*-β-glucopyranosyl-JA and 12-HSO_4-JA, include the indirect induction of tuber formation in *Solanum tuberosum* and *Helianthus tuberosus* plants (Simko *et al*., 1996; Sato *et al*., 2009; Koda and Okazawa, 1988; Seto *et al*., 2009; Matsuura *et al.,* 1993). Moreover, a specific enantiomer of JAG is active as a leaf-closing factor in *Albizzia* and *Samanea* by binding to specific motor cells responsible for nyctinastic movements mediated by rapid potassium fluxes in a COI1-JAZ independent pathway (Nakamura *et al.,* 2011) where the stereochemistry of the glycone moiety affects its biological activity and target affinity (Ueda *et al.,* 2015). This JA derivative has also been found to play a role in male flower determination in tassels of *Zea mays* (Acosta *et al.,* 2009). 12-HOJA, 12-HOJA-Ile and 12-HOOCJA-Ile were found to accumulate within 2-5 min in wound-induced leaves of *Arabidopsis* (Glauser *et al.,* 2008a) whereas 12-*O*-glucosyl-JA, 12-HOJA and 12-HSO_4-JA accumulated late in wound-induced leaves of *S. lycopersicum*. Moreover 12-HOJA and 12-HSO_4-JA downregulated the expression of wound-inducible genes (Miersch *et al.,* 2008). The naturally occurrence of jasmine ketolactone (JKL), a lactone of 12-HOJA in *Nicotiana attenuata* leaves, suggests the possibility that the inactive 12-HOJA-Ile can be converted into active derivatives. The latter based on the fact that two synthetic macrolactones of 12-HOJA-Ile, the (3*R*, 7*R*) and the (3*S*, 7*S*) diastereomers, were able to induce nicotine accumulation in a COI1-dependent manner (Jiménez-Alemán *et al.,* 2015). Nevertheless, hydroxylation, oxidation and sulfation of JA, JA-Ile and 11/12-HOJA are regarded as the attenuation of JA and JA-Ile signaling pathways, by decreasing their biological activity and causing a partially altered or decreased gene expression of JA-related responses (Miersch *et al.,* 2008). Other metabolic conversions like the methyl esterification of JA-Ile, reduce its ability to promote COI1-JAZ interaction in *Arabidopsis* plants indicating that esterification reversibly inactivates JAs (Fonseca *et al.,* 2009). In the case of MeJA, it is biologically inactive unless it is metabolized to JA and then conjugated to JA-Ile (Tamogami *et al.,* 2008). *S. lycopersicum* plants are able to metabolize airborne MeJA into JA, JA-Ile, JA-Val, 12-HOJA and 12-

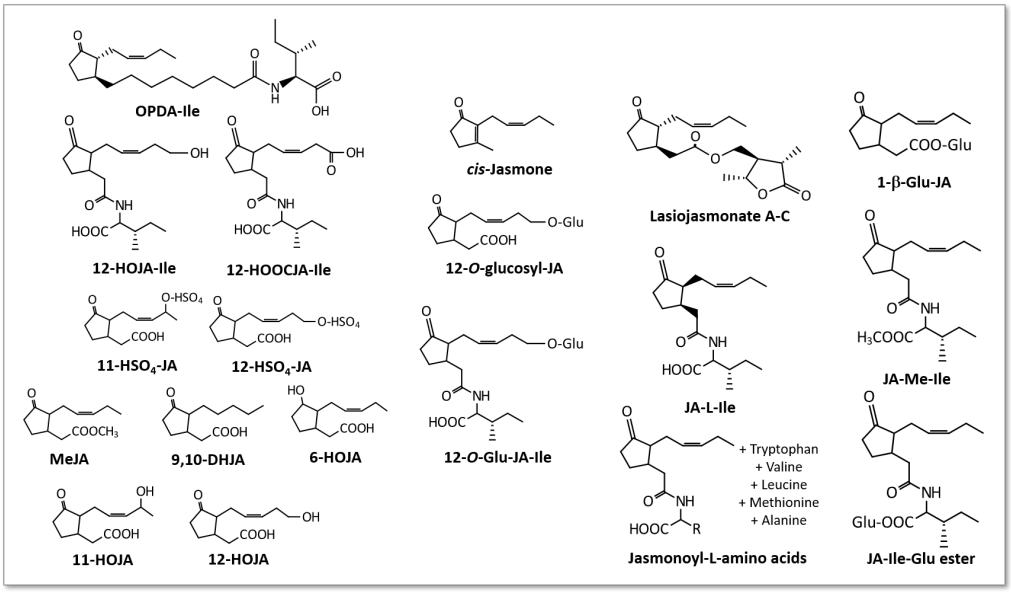

Figure 2. Some metabolic conversions of jasmonic acid, jasmonoyl-L-isoleucine (JA-Ile) and *oxo*-phytodienoic acid (OPDA). These include hydroxylation, conjugation to amino acids, oxidation, decarboxylation, methylation, reduction, sulfation, deconjugation, glucosylation, *O*-glucosylation and esterification. Biologically active jasmonates (JAs) are those conjugated to amino acids like isoleucine like JA-Ile and can be also glucosylated like 12-*O*-glucosyl-JA and 12-*O*-glucosyl-JA-Ile.

O-Glc-JA (Oki *et al.,* 2019). MeJA might regulate JA levels during fruit development in *Fragaria vesca* (Preuß *et al.,* 2014). Furthermore, glucosylation to JA/JA-Ile-Glc esters yields metabolites with unknown biological activity. Other biologically active JAs include jasmonoyl-L-tyrosine (JA-Tyr), jasmonoyl-L-valine (JA-Val), jasmonoyl-L-phenylalanine (JA-Phe), jasmonoyl-L-leucine (JA-Leu), jasmonoyl-L-alanine (JA-Ala), jasmonoyl-L-glutamine (JA-Gln) and jasmonoyl-L-tryptophan (JA-Trp), all involved in wounding and stress responses (Gapper *et al.,* 2002; Suza and Staswick, 2008; Kramell *et al.,* 1995a; 1995b; Staswick and Tiryaki, 2004; Staswick, 2009; Tamogami *et al.,* 1997). Their ability to promote interaction of *S. lycopersicum* COI1 and JAZ1/3 *in vitro* (Katsir *et al.,* 2008b) suggests that these compounds may be active signals in the wound response even though that the accumulation of JA-Ile is at least 10-fold higher. *cis*-Jasmone, one of the main components in plant volatiles, quickly accumulated in different elicited plant cell cultures (Mueller *et al.,* 1993). Although it was formerly believed to be an inactive JAs disposed through the gas phase (Koch *et al.,* 1997), it was later found to be released during insect herbivory by influencing a different signaling pathway from that of JA in *Arabidopsis* plants (Matthes *et al.,* 2010). *cis*-Jasmone has also a repellent activity against aphids (Bruce *et al.,* 2008) and induces the production of volatiles including the monoterpene (*E*)-β-ocimene, which in turn attracts parasitic insects (Birkett *et al.,* 2000).

There is quite some detailed knowledge on JA metabolism, although the steps in the inactivation of JA-Ile have been recently described. Inactivation of JA-Ile can occur via three possible ways *i.e.* either by *deconjugation* through hydrolysis of the jasmonoyl residue from the isoleucine moiety; by *oxidative inactivation* through a sequential ω-oxidation of the pentenyl site-chain of JA-Ile or by *hydrolysis* to JA. The enzymes responsible of the catalytic hydrolysis of the amide bond, IAR3 and ILL6, are located in the endoplasmic reticulum (ER) (Sánchez-Carranza *et al.,* 2016). These enzymes were identified as members of the indole-3-acetic acid (IAA) amido-hydrolase enzyme family and are essential for JA-homeostasis in *Arabidopsis* (Widemann *et al.,* 2013). On the other hand, the enzymes involved in the oxidative inactivation of JA-Ile were identified in *Arabidopsis* as belonging to the CYP94 cytochrome P450 family of enzymes and are also localized in the ER (Koo *et al.,* 2014). In *N. attenuata,* JIH1, a close homologue to IAR3, hydrolyzes JA-Ile to JA *in vitro* and its function was further confirmed in the RNA silenced *NaJIH1* gene plants where levels of JA-Ile increased significantly after herbivory (Woldemariam *et al*., 2012; 2014). The enzyme CYP94B3 is responsible for the accumulation of 12-HOJA-Ile after wounding (Koo *et al*., 2011; Kitaoka *et al*., 2011; Heitz *et al*., 2012) and can also catalyze the oxidation of JA-Ile to 12-HOOCJA-Ile, although CYP94C1 is mainly involved in this reaction (Heitz *et al*., 2012; Koo and Howe, 2012). More recently, additional enzymes such as CYP94B1 and CYP94B2 were found to inactivate JA-Ile. These two CYP94 enzymes can also catalyze the hydroxylation and oxidation of JA-Phe (Widemann *et al*., 2015), JA-Leu and JA-Val (Kitaoka *et al*., 2014). In this chapter we report on our study of the metabolic fate of exogenously applied JA; we screened cells and liquid media of cell suspension cultures of *Catharanthus roseus* (L.) G. Don to complete the picture of JA biosynthesis (Chapter 3, Chapter 5) and catabolism (present Chapter) after elicitation in our plant model system.

6.2 EXPERIMENTAL

6.2.1 Cell suspension cultures and elicitation with jasmonic acid

Cell suspension cultures of the *C. roseus* cell line CRPP were grown in 250 mL Erlenmeyer flasks containing 50 mL of Gamborg B5 medium (Gamborg *et al.,* 1968) supplemented with 30 g/L sucrose and 1.86 mg/L of 1-naphthalene acetic acid (NAA) and adjusted to pH 5.8 with 0.1 N KOH. Cell cultures were propagated on a rotary shaker (110 rpm) at 25 °C under continuous light (500-1500 lux) and were subcultured every three weeks by transferring 20 mL of the suspended cells to 50 mL of fresh medium. Four-day-old cell suspension cultures were treated with JA (7.18 µmol/flask; Sigma-Aldrich, St Louis, MO, USA) dissolved into 40% ethanol (v/v) and were harvested in quadruplicates at 0, 5, 30, 90, 360 and 1440 min after elicitation. Cells were filtered on Whatman filter paper under partial vacuum and biomass and media samples were immediately frozen in liquid nitrogen and kept at -80 °C; cells and growth media were lyophilized before any further processing.

6.2.2 Chemicals used for cell suspension cultures

The chemicals used for macro salts were: $CaCl_2$ (min. 99%), KH_2PO_4 (min. 99.5%), KNO_3 (min. 99%) and NH_4NO_3 (min. 99%) purchased from Merck (Darmstadt, Germany) and $MgSO_4$ obtained from OPG Farma (BUVA BV, Uitgeest, The Netherlands). The chemicals used for micro salts were: H_3BO_3, $MnSO_4.H_2O$, $ZnSO_4.7H_2O$, Na_2EDTA (Merck) and $FeSO_4.7H_2O$ (Brocades-ACF Groothandel NV, Maarssen, The Netherlands) dissolved into one solution and KI, $NaMoO_4.2H_2O$, $CuSO_4.5H_2O$ and $CoCl_2.6H_2O$ (Merck) dissolved into another solution to avoid insolubility problems. Thiamine-di-HCl was from Janssen Chimica (Geel, Belgium), pyridoxine-HCl was from Sigma-Aldrich Chemie (Steinheim, Germany), nicotinic acid (99.5%), glycine (99.7%) and NAA were from Merck (Darmstadt, Germany), sucrose (99.7%) and *myo*-inositol (99.7%) were from Duchefa Biochemie (Harlem, The Netherlands).

6.2.3 Extraction procedures

Approximately 100 mg of lyophilized plant material and lyophilized culture media were extracted twice with 2 mL of isopropanol, vortexed for 1 min, sonicated for 15 min and centrifuged for 15 min at 3,500 rpm at 10 °C. The extracts were dried under a gentle flow of N_2 gas, reconstituted into 1 mL of methanol-water (85:15 v/v) and subjected to SPE purification (Waters Sep-Pak C18, 1 mL, 100 mg; Milford, MA, USA), previously conditioned with 1 mL methanol and 1 mL methanol-water (85:15 v/v). Samples were loaded and eluted with 1 mL methanol-water (85:15 v/v). The solution (1 mL) was filtered (Sartorius RC 0.20 µm; Gottingen, Germany) and 1 µL was injected for UHPLC-TOF-MS analyses.

6.2.4 Quantification of jasmonates

External calibration curves of JA (Sigma-Aldrich, St Louis, MO, USA), JA-Ile (Cayman Chemical, Ann Arbor, MI, USA) and (±)-9,10-DHJA (OlChelm Ltd., Olomouc, Czech Republic) were built over different concentration levels. Working solutions were prepared in 1 mL of methanol-water (85:15 v/v). Peaks were identified based on chromatographic retention times and compared to their respective reference standards. Detection and quantification were based on peak area of each JAs. Results are shown in Table 1.

6.2.5 UHPLC-TOF-HRMS analyses

UHPLC-TOF-HRMS analyses were performed on a Micromass-LCT Premier time-of-flight spectrometer (Waters, MA, USA) with an electrospray interface and coupled with an Acquity UPLC system (Milford, MA, USA). ESI conditions: capillary voltage 2400 V, cone voltage 40 V, MCP detector voltage 2400 V, source temperature 120 °C, cone gas flow 20 L/h, desolvation gas flow 800 L/h. Detection was performed in positive and negative ion modes in the *m/z* range 100-1000 Da in a

centroid mode with a scan time of 0.5 s. For the dynamic range enhancement (DRE) lock mass, a 2 μg/mL solution of leucin-enkephalin (Sigma-Aldrich) was infused through the lock mass probe at a flow rate of 10 μg/min with a Shimadzu LC pump (LC-10ADvp, Duisburg, Germany). The separation was carried out on a Waters Acquity BEH C18 UPLC column 50 mm x 1.0 mm i.d. 1.7 μm with the following solvent system: A=0.1% (v/v) formic acid-water, B=0.1% (v/v) formic acid-acetonitrile. The flow rate was 300 μL/min using 2% of B for 4.8 min, 2-98% B in 4.9 min and holding 98% B for 6 min. All samples were analyzed once.

6.2.6 Data handling

Raw UHPLC–MS data were processed using MarkerLynx® software (Waters, Milford, MA, USA). Chromatograms for all JAs are shown in Figure 6.7.2. in the supplemental information section. Before statistical analysis, distributions were tested for normality using the Shapiro-Wilk test ($p < 0.05$). A one-way ANOVA corrected for multiple comparisons with Dunnett's test ($p < 0.05$) was applied to test significant differences in contents of each JAs compared to the same JAs at t=0 (or the earliest time point whenever a JAs was absent at t=0) in cells and in growth medium. ANOVA results are shown in Table 6.7.1 in the supplementary information section. Significant differences between JA-treated cells and JA-treated growth medium in each time point were analyzed with a two-tailed unpaired *t*-test with Welch's correction (*$p < 0.1$ and * $p < 0.05$). All statistical tests were performed with GraphPad Prism software (v. 8.4.3.686, La Jolla, CA, USA).

6.3 RESULTS AND DISCUSSION

6.3.1 Metabolism of jasmonates: hydroxylation and carboxylation

In our previous experiments, feeding cell suspension cultures of *C. roseus* with labeled JA did *not* induce the accumulation of *unlabeled* JA neither in cells nor in growth medium. Feeding cells with labeled dnOPDA showed, already after 5 min, the accumulation of *labeled* JA and *labeled* JA-Ile in cells and *labeled* JA in the growth medium after 30 min, but after 24 h no remaining labeled JAs were observed in the growth medium. Levels of labeled JA and labeled JA-Ile in cells showed a 10-fold decrease (Chapter 5). Apparently, cells are capable of a rapid production of JA, but also of a rapid catabolism and excretion. The present study aimed at mapping the catabolic and excretion pathways active in *C. roseus* cells by analyzing cells and their growth medium for JA, its catabolites and derivatives. Because JA metabolites in untreated and mock-treated cells fell below the detection level in most of the samples (data not shown), it suggests that these metabolites do not occur naturally in cells of *C. roseus* and especially, that the mock treatment did not induce their contents as it happened with fatty acids (FA) (Chapter 3). Consequently, all results shown in this chapter for JA metabolites, are the direct reflection of the metabolic fate of exogenously added JA. From 7.180 nmol/flask of JA

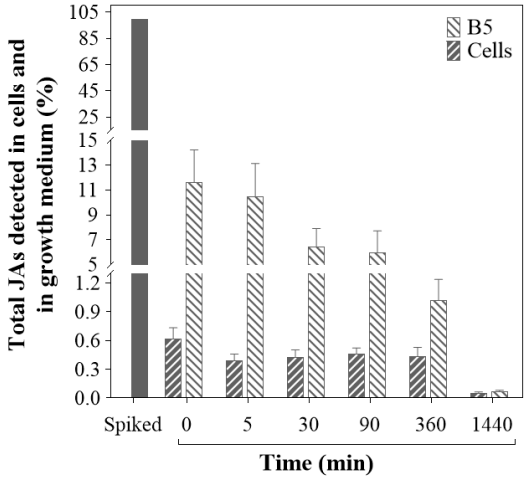

Figure 3. Percentage of total jasmonates detected in cells and growth medium (B5) of *Catharanthus roseus* measured by UHPLC-TOF-MS. Percentages were calculated from the sum of each jasmonate [nmol/flask] divided by the total amount of JA spiked [7180 nmol/flask]. Averaged data ± relative standard error of mean (SEM) of four biological replicates each analyzed once is shown. Significant differences in contents of total JAs in JA-treated cells compared to contents of total JAs in JA-treated growth medium in each time point are shown with a superscript (two-tailed unpaired *t*-test with Welch's correction, * $p < 0.1$ and ** $p < 0.05$; 0 min: t=1.116, df=4.018, p=0.3268; 5 min t=0.9674, df=3.004, p=0.4046; 30 min t=0.9983, df=5.035, p=0..3637; 90 min: t=1.022, df=5.020, p=0.3533; 360 min: t=0.5117, df=5.611, p=0.6284; 1440 min t=0.1340, df=3.539, p=0.9007).

fed to cells and growth medium, in the first time point (0 min) only 0.6 % of JAs was taken up by cells and 12.2 % remained in the growth medium (Fig. 3). From this time point onwards, JA levels in cells and growth medium decreased until JAs reached 0.04 % in both cells and medium. The profiles of JA, DHJA, 12-HOJA, 12-HOOCJA-Ile and 12-HOJA-Ile suggest that these are the major turnover metabolites in *C. roseus* cells. The possibility that the rest of the exogenous JA fed to each flask might have been converted to other JAs that were not measured such as glucosylated derivatives of JA, DHJA, 12-HOJA, 12-HOOOCJA and 12-HOOCJA-Ile and/or JA-Ile, cannot be excluded. At the time of these experiments, we only had access to 3 analytical standards *i.e.* JA, JA-Ile and DHJA, and in order to be able to compare amounts of all detected JAs in cells and growth medium, data in Figure 4 is expressed as AUC/flask for each JAs.

Of particular interest are changes of 12-HOJA in cells. This JAs was already observed at 30 min after induction reaching a maximum at 90 min (4-fold increase) and returning to negligible levels at 1440 min in cells (Fig. 4). Levels of 12-HOJA in the growth medium were relatively low at the early time points of the experiment; however, there was a 5.8-fold increase at 90 min in comparison to 0 min (Fig. 4). Contents of 12-HOJA and JA in cells had an inverse correlation supporting the idea that hydroxylation is one of the major inactivation mechanisms in *C. roseus* cells. Moreover, the presence of 12-HOJA in the medium at 0 min raises the question if extracellular oxidation also plays a role. The

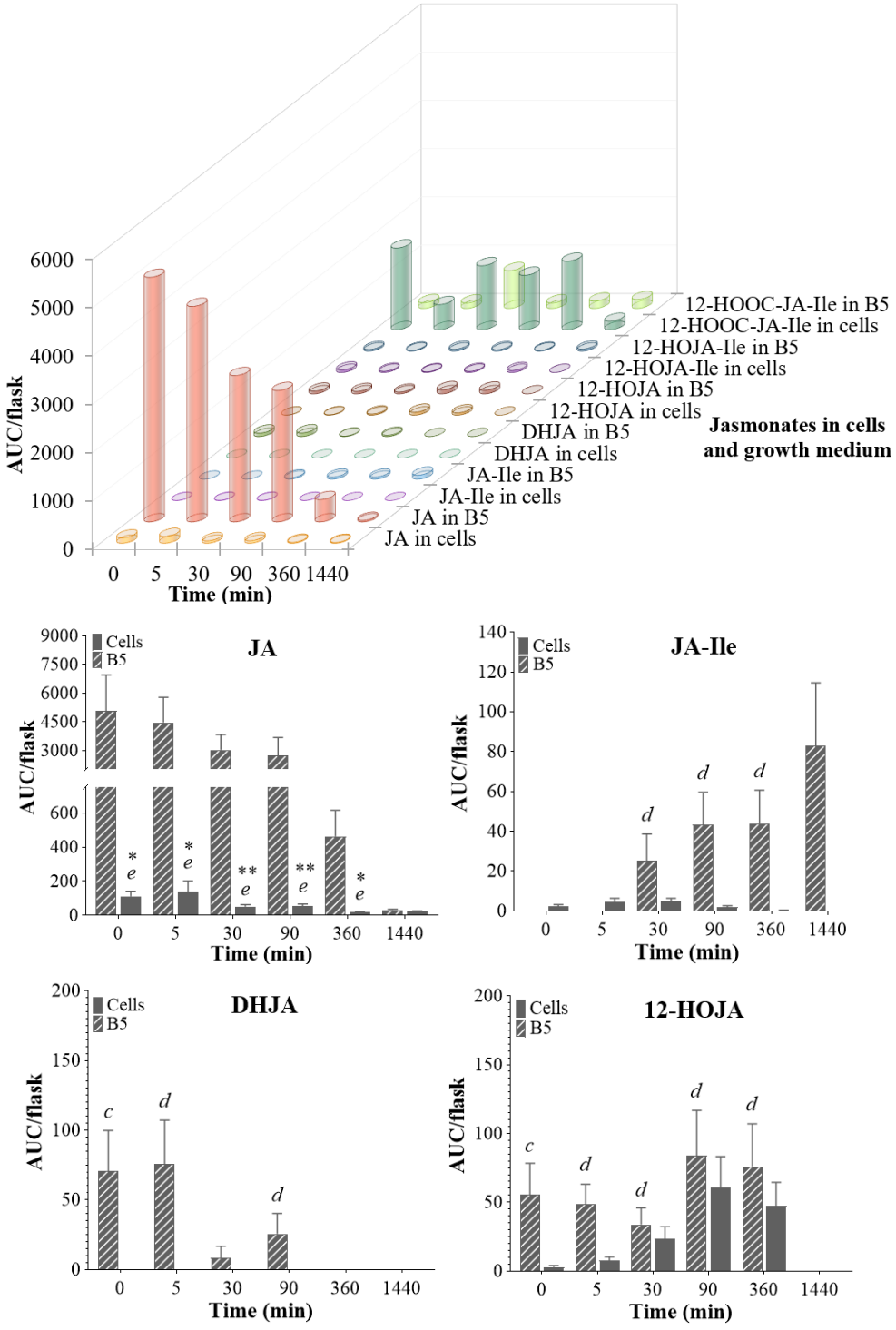

Figure 4. Continued

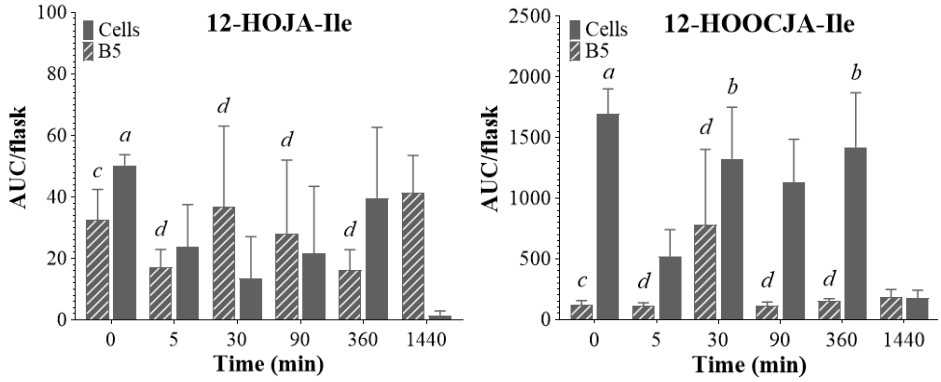

Figure 4. UHPLC-TOF-MS analysis of JA, JA-Ile, DHJA, 12-HOJA, 12-HOJA-Ile and 12-HOOCJA-Ile contents in 100 mg DW of cells and growth medium (B5) of *Catharanthus roseus* suspension cultures after JA-treatment measured by UHPLC-TOF-HRMS. Time is on the *x*-axis and area under the curve (AUC/flask) on the *y*-axis. Averaged data ± standard error of mean (SEM) of four biological replicates each analyzed once is shown. Significant differences in contents of each JAs compared to JA-treated at t=0 h and to the same or earliest time point (whenever there was no JAs present) in cells and in growth medium are marked with a superscript (one-way ANOVA with Dunnett's multiple comparison test, $p < 0.05$). Significant differences in contents of JA in JA-treated cells compared to contents of JA in JA-treated growth medium in each time point are shown with a superscript (two-tailed unpaired *t*-test with Welch's correction, * $p < 0.1$ and ** $p < 0.05$; 0 min: t=2.532, df=3.011, p=0.085; 5 min t=3.081, df=3.087, p=0.0521; 30 min t=3.623, df=3.007, p=0.036; 90 min: t=7.059, df=2.018, p=0.019; 360 min: t=2.686, df=3.005, p=0.0745; 1440 min not tested due to insufficient data).
[a] JA-treated significantly different from JA in JA-treated cells at t=0.
[b] JA-treated significantly different from JA in JA-treated cells at the same time point of observation.
[c] JA-treated significantly different from JA in JA-treated growth medium at t=0.
[d] JA-treated significantly different from JA in JA-treated growth medium at the same time point of observation.
[e] JA-treated cells significantly different from JA-treated growth medium at the same time point of observation.
No JA-Ile was detected in cells at 1440 min.
No DHJA was detected in cells at 360 and 1440 min.
No 12-HOJA was detected neither in cells nor in growth medium at 1440 min.

Table 1. UHPLC-TOF-MS analysis of JA, JA-Ile and DHJA contents in cells and growth medium of suspension cultures of *Catharanthus roseus* treated with JA (nmol/flask)

	JA		JA-Ile		DHJA	
Time (min)	**Cells**	**B5**	**Cells**	**B5**	**Cells**	**B5**
0	14.21±4.58	747.2±280.51	0.0001±0.0001	nd	nd	111.09±33.63
5	18.52±9.24	657.76±196.64	0.0007±0.0003	nd	nd	121.36±37.17
30	5.33±1.54	444.76±118.92	0.0008±0.0001	0.44±0.22	nd	15.17±0
90	5.68±1.9	531.72±63.31	nd	0.86±0.15	nd	42.89±0.16
360	0.61±0.25	63±22.86	nd	0.88±0.17	nd	nd
1440	0.94±0.51	0.92±0	nd	1.3±0.55	nd	nd

Averaged data ± standard error of mean (SEM) of four biological replicates each analyzed once by UHPLC-TOF-HRMS is shown.
B5: growth media, nd: not detected.

presence of a compound with a matching exact mass to that of 12-*O*-Glc-JA was noticed only in JA-treated cells in increasing amounts at 90-1440 min (data not shown), although its unambiguous

identification remains to be confirmed. Swiatek et al. (2004) reported similar results with tobacco BY-2 cells, where 2 h after feeding with JA and MeJA, the hydroxylated JAs 11-HOJA- and 12-HOJA were present, particularly in the liquid growth medium along with jasmonoyl-*O*-1-β-D-glucose, JA, hydroxyjasmonoyl-1-β-glucose and jasmonoyl-1-β-gentiobiose. Xia and Zenk (1993) fed cell suspension cultures of *Eschscholtzia californica* with JA and concluded that the major inactivation mechanisms were hydroxylation followed by glucosylation after finding 11-HOJA and jasmonoyl-1-*O*-β-D-glucoside along with DHJA. The same hydroxylated JAs in their glucosylated form were found in potato leaflets (Matsuura *et al.,* 2001). In wounded leaves of tobacco, the addition of JA resulted in the accumulation of JA, JA-Ile, 12-HOJA and 12-HOJA-glucoside in systemic leaves 2 h after treatment (Sato *et al.,* 2009).

DHJA was detected only in JA-treated growth medium, it was already present at 0 min, peaking at 5 min and not detected after 360 min (Fig. 4 and Table 1). DHJA had a 24-fold decrease at 30 min and a 4.2-fold decrease at 90 min, both when compared to values at 5 min. It is noteworthy that levels of DHJA at 0 min were higher than those of 12-HOJA in the growth medium at the same time point, but not higher than JA in the growth medium, suggesting an extracellular reaction of JA or with a component from the liquid growth medium. JA-Ile and JA also had an inverse behavior where contents of JA in cells had a 15.6-fold increase at 30 min in comparison to 0 min but then decreased to reach a non-detectable level at 90 min whereas JA-Ile in the growth medium showed an increase during the whole experiment, reaching the highest value at 1440 min (Fig. 4 and Table 1). This observation suggests that JA-Ile formed in the cells was excreted to the growth medium after 30 min. Oxidized and hydroxylated derivatives of JA-Ile 12-HOOCJA-Ile and 12-HOJA-Ile, were observed in cells and growth medium in our experiments. 12-HOOCJA-Ile was more abundant in cells than in the growth medium. Levels were similar throughout all the experiment except at 5 min and 1440 min (Fig. 4). Levels in the growth medium varied less if compared to cells. Levels of 12-HOJA-Ile were at least 20 times less than 12-HOOCJA-Ile throughout the experiment. 12-HOJA-Ile was also more abundant in cells at 0 min and 360 min decreasing to minimum levels at 1440 min (Fig. 4). Contents of 12-HOJA-Ile in growth medium remained similar throughout the experiment. However, because of their very low levels, their quantification was not possible. These carboxylated and hydroxylated derivatives were found to be formed in wounded leaves of *A. thaliana* 2-5 min after induction (Glauser *et al.,* 2008b) and are regarded as inactivated forms of JA-Ile as they are unable to promote *in vitro* JAZ-COI1 co-receptor assembly (Aubert *et al.,* 2015; Koo *et al.,* 2011).

Levels of JA in JA-treated cells reached their maximum at 5 min with a 1.3-fold increase in comparison to JA-treated cells at 0 min (Fig. 4 and Table 1). This observation agrees with our previous results, the accumulation of labeled JA after feeding with labeled dnOPDA peaking at 5 min (Chapter 5). In contrast, levels of JA in the growth medium slowly decreased over time reaching negligible levels after 360 min (Fig. 4 and Table 1). Furthermore, differences in contents of JA between the growth

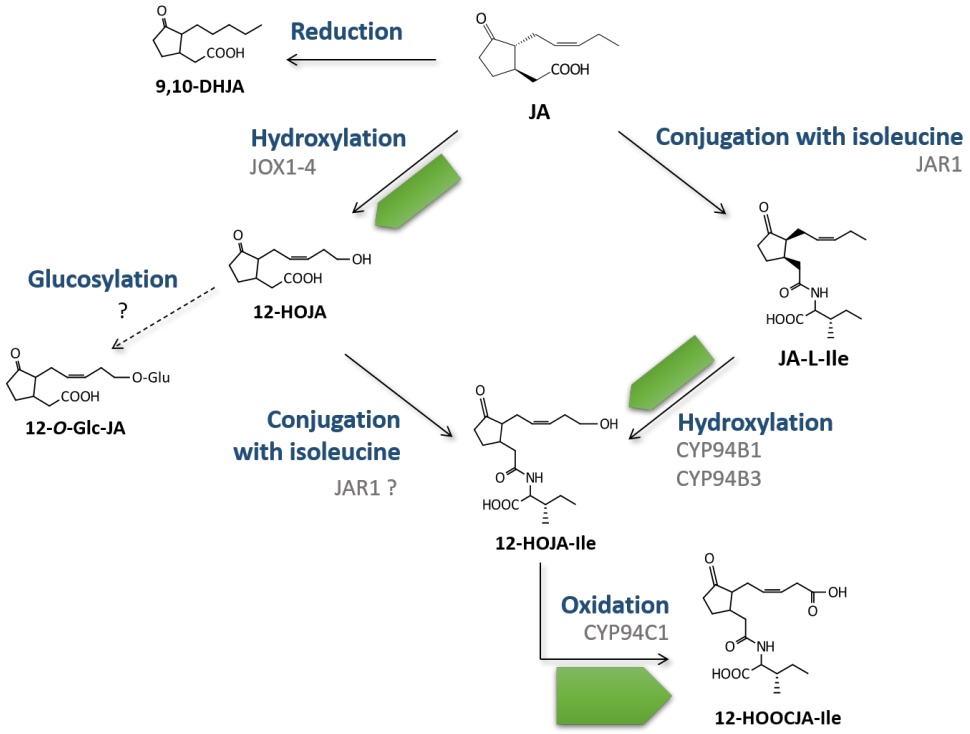

Figure 5. Model of the metabolic pathways of jasmonic acid found in cells and growth medium of *Catharanthus roseus* suspension cultures after JA-treatment measured by UHPLC-TOF-HRMS. Thickness of green arrows denote major conversions and thinner green arrows show minor ones. Dashed arrows show potential conversions of 12-hydroxyjasmonic acid (12-HOJA). Upon feeding cells with jasmonic acid (JA), the three main metabolic pathways were hydroxylation to 12-HOJA possibly catalyzed by JASMONATE-INDUCED OXYGENASES (JOX1-4) in *C. roseus* cells, conjugation with isoleucine to jasmonoyl-L-isoleucine (JA-L-Ile), catalyzed by JASMONATE RESISTANT1 (JAR1) and reduction to 9,10-dihydrojasmonic acid (DHJA). Subsequently, JA-Ile undergoes hydroxylation to 12-hydroxyjasmonoyl-L-isoleucine (12-HOJA-Ile) possibly catalyzed by the CYP94B1 and CYP94B3 enzymes in *C. roseus* cells and lastly, oxidation to 12-carboxyjasmonoyl-L-isoleucine (12-HOOCJA-Ile), possibly catalyzed by CYP94C1. We hypothesize if 12-HOJA could have been conjugated to 12-HOJA-Ile and further carboxylated to 12-HOOCJA-Ile or glucosylated to 12-*O*-glucosyl-JA (12-*O*-Glc-JA).

medium and cells were significant in all time points except for 1440 min (Fig. 5) suggesting the uptake of JA by cells from the growth medium rather than the *de novo* biosynthesis of JA.

Previous feeding experiments using cell suspension cultures of *C. roseus* have described hydroxylation and/or glucosylation events of exogenously added metabolites such as the conversion of exogenously fed Δ^9-tetrahydrocannabinol (Δ^9-THC) into different hydroxylated and glucosylated derivatives along with cannabinol (CBN) and its glycosylated derivative (Akhtar *et al.*, 2015); the accumulation of arbutin (hydroquinone-β-D-glucopyranoside) from exogenous hydroquinone (Inomata *et al.,* 1991); the hydroxylation of salicylic acid (SA) into 2,5-dihydroxybenzoic acid followed by its glycosylation of the hydroxyl group at C-5 (Mustafa *et al.,* 2009); the formation of desacetylcinobufagin

16-*O*-β-D-glucoside, 3-*epi*-desacetylcinobufagin 16-*O*-β-D-glucoside, 3-*oxo*-desacetylcinobufagin 16-*O*-β-D-glucoside and cinobufagin 3-*O*-β-D-glucoside from exogenously fed cinobufagin (Ye *et al.,* 2002): the conversion to 12β,13α-dihydroxytriptonide from exogenous triptonide (Ning *et al.,* 2004): the glucosylation of exogenously fed vanillin into glucovanillin (Yuana *et al.,* 2002) and vanillyl alcohol along with its benzyl and phenyl glucoside (Sommer *et al.,* 1997) and 4-formyl-2-methoxyphenyl-*O*-β-D-glucopyranoside in cell suspension cultures of *Coffea arabica* (Kometani *et al.,* 1993).

Glucosylation *in vitro* is a characteristic transformation in plant cells that is position-specific, that is at the β-linkage at C1 of the glucose moiety (Kometani *et al.,* 1993). This structural modification is an important regulation point in homeostasis because by changing the solubility of otherwise water-insoluble compounds, it inactivates them and facilitates their transport as it has been proven for the glucosides of salicylic acid, benzoic acid (Wasternack and Hause, 2013) and abscisic acid (Piotrowska and Bajguz, 2011). Glucosylation of JAs has been reported in wound-induction experiments in *S. lycopersicum* (Matsuura *et al.,* 2012; Sato *et al.,* 2011; Miersch and Wasternack, 2000) and *S. tuberosum* (Yoshihara *et al.,* 1996; Matsuura *et al.,* 2001; Matsuura and Yoshihara, 2003; Miersch *et al.,* 2008) and *in vitro* in cell suspension cultures of *S. peruvianum* (Schwarzkopf and Miersch, 1992). Together with hydroxylation, these further reactions might be part of a signal attenuation mechanism (Miersch *et al.,* 2008; Kitaoka *et al.,* 2014). The fact that particular derivatives of JA, *e.g.* 12-HOJA, 12-HSO_4-JA and 12-*O*-Glc-JA, occur in higher concentrations than JA in intact tissues, supports an inactivation function for these compounds (Miersch *et al.,* 2008).

The current understanding on phytohormone metabolism is still incomplete, while amino acid conjugation is crucial for activation and hence for the signaling pathway, other reversible modifications through *e.g.* esterification and glucosylation may be important for transport or as temporary form of storage, from which the active forms can be rapidly released by hydrolysis. The methylation of JA yields the volatile MeJA, which could be a messenger between plants and can more rapidly diffuse in leaves via the gas phase rather than via transport in the liquid phase. Hydroxylation and oxidation are irreversible inactivation events that at the end lead to signal attenuation. Considering the fast occurrence of metabolites in the medium extracellular conversions may occur. In addition, transport seems to play an important role, considering the high JA levels at 0 min in the cells. The catabolic enzymes seem to be constitutively expressed, regarding their immediate (0 min) activity.

6.4 CONCLUSIONS

In Chapter 5 we showed that feeding cells with *d5*-JA did not induce the accumulation of endogenous JA in cell suspensions of *C. roseus*. In agreement with that observation, JA was found to go down to basal levels within 1440 min. Here we show that the gradual decrease in JA levels in cells and the growth medium has an inverse correlation with levels of 12-HOJA. A similar pattern was found in JA-

Ile, while levels in cells increased within 30 min and went down to basal levels afterwards, in growth medium, levels of JA-Ile were only found after 30 min. Other metabolites like 12-HOJA-Ile and 12-HOOCJA-Ile were found mostly in cells although their presence in the growth medium points to their excretion. The unambiguous identification of 12-*O*-Glc-JA is to be confirmed. The 12-hydroxylation, 12-oxidation and reduction to DHJA seem to be the main catabolites of JA in cell suspension cultures of *C. roseus* (Fig. 4). These JAs were neither in untreated nor mock-treated cells present, which strongly suggests that these derivatives are the result of the metabolic fate of exogenously fed JA. Moreover, the sole presence of these metabolites shows their immediate conversion suggesting that the enzymes involved are constitutively present and active in cells of *C. roseus*. Based on the type of metabolites found, we expect the presence of the enzymes CYP94B3, CYP94B1, CYP94C1, JOX1-4 and JAR1, which would need further investigation. Finally, whereas JA, 12-HOJA, JA-Ile and DHJA were clearly detected as substrate and initial derivatives in this experiment, further oxidation, conjugation, hydroxylation and/or glucosylation of the various metabolites are most probably involved in cell suspensions of *C. roseus*, although this needs further testing.

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6.7 SUPPLEMENTAL INFORMATION

Table 6.7.1. ANOVA results for each test performed for each jasmonate in JA-treated cells and growth medium compared to JA in JA-treated cells and growth medium of suspension cultures of *Catharanthus roseus*

			Time (min)			
	0	**5**	**30**	**90**	**360**	**1440**
JAs Cells	$F_{(3,10)} = 16.56$ $p=0.0003$	$F_{(3,9)} = 1.837$ $p=0.2108$	$F_{(4,10)} = 4.593$ $p=0.0231$	$F_{(3,8)} = 2.508$ $p=0.1327$	$F_{(3,10)} = 6.557$ $p=0.0100$	$F_{(2,5)} = 1.522$ $p=0.3046$
JAs B5	$F_{(4,12)} = 5.083$ $p=0.0125$	$F_{(4,12)} = 7.835$ $p=0.0024$	$F_{(5,10)} = 6.744$ $p=0.0054$	$F_{(5,11)} = 46.33$ $p<0.0001$	$F_{(4,12)} = 5.238$ $p=0.0112$	$F_{(3,9)} = 2.187$ $p=0.1593$

JAs: jasmonates; B5: growth medium.

Figure 6.7.2. UHPLC profiles of 12-HOJA, 12-HOJA-Ile, 12-HOOC-JA-Ile, JA and DHJA detected in cells and/or growth medium of cell suspension cultures of *Catharanthus roseus.*

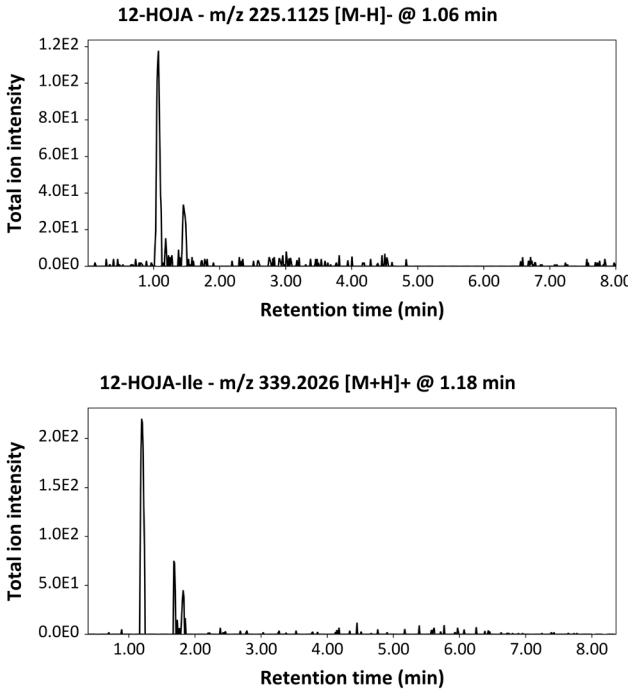
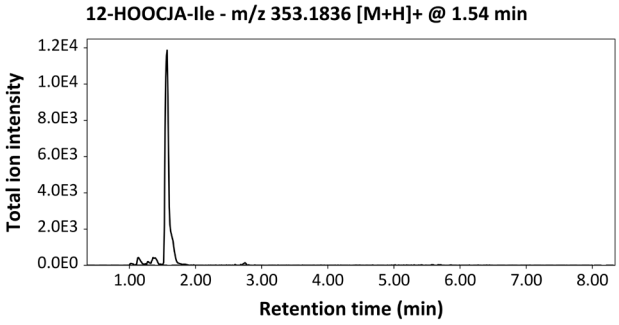
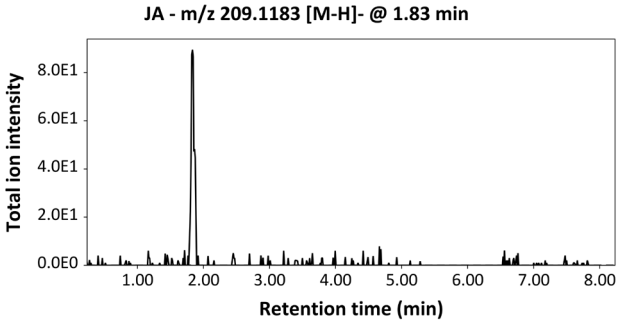
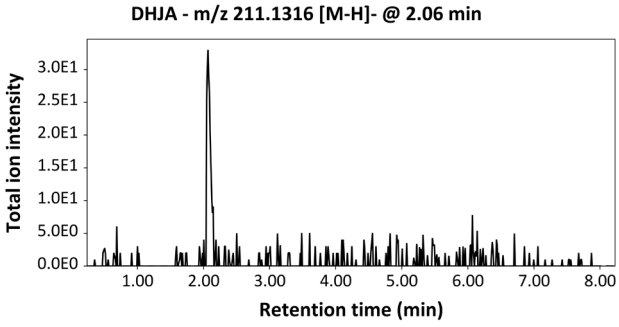
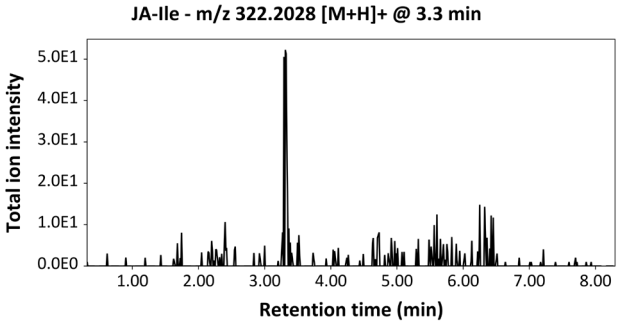


Figure 6.7.2. Continued


12-HOOCJA-Ile - m/z 353.1836 [M+H]+ @ 1.54 min
Total ion intensity
1.2E4
1.0E4
8.0E3
6.0E3
4.0E3
2.0E3
0.0E0
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
Retention time (min)


JA - m/z 209.1183 [M-H]- @ 1.83 min
Total ion intensity
8.0E1
6.0E1
4.0E1
2.0E1
0.0E0
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
Retention time (min)


DHJA - m/z 211.1316 [M-H]- @ 2.06 min
Total ion intensity
3.0E1
2.5E1
2.0E1
1.5E1
1.0E1
5.0E0
0.0E0
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
Retention time (min)


JA-Ile - m/z 322.2028 [M+H]+ @ 3.3 min
Total ion intensity
5.0E1
4.0E1
3.0E1
2.0E1
1.0E1
0.0E0
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
Retention time (min)

Chapter 7

General discussion & perspectives

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7.1 An integrated approach to understand the rapid JA-stress response

During its lifetime, a plant is permanently exposed to a number of environmental conditions which pose a continuous challenge to its survival, and to which it has to adjust accordingly. Environmental cues can include among others: exposure to UV light, suboptimal temperatures, drought, flooding, light quality, osmotic stress, exposure to heavy metals, wounding along with pathogen attack and herbivory, which can lead to decreased growth. Consequently, plants have evolved complex inducible systems that require a coordinated and integrated response that involves the perception of the external stress condition, the translation of such stimuli into an internal signal that will travel from the site of induction to the whole plant to set an "alarm state", and to accumulate enough amounts of messenger signals, which will eventually induce the expression of specific genes involved in the stress response. Such inducible signals are transmitted by small molecule messengers *i.e.* phytohormones like jasmonic acid (JA), that act through signal-transduction pathways to respond to the stress condition. JA along with its precursors and derivatives, *i.e.* jasmonates (JAs), classified as plant oxylipins, are biologically active both in plant development and in defense mechanisms, with roles in both healthy and stressed tissues.

Because in wound-induced experiments in *Arabidopsis thaliana* the JA biosynthetic precursors dinor-12-*oxo*-phytodienoic acid (dnOPDA) and/or 12-*oxo*-phytodienoic acid (OPDA) are rapidly released from galactolipid complexes in thylakoids, combined with rapidly increased endogenous levels of JA and jasmonoyl-L-isoleucine (JA-Ile) (Kourtchenko *et al.,* 2007; Browse, 2009a; 2009b), this suggests that JA can induce its own biosynthesis by a feedback loop mechanism. Further evidence comes from the fact that all JA biosynthetic genes are induced by JAs (Sasaki *et al.,* 2001). Nevertheless, the occurrence of mono- or digalactosyldiacylglycerol (MGDG and DGDG) and the phospholipids phosphatidylcholine (PC), phosphatidylethanolamine (PE) or phosphatidylglycerol (PG) containing either dnOPDA, OPDA, linolenic acid (C18:3) and/or (ω5*Z*)-etherolenic acid residues bound to galactolipids, has so far only been reported in members of the Brassicaceae, Convolvulaceae, Asteraceae, Lamiaceae and Linaceae plant families. Additionally, the enzymes 13-lipoxygenase (13-LOX), allene oxide synthase (AOS) and allene oxide cyclase (AOC) are able to *in situ* transform linoleic acid (C18:2), linolenic acid and roughanic acid (C16:3) esterified in MGDG and DGDG into dnOPDA or OPDA, thus suggesting the constitutive presence of these enzymes, and precluding gene expression of these and/or JA biosynthetic enzymes. However, neither the incorporation of these precursors released from galactolipids nor the *de novo* biosynthesis of JA into the JA-mediated stress response, have been proven yet. This prompts to the question: how do dnOPDA/OPDA esterified in arabidopsides contribute to the stress response, if at all? Originally, our results showed that after JA-treatment, C18:3 was indeed induced after 24 h of treatment suggesting that the *de novo* biosynthesis of JA did not take place. After reevaluating our multivariate data analysis (MVDA) approach and observing the strong effect of the mock-treatment exerted on C18:3, we concluded that under our experimental conditions, *i.e.* JA dissolved in ethanol, the mock treated and JA treated cells cannot be distinguished. In our

publication of 2014 in the *Molecules* journal (Goldhaber-Pasillas *et al*., 2014, retracted), we described the analysis of the fatty acids (FA), but further experiments showed that a calculation error was made, affecting only the quantitative values but not the qualitative ones. Moreover, a thorough analysis confirmed our conclusions that the FA C18-series has a time-dependent uptrend. Changes in FA profiles also occurred in the mock treatment (40 % v/v ethanol) (**Chapter 3**), thus suggesting the possibility of the post-translational event of SUMOylation (small ubiquitin-like modifier), that occurs during drought stress which is extensively described in *A. thaliana* (Benlloch and Lois, 2018). Interestingly, the JA biosynthetic genes *AOC3, AOS, DEFECTIVE ANTHER DEHISCENCE1* (*DAD1*)*, JASMONATE RESISTANT1* (*JAR1*)*, JASMONIC ACID CARBOXYL METHYLTRANSFERASE1* (*JMTI*) and *LOX3* are drought-inducible (Catala *et al.,* 2007). Therefore, we conclude that the use of JA without dissolution into an organic solvent is definitely encouraged, to bypass the strong effect observed with ethanol. In previous JA feeding and elicitation studies in plants and plant cell cultures, dimethyl sulfoxide (DMSO) has been often used as a solvent for JA and other substances. Although it has been published that DMSO does not induce the expression of the transcriptional factor (TF) OCTADECANOID-DERIVATIVE RESPONSIVE CATHARANTHUS AP2-DOMAIN3 (ORCA3) in *in vitro* cultures of *C. roseus* (Vom Endt *et al.,* 2007) and *A. thaliana* plants (Montiel *et al.,* 2011), there is also evidence that DMSO induces the expression of *TRYPTOPHAN DECARBOXYLASE* (*TDC*) and *STRICTOSIDINE SYNTHASE* (*STR*)*,* two TIA biosynthetic genes (Menke *et al.,* 1999), and ROS production (Pauw *et al.,* 2004), both cases in *C. roseus* cell suspension cultures. Consequently, the use of DMSO is also discouraged. Even if our results are inconclusive towards the undivided effect of JA without the mock on FA C18-series, MVDA clearly showed that the largest variation in FA profiles occurred at 24 h, thus partially answering the question that feeding cells with JA does not affect its precursor C18:3 and most especially, that these events do *not* fit into the fast JA-mediated early stress response, that can be better understood as occurring without JA biosynthetic genes expression. We attempted to understand the link between primary metabolism and JA in regard to possible changes in FA profiles, in a wider context. As our work shows that this possibility cannot be excluded, therefore it needs further research.

The lack of knowledge concerning the role of dnOPDA in the JA-mediated signaling pathway and the domination of knowledge generated about the early steps in the JA biosynthesis in plant models like *A. thaliana*, motivated us to explore the early stress response in a plant cell culture system like the cell suspension cultures of *Catharanthus roseus*. Short time-framed events intended to explore the uptake and biosynthetic fate of JA and its precursor dnOPDA, as well as their potential effect on the production of endogenous JA and unlabeled JAs, deuterium-labeled dnOPDA (*d*5-dnOPDA) and deuterium-labeled JA (*d*5-JA) were used. The deuterium-labeled JAs allowed us to monitor their incorporation in cells and to discriminate between endogenous JA-Ile and other JAs. Feeding with labeled dnOPDA resulted in the immediate accumulation of *labeled* JA and *labeled* JA-Ile in cells and

labeled JA in the growth medium, thus showing that the enzymes needed to convert (labeled) dnOPDA into (labeled) JA and JA-Ile, are constitutively present and active. On the other hand, feeding labeled JA did *not* result in the accumulation of neither endogenous unlabeled JA nor JAs, therefore disproving the putative feedback loop mechanism of exogenous JA fed to cell suspension cultures of *C. roseus* (**Chapter 5**). The fact that a multi-analytical platform with different ultra-high resolution abilities was needed to clearly distinguish each JA isotopologue from endogenous JA to avoid false positives, shows how dedicated extraction protocols for each plant material and analytical methods should be carefully validated. The preanalytical steps are of utmost importance, as the use of deuterium labeled phytohormones, proved to contribute to false positives. Consequently, the fact that under our experimental approaches using ^{1}H-NMR, GC-MS, GC-FID, TLC and UHPLC-HRMS/MS we could not confirm yet, the presence of arabidopside-like complexes in *C. roseus* when applying reported methods (**Chapter 5**), does not mean that such complexes do not occur in *C. roseus.* After all, questions like: do all plants have arabidopside-like complexes? Are the dnOPDA/OPDA esterified in galactolipids, entering the stress response? How does the rapid JA stress response work in non-*Arabidopsis* plants? are just some of the most important and intriguing questions in the field awaiting to be answered.

The emerging topic of JA's catabolism is giving a broader standpoint into the "switch-off" events of the JA-mediated stress response. Not only can JA be metabolized into a number of derivatives, but also JA-Ile and OPDA can undergo metabolic conversions into inactive derivatives, although the presence of OPDA-Ile in *A. thaliana*, raises the question regarding their biological role in the JA-mediated signaling pathway, and if JAR1 is responsible for the conjugation of OPDA with isoleucine. Sixteen conversion pathways have been described so far for JA and JA-Ile, and our results show that oxidation, conjugation, hydroxylation, reduction and glycosylation of both JAs were the major turnover metabolites, and it appears that these are the start of the major catabolic pathways in cell suspension cultures of *C. roseus* (**Chapter 6**). CYP enzymes, among others, were recently described to catalyze these reactions *e.g.* CYP94B3, CYP94B1, CYP94C1, IAA-ALANINE RESISTANT3 (IAR3) and JASMONATE-INDUCED OXYGENASE 1-4 (JOX), suggesting their presence in our cell suspension cultures. Given that a larger percentage of JAs was observed in the growth medium than in cells, one may ask: what is the minimum concentration of JA that can elicit the stress response in an *in vitro* model system like ours? How does the cell perceive an excess of JA? Why do some metabolites of JA occur more than others and do they have any biological activity? Apparently, the potential overdose of JA is quickly taken care of by irreversible events such as the hydroxylation, oxidation and reduction of JA, JA-Ile and 12-hydroxyjasmonic acid (12-HOJA) in cells and their excretion to the growth medium (**Chapters 5** and **6**).

The main goal of the work described in this PhD thesis was to get a better understanding of the basis of the rapid JA-mediated stress response in cell suspension cultures of *C. roseus*. Even if our

research did not deliver new information about a putative self-induction mechanism of JA, it disproved this hypothesis and instead, showed that fed dnOPDA is immediately converted to JA-Ile. Moreover, it contributed to expand the knowledge of the metabolic conversions that attenuate the signal propagation of JA *in vitro.* Especially, because it demonstrated the need to keep devising new, more specific methods for the analysis of other oxylipins present in *C. roseus*, and it raised the question about the possibility of other mechanisms involved in the fast stress response. Additionally, our work undertook the task to investigate the link between primary and secondary metabolism occurring during the JA-stress response (**Chapters 3, 5** and **6**). Due to the highly complex JA-mediated stress response, a holistic approach to evaluate and map all events taking place at the metabolite level is mandatory. Metabolic profiling of plant systems is a prerequisite for an integrated approach to study the effects of JA and its derivatives in cells, tissues and organs, especially outside plant model organisms. In this way, the field has greatly benefited from the development of more robust *omics* technologies as well as from analytical platforms such as hybrid quadrupole-orbitrap mass spectrometers with higher sensitivity and selectivity for phytohormone profiling, identification, confirmation and quantification. Particularly dose response studies seem of interest, as is the feeding with labeled JA and labeled dnOPDA which resulted in very different observations. It remains to be proven if feeding with dnOPDA induces the same set of genes in the JA-signaling pathway as JA or OPDA, as it is known that a differential gene expression is triggered by these two JAs in wounding and elicitation experiments. Both OPDA and JA are able to initiate the stress signal-related transduction pathways, although OPDA in a CORONATINE INSENSITIVE1 (COI1)-independent way. Further questions such as: what is the specific biological activity of each JAs, how many more are there, and which genes do they regulate? Are there other components in the JA signaling pathway? Why are some stress responses COI-JAZ-(in)dependent? Why does the extent of JA's effectiveness depend greatly on the plant species or its concentration? Why does the same JAs have different biological activities in different plants? All questions which are still unanswered.

7.2 Terpenoid indole alkaloids, the JA-late stress response in *C. roseus*

The JA-signaling pathway in *A. thaliana, Solanum lycopersicum, Nicotiana benthamiana* and *C. roseus* has been thoroughly studied for decades with major breakthroughs such as the inter- and intra-cellular localization of most enzymatic steps of terpenoid indole alkaloids (TIA) biosynthesis in *C. roseus* and the more complete elucidation of the seco-iridoid pathway, all published between 2013 and 2018 (Simkin *et al.,* 2013; Miettinen *et al.,* 2014; Dugé de Bernonville *et al.,* 2015; Qu *et al.,* 2018; Caputi *et al.,* 2018), which also means that involved genes are now publicly available in the GenBank, Medicinal Plant Genomics Resource and PhytoMetaSyn databases. In *C. roseus*, a plethora of information is available on the more than 130 different TIA present in plants, like the antitumor drugs vinblastine and vincristine, as well as the presence of some TIA in *in vitro* cell and tissue cultures. Also,

the biosynthesis is well defined to the enzyme and gene level. Based on this knowledge, the regulation of the biosynthesis has been studied in detail, resulting in the identification of the TF induced by JA like ORCA2 and ORCA3. For these studies, analytical platforms have been developed and designed to extract, isolate and identify the chemically different TIA, where some of them occur in very low amounts. The great clinical and industrial importance of *C. roseus* made it into one of the best studied medicinal plants and thus a suitable model system for JA-mediated plant stress response, including the effect on secondary metabolism. Concerning the late JA-mediated stress response in *C. roseus*, it depends on the signaling pathway that conveys the expression of several genes involved in TIA biosynthesis, taking place after 2 h of induction, where after a significant accumulation of TIA occurs after 24 h. Therefore, the early steps in the JA-mediated stress response include the fast and large accumulation of JAs as early as 30 sec after wounding in *A. thaliana* whereas the late stress response in *C. roseus* involves the increased accumulation of TIA like tabersonine, serpentine, catharanthine and α-TIA-3 after 24 of JA treatment (**Chapter 4**). The presence of 7 different α-methylene-indoline alkaloids (α-TIA 1-7) not previously reported to occur in cell suspension cultures of *C. roseus*, which were JA-inducible, just shows the richness and diversity of TIA in *C. roseus* cells. Likely candidates for some of the α-TIA are 19-hydroxytabersonine, akuammigine and lochnericine. Therefore, our work contributed to broaden the knowledge of JA-inducible minor TIA present in cell suspension cultures of *C. roseus* (**Chapter 4**). Their full identification could not be achieved by means of a HRMS-based platform, therefore reinforcing the undeniable need to develop further analytical platforms sensitive enough to elucidate the structures of the minor compounds of both the JA on TIA biosynthetic pathways.

7.3 Concluding remarks & perspectives

As an ideal plant model system, *C. roseus* served us to explore and understand more about the JA-mediated fast stress response. Our data clearly demonstrated that there are no negative results, just unexpected ones that challenged our scientific minds, redirected our focus, our analytical approaches and made us reformulate our research hypotheses. Moreover, this PhD thesis proved that this topic can be undertaken in a non-*Arabidopsis* plant model system and *in vitro*. Although we still need to test if one can expect the same or similar results *in planta* and *in vitro*, thus adding another layer of complexity to the already complex JA-stress response.

Whereas JA is considered an important element in plant stress signaling pathways and defense responses, most research efforts are focused on its role on plant growth, development and other physiological activities like fertility, sex determination, reproductive processes, fruit ripening and senescence, to name a few. With the challenge to improve plant resilience to environmental stresses and diseases, it is striking that there is not enough information available about the link of primary and secondary metabolism in plants in regard to JA. In connection to our results, there is an obvious need

to distinguish between a possible SUMOylation effect and JA induction on FA profiles. Increased levels of C18:3 were expected to occur in a shorter timeframe and instead, it was increased at the end of our observation points *along* with C18:3 in ethanol-treated cells. The expression of JA-biosynthetic genes should be tested, and JA *has* to be used undiluted in feeding experiments, together with the comprehensive analysis of oxylipins and metabolomics of unhydrolyzed extracts of *C. roseus*, this will give a better overview of the possible role of C18:3 during the early stress response.

The choice to use deuterium-labeled phytohormones justified our goals, it helped us to confirm that JA does not induce its own biosynthesis and that dnOPDA is converted to JA-Ile but it also questioned our analytical platforms and thinking. The overlap between *d*1-JA and JA accompanied by the lack of access to a higher-resolution mass spectrometer, could have falsely led us to claim that JA induces its own biosynthesis. Thus, collaboration in science, especially in the JA field, is a must. Moreover, the role of dnOPDA in the early and late JA-mediated stress responses in *C. roseus* awaits further research action like testing the differential gene expression triggered by JA and dnOPDA, and the effect of dnOPDA on TIA production, which raises the question whether dnOPDA, like OPDA, act through different signaling pathways; if fed dnOPDA later converted to JA-Ile is responsible for changes in TIA profiles, and if dnOPDA induces the expression of TIA biosynthetic genes. These questions could be answered with the monitoring of dnOPDA-induced cDNA macroarray analysis of *C. roseus* cells, to understand its effects at a deeper systems level. An interesting question is if priming with JA can induce epigenetic changes in plants of *C. roseus*, and if so, which defense-related genes are upregulated. Furthermore, the presence of available enzymes and/or precursors possibly involved in the rapid response, could be spatially and temporally localized with imaging techniques such as imaging and single-cell mass spectrometry, as it was through this technique that catharanthine and serpentine were found to occur in idioblasts and laticifers of *C. roseus.* Concerning the metabolic conversions of JA, JA-Ile and 11/12-HOJA observed in cells of *C. roseus,* our results point to the presence of CYP enzymes among others reported to catalyze these reactions. Further gene exploration for these enzymes in *C. roseus,* the exploration of new JAs along with their exact biological activity and role in signaling pathway, will greatly advance the current understanding of this often neglected topic in the JA-mediated stress response. Possibly, some of these JAs might play a role in mammalian inflammation or as antidepressants such as JA and its methyl ester and will provide insights into their pharmacological potential as plant-based drugs.

In conclusion, in this PhD thesis we established a different perspective for further studies of JA effects on FA, TIA and JAs in *in vitro* systems by showing which experiments, analytical standards and platforms should be used to avoid false positives. In addition, exploring the effects of JA with an *omics* approach will lead to a more comprehensive and integrated understanding of the profound effect that JA exerts in a plant cell, and should be part of a systems biology approach taking into account the total of metabolism in cells or plants. Thus, the research field of JA makes progress day by day, enabled

by the development and access to newer technologies. One finding after the next one will take us to more exciting questions that will challenge our imagination until we have fully dissected the intricate and fascinating world of the JA-stress response.

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SUMMARY

The main goal of this thesis was to get a better understanding of the basis of the rapid JA-mediated stress response. Through an integrated approach and using a high-resolution mass spectrometry-based (HRMS) platform, a targeted profiling was conducted to monitor the metabolism of JA using the model system of cell suspension cultures of *Catharanthus roseus.* Components in the JA-pathway involved in the primary metabolism such as the fatty acid (FA)-precursor C18:3; terpenoid indole alkaloids (TIA) belonging to the secondary metabolism and JA derivatives involved in its catabolism were examined.

Jasmonic acid and its derivatives are strong biological regulators with roles in both healthy and stressed tissues. **Chapter 1** gives a deep and thorough background on the biology of plant stress, covering the topics of phytohormones, their crosstalk, the biosynthesis of JA covering fatty acids (FA) and oxylipins, all metabolic conversions of JA reported until 2020, the general JA-signaling pathway and the effects of JA on terpenoid indole alkaloid (TIA) biosynthesis in *C. roseus*, including their spatial distribution *in planta*.

One of our first tasks was to establish and fine-tune extraction protocols and the analytical platform using GC-MS, to analyze FA in untreated cells and plants of *C. roseus*. **Chapter 2** describes that the degree of unsaturation and relative proportion of certain FA species varies between organs and the developmental stages of the plant. Based on variations observed in FA profiles in cell suspension cultures and intact organs of *C. roseus,* hierarchical cluster analysis (HCA) allowed the classification of two different stages of cell suspension cultures *i.e.* "young" and "old". Plant materials clustered in seeds as one group, characterized with high levels of C16:0, C18:0 and C18:1; roots was the second group with high levels of C16:0, C24:0 and C26:0; flower buds and leaves the third group, stems and flowers formed the fourth group. These last two groups were distinguished by similar levels of very long chain FA (VLCFA) and medium chain FA (MCFA).

The effects of JA on FA in cell suspension cultures of *C. roseus* are time dependent. PCA analysis distinguished three main events: one occurring before 30 min, a second taking place between 90 min and 360 min, where FA profile changes in these two first events were mostly associated to mock treatment. In the last event occurring after 1440 min, changes were caused by JA elicitation. An increasing tendency was observed in the accumulation of C18:0, C18:1, C18:2 and C18:3 after 1440 min in JA-treated cells, although without any significant change. No conclusions can be made yet on the effect of JA on FA profiles in *C. roseus* cell suspension cultures, as the 40 % ethanol solution (v/v) used in mock-treated cells has an elicitor-like effect on FA profiles and especially on the C18 series (**Chapter 3**). Mock treated cells may have gone through the post-translational modification process of

SUMOylation which is known to be activated by ethanol, although this needs further investigation in *C. roseus.*

An increased accumulation of some TIA in *C. roseus* occurred 1440 min after induction with JA. Analyses using ultra high-performance liquid chromatography (UHPLC) coupled to HRMS and a diode array detector (DAD), allowed the detection of 7 different α-methylene-indoline alkaloids (α-TIA 1-7) not previously reported in cell suspension cultures of *C. roseus*. Based on their exact mass and characteristic UV spectrum, the likely candidates are 19-hydroxytabersonine, akuammigine and lochnericine. The largest changes in TIA levels were observed 1440 min after JA treatment with increased levels of tabersonine, catharanthine, serpentine and α-TIA-1 and a significant decrease in levels of the precursor strictosidine in all treatments (**Chapter 4**).

Feeding experiments with labeled dnOPDA led to the immediate formation of *labeled* JA and *labeled* JA-Ile in cells and labeled JA in the growth medium, demonstrating that cells incorporate labeled dnOPDA and excreted labeled JA. Enzymes catalyzing these reactions must be constitutively present and active. Unlabeled JAs were neither observed in cells nor in the growth medium. The hypothesis that JA or dnOPDA could be present in bound forms to galactolipids and available for rapid release upon stress like in arabidopside-like complexes, could not be confirmed under our experimental conditions in *C. roseus* cells yet, when applying reported methods. The importance of using several analytical platforms for the analysis of labeled JA, allowed the identification of a false positive identification of unlabeled JA resulting from decomposition of the deuterium-labeled JA (**Chapter 5**).

The rapid decrease of JA levels after addition to the cells, parallels the accumulation of hydroxylated and carboxylated JAs such as 12-hydroxyjasmonic acid (12-HOJA), 12-hydroxyjasmonoyl-L-isoleucine (12-HOJA-Ile) and 12-carboxyjasmonoyl-L-isoleucine (12-HOOCJA-Ile) as well as 9,10-dihydrojasmonic acid (DHJA), the reduced version of JA. JA and 12-HOJA had an inverse correlation over time in cells and growth medium, a similar behavior for JA-Ile. Apparently, hydroxylation, oxidation and reduction of JA, JA-Ile and 12-HOJA are the start of the major catabolic pathway in cells of *C. roseus* (**Chapter 6**).

Altogether, these observations allowed a more systemic view of the JA induced stress response occurring in cell suspension cultures of *C. roseus,* a well-studied medicinal plant where the accumulation of TIA induced by JA is well known. The sequential steps in the stress response can be summarized in our proposed model where:

i. The late induction of C18:3 after JA-treatment in cells of *C. roseus*, does not fit into the early stress response.
ii. TIA accumulation in *C. roseus* cells is regarded as a late response in the JA signalling pathway.
iii. Upon feeding, enzymes start to immediately convert labeled dnOPDA into labeled JA after addition to the cells but labeled JA does not lead to the accumulation of unlabeled JA.

iv. dnOPDA does not induce a putative receptor in the plastid membrane which leads to the hydrolysis of (dn)OPDA (or its CoA ester) from galactolipids and/or free fatty acids. Labeled dnOPDA most likely goes through the oxidation system in the peroxisome yielding labeled JA. In contrast, labeled JA is immediately conjugated to JA-Ile by the cytosolic JA-amino synthetase 1 (JAR1). Labeled dnOPDA and labeled JA are excreted to the growth medium possibly by JA/JA-Ile TRANSPORTER 1 (JAT1).

v. Finally, exogenous JA, JA-Ile and 12-HOJA are irreversibly converted to their oxidated, hydroxylated and reduced metabolites, thus attenuating the stress response signal.

This model provides an integrated view on some of the early and late events in the stress response in a cell model system although there are still open questions regarding the mechanism of the fast liberation of JA precursor(s), and the identification and localization of the possible receptors involved. Further research on the fast response to JA should focus on the gene expression of enzymes involved in the release of the JA precursors and possible receptors for the JA signal molecules.

SAMENVATTING

De hoofddoelstelling van dit onderzoek was om een beter inzicht te krijgen in de basis van de snelle Jasmijnzuur (JA)-geïnduceerde stress-respons. Door middel van een geïntegreerde aanpak en met behulp van een massaspectrometrie-gebaseerd (HRMS) platform met hoge resolutie, werd een gerichte profilering uitgevoerd om de stofwisseling van JA met behulp van het modelsysteem van celsuspensiecultures van *Catharanthus roseus* te monitoren. Componenten in de JA-biosyntheseweg die bij het primaire metabolisme betrokken zijn, zoals de vetzuur-precursor C18:3, terpenoïde indoolalkaloïden (TIA) die tot het secundaire metabolisme behoren en JA-derivaten die betrokken zijn bij het katabolisme werden onderzocht.

Jasmijnzuur en afgeleiden daarvan zijn sterke biologische regulatoren en spelen zowel in gezond als in gestresst plantenweefsel een rol. **Hoofdstuk 1** geeft een diepgaande en grondige achtergrond van de biologie van plantenstress, waarbij de onderwerpen fytohormonen, hun interactie, de biosynthese van JA die vetzuren (FA) en oxylipinen omvat, worden behandeld, alle metabolische omzettingen van JA gerapporteerd tot 2020, de algemene JA- signaleringsroute en de effecten van JA op terpenoïde indoolalkaloïden (TIA) biosynthese in *C. roseus*, evenals TIA-biosynthese, inclusief hun ruimtelijke verdeling *in planta*.

Een van onze eerste taken was het opstellen en verfijnen van extractieprotocollen en het analytische platform met GC-MS, om FA in onbehandelde cellen en planten van C. roseus te analyseren. **Hoofdstuk 2** beschrijft dat de respons op stress in de celculturen van de *C. roseus* wordt gekenmerkt door de accumulatie van JA dat vervolgens de genexpressie van biosynthetische genen van TIA induceert en grote veranderingen teweegbrengt bij de FA-profielen. Veranderingen in de vetzuursamenstelling van membranen tonen een onmiddellijke respons in veranderende omstandigheden aan. De mate van onverzadiging of de relatieve verhouding van bepaalde soorten vetzuren (FA) verschilt tussen organen en verschilt afhankelijk van het ontwikkelingsstadium van de plant. Gebaseerd op de waargenomen verschillen van de FA-profielen van celsuspensiecultures en intacte organen van *C. roseus*, toonde hiërarchische clusteranalyse (HCA) verschillende stadia van celsuspensiecultures aan, *i.e.* "jonge" en "oude". Bovendien, C18:0, C18:2 en C18:3 hebben geen correlatie met de leeftijd van de cellen. Plantaardige materialen geclusterd met zaden als één groep, gekenmerkt door hoge niveaus van C16:0, C18:0 en C18:1; met wortels als tweede groep met hoge niveaus van C16:0, C24:0 en C26:0; met bloemknoppen als derde groep en met stengels en bloemen als vierde en laatste groep. Deze laatste twee groepen werden onderscheiden door hun vergelijkbare niveaus van FA (VLCFA) met zeer lange keten en FA (MCFA) met middellange keten.

De effecten van JA op FA in de celsuspensiecultuur van de *C. roseus* zijn tijdsgebonden. De PCA-analyse van de data onderscheidt drie belangrijke periodes: één vond plaats voor 30 minuten, een tweede vond plaats tussen 90 minuten en 360 minuten, waarbij veranderingen in het FA-profiel in deze twee eerste periodes meestal verband hielden met de controlebehandeling. In de laatste periode die plaatsvond na 1440 min, werden veranderingen veroorzaakt door JA-uitlokking. Een toenemende trend werd waargenomen in de accumulatie van C18:0, C18:1, C18:2 en C18:3 na 1440 min in met JA behandelde cellen, hoewel zonder noemenswaardige verandering. Er kunnen nog geen conclusies worden getrokken over het effect van JA op FA-profielen in *C. roseus* celsuspensieculturen, aangezien de 40% ethanoloplossing die wordt gebruikt in controlebehandelde cellen een elicitor-achtig effect heeft op FA-profielen en vooral op de C18-serie (**Hoofdstuk 3**). Mock-behandelde cellen hebben mogelijk het post-translationele modificatieproces van SUMOylation ondergaan waarvan bekend is dat het wordt geactiveerd door ethanol, hoewel dit verder onderzoek bij *C. roseus* nodig heeft.

Een verhoogde accumulatie van sommige TIA in *C. roseus* begint 1440 minuten na inductie met JA. Dankzij de analyses met de ultra high performance liquid chromatografie (UHPLC) gekoppeld aan een high resolutie MS en een diode array detector (DAD) konden 7 verschillende α-methylene-indoline alkaloïden (α-TIA 1-7) gedetecteerd worden die nog niet eerder zijn gerapporteerd in de celsuspensiecultuur van de *C. roseus*. Op basis van hun exacte massa en karakteristieke UV-spectrum zijn waarschijnlijke kandidaten 19-hydroxytabersoine, akuammigine en lochnericine. De grootste veranderingen in TIA-niveaus werden 1440 minuten na behandeling met JA waargenomen met verhoogde tabersonine-, catharanthine-, serpentine- en α-TIA-1-niveaus en een significante afname van de precursor strictosidine bij alle behandelingen (**Hoofdstuk 4**).

Voedingsexperimenten met gelabeld dnOPDA leidden tot de onmiddellijke vorming van *gelabeld* JA en *gelabeld* JA-Ile in cellen en gelabeld JA in het groeimedium, wat aantoont dat cellen gelabeld dnOPDA opnemen en gelabeld JA uitgescheiden. Enzymen die deze reacties katalyseren, moeten constitutief aanwezig en actief zijn. Ongelabelde JAs werden noch in cellen noch in het groeimedium waargenomen. De hypothese dat JA of dnOPDA aanwezig zou kunnen zijn in gebonden vormen aan galactolipiden en beschikbaar zou kunnen zijn voor snelle afgifte bij stress zoals in arabidopside-achtige complexen, kon onder onze experimentele omstandigheden in *C. roseus*-cellen nog niet worden bevestigd bij het toepassen van gerapporteerde methoden. Het belang van het gebruik van verschillende analytische platforms voor de analyse van gelabeld JA, maakte de identificatie mogelijk van een vals-positieve identificatie van ongelabelde JA als gevolg van het gebruik van deuterium-gelabeld JA (**Hoofdstuk 5**).

De snelle afname van JA-niveaus na toevoeging aan de cellen loopt parallel met de accumulatie van gehydroxyleerde en gecarboxyleerde JAs zoals 12-hydroxyjasmijnzuur (12-HOJA), 12-hydroxyjasmonoyl-L-isoleucine (12-HOJA-Ile) en 12-carboxyjasmonoyl -L-isoleucine (12-HOOCJA-Ile) en 9,10-dihydrojasmijnzuur (DHJA), de gereduceerde versie van JA. JA en 12-HOJA

hadden in de tijd een omgekeerde correlatie in cellen en groeimedium, een vergelijkbaar effect werd gevonden voor JA-Ile. Blijkbaar zijn hydroxylering, oxidatie en reductie van JA, JA-Ile en 12-HOJA het begin van de belangrijkste katabole route in cellen van *C. roseus* (**Hoofdstuk 6**).

Al met al maakten deze waarnemingen een meer systemisch beeld mogelijk van de door JA veroorzaakte stressreactie die optreedt in celsuspensieculturen van *C. roseus*, een goed bestudeerde medicinale plant waar de door JA geïnduceerde accumulatie van TIA goed bekend is. De opeenvolgende stappen in de stressreactie kunnen worden samengevat in ons voorgestelde model waar:

i. De late inductie van C18:3 na JA-behandeling in cellen van *C. roseus* past niet in de vroege stressreactie.
ii. TIA-accumulatie in *C. roseus*-cellen wordt beschouwd als een late respons in de JA-signaleringsroute.
iii. Na het voeden beginnen enzymen het gelabelde dnOPDA onmiddellijk om te zetten in gelabeld JA na toevoeging aan de cellen, maar gelabeld JA leidt niet tot de accumulatie van niet-gelabeld JA.
iv. dnOPDA induceert geen vermeende receptor in het plastidemembraan, wat leidt tot de hydrolyse van (dn)OPDA (of zijn CoA-ester) uit galactolipiden en/of vrije vetzuren. Gelabeld dnOPDA gaat hoogstwaarschijnlijk door het oxidatiesysteem in het peroxisoom en levert gelabeld JA op. Daarentegen wordt gelabeld JA onmiddellijk geconjugeerd aan JA-Ile door het cytosolische JA-aminosynthetase 1 (JAR1). Gelabeld dnOPDA en gelabeld JA worden mogelijk uitgescheiden op het groeimedium door JA/JA-Ile TRANSPORTER 1 (JAT1).
v. Ten slotte worden exogene JA, JA-Ile en 12-HOJA onomkeerbaar omgezet in hun geoxideerde, gehydroxyleerde en gereduceerde metabolieten, waardoor het stressreactiesignaal wordt verzwakt.

Dit model biedt een geïntegreerd beeld van enkele van de vroege en late gebeurtenissen in de stressrespons in een celmodelsysteem, hoewel er nog steeds open vragen zijn over het mechanisme van de snelle vrijgave van JA-precursor(s) en de identificatie en lokalisatie van de mogelijke receptoren. Verder onderzoek naar de snelle respons op JA zou zich moeten concentreren op de genexpressie van enzymen die betrokken zijn bij de vrijgave van de JA-voorlopers en mogelijke receptoren voor de JA-signaalmoleculen.

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CURRICULUM VITAE

Guitele Dalia Goldhaber Pasillas was born on November 24th, 1979 in Mexico City. She received her bachelor diploma in Biology from the National Autonomous University of Mexico (UNAM) in 2004. Throughout her undergraduate research project, she worked in the field of phytochemistry with the Mexican medicinal plant *Piqueria trinervia* used in the Mexican folk medicine to treat gastrointestinal disorders caused by enteropathogenic bacteria. Her work was performed in collaboration with the Institute of Chemistry, UNAM and the Faculty of Superior Studies Iztacala, UNAM, where she performed the bioguided isolation and identification of the secondary metabolites responsible for the antibacterial activity. In 2005 she continued with her MSc studies at the Botanical Garden of the Institute of Biology, UNAM and the Faculty of Chemistry, UNAM where she worked with the threatened Mexican plant species *Ligusticum porteri* under the supervision of Prof. dr. Robert Bye and the co-supervision of Prof. dr. Rachel Mata and Dr. Víctor Manuel Chávez Ávila. The main goal was to establish an *in vitro* protocol for the successful production of the secondary metabolites only available in the roots to alleviate the pressure of over collection in wild populations. She graduated with honors in 2008 and was postulated to the Gabino Barreda medal for the best postgraduate thesis of 2008. In 2009, she was awarded with a scholarship for her postgraduate studies abroad by the National Council of Science and Technology (CONACYT) and joined the group of the now Natural Products Laboratory lead by Prof. dr. Rob Verpoorte at Leiden University in The Netherlands. For her PhD studies she worked with the plant stress hormone jasmonic acid where her main goal was to understand the fast response of this phytohormone upon feeding with cell suspension cultures of the African medicinal plant and model system *Catharanthus roseus.* From 2014 to 2015, as a Marie Curie Experienced Researcher in Golm, Germany, she contributed to the identification of a new biomarker for X-linked adrenoleukodystrophy (X-ALD), a neonatal and fatal genetic disease, which was submitted as an official recommendation to the Dutch Ministry of Health in 2016, to update the neonatal screening panel in The Netherlands. From 2015 to 2016, as a research associate in Mannheim, Germany, she implemented a LC-MS-based metabolomics and lipidomics workflow to identify characteristic phospholipid signatures in resections from patients with hepatic metastases. From 2016 to 2017, as an analytical scientist in Katzrin, Israel, she contributed to the development of three new synthetic cannabinoids with proven anti-inflammatory activity. From 2017 to 2019, as an R&D scientist in Haifa, Israel, she developed 5 LC-MS/MS-based metabolomics, lipidomics and targeted profiling methods for flavonoids in *Cannabis* plants, that permitted the identification and validation of 5 new potential biomarkers in a mouse model system for diabetes, signature metabolites in a mouse model for dementia, and an integrative classification of *Cannabis* chemovars grown in Israel.

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- **Goldhaber-Pasillas GD**, Morin H, Wolfender JL, Verpoorte R (**2020**) Effects of dinor-12-*oxo*-phytodienoic acid feeding and study of the early steps of jasmonic acid biosynthesis in cell suspension cultures of *Catharanthus roseus*. *In preparation.*

- **Goldhaber-Pasillas GD**, Meiri D (**2020**) Flavonoid profile of *Cannabis* chemovars grown in Israel. *In preparation.*

- Joels G, **Goldhaber-Pasillas GD**, Meiri D (**2020**) Metabolomics and lipidomics profiling of mice preadipocytes treated with the *Cannabis* chemovar Kandy Kush. *In preparation.*

- Herzog K, **Goldhaber-Pasillas GD**, Schauer N, Waterham HR, Wanders RJA (**2019**) Comprehensive analysis of dried blood spots of patients with a PEX mutation and fed with cholic acid. *In preparation.*

- Herzog K, **Goldhaber-Pasillas GD**, Schauer N, Waterham HR, Wanders RJA (**2019**) Comprehensive and lipidomic profiling of human fibroblasts with a D-bifunctional protein peroxisomal dysfunction. *In preparation.*

- Roermund CWT, **Goldhaber-Pasillas GD** (**2019**) Comprehensive and lipidomic profiling of *fox1*, *pex11* and *pxa1/pxa2* mitochondria and peroxisomes yeast mutants defective in peroxisomal phospholipid transport. *In preparation.*

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