Regulation of Nectar Secretion and Volatile Emission in Plants by Jasmonates

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Gutachter

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Contents

1. General introduction

Plant-insect interactions

"Here, it takes all the running you can do to stay in the same place. If you want to get somewhere you must run at least twice as fast as that"
- (Red Queen, Through the looking glass, Lewis Caroll).

Like the red queen, both plants and herbivores constantly evolve in response to each other's defences, a perpetual change necessary to maintain the *status quo* (1). These competing interactions lead to an escalation of offensive and defensive measures - a scenario coined as evolutionary arms race (1-4). In a seminal paper in 1959, Fraenkel suggested that 'the food specificity of insects is based on presence or absence of these odd substances (secondary metabolites) in plants, which serve as repellents to insects' showing that plants manufacture an enormous variety of secondary compounds to protect themselves from insect herbivory (5,6) Later in 1964, Ehrlich and Raven proposed their model of plant-insect 'coevolution' by studying butterfly plant interactions in an attempt to account for the biological diversity of herbivores and host plants (2). In these 50 years since Fraenkel's initial proposition of his concept, understanding of plant-insect interactions has advanced exponentially in terms of evaluating ecophysiological functions and biochemical pathways involved in plant defence mechanisms (3, 7).

Plant defence strategies

Plants have developed a wide variety of defence mechanisms to protect themselves against herbivorous insects. These mechanisms can be constitutive (always present) or inducible (activated only upon attack). Expressing constitutive defences like thorns, spikes, phytochemical compounds or other feeding deterrents can be metabolically costly for the plant since these defences have to be maintained even in the absence of herbivore attack (8, 9). In contrast, inducible defences, which are produced after herbivory are more economical (10, 11). Plant defences can be further classified as 'direct' or 'indirect' depending on whether the plant controls the herbivores 'directly' by increasing the concentration of toxic phytochemicals or 'indirectly' by attracting the predators of the herbivores. Induced indirect plant defences thus involve a triangle of plant-herbivore-carnivorous arthropods and have been an interesting area of research (12, 13).

The focus of the present thesis is on indirect defence strategies of plants, which include the secretion of extrafloral nectar (EFN) and emission of volatile organic compounds (VOC). By secreting EFN or by emitting VOCs, plants signal an "alarm call" to carnivorous arthropods to locate their prey. EFN mainly comprises of an aqueous solution of sugars and small amounts of amino acids and is secreted in specialized organs called 'nectaries', which can be found in any vegetative or reproductive plant structures yet not involved in pollination (14, 15). Although the sugars in the nectar are known to be phloem-derived (15), what factors or mechanisms actually regulate this important secretory process is still very poorly understood (16). In contrast to floral nectar, EFN is not involved in the attraction of pollinators but is generally involved in recruiting arthropods, especially ants, which effectively safeguard the plant against herbivores (17). Another indirect defence strategy employed by many plants is the emission of VOCs, which provides chemical information to the natural enemies of the herbivores (18, 19). In general, VOCs comprise of terpenoids, C_6 and C_8 compounds. VOCs are synthesized *de novo* upon herbivore attack and are highly specific to the type of attacking insect, making them reliable host-location cues for carnivorous arthropods (20-22). In addition, several abiotic factors such as light, temperature, soil characteristics and water stress also affect the expression of these indirect defences (23-25). For example, it was shown that tobacco plants release temporally different volatile blends, which the lepidopteran insects use as cue to facilitate oviposition (26). Both VOC emission and EFN secretion are thus induced indirect defences against herbivores, which are influenced by biotic and abiotic factors (21-29). Such a customized elicitation of defences obviously involves a complex network of signal transduction pathways, which orchestrate these responses. Jasmonic acid (JA) and its related compounds (precursors and metabolites), collectively known as jasmonates are key signaling molecules involved in such herbivore-induced defence responses (30, 31). Indeed, exogenous application of JA has been shown to induce EFN secretion and VOC emission, similar to herbivore feeding implying that JA is involved in controlling these defence mechanisms (32, 33).

Signal transduction– Role of jasmonates

Effective reactions against herbivores require sequential identification of herbivore feeding, activation of signaling cascade and eventually defence responses. Among phytohormones involved in plant stress responses, JA has been shown to play a central role in regulating plant defence responses against herbivore attack (34). JA is not only important during plant stress but is also involved in reproductive development, carbon partitioning and senescence (30,35).

JA biosynthesis is initiated in response to biotic (herbivory or other tissue damage signals) or abiotic stress factors (see Fig. 1).

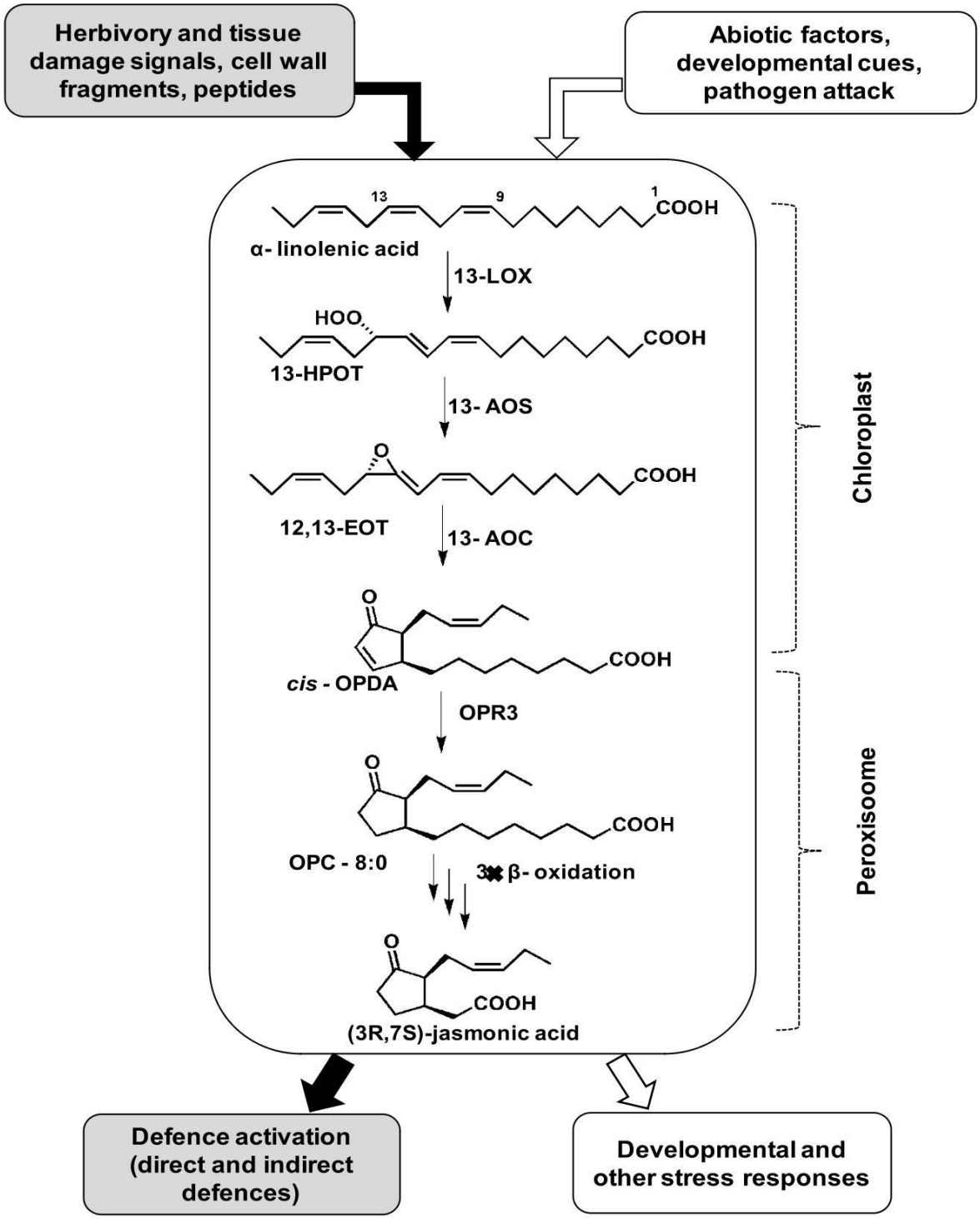

Figure 1: Biosynthetic pathway leading to jasmonic acid in higher plants, which is activated by herbivory or other signals and leads to defence gene activation (modified after 30, see text for details).

Jasmonates are derived from lipid oxidation pathways, which start with the release of fatty acid precursors from membrane lipids (36). α-linolenic acid, released by lipase activity from chloroplast membrane is the major precursor for numerous oxygenated compounds (called as oxylipins) including jasmonates. The first step is catalyzed by lipoxygenases, a family of non-heme iron containing dioxygenases, which insert molecular oxygen into α-linolenic in a regio- and stereo- specific manner to form (13*S*)-hydroperoxyoctadecatrienoic acid (13-HPOT) (see Fig. 1). The next step is the conversion of 13-HPOT by 13-allene oxide synthase (13-AOS) to an unstable allene oxide, 12,13-epoxyoctadecatrienoic acid (13-EOT) (37). 13-allene oxide cyclase (13-AOC) closes the cyclopentanone ring to yield *cis* (+)-12-oxophytodienoic acid (9*S*, 13S-OPDA) (38). Later, in peroxisomes, OPDA is reduced to 2'-(*Z*)-pentenyl cyclopentan-1-octanoic acid (OPC 8:0), a reaction catalyzed by OPDA reductase (OPR3) followed by three rounds of β -oxidation to yield JA (see Fig. 1). Although JA is an important signal molecule for triggering plant defence mechanisms, around 20 different derivatives of JA are also known (39-42). However, only a few enzymes active in converting JA into its metabolites have been identified so far (30). Some of the JA metabolites (12-OHJA and 12-O-Glc-JA) have been reported to function as an inactivate form of JA: a mechanism to turn off JA signaling (30, 40).

Active JA derivatives - activity by conjugation

A landmark in comprehending the JA signaling cascade in plants was the discovery of the F-box protein, coronatine-insensitive 1 (COI1), required for JA perception, which led to the idea that negative regulators of JA signaling are subject to ubiquitin-dependent degradation (43, 44). COI1 is associated with other proteins of SCF (Skip-Cullin-F box) complex and this SCF^{COI1} tags the unknown JA regulators for proteosomal destruction (30). The discovery of JAZ (jasmonate ZIM domain) proteins as these unknown JA regulators was a major breakthrough in understanding JA signaling (45, 46). In cells containing low levels of JA (or in an unstressed state), JAZs restrain the transcription factors (MYC2, a basic helix-loop-helix) that positively regulate JA responsive gene expression (see Fig. 2) (47).

In the first step, JA is enzymatically conjugated to isoleucine to form JA-Ile. Using yeast-hybrid strategy, Thines and coworkers (2007) showed that JA-Ile stabilizes the COI1-JAZ complex, and then SCF^{COI1} tags the JAZ proteins with ubiquitin for destruction (45). Extending this concept, Chini and coworkers (2007) showed that the carbonyl terminus of

JAZ protein is bound to MYC2 in *Arabidopsis thaliana* (46). After destruction of JAZ proteins, jasmonate-induced gene expression is up regulated leading to defence responses (see Fig. 2) (43-47). Analysis of JA derivatives that directly promote this COI1-JAZ interaction showed that COI1 binding to JAZs is stimulated by JA-Ile (43,47) whereas, on the other hand, JA, OPDA and MeJA were found to be inactive in these assays (45). Recently, a search for the most active stereoisomer of JA-Ile led to the identification of (+)-7-iso-JA-Ile as the most active form of this conjugate (41). These reports highlight the importance of JA-Ile as the bioactive jasmonate and underline the importance of structural requirements for activity. JA-Ile is the only known hormone, which is activated by conjugation while in other cases (auxin for example), conjugation inactivates the signal and helps to maintain hormone homeostasis (48). However, what factors regulate the biosynthesis of JA-Ile and in turn affect the indirect defence responses remains to be answered. Interestingly coronatine (see Fig. 2), a phytotoxin isolated from *Pseudomonas syringae* and its structural mimic, coronalon (6-ethyl indanoyl isoleucine conjugate, see Fig. 2), are reported to mimic JA induced defences such as VOC emission (32, 49, 50).

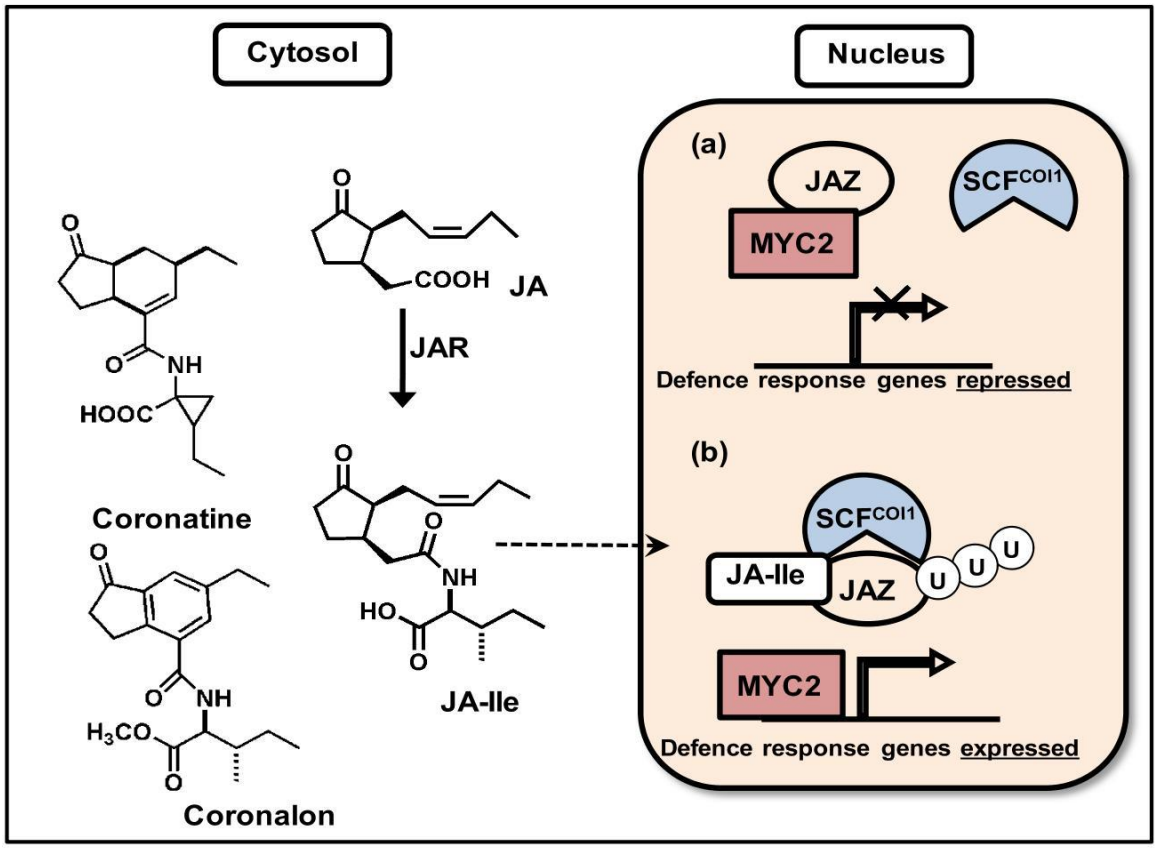

Figure 2. A model showing COI1-JAZ interactions known from *Arabidopsis thaliana*. JA-Ile promotes this interaction thereby enabling defence gene expression. (Adapted from (47)). (a) unstressed state in the absence of JA and (b) stressed state (eg. herbivory) in the presence of JA.

As described, plant hormones like JA and its derivatives play a prominent role in initiating plant defence responses. Although knowledge on the regulation of defences by jasmonates are available, some fundamental questions such as spatiotemporal distribution and evolutionary origin of these defences remain to be explored. For instance, in addition to evolving new defence strategies to overcome coevolving herbivore pressure, plants also must cope up with the fitness cost associated with phenotypically plastic traits like the induced indirect defences (10, 11). These defensive responses incur cost because in order to express these, plants must channelize resources from vegetative and reproductive growth (10, 11). Therefore, to achieve an optimal strategy, it is essential that the benefits of the defence are weighed against its cost. Given the conditions of limited resources and herbivore attack, the question is therefore not only how much should a plant invest into defence but more importantly how should these defences be distributed within a plant? Furthermore, abiotic factors impact plant defences to a great extent. For example, both EFN secretion and VOC
emission are influenced by light, water and seasonal variations (17-28). However, the interaction of abiotic factors with hormone signaling pathways, which regulate the expression of these defences, is not clearly understood. It is also worth mentioning that most of the studies on plant indirect defences have focused on higher plants like cotton, tomato, tobacco, and others (19, 26-31). Do primitive plants also feature these indirect defences? Very little information is available about lower plants in this respect, studying of which could provide insights into the evolution of indirect defence strategies in plants. The present work explores these questions by analyzing the spatiotemporal dynamics of indirect defences, regulation of floral nectar by jasmonates and also investigates the volatile emission from primitive fern species.

Aim of this thesis

The aim of the present work is to understand the spatiotemporal patterns of EFN secretion and VOC emission, interaction of jasmonate signalling with abiotic factors and to trace back the evolutionary origin of this jasmonate mediated indirect defences. Many pioneering reports have established that in addition to herbivory or damage, exogenous application of jasmonates can also up-regulate indirect defences in many different plant species and benefit the plants expressing these traits (33, 51, 52). Based on these studies, the present work employs an integrative approach to understand the overarching topic of jasmonate mediated regulation of nectar secretion (both EFN and floral) and VOC emission in plants. In details, the following questions were addressed mainly from a phytocentric perspective:

1. How is EFN secretion and VOC emission allocated within a plant? Are all plant parts equally defended? Using *Phaseolus lunatus* and *Ricinus communis* as study systems, these questions were answered.

2. EFN secretion is regulated by jasmonates; does the same apply to the secretion of floral nectar? This was investigated in *Brassica napus,* a close relative of *Arabidopsis thaliana,* in order to find parallels and differences in the respective signalling pathways.

3. What is the role of abiotic factors such as light quality and quantity in jasmonate-mediated EFN secretion? How does the light environment interact with signal transduction pathways? Using *P. lunatus* as the study system, the effect of changing light environment (day/night, quantity and quality) upon jasmonate-controlled EFN secretion was investigated.

4. JA-mediated regulation of plant indirect defences has been mostly studied in more derived, higher plant systems. Do ancient plants also emit volatiles in response to herbivory and jasmonates? These questions were tackled by studying volatile emission in *Pteridium aquilinum*, a primitive fern species.

Study systems- a brief description

Phaseolus lunatus (Fabaceae), Lima bean

Lima bean is a legume originating from central and South America and is of Andean or Mesoamerican genotype (53-55). The variety used in the current study is 'Jackson Wonder Bush', which belongs to the Mesoamerican genotype (56,57). Seeds collected from wild lima bean plants growing in the coastal area near Puerto Escondido in the state of Oaxaca, Mexico, were cultivated in the greenhouse for the present study. Wild forms of lima bean are self-compatible annuals or short-living perennials with mixed mating systems (58). Lima bean possesses extrafloral nectaries on its bracts at the stipules of the trifoliate leaves as well as at the petioles of individual leaflets (Fig. 3) (59). In addition, lima bean emits significantly increased amounts of about 12 different major VOCs after herbivore damage (32, 60). Both

defences are also inducible by exogenous jasmonate application; the VOCs blend is similar but not exactly identical to herbivore damage (61, 62). Lima bean is an attractive system to study regulatory mechanisms of indirect defences as it has both EF nectaries and emits VOCs. Only few other plants are known for which both these defences are described (63-65). Hence, lima bean is a suitable study system for investigating spatio-temporal variation of these indirect defences.

Brassica napus (Brassicacea), oilseed rape or canola

Canola originated from spontaneous hybridizations between turnip rape (*B. rapa* L; 2n = 20) and cabbage (*B. olearacea* L.;2n = 18) (66). The primary location of oilseed rape is believed to be in the Mediterranean region because both wild turnip and cabbage originated there (67). Canola was cultivated by ancient civilizations in Asia with early use recorded as early as 2000 BC in India and has been grown in Europe since the 13th century (67). Today, oilseed rape (Brassica and related species) is the second largest oilseed crop in the world providing 13% of the world supply (68). Rapeseed cultivars are classified as winter or spring types according to their vernalisation requirement to induce flowering (69). Winter rape 'Dwarf Essex' variety was used for the present work, which requires 6-8 weeks of vernalization (3-4 °C) to induce flowering. For this reason, in Europe, the winter variety oilseed rape are usually sown in early autumn and harvested late in the following summer (70). *B. napus* is important both as oilseed and honey crop (71). The flowers of *B. napus* are very attractive to honeybees, which ensure cross-pollination, while collecting pollen and nectar (72-74). Given the economical importance of *B. napus*, this was chosen as a study system to investigate the role of jasmonates on floral nectar secretion, which is a primary reward for the pollinators.

Pteridium aquilinum (L. Kuhn), Dennstaedtiaceae, bracken fern

Bracken fern has worldwide distribution throughout the tropical and temperate regions and is absent only in the arctic regions and tropical central America, making it one of the world's most widespread plant species (75, 76). It occurs frequently in the form of long-lived clones and has an invasive capacity due to its extensive rhizome system (77). Rhizomes are the main carbohydrate storage organs, which anchor the bracken to the soil (78). Bracken overwinters as underground rhizomes and this subterranean reserve is largely responsible for the persistence and rapid rate of vegetative encroachment of bracken by production of allelopathic chemicals (79, 80). Bracken is the most ancient plant, which possesses EF nectaries (see Fig. 3) (81) although no ecological benefits of EFN secretion has been shown

so far (81,82).Evolutionarily, bracken is not only very successful, but also is one of the oldest ferns with fossil records extending back to 55 million years (76). Further, the intense phytochemicals, present in the bracken have partly contributed to its evolutionary success (83, 84). These characteristics make bracken as a suitable study system for understanding the evolutionary origin of induced indirect defences.

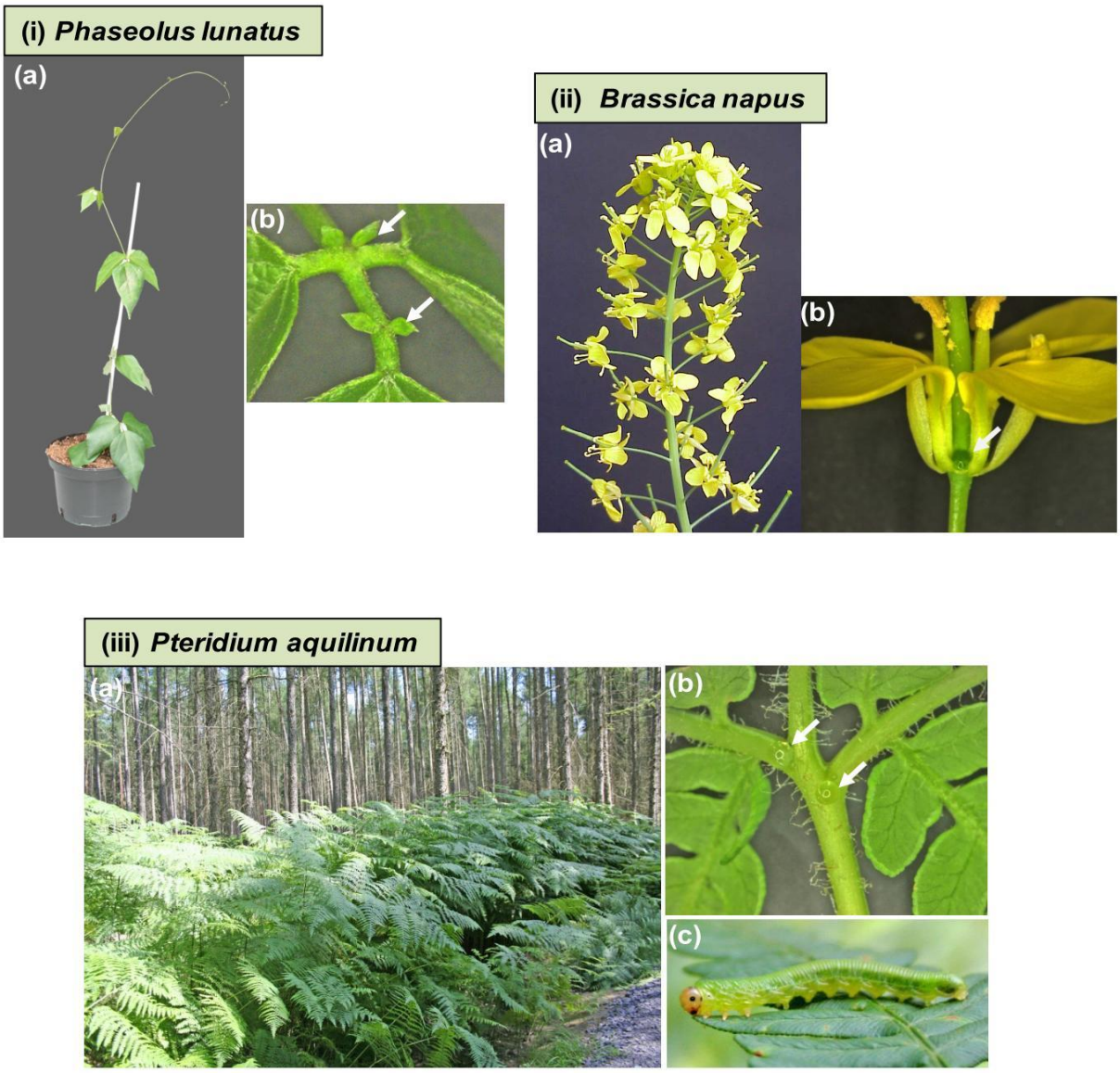

Figure 3. Study systems of the present work. (i) (a) *Phaseolus lunatus* (b) extrafloral nectaries (ii) (a) *Brassica napus* flowers (b) close–up of floral nectary (iii) (a) Natural growing site of *Pteridium aquilinum,* a forest about 15 km from Jena ,Germany (50°45`45.05``N and 11°40`34.85``E) (b) extrafloral nectaries of *P. aquilinum* (c) *Strongylogaster multifasciata* larva(Tenthredinidae, sawfly), a specialist herbivore of *P. aquilinum* found in its natural growing site. The arrows indicate nectaries.

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

Manuscript I

Testing the optimal defence hypothesis for two indirect defences: secretion of extrafloral nectar and emission of volatile organic compounds

Venkatesan Radhika, Christian Kost, Stefan Bartram, Martin Heil, Wilhelm Boland*

Planta, 2008, 228: 449-457

This manuscript analyzes the spatial variation in the allocation of two indirect defences, EFN and emission of VOCs in the light of the 'optimal defence hypothesis'. The results of this study show that the allocation of these indirect defences within a plant reflects the fitness value of the respective plant parts, younger leaves are better defended than older leaves. Further, the photosynthetic rate was found to increase with leaf age and pulse-labeling experiments were conducted to investigate the within-plant transport of photosynthetic assimilates. These experiments suggested transport of carbon from older to younger leaves, demonstrating that plants channel their resources optimally to maximize fitness.

I was responsible for experimental work, data evaluation and statistical analysis. Dr. Christian Kost helped with the experimental design and statistical analysis. Prof. Wilhelm Boland and Dr. Martin Heil contributed to designing of the experiments and analyzing the results. Stefan Bartram analyzed the samples of the labeling experiment with isotope ratio mass spectrometer. The first draft of the manuscript was written by me, which was corrected and refined by Dr. Christian Kost, Dr. Martin Heil and Prof. Wilhelm Boland.

Manuscript II

The role of jasmonates in floral nectar secretion

Venkatesan Radhika, Christian Kost, Wilhelm Boland and Martin Heil*

PlosOne, 2010, 5: (2) e9265

This manuscript describes the regulation of floral nectar secretion in *Brassica napus* by jasmonates. The results of this study show that the secretion of floral nectar, similar to its counterpart extrafloral nectar, is regulated by jasmonates. Blocking the jasmonate pathway led to a reduced production of floral nectar, which could be restored by exogenous application of jasmonates. Furthermore, the floral nectar secretion was not affected by leaf damage or herbivory, indicating a functional separation of defence signaling and reproductive nectar secretion.

I was responsible for planning and conducting the experiments as well as data analysis. Dr. Martin Heil conceived the idea and, together with Dr. Christian Kost, helped in experimental design. I wrote the first draft of the paper, which was modified after suggestions of Dr. Christian Kost, Dr Martin Heil and Prof. Wilhelm Boland.

Manuscript III

Orchestration of extrafloral nectar secretion by light *via* jasmonates

Venkatesan Radhika, Christian Kost, Axel Mithöfer, Wilhelm Boland*

Proceedings of the National Academy of Sciences, in preparation

This manuscript presents the effect of jasmonates on EFN secretion in different light environments. It was found that depending on the amount of light available, the response towards exogenous JA can change in terms of EFN secretion. Interestingly, JA induced EFN secretion during the light phase, whereas it suppressed EFN secretion during the dark phase. JA-Ile, on the other hand, induced EFN secretion in light but did not reduce EFN secretion during dark phase. Light quality in terms of changes in red (R): far-red (FR) light also influenced EFN secretion in response to jasmonate treatment. Analysis of endogenous levels of these phytohormones and inhibiting Ile biosynthesis in light phase revealed that probably JA-Ile is the active signal for regulation of this defense, whose formation might be light dependent.

I had the initial idea of measuring EFN under different light regimes. The experiments were designed by all coauthors. Performance of experiments and data evaluation was done by me. The first draft of the manuscript was written by me, and later refined and modified by Dr. Christian Kost, Dr. Axel Mithöfer and Prof. Wilhelm Boland.

Manuscript IV

Towards elucidating the differential regulation of floral and extrafloral nectar secretion

Venkatesan Radhika, Christian Kost, Wilhelm Boland and Martin Heil*

Invited article addendum, *Plant Signaling & Behavior, July 2010, Volume 5, Issue 7.*

This manuscript presents a comparison of floral and extrafloral nectar secretion in terms of ecological functions and the controlling signal cascades. Although these two nectar secretions serve different ecological roles in plants, one for defence (extrafloral) and the other for attraction of pollinators (floral), several similarities in the evolution and regulation of these secretions are dealt in this addendum. The article thus summarizes the current knowledge on these two types of nectar secretions focussing on hormonal regulation of nectar secretion. I was responsible for collection of references and writing the manuscript, which was corrected and modified by all the co-authors.

Manuscript V

Volatile emission in bracken fern (*Pteridium aquilinum*) is triggered by jasmonates but not herbivory – missing link or function?

Venkatesan Radhika, Christian Kost, Gustavo Bonaventure, Anja David, Wilhelm Boland*

Planta, submitted

This manuscript analyzes the volatile emission from the primitive bracken fern to understand the evolutionary origin of this defence in plants. Interestingly, volatiles could be induced by jasmonic acid and its derivatives, in the same way as it is known for higher plants. However, very low or negligible volatiles were emitted upon mechanical damage or herbivory by both generalist and specialist herbivores, which is in contrast to what is known from higher plants. Our results demonstrate that in ancient plants like bracken, the regulatory link between jasmonate signaling and volatile emission is missing which indicates a missing function or subsequent evolution of volatiles as an indirect defence strategy.

I was responsible for the experimental work including field collection of plants and insects, data evaluation and statistical analysis. Dr. Christian Kost and Prof. Wilhelm Boland helped in designing all the experiments and data analysis. Dr. Gustavo Bonaventure helped me in oxylipin analysis and manuscript correction. Anja David helped in field work of collecting the herbivores and volatile collection. The manuscript was written by me, modified and refined by Dr. Christian Kost, Dr. Gustavo Bonaventure and Prof. Wilhelm Boland.

Manuscript I

Testing the optimal defence hypothesis for two indirect defences:secretion of extrafloral nectar and emission of volatile organic compounds

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ORGINAL ARTICLE

Testing the optimal defence hypothesis for two indirect defences: extrafloral nectar and volatile organic compounds

Venkatesan Radhika · Christian Kost · Stefan Bartram · Martin Heil · Wilhelm Boland

Received: 16 December 2007 / Accepted: 2 May 2008 / Published online: 21 May 2008

Abstract Many plants respond to herbivory with an increased production of extrafloral nectar (EFN) and/or volatile organic compounds (VOCs) to attract predatory arthropods as an indirect defensive strategy. In this study, we tested whether these two indirect defences fit the optimal defence hypothesis (ODH), which predicts the within-plant allocation of anti-herbivore defences according to trade-offs between growth and defence. Using jasmonic acid-induced plants of *Phaseolus lunatus* and *Ricinus communis*, we tested whether the within-plant distribution pattern of these two indirect defences reflects the fitness value of the respective plant parts. Furthermore, we quantified photosynthetic rates and followed the within-plant transport of assimilates with ^{13}C labelling experiments. EFN secretion and VOC emission were highest in younger leaves. Moreover, the photosynthetic rate increased with leaf age, and pulse-labelling experiments suggested transport of carbon to younger leaves. Our results demonstrate that the ODH can explain the within-plant allocation pattern of both indirect defences studied.

Keywords Extrafloral nectar · Indirect defence · Ontogeny · Optimal defence hypothesis · Volatile organic compounds

Abbreviations

DMNT	(*E*)-4,8-dimethylnona-3,5,7-triene
EFN	Extrafloral nectar
IAEA	International Atomic Energy Agency
IRMS	Isotope ratio mass spectrometry
LSD	Least significant difference
JA	Jasmonic acid
MeSA	Methyl salicylate
ODH	Optimal defence hypothesis
PAR	Photosynthetic active radiation
PET	Polyethyleneterephthalate
TMTT	(*E*,*E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene
VBDB	Vienna Peedee belemnite
VOC	Volatile organic compounds

Introduction

Herbivores exert an immense selection pressure on plants, and the resulting arms-race has led to the evolution of an enormous variety of plant defences against herbivores (Walling 2000; Kessler and Baldwin 2002). Defences, which directly target the performance or survival of the herbivore, are generally referred to as '*direct*' defences. In contrast, plant traits that do not directly affect the herbivore but rather function via the attraction, nourishment or housing of predatory organisms, thereby increasing the predation pressure on herbivores, are termed '*indirect*' defences (Heil 2008). These plant defences, albeit often significantly contributing to the plant's ecological success, do however, not come without fitness costs (Gulmon and Mooney 1986).

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From an evolutionary perspective, any organism should respond to the resulting trade-offs in a way that maximises its reproductive output and minimises any investment in non-reproductive traits—even if they are essential for its survival. One example for such an evolutionary optimisation response that is generally regarded as a cost-saving strategy are herbivore-induced plant defences, which are activated only in case of an herbivore attack (Karban and Baldwin 1997; Dicke and Hilker 2003). The drawback of inducible defences, however, is the lag-time, which is the time required for the induction of the defence after the first contact with the herbivore, during which the plant remains vulnerable (Heil and Baldwin 2002; Zangerl 2003). Since most plant defences are neither consistently expressed throughout a plant's life nor evenly distributed within a plant (Zangerl and Rutledge 1996), several hypotheses have been suggested to predict their phenotypic variation depending on environmental or genetic factors (Karban and Baldwin 1997; Herms and Mattson 1992; Stamp 2003).

The optimal defence hypothesis (ODH) states that organisms evolved to allocate their defences in a way that maximises fitness (McKey 1974, 1979; Rhoades 1979). The underlying assumption is that defence is costly and thus, the spatio-temporal patterns of an adaptive defence allocation among plant parts should reflect the fitness-value of these organs (McKey 1974, 1979). In other words, the theoretical expectations of the ODH are that within a plant, young, still developing leaves should be better defended than older leaves.

However, physiological constraints may operate on plants, thereby causing them to deviate from these theoretical predictions. Empirical tests of the ODH are therefore required and many validating reports of this theory are indeed known for direct defences (Zangerl and Rutledge 1996; Ohnmeiss and Baldwin 2000; Barto and Cipollini 2005). Very little information, however, is available on the allocation pattern of indirect defensive strategies of plants.

This study aims at testing the predictions made by the ODH for two particularly widespread indirect defence traits: extrafloral nectar (EFN) and volatile organic compounds (VOCs), which are both involved in mediating the interaction between herbivore-damaged plants and members of the third trophic level (Arimura et al. 2005; Heil 2008). By offering EFN as a carbohydrate-rich reward (Bentley 1977; Koptur 1992) or by emitting VOCs that indicate the increased presence of potential prey to predators and parasitoids (Turlings et al. 1990; Pare and Tumlinson 1997), plants defend themselves indirectly against herbivores. Both EFN and VOCs are inducible traits, i.e. their production rate increases in response to herbivory or mechanical damage and this response is known to be regulated by the octadecanoid pathway, in which the phytohormone jasmonic acid (JA) plays a key role (Hopke et al. 1994; Heil et al. 2001). Exogenous treatment of plants with JA results in increased production rates of both EFN and VOCs, which closely resemble the plant's response induced by herbivore feeding in terms of quality and quantity (Dicke et al. 1999; Heil 2004).

We used lima bean (*Phaseolus lunatus* L., Fabacecae) and castor (*Ricinus communis* L., Euphorbiaceae) as experimental systems. Both plants bear extrafloral nectaries at the petioles of their leaves. In addition, lima bean releases VOCs after herbivory or when treated with JA that attract, e.g. carnivorous mites or parasitoid wasps under laboratory conditions (Dicke et al. 1999). At its natural growing site, JA-mediated EFN secretion has been shown to benefit the plant (Heil 2004; Kost and Heil 2005, 2008). In *R. communis*, herbivore or mechanical damage is known to increase EFN production (Wäckers et al. 2001). In the present investigation, we used JA to induce the production of EFN (both species) and VOCs (lima bean only) and tested the following predictions, which are derived from the ODH:

1. Both constitutive (i.e. untreated) and induced levels of EFN secretion and VOC emission are higher in younger leaves.
2. The ontogenetic pattern of indirect defence production (both EFN and VOCs) cannot be explained solely by the photosynthetic rate of the respective leaves.
3. Allocation of these defences to younger leaves is mediated by transporting newly assimilated carbohydrates from older source to younger sink leaves.

Materials and methods

Plant material and growth conditions

Plants of *P. lunatus* L. (lima bean) were cultivated from seeds derived from a native population growing in the coastal area near Puerto Escondido in the state of Oaxaca, Mexico. The parental plants have been used previously in field experiments on indirect plant defences (Heil 2004; Kost and Heil 2005, 2008). *Ricinus communis* L. plants (castor oil plant) were grown from seeds (Weber Seeds, Römhild, Germany) harvested from greenhouse-grown plants. Growing conditions were 20–22°C, 30–55% humidity during a 16 h photoperiod. Experiments were performed with 4-week-old plants (i.e. 5–6 leaf stage for *P. lunatus* and 4 leaf stage for *R. communis*). To study the ontogenetic pattern, both plants were grown in Klasmann clay substrate (Klasmann-Deilmann, Geeste, Germany). All experiments were performed in the greenhouse.

Numbering of leaves was based on their age as assessed by their insertion order into the main shoot. In *P. lunatus*, leaf 1 was the youngest, still unfolding leaf, leaves 2 and 3

were mostly unfolded, and leaves 4 and 5 were slightly to completely hardened leaves, respectively (Fig. 1a). The four leaves of *R. communis* were numbered accordingly (Fig. 1d).

Measurement of EFN secretion rates

To ensure that no nectar was present at the onset of the experiment, extrafloral nectaries were rinsed thoroughly with tap water and allowed to dry. EFN secretion was quantified one day after spraying either tap water (control treatment) or an aqueous solution of 1 mM JA (JA treatment) on all the leaves until runoff. Plants were treated twice at an interval of 30 min and after that leaves were allowed to dry for 1 h before plants were placed back into the greenhouse. The EFN produced after 24 h was quantified as the amount of secreted soluble solids (i.e. sugars and amino acids) using a temperature-compensated refractometer (ATAGO N-10E refractometer, Leo Kübler GmbH, Karlsruhe, Germany) as described by Heil et al. (2000, 2001). EFN was quantified as amount of soluble solids per dry weight of the secreting leaf material per 24 h.

Measurement of photosynthetic rate

The photosynthetic rate was measured with a portable open-mode photosynthesis system LI-6400 (LI-COR, Lincoln, NE, USA) using the 6400-15 *Arabidopsis* chamber on leaves attached to the plant. Measurements were taken between 9:00 a.m. and 2:00 p.m. using 360 µl l^{-1} CO_2 in the reference stream under approximately 900 µM m^{-2} s^{-1} PAR.

Collection and analysis of VOCs

The VOC emission as a function of leaf age in lima bean plants was measured by bagging all leaves individually in PET hoses (Toppits® 'Bratschlauch', Melitta, Minden,

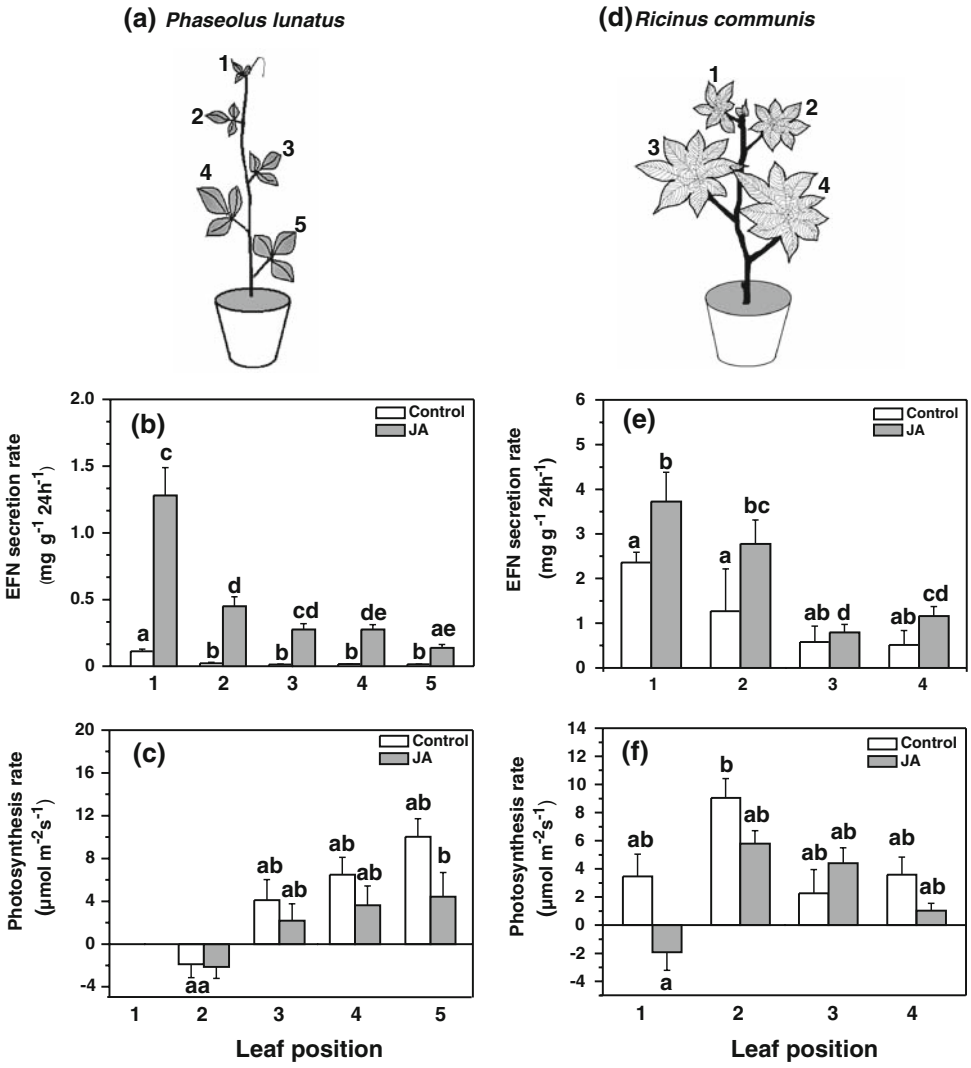

Fig. 1 **a** Numbering of differentially aged leaves, **b** ontogenetic variation of EFN secretion rate ($n = 9$), and **c** photosynthetic rate ($n = 6$), of untreated and jasmonic acid (*JA*)-induced *Phaseolus lunatus* plants. **d** Numbering of differentially aged leaves, **e** EFN secretion rate ($n = 7$), and **f** photosynthetic rate ($n = 7$) of untreated and JA-induced *R. communis* plants. EFN secretion rate is given in milligrams of soluble solids per g leaf dry weight per 24 h. The net photosynthetic rate is given as rates of CO_2 uptake in µmol m^{-2} s^{-1}. Due to the small size, the photosynthetic rate of the youngest leaf could not be measured in *P. lunatus*. *Different letters* denote significant differences between groups (global LSD post hoc for all factor combinations between leaf position and treatment after univariate ANOVA, $P < 0.05$). Data are presented as mean ± 95% CI

Germany) that do not emit detectable volatiles by themselves. VOCs emitted from each individual leaf were collected continuously for 24 h on charcoal traps (1.5 mg charcoal, Gränicher and Quartero, Daumazan sur Arize, France) by pulling air at about 500 ml min^{-1} using a 12 V vacuum pump (Gast Manufacturing, Benton Harbor, MI, USA). The traps were eluted with 2 × 20 µl of dichloromethane containing 200 ng $µl^{-1}$ of 1-bromodecane as an internal standard. The leaves were dried for dry weight determination. VOC samples were analysed on a Thermo Finnigan Trace GC-MS (Thermo, Bremen, Germany) equipped with a fused silica Alltech EC5 column (15 m × 0.25 mm internal diameter × 0.25 µm film thickness) using 1.5 ml min^{-1} helium as carrier gas. Separation was achieved under programmed conditions (45°C for 2 min, 10°C min^{-1} to 200°C, then 30°C min^{-1} to 280°C for 1 min; injector temperature: 220°C). MS analysis was performed on a TraceMS in electron impact full-scan mode at 70 eV with source temperature at 200°C and GC interface temperature at 280°C. Individual compounds were quantified with respect to the peak area of the internal standard and related to the dry weight of the leaf. The ten most dominantly emitted compounds, namely (*Z*)-3-hexenyl-acetate, (*E*)-*β*-ocimene, (*R*)-(−)-linalool, (*E*)-4,8-dimethylnona-1,3,7-triene (DMNT), (*E*)-2,6-dimethyloctatetraene ($C_{10}H_{14}$), methyl salicylate (MeSA), 2,6-dimethyl-3,5,7-octatriene-2-ol ($C_{10}H_{16}O$), *cis*-jasmone, (*E*)-*β*-caryophyllene, and (*E*,*E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT), were summed up to test for a putative effect of leaf position on total VOC emission.

Labelling experiment

In order to follow the internal transport of newly assimilated carbon, experiments were performed using synthetically premixed air containing $^{13}CO_2$ instead of $^{12}CO_2$ at a natural concentration of 380 ppm. In all cases, the air with $^{13}CO_2$ was purged for 24 h after induction with 1 mM JA solution. For each plant, each of five leaves were bagged individually in a PET hose (i.e. 'Bratschlauch', see above) and in each case, one of the five leaves was purged with labelled air, while all the other four leaves were purged with normal air. After 24 h, the ^{13}C content in the tissue of all five leaves as well as in the EFN secreted from this leaf was quantified using an isotope ratio mass spectrometer (IRMS). This procedure was applied to a total of eight replicates of four plants each, with one of the five leaves having experienced the $^{13}CO_2$-treatment until each leaf position within the four-plant group had received the ^{13}C treatment once. Due to technical reasons, we focussed this analysis on leaves in positions 1–3 and 5.

For IRMS measurements of EFN, nectar samples were filled in small 0.04 ml tin capsules for liquid samples (d: 3.5 mm, l: 5.5 mm; part. No. 184.9915.26, Lüdi AG, Flawil, Switzerland), dried in a desiccator filled with P_2O_5 as drying agent, and weighed before further analysis. For the solid leaf sample measurements, dried and powdered leaf material was weighed in 0.07 ml tin capsules (d: 4.0 mm, l: 6.0 mm; part. No. 176.1305.53, Lüdi AG). Capsules were sealed and combusted (oxidation at 1,020°C, reduction at 650°C) in a constant helium stream (80 ml min^{-1}) quantitatively to CO_2, N_2, and H_2O using an elemental analyzer (EuroEA CN2 dual, HEKAtech, Wegberg, Germany). After passing a water trap ($MgClO_4$), the gases were separated chromatographically at 85°C and transferred via an open split to a coupled isotope ratio mass spectrometer (IsoPrime, Micromass, Manchester, UK). Our laboratory working standard (acetanilide) has been calibrated on the VPDB scale using IAEA reference material, NBS 22, with a $\delta\ ^{13}C$ value of −29.78‰ (Werner and Brand 2001). All isotope ratios are given as $\delta\ ^{13}C$ values: $\delta\ ^{13}C\ (‰) = [(R_{sample}/R_{standard}) - 1] \times 10^3$, where R corresponds to the $^{13}C/^{12}C$ ratio of the sample and the standard.

Statistical analysis

All experiments were analysed with linear mixed-effect models with 'treatment' as fixed and 'plant individual' as random factor. Values of EFN secretion and total VOC emission have been log-transformed to meet the test assumptions of normality and homogeneity of variances. Global LSD post hoc tests were applied to the measured values for EFN secretion, VOC emission, and photosynthetic rates to test for between-group differences between all factor combinations of leaf position and treatment. All statistical analyses were performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). To control for multiple testing in comparing qualitative differences in the VOCs blend with leaf age, false-discovery rate (FDR) procedure was used.

Results

Ontogenetic pattern of EFN production

In both, *P. lunatus* and *R. communis,* the youngest leaf (i.e. leaf position 1) secreted the highest amount of EFN in undamaged controls as well as in JA-treated plants and the EFN secretion rate significantly decreased with leaf age (Fig. 1b, e, LSD post hoc test after univariate ANOVA: $P < 0.01$, $n = 9$ and 7, respectively). In case of lima bean, the mean amount of EFN secreted from the youngest leaf exceeded that of the oldest leaf about fivefold. In this species, the two youngest leaves secreted on average two times more EFN than the two older leaves. An analysis of the inducibility with respect to leaf age indicated that in both

plant species the youngest leaf did not only show the highest level of constitutive EFN secretion, but was also inducible to higher defence levels than the older leaves (LSD post hoc test after univariate ANOVA, $P < 0.05$, $n = 9$ in lima bean and $n = 7$ in *R. communis*).

Ontogenetic pattern of photosynthetic rate

Quantification of the photosynthetic rate indicated that younger leaves showed a lower gas exchange capacity than older ones in both control and JA-treated plants (Fig. 1c, f, LSD post-hoc test after univariate ANOVA: $P < 0.02$, $n = 6$ and 7, respectively), thus indicating a negative relation between EFN production and photosynthetic capacity. JA treatment did not significantly alter the photosynthetic rate in both plant species investigated (univariate ANOVA, $P > 0.05$, $n = 7$ in lima bean and $n = 6$ in *R. communis*).

Labelling experiment

Due to the absence of a positive relation between photosynthetic rate and EFN secretion (Fig. 1), we hypothesized that there should be a flow of photosynthates within the plant from older source to younger sink leaves. In labelling experiments with lima bean plants, in which one of five leaves was purged with artificial air containing $^{13}CO_2$ at 380 ppm while the other leaves were treated with natural air for 24 h, we measured the $^{13}C/^{12}C$-ratios of the EFN from each leaf (Fig. 2a) and the corresponding leaf tissue (Fig. 2b). After labelling leaf 1, no increased ^{13}C concentration in the tissues as well as EFN of the untreated leaves was observed. Treatment of leaves 2 and 3 showed for some replicates a clear, but for others only a slightly increased incorporation of ^{13}C into the younger leaves 1 or 1 and 2, respectively. In no case was a downstream transport, i.e., from the younger (1–3) to older (4–5) leaves, observed. The $\delta^{13}C$ values of downstream leaves were in all experiments close to the natural abundance level (−20 to −30‰; i.e. values of control plants). Labelling of leaf 5 led to a strong incorporation of ^{13}C in the tissues and the EFN of leaves 3, 2 and 1. The incorporation of ^{13}C in the leaf material was strongest in the most distal leaf number 1 and decreased continuously with increasing leaf age (i.e. the level of incorporation followed the leaf order $5^* \gg 1 > 2 > 3 > 4$, * = labelled leaf).

No increase of ^{13}C in the tissue and the EFN of leaf 4 could be detected, not even after treatment of leaf 5. In all experiments, the $\delta^{13}C$ values of leaf 4 were in the range of the natural abundance level. The amount of ^{13}C incorporated into EFN was on average about ninefold higher than that observed for the leaf tissue. Taken together, this experiment revealed a unidirectional transport of photosynthates from older source to younger sink leafs. Furthermore, no photosynthetic products were transported to leaf 4 and no transport occurred downstream to older leaves.

Ontogenetic pattern of VOC emission

Volatile organic compounds emitted from individual leaves were collected from uninduced controls and JA-treated lima bean plants and compared among leaf positions (Fig. 3). The total amount of VOCs released after induction from young leaves was significantly higher than the amounts emitted from older leaves (LSD post hoc test after univariate ANOVA, $P < 0.01$, $n = 8$). Constitutive VOC emission levels, however, were extremely low in leaf number 1 and virtually absent in all other leaves (Fig. 3). JA induction significantly increased the total VOC emission of leaves 1 and 2 over that of the older leaves 3, 4, and 5 (univariate ANOVA, $P < 0.01$, $n = 8$). Similar to our observation for the EFN secretion, the youngest lima bean leaf showed both the highest level of constitutive VOC emission and was inducible to higher levels than all the older leaves. Qualitative changes among differently aged leaves were observed in some of the main constituents of the emitted VOC blend (Fig. 3b, FDR-corrected univariate ANOVA: $P \leq 0.03$, $n = 8$). No significant difference was observed in levels of (*Z*)-*cis*-3-hexenyl acetate, MeSA, β-caryophyllene and TMTT emitted with leaf positions. In general, younger leaves (leaf positions 1–3) emitted more volatiles than the older leaves (leaf positions 4–5).

Discussion

The ODH predicts that the spatial allocation of defensive traits within a plant should favour more valuable and vulnerable plant parts (McKey 1974, 1979; Rhoades 1979). In line with these predictions, the young leaves of both lima bean and castor showed the highest level of the two indirect defences, EFN secretion and VOC emission (Figs. 1b, e, 3). Young leaves are generally important for future plant fitness since they already have caused high construction costs without having contributed very much yet to the plant's pool of photo-assimilates. Consequently, they have the highest future life span and can therefore be expected to contribute bulk to the prospective photosynthetic assimilation. Moreover, very young leaves usually still lack effective mechanical defences (Harper 1989) and indeed it has been shown for several plant species that young leaves, which are more nutritious (Slansky 1993), suffer more from herbivory than older ones within the same plant (Kursar and Coley 1991; Boege and Marquis 2006). Our results show that the young leaves are defended more, both before and after induction (Fig. 1). This observation is in line with the interpretation that EFN and VOCs are allocated based

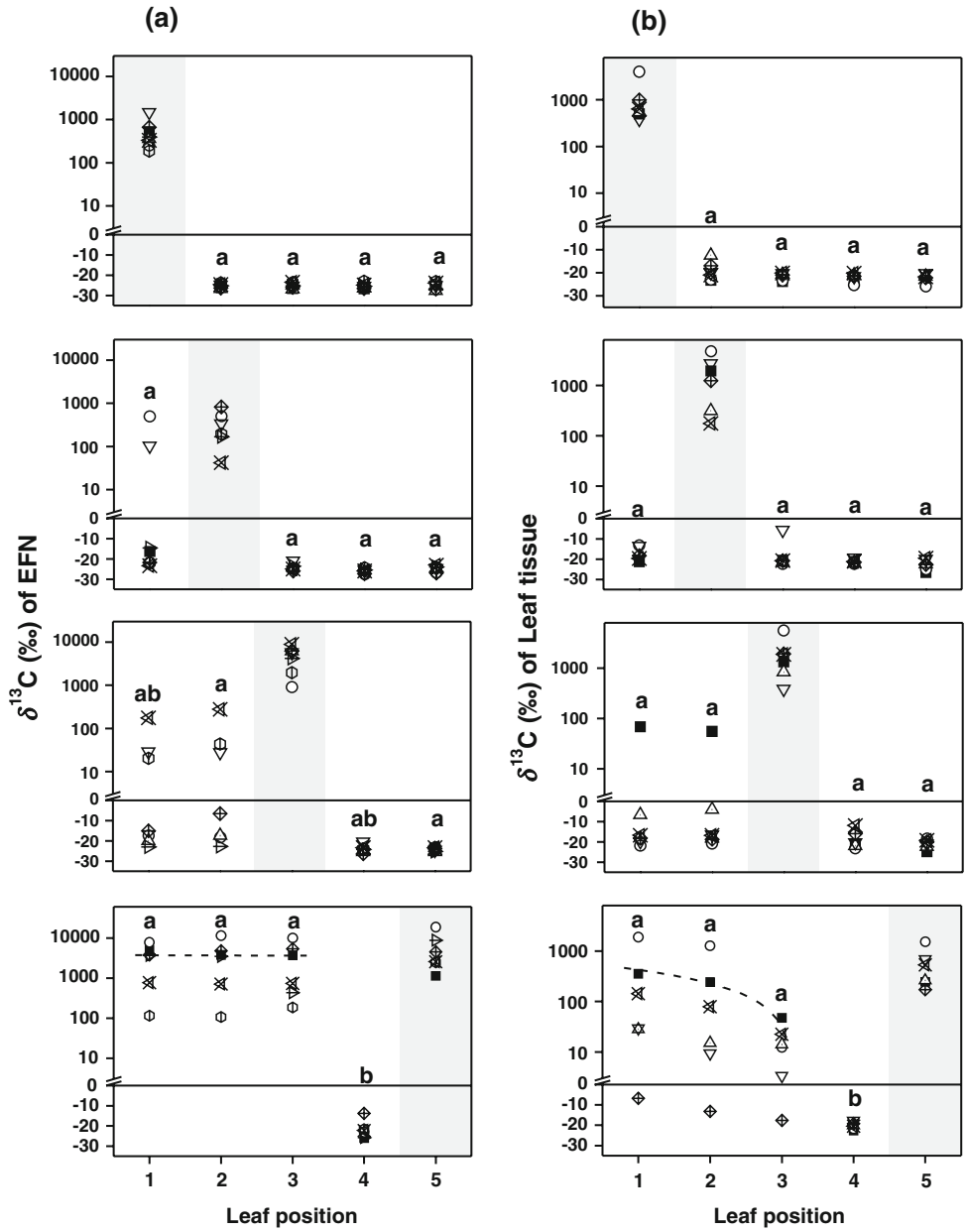

Fig. 2 Accumulation of ^{13}C in **a** EFN of lima bean plants when leaf position 1 ($n = 8$), 2, 3 ($n = 7$), and 5 ($n = 6$) were purged with air containing $^{13}CO_2$ (**a**), and leaf tissue ($n = 6$) when leaf positions 1, 2, 3 and 5 were purged with air containing $^{13}CO_2$ (**b**). Values are given as $\delta^{13}C$ (‰) with different *symbols* representing individual replicates. The *grey box* denotes the leaf position purged with air containing $^{13}CO_2$. The *dashed line* indicates the trend of the mean values of all replicates. *Different letters* indicate significant differences among leaf positions (LSD post hoc after univariate ANOVA, $P < 0.05$)

on the value and probability of attack of the leaves, as predicted by the ODH (Anderson and Agrell 2005). Also in the lima bean, which is a cyanogenic plant species, similar patterns have already been demonstrated for its direct defence, as young leaves were characterised by increased amounts of cyanide-containing precursors and higher capacities to release HCN per time unit than mature leaves (Ballhorn et al. 2005).

Our study lends support to previous findings where more valuable plant parts showed increased defence levels upon herbivore feeding (Heil et al. 2000; Wäckers and Bonifay 2004; Rostas and Eggert 2008). Furthermore, it is known that in myrmecophytes, ants preferably patrol and defend young leaves (Heil et al. 2001). In obligate ant-plants, however, this pattern is not necessarily caused by the spatial distribution of ant rewards (i.e. food bodies and EFN; Heil et al. 1997), but could also be achieved by special behavioural adaptations of the ants (Heil et al. 2004). In contrast, optimal distributions of defenders in facultative interactions with unspecialised animals require that the plants distribute the attractive traits accordingly (Downhower 1975; Heil et al. 2000).

Indeed, the overall emission rate of VOCs increased from young to older leaves, while the qualitative composition of the emitted blend changed only slightly. Both the quantitative and qualitative emission of VOCs have been shown to be highly variable depending on several interacting factors such as plant and herbivore species, type of damage (chewing vs. piercing-sucking) and abiotic factors

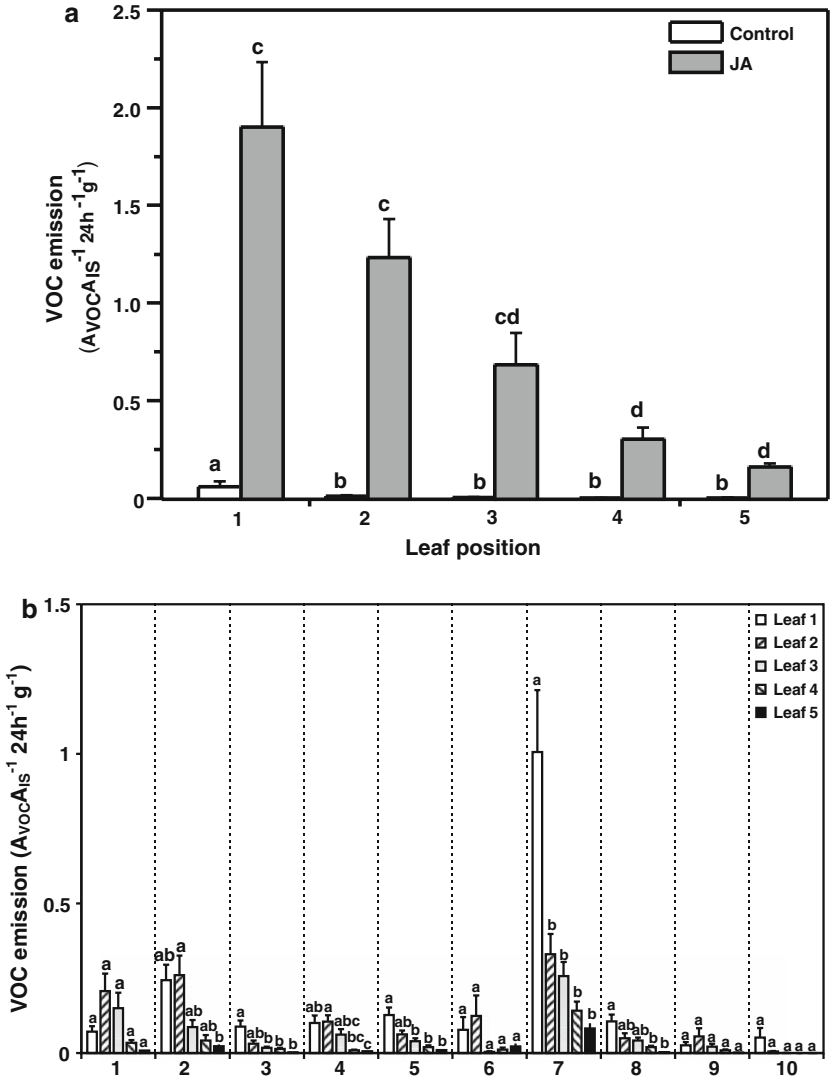

Fig. 3 **a** Ontogenetic variation of the total VOC emission (mean ± 95% CI) of lima bean plants ($n = 8$). The amount of emitted VOCs is given as peak area (A_{voc}) relative to the peak area of an internal standard (A_{IS}) per 24 h per g leaf dry weight. *Different letters* denote significant differences between groups (global LSD post hoc for all factor combinations between leaf position and treatment after univariate ANOVA, $P < 0.05$). **b** Mean (± 95% CI) relative amounts of volatiles emitted by JA-treated plants as determined by the ratio of peak area of the particular compound (A_{VOC}) to the peak area of the internal standard (A_{IS}) per 24 h per g dry weight. *Different letters* denote significant differences among leaf positions as determined with a FDR-corrected univariate ANOVA: $P \leq 0.03$, $n = 8$. *Tr* trace quantities (i.e. ≤0.005). Ten most dominant peaks of the TIC were chosen: *1* (3Z)-hexenyl acetate, *2* ocimene, *3* (*R*)-linalool, *4* DMNT, *5* $C_{10}H_{14}$, *6* methyl salicylate, *7* $C_{10}H_{16}O$, *8* *cis*-jasmone, *9* *β*-caryophyllene, *10* TMTT

like rainfall and light intensity (for review, see Arimura et al. 2005). Our finding that VOCs are emitted more from younger leaves could be interpreted as a strategy of a directional attraction of parasitoids or other arthropod predators to younger leaves (Hazarika et al. 2007)—a hypothesis that remains to be tested in future studies.

The consistent release patterns of EFN and VOCs give rise to the question whether both traits contribute equally to the plant's protection. Since it was recently shown for lima bean that VOCs act as airborne signals and induce EFN secretion in undamaged plant parts (Kost and Heil 2006; Heil and Silva Bueno 2007), an alternative scenario to the defense hypothesis could be that the primary function of the emitted VOCs is to induce EFN. In this case, parasitoids and other insect predators learning to associate increased VOC levels with an increased presence of herbivores could be a secondary function of the emitted VOCs.

Furthermore, the cost of these two indirect defences remains elusive, though VOCs have been estimated to cause low costs in corn plants (Hoballah et al. 2004). VOCs and EFN are carbon-based defences and thus might even compete for a common pool of metabolites. The amount of VOCs emitted ranges orders of magnitudes below the amount of carbohydrates that is secreted as EFN. In case of the lima bean for example, a young leaf emits only 1.9 ng/24 h g^{-1} dry weight of mainly carbon-based VOCs, while the same leaf secretes 1.3 mg EFN/24 h g^{-1} dry weight as sugars. It is thus likely that EFN accounts for higher metabolic costs than VOCs. However, further investigation is needed to fully understand the partitioning of plant metabolites for these two indirect defences and future studies must be directed to assess these costs and benefits of both VOC emission and EFN secretion under different herbivore pressures and inductive situations.

Despite being shaped by evolution as an adaptive response, the spatio-temporal distribution of defence traits within plants has to obey limitations in organ-wide or plant-wide resource availabilities. EFN and VOCs are primarily

carbon-based defences, and differences in photosynthetic C-assimilation among organs may thus also cause different production rates of these defensive traits. However, patterns in C-assimilation did not entirely match those observed for EFN and VOCs production, as older leaves were generally characterised by higher photosynthetic rates than younger leaves. On average, younger leaves showed a negative photosynthesis (Fig. 1c, f), i.e. respiration rate was higher than the rate of C-assimilation.

Leaf photosynthesis is the main source for the sugars secreted as EFN (Wardlaw 1990). Young, still developing leaves were characterised by low photosynthetic rates (Fig. 1c, f) and presumably had very low reserves for producing defensive compounds (Larson and Gordon 1969). Thus, they act as physiological sinks and import nutrients until they become competent enough to synthesize defence compounds on their own (Lalonde et al. 2004). Indeed, our ^{13}C labelling experiment in lima bean plants indicated a net transport of C assimilated by leaf 5 to younger leaves (1–3; Fig. 2) when all leaves were treated with JA. This result illustrates the transport of photosynthates within the plant from mature to young leaves, where protection is most essential. This finding is in line with previous studies showing that plants can metabolically reorganize in response to herbivory by reallocating resources to growing plant parts (Strauss and Agrawal 1999; Hui et al. 2003) as well as by making younger leaves stronger sinks for defensive metabolites (Arnold and Schultz 2002).

Transport of photosynthates depends on the vascular architecture, and studies have shown that the systemic induction of plant defences can depend on the way the leaves are connected by the vascular system (Davis et al. 1991; Orians et al. 2000; Schittko and Baldwin 2003; Orians 2005; Gomez and Stuefer 2006). In our study, we mimicked herbivory on a plant-wide level by spraying JA on all leaves. In this inductive situation, all the observed pattern could be explained with the ODH.

In summary, we have tested the predictions made by the ODH for two of the most widely distributed indirect plant defences, secretion of EFN and emission of VOCs. We have shown that the plant's induced defensive strategy involves channelling resources in a way that maximises the protection of its most valuable parts. This result is consistent with the ODH in that the youngest leaf, which is a greater contributor towards future plant fitness, enjoys higher defence levels by importing carbohydrates from older leaves. To our knowledge, this is the first report verifying that the within-plant distribution pattern of these two indirect defences does not simply reflect patterns of carbon assimilation, but actually represents an optimal defence strategy.

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Manuscript II

The role of jasmonates in floral nectar secretion

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The Role of Jasmonates in Floral Nectar Secretion

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Abstract

Plants produce nectar in their flowers as a reward for their pollinators and most of our crops depend on insect pollination, but little is known on the physiological control of nectar secretion. Jasmonates are well-known for their effects on senescence, the development and opening of flowers and on plant defences such as extrafloral nectar. Their role in floral nectar secretion has, however, not been explored so far. We investigated whether jasmonates have an influence on floral nectar secretion in oil-seed rape, *Brassica napus*. The floral tissues of this plant produced jasmonic acid (JA) endogenously, and JA concentrations peaked shortly before nectar secretion was highest. Exogenous application of JA to flowers induced nectar secretion, which was suppressed by treatment with phenidone, an inhibitor of JA synthesis. This effect could be reversed by additional application of JA. Jasmonoyl-isoleucine and its structural mimic coronalon also increased nectar secretion. Herbivory or addition of JA to the leaves did not have an effect on floral nectar secretion, demonstrating a functional separation of systemic defence signalling from reproductive nectar secretion. Jasmonates, which have been intensively studied in the context of herbivore defences and flower development, have a profound effect on floral nectar secretion and, thus, pollination efficiency in *B. napus*. Our results link floral nectar secretion to jasmonate signalling and thereby integrate the floral nectar secretion into the complex network of oxylipid-mediated developmental processes of plants.

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Introduction

Nectar is an aqueous plant secretion that mainly contains sugars and amino acids [1,2]. Many higher plants produce nectar in their flowers to attract insects or vertebrate pollinators, which transport pollen from one plant to another, thereby enabling outcrossing. Outcrossing contributes to the evolutionary success of angiosperms and lack of pollination often limits fruit yield [3]. Nectar rewards immensely influence pollinator behaviours such as visit frequency, number of flowers probed, probe time per flower, and also the movement of the pollinator after leaving the plant [4]. Flowers secreting more nectar are more successfully pollinated and higher levels of nectar may be one key to enhanced outcrossing in response to insect visitation [5]. Hence, floral nectar is involved in a highly important interaction among plants and animals. Despite these central ecological, evolutionary and economic functions, little is known on how plants control nectar secretion physiologically [6].

Variability in nectar secretion by environmental and physiological factors [7] and the dynamic regulation of nectar volume by reabsorption [8] and refilling of nectaries upon removal [9] have been reported [3]. Most recently, an extracellular invertase has been identified as a factor that is causally involved in nectar secretion in *Arabidopsis thaliana* flowers [10]. However, little is known about the hormonal regulation of floral nectar.

Here, we investigated whether jasmonates are involved in the control of flower nectar secretion. Jasmonates (term collectively used for all bioactive representatives of the jasmonate family) control central processes in plants such as root growth, defence, tendril coiling and reproduction [11,12]. In flowers, jasmonic acid (JA) plays multiple roles that are related to general developmental processes [13,14]. On the one hand, negative effects of jasmonate on flower opening and bud initiation have been reported for *Pharbitis nil* and *Nicotiana tabacum* [13,15]. On the other hand, JA appears to be necessary for pollen development and anther dehiscence in *Arabidopsis* [16]. Moreover, a tissue-specific synthesis of JA in flowers has been described [17–20]. Much less is known on the role of JA for nectar secretion. JA, its precursors and its derivatives orchestrate plant defence responses [12], including the secretion of extrafloral nectar [21,22], but their putative role in the regulation of floral nectar secretion has apparently never been considered.

To investigate whether floral nectar secretion is regulated via jasmonates, we used *Brassica napus* (canola or rapeseed) as experimental system. In this species, the nectar secretion is highest in fully-open flowers (Figure 1). *B. napus* is an important agricultural crop that attracts insect pollinators [23]. Nectar secretion has been shown to have positive effects on fruit ripening and seed germination rate, and it reduces the flowering period [24]. First, we investigated the relationship between ontogenetic changes in nectar secretion and endogenous JA levels. Assuming that the secretion of floral nectar secretion is affected by JA during flower development, we hypothesised that the temporal secretion pattern should correlate with the endogenous concentrations of JA in the flower tissue. We also predicted that any temporal changes in the JA content of the flowers should precede floral nectar secretion. Second, we exogenously applied to the flowers JA, the JA-amino acid conjugate jasmonoyl-isoleucine (JA-Ile), its mimic

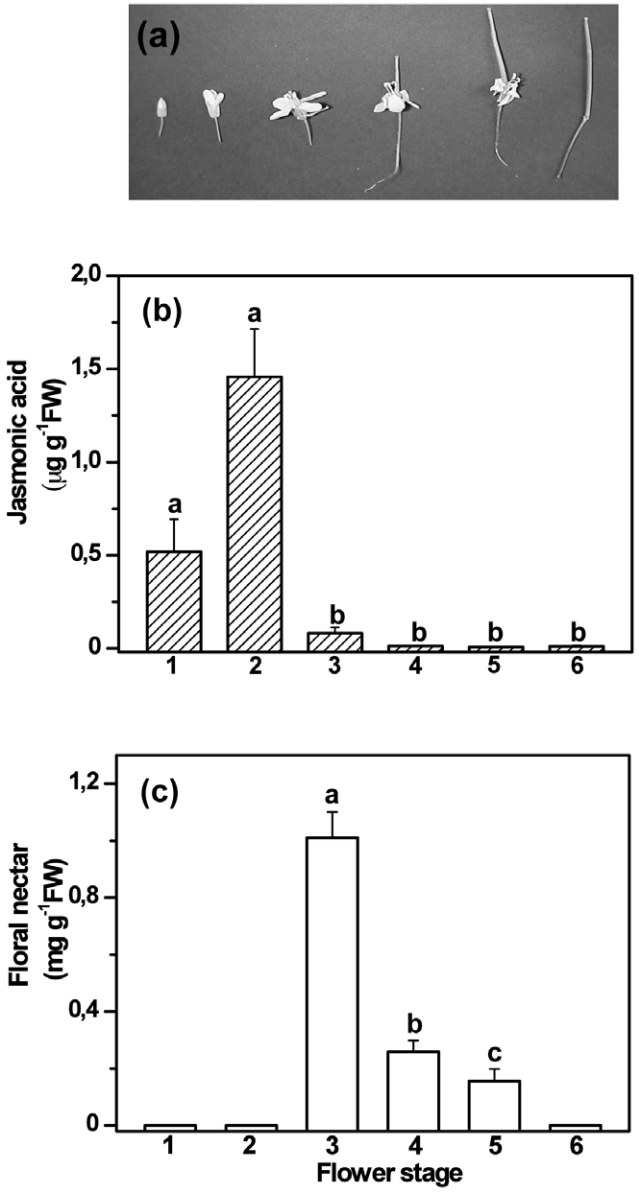

Figure 1. Ontogenetic changes of nectar secretion and endogenous JA in flower tissue. Panel A: Flower stages 1–6 as defined for the present study. Panel B: JA concentration (mean ± SE) is displayed in ng JA per g fresh mass. Different letters indicate significant differences among different stages (LSD post-hoc test after univariate ANOVA, $P<0.02$, $n = 5$). Panel C: Nectar secretion (mean ± SE) is given in mg soluble solids per g fresh mass of the flowers. Different letters indicate significant differences among stages (LSD post-hoc test after univariate ANOVA, $P<0.01$, $n = 10$). Only the flower stages with nectar secretion (3–5) were included in the post-hoc test in order to avoid inhomogeneity of variances due to zero-production in stages 1, 2 and 6.
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coronalon and phenidone (an inhibitor of endogenous JA synthesis). We predicted that application of JA or its mimics should induce EFN secretion, whereas phenidone should have an inhibitory effect. Finally, we investigated whether systemic, JA-dependent responses to leaf damage interfere with floral nectar secretion. Jasmonates are known to be systemically transported [21,25,26] and their application to – or induction in – leaves might therefore also affect floral nectar secretion. The results of our study represent a first step towards understanding the hormonal control of nectar secretion in flowers and its putative interference with other plant functions.

Results

Ontogenetic Changes in Nectar and Endogenous JA Levels

The developmental floral stages as defined for this study are presented in Fig 1. We classified the flowers morphologically into six stages starting from the very young bud (Stage 1) to the withered flower (Stage 6) as described in refs [27,37]. We distinguished the following six stages of flowers: stage 1 - loose bud, petals not expanded, stage 2 - corolla opening, beginning of anthers dehiscence, stage 3 - corolla fully expanded, full pollen exposure; stage 4 - corolla completely open after pollen exposure, stage 5 - shrivelled corolla, no pollen and stage 6 - withered corolla. Each flower remains open for about 3–4 days. Nectar secretion starts when the corolla is open in stage 2 and increases in the next stage when the corolla is fully expanded and the pollen is exposed and continues till stage 6 [37]. In our experiments, maximum amounts of nectar were produced when flowers were fully opened (stage 3, see Fig. 1, LSD post-hoc test after univariate ANOVA, $P<0.01$, $n = 10$). Endogenous JA levels showed a peak shortly before nectar secretion was highest (stage 2, see Fig. 1, LSD post-hoc test after univariate ANOVA, $P<0.02$, $n = 5$). The levels of endogenous OPDA (12-oxo-phytodienoic acid), the precursor of JA, were found to be approximately 25–50 ng per g fresh weight in stages 2, 3 and 4 and in the other stages of flower development the level of OPDA was lower than 20 ng.

Induction of Nectar by JA

Exogenous application of 1mM JA significantly increased nectar secretion after 24 h in comparison to control plants, which had been sprayed with water (Fig. 2a, LSD post-hoc test after univariate ANOVA, $P<0.01$, $n = 7$). Glucose and fructose were the major constituents of the nectar and the G:F ratio was in the range of 1.2–1.3 (Table 1). The sucrose concentrations were very low or undetectable. The nectar, thus, represents an hexose-dominated nectar according to the classification proposed by Baker & Baker [35]. No changes in nectar sugar composition were observed after JA treatment (Table 1). The effect of JA induction thus appears to be quantitative rather than qualitative. Next, we treated the flowers with phenidone, an inhibitor of lipoxygenases [38] that blocks endogenous JA synthesis. Phenidone treatment reduced nectar secretion to control levels after 24 h (Fig. 2a, LSD post-hoc after univariate ANOVA, $P<0.01$, $n = 7$), but high secretion rates could be restored by additional exogenous application of 1 mM JA following the phenidone treatment (Fig 2a). Application of phenidone did not lead to lower nectar levels than seen in control plants; hence attempts were made to treat plants with phenidone at early flowering stages (stage 1 or 2). However, this treatment led to delayed flower opening and not to a further decrease in nectar levels. Additionally, no significant reduction in the floral nectar secretion below control levels was observed when higher concentrations of phenidone (6 or 10 mM) were used.

JA Conjugates Induce Nectar Secretion

JA is transformed into a variety of metabolites such as methyl JA, hydroxyl JA and amino acid conjugates after its biosynthesis [12]. Recent reports on the jasmonate (ZIM) domain (JAZ) family of transcriptional repressors of jasmonate signaling have established that jasmonoyl isoleucine (JA-Ile) is a crucial regulatory signal for JA related responses [39–41]. In order to investigate

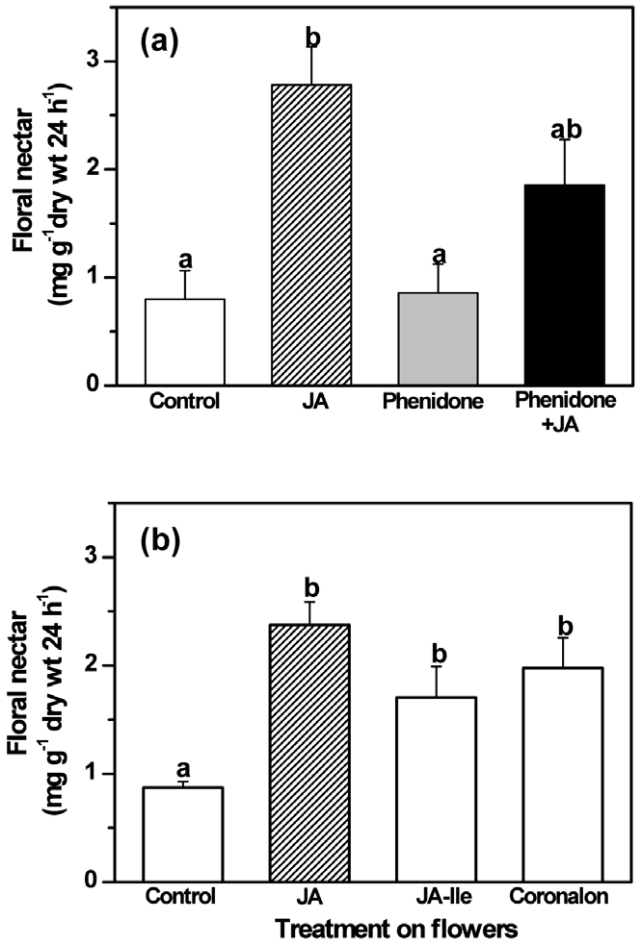

Figure 2. Changes in floral nectar secretion rate in response to different treatments. Panel A shows the consequences of an inhibition of *de novo* biosynthesis of JA. Different treatments (expected response in brackets) were: untreated (control levels), JA (increase), phenidone (reduced) and Phenidone + JA (restored). Nectar secretion rate (mean ± SE) is given as mg soluble solids per g dry mass of the flowers per 24 h. Panel B: Induction of nectar secretion with JA, JA-Ile and coronalon. Nectar secretion rate (mean ± SE) is given as mg soluble solids per g dry mass of the flowers per 24 h. Different letters indicate significant differences among treatments (LSD post-hoc test after univariate ANOVA, $P<0.01$, $n=7$ and 8, respectively).
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whether floral nectar secretion responds to known central regulatory factors of the octadecanoid signalling pathway, we treated the flower tissue with JA-Ile and its structural mimic coronalon [30,32]. Treatment with both JA-Ile and coronalon led to a significant increase in nectar secretion as compared to control plants (Fig 2b, LSD post hoc test after univariate ANOVA, $P<0.01$, $n=8$). There was no significant difference in the nectar production among the treatments with JA, JA-Ile and coronalon.

Signalling Conflicts between Anti-Herbivore Defence and Floral Nectar Secretion

To study whether systemic defence signalling interferes with the observed JA-mediated induction of floral nectar, we treated the leaves of *B. napus* with JA, mechanical damage and natural herbivores, treatments which are all known to increase endogenous JA levels [11,12,26]. No detectable effect on floral nectar secretion was observed when leaves of *B. napus* were subjected to

Table 1. Sugar composition of floral nectar after different treatments.

Treatment	Sugars (%)		G-F ratio
of leaves	**Glucose**	**Fructose**	
Tap water	56.6±5.8	43.3±4.8	1.3
JA	47.9±1.5	52.1±11.2	0.92
Mechanical damage	57.3±5.6	42.7±4.3	1.34
Specialist herbivore (*P.rapae*)	50.3±2.8	49.7±5.4	1.01
Generalist herbivore (*S. littoralis*)	56.7±5.6	43.2±5.5	1.31
of flowers			
Tap water	54.7±2.2	45.3±2.0	1.21
JA	55.9±3.7	44.1±3.0	1.27

Relative sugar concentration (mean ± SE) is given for 10 plant replicates. Nectar from 4–5 flowers per plant were pooled in all cases.
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application of JA, mechanical damage and leaf damage by generalist (*S. littoralis*) and specialist (*P. rapae*) herbivores (Fig. 3, LSD post-hoc test after univariate ANOVA, $P>0.05$, $n=10$). Even maximal herbivore damage afflicted by at least 2 larvae per every leaf did not affect nectar secretion in flowers. The nectar's sugar composition remained unchanged after all of these treatments (Table 1). Nectar was predominantly hexose-rich and the glucose:fructose ratio was 0.9–1.3, similar to the nectar composition that had been observed in the other experiments.

Discussion

As a first step to investigate whether the phytohormone jasmonic acid (JA) is involved in the secretion of floral nectar, we followed endogenous JA levels and the amounts of nectar secreted during flower ontogeny in *Brassica napus* plants. A burst of endogenous JA preceded the maximal nectar secretion, suggesting

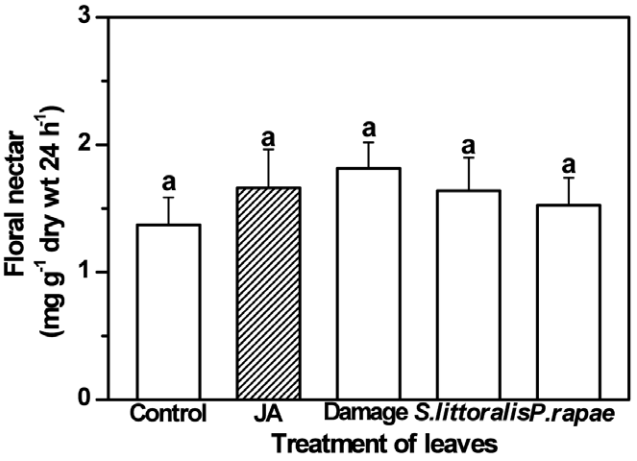

Figure 3. Nectar secretion rate in response to natural and mimicked leaf herbivory. Herbivory of leaves was mimicked by the exogenous application of JA, mechanical damage, or inflicted by either generalist (*Spodoptera littoralis*) or specialist (*Pieris rapae*) herbivores. Nectar secretion rate (mean ± SE) is given as mg soluble solids per g dry mass of the flowers per 24 h. No significant differences among treatments could be detected (LSD post-hoc test after univariate ANOVA, $P>0.05$ for all comparisons, $n=10$).
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that JA controls nectar secretion in flowers in the same way as it induces the secretion of defensive extrafloral nectar [22]. The observation that exogenous application of JA to the flowers of *B. napus* significantly increased the production rate of floral nectar corroborated this interpretation. When endogenous JA synthesis was inhibited at the stage of highest nectar secretion by application of phenidone, nectar secretion decreased to control levels. Phenidone only inhibits one early enzymatic step in the octadecanoid cascade [38] and thus reduces the *de novo* synthesis of endogenous JA, but it does not affect JA-concentrations that are already present in the tissue [22]. Our results indicate, therefore, that basal JA levels were sufficient to allow a background nectar production. Even higher concentrations of phenidone (up to 10 mM) did not significantly reduce nectar secretion further and high nectar secretion could be restored when JA was applied in addition to phenidone (Fig 2a). Both observations exclude a direct inhibitory effect of phenidone on nectar secretion and support a positive effect of JA or its derivatives on nectar secretion rates in *Brassica napus* flowers.

The endogenous JA level peaked in the flower stage 2 (Fig. 1), which precedes the stage with the highest nectar secretion (stage 3). Because JA is subject to natural turnover rates, blocking the *de novo* synthesis of JA using phenidone at earlier stages of flower development (stages 1 and 2) likely would have reduced the JA levels in the following stages even below the levels that ocurred in control plants. Unfortunately, applying phenidone to earlier stages of flowering such as stage 1 or 2 delayed or even ceased flower opening and was, thus, not feasible in the context of the present study. Jasmonic acid is a multifunctional growth regulator in plants that modulates many developmental processes [12] and has repeatedly been reported in the context of flower development. In *Arabidopsis thaliana*, flower development is linked to JA biosynthesis [34] as shown, for example by *coi1* mutants, which are defective in JA-signalling and male sterile [18]. The triple mutant *fad3fad7fad8* has also been shown to have an anther-dehiscence defective phenotype: this mutant lacks the fatty acid desaturase, which catalyses the removal of two hydrogen atoms from linolenic acid to generate the free linolenic acid, an important precursor for JA biosynthesis [19]. Recently Sanders *et al.* have reported a similar result in the mutant of DELAYED DEHISCENCE 1, that encodes an enzyme, 12-oxophytodienoate reductase, which catalyzes the formation of the JA-precursor OPDA [20]. Unfortunately, none of these studies reported nectar secretion rates, likely due to the small size of *Arabidopsis* flowers. Furthermore, far-red light inhibited flower opening in *Pharbitis nil* [13] and the same wavelength can inhibit the sensitivity of JA-regulated genes to jasmonates and thus, suppress their expression even when JA is present [42,43]. In a recent study on *Brassica napus*, exogenous application of MeJA at early stages of flower development affected flowering time, flower morphology and the number of open flowers [44]. Similarly, exogenous MeJA interfered with normal flower development in *Chenopodium rubrum* [45]. In our study, we found (i) that increased JA levels preceded the highest nectar secretion rate, (ii) that inhibiting endogenous JA synthesis at early stages of flower development negatively interfered with flower development and (iii) that exogenous JA at the stage of highest natural nectar secretion further increased secretion rates. All these observations are in line with our interpretation that JA at earlier flowering stages is essential for normal flower development and at later stages involved in the control of nectar secretion.

Are the increases in nectar secretion seen after elicitor treatment in our study within a natural range? Quantitative dose-response relationships were found in the induction of extrafloral nectar production in *Macaranga tanarius* plants that were sprayed with JA [22]. In our study, the concentration of elicitors was 1mM in all cases and the same concentration elicited responses within natural ranges when used to induce other species, whereas higher concentrations are known to have phytotoxic effects [46–49]. We, thus, conclude that the maximum rates of nectar secretion, which we observed in JA-treated flowers, were still within ranges that may also occur in nature.

Research on jasmonate signalling recently experienced a significant breakthrough with the discovery of a family of JAZ (jasmonate ZIM-domain) proteins [39,40]. Jasmonic acid does not directly induce gene activity, rather, the JA-amino acid conjugate jasmonoyl–isoleucine (JA-Ile, see ref [50] binds to the COI1 (coronatin-insensitive 1)-unit of an E3 ubiquitin ligase complex termed SCF^{COI1} (for Skip/Cullin/Fbox – COI1), which targets JAZ-proteins for ubiquitination and thus their rapid degradation [39]. When we treated the flowers with JA-Ile and its structural mimic coronalon, an increased nectar flow was observed. These results demonstrate that the signalling cascades, which control floral nectar secretion, are very similar to those involved in jasmonate-responsive gene expression in tomato and *Arabidopsis* [41,50].

Plants do not only interact with pollinators, but also with other insects, many of which are detrimental to the plant since they feed on plant tissue. One of the remarkable features of plant defences against these herbivores is that they are often inducible, with JA acting as the central signalling molecule. Considerable evidence exists to support the systemic induction of defence responses in plants when only certain plant parts are attacked [51] and recent data [26] support that jasmonates can move through phloem and xylem to induce defences in distant plant parts. Such a long-distance transport of JA or other jasmonates could cause signalling conflicts between leaves and flowers. Does, therefore, damaging the leaves of *B. napus* and the resulting release of jasmonates from damaged leaves interfere with the nectar secretion in flowers? Increasing nectar secretion in flowers in response to leaf herbivory would demand more resources to flowers, which could otherwise be allocated to leaf defences. On the other hand, decreasing nectar secretion would lower the chance of pollination, which becomes even more essential in time of leaf damage or stress. Recently, Bruinsma *et al* investigated effects of JA treatment on leaves of *B. nigra* upon pollinator preferences [49]. They observed no change in pollinator preference and rates of flower visitation, but saw a decreased nectar secretion in JA treated plants. In our case, we found no difference in floral nectar secretion with different treatments on leaves. However, in their study, Bruinsma *et al.* collected nectar after 2 days of treatment, a time span that possibly was enough to reduce photosynthetic activity that thereby result in a shortage of resources required for nectar production. In our study, there was no detectable effect on the floral nectar production by damage to the leaves in a 24 h time period. As it would be expected from an evolutionary point of view, defence signalling in response to leaf herbivory does not directly interfere with the regulation of floral nectar secretion.

Conclusions

One of the major links between pollinator behaviour and plant reproductive success or crop productivity is floral nectar, whose regulation is understudied. We demonstrate that floral nectar secretion is regulated by jasmonates, plant hormones that so far have been mainly discussed in the context of plant development and defence activation. Which physiological and genetic processes are involved in the jasmonate-responsive nectar secretion remains, however, to be elucidated. The changes that we observed were

quantitative, rather than qualitative ones. The jasmonate-mediated up-regulation of nectar secretion is, thus, unlikely to impair the attractiveness of nectar to pollinators, opening interesting perspectives for crops whose pollination is nectar-limited. We also found that induction of jasmonate-dependent defence responses in leaves did not directly interfere with floral nectar secretion. The mechanisms, however, by which plants achieve this highly important functional separation remain to be elucidated. Research on jasmonate signalling in plants has recently experienced major developments, and the finding of its role in the regulation of floral nectar secretion shows that important functions of jasmonates are still being discovered.

Materials and Methods

Plant Material and Induction of Flowers

Brassica napus (cv. Dwarf essex) plants were grown in Klasmann clay substrate (Klasmann-Deilmann, Geeste, Germany) under 16 h day conditions. The plants used for the experiments were 4–5 weeks old. The flowers of the plant under study have been divided into six developmental stages based on visual observation [27] as seen in Figure 1a. Each stage lasts for about 3–4 days. Nectaries of brassicacean plants are usually present in the filament bases between sepals and stamens. In *B. napus* flowers, four nectaries develop in a circle surrounding the base of the filaments [27,28], two of which are present at the inner side of the two short filaments and two at the outer side. The nectaries at the inner side are known as lateral nectaries and the ones on the outer side as median nectaries. The median nectaries are inactive or secrete very little nectar. In our study, we collected nectar from all the nectaries.

For all experiments with fully-opened flowers (stage 3), flowers that were open for 1d were used. An aqueous solution of 1 mM JA was sprayed on the flowers until run-off and the same amount of tap water was sprayed on control plants. The spraying was repeated after 30 min, and then the flowers were left to absorb for one hour. For phenidone (1-phenyl-3-pyrazolidinone) treatment, an aqueous solution of phenidone (2 mM, Sigma-Aldrich, Germany) was sprayed two times as described for JA. The same concentration inhibited endogenous JA synthesis without causing phytotoxicity in earlier studies [22,29]. 'Phenidone + JA' treated flowers received an additional spray of 1 mM JA two times after the final phenidone application. A similar procedure was used for other induction experiments with aqueous solutions of JA-Ile (1 mM) and coronalon (100 µM) [30,31]. JA-Ile and coronalon were synthesized according to literature procedures [30,32].

Rearing of Herbivores and Induction of Leaves

The generalist herbivore, *Spodoptera littoralis* Boisd. (Lepidoptera, Noctuidae) was reared at 22–24°C under 14–16 h photoperiod in plastic boxes and fed on artificial diet (500 g of ground white beans soaked overnight in 1.2 l water, 9 g vitamin C, 9 g paraben, 4 ml formalin and 75 g agar boiled in 1 l of water). The specialist herbivore, *Pieris rapae* was maintained on Brussels sprout plants (*Brassica oleracea* convar. *fruticosa* var. *gemifera* cv. Rosella) at 22°C under a 16 h photoperiod. Third-instar larvae of both herbivores were allowed to feed on all leaves of the experimental plant for 24 h by placing them in clip cages (~4.9 g, 56 mm diameter made of transparent plastic) with at least 2 larvae per cage. 'Damaged' leaves were wounded by puncturing all the leaves with a pattern wheel (approximately 100 holes per leaf). Similar to the treatment on flower tissues, JA (1 mM) and tap water (control) was sprayed on all leaves. All flowers were bagged in PET foil (Toppits® 'Bratschlauch', Melitta, Minden, Germany) to prevent direct induction of the flowers by any airborne cue that might be released from the leaves in response to these treatments.

Nectar Quantification

The concentration of floral nectar was measured immediately after collection using a temperature compensated refractometer (ATAGO N-10E refractometer, Leo Kübler GmbH, Karlsruhe, Germany) and the nectar volume was quantified using 5 µl microcapillaries as described in [33]. The nectar was quantified as amount of soluble solids per g dry weight of the secreting flower material per 24 h. All experiments were conducted in a climate-controlled greenhouse. Since nectar secretion was highest in the fully opened flowers, all experiments were conducted with flowers of this stage. Application of phenidone to flowers at earlier stages led to delayed or complete cessation of flower opening, probably because JA is a ubiquitous phytohormone involved in several processes, including flower development [17–20,34]. Therefore, the treatment was done to fully opened flowers only.

Nectar sugar composition was analysed by gas chromatography-mass spectrometry (GC-MS). Nectars were lyophilized and silylated using N-methyl-N(trimethylsilyl)-triflouroacetamide (MSTFA). 50µl of this reagent was added to nectar samples in 100 µl of dry pyridine and the mixture was heated to 60°C for 1 h for completion of the reaction. The silylated derivatives were analyzed by GC-MS. Sugar standards (Sigma-Aldrich, Germany) were prepared similarly and the chromatographic analysis was run twice for each sample. Samples were analyzed on a GC-Trace-MS (Thermo Finnigan) using a DB-5 column (15 m×0.25 mm×0.25 µm; AllTech, Unterhaching, Germany). The temperature program for the separation started with 40°C isothermal for 3 min followed by an increase to 120°C at a rate of 10°C min^{-1} for 2 min and then an increase by 7°C min^{-1} to 250°C. The split ratio was maintained at 1:10 with an inlet temperature of 220°C. Both glucose and fructose concentrations were determined and their relative proportions calculated [35].

Determination of Endogenous JA Levels

In order to compare differences in the levels of endogenous JA among various floral stages, flower tissues of approximately the same fresh weight from all 6 developmental stages (Fig. 1a) were collected and the phytohormone extracted. Endogenous concentrations of JA were quantified by GC-MS as its pentafluorobenzyl (PFB)-oxime using a Finnigan GCQ ion trap mass spectrometer (Thermoelectron, Bremen, Germany) following the procedure of Schulze *et al.* [36].

Statistical Analysis

All experiments were analysed with linear mixed-effect models with 'treatment' as fixed and 'plant individual' as random factor. LSD post-hoc tests were performed to test for between-group differences. The following variables were transformed (transformation given in brackets) to meet the assumptions of homogenous variance: endogenous JA (log x) and nectar induction experiment by JA-Ile and coronalon (1/x). All statistical analyses were performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA).

Author Contributions

Conceived and designed the experiments: VR CK WB MH. Performed the experiments: VR CK. Analyzed the data: VR CK. Wrote the paper: VR CK WB MH.

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Manuscript III

Orchestration of extrafloral nectar secretion by light *via* jasmonates

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Abstract

Plants perceive changes in their environment as cues to control and adjust their physiological responses accordingly to achieve fitness. Whether and how plants use such cues and orchestrate their defence responses against herbivores is still unclear. We addressed this question by studying the secretion of extrafloral nectar (EFN), an indirect defense mechanism against herbivory, which is regulated via the octadecanoid-signaling pathway. In lima bean (*Phaseolus lunatus*), a temporal pattern in EFN secretion was observed: plants constitutively produced high amounts of EFN at night as compared to day. Depending on the light environment, jasmonic acid (JA) treatment had different effects on EFN secretion: induction during light phase and suppression during the dark phase. In contrast, relative to control plants treatment with the isoleucine-JA-conjugate (JA-Ile), increased EFN secretion in light phase, yet did not change the secretion rate under dark conditions. In light-exposed plants, inhibition of Ile biosynthesis significantly decreased the EFN secretion, corroborating the hypothesis that probably JA-Ile is the actual signal. Moreover, methyl jasmonate, a derivative of JA in which the free acid moiety is unavailable for conjugation to JA, did neither induce EFN production under light nor supressed EFN secretion in dark conditions. Alterations of the light spectral quality (measured as ratio of red (R) to far-red (FR) radiation) strongly affected EFN secretion: exposure to 10:90 R:FR increased EFN secretion by JA-Ile treatment but not with JA. When exposed to FR only, plants treated with both JA and JA-Ile reduced EFN secretion rate whereas at 50:50 R:FR ratio, both JA and JA-Ile induced EFN secretion. We conlcude that plants temporally orchestrate EFN secretion and this regulation of EFN secretion is mediated by JA-Ile biosynthesis.

Key words

Extrafloral nectar, light, jasmonic acid, jasmonic acid-isoleucine, far red radiation, orchestration.

Introduction

In their natural environment, plants continuously experience daily (day/night) and seasonal environmental fluctuations, which provoke plastic, adaptive responses that allow plants to cope with these changes. Being sessile and obligate photoautotrophs, plants have evolved to anticipate predictable changes in the light environment and synchronize their physiological processes such as photosynthesis, stomatal movements and flowering to these changes (1). This intricate synchronization apparently involves overlap with the underlying signal transduction pathways and evidence supporting such crosstalk involving hormones like abscisic acid, auxin, cytokinins and brassinosteroids is well established (2, 3). Although modulation in hormonal biosynthesis could be implied as a consequence of such interactions, changes in responsiveness to hormonal treatment as a function of day/night cycle is understudied.

Light is most powerful and best characterized entrainment stimulus (4). Light not only delivers the energy to fuel a plant's metabolism during daytime, but also serves as a cue for the risk of herbivory, because it also strongly affects feeding patterns of herbivores (5, 6). Being able to integrate information from the abiotic environment and regulate its defense responses accordingly is considered a huge selective advantage given that the cue used accurately predicts the risk of herbivory (7). When attacked by herbivores, plants initiate defenses which can affect the attacking herbivore either directly (e.g. chemical defenses or physical barriers) or indirectly by attracting predatory insects (via e.g. the emission of volatile organic compounds or the secretion of extrafloral nectar) to the herbivore-attacked plants (8). All known inducible anti-herbivore defenses are regulated by jasmonic acid (JA) (9, 10), the key phytohormone of the octadecanoid-signaling pathway, known for its role in many plant processes, including responses to biotic and abiotic stresses (11-14). Recent reports on JAZ (jasmonate ZIM-domain) proteins as repressors, which are targeted for proteosomal degradation in response to jasmonates led to the discovey that JA-Ile is the active form of the hormone (12, 15-17). In *Arabidopsis,* JAR1 catalyzes the biochemical activation of JA *via* adenylation and subsequent conjugation with amino acids (18), which eventually activates the downstream defense responses (12). However, which abiotic factors actually regulate the jasmonate-mediated responses is yet not fully understood.

The link between jasmonate signaling and light environment has been studied extensively in the context of shade-avoidance and competition (19-22). Far red light (FR), detected by phytochromes, is the main signal that plants use to sense the presence of neighbors and to down-regulate anti-herbivore defenses (23) and many studies have reported

an interaction between JA and FR responses. For example, in *Arabidopsis*, it was shown that mutants devoid of the phytochrome chromophore were characterized by higher JA levels and a constitutive expression of JA-inducible genes (24). Even though it is known that jasmonate signaling is sensitive to changes in the light, the mechanistic bases of how exactly the light environment affects jasmonate-regulated stress responses are poorly understood.

Light signals can vary in quantity, quality, direction and duration, and any variation will affect photosynthetic efficiency. Since photosynthesis is also the main source of the building blocks required for the formation of defensive compounds, the availability of light should affect the production of such compounds via either suppression on a regulatory level or simply the shortage of the required precursors. This should hold true for mainly carbon-based defenses such as the secretion of extrafloral nectar (EFN). Extrafloral nectar is an aqueous solution that contains mainly sugars, secreted from specialized organs, the so-called nectaries (25). Lima bean (*Phaseolus lunatus* L., Fabaceae), the model system used in this study possesses extrafloral nectaries at the stipules of the trifoliate leaves as well as at the petioles of the individual leaflets (26). Previous studies have established that EFN secretion in lima bean is inducible in response to herbivory and acts as an effective defense against herbivores (27). Several studies discussing the anatomy, morphology, composition and defensive function of EFN secretion exist; however, the mechanism of regulation remains to be explored (8, 10, 28). Here, we investigated the functional relationship between EFN secretion, jasmonate signaling and light availability with the aim of understanding how plants orchestrate changes in light environments and this jasmonate-mediated indirect defense mechanism.

Results

Jasmonate responsiveness in terms of EFN secretion critically depends on light

Analyzing EFN secretion during a normal day-night cycle in lima bean, we observed that control plants exposed to a period of 16/ 8 light/ dark cycle (similar to natural conditions) secreted maximum EFN during the night (10 pm), whereas JA-treated plants secreted the maximum EFN in the morning (10 am) (Fig. 1) when exposed to the same light conditions. This observation gave us a first clue that depending on the time of treatment (day or night), plants secrete high or low EFN in response to JA. As a next step, to investigate the effect of prolonged light and dark conditions on jasmonate-controlled EFN secretion, lima bean plants treated with JA and its isoleucine conjugate, JA-Ile, were exposed for 24 h of complete darkness or light and the EFN secretion rate was measured. In general, EFN secretion was

enhanced in the 24 h dark compared to 24 h light conditions (Fig. 2) similar to the previous observation under normal day/night conditions (Fig. 1). Moreover, we found that in the dark, the rate of EFN secretion was significantly reduced in plants treated with JA, whereas the rate was unchanged in plants treated with JA-Ile, probably because already the control plants had reached the maximum EFN secretion rate which could not be increased any further (Fig. 2a). In light, on the other hand, the reduced rate of EFN secretion increased significantly in plants treated with both JA and JA-Ile (Fig. 2b). This result suggested that in untreated plants, in dark, the rate of EFN secretion is probably regulated by a signal other than jasmonates. However, the negative effect of JA upon rates of EFN secretion in plants exposed to prolonged darkness could be a consequence of the concentration applied. To verify this, the rates of EFN secretion were measured in plants treated with even lower concentrations of JA and exposed to prolonged dark period (Fig. S1). This experiment confirmed that concentrations of JA as low as 100 µM inhibited EFN secretion in plants exposed to the dark. From these results, we conclude that there is an underlying additional control of EFN secretion, which modulates jasmonate responsiveness as a function of light conditions.

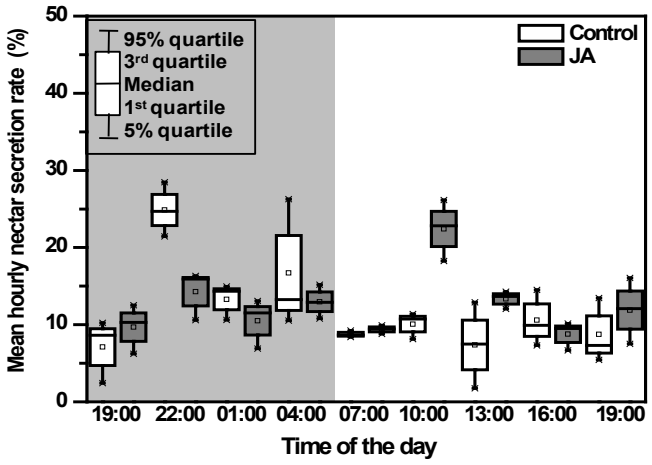

Figure 1 EFN secretion pattern in plants before and after JA treatment during 24h day/night cycle. EFN secretion rates were monitored every 3 h for 24 h in plants kept at 27.5 °C temperature and 65% humidity. After treatment, plants were exposed to 16/8 light/dark regime. Rates are expressed as percentage of total EFN secretion in plants measured in three independent experiments per treatment.

Induction of EFN secretion by jasmonates depends on light quality but not quantity

Light is the primary regulator of plant processes, and plants respond to changes in light both qualitatively and quantitatively. After establishing the differential effect of JA and JA-Ile upon EFN secretion in plants exposed to light and to dark, we asked whether this effect

depends on either the quantity of light to which plants are exposed or the light's spectral quality. We evaluated the effect of light intensity on EFN secretion by exposing plants to increasing light intensities starting with darkness and increasing exposure stepwise to 100% light after JA treatment (Fig. 3). Even when exposed to only 25% light, EFN secretion in JA-treated plants was significantly higher than in control plants and however, further

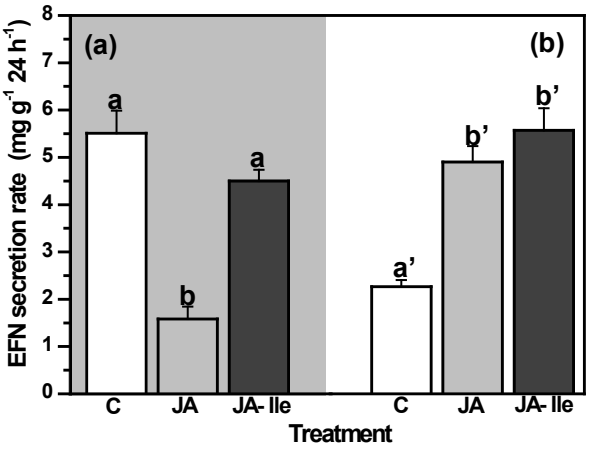

Figure 2 Extrafloral nectar secretion rates in plants exposed to dark and light conditions after JA and JA-Ile treatments**.** Changes (mean ± 95% confidence) are expressed as mg soluble solids per g fresh weight in plants exposed to (a) 24 h dark conditions (LSD posthoc after univariate ANOVA, $P < 0.02$, n= 5) and (b) 24 h light conditions (50%) (LSD posthoc after univariate ANOVA, $P < 0.02$, n= 6) at 27.5 ^{o}C and 65% humidity in both cases. Different letters indicate significant differences among treatments.

increasing the light intensity to 50% and 100% did not result in an even higher amount of EFN produced (Fig. 3). This result indicates that even though the induction of EFN secretion by JA is light-dependent, the induction effect does not seem to be limited by the availability of light.

But do changes in the spectral light quality modulate the plant's jasmonate-controlled EFN secretion? To address this issue, we treated plants with JA and JA-Ile and measured the rate of EFN secretion after 24 h of exposure to different ratios of R and FR radiation (Fig. 4). Treatment with both JA and JA-Ile significantly reduced EFN secretion in plants exposed to 100% FR light. When the ratio of R to FR radiation was increased to 10:90, the rate of EFN secretion was significantly lower in JA-treated plants than in JA-Ile-treated plants; however, the rate of EFN secretion was similar in JA-Ile-treated plants and control plants (Fig. 4). It is worth mentioning that in plants exposed to 24h darkness, JA reduced EFN secretion while JA-Ile did not in comparison to the control plants (Fig. 2). This is comparison to the plants

exposed to 10:90 R:FR or 100% FR radiation indicates that light quality signals are important for this modulation. It is interesting that the control plants also behave differently in these cases. Further increasing the R:FR ratio to 50:50 restored the inductive effect of both JA and JA-Ile (Fig. 4). In sum, our results demonstrate that the regulation of EFN secretion by jasmonates is strongly affected by light quality, yet not light quantity.

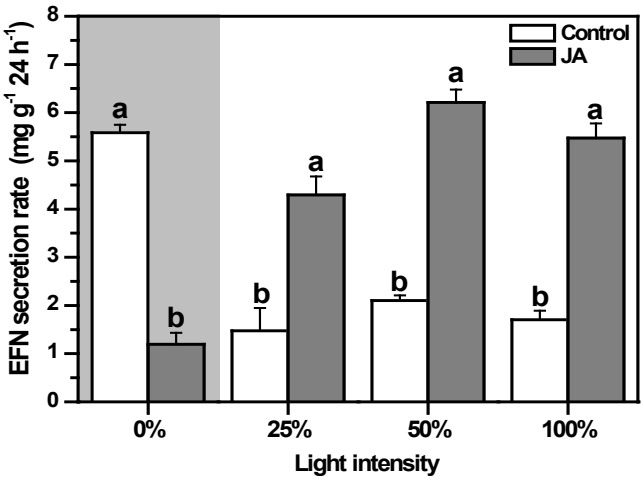

Figure 3 Changes in extrafloral nectar (EFN) secretion rates in plants exposed to increasing light intensities. EFN secretion rates (mean ± 95% confidence interval) are expressed as mg per gram fresh weight of the leaf tissue measured at 27.5° C and 65% humidity (LSD posthoc after univariate ANOVA, $P < 0.03$, $n = 8$).

Biosynthesis of phytohormones is light-dependent

Jasmonates are synthesized *de novo* from linolenic acid via the octadecanoid pathway following herbivory or mechanical damage (29). To explore whether the biosynthesis of these phytohormones relies on the availability of light, we investigated the synthesis of JA and JA-Ile in both mechanically damaged and control plants in dark and light at various time points (Fig. 5). Wounding resulted in significantly increased levels of both JA and JA-Ile and the maximum levels were reached after about an hour of wounding (Fig. 5). Interestingly, JA-levels of wounded leaves did not show a significant change in dark and light phases whereas JA-Ile levels were significantly higher in the light as compared to the dark phase (Fig.5).

Additionally, a kinetic study was designed and carried out in untreated plants exposed to varying amounts of light quality from a ratio of 10:90 R to FR radiation (which closely resembled the pattern of EFN secretion in plants exposed to the dark) to a ratio of 50:50 R: FR radiation (which resembled the pattern of EFN secretion in plants exposed to 50% light treatment) (Fig. S2). Exposing plants to 50:50 R:FR radiation even for 5 min was sufficient

to trigger hormone biosynthesis and reached a maximum at 30 min of exposure after mechanical wounding (Fig S2).

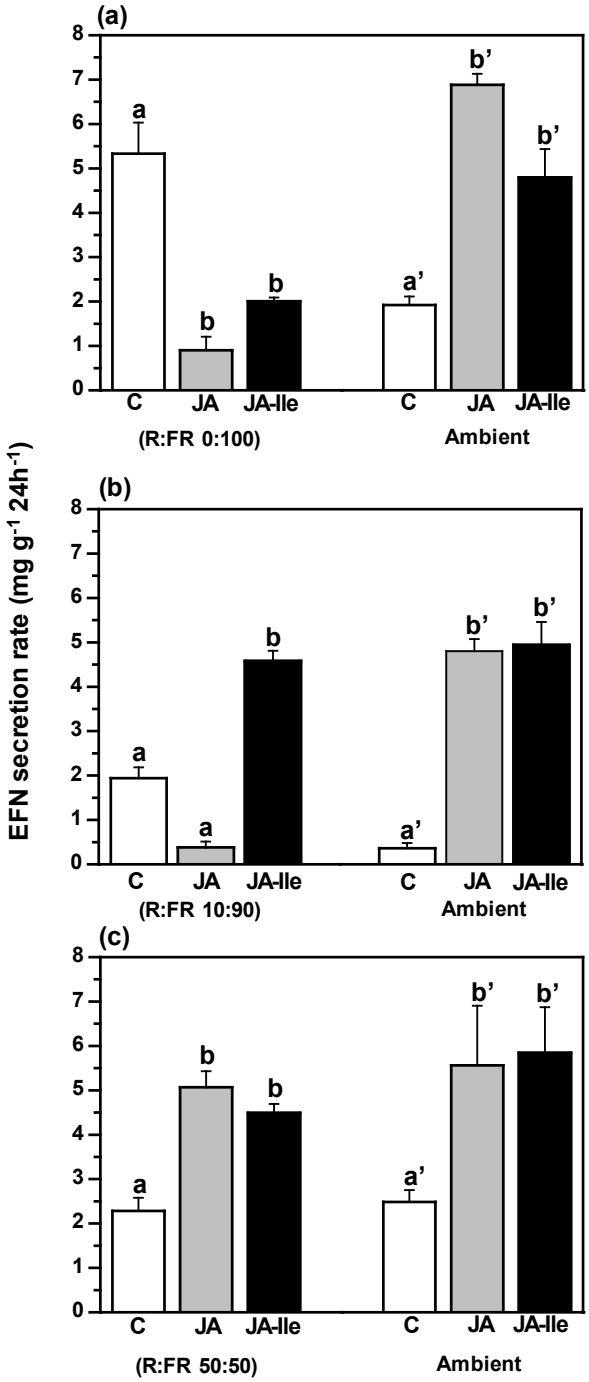

Figure 4. Extrafloral nectar (EFN) secretion rates in plants treated with JA and JA-Ile and exposed to different ratios of red (R) and far red (FR) radiation. Changes in mean EFN secretion rates (± 95% confidence interval) are expressed as mg soluble solids per g fresh weight of leaf tissue. Plants exposed to different R:FR ratios are compared to plants exposed to ambient white light conditions (a) 100% FR (LSD posthoc after univariate ANOVA, $P < 0.01$, $n = 4$), (b) 10:90 R:FR (LSD posthoc after univariate ANOVA, $P < 0.03$, $n = 4$) and (c) 50:50 R:FR (LSD posthoc after univariate ANOVA, $P < 0.04$, $n = 4$).

Jasmonic acid-isoleucine conjugate is critical for EFN secretion

The finding that in dark, JA seems to have an inhibitory effect on EFN production while JA-Ile does not, suggested that probably JA-Ile rather than JA is the active signaling compound that induces EFN secretion in the lima bean. Assuming that molecules with similar structures also possess similar biological activities, we used coronalon (COR; i.e. 6-ethyl indanoyl isoleucine conjugate), a structural mimic of JA-Ile, to test this hypothesis. This compound is known to be functionally more active than JA in inducing plant defence responses even at lower concentrations (30). Application of COR to plants exposed to either light or dark conditions resulted in a EFN secretion pattern that resembled that of plants treated with JA-Ile: COR-treated plants showed a high EFN secretion rate in the light, whereas COR treatment had no effect in the dark (Fig. 6a).

If JA-Ile is the active compound that triggers EFN secretion, formation of the compound by a conjugation reaction between isoleucine and JA should be the critical step. In this case, a free acid moiety must be present to form the isoleucine conjugate. Consequently, blocking the acid moiety should inhibit the induction of EFN secretion even in the presence of light. We tested this hypothesis using the methyl ester of JA (MeJA) for inducing EFN in plants exposed to both light and dark conditions. MeJA did not induce EFN in plants exposed to the light (Fig. 6b), implying that the presence of free JA (i.e. the non-methylated form) is important for conjugation with Ile and, as a consequence, for the induction of EFN. MeJA had no significant effect on rates of EFN secretion in plants exposed to dark (Fig. 6b).

Another test of verifying whether the formation of JA-Ile is light-dependent, is to wound plants in the dark. It is known that JA is synthesized *de novo* in response to herbivory or mechanical damage (29, 31). Not observing an increased EFN secretion rate in dark conditions would therefore support the hypothesis that the conjugation that forms JA-Ile from JA is light-dependent.

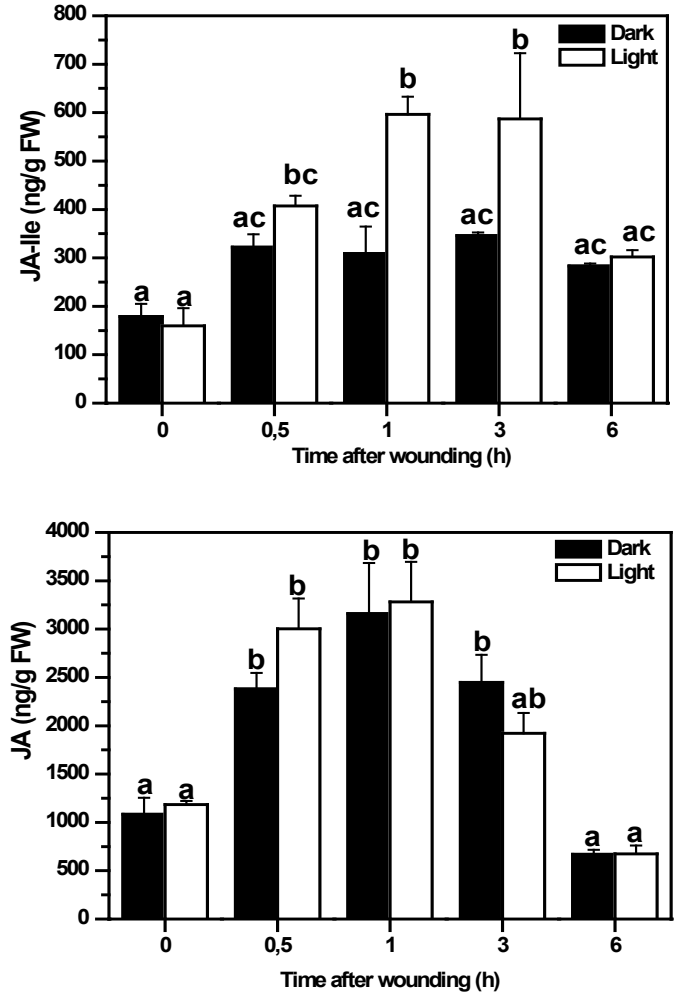

Figure. 5. Kinetics of (a) JA-Ile and (b) JA levels (mean ± 95% confidence interval) in plants kept in darkness for 24 h and wounded with a pattern wheel, just before the light was switched on (LSD post hoc after univariate ANOVA; $P < 0.03$, $n = 3$ for each time point and treatment).

Indeed mechanically wounded plants that were kept in the dark showed no increase in EFN secretion relative to control plants (Fig S3). Further, to test whether the availability of Ile as part of the JA-Ile conjugate is important for EFN secretion, its biosynthesis was inhibited by treating plants which had been exposed to light with the herbicide chlorosulfuron; and the rate of EFN secretion was measured after 24h.

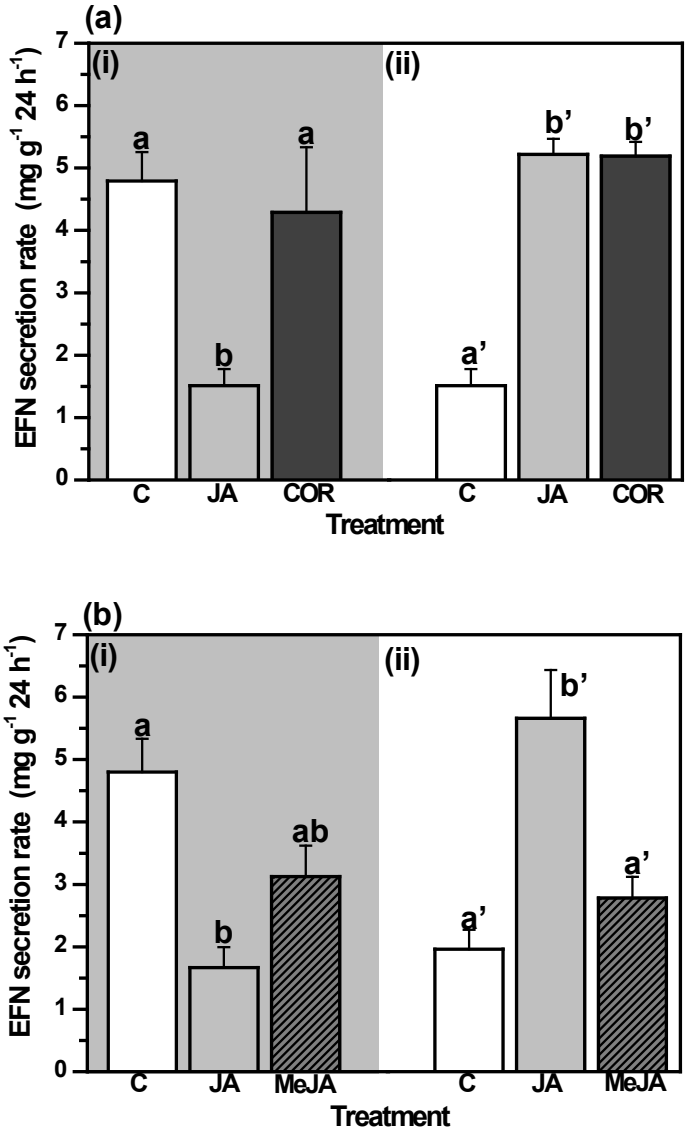

Figure 6 Extrafloral nectar (EFN) secretion rates in plants treated with MeJA and COR. Changes (Mean ± 95% confidence interval) are expressed as mg soluble solids per g fresh mass in plants treated **(a)** with COR and exposed to dark (i) and light (ii) conditions (LSD posthoc after univariate ANOVA, $P < 0.01$, $n = 5$) and **(b)** with MeJA and exposed to dark (i) and light (ii) conditions (LSD posthoc after univariate ANOVA, $P < 0.03$, $n= 5$).

Chlorosulfuron blocks the acetolactate synthase, which inhibits the biosynthesis of branched chain amino acids (32). Inhibiting Ile biosynthesis significantly reduced the rate of EFN secretion in plants exposed to light (Fig. 7a), though the rate could be restored by the exogenous application of JA, JA-Ile or COR. An analysis of the amount of free amino acids in the leaf tissue of inhibitor-treated plants revealed significantly reduced Ile levels relative to the leaf tissue of control plants (Fig. 7b). The analysis of free amino acids in leaf tissues

during the exposure to prolonged light and dark period revealed that several amino acids including Ile are present at higher concentrations during the night than during the day (Fig. S4). Collectively, these results provide additional evidence that the presence of light and not only the availability of JA or Ile is the limiting factor for the rate of EFN secreted in plants exposed to the dark.

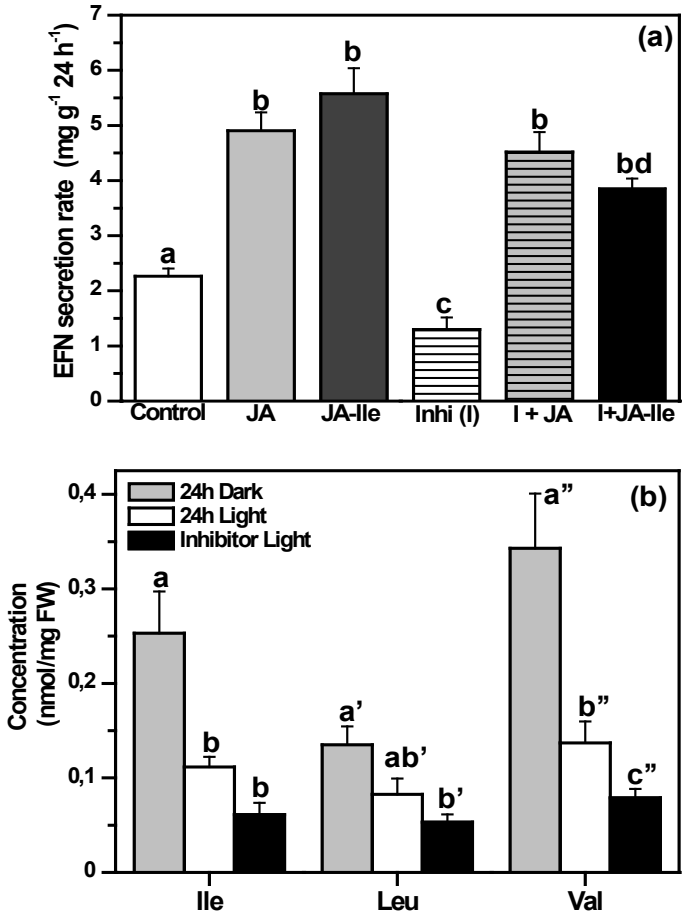

Figure 7. (a) Changes in mean EFN secretion (± 95% confidence interval) rates expressed as mg soluble solids per g fresh mass in plants treated with JA, JA-Ile, Ile-inhibitor (I) chlorsulfuron (1µM), JA and JA-Ile treatments after inhibitor application and exposed to light conditions (LSD posthoc after univariate ANOVA, $P < 0.01$, $n = 6$); and (b) levels of branched chain amino acids expressed as nmoles per mg fresh weight of leaf tissue in plants exposed to 24h dark and light conditions, and in plants treated with inhibitor and exposed to light conditions (LSD post-hoc after univariate ANOVA, $P < 0.04$, $n = 6$).

Discussion

Plants perceive fluctuations in light intensity, spectral quality and periodicity through phytochromes and acclimatize accordingly, making light availability the most important entrainment stimulus (22). Light signals can modulate plant responses by interacting with the biosynthetic pathways, involving perception and signaling mechanisms (2, 3). Whether or not light signals can interfere with responsiveness to defence elicitor hormones was the major focus of this study. We found that applying JA of same concentration can have different effects on the focal defence response depending on the light environment: induction of EFN secretion in light and suppression in dark. This observation can be compared to the phenomenon of "gating" (33), which has been reported for other phytohormones such as auxin, gibberillic acid and ethylene (for review see (3)). EFN secretion in lima bean showed an inherent temporal pattern, highest at night in untreated plants while upon jasmonic acid (JA) treatment maximum EFN secretion was observed in day. This inherent temporal variation in EFN secretion was found to be modulated by JA treatment. Interestingly, JA-Ile induced EFN secretion in plants exposed to prolonged light but did not reduce EFN secretion in plants kept in prolonged darkness like JA. Based on these observations, we hypothesize that in dark, JA is incapable of inducing EFN secretion probably because the conjugation to isoleucine to form the active molecule is limited in dark phase (Fig. 8). Consistent with this hypothesis, we found that inhibiting Ile biosynthesis in light phase significantly reduced EFN secretion and coronalon induced EFN secretion in light and had no effect in the dark phase. Further, it is known that silencing threonine deaminase (TD), an enzyme which catalyzes the first committed step in Ile biosynthesis, leads to plants that are susceptible to herbivore attack due to reduced defense levels (34). Our results are in line with these observations and underline the importance of Ile in the regulation of defence responses. When plants were treated with MeJA, EFN secretion was not enhanced (Fig 5b). Induction in response to MeJA treatment is not due to the activity of MeJA itself, but is caused by the hydrolysis of methyl jasmonate, which liberates the free acid (35). Many plants, however, are incapable of performing this hydrolysis; in these plants, MeJA is inactive. This is also the case for lima bean. In sum, the presence of a free carboxyl group, which is essential for the subsequent conjugation with amino acid is also essential for the induction of EFN in the presence of light. Further, our analyses of endogenous levels of phytohormones clearly showed that the biosynthesis of JA-Ile depends on the availability of light (Fig. 5). Taken together, we hypothesize that light availability acts to regulate jasmonate responsiveness and subsequent EFN secretion by modulating the formation of the Ile conjugate.

Interactions between light and herbivory have been studied primarily in the context of diurnal variation in herbivore behavior and host plant chemistry (36-39). One well-studied indirect plant defense against herbivores, which varies diurnally, is the emission of volatile organic compounds (VOCs). VOCs emission has been repeatedly shown to be light dependent, and VOCs released during the day differ from VOCs released during night, making the release of VOCs, a reliable cue for parasitoids (40, 41). Recently, it was shown by Arimura et al. (42) that continuous mechanical damage during day or night can lead to increased JA levels, but the emission of the volatile compound ocimene starts only during the day because photosynthesis is the source for the formation of its precursors by the 2-C-methyl-D-erythritol 4-P pathway. The secretion of EFN is an indirect defense strategy similar to the emission of VOCs and involves attracting parasitoids to prey on herbivores. The regulation of EFN secretion, like the regulation of volatile emission, is mediated by the octadecanoid-signaling pathway (9). However, the role of the light environment on variation in EFN secretion is not as well characterized as its role in VOC emission. Temporal variation in EFN secretion has been studied in *Macaranga tanarius* where the EFN secretion was found to peak during dusk (43) and other similar studies have interpreted such variation as an adaptive strategy to the occurrence of plant herbivores (44, 45). However, validation that that such temporal variation in EFN production is actually correlated to herbivore or ant activity needs careful studies under natural growing conditions.

In our study, we found that JA and JA-Ile differently affect the rate of EFN secretion in plants exposed to dark and light conditions. A possible mechanism of this light-dependent regulation could be photosynthesis, which provides energy and metabolic precursors for the production of sugars and defensive compounds. JA is known to inhibit photosynthesis-related genes, and JA defense signaling and phytochrome-mediated light signaling are antagonistic to each other (24). In *Arabidopsis*, JAR1 (JASMONATE RESISTANT 1) catalyzes the formation of JA-Ile by the pyrophosphorylysis of ATP via an enzyme-bound acyl-AMP intermediate, the adenylate (18, 46). JA-Ile is formed by the activation of JA via adenylation which involves ATP and is a highly energy-demanding process (18, 47). However, it is likely that JA-Ile is not the sole activator of anti-herbivore defence mechanisms. Studies reporting activity of other metabolites in the absence of JA are known, for example, opr3 mutants impaired in converting OPDA to JA were shown to be defective in fertility not in pathogen resistance in *Arabidopsis thaliana* (48). In *Nicotiana attenuata*, JA-Ile was capable of recovering resistance to *Manduca sexta* in JAR4/6 silenced plants but only to a lesser extent in LOX3 silenced plants showing that JA and JA-Ile play different roles in herbivore

resistance (49). Our results demonstrate that light signals can interact with jasmonate responsiveness of plants and thereby regulate EFN secretion. This fine tuning of induced indirect defence ensures effective and optimal defence during both dark and light phases.

Plant-herbivore interactions in the context of changing light environments have largely focused on the changes in light availability caused by plant canopies; these changes have a major effect on spectral balance in terms of R:FR ratios (39). In shade, when the FR component is enriched, leaf tissues are more favorable for herbivore feeding because they contain fewer defensive compounds (39). In *N. attenuata*, FR is known to induce the down-regulation of chemical defenses such as the herbivore-induced accumulation of phenolics (23) and in *A. thaliana,* FR improves tissue quality and reduces plants' sensitivity to JA (21). These studies were conducted to understand how FR signals can help plants better compete and how plants solve the dilemma of competition *versus* herbivory. In our investigation, however, we studied the effect of different ratios of R to FR radiation, asking how the quality of light spectra modulates jasmonate-mediated EFN secretion. We found that in plants exposed to 100% FR radiation, neither JA nor JA-Ile induces EFN secretion, and that as the ratio of R to FR radiation is increased 10:90, JA causes reduction but JA-Ile does not. When the ratio of R to FR radiation was increased to 50:50, both JA and JA-Ile induced EFN secretion. In summary, plants modulate their sensitivity to jasmonates as a function of light and this correlates with increase or decrease in EFN secretion rate. We speculate that JA-Ile, whose formation is probably light-controlled, is the active signal for this indirect defence. More research on the effect of light conditions on jasmonate signaling is necessary to understand how plants fine-tune their signaling pathways in response to changes in environmental conditions. Furthermore, field studies regarding day-night changes in ant protective and herbivore behavior would help in interpreting the evolution and ecological function of day/night patterns of EFN secretion.

Materials and methods

Plant growth and light conditions

Plants of *Phaseolus lunatus* (Lima bean) were cultivated from seeds derived from a native population growing in the coastal area near Puerto Escondido in the state of Oaxaca, Mexico. The parental plants were used previously in field experiments (27). Plants were grown in climate chambers (Snijders Microclima MC1000E, Snijders Scientific, Tilburg, Netherlands) in the greenhouse at 27° C, 65% humidity, in a 16 h photoperiod. Experiments were

performed with 4-week-old plants (i.e., 5-6 leaves per plant). For artificial night experiments, the plants were kept at the same temperature and humidity in complete darkness for 24 h. Diurnal changes in EFN secretion were monitored continuously for a period of 24 h under the 16h photoperiod (457.1 µmol) at 27° C and 65% humidity. For experiments regarding EFN secretion at increasing light intensities, plants were exposed to 0% (0.02 µmol), 25% (241.7 µmol), 50% (451.7 µmol), 100% (712.8 µmol) (measured using a LI-COR 250A light meter, Li-COR Biosciences GmbH, Bad Homburg, Germany) at 27° C and 65% humidity in a climate chamber. For experiments with different R:FR ratios of light, the plants were kept in growth chambers containing LED lampbanks (CLF floralLED series, CLF Plant Climatics GmbH, Emersacker, Germany, with overall light intensity up to 450 µmol/ m^2 s) as the light source, where each light wavelength can be programmed to desirable intensities ranging from 1 to 100%.

EFN measurements

At the beginning of the experiment the extrafloral nectaries were washed thoroughly with tap water and allowed to dry in order to ensure all nectar was completely removed. EFN secretion was then induced by spraying an aqueous solution of the focal inducer (1mM) on the leaves until run-off. Plants were treated twice with the desired compound at an interval of 30 min, and after that leaves were allowed to dry for 1 h before plants were placed back into the climate chambers or the greenhouse. The EFN secreted 24 h after the treatment was quantified as the amount of soluble solids (i.e. sugars and amino acids). The concentration of EFN was measured immediately upon removal from the nectary using a temperature-compensating refractometer (ATAGO N-10E refractometer, Leo Kübler GmbH, Karlsruhe, Germany) and the nectar volume was quantified directly using 5 µl micro-capillaries as described (9, 43). EFN measurements from all nectaries of an individual leaf were pooled. The EFN was quantified as the amount of soluble solids per dry weight of leaf material secreted in 24 h.

Phytohormone and amino acid analysis

Analysis and quantification of phytohormones were performed using standard LCMS protocols (49). Amino acid analysis was carried out after derivatization with mercaptoethanol and O-phthaldialdehyde, a method with which cysteine and proline cannot be detected (50, 51).

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Supplementary figures

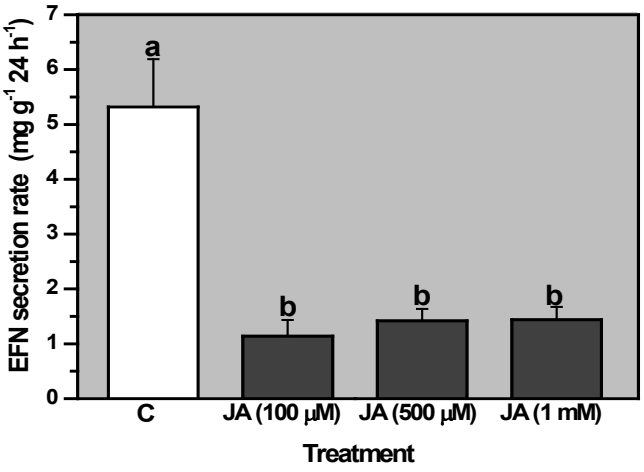

Figure S1 Concentration-dependent effect of JA on extrafloral nectar (EFN) secretion rates in plants exposed to dark conditions. Changes in mean EFN secretion rates (± 95% confidence interval) expressed as mg soluble solids per g fresh mass in plants treated with different JA concentrations and exposed to dark conditions at 27.5 ^{o}C and 65% humidity (LSD posthoc after univariate ANOVA, $P < 0.03$, $n = 4$).

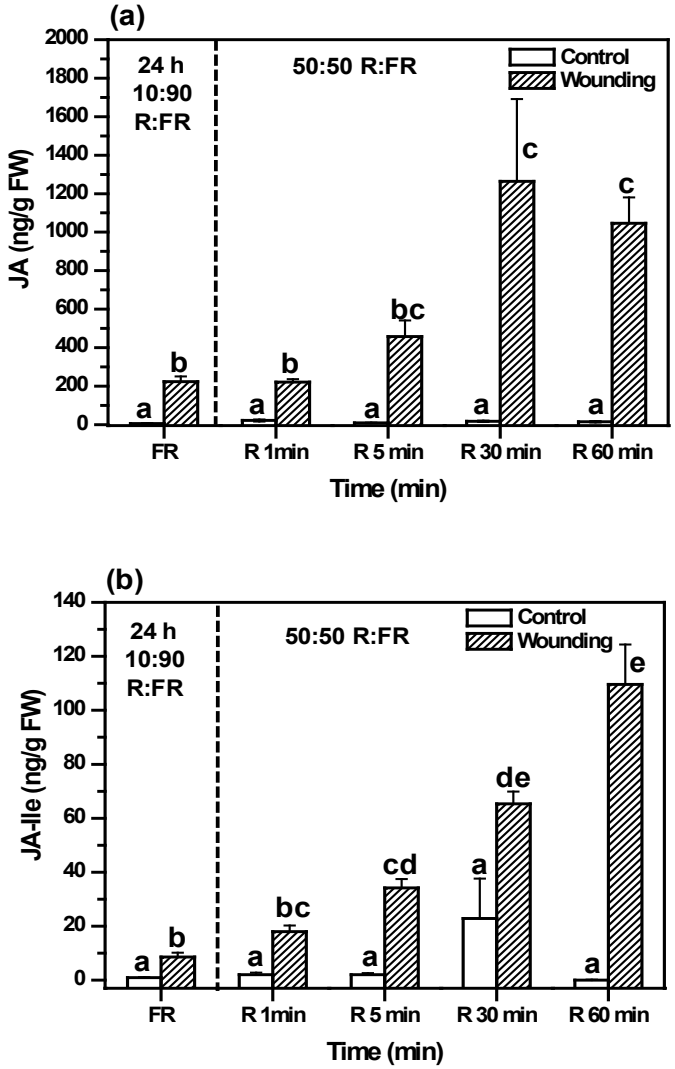

Figure S2. Kinetics of phytohormones in plants exposed to different R:FR ratios. Levels of (a) JA and (b) JA-Ile in plants kept for 24 h at a 10:90 R:FR ratio and wounded with a pattern wheel just before the ratio was changed to 50:50 R:FR (LSD post hoc after univariate ANOVA; $P < 0.01$, $n = 3$ for each time point and treatment after Bonferroni correction for multiple comparisons).

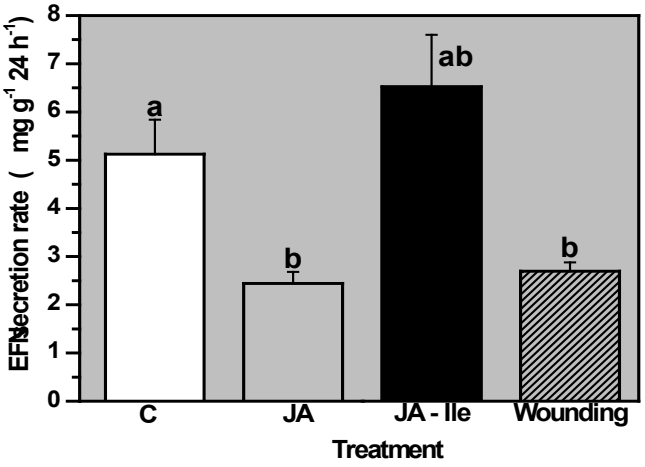

Figure S3 Extrafloral nectar (EFN) secretion rates after mechanical damage to plants exposed to dark conditions. Mean EFN secretion (± 95% confidence interval) rates expressed as mg soluble solid per gram leaf fresh weight after various treatments to plants exposed to dark conditions (LSD posthoc after univariate ANOVA, $P < 0.03$, $n = 5$). The wounding was done using pattern wheel and care was taken to ensure complete darkness while wounding.

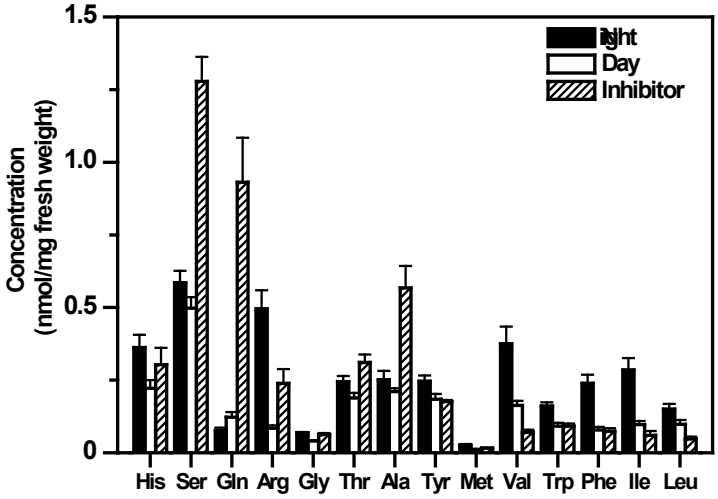

Figure. S4 Levels of free amino acids expressed as nmoles per mg fresh weight of leaf tissue from plants exposed to 24 h dark and light conditions, and plants treated with inhibitor and exposed to light conditions. Concentrations of these amino acids were measured in three independent experiments and are expressed as means (± 95% confidence interval).

Manuscript IV

Towards elucidating the differential regulation of floral and extrafloral nectar secretion

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Nectar is a rich source of sugars that serves the attraction of pollinators (floral nectar) or predatory arthropods (extrafloral nectar). We just begin to understand the similarities and differences that underlie the secretory control of these two important types of plant secretions. Jasmonates are phytohormones, which are well documented to be involved in plant developmental processes and plant defence responses against herbivores, including the secretion of extrafloral nectar. Recently, jasmonates have also been implicated in the regulation of floral nectar secretion in *Brassica napus*. Due to a trade-off between reproduction and defence, however, plants need to functionally separate the regulation of these two secretory processes. In line with this prediction, externally applying jasmonates to leaves did indeed not affect floral nectar secretion. Here we compare the current knowledge on the regulation of floral and extrafloral nectar secretion to understand similarities and dissimilarities between these two secretory processes and highlight future research directions in this context.

Jasmonic acid (JA) and other JA-derived compounds (jasmonates) control both plant developmental processes such as flowering[1,2] and anther dehiscence[3] and activate plant defence responses against herbivores.[4] For example, JA induces extrafloral nectar (EFN) secretion in various plant species from different families.[5-7] Recently, jasmonates have also been implicated in the secretion process of floral nectar (FN).[8] Floral nectar and extrafloral nectar share many chemical and functional properties[9] and apparently there is some similarity in the regulation of EFN and FN secretion. Here we compare the current knowledge on the regulation of these two processes (Table 1) and highlight future research directions.

Extrafloral nectar is an indirect defence trait that is used by many plant species to attract and nourish predatory arthropods, especially ants, which serve the nectar-secreting plants as 'bodyguards'[10] by effectively reducing the herbivore pressure on the EFN-secreting plant.[11,12] External application of JA induces EFN secretion in many plant species, including *Phaseolus lunatus*[12], *Macaranga tanarius*[5] and several *Acacia* species[13] - an effect that is similar to the induction caused by herbivore feeding[5]. Blocking JA biosynthesis with phenidone, an inhibitor that reduces the fatty acid hydroperoxide formed by the lipoxygenase catalyzing the first step in the octadecanoid signalling pathway[14], reduces EFN secretion.[5,13]. While EFN serves defensive functions, floral nectar attracts plant pollinators and therefore significantly contributes to a plant's reproductive success.[15,16]. The adaptive significance of floral nectar for mediating plant-pollinator interactions has been well studied.[17,18] Besides very few studies, however, that investigated the effect of various growth regulators on FN

secretion, our understanding of the physiological processes that regulate this trait remains rather poor.[19] Recently, it was discovered that exogenous application of JA increased FN secretion in oilseed rape (*Brassica napus*).[8] Further, blocking JA biosynthesis with phenidone effectively reduced FN secretion, an effect that could be restored by an additional JA treatment.[8] Thus, major regulatory mechanisms appear to control the secretion of both, FN and EFN. How similar are the two mechanisms, and how can the plant physiologically separate the secretion of EFN and FN? Both types of secretion function in ecologically very different contexts and, thus, clearly need to be controlled independently.

One option would be the involvement of other jasmonates. Although JA is an important signal on its own, around 20 different JA-derived metabolites are also known to be involved in defence signalling.[20,21] Even metabolic precursors of JA may elicit different defensive phenotypes[22,23], which opens interesting possibilities for a fine-tuning of jasmonate-dependent responses. In particular, the JA-amino acid conjugate jasmonoyl isoleucine (JA-Ile) has recently been discovered as functioning as the central signalling molecule of the jasmonate pathway.[23-25] Both JA-Ile and its structural mimic, coronalon, induced FN synthesis when applied to *Brassica napus* flowers.[8] The role of JA-Ile in EFN secretion, however, has yet to be studied.

What about other triggers? Exogenous application of auxin can strongly reduce floral nectar secretion in *Euphorbia pulcherrima* and *Antirrhinum majus.*[26,27] In another study, a similar reduction of FN production has been reported from snapdragon flowers upon indole acetic acid (IAA) treatment.[28] In the same study, the distribution of (^{14}C) sucrose in flowers and nectar suggested that IAA acts on the secretory process in the nectary cells, rather than on the mobilization of sugars to the nectary.[28] Recently, it was shown in *Arabidopsis thaliana* that IAA blocks FN secretion until the onset of anthesis.[29] Moreover, exogenous application of gibberellic acid (GA3), naphthalene acetic acid (NAA), indole butyric acid (IBA) and IAA to *Brassica campestris* and *Brassica oleracea* resulted in an induction of floral nectar, among which GA3 showed the strongest inducing effect in terms of nectar amount, sugar content and pollinators attracted.[30] In *A. thaliana*, an extracellular invertase has been reported to be causally involved in the mobilization of starch deposits and thus, floral nectar secretion[31], but the hormonal control of this enzyme remains to be studied.

JA and its derivatives not only induce FN and EFN secretion, but can elicit another indirect defence strategy: volatile organic compounds (VOCs)[32], which are released upon herbivore attack or exogenous JA treatment.[33] Besides their role for attracting predatory arthropods to herbivore-damaged plants, VOCs also function as a signal that is externally

transmitted via the gas-phase and which systemically induces the EFN secretion of both the emitting plant[34] and also of different, neighbouring plant individuals.[35] Whether VOCs also affect the secretion rate of FN, however, has never been studied.

In addition to JA, coronatine[36], a phytotoxin isolated from the pathogenic bacterium *Pseudomonas syringae*, triggers VOC emission in many plant species.[36] Although coronatine and its structural mimic coronalon[37] induce VOC emission[22] and FN secretion[8], it is not known to date whether these compounds also induce EFN secretion. Floral herbivory (florivory) has been reported to reduce floral nectar and the number of pollinator visits.[38] However, its effect on EFN secretion has not been studied to date. Also floral volatiles, which are attractive to pollinators, are altered qualitatively and quantitatively by florivory in *Pastinaca sativa*.[39] Similarly, herbivore-induced volatiles that induce EFN, could also affect FN secretion (Table 1).

Although many gaps in our knowledge remain to be filled, it becomes apparent that - despite the different ecological functions of FN and EFN - there exist some similarities in their regulation (Table 1). Deepening our understanding on the regulatory role of jasmonates and other phytohormones for both FN and EFN secretion and elucidating how these pathways are interconnected, yet functionally separated, will provide interesting insights into the physiological basis of these processes and ultimately into the evolutionary constraints and trade-offs that shaped this regulatory separation. In particular, future work should address the following questions: (1) How do plants achieve and maintain the regulatory separation of FN and EFN secretion, although these two pathways obviously share some signalling molecules? (2) Do other phytohormones (JA-Ile, IAA, GA3, etc.) also affect EFN production? (3) Do herbivore-induced VOCs elicit FN secretion?

Answering these questions requires a combination of different, yet complementary methodologies: Labelling experiments, for example with ^{13}C, would allow to investigate whether or not the functional separation of FN and EFN secretion is achieved by a strictly local (i.e. in herbivore-wounded tissues/ flowers) production of the responsible jasmonates. Moreover, gaining a deeper understanding requires also combining the more classical approach of using well-characterized, specific inhibitors[40] and external application of phytohormones with modern technologies that analyse a plant's transcriptome, proteome and metabolome. Finally, using mutants that lack certain key genes such as those involved in the JA signalling cascade like *coi1* (*coronatine insensitive 1;* defective in all JA-related responses[41]) or *jar1* (*jasmonic acid resistant 1* , impaired in the biosynthesis of JA-Ile[42]) will provide mechanistic insight into the regulation of nectar secretion.

Table 1. A comparison of floral and extrafloral nectar production

	Floral nectar	**Extrafloral nectar**
Function	Pollination or attraction[43]	Indirect defence[12,44,45]
Consumers	Birds, insects and other pollinators[46]	Arthropods, especially ants[11,46]
Elicitor/ inhibitor		
Jasmonic acid	Increases secretion[8]	Increases secretion[5]
Jasmonoyl isoleucine	Increases secretion[8]	Not known
Coronalone	Increases secretion[8]	Not known
Phenidone [1]	Flower treatment: reduces secretion[8]	Leaf treatment: reduces secretion[5]
Gibberillic acid	Increases secretion[30]	Not known
Indole acetic acid	Decreases secretion[26,27]	Not known
Ethylene	Not known	Not known
Herbivory	No effect[8] /decreases secretion [47]	Increases secretion[5,11]
Florivory	Decreases secretion[38]	Not known
Plant volatiles:		
Herbivore induced	Not known	Increases secretion[34,35]
Florally emitted	Not known	Not known

[1] inhibits JA biosynthesis

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Manuscript V

Volatile emission in bracken fern (*Pteridium aquilinum*) is induced by jasmonates but not by herbivory

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Abstract

The induced emission of volatile organic compounds (VOCs) from herbivore-damaged plants is generally believed to function as a plant's 'cry for help' to attract predators of their herbivores. Although the jasmonate-mediated regulation of VOC emission has been extensively investigated in higher plants, only little is known about VOC production and its regulation in lower plants. Here, we investigate whether the emission of VOCs from the evolutionary ancient bracken fern *Pteridium aquilinum* is regulated by the octadecanoid signaling pathway. When treated with jasmonic acid (JA), bracken responded with the emission of a blend of VOCs that are mainly comprised of terpenoids. Likewise, treatment with the JA precursors OPDA and linolenic acid also induced VOC emission, albeit in lower amounts than JA. Qualitatively and quantitatively similar VOC blends were released upon treatment with other elicitors such as coronalon and alamethicin. Interestingly, either single or continuous mechanical wounding of fronds, as well as feeding of both generalist and specialist herbivores, induced only very low levels of terpenoid emission. The terpenoid emission upon JA treatment could be blocked with fosmidomycin and mevinolin, inhibitors of the MEP and MVA pathways, respectively. This result indicated that similar to higher plants, terpenoid VOCs were produced via these pathways in bracken fern. In sum, these results suggest that the biosynthetic machinery for VOC emission was already present when the regulatory link between herbivory and the octadecanoid pathway evolved.

Keywords

Bracken fern, volatile organic compounds, jasmonic acid, herbivory, evolution, *Pteridium aquilinum*.

Introduction

The emission of volatile organic compounds (VOCs) is a well known indirect defence mechanism, by which plants recruit antagonists (predators and parasitoids) of their herbivores (Dicke et al. 1999; Kessler and Baldwin 2001; Pare and Tumlinson 1999). VOCs are generally believed to function as an 'alarm signal' that is generated by plants in distress and depending on the type of stress (herbivore-/ pathogen-attack or tissue damage) and the plant species, quantitatively and qualitatively different bouquets are released (Halitschke et al. 2008; Kant et al. 2009). Terpenoids are the most abundant and structurally diverse class of VOCs released upon herbivore damage by many higher plants (Pare and Tumlinson 1999). These can be a mixture of monoterpenes (C10), sesquiterpenes (C15) and homoterpenes (C11, C16), all of which are synthesized from a basic C5 unit, isopentenyl diphosphate (IPP) and dimethyallyl diphosphate (DMAPP) via either the cytosol-localized mevalonate (MVA) or plastid-localized methylerythritol (MEP) pathway (Arigoni et al. 1997; Lichtenthaler et al. 1997; McGarvey and Croteau 1995; Piel et al. 1998). A huge body of literature is available on the regulation of VOC emission via the octadecanoid pathway as well as on the chemical elicitors that are capable of triggering this indirect defence in many higher plant species such as *Phaseolus lunatus*, *Gossypium hirsutum*, *Populus simoniix*, *Nicotiana attenuata* *Solanum tuberosum* and *Zea mays* (Hopke et al. 1994; Pare and Tumlinson 1999; van Poecke and Dicke 2004; Wasternack 2007). In contrast, very little is known on the events that activate this indirect defence in lower plants such as ferns (Boland et al. 1995; Imbiscuso et al. 2009).

In higher plants, oxylipin molecules such as jasmonic acid (JA), its precursor 12-oxophytodienoic acid (OPDA) and other JA derivatives like jasmonyl-isoleucine (JA-Ile) and methyljasmonate (MeJA) have been implicated as major regulators of VOC emission (Arimura et al. 2005; Farmer et al. 2003; van Poecke and Dicke 2004): both herbivory and continuous mechanical wounding (Mithöfer et al. 2005) result in an endogenous accumulation of JA leading to an increased VOC emission (Arimura et al. 2005). Interestingly, exogenous application of JA also triggers the emission of VOCs, rendering the use of this elicitor a powerful methodological tool for the study of this indirect defence. The qualitative and quantitative composition of the VOC blend emitted upon JA-treatment strongly resembles the one released after wounding or herbivory (Dicke et al. 1999; Hopke et al. 1994; Kost and Heil 2008). In addition to precursors or derivatives of jasmonic acid, several other low molecular weight compounds of microbial, fungal or insect origin represent another class of elicitors that trigger VOC emission in higher plants (Dudareva et al. 2006; Engelberth et al. 2001; Koch et al. 1999). In many cases, this effect is due to the fact that the structures of these

compounds resemble endogenous plant signals. Particularly well studied in this context is the phytotoxin coronatine, an amino acid conjugate, which is produced by certain pathovars of *Pseudomonas syringae* and elicits VOC production in many higher plants (Boland et al. 1995; Koch et al. 1999). Its structural analogue 6-ethyl indanoyl isoleucine conjugate, (i.e. coronalon, COR (2,6-ethyl-1-oxo-indane 4-carbonyl)-amino-3-methyl-pentanoic acid methyl ester)) is known to be an even more powerful elicitor of VOC production than JA even at lower concentrations (Schüler et al. 2004). Furthermore, VOC emission is also elicited by hydrolytic enzymes released from invading fungi (Croft et al. 1993; Huang et al. 2003) and several reports exist that indicate the induction of plant defences in response to fungal cell wall fragments via the activation of the octadecanoid signalling pathway (Gundlach et al. 1992; Rose et al. 1996). For example, the plant parasitic fungus *Trichoderma viride* produces a number of ion-channel forming peptides, alamethicin (ALA) being a major compound of this mixture (Brewer et al. 1987). ALA has been shown to evoke VOC emission in *Phaseolus lunatus* via the octadecanoid pathway and this ALA-induced VOC blend was found to resemble the blend released upon treatment with early octadecanoids (Engelberth et al. 2001; Koch et al. 1999).

Ferns are the most ancient of extant plant groups with fossil records predating the early Devonian era (about 400 million years ago) (Schneider et al. 2004; Smith 1972). They have been thriving on this planet for about 200 million years before the first flowering plants evolved (Cooper- Driver 1978). Bracken fern (*Pteridium aquilinum* (L.) Kuhn, Dennstaediaceae), the study system of the present investigation, is considered one of the world's most widespread plants and the most common fern occupying a variety of habitats (Harper 1977). Bracken has survived several ecological challenges for a long period of time and this success may be partly attributed to its extensive defenses which include a diverse number of secondary compounds like sesquiterpene indanones, cyanogenic glycosides (Cooper-Driver 1976; Schreiner et al. 1984), phytoecdysteroids (Jones and Firn 1978) and tannins (Tempel 1981), due to which only few insects utilize this species as a food source (Balick et al. 1978; Cooper- Driver 1978; Cooper-Driver 1990). Although the presence of such extensive direct defences is well documented for this fern, it is completely unclear whether primitive plants like ferns also employ indirect defence strategies such as VOC emission.

To fill this gap, we studied the emission of VOCs in the phylogenetically ancient bracken fern to unravel whether bracken does emit VOCs at all, and if so, whether the same regulatory events that induce this trait in higher plants, are already present in *P. aquilinum.* In

this way, it is not only possible to gain inside into the chemical ecology of anti-herbivore defences in bracken, but also into a plant species that likely represent an evolutionary ancestor of the modern angiosperms. Hence, studying VOC emission in lower plants such as bracken fern can shed light on the evolution of VOC emission and the ancestral function of this trait. These analyses are greatly aided by the wealth of information that is available on the VOC emission in higher plants (Dicke et al. 2003; van Poecke and Dicke 2004), such as the response to certain elicitor treatments, the biosynthetic pathways and regulating phytohormones involved, as well as the quantitative and qualitative composition of the VOC blends emitted upon different treatments. In this study, we investigated the VOC emission in *Pteridium aquilinum* addressing the following questions:

1. Does the ancient bracken fern emit VOCs upon treatment with JA and other elicitors known to induce VOC production in higher plants (OPDA, linolenic acid, coronalon and alamethicin)?
2. If terpenoids are produced, is their allocation to biosynthetic pathways (MEP and MVA pathways) comparable to higher plants?
3. Does bracken release a similar VOC blend after simple or continuous wounding and upon herbivory by generalist (*Spodoptera littoralis*) and a specialist herbivore (*Strongylogaster multifasciata*)?
4. How do the endogenous levels of oxylipins change upon damage and herbivory?

Materials and methods

Plant and insect material

Pteridium aquilinum (L.) Kuhn, Dennstaediaceae were collected as fragments of rhizomatous underground stems from a forest about 15 km from Jena (Germany, 50°45`45.05``N and 11°40`34.85``E), and the whole plants were brought to the greenhouse for further propagation. Experiments were done on plants vegetatively propagated from these in the greenhouse and grown at a temperature of 27 – 30 °C and 45 - 50% humidity, under 16 h photoperiod in Klasmann clay substrate (Klasmann-Deilmann, Geeste, Germany).

The generalist herbivore, *Spodoptera littoralis* Boisd. (Lepidoptera, Noctuidae) was reared on artificial diet (500 g of ground white beans soaked overnight in 1.2 l water, 9 g vitamin C, 9 g paraben, 4 ml formalin and 75 g agar boiled in 1 l of water). Larvae of the specialist herbivore *Strongylogaster multifasciata* (Geoffroy, 1785) (Tenthredinidae) were collected in the same field as the plants between May – June 2009, identified following

Lorenz & Krauz (Lorenz 1957) and maintained until use (roughly 3-5 days) on fresh fronds of *P. aquilinum*.

Plant treatments

For elicitor treatments, JA (1 mM), OPDA (1 mM), linolenic acid (2 mM) and Coronalon (100 µM) were sprayed as aqueous solution onto the surface of the fern fronds. These concentrations were chosen based on previous literature reports in which these compounds were shown to induce VOC emission in other plant species at the respective concentration (Engelberth et al. 2001; Koch et al. 1999; Lauchli et al. 2002). This procedure was repeated again after 30 min and then the plants were allowed to dry. The ALA treatment was applied by placing plantlets for 24 h in ALA solution at a concentration of 10 µg ml^{-1} water (ALA, Sigma, St. Louis). ALA was initially dissolved in methanol at 10 mg ml^{-1} concentration and this stock solution was diluted in tap water to obtain the desired final concentration. Fronds were damaged mechanically by puncturing 2-3 rows of holes with a pattern wheel. Continuous mechanical damage was inflicted using the MecWorm system (Mithöfer et al. 2005) for 24 h programmed to punch 10 holes per minute and VOCs were collected simultaneously.

Oral secretions (OS) were collected from third instar larvae of *Spodoptera littoralis* grown on *P. aquilinum* diet or artificial diet, or from field-collected *Strongylogaster multifasciata* larvae. To reproducibly mimic feeding of an herbivore, 20 µl of the OS was diluted 1:1 with de-ionized water and applied to the mechanically damaged fronds. Application of the same amount of water to mechanical wounds served as a control in all experiments.

Inhibition of VOC emission

Fosmidomycin was used to inhibit the DXP-reductoisomerase of the MEP pathway (Kuzuyama et al. 1998) and mevinolin to block the HMGR-CoA reductase, the main enzyme of the MVA pathway (Alberts et al. 1980). For inhibitor treatments, plantlets were cut and immediately placed in 100 µM of fosmidomycin (synthesized following a patent of the Fujisawa Pharamaceutical Company, (Kamiya 1980)) or mevinolin (Fluka Chemie GmbH, Buchs, Switzerland) solution for 24 h prior to elicitation of VOCs by JA. Before use, the lactone of mevinolin was converted into open acid form according to literature procedure (Kita et al. 1980).

Oxylipin analysis

Phytohormone analysis was performed by homogenizing approximately 200 mg of frozen tissue in 1 ml of ethylacetate spiked with the respective deuterated internal standards (100 ng). Homogenates were centrifuged for 15 minutes at 4 °C and the organic phase collected. The remaining plant material was re-extracted in 0.5 ml ethylacetate, organic layers were combined and samples evaporated under nitrogen. The residue was re-suspended in 70% methanol, centrifuged and analyzed by liquid chromatography-mass spectrometry (Wang et al. 2008). To analyze the hydroperoxide, 500 mg of frozen tissue was homogenized in an ice-cold mixture of chloroform/ methanol (2:1 v/v) spiked with 5 ng of 15-hydroperoxy-eicosadienoic acid (Cayman Chemicals, IBL International GmbH, Hamburg, Germany). Then, 1.25 ml of chloroform was added and centrifuged at 2,000 rpm for 15 minutes at 4 °C and the phases were separated. The water phase was re-extracted with 2 ml of hexane. Hexane and chloroform layers were combined and the solvents were evaporated under nitrogen stream. The samples were then re-suspended in 70% methanol and after centrifugation, analyzed by liquid chromatography (ESI) - tandem mass spectrometry. Free fatty acids analysis was performed by gas chromatography-mass spectrometry (GC-MS) as previously described (Kallenbach et al. 2010).

VOC collection and analysis

Treated fronds were bagged individually in a PET foil 'Bratschlauch' (Toppits® 'Bratschlauch', Melitta, Minden, Germany) that does not emit detectable volatiles by itself. VOCs emitted from each frond were collected continuously for 24 h on charcoal traps (1.5 mg charcoal, Gränicher & Quartero, Daumazam sur Azize, France) by pulling air at about 500 ml min^{-1} using a 12 V vacuum pump (Gast Manufacturing, Benton Harbor, USA). The traps were eluted with 2 × 20 µl dichloromethane containing 200 ng μl^{-1} of 1-bromodecane as an internal standard. Leaves were dried for dry weight determination. VOCs samples were analysed on a Thermo Finnigan Trace GC-MS (Thermo, Bremen, Germany) equipped with a fused silica Alltech EC5 column (15 m × 0.25 mm internal diameter × 0.25 µm film thickness) using 1.5 ml min^{-1} helium as carrier gas. Separation was achieved under programmed conditions (45 °C for 2 min, 10 °C min^{-1} to 200 °C, then 30 °C min^{-1} to 280 °C for 1 min; injector temperature: 220 °C). MS analysis was performed in electron impact full-scan mode at 70 eV with source temperature at 200 °C and GC interface temperature at 250 °C. Compounds were identified tentatively by comparison to the NIST database and subsequently collated with spectra from

reference compounds. Individual compounds were quantified with respect to the peak area of the internal standard and related to the dry weight of the frond.

Statistical analysis

Differences between treatments were evaluated with the 'general linear model' command of SPSS with 'treatment' as fixed and 'plant individual' as random factor. For multiple comparisons, LSD post hoc test tests were used when the variances were homogeneous and Tamhane's T2 post hoc test if this assumption was violated. All statistical analyses were done using SPSS 17.0 (SPSS Inc., Chicago, USA).

Results

VOC emission from P. aquilinum *after various treatments*

The total amount of VOCs released by bracken upon treatment with various elicitors (i.e. JA, coronalon, OPDA, linolenic acid, and alamethicin) was analyzed and compared to the effect of mechanical wounding and herbivore damage (Fig. 1).The total amount of VOCs emitted was generally higher in elicitor-treated plants relative to mechanically damaged and control plants (Fig. 1, LSD posthoc test after univariate ANOVA, $P < 0.02$, $n \geq$ 3-6 per treatment). Among all treatments, coronalon (COR) induced the highest production levels of VOCs. Damaging the fern fronds using a pattern wheel (single event) induced low VOC emission. To verify whether this result was merely the consequence of the low damage level, we employed a mechanical device (i.e. 'Mecworm' (Mithöfer et al. 2005)) to inflict a continuous and long-lasting damage that mimics insect feeding. However, Mecworm treatment did not significantly increase VOC release as compared to simple wounding (LSD posthoc test after univariate ANOVA, $P > 0.05$, Fig. 1, $n \geq$ 3-6). Interestingly, even treatment of fronds with the larvae of specialist feeder, *Strongylogaster multifasciata*, as well as with the generalist herbivore *Spodoptera littoralis* did not increase VOC emission rates in the treated plants (Fig.1).

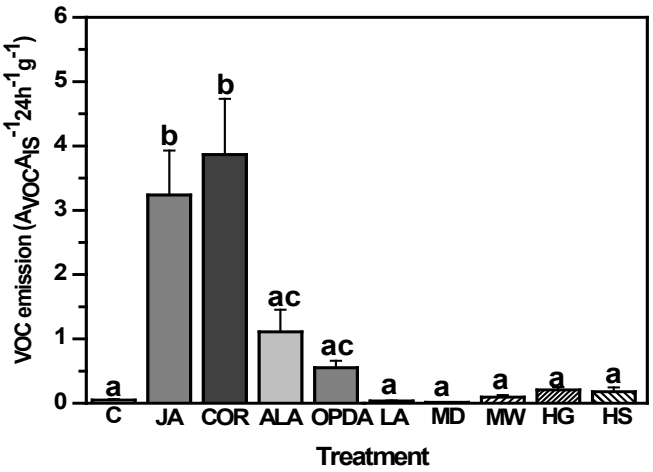

Figure 1. Total mean amounts (± 95% confidence interval) of volatile organic compounds emitted by *P.aquilinum* after various treatments: C (control, tap water); JA (Jasmonic acid, 1 mM); COR (coronalon, 100 μM); ALA-(alamethicin,10 μgml^{-1}); OPDA (12-oxophytodienoic acid, 1mM); LA (linolenic acid, 2 mM); MD (simple mechanical damage by pattern wheel); MW (mechanical damage by Mecworm); HG (damage by generalist herbivore, *S. littoralis*); HS (damage by specialist herbivore, *S. multifasciata*). The relative amounts of volatiles were determined as the ratio of peak area of a particular compound (A_{VOC}) to the peak area of an internal standard (A_{IS}) per gram dry weight. Different letters indicate significant difference between the treatments (LSD posthoc test after univariate ANOVA, $P < 0.02$, $n \geq 3$-5).

Also the qualitative composition of the VOC blends differed strongly upon the different treatments (Fig. 2). Nine dominant compounds were identified: 1-octen-3-ol and 3-octanol (both C_8 alcohols), p-cymene, limonene, γ-terpinene, linalool and α-terpineol (monoterpenes), (*E*)-(β)-farnesene (sesquiterpene), as well as nonanal in trace amounts. (*E*)-β-farnesene was the most dominant compound emitted after JA or coronalon treatment (Fig. 2a). 1-Octen 3-ol and 3-octanone were released after both damage- and elicitor treatments and to a small extent also from control plants (Fig. 2). ALA induced a VOC blend that closely resembled the one induced by treatment with the precursor of JA, OPDA, with the exception that (*E*)-β-farnesene was detected after ALA- but not after OPDA treatment. Limonene emission was detected after all elicitor treatments, except after linolenic acid- and damage treatments. In contrast, herbivory by a generalist herbivore induced the emission of limonene, yet in small amounts (Fig. 2b). In summary, bracken responded to JA and elicitor treatments with a characteristic emission pattern of VOCs, but neither mimicked nor natural herbivory induced such a VOC profile.

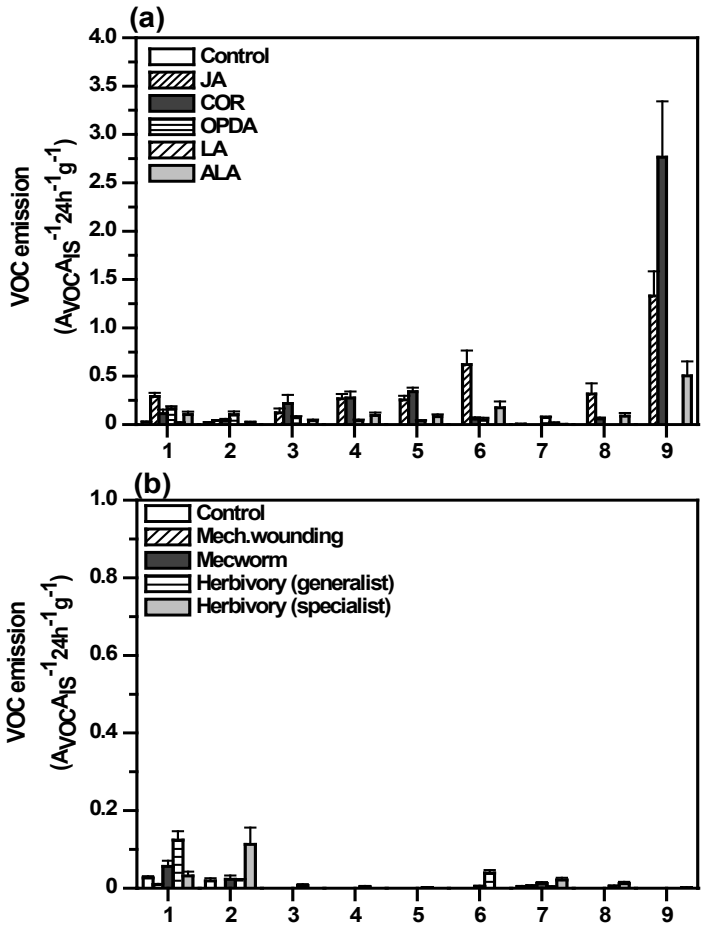

Figure 2. Qualitative differences in the mean VOC (± 95% confidence interval) emission after various treatments (same as Fig.1). (a) Elicitor treatments (C, JA, OPDA, COR, LA and ALA) and (b) Damage treatments (C, MD, MW, HG and HS). The relative amounts of volatiles were determined as the ratio of peak area of a particular compound (A_{VOC}) to peak area of an internal standard (A_{IS}) per gram dry weight. Nine compounds were identified from the VOCs blends: 1: 1-octen-3-ol, 2:3-octanol, 3: p-cymene, 4: limonene, 5: γ-terpinene; 6: linalool; 7: nonanal; 8: α-terpineol; 9: (E) β-farnesene.

VOC emission after inhibitor treatment

Since bracken produced both mono- and sesquiterpenes in response to JA treatment, we investigated the allocation of metabolic pathways involved in the production of these compounds using specific inhibitors (fosmidomycin and mevinolin). Treatment with either inhibitor did not affect the emission rates of C_8 volatiles, namely 1-octen-3-ol and

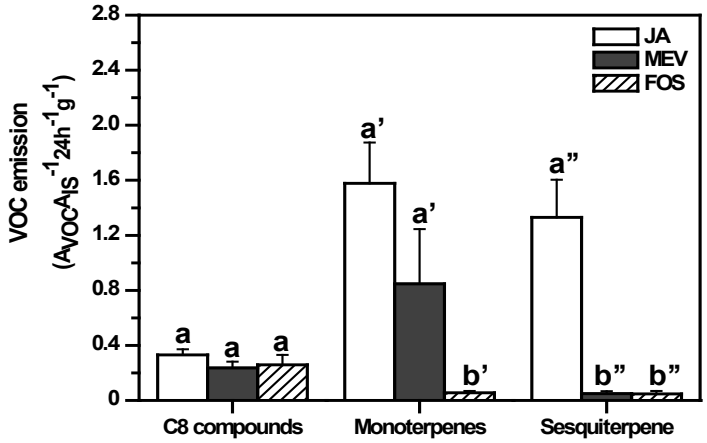

Figure 3. VOC emission upon application of inhibitors, fosmidomycin (FOS) and mevinolin (MEV) prior to JA treatment. Data represents mean (± 95% confidence interval) of five individual replicates in each treatment. Different letters indicate significant difference between the treatments (LSD posthoc test after univariate ANOVA, $P < 0.02$). VOCs emitted in response to treatments are grouped according to their chemical class (see figure 1, compounds 1-2 (C8); 3-8 (monoterpenes) and 9 (sesquiterpene)).

3-octanol (LSD posthoc test after univariate ANOVA, $P > 0.05$, $n = 5$) (Fig. 3), compared to JA-treated plants, whereas the amounts of monoterpenes emitted after inhibition with fosmidomycin were significantly lower compared to JA-treated plants (LSD posthoc test after univariate ANOVA, $P < 0.02$, $n = 5$). Fosmidomycin was more effective in blocking monoterpene production than mevinolin, indicating that the MEP pathway accounted for the formation of these compounds (Fig. 3). On the other hand, emission of (*E*)-β-farnesene was supressed by both inhibitors by almost 90% as compared to JA treatment, which suggests that the plastid-derived MEP pathway supports the formation of both mono and sesquiterpene synthesis in bracken. These results show that the mode of allocation for VOC emission in bracken is comparable to higher plants.

Oxylipin analysis

To unravel whether herbivory or tissue damage results in increased levels of endogenous JA as is known from higher plants, we monitored changes in the endogenous JA levels and its immediate precursors after both treatments. Initially, a kinetic study was conducted by measuring changes in the levels of the phytohormone JA, and its precursor 12-oxophytodienoic acid (OPDA) upon wounding as a function of time (Fig. 4a). Internal JA levels of the fronds reached a maximum after 30 minutes of wounding, while no major burst in OPDA levels was detected (Fig. 4a). C_{16} dinor-OPDA (dnOPDA) could not be detected. Further, the endogenous levels of 13-hydroperoxide (HPOT) were quantified at different times after wounding. Even though 13-HPOT levels increased with time after mechanical wounding, no major peak in the levels of this compound could be detected within 40 min (Fig. 4b). Furthermore, to analyze components of the octadecanoid signalling cascade upstream of JA biosynthesis, we also quantified the levels of free fatty acids (FFAs) in bracken before and 30 min after wounding. In unwounded tissue, total FFAs accumulated to about 8.8 ± 0.35 (mean ± 95% confidence interval) μmol (g fresh weight)$^{-1}$, of which saturated FFAs (16:0 and 18:0) accounted for 34% and unsaturated FFA ($16:1^{\Delta7}$,$16:2^{\Delta7,12}$,$16:3^{\Delta7,10,13}$,$18:1^{\Delta9}$,$18:2^{\Delta9,12}$,$18:3^{\Delta9,12,15}$) constituted 65% of the total amount of FFAs. Mechanical damage did not significantly increase the FFA content of the leaves 30 min after the stimulus, including the levels of linolenic acid ($18:3^{\Delta9,12,15}$), the major precursor for JA biosynthesis (Fig. 4c).

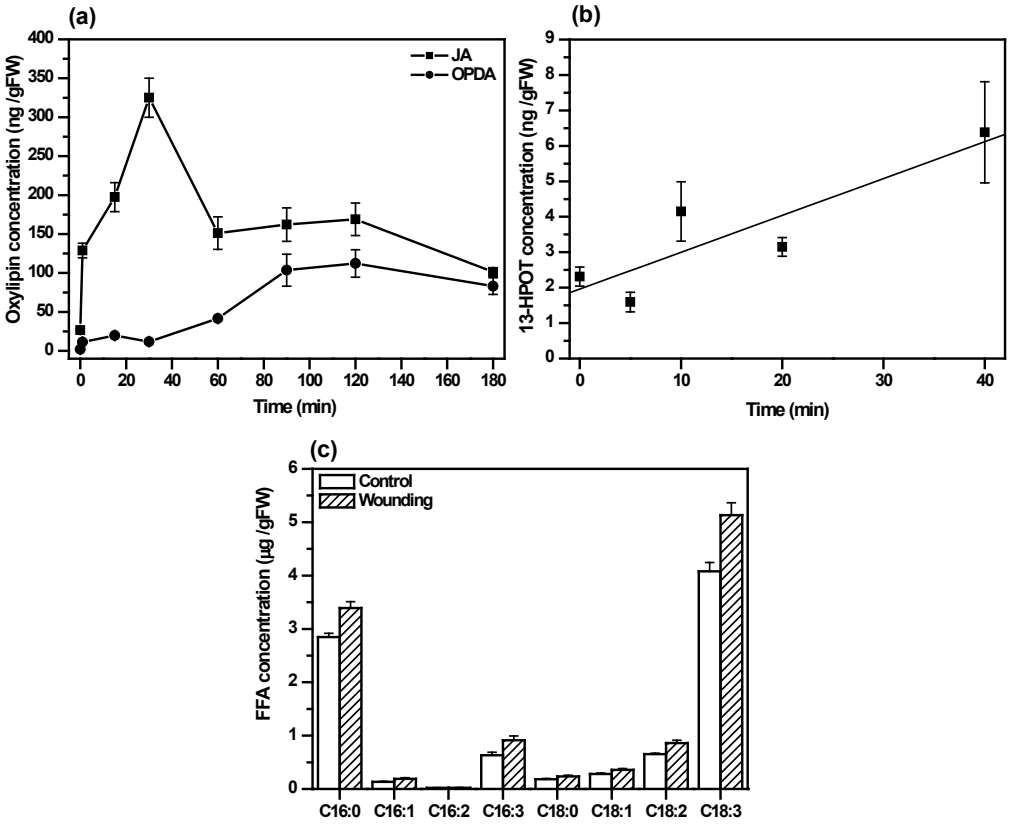

Figure 4. Analysis of oxylipins (a) Quantification and time course of JA and OPDA levels after simple mechanical wounding of three individual replicates in each time point (mean ± 95% confidence interval). (b) Kinetics of 13-HPOT after mechanical wounding. Data represents mean ± 95% confidence interval of four replicates at each time point. (c) Free fatty acid content of bracken fern before and after mechanical damage for 30 min presented as (mean ± 95% confidence interval) µg per gram fresh weight of the tissue of three individual replicates.

To analyze the effect of herbivory on endogenous JA levels, oral secretions (OS) from generalist herbivores (*Spodoptera littoralis*; reared on artificial as well as fern diet; Fig. 5a) and specialist herbivores (*Strongylogaster multifasciata*; reared on fern diet; Fig. 5b) were applied to mechanically damaged fronds. Interestingly, JA levels did not increase significantly relative to wounded tissue before and after generalist or specialist OS application (Fig. 5, Tamhane's T2 posthoc test: $P > 0.05$, $n = 5$).

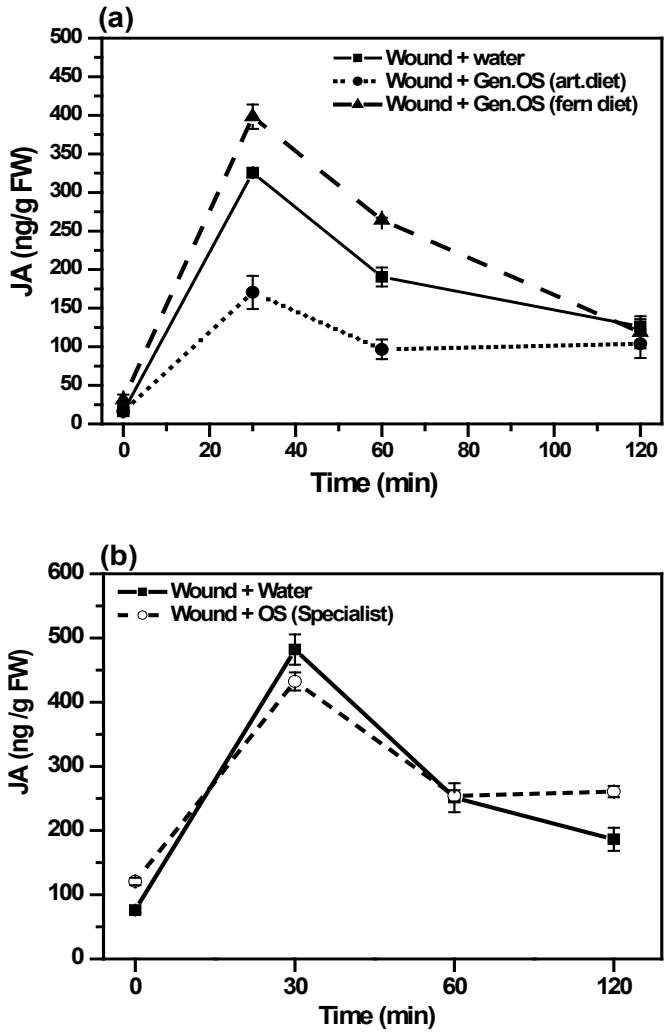

Figure 5. Quantification and time course of endogenous JA levels (mean ± 95% confidence interval) after herbivore treatment (a) JA levels after wounding only, wounding and application of oral secretion (OS) collected from generalist herbivore (Gen. OS), *Spodoptera littoralis* reared on artificial diet and fern diet to the mechanical wounds. (b) Endogenous JA levels after mechanical wounding and wounding + application of oral secretions collected from specialist herbivore (Spl. OS), *Mamestra brassicae* reared on fern diet. Data represents five individual replicates at each time point and treatment.

Discussion

The aim of this study was to investigate whether the emission of VOCs in the evolutionary ancient fern *P. aquilinum* is regulated by jasmonates like in higher plant species and also to compare VOC emission in bracken after various treatments (elicitors and damage) with known data from higher plants. Our results indicate that even though the fern responded to exogenous JA treatment with an increased VOC emission similar to higher plants, relatively

low amounts were released upon mechanical damage or herbivory, in contrast to higher plants like *P. lunatus* for instance (Figs. 1 and 2). Treatment with JA-related compounds such as OPDA and linolenic acid induced VOC emission, albeit in lower amounts as compared to JA treatment. In sum, although we could detect endogenous JA levels after simple mechanical wounding and herbivory (Fig. 4), these treatments did not induce VOC emission thereby indicating that the regulatory link that connects VOC emission to octadecanoid pathway in higher plants is probably missing in this evolutionarily ancient fern.

For many higher plants, it is well known that insect feeding induces the emission of a VOC blend that strongly resembles the one released upon exogenous application of JA (Arimura et al. 2005; Bruinsma et al. 2009; Dicke et al. 1999; Kost and Heil 2008; Zhang et al. 2009). In our study, however, mechanical damage or herbivory induced an emission of VOCs that was 'on average' one magnitude lower than after JA treatment. In order to rule out the possibility that the observed low VOC emission was due to a too low damage intensity, we used a computer-controlled device ('Mecworm'), which mechanically damages plant tissues by mimicking insect feeding in terms of damage duration and intensity (Mithöfer et al. 2005). Interestingly, even continuous damage inflicted by this instrument did not elicit a strong VOC emission in the fern (Fig. 2).

When the ferns were treated with COR, higher amounts of VOCs were released relative to JA-treated ferns (Fig. 2a), with the emitted blends being qualitatively almost identical. This is in stark contrast to what is known from higher plant species, in which elicitation with COR induces a much more complex spectrum of VOCs than JA (Schüler et al. 2004). Furthermore, in *Phaseolus lunatus,* treatment with ALA mainly induces homoterpenes and the emitted VOC blend resembles OPDA-treated rather than JA-treated plants (Koch et al. 1999). In the present study, ALA treatment elicited a VOC profile that was more similar to OPDA-treated than JA-treated plants, thereby corroborating previous findings in higher plants. The major compound we observed upon JA- and COR-treatment was (*E*)-β-farnesene, which is a sesquiterpene released by many different plant species (Fig. 2a). Previous studies have shown an increased emission of this compound in response to simple mechanical damage (McAuslane and Alborn 1998), herbivory (Pare and Tumlinson 1999; Rose and Tumlinson 2004) and JA treatment (Rodriguez-Saona et al. 2001; Schmelz et al. 2001).

VOCs are known to be predominantly synthesized via the MEP or MVA pathways in higher plants and studies using inhibitors of either pathways to dissect the origin of the mono- or sesqui-terpenes have shown that there can be crosstalk between the two pathways (Laule et al. 2003). For example, in lima bean, herbivory stimulates the emission of homoterpene

DMNT through the cytosolic MVA pathway (Bartram et al. 2006). When this pathway was blocked, the MEP pathway was found to compensate and treatments of different elicitors was found to channel biosynthesis of DMNT via MEP or MVA pathways (Bartram et al. 2006; Jux et al. 2001). In another study using *Antirrhinum majus*, it was reported that the MEP pathway can provide substrates for both sesquiterpene and monoterpene biosynthesis respectively (Dudareva et al. 2005). In bracken, we found that fosmidomycin can effectively block both monoterpene and sesquiterpene emission and mevinolin, on the other hand, could inhibit sesquiterpene biosynthesis effectively, which is in line with previous reports.

In summary, in the fern species studied here, elicitors like JA, COR OPDA, linolenic acid and ALA induced emission of VOCs similar to higher plants albeit differences in the complexity of the VOCs spectra whereas mechanical damage or herbivory resulted in much reduced emission levels. A possible ecological explanation for this could be that bracken fern depends more on direct than indirect defences to protect itself from herbivore feeding. Indeed, bracken is known to be highly toxic and generally unattractive to insects or mammalian herbivores (Balick et al. 1978; Cooper-Driver 1985; 1990; Cooper-Driver et al. 1977). Among the few insects feeding on bracken, a predominance of sawflies has been reported (Cooper-Driver 1978; Smith 2005). Consistent with these reports, *S. multifasciata* was observed to be a herbivore of bracken in its natural growing site and was used for our experiments. However, larval feeding of this herbivore resulted in much lower emission levels of VOC than was released from elicitor-treated ferns (Fig. 2).

The induction of endogenous levels of JA in response to mechanical damage and herbivory was not different in our study and did not exceed 500 ng (g FW)$^{-1}$ (Figs. 4 and 5). The observation that exogenous JA treatment could invoke VOCs while herbivory or simple damage could not; together with the result that the endogenous JA levels remained similar for mimicked and natural herbivory implies that the lack of VOC emission after herbivory could be due to low internal JA levels. Probably, the endogenous JA level does not exceed a "threshold" value required for the biosynthesis and release of VOCs, which could be attained by external application. However, whether the reduced VOC emission in response to herbivory in bracken is actually due to this endogenous 'threshold' problem needs further studies.

Interestingly, another lower plant *Ginkgo biloba*, has also been shown to produce increased amounts of VOCs upon JA treatment, but failed to emit any volatiles after tissue damage (Van Den Boom et al. 2004). Our results are in line with these observations, thereby indicating that in ancient plants, the biosynthetic machinery needed for the emission of VOCs

is already active. However, whether other biotic or abiotic stress factors can activate these responses remains elusive. For example, the observations that COR, a structural mimic of coronatine, which is derived from the phytopathogenic bacterium *Pseudomonas syringae*, and ALA, an ion-channel forming peptide originally isolated from the fungus *Trichoderma viride* induced VOC emission in bracken, may point into the direction of VOC emission functioning primarily as a direct defence against phytopathogens (Holopainen 2004). However, this hypothesis needs further testing. Unfortunately, attempts to infect bracken with *P. syringae* were unsuccessful and did therefore not result in increased production rates of VOC (data not shown). These preliminary experiments highlight the need to identify pathogens that also infect bracken, to test the abovementioned hypothesis.

Furthermore, the main proportion of insects that attack fern plants are phloem feeders (Shaposhnikov 1987), whose effects on VOC emission need to be evaluated. Aphids are known to induce VOC emission in barley plants (Ninkovic et al. 2001) and recently, it was demonstrated that the octadecanoid pathway, specifically the *COI1* gene is required for the production of aphid induced VOC emission in *Arabidopsis thaliana* (Girling et al. 2008). Similar studies in bracken are necessary to understand the functional significance of VOC emission in ferns.

Emission of VOCs by plants has been a topic of debate since many years and a functional explanation of plant VOCs has been sought intensively (Dudareva et al. 2006; Gang 2005). Although the emission of VOCs in plants is traditionally assumed to function as a defence against herbivores (Fraenkel 1959, Dicke et al. 1991), it is also known that VOC can serve other purposes such as antibiotics against plant pathogens or protection against abiotic stresses such as UV-B radiation and ozone (Holopainen 2004). For example, it is known that mosses emit isoprene, which provides thermo tolerance against temperature fluctuations (Hanson et al. 1999). Gymnosperms, such as conifers store and emit monoterpenes upon ozone exposure, which may serve as an exogenous protection against ozone (Loreto et al. 2004).

Although it might be difficult to reconstruct the evolutionary origin and ancestral function of VOC emission, studies on phylogenetically ancient plant species can help to answer these questions. Following this approach, our results indicate that the biosynthetic machinery for VOC emission that is regulated via the octadecanoid pathway is already present in *P. aquilinum*. However, and in contrast to what is know from higher plants, it is not linked to mechanical wounding or herbivore damage. This finding suggests that VOCs likely serve a different ecological function in bracken.

Currently, virtually all knowledge that is available on indirect defences such as VOC emission, stems from higher plants such as cotton, tobacco, tomato, soybean, lima bean and maize (van Poecke and Dicke 2004). In light of the abovementioned findings in bracken, it will be very interesting to also investigate VOC emission in other plant species, which are more derived than ferns, such as conifers or gnetales. Understanding the regulation of VOC emission in these plant species will help to trace back the point in the evolutionary time at which the plant-internal recognition mechanisms for herbivore damage and the downstream octadecanoid signalling pathway were linked to the VOC producing machinery.

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8. General discussion

This thesis aims at contributing to the understanding of jasmonate-mediated indirect defence mechanisms in plants and extends the knowledge on regulation of indirect defence responses by including also the role of abiotic factors. Furthermore, it provides novel insights into the evolutionary origin of these defences. When lima bean is attacked by a herbivore, which can be mimicked by exogenous jasmonic acid (JA) application, it distributes its indirect defences (EFN secretion and VOC emission) in an optimal manner in such a way that plant parts with higher expected future fitness value are defended more strongly (Manuscript I). Extending this spatial variation, temporal patterns of EFN secretion were investigated in same study system (Manuscript III). The JA-mediated control of EFN secretion was found to be light-dependent and JA-Ile was identified as the active signal molecule controlling this indirect defence (Manuscript III). Additionally, JA was also demonstrated to control reproductive floral nectar secretion in *Brassica napus* (Manuscript II & IV). Further, JA regulation of VOC emission was studied in the evolutionary ancient bracken fern, *Pterdium aquilinum* (Manuscript V) to understand the evolutionary origin of this defence trait.

The following discussion integrates the findings from the present work, describing the ecology and regulation of spatio-temporal variation in indirect defences, suggesting how results from this thesis help in answering some functional questions in plant-insect interactions. Moreover, the role of indirect defences from an evolutionary perspective and potential future directions are also discussed.

Variability in plant defences – changes in space and time

In 1965, Bradshaw defined plasticity as ‘shown by a genotype when its expression is able to be altered by environmental influences’ (85). This environment–induced phenotypic variation in plants is often considered to be a functional response to maximize fitness (86). Assessing variability in plant morphology and physiology is therefore critical to understand the function of phenotypic plasticity. For example, the chemical characteristics of a plant show seasonal variation. Moreover, plants may differ in their quality as food source for herbivores whether between species, between individuals of same species as well as within parts of the same plant. Dissimilarities in biomass partitioning, environmental conditions, differences in history of relationships with herbivores or genetic differences have been invoked to explain this high

variability in plant characteristics, thus creating a nutritional mosaic for the foraging herbivores (87). Several hypotheses have been put forward to explain the distribution of plant defences and studies reporting evidence in favor and against these hypotheses are known (88). These theories largely predict variability in direct defences which confer a 'bottom-up' control on herbivores, does the basic hypothesis hold true also for indirect defenses ('top-down' control)? In the following, the variability in indirect defenses, causal factors and ecological consequences are discussed.

Induced responses to herbivory that result in changes in the quality and/or quantity of EFN have been reported in many plant systems such as *Gossypium hirsutum* (63), *Impatiens sultani* (89), *Ricinus communis* (90), *Passiflora incarnate* (91, 92), *Macaranga tanarius* (93) and *Phaseolus lunatus* (51) (for review see (94). How does EFN secretion vary within a plant? In the present work, it was revealed that upon plant-wide attack as mimicked by JA application, the indirect defences were optimally allocated to the most expected valuable plant parts, which in line with the predictions of the optimal defence theory (ODT) (Manuscript I). Although the ecological significance of the present findings remains to be demonstrated, there exist reports in other plant systems, where ants, the most important arthropods attracted by EFN secretion patrol and accumulate on young leaves (95, 96). In case of lima bean, correlating the spatial distribution of EFN secretion to ant activity remains to be studied. Additionally, EFN secretion in some plant species follows circadian rhythms while in some cases, it is constant throughout day or night (97-99). In the current study, it was found that lima bean secretes higher EFN at night as compared to the day (Manuscript III). Since very few studies have addressed the temporal dynamics of EFN secretion, the ecological advantages of temporal patterns in EFN secretion remains to be explored (93). However, in several studies, long term temporal patterns in EFN production have been interpreted as an adaptation to either occurrence of herbivores or defenders (14, 100).

The emission of volatile organic compounds can vary both qualitatively and quantitatively depending on the leaf developmental stage, type of herbivore, genotype or cultivar as well as abiotic fluctuations such as light intensity, water and nutrient availability (101, 102). In addition, diurnal patterns in VOC emission have been demonstrated. In lima bean, for instance, it was demonstrated that the VOC, ocimene is released only during the photophase (57). Continuous nocturnal mechanical damage invoked very low amounts of ocimene only, which was attributed to the limited supply of substrates necessary for the biosynthesis of ocimene in the dark phase (57). In contrast, it was shown that in response to herbivory, *Nicotiana tabacum* releases several volatiles exclusively at night, which were

shown to repel female moths (26). The ecological function of herbivore-induced volatiles has also been demonstrated in case of volatiles released from lima bean plants when attacked by *Tetranychus urticae*. The induced volatiles were attractive for the carnivorous mite, *Phytoseiulus persimilus*, which removed the two-spotted spider mites from the plant (103, 104). Several similar volatile-mediated interactions between plants and carnivores are reported in case of plant-caterpillar-parasitic wasp tritrophic systems ((105), for review see (106)). In addition to being an indirect defence against herbivores, VOC emissions are also known to participate in plant-plant signaling and have also been shown to serve as an intra-plant cue for inducing EFN secretion in lima bean (107, 108). In the present study, VOC emission was higher in younger leaves (Manuscript I), however, the putative effect of VOC acting as a mobile signal for induction of EFN secretion within the plant could not be disentangled, which should be addressed in future studies. What is the benefit of spatiotemporal variation in defence responses? Plant defences are generally considered to be costly and therefore plants must attain a balance to ensure protection without compromising growth. One obvious advantage of spatiotemporal patterns in defence responses could be that the plant can save its resources by expressing defences intensely at most valuable part or at times when the defenders are most active.

What is the cost of producing these defences? The primary benefit of inducible defences is the economy since expressing defence traits need expenditure of metabolic energy which can be otherwise used for growth and reproduction (10, 11). It is reported that some plant species have lost nectaries in ecosystems without mutualistic ant species which implies that secreting EFN in the absence of nectar feeders can be costly (14, 109). In another study, the expenditure of EFN secretion was calculated to account for 1% of the total energy investment in the neotropical tree, *Ochrima pyrimidale* (110). However, in *Acacia* species, constitutive EFN secretion was found to be the more derived state than induced secretion indicating that constitutive secretion might be costly and benefits the nectar-secreting plant only when the defenders are present permanently (obligate ant-plants) (111). In general, the cost of producing EFN is assumed to be low as observed in the above mentioned studies; however, this has not been really tested in many studies. The actual cost of EFN secretion could arise depending on the resource availability (water and nutrients), which needs more investigation. In addition to the direct costs of diverting primary metabolites to EFN, it can also entail indirect (ecological) costs as EFN secretion can itself attract herbivores (112). When herbivores are attracted and retained by EFN, herbivory on nectar-bearing plant can increase (29, 112, 113). In many plants, a baseline level of EFN is secreted constitutively (63,

93) to ensure prophylactic protection e.g. prevention of herbivore oviposition or removal of herbivore eggs (114) even before the herbivores arrive. In this case, the cost-saving benefit of inducible EFN secretion is countered by loss of this prophylactic protection (87). In evolutionary context, selection should favor attributes that increase the benefit (protection from herbivores) and reduce the cost (EFN secretion) (14). Since increased attraction of ants might result from increasing nectar secretion, this would further increase the cost of this defence. Therefore, if a plant can control the time and/or location of EFN production by secreting higher amounts of EFN at more vulnerable plant parts at time when the herbivores are active; this would reduce the cost of protection.

The cost of producing volatiles is reported to be high (115, 116), yet there are reports where the cost of volatile production were estimated to be low (117-119). The biosynthesis and storage of terpenoids is expected to entail higher costs as compared to the maintenance of terpenoid pools in plants (115). Nonetheless, quantification of metabolic costs in producing defences still remains a topic of debate since experimental evidence is scarce and controversial (11, 87, 117). In maize plants, the cost of induced volatile emission in response to caterpillar (*Spodoptera littoralis*) regurgitant treatment was detected only in young plants (120). Very low or no cost of volatile production (in terms of seed dry weight) was detected in mature plants, which was reported to compensate for their metabolic investment in the earlier developmental stage (120). Metabolic adaptations like sharing of biosynthetic enzymes between different pathways involved in terpenoid production or use of single enzyme to make many products have been proposed to reduce terpenoid costs (115). The high metabolic cost involved in plant defences with the frequent absence of measurable fitness costs for defence as a whole, itself indicates indirectly that plants must have evolved mechanisms for reducing the costs of defences (121).

Regulation of indirect defences

Parallel to functional interpretation of allocation patterns, it is also very important to consider the underlying mechanisms that control these patterns in indirect defences. EFN secretion has been described as a passive process by some researchers and extrafloral nectaries are considered to have originated as sugar valves through which plants excrete surplus sugars (14, 122). This theory is in accordance with the fact that activity of extrafloral nectaries often correlates with local requirements for nutrients and assimilates (sink strength) (Manuscript I

and (123)). Furthermore, literature reports demonstrate that vascular architecture can create variation of nutrients, which can lead to spatial variability in plant defences (124, 125).

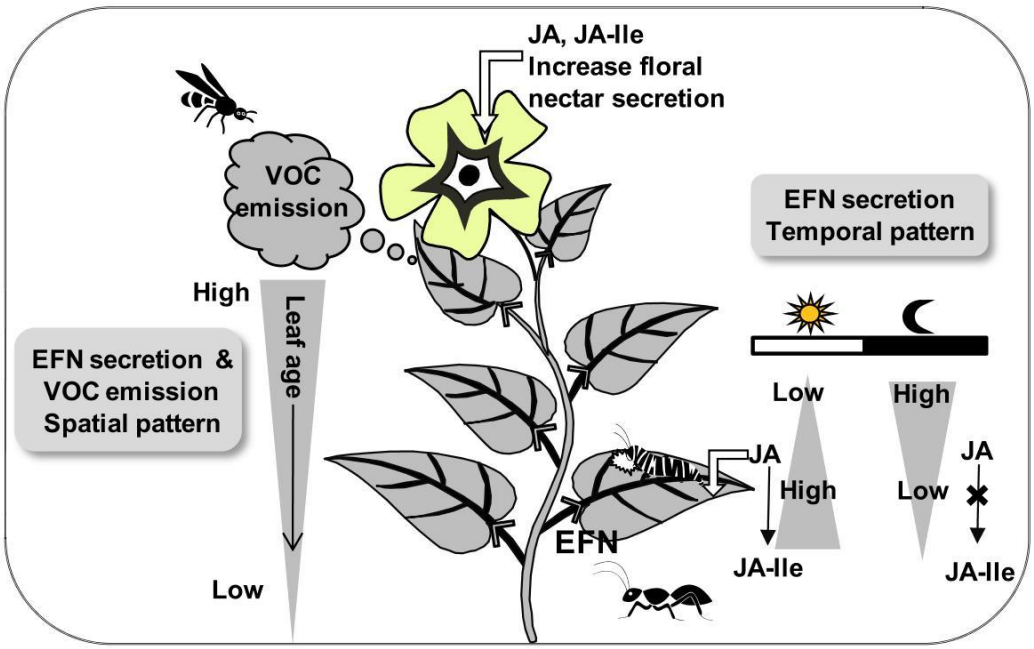
How do plants modulate their defences temporally? How do plants 'know' whether it is day or night and when to respond? Light is the most important external stimulus that entrains the innate clock of plants to environmental day and night cycle (126). Such synchronization requires intimate overlap with signaling cascades to bring about the necessary specific changes accordingly. In fact, in 1937 (127), it was noted that the sensitivity of plants to auxin varies over the day indicative of what is presently known as the gating of auxin signaling and hence suggest a link between phytohormone signaling and the circadian clock (128). Such interactions are also known for other phytohormones like abscisic acid, cytokinins and ethylene (129). Predictable daily rhythms in light necessitate rhythms of uptake and use of water and carbon by plants (130). Therefore strong evolutionary pressures favor physiological rhythms like stomatal responses, hypocotyl elongation and cold signaling, which obviously require corresponding rhythmic hormonal biosynthesis that regulates such processes (129, 131). Indeed, circadian rhythms in endogenous hormonal biosynthesis are known, for example, the levels of indole acetic acid (IAA) and its conjugates vary in a circadian manner in *Arabidopsis thaliana* (132). Additionally many auxin-induced genes are regulated by circadian rhythm (128, 133). Further, as mentioned earlier, rhythmic sensitivity to auxin and ABA are also known (129), however, the mechanism of this regulation is not known (134). In the present study, it was shown that jasmonate mediated regulation of EFN secretion is light dependent, indicating a network, in which abiotic factors (light availability); jasmonate signaling and indirect defences are tightly integrated. What is the effect of other biotic factors like temperature or soil characteristics? Indeed, it is reported that at elevated as well as at low temperatures, plant have less immunity to pathogens (135, 136). Also, the effect of plant allelochemicals on insect herbivores and their predators has been shown to be a function of temperature (137, 138). Similarly, abiotic factors play a major role in influencing herbivore-induced VOC emissions (21, 23, 25). For example, *Zea mays* released higher VOC levels in dry soil than in wet soil and at an optimal temperature range of 22 – 27° C (23). Hence abiotic factors are important parameters, which impact the intensity and variability of plant defence responses.

So far, the role of jasmonates in plant defence responses have been discussed, but in addition, jasmonates are also important for plant developmental processes such as flowering, senescence, seed germination, anther dehiscence, tendril coiling and root growth (30, 139, 140). Since plants are simultaneously visited by detrimental herbivores and beneficial

pollinators and both visitors can have immense impact on plant fitness, how do plants cope with herbivory without compromising its reproductive fitness? Although the effect of each visitor (pollinator and herbivore) is known individually, few studies have dealt with interactive effects. This relationship between attraction or defence traits can occur through a number of mechanisms. For example, ecological or pleiotropic effects may result in trade-offs between attraction and defense characteristics (141). The genes or the common precursors along the metabolic pathway of anti-herbivore defence might be linked or impact floral attractive traits or *vice versa* (141-143). For instance, in a recent study, it was demonstrated that JA drives herbivore-induced changes in flower-opening times in tobacco, suggesting that herbivore-induced changes in floral or flowering traits can positively affect plant fitness (144). In line with these findings, in the present work, JA was revealed to regulate floral nectar (FN) secretion, the primary reward for pollinators in *Brassica napus* (Manuscript II & IV). The discovery of jasmonates as a common signal for floral and extrafloral nectar secretion opens new perspectives and forms a basis for future studies addressing the mechanisms by which plant achieve a functional discrimination between stress and sex.

Indirect defences – an evolutionary perspective

There are two hypothesis, which explain the evolution of nectar (FN and EFN) secretions: 1) 'the leaky phloem hypothesis'-according to which, the high hydrostatic pressure of the phloem coupled with the structural weakness of the expanding tissue can lead to a ‚leak' in the phloem solution resulting in nectar (14) and 2) ‚the surplus sugar excretion hypothesis', according to which nectar might have originated as an excretion of excess sugar from the phloem due to the high transpiration rate of the flowers (for review see (145)). Both hypotheses are complementary to each other (145). For example, the leaky phloem hypothesis could be relevant for nectar secretions in non-reproductive organs and the sugar excretion hypothesis might hold true for more derived plant species with flowers (145).



Scheme 1. A schematic illustration showing major findings of the present work (Manuscript I, II and III). The scheme shows a plant featuring floral as well as extrafloral nectar (EFN) and also capable of emitting VOCs. EFN and VOCs induced upon herbivory are attractive to carnivorous arthorpods like wasps and ants respectively. The left hand side shows the spatial pattern in the distribution of EFN secretion and VOC emission within the plant after JA treatment, which mimics herbivore attack; young leaves produce more of these defences than older leaves (Manuscript I). The floral nectar secretion is also controlled by JA; higher floral nectar secretion was observed upon treatment with JA and its derivative, JA-Ile (Manuscript II & IV). The right hand side of the scheme depicts the temporal pattern in EFN secretion before and after JA and JA-Ile treatments; in the absence of any treatment, EFN secretion is higher at night. Upon JA treatment or wounding at night, EFN secretion is lowered probably due to the low levels of JA-Ile formation indicating JA-Ile as the active signal for the regulation of EFN secretion (Manuscript III).

However, these hypotheses consider physiological reasons for the evolution of nectar secretion, whereas in addition, ecological significance of this secretion needs also to be taken into account because both floral and extrafloral nectar confer a huge selective advantage to the secreting plant either by attracting predators (EFN) or pollinators (FN) (51, 146). Therefore, to understand the origin of nectar secretion, it is important to gain insight into the signalling pathways controlling the nectar secretion, as well as the nectar-mediated ecological interactions. The oldest extant plant species known to bear nectaries is the bracken fern (*Pteridium aquilinum*) (147) and, EFN secretion in bracken could neither be induced by jasmonates nor by herbivory (VR, unpublished results). Nectar secretion is more common in Angiosperms, dating back to late Cretaceous when both floral and extrafloral nectaries were

present (146). Future studies on understanding regulatory mechanism of nectar production in plant systems featuring both extrafloral and floral nectaries (eg. Euphorbiacea, Bignoniacea, Passifloracea) will help to explore how plants achieve and maintain discrimination between these two important secretory processes.

How did herbivore-induced VOC emissions evolve? Although several fascinating functions of plant VOCs are being discovered the basic question of why must plants produce such a diverse VOC blend still remains under debate and VOC emission are discussed to have originated as a direct defence, which later might have evolved into an indirect defence mechanism (148-151). The present study adds a new dimension to this whole debate because the bracken fern is capable of synthesizing VOCs in response to known elicitors (see Table 1) but VOC emission was not elicited upon damage treatments, thus lending support to the idea of subsequent evolution (Manuscript IV & V, see also Table 1). In a seminal review, Jones & Firn (1991) established that plants actually contain a huge number of inactive secondary compounds and the evolution of plant defence might have proceeded independent of consumer adaptation (152). Although the 'raison d'être' of plant VOCs is generally assumed to be against herbivory, it is known that the radiation of insects occurred much later than evolution of plant traits and there is a lack of evolutionary feedback from insects to plants (5, 153). Additionally it is known that VOCs can serve other purposes than defence against herbivores such as antibiotics in plant-pathogen interactions, attractants in plant-pollinator interactions, in plant-plant communication, thermo-tolerance or even as a direct defence mechanism (107,150). The present study shows that the ability to synthesize VOCs in response to jasmonates exists in archaic species but whether or not these emissions are useful in repelling herbivores directly is not known. Also the question of whether or not VOC emission cost effective against herbivory should be considered (154,155). Future studies should aim at assessing cost-benefit paradigm of VOCs including the cost of emitting VOCs to attract natural enemies in the absence of natural enemies of the herbivores.

Table 1. A comparison of VOC emission from bracken (Manuscript V) and lima bean

Treatment	Terpenoid emission		References
	P. aquilinum[a] (Manuscript V)	***P. lunatus*** (see Refs)	
Jasmonic acid	++	++	(32, 62)
12-oxophytodienoic acid	+	++ (including TMTT)	(156)
Linolenic acid	tr	only DMNT & TMTT	(156)
Coronalon	++	++	(157)
Alamethicin	+	Homoterpenes & MeSA	(158)
Mechanical damage	tr	++	(57, 159)
Herbivory	tr	++	(61, 159)
Fosmidomycin	mono & sesquiterpene blocked	monoterpenes blocked	(160, 161)
Mevinolin	only sesquiterpene inhibited	DMNT blocked	(160, 161)

[a]Homoterpenes (DMNT or TMTT) were not emitted after any of the given treatments (++ >40%); + 10-25%; tr <10%)

Future perspectives

In a classic case of 'the enemy of my enemy is my friend', plants attacked by herbivores activate indirect defences that are attractive to carnivores, the natural enemies of the herbivores. EFN secretion and VOC emission, the focal defence responses of the present work represent such indirect strategies, both of which are reorganized functionally after herbivore attack (Manuscript I). Investigation of plant-herbivore interactions from a phytocentric perspective crucially depends on mechanistic knowledge of plant's signal transduction pathways like for example, the jasmonate signaling cascade, which regulates these defences. Manipulating plant responses by modifying key components of the cascade using either inhibitors or genetic approaches would help in further dissecting the complexity of these responses. Although in the present work, such an attempt was made using inhibitors (Manuscript II, III & V); further studies on plants, genetically manipulated to express particular defense traits subject to different environments would help in understanding functional significance of particular traits. In the arms race between plants and herbivores, patterns in plant defences can drastically influence feeding behaviour of herbivores. For example, feeding patterns of herbivores might actually reflect the plant's rhythmicity in defence expression. Therefore understanding the circadian control of plant defence responses is vital. Future studies should also consider the effects of other community members like

pathogens and competitors on these interactions and their overlapping signal cascades such as between JA and SA pathways or between light (far red) and defence signaling. Taken together, the present work provides answers to the questions initially posed (see aims of this thesis in the introduction) namely:

1) Are indirect defences equally distributed within a plant? - **No**, indirect defences are optimally allocated to younger plant parts, which reflects the supposed future fitness value (Manuscript I)

2) Do jasmonates also control reproductive floral nectar secretion? - **Yes** (Manuscript II & IV)

3) Does light environment interact with JA signaling? - **Yes** , light plays a major role in influencing jasmonate regulation of EFN secretion (Manuscript III) and

4) Do ancient plants emit VOCs in response to jasmonates and herbivory? - **Yes and No** , bracken emits VOCs when treated with jasmonates, but not upon herbivory (Manuscript V).

9. Summary

Plants have evolved a multitude of protective traits to cope with abiotic and biotic stress factors. These defensive traits can either directly affect the herbivores, such as toxins or other deterrents (i.e. 'direct defences'), or act indirectly by recruitment of the herbivores' predators (i.e. 'indirect defences'). The present thesis focuses on inducible indirect defence strategies of plants, namely the secretion of extrafloral nectar (EFN) and the emission of volatile organic compounds (VOC). Both defence mechanisms are inducible upon herbivore damage and are regulated by the octadecanoid pathway, in which the phytohormone, jasmonic acid (JA) acts as the central signaling molecule. The present work aims at elucidating the role of jasmonates (JA and its derivatives) for regulating nectar secretion (i.e. both floral and extrafloral nectar) and VOC emission as well as understanding the ecological and evolutionary constraints involved in shaping plant indirect defence traits. These basic aspects were illuminated from various angles using different model systems particularly suited to address individual questions. The first goal was to gain insight into factors that determine the spatial distribution patterns of EFN and VOCs in response to jasmonate induction (Manuscript I). Plant defense responses are critically affected by abiotic factors such as light availability, which allows a plant to adapt to changing environmental conditions. Therefore, the next aim was to investigate the impact of light on the JA-dependent EFN secretion (Manuscript III). EFN secretion plays a vital role in the plant defence, while floral nectar (FN) is an important reward for pollinators. To identify differences and similarities in the regulatory processes that underlie nectar secretion, the role of jasmonates in controlling FN secretion was explored (Manuscript II & IV). Finally, most of the literature on indirect plant defences is derived from higher plant species and very few studies so far have also analyzed lower plants in this context. To bridge this gap and to scrutinize the role of jasmonates for regulating indirect defences also in lower plants, VOC emission in response to jasmonates and herbivory was investigated in the ancient bracken fern (*Pteridium aquilinum*) (Manuscript V).

Spatial distribution of indirect defences reflect an optimal defence strategy

Optimal defence hypothesis (ODH) predicts defences within a plant should be allocated such that the more valuable and vulnerable parts are defended more intensely. Upon herbivory, lima bean defends itself by secreting EFN or by emitting VOCs. However, are all plant parts defended equally? Analysis of EFN and VOC production as a function of leaf age revealed that younger leaves produced more EFN and emitted more VOCs as compared to older

tissues, which is in agreement with the predictions made by the ODH. Although younger leaves exhibited lower rates of photosynthetic assimilation (i.e. the main source for the building blocks of the two defences) than mature leaves, the production of these two indirect defences was most intensive in younger tissues. Experiments using labeled $^{13}CO_2$ suggested that the photosynthates necessary for these defences are transported from older to younger leaf tissues, where tissue loss probably has more severe fitness consequences. These results indicate that allocation to indirect defences within a plant follows an optimal defence strategy.

Floral nectar secretion is regulated by jasmonates

EFN secretion is an indirect defence mechanism, whose production is controlled by jasmonates. However, it was not known whether jasmonates also regulate the secretion of floral nectar that is mainly secreted for pollinator attraction. To address this question, the role of jasmonates for floral nectar secretion was investigated in *Brassica napus*. Here it was found that - similar to EFN - jasmonates were involved in the regulation of floral nectar secretion. Exogenous application of JA, jasmonoyl isoleucine (JA-Ile), and coronalon (i.e. structural mimic of JA-Ile) to flowers enhanced the secretion rate of floral nectar, whereas inhibiting JA biosynthesis reduced nectar secretion levels. However, treating leaves with jasmonates did not affect the floral nectar secretion, which indicates a functional regulatory separation between leaf herbivory and floral nectar secretion. These results suggest that jasmonates are not only important regulators of plant defences against herbivores, but are also involved in controlling the floral nectar secretory process.

Regulation of EFN secretion by jasmonates is light-dependent

In addition to coping with herbivores, plants must also coordinate their responses with changing abiotic conditions, with the availability of light being one of the most important factors. To understand the influence of light conditions on jasmonate-regulated indirect defences, EFN secretion induced by jasmonates, was studied in plants exposed to light regimes that differed both qualitatively and quantitatively. Under normal day-night conditions, EFN secretion in untreated *P. lunatus* plants followed a temporal pattern and peaked in the night. JA treatment, however, had different effects on the EFN production, depending on the light environment: induction under light and suppression under dark conditions. Interestingly, JA-Ile application did not reduce EFN secretion in the dark like JA, but it induced EFN secretion under light conditions. In plants exposed to a ratio of 10:90 (R:FR) radiation, JA- Ile but not JA induced EFN secretion. At 100% FR both JA and JA-Ile

reduced EFN secretion. Inhibition of Ile biosynthesis led to a reduced EFN secretion, even under light conditions and biosynthesis of JA-Ile was found to be light-dependent. These results imply an interaction between light quality and jasmonate signaling, which results in a tightly controlled modulation of the defence in response to the light regime to which a plant is exposed.

Volatile emission in the evolutionary ancient fern *Pteridium aquilinum* is triggered by jasmonates but not linked to herbivory

With the aim to understand the evolutionary origin of the regulation of indirect defences, VOC emissions were studied in the evolutionary ancient fern species *Pteridium aquilinum*. The results indicated that this fern could produce volatiles in response to elicitors that are known to activate VOC emission in higher plants (JA, JA-Ile, alamethicin, coronalon, OPDA (precursor of JA) and linolenic acid). However, in contrast, no volatiles were emitted upon mechanical damage or herbivory (generalist and specialist) which suggest a different, yet unclear, ecological function of VOCs compared to higher plants as well as a different signalling pathway in response to herbivory. Further, no significant changes in the endogenous oxylipin (JA and its precursors) levels were observed before and after herbivory. In sum, these results suggest that the biosynthetic machinery for VOC emission was already present when the regulatory link between herbivory and the jasmonate signaling pathway evolved.

10. Zusammenfassung

Pflanzen haben eine Vielzahl von Verteidigungsmerkmalen entwickelt, um abiotischen und biotischen Stressfaktoren gewachsen zu sein. Diese Verteidigungsmerkmale richten sich entweder direkt gegen Herbivoren, wie beispielsweise Toxine oder andere Abwehrsubstanzen („direkte Verteidigung“), oder aber sie wirken indirekt, indem sie Raubinsekten, also die Feinde der Herbivore, anlocken („indirekte Verteidigung“). Die vorliegende Doktorarbeit konzentriert sich auf induzierbare indirekte Verteidigungsstrategien von Pflanzen, und zwar die Sekretion von extrafloralem Nektar (EFN) und die Emission von flüchtigen organischen Verbindungen (*volatile organic compounds*, VOCs). Beide Abwehrmechanismen sind induzierbar durch Insektenfraß und werden durch den Octadecanoid-Signalweg reguliert, in dem das Phytohormon Jasmonsäure (*jasmonic acid*, JA) als zentrales Signalmolekül fungiert. Die vorliegende Arbeit möchte dazu beitragen, die Rolle von Jasmonaten (JA und ihre Derivate) im Hinblick auf die Regulierung der Nektarsekretion (sowohl von floralem als auch extrafloralem Nektar) und auf die VOC-Emission aufzuklären sowie das Verständnis der ökologischen Bedingungen und evolutionären Voraussetzungen bei der Ausbildung von indirekten pflanzlichen Verteidigungsmerkmalen besser zu verstehen. Grundlegende Aspekte werden von unterschiedlichen Blickwinkeln beleuchtet, wobei verschiedene Modellsysteme verwendet werden, die geeignet sind, sich gezielten Fragestellungen zu widmen. Eine erste Zielstellung war die Untersuchung möglicher Faktoren, die die JA-abhängige Akkumulation und Verteilung von extrafloralem Nektar und Bildung von VOCs beeinflussen (Manuskript I). Pflanzenabwehrreaktionen werden insbesondere durch abiotische Faktoren gesteuert, beispielsweise durch Licht, wodurch eine optimale Adaptation der Pflanze an ihre gegenwärtige Umwelt gewährleistet wird. Daher wurde die Rolle von Licht bei der Jasmonat-abhängigen EFN-Sekretion erforscht (Manuskript III). EFN-Sekretion spielt eine lebenswichtige Rolle bei der pflanzlichen Verteidigung, während floraler Nektar (FN) eine Belohnung für Bestäuber darstellt. Um wesentliche Unterschiede und Ähnlichkeiten in regulatorischen Prozessen, die der Nektarbildung zugrundeliegen, zu identifizieren, wurde die Rolle von Jasmonaten bei der Kontrolle der FN-Sekretion untersucht (Manuskripte II & IV). Die meiste Literatur über indirekte Verteidigungsmechanismen bezieht sich auf höhere Pflanzen, während bislang sehr wenige Studien niedere Pflanzen in diesem Zusammenhang untersucht haben. Um diese Lücke zu schließen und um die Rolle von Jasmonaten bei der Regulierung der indirekten Verteidigung auch bei niederen Pflanzen eingehend zu prüfen,

wurde die VOC-Emission als Reaktion auf Jasmonate und Herbivorie beim Adlerfarn (*Pteridium aquilinum*) untersucht (Manuskript V).

Die räumliche Verteilung von indirekten Verteidigungsmechanismen stellt eine optimale Abwehrstrategie dar

Die Hypothese optimaler Verteidigung (*optimal defence hypothesis*, ODH) fordert, dass Verteidigungsreaktionen innerhalb einer Pflanze so verteilt werden, dass die lebenswichtigen und leichter verwundbaren Pflanzenteile intensiver verteidigt werden als andere Pflanzenteile. Die Limabohne reagiert auf Insektenfraß, indem sie EFN produziert oder flüchtige organische Verbindungen (VOCs) in die Umgebung abgibt. Werden jedoch alle Pflanzenteile in gleicher Weise verteidigt? Die Analyse der EFN- und VOC-Produktion abhängig vom Blattalter zeigte, dass jüngere Pflanzen mehr EFN produzierten und mehr VOCs abgaben im Vergleich zu älterem Blattgewebe; dieses Ergebnis entspricht der Vorhersage der ODH. Obwohl jüngere Blätter eine geringere Fotoassimilationsrate aufwiesen als ältere, reife Blätter (d.h., die Hauptquelle der Bausteine für die zwei Abwehrreaktionen EFN und VOCs in jungen Blättern gemindert ist), war die Steigerung der zwei indirekten Verteidigungslinien in jüngerem Gewebe am intensivsten. Experimente, in denen markiertes $^{13}CO_2$ verwendet wurde, weisen darauf hin, dass die Fotosyntheseprodukte, die für die Abwehrreaktionen notwendig sind, vom älteren in das jüngere Pflanzengewebe transportiert werden, wo ein durch Herbivorie verursachter Gewebeverlust wahrscheinlich gravierendere Fitnessverluste für die Pflanze als Ganzes zur Folge hätte als wenn altes Blattgewebe befallen würde. Diese Ergebnisse weisen darauf hin, dass die Verteilung von Abwehrreaktionen innerhalb einer Pflanze dem Prinzip der optimalen Verteidigung folgt.

Die Bildung von floralem Nektar wird von Jasmonaten reguliert

Die Sekretion von extrafloralem Nektar (EFN) ist ein indirekter Abwehrmechanismus, der von Jasmonaten gesteuert wird. Jedoch war bisher nicht bekannt, ob Jasmonate auch die Sekretion von Blütennektar regulieren, der vor allem deswegen gebildet wird, um Bestäuber anzulocken. Um diese Frage zu beantworten, wurde die Rolle von Jasmonaten bei der Bildung von floralem Nektar in *Brassica napus* untersucht. Die Untersuchungen ergaben, dass – ähnlich wie bei EFN – Jasmonate an der Regulierung der Blütennektarbildung beteiligt ist. Das Besprühen der Blüten mit JA, Jasmonoyl-Isoleucin (JA-Ile) und Coronalon (einem strukturellen Analog von JA-Ile) förderte die Sekretionsrate von floralem Nektar, während eine gehemmte JA-Biosynthese eine verringerte Sekretion von Blütennektar zur Folge hatte.

Allerdings hatte eine Jasmonat-Behandlung von Blättern keinen Einfluss auf die Bildung von floralem Nektar, was auf eine funktionale Trennung der Regulierung von Fraßschaden an Blättern und der Produktion von Blütennektar hinweist. Die Ergebnisse lassen vermuten, dass Jasmonate nicht nur wichtige Regulatoren der pflanzlichen Verteidigung gegen Herbivoren sind, sondern auch eine Rolle bei der Sekretion von floralem Nektar spielen.

Die Regulierung der EFN-Sekretion durch Jasmonate ist lichtabhängig

Pflanzen müssen sich nicht nur gegen Herbivorie wehren, sondern ihre Reaktionen mit wechselnden abiotischen Bedingungen koordinieren, von denen die Verfügbarkeit von Licht einer der bedeutendsten Faktoren ist. Um den Einfluss verschiedener Lichtbedingungen auf Jasmonat-regulierte indirekte Verteidigungsmechanismen zu bestimmen, wurde die Jasmonat-induzierte EFN-Produktion in Pflanzen untersucht, die Lichtverhältnissen ausgesetzt waren, welche sich sowohl qualitativ als auch quantitativ unterschieden. Unter normalen Tageslichtbedingungen folgte die EFN-Sekretion in unbehandelten Limabohnenpflanzen (*Phaseolus lunatus*) einem zeitlichen Muster und erreichte einen Höchststand in der Nacht. Eine Behandlung mit JA zeigte dabei unterschiedliche Effekte auf die EFN-Produktion, abhängig von der Lichtumgebung: Im Licht wurde die Sekretion von extrafloralem Nektar induziert, bei Dunkelheit hingegen unterdrückt. Interessanterweise reduzierte die Behandlung mit JA-Ile die EFN-Produktion in der Dunkelheit nicht, im Gegensatz zur Behandlung mit JA, aber sie induzierte die Bildung von extrafloralem Nektar unter hellen Bedingungen. In Pflanzen, die einer Rotlichtbestrahlung mit einem Verhältnis von 10:90 (R:FR, wobei R für *red*, FR für *far red* steht) ausgesetzt worden waren, löste JA- Ile, nicht aber JA die EFN-Sekretion aus. Bei 100% FR drosselten sowohl JA also auch JA-Ile die Sekretion von EFN. Eine Hemmung der Ile-Biosynthese führte auch zu einer verminderten EFN-Sekretion sogar unter Lichtbedingungen, und zusätzlich stellte sich heraus, dass die Biosynthese von JA-Ile lichtabhängig ist. Diese Ergebnisse – zusammen mit Experimenten, in denen Pflanzen unterschiedlichen Lichtqualitäten ausgesetzt worden waren (R:FR Verhältnis) – implizieren, dass es eine Wechselwirkung zwischen Lichtqualität und Jasmonat-Signaltransduktion gibt, die in einer eng regulierten Anpassung der Abwehr gegen Schädlinge an die Lichtverhältnisse, denen eine Pflanze ausgesetzt ist, resultiert.

Die Emission von flüchtigen Verbindungen (VOCs) beim Adlerfarn *Pteridium aquilinum*, einer ursprünglichen, niederen Pflanze, wird von Jasmonaten ausgelöst, ist aber nicht an Herbivorie gekoppelt

Mit dem Ziel, den evolutionären Ursprung der Regulierung indirekter Verteidigung zu verstehen, wurde die VOC-Emission in der, evolutionsbiologisch betrachtet, alten Farnart *Pteridium aquilinum* untersucht. Die Ergebnisse weisen darauf hin, dass dieser Farn flüchtige Verbindungen als Reaktion auf Elizitoren produzieren kann, von denen man weiß, dass sie die VOC-Emission auch in höheren Pflanzen aktivieren (JA, JA-Ile, Alamethicin, Coronalon, OPDA (Vorläufer von JA) und Linolensäure). Allerdings wurden die flüchtigen Substanzen im Farn interessanterweise nicht nach mechanischer Verwundung oder Insektenfraß (durch Generalisten oder Spezialisten) gebildet, was nahelegt, dass es sich hier um eine andere, noch unklare ökologische Funktion von VOCs im Vergleich zu ihrer Rolle in höheren Pflanzen handelt und dass es als Reaktion auf Fraßinsekten einen anderen Signalweg geben müsste. Weiterhin konnten keine signifikanten Änderungen der endogenen Oxylipin- Gehalte (JA und ihre Vorläufer) vor und nach Herbivorie beobachtet werden. Zusammengefasst deuten diese Ergebnisse darauf hin, dass die Biosynthesestufen der VOC-Emission im Farn bereits vollständig vorhanden waren, bevor das regulative Bindeglied zwischen Herbivorie und Jasmonat-Signaltransduktion evolvierte.

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13. Eigenständigkeitserklärung

Entsprechend der geltenden, mir bekannten Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich -Schiller-Universität Jena erkläre ich, daß ich die vorliegende Dissertation eigenständig angefertigt und alle von mir benutzten Hilfsmittel und Quellen angegeben habe. Personen, die mich bei der Auswahl und Auswertung des Materials sowie bei der Fertigstellung der Manuskripte unterstützt haben, sind am Beginn eines jeden Kapitels genannt. Es wurde weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte für Arbeiten, welche im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen, geldwerte Leistungen erhalten. Die vorgelegte Dissertation wurde außerdem weder als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung noch als Dissertation an einer anderen Hochschule eingereicht.

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Radhika Venkatesan

Jena,

12. Acknowledgments


Picture by Radhika Venkatesan

This thesis marks the end of my journey which I started from my home country with many doubts and fear of working in a distant, new country. Now when I look back at this exciting, motivating and at times exhausting and difficult journey, I reflect that I could not have completed this journey without the support of some special people to whom I owe my warm gratitude.

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14. Curriculum vitae

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Publications

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3. **Radhika V**, Kost.C, Heil.M, Boland. W (2008) Testing the optimal defence hypothesis for two indirect defences: extrafloral nectar secretion and volatile organic compounds. *Planta*, 228; 449-457.
4. **Radhika V**, Subramanian.S, Natarajan.K.A, (2006) Bioremediation of Zinc using *Desulfotomaculum nigrificans*: Bioprecipitation and Characterization Studies. *Water research*, 40(19): 3628-3636.
5. **Radhika V**, Kost.C, Bonaventure G, David A, Boland. W. Volatile emission in bracken (*Pteridium aquilinum*) is induced by jasmonates but not by herbivory. *Planta* (submitted)
6. **Radhika V**, Kost.C, Mithöfer A, Boland. W. Orchestration of extrafloral nectar secretion by light via jasmonates (in preparation).

Oral Presentations

1. **Radhika V**, Kost C, Mithöfer A & Boland W (October 2009) Day or Night? Nectar is Right. *38th Doktoranden Workshop, Naturstoffe-Chemie, Biologie und ökologie* , Leibniz Institut für Pflanzenbiochemie, Halle, Germany.
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Poster Presentations

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