OXYLIPIN PHYTOHORMONES IN PLANT-INSECT INTERACTIONS: ACTION AND METABOLISM OF JASMONIC ACID AND 12-OXOPHYTODIENOIC ACID IN PLANTS AND INSECTS

Dissertation

zur Erlangung des akademischen Grades
doctor rerum naturalium (Dr. rer. nat.)

vorgelegt dem Rat der Chemisch-Geowissenschaftlichen Fakultät
der Friedrich-Schiller-Universität Jena

von Diplom Chemikerin
Paulina Anna Dąbrowska

geboren am 02.05.1980 in Warschau

Gutachter:

1. Prof. Dr. Wilhelm Boland	Department of Bioorganic Chemistry, Max Planck Institute for Chemical Ecology, Jena
2. Prof. Dr. Rainer Beckert	Institut für Organische Chemie und Makromolekulare Chemie, Friedrich Schiller Universität, Jena
3. Prof. Dr. Dr. h. c. mult. Wittko Francke	Abteilung für Organomeereschemie, Universität Hamburg, Hamburg

Tag der öffentlichen Verteidigung: 25.03.2009

“I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale.”

Maria Skłodowska - Curie (1867 – 1934)

Contents

Abbreviations and symbols

12,13-EOT	12,13–epoxyoctadecatrienoic acid
13-HPOT	(13*S*)-hydroperoxyoctadecadienoic acid
ABC transporter	ATP-binding cassette transporter
ACC	1-amino cyclopropane-1-carboxylic acid
ACX1	acyl-CoA oxidase 1
AOC	alleneoxide cyclase
AOS	alleneoxide synthase
BA2H	benzoic acid-2-hydroxylase
CoA	coenzym A
COI1	CORONATINE INSENSITIVE 1
CTS	COMATOSE
DAD	delayed anther dehiscence (mutant name)
ET	ethylene
FAC	fatty acid amino acid conjugates
f.i.	for instance
Fig.	figure
GSH	reduced glutathione
GST	glutathione *S*-transferase
HR	hypersensitive response
ICS	isochorismate synthase
IPL	isochorismate puryvate lyase
JA	jasmonic acid
JAMe	jasmonic acid methyl ester
JA-Ile	jasmonoyl-L-isoleucine
JAZ	JASMONATE ZIM-DOMAIN
KAT	L-3-ketoacyl CoA thiolase
LOX	lipoxygenase
MAPK	mitogen-activated protein kinase
MFP	multifunctional protein
NPR1	NON EXPRESSOR OF PR1
NCI	negative chemical ionization
OPC 8:0	8-[(1*S*,2*S*)-3-Oxo-2-{(*Z*)-pent-2-enyl}cyclopentyl]octanoate
OPDA	*cis*-(+)-12-oxophytodienoic acid

OPR	OPDA-reductase
PAL	phenylalanine ammonia-lyase
PAPS	3'-phosphoadenosine-5'-phosphosulfate
PFBHA	*O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride
PFBO	*O*-(2,3,4,5,6-pentafluorobenzyl)oxime
PIs	proteinase inhibitors
PIN	wound induced proteinase inhibitors
PR	pathogenesis related (proteins)
PUFA	polyunsaturated fatty acid
ROS	reactive oxygen species
SA	salicylic acid
SABP	salicylic acid binding protein
SAR	systemic acquired resistance
ST	sulfotransferase
Tab.	table
TD	threonine deaminase

1. General Introduction

In his very early attempts to segregate all living things, Aristotle distinguished the kingdom of plants, regarded as non-moving organisms confined to one habitat place, from the kingdom of mobile animals. The seemingly trivial fact that plants are unable to run away from their enemies is also one of the main reasons that flora was forced to evolve a set of sophisticated defensive strategies. As early as 1888 Jenaer biologist Ernst Stahl suggested that the enormous variety of protective strategies plants have, including an impressive amount of chemicals, was shaped and optimized under the selection pressure of the animal kingdom.[1, 2] Obviously, in the course of co-evolution plants' enemies, such as insect herbivores or pathogens, developed corresponding counter-adaptations.

1.1. Phytohormones regulating plants' defenses

Plant defensive strategies can be generally divided into two major groups: energetically costly, but always present constitutive defenses, and the more economical inducible defenses.[3] Constitutive defenses include mechanical protection (thorns, spikes, trichomes)[4], defenses mediated by deterrent or toxic secondary metabolites (alkaloids, glucosinolates, terpenoids and phenolics) [5, 6] and compounds that inhibit digestion, for example, proteinase inhibitors (PIs).[7] Less evident inducible defenses have gained attention only recently.[8] They include plant protective means that are activated exclusively upon attack. Next to the induced synthesis of secondary metabolites [8, 9], one of the most prominent examples of plant induced defense is herbivore-elicited volatile emission and the secretion of extrafloral nectar.[10-12]

The success of inducible plant defenses depends highly on the efficient and fast recognition of the attack, which in turn is relative to the signaling cascade responsible for the alteration of gene expression. The important role of signals mediating/regulating plant stress responses is carried out by a set of phytohormones; among these, a crucial role is assigned to jasmonic acid (JA) (**1**) and its precursors and derivatives (collectively known as jasmonates), salicylic acid (SA) (**2**) and ethylene (ET).[13, 14]

1.1.1. Jasmonates

The JA-mediated wound response to herbivore feeding can lead to the volatile emission as well as to the formation of defense secondary metabolites or defense proteins. A correlation between JA and the induction of phytoalexin biosynthesis (including the biosynthesis of flavonoids, alkaloids, terpenoids) has been demonstrated.[9, 15-17] Another example of JA-linked response is the induced formation of PINs, leucine aminopeptidases and threonine deaminase (TD) in tomato [13, 18, 19], which are thought to inhibit proteolytic degradation in the midgut of herbivores. Whereas both of these factors have an immediate effect on a feeding herbivore and thus are part of plants' direct defense, the emission of volatiles can constitute a part of direct or indirect defense. Some components of released volatile blends are directly repelling [20, 21], whereas some attract herbivores' enemies - predatory arthropods [22-27] - and in this way play an indirect role in plants' defense. Moreover, evidence accumulates suggesting that volatiles play a role in plant-plant communication [28-30] and in within-plant signaling [31], which also contributes to plant defense strategies. Another interesting example of a JA-mediated indirect defense response to herbivory was recently shown for lima bean (*Phaseolus lunatus*), which secretes a sweet extrafloral nectar; the nectar in turn attracts ants and these reduce the rate of herbivory.[11, 12]

The collective term 'jasmonates' describes a group of compounds belonging to the oxylipin family - lipid-derived metabolites in plants. The first physiological effects of this compound class were discovered for jasmonic acid (**1**) and its methyl ester in 1980, where they were shown to act as senescence-promoting [32] and growth-inhibiting agents.[33] Jasmonates were later found to play an important role in many other processes involving plant growth and development, i.e. seed germination, flower formation, reproductive development.[34] On the other hand, what really secured a lasting interest in this phyotohormone group was their signaling function in the responses of plants to abiotic (ultraviolet radiation [35], ozone [36], salt stress [37]) and biotic stresses, such as herbivore wounding or pathogen attack (see **Fig.1** for overview).[38]

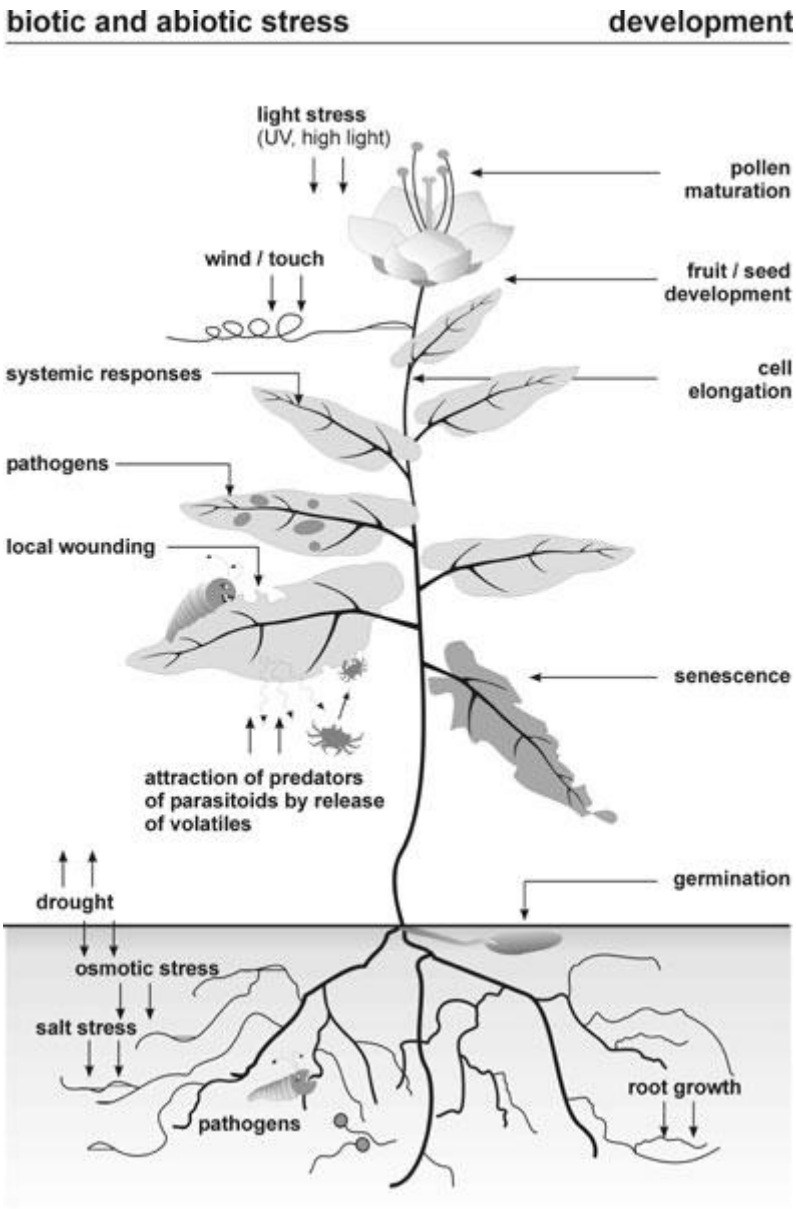


Figure 1. Overview of the biotic and abiotic stress responses and developmental processes in which jasmonic acid and jasmonates function as signals (after Wasternack 2004 [39]).

JA biosynthesis was elucidated in the early 80's by Vick and Zimmerman.[40-42] Like other oxylipins, jasmonates originate from α-linolenic acid (18:3) (**3**) released from chloroplast membranes. While phopspholipid-hydrolyzing enzymes belong to at least five different families [43], a direct link to JA biosynthesis has so far been proven only for phospholipase A_2 [44] and DAD-like phospholipase A_1.[45] The free fatty acid (however activity of 13-LOX with PUFAs esterified to phospholipids has also been demonstrated [46, 47]) is subsequently oxidized by the action of 13-lipoxgenase (13-LOX) to hydroperoxide – (13*S*)-hydroperoxyoctadecadienoic acid (13-HPOT) (**4**) and further down to the unstable 12,13–epoxyoctadecatrienoic acid (12,13-EOT) (**5**) by

13-alleneoxide synthase (13-AOS) (**Fig.2**).[48-50] The next enzyme of the biosynthetic pathway, the alleneoxide cyclase (AOC), closes the cyclopentenone ring and establishes the configuration of *cis*-(+)-(9*S*,13*S*)-12-oxophytodienoic acid (OPDA) (**6**).[51, 52] OPDA is the final product of the plastid-located part of JA biosynthesis. The subsequent step, reduction of the ring localized double bond, is catalyzed by peroxisomal OPDA reductase (OPR 3).[53, 54] Though there are hints correlating the import of OPDA or its CoA ester into peroxisomes with the ABC transporter COMATOSE (CTS), it is still not exactly known how the transport of the OPDA between chloroplast and peroxisomes takes place.[55] The three subsequent cycles of β-oxidation shorten the side chain of the 8-[(1*S*,2*S*)-3-Oxo-2-{(*Z*)-pent-2-enyl}cyclopentyl]octanoate (OPC 8:0) intermediate (**7**) to form the final product of the pathway – (+)-7-*iso*-jasmonic acid (**1**). Recently a specific onset of three enzymes of this β–oxidation has been identified: acyl-CoA oxidase (ACX1) [56], multifunctional protein (MFP) [57] and a L-3-ketoacyl CoA thiolase (KAT).[58, 59] Interestingly, evidences increases that the β-oxidative steps take place only with the corresponding CoA esters of OPDA and/or OPC 8:0 [57, 60], whose formation is catalyzed by the 4-coumarate:CoA ligase like (4-Cl-like) enzymes.[60]

Though the involvement of the jasmonic acid in signal transduction has been long established, detailed knowledge about how jasmonates regulate expression of genes coding for enzymes of secondary metabolism remained vague till recently. It was known that the F-box protein coronatine insensitive 1 (COI1) is required for the perception of jasmonates, based on the discovery of the first JA-insensitive mutant using the bacterial toxin coronatine (**8**), which is structurally and functionally similar to the JA-Ile (**9**).[34] This protein forms a part of an enzyme complex called SCF^{COI1}, which was predicted to tag unknown regulators of jasmonate signaling with ubiquitin, leading in turn to their destruction. These unknown regulators have been newly identified as JAZ proteins.[61, 62] JAZ proteins are normally bound to transcription factors (MYC2) and inhibit their activity (**Fig.3a**). In response to attack, however, JA bound to isoleucine in form of JA-Ile (**9**) stabilizes the interaction between COI1 and JAZ. At this stage, JAZ proteins are probably already marked for destruction by ubiquitin. After JAZ proteins are destroyed they liberate the transcription factors (MYC2), which in turn allow the transcription of genes responsible for producing proteins involved in defense or developmental reactions (**Fig.3b**). There are also indications as to the second type of regulatory loop based on the activity of JAZ pro -

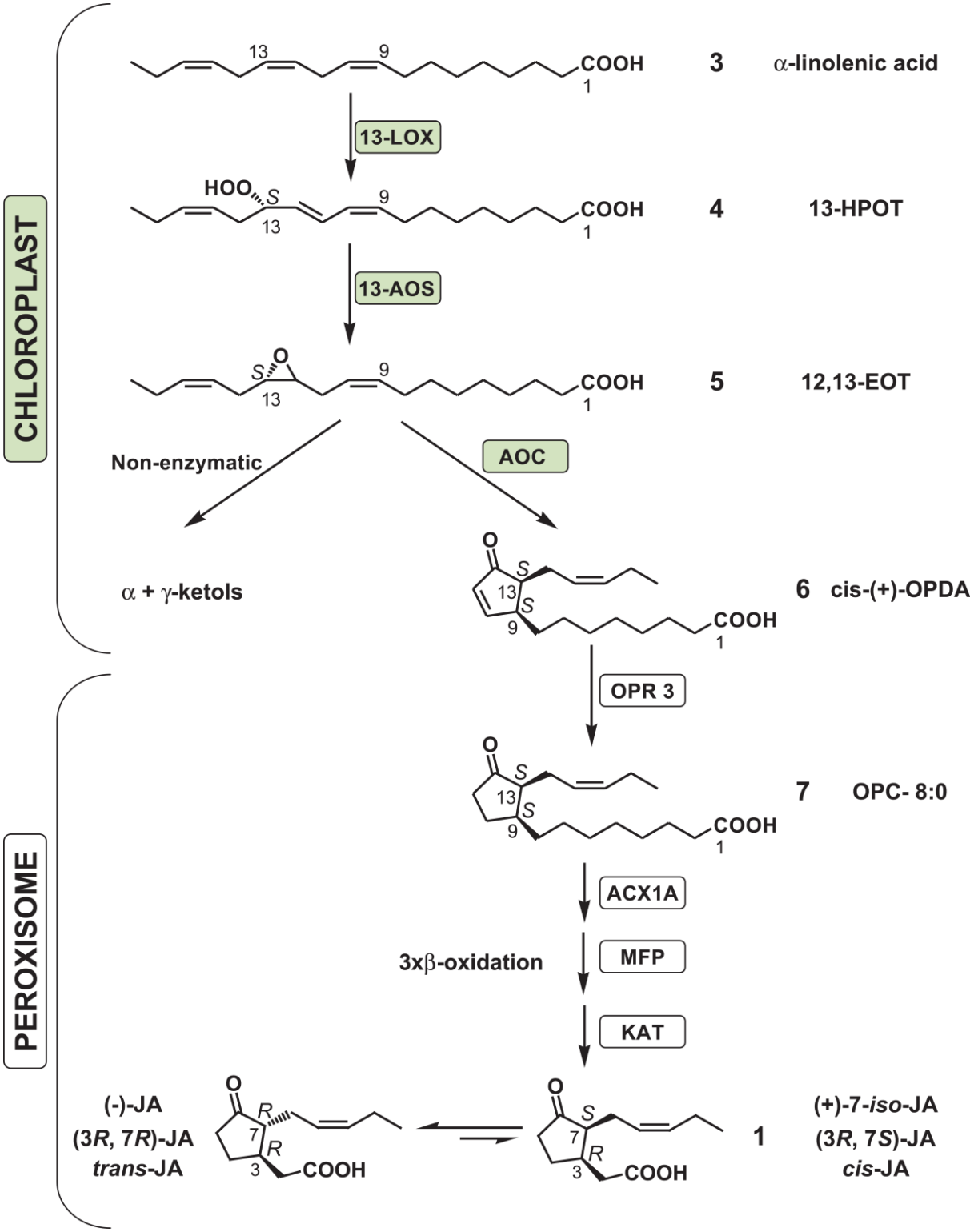


Figure 2. The JA biosynthetic pathway with important enzymes and intermediates, showing the location of the transformations in plants (modified after Wasternack 2007 [63]).

- teins, in which JA signaling activates JAZ gene transcription and leads to the down-regulation of jasmonate action.[64]

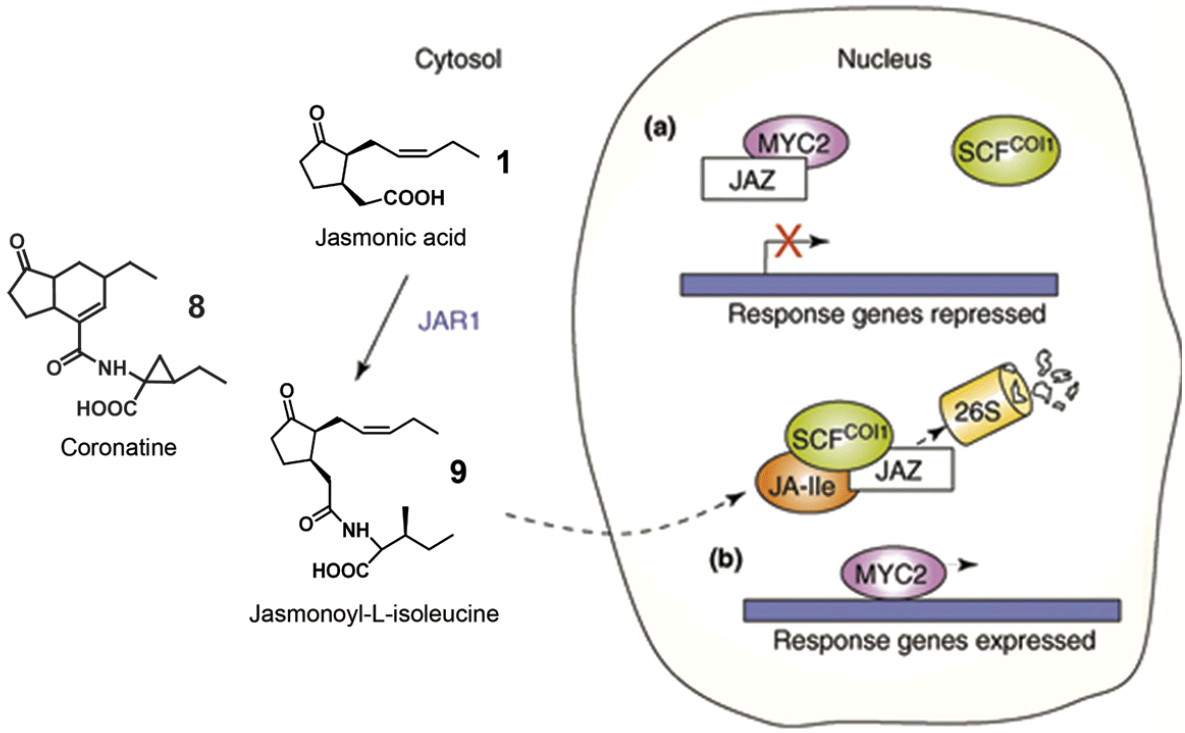


Figure 3. Model for COI1-JAZ jasmonate signalling in *Arabidopsis:* a) JA-Ile promotes SCF^{COI1} interaction with JAZ transcriptional repressors; b) this interaction leads to JAZ ubiquitination and subsequent degradation by 26S proteasome, which releases the MYC2 transcription factors (modified after Staswick 2007 [65]).

As the mechanism shows, not only JA is essential for signal transduction, but so is the JA derivative – JA-Ile. Amino acid conjugates of jasmonic acid were initially thought to be products of JA metabolism, and their formation was correlated with JA inactivation (**Fig.4**).[66, 67] The elucidation of their role points to the possibility that other naturally occurring jasmonates are also involved in important biological activities.

Especially interesting in this regard is the immediate precursor of (**1**), 12-oxophytodienoic acid (OPDA) (**6**). OPDA has been reported several times to play an important role in plant defence mechanisms, mainly by fine-tuning JA's responses, and by eliciting different effects that are independent from JA.[54, 68-73] In contrast to JA, (**6**) contains aṇ α,β-unsaturated carbonyl structure, which is highly reactive in the Michael addition reactions and thus makes OPDA potentially toxic.[74] The electrophilic properties of (**6**) have been addressed in a number of reports.

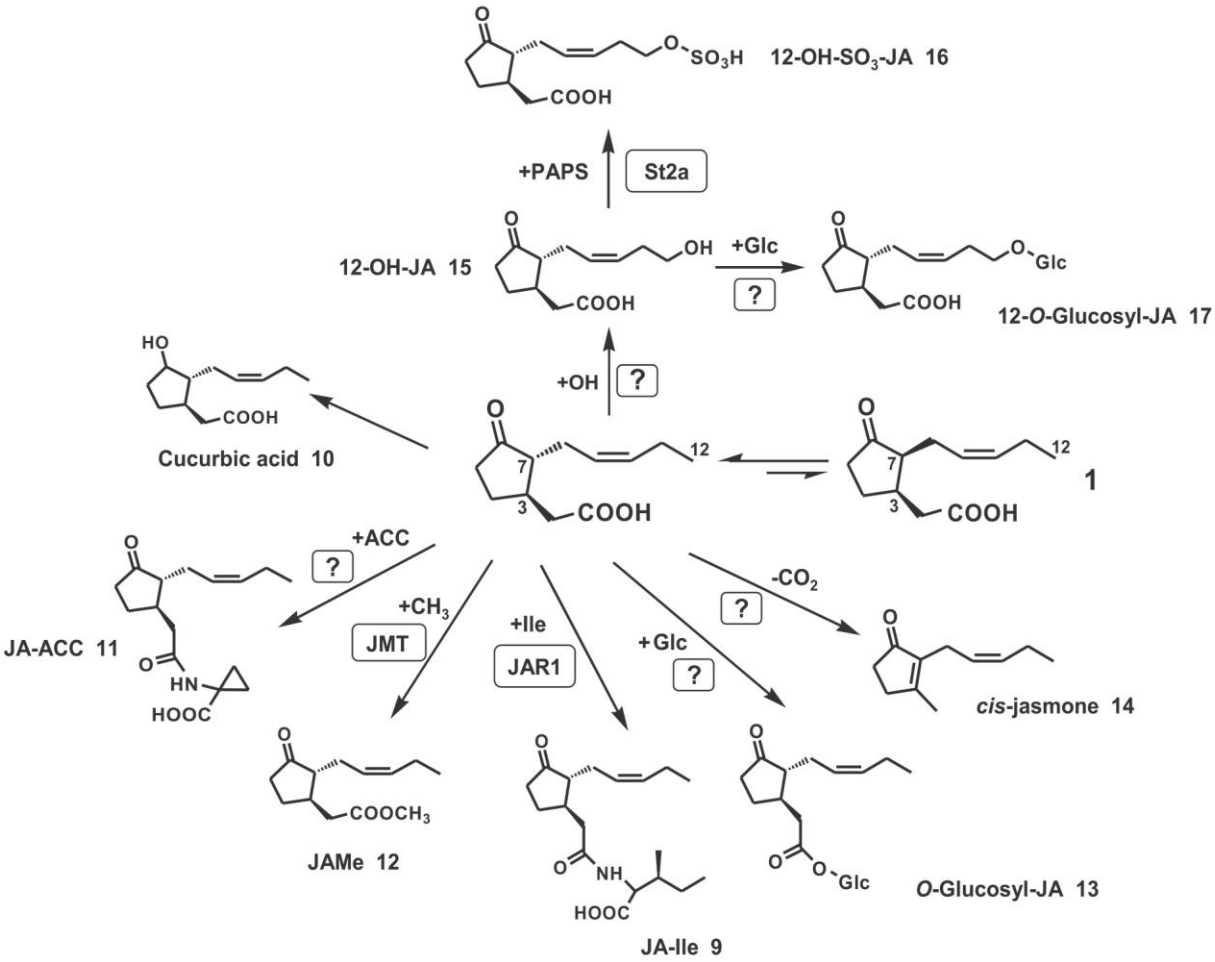
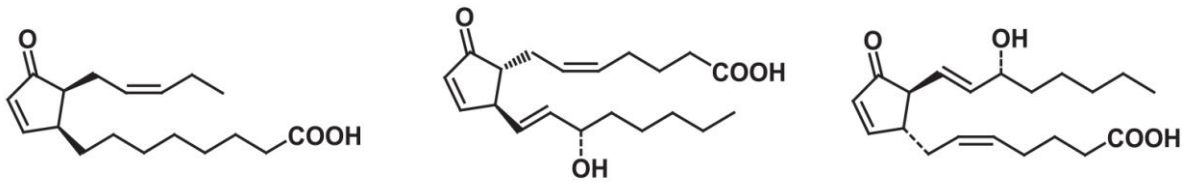


Figure 4. The metabolic fate of jasmonic acid (modified after Wasternack 2007 [63]). Newly biosynthesized *cis*-JA is easily transformed to *trans*-JA by isomerization, resulting in a molar equilibrium of about 9:1.[66, 75] *Trans*-JA can be further metabolized to 1) cucurbic acid (**10**) by reducing the keto group [66, 76] and further to cucurbic acid-*O*-glucoside [66], 2) JA-ACC (**11**) by conjugating the carboxylic acid side-chain to ethylene precursor 1-amino cyclopropane-1-carboxylic acid (ACC) [67], 3) JAMe (**12**) by JA methyl transferase (JMT) [77], which can act as an endogenous as well as a volatile signal, 4) JA-Ile (**9**) by JAR1 [67] (*A. thaliana*) or by JAR4 [78], 5) jasmonoyl-1-β-glucose (**13**) and similar derivatives [79], 6) *cis*-jasmone (**14**) by decarboxylation [80], 7) 12-OH-JA (**15**) by hydroxylation at C-12 of the pentenyl side chain and further to (**16**) or (**17**) by corresponding sulfation or glucosylation.[66, 79, 81, 82] Most of these transformations reduce the activity of (**1**). The effect, however, can be just opposite, as in the case of (**9**), or the exact activity and correlated function of the putative metabolite may not yet be fully elucidated (see *cis*-jasmone [83]).

Stinzi *et al*. [69] observed an induction of GST1 (glutathione *S*-transferase) gene in *A*. *thaliana opr3* mutant exclusively after treatment with OPDA, which was attributed to the chemical properties of the cyclopentenone ring. The conjugation of OPDA with glutathione, indicated by the induction of the GST1 gene, was proven by Davoine *et al*.[84, 85] The authors found an OPDA-GSH adduct and showed that it

accumulated transiently in response to cryptogein elicitation in tobacco leaves. Also, more recently, the *in vivo* accumulation of the OPDA-GSH conjugate was reported in leaves of *Arabidopsis thaliana*, following a *Pseudonomas syringae* infection.[86] According to both groups, the conjugation of OPDA with glutathione represents one of the metabolic disposal routes for the products of the lipoxygenase pathway. Additionally, uniquely, OPDA was found in *A. thaliana* not only as free acid (or its methyl ester) but also esterified to galactolipids in the form of the so-called arabidopsides.[87-90] The diversity of esterified OPDA and its large amounts found in lipid membranes, which can be easily released in response to wounding, raise the question as to its biological functions. One of the arabidopside types – arabidopside E - was recently found to accumulate up to 8% of the total lipid content in the defense reaction to bacterial pathogen and to inhibit bacterial growth *in vitro*.[91]

The octadecanoid-derived signaling pathway in plants has a parallel system in mammals, where prostaglandins and related eicosanoids are being synthesized from arachidonic acid (20:4). The similarities refer not only to the structural features (**Fig.5**), but also to the function of the resulting compounds. Prostaglandins in mammalian organisms, like jasmonates in plants, are responsible for mediating and regulating inflammatory events and pain responses.[92] It is thus reasonable to assume that since in both, plants and animals, the defense systems are based on lipid-derived signaling compounds, they might have developed from common ancestral organisms along a divergent evolutionary path.[93]



***cis*-(+)-12-oxophytodienoic acid 6** **Prostaglandin A_2 18** **Prostaglandin J_2 19**

Figure 5. Example of structural similarities between octadecanoid-derived plant signals and eicosanoid-derived animal signals.

1.1.2. Oxylipin-related signals

Next to the thoroughly studied jasmonate pathway, there are several other biosynthetic pathways, all originating from linole(n)ic acid. These lead to a myriad of oxygenated fatty acid compounds, collectively known as oxylipins. Though the primary role in plant defense responses is attributed to the jasmonates, it is

becoming obvious that an adequate reaction to many differing stress stimuli can require corresponding differential signals. Generally oxylipins can be formed either in enzymatic or non-enzymatic pathways. In the case of enzymatic pathways, the substantial branching leading to various compounds happens on the level of LOX-generated hydroperoxides. They can be further transformed either by the allene oxide synthase pathway mentioned above (leading to OPDA and JA), or by the peroxygenase pathway (which might generate epoxides, epoxy alcohols), or by lyase pathway (leading to aldehydes and oxo-acids) (for a detailed review, see Blee 1998[94] and 2002 [95]). The functions of all oxylipin compounds have not been yet elucidated. Nevertheless almost undisputed is the significance of aldehydes – products of the lyase pathway, e.g. leaf aldehyde (2(*E*)-hexenal), which can serve as insect attractant [96] but also exhibit antifungal activities [97] or traumatin (12-oxo-10(*E)*-dodecanoic acid); the latter was suggested to trigger cell division near the wounding site, leading to the development of a protective "layer" around it.[98] Non-enzymatically formed oxylipins are also referred to as phytoprostanes (as analogues of mammalians' isoprostanes) [99, 100] and have been reported to accumulate in plants in response to oxidative stress and to induce phytoalexin accumulation [101] (for a detailed review see Müller 2004 [102]).

1.1.3. Salicylic acid

While the function of jasmonates is usually correlated with plants' responses to herbivores, wounding stress and necrotrophic pathogens, SA is known to act as a signal for systemic acquired resistance (SAR) [103] and hypersensitive response (HR) in plants infected with biotrophic pathogens.[104, 105] A hypersensitive response is characterized by events that help to locally restrict pathogen growth, such as the localized death (necrosis) of host tissue around the site of infection. SAR, on the other hand, respresents the long-term development of enhanced resistance to a secondary infection, which is manifested throughout the plant.[106-108] Naturally both of these responses involve a significant amount of biochemical and molecular changes, as for instance: the synthesis of hydroxyproline-rich glycoproteins, cellulose, lignin, meant to fortify the cell walls; or the synthesis of pathogenesis-related (PR) proteins, which include e.g. β-1,3-glucanases or chitinases that degrade the cell walls of microbes.[109] Like jasmonates, which act as a general signals to mediate the wound response, SA was shown to induce PR proteins in a wide range of plants (for review

see Raskin 1992 [110], Klessig and Malamy 1994 [111], Loake and Grant 2007 [112]) and to act as a traveling signal for SAR.[113-116]

Recent findings shed light on the biosynthesis of SA. It seems that the prevalent amounts of (**2**) are synthesized from chorismate (**20**) (originating from shikimate pathway) via isochorismate (**21**).[117, 118] Nevertheless, an earlier proposed route [119, 120] starting from phenylalanine (**22**) through *trans*-cinnamic acid (**23**) (involving phenylalanine ammonia-lyase (PAL)), and benzoic acid (**24**) may also be operative (**Fig.6**). Free salicylic acid is regarded as a signalling molecule. Its glucose esters and glucosides, which are detected as well in plant material, are considered to represent a storage form of a phytohormone.[121] A methylation of (**2**) [122] results in the formation of volatile methyl salicylate, which like JAMe can act as endogenous or as volatile signal.[123-125]

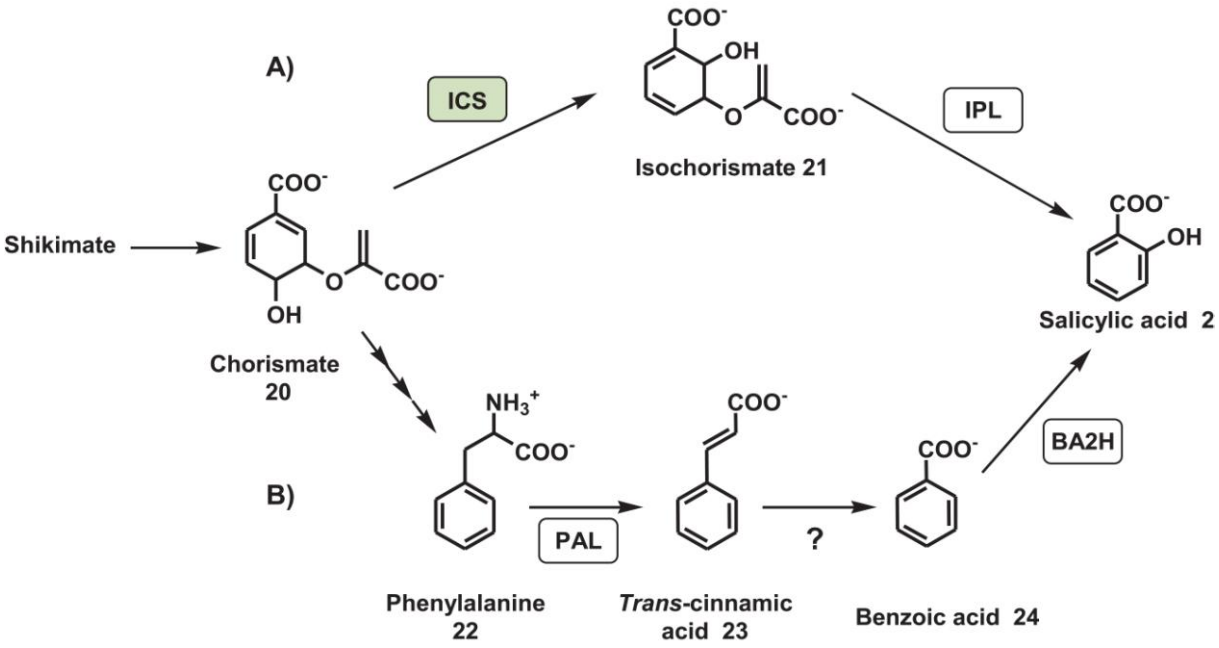


Figure 6. Proposed pathways for biosynthesis of SA in plants: A) from chorismate via chloroplast-localized isochorismate synthase (ICS) to isochorismate, which is then transformed by isochromate pyruvate lyase (IPL) to SA; B) from phenylalanine via phenylalanine ammonia lyase (PAL) to *trans*-cinnamic acid, which is further converted to benzoic acid, subsequently hydroxylated to SA by the activity of benzoic acid-2-hydroxylase (BA2H).

The exact mode of action of SA is not fully elucidated.[126] It is known that SA signalling is mediated by at least two pathways: NPR1 (NON EXPRESSOR OF PR1) dependent and NPR1 independent. It has been shown that SA enables and enhances the interaction of the NPR1 protein with a specific transcription factor, which in turn leads to the expression of PR genes.[127, 128] The NPR1 independent

pathway seems to be very complicated and its actual course is uncertain.[112, 126] Moreover, SA can specifically bind to a variety of plant proteins; its binding affects their activity, as in the case of SA-binding proteins (SABP).[129, 130] These proteins have been shown to possess catalase activity, which is blocked after SA binding.[129, 131] Inhibited catalase activity can in turn lead to the accumulation of H_2O_2 and other reactive oxygen species (ROS), which can elicit various defence responses [132], including the activation of PR genes.[105, 111]

1.1.1.4. Signalling network in stressed plants

The signalling network in plants is activated in response to various stress stimuli. In the case of a herbivore attack it is the inflicted wound and the insect's oral secretion which acts as an additional elicitor.[10, 133, 134] Different elements of the caterpillar's regurgitate have been shown to activate plant responses, e.g. β-glucosidase from *Pieris brassicae* [135], glucose oxidase [136] or the extensively studied fatty acid amino acid conjugates (FACs), such as volicitin (*N*-(17-hydroxylinolenoyl)-L-glutamine).[137-140] The reception of this primary signal is then followed by a cascade of different signal transduction events, including the depolarization of membrane potentials and intracellular calcium influx [141], the generation of ROS [142] or an induction of mitogen-activated protein kinases (MAPK) [143], both of which have been proposed to be the early activators of JA and SA pathways. Unfortunately our knowledge about these early signalling steps is still incomplete.[26]

JA- and SA-mediated stress responses employ a set of different elements in their mode of action. Since the activation of defense strategies is energetically costly [144, 145], plants adjust the jasmonic acid, ethylene and salicylic acid pathways in ways that are most economically tailored to particular attackers. The interactions between JA, SA end ET can therefore be antagonistic, cooperative or synergistic, depending on the plant species and the combination of invaders (for review see Rojo 2003 [146], Koornneef 2008 [147]).

In most of the cases, JA and the ethylene signaling pathway act synergistically [148, 149], especially in response to pathogens.[150-152] It has been shown that the expression of the ETHYLENE RESPONSE FACTOR (ERF1) requires both JA and ethylene signaling and that ethylene and JA treatment have a synergistic effect on the expression of ERF1.[153]

On the other hand, most of the reported cases of cross-talk between the SA and JA pathways are negative interactions.[154-156] SA was thought to block JA biosynthesis in the same way the salicylic acid derivatives block the prostaglandin synthesis in animals.[157-159] The actual mechanism seems to be more complicated than expected.[146, 147, 160] In addition SA suppresses JA-dependent signaling.[161, 162] Over the years, several molecular components of the SA/JA cross-talk have been elucidated (for a recent review see Koornneef 2008 [147]). Cases of cooperative and synergistic interactions between JA/ET and SA pathways have also been reported.[148, 163-168] It may be that the outcome of the interactions among the pathways is concentration or organ specific, which would allow responses to different attackers to be fine-tuned.[147, 166]

1.2. Insect counter-adaptations

The concept of a co-evolutionary arms race between insect and plant kingdoms, originally proposed by Ernst Stahl [1] and then propagated by Ehrlich and Raven [169], has been referred to repeatedly over the last 30 years.[170-174] The study of plant-insect co-evolution has focused primarily on the biochemical adaptations of insects to plants' secondary metabolites.[174, 175] Herbivore adaptations to plant defenses can, however, expand to include mechanical adaptations [176], behavioral adaptations [177, 178], use of microbial symbionts [179] or host manipulation.[178] In order to realize a variety of these strategies I would like to bring up several examples.

One of the most economical solutions used by herbivores is simply to avoid ingesting dangerous compounds with special feeding strategies, as in the case of red milkweed beetles (*Tetraopes tetrophthalmus)*, which feed on the leaves of the common milkweed (*Asclepias syriaca*).[178] Since the plant contains significant amounts of harmful latex, the larvae starts feeding by cutting through latex ducts, which in turn depletes the amount of dangerous leaf exudates. If the ingestion of the toxic substance is inevitable, insects also employ various strategies. A relatively uncomplicated solution is a neutralizing strategy employed by a privet moth larvae (*Brahmaea wallichii*), a specialist on privet trees (*Ligustrum obtusifolium*). The insect selectively secretes high amounts of free glycine, a neutralizer of the defense chemical oleuropein, into its digestive juice.[180] In this case, glycine competes in an alkylation reaction with the amino residue in the side chain of lysine and thereby inhibits the protein-degenerating activity of oleuropein, a phenolic secoiridoid

glycoside. Some insects have learned to use their microbial symbionts to lower the defensive mechanism of the host plant, as is the case for several species of bark beetles (*Ips typographus*), which introduce the blue stain fungi (genera: *Ceratocystis*, *Ophiostoma*) into trees before feeding. The pathogen helps to minimize the defense mechanism of the host tree – the Norway spruce (*Picea albies*).[179] Even more exquisite are adaptations that allow insects to sequester toxic substances, store them and use them for their own defense. One of the first examples of such an adaptation was described for the larvae and adults of monarch butterfly (*Danaus plexippus*), whose Na^+/K^+ ATPases are insensitive to cardenolides of *Asclepia spp*.[181] These compounds are ingested and stored for the insect's defense. Along secondary metabolites' plants' constitutive defenses include proteinase inhibitors, for which insects have also evolved corresponding adaptations [182], for example, the larvae of the generalist fall armyworm (*Spodoptera frugiperda*) have an altered complementation of proteoloytic enzymes in the midgut, where they express higher amounts of trypsine, less susceptible to the action of soybean PIs.[183]

Indisputably, however, the use of specialized enzymes to detoxify compounds is from a biochemical point of view the most interesting way of insects counter adaptations to plant-derived deterrent or toxic substances. In this regard detoxification enzymes include P450s [184], oxidases (flavin monooxygenases, dehydrogenases), hydrolases (carboxyesterases, epoxide hydrolases), group transfer enzymes (glutathione *S* transferases (GSTs), glycosyl transferases, sulfotransferases). The largest of these groups of detoxification enzymes are the P450s. To date the most thoroughly studied example of their involvement is the metabolism of furocoumarins (i.e. xanthotoxin) by the group of a specialist caterpillars from the genus *Papilio* (black swallowtail (*Papilio polyxenes*)), through the action of P450 cytochrome monooxygenase.[185-187] Interestingly, a similar mechanism, based on the action of the same enzyme class, has been reported for a generalist insect, *Helicoverpa zea*, which "learned" to feed on *Apiaceae* and *Rutaceae* plants containing furocoumarins.[188, 189] Another interesting example of specialized detoxifying enzymes comes from the larvae of arctiid moth (*Tyria jacobaeae*), which feed exclusively on the pyrrolizidine alkaloid (PA)-containing ragwort (*Senecio jacobea*). Ingested PAs are efficiently *N*-oxidized in the hemolymph of *T. jacobeae* by senecionine *N*-oxygenase, a flavin-dependent monooxygenase.[190] In addition, specialists on plants with glucosinolate-myrosinase defense systems have developed

different detoxification proteins: diamondback moth (*Plutella xylostella)* larvae possess specific glucosinolate sulfatase, which desulfates glucosinolates, producing metabolites that no longer act as substrates for myrosinases.[191] In contrast the pierid butterfly (*Pieris rapae*) features nitrile-specifier protein, which promotes the formation of nitrile breakdown products; instead of toxic isothiocynates arising from myrosinase catalyzed glucosinolate hydrolysis.[192]

1.2.1. Insect GSTs

Insects also make use of a special group of multifunctional detoxifying enzymes known as glutathione *S*-transferases (EC 2.5.1.18) (for review see Yu 1996 [193], Ranson and Hemingway 2005 [194]). In the insect kingdom their importance is associated with resistance to insecticides and allelochemicals [193, 194], as GSTs can metabolize insecticides by conjugation with reduced glutathione; this process produces water-soluble metabolites that are more readily excreted, as in the case of organophosphorous insecticides.[195] In addition to detoxifying insecticides, GSTs are used by lepidopteran insects to metabolize various toxic allelochemicals, including isothiocyanates [196], organocyanates [197] or a variety of α,β- unsaturated compounds, for instance *trans*-cinnamaldehyde [198] 2(*E*)-hexanal, or benzaldehyde.[199] In addition to their “regular” function, catalyzing interactions between reduced glutathione (GSH) and electrophilic agents, many GSTs have other targets, for example, reduction of trinitroglycerin, thiolysis of 4-nitrophenyl acetate, isomerization of maleylacetoacetate or Δ^5–3- ketosteroids, as well as converting PGH_2 to PGD_2.[200]

The diverse functions of GSTs are also clearly displayed in their genetic characteristics. Insect cytosolic GSTs alone have been assigned to at least six different classes: Delta, Epsilon, Omega, Sigma, Theta and Zeta [201, 202]; among these, Delta and Epsilon include the majority of GSTs implicated in xenobiotic metabolism.[202] Omega, Sigma, Theta and Zeta classes have on the other hand a much wider taxonomic distribution and play more likely essential housekeeping roles.[201, 203]

Since insect GSTs metabolize various toxic allelochemicals, they can be expected to play an important role in the feeding strategies of lepidopterans. The highly polyphagous insects are thought to possess multiple GSTs, which help detoxify the diverse toxic allelochemicals found in their host plants. On the other

hand, specialist insects, which have a narrow range of host plant species and so encounter more specific allelochemicals, are thought to have fewer GSTs.[193]

1.2.1. Plant “signaling” molecules

Understanding the development of insects’ adaptations focused primarly on toxic plants’ secondary metabolites. These are difficult for the insects to avoid if they determine a part of a constitutive defense. However, when these defenses are activated by external attack - meaning, they are a piece of plant-induced defenses - insects which are able to “sense” their presence and thus have time to activate their own defense systems would be privileged. The signaling role in plants is carried out as previously explained (see 1.1.1) by plant hormones, namely JA (and jasmonates) and SA. Since in the course of feeding on plants, insects ingest their hormones, these represent good candidates for early cues warning herbivores about accumulating plant toxins. In course of evolution, in other words, plant signaling molecules could also have evolved into insect signaling molecules. This intriguing hypothesis was first proposed by the Berenbaum group in 2002.[204] They observed a significant upregulation in transcripts of four cytochrome P450 genes of the corn earworm (*Helicoverpa zea*) in response to feeding on artificial diet implemented with either JA or SA. Though there are some weak points in both their experimental setup (which relied on high concentrations of JA) and in their subsequent reasoning (only the transcripts of four very general P450s were measured), researchers in this study asked a very important question about possibility of advanced “eavesdropping” of insects on plant defensive system.

1.3. Goals of this study

Given the indisputable importance of phytohormones, especially jasmonates, in plant responses to stress, it is crucial to be able to monitor changes in their accumulation throughout the stimulus events. Several analytical methods have been introduced to reliably quantify plant hormones.[75, 205-209] Since in all cases the compounds of interest have to be extracted from the plant material, many of these methods confront the analytes with harsh extraction conditions such as heat [205, 210] or acidic pH.[207] Such treatments can result in unpredictable structural changes, for example, isomerizations, which modify the nature of the original compound and therefore falsify the extraction results. Moreover, amounts of phytohormones present

in the plant material are often limited to ranges of few ng per gram of fresh weight, which puts additional requirements on the extraction procedures.

In previous work, Dr. Birgit Schulze established a reliable analytical method for analysis and quantification of the significant phytohormones in plant material.[211] This approach is based on *in situ* derivatization of the analytes with *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA), which readily reacts with carbonyl groups of the jasmonates to form stable imines. This modification secures the stereochemical identity of the original analyte throughout later extraction procedure. Moreover, the PFB moiety, due to its high electronegativity, allows the "negative chemical ionization" (NCI) modus to be used in the subsequent mass spectrometry analysis, which in turn greatly enhances the sensitivity of the method. A further purification step implements aminopropyl cartridges; these selectively bind the carboxyl functions of the jasmonates, which enriches the sample and purifies it from the irrelevant substances such as chlorophyll.

The development of a reliable analytical method allowed more detailed studies of jasmonates to be made. These studies include:

(1) Determination of jasmonates signaling regulation in different plant families and species in response to various stress stimuli, i.e.: characterization of time-dependent accumulation patterns. Interesting is also comparison of jasmonate's accumulation in response to continuous mechanical wounding (implemented with a recently developed mechanical caterpillar, MecWorm) and in response to herbivore feeding – a study which could help separate the impact of simple wounding event from the impact of additional herbivore elicitors such as FACs, thus clarifying the plant's signaling network.

(2) Identification of the potential involvement of jasmonates in plant-insect interactions, following the idea proposed by Li *et al*.[204] Analysis of the profile of the plant-derived phytohormones in the insect gut could help asses their putative role and mode of action in the insects' "eavesdropping" on plant defensive system. Moreover, considering the structural similarities between jasmonates and prostaglandins, an investigation of the fate of plant octadecanoids in the herbivore gut could shed a light on their possible interference with eicosanoid related receptors in insects.

2. Thesis outline - List of articles and manuscripts and author's contribution

Unpublished results Part I

Insect Stealth feeding: A Specialist Herbivore Manipulates Plant Defense Responses

Heiko Vogel, Paulina Dąbrowska, Axel Mithöfer, Jürgen Kroymann, Maritta Kunert, Wilhelm Boland

in preparation

Unpublished results Part I present the results of a set of experiments performed with a mechanical caterpillar, MecWorm. The goal of this study was to investigate the role of mechanical wounding in JA mediated response to herbivory on the level of gene expression and the phyto-hormone amounts on the example of *A. thaliana*. The effects of continuous mechanical wounding were compared with the wounding produced by a specialist insect – diamond back moth (*Plutella xylostella*). Interestingly, significant discrepancies between these two different modes of wounding were found relating to gene expression and levels of jasmonic acid and its immediate precursor OPDA.

I and Maritta Kunert were responsible for extraction of the plant material and quantification of the phytohormone levels.

Unpublished results Part II

The phytohormone precursor OPDA is isomerized in the insect gut by a single, specific Glutathione *S*-transferase

Paulina Dąbrowska, Dalial Freitak, Heiko Vogel, David G. Heckel, Wilhelm Boland

In preparation

These results embrace the work on the identification of an enzyme responsible for the isomerization of 12-oxophytodienoic acid – a phenomenon described in Article III. A hypothesis on the involvement of Glutathione *S*-transferases in this transformation process is tested and confirmed by proving the OPDA isomerization activity of purified GST fractions from the gut of two insect species: *Spodoptera littoralis* and *Helicoverpa armigera*. Further identification of the specific OPDA isomerase is based on heterologous expression of 16 putative GST proteins from *Helicoverpa armigera* in *E. coli* and subsequent incubation of the expressed proteins with 12-oxophytodienoic acid. Bioassays suggest the presence of a putative OPDA isomerase candidate within the tested proteins.

The described work was performed in collaboration with Dalial Freitak, who performed substantial parts of protein expression in *E. coli* and Dr. Heiko Vogel, who provided expertise on biochemical aspects of this work. I planned the experiments, performed OPDA incubation assays and worked on protein expression with Mrs. Freitak.

Article I

Effects of Feeding Spodoptera littoralis on Lima Bean Leaves IV: Diurnal and Nocturnal Damage Differentially Initiate Plant Volatile Emission

Gen Arimura, Sabrina Köpke, Maritta Kunert, Veronica Volpe, Anja David, Peter Brand, Paulina Dąbrowska, Massimo Maffei, Wilhelm Boland

Plant Physiology (2008) **146**, 965-973

This manuscript describes a study on JA-mediated volatile emission (i.e. β-ocimene and 3(*Z*)-hexenyl acetate) from leaves of lima bean (*Phaseolus lunatus*) continuously damaged with the mechanical caterpillar - Mec Worm. Qualitative and quantitative differences between nocturnal and diurnal volatile emission were compared and correlated with the differences in JA accumulation and expression of the *Pl*OS gene (*P. lunatus* β-ocimene synthase). Quite surprisingly, nocturnal damage caused significantly higher levels of JA along with enhanced expression of the *Pl*OS gene. Results from this study clearly show that the expression of the “volatile-producing” *Pl*OS gene is controlled by damage-dependent JA levels.

I and Peter Brand were responsible for plant material extraction, quantification of the JA levels and evaluation of the results. I wrote the part of the experimental section concerning JA extraction and quantification procedure.

Article II

Functional Identification and Differential Expression of 1-Deoxy-D-Xylulose 5-Phosphate Synthase and Other MEP Pathway Genes in Induced Terpenoid Resin Formation of Norway spruce (*Picea abies*)

M. Phillips, M.H. Walter, S. Ralph, P. Dąbrowska, K. Luck; E.M. Urós, W. Boland, D. Strack, M. Rodríguez-Concepción, J. Bohlmann, J. Gershenzon

Plant Molecular Biology (2007) **65**, 243-257

This manuscript presents a detailed study of the 1-deoxy-D-xylulose 5-phopsphate synthase (DXS), an enzyme, which catalyzes the first step of the biosynthetic pathway leading to terpenoids and terpenoid-based oleo-resins; these are one of constitutive and inducible defenses against herbivores in conifers. The main part of this work was conducted with Norway spruce (*Picea* albies) cell cultures, which were treated with different elicitors (i.e.: chitosan). The up-regulation of synthase gene transcripts, the extent of enzyme activity and the accumulation of induced jasmonates (JA and OPDA) were then observed and measured.

In this cooperative project I was responsible for extraction and quantification of JA and OPDA in cell culture samples. I wrote the part of the experimental section as well as the part of results concerning the found amounts of octadecanoic signals.

Article III

Rapid Enzymatic Isomerization of 12-Oxophytodienoic Acid in the Gut of Lepidopteran larvae

Birgit Schulze, Paulina Dąbrowska, Wilhelm Boland

ChemBioChem (2007) **8**, 208-216

This manuscript is a detailed study on the fate of plant-derived oxylipins in the gut of a feeding herbivore. The pattern of oxylipins produced in the leaves of the lima bean (*Phaseolus lunatus*) plants in response to feeding by Egyptian cotton leaf worm (*Spodoptera littoralis*) was compared with the pattern of these compounds ingested and excreted by the insect. Surprisingly, unlike the majority of oxylipins, 12-oxophytodienoic acid (OPDA) – the immediate precursor of important plant hormone – jasmonic acid was not detectable in the insect's gut. Instead, the structurally related double bond isomer tetrahydrodicranenone B (*iso*-OPDA) was identified. Experiments such as feeding studies with deuterium labeled OPDA proved that the observed OPDA isomerization is catalyzed by an unknown gut enzyme.

The manuscript was based on the previous work from Dr. Birgit Schulze, who measured spatial distribution of oxylipins in the herbivore damaged leaves and performed initial analysis of oxylipins in the insect frass and regurgitate, which led to the finding of *iso*-OPDA. I was responsible for the synthesis of Tetrahydrodicranenone B for structure confirmation, for the *in vitro* assays of OPDA isomerization, for the feeding experiments and analysis of the results. The synthesis of deuterium labeled OPDA used for feeding experiments was performed by me and Dr. Schulze. The first draft of the manuscript was written by Dr. Birgit Schulze and later refined by Prof. Wilhelm Boland.

Article IV

Iso-OPDA: An Early Precursor of *cis*-Jasmone in Plants?

Paulina Dąbrowska, Wilhelm Boland

ChemBioChem (2007) **8**, 2281-2285

This manuscript presents a hypothesis concerning a novel biosynthetic pathway leading to an important plant volatile compound, *cis*-jasmone. The proposal is based on an experimental observation, in which the administration of aqueous emulsions of Tetrahydrodicranenone B (*iso*-OPDA) to plantlets of several plant species resulted in the emission of *cis*-jasmone. A similar result was obtained with a yeast culture (*Saccharomyces cerevisiae).* Accordingly, the transformation of *iso*-OPDA to *cis*-jasmone is relatively general and requires only a functional peroxisomal β-oxidation system. The novel pathway implies that the jasmonic acid pathway branches, using the putative plant isomerase (parallel to insect's) converting 12-oxophytodienoic acid to *iso*-OPDA, which is further degraded by β-oxidation and subsequently decarboxylated to give *cis*-jasmone.

I discovered the transformation of *iso*-OPDA to *cis*-jasmone in plant tissues and performed all the initial experiments. The first draft of the manuscript was written by me and then modified by Prof. Wilhelm Boland.

3. Unpublished results Part I

Insect Stealth feeding: A Specialist Herbivore Manipulates Plant Defense Responses

Heiko Vogel, Paulina Dąbrowska, Axel Mithöfer, Jürgen Kroymann, Maritta Kunert, Wilhelm Boland

In preparation

Plants as immobile organisms are challenged by a multitude of pathogens and insect herbivores and thus need to recognize the attacker to “organize” an appropriate defence response. To separate physical and chemical signals originating from insects during herbivory, a mechanical device (MecWorm) was developed, which is programmable to replicate very closely the pattern, time course, and quantity of damage caused by different insects. With help of this device we were able to discover that the impact of continuous mechanical damage on the plant defence response has been largely underestimated, necessitating a very cautious rating of the different individual stimuli emanating from insects during herbivory.

Moreover, we could show that larvae of the crucifer specialist, *Plutella xylostella* (Diamondback Moth, Lepidoptera: *Plutellidae*) when feeding on *Arabidopsis thaliana,* suppresses the production of Jasmonic acid (JA) and its precursor 12-oxophytodienoic acid (OPDA), the major phytohormone orchestrating a plethora of defence responses in plants. Our results demonstrate that *Plutella xylostella* can strongly interfere with plants defence responses both at the transcriptional and the phytohormone level. These findings shed light on evolution of plant-insect interactions and suggest highly sophisticated and complex co-evolutionary interactions.

Results

Global Expression analysis in MecWorm-wounded versus *P. xylostella* attacked leaves. To assess the relative contribution of insect-mimicking wounding on induced transcript changes, we compared transcript profiles from *A. thaliana* leaves damaged by *P. xylostella* larvae (a worldwide pest on cruciferous plants), and by MecWorm *versus* undamaged control plants (**Fig.1**). All plants were 4.5 weeks old at the time of the experiments and were raised under the same environmental conditions. Experiments were conducted for 1, 3, and 9 hours, each with 3 biological replicates and randomized between the treatments. Transcript profiles were recorded with *Arabidopsis* whole-genome arrays from Agilent (**Fig.4A**, Tab.1 in Supplementary material 15.1.). We observed a massive impact of both the MecWorm treatment and *P. xylostella* herbivory on the plant transcriptome. After 9 h for example a change in transcript levels of > 5500 genes compared to control plants was detected. Among these, about 70% constituted the same genes identically regulated by MecWorm and *P. xylostella* (**Fig.2**). Most importantly, continuous MecWorm damage reproduced

transcriptional changes for many previously herbivory-assigned genes in a quantitative, time-dependent, and fully repeatable manner (**Fig.4A**, Tab.1 in Supplementary material 15.1.). For selected genes (**Fig.4** below) covering a wide range of basal expression levels in control plants, we confirmed transcript patterns with quantitative RealTime PCR in two further, independent biological replicates (**Fig.4B**, Tab.1 in Supplementary material 15.1.). These genes included *LOX2*, *VSP2*, *TRP1*, two transcription factor genes, a jasmonate-linked *AP2*-like gene, and the coronatine-linked *CORI3* (**Fig.4B**). Hence, in contrast to previously published results [212] these genes are clearly responding to the mechanical impact of the mimicked feeding process and not necessarily to insect-derived chemical cues.

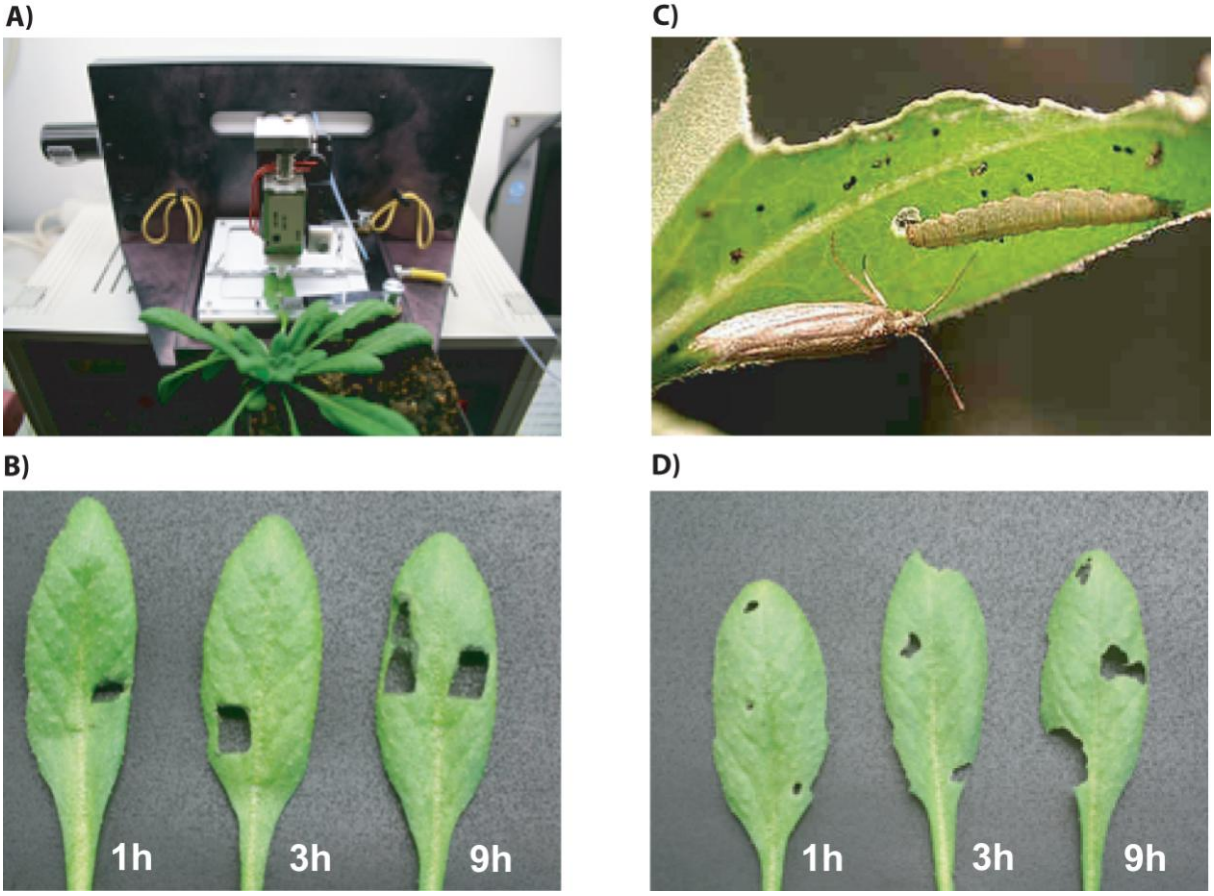


Figure 1. Comparison of the damage inflicted by MecWorm device (A, B) and *P.xylostella* larvae (C, D) on 4.5 week old *A. thaliana* leaves for 1, 3 and 9 hours.

Transcript levels of other genes, however, differed strikingly between MecWorm and *P. xylostella* damage (**Fig.2**), demonstrating that insect-derived chemical cues do play a role in eliciting changes in the plant's transcriptome during insect herbivory. For example, transcript levels from stress-related heat-shock response genes were strongly induced by MecWorm damage but remained nearly unchanged during *P. xylostella* herbivory (Tab.1 in Supplementary material 15.1.), indicating that insect -

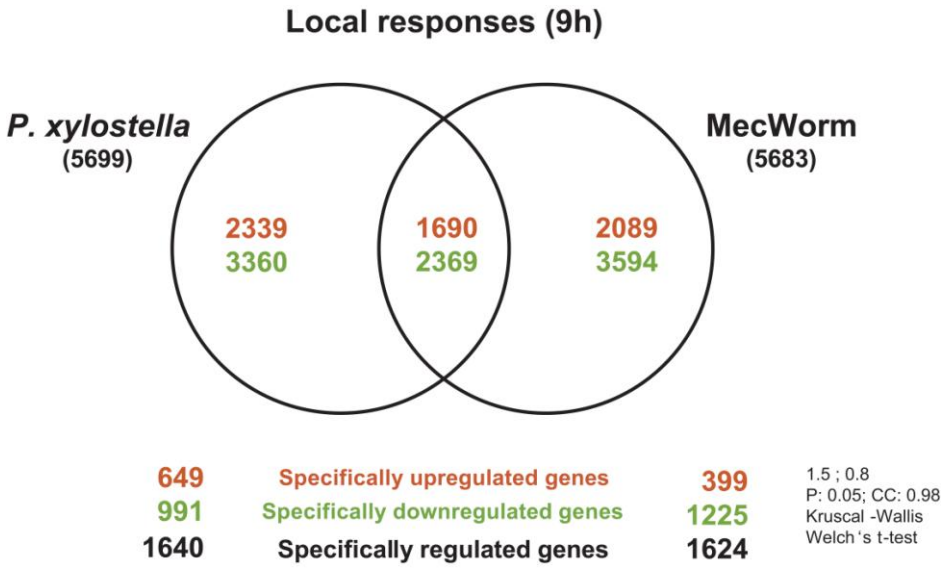


Figure 2. Comparison of the global changes in the transcripts of *A. thaliana* after 9h damage by *P. xylostella* and by MecWorm in locally wounded leaves. Red numbers indicate upregulated genes, green downregulated. From around 5700 differentially regulated genes, c.a. 30% is specifically regulated by MecWorm and *P. xylostella*.

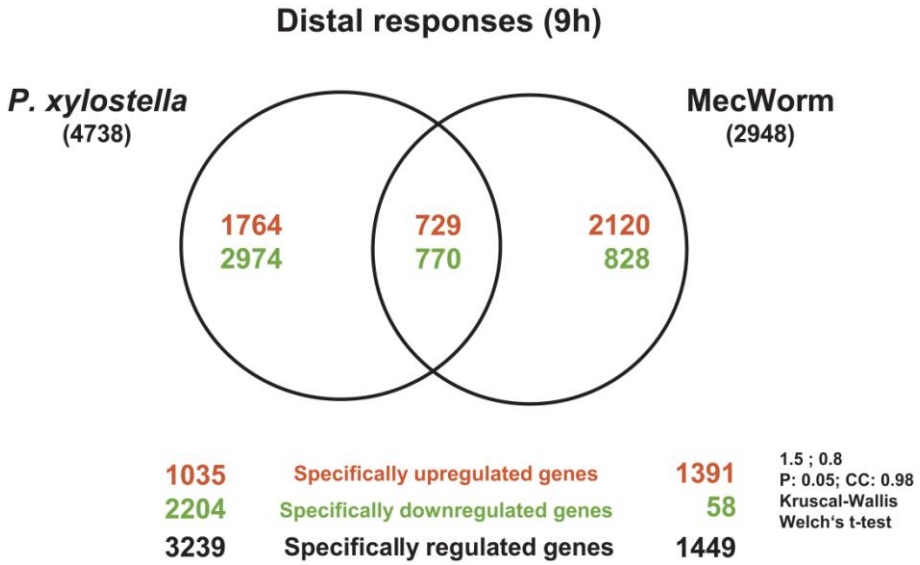


Figure 3. Comparison of the global changes in the transcripts of *A. thaliana* after 9h damage by *P. xylostella* and by MecWorm in distal leaves. Red numbers indicate upregulated genes, green downregulated. *P. xylostella* feeding differentially regulated significantly higher amounts of genes than MecWorm treatment.

- derived chemical factors repressed damage-induced heat-shock gene expression. Even more striking discrepancies were observed in distal responses, in case of which *P. xylostella* feeding caused a change in transcripts of significantly higher amounts of

genes than MecWorm treatment (around 1.5 times more). Moreover, predominant number of these genes (70%) was specifically regulated (**Fig.3**).

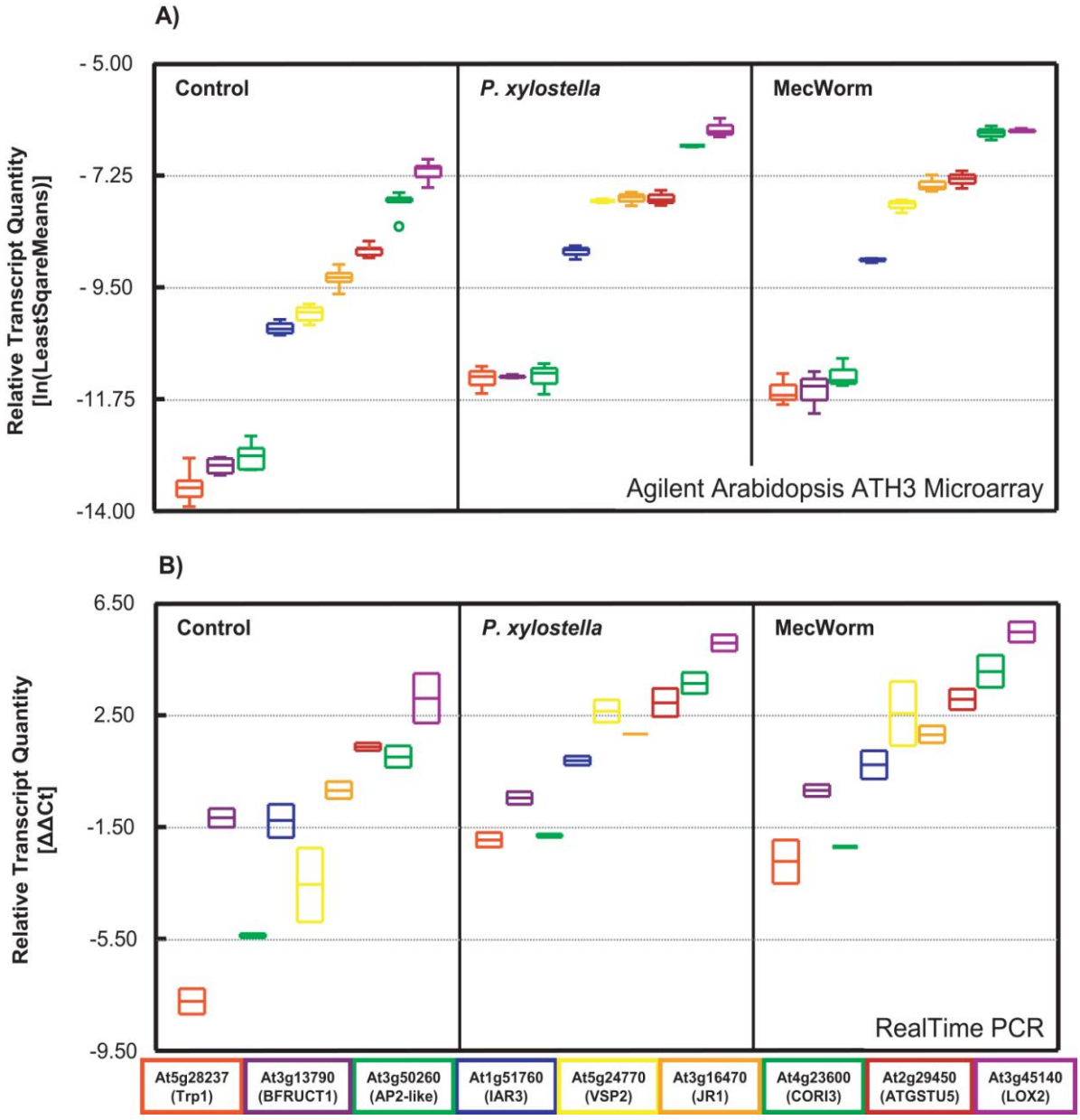


Figure 4. *P. xylostella versus* MecWorm: A) Relative transcript quantities for selected genes (below) in untreated controls, after 3 h of *P. xylostella* herbivory or 3 h of MecWorm damage, determined in microarray hybridization experiments; B) Relative transcript quantities measured with RT-PCR. Log-transformed data for A) and B) were taken from Table 1 in Supplementary material 15.1.

Phytohormone content analysis in locally treated *Arabidopsis thaliana* leaves. To investigate the dynamics of JA, OPDA, and SA production in differentially treated *A. thaliana* leaves and to compare the amounts with the changes in the correlated gene transcripts, we monitored the production of these phytohormones after

MecWorm treatment and feeding of *P. xylostella*. Time points (1, 3, 9 h) for harvesting plant tissue were identical with the ones used for the array experiments. For each experiment locally wounded leaves and untreated control were harvested and shock frozen with liquid nitrogen.

MecWorm treatment induced a clear and strong increase in the production of JA (**Fig.5A**). Already 1 h of wounding time resulted in significant increase of the level of the phytohormone in local leaves when compared to non-treated controls. Longer wounding time corresponding to larger damaged area, namely 3 and 9 h treatment, consequently revealed stronger increase of JA amounts. In comparison, only 9 h of *P. xylostella* feeding produced significantly higher JA amounts compared with control levels. Moreover, the magnitude of JA production was overall much higher in response to MecWorm treatment. Thanks to the analytical method used for JA analysis, we were also able to determine the percentage of *cis*-JA (**Fig.5B**), which is the *de novo* synthesized epimer of JA in the LOX pathway. While the share of *cis*-JA after MecWorm treatment was elevated to around 35-40%, in case of *P. xylostella* feeding the quota of the more active isomer stayed in the low range, which is comparable with the thermodynamical equilibrium between both JA epimers [75].

For OPDA, the biosynthetic precursor of JA, we observed a very similar pattern (**Fig.5C**). Increased OPDA amounts after MecWorm treatment were found after 3 h onward, whereas phytohormone levels after *P. xylostella* feeding were only slightly elevated after 9 h. Additionally, the absolute amounts of OPDA which accumulated after caterpillar feeding were substantially lower than those found after MecWorm wounding.

These initial results suggest a striking discrepancy between MecWorm treatment and herbivore feeding. However, to evaluate the possibility that caterpillars produced lower and not always reproducible damage on the leaf when compared to the computer controlled MecWorm system, we additionally investigated JA (**Fig.6A**) and OPDA (**Fig.6B**) amounts in leaves that have been wounded with MecWorm for 9 h but with lower “wounding frequency”. This treatment, based on extending the delay time between single needle punches, produced within the 9 h treatment three differently damaged areas: 1 (corresponding to 26 sec delay between needle punches and equal to 9 h initial experiment treatment), 0.5 (corresponding to 52 sec delay) and 0.25 (corresponding to 104 sec delay). In case of 0.5 damage amounts of

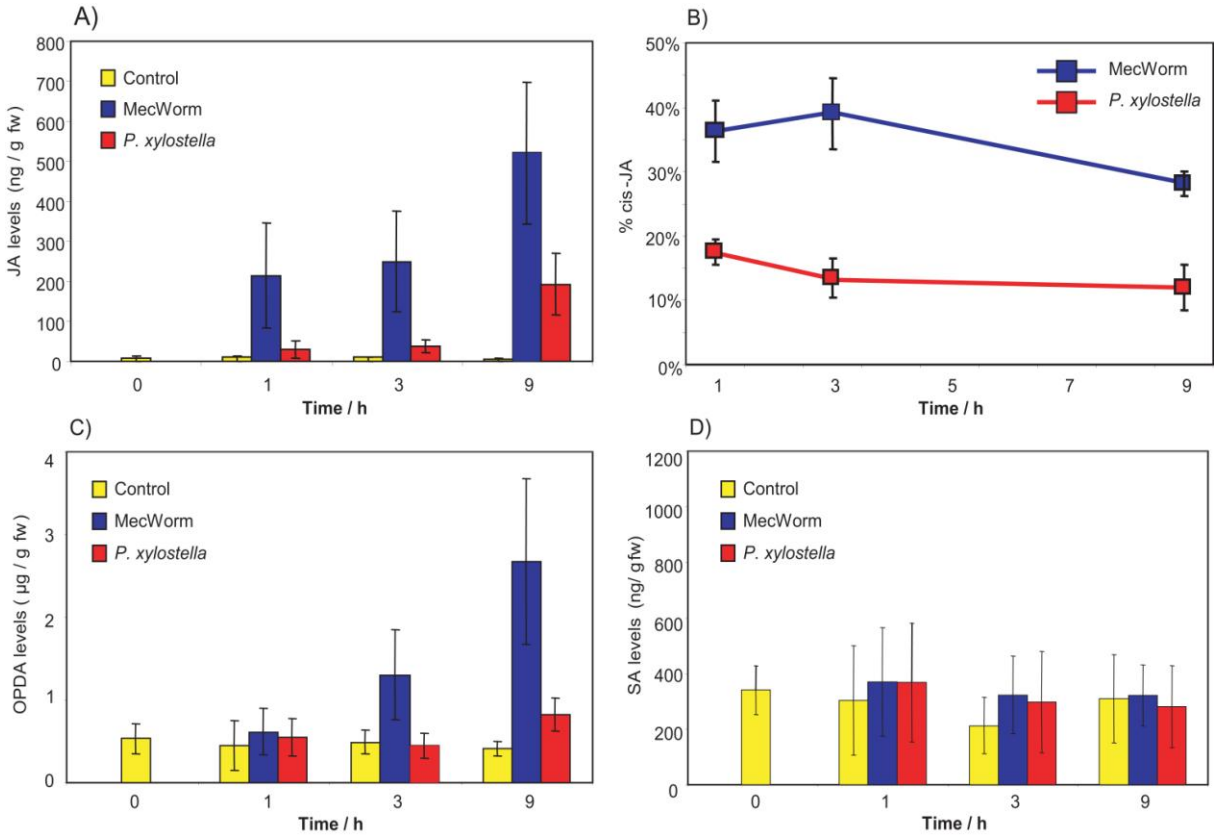


Figure 5. Signal signature upon MecWorm treatment and *P. xylostella* feeding. A) JA levels in local leaves of *A. thaliana* at different time points; B) Percentages of *cis*-JA in local leaves of *A. thaliana* at different time points. Values exhibit the share of *cis* epimer in total amount of JA; C) Levels of OPDA in local leaves of *A. thaliana* at different time points; D) Levels of SA in local leaves of *A. thaliana* at different time points. In all cases values presented are means (± standard deviation SD) of five repeats.

both JA and OPDA were still significantly higher than corresponding levels of these phytohormones after 9 h *P. xylostella* feeding. On the basis of this set of experiments we were able to calculate a linear correlation between wounded leaf area and corresponding amounts of accumulated JA and OPDA (**Fig.7**). The correlation found for JA is far more accurate (R^2 = 0.5065) than the one for OPDA (R^2 = 0.345). Furthermore, neither MecWorm nor *P. xylostella* induced a significant increase in SA accumulation (**Fig.5D**).

Materials and Methods

Plant and Insect Materials and Growth Conditions. *A. thaliana* seeds (ecotype Columbia) were obtained from the Nottingham *Arabidopsis* Stock Center. Seeds were sown on a Mini-Tray: vermiculite (3:1) soil mix (Einheitserdenwerk, Fröndenberg, Germany) and cold stratified for 7 days at 4 °C. Afterwards, plants

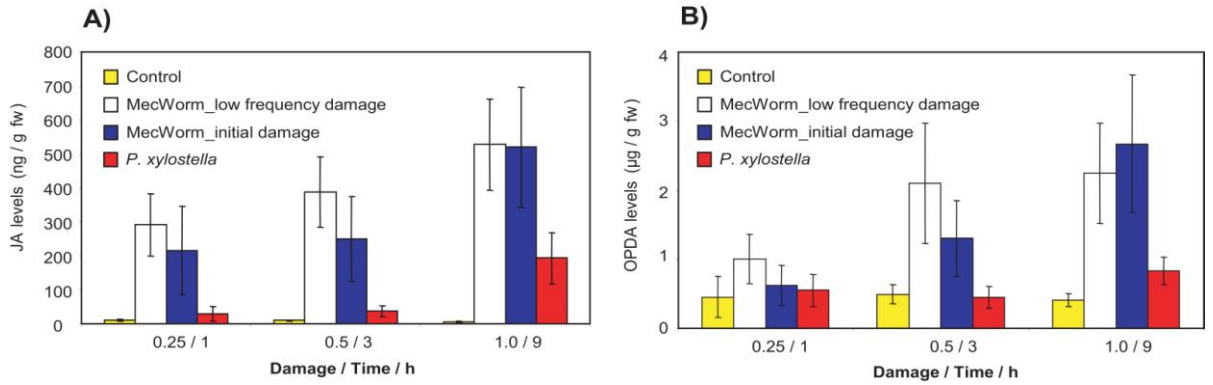


Figure 6. Comparison of signal signature between initial MecWorm treatment and *P. xylostella* feeding (Fig.5) with modified MecWorm treatment for 9 h: A) Comparison of JA levels; B) Comparison of OPDA levels. In all cases values presented are means (± standard deviation SD) of five repeats.

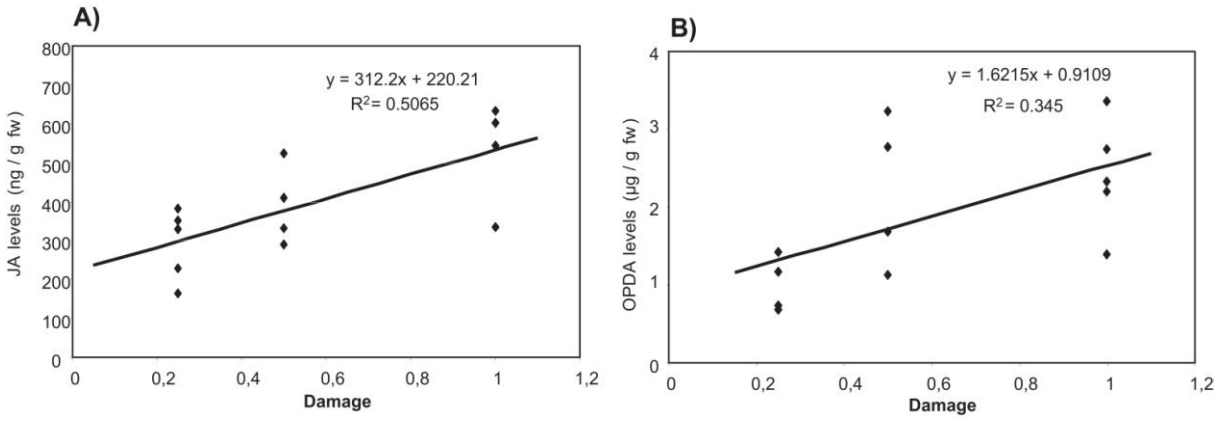


Figure 7. Linear correlation between wounded leaf area and corresponding levels of: A) JA; B) OPDA, where 1 is considered damage produced with initial MecWorm treatment for 9 h.

were placed in ventilated growth rooms with constant air flow and 40 % humidity at 23 °C. Plants were grown at a distance of 30 cm from fluorescent light banks with four bulbs of cool white and four bulbs of wide spectrum lights at a 14 h light/10 h dark photoperiod. Grow domes were removed after 5 days under lights and plants were fertilized once with 1 ml of Scotts Peters Professional Peat Lite Special 20N:10P:20K with trace elements and 1 liter water per flat, added to the bottom of the tray. Approximately 6 days after germination, plants were transferred toindividual pots (7.5 x 7.5 cm²) and were grown for 22 days under strict light, temperature and humidity control.

Eggs of the Diamondback moth (*Plutella xylostella*) (G-88 strain) were originally obtained from the New York State Agricultural Experimental Station (Geneva, NY), and a colony was maintained at the MPI in Jena. Larvae were reared on a wheat germ based artificial diet according to published procedures [213] at 27 °C and 16 h

light/8 h dark cycles. Herbivory screens were performed with fourth-instar *P. xylostella* larvae.

Plant treatment. All induction experiments were performed 4 weeks post germination. All plants were at a vegetative growth stage and pre-bolting. For each experiment, control plants were included and subjected to the same environmental conditions (except for the respective experimental trigger) as treated plants. Insect herbivory screens were carried out with two larvae per rosette leaf. Mechanical wounding was performed with MecWorm.[214] Rosette leaves were damaged continuously during experiments, inflicting damage on a leaf area comparable to insect herbivory at the various time points. Details are stated in the particular experiments. Leaf material was immediately frozen in liquid nitrogen and stored at -80 °C. Experiments were conducted for 1, 3, and 9 hours, each with three biological replicates and randomized between the treatments.

Microarray Preparation. Leaf material was ground to a fine powder in liquid N_2, and total RNA was isolated using the TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturers´ protocol. An additional DNAse (Turbo DNAse, Ambion) treatment was included prior to the second purification step to eliminate any contaminating DNA. A second purification step was performed with RNeasy columns (Qiagen, Hilden, Germany) to eliminate contaminating polysaccharides, proteins and the DNAse enzyme. RNA integrity was verified on an Agilent 2100 Bioanalyzer using the RNA Nano chips (Agilent Technologies, Palo Alto, CA). RNA quantity was determined photospectrometrically.

Total RNA was amplified using the Agilent low input linear amplification kit according to the process outlined by the manufacturer (Agilent Technologies). 1-5 µg of amplified target cRNA was labeled with either cy5 or cy3 using the Micromax kit (Perkin Elmer, Boston, MA). The labeled material was passed through zymo RNA Clean-up Kit-5 columns (Zymo Research Corporation, CA) to remove any un-incorporated label and eluted in 15-20 µl of RNAse-free water (Ambion, Austin, TX). Concentration of labeled cRNA and label incorporation was determined by Nanodrop-1000 spectrophotometer analysis. All of the labeling and post labeling procedures were conducted in ozone-free enclosure to ensure the integrity of the label. Labeled material was setup for fragmentation reaction, hybridized overnight in the rotating oven at 60° C in an ozone-free room, followed by washing steps. All

conditions were according to the Agilent protocol. Arrays were scanned using the Agilent scanner. Agilents feature extraction software (Version 7.5) was used for extracting array data. Further analysis was done using Rosetta Luminator and GeneSifter software.

Analysis of JA, OPDA, and SA in locally treated *Arabidopsis thaliana* leaves. After completion of single experiments, plant material was weighed and shock frozen with liquid nitrogen and samples were kept at -80˚C until used. Jasmonic acid (JA), 12-Oxophytodienoic acid (OPDA), and Salicylic acid (SA) were analyzed in plant material according to modified protocols from Schulze *et al*.[211] Briefly, frozen plant material was mixed with a methanol/BHT (2,6-di-*tert*-butyl-4-methylphenol, Sigma-Aldrich, Taufkirchen, Germany) solution (2.5 ml, 0.05% BHT), followed by addition of derivatization agent - PFBHA (Pentafluorobenzylhydroxylamine, 2 ml, 0.05 M in methanol, Sigma-Aldrich). For quantification: 9,10-[$^2H_2$]-dihydrojasmonic acid (250 ng), [$^2H_2$]-dihydrodicranenone B (250 ng) and [3,4,5,6-$^2H_4$]-salicylic acid (500 ng) were added as internal standards. Next the mixture, cooled on ice and kept under argon atmosphere, was homogenized for 5 min with a high performance dispenser at 24,000 rpm (Ultra-Turrax T-25, IKA-Werk, Germany). For completing the derivatization, samples were shaken for 2 h at RT. Samples were then transferred to 50 ml centrifuge glass tubes and acidified with 0.1 M HCl (pH~3). The methanol/water phase was quantitatively extracted with hexane (3 x 5 ml) and dichloromethane (3 x 5 ml). The combined layers were subsequently passed through preconditioned (methanol, 5 ml; hexane, 5 ml/methanol, 5 ml; dichloromethane, 5 ml) Chromabond aminopropyl cartridges (0.5 g for hexane layers, 1 g for dichloromethane layers, Macherey-Nagel, Düren, Germany). Cartridges were washed with: *i*-propanol:dichloromethane (5 ml, 2:1, v:v) for hexane phases and dichloromethane (5 ml) for dichloromethane phases and in both cases eluted with diethyl ether:formic acid (10 ml, 98:2, v:v). The solvent was removed under a gentle stream of argon. The residue from the hexane phases was treated with the ethereal solution of diazomethane and after removal of diazomethane, was re-dissolved in 30 µl of dichloromethane. The residue from dichloromethane phases was treated with 50 µl of MTBSTFA (*N*-*tert*-Butyldimethylsilyl-*N*-methyltrifluoroacetamide, Macherey-Nagel) for 1 h at 100^{o}C.

The hexane phases samples were analyzed on a Finnigan GCQ Instrument (Thermoelectron, Bremen, Germany) running in a CI negative ion mode (NCI), as

described in Schulze *et al.*[211] The dichloromethane phase samples were analyzed on a Finnigan Trace MS in SIM mode.

For quantification characteristic fragment ions of the PFB-oximes of JA and OPDA were used: m/z 399 for JA and m/z 403 for 9,10-[$^2H_2$]-dihydrojasmonic acid, m/z 481 for OPDA and m/z 483 for [$^2H_2$]-dihydrodicraneone B, m/z 309 for SA and m/z 313 for [3,4,5,6-$^2H_4$]-salicylic acid. Amounts were calculated in respect to weight of collected fresh material.

For calibration, known amounts of JA, OPDA, and SA were added to the plant material (0.225 g) and the extraction procedure was performed. Calibration curves were obtained by plotting the peak area ratio compound/standard against added amount of the compound.

4. Unpublished results Part II

The phytohormone precursor OPDA is isomerized in the insect gut by a single, specific Glutathione S-transferase

Paulina Dąbrowska, Dalial Freitak, Heiko Vogel, David G. Heckel, Wilhelm Boland

In preparation

Oxylipins play important roles in stress signalling in plants. 12-oxophytodienoic acid (OPDA) is a biosynthetic precursor of jasmonic acid (JA), a key phytohormone in the induction of plant anti-herbivory defences. OPDA itself may modulate the effects of JA. When consumed by Lepidopteran larvae, plant-derived OPDA undergoes isomerization to *iso*-OPDA in the midgut (see Article III). We previously reported that in contrast to OPDA epimerization, this isomerization is enzyme-dependent, and here we investigate the role of glutathione transferases (GSTs) in the process. Purified GST fractions from the gut of Egyptian cotton leafworm (*Spodoptera littoralis*) and cotton bollworm (*Helicoverpa armigera*) both exhibited strong OPDA isomerization activity, accompanied by transient appearance of a glutathione-OPDA conjugate. Only one of 16 cytosolic GST proteins from the larval gut of cotton bollworm, GST-16, catalyzed OPDA isomerization when expressed in *E.coli.* The α,β-unsaturated keto structure of OPDA, a reactive electrophile species, represents a suitable target for nucleophilic reagents in Michael addition of the abundant nucleophile glutathione (GSH). Addition and subsequent elimination of GSH leading to double-bond isomerization occurs in mammalian steroid biosynthesis and aromatic amino acid catabolism. A similar isomerization is seen in the conversion of the structurally similar prostaglandin A_1 to prostaglandin B_1, although GSTs have not been implicated in this process. GST-16 could have a specific function in transforming an endogenous prostaglandin. Alternatively, if plant-produced OPDA interferes with prostaglandin signaling pathways of its insect herbivores, GST-16 could be playing an unusual detoxicative role.

Results

Glutathione stimulates isomerization. A crude homogenate of larval midgut tissue of *S. littoralis* possesses the ability to isomerize *cis*-OPDA to *iso*-OPDA.[215] Addition of reduced glutathione clearly enhances the rate of isomerization (**Fig.1A,B**). Control experiments in the absence of midgut homogenate, in which we incubated *cis*-OPDA with excess of GSH in different pH buffers (cis-OPDA/ GSH 1:10-20, pH 7, 9, 10.5), did not show OPDA isomerization (**Fig.1C**) and thus excluded the possibility that GSH alone could be responsible for isomerization. An excess of GSH prompted only epimerization of *cis*-OPDA to *trans*-OPDA, which is also seen following treatment of *cis*-OPDA with bovine serum albumin.[215]

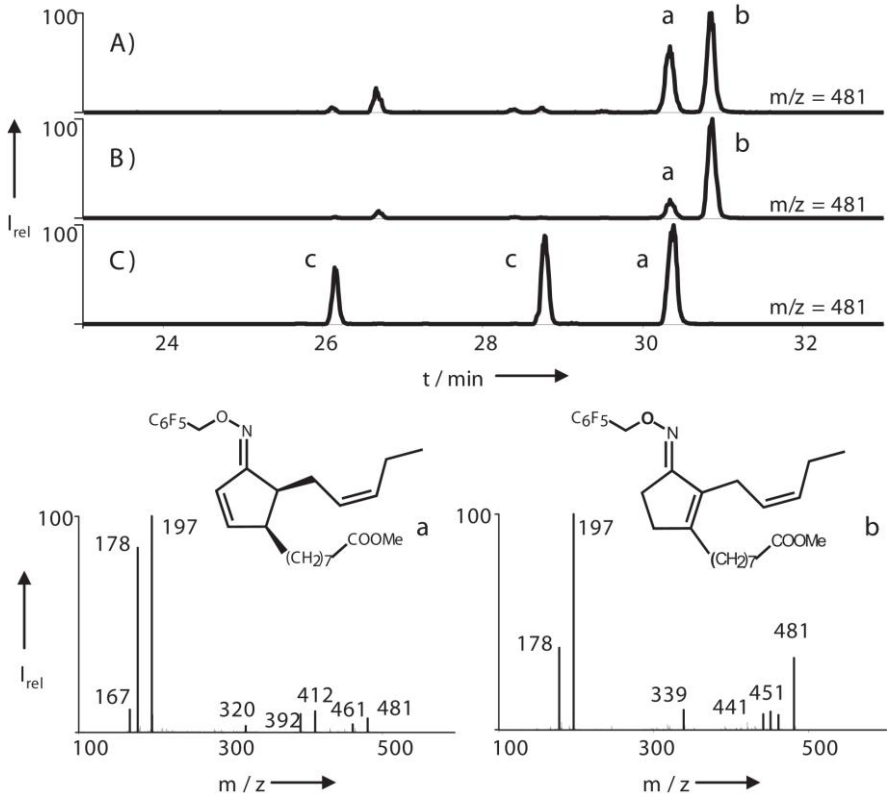


Figure 1. Incubation assays of *cis*-OPDA with: A) crude midgut homogenate from *S. littoralis*; B) crude midgut homogenate with addition of reduced glutathione, C) excess of glutathione in pH 10.5. Identification of compounds: a: *cis*-OPDA, b: *iso*-OPDA, c: *trans*-OPDA represented by the *syn* and *anti* isomers of their PFB oximes. Reference mass spectrum of PFB oximes of *cis*-OPDA and *iso*-OPDA.

Semipurified glutathione transferases catalyze OPDA isomerization. GST enzymes were enriched from crude midgut homogenate from *S. littoralis* using glutathione affinity columns. The molecular mass of purified GST fraction was estimated to be ~27 kDa by SDS page (**Fig.2A**), which is in agreement with literature data.[216] MALDI-TOF analysis of the elute fraction confirmed GST identity with the GST from *Spodoptera littoralis* (mW 26219 Da, pI 6.1278). In initial purification trials we used relatively low amounts of midgut homogenate powder (~15-20 mg). For subsequent activity assays we employed fractions resulting directly from the cartridge preparations. 2 h incubations of *cis*-OPDA with elute and flow through fractions revealed formation of *iso*-OPDA only in the assays with elute fractions (**Fig.3A,B**). Similar results were obtained from semi-purified GSTs from cotton bollworm (*H. armigera)*. The molecular mass of the purified GST fraction was in this case estimated to be ~25 kDa by SDS page (**Fig.2B**), with MALDI-TOF analysis confirming the GST identity. The OPDA isomerization activity occurred exclusively in the elute fraction, containing the mixture of GST proteins (**Fig.3C,D**).

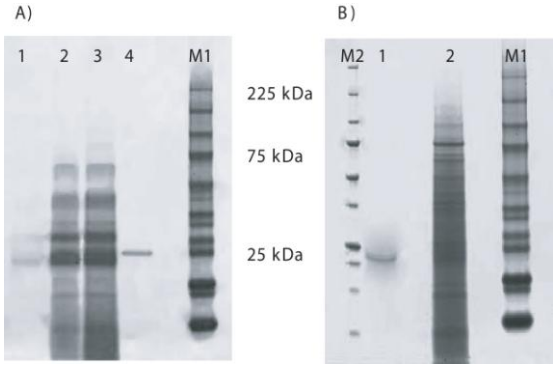


Figure 2. SDS Pages of purification of GST fraction on GST Bind™ Fractogel Cartridges from: A) *S. littoralis*, line assignment: 1 – wash fraction, 2,3 – flow through fraction, 4 – elute fraction, M1 - Rainbow marker (Amersham); B) *H. armigera*, line assignment: M2 - Precision Plus Protein Unstained Standard (BioRad), 1 – elute fraction, 2 – flow through fraction.

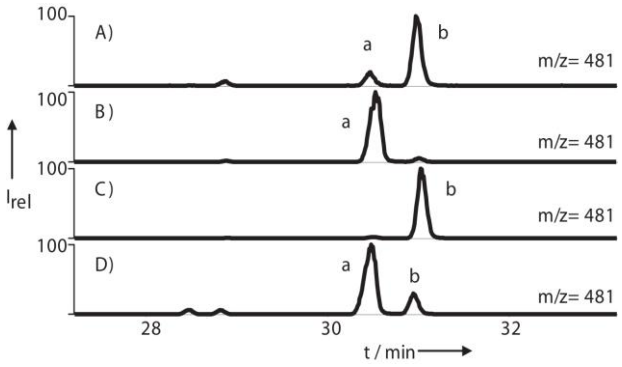


Figure 3. Incubation assays of *cis*-OPDA with: A) elute fraction and B) flow through fraction from the purification of the gut homogenate powder from *S. littoralis* on GST affinity cartridges; C) elute fraction and D) flow through fraction from the purification of the gut homogenate powder from *H. armigera* on GST affinity cartridges.

Having an active GST fraction enabled us also to perform kinetic observations, in which we measured decrease in UV absorbance of OPDA maximum over time, indicating the shift of the double bond position (**Fig.4**). The transformation seems to be most rapid in the first 20 min (50% conversion), after which the absorbance decrease is becoming distinctly slower.

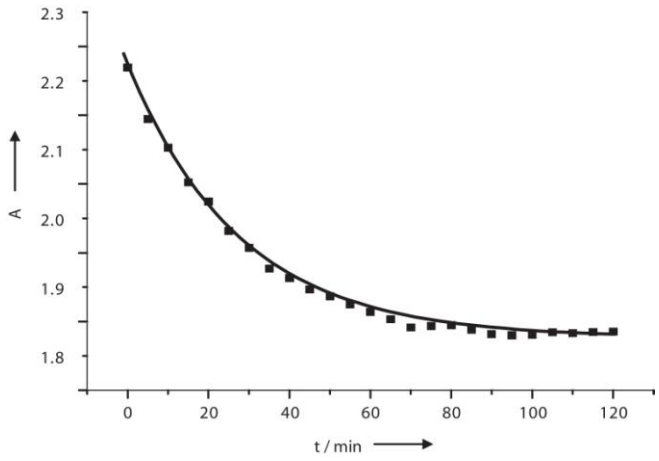


Figure 4. Change in UV absorbance of OPDA maximum (at λ = 230nm in phosphate buffer) over time indicating the shift of the double bond position.

To investigate some specific GSTs for their ability to isomerize OPDA, we incubated *cis*-OPDA with commercially available GST from equine liver, and with GST-X01 from *H. armigera* expressed in *E. coli* (generous gift from Dr. Choon Wei Wee) at pH 7 and

with differing ratios of GSH. None of these enzymes produced any traces of *iso*-OPDA. Also albumin did not catalyze formation of iso-OPDA.

In order to further characterize the observed OPDA isomerase activity [194, 200], we separated cytosolic and microsomal protein fractions from the pooled guts of *H. armigera* (see Supplementary material 15.2. for details). Most activity was observed in the cytosolic fraction, which was then further purified on the GST affinity cartridges. As expected *cis*-OPDA was fully isomerized only in the assay with elute fraction, containing the GSTs.

Glutathione-OPDA conjugation accompanies OPDA isomerization. In order to obtain reference material for analysis we synthesized the glutathione-OPDA adduct, starting with *cis*-OPDA according to the general protocol of Blackburn et al.[217] Although HPLC separation resulted in only a single peak with the mass spectrum corresponding to the expected GS-OPDA adduct (see Supplementary material 15.2., Fig.1.) [85, 86], NMR data clearly showed a mixture of at least two different GS-OPDA diastereomers (see Supplementary material 15.2., Fig.2., 3., 4., Tab.1). This material sufficed however for our feeding experiments and as a reference for analysis. We monitored GS-OPDA conjugate formation and *iso*-OPDA appearance in insect samples and semi-purified GST fractions. Since the simultaneous HPLC-MS analysis of the oxylipins and the GS-oxylipin conjugates was unreliable due to relatively different polarity of these two compound classes, we split each sample and analyzed one aliquot with HPLC-MS and the other on GC-MS following the usual derivatization with PFBHA. Incubation of *cis*-OPDA with GSH and purified GST fraction from *H. armigera* in pH 7 resulted in very rapid formation of the conjugate (**Fig.5**). Surprisingly, analysis of the insect frass and gut content (from the *cis*-OPDA feeding experiments) did not reveal substantial amounts of the glutathione-OPDA conjugate, but as before relatively high amounts of *iso*-OPDA (**Fig.6**). This suggests that the conjugate, once formed, is cleaved *in vivo* to yield the isomer. To more directly examine the role of the conjugate, we incubated the synthesized glutathione-OPDA with the semipurified GST fraction from *H. armigera*. This did result in the production of *iso*-OPDA but at a lower amount than in the original assays (~13%, **Fig.7**). This is likely due to the formation of a significant amount of *trans*-OPDA during the chemical synthesis of the conjugate, which cannot be converted to *iso*-OPDA. Likewise other diastereomers of the conjugate could have been formed during our chemical synthesis, which are thermodynamically favored but not permissive of the

enzymatically controlled process of conjugation and release that promotes isomerization.

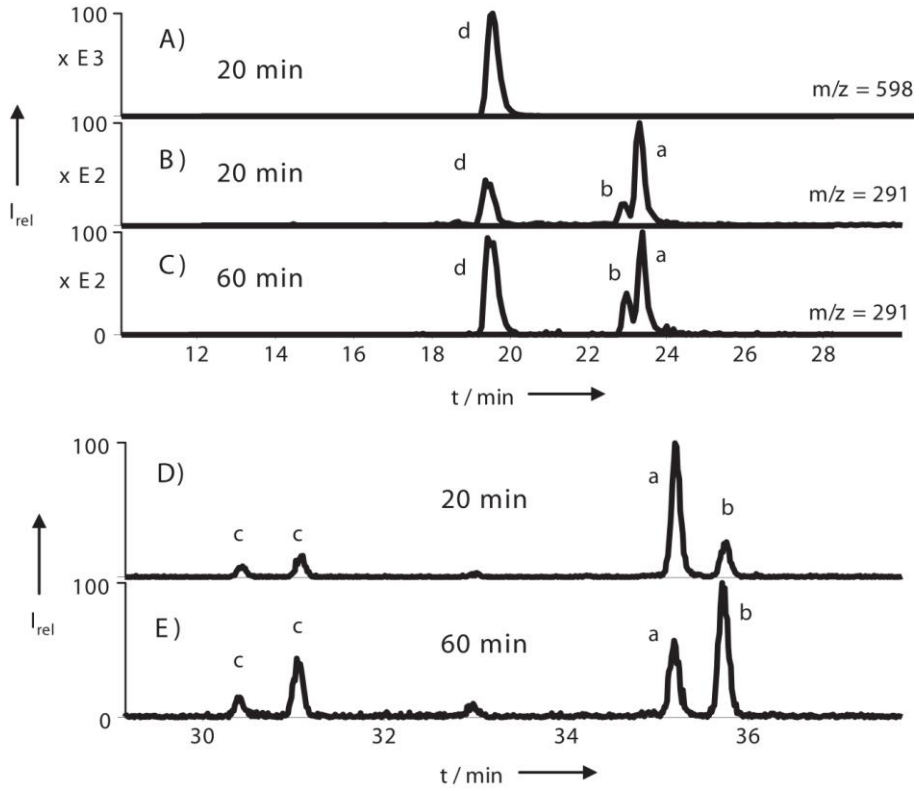


Figure 5. Cytosolic GST fraction assays with *cis*-OPDA (a), showing: HPLC-MS runs (A), B), C)) from water aliquot of mixture and parallel GC-MS chromatograms (D), E)) from derivatized aliquot; A) formation of OPDA-GSH conjugate (d) after 20 min of incubation; B),D) indication of formation of *iso*-OPDA (b) after 20 min of incubation; C),E) increased amounts of *iso*-OPDA (b) after 60 min of incubation. 100% conversion was not observed while the amount of protein fraction added was lower than in original assays.

A single GST from *H. armigera* catalyzes OPDA isomerization. To visualize the different GST proteins present in the cytosolic fraction we separated the semipurified GSTs on 2D SDS PAGE (see Supplementary material 15.2. for details). As a result we obtained roughly 12 separate protein spots (**Fig.8**): 1 in pİ range of 3 (spot 1), 1 in a pİ range of 10-11 (spot 12), and around 10 in a pİ range between pİ 5 and pİ 8 (spots 2-11). As posttranslational modification of GSTs is considered very rare [200] we expected that 12 separate spots would correlate with 12 different GST proteins. MALDI TOF analysis gave good hits for most of the spots.

To identify potential candidate genes for these proteins, we screened our in-house *Helicoverpa armigera* cDNA libraries for GST sequences based on both keyword (GST) and BLAST searches with available insect GST sequences.

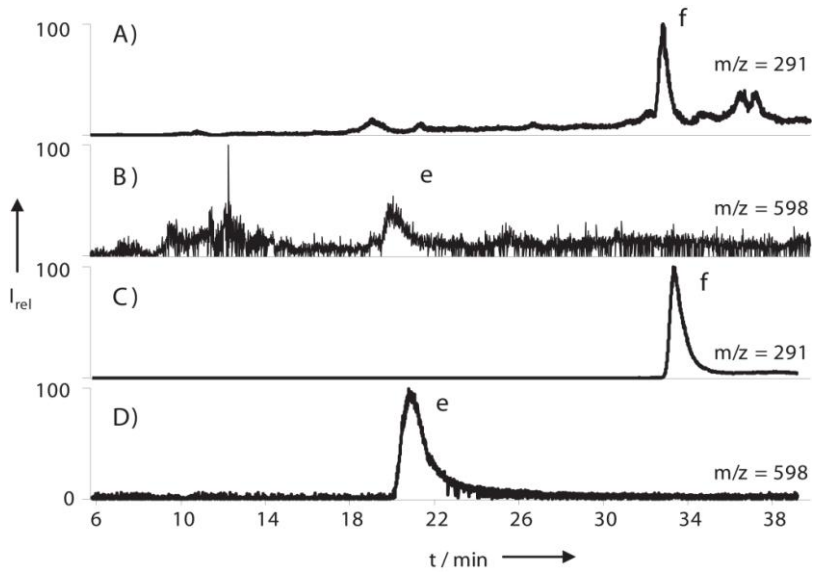


Figure 6. micro-HPLC chromatogram of the water/acetonitrile extract from the insect frass after feeding on *cis*-OPDA enriched diet: A) trace of m/z 291 reveals a significant peak of *iso*-OPDA, B) trace of m/z 598 shows only small traces of OPDA-GSH; C) *iso*-OPDA standard; D) OPDA-GSH standard.

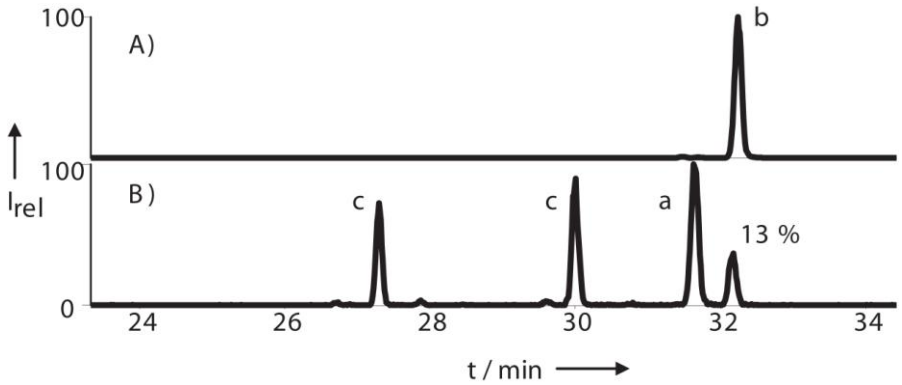


Figure 7. Cytosolic GST fraction assays with: A) *cis*-OPDA (a), showing full conversion to *iso*-OPDA (b); B) synthetically prepared OPDA-GSH conjugate, showing only 13% conversion to *iso*-OPDA.

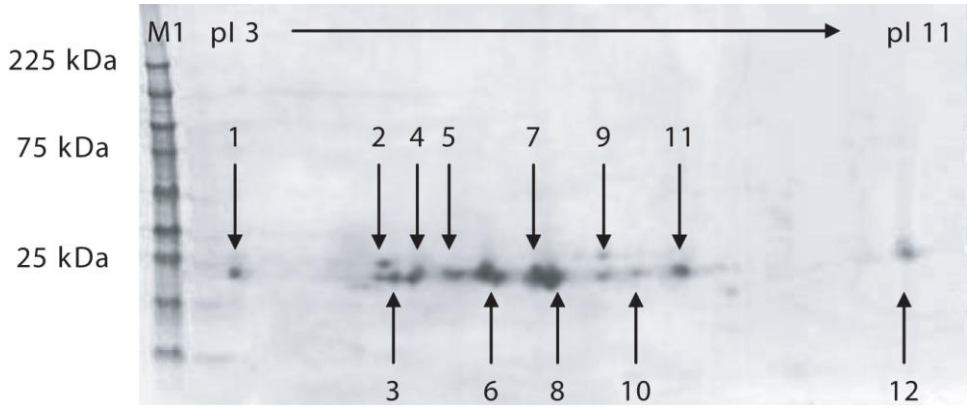


Figure 8. 2D SDS Page of the purified GST cytosolic fraction from *H*. *armigera*. Spots picked for MALDI TOF analysis are numbered. M1 - Rainbow marker (Amersham).

The six GST family classes identified in other insect species are all represented in our libraries and the majority of the GSTs belong to the insect-specific Delta and Epsilon classes. As the conjugating activity was identified in gut tissue, we additionally searched tissue-specific libraries for GST genes expressed and removed those that were present only in tissues other than *H. armigera* midguts. Out of the total 40 GSTs, 18 were identified to be expressed in midgut tissue. As we had identified the conjugating activity in the cytosolic fraction, we omitted two microsomal midgut GSTs from further expression analysis. In total 16 cytosolic glutathione *S*-transferase (GST) gene sequences were cloned and expressed in *E. coli* (for primers used for cloning GST 16 see Supplementary material 15.2., Tab.2.) (**Fig.9**). OPDA isomerization activity assays were performed with lysed *E. coli* cells mixtures in phosphate buffer (pH 7) with an excess of reduced GSH. Samples from all assays were measured on GC-MS after the usual derivatization. From 16 expressed proteins there were 4 GST proteins which showed epimerization activity (transformation of *cis*-OPDA to *trans*-OPDA), namely: GST 1, 5, 6, 17 (**Fig.10**) but only a single one (GST 16, Accession Number: FJ546089) showed OPDA isomerization activity. The remaining 11 GSTs did not modify the substrate. Unfortunately further purification of GST-16 (on GST affinity columns) resulted in unexpected loss of isomerization activity, therefore impeding more detailed biochemical characterization of the putative OPDA isomerase.

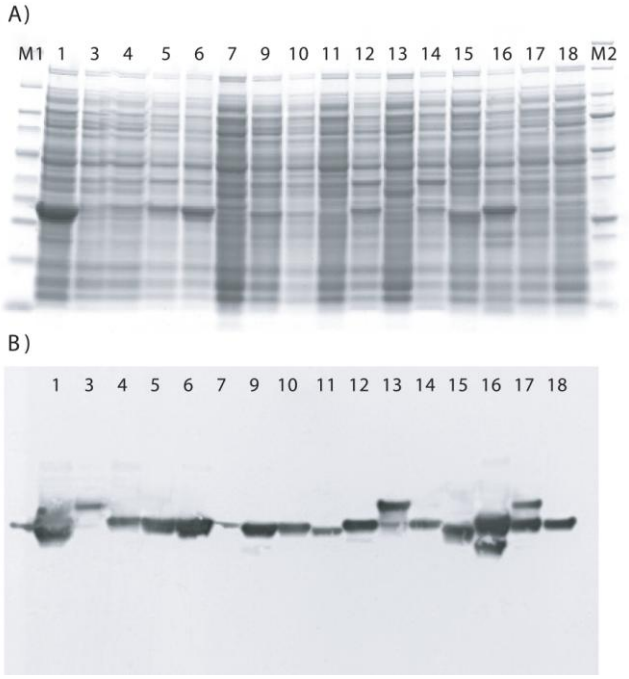


Figure 9. Expression of 16 expressed GST proteins: A) 1D SDS PAGE of crude *E. coli* lysates; B) Western Blot.

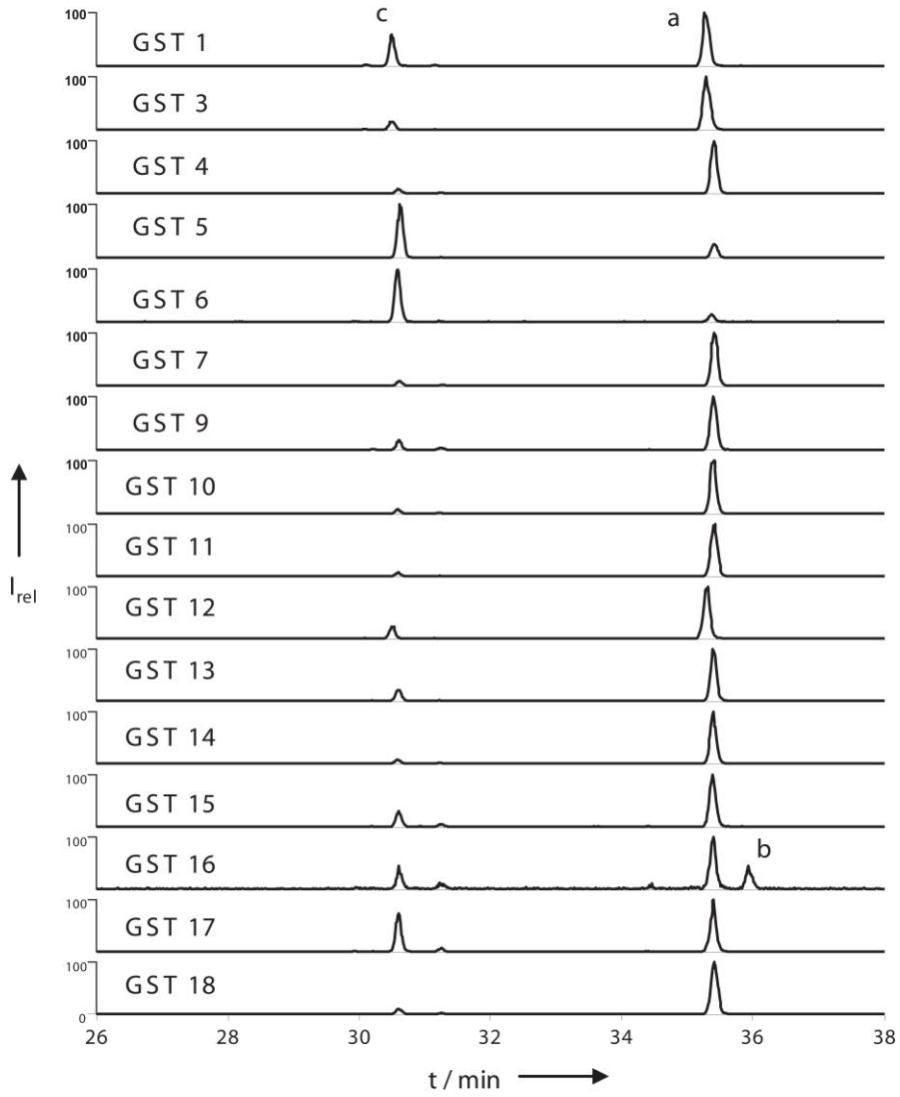


Figure 10. Activity assays with crude *E. coli* lysates of expressed GST proteins for *cis*-OPDA (a) isomerization. *iso*-OPDA (b) formation only with GST 16. GST 1, 5, 6 and 17 show epimerization activity with formation of *trans*-OPDA (c).

Distribution of OPDA-isomerization-ability in insects. Not all lepidopteran insect species possess the ability to isomerize plant derived OPDA, f.i.: the Brassicacae specialist *Pieris rapae* isomerized only around 14% of OPDA in contrast to over 80% isomerization rates in Spodoptera species.[215] In order to get a better understanding about the scope of OPDA isomerization ability within the insect class we performed feeding experiments with several species from different insect families. The results are summarized in **Tab.1**. Where possible we performed feeding experiments with OPDA enriched artificial diet (*Helicoverpa assulta*, *Manduca sexta*, *Plutella xylostella*, *Bombyx mori*), but in case of two insect species, for which artificial diet was not available (*Plagiodera versicolora*), or the feeding experiments proved to be difficult (*Galleria mellonella*) we chose either feeding on preferred plant species (willow

leaves) or incubation essay with dissected gut tissue. These experiments do not provide as exact answers as the OPDA enriched diet feedings, but still provide an estimation of OPDA isomerization ability. The remarkable differences in OPDA isomerization ability found within the insects (**Tab.1**) additionally support the hypothesis about the functional specificity of one GST protein capable of OPDA transformation. As a possible explanation of this differences we could come back to already suggested hypothesis [193], that the generalistic, highly polyphagous insects like *Spodoptera littoralis* or *Helicoverpa armigera*, tend to evolve multiple, differentially functionalized GSTs, which may help detoxify the diversity of allelochemicals found in their host plants. At the same time specialist insects, encountering more specific plant derived metabolites need a less elaborate choice of GSTs.[193]

Table 1. OPDA isomerization ability within different insect families and species.

Insect species	Order	Family	OPDA	*iso*-OPDA
Helicoverpa assulta	*Lepidoptera*	*Noctuidae*	20%	80%
Manduca sexta	*Lepidoptera*	*Sphingidae*	27%	73%
*Bombyx mori**	*Lepidoptera*	*Bombycidae*	100%	0%
*Galleria mellonella***	*Lepidoptera*	*Pyralidae*	100%	0%
Plutella xylostella	*Lepidoptera*	*Plutellidae*	100%	0%
*Plagiodera versicolora****	*Coleoptera*	*Chrysomelidae*	100%	0%

* Feeding experiment with OPDA enriched artificial diet and OPDA incubation with dissected gut tissue; ** OPDA incubation with dissected gut tissue; *** Feeding experiment on willow leaves. Frass was collected from adult beetles, not larvae. Willow leaves were extracted after mechanical wounding to prove accumulation of OPDA.

5. Article I

Effects of Feeding Spodoptera littoralis on Lima Bean Leaves IV: Diurnal and Nocturnal Damage Differentially Initiate Plant Volatile Emission

Gen Arimura, Sabrina Köpke, Maritta Kunert, Veronica Volpe, Anja David, Peter Brand, Paulina Dąbrowska, Massimo Maffei, Wilhelm Boland

Plant Physiology (2008) **146**, 965-973

Effects of Feeding *Spodoptera littoralis* on Lima Bean Leaves: IV. Diurnal and Nocturnal Damage Differentially Initiate Plant Volatile Emission[1][W][OA]

Gen-ichiro Arimura[2], Sabrina Köpke[2], Maritta Kunert[2], Veronica Volpe, Anja David, Peter Brand, Paulina Dabrowska, Massimo E. Maffei, and Wilhelm Boland*

Department of Bioorganic Chemistry, Max Planck Institute for Chemical Ecology, D–07745 Jena, Germany (G.-i.A., S.K., M.K., V.V., A.D., P.B., P.D., W.B.); and Department of Plant Biology and Centre of Excellence for Plant and Microbial Biosensing, University of Turin, I–10125 Turin, Italy (V.V., M.E.M.)

Continuous mechanical damage initiates the rhythmic emission of volatiles in lima bean (*Phaseolus lunatus*) leaves; the emission resembles that induced by herbivore damage. The effect of diurnal versus nocturnal damage on the initiation of plant defense responses was investigated using MecWorm, a robotic device designed to reproduce tissue damage caused by herbivore attack. Lima bean leaves that were damaged by MecWorm during the photophase emitted maximal levels of β-ocimene and (*Z*)-3-hexenyl acetate in the late photophase. Leaves damaged during the dark phase responded with the nocturnal emission of (*Z*)-3-hexenyl acetate, but with only low amounts of β-ocimene; this emission was followed by an emission burst directly after the onset of light. In the presence of $^{13}CO_2$, this light-dependent synthesis of β-ocimene resulted in incorporation of 75% to 85% of ^{13}C, demonstrating that biosynthesis of β-ocimene is almost exclusively fueled by the photosynthetic fixation of CO_2 along the plastidial 2-C-methyl-D-erythritol 4-P pathway. Jasmonic acid (JA) accumulated locally in direct response to the damage and led to immediate up-regulation of the *P. lunatus* β-ocimene synthase gene (*PlOS*) independent of the phase, that is, light or dark. Nocturnal damage caused significantly higher concentrations of JA (approximately 2–3 times) along with enhanced expression levels of *PlOS*. Transgenic *Arabidopsis thaliana* transformed with *PlOS* promoter::β-glucuronidase fusion constructs confirmed expression of the enzyme at the wounded sites. In summary, damage-dependent JA levels directly control the expression level of *PlOS*, regardless of light or dark conditions, and photosynthesis is the major source for the early precursors of the 2-C-methyl-D-erythritol 4-P pathway.

Herbivore-induced plant volatiles (HIPVs) attract herbivore natural enemies and, hence, benefit plants indirectly. Like floral volatiles (Dudareva et al., 2003, 2005), HIPVs display diurnal or nocturnal rhythms that may be coordinated with the habits of the parasitoids that are attracted to them (Loughrin et al., 1994; Turlings et al., 1995; Arimura et al., 2004a, 2005). Whereas floral scents or light-dependent isoprene emissions are constitutively produced according to circadian rhythms (Dudareva et al., 2005; Loivamäki et al., 2007; Roeder et al., 2007), the release of HIPVs is a phenotypically plastic response of plants to herbivory that is influenced by insect performance and environmental factors (e.g. photoperiod and temperature). Herbivorous insects likewise depend on plant volatiles for information about their food plants and the surrounding environment so that they can modulate their activity accordingly. For example, caterpillars of *Mythimna separata* behaved as if they were in the dark when exposed during the day to volatiles emitted from host plants (infested or uninfested) in the dark (Shiojiri et al., 2006). Likewise, regardless of the amount of light available, caterpillars behaved as if they were in the light when exposed to volatiles emitted from plants during the photophase (Shiojiri et al., 2006). Moreover, volatile compounds released at night from lepidopteran-infested tobacco (*Nicotiana tabacum*) plants were repellent to conspecific female moths (*Heliothis virescens*; De Moraes et al., 2001). These examples clearly demonstrate that plants are able to generate temporally different volatile blends that address different organisms at different times of the day in order to optimize defense. Whereas many elements of damage recognition, signal transduction, de novo biosynthesis, and metabolic regulation involved in HIPV biosynthesis are known (Arimura et al., 2005), only little is known about the mechanisms of the temporal variations underlying the synthesis and release of HIPVs. In particular, the question of whether HIPV emissions are controlled by circadian clocks or by environmental factors, such as the insect's activity level or the amount of light, remain to be addressed.

Here, we report on the results of diurnal and nocturnal leaf damage using the previously developed

[1] This work was supported in part by the Center of Excellence for Plant and Microbial Biosensing of the University of Turin.

[2] These authors contributed equally to the article.

* Corresponding author; e-mail boland@ice.mpg.de.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Wilhelm Boland (boland@ice.mpg.de).

[W] The online version of this article contains Web-only data.

[OA] Open Access articles can be viewed online without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.107.111088

MecWorm device to mimic herbivore-caused tissue damage in a completely reproducible and quantifiable manner. The continuous mechanical damage effected by the robotic device was able to induce in lima bean (*Phaseolus lunatus*) leaves the emission of volatiles whose profiles perfectly matched those of the HIPVs induced by feeding larvae of *Spodoptera littoralis* (Mithöfer et al., 2005) The volatile spectrum comprised monoterpenes, sesquiterpenes, and homoterpenes along with fatty acid-derived volatiles, such as (Z)-3-hexenyl acetate (Hex-Ac). The major compounds from the group of terpenoids were (*E*)-β-ocimene (69%), linalool (4%), and (*E*)-4,8-dimethyl-1,3,7-nonatriene (11%). Because this computer-controlled mechanical induction process (1) does not depend on undefined components of insect salivary secretions (Paré and Tumlinson, 1999), (2) can be fully reproduced at any time of the day, and (3) can be adjusted to predefined damage levels, this approach is ideally suited for a comparative study of the effects of diurnal or nocturnal damage on the emission of HIPVs.

Using this fully reproducible mode of mechanical damage, we present evidence that temporally different instances of leaf damage to the same plant during either the light phase (day) or the dark phase (night) play a decisive role in the control of HIPV emission. We demonstrate that continuous mechanical damage during the day or night is associated with an enhanced level of jasmonic acid (JA). This JA production is closely followed by up-regulation of the transcript level of the gene coding for the (*E*)-β-ocimene synthase. During the photophase, damage by MecWorm leads to the instantaneous emission of (*E*)-β-ocimene. In the dark, however, a very weak nocturnal emission is followed by a rather brief emission burst after the onset of the photophase. Studies with $^{13}CO_2$ demonstrate that the early steps of the terpenoid pathway along the 2-C-methyl-D-erythritol 4-P (MEP) pathway appear to be responsible for the delayed response. We propose a schematic view of the elements of signaling and metabolic regulation required for the herbivore-induced β-ocimene emission and the light-independent Hex-Ac.

RESULTS

Functional Characterization of the β-Ocimene Synthase Gene in Lima Bean

The monoterpene (*E*)-β-ocimene, emitted from lima bean leaves after damage by the feeding larvae of *S. littoralis*, was one of the major volatiles (64%–69% of the total volatiles; Arimura et al., 2007). To explore the regulatory mechanisms for herbivore-induced β-ocimene emission, we first isolated the full-length clone of the cDNA of the β-ocimene synthase from lima bean (*P. lunatus* ocimene synthase [*PlOS*]; GenBank accession no. EU194553). To functionally characterize *PlOS*, the recombinant protein was expressed in *Escherichia coli*. Assays with geranyl diphosphate as a substrate produced two monoterpene hydrocarbons, namely, (*E*)-β-ocimene (98%) and its (Z)-isomer (2%; Fig. 1). This ratio corresponds to the composition of β-ocimene isomers emitted from the lima bean plants after 1 d of damage by the feeding larvae of *S. littoralis* (E:Z = 97:3). Neither farnesyl diphosphate nor geranylgeranyl diphosphate was converted by the recombinant protein.

Rhythmic Emission of Herbivore-Induced Volatiles β-Ocimene and Hex-Ac

Volatiles in the headspace of induced plants were analyzed with zNose, which combines a high time resolution (10 min) with the ability to run unattended over prolonged periods (Kunert et al., 2002). Figure 2A demonstrates the time course of the emission of the two selected HIPVs, Hex-Ac and β-ocimene. Emission was low on the first day after damage by feeding larvae of *S. littoralis*; strong diurnal emissions of both compounds followed on the second day (14/10 light-dark [LD] cycle). This was accompanied by up-regulation of the *PlOS* transcript. Maximal transcript levels of *PlOS* were observed during the photophase (7 AM–9 PM) of a LD cycle. To test whether herbivore-induced terpenes are regulated according to circadian rhythms, we introduced an additional dark (AD) period for 4 h during the second and third photophases and investigated its effect on the *PlOS* transcript level and β-ocimene emission (Fig. 2B). Although β-ocimene was permanently emitted during the full photoperiod of Figure 2A, during the AD period (Fig. 2B) volatile emissions were reduced within 40 to 60 min following the onset of darkness. This clearly demonstrates that the herbivore-induced emission of β-ocimene is strictly linked to light and, unlike floral scents (Dudareva et al., 2005; Roeder et al., 2007), is not under the control of the circadian clock. Most important, the transcript level of the *PlOS* gene was affected by the AD period and dropped to very low levels at the end of AD, whereas transcript levels of *PlOS* remained high in the light

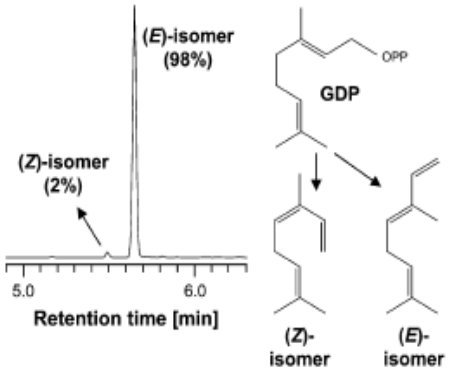


Figure 1. Gas chromatographic separation of the β-ocimene isomers formed by the recombinant PlOS enzyme with GDP as substrate.

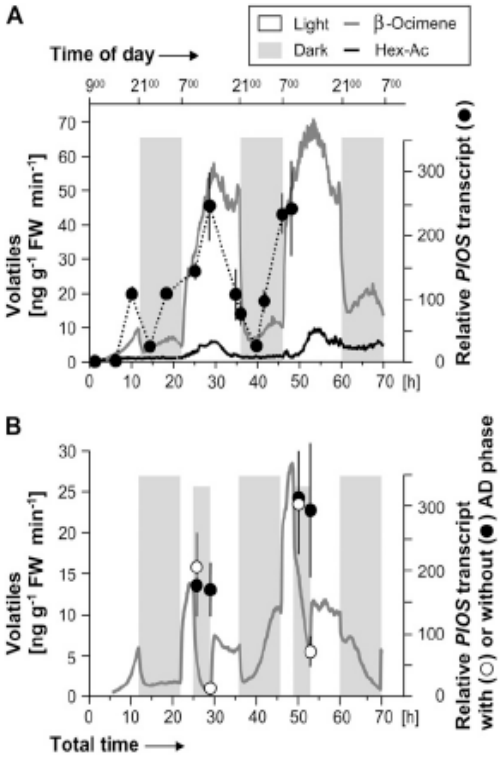


Figure 2. Emission of the volatiles β-ocimene and Hex-Ac and transcript levels of *PlOS* in damaged lima bean leaves. Damage conditions: larvae of *S. littoralis* under a 14:10 LD cycle (A) and under a LD cycle set with 4 h of additional darkness (AD) during the second (25–29 h) and third (49–53 h) photoperiods (B). Time of day is shown across the top *x* axis, and total elapsed time is shown on the bottom *x* axis. Headspace analyses were performed in triplicate. Emission is expressed as ng min^{-1} g^{-1} FW. Data regarding to the transcript levels represent the mean ± SE (n = 4–5).

period without AD ($P < 0.05$; ANOVA; see time points 29 and 53 h; $P < 0.05$). Note that the feeding performance of *S. littoralis* did not change even after the onset of the AD period.

Effects of Diurnal and Nocturnal Damage on Volatile Emissions

To determine temporal relation between leaf damage and emission of HIPVs, we used the recently developed MecWorm (Mithöfer et al., 2005); with MecWorm, we could evaluate differences between induction processes during the light and dark periods. Continuous mechanical damage for 6 h during the photophase induced the rhythmic emission of volatiles. β-Ocimene and Hex-Ac (Fig. 3A) were chosen to represent the groups of terpenoids and fatty acid-derived compounds and monitored over several days. In contrast to the typical delay observed after insects fed (Fig. 2A) with MecWorm, volatiles were emitted almost instantaneously. Moreover, both compounds displayed different emission maxima: Emission of Hex-Ac closely followed the damage period, whereas emission of β-ocimene increased further after the damage had ended. Emission remained high until to the onset of the dark period followed by a sharp decrease of β-ocimene production. Emission profiles of both compounds were entirely different when MecWorm (6 h) was applied during the dark phase. Whereas Hex-Ac emission closely followed the damage period (albeit at a lower level than during the photophase), the nocturnal emission of β-ocimene started only sluggishly and was followed by a very strong emission burst at the beginning of the photophase (Fig. 3B). Unlike diurnal damage, which resulted in strong β-ocimene emissions over the full light period, nocturnally induced β-ocimene production ceased after the initial burst and rapidly dropped to the basal level at the onset of the dark phase. In addition to the rather low emission of Hex-Ac during the night, nocturnal damage resulted in additional, albeit weak, emissions of Hex-Ac during the photophase. Interestingly, the total amount of β-ocimene emitted after nocturnal damage was higher than the amount released after the damage that occurred in the light.

Evidence for de Novo Biosynthesis of β-Ocimene during the Early Morning Burst

The supply of C_5 precursors for monoterpene biosynthesis via the plastidial MEP pathway is directly linked to CO_2 fixation (Paré and Tumlinson, 1997). The Calvin cycle delivers the starting substrates for the MEP pathway, namely, glyceraldehyde-3-P and pyruvate. To assess how fast and to what extent CO_2 fixation contributed to the light-dependent emission of β-ocimene in induced plants, we supplied air with $^{13}CO_2$ (at 380 μg mL^{-1}) to damaged lima bean leaves and monitored the incorporation of ^{13}C into β-ocimene (Fig. 4). $^{13}CO_2$ was introduced 30 min before the end of the dark phase (6:30 AM–7 AM), and the emitted volatiles were collected at 30-min intervals and analyzed by mass spectroscopy. Analysis showed that, during the first 30 min (7 AM–7:30 AM), (*E*)-β-ocimene had incorporated almost 75% of ^{13}C. This level rose during the second interval (7:30 AM–8 AM) to about 85%. After 90 min, $^{13}CO_2$ gas was exchanged with ambient air ($^{12}CO_2$) and the degree of labeling in (*E*)-β-ocimene leveled off to an average of 25%. We therefore conclude that the light-dependent synthesis of β-ocimene was strictly linked to the photosynthetic fixation of CO_2. This differs from previous findings, namely, that monoterpenes and sesquiterpenes are not labeled with the $^{13}CO_2$ gas in tomato (*Solanum lycopersicum*) plants infested with tobacco hornworms during the photophase (Farag and Paré, 2002). As mentioned above, the induced emission of Hex-Ac depended strictly on the timing of damage, regardless of light or dark conditions (Fig. 3, A and B). Because Hex-Ac is derived from the oxidative degradation of lipids from the plasma membrane rather than from de novo biosynthesis, no

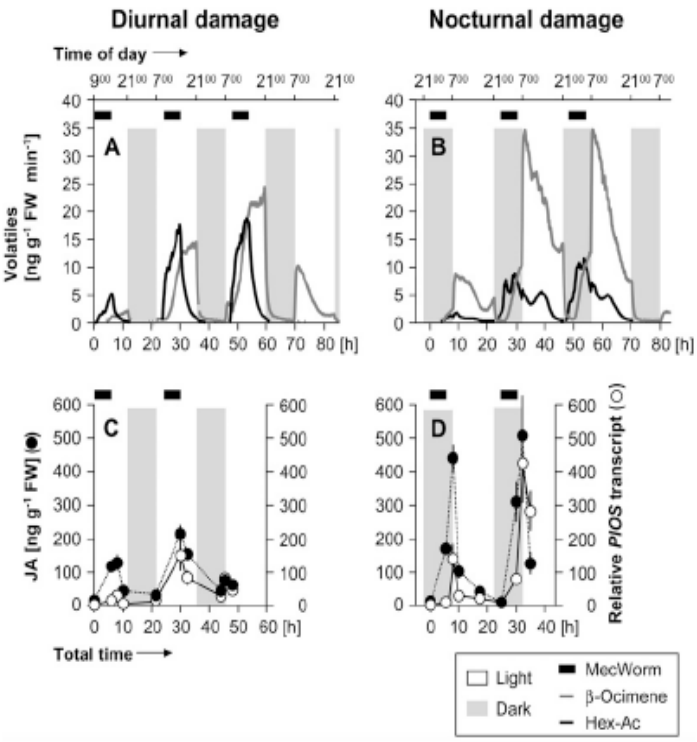


Figure 3. Emissions of β-ocimene and Hex-Ac (A and B) and levels of JA and *PlOS* transcripts (C and D) in MecWorm-treated leaves. Damage program: 6 h during either the photophase (A and C) or the dark phase (B and D) according to a 14:10 LD cycle. Damage periods started 2 h after the onset of the light or dark phase with punching every 5 s for 6 h (9 AM–3 PM during the photophase and from 11 PM–5 AM during the night). Headspace analyses were performed in triplicate. Emission is expressed as ng min^{-1} g^{-1} FW. JA and transcript levels represent the mean ± SE (n = 3–4).

incorporation of $^{13}CO_2$ into Hex-Ac was observed (data not shown).

Effect of Diurnal and Nocturnal Damage on JA Production and Gene Regulation

Damage by feeding herbivores and the external application of JA (0.5 mM) to lima bean leaves (Dicke et al., 1999) significantly induced the *PlOS* transcript (Fig. 5) and resulted in β-ocimene emission. In line with these observations, continuous wounding with MecWorm triggered rapid production of the phytohormone during both the light and dark phases, which was accompanied by a correspondingly elevated level of the *PlOS* transcript (Fig. 3, C and D). A close connection between the increased level of JA and the elevated transcript level of *PlOS* was observed for both diurnal and nocturnal damage. When leaves were damaged by MecWorm for 6 h starting at 11 PM (dark phase), induced JA levels were significantly higher (approximately 500 ng JA g^{-1} leaf fresh weight) than after they had been damaged for 6 h during the day starting at 9 AM (approximately 200 ng JA g^{-1} fresh weight). On average, the nocturnal levels of JA we observed were 2 to 3 times higher than those measured after damage during the photophase (Fig. 3, C and D). Interestingly, the day-induced JA level dropped immediately after the mechanical wounding ended, whereas nocturnally induced JA continued to increase for about 2 h after the mechanical wounding ended. Manual wounding (single events) raised the level of JA only moderately after 30 min (approximately 100 ng g^{-1} fresh weight; Supplemental Fig. S1) along with the transcript levels of *PlOS* after 6 h (Fig. 5); emission of (*E*)-β-ocimene as a consequence of manual wounding was only occasionally observed. Treatment of the leaves with the phytohormone salicylic acid or the channel-forming fungal elicitor alamethicin, which stimulates internal salicylic acid biosynthesis (Engelberth et al., 2001), did not affect transcript levels of *PlOS*.

Localization of JA and *PlOS* Transcript Accumulation in Damaged Leaves

Localization patterns of JA and the *PlOS* transcript in leaf sections in relation to the first and second instances of damage in the dark were then investigated (Fig. 6A). Thirty-two hours after onset of the first nocturnal damage by MecWorm, high JA levels were found in segments 2 and 3. Increased JA levels in segments 2 and 3 reflect the increased JA levels of the consecutive treatments shown in Figure 3D. JA and *PlOS* transcript levels in undamaged neighbor segments 1 and 4 corresponded to the resting level, showing that JA accumulated only locally at the wounding site. *PlOS* expression showed a similar localization pattern; the difference in expression levels in leaf sections 2 and 3 was statistically insignificant. That *PlOS* was expressed only at the sites of wounding was further demonstrated with transgenic Arabidopsis (*Arabidopsis thaliana*) stably transformed with *PlOS* promoter::GUS. As shown in Figure 6A, 2 h of manual

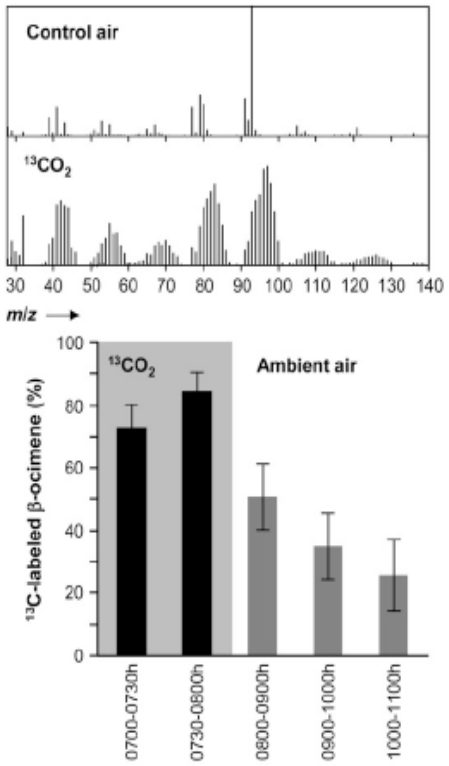


Figure 4. Pulse labeling of (*E*)-β-ocimene after administration of $^{13}CO_2$. Ambient air in the cabinet was exchanged by synthetic air containing $^{13}CO_2$ gas (380 μg mL^{-1}) 0.5 h before the onset of the light phase. Headspace analysis was started at its onset. The shaded box corresponds to the presence of $^{13}CO_2$. One hour after the onset of the photophase, the cabinet was purged with ambient air to remove $^{13}CO_2$. Collection periods for volatiles are indicated. Mass spectra of β-ocimene from leaves in ambient air or $^{13}CO_2$-containing air are shown on top. Data represent the mean ± SE (n = 4).

wounding led to the expression of the inducible GUS reporter gene only at the wounding sites (Fig. 6B). Treatment with MecWorm led to the same result (Fig. 6C). Overall, these results indicate that JA-induced *PlOS* expression is localized at the wounding site, thus facilitating terpenoid emission locally and temporally. Similar herbivory-linked local patterns were observed for *TPS* genes *AtTPS12* (*At4g13280*), *AtTPS13* (*At4g13300*; Ro et al., 2006), and *LjEβOS* (Arimura et al., 2004b).

DISCUSSION

Analyzing the volatiles from lima bean plants damaged by MecWorm for 6 h during the day or night over a period of 3 d, we observed a clear difference between the emission of the terpenoid β-ocimene and the emission of the fatty acid-derived volatile Hex-Ac. However, emission patterns for both compounds were clearly periodic (Fig. 7). The total amount of volatiles increased during the day (independent of whether damage occurred in the light or dark), whereas the amount dropped at night. This pattern corresponded to the results of Loughrin et al. (1994) and Turlings et al. (1995), who studied corn (*Zea mays*) and cotton (*Gossypium hirsutum*) plants infested by caterpillars. However, our study has revealed additional details due to two major technical improvements. First, the completely reproducible mechanical damage inflicted by MecWorm when applied during the day or night to the same plant species allowed us to compare even subtle differences in the biosynthesis of HIPVs, JA, and the expression level of *PlOS*. Second, the high time resolution of zNose (Kunert et al., 2002) allowed close correlation to be made between the emission profiles of individual volatiles and their gene expression, as well as their levels of JA.

In lima bean, herbivore-induced emission of β-ocimene was found to be exclusively linked to light and not to the circadian clock. Rapidly diminishing levels of β-ocimene after an AD period was introduced into the photophase strikingly demonstrated this link. The light-dependent β-ocimene emission was hampered by the level of the early terpenoid precursors delivered to the MEP pathway by the photosynthetic fixation of CO_2. Because the monoterpene β-ocimene is synthesized in the lima bean via the MEP pathway (Piel et al., 1998; Bartram et al., 2006), the very high degree of ^{13}C-labeling of the monoterpene (70% within the first 30 min and 85% in the second 30 min after the beginning of the light phase) synthesized de novo from $^{13}CO_2$ after the onset of the light period strongly supported this view. Thus, the metabolic flux from photosynthesis to terpenoid biosynthesis is a key regulatory element of the light-dependent emission of β-ocimene. This view is complemented by the observation that, after the first instance of damage, the high

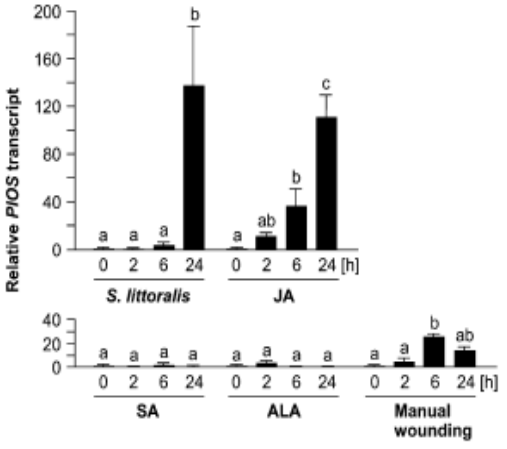


Figure 5. Effect of herbivory, wounding, and chemical treatment on the expression level of *PlOS* in lima bean leaves. Chemical treatments: JA (0.5 mM), salicylic acid (SA; 0.5 mM), and alamethicin (ALA; 1 mM). Means with small letters are significantly different according to ANOVA followed by Scheffe's test ($P < 0.05$). Data represent the mean ± SE (n = 4).

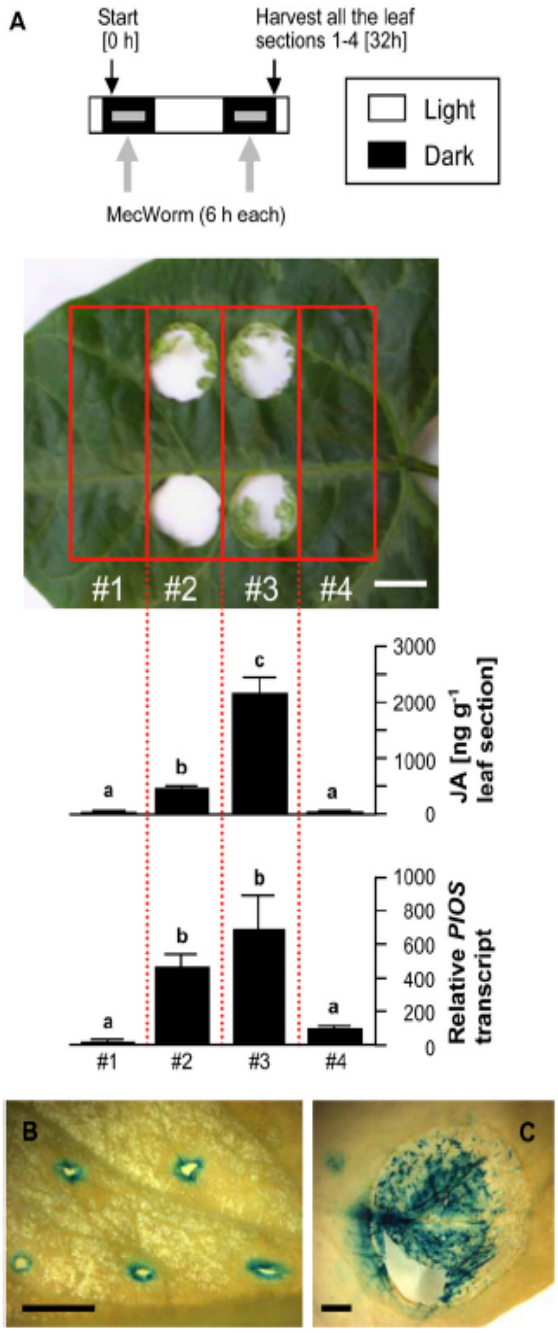


Figure 6. Localization of *PlOS* transcript and accumulation of JA in mechanically damaged lima bean leaves. A, Leaf damaged for 6 h by MecWorm during the first and second dark phases. See Figure 3D for details. Leaf segments were harvested 32 h after initiation of the first MecWorm damage. Sections 2 and 3 correspond to the first and the second nocturnal damage. Undamaged sections 1 and 4 served as control. Data represent the mean ± SE (n = 3–4). Scale bar = 10 mm. Means followed with small letters are significantly different according to ANOVA followed by Scheffe's test ($P < 0.05$). B and C, Wound-induced expression of *PlOS* promoter::*GUS* reporter gene constructs in leaves of 6-week-old Arabidopsis plants. Wounding by a needle (B) or MecWorm treatment (C) induced GUS activity after 2 h in areas in close proximity to wounding sites. Histochemical GUS assays were performed with two independent lines. Scale bar = 2 mm.

transcript level of nocturnally induced *PlOS* followed the high nocturnal level of JA. Also, all other enzymes along the MEP pathway have to be produced during the dark phase. The metabolic flow-through system begins to operate as soon as the very first photosynthates are delivered, and β-ocimene can be emitted within minutes after the onset of the light period. On the other hand, light seems also to be essential to maintain high levels of *PlOS* expression because an additionally introduced dark phase rapidly reduced *PlOS* expression levels in herbivore-damaged leaves within 4 h after the dark period began (Fig. 2B). Similar observations were made for the isoprene synthase, the 1-deoxy-D-xylulose 5-P reductoisomerase from gray poplar (*Populus* x *canescens*; Loivamäki et al., 2007), and the germin-like protein from mustard (*Sinapsis alba*; Heintzen et al., 1994). In both plants, the corresponding mRNAs were no longer detected in the dark even when expression was under the control of the endogenous clock.

In addition to increasing levels of JA and the presence/absence of light, other factors, such as the impact of an endogenous clock, seem to contribute to regulating *PlOS*. Screening the database PLACE (http://www.dna.affrc.go.jp/PLACE/signalscan.html) for cis-acting regulatory DNA elements revealed the presence of a circadian element (CAANNNNATC; Piechulla et al., 1998) in the *PlOS* promoter. Moreover, a T/G-box AACTGG motif is present in the promoter fragment, which is known to be a JA-responsive MYC2 transcription factor (Boter et al., 2004). Altogether, these findings suggest that *PlOS* is regulated by multiple elements, including JA and the clock. Such a complex regulatory mode easily explains the diurnal regulation of *PlOS* expression in lima bean leaves in response to feeding *S. littoralis* (Fig. 2A). In herbivore-damaged lima bean leaves, the level of endogenous JA stays consistently high and shows no diurnal pattern (Supplemental Fig. S1). However, rapidly fluctuating JA levels, which are typical for the onset of herbivory or during the application of MecWorm, seem to override other factors (Fig. 3, C and D).

Another major difference between nocturnal and daytime damage was observed in the levels of JA. JA is the major plant hormone involved in the up-regulation of volatiles, in particular, terpenoid biosynthesis (Arimura et al., 2005). Unexpectedly, levels of JA were 2 to 3 times higher after nocturnal damage than they were after daytime damage. As shown in Figure 3C, during the day JA production stopped immediately once the damage period ended, whereas JA production at night increased levels for 2 h after MecWorm treatment ended. Because both damage regimes destroyed the same amount of leaf tissue, which in turn should have generated the same amount of JA, we postulate that certain JA-modifying steps exist that occur only during the light phase. Candidates that might be responsible for effecting different regulatory processes according to the different light regimes include (1) inactivation of JA by hydroxylation, (2) transformation into a sulfate

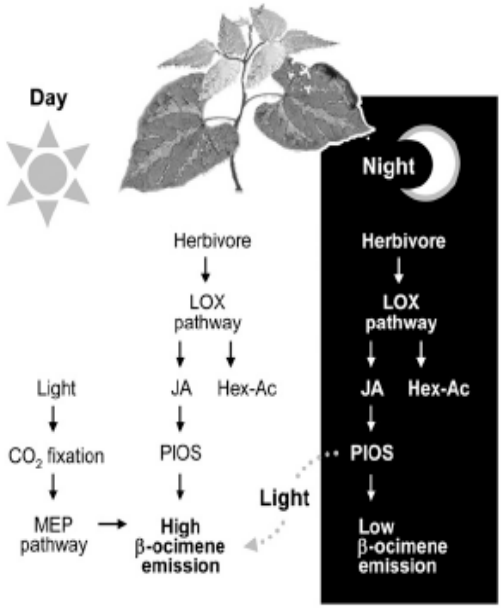


Figure 7. Schematic representation of the signaling and metabolic pathways required for herbivore-induced β-ocimene and Hex-Ac emissions in lima bean leaves in a daily cycle. LOX, Lipoxygenase.

ester or glucoside, and (3) conjugation of the phytohormone to amino acids reviewed by Liechti and Farmer (2006). Moreover, the higher diurnal emission of Hex-Ac (Fig. 3, A and B), the compound that competes with JA for the common precursor α-linolenic acid, may also limit JA production during the light phase. Only comprehensive profiling of the different JA derivatives and all other products derived from α-linolenic acid will clarify which compound is responsible.

Production of JA and the emission of the fatty acid-derived volatile Hex-Ac correlated closely with MecWorm damage, regardless of whether damage occurred under light or dark conditions (Fig. 3, A and B). Besides the low nocturnal emission of Hex-Ac (Fig. 3B), a second emission peak occurred during the day. This biphasic emission of Hex-Ac corresponded to previous observations in Arabidopsis. Enhanced transcript levels of CHAT (At3g03480) coincided with the highest levels of Hex-Ac, which were emitted several hours after wounding. However, the first emission of Hex-Ac was observed just 5 min after wounding (Loreto et al., 2006; D'Auria et al., 2007). The release of (Z)-3-hexenal, another initially formed green-leaf volatile, occurred within 20 s after the damage of Arabidopsis leaf tissue (Matsui et al., 2000). The immediate release of green-leaf volatiles is due to the presence of a functional array of enzymes (phospholipase, lipoxygenase, and fatty acid hydroperoxide lyase) and their contact with α-linolenic acid or the corresponding 13-hydroperoxide in the damaged tissue (Matsui et al., 2000). Thus, biphasic emission of Hex-Ac from damaged lima bean leaves has two elements, namely, the immediate wound response based on constitutively expressed enzymes and induced biosynthesis, which is mediated by enhanced levels of JA (Matsui, 2006; D'Auria et al., 2007).

Both Hex-Ac and β-ocimene, volatiles focused on in this research, are known to attract carnivorous arthropods and thus function as indirect plant defenses by increasing predation pressure on attacking herbivores (Dicke et al., 1990) and by plant-plant interactions (Arimura et al., 2000, 2001; Köllner et al., 2004). Particularly intriguing is the recent observation by Heil and Silva Bueno (2007) that the induced volatiles serve as an information highway, speeding communication between remote parts of the attacked plant or bridging vascular unconnected leaves (Orians, 2005). The immediate damage-linked release of Hex-Ac, which has been shown to induce nectar flow in neighboring areas of the same and other plants, is very important. Any damage by an herbivore allows endangered plant areas to transport information on the herbivore attack without the need for delayed induction. Also, the early-morning terpenoid emission burst following nocturnal leaf damage may have an important biological function, namely, to massively attract the natural enemies of herbivores that are present during the morning hours (Kaas et al., 1993; Hirose et al., 2003). Current findings will have to be complemented by creative field experiments and comparative approaches to better understand emerging areas, such as the macroecology or community ecology, of diurnal plant-insect interactions in the ecosystem.

MATERIALS AND METHODS

Plants and Caterpillars

Lima bean (*Phaseolus lunatus* 'Ferry Morse' var. Jackson Wonder Bush) was grown in soil. Individual plants were grown in plastic pots in a growth chamber at 23°C (160 μE m^{-2} s^{-1} during a 14-h photoperiod; relative humidity 60%) for 2 weeks. Larvae of *Spodoptera littoralis* Boisd. (Lepidoptera, Noctuidae) were reared on an artificial diet in a plastic box (25°C ± 1°C; 14:10 LD; Bergomaz and Boppré, 1986).

Plant Treatments

Primary leaves from intact lima bean plants were used for all treatments and analyses. For *S. littoralis* infestation, three second- and third-instar larvae were placed on a leaf. Manual wounding was performed by punching 18 6-mm-diameter holes into a leaf for a total of 508 mm^2 of damaged area. MecWorm operation was programmed to generate within 6 h circles of damaged leaf area in the primary leaf, yielding 265 mm^2 of total damaged area (two circle sectors; ϕ = 13.0 mm) using 12 punches min^{-1}. For chemical treatment, JA or salicylic acid (0.5 mM, pH 5.8–6.0) in 2 mL of water was sprayed onto intact plants in plastic pots. Alamethicin (1 mM) was applied to the petioles of detached plantlets in aqueous solutions. All treatments, except MecWorm damage in the darkness, started at 9 AM. During treatments, temperature was maintained at 25°C ± 1°C with a photoperiod of 14 h (160 μE m^{-2} s^{-1}). The light period ran from 7 AM to 9 PM.

Labeling Experiments with $^{13}CO_2$

Leaves were damaged by MecWorm during the first and second dark phases (11 PM–5 AM) and covered by a Plexiglas cabinet (approximately 500 mL) with air constantly passing through the system at 50 mL min^{-1}. For pulse

labeling, synthetic air with 380 μg mL^{-1} $^{13}CO_2$, 20.9% O_2, and 79% N_2 (Westfalen A.G.) was introduced into the cabinet with a starting flow of 200 mL min^{-1} for 10 min (6 AM–6:10 AM) to replace the natural $^{12}CO_2$ followed by a reduced flow at 50 mL min^{-1}. The emitted volatiles were collected by solid-phase microextraction on a polydimethylsiloxane 100 fiber (Supelco). Samples were analyzed on an Agilent 6580 gas chromatograph coupled to a Micromass GCT time-of-flight mass spectrometer (Micromass UK Ltd.). Separation was performed on a Zebron ZB-5ms capillary column (30 m × 0.25 mm i.d. × 0.25 μm; Phenomenex). Helium at a flow rate of 1.0 mL min^{-1} served as carrier gas and a split-mode injection (1:10) was employed. The gas chromatograph injector, transfer line, and ion source were set at 220°C, 280°C, and 280°C, respectively. Spectra were taken in the total ion-scanning mode at 70 eV. Compounds were eluted under programmed conditions starting at 40°C (2-min isotherm) followed by heating at 15°C min^{-1} to 200°C, then at 40°C min^{-1} to 280°C. The injected amount was 1 μL. The intense fragment ions at *m/z* 93 (control plants) and at *m/z* 94 to 100 ($^{13}CO_2$-fed plants) were used to analyze and calculate the incorporation rate of ^{13}C. The fragment ions at *m/z* 94 to 100 correspond to the incorporation of one to seven ^{13}C-atoms into β-ocimene (see Fig. 4).

zNose

A primary leaf attached to an intact lima bean plant was enclosed in a Plexiglas cabinet (552 mL) where it was subjected to insect and MecWorm damage (Mithöfer et al., 2005). zNose (model 4100; Electronic Sensor Technology) was connected to the cabinet via a stainless steel needle attached to the Luer inlet on the instrument. The detector (surface acoustic wave detector) of zNose was operated at 30°C (Kunert et al., 2002). Prepurified air (ZeroAir generator [Parker Hannifin Corp.] and an additional charcoal filter) was passed through the plastic cabinet at 40 mL min^{-1}. Every 10 min, zNose trapped an aliquot of the headspace volatiles for 60 s on an internal Tenax precolumn at 30 mL min^{-1} followed by separation on the main column under programmed conditions from 40°C to 175°C at 5°C s^{-1}. A DB5 column (1-m × 0.25-mm × 0.25-μm film; Electronic Sensor Technology) was used. β-Ocimene and Hex-Ac were quantified after calibration with authentic standards and are presented as ng min^{-1} g^{-1} fresh weight.

Analysis of JA

Plant material was weighed (0.2–0.25 g) and shock frozen with liquid nitrogen. For analyses of spatial patterns of JA, we mixed two samples corresponding to each leaf section to obtain 0.2 g of leaf sections. JA was quantified in plant material according to a modified protocol from Schulze et al. (2006). Frozen plant material was mixed with methanol/2,6-di-tert-butyl-4-methylphenol (BHT; Sigma-Aldrich) solution (2.5 mL, 0.05% BHT), followed by the addition of the derivatization agent pentafluorobenzylhydroxylamine hydrochloride, 2 mL, 0.05 M in methanol (Sigma-Aldrich). For quantification, 9,10-[$^2H_2$]-dihydrojasmonic acid (250 ng) was added as an internal standard. Next, the mixture, cooled on ice and kept under argon atmosphere, was homogenized for 5 min with a high-performance dispenser at 24,000 rpm (Ultra-Turrax T-25; IKA-Werk). Derivatization was completed by shaking the samples for 2 h at room temperature. After acidification with 0.1 M HCl (approximate pH 3), the methanol/water phase was quantitatively extracted with hexane (3 × 5 mL). The combined organic layers were subsequently passed through preconditioned (methanol, 5 mL; hexane, 5 mL) Chromabond NH_2 cartridges (3 mL/0.5 g; Macherey-Nagel). Cartridges were washed with *i*-propanol:dichloromethane (5 mL, 2:1 [v/v]) and eluted with diethyl ether:formic acid (10 mL, 98:2 [v/v]). The solvent was then removed under a gentle stream of argon and the sample was esterified with an etheral solution of diazomethane for 5 min. After removal of excess diazomethane, the sample was taken up in 45 μL of dichloromethane. Samples were analyzed on a Finnigan GCQ Instrument (Thermoelectron) running in a CI-negative ion mode, as described by Schulze et al. (2006).

cDNA Cloning and Terpene Synthase Assay

A whole leaf was crushed in liquid N_2 and the tissue (100 mg) was used for RNA extraction. First-strand cDNA was synthesized using the SuperScript II (Invitrogen) oligo(dT)$_{12-18}$ primer and 1 μg of total RNA at 50°C for 50 min. For PCR, primers for the *PIOS* cDNA fragment were designed using a *LjEβOS* cDNA sequence (GenBank accession no. AY579970). PCR was run for 2 min at 95°C, 35 cycles of 15 s at 94°C, 30 s at 50°C, and 60 s at 72°C. Further cloning of 5′- and 3′-ends was accomplished by RACE-PCR using the first-choice RLM-RACE kit (Ambion) according to the manufacturer's protocol. For functional identification of the full-length genes, the region of an open reading frame of *PIOS* was subcloned into pHis-8-3 expression vectors (Jez et al., 2000). Terpene synthase enzyme assays were performed according to Arimura et al. (2008).

Real-Time PCR

First-strand cDNA was synthesized from isolated total RNA (see above) using SuperScript II reverse transcriptase, oligo(dT)$_{12-18}$ primer, and 1 μg of total RNA at 42°C for 50 min. Real-time PCR was done on a Mx3000P real-time PCR system (Stratagene). The process was performed with 25 μL of reaction mixture containing 12.5 μL of 2× Brilliant SYBR Green QPCR master mix (Stratagene), cDNA (1 μL from 20 μL of each reverse transcriptase product pool), 100 nM primers, and 30 mM ROX as a passive reference dye. Initial polymerase activation: 10 min at 95°C; 40 cycles of 30 s at 95°C, 60 s at 55°C, and 30 s at 72°C. PCR conditions were determined by comparing threshold values in a dilution series of the RT product, followed by non-RT template control and nontemplate control for each primer pair. Relative RNA levels were calibrated and normalized with the level of *PlACT1* mRNA (GenBank accession no. DQ159907). Primers are shown in Supplemental Table S1.

Cloning the *PlOS* Promoter and *PlOS* Promoter::GUS Fusion Construct

Genomic DNA was extracted from lima bean leaves using the Qiagen plant DNA extraction kit. Genomic DNA was digested with *Eco*RV and then circularized with T4 DNA ligase (New England Biolabs). Nested, inverse PCR was performed with circularized genomic DNA as the template, using *Taq* DNA polymerase (New England Biolabs). PCR protocol: 40 cycles of 15 s at 95°C, 30 s at 55°C, and 60 s at 72°C. The PCR product was ligated to a TOPO TA cloning kit (Invitrogen). Primers are shown in Supplemental Table S1. The 517-bp *PlOS* promoter region upstream of the start codon (GenBank accession no. EU194554) was reamplified by PCR using *Pfu* DNA polymerase with a pair of primers introducing restriction sites. The amplified promoter fragments were digested with *Bam*HI and *Eco*RI, and digested fragments were ligated to the corresponding site of pCAMBIA1391 upstream of the *GUS* gene (Cambia). Constructs were transformed into the *Agrobacterium* GV3101 strain, which was then used to transform Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia-0 according to the floral-dip method (Clough and Bent, 1998). Arabidopsis T_1 or T_2 lines were used for histochemical analysis according to the method described by Jefferson et al. (1987).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers EU194553 and EU194554.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Levels of JA in leaves after feeding by *S. littoralis* or manual wounding in a LD cycle.

Supplemental Table S1. Primers used for this study.

ACKNOWLEDGMENTS

We thank Dr. A. Mithöfer and Dr. S. Bartram for valuable discussions and H. Uchtenhagen and S. Garms for technical assistance.

Received October 16, 2007; accepted December 10, 2007; published December 28, 2007.

LITERATURE CITED

Arimura G, Garms S, Maffei M, Bossi S, Schulze B, Leitner M, Mithöfer A, Boland W (2008) Herbivore-induced terpenoid emission in *Medicago truncatula*: concerted action of jasmonate, ethylene and calcium signaling. Planta 227: 453–464

Arimura G, Huber DPW, Bohlmann J (2004a) Forest tent caterpillars (*Malacosoma disstria*) induce local and systemic diurnal emissions of

terpenoid volatiles in hybrid poplar (*Populus trichocarpa* x *deltoides*): cDNA cloning, functional characterization, and patterns of gene expression of (-)-germacrene D synthase, *PtdTPS1*. Plant J 37: 603–616

Arimura G, Kost C, Boland W (2005) Herbivore-induced, indirect plant defences. Biochim Biophys Acta 1734: 91–111

Arimura G, Ozawa R, Horiuchi J, Nishioka T, Takabayashi J (2001) Plant-plant interactions mediated by volatiles emitted from plants infested by spider mites. Biochem Syst Ecol 29: 1049–1061

Arimura G, Ozawa R, Kugimiya S, Takabayashi J, Bohlmann J (2004b) Herbivore-induced defense response in a model legume. Two-spotted spider mites induce emission of (*E*)-β-ocimene and transcript accumulation of (*E*)-β-ocimene synthase in *Lotus japonicus*. Plant Physiol 135: 1976–1983

Arimura G, Ozawa R, Shimoda T, Nishioka T, Boland W, Takabayashi J (2000) Herbivory-induced volatiles elicit defence genes in lima bean leaves. Nature 406: 512–515

Arimura G, Volpe V, Kunert M, Maffei M, Boland W (2007) Terpenoid biosynthesis and signaling in legume plants in response to herbivorous damage. *In* A Ramina, C Chang, J Giovannoni, H Klee, P Perata, E Woltering, eds, Advances in Plant Ethylene Research. Springer, Dordrecht, The Netherlands, pp 353–358

Bartram S, Jux A, Gleixner G, Boland W (2006) Dynamic pathway allocation in early terpenoid biosynthesis of stress-induced lima bean leaves. Phytochemistry 67: 1661–1672

Bergomaz R, Boppré M (1986) A simple instant diet for rearing Arctiidae and other moths. J Lepid Soc 40: 131–137

Boter M, Ruíz-Rivero O, Abdeen A, Prat S (2004) Conserved MYC transcription factors play a key role in jasmonate signaling both in tomato and *Arabidopsis*. Genes Dev 18: 1577–1591

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J 16: 735–743

D'Auria JC, Pichersky E, Schaub A, Hansel A, Gershenzon J (2007) Characterization of a BAHD acyltransferase responsible for producing the green leaf volatile (*Z*)-3-hexen-1-yl acetate in *Arabidopsis thaliana*. Plant J 49: 194–207

De Moraes CM, Mescher MC, Tumlinson JH (2001) Caterpillar-induced nocturnal plant volatiles repel conspecific females. Nature 410: 577–580

Dicke M, Gols R, Ludeking D, Posthumus MA (1999) Jasmonic acid and herbivory differentially induce carnivore-attracting plant volatiles in lima bean plants. J Chem Ecol 25: 1907–1922

Dicke M, van Beek TA, Posthumus MA, Ben Dom N, van Bokhoven H, de Groot AE (1990) Isolation and identification of volatile kairomone that affects acarine predator-prey interactions. J Chem Ecol 16: 381–396

Dudareva N, Andersson S, Orlova I, Gatto N, Reichelt M, Rhodes D, Boland W, Gershenzon J (2005) The nonmevalonate pathway supports both monoterpene and sesquiterpene formation in snapdragon flowers. Proc Natl Acad Sci USA 102: 933–938

Dudareva N, Martin D, Kish CM, Kolosova N, Gorenstein N, Fäldt J, Miller B, Bohlmann J (2003) (*E*)-β-Ocimene and myrcene synthase genes of floral scent biosynthesis in snapdragon: function and expression of three terpene synthase genes of a new terpene synthase subfamily. Plant Cell 15: 1227–1241

Engelberth J, Koch T, Schüler G, Bachmann N, Rechtenbach J, Boland W (2001) Ion channel-forming alamethicin is a potent elicitor of volatile biosynthesis and tendril coiling. Cross talk between jasmonate and salicylate signaling in lima bean. Plant Physiol 125: 369–377

Farag MA, Paré PW (2002) C_6-Green leaf volatiles trigger local and systemic VOC emissions in tomato. Phytochemistry 61: 545–554

Heil M, Silva Bueno JC (2007) Within-plant signaling by volatiles leads to induction and priming of an indirect plant defense in nature. Proc Natl Acad Sci USA 104: 5467–5472

Heintzen C, Fischer R, Melzer S, Kappeler K, Apel K, Staiger D (1994) Circadian oscillations of a transcript encoding a germin-like protein that is associated with cell walls in young leaves of the long-day plant *Sinapis alba* L. Plant Physiol 106: 905–915

Hirose Y, Ehler LE, Hirose Y (2003) Influence of host age on patch use by a quasi-gregarious egg parasitoid. Popul Ecol 32: 789–796

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6: 3901–3907

Jez JM, Ferrer JL, Bowman ME, Dixon RA, Noel JP (2000) Dissection of malonyl-coenzyme A decarboxylation from polyketide formation in the reaction mechanism of a plant polyketide synthase. Biochemistry 39: 890–902

Kaas JP, Ramaswamy SB, Elzen GW (1993) Behavioral time budget and periodicity exhibited by *Microplitis croceipes* in field cages with *Heliothis virescens* on spring host plants. Entomophaga 38: 143–154

Köllner TG, Schnee C, Gershenzon J, Degenhardt J (2004) The variability of sesquiterpenes emitted from two *Zea mays* cultivars is controlled by allelic variation of two terpene synthase genes encoding stereoselective multiple product enzymes. Plant Cell 16: 1115–1131

Kunert M, Biedermann A, Koch T, Boland W (2002) Ultrafast sampling and analysis of plant volatiles by a hand-held miniaturised GC with pre-concentration unit: kinetic and quantitative aspects of plant volatile production. J Sep Sci 25: 677–684

Liechti R, Farmer EE (2006) Jasmonate biochemical pathway. Sci STKE 2006: cm3

Loivamäki M, Louis S, Cinege G, Zimmer I, Fischbach RJ, Schnitzler JP (2007) Circadian rhythms of isoprene biosynthesis in grey poplar leaves. Plant Physiol 143: 540–551

Loreto F, Barta C, Brilli F, Nogues I (2006) On the induction of volatile organic compound emissions by plants as consequence of wounding or fluctuations of light and temperature. Plant Cell Environ 29: 1820–1828

Loughrin JH, Manukian A, Heath RR, Turlings TC, Tumlinson JH (1994) Diurnal cycle of emission of induced volatile terpenoids by herbivore-injured cotton plant. Proc Natl Acad Sci USA 91: 11836–11840

Matsui K (2006) Green leaf volatiles: hydroperoxide lyase pathway of oxylipin metabolism. Curr Opin Plant Biol 9: 274–280

Matsui K, Kurishita S, Hisamitsu A, Kajiwara T (2000) A lipid-hydrolysing activity involved in hexenal formation. Biochem Soc Trans 28: 857–860

Mithöfer A, Wanner G, Boland W (2005) Effects of feeding *Spodoptera littoralis* on lima bean leaves. II. Continuous mechanical wounding resembling insect feeding is sufficient to elicit herbivory-related volatile emission. Plant Physiol 137: 1160–1168

Orians C (2005) Herbivores, vascular pathways, and systemic induction: facts and artifacts. J Chem Ecol 31: 2231–2242

Paré PW, Tumlinson JH (1997) De novo biosynthesis of volatiles induced by insect herbivory in cotton plants. Plant Physiol 114: 1161–1167

Paré PW, Tumlinson JH (1999) Plant volatiles as a defense against insect herbivores. Plant Physiol 121: 325–332

Piechulla B, Merforth N, Rudolph B (1998) Identification of tomato *Lhc* promoter regions necessary for circadian expression. Plant Mol Biol 38: 655–662

Piel J, Donath J, Bandemer K, Boland W (1998) Mevalonate-independent biosynthesis of terpenoid volatiles in plants: induced and constitutive emission of volatiles. Angew Chem Int Ed 37: 2478–2481

Ro DK, Ehlting J, Keeling CI, Lin R, Mattheus N, Bohlmann J (2006) Microarray expression profiling and functional characterization of *AtTPS* genes: duplicated *Arabidopsis thaliana* sesquiterpene synthase genes *At4g13280* and *At4g13300* encode root-specific and wound-inducible (*Z*)-gamma-bisabolene synthases. Arch Biochem Biophys 448: 104–116

Roeder S, Hartmann AM, Effmert U, Piechulla B (2007) Regulation of simultaneous synthesis of floral scent terpenoids by the 1,8-cineole synthase of *Nicotiana suaveolens*. Plant Mol Biol 65: 107–124

Schulze B, Lauchli R, Sonwa MM, Schmidt A, Boland W (2006) Profiling of structurally labile oxylipins in plants by in situ derivatization with pentafluorobenzyl hydroxylamine. Anal Biochem 348: 269–283

Shiojiri K, Ozawa R, Takabayashi J (2006) Plant volatiles, rather than light, determine the nocturnal behavior of a caterpillar. PLoS Biol 4: e164

Turlings TC, Loughrin JH, McCall PJ, Rose US, Lewis WJ, Tumlinson JH (1995) How caterpillar-damaged plants protect themselves by attracting parasitic wasps. Proc Natl Acad Sci USA 92: 4169–4174

6. Article II

Functional Identification and Differential Expression of 1-Deoxy-D-Xylulose 5-Phosphate Synthase and Other MEP Pathway Genes in Induced Terpenoid Resin Formation of Norway spruce (*Picea abies*)

M. Phillips, M.H. Walter, S. Ralph, P. Dabrowska, K. Luck, E.M. Urós, W. Boland, D. Strack, M. Rodríguez-Concepción, J. Bohlmann, J. Gershenzon

Plant Molecular Biology (2007) **65**, 243-257

Plant Mol Biol (2007) 65:243–257
DOI 10.1007/s11103-007-9212-5

Functional identification and differential expression of 1-deoxy-D-xylulose 5-phosphate synthase in induced terpenoid resin formation of Norway spruce (*Picea abies*)

Michael A. Phillips · Michael H. Walter · Steven G. Ralph · Paulina Dabrowska · Katrin Luck · Eva Maria Urós · Wilhelm Boland · Dieter Strack · Manuel Rodríguez-Concepción · Jörg Bohlmann · Jonathan Gershenzon

Received: 26 April 2007 / Accepted: 16 July 2007 / Published online: 9 August 2007

Abstract Conifers produce terpenoid-based oleoresins as constitutive and inducible defenses against herbivores and pathogens. Much information is available about the genes and enzymes of the late steps of oleoresin terpenoid biosynthesis in conifers, but almost nothing is known about the early steps which proceed via the methylerythritol phosphate (MEP) pathway. Here we report the cDNA cloning and functional identification of three Norway spruce (*Picea abies*) genes encoding 1-deoxy-D-xylulose 5-phosphate synthase (DXS), which catalyzes the first step of the MEP pathway, and their differential expression in the stems of young saplings. Among them are representatives of both types of plant *DXS* genes. A single type I *DXS* gene is constitutively expressed in bark tissue and not affected by wounding or fungal application. In contrast, two distinct type II *DXS* genes, *PaDXS2A* and *PaDXS2B*, showed increased transcript abundance after these treatments as did two other genes of the MEP pathway tested, 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) and 4-hydroxyl 3-methylbutenyl diphosphate reductase (HDR). We also measured gene expression in a Norway spruce cell suspension culture system that, like intact trees, accumulates monoterpenes after treatment with methyl jasmonate. These cell cultures were characterized by an up-regulation of monoterpene synthase gene transcripts and enzyme activity after elicitor treatment, as well as induced formation of octadecanoids, including jasmonic acid and 12-oxophytodienoic acid. Among the Type II *DXS* genes in cell cultures, *PaDXS2A* was induced by treatment with chitosan, methyl salicylate, and *Ceratocystis polonica* (a bark beetle-associated, blue-staining fungal pathogen of Norway spruce). However, *PaDXS2B* was induced by treatment with methyl jasmonate and chitosan, but was not affected by methyl salicylate or *C. polonica*. Our results suggest distinct functions of the three *DXS* genes in primary and defensive terpenoid metabolism in Norway spruce.

Keywords *Ceratocystis polonica* · Conifer defense · Fungal elicitor · Isoprenoid biosynthesis · Methyl jasmonate · Terpenoid synthase · Oleoresin · Conifer genomics

Electronic supplementary material The online version of this article (doi:10.1007/s11103-007-9212-5) contains supplementary material, which is available to authorized users.

M. A. Phillips (✉) · K. Luck · J. Gershenzon
Max Planck Institut für Chemische Ökologie, Abteilung Biochemie, Hans Knöll Str. 8, Jena 07745, Germany
e-mail: mphillips@ice.mpg.de

M. H. Walter · D. Strack
Leibniz-Institut für Pflanzenbiochemie, Abteilung Sekundärstoffwechsel, Weinberg 3, Halle (Saale) 06120, Germany

S. G. Ralph · J. Bohlmann
Michael Smith Laboratories, University of British Columbia, Vancouver, Canada

P. Dabrowska · W. Boland
Max Planck Institut für Chemische Ökologie, Abteilung Bioorganische Chemie, Hans Knöll Str. 8, Jena 07745, Germany

E. M. Urós · M. Rodríguez-Concepción
Departament de Bioquímica i Biología Molecular, Facultat de Biologia, Universitat de Barcelona, Av. Diagonal 645, 08282 Barcelona, Spain

E. M. Urós · M. Rodríguez-Concepción
Consorci CSIC-IRTA de Genetica Molecular Vegetal, Jordi Girona 18-26, 08034 Barcelona, Spain

Introduction

One of the most characteristic features of conifers is their production of a viscous and pungent oleoresin which is thought to serve as a defense against herbivores and pathogens (Phillips and Croteau 1999; Franceschi et al. 2005; Keeling and Bohlmann 2006a,b). Composed of mono-, sesqui-, and diterpenes, oleoresin is present constitutively in many conifer species, but accumulation is also induced upon herbivore or pathogen attack. Induction involves the differentiation of traumatic resin ducts or resin blisters (Phillips and Croteau 1999; Martin et al. 2002), enhanced biosynthesis of terpenoid constituents (Martin et al. 2002; Miller et al. 2005; Zeneli et al. 2006), and emission of terpenoid volatiles from needles (Martin et al. 2003).

Picea spp. are a well-established system for investigating the molecular, biochemical and ecological aspects of constitutive and induced oleoresin defenses in conifers (Martin et al. 2002; Byun-McKay et al. 2003; Martin et al. 2003, 2004; Miller et al. 2005; Schmidt et al. 2005; Erbilgin et al. 2006; Keeling and Bohlmann 2006a, b; Ralph et al. 2006; Zeneli et al. 2006). Both the octadecanoid and ethylene signaling pathways are known to be involved in the insect and wound-induced defense response in spruce (Franceschi et al. 2002; Martin et al. 2002; Hudgins et al. 2003; Hudgins and Franceschi 2004; Zhao et al. 2004; Huber et al. 2005; Hudgins et al. 2006; Ralph et al. 2007). Treatment of Sitka (*P. sitchensis*) or Norway spruce (*P. abies*) with methyl jasmonate induces oleoresin accumulation (Franceschi et al. 2002; Martin et al. 2002; Miller et al. 2005), and this has been used to study the ecological roles of terpenoid defenses. For example, methyl jasmonate-treated trees were more resistant to colonization by the bark beetle, *Ips typographus* (Erbilgin et al. 2006), and the bark beetle-associated fungus, *Ceratocystis polonica* (Zeneli et al. 2006). Further understanding of the defensive function of conifer oleoresin would benefit greatly from more knowledge of the pathway of oleoresin terpenoid biosynthesis and how this is regulated.

The biosynthesis of oleoresin terpenes in conifers involves three stages: (1) the production of the central C_5 intermediates, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), by the methylerythritol phosphate (MEP) and mevalonate pathways, (2) the condensation of IPP and DMAPP into C_{10}, C_{15}, and C_{20} prenyl diphosphates, the precursors of mono-, sesqui- and diterpenes, respectively, and (3) the late cyclization and oxidation steps catalyzed by terpenoid synthases (TPS) and cytochrome P450 dependent monooxygenases (CYP450) which determine the particular carbon skeleton and oxidation pattern of the product (reviewed in Croteau 1987). The third stage has been well studied, starting with pioneering work on the molecular biology and enzymology of TPS and CYP450 activities in grand fir (*Abies grandis*) (Steele et al. 1995; Bohlmann et al. 1997; Steele et al. 1998a). More recently, inducible *TPS* gene expression was found to be responsible for much of the chemical diversity of traumatic oleoresin in Norway (Fäldt et al. 2003; Martin et al. 2004) and Sitka spruce (Miller et al. 2005). In addition, CYP450 gene expression was demonstrated to be involved in traumatic diterpene resin acid biosynthesis in loblolly pine (*Pinus taeda*) (Ro et al. 2005; Ro and Bohlmann 2006). In contrast, only limited knowledge is available on the role of second stage enzymes, the isoprenyl diphosphate synthases, in conifer oleoresin formation (Martin et al. 2002; Schmidt et al. 2005). To the best of our knowledge, there are no reports on the possible regulatory function of genes and enzymes of the first stage. However, recent microarray gene expression analysis of all known steps of the terpenoid oleoresin pathway in Sitka spruce revealed substantial induction of transcript for some of the early pathway steps upon insect attack (Ralph, et al. 2006; S. Ralph and J. Bohlmann, unpublished results).

To learn more about the first stage of terpenoid biosynthesis in conifer oleoresin production, we began by investigating the first step in the MEP pathway of terpenoid biosynthesis, which is catalyzed by 1-deoxy-D-xylulose 5-phosphate synthase (DXS) (Sprenger et al. 1997; Lange et al. 1998; Lois et al. 1998; Rodriguez-Concepcion and Boronat 2002). The MEP pathway is thought to be much more important than the mevalonate pathway in providing precursors for both monoterpenes and diterpenes (Lange and Ghassemian 2003), the major components of spruce oleoresin. In addition, previous studies have shown that DXS is a highly regulated enzyme that is a significant rate-determining step of the MEP pathway in other plant species (Lois et al. 2000; Walter et al. 2000; Estevez et al. 2001). Two types of *DXS* genes have been characterized based on sequence properties and expression pattern: one type (type I) which is constitutively expressed in photosynthetic tissues and is probably involved in supplying substrate for primary isoprenoids, such as carotenoids and phytol, and a second type (type II) which appears to be involved in supplying substrate for specialized terpenoids involved in ecological interactions (Walter et al. 2002). Here we report the cDNA cloning, functional identification, and expression analysis of three *DXS* genes from Norway spruce and their different roles in terpenoid biosynthesis.

Oleoresin formation in conifers has been a difficult process to study at the molecular and biochemical levels because it typically occurs in large, slow-growing trees and is restricted to specific tissues and stages of development, or only occurs after initial herbivore or pathogen attack (Martin et al. 2002; Franceschi et al. 2005). To overcome these problems, we established a Norway spruce cell

culture system to study terpene formation and examined the expression of *DXS* and other genes of terpene biosynthesis after induction.

Materials and methods

Plant growth conditions and treatments

Norway spruce (*P. abies* L. Karst) saplings of clonal lines 3166-728 and 1015-903 were propagated from lateral branches at the Niedersächsische Forstliche Versuchsanstalt (Escherode, Germany) and grown in a walk-in growth chamber under lighting conditions as reported previously (Martin et al. 2002). After 6 months, saplings were transferred to 2 l pots with a 2:3 (v/v) mixture of peat:universal planting mix and grown in a greenhouse at 24°C with mechanical misters supplying 10 s of mist per hour. Saplings were 2–3 years old (60–90 cm tall) when treatments were carried out as follows. Methyl jasmonate (MeJA, Aldrich, Steinheim, Germany) was sprayed onto saplings as a 25 mM solution containing 0.2% Tween 20 as described previously (Martin et al. 2002). Control saplings were sprayed with a Tween 20 solution only. Mechanical wounding consisted of a series of horizontal razor blade scores along the entire length of the trunk, approximately 3–4 per cm. After 2 h, saplings were wounded a second time. For combined treatments, the freshly wounded trunks were treated with either a chitosan solution (10 mg/ml) or a *C. polonica* spore culture using a fine tipped paint brush. Saplings used for time course measurements were harvested at 1, 3, 7, and 10 days post-treatment, while those used for single time point treatments were harvested at day 3. A minimum of six saplings was harvested from each group. After harvesting of stems, the outer bark and cambium were separated from the xylem (Martin et al. 2002) and the former frozen in liquid nitrogen prior to RNA extraction.

Norway spruce cell culture: establishment, treatments, and analysis

Somatic embryogenic cultures of Norway spruce were initiated from mature seeds, propagated as callus on solid EDM6 medium (Bishop-Hurley et al. 2001) and subcultured every 7 days. Actively growing calli were then transferred to 30 ml liquid EDM6 media and grown at 24°C in darkness with gentle shaking and sub-culturing at 10–12 day intervals. For induction experiments with defined elicitors, MeJA, chitosan (Sigma Chemicals, St. Louis, MO, USA), or methyl salicylate (MeSA) (Sigma Chemicals) was added to a final concentration of 50 µM, 50 µg/ml, and 50 µM, respectively, 10 days after subculture. For induction experiments with a fungal pathogen, *C. polonica* spore cultures (isolate 93–208/115, courtesy of the Culture Collection of the Norwegian Forest Research Institute, Ås, Norway) were diluted 1:100 into spruce liquid cultures for induction. *C. polonica* spore cultures were prepared by transferring a 5 mm disk of mycelium onto a 9 cm sterile Petri dish containing 4% malt extract (w/v), 0.4% yeast extract (w/v), and 1.5% agar (w/v) at pH 7.0, incubating the sealed plate at 22°C in darkness for 8 days, and then gently washing the plate surface with 2 ml solution of 0.9% NaCl and 0.5% Tween 20, followed by 10 ml 0.9% NaCl to collect the spores. Induced tissue from liquid Norway spruce cell cultures was harvested by filtration on a side-arm flask equipped with a Buchner funnel and No. 2 Whatman filter paper. For monoterpene analysis of cell cultures, a single 30 ml culture was used to inoculate 200 ml fresh liquid EDM6 and grown under the same conditions for 3 days. Two grams of sterile XAD4 Amberlite resin (Sigma Chemicals) were then added to the culture along with isobutylbenzene (Acros, Geel, Belgium) as internal standard (1 µg/ml final concentration), and either MeJA (50 µM final concentration), MeSA (50 µM final concentration), chitosan (50 µg/ml final concentration), or 2 ml *C. polonica* spore culture were added as elicitors. After 1 week, the cells were separated from the XAD4 resin by centrifugation, and the resin was vacuum filtered on Whatman No. 2 paper. The dried resin was extracted with 1 ml pentane with vigorous shaking for 2 h, and the solvent then passed over a Pasteur pipette column containing 100 mg each silica gel and $MgSO_4$ and concentrated to 100 µl under a gentle nitrogen stream. The concentrated extracts were transferred to a GC vial and analyzed by electron impact GC-MS using an Agilent 6890 gas chromatograph (Palo Alto, CA, USA) equipped with a 5973 mass selective detector as previously described (Phillips et al. 2003). For monoterpene synthase enzyme assays, triplicate cultures were induced with 50 µM MeJA in 0.2% Tween 20 (Sigma Chemicals) or 0.2% Tween 20 alone (control) and then harvested at the indicated time points by vacuum filtration for extraction and assay as described below.

RNA isolation and cDNA synthesis

RNA from cultured cells was obtained with the Plant RNeasy kit (QIAGEN, Hilden, Germany) using 100 mg tissue homogenized in a chilled 2 ml glass Tenbroek homogenizer containing 450 µl RLT buffer (QIAGEN) and 0.14 M b-mercaptoethanol. Total RNA from spruce bark and needles was isolated with the Invisorb Spin Plant RNA kit (Invitek, Berlin, Germany). Contaminating DNA was removed via on-column Dnase I digestion (QIAGEN) in both cases. Total RNA quality and concentration were

determined using an Agilent Bioanalyzer 2100 (Palo Alto, California, USA) and RNA Nano 6000 LabChips.

cDNA library construction and screening

PolyA+ RNA was purified from total RNA isolated from MeJA-treated Norway spruce liquid culture using poly(dT)$_{25}$ coated magnetic Dynabeads (Dynal Biotech, Oslo, Norway). Residual rRNA contamination was determined using an RNA Pico6000 LabChip (Agilent). Five micrograms purified mRNA was used to construct a λZAPII-cDNA library (Stratagene, La Jolla, CA, USA) according to manufacturer's instructions. cDNAs were separated on a CL-2B Sepharose (Stratagene) size exclusion column, ligated into the lambda ZAP vector (Stratagene), and packaged using Gigapack Gold III packaging extract (Stratagene). Average insert size in the amplified and titered library was judged by T3 and T7 primed PCR amplification of randomly selected phage plaques. The library was screened by replica filter hybridization (Bohlmann et al. 1997) using a mixture of three putative spruce *DXS* cDNA fragments, 600, 800, and 1,400 bp in length, identified in the database and clone bank of TREENOMIX:Conifer Forest Health EST sequencing project (http://www.treenomix.ca; Ralph et al. 2006). PCR amplified and gel purified *DXS* fragments were diluted to 20 ng/μl and labeled with 5 μl [α-^{32}P]dCTP (10 μCi/μl, Hartmann Analytic, Braunschweig, Germany) using a MegaPrimer DNA Labeling System (Amersham Pharmacia Biotech, Uppsala, Sweden). λ phage plaques hybridizing with the *DXS* probes were isolated in two rounds of screening under high stringency conditions (overnight hybridization at 65°C in 5× SSC followed by three washes for 15 min at 65°C and 1×, 0.5×, and 0.1× SSC, or until radioactivity as judged by a hand held Geiger counter was below 10 times background) starting with 300,000 phage plaques. The in vivo excised phagemids were transfected into *E. coli* SOLR cells to obtain pBluescript plasmids, which were sequenced using M13 and M13R on an ABI Prism® 3100 sequencing system.

Functional expression and characterization of recombinant DXS proteins

Based on TargetP and ChloroP predictions of plastid transit peptide truncation sites, pseudomature forms of each *DXS* cDNA lacking the sequence for the predicted transit peptide were PCR amplified with primers bearing attB sites for cloning into Gateway® entry clones with BP Clonase II and pDONR207 (Invitrogen, Carlsbad, CA, USA). Sequences of the attB primers used are provided in Supplement Table S1. Entry clones were obtained using manufacturer's protocols and then transferred into a Gateway-compatible pET32 vector using LR Clonase II (Invitrogen). Single colonies carrying each spruce *DXS* expression construct were used to start cultures in 5 ml LB medium containing 50 μg/ml carbenicillin for plasmid preparations. Purified destination vectors were fully sequenced and then used in a bacterial complementation assay with a DXS-deficient strain of *E. coli* engineered to utilize exogenously supplied mevalonate as a source for isoprenoid biosynthesis (Campos et al. 2001). DXS activity was confirmed by transforming the DXS-deficient strain with the plasmids and then growing transformed cells on mevalonate-free media.

Quantitative real-time PCR

For measurements of transcript abundance, 1 μg total RNA from culture or saplings was converted to cDNA in a 20 μl reverse transcription reaction using SuperScript III RT (Invitrogen) and 50 pmol anchored poly dT primer (Supplement Table S1), then diluted 1:20 with sterile water. Quantitative real-time PCR was performed using a Stratagene Mx3000P and Brilliant SYBR® Green assays in 20 μl containing 1 μl diluted template, 10 pmol each forward and reverse primer, and Taq SYBR Green mix prepared according to manufacturer's instructions (Stratagene). All primers used for quantitative PCR for Norway spruce *DXS* genes and reference genes are shown in Supplement Table S1. Primer design was performed with BeaconDesigner 5.0 (PremierBiosoft, Palo Alto, USA). All primers were HPLC purified. Six amplicons from each primer pair were cloned and sequenced to confirm primer specificity. Primer efficiencies and fold change calculations were performed according to the Pfaffl method (Pfaffl 2001). Possible primer cross-hybridization between the three similar *PaDXS* sequences was tested by a real-time PCR competition assay using dilute, purified plasmids encoding each *DXS* cDNA as template in all possible combinations with each *DXS* primer pair. In addition to non-template (water) controls, non-RT controls were used as templates in real-time PCR assays to detect the presence of genomic DNA contamination. Reference genes used for relative quantification were Norway spruce tubulin or Norway spruce ubiquitin. Significance for fold change expression between treatment and control was tested using a one-sample *t*-test. *P*-values were calculated using Origins v.7.03.

Analysis and quantification of jasmonic acid (JA) and 12-oxophytodienoic acid (OPDA)

Following the method described by Schulze et al. (2006), 3 g frozen cell culture material were mixed with 3 ml of a

pentafluorobenzylhydroxylamine solution, 0.05 M in methanol (Sigma-Aldrich, Taufkirchen, Germany) followed by addition of 9,10-[$^2H_2$]-dihydrojasmonic acid (250 ng) and [$^2H_2$]-dihydrodicranenone B (250 ng) as internal standards. Tissue was homogenized for 5 min with a high performance polytron disrupter at 24,000 rpm (Ultra-Turrax T-25, IKA-Werk, Germany). Samples were derivatized while shaking for 2 h at room temperature and then transferred to 10 ml glass centrifuge tubes. Water (3 ml) was added, and the solutions were adjusted to pH 3 with HCl. The methanol–water mixture was extracted with hexane (3 × 10 ml), and the organic phase was collected following phase separation by centrifugation. Combined hexane layers were subsequently passed through Chromabond aminopropyl cartridges (0.5 g, Macherey-Nagel, Düren, Germany) preconditioned with 5 ml each methanol and hexane. The cartridges were washed with *i*-propanol:dichloromethane (5 ml, 2:1, (v/v)) and eluted with diethyl ether:formic acid (10 ml, 98:2, (v/v)). The samples were dried under a gentle stream of argon and the residue treated with an ethereal solution of diazomethane. After removal of diazomethane, the obtained residue was redissolved in 30 µl of dichloromethane. Derivatized samples were analyzed on a Finnigan GCQ instrument (Thermoelectron, Bremen, Germany) running in negative CI mode, as previously described (Schulze et al. 2006). Characteristic fragment ions of the perfluorobenzyl oximes of JA and OPDA were used for quantification (JA, *m/z* 399; 9,10-[$^2H_2$]-dihydrojasmonic acid, *m/z* 403; OPDA, *m/z* 481; [$^2H_2$]-dihydrodicraneone B, *m/z* 483). Tissue matrix effects were measured with a calibration curve based on known amounts of JA and OPDA added to non-elicited cell culture material.

Monoterpene synthase assays

Assays were performed as previously described (Bohlmann et al. 1997; Martin et al. 2002; Phillips et al. 2003) with minor modifications. In brief, approximately 200 mg cells were homogenized in a chilled 2.0 ml glass Tenbroek homogenizer in 1.0 ml cold assay buffer (100 mM HEPES pH 8.0, 10% (v/v) glycerol, 2 mM dithiothreitol, 0.5 mM $MnCl_2$, 100 mM KCl), gently mixed at 4°C for 30 min, and finally centrifuged for 30 min at 16,000*g* in a microcentrifuge at 4°C. Supernatants were filtered through a 0.2 µm syringe filter. A 10 µl aliquot was diluted 1:10 into fresh cold assay buffer containing 0.5 µl [1-^{3}H] geranyl pyrophosphate (20 Ci/mmol, 1 mCi/ml, American Radiolabeled Chemicals, St. Louis, MO, USA) and a 1.0 ml hexane overlay. Incubations were carried out at 30°C for 20 min, then vortexed and centrifuged for 1 min to separate phases. The organic (upper) phase was aspirated and passed over a glass column containing glass wool, silica gel 60 (Merck, Darmstadt, Germany), and $MgSO_4$ into a scintillation vial containing 2 ml Lumasafe LSC cocktail (Lumac B.V., Groningen, Netherlands). The extraction was repeated once, and organic phases were pooled and counted by scintillation counting. Protein concentrations were determined by Bradford assay (Biorad, Hercules, CA, USA).

Results

Norway spruce contains both type I and type II *DXS* genes

To investigate a possible role of *DXS* and other MEP pathway genes in Norway spruce terpenoid oleoresin formation, we first mined the nearly 200,000 Sitka spruce, white spruce (*P. glauca*), and interior hybrid spruce (*P. glauca* × *engelmannii*) sequences in the EST database developed by the TREENOMIX:Conifer Forest Health Project (http://www.treenomix.ca; Ralph et al. 2006) for sequences with similarities to known genes of the plant MEP pathway. This search revealed three distinct contigs of ESTs for candidate spruce *DXS* genes. Partial cDNA clones corresponding to the three *DXS* candidates were then used to screen a cDNA library made from methyl jasmonate (MeJA)-treated Norway spruce cell cultures yielding full length cDNAs for three distinct genes (Fig. 1). All three *P. abies DXS* genes (*PaDXSs*) showed features similar to known *DXS* genes from other plant species, including the presence of an N-terminal targeting sequence, a highly conserved His at position 127 (relative to *PaDXS1*) thought to be involved in proton transfer (Lois et al. 1998), and a conserved thiamine pyrophosphate binding domain typical of transketolase-like sequences (positions 212–244). Comparison of their deduced amino acid sequences to known DXS sequences of plant origin indicated that one sequence, PaDXS1, grouped with type I *DXS* genes, while the other two, PaDXS2A and PaDXS2B, represented two distinct subclasses of type II *DXS* genes (Fig. 2). Specifically, mature PaDXS1 exhibits 83% identity (88% similarity) to MtDXS1 from *Medicago truncatula* (Walter et al. 2002) but only 73% identity (80% similarity) to MtDXS2. Mature PaDXS2A is 78% identical (86% similar) to MtDXS2 and only 73% identical (81% similar) to MtDXS1. Likewise, PaDXS2B has a 80% identity score (85% similarity) to MtDXS2 and displays only 76% identity (82% similarity) to MtDXS1. As for the relationships within spruce DXS sequences, the mature form of PaDXS1 shows 74% and 75% amino acid identity (87% and 85% similarity) with those of PaDXS2A and PaDXS2B, respectively, while the two type II PaDXS share 80% identity (89% similarity).

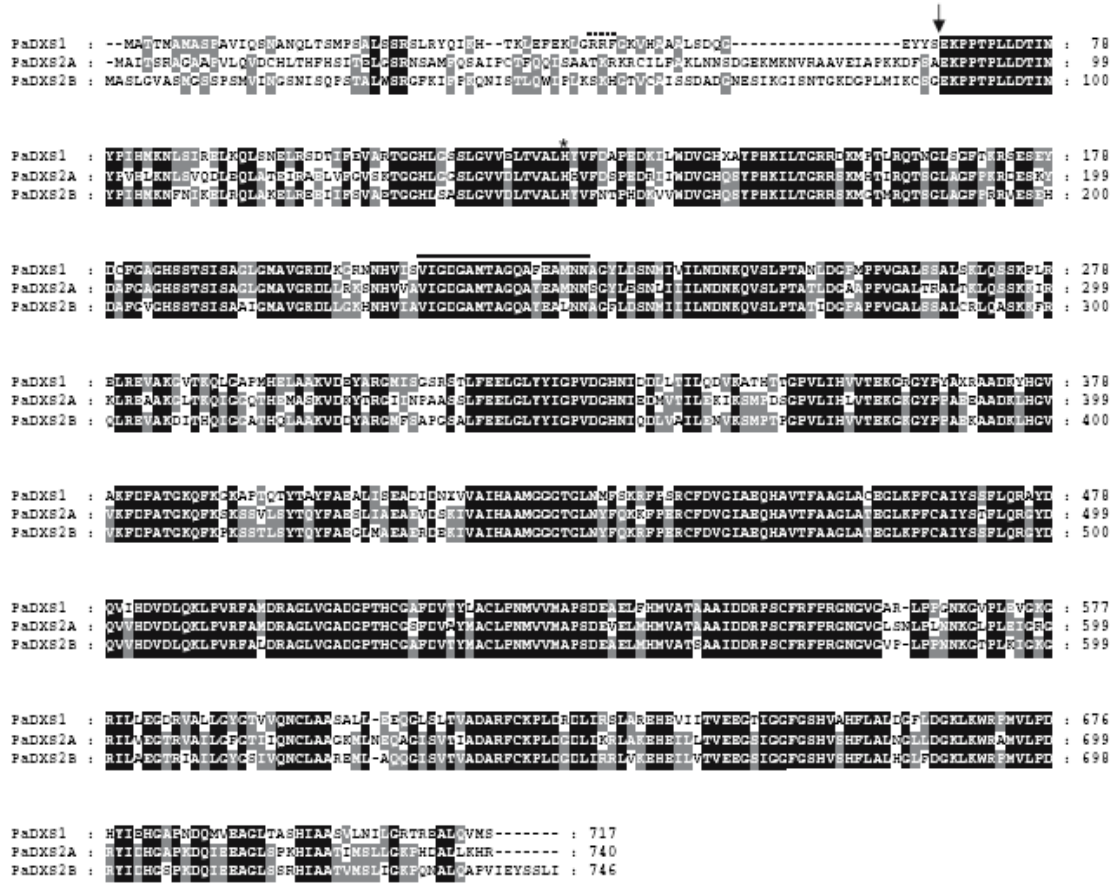


Fig. 1 Alignment of amino acid sequences of Norway spruce DXS proteins deduced from cDNAs. Alignments were performed with VectorNTI 10.0 (gap opening penalty 10, gap extension penalty 0.1). Residues conserved among all three genes are shown in black, while residues conserved in two out of the three are shown in grey. The asterisk indicates the conserved His residue thought to be involved in proton transfer. The horizontal line denotes part of the TPP binding motif conserved among DXS proteins. The dashed line denotes the N domain of the presumed transit peptide and the arrow indicates the presumed transit peptide cleavage site. This last was the truncation site used for bacterial expression and complementation

Predictive algorithms suggested plastid localization for PaDXS1 (Predotar, 0.96; TargetP, 0.73) and PaDXS2B (Predotar, 0.92; TargetP, 0.87), while predictions for PaDXS2A were less clear (Predotar: 0.14 plastid, 0.82 elsewhere; TargetP: 0.22 plastid, 0.24 mitochondria). Because the targeting peptides of all known DXS sequences are bipartite, it is likely their ultimate destination is the thylakoid lumen (Krushkal et al. 2003). The amino acid sequence of PaDXS1 shows the presence of tandem Arg residues at positions 48–49 of the transit peptide N-domain (Fig. 1), followed by moderately hydrophobic residues in the H-domain and a Lys at position 68 before a conserved PPT motif, which suggests participation of the ΔpH/tat targeting pathway (Mori and Cline 2001). PaDXS2A and PaDXS2B lack the tandem Arg sequences and have instead KR and KH in the same positions (Fig. 1). They both contain numerous basic residues in the H-C domain, making these sequences incompatible with the sec targeting pathway (Mori and Cline 2001). Thus, all three spruce DXS genes encode transit peptides with features consistent with targeting to the thylakoid lumen via the ΔpH/tat translocation pathway.

All three Norway spruce DXS genes encode functional proteins

Functional identification of proteins encoded by the three *PaDXS* cDNAs was accomplished via complementation of a DXS-deficient *Escherichia coli* strain engineered to utilize mevalonate for isoprenoid biosynthesis (Campos et al. 2001). In the absence of mevalonate, only cells transformed with a plasmid bearing a functional *DXS* gene are viable. Since *E. coli* lacks subcellular compartments typical

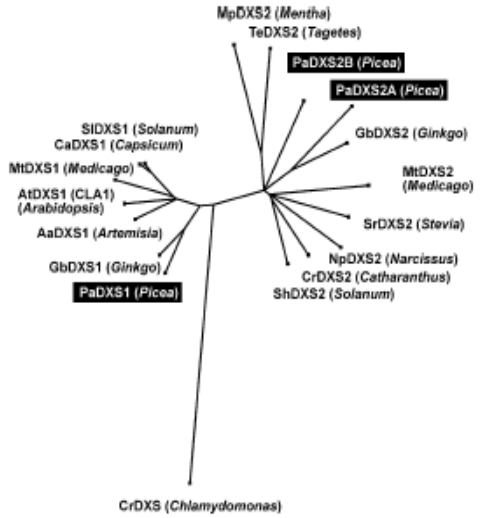


Fig. 2 Similarity tree of amino acid sequences of mature DXS proteins excluding transit peptides. The tree was generated using the programs Distances and Splits of the HUSAR analysis package using default values. The DXS of the green alga *Chlamydomonas reinhardtii* (CrDXS, AJ007559) was used as an outgroup. Novel sequences resulting from this work are highlighted. The following plant sequences were included: CaDXS1 (TKT2), *Capsicum annuum*, accession number Y15782; SlDXS1, *Solanum lycopersicum*, AF143812; MtDXS1, *Medicago truncatula*, AJ430047; AtDXS1 (CLA1), *Arabidopsis thaliana*, U27099; AaDXS1, *Artemisia annua*, AF182286; GbDXS1, *Ginkgo biloba*, AY505128; PaDXS1, *Picea abies*, EF688331; MpDXS2, *Mentha piperita*, AF019383; TeDXS2, *Tagetes erecta*, AF251020; PaDXS2B, *Picea abies*, EF688333; PaDXS2A, *Picea abies*, EF688332; GbDXS2, *Ginkgo biloba*, AY494185; MtDXS2, *Medicago truncatula*, AJ430048; SrDXS2, *Stevia rebaudiana*, AJ429232; NpDXS2, *Narcissus pseudonarcissus*, AJ279018; CrDXS2, *Catharanthus roseus*, AJ011840; ShDXS2, *Solanum habrochaites*, AY687353

of plant cells, we used truncated forms of the three *PaDXS* cDNAs lacking the putative transit peptide for complementation tests. All three *PaDXS* cDNAs, *PaDXS1*, *PaDXS2A*, and *PaDXS2B*, complemented the *E. coli* dxs$^-$ strain, allowing growth in the absence of mevalonate, while vector-transformed controls required addition of mevalonate for growth (Fig. 3). These results clearly prove that *PaDXS1*, *PaDXS2A*, and *PaDXS2B* encode functional DXS proteins. Although we cannot exclude the possibility that additional *DXS* genes may exist in the Norway spruce genome, the fact that no additional contigs of ESTs for *DXS*-like genes were found in the TREENOMIX database of more than 200,000 spruce ESTs, which covers a diverse array of tissues and treatments (Ralph et al. 2006), and the fact that no additional *DXS* genes were found during screening of 300,000 phage plaques from our original cDNA library, make it unlikely that *DXS* genes other than those represented by *PaDXS1*, *PaDXS2A*, and *PaDXS2B* contribute much to constitutive or induced terpenoid biosynthesis in spruce.

The *DXS* genes are differentially expressed in sapling stems

It has previously been shown that mechanical wounding or treatment of conifer trees with MeJA results in increased transcript levels of late genes of terpenoid biosynthesis, specifically members of the large *TPS* gene family and CYP720-type *CYP450* genes, leading to induced oleoresin formation (Steele et al. 1998b; Fäldt et al. 2003; Miller et al. 2005; Ro et al. 2005). To investigate the role of each of the three *PaDXS* genes in induced (traumatic) oleoresin formation in Norway spruce, we analyzed transcript levels via quantitative real-time PCR (qRT-PCR) using gene-specific primers for *PaDXS1*, *PaDXS2A*, and *PaDXS2B*. The specificity of each pair of *DXS* primers was confirmed in qRT-PCR SYBR Green assays in which each primer pair was individually tested using plasmids encoding each of the three cloned *PaDXS* cDNAs as template in separate reactions. In each case, *DXS* primers produced a C_t value at least 15 C_ts lower for the intended target than for the other two *DXS* cDNAs at similar concentrations. Cloning and sequencing of multiple independent qRT-PCR products obtained with each primer pair and total cDNA templates further confirmed these primer specificities.

In an initial time course analysis, we measured gene-specific changes of *DXS* transcript levels in RNA samples isolated from whole bark peels of Norway spruce saplings treated by mechanical wounding (Fig. 4A) or MeJA (Fig. 4B). Trees were harvested for RNA isolation at 1, 3, 7, and 10 days after treatment. *PaDXS1* transcript levels responded only slightly and transiently to wounding or MeJA (Fig. 4). In contrast, transcripts for both *PaDXS2A* and *PaDXS2B* in wounded and MeJA-treated trees had increased rapidly by the first time point measured and maintained levels well above those found in non-treated control trees over the ten-day time course (Fig. 4). Treatment with MeJA produced greater effects than mechanical wounding, and the timing of *PaDXS2A* and *PaDXS2B* transcript changes also appeared to be somewhat different when comparing MeJA treatment with wounding.

Transcripts of *DXS* type II and other MEP pathway genes are induced by wounding and fungal treatments in Norway spruce stems

Given the strong induction of *PaDXS2A* and *PaDXS2B* transcripts in stems 3 d after mechanical wounding (Fig. 4A), we chose this time point to compare the effects of wounding and fungal infection on the expression of the *DXS* genes using three different treatments. The first treatment consisted of mechanical wounding of the stems with a razor blade. The second simulated fungal infection

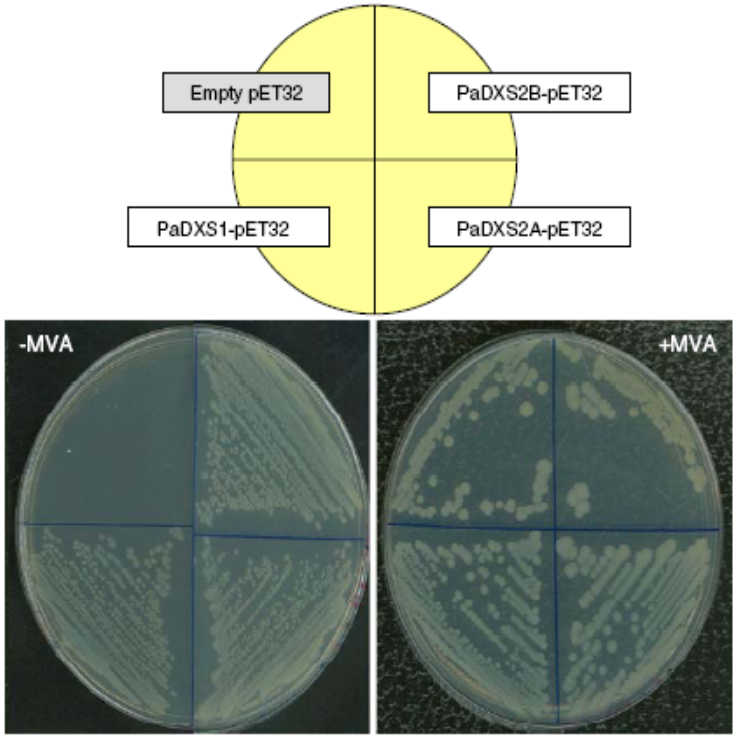


Fig. 3 Functional expression of Norway spruce *DXS* genes in *E. coli*. A DXS deficient strain of *E. coli* engineered to utilize mevalonate as a source of isoprenoids was transformed either with plasmids bearing individual spruce *DXS* genes as indicated or empty vector (negative control). Growth on media lacking mevalonate indicates an active *DXS* gene

by mechanical wounding followed by application of a chitosan solution (Miller et al. 1986; Croteau et al. 1987). The third treatment involved actual fungal inoculation accomplished by wounding followed by application of a *C. polonica* spore solution. *C. polonica* is a blue-stain pathogen of Norway spruce that is nearly always associated with the attack of the bark beetle *I. typographus*.

Wounding and fungal infection led to an increase in steady-state transcript levels from *PaDXS2A* and *PaDXS2B*, but not for *PaDXS1* (Fig. 5A), demonstrating that the lack of response of *PaDXS1* previously observed in the time course assays with wounded or MeJA-treated trees (Fig. 4) was not altered by addition of fungal elicitors. *PaDXS2A* showed a three- to fivefold increase in transcript abundance in response to mechanical wounding and fungal treatment, while induction of *PaDXS2B* transcripts was nearly twice this amount. Although treatment of wounded trees with chitosan did not increase transcript abundance beyond that of wounding alone, *C. polonica* inoculation of wounded stems caused a slight, yet reproducible increase in both *PaDXS2A* and *PaDXS2B* beyond that observed for wounding alone ($P < 0.01$).

We extended this experiment by analyzing the transcript levels of putative genes encoding two subsequent steps of the MEP pathway, 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) and 4-hydroxyl 3-methylbutenyl diphosphate reductase (HDR). The sequence for DXR was obtained from the TREENOMIX:Conifer Forest Health EST database (http://www.treenomix.ca), while a full length HDR was obtained by homology-based cDNA library screening (M. Phillips, unpublished results). Mechanical wounding and fungal treatment caused an up-regulation of transcript levels for genes encoding both DXR and HDR (Fig. 5A).

Elicitor treatment triggers monoterpene accumulation in Norway spruce cell suspension cultures and increases in jasmonic acid and OPDA

To distinguish between the effects of wounding and fungal infection on *DXS* expression, we sought an experimental system that would allow application of fungal elicitors without mechanical wounding, a difficult challenge with intact trees and their protective bark covering. However, an inducible cell culture system would meet these requirements and allow the regulation of terpene formation to be investigated apart from specific cell types. We established a Norway spruce cell suspension from calli of an embryogenic culture that had been initiated from mature seeds. Despite the lack of specialized, resin forming cells typical of conifer stems in the culture (Keeling and Bohlmann 2006b), we detected small amounts of monoterpenes resembling the oleoresin compounds of intact trees. Using a sterilized XAD4 resin overlay to trap and concentrate monoterpenes, we reproducibly measured α-pinene,

Plant Mol Biol (2007) 65:243–257 251

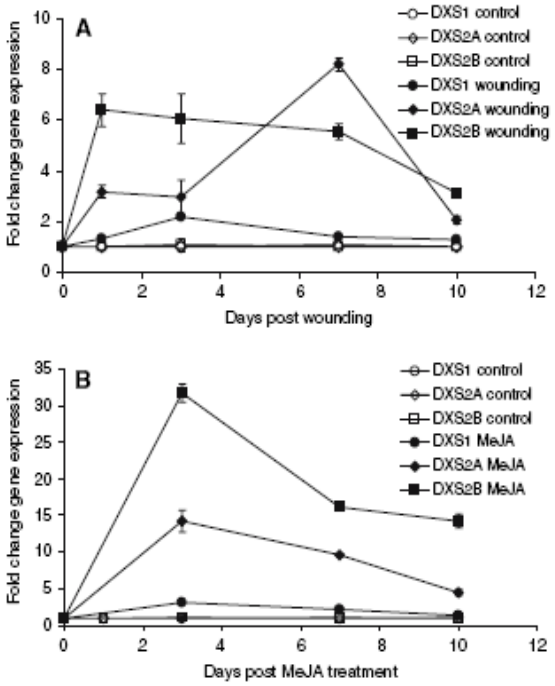


Fig. 4 Changes in steady-state transcript levels of different *DXS* genes in Norway spruce saplings following mechanical wounding (**A**) or MeJA treatment (**B**). A minimum of six saplings per replicate were harvested and pooled, used in RNA extractions, and analyzed by qRT-PCR in triplicate ($n = 12$). Control saplings for the MeJA treatment were sprayed with 0.2% (v/v) Tween 20. Control saplings for the wounding treatment were not handled at all. The normalizer gene used was ubiquitin. Error bars indicate standard deviations

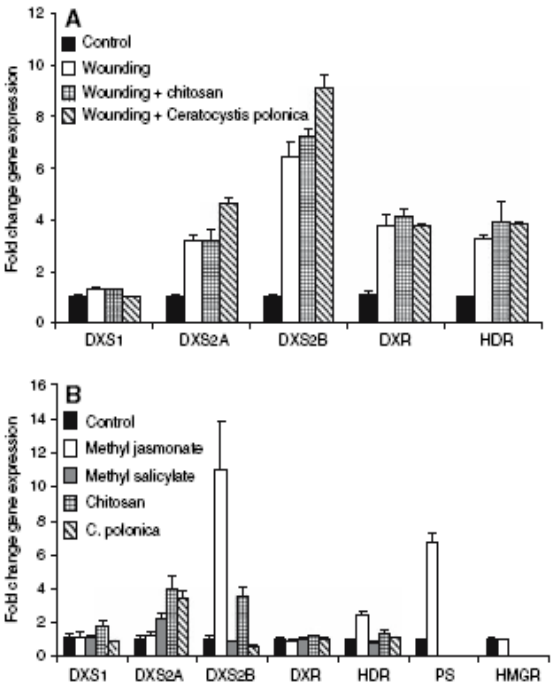


Fig. 5 Steady-state transcript levels of isoprenoid biosynthetic genes in Norway spruce saplings (**A**) and suspension cultured cells (**B**) after various treatments as determined by qRT-PCR ($n = 12$). Saplings were harvested 3 days after treatment, while cultured cells were harvested 18 h after treatment. The normalizer gene used was ubiquitin. Fold changes were calculated according to the efficiency corrected method (Pfaffl 2001). DXR (1-deoxyxylulose 5-phosphate reductoisomerase) and HDR (1-hydroxy-2-methyl-2-butenyl 4-diphosphate reductase) catalyze the second and seventh steps, respectively, of the MEP pathway and have previously been shown to be regulated steps. PS ((−)-α/β-pinene synthase) was functionally characterized previously (Martin et al. 2004) and HMGR (3-hydroxy 3-methylglutaryl CoA reductase) was identified from an EST collection (Ralph et al. 2006; http://www.treenomix.ca) based on sequence similarity. Error bars indicate standard deviations

β-pinene, camphene, limonene, and myrcene released from the cultured cells following addition of MeJA to the growth medium (Fig. 6). Based on comparison to the internal standard, all were detected at concentrations corresponding to 25–50 ng/ml culture. These monoterpenes were absent in all controls, including cells not treated with MeJA, MeJA-induced cells grown in the absence of XAD4 resin, and XAD4 resin incubated in culture medium without cells (data not shown).

This inducible cell culture provided an excellent opportunity to learn more about the signaling pathway involved in triggering oleoresin terpene formation in conifers. Given the dramatic effects of MeJA on resin production in Norway spruce (Martin et al. 2002) and the elevated transcript levels of octadecanoid pathway genes observed on insect attack in Sitka spruce (Miller et al. 2005; Ralph et al. 2006), we searched for jasmonic acid (JA) and other oxylipins after elicitation with chitosan. Compared to unelicited controls (0 h), JA levels more than doubled 2 h after application of chitosan (Fig. 7A), consistent with previous reports of induced JA formation in conifer cell cultures (Blechert et al. 1995). In addition to the increase in JA levels, the isomeric composition shifted towards a greater proportion of the less stable *cis*-JA (18.4% in controls rising to 70.0% at 2 h after treatment) compared to the *trans*-isomer, suggesting *de novo* JA biosynthesis in induced cells. Another oxylipin detected was 12-oxophytodienoic acid (OPDA), a JA precursor potentially active in defense signaling. Levels of OPDA increased more than ten-fold (4 h) after chitosan elicitation (Fig. 7B).

Elicitor treatment stimulates monoterpene synthase activity in cultured cells

To measure the activation of terpene biosynthesis in cultured Norway spruce cells, we monitored TPS enzyme activity by measuring the conversion of various prenyl

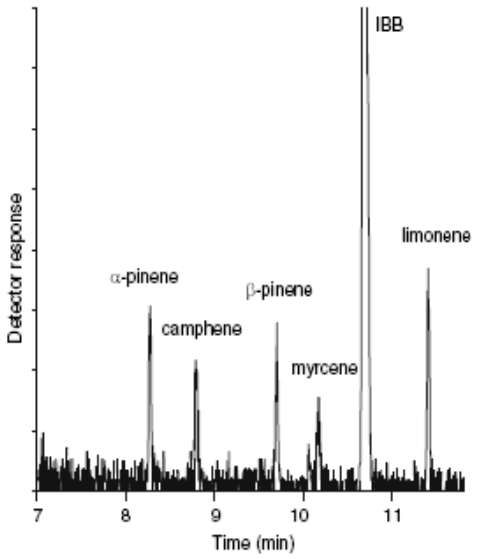


Fig. 6 Profile of monoterpene products accumulated in Norway spruce cell suspension culture after application of MeJA to the growth medium. Depicted is the total ion trace of GC-MS analysis performed on a pentane extract of XAD4 resin beads which had been co-incubated with cultured spruce cells as a monoterpene trap. Compounds were identified by their mass spectra and retention times compared with authentic standards. IBB, isobutylbenzene, was used as an internal standard

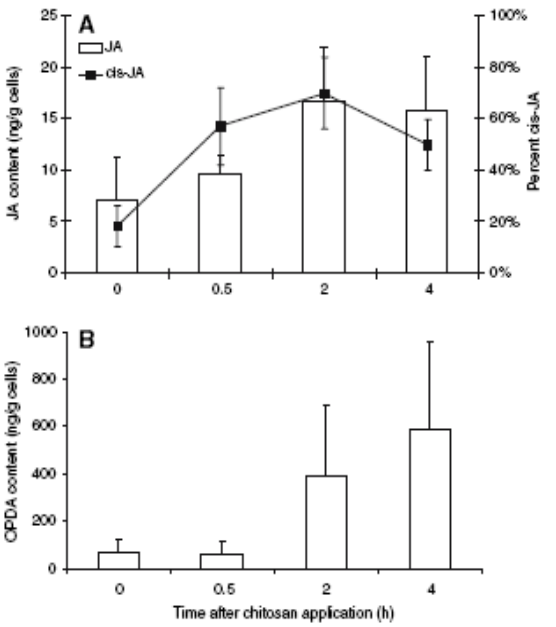


Fig. 7 Content of jasmonic acid (JA) and 12-oxophytodienoic acid (OPDA) in Norway spruce cell suspension cultures following treatment with chitosan, a fungal cell wall component. Results are expressed per gram fresh weight of cells. The ratio of *cis*-jasmonic acid (*cis* JA) to *trans*-jasmonic acid is an indicator of *de novo* biosynthesis since JA biosynthesized in the *cis* form tautomerizes to the more stabile *trans* isomer

diphosphate substrates to terpene products. Mono-TPS activity (conversion of geranyl diphosphate to monoterpenes) was readily detectable under constitutive conditions, and increased substantially after application of chitosan or MeJA (Fig. 8A). A time course of mono-TPS activity after application of MeJA showed a detectable increase in enzyme activity in as little as two hours (Fig. 8B). Mono-TPS activity continued to rise after 2 h and reached levels approximately five times higher than those measured in control cells after 48 h. In contrast to mono-TPS activity, neither sesqui-TPS (farnesyl diphosphate to sesquiterpenes) nor di-TPS (geranylgeranyl diphosphate to diterpenes) activity was found in control or MeJA-induced cell cultures, nor were any sesquiterpenes or diterpenes detected in organic extracts (data not shown). Taken together, the formation of jasmonates, the induction of monoterpene synthase activity and the accumulation of monoterpenes suggest that at least a portion of the signaling and metabolic pathways of induced terpenoid defense are functional in cultured Norway spruce cells.

Type II *DXS* genes are differentially induced in cell cultures by fungal treatment, elicitors, and signaling compounds

The expression of *PaDXS* and other isoprenoid biosynthetic genes was measured in our inducible, terpene-producing cell cultures to compare and extend the results obtained with intact plants. The use of cell cultures allowed us to test the effect of certain signaling compounds on Norway spruce tissue in a more direct fashion than by application to mechanical wounds of intact saplings. Transcript levels were analyzed 18 h after treatment of cells. In agreement with the results obtained from intact saplings (Fig. 5A), *PaDXS1* transcript levels were not affected by any of the treatments tested on cell cultures (Fig. 5B), suggesting again that *PaDXS1* does not encode a regulated step in the formation of induced terpenoid defenses in Norway spruce. Similarly, transcript levels of *DXR* were also not affected, and a response of *HDR* transcript was detected only in response to MeJA treatment ($n = 12$, $P < 0.01$) (Fig. 5B).

In contrast, the type II *DXS* genes, *PaDXS2A* and *PaDXS2B*, were both activated by some of the signaling compounds and elicitors tested, but not in a similar manner. *PaDXS2A* transcript levels were not affected by MeJA but increased approximately fourfold in response to chitosan and *C. polonica* spores (Fig. 5B). *PaDXS2A* also responded slightly to treatment with methyl salicylate (MeSA) ($n = 12$, $P < 0.01$) consistent with its activation by fungal elicitors. On the other hand, transcript levels of *PaDXS2B* responded strongly (more than 10-fold increase) to MeJA, but were not affected by MeSA or *C. polonica* spores.

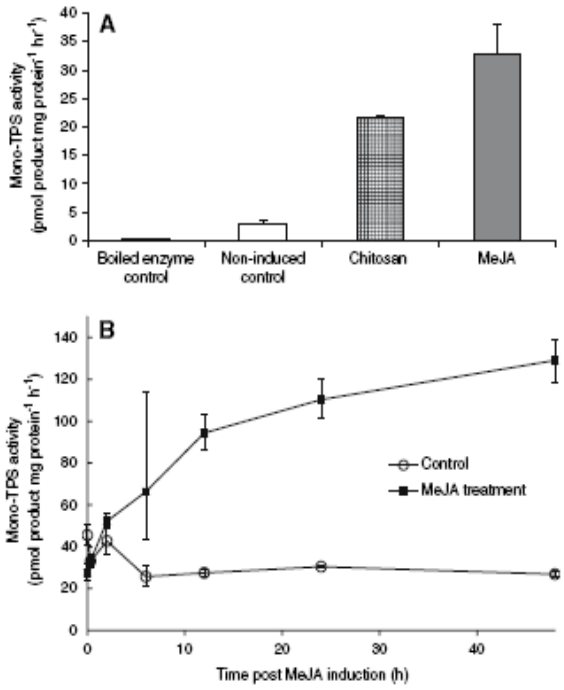


Fig. 8 The effect of elicitors on the induction of monoterpene synthase activity in Norway spruce cell suspension cultures. Chitosan (50 μg ml^{-1}) or MeJA (50 μM) was applied (**A**), and activity was measured by the conversion of GPP to monoterpene products 18 h after induction. The product spectrum was similar to that in Fig. 6. A time-course of monoterpene synthase activity in Norway spruce cell cultures after induction with 50 μM MeJA (**B**) was conducted separately. Error bars in A and B indicate standard deviations

Chitosan was the only common elicitor for both type II *DXS* genes.

Among other genes studied, transcripts of the monoterpene synthase, (−)-α/β-pinene synthase (PS) (Martin et al. 2004) responded to MeJA like *PaDXS2B* (Fig. 5B), indicating that genes of other stages of terpene biosynthesis may be coordinately regulated with specific DXS2-type isogenes. The lack of response of the mevalonate pathway gene, HMG-CoA reductase (HMGR; Fig. 5B) upon MeJA treatment supports the dominant role of the MEP rather than the mevalonic acid pathway in supplying substrate for monoterpene formation in Norway spruce.

Discussion

Transcription of MEP pathway genes regulates defensive oleoresin formation in Norway spruce

Despite the intensive study of genes and enzymes participating in oleoresin terpene biosynthesis in conifers, almost no information is available about the role of the MEP pathway in this process. The MEP pathway, along with the mevalonate pathway, constitutes the first stage of terpene biosynthesis, producing the C_5 intermediates, IPP and DMAPP, from basic precursors, such as glyceraldehyde-3-phosphate and pyruvate. Here we have isolated and functionally characterized three genes from Norway spruce encoding DXS, the first step of the MEP pathway, and examined their expression and the expression of other MEP pathway genes after wounding and simulated pathogen attack.

We observed a general increase in transcripts of all three MEP pathway genes studied in response to mechanical wounding, fungal elicitors, and treatment with defensive signaling compounds known to activate terpene oleoresin formation (Martin et al. 2002; Phillips et al. 2006). These results suggest that the MEP pathway plays an important role in regulating the formation of oleoresin components, and that this regulation is at least partly at the level of transcription. Among the steps of the MEP pathway, DXS appears to have particular regulatory significance since there is a small family of *DXS* genes, differentially responsive to various wounding treatments and biotic stresses. In addition, *DXS* transcripts collectively increase to a much greater extent than transcripts for the two other MEP pathway genes examined, *DXR* and *HDR*.

Type II but not Type I *DXS* genes are activated by wounding, fungi, and signaling compounds

The two types of *DXS* genes were previously reported in plants based on differences in sequence and expression pattern (Walter et al. 2002). Both were found in Norway spruce. Type I genes have been implicated in primary terpenoid metabolism due to their constitutive expression in photosynthetic tissues, consistent with a role in providing precursors for chlorophyll and carotenoid biosynthesis. Here we found *PaDXS1* to be constitutively expressed under all conditions, and not induced by any wounding, fungal elicitor or defensive signal, indicating it is likely to be involved in primary metabolism (Walter et al. 2002).

In contrast, type II *DXS* genes have been shown to be active in isoprenoid metabolism in specialized tissues, such as in the apocarotenoid-accumulating roots of legumes following mycorrhizal colonization (Walter et al. 2002), in the monoterpene synthesizing gland cells of mint leaf trichomes (Lange et al. 1998), or in tissues forming diterpenes in *Ginkgo biloba* (Kim et al. 2006). Here we demonstrated for the first time the involvement of type II *DXS* in inducible terpene defenses. The two type II *DXS* genes of Norway spruce respond to a variety of stimuli previously associated with the activation of induced resin defenses in conifers, including fungal infection,

mechanical wounding, chitosan and MeJA treatment (Steele et al. 1995; Martin et al. 2002, 2005), and their up-regulation is linked to the activation of other genes involved in the defense response, such as terpene synthases. While some small differences were noted in the timing and extent of the responses of *PaDXS2A* and *PaDXS2B* to various forms of defense induction in saplings, these differences were much more pronounced in cell suspension culture. For example, while *PaDXS2B* was highly activated by MeJA in culture, *PaDXS2A* was completely unresponsive. Methyl salicylate (MeSA) had the opposite effect, producing a slight induction of *PaDXS2A* ($n = 12$, $P < 0.01$) but not of *PaDXS2B*. Treatment with live *C. polonica* spores resembled MeSA with a slight induction of *PaDXS2A* and a slight repression of *PaDXS2B* ($n = 12$, $P = 0.05$). Chitosan was the only treatment that had a similar effect on both genes in cell culture.

The distinct expression patterns of the two type II *DXS* genes in spruce cell culture may be indicative of separate jasmonate- and salicylate-mediated signaling pathways in gymnosperms homologous to those known in angiosperms (Kunkel and Brooks 2002; Glazebrook et al. 2003). Jasmonates have been demonstrated to induce defense signaling and accumulation in a variety of gymnosperm species, especially in cell cultures (Ketchum et al. 1999; Kozlowski et al. 1999; Lapointe et al. 2001; Khosroushahi et al. 2006). However, only rarely has an attempt been made to measure endogenous jasmonate levels in gymnosperms (Blechert et al. 1995). In this investigation, we found measurable levels of jasmonic acid in Norway spruce cell suspension cultures that increased 2 h after treatment with chitosan. The presence of OPDA, an intermediate in jasmonic acid biosynthesis, at levels more than 30 times higher than JA is notable because this octadecanoid has been postulated to serve as a signal independent of JA (Stintzi et al. 2001; Taki et al. 2005; Buseman et al. 2006). To our knowledge, this is the first report of OPDA in a gymnosperm. There is also evidence for salicylic acid (SA) as an endogenous signal in gymnosperms. Increases in bound SA have been observed in Norway spruce seedling roots inoculated with *Pythium* sp. (Kozlowski and Metraux 1998) and increases in both free and bound SA have been detected in seedlings treated with MeJA (Kozlowski et al. 1999).

In angiosperm defense signaling, jasmonate is more commonly associated with responses to herbivores and salicylic acid with responses to pathogens. Our results on *DXS* expression in spruce cell culture show the rudiments of a similar pattern in that one transcript (*PaDXS2A*, but not *PaDXS2B*) is inducible by both MeSA and *C. polonica* spore treatment, while the other transcript (*PaDXS2B*, but not *PaDXS2A*) is inducible by MeJA. However, both genes respond similarly to chitosan, and the response pattern in saplings is not qualitatively different. This may not be too surprising because herbivore and pathogen attack often occur simultaneously in gymnosperms. Bark beetle attacks, for example, are nearly always associated with fungal invasion (Bohlmann et al. 1997; Phillips and Croteau 1999; Franceschi et al. 2005; Keeling and Bohlmann 2006a, b), so responses to insects, pathogens and wounding may appear to be similar. Studies with mature Norway spruce showed increased formation of traumatic resin ducts after inoculation with *C. polonica*. However, control trees in which a wound was made for insertion of sterile agar plugs also developed more traumatic ducts than non-wounded controls indicating similar response to pathogens and wounding (Nagy et al. 2000).

Inducible accumulation of terpenes in Norway spruce cell suspension cultures provides a convenient experimental system for studying induced oleoresin formation

Despite the enormous differences in tissue organization between spruce cell suspension cultures and intact spruce trees, suspension cultures also exhibit induced terpene formation making them a useful system for a detailed dissection of this process. A wide range of elicitors can be tested on suspension cultures in known concentrations, and experiments may allow separation of specific wound and pathogen-specific pathways. In addition, suspension cultures allow uniform stimulation of a large population of cells which should facilitate isolation of components of the signaling pathway. In our cultures, treatment with MeJA led to the accumulation of a similar profile of monoterpenes as in intact trees. Such accumulation is likely a result of increased biosynthesis since MeJA treatment also caused an increase in monoterpene synthase activity, as measured by in vitro enzyme assays, and an increase in the transcript level of one of the major monoterpene synthases as measured by qRT-PCR. Norway spruce cultures were previously described as incapable of *de novo* monoterpene biosynthesis (Lindmark-Henriksson et al. 2003, 2004), but this conclusion may be blamed on the difficulty of detecting low levels in culture.

Measurements of gene expression in our cultures demonstrated that genes encoding key steps in the MEP pathway were stimulated by MeJA. However, such an increase was not observed for HMGR, a key gene of the mevalonate pathway, suggesting that the MEP pathway is primarily responsible for providing precursors for induced oleoresin biosynthesis. Interestingly, the gene expression pattern measured in suspension culture differed from that observed in intact saplings. For example, *PaDXS2B* responded significantly to MeJA in culture and in saplings,

while *DXR* and *HDR* only responded in saplings. These observations suggest that the lack of differentiated cells and specialized anatomical structures in the suspension cultures may limit transcriptional activation. Incomplete activation of the MEP pathway may be responsible for the low levels of monoterpenes detected in cultures, in comparison to intact plants, and the lack of sesquiterpene and diterpene accumulation in cultures.

In spite of its importance in terpenoid biosynthesis, the MEP pathway was first discovered slightly more than ten years ago (Rohmer et al. 1993). Hence there are still many unanswered questions about the enzymology of the pathway and its regulation, and about which terpenes are formed from MEP pathway products, and how this pathway complements the mevalonate pathway, the other route for producing the C_5 units of terpenes. We have now demonstrated an important function of the MEP pathway in controlling production of terpene metabolites in conifer oleoresin, using Norway spruce as a model. Knowledge of how oleoresin formation is regulated should contribute to a greater understanding of conifer defense and facilitate our ability to manage many of the destructive pests of conifer forests.

Acknowledgements We thank Kerstin Manke (IPB, Halle) for skillful technical assistance, Xue Mei Niu (Hans Knöll Institute, Jena) for the preparation of *C. polonica* spore cultures and Ms. Sharon Jancsik (UBC Michael Smith Laboratories) for maintaining the EST/FLcDNA-collections of the TREENOMIX:Conifer Forest Health Project. We also thank Paal Krokene of the Norwegian Forest Research Institute (Ås, Norway), for providing the *C. polonica* strain. The research reported in this paper was supported with funds from the Max Planck Society (to JG), Genome British Columbia and Genome Canada in support of the TREENOMIX:Conifer Forest Health Project (grant to JB), and the Natural Sciences and Engineering Council of Canada (NSERC, grant to JB). JB is an NSERC E.W.R. Steacie Memorial fellow.

References

Bishop-Hurley SL, Zabkiewicz RJ, Grace L, Gardner RC, Wagner A, Walter C (2001) Conifer genetic engineering: transgenic *Pinus radiata* (D. Don) and *Picea abies* (Karst) plants are resistant to the herbicide Buster. Plant Cell Rep 20:235–243

Blechert S, Brodschelm W, Holder S, Kammerer L, Kutchan TM, Mueller MJ, Xia ZQ, Zenk MH (1995) The octadecanoic pathway: signal molecules for the regulation of secondary pathways. Proc Natl Acad Sci USA 92:4099–4105

Bohlmann J, Steele CL, Croteau R (1997) Monoterpene synthases from Grand fir (*Abies grandis*)—cDNA isolation, characterization, and functional expression of myrcene synthase, (−)(4*S*)-limonene synthase, and (−)-(1*S*,5*S*)-pinene synthase. J Biol Chem 272:21784–21792

Buseman CM, Tamura P, Sparks AA, Baughman EJ, Maatta S, Zhao J, Roth MR, Esch SW, Shah J, Williams TD, Welti R (2006) Wounding stimulates the accumulation of glycerolipids containing oxophytodienoic acid and dinor-oxophytodienoic acid in Arabidopsis leaves. Plant Physiol 142:28–39

Byun-McKay SA, Hunter WL, Godard KA, Wang SX, Martin DM, Bohlmann J, Plant AL (2003) Insect attack and wounding induce traumatic resin duct development and gene expression of (−)-pinene synthase in Sitka spruce. Plant Physiol 133:368–378

Campos N, Rodriguez-Concepcion M, Sauret-Gueto S, Gallego F, Lois LM, Boronat A (2001) *Escherichia coli* engineered to synthesize isopentenyl diphosphate and dimethylallyl diphosphate from mevalonate: a novel system for the genetic analysis of the 2-C-methyl-D-erythritol 4-phosphate pathway for isoprenoid biosynthesis. Biochemical J 353:59–67

Croteau R (1987) Biosynthesis and catabolism of monoterpenoids. Chem Rev 87:929–954

Croteau R, Gurkewitz S, Johnson MA, Fisk HJ (1987) Biochemistry of oleoresinosis—Monoterpene and diterpene biosynthesis in Lodgepole pine saplings infected with *Ceratocystis clavigera* or treated with carbohydrate elicitors. Plant Physiol 85:1123–1128

Erbilgin N, Krokene P, Christiansen E, Zeneli G, Gershenzon J (2006) Exogenous application of methyl jasmonate elicits defenses in Norway spruce (*Picea abies*) and reduces host colonization by the bark beetle *Ips typographus*. Oecologia 148:426–436

Estevez JM, Cantero A, Reindl A, Reichler S, Leon P (2001) 1-deoxy-D-xylulose-5-phosphate synthase, a limiting enzyme for plastidic isoprenoid biosynthesis in plants. J Biol Chem 276:22901–22909

Fäldt J, Martin D, Miller B, Rawat S, Bohlmann J (2003) Traumatic resin defense in Norway spruce (*Picea abies*): methyl jasmonate-induced terpene synthase gene expression, and cDNA cloning and functional characterization of (+)-3-carene synthase. Plant Mol Biol 51:119–133

Franceschi VR, Krekling T, Christiansen E (2002) Application of methyl jasmonate on *Picea abies* (Pinaceae) stems induces defense-related responses in phloem and xylem. Am J Bot 89:578–586

Franceschi VR, Krokene P, Christiansen E, Krekling T (2005) Anatomical and chemical defenses of conifer bark against bark beetles and other pests. New Phytol 167:353–375

Glazebrook J, Chen WJ, Estes B, Chang HS, Nawrath C, Metraux JP, Zhu T, Katagiri F (2003) Topology of the network integrating salicylate and jasmonate signal transduction derived from global expression phenotyping. Plant J 34:217–228

Huber DP, Philippe RN, Godard KA, Sturrock RN, Bohlmann J (2005) Characterization of four terpene synthase cDNAs from methyl jasmonate-induced Douglas-fir, *Pseudotsuga menziesii*. Phytochemistry 66:1427–1439

Hudgins JW, Franceschi VR (2004) Methyl jasmonate-induced ethylene production is responsible for conifer phloem defense responses and reprogramming of stem cambial zone for traumatic resin duct formation. Plant Physiol 135:2134–2149

Hudgins JW, Christiansen E, Franceschi VR (2003) Methyl jasmonate induces changes mimicking anatomical defenses in diverse members of the Pinaceae. Tree Physiol 23:361–371

Hudgins JW, Ralph SG, Franceschi VR, Bohlmann J (2006) Ethylene in induced conifer defense: cDNA cloning, protein expression, and cellular and subcellular localization of 1-aminocyclopropane-1-carboxylate oxidase in resin duct and phenolic parenchyma cells. Planta 224:865–877

Keeling CI, Bohlmann J (2006a) Diterpene resin acids in conifers. Phytochemistry 67:2415–2423

Keeling CI, Bohlmann J (2006b) Genes, enzymes and chemicals of terpenoid diversity in the constitutive and induced defence of conifers against insects and pathogens. New Phytol 170:657–675

Ketchum RE, Gibson DM, Croteau RB, Shuler ML (1999) The kinetics of taxoid accumulation in cell suspension cultures of *Taxus* following elicitation with methyl jasmonate. Biotechnol Bioeng 62:97–105

Khosroushahi AY, Valizadeh M, Ghasempour A, Khosrowshahli M, Naghdibadi H, Dadpour MR, Omidi Y (2006) Improved Taxol

production by combination of inducing factors in suspension cell culture of *Taxus baccata*. Cell Biology Intl 30:262–269

Kim SM, Kuzuyama T, Chang YJ, Song KS, Kim SU (2006) Identification of class 2 1-deoxy-D-xylulose 5-phosphate synthase and 1-deoxy-D-xylulose 5-phosphate reductoisomerase genes from *Ginkgo biloba* and their transcription in embryo culture with respect to ginkgolide biosynthesis. Planta Medica 72:234–240

Kozlowski G, Metraux JP (1998) Infection of Norway spruce (*Picea abies* (L) Karst) seedlings with *Pythium irregulare* Buism and *Pythium ultimum* Trow: histological and biochemical responses. Eur J Plant Path 104:225–234

Kozlowski G, Buchala A, Metraux JP (1999) Methyl jasmonate protects Norway spruce *Picea abies* (L.) Karst. seedlings against *Pythium ultimum* Trow. Physiol Mol Plant Path 55:53–58

Krushkal J, Pistilli M, Ferrell KM, Souret FF, Weathers PJ (2003) Computational analysis of the evolution of the structure and function of 1-deoxy-D-xylulose-5-phosphate synthase, a key regulator of the mevalonate-independent pathway in plants. Gene 313:127–138

Kunkel BN, Brooks DM (2002) Cross talk between signaling pathways in pathogen defense. Curr Opin Plant Biol 5:325–331

Lange BM, Ghassemian M (2003) Genome organization in *Arabidopsis thaliana*: a survey for genes involved in isoprenoid and chlorophyll metabolism. Plant Mol Biol 51:925–948

Lange BM, Wildung MR, McCaskill D, Croteau R (1998) A family of transketolases that directs isoprenoid biosynthesis via a mevalonate-independent pathway. Proc Natl Acad Sci USA 95: 2100–2104

Lapointe G, Luckevich MD, Seguin A (2001) Investigation on the induction of 14–3-3 in white spruce. Plant Cell Rep 20:79–84

Lindmark-Henriksson M, Isaksson D, Sjodin K, Hogberg HE, Vanek T, Valterova I (2003) Transformation of alpha-pinene using *Picea abies* suspension culture. J Nat Prod 66:337–343

Lindmark-Henriksson M, Isaksson D, Vanek T, Valterova I, Hogberg HE, Sjodin K (2004) Transformation of terpenes using a *Picea abies* suspension culture. J Biotechnol 107:173–184

Lois LM, Campos N, Putra SR, Danielsen K, Rohmer M, Boronat A (1998) Cloning and characterization of a gene from *Escherichia coli* encoding a transketolase-like enzyme that catalyzes the synthesis of D-1-deoxyxylulose 5-phosphate, a common precursor for isoprenoid, thiamin, and pyridoxol biosynthesis. Proc Natl Acad Sci USA 95:2105–2110

Lois LM, Rodriguez-Concepcion M, Gallego F, Campos N, Boronat A (2000) Carotenoid biosynthesis during tomato fruit development: regulatory role of 1-deoxy-D-xylulose 5-phosphate synthase. Plant J 22:503–513

Martin D, Tholl D, Gershenzon J, Bohlmann J (2002) Methyl jasmonate induces traumatic resin ducts, terpenoid resin biosynthesis, and terpenoid accumulation in developing xylem of Norway spruce stems. Plant Physiol 129:1003–1018

Martin DM, Gershenzon J, Bohlmann J (2003) Induction of volatile terpene biosynthesis and diurnal emission by methyl jasmonate in foliage of Norway spruce. Plant Physiol 132:1586–1599

Martin DM, Faldt J, Bohlmann J (2004) Functional characterization of nine Norway spruce TPS genes and evolution of gymnosperm terpene synthases of the TPS-d subfamily. Plant Physiol 135:1908–1927

Miller RH, Berryman AA, Ryan CA (1986) Biotic elicitors of defense reactions in Lodgepole pine. Phytochemistry 25:611–612

Miller B, Madilao LL, Ralph S, Bohlmann J (2005) Insect-induced conifer defense. White pine weevil and methyl jasmonate induce traumatic resinosis, *de novo* formed volatile emissions, and accumulation of terpenoid synthase and putative octadecanoid pathway transcripts in Sitka spruce. Plant Physiol 137:369–382

Mori H, Cline K (2001) Post-translational protein translocation into thylakoids by the Sec and Delta pH-dependent pathways. Biochim Biophys Acta 1541:80–90

Nagy NE, Franceschi VR, Solheim H, Krekling T, Christiansen E (2000) Wound-induced traumatic resin duct development in stems of Norway spruce (Pinaceae): Anatomy and cytochemical traits. Am J Bot 87:302–313

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:2002–2007

Phillips MA, Croteau RB (1999) Resin-based defenses in conifers. Trends Plant Sci 4:184–190

Phillips MA, Wildung MR, Williams DC, Hyatt DC, Croteau R (2003) cDNA isolation, functional expression, and characterization of (+)-alpha-pinene synthase and (−)-alpha-pinene synthase from loblolly pine (*Pinus taeda*): stereocontrol in pinene biosynthesis. Arch Biochem Biophys 411:267–276

Phillips M, Bohlmann J, Gershenzon J (2006) Molecular regulation of induced terpenoid biosynthesis in conifers. Phytochem Rev 5:179–189

Ralph SG, Yueh H, Friedmann M, Aeschliman D, Zeznik JA, Nelson CC, Butterfield YSN, Kirkpatrick R, Liu J, Jones SJM, Marra MA, Douglas CJ, Ritland K, Bohlmann J (2006) Conifer defence against insects: microarray gene expression profiling of Sitka spruce (*Picea sitchensis*) induced by mechanical wounding or feeding by spruce budworms (*Choristoneura occidentalis*) or white pine weevils (*Pissodes strobi*) reveals large-scale changes of the host transcriptome. Plant Cell Environ 29:1545–1570

Ralph SG, Hudgins JW, Jancsik S, Franceschi VR, Bohlmann J (2007) Aminocyclopropane carboxylic acid synthase is a regulated step in ethylene-dependent induced conifer defense. Full-length cDNA cloning of a multigene family, differential constitutive, and wound- and insect-induced expression, and cellular and subcellular localization in spruce and Douglas fir. Plant Physiol 143:410–424

Ro DK, Bohlmann J (2006) Diterpene resin acid biosynthesis in loblolly pine (*Pinus taeda*): Functional characterization of abietadiene/levopimaradiene synthase (PtTPS-LAS) cDNA and subcellular targeting of PtTPS-LAS and abietadienol/abietadienal oxidase (PtAO, CYP720B1). Phytochemistry 67:1572–1578

Ro DK, Arimura G, Lau SY, Piers E, Bohlmann J (2005) Loblolly pine abietadienol/abietadienal oxidase PtAO (CYP720B1) is a multifunctional, multisubstrate cytochrome P450 monooxygenase. Proc Natl Acad Sci USA 102:8060–8065

Rodriguez-Concepcion M, Boronat A (2002) Elucidation of the methylerythritol phosphate pathway for isoprenoid biosynthesis in bacteria and plastids. A metabolic milestone achieved through genomics. Plant Physiol 130:1079–1089

Rohmer M, Knani M, Simonin P, Sutter B, Sahm H (1993) Isoprenoid biosynthesis in bacteria—a novel pathway for the early steps leading to isopentenyl diphosphate. Biochemical J 295:517–524

Schmidt A, Zeneli G, Hietala AM, Fossdal CG, Krokene P, Christiansen E, Gershenzon J (2005) Induced chemical defenses in conifers: Biochemical and molecular approaches to studying their function. In: Romeo JT (ed) Recent advances in phytochemistry 39: chemical ecology and phytochemistry of forest ecosystems, Vol 39. Elsevier, Amsterdam, pp 1–28

Schulze B, Lauchli R, Sonwa MM, Schmidt A, Boland W (2006) Profiling of structurally labile oxylipins in plants by in situ derivatization with pentafluorobenzyl hydroxylamine. Anal Biochem 348:269–283

Sprenger GA, Schorken U, Wiegert T, Grolle S, deGraaf AA, Taylor SV, Begley TP, Bringer-Meyer S, Sahm H (1997) Identification of a thiamin-dependent synthase in *Escherichia coli* required for the formation of the 1-deoxy-D-xylulose 5-phosphate precursor to isoprenoids, thiamin, and pyridoxol. Proc Natl Acad Sci USA 94:12857–12862

Steele CL, Lewinsohn E, Croteau R (1995) Induced oleoresin biosynthesis in grand fir as a defense against bark beetles. Proc Natl Acad Sci USA 92:4164–4168

Steele CL, Crock J, Bohlmann J, Croteau R (1998a) Sesquiterpene synthases from grand fir (*Abies grandis*)—Comparison of constitutive and wound-induced activities, and cDNA isolation, characterization and bacterial expression of delta-selinene synthase and gamma-humulene synthase. J Biol Chem 273: 2078–2089

Steele CL, Katoh S, Bohlmann J, Croteau R (1998b) Regulation of oleoresinosis in grand fir (*Abies grandis*). Differential transcriptional control of monoterpene, sesquiterpene, and diterpene synthase genes in response to wounding. Plant Physiol 116: 1497–1504

Stintzi A, Weber H, Reymond P, Browse J, Farmer EE (2001) Plant defense in the absence of jasmonic acid: the role of cyclopentenones. Proc Natl Acad Sci USA 98:12837–12842

Taki N, Sasaki-Sekimoto Y, Obayashi T, Kikuta A, Kobayashi K, Ainai T, Yagi K, Sakurai N, Suzuki H, Masuda T, Takamiya K, Shibata D, Kobayashi Y, Ohta H (2005) 12-Oxo-phytodienoic acid triggers expression of a distinct set of genes and plays a role in wound-induced gene expression in Arabidopsis. Plant Physiol 139:1268–1283

Walter MH, Fester T, Strack D (2000) Arbuscular mycorrhizal fungi induce the non-mevalonate methylerythritol phosphate pathway of isoprenoid biosynthesis correlated with accumulation of the 'yellow pigment' and other apocarotenoids. Plant J 21:571–578

Walter MH, Hans J, Strack D (2002) Two distantly related genes encoding 1-deoxy-D-xylulose 5-phosphate synthases: differential regulation in shoots and apocarotenoid-accumulating mycorrhizal roots. Plant J 31:243–254

Zeneli G, Krokene P, Christiansen E, Krekling T, Gershenzon J (2006) Methyl jasmonate treatment of large Norway spruce (*Picea abies*) trees increases the accumulation of terpenoid resin components and protects against infection by *Ceratocystis polonica*, a bark beetle-associated fungus. Tree Physiol 26:977–988

Zhao J, Zheng SH, Fujita K, Sakai K (2004) Jasmonate and ethylene signalling and their interaction are integral parts of the elicitor signalling pathway leading to beta-thujaplicin biosynthesis in *Cupressus lusitanica* cell cultures. J Exp Bot 55:1003–1012

7. Article III

Rapid Enzymatic Isomerization of 12-Oxophytodienoic Acid in the Gut of Lepidopteran larvae

Birgit Schulze, Paulina Dabrowska, Wilhelm Boland

ChemBioChem (2007) **8**, 208-216

CHEMBIOCHEM

DOI: 10.1002/cbic.200600379

Rapid Enzymatic Isomerization of 12-Oxophytodienoic Acid in the Gut of Lepidopteran Larvae

Birgit Schulze, Paulina Dąbrowska, and Wilhelm Boland*[a]

*In response to feeding larvae of the Mediterranean climbing cutworm (*Spodoptera littoralis*), leaves of the lima bean (*Phaseolus lunatus*) produce fatty acid-derived signaling compounds (oxylipins). The major products are the phytohormones jasmonic acid and its biosynthetic precursor 12-oxophytodienoic acid (OPDA), along with 13-hydroxy-12-oxooctadeca-9,15-dienoic acid, 9-hydroxy-12-oxooctadeca-10,15-dienoic acid (α- and γ-ketol), as well as unsaturated aldehydes. Oxylipin production is highest at the feeding zone of the insect and decreases with distance from the damaged area. Accordingly, the feeding insect experiences high local concentrations of oxylipins, which are taken up into the alimentary canal and are finally excreted with the feces. In contrast to most other oxylipins, OPDA was not detectable in the insect's gut; instead the structurally related tetrahydrodicranenone B (iso-OPDA) was identified. Feeding experiments with deuterium-labeled OPDA proved that the isomerization is catalyzed by an enzyme from the insect's gut tissue. The phenomenon appears to be widespread among Lepidopteran larvae.*

Introduction

Plants have evolved effective defense strategies against herbivorous attackers by relying on constitutive and induced synthesis of toxic secondary metabolites or volatiles that mediate indirect defenses from higher trophic levels.[1] Inducible defenses save resources and reduce the danger of resistance, since they are only activated upon attack. In consequence, the reliable recognition of herbivores followed by an effective signaling chain that mediates the alteration of gene expression is an important prerequisite for the success of inducible defense mechanisms. So far, a large set of plant hormones and signaling compounds that regulate plant stress responses have been identified. Among the fatty acid-derived compounds, so called oxylipins, the phytohormones jasmonic acid (JA) and 12-oxophytodienoic acid (OPDA) seem to play a major role.[2,3] In addition, several minor and less well studied oxylipins have been shown to be involved in signaling, coordination, and fine tuning of plant-defense reactions. The fatty acid-derived oxidation products can also serve as defensive compounds themselves. This is immediately obvious for compounds containing reactive structural elements such as α,β-unsaturated aldehydes or ketones, which can act as Michael acceptors with glutathione or nucleophilic amino acid residues of proteins and nucleic acids.[4,5] For example, fatty acid-derived aldehydes act as a direct defense against aphids,[6] or as teratogenic defense compounds in the trophic interaction of diatoms with phytoplankton consumers.[7]

However, herbivores have evolved strategies to combat the plant's defensive arsenal. An economic and efficient counter defense demands an early and reliable detection system of the plant's defensive compounds to activate detoxification mechanisms. In analogy to the plant's induced defense, the herbivore is best protected when it recognizes early cues, such as compounds involved in the early events of upregulation of the plant's induced defense. In line with this, Li et al. reported that typical plant hormones such as salicylic acid and JA can be sensed by insects and trigger expression of genes in the gut tissue that code for typical detoxification catalysts from the family of P450 enzymes.[8] This ability to recognize the plant's phytohormone levels allows the insects to have their detoxification ready when the induced plant defense becomes effective.

In the mutual interaction between plants and their invertebrate herbivores, fatty acid-derived metabolites are perfect candidates for mediating recognition between different kingdoms.[9] They are ubiquitous in cell membranes and utilized by plants and animals to generate physiologically active compounds, including powerful signaling molecules. Accordingly, oxylipins are ideal candidates for cross-kingdom signals, with signal recognition and processing taking place at the damaged zone of the attacked leaf and within the insect after ingestion of the plant material.

To explore the molecular basis and the extent of such recognition processes, we simultaneously analyzed the spatial distribution of several members of the oxylipin family in lima bean plants (*Phaseolus lunatus*) attacked by *Spodoptera littoralis*. We demonstrate that plants respond with an enhanced synthesis of an array of oxylipins that display different accumulation patterns in the attacked or distant leaves. Moreover, we demonstrate that the compounds are differentially treated or metabo-

[a] Dr. B. Schulze, P. Dąbrowska, Prof. W. Boland
Max Planck Institute for Chemical Ecology
Hans-Knöll-Strasse 8, 07745 Jena (Germany)
Fax: (+49) 3641-571202
E-mail: boland@ice.mpg.de

lized in the insect's digestive tract. In particular, the JA precursor OPDA is enzymatically transformed into the structurally very different tetrahydrodicranenone B (*iso*-OPDA) by a double-bond shift. This is the first example of an active transformation of a phytohormone by an herbivore and might be important for our understanding of the interactions between plants and their herbivores.

Results and Discussion

Spatial distribution of oxylipins in caterpillar-damaged leaves

To monitor the spatial pattern of oxylipin signals radiating through the plant from the biting zone of the attacking insect, larvae of *S. littoralis* were allowed to feed for 24 h on one of the two primary leaves of a lima bean plantlet. Then, samples were taken at different distances from the damaged area and from the neighboring, undamaged primary leaf. To cover a broad spectrum of oxylipins, including structurally sensitive dienoic aldehydes, ketones and hydroxylated ketols, the oxo group of these oxylipins was derivatized in situ with pentafluorobenzyl hydroxylamine (PFBHA) to yield stable pentafluorobenzyl oximes (PFB oximes), as described previously.[10] During the feeding process, a clear spatial gradient of oxylipin accumulation developed (Figure 1). JA biosynthesis was induced locally at the feeding zone of the caterpillar; levels increased 20-fold from 40 ng g^{-1} fresh weight (frw) to an average of 800 ng g^{-1} frw, with a maximum directly at the feeding site and radiated from there approximately 10–20 mm into the undamaged tissue. No accumulation of JA was observed in the neighboring leaves. Analogously to JA, OPDA levels increased fivefold when compared to the resting level. A slight increase in OPDA was also observed in the neighboring leaf tissue. Since OPDA is not only a precursor for JA but can act as an JA-independent signaling molecule,[11–13] its accumulation might be relevant for the induction of a certain subset of defense responses in the insect's gut tissue.

The accumulation of 13-hydroxy-12-oxooctadeca-9,15-dienoic acid and 9-hydroxy-12-oxooctadeca-10,15-dienoic acid (α- and γ-ketol) near to the feeding site of the herbivore (Figure 1) is remarkable. Although, the ketols have been reported as signals for flower formation,[14,15] they have not yet been considered or demonstrated as being involved in plant–insect interactions. The ketols result from parallel transformations of the highly reactive allene oxide intermediate en route to OPDA and JA and are, thus, principal candidates for fine tuning plant-stress reactions. Moreover, the amounts of oxidatively degraded fatty acid fragments such as 11-oxoundec-9-enoic acid and 13-oxotrideca-9,11-dienoic acid were increased in the damaged leaves (Figure 1). The latter share many structural features with the wound hormone traumatin and might represent bioactive compounds as well. As these structures carry an inherent toxicity due to the α,β-unsaturated carbonyl group, they might also be well suited as direct defense metabolites, for example, as antibiotics.[16]

Oxylipins in the insect's gut

Since analysis of the spatial distribution of oxylipins has shown that these compounds predominantly originate at the bite zone of the herbivore, they will be ingested by the feeding larvae. To verify this assumption, larvae of *S. littoralis* were reared on lima bean leaves for 24 h, and the ingested and digested leaf material was obtained by collecting the caterpillar's regurgitant and frass. For some compounds, surprising differences between the oxylipin signature of the plant tissue, the regurgitant, and the frass were found (Table 1). In comparison

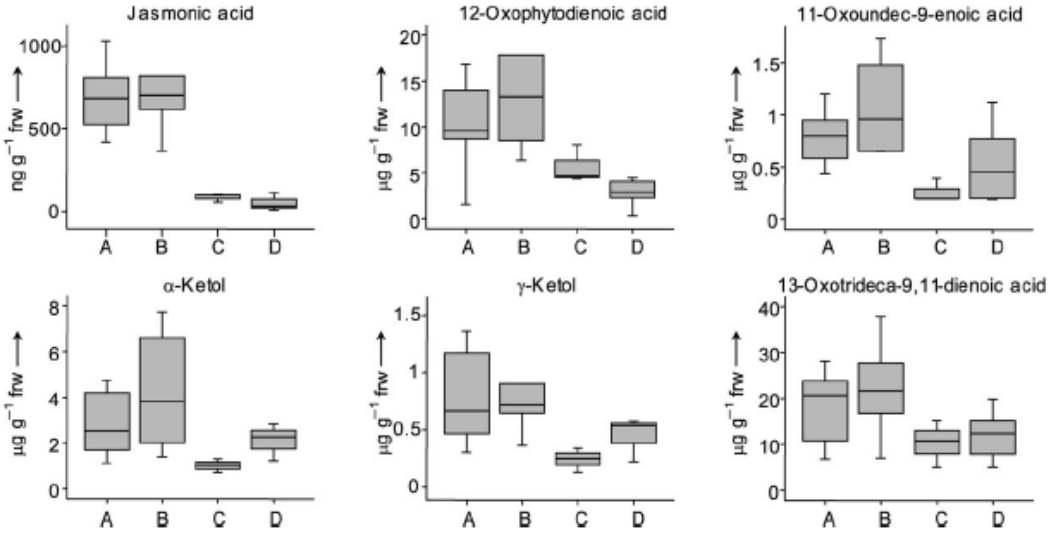
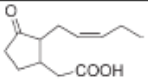
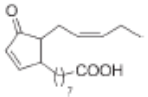
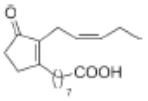
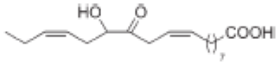
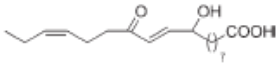
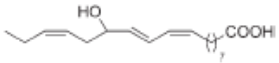
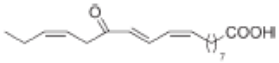
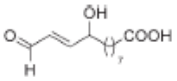
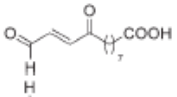
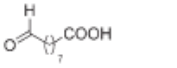
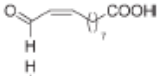
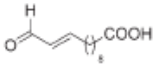
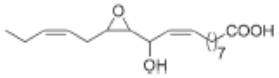


Figure 1. Oxylipin profiles in lima bean plantlets after 24 h of feeding of *S. littoralis* larvae. A) 0–10 mm from the bite zone ($n=6$); B) 10–20 mm from the bite zone ($n=6$); C) opposite primary leaf ($n=3$); D) control, primary leaves from undamaged plantlets ($n=4$). Bars indicate the highest and lowest values.

Table 1. Oxylipins in lima bean leaves, regurgitant, and frass of *S. littoralis* larvae after 24 h of feeding.

Oxylipin		*P. lunatus*	*S. littoralis*	
Name	Structure	Leaf	Regurgitant	Frass
jasmonic acid (JA)		+	+	+
12-oxophytodienoic acid (OPDA)		+	-	-
tetrahydrodicranenone B (*iso*-OPDA)		-	+	+
13-hydroxy-12-oxooctadeca-9,15-dienoic acid (α-ketol)		+	+	+
9-hydroxy-12-oxooctadeca-10,15-dienoic acid (γ-ketol)		+	-	-
13-hydroxyoctadeca-9,12,15-trienoic acid (13-HOTE)		+	+	-
13-oxooctadeca-9,12,15-trienoic acid (13-KOTE)		+	+	+
9-hydroxy-12-oxododec-10-enoic acid		+	+	+
9,12-dioxododec-10-enoic acid		+	-	-
9-oxononanoic acid		+	+	+
11-oxoundec-9-enoic acid		+	+	+
traumatin		+	+	+
13-oxotrideca-9,11-dienoic acid		+	+	+
12,13-epoxy-11-hydroxyoctadeca-9,15-dienoic acid		+	+	+

to the leaf material, free fatty acids were abundant in the regurgitant and the frass. Terminal oxo acids, resulting from oxidative degradation of the C_{18} precursor fatty acids, for example, 9-hydroxy-12-oxododec-10-enoic acid, 9-oxononanoic acid, 11-oxoundec-9-enoic acid, traumatin, and 13-oxotrideca-9,11-dienoic acid were found in the bite zone of the leaf, in the regurgitant, and the frass, although the α,β-unsaturated carbonyl compounds could principally react with glutathione, proteins, or other biomolecules in the insect's gut. In line with this, the conjugated 13-oxooctadeca-9,12,15-trienoic acid (13-KOTE) also survived the gut passage and occurred in the regurgitant and frass. In contrast, the highly reactive 9,12-dioxododec-10-enoic acid was not present in the regurgitant and frass. Furthermore, γ-ketol, present in the damaged leaf tissue, was not detected in the regurgitant and frass, while the α-ketol was found in all samples. Additionally, 13-oxotrideca-9,11-dienoic acid, which is present in the leaf predominantly as the *Z* isomer, suffered in the alkaline milieu of the insect's gut (pH 9–10.5),[17] isomerizing to the *E* isomer.

JA was present in high amounts in the caterpillar's regurgitant and even seemed to accumulate in the insect's frass (Figure 2, left). As the plant-derived JA has been reported to be a compound that induces detoxification enzymes (such as P450) in insects,[8] this finding prompted us to investigate the origin of the JA in more detail, since, besides free JA, its amino acid conjugates (for example, JA-isoleucine) play an important role as plant signaling molecules.[18] In order to test whether the accumulation of JA in the frass of *S. littoralis* larvae could be due to the cleavage of such conjugates in the caterpillar's digestive system, we fed deuterium-labeled JA-isoleucine

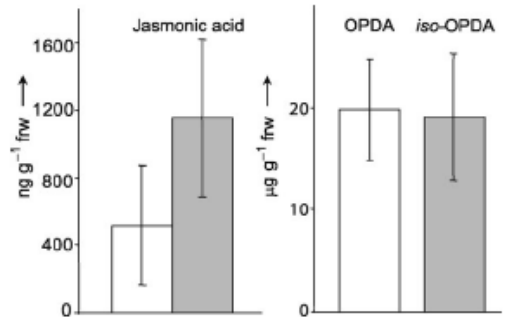
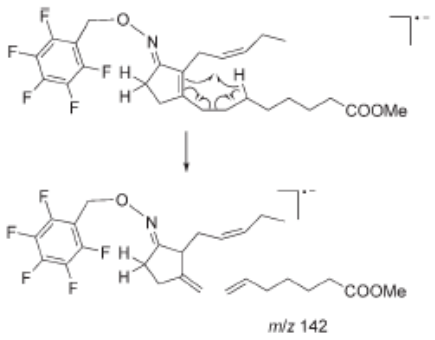


Figure 2. Oxylipin levels in *S. littoralis* damaged lima bean leafs (□) and in the frass of the larvae (■) after 24 h of feeding. Left: JA ($n=5$). Right: OPDA in the attacked lima bean leaves and *iso*-OPDA in the caterpillar's frass ($n=7$). Error bars represent standard deviation.

([2,2,5,5,7-$^2H_5$]-JA-Ile) to larvae of *S. littoralis*. However, the labeled conjugate proved to be stable against hydrolysis, hence, the cleavage of this and other related amino acid conjugates is not dominant in the insect's gut.

Most surprisingly, OPDA was completely absent in the regurgitant and the frass of *S. littoralis* larvae. This was particular remarkable since OPDA was the most abundant oxylipin in the damaged leaf tissue. However, since the negative chemical ionization (CI-MS) spectra of the PFBO oximes of OPDA and other oxylipins display characteristic fragmentation patterns, the fate of the ingested OPDA was easily unraveled. Comparison of the mass spectrum of an unknown but closely eluting compound with that of the OPDA–PFB oxime, revealed similar spectra for both compounds with prominent ions at $[M-20]^-$ ($[M-HF]^-$) and at $[M-50]^-$ ($[M-HF-NO]^-$)[19] A fragment at *m/z* 339 was characteristic for the unknown compound (Figure 3). Assuming the same basic cyclopentenone structure for both compounds, only a location of the double bond between the two side chains of the cyclopentenone would allow a β-cleavage of the side chain to result in the observed loss of methyl 6-heptanoate ($[M-20-142]^-$, generating *m/z* 339; Scheme 1). Due to this difference in the fragmentation patterns, the unknown compound was assigned as tetrahydrodicranenone B (*iso*-OPDA; Figure 3) and confirmed by a synthetic reference.[20] This clearly underlines the diagnostic value of the fragmentation patterns of PFB oximes of oxylipins in either negative CI or positive EI-MS, as described recently.[10]



Scheme 1. Diagnostic fragmentation of the PFB oxime of *iso*-OPDA during negative chemical ionization mass spectroscopy. Only *iso*-OPDA shows the characteristic loss of the side chain fragment 6-heptenoic acid methyl ester (*m/z* 142). An additional loss of HF[19] generates the characteristic fragment ion at *m/z* 339.

The above finding suggested that the plant-derived OPDA might be isomerized in the insect gut to give *iso*-OPDA (Scheme 2). The transformation proceeded quantitatively, since no traces of OPDA were detectable. In contrast to JA, which accumulated in the caterpillar's frass, the amount of *iso*-OPDA detected in the frass of *S. littoralis* was, in the first instance, comparable to the amount of ingested OPDA from the lima bean leaf (Figure 2, right). *Iso*-OPDA had been previously found in the Japanese moss *Leucobryum scabrum* and in barley leaves (*Hordeum vulgare*),[21,22] but not in healthy or herbivore-infested lima bean leaves. To confirm that the *iso*-OPDA formation is, indeed, derived from the ingested plant-derived OPDA, we synthesized deuterium-labeled OPDA ([18,18,18,17,17-$^2H_5$]-OPDA) from deuterated linolenic acid using a flax seed enzyme extract and following a protocol of Kramell et al.[22] This approach simultaneously afforded labeled OPDA along with labeled α- and γ-ketol. When the labeled OPDA was fed together with an artificial diet to larvae of *S. littoralis*, a complete conversion into [18,18,18,17,17-$^2H_5$]-*iso*-OPDA was observed (Figure 4).

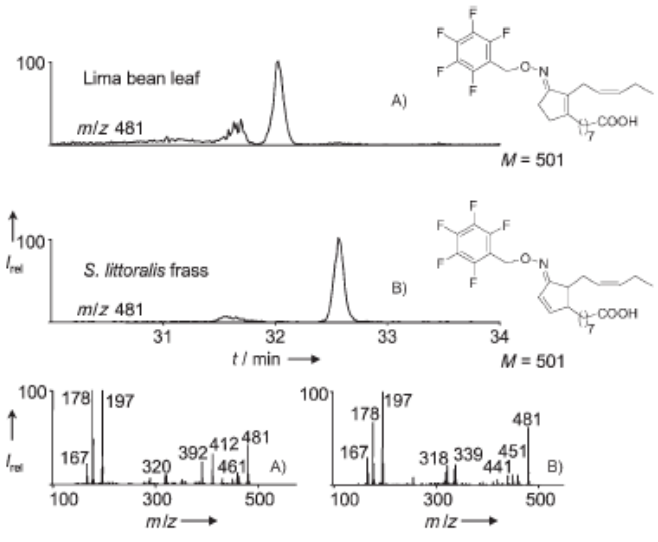
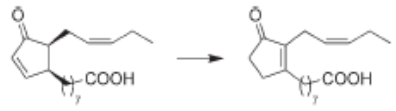


Figure 3. Chromatographic separation of OPDA isomers. The common fragment at *m/z* 481 was used to monitor the isomers. A) OPDA-PFB oxime from leaf material; B) *iso*-OPDA-PFB oxime from the frass of *S. littoralis*. For a discussion of the diagnostic fragmentation pattern of the OPDA isomers see the text.

CHEMBIOCHEM W. Boland et al.



Scheme 2. Isomerization of *cis*-OPDA to *iso*-OPDA in the caterpillar's gut.

The failure of the isomerization of OPDA to *iso*-OPDA by the alkaline medium or strong organic bases suggested a biocatalytic transformation by an enzyme present in the insect's regurgitant or in the epithelial cells of the gut. The catalytic properties of the digestive fluid were tested by treatment of OPDA with the regurgitant of *S. littoralis* larvae; however no *iso*-OPDA was detected. Moreover, treatment of OPDA with

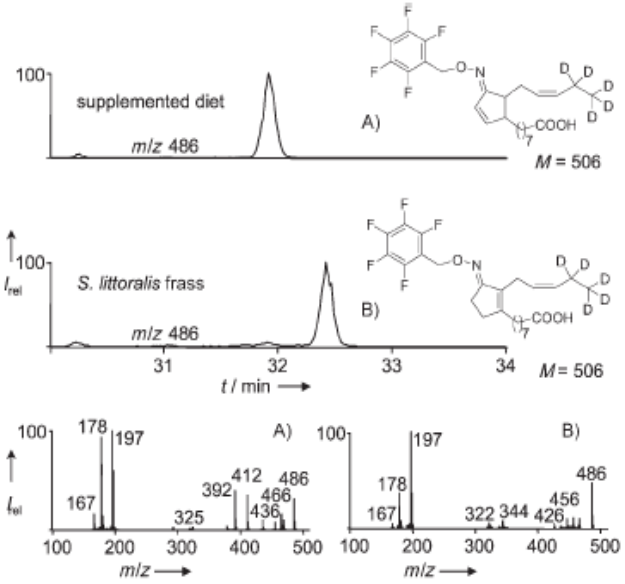
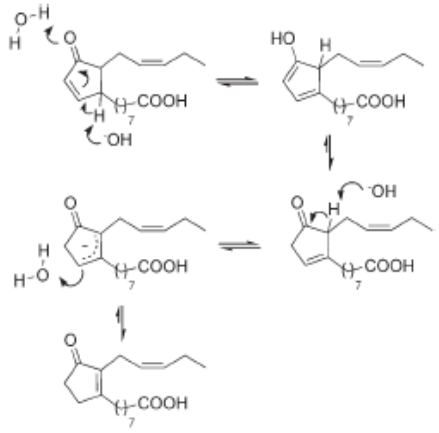


Figure 4. Isomerization of deuterium labeled *cis*-OPDA in the insect's gut. A) deuterium labeled *cis*-OPDA recovered from artificial diet. B) *iso*-OPDA extracted from the frass of *S. littoralis* larvae after 24 h of feeding on the artificial diet. Both compounds were analyzed as their PFB oximes. For a discussion of the diagnostic fragmentation pattern of the OPDA-isomers see the text.



Scheme 3. Proposed mechanism for the isomerization of *cis*-OPDA to *iso*-OPDA by the gut tissue.

bovine serum albumin (BSA) only resulted in epimerization of *cis*-OPDA to *trans*-OPDA, analogously to the previously reported epimerization of *cis*-JA into *trans*-JA[24] and the treatment of OPDA with the organic bases DBN or DBU (vide supra). However, after a 2-h incubation of OPDA with isolated and cleaned gut tissue of *S. littoralis* at RT, an almost quantitative isomerization of OPDA to

In vitro assays of OPDA isomerization

Isomerization of OPDA to *iso*-OPDA is known to be a thermally induced reaction during gas chromatography.[23] Owing to the in situ derivatization of all oxo-oxylipins with PFBHA to their PFB oximes, epimerization or isomerization of OPDA during extraction and gas-chromatographic separation are prevented. On the other hand, the alkaline nature of the gut of lepidopteran larvae (pH 9–10.5)[17] could account for the observed isomerization (Scheme 3). To test for a nonenzymatic isomerization of OPDA to *iso*-OPDA, OPDA was incubated in various buffers of different pH values (pH 3, 5, 7, and 10). Additionally, OPDA was treated with the bases DBN (1,5-diazabicyclo-[4.3.0]non-5-ene) and DBU (1,5-diazabicyclo[4.3.0]undec-7-ene) in various molar ratios (OPDA/DBN or DBU 1:1, 1:5). Neither alkaline nor acidic pH resulted in isomerization of OPDA to *iso*-OPDA, and with the stronger bases DBN or DBU, only low amounts of *iso*-OPDA along with epimerization of the authentic *cis*-OPDA to *trans*-OPDA was observed (Figure 5). Higher concentrations (OPDA/DBN or DBU, 1:10) led to decomposition of OPDA.

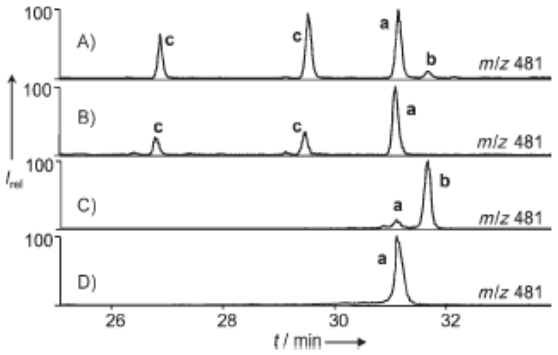
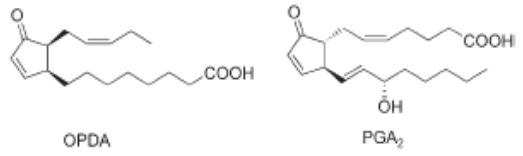


Figure 5. Isomerization of *cis*-OPDA by chemical reagents and tissue preparations. A) treatment of *cis*-OPDA with DBN (OPDA/DBN, 1:1); B) treatment of *cis*-OPDA with bovine serum albumin. Identification of compounds: a: *cis*-OPDA, b: *iso*-OPDA, c: *trans*-OPDA represented by the *syn* and *anti* isomers of their PFB oximes. DBN and BSA preferentially epimerize *cis*-OPDA to the corresponding *trans* isomer. C) Tissue preparations from the insect gut transform *cis*-OPDA to *iso*-OPDA; epimerization is not observed. D) Preparations from boiled tissue have no effect on *cis*-OPDA.

iso-OPDA was observed. This reaction was not observed when the gut tissue was boiled prior to the experiment (Figure 5D). Hence, the catalytic activity can be attributed to an enzyme integrated or attached to the gut tissue.

Enzyme specificity and occurrence in Lepidoptera

To test the structural requirements for this biotransformation, feeding experiments were conducted with a mixture of *cis*/*trans*-OPDA and the structurally related prostaglandin A_2 (PGA_2; Scheme 4), for which a similar rearrangement of the



Scheme 4. Structural similarity of *cis*-OPDA and prostaglandin A_2 (PGA_2). Common structural elements suggest that plant-derived *cis*-OPDA might interfere with prostaglandin-linked effects in the insect.

double bound to form prostaglandin B_2 (PGB_2) is known.[25] Analysis of the regurgitant and frass of *S. littoralis* fed a supplemented artificial diet indicated a clear preference for the isomerization of *cis*-OPDA over *trans*-OPDA (Figure 6). In line with

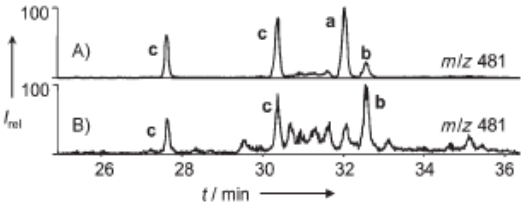


Figure 6. Gut passage and transformation of OPDA isomers. A) Artificial diet supplemented with a mixture of a: *cis*-OPDA, b: *iso*-OPDA, and c: *trans*-OPDA. B) Analysis of the frass of the feeding *S. littoralis* larvae after 24 h. *cis*-OPDA (a) was largely converted to *iso*-OPDA (b); *trans*-OPDA (c) remained unaffected.

this, no conversion of the *trans*-disubstituted prostaglandin PGA_2 to PGB_2 was observed, although the additional double bond should facilitate isomerization. Other oxylipins, such as deuterium-labeled α-ketol ([18,18,18,17,17-$^2H_5$]-α-ketol) and γ-ketol ([8,18,18,17,17-$^2H_5$]-γ-ketol) passed the gut without change and were excreted with the frass. Thus, the catalytic activity in the caterpillar's gut appears to be specific for the transformation of the plant-derived *cis*-OPDA.

To localize the enzymatic activity, we dissected the gut of *S. littoralis* larvae that had previously been fed an OPDA-supplemented artificial diet. Already in the foregut of the larvae, OPDA was found to be almost quantitatively converted to *iso*-OPDA (Figure 7); this suggests that the isomerization takes place immediately after ingestion of the plant material. Since *S. littoralis* is a generalist insect, we next addressed the question whether insects specialized on a certain host plant possess the same enzymatic activity. Moreover, information on the occurrence of the process within Lepidoptera is important to allow conclusions on the significance of this isomerization for the insects. As generalists, *Spodoptera littoralis*, *Spodoptera exigua*, *Helicoverpa armigera*, and *Trichoplusia ni* were tested. We choose *Pieris rapae* as a specialist adapted to Brassicaceae. As seen from Figure 8, the enzyme activity is widespread among the lepidopterans. All generalists effectively transformed OPDA to *iso*-OPDA (ca. 90% isomerization), but the specialist *P. rapae* showed only a low conversion of OPDA ingested from its food plant *Arabidopsis thaliana* (17% isomeriza-

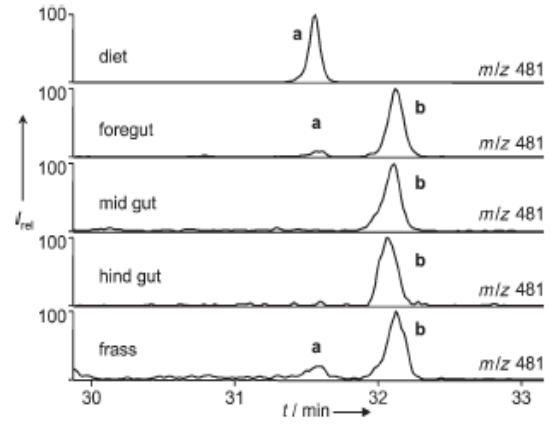


Figure 7. Localization of the enzymatic activity in the insect's gut. Upper line: *cis*-OPDA (a) reextracted from the artificial diet. Lower lines: The oxylipin profile of the different preparations from the fore gut to the hind gut and the frass demonstrates effective isomerization to *iso*-OPDA (b) already in the fore gut (>90% conversion). No *cis*-OPDA was found in the mid-gut or hind-gut segments.

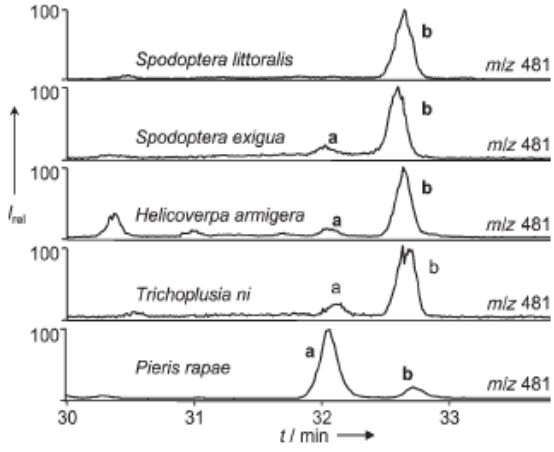


Figure 8. Isomerization of *cis*-OPDA in different lepidopteran species. Larvae of the generalist herbivores *S. littoralis*, *S. exigua*, *H. armigera* and *T. ni* efficiently transform ingested *cis*-OPDA (a) to the corresponding *iso*-OPDA (b). The specialist *P. rapae* was allowed to feed on *A. thaliana* to encounter plant-derived *cis*-OPDA. All data represent analyses of the excreted frass after 24 h of feeding.

tion). Unlike in the Spodoptera larvae, we observed no isomerization of OPDA with gut tissue from *P. rapae*. Since, in a recent study, fatty acids and various oxo acids such as 13-KOTE, JA, and *cis*-OPDA were found in the regurgitant of *P. rapae*,[26] this insect apparently does not posses the same level of isomerizing capacity as the generalist lepidopterans.

We have shown in this study that many lepidopteran larvae possess an enzyme in their gut tissue that effectively and specifically catalyzes the isomerization of plant-derived *cis*-OPDA to *iso*-OPDA (Figure 8). The transformation of OPDA to *iso*-OPDA induces major changes in the structure and reactivity of the molecule. While the orientation of the substituents at the ring in *cis*-OPDA results in a bent structure, the shift of the double bound flattens the molecule and generates a planar tetrasubstituted double bond that no longer matches up with the interaction sites of *cis*-OPDA. Therefore, receptors that recognize the characteristic architecture of OPDA no longer respond to *iso*-OPDA, as shown previously in barley.[22] The same observation was made for JA, which lost the ability to induce volatile biosynthesis in lima beans after introduction of a central ring double bond between the two side chains.[27] The planar prostaglandins of the PGB series are also found to be less active than their precursors in the PGA series.[28] Accordingly, *cis*-OPDA, JA, and PGA are important signaling compounds that can be inactivated by a shift of the ring double bond. The isomerization of PGA to PGB in mammals is known to be catalyzed by specific enzymes and by albumin (vide supra).[28,29]

Insects produce and utilize prostaglandins, as has been exemplified by midgut preparations of *Manduca sexta*, which produced several prostaglandins including PGA and PGB.[30] However, the physiological role and action of the prostaglandins produced in the insect's alimentary canal remain to be established. Since prostaglandins mediate the insect's immune responses against pathogens, interference by the structurally related OPDA could affect vital functions and might explain the necessity for an immediate isomerization of *cis*-OPDA to *iso*-OPDA in the foregut.[31] Future work on the effect of *cis*-OPDA on the physiology of insects and the isolation of the involved enzymes from gut tissue are necessary to understand the functional significance of the isomerization process.

Experimental Section

Plants and caterpillars: Lima beans (*Phaseolus lunatus* L. cv. Ferry Morse var. Jackson Wonder Bush) were grown in sterilized pot soil under greenhouse conditions (21–23 °C, 50–60% humidity, 14 h light, approximately 300 $\mu E\,m^{-2}s^{-1}$). Experiments were conducted on 12- to 14-day-old plantlets that had two fully developed primary leaves.

Larvae of all lepidopterans were hatched from eggs. *Spodoptera littoralis*, *Spodoptera exigua*, *Helicoverpa armigera*, and *Trichoplusia ni* were reared on an agar-based optimal diet,[32] while *Pieris rapae* was reared on Chinese cabbage leaves. The temperature was kept at 23–25 °C, and a regime of 16 h light and 8 h darkness was followed.

Oxylipins: 12,13-Epoxy-11-hydroxyoctadeca-9,15-dienoic acid methyl ester, traumatin, and [17,17,18,18,18-$^2H_5$]-linolenic acid were purchased from Larodan Fine Chemicals (Malmö, Sweden). [9,10-$^2H_2$]-Dihydrojasmonic acid was obtained by hydrogenation of JA with deuterium by following the procedure of Koch et al.[11] Labeled jasmonic acid isoleucine conjugate ([2,2,5,5,7-$^2H_5$]-JA-Ile) was synthesized from deuterated JA[27] and conjugated to isoleucine.[33] Tetrahydrodicranenone B (*iso*-OPDA) and [15,16-$^2H_2$]-tetrahydrodicranenone B were synthesized according to the protocol of Lauchli et al.[20] 11-Oxoundec-9-enoic acid and [9,10-$^2H_2$]-11-oxoundec-9-enoic acid were synthesized as described by Schulze et al.[10] 13-Oxotrideca-9,11-dienoic acid was obtained according to Adolph et al.[34] 13-Hydroxyoctadeca-9,12,15-trienoic acid (13-HOTE) and 13-oxooctadeca-9,12,15-trienoic acid (13-KOTE) were obtained from 13-hydroperoxyoctadeca-9,12,15-trienoic acid by reduction and oxidation as described.[35] A mixture of 9-hydroxy-10-oxostearic acid and 10-hydroxy-9-oxostearic acid was obtained by oxidizing *threo*-9,10-dihydroxystearic acid (Sigma–Aldrich) with Bobitt's reagent.[36] *cis*-OPDA, 13-hydroxy-12-oxooctadeca-9,15-dienoic acid (α-ketol), and 9-hydroxy-12-oxooctadeca-10,15-dienoic acid (γ-ketol) were synthesized by using a modified Zimmermann–Feng approach.[22,37] In brief, flax seeds were homogenized in acetone and diethyl ether to obtain a fine powder. The powder (7 g) was extracted with phosphate buffer (70 mL, 0.1 M, pH 7) to yield a crude enzyme extract. Then, a solution of linolenic acid in borate buffer (250 mg in 100 mL, 0.1 M, pH 9) was added, and the mixture was stirred for 2 h at RT. The reaction mixture was then extracted with dichloromethane, and the products were purified by preparative HPLC equipped with a Gilson 115 UV detector and a Gilson 206 fraction collector (Gilson International, Bad Camberg, Germany). Chromatography was performed on RP18 (LichroCART 250 × 10 mm × 5 μm Purospher STAR, VWR, Darmstadt, Germany) by using methanol/water (75:25, 0.05% acetic acid) with detection at 223 nm.[22] [17,17,18,18,18-$^2H_5$]-OPDA, [17,17,18,18,18-$^2H_5$]-α-ketol, and [17,17,18,18,18-$^2H_5$]-γ-ketol were synthesized from [17,17,18,18,18-$^2H_5$]-linolenic acid by using the same approach. A mixture of the *cis*- and *trans*-OPDA isomers was obtained by treating *cis*-OPDA with DBN (1:1 molar ratio of OPDA and DBN for 2 h at RT).

Analysis of plant material, caterpillar's regurgitant, and frass: The oxylipin profile of plant material, regurgitant, and frass was analyzed according to Schulze et al.[10] In brief, the sample was mixed with PFBHA (1–3 mL, 0.05 M in methanol). For quantification of oxylipins, [9,10-$^2H_2$]-dihydrojasmonic acid (150 ng), [15,16-$^2H_2$]-tetrahydrodicranenone B (250 ng), [9,10-$^2H_2$]-11-oxoundec-9-enoic acid (250 ng), and a mixture of 9-hydroxy-10-oxostearic acid and 10-hydroxy-9-oxostearic acid (500 ng) were added as internal standards. The samples were cooled on ice and homogenized for 5 min with a high-performance dispenser (24000 rpm, Ultra-Turrax T-25, IKA-Werk, Germany). To complete the derivatization, samples were shaken for 2 h at RT. After acidification, PFB oximes were first extracted with hexane and then with dichloromethane. The combined extracts were separately passed through preconditioned aminopropyl cartridges (Chromabond, Macherey & Nagel, Düren, Germany, 0.5 g for the hexane phase and 1 g for the dichloromethane phase). The cartridges were washed with propan-2-ol/dichloromethane (5 mL, 2:1) or dichloromethane (5 mL), respectively, and eluted with diethyl ether/formic acid (10 mL, 98:2). The solvent was removed under a gentle stream of argon, and the residue was treated with an ethereal solution of diazomethane. The solvent was removed after 5 min, and the sample was dissolved in dichloromethane (30 μL). The samples from extracts with dichloromethane were additionally treated with *N*-methyltrimethylsilyltrifluoroacetamide (MSTFA, 30 μL) for 1 h at 40 °C. Samples were ana-

lyzed on a Finnigan GCQ Instrument (Thermoelectron, Bremen, Germany) running in CI negative-ion mode with methane as reagent gas.[10] The instrument was equipped with a nonpolar guard column (3 m, Supelco, Taufkirchen, Germany) linked to a RTX-200 capillary column (30 m × 0.25 mm × 0.25 μm; Restek, Bad Homburg, Germany). Helium at a constant rate of 40 cm s^{-1} served as carrier gas. Separation of the compounds was achieved under programmed conditions from 100 to 210 °C at 10 °C min^{-1}, then at 1 °C min^{-1} to 237 °C, followed by rapid heating (40 °C min^{-1}) to 300 °C; this temperature was maintained for 2 min prior to cooling.

Oxylipins were identified by comparison of mass spectra and retention times with authentic standards. 9-Hydroxy-12-oxododec-10-enoic acid and 9,12-dioxododec-10-enoic acid were identified by comparing their mass spectra with the literature.[38] The mass spectra of all compounds analyzed can be taken from Schulze et al.[10]

Characteristic fragment ions of the PFB oximes were used for quantification: *m/z* 399 for JA, *m/z* 403 for [9,10-$^2H_2$]-dihydrojasmonic acid, *m/z* 481 for OPDA and *iso*-OPDA, *m/z* 483 for [15,16-$^2H_2$]-tetrahydrodicranenone B, *m/z* 357 for 11-oxoundec-9-enoic acid, *m/z* 359 for [9,10-$^2H_2$]-11-oxoundec-9-enoic acid, *m/z* 252 for 13-oxotrideca-9,11-dienoic acid, *m/z* 320 for α- and γ-ketol, and *m/z* 324 + 414 for 9-hydroxy-10-oxostearic acid and 10-hydroxy-9-oxostearic acid. The response curves of all relevant oxylipins were obtained by adding known amounts of analytes to samples containing leaf tissue (0.5 g) prior to derivatization and workup. The area ratio of the analyte to the corresponding standard was plotted against the amount of added compound. [9,10-$^2H_2$]-Dihydrojasmonic acid served as internal standard for JA, [15,16-$^2H_2$]-tetrahydrodicranenone B for OPDA, and [9,10-$^2H_2$]-11-oxoundec-9-enoic acid for 11-oxoundec-9-enoic acid and 13-oxotrideca-9,11-dienoic acid.[10] α-Ketol and γ-ketol were calibrated against the internal standards 9-hydroxy-10-oxostearic acid and 10-hydroxy-9-oxostearic acid.

Caterpillar feeding on lima bean: 2nd–3rd instar *S. littoralis* larvae were placed on intact plantlets of *P. lunatus* (four on each plant). Caterpillar-damaged leaves were harvested after 24 h. To monitor the oxylipins in different regions of the damaged leaf, the leaf material surrounding the feeding areas (ca. 10 mm) was cut off with a razor blade followed by a second section of ca. 10 mm. The cutting with the razor blade was done quickly, and the leaf strips were immediately frozen in liquid nitrogen to avoid spreading and scrambling of the signal due to wound response. Additionally, the neighboring leaves and noninduced control leaves were collected, and samples were stored at −80 °C until they were subjected to the workup procedure.

Regurgitant of the caterpillars was collected after 24 h of feeding by holding the insect with a pair of spring steel forceps and gently touching the mandibles with a small glass capillary. The regurgitant (usually about 300–700 μL) was immediately covered with PFBHA (1 mL, 0.05 M in methanol) to derivatize labile compounds. Frass (0.5 g) from the caterpillars was collected, extracted, and purified according to the protocol for oxylipins.

Feeding and recovery of oxylipins: Artificial diet was soaked with oxylipin/water solutions to achieve concentrations of 6–30 μg g^{-1} in the diet. Prostaglandin A_2 (PGA_2) and JA-Ile were similarly applied to the artificial diet (6 and 1 μg per g diet, respectively). Caterpillars were starved for 2 h prior to the start of the experiment and placed separately in small Petri dishes. The insects were allowed to feed on the oxylipin-supplemented diet for 24 h. Then, the frass (0.5 g) and the remaining diet (0.5 g) were collected. Larvae of *P. rapae* refused the artificial diet and were reared on *A. thaliana*, thus ingesting the plant-derived OPDA. After 24 h of feeding on OPDA-supplemented diet, *S. littoralis* larvae were frozen at −80 °C and dissected with a sharp scalpel to remove the gut. The gut was separated into foregut, midgut, and hindgut. Gut segments from up to 20 insects were pooled to gain sufficient material for analysis.

In vitro isomerization of OPDA: *cis*-OPDA (10–20 μg) was dissolved in phosphate buffers of different pH (pH 3, 5, 7, 10) and allowed to react at RT for 4 or 10 h. Additionally, OPDA was treated with different molar ratios of DBN and DBU in CH_2Cl_2 for 2 and 4 h at RT (OPDA/DBN or DBU, 1:1, 1:5, and 1:10). The reaction mixture was derivatized with PFBHA (0.5 mL; 0.5 M in methanol) and analyzed as described above.

To analyze the effect of the regurgitant from *S. littoralis* or that of albumin on OPDA, *cis*-OPDA (ca. 10–20 μg) was dissolved in pooled samples of regurgitant or in a solution of bovine albumin (1 mL, 1% protein concentration in potassium phosphate buffer (pH 7) or sodium borate buffer (pH 9)) and allowed to react for 2 h at RT. The effect of isolated gut tissue was investigated by placing five cleaned guts from 5th instar larvae of *S. littoralis* into Ringer's solution (2 mL, pH 7 containing 20 μg of *cis*-OPDA) for 2 h at RT. The experiment was repeated with *S. littoralis* guts boiled for 15 min in 100 °C. Samples were analyzed as described.

Acknowledgements

We thank Prof. C. Wasternack and Dr. Otto Miersch (IPB, Halle, Germany) for helpful advice, Dr. Jian-Wen Tan for the synthesis of the labeled jasmonic acid conjugate, and Prof. G. Spiteller for fruitful discussions on the interpretation of mass spectra. We are indebted to Dr. G. Trautmann (Bayer AG, Monheim, Germany) for supplying us with egg clutches of S. littoralis, S. exigua *and* H. armigera *and Dr. Heiko Vogel for the supply of* T. ni *and* P. rapae *larvae. We thank the Deutsche Forschungsgemeinschaft, Graduiertenkolleg GRK 266 (to B.S.) and the Max-Planck-Gesellschaft, International Max Planck Research School IMPRS (to P.D.) for financial support.*

Keywords: bioorganic chemistry · double-bond isomerization · oxophytodienoic acid · phytochemistry · plant–insect interactions

[1] G. Arimura, C. Kost, W. Boland, *Biochim. Biophys. Acta Mol. Cell Biol. Lipids* **2005**, *1734*, 91–111.
[2] M. H. Beale, J. L. Ward, *Nat. Prod. Rep.* **1998**, *15*, 533–548.
[3] R. Halitschke, I. T. Baldwin, *J. Plant Growth Regul.* **2004**, *23*, 238–245.
[4] C. Davoine, T. Douki, G. Iacazio, J. L. Montillet, C. Triantaphylidès, *Anal. Chem.* **2005**, *77*, 7366–7372.
[5] S. H. Lee, M. V. S. Elipe, J. S. Arora, I. A. Blair, *Chem. Res. Toxicol.* **2005**, *18*, 566–578.
[6] G. Vancanneyt, C. Sanz, T. Farmaki, M. Paneque, F. Ortego, P. Castañera, J. J. Sánchez-Serrano, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 8139–8144.
[7] G. Pohnert, *ChemBioChem* **2005**, *6*, 946–959.
[8] X. C. Li, M. A. Schuler, M. R. Berenbaum, *Nature* **2002**, *419*, 712–715.
[9] J. C. Schultz, H. M. Appel, *Ecology* **2004**, *85*, 70–77.
[10] B. Schulze, R. Lauchli, M. Mekem Sonwa, A. Schmidt, W. Boland, *Anal. Biochem.* **2006**, *348*, 269–283.
[11] T. Koch, T. Krumm, V. Jung, J. Engelberth, W. Boland, *Plant Physiol.* **1999**, *121*, 153–162.

[12] S. Blechert, W. Brodschelm, S. Hölder, L. Kammerer, T. M. Kutchan, M. J. Mueller, Z. Q. Xia, M. H. Zenk, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 4099–4105.
[13] B. A. Stelmach, A. Müller, E. W. Weiler, *Phytochemistry* **1999**, *51*, 187–192.
[14] A. N. Grechkin, L. S. Mukhtarova, M. Hamberg, *Biochem. J.* **2000**, *352*, 501–509.
[15] M. Yokoyama, S. Yamaguchi, S. Inomata, K. Komatsu, S. Yoshida, T. Iida, Y. Yokokawa, M. Yamaguchi, S. Kaihara, A. Takimoto, *Plant Cell Physiol.* **2000**, *41*, 110–113.
[16] I. Prost, S. Dhondt, G. Rothe, J. Vicente, M. J. Rodriguez, N. Kift, F. Carbonne, G. Griffiths, M. T. Esquerré-Tugayé, S. Rosahl, C. Castresana, M. Hamberg, J. Fournier, *Plant Physiol.* **2005**, *139*, 1902–1913.
[17] G. W. Felton, S. S. Duffey, *J. Chem. Ecol.* **1991**, *17*, 1821–1836.
[18] P. E. Staswick, I. Tiryaki, *Plant Cell* **2004**, *16*, 2117–2127.
[19] F. F. Hsu, S. L. Hazen, D. Giblin, J. Turk, J. W. Heinecke, M. L. Gross, *Int. J. Mass Spectrom.* **1999**, *187*, 795–812.
[20] R. Lauchli, W. Boland, *Tetrahedron* **2003**, *59*, 149–153.
[21] T. Ichikawa, K. Yamanda, M. Namikawa, K. Sakai, K. Kondo, *J. Hattori Bot. Lab.* **1984**, *56*, 209–213.
[22] R. Kramell, O. Miersch, R. Atzorn, B. Parthier, C. Wasternack, *Plant Physiol.* **2000**, *123*, 177–187.
[23] B. A. Vick, D. C. Zimmerman, D. Weisleder, *Lipids* **1979**, *14*, 734–740.
[24] M. J. Mueller, W. Brodschelm, *Anal. Biochem.* **1994**, *218*, 425–435.
[25] D. S. Straus, C. K. Glass, *Med. Res. Rev.* **2001**, *21*, 185–210.
[26] M. J. Mueller, L. Mène-Saffrané, C. Grun, K. Karg, E. E. Farmer, *Plant J.* **2006**, *45*, 472–489.
[27] T. Koch, K. Bandemer, W. Boland, *Helv. Chim. Acta* **1997**, *80*, 838–850.
[28] R. L. Jones, *Biochem. J.* **1970**, *119*, 64P–65P.
[29] J. S. Yang, C. E. Petersen, C. E. Ha, N. V. Bhagavan, *Protein Sci.* **2002**, *11*, 538–545.
[30] K. Büyükgüzel, H. Tunaz, S. M. Putnam, D. Stanley, *Insect Biochem. Mol. Biol.* **2002**, *32*, 435–443.
[31] D. Stanley, *Annu. Rev. Entomol.* **2006**, *51*, 25–44.
[32] R. Bergomaz, M. Boppré, *J. Lepidopterists' Soc.* **1986**, *40*, 131–137.
[33] D. Spiteller, N. J. Oldham, W. Boland, *J. Org. Chem.* **2004**, *69*, 1104–1109.
[34] S. Adolph, S. A. Poulet, G. Pohnert, *Tetrahedron* **2003**, *59*, 3003–3008.
[35] T. Koch, M. Hoskovec, W. Boland, *Tetrahedron* **2002**, *58*, 3271–3274.
[36] J. M. Bobbitt, *J. Org. Chem.* **1998**, *63*, 9367–9374.
[37] D. C. Zimmerman, P. Feng, *Lipids* **1978**, *13*, 313–316.
[38] B. A. W. Gallasch, G. Spiteller, *Lipids* **2000**, *35*, 953–960.

Received: September 14, 2006
Published online on December 29, 2006

8. Article IV

iso-OPDA: An Early precursor of _cis_-Jasmone in plants?

Paulina Dąbrowska, Wilhelm Boland

ChemBioChem (2007) **8**, 2281-2285

FULL PAPERS

DOI: 10.1002/cbic.200700464

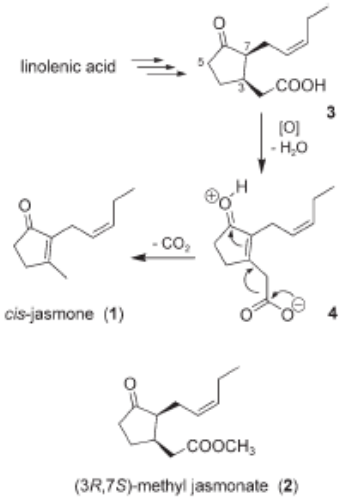
iso-OPDA: An Early Precursor of *cis*-Jasmone in Plants?

Paulina Dąbrowska and Wilhelm Boland*[a]

cis-Jasmone is a highly appreciated fragrance and plant-derived signal molecule that controls pollination, attracts parasitoids of attacking herbivores, and serves as an intra- and interspecific signal that controls gene expression. cis-Jasmone is produced from linolenic acid along the jasmonic acid cascade. In addition to the conversion of jasmonic acid into cis-jasmone, a novel pathway might exist that converts cis-oxophytodienoic acid (OPDA), an early precursor of jasmonic acid, into iso-OPDA. The planar iso-OPDA is degraded by β-oxidation to 3,7-didehydrojasmonic acid, which yields cis-jasmone by spontaneous decarboxylation. The degradation of iso-OPDA to cis-jasmone is demonstrated for many plant species and the yeast Saccharomyces cerevisiae.

Introduction

Since antiquity the jasmonoids have belonged to the most desirable scents for perfumes and cosmetics. Even Plinius the elder mentioned the use of jasmine as an ingredient for perfumes.[1] The essential oil of jasmine comprises more than a hundred components, but the most important contributions come from *cis*-jasmone **1** and the *cis* isomer of methyl jasmonate **2**, Scheme 1.[2] *cis*-Jasmone was originally discovered and characterised as a minor (3%) constituent of jasmine absolute.[3] The same compound is also present in the absolutes of neroli (*Citrus bigaradia*), jonquil (*Narcissus jonquilla* L.), bergamot (*Citrus bergamia*), and the *Pittosporum* family.[3,4] Moreover, *cis*-jasmone belongs to the odour profile of peppermint oil (*Mentha piperita*), tea (*Camila sinensis*), orange (*Citrus sinensis*) and, of course, jasminum leaves.[5,6] The same compound is found in the headspace of the floral scents of *Actinidia*, *Lonicera*, *Magnolia*, *Nelumbo*, *Nicotiana*, *Olea*, and *Philadelpus* spp.[7] In the insect kingdom, *cis*-jasmone functions as a pheromone of the olive fly[8] and is present in the aphrodisiac cocktail of the Danaid butterflies *Amauris ochlea*, *A. albimaculata*, and *A. damocles*.[9]



Scheme 1. Biosynthesis of *cis*-jasmone from jasmonic acid and structure of (3*R*,7*S*)-methyl jasmonate.

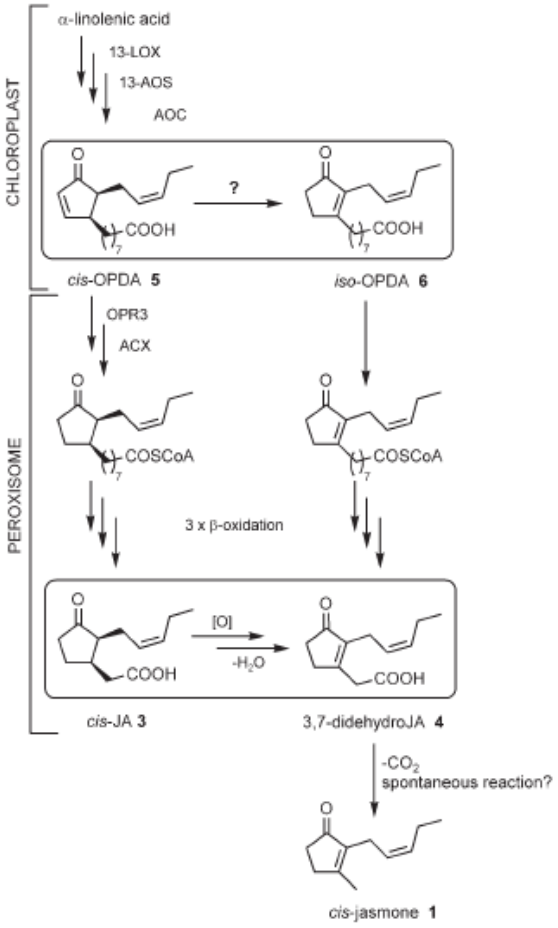
In recent years, *cis*-jasmone has gained additional attention because the compound is released in small amounts from herbivore-damaged leaves.[10,11] The compound is repellent to aphids and it attracts the pest's parasitoids.[12,13] Moreover, *cis*-jasmone induces the selective production of secondary metabolites that are capable of reducing the pest's development.[14] External application of the compound to leaves of *Arabidopsis thaliana* specifically up-regulated a set of characteristic genes that were not correlated with typical jasmonic-acid-induced responses, in particular the oxophytodienoic reductase gene—OPR1; this attributes a unique and characteristic function of the plant defence complex to *cis*-jasmone.[14]

While the compound itself is long known, only a single experimental approach to the biosynthetic pathway that leads from linolenic acid via the jasmonic acid pathway to *cis*-jasmone has been reported.[5]

The initial proposal of Demole[15] takes into account the close structural resemblance of *cis*-jasmone and methyl jasmonate and postulates an acid-induced decarboxylation of an unsaturated derivative of jasmonic acid. Administration of a deuterium-labelled jasmonic acid **3** to various plants[5] confirmed that jasmonic acid can indeed act as a precursor for the volatile semiochemical *cis*-jasmone (Scheme 1). It has been sug-

[a] Dipl.-Chem. P. Dąbrowska, Prof. Dr. W. Boland
Max Planck Institute for Chemical Ecology
Hans-Knöll-Strasse 8, 07745 Jena (Germany)
Fax: (+49) 3641-571202
E-mail: Boland@ice.mpg.de

gested, that the transformation of jasmonic acid **3** to the planar unsaturated 3,7-didehydrojasmonic acid **4**, followed by decarboxylation to *cis*-jasmone might represent a mode of inactivation and disposal of the phytohormone to the gas phase.[5,16] Unlike the phytohormone **3**, the unsaturated intermediate **4** proved to be biologically inactive. By addressing the fate of plant-derived oxylipins in the gut of herbivorous insects we recently discovered an enzyme-catalysed double bond isomerisation of *cis*-OPDA **5**, which is an early precursor of jasmonic acid, to the planar *iso*-OPDA **6** (Scheme 2), which resembles the structure of the biologically inactive 3,7-didehydrojasmonic acid **4** (Scheme 1).[17] To test for analogous inactivating effects of the planarisation on the biological activity of *cis*-OPDA **5**,[18] we applied *iso*-OPDA **6** to selected plants and observed an unexpected, but strong emission of *cis*-jasmone **1**. Because *iso*-OPDA **6**, or tetrahydrodicranenone B, was previously reported as a constituent of a Japanese moss (*Dicranum majus*),[19] and because **6** accumulated in sorbitol-stressed barley leaves,[20] we investigated whether *iso*-OPDA **6** could serve as a widespread and early precursor for an alternative biosynthetic pathway that leads to *cis*-jasmone **1**. Here we report the first data that demonstrates that many plants and even yeast are able to use *iso*-OPDA **6** for the production of the volatile fragrant *cis*-jasmone **1**, as was postulated by us previously.[21]



Scheme 2. Proposed biosynthetic pathways that lead to *cis*-jasmone. Abbreviations: LOX, lipoxygenase; AOS, allene oxide synthase; AOC, allene oxide cyclase; OPR, OPDA reductase; ACX, acyl-CoA oxidase; OPDA, 12-oxophytodienoic acid; *iso*-OPDA, tetrahydrodicranenone B; JA, jasmonic acid.

Results and Discussion

Administration experiments

Sorbitol stress in barley (*Hordeum vulgare* cv. Salome) has been correlated with temporarily changing levels of octadecanoids and jasmonates. GC-MS analyses revealed a sequential increase of free α-linolenic acid, *cis*-OPDA, and jasmonic acid along with their methyl esters (see Scheme 2). Individual oxylipins could be correlated with a characteristic pattern of gene expression.[20] *iso*-OPDA **6** accumulated as a minor product, but no biological activity was found to be associated with this compound.

To test whether or not the planar *iso*-OPDA is really an inactive compound, we administered a solution of **6** in DMSO/water (see the Experimental Section) to freshly cut plantlets of the lima bean (*Phaseolus lunatus*) and assayed **6** for its ability to induce volatile biosynthesis, as has been shown previously for jasmonic acid **3** and *cis*-OPDA **5**.[18]

Consistent with the inactivity of 3,7-didehydrojasmonic acid (**4**), also after application of the methyl ester of **6** (=*iso*-OPDAMe **7**) no induction of volatile biosynthesis was observed with the remarkable exception of *cis*-jasmone formation (Figure 1). Control experiments without added *iso*-OPDAMe showed no production of *cis*-jasmone **1**. To confirm this result, the experiments were repeated with [$^2H_2$]-*iso*-OPDAMe **7**, which consequently led to the emission of [$^2H_2$]-*cis*-jasmone (Figure 1). Accordingly, the methyl ester **7** is cleaved in the plant tissue and is subsequently processed by β-oxidation to the level of the 3,7-didehydrojasmonic acid **4**; this is followed by decarboxylation.

In order to assess whether this transformation is widespread in plants, we investigated selected plant species for their ability to convert *iso*-OPDAMe **7** to *cis*-jasmone **1**. Results clearly show that the process occurs throughout many, distinctively different plant families (Table 1). Interestingly, even leaves of *Arabidopsis thaliana*, which are known to emit very low amounts of only a few volatiles produced substantial amounts of [$^2H_2$]-*cis*-jasmone **1** after treatment with labelled *iso*-OPDAMe. Also, the leaves of Gingko biloba, which failed to convert jasmonic acid **3** to *cis*-jasmone[5] transformed *iso*-OPDAMe to *cis*-jasmone **1**.

The prevalence of the conversion process suggests that the mechanism that is responsible for the degradation and conversion of the C18-oxylipin, *iso*-OPDA **6** to the C11-fragrance *cis*-jasmone **1** has to be operative in very different plant species. By analogy to the biosynthesis of jasmonic acid **3** from *cis*-OPDA **5** it is reasonable to assume that also the isomeric *iso*-

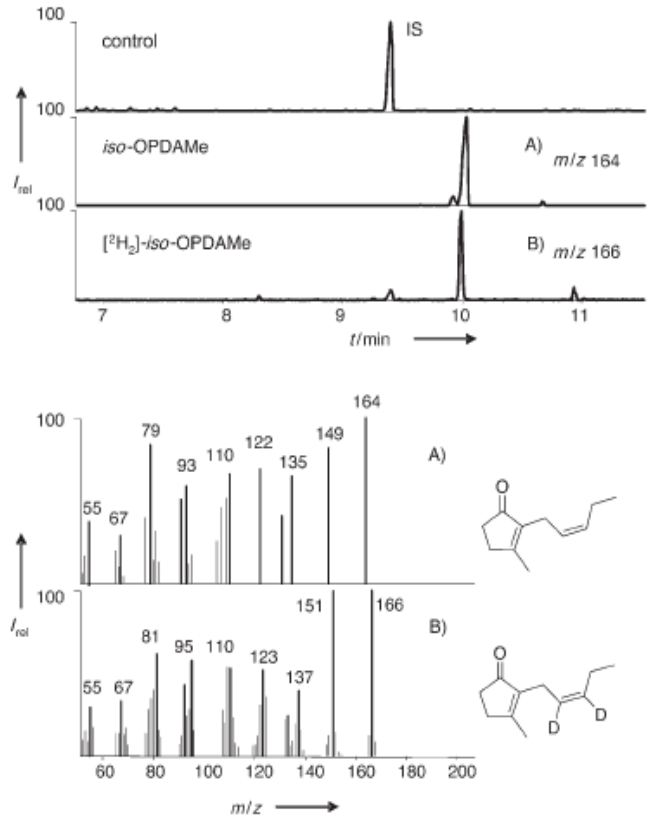


Figure 1. Emission of *cis*-jasmone **1** from *iso*-OPDAMe-treated lima bean leaves. Freshly cut plantlets of lima bean leaves in water (control) and in aqueous solutions (2.5 mL, 1.7 mM) solutions of: A) *iso*-OPDAMe; B) [$^2H_2$]-*iso*-OPDAMe. Mass spectra of metabolites: A) *cis*-jasmone; B) [$^2H_2$]-*cis*-jasmone.

Table 1. Conversion of *iso*-OPDA to *cis*-jasmone in selected plant species.

Plant species	Plant family	Formation of cis-jasmone 1
Phaseolus lunatus	Fabaceae	+
Arabidopsis thaliana[a]	Brassicaceae	+
Gingko biloba	Ginkgoaceae	+
Nicotiana attenuata	Solanaceae	+
Camelia sinensis[b]	Theaceae	+
Jasminum officinalis[c]	Oleaceae	+
Jasminum officinalis[d]	Oleaceae	+

[a] Ecotype: Columbia. [b] Tested cultivar: Indian Yakitiba. [c] Leaves. [d] Flowers.

OPDA **6** will be channelled into the peroxisomal β-oxidation (Scheme 2) after export from the chloroplast.

We assume that due to the high structural similarities between *cis*-OPDA **5** and *iso*-OPDA **6**, the latter is processed analogously to the jasmonic acid pathway to yield 3,7-didehydrojasmonic acid **4** (Scheme 2). The final step of the transformation, namely the conversion of **4** to *cis*-jasmone **1** might even proceed spontaneously because protonation of **4** is sufficient to induce the terminal decarboxylation that leads to **1**.[5]

Peroxisomes are the main sites of fatty acid degradation in plants and these organelles are involved in the β-oxidation of *cis*-OPDA.[22–24] Degradation of saturated fatty acids by the β-oxidation pathway requires the presence of four enzymes. The first enzyme is an acyl-CoA oxidase (ACX), which converts acyl-CoAs to *trans*-2-enoyl-CoAs. The 2-enoyl-CoA hydratase converts the *trans*-2-enoyl-CoA to the *S* isomer of 3-hydroxyacyl-CoA, which is subsequently oxidised to 3-ketoacyl-CoA by a dehydrogenase. The last enzyme, the 3-ketothiolase, cleaves 3-ketoacyl-CoA and generates acetyl-CoA along with the C2-shortened acyl-CoA (Scheme 2). In *A. thaliana*, two 4-coumarate ligase-type proteins, namely At4g05160 and At5g63380 selectively contribute to the jasmonic acid biosynthesis by converting *cis*-OPDA **5** into the corresponding CoA-ester that is required for the first desaturation step.[25,26]

It is reasonable to assume that other plants possess a similar system of a selective degradation of *cis*-OPDA because the jasmonate pathway is a central signalling pathway in plants. Accordingly, all the selected test plants from different families were able to convert *iso*-OPDA into *cis*-jasmone (Table 1). On the other hand, oxylipin analysis[17] of the test plants and mechanically wounded test plants gave no evidence for the accumulation of *iso*-OPDA **6**. Also, the flowers of *Jasminum officinalis*, which constitutively emit *cis*-jasmone **1**, did not contain *iso*-OPDA. We therefore assume that **6** is rapidly channelled into β-oxidation and does not accumulate in the plants.

Yeasts, for example *Saccharomyces cerevisiae*, also possess a peroxisomal β-oxidation pathway, but lack the plant-typical jasmonate pathway.[27] To test whether or not a specific enzymatic system is required for the transformation of *iso*-OPDA **6** to *cis*-jasmone **1** we added **6** to a growing culture of *S. cerevisiae* (see the Experimental Section). Analysis of the gas phase clearly demonstrated that even the yeast could efficiently convert *iso*-OPDA into *cis*-jasmone **1**, but a corresponding experiment with a growing *E. coli* culture failed to provide *cis*-jasmone (see the Experimental Section).

According to Scheme 2 two independent pathways might contribute to the production of *cis*-jasmone **1** in plants. Both originate from α-linolenic acid, which is converted in the chloroplast by a well-established sequence into *cis*-OPDA.[28] After export to the cytosol the double bond of the cyclopentenone is reduced, and after transfer into the peroxisome, three consecutive β-oxidations yield jasmonic acid **3**. The introduction of a central double bond and subsequent decarboxylation[5,15] represent a late pathway to the plant semiochemical *cis*-jasmone **1**. In line with our current findings, an early isomerisation of *cis*-OPDA **5** to *iso*-OPDA **6** might provide an alternative route to *cis*-jasmone **1** that is apparently operative in many plants and even in yeast. This novel pathway requires only a hitherto unknown isomerase and a functional β-oxidation pathway that tolerates the planar stereochemistry of the cyclo-

CHEMBIOCHEM W. Boland and P. Dąbrowska

pentenoid moiety. Because a yeast that does not possess a genuine production of *cis*-jasmone **1** is also able to convert *iso*-OPDA **6** into **1**, we postulate that the final decarboxylation proceeds, indeed, as a spontaneous process that does not require support by a specific enzyme.[5]

Conclusions

cis-Jasmone **1** is an important signal molecule that is released from flowers to attract pollinators, and it is emitted from herbivore-damaged vegetative tissue to attract the parasitoids of the herbivore.[12] *cis*-Jasmone **1** also functions as an intra- and interplant signal that controls gene expression.[14, 29] According to Scheme 2 the biosynthesis of **1** starts with the activation of linolenic acid, either by an endogenous program that controls the emission of the flower volatile, or after induction, by herbivory. Here we provide the first evidence that two different pathways might exist in plants that lead to *cis*-jasmone **1**. Plants that possess an enzyme that converts *cis*-OPDA **5** into the planar *iso*-OPDA **6** are principally able to channel **6** into the peroxisomal β-oxidation to generate 3,7-didehydrojasmonic acid **4**; this provides *cis*-jasmone **1** after decarboxylation. Because this process also occurs in the yeast *S. cerevisiae*, which has no operative pathway for this fragrance, the decarboxylation most likely occurs as a spontaneous reaction.[5] Another pathway leads from the phytohormone jasmonic acid **3** to the central intermediate 3,7-didehydrojasmonic acid **4**. The essential enzymes, namely the isomerase for the production of *iso*-OPDA **6** from **5** and those that are required for the introduction of a double bond into jasmonic acid **3** are not yet known. However, both pathways appear to be tightly regulated because the emission of flower volatiles and of herbivore-induced volatiles follow a typical circadian rhythm.[30]

The pathway that involves the isomerisation of *cis*-OPDA **5** to *iso*-OPDA **6** is of particular interest, because this route does not require specific enzymes for the conversion of **6** to *cis*-jasmone **1**. Therefore, overexpression of the isomerase in appropriate plant systems could provide an interesting biotechnological route to the industrially important fragrant *cis*-jasmone **1**. The same strategy could also increase the resistance of plants because *cis*-jasmone **1** is able to reduce the fecundity of attacking herbivores and to attract their natural parasitoids.[12–14]

Experimental Section

Synthesis of iso-OPDAMe and [$^2H_2$]-*iso*-OPDAMe: 3-Iodo-2-pent-2-ynyl-cyclopent-2-enone was obtained by following the method of Lauchli et al.[21,31] from commercially available 1,3-cyclopentanedione. Hydrogenation and deuteration of methyl 8-(3-oxo-2-pent-2-ynyl-cyclopent-1-enyl)-octanoate were performed as described previously.[21]

Synthesis of methyl 8-(3-oxo-2-pent-2-ynyl-cyclopent-1-enyl)-octanoate: A suspension of zinc powder (0.298 mg, 4.5 mmol) and iodine (41 mg, 0.2 mmol) in dry *N*,*N*-dimethylacetamide (3 mL) was placed in a predried Schlenk flask (20 mL) and stirred. After 10 min, 8-iodooctanoate (0.66 g, 2.4 mmol) was added. The suspension was stirred at 70 °C, and samples were periodically removed, treated with NH_4Cl and analysed by GC-MS. When the complete conversion to 8-iodooctanoate (~4 h) was apparent, the Zn dust was allowed to settle, and the supernatant was transferred by syringe to another Schlenk flask (20 mL) that contained $Pd[PPh_3]_4$ (~2 mg) and 3-iodo-2-pent-2-ynylcyclopent-2-enone (130 mg, 0.47 mmol) in dry THF (2 mL). The reaction mixture was stirred at room temperature overnight, hydrolysed with sat. NH_4Cl, and extracted with diethyl ether (3×10 mL). The combined organic layers were dried (Na_2SO_4) and concentrated in vacuo. The oily residue was purified by flash chromatography on silica gel by using petrol ether/ethyl acetate (3:1, *v*/*v*) for elution. Yield: 78 mg (54 %) of yellow oil. Spectroscopic data were in agreement with literature.[21]

Induction experiments and volatile collection: *Iso*-OPDAMe **7** and [$^2H_2$]-*iso*-OPDAMe [$^2H_2$]-**7** were dispersed in tap water (at 1.7 μmol mL^{-1}) by sonication for ca. 3 min. Alternatively, a stock solution in DMSO (25 mg of *iso*-OPDAMe **7** or [$^2H_2$]-*iso*-OPDAMe [$^2H_2$]-**7** in 96 μL DMSO) was dispersed in tap water (final concentration 1.7 μmol mL^{-1}) and sonicated. Both procedures and compounds resulted in stable emulsions. Emulsions without DMSO were stable for 2 days, which was sufficient to complete the experiments; emulsions with DMSO proved to be significantly more stable. In all experiments with DMSO-derived emulsions, control experiments were carried out with tap water that contained equivalent amounts of DMSO but no test compound. Freshly detached leaves, small plantlets (lima bean) or flowers (*Jasminum officinalis*, *Narcissus jonquilla*) were immediately placed into the emulsions (2.5 mL in small vials, closed with aluminum foil caps) and placed in dessicators (250 mL) for volatile collection.[32] For microbial transformations: *iso*-OPDAMe was added to a liquid culture of *E. coli* (*E. coli* K12) in standard LB medium (2.5 mL, 1.7 μmol mL^{-1}). The yeast (INVSc1 Strain, Invitrogen) in liquid YPD medium (Y 1375, Sigma–Aldrich) was treated with *iso*-OPDAMe (2.0 mL, 1.7 μmol/mL) with slow stirring. Control experiments were performed with a heat-inactivated yeast culture (100 °C, 15 min) and failed to produce *cis*-jasmone. After addition of *iso*-OPDA to the growing microbial cultures, the produced volatiles were collected for ca. 28 h by absorption on charcoal filters (1.5 mg, CLSA Filter, Gränicher & Quartero, Daumazan sur Arize, France).[32] After desorption with dichloromethane (2×20 μL), that contained 1-bromodecane (200 ng mL^{-1}) as an internal standard, the samples (1 μL) were analysed on a Finnigan Trace Instrument in split mode (1:10), equipped with EC-5 column (15 m×0.25 mm×0.25 μm, Alltech, Unterhaching, Germany). Volatiles were eluted under programmed conditions from 45 °C (2 min) followed by 10 °C min^{-1} to 200 °C and 30 °C min^{-1} to 280 °C. Helium at a flow rate of 1.5 mL min^{-1} served as a carrier gas. The GC injector, transfer line, and ion source were set at 220 °C, 280 °C, and 280 °C, respectively. Spectra were taken in the total-ion-scanning (TIC) mode at 70 eV.

Oxylipin analysis: Control leaves were shock frozen with liquid nitrogen. Sample leaves were artificially wounded with a razor blade, kept in water for 90 min (optimal time for oxylipin wound-induced accumulation) and then shock frozen. Flower material was analysed without pre-treatment. Extraction and derivatisation of oxylipins was achieved according to the protocol of Schulze et al.[33] In brief, sample tissue (0.25 g of plant or flower) was frozen in liquid nitrogen. The crushed sample (0.25 g) was covered with 0.05 M pentafluorobenzylhydroxylamine hydrochloride (PFBHA) in MeOH (2 mL) and MeOH (2.5 mL). 9,10-[$^2H_2$]-Dihydrojasmonic acid (250 ng) was added as an internal standard for the analysis. Samples were then homogenised with a high performance dispenser at 24000 rpm (Ultra-Turrax T-25, IKA Werk, Staufen, Germany) fol-

lowed by shaking for 2 h at RT. After transfer to centrifuge glass tubes the sample was acidified with 0.1 M HCl (pH ~3). The MeOH/water phase was carefully extracted with hexane (3 × 5 mL). The combined hexane layers were passed through preconditioned aminopropyl cartridges (0.5 g, Macherey–Nagel). After washing (isopropanol/dichloromethane 2:1, *v/v*) the oxylipins were eluted (diethyl ether/formic acid 98:2, *v/v*) and derivatised with an ethereal solution of diazomethane after removal of solvents. Samples were analysed on a Finnigan GCQ Instrument by running in CI-negative ion mode (NCI).[33]

Acknowledgements

We thank Doreen Schachtschabel for helpful discussions, Anja David for help with volatile collection, Andreas Habel for supplying us with yeast culture and Dr. Dieter Spiteller for E. coli *culture. P.D. is thankful for a PhD stipend from the Max Planck Gesellschaft, International Max Planck Research School IMPRS.*

Keywords: bioorganic chemistry · biosynthesis · *cis*-jasmone · oxidation · oxylipins

[1] G. Ohloff in *Irdische Düfte, Himmlische Lust: Eine Kulturgeschichte der Duftstoffe*, Birkhaeuser, Basel, **1992**, p. 107.
[2] T. E. Acree, R. Nishida, H. Fukami, *J. Agric. Food Chem.* **1985**, *33*, 425–427; H. Kiyota, S. Takigawa, S. Kuwahara, *Helv. Chim. Acta* **2004**, *87*, 1854–1859.
[3] L. Ruzicka, M. Pfeiffer, *Helv. Chim. Acta* **1933**, *16*, 1208–1214.
[4] D. Joulain, *Parfums Cosmet. Aromes* **1975**, *4*, 33–45.
[5] T. Koch, K. Bandemer, W. Boland, *Helv. Chim. Acta* **1997**, *80*, 838–850.
[6] *Dictionary of Natural Products, Vol. 4*, Chapman & Hall, London, **1994**, p. 3964.
[7] J. T. Knudsen, L. Tollsten, L. G. Bergström, *Phytochemistry* **1993**, *33*, 253–280.
[8] W. L. Roelofs, T. E. Acree, R. Nishida, T. C. Baker, *Chem. Eng. News* **1983**, *61*, 34.
[9] S. Schulz, M. Boppre, R. I. Vanewright, *Philos. Trans. R. Soc. London Ser. B* **1993**, *342*, 161–181.
[10] J. H. Loughrin, A. Manukian, R. R. Heath, J. H. Tumlinson, *J. Chem. Ecol.* **1995**, *21*, 1217–1227.
[11] C. Kost, M. Heil, *J. Ecol.* **2006**, *94*, 619–628.
[12] M. A. Birkett, C. A. M. Campbell, K. Chamberlain, E. Guerrieri, A. J. Hick, J. L. Martin, M. Matthes, J. A. Napier, J. Pettersson, J. A. Pickett, G. M. Poppy, E. M. Pow, B. J. Pye, L. E. Smart, G. H. Wadhams, L. J. Wadhams, C. M. Woodcock, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 9329–9334.
[13] T. J. A. Bruce, J. L. Martin, J. A. Pickett, B. J. Pye, L. E. Smart, L. J. Wadhams, *Pest Manage. Sci.* **2003**, *59*, 1031–1036.
[14] J. A. Pickett, M. A. Birkett, M. C. B. Moraes, T. J. A. Bruce, K. Chamberlain, R. Gordon-Weeks, M. C. Matthes, J. A. Napier, L. E. Smart, L. J. Wadhams, C. M. Woodcock, *Allelopathy J.* **2007**, *19*, 109–117.
[15] E. P. Demole in *Fragrance Chemistry*, Academic Press, New York, **1982**, p. 349.
[16] C. C. von Dahl, I. T. Baldwin, *Physiol. Plant.* **2004**, *120*, 474–481.
[17] B. Schulze, P. Dabrowska, W. Boland, *ChemBioChem* **2007**, *8*, 208–216.
[18] T. Koch, T. Krumm, V. Jung, J. Engelberth, W. Boland, *Plant Physiol.* **1999**, *121*, 153–162.
[19] T. Ichikawa, M. Namikawa, K. Yamada, K. Sakai, K. Kondo, *Tetrahedron Lett.* **1983**, *24*, 3337–3340.
[20] R. Kramell, O. Miersch, R. Atzorn, B. Parthier, C. Wasternack, *Plant Physiol.* **2000**, *123*, 177–187.
[21] R. Lauchli, W. Boland, *Tetrahedron* **2003**, *59*, 149–153.
[22] B. Gerhardt, *Prog. Lipid Res.* **1992**, *31*, 417–446.
[23] H. Kindl, *Biochimie* **1993**, *75*, 225–230.
[24] S. Reumann, C. L. Ma, S. Lemke, L. Babujee, *Plant Physiol.* **2004**, *136*, 2587–2608.
[25] C. Delker, B. K. Zolman, O. Miersch, C. Wasternack, *Phytochemistry* **2007**, *68*, 1642–1650.
[26] K. Schneider, L. Kienow, E. Schmelzer, T. Colby, M. Bartsch, O. Miersch, C. Wasternack, E. Kombrink, H. P. Stuible, *J. Biol. Chem.* **2005**, *280*, 13962–13972.
[27] I. J. van der Klei, M. Veenhuis, *Trends Microbiol.* **1997**, *5*, 502–509.
[28] F. Schaller, A. Schaller, A. Stintzi, *J. Plant Growth Regul.* **2004**, *23*, 179–199.
[29] M. Heil, J. C. Silva Bueno, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 5467–5472.
[30] R. Kaiser in *Perfumes: Art, Science and Technology* (Eds.: P. M. Müller, D. Lamparsky), Elsevier, London, **1991**, pp. 213–250.
[31] A. Schmidt, W. Boland, *J. Org. Chem.* **2007**, *72*, 1699–1706.
[32] J. Donath, W. Boland, *Phytochemistry* **1995**, *39*, 785–790.
[33] B. Schulze, R. Lauchli, M. M. Sonwa, A. Schmidt, W. Boland, *Anal. Biochem.* **2006**, *348*, 269–283.

Received: August 8, 2007
Published online on November 21, 2007

9. General Discussion

The present thesis contributes a set of new and important findings to the field of plant oxylipins and to the role of these compounds in plant-insect interactions. Our results provide evidence for several different hypotheses: 1) JA and OPDA are universal stress signals common among very different plant species (Unpublished results Part I, Article I, Article II), 2) continuous mechanical wounding is an important component of the JA-mediated wound response (Unpublished results Part I, Article I), 3) the specialist insect *Plutella xylostella* seems to be able to influence/suppress the plant's oxylipin-signaling network (Unpublished results Part I), 4) generalist insects may perceive plant-signaling compounds (Article III, Unpublished results Part II), 5) an important volatile signal, *cis*-jasmone, can be synthesized in plants with help of a plant analogue of insect OPDA isomerase (Article IV).

9.1. Jasmonates – universal stress signals in plant kingdom

The versatility of jasmonates, which are stress signals in the plant kingdom, has been repeatedly confirmed.[63, 218] As this thesis clearly shows, JA is involved in a signaling network of very different species throughout the plant kingdom (angiosperms: *A. thaliana* (*Brassicaceae*), *P. lunatus* (*Fabaceae*), gymnosperms: *P. abies* (*Pinaceae*)). The JA-mediated signaling is elicited not only by herbivore damage but also by continuous wounding (Unpublished results Part I, Article I) and fungal elicitors such as chitosan (Article II). Along with JA accumulation, elevated levels of an immediate precursor of JA - OPDA were found in differently stressed plants of various species. The case of Norway spruce is the first report of OPDA's presence in gymnosperms (Article II).

JA is also, as mentioned (see 1.1.1), responsible for up-regulating volatiles, in particular, terpenoid biosynthesis.[26] Since volatile emission is supposed to be periodic [24, 219] we investigated correlations between the emission of separate volatile compounds and JA levels in lima bean leaves after damage was inflicted during the day and at night (Article I). Using MecWorm, we were able to apply completely reproducible damage in both periods, a feature that would have been impossible using feeding herbivores. Transcripts of the *Pl*OS gene, which is involved in biosynthesis of an important volatile compound, β-ocimene, closely followed the levels of JA, indicating JA is directly involved in its biosynthesis. Interestingly,

however, the resulting emission of β-ocimene required the presence of light, so that the nocturnal damage was characterized by only low emission rates of this compound. Furthermore, quite unexpectedly, analyzed levels of JA were significantly higher after nocturnal damage than they were after daytime damage. These results suggest that certain JA-modifying/metabolizing steps occur only during the light phase. Comprehensive profiling of different JA derivatives and/or other products derived from α-linolenic acid are needed to further clarify this interesting observation (Article I).

Establishing the presence and levels of JA and OPDA in all mentioned experiments was possible thanks to the reliable analytical method [211], which proved to be robust for different plant tissues and even cell cultures (Unpublished results Part I, Article I, Article II, Article III, Unpublished results Part II). This analytical method was expanded to help quantify another important plant hormone, SA (Unpublished results Part I).

9.2. Role of continuous mechanical wounding in elicitating plants' defense responses

Contrary to many previous studies, which inadequately mimicked the herbivore wounding by single or sporadic squeezing damaging events, cutting off leaf material, or punching holes into leaves, experiments presented in Unpublished results Part I and Article I were conducted with the mechanical caterpillar, MecWorm, which produces reproducible, continuous mechanical damage. This device has already been proven to elicit effects matching in terms of volatile emission the ones induced by a feeding herbivore [214] and thus undermined a general belief that plants' recognition of feeding insects is based predominantly on chemical cues, such as FACs.

Analysis of differential gene expression in *A. thaliana* after wounding inflicted by MecWorm (Unpublished results Part I) showed clearly, in contrast to results published earlier [212], that genes related to jasmonate signaling (such as: LOX2, CORl3) were strongly upregulated. This observation agrees with previous studies using MecWorm [214] and proves that continuous mechanical damage can by itself elicit the JA-mediated defense network.

Simultaneous analysis of JA and OPDA (Unpublished results Part I) confirmed the results of the transcriptional profiling, showing a strong accumulation in levels of both signals over the course of wounding. Interestingly, thanks to the reproducibility of the MecWorm treatment, another important observation was possible: there was a linear correlation between the amounts of JA and OPDA and the extent of mechanical damage. This finding strongly implies that the signal for the biosynthesis of jasmonic acid is correlated with the disruption of separate, single cells.

9.3. The specialist insect *Plutella xylostella* can influence JA signaling in *Arabidopsis thaliana*

During co-evolution insects learned to deal with plant defense responses not only by fighting off the secondary metabolites but also by manipulating the plant itself (see 1.2). Since jasmonates play a primary role in inducing plants' defensive arsenal (see 1.1.1), the ability to manipulate these signals could be highly advantageous for the herbivore. Results of the studies presented in Unpublished results Part I imply that a specialist herbivore, the diamondback moth (*P. xylostella*), is able to significantly suppress the biosynthesis of JA. This notion has been previously proposed in a study by Vogel *et al*. (2007).[220] Amounts of JA and OPDA over the course of 9 hours of feeding did not rise drastically, as in the case of continuous mechanical wounding or feeding of the generalist herbivore – *Spodoptera littoralis*.[221] Furthermore, the percentage of *cis*-JA, which is the *de novo* biosynthesized epimer of JA, stayed at a relatively low level in the case of *P. xylostella;* the corresponding values for MecWorm wounding were 2 to 3 times higher. The observed suppression of JA biosynthesis cannot have resulted either from elevated amounts of SA, the reported antagonist of JA pathway [157, 222], or from the significantly lower levels of damage inflicted by the caterpillars (Unpublished results Part I). It is also puzzling that, according to gene expression analysis, the jasmonate-related genes of the feeding diamondback moth were upregulated (Unpublished results Part I and Ehlting *et al.* 2008 [223]), similar to continuous mechanical wounding, which resulted in elevated amounts of JA.

The most obvious explanation for these discrepancies and for the fact that transcript levels of 30% of all analyzed genes between MecWorm wounding and *P. xylostella* feeding differed significantly (Unpublished results Part I) seems to be an insect-derived chemical cue. Other explanations should not be ignored, however,

especially since the impact of the putative chemical would have to occur at questionably high levels. The amount of salivary secretion produced by *P. xylostella* larvae does not exceed a few picoliters. Even considering that the putative chemical signal accumulates in the course of feeding, extremely high levels would be needed to suppress the biosynthesis of JA on an enzyme level. This is why in order to fully understand the intricacies of the interaction between the specialist herbivore *P. xylostella* and its host plant, *A. thaliana,* more studies are essential, including careful analysis of the insect's salivary secretions and of the differences in gene expression between herbivore feeding and MecWorm wounding using *P. xylostella* regurgitate.

With regard to new findings concerning how JA acts (see 1.1.1), particularly the findings that stress the importance of JA-Ile in the signal transduction, it is worth noting that the analyses presented in this thesis (Unpublished results Part I) did not quantify the JA-amino acid conjugates. It is thus also possible that these undefined elements play an unknown role in the process.

9.4. Generalist insects recognize plant-signaling molecules

Results presented in Article III and Unpublished results Part II support the hypothesis proposed initially by Li *et al*.[204] Using the example of a generalist herbivore corn earworm (*H. zea),* authors of this report have shown an upregulation of insect genes encoding for a group of general detoxification enzymes - namely P450s - in response to feeding of two major plant hormones, JA and SA. This observation lead to the hypothesis that especially polyphagous insects, encountering very diverse plant defense compounds, may have evolved an ability to "eavesdrop" on plants' signaling molecules, which allowed them to gain time to prepare their detoxification mechanisms (see 1.2.1). Levels of 12-oxophytodienoic acid in the insect gut not only correlates very well with the proposed hypothesis, but provides even more convincing evidences supporting it. Mere upregulation of general detoxification enzymes could be a universal strategy used by herbivores (authors of the mentioned report did not look for a possible change in expression of any other general detoxyfication genes). In contrast findings presented in this thesis show that generalist insects dispose of a specific enzyme designed to precisely isomerize the immediate precursor of the major wound-response-mediating phytohormone (Unpublished results Part II).

On the other hand, correlating OPDA isomerization with the activity of Glutathione *S*-transferases complies with several reports from both plant and prostaglandin studies. Treating *A*. *thaliana* with 12-oxophytodienoic acid or structurally related phytoprostanes resulted in the upregulation of GST-related genes [69], and an accumulation of OPDA-GSH conjugates has been reported in cryptogein-elicited tobacco [84, 85] and pathogen-infected Arabidopsis plants.[86] A structurally related isomerization of the double bond has been observed for prostaglandin A_1 and A_2.[224, 225] Isomerization activity has been observed in the plasma of several mammalian species, but the responsible enzyme, PGA_1-Δ-isomerase (EC 5.3.3.9), was never isolated and fully characterized.[226-228] It has, however, been proposed that the mechanism of the putative enzyme resembles the mode of action of ketosteroid isomerases [227, 229], which are associated with mammalian GSTs.[230, 231]

While the advantages for herbivores of OPDA isomerization - namely, the early recognition of defense responses in plants - are relatively obvious, the reason for and significance of this extremely specific transformation remain unknown. One explanation according to Li *et al*.[204] is that insects in the course of co-evolution have "learned" to recognize OPDA. Evidence supporting this notion is the fact that the ability to isomerize OPDA is associated with generalist, polyphagous insects only, not with specialists (Unpublished results Part II), which means that the responsible enzyme appeared as a separate trait in a certain moment of evolution. This reasoning does not, however, clarify the mechanistic basis and the need of the transformation.

Another explanation could be associated with the reactive α,β–unsaturated double bond system of OPDA, which can be correlated with its putative toxicity. It has been shown for jasmonates that flattening the cyclopentenone ring - this happens when the double bond is introduced between the substituents - leads to a decrease in activity.[80, 232] In the case of OPDA, such a change transforms the molecule to more substituted and therefore less reactive Michael acceptor. This hypothesis implies the existence of receptors to which OPDA can bind and cause "toxic" effects.

9.4.1. OPDA interference with putative prostaglandin receptors in insects

In the search for putative OPDA receptors in insects, one has to bring up the closest structural "cousins" of jasmonates in animal kingdom, the prostaglandins. With regard to structural and functional similarity, the following question may be

asked: Is OPDA isomerization in insects a way of avoiding interference between plant-derived octadecanoic signals and animal-related eicosanoid signals?

The presence of eicosanoids in insects has been proven.[233, 234] Eicosanoids have been repeatedly proposed to play a role in important physiological processes, for example, reproduction [233, 235, 236] and immune responses.[234, 237-239] The majority of these studies argue that prostaglandins are involved, based on indirect evidence (for review see Stanley-Samuelson 2006 [240]), such as higher mortality rates among bacterially infected tobacco hornworm (*Manduca sexta*) larvae injected with prostaglandin biosynthesis inhibitors [237], or the ability of isolated body tissues to transform arachidonic acid to several different prostaglandins.[241, 242] Only few reports present analytical proof of the presence of prostaglandins in insects [234] and practically nothing is known about prostaglandin receptors in insects.[236] This limited and vague knowledge about the role and function of prostaglandins in insects can not provide competent arguments in the discussion of the proposed hypothesis.

Interestingly, however, preliminary experimental results indicate that OPDA has an effect on insects' development. In attempt to pin down the possible negative/toxic effect of OPDA, we carried out experiments in which aqueous solutions of OPDA were injected into the fat body of the 4-th instar *Spodoptera littoralis*. In this way a "deactivating" isomerization taking place in the insect gut was avoided and the potential effect of OPDA revealed. In the trial size of 66 caterpillars, the OPDA-injected individuals pupated statistically 1 day earlier than the control group, which was injected with water. A similar effect was not observed for insects injected with water solutions of tetadehydrodicranenone B - *iso*-OPDA.

This preliminary result can only be interpreted cautiously, since the injected amounts of OPDA (0.5 μg per larvae) were relatively high when compared with the ones naturally encountered by caterpillars. Moreover, early pupation is a very general insect stress response, and without further studies it's almost impossible to assess the exact significance of this finding and its correlation to prostaglandin role in insects.

9.5. Putative significance of OPDA isomerase for plants

The hypothesis presented in Article IV concerning the presence and function of a putative OPDA isomerase in plants is controversial, mainly due to the fact that

strong evidence for *iso*-OPDA's presence in plant tissue is missing. Tetrahydrodicranenone B was only once reported to accumulate in sorbitol-stressed barley leaves.[232] In extracts from several different plant and flower tissues (Article IV), no traces of *iso*-OPDA were detected. It is, however, possible that the isomerized OPDA is immediately channeled through the β-oxidation cycles to form *cis*-jasmone and in this way never accumulates in plants.

Considering structural similarities, it is clear that the *cis*-jasmone has to originate from jasmonates. The biosynthetic pathway starting from jasmonic acid was established several years ago [80], but the putative enzymes involved (leading from jasmonic acid to didehydrojasmonic acid) have not yet been identified.[63] From a strictly theoretical point of view, isomerization of the double bond position is energetically more favorable than the oxidation/elimination cycle, which also makes the hypothetical pathway proposed in the Article V more profitable.

The role of *cis*-jasmone, which was proposed to be "the volatile sink" for jasmonic acid [80], was recently re-discovered and supports the importance of *cis*-jasmone in plant defense.[20, 243] This volatile signal is directly repellent to aphids and at the same time attractive to aphid antagonists, such as the seven-spot ladybird (*C. semptepunctata).* The positive effects of *cis*-jasmone have been shown not only in laboratory experiments but also in field studies.[20, 83, 244] Furthermore, *cis*-jasmone was shown to induce a unique set of genes in *A. thaliana*, very different from responses to structurally similar methyl jasmonate.[245] Overall, the importance of and interest in field applications of *cis*-jasmone have increased dramatically in the last few years.

Results presented in this thesis help fill in the details of this trend. In particular, correlating OPDA isomerization activity with the group of glutathione *S*-transferases can help to identify a putative, corresponding enzyme in the plant genome. Although today the idea of cloning insect OPDA isomerase into plants is only a theoretical possibility, such a transformation would give plants, which do not normally dispose of *cis*-jasmone, the ability to biosynthesize this volatile signal along with jasmonates.

10. Summary

During co-evolution plants have evolved a myriad of different responses to defend themselves against various enemies such as insects or pathogens. These defense strategies can either be constitutively expressed (spikes, thorns, secondary metabolites) or induced in response to attack. The activation of such defense mechanisms is mediated by a set of signals in which jasmonates play an essential role.

The goal of this thesis was to study the importance of JA signaling in plant responses to different stress stimuli (herbivore feeding, continuous mechanical wounding, treatment with fungal elicitors) and to establish whether, in the course of evolutionary adaptations, insects have evolved ways to perceive or manipulate this primary defensive plant signal.

In the course of research dedicated to pursuing these objectives, the following observations were made:

Jasmonates are universal stress signals in plant kingdom. Confirming established knowledge, the results of this thesis showed the universal involvement of jasmonates in the general plant response reaction. JA and OPDA levels accumulated after elicitation with herbivore feeding (*Spodoptera littoralis*), continuous mechanical wounding (MecWorm), and treatment with the fungal elicitor chitosan in different plant species: *Arabidopsis thaliana*, *Phaseolus lunatus*, *Picea abies*. Interesting differences were found in the accumulation of JA in response to mechanical wounding at night and during the day. This finding may be of great significance for understanding ecological interactions between plants and insects.

Continuous mechanical wounding is sufficient to upregulate genes hitherto correlated with herbivore-specific responses. Differential gene expression analysis of the leaves of *A. thaliana* damaged with mechanical caterpillar – MecWorm - showed that continuous mechanical damage is sufficient to upregulate plant genes previously correlated with herbivore-specific responses. Moreover, analysis of phytohormone levels in these leaves enabled a linear correlation to be established between the amounts of damage and the amounts of accumulated JA, which suggests that the biosynthesis of jasmonic acid depends on the disruption of separate, single cells.

Specialist insect *Plutella xylostella* can influence JA signaling in *Arabidopsis thaliana.* Comparing the levels of JA and OPDA in leaves of *A.thaliana* damaged by a specialist herbivore (the diamondback moth) and the mechanical caterpillar revealed significant differences, which imply that *P. xylostella* may be able to suppress the biosynthesis of JA, the major stress responsive plant signaling molecule. Further studies are necessary to elucidate the character of insects' influence on the plant physiology.

Generalist insects recognize the plant signaling molecule 12-oxophytodienoic acid. Generalist insects (*Spodoptera littoralis, Helicoverpa zea* and others) were found to posses an ability to enzymatically isomerize plant signaling molecule – 12-oxophytodienoic acid, an early precursor of JA. The activity of the putative enzyme was correlated with the group of glutathione *S*-transferases. The subsequent expression of candidate genes in *E. coli* revealed one enzyme that exhibited OPDA isomerization activity. Specialist insects seem not to possess the relevant enzyme.

Alternative biosynthetic pathway leading to *cis*-jasmone can be useful for future crop protection. Defining the plant's ability to convert *iso*-OPDA to *cis*-jasmone and finding at the same time a candidate gene for OPDA isomerase allows theoretical speculation about specific genetic modifications, namely, those that would enable plants to biosynthesize *cis*-jasmone along with JA in response to external attack.

Zusammenfassung

Im Laufe der Coevolution mit anderen Organismen haben Pflanzen zahlreiche Schutzmechanismen entwickelt, um sich gegen unterschiedliche Fraßfeinde, wie Insekten oder Pathogene, zu verteidigen. Diese Abwehr erfolgt entweder konstitutiv in Form von beispielsweise Dornen, Stacheln oder Sekundärmetaboliten oder induziert als Antwort auf einen Angriff. Die Aktivierung dieses Abwehrmechanismus erfolgt durch eine Reihe von Signalen, wobei Jasmonate eine essentielle Rolle spielen.

Das Ziel dieser Arbeit war es zu untersuchen, wie wichtig das Jasmonsäure „signaling“ für die Antworten der Pflanzen auf verschiedene Stressstimuli, wie Fraß durch Herbivoren, kontinuierliche mechanische Verwundung oder Behandlung mit fungalen Elizitoren ist. Des Weiteren sollte festgestellt werden, ob Insekten im Laufe der coevulotionären Anpassung einen Weg zur Wahrnehmung und Manipulation der pflanzlichen Abwehrsignale gefunden haben.

Im Zuge der Forschungsarbeiten zur Beantwortung der obengenanten Fragestellung wurden folgende Beobachtungen gemacht:

Jasmonate sind universelle Stresssignale des Pflanzenreiches. Im Rahmen dieser Arbeit konnte bestätigt werden, dass Jasmonate stets an der generellen Abwehrreaktion der Pflanzen beteiligt sind. Es konnte ein Anstieg des JA- und OPDA-Gehaltes, ausgelöst durch folgende Elizitoren, beobachtet werden: Herbivorenfraß (*Spodoptera littoralis*), kontinuierliche mechanische Verwundung (MecWorm) und Behandlung mit fungalen Elizitoren (Chitosan). Untersucht wurden verschiedene Pflanzenarten: *Arabidopsis thaliana, Phaseolus lunatus, Picea abies*. Außerdem konnten interessante Unterschiede in der Menge der akkumulierten JA nach kontinuierlicher mechanischer Verwundung am Tag und in der Nacht festgestellt werden. Diese Beobachtung könnte von großer Bedeutung für die Regulation der ökologischen Interaktionen zwischen Insekten und Pflanzen sein.

Kontinuierliche mechanische Verwundung ist ausreichend, um jene Gene hoch zu regulieren, die auch für die bereits beobachtete Antwort auf Herbivorenfraß verantwortlich sind. Zusätzlich ermöglichte die Analyse des Phytohormonniveaus eine lineare Korrelation zwischen der Menge der akkumulierten JA und der Fläche

der Blattverletzung. Dieser quantitative Zusammenhang lässt vermuten, dass die JA-Biosynthese von der Verletzung der separaten Zellen abhängig ist.

Der Spezialist *Plutella xylostella* kann das JA „signalling“ in *A. thaliana* beeinflussen. Ein Vergleich des JA- und OPDA-Niveaus von *A. thaliana* Blättern nach einer Verwundung durch die Kohlschabe zum einen und Mec Worm zum anderen zeigte signifikante Unterschiede. Dies lässt vermuten, dass *P. xylostella* in der Lage ist, die Biosynthese von JA, dem wichtigsten Signalmolekül der Pflanzenabwehr, zu unterdrücken. Um den Modus dieses Einflusses auf die Physiologie der Pflanze zu erklären, sind weitere Untersuchungen nötig.

Generalisten können das pflanzlichen Signalmolekül 12-Oxophytodiensäure erkennen. Es wurde gezeigt, dass Generalisten, wie *Spodoptera littoralis* oder *Helicoverpa zea* fähig sind, das pflanzliche Signalmolekül OPDA enzymatisch zu isomerisieren. Das hierfür verantwortliche Enzym steht offensichtlich mit der Gruppe der Glutathion-S-Transferasen (GST) in Zusammenhang. Expression der Kandidat-Gene in *E. coli* zeigte nur ein Enzym, das die Isomerisierung von OPDA katalysierte. Spezialisten scheinen dieses relevante Enzym nicht zu besitzen.

Ein alternativer biosynthetischer Weg zum *cis*-Jasmon könnte zukünftig Anwendung im Pflanzenchutz finden. Mit der Entdeckung der OPDA-Isomerase und basierend auf der Tatsache, dass Pflanzen in der Lage sind, *iso*-OPDA zum *cis*-Jasmon umzuwandeln, wäre eine genetische Modifikation denkbar, nach der es Pflanzen möglich sein sollte, *cis*-Jasmon zu produzieren, um so auf einen externen Angriff zu reagieren.

11. References

[1] E. Stahl, *Jenaer Zeitschr. Med. Naturwiss.* **1888**, *22*, 557-684.

[2] T. Hartmann, *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 4541-4546.

[3] R. Karban, A. A. Agrawal, M. Mangel, *Ecology* **1997**, *78*, 1351-1355.

[4] K. von Marilaun A, *The Natural History of Plants*, Blackie, London, **1894**.

[5] U. Wittstock, J. Gershenzon, *Curr. Opin. Plant Biol.* **2002**, *5*, 300-307.

[6] D. F. Rhoades, *Evolution of plant chemical defense against herbivores*, Academic Press, Boston, **1979**.

[7] R. M. Broadway, S. S. Duffey, *J. Insect Physiol.* **1986**, *32*, 827-833.

[8] R. Karban, I. T. Baldwin, in *Induced responses to herbivory*, University of Chicago Press, **1997**, 319.

[9] H. Gundlach, M. J. Muller, T. M. Kutchan, M. H. Zenk, *Proc. Natl. Acad. Sci. U. S. A.* **1992**, *89*, 2389-2393.

[10] P. W. Pare, J. H. Tumlinson, *Nature* **1997**, *385*, 30-31.

[11] M. Heil, D. McKey, *Annu. Rev. Ecol. Evol. Syst.* **2003**, *34*, 425-453.

[12] C. Kost, M. Heil, *J. Ecol.* **2006**, *94*, 619-628.

[13] L. L. Walling, *J. Plant Growth Regul.* **2000**, *19*, 195-216.

[14] G. A. Howe, G. Jander, *Annu. Rev. Plant Biol.* **2008**, *59*, 41-66.

[15] M. J. Müller, W. Brodschelm, E. Spannagl, M. H. Zenk, *Proc. Natl. Acad. Sci. U. S. A.* **1993**, *90*, 7490-7494.

[16] S. Blechert, W. Brodschelm, S. Holder, L. Kammerer, T. M. Kutchan, M. J. Mueller, Z. Q. Xia, M. H. Zenk, *Proc. Natl. Acad. Sci. U. S. A.* **1995**, *92*, 4099-4105.

[17] A. A. Agrawal, *Curr. Opin. Plant Biol.* **2000**, *3*, 329-335.

[18] H. Chen, B. C. McCaig, M. Melotto, S. Y. He, G. A. Howe, *J. Biol. Chem.* **2004**, *279*, 45998-46007.

[19] H. Chen, C. G. Wilkerson, J. A. Kuchar, B. S. Phinney, G. A. Howe, *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102*, 19237-19242.

[20] M. A. Birkett, C. A. M. Campbell, K. Chamberlain, E. Guerrieri, A. J. Hick, J. L. Martin, M. Matthes, J. A. Napier, J. Pettersson, J. A. Pickett, G. M. Poppy, E. M. Pow, B. J. Pye, L. E. Smart, G. H. Wadhams, L. J. Wadhams, C. M. Woodcock, *Proc. Natl. Acad. Sci. U. S. A.* **2000**, *97*, 9329-9334.

[21] C. M. De Moraes, M. C. Mescher, J. H. Tumlinson, *Nature* **2001**, *410*, 577-580.

[22] J. Takabayashi, M. Dicke, *Trends Plant Sci.* **1996**, *1*, 109-113.

[23] T. C. J. Turlings, F. L. Wackers, L. E. M. Vet, W. J. Lewis, J. H. Tumlinson, in *Insect learning: Ecological and evolutionary perspectives*, Chapman and Hall Ltd., **1993**, pp. 51-78.

[24] T. C. J. Turlings, J. H. Loughrin, P. J. McCall, U. S. R. Rose, W. J. Lewis, J. H. Tumlinson, *Proc. Natl. Acad. Sci. U. S. A.* **1995**, *92*, 4169-4174.

[25] C. M. De Moraes, W. J. Lewis, P. W. Pare, H. T. Alborn, J. H. Tumlinson, *Nature* **1998**, *393*, 570-573.

[26] G. Arimura, C. Kost, W. Boland, *Biochim. Biophys. Acta Mol. Cell Biol. Lipids* **2005**, *1734*, 91-111.

[27] A. Kessler, I. T. Baldwin, *Science* **2001**, *291*, 2141-2144.

[28] I. T. Baldwin, J. C. Schultz, *Science* **1983**, *221*, 277-279.

[29] I. T. Baldwin, R. Halitschke, A. Paschold, C. C. von Dahl, C. A. Preston, *Science* **2006**, *311*, 812-815.

[30] G. Arimura, R. Ozawa, T. Shimoda, T. Nishioka, W. Boland, J. Takabyashi, *Nature* **2000**, *406*, 512-515.

[31] M. Heil, J. C. Silva Bueno, *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 5467-5472.

[32] J. Ueda, J. Kato, *Plant Physiol.* **1980**, *66*, 246-249.

[33] W. Dathe, H. Ronsch, A. Preiss, W. Schade, G. Sembdner, K. Schreiber, *Planta* **1981**, *153*, 530-535.

[34] B. J. F. Feys, C. E. Benedetti, C. N. Penfold, J. G. Turner, *Plant Cell* **1994**, *6*, 751-759.

[35] R. Liechti, E.E. Farmer, *Sci STKE* **2006**, *203*.

[36] M. V. Rao, H. Lee, R. A. Creelman, J. E. Mullet, K. R. Davis, *Plant Cell* **2000**, *12*, 1633-1646.

[37] G. Abdala, O. Miersch, R. Kramell, A. Vigliocco, E. Agostini, G. Forchetti, S. Alemano, *Plant Growth Regul.* **2003**, *40*, 21-27.

[38] E. E. Farmer, E. Almeras, V. Krishnamurthy, *Curr. Opin. Plant Biol.* **2003**, *6*, 372-378.

[39] C. Wasternack, *J. Plant Growth Regul.* **2004**, *23*, 167-169.

[40] D. C. Zimmerman, P. Feng, *Lipids* **1978**, *13*, 313-316.

[41] B. A. Vick, P. Feng, D. C. Zimmerman, *Lipids* **1980**, *15*, 468-471.

[42] B. A. Vick, D. C. Zimmerman, *Biochem. Biophys. Res. Commun.* **1983**, *111*, 470-477.

[43] P. Dormann, in *Plant Lipids: Biology, Utilisation and Manipulation*, Blackwell Publ, Malden, **2005**, pp. 123-161.

[44] J. Narvaez-Vasquez, J. Florin-Christensen, C. A. Ryan, *Plant Cell* **1999**, *11*, 2249-2260.

[45] S. Ishiguro, A. Kawai-Oda, J. Ueda, I. Nishida, K. Okada, *Plant Cell* **2001**, *13*, 2191-2209.

[46] A. R. Brash, C. D. Ingram, T. M. Harris, *Biochemistry* **1987**, *26*, 5465-5471.

[47] I. Feussner, C. Wasternack, *Annu. Rev. Plant Biol.* **2002**, *53*, 275-297.

[48] W. C. Song, C. D. Funk, A. R. Brash, *Proc. Natl. Acad. Sci. U. S. A.* **1993**, *90*, 8519-8523.

[49] G. A. Howe, A. L. Schilmiller, *Curr. Opin. Plant Biol.* **2002**, *5*, 230-236.

[50] M. Stumpe, I. Feussner, *Phytochemistry Reviews* **2006**, *5*, 347-357.

[51] J. Ziegler, M. Keinanen, I. T. Baldwin, *Phytochemistry* **2001**, *58*, 729-738.

[52] P. Zerbe, E. W. Weiler, F. Schaller, *Phytochemistry* **2007**, *68*, 229-236.

[53] F. Schaller, C. Biesgen, C. Mussig, T. Altmann, E. W. Weiler, *Planta* **2000**, *210*, 979-984.

[54] J. Strassner, F. Schaller, U. B. Frick, G. A. Howe, E. W. Weiler, N. Amrhein, P. Macheroux, A. Schaller, *Plant J.* **2002**, *32*, 585-601.

[55] F. L. Theodoulou, K. Job, S. P. Slocombe, S. Footitt, M. Holdsworth, A. Baker, T. R. Larson, I. A. Graham, *Plant Physiol.* **2005**, *137*, 835-840.

[56] C. Y. Li, A. L. Schilmiller, G. H. Liu, G. I. Lee, S. Jayanty, C. Sageman, J. Vrebalov, J. J. Giovannoni, K. Yagi, Y. Kobayashi, G. A. Howe, *Plant Cell* **2005**, *17*, 971-986.

[57] C. Delker, B. K. Zolman, O. Miersch, C. Wasternack, *Phytochemistry* **2007**, *68*, 1642-1650.

[58] C. Wasternack, I. Stenzel, B. Hause, G. Hause, C. Kutter, H. Maucher, J. Neumerkel, I. Feussner, O. Miersch, *Journal of Plant Physiology* **2006**, *163*, 297-306.

[59] M. C. Castillo, C. Martinez, A. Buchala, J. P. Metraux, J. Leon, *Plant Physiol.* **2004**, *135*, 85-94.

[60] K. Schneider, L. Kienow, E. Schmelzer, T. Colby, M. Bartsch, O. Miersch, C. Wasternack, E. Kombrink, H. P. Stuible, **2005**, *280*, 13962-13972.

[61] B. Thines, L. Katsir, M. Melotto, Y. Niu, A. Mandaokar, G. H. Liu, K. Nomura, S. Y. He, G. A. Howe, J. Browse, *Nature* **2007**, *448*, 661.

[62] A. Chini, S. Fonseca, G. Fernandez, B. Adie, J. M. Chico, O. Lorenzo, G. Garcia-Casado, I. Lopez-Vidriero, F. M. Lozano, M. R. Ponce, J. L. Micol, R. Solano, *Nature* **2007**, *448*, 666-U664.

[63] C. Wasternack, *Ann. Bot.* **2007**, *100*, 681-697.

[64] E. E. Farmer, *Nature* **2007**, *448*, 659-660.

[65] P. E. Staswick, *Trends Plant Sci.* **2008**, *13*, 66-71.

[66] G. Sembdner, B. Parthier, *Annu. Rev. Plant Physiol. Plant Molec. Biol.* **1993**, *44*, 569-589.

[67] P. E. Staswick, I. Tiryaki, *Plant Cell* **2004**, *16*, 2117-2127.

[68] B. A. Stelmach, A. Müller, P. Hennig, D. Laudert, L. Andert, E. W. Weiler, *Phytochemistry.* **1998**, *47*, 539-546.

[69] A. Stintzi, H. Weber, P. Reymond, J. Browse, E. E. Farmer, *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98*, 12837-12842.

[70] N. Taki, Y. Sasaki-Sekimoto, T. Obayashi, A. Kikuta, K. Kobayashi, T. Ainai, K. Yagi, N. Sakurai, H. Suzuki, T. Masuda, K. Takamiya, D. Shibata, Y. Kobayashi, H. Ohta, *Plant Physiol.* **2005**, *139*, 1268-1283.

[71] C. Ribot, C. Zimmerli, E. E. Farmer, P. Reymond, Y. Poirier, *Plant Physiol.* **2008**, *147*, 696-706.

[72] T. Koch, T. Krumm, V. Jung, J. Engelberth, W. Boland, *Plant Physiol.* **1999**, *121*, 153-162.

[73] J. Fliegmann, G. Schüler, W. Boland, J. Ebel, A. Mithöfer, *Biol. Chem.* **2003**, *384*, 437-446.

[74] S. Vollenweider, H. Weber, S. Stolz, A. Chételat, E. E. Farmer, *Plant J.* **2000**, *24*, 467-476.

[75] M. J. Müller, W. Brodschelm, *Anal. Biochem.* **1994**, *218*, 425-435.

[76] G. Sembdner, C. Bruckner, A. Kehlen, H. D. Knofel, R. Kramell, A. Meyer, *Biol. Chem. Hoppe-Seyler* **1991**, *372*, 754-755.

[77] H. S. Seo, J. T. Song, J. J. Cheong, Y. H. Lee, Y. W. Lee, I. Hwang, J. S. Lee, Y. D. Choi, *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98*, 4788-4793.

[78] J. H. Kang, L. Wang, A. Giri, I. T. Baldwin, *Plant Cell* **2006**, *18*, 3303-3320.

[79] A. Swiatek, W. Van Dongen, E. L. Esmans, H. Van Onckelen, *Plant Physiol.* **2004**, *135*, 161-172.

[80] T. Koch, K. Bandemer, W. Boland, *Helv. Chim. Acta.* **1997**, *80*, 838-850.

[81] S. K. Gidda, O. Miersch, A. Levitin, J. Schmidt, C. Wasternack, L. Varin, *J. Biol. Chem.* **2003**, *278*, 17895-17900.

[82] G. Glauser, E. Grata, L. Dubugnon, S. Rudaz, E. E. Farmer, J. L. Wolfender, *J. Biol. Chem.* **2008**, *283*, 16400-16407.

[83] T. J. A. Bruce, J. L. Martin, J. A. Pickett, B. J. Pye, L. E. Smart, L. J. Wadhams, *Pest Manag. Sci.* **2003**, *59*, 1031-1036.

[84] C. Davoine, T. Douki, G. Iacazio, J. L. Montillet, C. Triantaphylides, *Anal. Chem.* **2005**, *77*, 7366-7372.

[85] C. Davoine, O. Falletti, T. Douki, G. Iacazio, N. Ennar, J. L. Montillet, C. Triantaphylides, *Plant Physiol.* **2006**, *140*, 1484-1493.

[86] S. Mueller, B. Hilbert, K. Dueckershoff, T. Roitsch, M. Krischke, M. J. Mueller, S. Berger, *Plant Cell* **2008**, *20*, 768-785.

[87] B. A. Stelmach, A. Müller, P. Hennig, S. Gebhardt, M. Schubert-Zsilavecz, E. W. Weiler, *J. Biol. Chem.* **2001**, *276*, 27.

[88] Y. Hisamatsu, N. Goto, K. Hasegawa, H. Shigemori, *Tetrahedron Lett.* **2003**, *44*, 5553-5556.

[89] Y. Hisamatsu, N. Goto, M. Sekiguchi, K. Hasegawa, H. Shigemori, *Journal of Natural Products* **2005**, *68*, 600-603.

[90] C. M. Buseman, P. Tamura, A. A. Sparks, E. J. Baughman, S. Maatta, J. Zhao, M. R. Roth, S. W. Esch, J. Shah, T. D. Williams, R. Welti, *Plant Physiol.* **2006**, *142*, 28-39.

[91] M. X. Andersson, M. Hamberg, O. Kourtchenko, A. Brunnstrom, K. L. McPhail, W. H. Gerwick, C. Gobel, I. Feussner, M. Ellerstrom, *J. Biol. Chem.* **2006**, *281*, 31528-31537.

[92] F. Marks, G. Fuerstenberger, *Prostaglandins, Leukotriens and Other Eicosanoids: from biogenesis to clinical application*, Wiley-VCH, Weinheim; New York; Chichester; Brisbane; Singapore; Toronto, **1999**.

[93] D. R. Bergey, G. A. Howe, C. A. Ryan, *Proc. Natl. Acad. Sci. U. S. A.* **1996**, *93*, 12053-12058.

[94] E. Blée, *Prog. Lipid. Res.* **1998**, *37*, 33-72.

[95] E. Blée, *Trends Plant Sci.* **2002**, *7*, 315-321.

[96] L. M. Riddiford, *Science* **1967**, *158*, 139-&.

[97] S. F. Vaughn, H. W. Gardner, *J. Chem. Ecol.* **1993**, *19*, 2337-2345.

[98] B. A. Vick, *Lipid Metabolism in Plants*, CRC Press, Boca Raton, Ann Arbor, London, Tokyo, **1993**.

[99] M. J. Müller, *Chem. Biol.* **1998**, *5*, R323-R333.

[100] I. Thoma, M. Krischke, C. Löffler, M. J. Müller, *Chemistry & Physics of Lipids* **2004**, *128*, 135-148.

[101] I. Thoma, C. Löffler, A. K. Sinha, M. Gupta, M. Krischke, B. Steffan, T. Roitsch, M. J. Müller, *Plant J.* **2003**, *34*, 363-375.

[102] M. J. Müller, *Curr. Opin. Plant Biol.* **2004**, *7*, 441-448.

[103] J. A. Ryals, U. H. Neuenschwander, M. G. Willits, A. Molina, H. Y. Steiner, M. D. Hunt, *Plant Cell* **1996**, *8*, 1809-1819.

[104] L. A. J. Mur, Y. M. Bi, R. M. Darby, S. Firek, J. Draper, *Plant J.* **1997**, *12*, 1113-1126.

[105] L. A. J. Mur, I. R. Brown, R. M. Darby, C. S. Bestwick, Y. M. Bi, J. W. Mansfield, J. Draper, *Plant J.* **2000**, *23*, 609-621.

[106] K. S. Chester, *Q. Rev. Biol.* **1933**, *8*, 275-324.

[107] A. F. Ross, *Virology* **1961**, *14*, 329.

[108] A. F. Ross, *Virology* **1961**, *14*, 340.

[109] E. R. Ward, S. J. Uknes, S. C. Williams, S. S. Dincher, D. L. Wiederhold, D. C. Alexander, P. Ahlgoy, J. P. Metraux, J. A. Ryals, *Plant Cell* **1991**, *3*, 1085-1094.

[110] I. Raskin, *Annu. Rev. Plant Physiol. Plant Molec. Biol.* **1992**, *43*, 439-463.

[111] D. F. Klessig, J. Malamy, *Plant Mol.Biol.* **1994**, *26*, 1439-1458.

[112] G. Loake, M. Grant, *Curr. Opin. Plant Biol.* **2007**, *10*, 466-472.

[113] J. Malamy, J. P. Carr, D. F. Klessig, I. Raskin, *Science* **1990**, *250*, 1002-1004.

[114] J. P. Metraux, C. Nawrath, T. Genoud, *Euphytica.* **2002**, *124*, 237-243.

[115] N. Yalpani, P. Silverman, T. M. A. Wilson, D. A. Kleier, I. Raskin, *Plant Cell* **1991**, *3*, 809-818.

[116] T. Gaffney, L. Friedrich, B. Vernooij, D. Negrotto, G. Nye, S. Uknes, E. Ward, H. Kessmann, J. Ryals, *Science* **1993**, *261*, 754-756.

[117] M. C. Wildermuth, J. Dewdney, G. Wu, F. M. Ausubel, *Nature* **2001**, *414*, 562-565.

[118] M. A. Strawn, S. K. Marr, K. Inoue, N. Inada, C. Zubieta, M. C. Wildermuth, *J. Biol. Chem.* **2007**, *282*, 5919-5933.

[119] N. Yalpani, J. Leon, M. A. Lawton, I. Raskin, *Plant Physiol.* **1993**, *103*, 315-321.

[120] B. MauchMani, A. J. Slusarenko, *Plant Cell* **1996**, *8*, 203-212.

[121] J. V. Dean, L. A. Mohammed, T. Fitzpatrick, *Planta* **2005**, *221*, 287-296.

[122] F. Chen, J. C. D'Auria, D. Tholl, J. R. Ross, J. Gershenzon, J. P. Noel, E. Pichersky, *Plant J.* **2003**, *36*, 577-588.

[123] M. Seskar, V. Shulaev, I. Raskin, *Plant Physiol.* **1998**, *116*, 387-392.

[124] V. Shulaev, P. Silverman, I. Raskin, *Nature* **1997**, *385*, 718-721.

[125] J. Huang, Y. J. Cardoza, E. A. Schmelz, R. Raina, J. Engelberth, J. H. Tumlinson, *Planta* **2003**, *217*, 767-775.

[126] J. Shah, *Curr. Opin. Plant Biol.* **2003**, *6*, 365-371.

[127] W. H. Fan, X. N. Dong, *Plant Cell* **2002**, *14*, 1377-1389.

[128] C. M. Pieterse, L. Van Loon, *Curr. Opin. Plant Biol.* **2004**, *7*, 456-464.

[129] Z. X. Chen, J. W. Ricigliano, D. F. Klessig, *Proc. Natl. Acad. Sci. U. S. A.* **1993**, *90*, 9533-9537.

[130] D. H. Slaymaker, D. A. Navarre, D. Clark, O. del Pozo, G. B. Martin, D. F. Klessig, *Proc. Natl. Acad. Sci. U. S. A.* **2002**, *99*, 11640-11645.

[131] U. Conrath, Z. X. Chen, J. R. Ricigliano, D. F. Klessig, *Proc. Natl. Acad. Sci. U. S. A.* **1995**, *92*, 7143-7147.

[132] L. D. Keppler, C. J. Baker, *Phytopathology.* **1989**, *79*, 555-562.

[133] T. C. J. Turlings, J. H. Tumlinson, W. J. Lewis, *Science* **1990**, *250*, 1251-1253.

[134] W. Boland, Z. Feng, J. Donath, A. Gäbler, *Naturwissenschaften* **1992**, *79*, 368-371.

[135] L. Mattiacci, M. Dicke, M. A. Posthumus, *Proc. Natl. Acad. Sci. U. S. A.* **1995**, *92*, 2036-2040.

[136] R. O. Musser, S. M. Hum-Musser, H. Eichenseer, M. Peiffer, G. Ervin, J. B. Murphy, G. W. Felton, *Nature* **2002**, *416*, 599-600.

[137] T. Alborn, T. C. J. Turlings, T. H. Jones, G. Stenhagen, J. H. Loughrin, J. H. Tumlinson, *Science* **1997**, *276*, 945-949.

[138] R. Halitschke, U. Schittko, G. Pohnert, W. Boland, I. T. Baldwin, *Plant Physiol.* **2001**, *125*, 711-717.

[139] D. Spiteller, W. Boland, *J. Org. Chem.* **2003**, *68*, 8743-8749.

[140] A. Mithöfer, W. Boland, *Plant Physiol.* **2008**, *146*, 825-831.

[141] M. Maffei, S. Bossi, D. Spiteller, A. Mithöfer, W. Boland, *Plant Physiol.* **2004**, *134*, 1752-1762.

[142] T. Jabs, M. Tschope, C. Colling, K. Hahlbrock, D. Scheel, *Proc. Natl. Acad. Sci. U. S. A.* **1997**, *94*, 4800-4805.

[143] S. Seo, N. Okamoto, H. Seto, K. Ishizuka, H. Sano, Y. Ohashi, *Science* **1995**, *270*, 1988-1992.

[144] I. T. Baldwin, *Proc. Natl. Acad. Sci. U. S. A.* **1998**, *95*, 8113-8118.

[145] E. Rojo, J. Leon, J. J. Sanchez-Serrano, *Plant J.* **1999**, *20*, 135-142.

[146] E. Rojo, R. Solano, J. J. Sanchez-Serrano, *J. Plant Growth Regul.* **2003**, *22*, 82-98.

[147] A. Koornneef, C. M. J. Pieterse, *Plant Physiol.* **2008**, *146*, 839-844.

[148] Y. Xu, P. F. L. Chang, D. Liu, M. L. Narasimhan, K. G. Raghothama, P. M. Hasegawa, R. A. Bressan, *Plant Cell* **1994**, *6*, 1077-1085.

[149] I. Penninckx, B. Thomma, A. Buchala, J. P. Metraux, W. F. Broekaert, *Plant Cell* **1998**, *10*, 2103-2113.

[150] B. Thomma, I. Nelissen, K. Eggermont, W. F. Broekaert, *Plant J.* **1999**, *19*, 163-171.

[151] B. Thomma, I. Penninckx, W. F. Broekaert, B. P. A. Cammue, *Curr. Opin. Immunol.* **2001**, *13*, 63-68.

[152] M. Berrocal-Lobo, A. Molina, R. Solano, *Plant J.* **2002**, *29*, 23-32.

[153] O. Lorenzo, R. Piqueras, J. J. Sanchez-Serrano, R. Solano, *Plant Cell* **2003**, *15*, 165-178.

[154] C. M. J. Pieterse, J. A. Van Pelt, S. C. M. Van Wees, J. Ton, K. M. Leon-Kloosterziel, J. J. B. Keurentjes, B. W. M. Verhagen, M. Knoester, I. Van der Sluis, P. Bakker, L. C. Van Loon, *Eur. J. Plant Pathol.* **2001**, *107*, 51-61.

[155] M. J. Stout, J. S. Thaler, B. Thomma, *Annu. Rev. Entomol.* **2006**, *51*, 663-689.

[156] S. H. Spoel, J. S. Johnson, X. Dong, *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 18842-18847.

[157] H. Peña-Cortés, T. Albrecht, S. Prat, E. W. Weiler, L. Willmitzer, *Planta* **1993**, *191*, 123-128.

[158] K. Harms, I. Ramirez, H. Peña-Cortés, *Plant Physiol.* **1998**, *118*, 1057-1065.

[159] D. Laudert, E. W. Weiler, *Plant Journal.* **1998**, *15*, 675-684.

[160] S. H. Spoel, A. Koornneef, S. M. C. Claessens, J. P. Korzelius, J. A. Van Pelt, M. J. Mueller, A. J. Buchala, J. P. Metraux, R. Brown, K. Kazan, L. C. Van Loon, X. N. Dong, C. M. J. Pieterse, *Plant Cell* **2003**, *15*, 760-770.

[161] S. H. Doares, J. Narvaezvasquez, A. Conconi, C. A. Ryan, *Plant Physiol.* **1995**, *108*, 1741-1746.

[162] T. Niki, I. Mitsuhara, S. Seo, N. Ohtsubo, Y. Ohashi, *Plant Cell Physiol.* **1998**, *39*, 500-507.

[163] N. Mitter, K. Kazan, H. M. Way, W. F. Broekaert, J. M. Manners, *Plant Sci.* **1998**, *136*, 169-180.

[164] R. M. P. van Poecke, M. Dicke, *J. Exp. Bot.* **2002**, *53*, 1793-1799.

[165] P. Kenton, L. A. J. Mur, R. Atzorn, C. Wasternack, J. Draper, *Mol. Plant-Microbe Interact* **1999**, *12*, 74-78.

[166] L. A. J. Mur, P. Kenton, R. Atzorn, O. Miersch, C. Wasternack, *Plant Physiol.* **2006**, *140*, 249-262.

[167] P. M. Schenk, K. Kazan, I. Wilson, J. P. Anderson, T. Richmond, S. C. Somerville, J. M. Manners, *Proc. Natl. Acad. Sci. U. S. A.* **2000**, *97*, 11655-11660.

[168] S. C. M. van Wees, E. A. M. de Swart, J. A. van Pelt, L. C. van Loon, C. M. J. Pieterse, *Proc. Natl. Acad. Sci. U. S. A.* **2000**, *97*, 8711-8716.

[169] P. R. Ehrlich, P. H. Raven, *Evolution* **1964**, *18*, 586-608.

[170] G. S. Fraenkel, *Science* **1959**, *129*, 1466-1470.

[171] M. Berenbaum, P. Feeny, *Science* **1981**, *212*, 927-929.

[172] J. X. Becerra, *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 7483-7488.

[173] J. Thomson, *What we know and do not know about coevolution: insect herbivores and plants as a test case*, Blackwell Science, London, **1999**.

[174] R. I. Krieger, P. P. Feeny, C. F. Wilkinson, *Science* **1971**, *172*, 579.

[175] R. L. Lindroth, in *Insect-plant interactions. Volume 3.* (Ed.: E. A. Bernays), CRC Press, Boca Raton, Florida, **1991**, pp. 1-33.

[176] E. A. Bernays, *Philos. Trans. R. Soc. Lond. Ser. B-Biol. Sci.* **1991**, *333*, 257-264.

[177] R. Shroff, F. Vergara, A. Muck, A. Svatos, J. Gershenzon, *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 6196-6201.

[178] D. E. Dussourd, *J. Insect Behav.* **1999**, *12*, 501-515.

[179] P. Krokene, H. Solheim, *Phytopathology.* **1998**, *88*, 39-44.

[180] K. Konno, C. Hirayama, H. Shinbo, *J. Insect Physiol.* **1997**, *43*, 217-224.

[181] G. L. Vaughan, A. M. Jungreis, *J. Insect Physiol.* **1977**, *23*, 585-589.

[182] R. M. Broadway, *J. Insect Physiol.* **1995**, *41*, 107-116.

[183] L. Paulillo, A. R. Lopes, P. T. Cristofoletti, J. R. P. Parra, W. R. Terra, M. C. Silva, *J. Econ. Entomol.* **2000**, *93*, 892-896.

[184] R. Feyereisen, *Annu. Rev. Entomol.* **1999**, *44*, 507-533.

[185] M. B. Cohen, M. R. Berenbaum, M. A. Schuler, *J. Chem. Ecol.* **1989**, *15*, 2347-2355.

[186] W. Li, M. R. Berenbaum, M. A. Schuler, *Insect Biochemistry and Molecular Biology* **2001**, *31*, 999-1011.

[187] W. M. Li, M. A. Schuler, M. R. Berenbaum, *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100*, 14593-14598.

[188] X. C. Li, M. R. Berenbaum, M. A. Schuler, *Insect Mol. Biol.* **2002**, *11*, 343-351.

[189] M. Sasabe, Z. M. Wen, M. R. Berenbaum, M. A. Schuler, *Gene* **2004**, *338*, 163-175.

[190] C. Naumann, T. Hartmann, D. Ober, *Proc. Natl. Acad. Sci. U. S. A.* **2002**, *99*, 6085-6090.

[191] A. Ratzka, H. Vogel, D. J. Kliebenstein, T. Mitchell-Olds, J. Kroymann, *Proc. Natl. Acad. Sci. U. S. A.* **2002**, *99*, 11223-11228.

[192] U. Wittstock, N. Agerbirk, E. J. Stauber, C. E. Olsen, M. Hippler, T. Mitchell-Olds, J. Gershenson, H. Vogel, *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 4859-4864.

[193] S. J. Yu, *Zoological Studies* **1996**, *35*, 9-19.

[194] H. Ranson, J. Hemingway, *Glutathione Transferases*, Elsevier BV, **2005**.

[195] H. S. Huang, N. T. Hu, Y. E. Yao, C. Y. Wu, S. W. Chiang, C. N. Sun, *Insect Biochemistry and Molecular Biology* **1998**, *28*, 651-658.

[196] R. W. Wadleigh, S. J. Yu, *J. Chem. Ecol.* **1988**, *14*, 1279-1288.

[197] R. W. Wadleigh, S. J. Yu, *J. Econ. Entomol.* **1988**, *81*, 776-780.

[198] K. Usui, J. Fukami, T. Shishido, *Pesticide Biochemistry and Physiology* **1977**, *7*, 249-260.

[199] R. W. Wadleigh, S. J. Yu, *Insect Biochemistry* **1987**, *17*, 759-764.

[200] J. D. Hayes, J. U. Flanagan, I. R. Jowsey, *Annual Review of Pharmacology and Toxicology* **2005**, *45*, 51-88.

[201] Y. C. Ding, F. Ortelli, L. C. Rossiter, J. Hemingway, H. Ranson, *Bmc Genomics* **2003**, *4*, 35.

[202] H. Ranson, C. Claudianos, F. Ortelli, C. Abgrall, J. Hemingway, M. V. Sharakhova, M. F. Unger, F. H. Collins, R. Feyereisen, *Science* **2002**, *298*, 179-181.

[203] P. G. Board, M. Coggan, G. Chelvanayagam, S. Easteal, L. S. Jermiin, G. K. Schulte, D. E. Danley, L. R. Hoth, M. C. Griffor, A. V. Kamath, M. H. Rosner, B. A. Chrunyk, D. E. Perregaux, C. A. Gabel, K. F. Geoghegan, J. Pandit, *J. Biol. Chem.* **2000**, *275*, 24798-24806.

[204] X. C. Li, M. A. Schuler, M. R. Berenbaum, *Nature* **2002**, *419*, 712-715.

[205] E. A. Schmelz, J. Engelberth, H. T. Alborn, P. O'Donnell, M. Sammons, H. Toshima, J. H. Tumlinson, *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100*, 10552-10557.

[206] M. J. Mueller, L. Mene-Saffrane, C. Grun, K. Karg, E. E. Farmer, *Plant J.* **2006**, *45*, 472-489.

[207] H. Weber, B. A. Vick, E. E. Farmer, *Proc. Natl. Acad. Sci. U. S. A.* **1997**, *94*, 10473-10478.

[208] X. Q. Pan, R. Welti, X. M. Wang, *Phytochemistry* **2008**, *69*, 1773-1781.

[209] I. Stenzel, B. Hause, O. Miersch, T. Kurz, H. Maucher, H. Weichert, J. Ziegler, I. Feussner, C. Wasternack, *Plant Mol.Biol.* **2003**, *51*, 895-911.

[210] E. A. Schmelz, J. Engelberth, J. H. Tumlinson, A. Block, H. T. Alborn, *Plant J.* **2004**, *39*, 790-808.

[211] B. Schulze, R. Lauchli, M. M. Sonwa, A. Schmidt, W. Boland, *Anal. Biochem.* **2006**, *348*, 269-283.

[212] P. Reymond, N. Bodenhausen, R. M. P. Van Poecke, V. Krishnamurthy, M. Dicke, E. E. Farmer, *Plant Cell* **2004**, *16*, 3132-3147.

[213] A. M. Shelton, R. J. Cooley, M. K. Kroening, W. T. Wilsey, S. D. Eigenbrode, *J. Entomol. Sci.* **1991**, *26*, 17-26.

[214] A. Mithöfer, G. Wanner, W. Boland, *Plant Physiol.* **2005**, *137*, 1160-1168.

[215] B. Schulze, P. Dabrowska, W. Boland, *Chembiochem* **2007**, *8*, 208-216.

[216] L. Lagadic, A. Cuany, J. B. Berge, M. Echaubard, *Insect Biochemistry and Molecular Biology* **1993**, *23*, 467-474.

[217] M. Blackburn, B. Ketterer, D. J. Meyer, A. M. Juett, A. W. Bull, *Chem. Res. Toxicol.* **1997**, *10*, 1364-1371.

[218] M. H. Beale, J. L. Ward, *Nat. Prod. Rep.* **1998**, *15*, 533-548.

[219] J. H. Loughrin, A. Manukian, R. R. Heath, J. H. Tumlinson, *J. Chem. Ecol.* **1995**, *21*, 1217-1227.

[220] H. Vogel, J. Kroymann, T. Mitchell-Olds, *PLoS ONE* **2007**, e1081.

[221] H. U. Stotz, T. Koch, A. Biedermann, K. Weniger, W. Boland, T. Mitchell-Olds, *Planta* **2002**, *214*, 648-652.

[222] G. W. Felton, K. L. Korth, *Curr. Opin. Plant Biol.* **2000**, *3*, 309-314.

[223] J. Ehlting, S. G. Chowrira, N. Mattheus, D. S. Aeschliman, G. I. Arimura, J. Bohlmann, *Bmc Genomics* **2008**, *9*.

[224] R. L. Jones, *Biochem. J.* **1970**, *119*, 64.

[225] H. Polet, L. Levine, *Biochem. Biophys. Res. Commun.* **1971**, *45*, 1169.

[226] H. Polet, L. Levine, *J. Biol. Chem. 276(30):28628, 2001 Jul 27.* **1975**, *250*, 351-357.

[227] R. L. Jones, S. Cammock, E. W. Horton, *Biochimica et Biophysica Acta* **1972**, *280*, 588-601.

[228] H. Polet, L. Levine, *Arch. Biochem. Biophys.* **1975**, *168*, 96-103.

[229] S. K. Malhotra, H. J. Ringold, *J. Am. Chem. Soc.* **1965**, *87*, 3228.

[230] A. M. Benson, P. Talalay, J. H. Keen, W. B. Jakoby, *Proc. Natl. Acad. Sci. U. S. A.* **1977**, *74*, 158-162.

[231] A. S. Johansson, B. Mannervik, *J. Biol. Chem.* **2001**, *276*, 33061-33065.

[232] R. Kramell, O. Miersch, R. Atzorn, B. Parthier, C. Wasternack, *Plant Physiol.* **2000**, *123*, 177-187.

[233] D. Destephano, U. E. Brady, R. E. Lovins, *Prostaglandins* **1974**, *6*, 71-79.

[234] R. A. Jurenka, V. K. Pedibhotla, D. W. Stanley, *Arch. Insect Biochem. Physiol.* **1999**, *41*, 225-232.

[235] W. Loher, I. Ganjian, I. Kubo, D. Stanleysamuelson, S. S. Tobe, *Proc. Natl. Acad. Sci. U. S. A.* **1981**, *78*, 7835-7838.

[236] M. N. Medeiros, D. M. P. Oliveira, G. O. Paiva-Silva, M. A. C. Silva-Neto, A. Romeiro, M. Bozza, H. Masuda, E. A. Machado, *Insect Biochemistry and Molecular Biology* **2002**, *32*, 537-545.

[237] D. W. Stanleysamuelson, E. Jensen, K. W. Nickerson, K. Tiebel, C. L. Ogg, R. W. Howard, *Proc. Natl. Acad. Sci. U. S. A.* **1991**, *88*, 1064-1068.

[238] J. S. Miller, T. Nguyen, D. W. Stanleysamuelson, *Proc. Natl. Acad. Sci. U. S. A.* **1994**, *91*, 12418-12422.

[239] E. Buyukguzel, H. Tunaz, D. Stanley, K. Buyukguzel, *J. Insect Physiol.* **2007**, *53*, 99-105.

[240] D. W. Stanley, J. S. Miller, *Entomol. Exp. Appl.* **2006**, *119*, 1-13.

[241] D. W. Stanleysamuelson, C. L. Ogg, *Insect Biochemistry and Molecular Biology* **1994**, *24*, 481-491.

[242] K. Buyukguzel, H. Tunaz, S. M. Putnam, D. Stanley, *Insect Biochemistry and Molecular Biology* **2002**, *32*, 435-443.

[243] J. A. Pickett, G. M. Poppy, *Trends Plant Sci.* **2001**, *6*, 137-139.

[244] W. Powell, J. A. Pickett, *Pest Manag. Sci.* **2003**, *59*, 149-155.

[245] T. J. A. Bruce, M. C. Matthes, K. Chamberlain, C. M. Woodcock, A. Mohib, B. Webster, L. E. Smart, M. A. Birkett, J. A. Pickett, J. A. Napier, *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 4553-4558.

[246] S. W. Baertschi, C. D. Ingram, T. M. Harris, A. R. Brash, *Biochemistry* **1988**, *27*, 18-24.

[247] D. Laudert, P. Hennig, B. A. Stelmach, A. Muller, L. Andert, E. W. Weiler, *Analytical Biochemistry.* **1997**, *246*, 211-217.

12. Selbständigkeitserklärung

Jena, 18.12.2008

Hiermit erkläre ich, Paulina Anna Dąbrowska, dass ich die vorliegende Arbeit selbständig und unter Verwendung der angegebenen Hilfsmittel, persönlichen Mitteilungen und Quellen angefertigt habe.

…….

Paulina Anna Dąbrowska

13. Acknowledgements

This work like most of other PhD projects would not be possible without substantial help of many people, who contributed to it in many different, but always important ways.

My special thanks go in first hand to my PhD supervisor – **Prof. Dr. Wilhelm Boland**, who always provided me with his inexhaustible ideas and enthusiasm along the way.

I'm grateful to **Prof. Dr. Reiner Beckert** for consulting this study and giving me possibility to present this thesis at the Faculty of Chemical and Earth Sciences at the Friedrich Schiller University, Jena.

I'm also thankful to Max Planck Gesellschaft for the financial support in frames of **International Max Planck Research School** Programme.

I would like to specially acknowledge **Dr. Birgit Schulze** for her invaluable help in the beginnings of my work, which extended from equipping me with the essential literature, through introduction to the exciting field of oxylipin extraction on explaining me analytical instrument wonders finishing. Her amazing patience and above all her experience enabled me fast understanding of a very complex subject and saved me from the most frustrating failures.

I owe a special gratitude to **Dalial Freitak**, who not only shared a substantial part of my life in Jena as a best friend but also proved to be an excellent cooperation partner and at the same time a molecular biology tutor.

I am very grateful to **Dr. Maritta Kunert** and **Anja David** for their support, help and expertise during my first experiments in the Institute as well as for sharing in the later stages responsibility and loads of work in the "MecWorm-Arabidopsis project".

This work would also not be possible without strong support from **Dr. Dieter Spiteller**, who not only patiently listened to my problems but most importantly provided useful ideas for their solutions. He also saved me several times from depth of desperation resulting from mass spectrometer breakdowns.

I am indebted to **Dr. Heiko Vogel**, who believed in me developing a feeling for molecular biology and who patiently convinced me to the "biologist" point of view. Thanks to his ideas and expertise I had an opportunity to work on an exciting and challenging project.

With “Molto Grazie!” I would also like to thank **Fabio Rui** for being irreplaceable lab and PhD companion.

I would like to sincerely thank all friends and colleagues from the Max Planck Institute, in particular:

Anna Fontana for her endless support and making my life in Jena worthwhile; **Andrea Fiedler** for listening to my scientific and non-scientific problems and for her practical help in my first days in Institute and Jena; **Andreas Habel** for his chemical experience, which was always there when I needed it; **Angelika Berg** for her invaluable care for caterpillars and plants; **Annika Schmidt** for helpful tips concerning the synthesis of Tetrahydrodicranenone B; **Alexie Papanicoloau** for his refreshingly sharp tongue; **Axel Mithöfer** for his trust in handing me over the phytohormone analysis of the “MecWorm-Arabidopsis project”; **Brent Sørensen** for amazing Canadian pancakes and inspiring me to make more sports; **Christian Kost** for interesting scientific discussions in “the women office”; **Daniel Veit** for patience and professional help with various technical problems; **Doreen Schachtschabel** for chemical synthesis related tips and cheerful company on conferences in Irsee and PennState; **Fredd Vergara** for sharing with me his football fascinations; **Gen Arimura** for helpful scientific discussions and contagious life optimism; **Hanna Fisher** and **Andrew Heidel** for a lot of fun on common movie nights; **Ivana Radojcic** for support, optimism and teaching me how to be “perfectly organized” in science and life; **Jana Becher** for being an indispensable office, social and “life discussing” companion as well as her free Adobe Photoshop leads and proof-reading this thesis; **Jeanette Kley** for cheerful company and inspiring me to try dancing classes; **Jeffrey Wee** for his helpful tips concerning GSTs expression; **Juliette Courtiade** for giving me home in Kefersteinstrasse 17 and for her invaluable company in countless common enterprises; **Karin Groten** for her amazing administrative help in my first days in Institute and Jena; **Kerstin Ploss** for ice-skating experiences and understanding in frustrations caused by GCQ; **Kusuma Jitsaeng** for a cheerful company on common parties, **Matthias Funke** for a cheerful company; **Michael Philips** for giving me a possibility to work on a part of his project; **Peter Brand** for being the fastest learning technician ever; **Radhika Venkatesan** for being the best “partnering student” during IMPRS recruitments and her company on the conference in London; **Roy Kirsch** for an exquisite musical education; **Sabrina Disher** and **Sindy Frick** for cheerful company in the lab; **Sebastian Schöne** for providing a fine

lunch company; **Shirsha Mitra** for her support, optimism, joyful time spend together in the guest house and interesting Indian cuisine experiences; **Stefan Garms** and **Stefan Bartram** for helpful discussions and technical support; **Thomas Wichard** for sharing long, very wise and funny discussions and for his invaluable company on the conferences in Copenhagen and Berlin; **Yannick Pauchet** for helpful hints concerning molecular biology and 2D gel electrophoresis and unforgettable French accent.

I am also deeply indebted to all members of institute service facilities: NMR group and **Renate Ellinger** and **Bernd Schneider** for their help with recording and elucidation of my NMR spectra, MS and Proteomics group with **Alexander Muck, Antje Loele** and **Aleš Švatos** for their help with my MALDI-MS measurements of protein samples, Greenhouse team and **Andreas Weber** for taking care of countless Arabidopsis seedlings, EDV group and **Martin Niebergall** for his computer expertise, Library team and **Simone Bieniek** for helping me with getting important articles, all members of Haustechnik and Administration.

From the important people “outside” Institute I would like to especially thank: **Bationa Shahollari** for her support and funny game evenings; **Christian Dimkpa** for giving me a chance to work with him on siderophores project; **Emily Wheeler** for amazing help with correcting my English in countless scientific contributions, including this thesis; **Ellen** and **Christian Hasher** for teaching me German in many different and inspiring ways; **Enrico Tomelleri** for his optimism and strange winter expedition to Oberhof; **Klaus König** for keeping my mind and body in optimal condition during Morgenpraxis yoga classes; **Kristina Trusilova** for her support, discussions, encouraging me to do many new things in my life and for taking me on Spanish classes, which quite unexpectedly changed my life; **Mirek** and **Albi Rękas** for our Sunday “polish connection” meetings; **Valentina Sicardi** for her cheerful company on the evenings out.

I owe special thanks to **David** for his love, patience, support and for giving me a non-work related life.

Above all I would like to thank **my parents** for their never ending support during my PhD years and their love.

14. Curriculum vitae

Personal data

Name:	Paulina Anna Dabrowska
Date of birth:	02.05.1980
Place of birth:	Warszawa, Poland
Home address:	Camburgerstr.17, 07743 Jena, Germany
Marital status:	unmarried
E-mail:	pdabrowska@poczta.onet.pl, pdabrowska@ice.mpg.de

Scientific career

since 01/2005	PhD student in the Max Planck Institute for Chemical Ecology in Jena and Friedrich-Schiller University Jena
10/2003 – 07/2004	Diploma thesis: *Synthesis and enzyme catalyzed resolution of β-hydroxynitrones* on Warsaw University of Technology, Faculty of Chemistry, under supervision of Prof. Jan Plenkiewicz (Department of Drugs Technology and Biotechnology)
10/1999 – 06/2003	Warsaw University of Technology, Diploma in chemistry; engineer degree, specialization in Drugs Technology and Biotechnology

Education

09/1995 – 06/1999	Public high school (XVLI Liceum im. Joachima Lelewela), Warsaw, (Abitur)
09/1987 – 06/ 1995	Public primary school (Szkoła Podstawowa nr. 65 im. Władysława Orkana), Warsaw

Practical training and stays abroad

03/2004 – 05/2004	3 months research project at the University Paris – Sud in frame of Erasmus student exchange programme: *Enzymatic synthesis of D-glucosaminic acid from D-glucosamine*
08/2003	1 month professional apprenticeship in a pharmaceutical company - Polfa Tarchomin S.A., Warsaw

Scientific publications

1) Dimkpa CO, Svatoš A, **Dąbrowska P**, Schmidt A, Boland W, Kothe E (2008) Involvement of Siderophores in the Reduction of Metal-induced inhibition of Auxin Synthesis in Streptomyces. *Chemosphere,* **74**, 19-25.

2) Arimura G, Köpke S, Kunert M, Volpe V, David A, Brand P, **Dąbrowska P**, Maffei M, Boland W (2008) Effects of Feeding *Spodoptera littoralis* on Lima Bean Leaves IV: Diurnal and Nocturnal Damage Differentially Initiate Plant Volatile Emission. *Plant Physiology*, **146**, 965-973.

3) **Dąbrowska P**, Boland W (2007) *iso*-OPDA – an early precursor of *cis*-jasmone in plants? *ChemBioChem*, **8**, 2281 – 2285.

4) Philips M, Walter MH, Ralph S, **Dąbrowska P**, Luck K, Urós EM, Boland W, Strack D, Rodríguez-Concepción M, Bohlmann J, Gershenzon J (2007) Functional Identification and Differential Expression of 1-Deoxy-D-Xylulose 5-Phosphate Synthase and Other MEP Pathway Genes in Induced Terpenoid Resin Formation of Norway spruce (Picea abies). *Plant Molecular Biology*, **65**, 243-257.

5) Schulze B, **Dąbrowska P**, Boland W (2007) Rapid enzymatic isomerization of 12-oxophytodienoic acid in the gut of lepidopteran larvae. *ChemBioChem*, **8**, 208-216.

Other Publications

6) Wielechowska M, **Dąbrowska P**, Plenkiewicz J (2006) Lipase-catalyzed separation of the enantiomers of 1-arylidenamino-3-aryloxypropan-2-ol-N-oxides. Preparation of optically active nitrones. *Tetrahedron Asymmetry*, **17**, 1786-1792.

7) Dąbkowska K, **Dąbrowska P**, Drabik J, Kopczuk D, Plenkiewicz J, Strosznajder JB, Wielechowska M (2005) Preparation of 1-Arylideneamino- and 1-Alkylideneamino-3-phenoxypropan-2-ol N-oxides - A New Type of Nitrones. *Synthetic Communications*, **35**, 1455-1459.

In preparation

Dąbrowska P, Freitak D, Vogel H, Heckel DG, Boland W, The phytohormone precursor OPDA is isomerized in the insect gut by a single, specific Glutathione *S*-transferase.

Oral presentations

Dąbrowska P, Freitak D, Vogel H, Boland W (2008) Enzyme assisted isomerization of 12-oxophytodienoic acid in the insect gut. *25th ANNUAL ISCE MEETING*, State College, USA.

Dąbrowska P, Freitak D, Vogel H, Boland W (2008) Enzyme assisted isomerization of 12-oxophytodienoic acid in the insect gut and its significance for the herbivore. *20.Irseer Naturstofftage der DECHEMA e.V.*, Irsee, Germany.

Dąbrowska P, Schulze B, Freitak D, Vogel H, Boland W (2007) Enzyme assisted isomerization of 12-oxophytodienoic acid in the insect gut and its significance for the herbivore. *3rd European Symposium on Plant Lipids of European Federation for the Science and Technology of Lipids*, York, United Kingdom.

Dąbrowska P, Boland W (2006) Plant derived oxylipins in the insect gut. *32. Doktorandenworkshop, Naturstoffe: Chemie, Biologie und Ökologie*, Bayreuth, Germany.

Poster Presentations

Dąbrowska P, Freitak D, Vogel H, Boland W (2008) Enzyme assisted isomerization of 12-oxophytodienoic acid in the insect gut and its significance for the herbivore. *Multitrophic Interactions*, Göttingen, Germany.

Dąbrowska P, Freitak D, Vogel H, Boland W (2008) Enzyme assisted isomerization of 12-oxophytodienoic acid in the insect gut and its significance for the herbivore. *20. Irseer Naturstofftage der DECHEMA e.V.*, Irsee, Germany.

Dąbrowska P, Schulze B, Boland W (2006) Oxylipins in attacked plant leaves and their metabolism in feeding insects. *2nd International Conference Nonmammalian Eicosanoids, Bioactive Lipids and Plant Oxylipins*, Berlin, Germany.

Schulze B, **Dąbrowska P**, Boland W (2005) Profiling of labile plant oxylipins by *in situ* derivatization with pentafluorobenzylhydroxylamine. *2nd European symposium on Plant Lipids of European Federation for the Science and Technology of Lipids*, Copenhagen, Denmark.

15. Supplementary material

15.1. Unpublished results Part I

Expression analysis by RealTime PCR. For real-time PCR RNA quantity and integrity was additionally determined with RNA Nano LabChips run on an Agilent 2100 Bioanalyzer (Agilent). Subsequently, 400 ng of DNA-free total RNA was converted into single-stranded cDNA using a mix of random and oligo-dT_{20} primers according to the ABgene protocol (ABgene, UK). Gene-specific primers were designed on the basis of sequence obtained for selected Arabidopsis genes and several additional genes as potential house-keeping genes to serve as the endogenous control (normalizer). Q-RT-PCR was done in optical 96-well plates on a MX3000P Real-Time PCR Detection System (Stratagene, USA) using the Absolute QPCR SYBR green Mix (ABgene) to monitor double-stranded DNA synthesis in combination with ROX as a passive reference dye included in the PCR master mix. A dissociation curve analysis was performed for all primer/probe pairs, and all experimental samples yielded a single sharp peak at the amplicon's melting temperature. Furthermore, we tested four genes as invariant endogenous controls in the assay to correct for sample-to-sample variation in RT-PCR efficiency and errors in sample quantitation, and found both *RPS18B* and *EF-1α* performed best as an endogenous control ('normalizer'). The dynamic range of a given primer/probe system and its normalizer was examined by running triplicate reactions of tenfold-dilution series (five different RNA concentrations). The resulting standard curve was a nearly straight line for both the target and normalizer realtime PCRs for the same range of total RNA concentrations. For all PCRs, efficiency was between 94% and 106%. Since target and normalizer had similar dynamic ranges, we used the comparative quantitation method (ΔΔCt) to contrast MecWorm *versus* controls (M-C), *P. xylostella versus* controls (P-C), and *P. xylostella versus* MecWorm treatments (P-M), and transformed to absolute values with $2^{-\Delta\Delta Ct}$ for obtaining fold changes between MecWorm and controls (M *vs* C), *P. xylostella* and controls (P *vs* C), and *P. xylostella* and MecWorm (P *vs* M). All of the assays were run in triplicate (biological replication) and quadruplicate (technical replication) to control for overall variability. Relative fold changes for each gene were set to 1 for the control treatment (non-treated control plants).

Microarray hybridizations and raw data processing were performed by MOgene (St. Louis, MO) according to the instructions of the array manufacturer. In the table (**Tab.1**), the first column depicts genes with The Arabidopsis Information Resource (TAIR) identifier and a short gene product description. Least square means were obtained for controls (C), MecWorm (M) and *P. xylostella* (P) treatments after standardization (Raw data/Total Raw Data). For comparisons between MecWorm and controls (M/C), *P. xylostella* and controls (P/C), and *P. xylostella* and MecWorm (P/M), fold change, F ratio, and statistical significance for differential transcript accumulation are indicated.

Table 1. Transcript profiling with Agilent Arabidopsis ATH3 microarrays and with RealTime PCR.

	Agilent Arabidopsis ATH3 Microarray									RealTime PCR														
	Least Square Means (Raw Data/Total Raw Data)			Fold Change	F ratio; P (df = 1,7)		Fold Change	F ratio; P (df = 1,7)		Fold Change	F ratio; P (df = 1,4)		Least Square Means $Ct_{(Control\ Gene = RPS18)}$			Least Square Means $Ct_{(Test\ Gene)}$			$\Delta Ct_{(Test\ Gene - Control\ Gene)}$			$\Delta\Delta Ct$			$2^{-\Delta\Delta Ct}$ = Fold Change		
Treatment	**C**	**M**	**P**										**C**	**M**	**P**	**C**	**M**	**P**	**C**	**M**	**P**						
N	**6**	**3**	**3**										**2**	**2**	**2**	**2**	**2**	**2**									
Comparison					**M/C**			**P/C**			**P/M**											**M-C**	**P-C**	**P-M**	**M *vs* C**	**P *vs* C**	**P *vs* M**
Gene																											
At1g51760 (IAR3)	3.3E-05	1.3E-04	1.5E-04	3.9	773.4 <0.0001		4.6	228.7 <0.0001		1.2	3.9 0.1184		23.1	22.6	22.4	24.4	21.8	21.5	1.3	-0.7	-0.9	-2.0	-2.1	-0.1	4.0	4.4	1.1
At3g50260 (AP2 transcr. factor)	2.6E-06	1.3E-05	1.3E-05	5.1	44.5 0.0003		4.9	47.1 0.0003		1.0	0.0 0.9166		22.7	22.2	22.4	28.1	24.4	24.2	5.4	2.2	1.8	-3.2	-3.6	-0.4	9.0	12.0	1.3
At4g23600 (CORI3)	4.2E-04	1.7E-03	1.3E-03	4.1	159.2 <0.0001		3.1	282.4 <0.0001		0.8	8.7 0.0420		23.1	22.6	22.4	22.1	18.5	18.7	-1.0	-4.1	-3.7	-3.0	-2.6	0.4	8.2	6.2	0.8
At5g24770 (VSP2)	4.5E-05	3.9E-04	4.3E-04	8.8	303.7 <0.0001		9.6	2668.3 <0.0001		1.1	1.1 0.3497		23.1	22.6	22.4	26.7	20.0	19.7	3.6	-2.6	-2.6	-6.1	-6.2	-0.1	69.6	73.8	1.1
At3g16470 (JR1)	9.2E-05	6.0E-04	4.5E-04	6.6	156.9 <0.0001		4.9	203.0 <0.0001		0.8	4.7 0.0976		23.1	22.6	22.4	23.3	20.8	20.5	0.2	-1.8	-1.8	-2.0	-2.0	0.0	4.0	4.0	1.0
At5g28237 (Tryptophan synthase, β)	1.4E-06	9.6E-06	1.2E-05	6.7	41.8 0.0004		8.5	73.9 <0.0001		1.3	1.0 0.3840		23.1	22.6	22.4	30.8	25.3	24.3	7.7	2.7	2.0	-5.0	-5.8	-0.8	31.8	54.7	1.7
At2g29450 (ATGSTU5)	1.6E-04	6.7E-04	4.5E-04	4.2	123.1 <0.0001		2.8	103.6 <0.0001		0.7	7.5 0.0516		22.7	22.2	22.4	21.4	19.2	19.4	-1.4	-3.1	-3.0	-1.7	-1.6	0.1	3.3	3.0	0.9
At3g45140 (LOX2)	7.9E-04	1.8E-03	1.8E-03	2.2	111.3 <0.0001		2.3	42.2 0.0004		1.0	0.2 0.7025		22.7	22.2	22.4	19.6	16.7	17.3	-3.1	-5.5	-5.1	-2.4	-2.0	0.4	5.2	4.0	0.8
At3g13790 (BFRUCT1)	2.1E-06	1.0E-05	1.2E-05	4.8	28.2 0.0011		5.9	212.0 <0.0001		1.2	1.1 0.3458		22.7	22.2	22.4	23.9	22.4	22.8	1.2	0.2	0.5	-1.0	-0.7	0.3	2.0	1.6	0.8
At1g07400 (HSP17.8-CI)	2.4E-05	3.4E-04	2.6E-05	14.0	75.9 <0.0001		1.0	0.0 0.8687		$1/_{13.3}$	33.4 0.0045																
At1g52560 (HSP26.5-P)	1.6E-06	6.7E-06	1.9E-06	4.2	197.1 <0.0001		1.2	1.0 0.3604		$1/_{3.5}$	63.7 0.0013																
At1g53540 (HSP17.6C-CI)	1.2E-05	2.4E-04	1.1E-05	20.1	247.8 <0.0001		0.9	2.2 0.1780		$1/_{22.3}$	119.4 0.0004																
At1g59860 (HSP17.6A-CI)	2.2E-05	1.1E-04	2.2E-05	5.2	107 <0.0001		1.0	0.0 0.9735		$1/_{5.2}$	47.2 0.0024																
At1g72070 (TCJ2)	1.4E-06	9.1E-06	1.8E-06	6.6	236.2 <0.0001		1.3	3.4 0.1057		$1/_{5.2}$	86.5 0.0007																
At1g74310 (HSP101)	6.0E-06	2.5E-05	6.7E-06	4.2	418.1 <0.0001		1.1	0.7 0.4419		$1/_{3.8}$	180.9 0.0002																
At1g80920 (HSP40)	1.7E-04	9.6E-04	3.2E-04	5.8	187.5 <0.0001		1.9	28.3 0.0011		$1/_{3.0}$	43.4 0.0027																
At2g20560 (HSP40)	1.0E-05	3.8E-05	1.0E-05	3.7	31.9 0.0008		1.0	0.0 0.9467		$1/_{3.8}$	14.8 0.0184																
At2g26150 (ATHSFA2)	5.2E-06	1.6E-05	5.6E-06	3.1	214.2 <0.0001		1.1	0.4 0.5340		$1/_{2.9}$	70.0 0.0011																
At2g29500 (HSP17.6B-CI)	2.8E-05	3.0E-04	2.6E-05	10.5	46.1 0.0003		0.9	0.5 0.4877		$1/_{11.7}$	20.3 0.0108																
At2g32120 (HSP70)	1.1E-05	4.4E-05	1.1E-05	4.0	669.2 <0.0001		1.1	0.5 0.4857		$1/_{3.8}$	383.8 <0.0001																
At3g12580 (HSP70)	5.3E-05	2.4E-04	4.3E-05	4.6	86.6 <0.0001		0.8	1.9 0.2152		$1/_{5.6}$	44.0 0.0027																
At3g46230 (HSP17.4-CI)	5.8E-06	2.3E-05	6.1E-06	4.0	139.4 <0.0001		1.0	0.1 0.7975		$1/_{3.8}$	65.3 0.0013																
At4g10250 (HSP22.0-ER)	9.3E-07	7.9E-06	9.3E-07	8.5	548.0 <0.0001		1.0	0.0 0.9812		$1/_{8.6}$	434.3 <0.0001																
At4g11660 (HSF7)	3.5E-06	8.0E-06	2.9E-06	2.3	58.1 0.0001		0.8	5.0 0.0608		$1/_{2.8}$	42.7 0.0028																
At4g25200 (HSP23.6-M)	1.4E-06	1.4E-05	9.5E-07	10.1	71.7 <0.0001		0.7	1.6 0.2457		$1/_{15.2}$	34.7 0.0042																
At4g36990 (HSF4)	1.7E-05	3.9E-05	2.1E-05	2.4	62.5 <0.0001		1.3	7.5 0.0286		$1/_{1.8}$	16.4 0.0155																
At5g12030 (HSP17.7-CII)	3.1E-06	1.9E-05	3.0E-06	6.1	132.0 <0.0001		1.0	0.0 0.8341		$1/_{6.3}$	60.9 0.0015																
At5g23240 (HSP40)	3.2E-06	1.7E-05	3.7E-06	5.5	198.3 <0.0001		1.2	0.2 0.6648		$1/_{4.6}$	38.1 0.0035																
At5g52640 (HSP81-1)	4.9E-05	1.8E-04	3.9E-05	3.7	95 <0.0001		0.8	5.2 0.0571		$1/_{4.6}$	50.5 0.0021																
At5g62020 (HSF6)	1.2E-05	3.9E-05	1.8E-05	3.2	122.3 <0.0001		1.5	25.5 0.0015		$1/_{2.2}$	38.4 0.0035																

15.2. Unpublished results Part II

Materials and Methods

Separating cytosolic and microsomal protein fractions. Larval midguts (30 *H. armigera* larva, 4-th instar) were dissected and split longitudinally, washed in ice-cold 0,1M phosphate buffer and submerged into ice-cold homogenization buffer (0,1M PBS, 1mM EDTA, 1mM DTT, 1mM PTU, 5mM Protease Inhibitor – Pierce). Midguts were homogenized with a Polytron homogenizer (Brinkman Industries). The homogenate was centrifuged at 4°C 10,000 g for 10 min and the supernatant was filtered through the glass wool to get rid of any possible debris. For obtaining the microsomal fraction filtrate was centrifuged at 4°C 100,000g for 60 min. Microsomal pellet was dissolved in buffer (0,1M PBS, 20% glycerol, 0,1mM EDTA, 0,1mM DTT, 0,1M PTU, 1% Triton X-100, 5mM Protease Inhibitor – Pierce).

GSTs visualization and separation on 1D and 2D SDS pages. To visualize proteins present in the elution fraction, Sodium Dodecyl Sulphate Polyacrylamide Gradient Gel Electrophoresis (SDS-PAGE) was performed in a XT-MES buffer system. We used both 1D and 2D SDS electrophoresis. Samples were transferred to new tubes, loading buffer was added to the samples, heat denatured and loaded on a 4 – 12 % Bis-Tris Criterion XT Precast Gel (BioRad). Gels were run at 80V for ~ 3.5 hours or until the dye front reached the gel end. On the gels, two different protein markers were used. Rainbow marker (Amersham) served as a running control marker and the Precision Plus Protein Unstained Standard (BioRad) for precise protein molecular weight estimation. After the run was complete, gels were washed 3 times for 15 min, followed by staining with Coommassie blue (Imperial Blue, Pierce) for 2-3 hours, then de-stained overnight. In the case of 2D gels, proteins were fixed on gel after electrophoresis step in 40% (v/v) ethanol and 10% (v/v) acetic acid for 60 min and stained overnight with Colloidal Coomassie staining.

MALDI-TOF analysis of the proteins. For protein identification, spots were manually cut out from SDS-gels, transferred to 96-well microtiterplates (MTP) and processed on an automatic Ettan TA Digester (GE Healthcare). The gel plugs were rinsed with 50 mM ammonium bicarbonate/50% acetonitrile three times for 20 min to remove the Coomassie stain. The gel plugs were then air-dried and digested with trypsin overnight at 37°C. The resulting peptides were extracted from the gel plugs, collected in an MTP and vacuum-dried. Samples were submitted for MALDI-TOF

mass spectrometry and *de novo* sequencing by Q-TOF to our in-house MassSpec service group.

HPLC-MS^2 analysis and purification of the GS-OPDA conjugate. Qualitative HPLC-MS^2 measurements and purification of GS-OPDA conjugate were performed on HPLC system Agilent HP1100 coupled with LTQ mass spectrometer (Thermo Finnigan, Bremen, Germany). HPLC separation was achieved on a Phenomenex column Synergy polar RP (250 mm x 2 mm, 4 mm) (Phenomenex, Aschaffenburg, Germany) by using gradient of elution at 0.2 mLmin^{-1} (solvent A: H_2O, 0.5% AcOH; solvent B: MeCN, 0.5% AcOH) starting with 20% B (3 min), programmed to 100% B in 20 min. Elution with 100% B was maintained for 5 min prior to equilibration with the initial solvent mixture. MS^1 and MS^2 analyses were performed initially in negative and positive ESI mode (**Fig.1**). For subsequent qualitative analysis and purification of the conjugate ESI negative mode was used. Analysis of the gut content and feces samples for the GS-OPDA conjugate was performed on microHPLC DIONEX system Ultimate 3000 (DIONEX, Germering, Germany) hooked to a Thermo Finnigan LTQ mass spectrometer and equipped with Phenomenex Luna C18 (20 mm x 0.30 mm, 5 μm) column, using already described solvent gradient of elution at 4 μLmin^{-1}. NMR spectroscopy was performed with a cryo probe on a Bruker AV-500 MHz NMR spectrometer (see below).

NMR and MS spectra of synthesized OPDA-GSH. Since our starting material - *cis*-OPDA was obtained via the flaxseed extract preparation, which generates a mixture of *cis*-(+)-(9*S*,13*S*) and cis-(-)-(9*R*,13*R*)-OPDA (in our case modest excess of *cis*-(+)(9*S*,13*S*) on the basis of CD spectrum).[246, 247] Subsequent chemical synthesis can result in unspecific formation of theoretically eight possible isomers of the conjugate. We know however from previous feeding experiments that this *cis*-OPDA enantiomeric mixture is being fully isomerized by the insects. Moreover the plant derived OPDA is optically pure (*cis*-(+)-(9*S*,13*S*)-OPDA) and thus we conclude that it is not the absolute configuration of the side chains which is crucial for the transformation, but the *cis* relationship between them. To simplify the analysis we focused only on the relations of the side chains and the GSH moiety to each other and to the cyclopentanone ring plane (since the NMR spectra of *cis*(+) and *cis*(-)-OPDA are identical, we analyzed the spectra for orientation of the side chains and the GSH moiety relative to each other and to the cyclopentanone ring plane). Basic

reaction conditions and the excess of GSH cause almost immediate epimerization on C13 of *cis*-OPDA

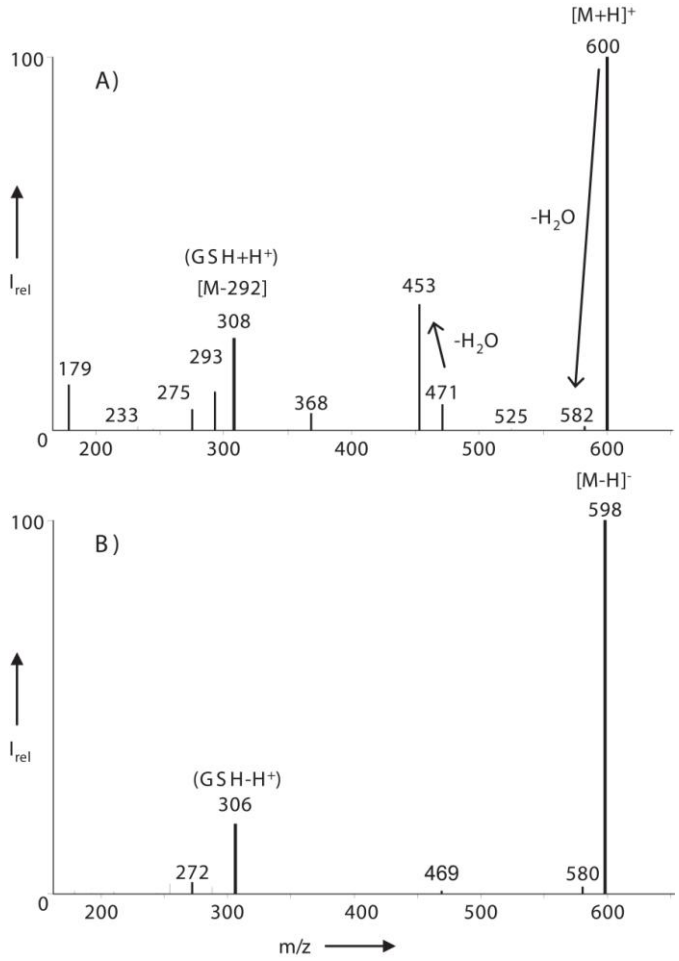


Figure 1. MS^2 spectra of OPDA-GSH: A) in positive mode (Collision Energy 27%), fragmentation spectrum of pseudomolecular ion $[M+H]^+$ m/z 600, B) in negative mode ($[M-H]^-$ m/z 598). Main fragmentations in positive mode are in agreement with literature.[84, 85]

to *trans*-OPDA. The other chiral center of the OPDA-GSH is generated by conjugation, where the attack of nucleophilic sulphur can occur either from “above” or from “below” of the cyclopentenone ring plane. Taking in consideration the steric hindrance of the initial *cis*-OPDA side chains the preferential attack of the GSH should occur anti to the substituent. **Fig.2** represents a partial 2D HSQC spectrum of the purified compound with assigned peak signals (detailed signal assignments and 2D spectrum presented in **Tab.1**, **Fig.3** with relative stereochemistry on example of *cis*-(+)-OPDA), in which there are two distinct pairs of signals, corresponding to olefinic carbons (15 and 16) of the side chain. These “double” signals could be explained by a mixture of two diastereomers: *cis* (Diastereomer B) and *trans*

(Diastereomer A) enantiomers of the side chains. Thanks to the 2D HSQC spectrum an estimation of the enantiomer-ratio was possible (A:B = 1.6:1) on the basis of the ^{1}H spectrum integrals (**Fig.2**). In contrast, elucidation of the stereochemistry on the C10 turned out to be more complicated. Using the information from ROESY spectra we could identify the interaction between the signals of 10A and 10B protons with the combined signals of protons 5-8 of the side chain (**Fig.4**), which is only possible when the GSH moiety is situated on the opposite side of the cyclopentanone ring plane than the side chain at C9.

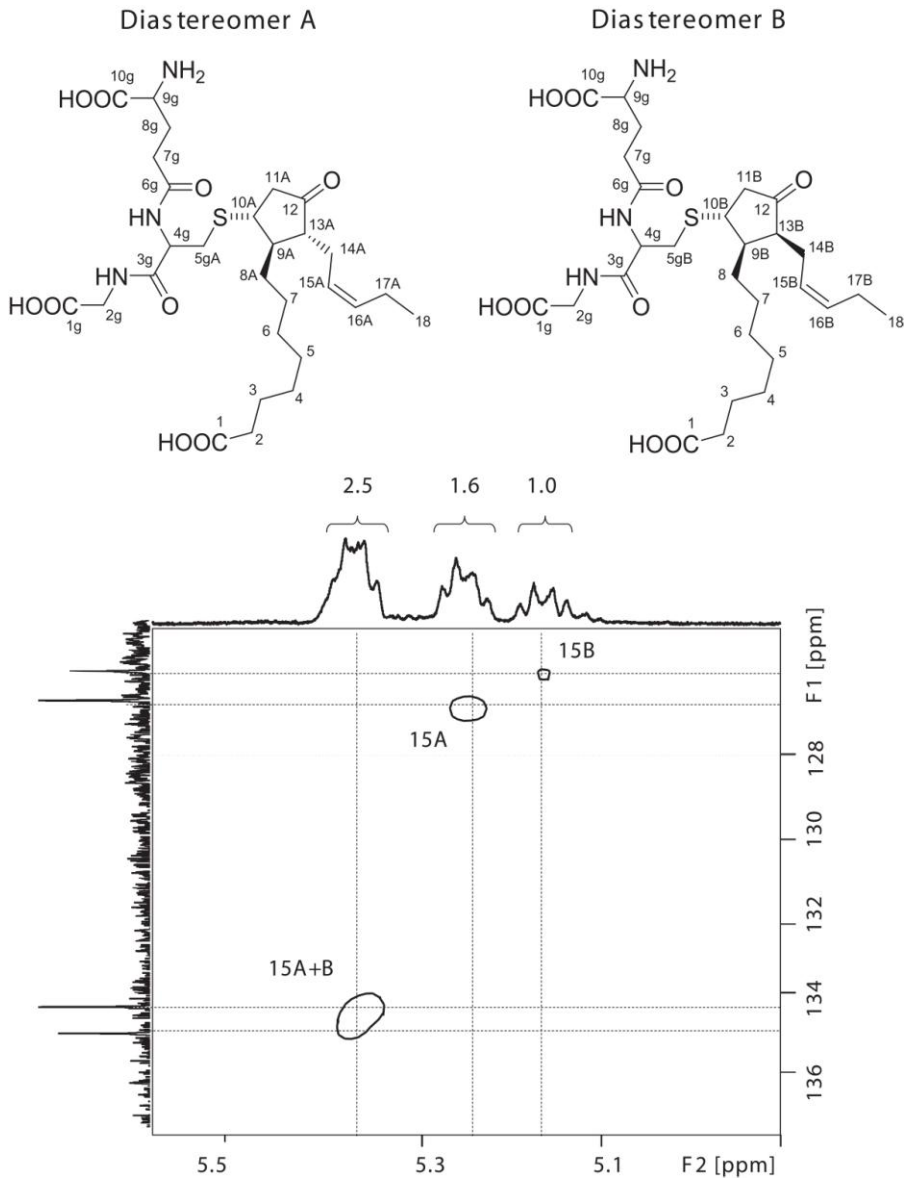


Figure 2. Part of the 2D HSQC spectrum of the OPDA-GSH. Ratio of the *cis*, *trans* side chain – OPDA isomers shown. Structures and labelling of carbon atoms in two OPDA-GSH diastereomers identified in the synthesis product mixture with the example of relative stereochemistry of *cis*(+)-OPDA as starting material.

Table 1. Assignment of the ^{1}H and ^{13}C signals of two OPDA-GSH diastereomers (A and B) on the basis of 2D HSQC spectrum.

Carbon/ associated proton number	^{1}H chemical shift / ppm	^{13}C chemical shift / ppm
1	-	178.0
2	2.23	34.5
3	1.49	25.3
4	1.49	29.3
5,6,7,8	1.15 – 1.35	26-31
8A	1.15 – 1.35	28 (?)
9A	2.12	45.8
9B	1.75 m	46.9
10A	3.34	43.2
10B	3.06	44.2
11A	2.68/2.22	44.3
11B	2.83/2.12	47.7
12	-	~221.0
13A	2.61	51.9
13B	2.08	54.5
14A	2.19/2.06	22.8
14B	2.27/1.96	26.8
15A	5.25	126.9
15B	5.16	126.2
16A	5.37	134.2
16B	5.37	134.8
17	1.96	21.3

18	0.87	14.5
5gA	2.97/2.78	33.6
5gB	2.89/2.68	32.7
4g	4.45	54.2
3g	-	172.4
2g	3.74	42.9
1g	-	174.5
6g	-	175.2
7g	2.40	32.6
8g	2.02	27.2
9g	3.59	55.0
10g	-	174.0

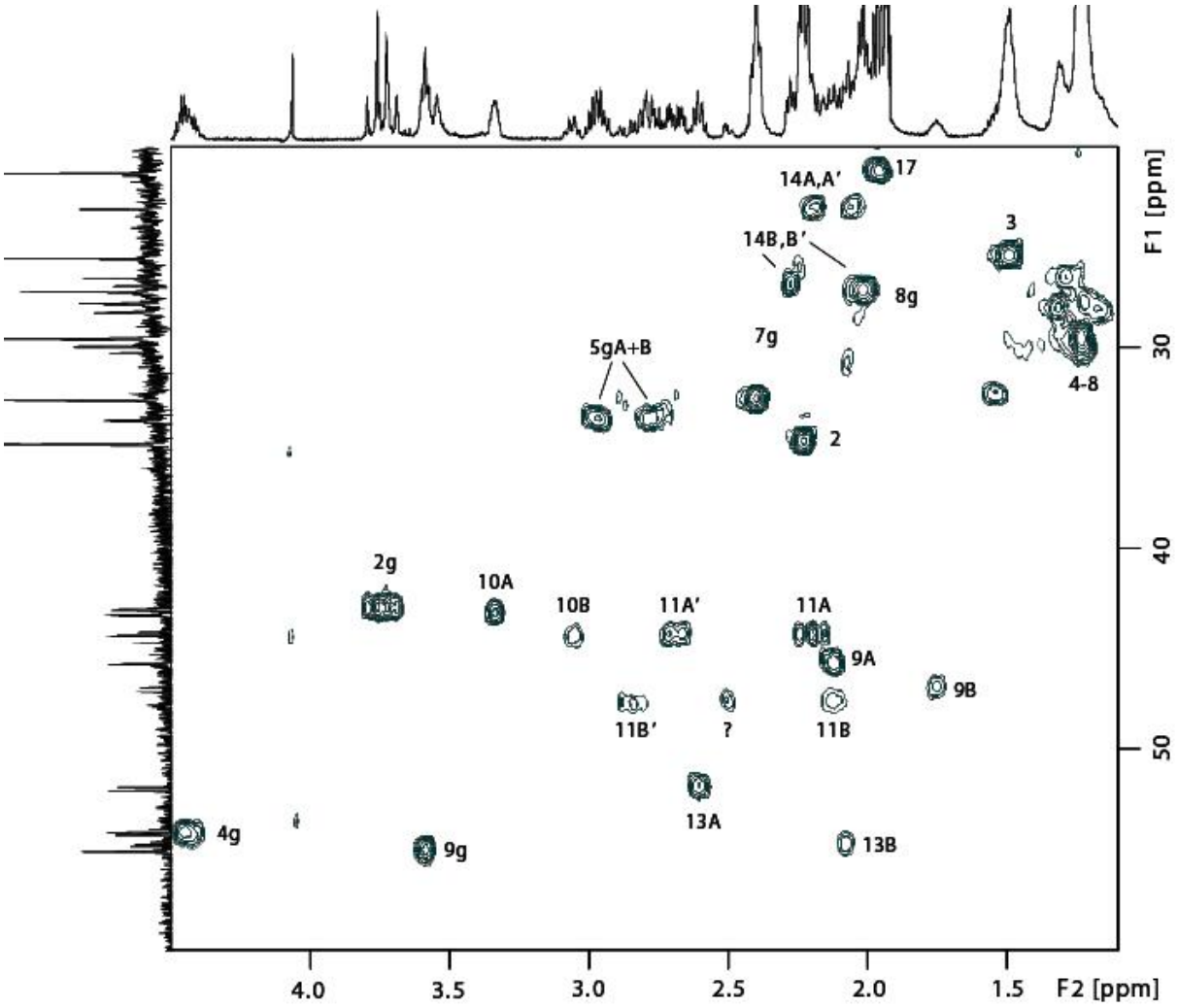


Figure 3. Partial 2D HSQC spectrum with signal assignments.

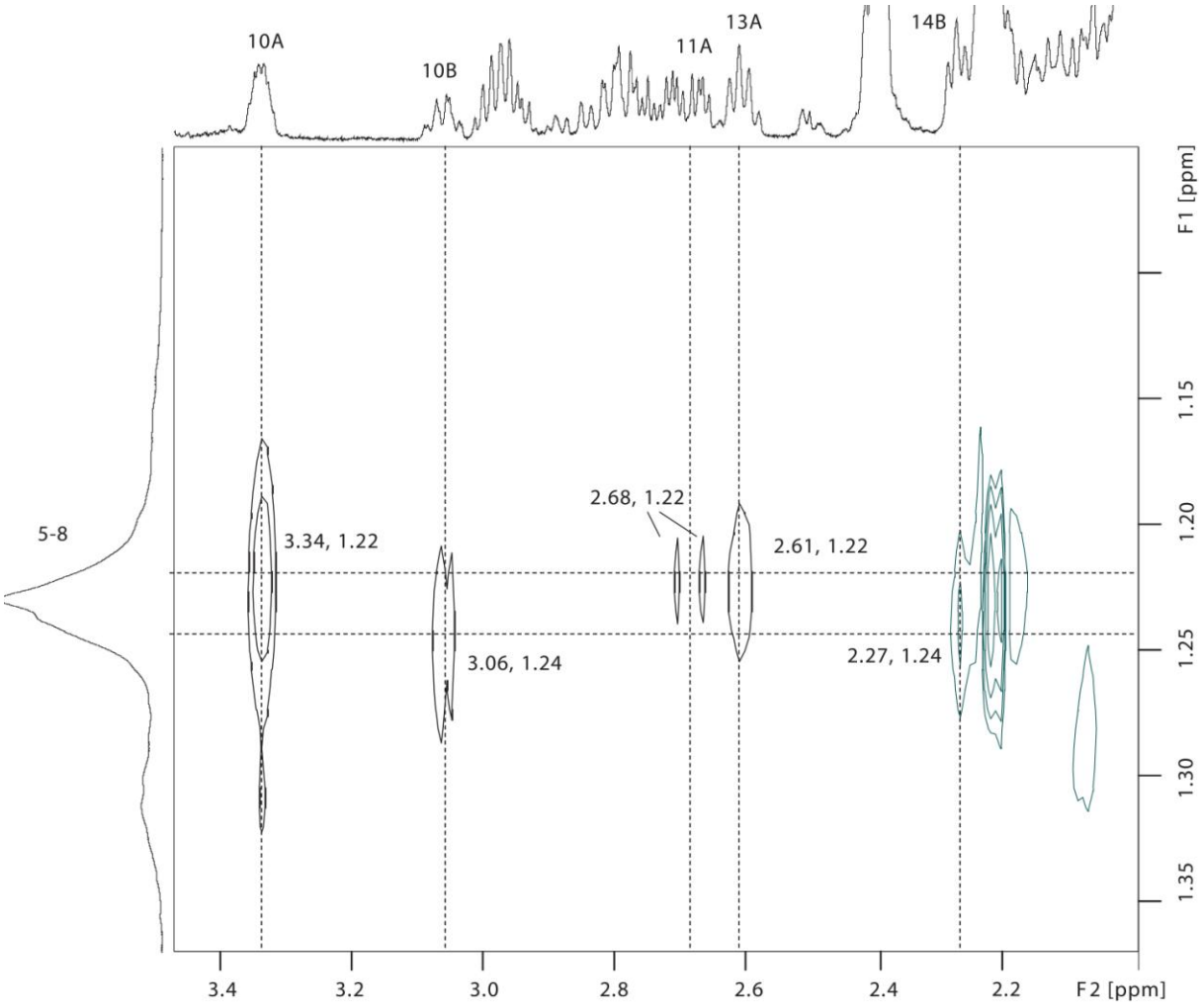


Figure 4. Partial ROESY spectrum with signal assignments.

Structural information from ROESY spectrum:

Diastereomer A:

3.34 (H10A) ---- 1.22 (CH_2 side chain at C9, 5-8)
2.61 (H13A) ---- 1.22 (CH_2 side chain at C9, 5-8)
2.68 (H11A) ---- 1.22 (CH_2 side chain at C9, 5-8)

} H13A, H10A, H11A and side chain at C9 on the same side of cyclopentanone ring plain

Diastereomer B:

3.06 (H10B) ---- 1.24 (CH_2 side chain at C9, 5-8)
2.27 (H14B) ---- 1.24 (CH_2 side chain at C9, 5-8)

} Side chains at H13B and H9 and H10B on the same side

Table 2. Primers used for expression of GST 16.

Gene	Forward Primer	Reverse Primer
GST 16	ATGGGTTTGACAGTATACAAA	GGCCAATTTCAATTTCTCGAG