How elms respond to insect egg deposition: Investigations of an ecological phenomenon by chemical and molecular approaches

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Meinen

Eltern und Großeltern

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The thesis is based on the following publications and a manuscript:

1. Büchel K, Malskies S, Mayer M, Fenning TM, Gershenzon J, Hilker M and Meiners T (2011) **How plants give early herbivore alert: Volatile terpenoids attract parasitoids to egg-infested elms**. *Basic and Applied Ecology*, 12(5):403–412

   Authors' contributions: KB and TM designed the experiments. KB developed and conducted chemical inhibitions of elm terpenoid biosynthesis, headspace collections of elm volatiles and olfactometer biotests with egg parasitoids. SM conducted biotests with individual volatile compounds, and MM did the field experiments. KB and TM analysed the data. TF and KB generated elm clonal material. KB wrote the first draft of the manuscript. It was mainly revised by TM. MH, JG and TF contributed to the revision.

2. Büchel K, McDowell E, Nelson W, Descour A, Gershenzon J, Hilker M, Soderlund C, Gang DR, Fenning TM and Meiners T (2012) **An elm EST database for identifying leaf beetle egg-induced defense genes**. *BMC Genomics*, 13:242

   Authors' contributions: KB, TM and TF designed the experiments. TF and KB generated elm clonal material. KB carried out plant treatments, RNA extractions, and provided the RNA samples. cDNA libraries were developed by EMD. DG coordinated sequencing. CS, AD and WN performed sequence alignment, assembling, annotation and database construction. Data were analysed by KB with assistance by TM and DG. KB wrote the first draft of the manuscript. It was mainly revised by TM. MH, JG and TF contributed to the revision.

3. Büchel K, Austel N, Mayer M, Gershenzon J, Fenning TM, and Meiners T (2013) **Smelling the tree and the forest: elm background odours affect egg parasitoid orientation to herbivore induced terpenoids**. *Biocontrol*, DOI 10.1007/ s10526-013-9544-9.

   Authors' contributions: KB designed the experiments in cooperation with TM. KB developed and conducted headspace collections of elm leaf volatiles, NA conducted biotests studying the parasitoid´s olfactorey response to individual volatile compounds, and MM did the field experiments. KB analysed the data with the help of TM. MH and JG contributed reagents/materials/analysis tools and lab space. TF and KB generated elm clonal material, and TM collected the insects. KB wrote the first draft of the manuscript. It was mainly revised by TM. JG and TF contributed to revision.

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   Authors' contributions: KB wrote the first draft of the manuscript. It was mainly revised by TM and MH. JG and TF contributed to the revision.

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All other co-authors of the manuscripts have been diploma students of the lab Applied Zoology / Animal Ecology, Freie Universität Berlin, Germany.

Content

Chapter 1

General introduction and thesis outline

Plants are exposed to a multitude of herbivorous insect species that exploit plants as oviposition sites and food source. They have developed a huge variety of mechanisms to defend themselves against herbivores and thus, to ensure their survival. Plant defence mechanisms have co-evolved in close interaction with herbivorous insects over a long time and are often very specific in their impact against the different insect species (Schoonhoven et al. 2005).

Constitutive plant defences are always present whereas inducible defences are only elicited by attack. Defences of both categories can act either directly or indirectly against the stressors. Physical barriers such as bark and trichomes represent the first effective constitutive defence mechanisms. Constitutive and inducible chemical defence may be provided by secondary plant metabolites (e.g. terpenoids, phenolics, alkaloids) that act as repellents, anti-digestive compounds, or toxins directly against herbivores (Howe and Jander 2008; Mithöfer and Boland 2012; Wittstock and Gershenzon 2002). Constitutive and inducible plant defence can also act indirectly against herbivorous arthropods; especially the attack-induced release of volatile organic compounds that attract predators or parasitoids of herbivores from higher trophic levels has been investigated by numerous studies (Dicke and Baldwin 2010; Hilker and Meiners 2006; Arimura et al. 2005).

Plants are able to show defensive responses that are specific for the type of attack; they differentiate between artificial wounding, attack by pathogens, insect herbivore feeding or insect egg deposition. Compounds (elicitors) that are specifically associated with the different attackers inform the plant on the attacker type (Wu and Baldwin 2009). Fine-tuning of the defence reactions specifically to the encountered attacker is controlled by downstream signalling pathways and the cross-talk between them (Verhage et al. 2010; Walling 2009). Many defence-signalling pathways are mediated by phytohormones like jasmonic acid, salicylic acid, and ethylene. Jasmonic acid is synthesized *via* the octadecanoid pathway and is one of the best studied hormones involved in defence reactions (Robert-Seilaniantz et al. 2011; Wasternack 2007; Farmer et al. 2003).

The first study demonstrating indirect induced plant defence against insect eggs was a study of the European field elm *Ulmus minor* Mill. (Ulmaceae), where eggs of the

elm leaf beetle *Xanthogaleruca luteola* (Müller) (Coleoptera: Chrysomelidae) induced elm leaf volatiles which attracted the egg parasitoid *Oomyzus gallerucae* (Hymenoptera: Eulophidae), a wasp specialized on elm leaf beetle eggs (Meiners and Hilker 1997). During the last decade indirect induced defence against insect egg laying has been demonstrated in several tritrophic systems. In addition to oviposition-induced changes of plant volatile emissions, insect egg deposition was also shown to induce changes of plant surface chemistry which arrests egg parasitoids, and thus, enhances the parasitoid´s foraging efficiency (reviewed by Hilker and Meiners 2010; Blenn et al. 2012).

So far, only a few studies addressed the question which of these egg-induced plant volatiles are relevant for egg parasitoid attraction. Terpenoids and green leaf volatiles (C6-aldehydes, -alcohols, and their esters) represent major classes of herbivore-induced plant volatiles. Despite the enormous variety of plant volatiles, parasitoids use relatively few ubiquitous plant volatile compounds for host location (Pichersky and Gershenzon 2002; McCormick et al. 2012). A few oviposition-induced plant terpenoids have been demonstrated to play a key role for attraction of egg parasitoids to egg-laden leaves, i.e. (*E*)-*ß*-caryophyllene in bean ssp. (Colazza et al. 2004a, b) and (*E*)-*β*-farnesene (in the background of other pine terpenoids) in Scots pine (*Pinus sylvestris*) (Beyaert et al. 2010). The odour of egg-laden elm leaves damaged by feeding of the elm leaf beetle consists mainly of terpenoids – among them also (*E*,*E*)-*α*-farnesene and (*E*)-*ß*-caryophyllene, and several green leaf volatiles. However, when I started this PhD thesis it was unknown which volatiles were responsible for parasitoid attraction (Wegener et al. 2001). The orientation of parasitoids to their host may be determined by quantitative as well as by qualitative differences between the egg-induced leaf odour blend and odour of egg-free leaves. While some individual components of plant odour blends may be attractive, others may be even repellent or have no effect on carnivorous enemies of the herbivores (Schroeder and Hilker 2008; McCormick et al. 2012). Parasitoids are able to even recognize host-specific plant odour that is released in highly variable habitat background odour. Numerous abiotic and biotic factors influence both the parasitoid´s olfactory perception as well as the odour profiles that are encountered by a parasitoid (Hilker and McNeil 2008; Takabayashi et al. 1994; Wäschke et al. 2013).

Direct plant defences induced by insect eggs have so far been reported only for herbaceous crop species. These egg-induced direct defences include the production of ovicidal substances (rice) killing the eggs, growth of neoplasms (pea) detaching the eggs or impairing access of hatching larvae to leaf tissue, and development of necrotic zones at the

site of egg deposition (cabbage, potato, black mustard plants); eggs easily fall off from necrotic tissue (reviewed by Hilker and Meiners 2011).

Knowledge on how plants are able to respond to insect egg laying at the molecular level is scarce. Eggs of *Pieris brassicae* have been shown to cause considerable changes in the plant's transcriptome of Brussels sprouts (*Brassica oleracea* var. *gemmifera*) and *Arabidopsis thaliana* (Little et al. 2007; Fatouros et al. 2008). To date, only studies of Brussels sprouts and Scots pine (Köpke et al. 2010) have addressed the role of egg-induced transcriptional changes in indirect plant defence.

From an ecological and evolutionary research perspective the *U. minor* - *X. luteola* - *O. gallerucae* tritrophic system is optimal for studying indirect egg-inducible defence mechanisms. This system has been almost unaffected by breeding for agriculture and forestry, and it is supposed that the species of this system co-evolved for a long time period because of high species specificity of the elm´s defence response to eggs (Meiners and Hilker 2000) and the high degree of specialization of the elm leaf beetle on elm and *O. gallerucae* on elm leaf beetle eggs (Meiners et al. 2000).

The original natural range of the European field elm *Ulmus minor* (Ulmaceae) extends predominantly within Southern Europe. However, through cultivation it occurs throughout the temperate world. Prior to the widespread occurrence of the Dutch elm disease caused by a fungus, elms were also frequently planted within urban areas because of their environmental tolerance (Richens 1983; Heybroek 1993).

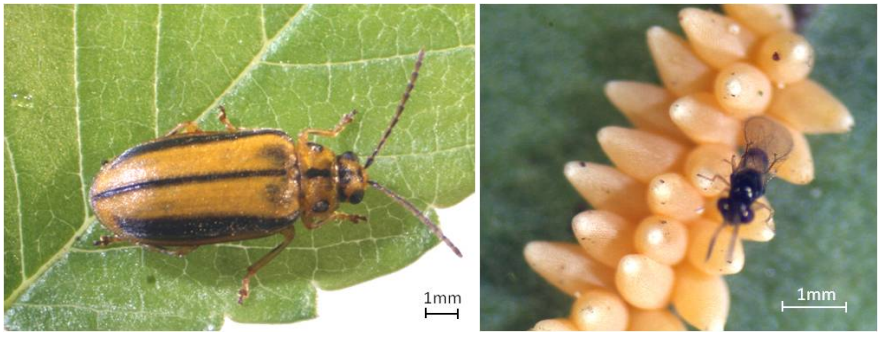

Figure 1. Elm leaf beetle *Xanthogaleruca luteola* on leaf of *Ulmus minor* (left) and egg parasitoid *Oomyzus gallerucae* on eggs of *X. luteola* (right).

The elm leaf beetle *X. luteola* can defoliate entire trees and is recognized as a major urban and forest pest in the USA and Australia. In Europe, eggs of the indigenous *X. luteola* are often heavily attacked by the chalcidoid egg parasitoid *Oomyzus gallerucae*. In the USA and Australia, the egg parasitoid *O. gallerucae* does not occur, and thus, the lack of this natural antagonist leads to high densities of elm leaf beetle populations which may almost

completely defoliate the trees in these regions (Kielbaso and Kennedy 1983; Dahlsten et al. 1994) (Fig.1).

The questions addressed in this thesis are based on previous research which demonstrated that elms show a distinct eco-physiological response to egg deposition by the elm leaf beetle. In short, these previous results are the following:

Undamaged elm leaves emit only small amounts of volatiles which are not attractive to the egg parasitoid *O. gallerucae.* Odour from elm leaves laden with elm leaf beetle eggs and damaged by beetle feeding activity is attractive to *O. gallerucae*, whereas odour from feeding-damaged, but egg-free leaves does not attract the parasitoid (Meiners and Hilker 1997; Meiners and Hilker 2000). Volatiles attractive to the egg parasitoid are emitted both from the site of egg deposition and from adjacent leaf tissue. Female beetles scratch the lower leaf surface by gnawing shallow grooves in the leaf epidermis and then glue their eggs with the help of oviduct secretion onto these grooves. Neither artificial wounding of the leaf surface nor artificial application of oviduct secretion onto an undamaged leaf does cause the release of attractive volatiles from elm leaves. The elicitor of the oviposition-induced defence response was detected in the oviduct secretion that needs to be applied onto epidermal leaf wounds to trigger leaf volatile emission that attracts the egg parasitoid. The oviposition-induced attraction of egg parasitoids can also be elicited by applying jasmonic acid or methyl jasmonate onto elm leaves. This treatment leads almost exclusively to the emission of the sesquiterpenoids (*E*,*E*)-*α*-farnesene and (*E*)-*β*-caryophyllene as major compounds. The induction of elm leaf volatiles attractive to *O. gallerucae* egg parasitoids was demonstrated on a time scale of a few hours up to five days after leaf treatment. This time period exactly matches the time between egg deposition and hatching of larvae (Meiners and Hilker 2000; Meiners unpublished data; Wegener et al. 2001; Hilker and Meiners 2006).

Thesis outline

The main goal of this thesis is to deepen our knowledge on how the so fine-tuned interactions between the field elm *U. minor*, the herbivorous leaf beetle *X. luteola*, and the egg parasitoid *O. gallerucae* are modulated. I have combined techniques used in chemical ecology and molecular biology to reach this aim. My research focused on the four main questions outlined in Figure 2. While questions 1 – 3 were investigated experimentally by using the tritrophic system of the elm *U. minor,* the elm leaf beetle *X. luteola* and the egg parasitoid *O. gallerucae*, question 4 was addressed by literature studies.

1. *Do terpenoids emitted from oviposition-induced elm leaves play a role in mediating indirect elm defence?*

The egg parasitoids are known to be attracted by odour of egg-infested elms; this odour is composed of mainly terpenoids. The study presented in **chapter 2** investigated the role of oviposition-induced terpenoids emitted from elm leaves for orientation of the egg parasitoid *O. gallerucae*. Laboratory and field studies were conducted. Bioassays studying the parasitoid´s olfactory orientation and chemical analyses of elm volatiles were used to test which terpenoids mediate indirect defence.

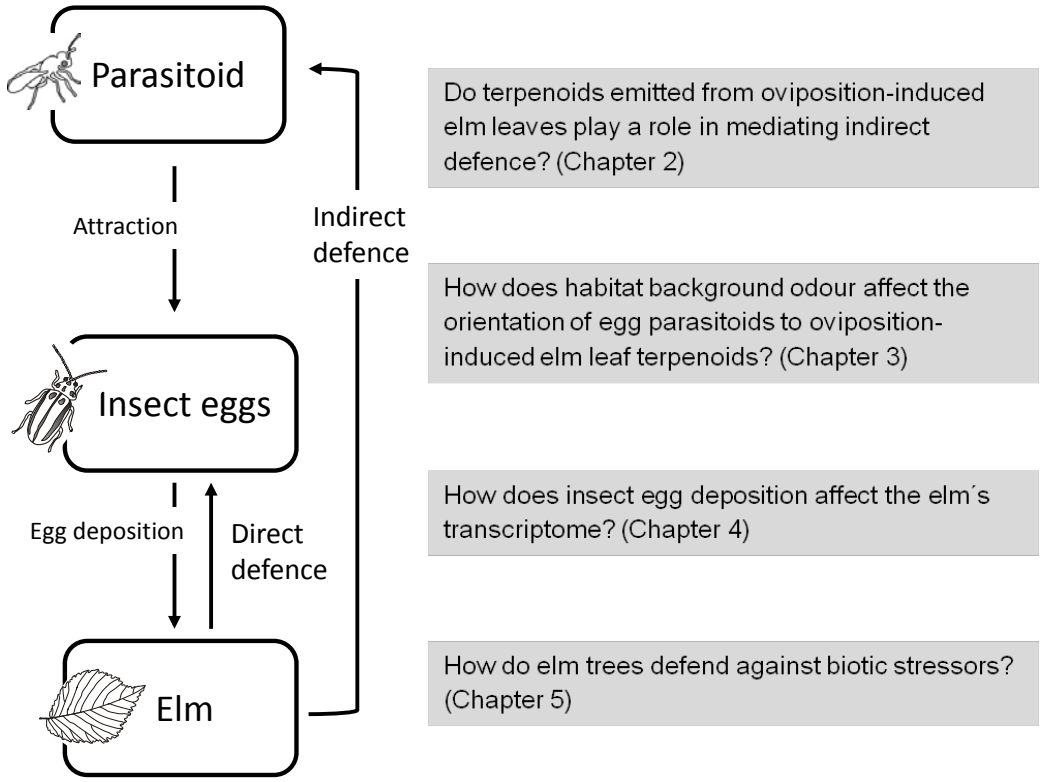

Figure 2. Overview of the general issues investigated in this dissertation

2. *How does habitat background odour affect the orientation of egg parasitoids to oviposition-induced elm leaf terpenoids?*

To further elucidate the factors that affect host location behaviour of *O. gallerucae*, **chapter 3** explores the question how habitat background odour affects egg parasitoid orientation to oviposition-induced elm and its terpenoids. Chemical analyses were used to determine the composition of habitat background odour, i.e. of egg-free feeding-damaged elm and non-infested elm. Lab and field experiments demonstrated how the egg parasitoid distinguished between different odorous backgrounds.

3. *How does insect egg deposition affect the elm´s transcriptome?*

In **chapter 4** of this thesis I investigated *in silico* how egg deposition by the elm leaf beetle affects transcript levels of genes encoding enzymes involved in the primary and secondary metabolism of *U. minor*. Elm EST data were obtained from RNA isolated from differently treated elm leaves. Comparative analysis of hundreds of transcripts of genes revealed differences in the transcript signature of elm leaves treated with egg laying and feeding by elm leaf beetles, with only feeding, with artificial transfer of egg clutches, and with methyl jasmonate in comparison to untreated elms.

4. *How do elm trees defend against biotic stressors?*

Knowledge given in this thesis is embedded into a broader context in **chapter 5**. The review highlights knowledge of direct and indirect elm (*Ulmus* spp.) defences against biotic stressors focusing on morphological, chemical and molecular aspects.

The summary of this doctoral thesis is given in English and in German in **chapter 6**.

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Chapter 2

How plants give early herbivore alert: Volatile terpenoids attract parasitoids to egg-infested elms

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How plants give early herbivore alert: Volatile terpenoids attract parasitoids to egg-infested elms

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Abstract

Plants can defend themselves against insect attack prior to larval feeding damage by responding to eggs laid on their leaves. Insect egg deposition can induce leaves to release a complex blend of volatiles attracting egg parasitoids which kill the eggs. Only a few studies have addressed the question which of these egg-induced plant volatiles are relevant for parasitoid attraction. Egg deposition by the elm leaf beetle *Xanthogaleruca luteola* on leaves of the European field elm *Ulmus minor* is known to induce the emission of a blend consisting mainly of terpenoids and some green leaf volatiles, which attracts a specialised egg parasitoid of *X. luteola*, the eulophid wasp *Oomyzus gallerucae*. Here, we investigated the role of oviposition-induced terpenoids from elm leaves for parasitoid attraction. Quantitative GC–MS analyses showed that inhibition of terpene biosynthesis in leaves by treatment with cerivastatin® and fosmidomycin reduced emission of (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), (*E*)-β-caryophyllene and a yet unidentified oxygenated sesquiterpene, but unexpectedly also that of green leaf volatiles (GLVs). Laboratory olfactometer assays revealed that inhibitor treatment rendered oviposition-induced elm leaves unattractive for the parasitoids. Further bioassays showed that single terpenoids *per se* attracted the parasitoids. Although the only tested GLV 1-hexanol was not attractive in olfactometer tests, we cannot rule out that other GLVs might play a role in parasitoid attraction. When attractiveness of DMNT was tested in the field, parasitoids were attracted to DMNT-baited traps in the presence of background odour emitted by a natural elm stand. We conclude that elms alert their egg parasitoid "helpers" after elm leaf beetle oviposition by means of one or more terpenoid volatiles.

Zusammenfassung

Pflanzen können sich gegen Insektenbefall schon vor Beginn von Larvenfraß schützen, indem sie auf Eiablagen an ihren Blättern reagieren. Eiablagen von Insekten können in Blättern die Abgabe von komplexen Duftgemischen induzieren, die Parasitoide anlocken, welche die Eier abtöten. Nur wenige Studien haben sich bisher der Frage gewidmet, welche der eiablageinduzierten Duftkomponenten für die Anlockung von Parasitoiden relevant sind. Eiablagen des Ulmenblattkäfers *Xanthogaleruca luteola* auf Blätter der Feldulme *Ulmus minor* können bekanntlich die Emission eines Duftgemisches induzieren, das hauptsächlich aus Terpenoiden und einigen grünen Blattdüften besteht und anlockend auf einen Eiparasitoiden von *X. luteola* wirkt, i.e.

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auf die eulophide Wespe *Oomyzus gallerucae*. Wir haben die Rolle der eiablageinduzierten Terpenoide von Ulmenblättern für die Anlockung dieser Parasitoide untersucht. Quantitative GC–MS Analysen zeigten, dass eine Hemmung der Terpenbiosynthese in Blättern durch Behandlung mit Cerivastatin und Fosmidomycin die Emission von einigen Terpenoiden wie (*E*)-4,8-dimethyl-1,3,7-nonatrien (DMNT), (*E*)-β-Caryophyllen und einem nicht identifizierten oxygenierten Sesquiterpen, aber unerwarteterweise auch von einigen grünen Blattdüften signifikant reduzierte. Olfaktometertests im Labor ergaben, dass eiablageinduzierte Ulmenblätter ihre Attraktivität für Parasitoide nach Inhibitorbehandlung verloren. Weitere Tests zeigten, dass einzelne Terpenoide *per se* die Parasitoide anlocken. Auch wenn die einzige getestete grüne Blattduftkomponente 1-hexanol in Olfaktometertests keine Attraktivität zeigte, kann eine Funktion anderer Grünblattdüfte für die Parasitoidenanlockung nicht ausgeschlossen werden. Eine Untersuchung der Attraktivität von DMNT im Freiland zeigte, dass Parasitoide zu Fallen mit DMNT in Gegenwart von Hintergrundduft (natürlicher Ulmenbestand) angelockt werden. Wir schlussfolgern, dass Ulmen die Eiparasitoide als "Helfer" bei der Insektenabwehr mit Hilfe von einer oder mehreren terpenoiden Duftkomponenten alarmieren.

Keywords: Indirect plant defence; Multitrophic interaction; Oviposition induced volatiles; Background odours; Olfactometer bioassay; Field trap; Inhibitior; DMNT; GLVs

Introduction

Plants attacked by herbivorous arthropods have developed a multitude of direct and indirect defence strategies (Howe & Jander 2008). Many plants defend themselves indirectly against herbivores by the emission of volatile compounds that inform herbivore enemies about the presence of prey. Carnivorous arthropods are attracted and reduce the number of herbivores, thus protecting the plant from damage (Bruinsma & Dicke 2008; but see also Allison & Hare 2009). Most of the knowledge on indirect plant defences originates from studies on feeding-induced volatiles (Dicke, van Poecke, & de Boer 2003). However, in addition to feeding, insect egg deposition can also induce indirect plant defence and may elicit the emission of leaf volatiles which attract egg parasitoids (Hilker & Meiners 2006, 2010). Induction of plant volatiles by insect egg deposition has been shown both in trees (elm, pine) and herbaceous crops (bean, cabbage) (Meiners & Hilker 2000; Hilker, Kobs, Varama, & Schrank 2002; Colazza, McElfresh, & Millar 2004; Conti et al. 2008).

Terpenoids and green leaf volatiles (GLVs) represent major classes of herbivore-induced plant volatiles (Arimura, Matsui, & Takabayashi 2009). Terpenoids constitute the most abundant and structurally diverse group of plant secondary metabolites and are released by a multitude of higher plants (Cheng et al. 2007). For example, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), (*E*)-β-caryophyllene and (*E*,*E*)-α-farnesene are plant terpenoids attractive to carnivorous enemies of herbivorous arthropods (Dicke, Sabelis, Takabayashi, Bruin, & Posthumus 1990; Arimura, Kost, & Boland 2005).

For attraction of egg parasitoids to egg-laden leaves, oviposition-induced plant terpenoids may play a key role. For example, *Trissolcus basalis* (Wollaston) (Hymenoptera: Scelionidae) responds positively to odours of oviposition-induced bean plants; the attractive odour is characterised by high amounts of (*E*)-β-caryophyllene. Thus, this terpenoid might be of importance as synomone (Colazza et al. 2004). The sesquiterpene (*E*)-β-farnesene released from oviposition-induced pine is a key compound for attracting the egg parasitoid *Closterocerus ruforum* in the background of other pine terpenoids (Mumm & Hilker 2005; Beyaert et al. 2010). In addition to terpenoids, also green leaf volatiles may be important for host location by egg parasitoids. The egg parasitoid *Trichogramma chilonis* shows a positive response to the GLV (*Z*)-3-hexenyl acetate and hexyl acetate (Reddy, Holopainen, & Guerrero 2002), i.e. volatiles that are also known to be emitted in small amounts by uninfested plants.

Numerous studies have shown the attraction of enemies of herbivorous insects by complex mixtures of volatiles typically emitted from plants. Individual compounds can also be relevant for attraction. While some individual components of plant odour blends can be attractive, others may be even repellent or have no effect on herbivore enemies (Schroeder & Hilker 2008). To determine the role of individual volatiles, researchers have attempted to fractionate volatile blends (D'Alessandro, Brunner, von Merey, & Turlings 2009), alter them by genetic engineering (Schnee et al. 2006) or by treating plants with synthetic chemicals that induce production of terpenes (Martin, Gershenzon, & Bohlmann 2003). Another approach is the use of specific inhibitors of plant terpenoid (Mumm, Posthumus, & Dicke 2008) or phenolic (D'Alessandro, Held, Triponez, & Turlings 2006) biosynthesis or of the octadecanoid pathway (Bruinsma et al. 2010) to manipulate volatile composition. Fosmidomycin (3-[formyl (hydroxy) amino] propoylphosphonic acid) inhibits terpenoid biosynthesis by inhibition of 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), an important enzyme of the MEP terpenoid biosynthesis pathway (Towler & Weathers 2007). Cerivastatin®, a compound used as cholesterol blocker in human medicine, inhibits terpenoid biosynthesis by affecting 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), which catalyses a rate-limiting step in the MVA terpenoid biosynthesis pathway in plants (Bartram, Jux, Gleixner, & Boland 2006).

In the present study, we have employed the terpene biosynthesis inhibitors cerivastatin and fosmidomycin to investigate the role of oviposition-induced terpenoid volatiles

for attraction of egg parasitoids. The tritrophic system studied consisted of the European field elm *Ulmus minor* (Ulmaceae), the elm leaf beetle *Xanthogaleruca luteola* (Coleoptera: Chrysomelidae), and the egg parasitoid *Oomyzus gallerucae* (Hymenoptera: Eulophidae) which is highly specialised on eggs of the elm leaf beetle. Odour from elm leaves laden with elm leaf beetle eggs and damaged by beetle feeding activity is attractive to *O. gallerucae*, whereas odour from feeding-damaged, but egg-free leaves does not attract the parasitoid. Egg deposition by the elm leaf beetle onto leaves or leaf treatment with jasmonic acid (JA) induces volatiles attracting the egg parasitoids (Meiners & Hilker 2000). Undamaged elm leaves emit only small amounts of volatiles which are not attractive to the egg parasitoid. Elm leaves with eggs and damaged by feeding or leaves treated with JA emit volatile blends containing the sesquiterpenes (*E*,*E*)-α-farnesene and (*E*)-β-caryophyllene and the homoterpene DMNT as major compounds (Wegener, Schulz, Meiners, Hadwich, & Hilker 2001). These previous findings suggest that the egg parasitoid *O. gallerucae* perceives information on the presence of host eggs by oviposition-induced elm terpenoids, but it is not clear which terpenoids are behaviourally active and how they are perceived in a natural odour environment.

To study the role of terpenoids for attraction of egg parasitoids we compared the attractiveness of odour released from elm subjected to leaf beetle oviposition and feeding and treated with terpenoid biosynthesis inhibitors to that released from elm subjected to oviposition and feeding without inhibitor treatment by an olfactometer bioassay. Furthermore, the odour of these two experimental groups was compared by GC–MS. Individual terpenes dominant in the attractive odour were tested for their attractiveness in the olfactometer. To elucidate whether parasitoids respond to an individual terpenoid also in the presence of an odour background of a natural elm stand, we performed a field test with traps baited with the homoterpene DMNT. This homoterpene was found to be significantly and most reduced by treatment of elm with terpene biosynthesis inhibitors. The results demonstrate the involvement of specific volatile terpenoids in egg parasitoid attraction.

Materials and methods

Plants and insects

All plants originated from a shoot culture of a single genotype of the European field elm, *U. minor*, and are referred to as *U. minor* cv. 'Dahlem' (see Appendix A for details). All experiments were conducted with 3- to 4-month-old elm plants with 15–20 leaves. The height of the plants was ca. 50 cm.

Adults and eggs of the elm leaf beetle, *X. luteola*, were collected in May in the years 2005 to 2007 in the environs of Montpellier, Perpignan (France), and Parlavà (Spain). Adult beetles and hatching larvae were reared in cages (40 cm × 40 cm × 70 cm) on elm plants in the greenhouse (15–34 °C, 55–75% RH, 16 h light:8 h dark). Adult parasitoids eclosing from the beetle eggs were fed with diluted honey and kept in Petri-dishes at 10 °C (16 h light:8 h dark). A few days prior to testing, parasitoids were transferred to warmer conditions (22 °C, 16 h light:8 h dark). Only 5- to 10-day-old female parasitoids inexperienced with host eggs were studied.

Plant treatments

During treatments all plants were kept in a climate chamber (22 °C, 55% RH, 150–200 μmol m^{-2} s^{-1} PAR, 16 h light:8 h dark).

Plant infestation by beetles: Elm plants used for the different experiments were induced for 48–72 h by beetle feeding and oviposition. Volatiles from twigs with eggs and feeding damage are known to attract the parasitoids for up to 72 h after egg deposition, whereas plants without eggs, but feeding damage do not attract the parasitoids (Meiners & Hilker 1997). For induction, 7–15 female *X. luteola* were encaged in microperforated plastic bags (180 mm × 350 mm, Weber Packaging GmbH, Germany) on elm plants ca. 20 cm below the top where they could feed and lay eggs

Plant treatment by inhibitors: Fosmidomycin (FR-31564, Invitrogen, USA) and cerivastatin® (a gift from Bayer AG, Germany) used for inhibition of elm terpenoid biosynthesis were prepared as 60 μM stock solutions in distilled water and stored at 4 °C. Hydroponic elm plants were watered with 300 ml of 30 μM fosmidomycin or cerivastatin® solution. Pots were covered with aluminium foil to protect the UV-sensitive solution against light. The plants were induced by beetle feeding and oviposition for 72 h and at the same time treated with the chemical solution. To control whether the terpene biosynthesis inhibitors, cerivastatin® and fosmidomycin, did not only affect terpenoid biosynthesis, but also photosynthesis, we compared chlorophyll fluorescence of 6 inhibited *versus* 6 non-inhibited plants, both of which had been attacked by feeding and ovipositing elm leaf beetles. Chlorophyll fluorescence was measured using a portable mini-PAM (pulse-amplitude-modulation) fluorometer (Heinz Walz GmbH Effeltrich, Germany).

Plant volatile collection and chemical analysis of plant volatiles

Volatiles of differently treated elm plants were collected using a dynamic headspace collection system (see Appendix A). The volatiles from inhibited plants ($N = 6$) and non-inhibited plants ($N = 5$) were collected for a period of 6 h. All collections were performed between 9:00 a.m. and 3:00 p.m. to reduce differences due to possible diurnal rhythm of volatile emission. Plant volatiles were extracted from the charcoal filter with 25 μl dichloromethane containing 25 ng μl^{-1} n-tridecane (Sigma–Aldrich) as an

internal standard. Extracts were analysed by coupled gas chromatography–mass spectrometry on a Fisons 8060 GC system and MD 800 quadrupole MS (see Appendix A). Plant volatiles were quantified by comparing the peak areas of individual compounds with the peak area resulting from the co-injection of the internal standard. Compounds that were detected in at least 3 of the 5 replicates of egg- and feeding-induced non-inhibited plants were included in the statistical analysis and calculated as nanograms released per gram fresh weight of the aerial parts of the plant.

Laboratory bioassays

For all bioassays a four-arm airflow olfactometer as described by Meiners and Hilker (1997) was used (see Appendix A for details).

When testing the parasitoid's response to plant odour, a potted elm plant was placed in a glass cylinder (250 ml) with an open bottom. The bottom was enclosed in a polyvinyl acetate oven bag (see above), so that odour from the soil was excluded. The opening in the top was connected to the test field of the olfactometer, while the three other fields of the arena were provided with charcoal-filtered humidified air. The test and control odour plant sources were renewed after testing the response of 6 parasitoids.

When testing synthetic reference compounds, 10 ng (in 10 μl hexane) of the compound was spotted on a filter paper (94 mm Ø, Melitta, Minden, Germany). This amount was within the range of the average amounts of the respective volatile compound collected from the elm plants (see Appendix A, olfactometry, for details on the calculation) during 5 min ((*E*)-*β*-caryophyllene 10.2 ng, (*E*,*E*)-*α*-farnesene 11.5 ng, DMNT 5.5 ng, *α*-farnesene 1.8 ng, 1-hexanol 12.2 ng). After solvent evaporation (20 s), the sample was placed in a conical glass jar (250 ml). Individual test standard compounds were renewed for every parasitoid individual tested. Two opposite fields of the four arm-olfactometer were supplied with odour from a standard compound, the two control fields with 10 μl hexane.

A bioassay was started by placing a female parasitoid into the centre of the olfactometer arena. For a period of 300 s, we recorded the parasitoid's residence time in each of the four olfactometer odour fields using the Observer programme 3.0 (Noldus, Wageningen, Netherlands). The experiments were performed at 22–25 °C, 70–80% RH and 16 μmol m^{-2} s^{-1} PAR (light above arena). The olfactometer was cleaned with ethanol after each run. In total, $N = 29$ to 33 parasitoids were tested per plant treatment or per synthetic reference compound and used as individual datapoints.

Field experiment

To test the attractiveness of DMNT towards *O. gallerucae* in the field, traps baited with DMNT were placed in a naturally grown elm stand. The stand was located close to Teyran (Montpellier, Southern France) where we usually collected elm leaf beetle eggs parasitised by *O. gallerucae*. The field experiment was conducted during July/August. Blue sticky traps (25 cm × 10 cm, Katz Biotech AG, Germany) were attached to bamboo sticks at heights of 1–1.5 m. The stick with the trap was placed at a distance of 0.5 m from the next elm (2–4 m height). The traps were baited with either 10 μl hexane (solvent only – control) or 30 ng DMNT in 10 μl hexane (test). The solvent and the test solution were applied on 10 cotton balls (1 cm Ø) which were attached to each trap. Test and control traps were mounted as pairs at a distance of 1.2 m from each other ($N = 20$ pairs were tested). The distance to the next pair was at least 2 m. The traps were collected after 24 h, and the trapped parasitoids were identified and counted under a stereomicroscope. Other species captured by the traps were low in individual numbers on most traps; we did not identify them to the species level. The main bycatch was a large dipteran species which seemed to be attracted optically to baited and unbaited traps.

Statistical analysis

All statistics was performed using Statistica (StatSoft Inc., 1999, Tulsa, USA). Normal distribution was tested by the Shapiro–Wilk test and homogeneity of variance by Levene's test. Because the condition of normality was not met in most cases, non-parametric tests were used. Differences in quantities of individual compounds between the different treatments were compared by Mann–Whitney *U* tests. The Wilcoxon one-sample test evaluated whether the time spent by the parasitoids in the test odour differed significantly from the null hypothesis (150 s for experiments with synthetic standards offered in two olfactometer fields, 75 s for experiments with odour from treated plants offered in one field of the four-arm-olfactometer assuming equally long residence times in all four olfactometer fields during an observation period of 300 s). Field trapping data were analysed by a Wilcoxon-test for matched pairs.

Results

Attraction of egg parasitoids to elm after inhibition of terpene biosynthesis

When the parasitoids were exposed to odour from elm plants induced by elm beetle oviposition and feeding, but not treated with terpene biosynthesis inhibitors, they spent significantly more time (median = 125 s) in the test field supplied with this odour than expected (75 s) ($Z = 2.35$, $N = 29$, $p = 0.02$, Fig. 1). However, when the odour from herbivore-induced elm treated with inhibitors was offered, the egg parasitoids stayed only 35 s (median) in the olfactometer field supplied with this odour; this residence time did not differ from the time expected ($Z = 0.15$, $N = 33$, $p = 0.88$).

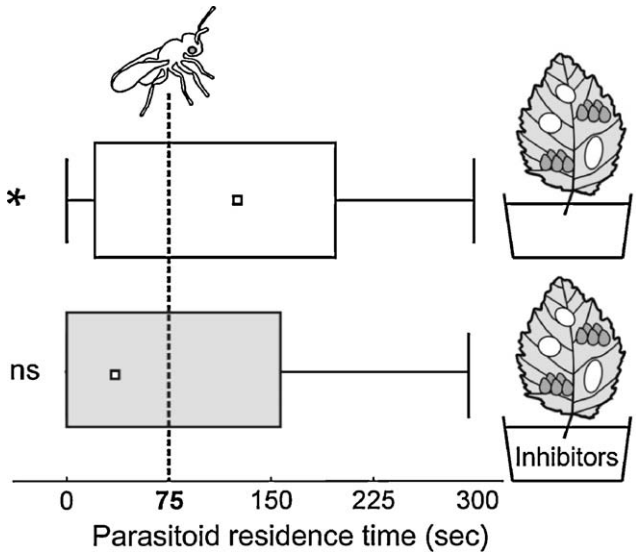

Fig. 1. Olfactometer residence time of *Oomyzus gallerucae* in odour of oviposition- and feeding-induced *Ulmus minor* plants treated with (grey bar; $N = 6$ plants, 33 parasitoids) or without terpenoid biosynthesis inhibitors (white bar; $N = 5$ plants, 29 parasitoids). Median, 75th and 25th percentiles, minimum and maximum value, and mean expected value (dashed line = 75 s) are shown. Wilcoxon one-sample test: $^*P \leq 0.05$, ns $P > 0.05$.

Thus, inhibition of terpenoid biosynthesis in herbivore-induced elm plants changed the volatile blend in such a way that it was no longer attractive towards the egg parasitoids.

In order to find out whether treatment of plants had affected the beetles' oviposition and feeding activity and thus, could indirectly affect attractiveness of these plants to the parasitoids, we controlled for the feeding damage and recorded the number of eggs on both inhibited and non-inhibited elm at the end of the treatment period (72 h). Feeding damage was in the range of previous calibration measurements (10–15% of the total leaf area), and the extent of damage did not differ between inhibited and non-inhibited elm. Neither did inhibitor treatment affect the number of egg clutches laid on elm. Elm leaf beetle females deposited a similar number of egg clutches on non-inhibited plants (13.2 ± 5.4 clutches per plant) and on inhibited plants (14.0 ± 2.6 clutches per plant) ($t = 0.32$, $df = 9$, *ns*, Student's *t*-test).

Inhibition of terpenoid biosynthesis

Oviposition- and feeding-induced elm plants that were not treated with terpenoid biosynthesis inhibitors (control plants) consistently released 31 volatiles, of which 28 occurred in quantifiable amounts (Table 1, egg and feeding). Thirteen compounds were terpenoids. The main terpenoids emitted by these elms were the C_{11} homoterpene DMNT, the sesquiterpenes (*E*)-β-caryophyllene, (*E*,*E*)-α-farnesene, and an unknown oxygenated sesquiterpene. In contrast, oviposition- and feeding-induced elms that were chemically treated with the terpenoid biosynthesis inhibitors cerivastatin® and fosmidomycin (Table 1, inhibitors) released 9 terpenoid compounds. Nearly all compounds detected in the headspace of herbivore-induced control plants were also found in the headspace of induced, inhibitor-treated plants, except for the monoterpenes α- and β-pinene and sabinene, and the sesquiterpene (*Z*,*E*)-α-farnesene, which were emitted only by control plants.

When comparing the quantitative emission of individual volatile compounds released from herbivore-induced, inhibitor-treated elm plants and herbivore-induced ones without inhibitor treatment (Table 1), we found significantly lower emission of DMNT, (*E*)-β-caryophyllene and the oxygenated sesquiterpene from inhibited elms than from non-inhibited ones. Furthermore, the emission of six green leaf volatiles (GLVs; (*E*)-2-hexenal, (*Z*)-3-hexenol, 1-hexanol, (*Z*)-3-hexenyl isobutyrate, (*Z*)-3-hexenyl butyrate and (*Z*)-3-hexenyl 2-methylbutyrate) was significantly reduced in inhibited plants compared to non-inhibited ones. The GLVs hexyl acetate and (*E*)-2-hexenyl acetate and the sesquiterpene (*E*,*E*)-α-farnesene tended to be emitted in lower quantities by inhibitor-treated plants.

The net assimilation of inhibited plants ($19.0 \pm 5.2\ \mu mol\ m^{-2}\ s^{-1}$) was slightly lower than those of non-inhibited plants ($20.8 \pm 5.8\ \mu mol\ m^{-2}\ s^{-1}$), but these differences were not significant ($t = 0.57$, $df = 10$, *ns*, Student's *t*-test) indicating that photosynthesis was not affected by treatment with fosmidomycin and cerivastatin®.

Response of egg parasitoids to single terpenoids

To investigate the egg parasitoid's olfactory response to specific elm leaf beetle-induced volatiles, we offered singly three sesquiterpenes, one homoterpene and one GLV in a four-field olfactometer to *O. gallerucae*. The sesquiterpenes (*E*,*E*)-α-farnesene, (*E*)-β-caryophyllene and the homoterpene DMNT were selected for the olfactometer bioassays as most abundant terpenoid compounds in the odour of elm leaves on which eggs were deposited and that were damaged by feeding (Table 1). The sesquiterpene α-humulene was tested because of its structural similarity to β-caryophyllene and because another eulophid wasp (*Chlosterocerus ruforum*) is known to respond to this sesquiterpene both electrophysiologically and behaviourally (Beyaert et al. 2010). 1-Hexanol was selected as the GLV reduced most (twelvefold) by inhibitor application (Table 1). The egg parasitoids responded positively to DMNT ($Z = 3.1$, $N = 28$, $p = 0.002$) and all sesquiterpenes tested (β-caryophyllene: $Z = 3.2$, $N = 21$, $p < 0.001$; (*E*,*E*)-α-farnesene: $Z = 2.3$, $N = 32$, $p = 0.021$; α-humulene: $Z = 2.2$, $N = 29$, $p = 0.028$) (Fig. 2). In contrast, the egg parasitoid showed a tendency to avoid 1-hexanol ($Z = 1.9$, $N = 29$, $p = 0.053$).

Table 1. Comparison of volatiles[a] detected in the headspace of egg- and feeding-induced *Ulmus minor* plants (egg and feeding) that were otherwise untreated or additionally treated with terpenoid biosynthesis inhibitors.

Compound	RI	Egg and feeding ($N=5$)	Egg and feeding + inhibitors ($N=6$)	P
Monoterpenes				
α-Pinene	931	tr	nd	–
Sabinene	971	0.05 (0–0.06)	nd	ns
β-Pinene	972	tr	nd	–
β-Myrcene	989	0.14 (0.06–0.2)	0.06 (0.05–0.09)	ns
Limonene	1027	0.2 (0.17–0.32)	0.1 (0.06–0.11)	ns
Linalool	1100	0 (0–0.08)	0 (0–0.01)	ns
Homoterpene				
(*E*)-4,8-Dimethyl-1,3,7-nonatriene	1112	2.1 (1.6–4.0)	0.34 (0.28–0.38)	**
Sesquiterpenes				
(*E*)-β-Caryophyllene	1417	3.7 (2.6–5.8)	1.0 (0.5–2.1)	*
α-Humulene	1453	0.67 (0.56–1.11)	0.29 (0.11–0.51)	ns
Germacrene D	1478	0.39 (0.26–0.067)	0.32 (0.16–0.65)	ns
(*Z*,*E*)-α-Farnesene	1489	tr	nd	–
(*E*,*E*)-α-Farnesene	1503	3.1 (2.8–3.2)	0.64 (0.41–2.15)	ns
Ox. sesquiterpene, $m/z=202$, 159b, 67	1584	8.5 (6.3–12.0)	2.1 (0.6–5.6)	*
Aromatics				
Methyl benzoate	1092	0.01 (0–0.06)	0.03 (0.02–0.05)	ns
Phenyl acetonitrile	1134	0.39 (0.17–0.42)	0.37 (0.09–0.54)	ns
Ethyl benzoate	1169	0.05 (0–0.13)	0.05 (0.05–0.09)	ns
Methyl salicylate	1189	0.28 (0.11–0.93)	0.09 (0.02–0.23)	ns
GLV				
(*E*)-2-Hexenal	848	2.12 (1.0–11.2)	0.18 (0.16–0.51)	*
(*Z*)-3-Hexenol	849	12.5 (5.3–10.9)	2.4 (1.6–3.4)	*
(*E*)-2-Hexenol	861	1.8 (0.5–5.0)	0.17 (0.14–0.41)	ns
1-Hexanol	869	1.2 (0.9–9.8)	0.1 (0.09–0.23)	*
(*Z*)-3-Hexenyl acetate	1006	42.5 (39.4–53.2)	30.0 (21.8–33.1)	ns
Hexyl acetate	1013	0.67 (0.52–1.87)	0.24 (0.19–0.28)	ns
(*E*)-2-Hexenyl acetate	1016	1.8 (1.0–5.5)	0.67 (0.39–1.29)	ns
(*Z*)-3-Hexenyl propionate	1100	0.04 (0.03–0.28)	0.02 (0.01–0.02)	ns
(*Z*)-3-Hexenyl isobutyrate	1144	0.21 (0.19–0.78)	0.06 (0.05–0.1)	*
(*Z*)-3-Hexenyl butyrate	1185	3.3 (5.4–1.0)	1.0 (0.9–1.3)	*
(*Z*)-3-Hexenyl 2-methylbutyrate	1230	0.95 (0.94–4.65)	0.64 (0.39–0.87)	*
(*Z*)-3-Hexenyl 3-methylbutyrate	1236	0.25 (0.15–0.95)	0.13 (0.04–0.29)	ns
(*Z*)-3-Hexenyl benzoate	1568	0.15 (0–1.49)	0 (0–0)	ns
Unknown				
Unknown 1, $m/z=85$, 57b	1064	3.2 (1.6–3.5)	0.79 (0.43–1.72)	ns

GLV = green leaf volatile; RI: retention index; m/z: mass-to-charge ratio; b: base peak; tr: compound detected only in traces in single samples; nd: not detected in all replicates.

[a] Quantities of compounds (median and 25–75% quartile ranges) in ng/gFW (fresh weight)/μg solvent; Mann–Whitney U-test.

* $P \leq 0.05$.

** $P \leq 0.01$.

ns $P > 0.05$.

Attraction to DMNT in the presence of background odours

Traps baited with DMNT were placed close to trees in elm stands to provide a natural background odour for *O. gallerucae*. After 24 h traps baited with DMNT attracted significantly more parasitoids than the control traps treated with the solvent only ($Z=3.83$, $N=20$, $p=0.001$). While the 20 control traps caught 0–7 wasps each (median 1.5; total 41), the 20 traps baited with DMNT caught 2–24 parasitoids (median 5, total 131) (Fig. 3).

Discussion

Our study aimed to elucidate the role of terpenoids for indirect plant defence induced by egg deposition of an herbivorous insect. Elm foliage induced by eggs of the elm leaf beetle and its feeding damage is known to attract egg parasitoids by release of induced volatiles (Meiners & Hilker 1997, 2000), among them several terpenoids (Wegener et al. 2001). Chemical inhibition of terpenoid biosynthesis reduced emission of several terpenoids and GLVs from herbivore-induced elm leaves and led to a loss of attractiveness of

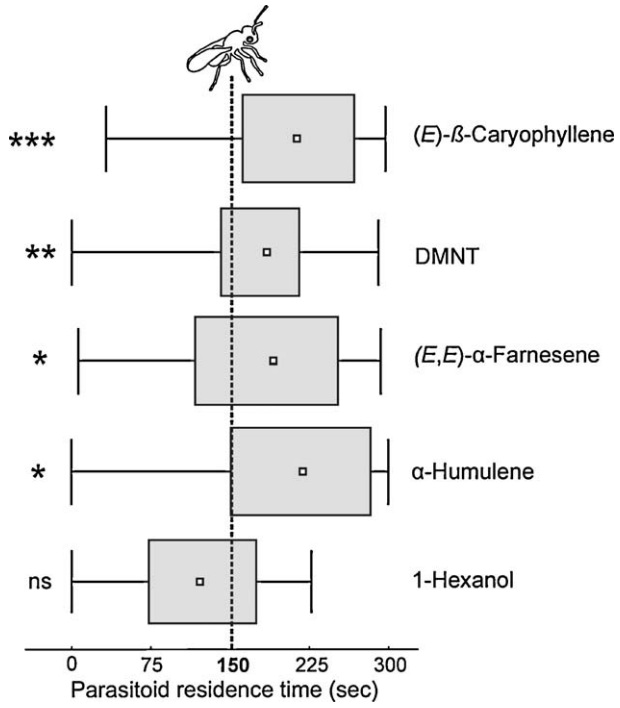

Fig. 2. Olfactometer residence time of *Oomyzus gallerucae* in odour of individual volatile compounds: (1) (*E*)-*β*-caryophyllene, $N = 21$ (parasitoids); (2) DMNT, $N = 28$; (3) (*E*,*E*)-*α*-farnesene, $N = 32$; (4) *α*-humulene, $N = 29$ and (5) 1-hexanol, $N = 29$. Median, 75th and 25th percentiles, minimum and maximum value, and mean expected value (dashed line = 150 s) are shown. Wilcoxon one-sample test: $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P < 0.001$, ns $P > 0.05$.

egg-laden elm leaves towards the parasitoids. Olfactometer bioassays testing the parasitoids' response to single terpenoid reference compounds of which emission was reduced by chemical inhibition revealed that each of these terpenoids

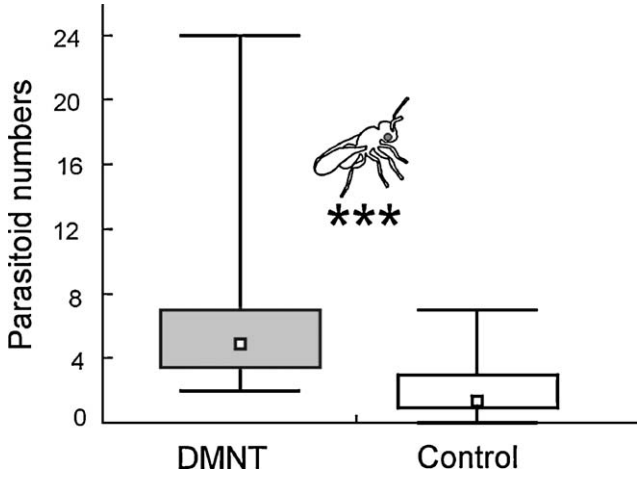

Fig. 3. Number of parasitoids (median, 75th and 25th percentiles, minimum and maximum) caught in traps baited with DMNT (+solvent) or solvent only (control). $N = 20$ traps of each type. DMNT- and control traps were located pairwise in elm tree stands. Trapping period: 24 h. Wilcoxon matched-pairs-test: $^{***}P \leq 0.001$.

per se can attract the parasitoid. Hence, our results show for the first time the relevance of individual terpenoids in oviposition-induced plant defence. In addition to terpenoids, other compound classes (GLV) may also contribute to the attractiveness of egg-laden elm (see below).

The chemical inhibition of terpenoid biosynthesis did not affect the overall photosynthetic performance of elm in our study (but see Possell, Ryan, Vickers, Mullineaux, & Hewitt 2010). Thus, our results indicate that unattractiveness of chemically inhibited elm was most likely due to changes in volatile emission rather than to changes in primary carbon metabolism or stomatal opening and closing.

The reduction of the emission of single GLVs was unexpected. GLVs are not synthesised by the terpenoid pathways, and the emission should therefore not be affected by inhibitors of the terpenoid biosynthesis. Thus, the reduction of GLV emission from inhibitor-treated elm remains puzzling.

Since the inhibitor-treatment did not only affect the emission of terpenoids, but also the release of GLVs, we cannot conclude that the unattractiveness of inhibitor-treated herbivore-induced elm was only due to a change of emission of terpenes. Other egg parasitoid species who are generalists at the plant level make use of GLVs for host orientation (Romeis, Babendreier, Wäckers, & Shanower 2005). Nevertheless, our data show that single terpenoids may be crucial for attraction of *O. gallerucae*. Furthermore, two findings suggest that GLVs do not function as attractive synomones for *O. gallerucae*: (1) egg-free elms induced by feeding are emitting high amounts of GLVs (Wegener et al. 2001), but are not attractive to the parasitoids (Meiners & Hilker 1997, 2000); (2) 1-hexanol, the GLV of which emission was reduced most by inhibitor treatment in this study was not attractive to the parasitoids. A recent study showed that reduction of (*Z*)-3-hexenyl acetate in the odour of an African grass laden with stemborer eggs (*Chilo partellus*; Lepidoptera) led to a shift of ratios of plant volatile compounds which rendered the odour attractive to the braconid larval parasitoid, *Cotesia sesamiae*. Hence, reduction of GLV in the odour of egg-laden plants might contribute to their attractiveness to larval parasitoids (Bruce, Midega, Birkett, Pickett, & Khan 2010).

Chemical inhibition of plant terpenoid biosynthesis in cut Lima beans revealed that monoterpenes play an important role in attracting predatory mites to spider-mite infested plants (Mumm et al. 2008). Inhibition of terpenoid biosynthesis in elm significantly reduced the emission of several sesquiterpenes. The emission of monoterpenes, however, was not significantly affected, even though the treatment by the inhibitors fosmidomycin and cerivastatin was expected to reduce both mono- and sesquiterpene biosynthesis. In poplar, incomplete inhibition of terpenoid biosynthesis by fosmidomycin has been shown several times (e.g. Possell et al. 2010). Incomplete inhibition in our case might have been caused by the fact that the roots of the 3- to 4-month-old plants did not take up sufficient quantities of fosmidomycin and thus, too low quantities of fosmidomycin

reached the leaf plastids where the MEP pathway producing mainly monoterpenes, diterpenes, and tetraterpenes is located. Based on their experiments with *Artemisia annua* L. Towler and Weathers (2007) suggested that maturating plants compensate chemical inhibition to a greater extent than seedlings because of higher biomass in a later developmental stage.

The emission of the homoterpene DMNT (and to some degree also that of the sesquiterpenes (*E*)-β-caryophyllene and (*E*,*E*)-α-farnesene) from plants after herbivore feeding is well known to play an important role in the attraction of parasitoids or predators of feeding larvae or adults (Vet & Dicke 1992). Our olfactometer bioassays clearly show the importance of sesquiterpenes for parasitoid-mediated defence against eggs by (1) the non-attractiveness of the odour of chemically inhibited elm (with reduced total sesquiterpene emission) to egg parasitoids and (2) the attractiveness of individual sesquiterpenes. Comparative analyses of the headspace of oviposition-induced plants and their respective controls suggested that sesquiterpenes also play a role in the attraction of egg parasitoids in tritrophic systems other than the one investigated here. For example, bean plants (*Vicia faba* L. and *Phaseolus vulgaris* L.) induced by feeding and oviposition of *Nezara viridula* (L.) (Heteroptera: Pentatomidae) release a volatile blend with higher quantities of (*E*)-β-caryophyllene than plants without eggs. Only odour from feeding- and oviposition induced plants attract the egg parasitoid *T. basalis* (Wollaston) (Hymenoptera: Scelionidae) (Colazza et al. 2004), whereas odour from feeding-induced plants (without eggs) is not attractive. These findings suggest that (*E*)-β-caryophyllene plays a key role in the attraction of the egg parasitoid. Another example for the relevance of sesquiterpenes in attraction of egg parasitoids to oviposition-induced plants is provided by studies of the tritrophic system of pine, pine sawflies, and egg parasitoids attacking the sawfly eggs. The sesquiterpene (*E*)-β-farnesene released from oviposition-induced pine has been shown to be a key compound for attraction of egg parasitoids to pine sawfly eggs if the parasitoids perceive the sesquiterpene in admixture with other pine terpenoids; (*E*)-β-farnesene *per se* without terpenoid background odour is not attractive (Mumm & Hilker 2005; Beyaert et al. 2010).

The ecological relevance of (terpenoid) background odour and of plant odour diversity for olfactory orientation of insects has been discussed further in detail by Schroeder and Hilker (2008) and Randlkofer, Obermaier, Hilker, and Meiners (2010). Our results show that DMNT plays a role in the indirect defence of elm against leaf beetle eggs since chemically inhibited elm (with reduced DMNT emission) was no longer attractive to the egg parasitoids, and DMNT *per se* (with and without background odour) was shown to attract the parasitic wasps. Interestingly, α-humulene, a minor volatile sesquiterpene released from induced elms, also attracted the parasitoids. The relevance of herbivore-induced minor plant volatiles for parasitoid behaviour has been shown in several studies of tritrophic interactions between plants, herbivores and parasitoids (D'Alessandro et al. 2009). In contrast, our study is the first confirming the attractiveness of individual terpenoids released from oviposition-induced plants. The terpenoids that attract the parasitoid of elm leaf beetle eggs are ubiquitous volatile compounds that do not occur singly in nature. The parasitoid will encounter them in admixture with other volatiles. Our results suggest that the egg parasitoid responds positively to enhanced quantities of a single terpenoid or mixtures of terpenoids. Even though the terpenoids found to be attractive for *O. gallerucae* are ubiquitous volatiles which might also be released by other (induced or non-induced) plant species, the parasitoid might afford to rely on a single volatile when searching for host eggs since this species shows high habitat fidelity and will hardly leave the elm stand. Therefore, the chance to encounter an elm will be high, and the increased amounts of single terpenoids in the odour of egg-laden elm will enable the parasitoid to distinguish between elms with and without host eggs.

Future studies need to further elucidate the relevance of individual herbivore-induced plant volatile compounds for host location of enemies of herbivorous arthropods in natural odourous environments. The finding that egg parasitoids of elm leaf beetle eggs can use single, oviposition-induced elm terpenoids for host search render the tritrophic system of elm, elm leaf beetles and egg parasitoids a suitable model to further study biological control of herbivores *via* induction of the attractive plant volatiles by e.g. plant treatment with phytohormones or by genetic plant manipulation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.baae.2011.06.002.

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Chapter 3

Smelling the tree and the forest: elm background odours affect egg parasitoid orientation to herbivore induced terpenoids

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Chapter 4

An elm EST database for identifying leaf beetle egg-induced defense genes

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An elm EST database for identifying leaf beetle egg-induced defense genes

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Abstract

Background: Plants can defend themselves against herbivorous insects prior to the onset of larval feeding by responding to the eggs laid on their leaves. In the European field elm (*Ulmus minor*), egg laying by the elm leaf beetle (*Xanthogaleruca luteola*) activates the emission of volatiles that attract specialised egg parasitoids, which in turn kill the eggs. Little is known about the transcriptional changes that insect eggs trigger in plants and how such indirect defense mechanisms are orchestrated in the context of other biological processes.

Results: Here we present the first large scale study of egg-induced changes in the transcriptional profile of a tree. Five cDNA libraries were generated from leaves of (i) untreated control elms, and elms treated with (ii) egg laying and feeding by elm leaf beetles, (iii) feeding, (iv) artificial transfer of egg clutches, and (v) methyl jasmonate. A total of 361,196 ESTs expressed sequence tags (ESTs) were identified which clustered into 52,823 unique transcripts (Unitrans) and were stored in a database with a public web interface. Among the analyzed Unitrans, 73% could be annotated by homology to known genes in the UniProt (Plant) database, particularly to those from *Vitis*, *Ricinus*, *Populus* and *Arabidopsis*. Comparative *in silico* analysis among the different treatments revealed differences in Gene Ontology term abundances. Defense- and stress-related gene transcripts were present in high abundance in leaves after herbivore egg laying, but transcripts involved in photosynthesis showed decreased abundance. Many pathogen-related genes and genes involved in phytohormone signaling were expressed, indicative of jasmonic acid biosynthesis and activation of jasmonic acid responsive genes. Cross-comparisons between different libraries based on expression profiles allowed the identification of genes with a potential relevance in egg-induced defenses, as well as other biological processes, including signal transduction, transport and primary metabolism.

Conclusion: Here we present a dataset for a large-scale study of the mechanisms of plant defense against insect eggs in a co-evolved, natural ecological plant–insect system. The EST database analysis provided here is a first step in elucidating the transcriptional responses of elm to elm leaf beetle infestation, and adds further to our knowledge on insect egg-induced transcriptomic changes in plants. The sequences identified in our comparative analysis give many hints about novel defense mechanisms directed towards eggs.

Background

Trees grow under a multitude of abiotic and biotic stresses. Although the suite of genes in trees is similar to that in herbaceous and crop plants, the ecological survival strategies of trees and especially the regulation mechanisms of their secondary metabolic processes are likely to differ from those of herbaceous plants, because of the different life times and size of these types of plants [1-4]. The advent of high-throughput sequencing technologies enables a broad snapshot of the molecular-genetic processes in plant, and have already been used to reveal the large scale transcriptional alterations that occur in plant–insect interactions [5,6]. However, most of the current knowledge about plant defense mechanisms against herbivorous insects has been obtained from studies with herbaceous annuals or short-lived perennials, with few studies of the modulation of complex tree defensive responses.

From an ecological and evolutionary research perspective, the optimal tree species for studying defense

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mechanisms would be one that has been unaffected by breeding for agriculture and forestry, and that is attacked by a highly specialized pest organism. Such conditions can be found for the field elm (*Ulmus minor)* and its closely co-evolved herbivore, the elm leaf beetle (*Xanthogaleruca luteola*) [7,8].

Plants have developed various mechanisms to defend themselves against herbivorous insects [9,10]. In addition to nonspecific, constitutively expressed physical and chemical barriers (e.g. trichomes, thick cell walls, adverse secondary metabolites), plants employ specific induced defenses in response to insect feeding or even egg laying [11,12].

In contrast to feeding, insect egg laying causes minimal damage to plants, dependent on the egg laying behavior of herbivorous insects, which can be quite distinct in different species [13,14]. Direct defenses against insect eggs have been reported for crop and herbaceous species including the production of ovicidal substances [15], growth of neoplasms [16], development of necrotic zones [17,18]. Indirect defense against insect egg laying includes induced changes of plant volatile emissions or modifications of the plant surface chemistry attracting or arresting egg parasitoids, which in turn kill the eggs of the herbivores [19,20].

The first study demonstrating indirect defense against insect eggs was a study of the field elm, where eggs of the elm leaf beetle induced volatiles which attract the egg parasitoid *Oomyzus gallerucae,* a tiny eulophid wasp specialized on elm leaf beetle eggs [21]. Elm leaf beetles often feed and lay eggs on the same plant and are known to remove the leaf epidermis prior to egg laying by scratching the leaf surface with their mouthparts. Experimental simulation of this egg laying sequence by transferring eggs or oviduct secretion on scratched elm leaves or treatment with jasmonic acid (JA) or methyl jasmonate (MeJA) also elicited indirect defense responses in field elms ([8,21], Meiners T. unpublished data). A recent study further showed that terpenoids present in the odor of egg-induced elm leaves are relevant for attraction of the egg parasitoids [22]. Induction of attractive plant volatiles by insect egg laying has been shown in one other tree species and two herbaceous crops [8,23-25].

The natural range of the European field elm *Ulmus minor* (Ulmaceae) extends predominantly within Southern Europe. However, through cultivation it occurs throughout the temperate world. Elms are greatly valued for their timber qualities and prior to the Dutch elm disease outbreaks, elms were also frequently planted within urban areas because of their environmental tolerance [26,27]. Many insects including moths, gall mites, and beetles feed on field elms. The elm leaf beetle *X. luteola* can defoliate entire trees and is recognized as a major urban and forest pest in the USA and Australia [28,29]. The recently published EST sequences for *U. americana* is to our knowledge, the only other gene expression study of any *Ulmus* species, where 535 ESTs (grouped into 314 unique transcripts) were identified after trees (hard calli) were exposed to the fungal pathogen *Ophiostoma novo-ulmi*, which is the causative agent of Dutch elm disease [30].

Knowledge on how plants are able to respond at the molecular level towards egg laying is scarce. Specific transcriptional changes of a wide range of genes involved in several metabolic processes have been shown in Brussels sprouts (*Brassica oleracea* var. *gemmifera*) and *Arabidopsis thaliana* in response to *Pieris brassicae* egg laying [31,32]. The formation of neoplasms on pea pods after egg laying by bruchid beetles is associated with the upregulation of genes *inter alia* encoding enzymes involved in the octadecanoid pathway [33]. Scots pine (*Pinus sylvestris*) responds to eggs laid by the pine sawfly by enhancing the transcription of sesquiterpene synthase genes [34].

Inducible defenses might start with the perception of insect attack by the plants. Compounds released onto the leaves by the female insect with her eggs (e.g. oviduct secretion or accessory glandular secretion attaching the eggs to leaf tissue) or substances released into plant wounds during feeding (saliva- or regurgitate-derived compounds) most likely convey the information indicating an "insect attack", and so trigger a cascade of plant reactions, followed by downstream signaling pathways that mediate specific gene expression leading to the biosynthesis of metabolites which are responsible for the direct and indirect defenses [11,35].

It has been suggested that plants orchestrate their defense reactions against different insect herbivores by a cross-talk between phytohormone pathways, with the octadecanoid signal-transduction pathway playing a key role in this process [36-38]. However, although jasmonic acid (JA) is known to induce indirect defenses in plants *via* the production of volatiles that attract egg parasitoids, the headspace profiles of egg-induced plants and JA-treated ones differ from each other indicating that other plant hormones are also involved in the orchestration of defenses that signal the presence of eggs to egg parasitoids [39,40].

Herbivore eggs have been shown to induce changes in the plant's primary and secondary metabolism and can cause dramatic changes in the plant's transcriptome [31,32]. To date, however, only two studies of Scot pine and Brussels sprouts have addressed the role of egg-induced transcriptional changes in indirect defenses [32,34,41].

We have shown previously that elms can produce a distinct eco-physiological response to the egg laying activities of elm leaf beetle even in the absence of

herbivory [8]. The elegant subtlety of these responses and the co-evolved species specificity predestinate this natural ecological *U. minor - X. luteola - O. gallerucae* system for studying egg-induced transcriptional changes in plants. Here we present the first time a large-scale study of insect egg-induced defense in a natural ecological plant–insect-system.

For identification of egg-induced genes in the field elm, five cDNA libraries were constructed from young elm trees of a single clone. Leaves were harvested after different time periods and different treatments with feeding and/or egg laying by the elm leaf beetle, artificial transfer of egg clutches (to distinguish between egg laying and feeding effects), and spraying with MeJA. A total of 361,196 expressed sequence tags (ESTs) were pyrosequenced and assembled into unique transcripts (Unitrans). Here we report the comparative analysis of 21,490 Unitrans (each represented by at least two ESTs) in order to detect differences in functionally annotated gene transcript abundances. This EST collection represents the first large genomic resource for the European field elm, and the database is now available with a public web interface (www.agcol.arizona.edu/pave/elm), where it is possible to query the different elm libraries based on ESTs, Unitrans, UniProt IDs / descriptions, Protein Families (Pfam), Enzyme Commission numbers (EC) and Gene Ontology terms (GO).

Results

Sequencing of elm after treatment with leaf beetles

Non-normalized total RNA was isolated from leaves of clonal *U. minor* plants that had been exposed to one of five separate treatments: untreated intact elm leaves (C = control), leaves with egg laying and feeding by the elm leaf beetle, *Xanthogaleruca luteola* (EF), leaves with feeding alone by adult *X. luteola* (F), scratched leaves (removal of leaf epidermis to mimic natural egg laying) with manually transferred egg clutches to the scratched site (E); and leaves sprayed with methyl jasmonate (MeJA). Random cDNAs were synthesized from each of these mRNA samples and 454 pyrosequenced. An additional three samples, consisting of mixtures of cDNA libraries, were also sequenced to increase sequence coverage for detected genes (Table 1). After pre-processing, clustering and assembling, we obtained 21,490 Unitrans (unique transcripts) represented by at least two ESTs plus 31,333 Unitrans (singletons) represented by one EST to give a total of 52,823 Unitrans. The elm sequencing libraries obtained from the single treatments contained between 811 Unitrans (≥ 2 EST) (E) and 2,272 Unitrans (≥2 EST) (MeJA), with ~20% singletons per library, while for the mixed libraries between, 12,402 Unitrans (≥2 EST) (E) and 15,083 Unitrans (≥2 EST) (EF + F) were obtained with ~40% singletons per library. As is typical for singletons derived from 454 sequencing, many appeared to represent real gene transcripts, whereas the origin of others is questionable and may well be artifacts. For further analysis Unitrans whose sequence quality was sufficient (plant UniProt annotated with E-value ≤1e-20 threshold) were used. A total of 60% of the Unitrans were between 200–400 nt in length and 71% consist of 2–5 ESTs (see Additional files 1 and 2). Most Unitrans (≥2 EST) showed an open reading frame size in the range of 51-100 (singletons 1-50) (Additional file 3). Thus, although this is the first large-scale sequencing project for this genus, it is almost certainly not a complete representation of all genes expressed in these tissues.

Functional annotation of sequenced transcripts

Among the total number of Unitrans ≥2 ESTs (21,490), 8,780 (41%) were annotated using BLASTx against the

Table 1 Sequencing output of elm libraries

cDNA Libraries[a]	ESTs	Unitrans[b] ≥ 2 ESTs	Library specific Unitrans[c]	Singletons (%)[d]
Untreated control (C)	2132	836	31	174 (17)
Egg & feeding (EF)	1921	826	50	211 (20)
Feeding (F)	4725	1453	65	326 (18)
Methyl jasmonate (MeJA)	7080	2272	153	679 (23)
Transferred eggs (E)	2133	811	40	188 (19)
Mix[e] EF + F	169672	15083	2844	11560 (43)
C + MeJA + E	71239	9141	860	8043 (47)
C + EF + F + MeJA + E	98210	12402	2249	9755 (44)
Tag unidentifiable	4084	597	200	397 (40)
Total	**361196**	**21490**	**–**	**31333 (59)**

[a] Libraries of differently treated elms: C = Control (untreated *Ulmus minor* leaves); EF = Egg laying & feeding (=leaves with elm leaf beetle eggs and feeding damage by female beetles [natural situation]; F = Feeding (=leaves with feeding damage by male beetles); Methyl jasmonate (= leaves treated with 50 μmol MeJA), E = Transferred eggs (= leaves that had been scratched and had eggs artificially placed on them); [b] Number of Unitrans (unique transcripts) ≥2 ESTs that contain at least one EST of one of the libraries; [c] Number of Unitrans ≥2 ESTs that contain ESTs of only the particular library; [d] Percent singletons in relation to all unique transcripts (Unitrans ≥2 ESTs + singletons); [e] New sequencing runs of combined libraries.

plant taxonomic database of the UniProt protein function and sequence database platform with an E-value threshold of ≤1e-20. Not surprisingly, the most abundant gene products with known function in the elm leaf EST database included genes involved in photosynthesis (Additional file 4). The top four plant genera to which 73% of the Unitrans were annotated using the Plant UniProt database included *Vitis, Ricinus, Populus* and *Arabidopsis* (Additional file 5). The resulting annotated Unitrans were grouped into nine different functional categories based on their Gene Ontology term (GO term, Figure 1). Most Unitrans belonged to the categories "cellular process or metabolic process" (90.5%), whereas 0.5% fell into the category "defense response".

Changes in transcript abundances among treatments

The sequencing was performed with the aim of detecting leaf beetle egg-induced defense genes and associated regulatory elements, based on the assumption that changes in abundances of mRNA species are reflected by differences in the number of ESTs that encode particular genes. It is possible for abundances of a given transcript to be falsely low in a sequenced library due to poor quality sequence, insufficient sequence depth, misassembled Unitrans or misidentification of the best organism match for a Unitrans due to sequencing/assembly errors. Hence the R statistic was applied to the elm database and used as an initial statistical screening tool [42]. The library counts were displayed as parts per 10,000 (pptt) or parts per 1,000 (ppt), which normalizes transcript abundances based on their library size. This prevents over-evaluation of high transcript numbers in a large library relative to low numbers of transcript in a smaller library.

The five treatments were compared using relative EST abundance per annotated GO functional category (i.e., summed across all Unitrans annotated to that category). To obtain a broad overview of the transcriptomic responses in major plant physiological processes, nine GO categories were selected and four of them were considered as significantly differentially expressed in the respective treatment compared to untreated elms (C) (Figure 2a). For the GO term categories "photosynthesis" and "electron transport + energy", the comparison indicated a decrease in transcript abundances for egg-induced (EF) as well as MeJA treated plants. Chlorophyll a-b binding proteins (Unitrans: elm_00108, data not shown) were mostly responsible for the differential transcript abundances between treatments. For almost all categories, MeJA treated plants showed transcript abundance patterns similar to EF treated plants, suggesting that MeJA does indeed play a significant role in the plant's response to egg laying. Likewise, similar patterns of transcript abundances were observed between untreated plants (C), feeding-induced plants (F), and plants with the experimental imitation of the egg laying event by transfer of egg clutches (E). For the category "transport" E and MeJA treated plants showed increased transcript levels in comparison to the other treatments. Feeding-induced plants showed decreased transcript levels in comparison to the other treatments only for the category "amino acid metabolism". In "carbohydrate metabolism" and "signal transduction" a significant increase in transcriptional changes was determined only for egg-induced plants. For these categories no single Unitrans is responsible for the changed transcript pattern. For the category "fatty acid biosynthesis", the largest group of ESTs responsible for differences between treatments matched a lipoxygenase (Unitrans: elm_00084, data not

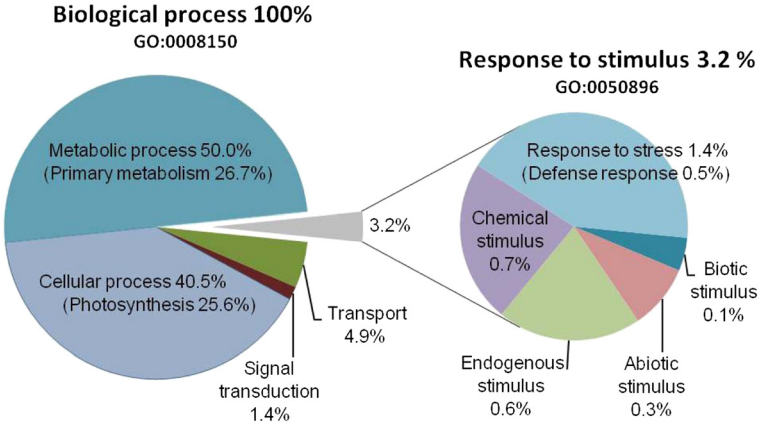

Figure 1 Functional distribution of all annotated Unitrans (>2 ESTs) based on their predicted Gene Ontology (GO) term. All GOs shown had at least 200 ESTs and an E value threshold of ≤1e-40 after annotation by the UniProt database. The left chart represents the five main groups of biological processes (GO first level), the right chart represents subcategories for "Response to stimulus" (GO second level), and "Response to stress" (in brackets, GO third level).

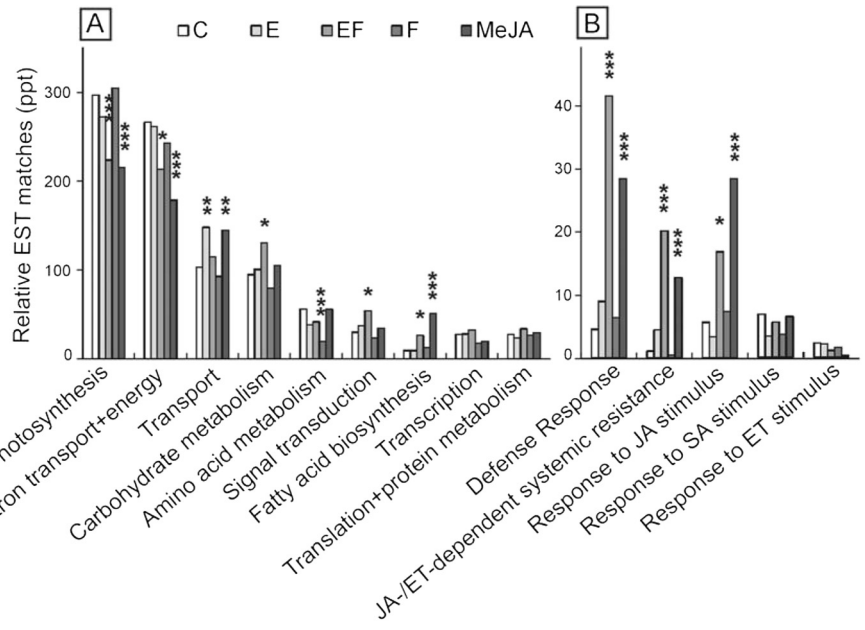

Figure 2 Functional distribution of elm leaf EST matches from the five different single libraries based on their predicted Gene Ontology (GO) term. All GOs shown had at least 200 ESTs and an E value threshold of ≤1e-40 after annotation by the UniProt database. The GO terms from each library are divided into two groups: (A) important plant physiological processes and (B) defense-related processes including response to jasmonic acid (JA), ethylene (ET) and salicylate (SA). The libraries are: EF (egg laying & feeding), F (feeding), MeJA (methyl jasmonate), E (artificial scratching & egg transfer) and C (untreated control). The y-axis indicates relative EST matches by parts per thousand (ppt) relative to the library size. For statistical analysis EST abundances by library were compared pairwise by GO category. Asterisks denote treatments in which ESTs were differentially expressed relative to the control treatment; *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001; Fisher`s exact test.

shown), which is a key enzyme in JA biosynthesis. The strongest increase of lipoxygenase-related ESTs was observed for MeJA treated plants.

Focusing on defense-related processes a well as the jasmonic acid (JA), ethylene (ET)- and salicylic acid (SA) pathways, five further categories were selected and three of them revealed R statistic values >3 for at least one pair-wise comparison of EST abundances by treatment (Figure 2b). For egg-induced plants (EF), the GO analysis indicated a particular increase in the proportion and variety of expressed genes involved in the "defense responses" and the "responses to jasmonic acid / ethylene dependent systemic resistance". In both cases class I chitinases (Unitrans: elm_00100, data not shown) appeared to be responsible for much of the observed differential expression. Lipoxygenases appeared to be responsible for differential expression in the category "response to JA stimulus", which is consistent with the result in the category "fatty acid biosynthesis". On the other hand, GO analysis indicated no significant differences between the compared treatments in transcript abundances involved in transport, carbohydrate metabolism, signal transduction, translation, transcription, ET- and SA-pathways (Figure 2a and b).

The distribution of Unitrans ≥ 2 ESTs between the different treatments annotated against the plant taxonomic UniProt database is shown in the Venn diagrams of Figure 3. Focusing on the analysis of the "egg"-induced treatment (E) and the mixed library EF + F, the pairwise intersections between the C, E and EF treatments are about 30% of the Unitrans (Figure 3A). When including data from the other treatments, half of the Unitrans for the EF or F treatments overlap with MeJA (Figure 3B). Interestingly around 90% of the C and F treatment Unitrans overlap with the those from the (10–17 fold larger) mixed sample EF + F (Figure 3C). This suggests that many of the assignments that are apparently unique to one treatment may well be shared with other treatments, but insufficient sequence coverage prevented detection in these other samples. We have highlighted (in parentheses) those transcripts assigned to the gene ontology category "defense response" in the Venn diagrams (Figure 3, A–C). As expected, only a small number of Unitrans from the untreated plants (C) were found to be assigned to this category. All Unitrans related to defense were detected in treatments that include induction by eggs (E, EF and EF + F). Here the Unitrans number increased with the library size. Table 2 shows a list of Unitrans with predicted gene functions belonging to the GO category "defense response".

For visualization of metabolic pathways represented by gene transcripts, maps were reconstructed with the iPath

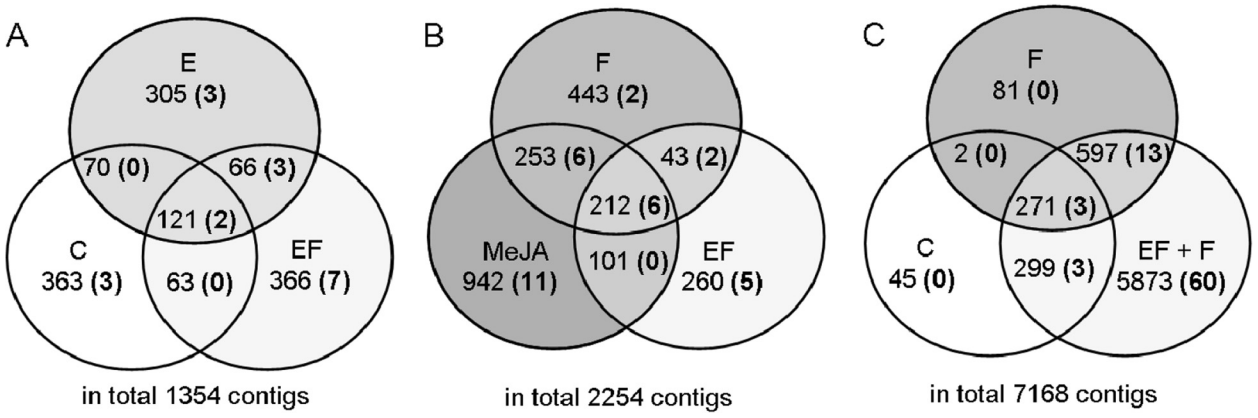

Figure 3 Comparison of Unitrans abundances (E value thresholds ≤1e-20) for genes differentially expressed in *Ulmus minor* plants subjected to various treatments: A) *Xanthogaleruca luteola* feeding together with egg laying (EF), artificial scratching with transferred egg clutches (E), untreated control (C); **B)** *X. luteola* feeding (F), methyl jasmonate (MeJA) and EF; **C)** mixed library (EF + F), C and F; genes in brackets were classified in terms of the Gene Ontology (GO) category "defense response" (E-value ≤ 1e-20). The Unitrans belonging to each category are listed in Additional files 6, 7, and 8.

software [43], using enzymes corresponding to the annotated Unitrans. The enzymes are designated by the usual enzyme commission (EC) nomenclature. Cross-comparisons among treatments (EF, F, C, E, and MeJA) demonstrate that most enzymes are only expressed in one of the two compared treatments below (Additional file 9). Because library size had a strong influence on the extent of the annotated and mapped enzymes, we mapped the largest library, EF + F, in which most transcripts of the other libraries occur (for data on F and C libraries see Venn diagram in Figure 3C and for MeJA, EF libraries data not shown). We used the 451 EC numbers of the EF + F library to generate a metabolic map to examine putative biochemical pathways present in feeding- and egg-induced *U. minor*, and also highlighted those putative enzymes preferentially expressed in egg-induced plants (Figure 4). Enzymes associated with primary metabolism (carbohydrate-, amino acid-, nucleotide-, energy- and lipid metabolism) are predominant, whereas enzymes associated with secondary metabolism (e. g. phenylpropanoid, flavonoid, and terpenoid biosyntheses) are much less prevalent.

To elucidate the molecular basis for the biosynthesis of volatiles involved in indirect defenses of elm to leaf beetles, we mainly focused on terpenoid metabolism comparing the different treatments with iPath, a web-based tool for the visualization of metabolic pathways. According to the different iPath maps, the enzymes involved in terpenoid biosynthesis were most frequently observed in the large treatment combination EF + F (Figure 4, Additional file 9). Several transcripts involved in terpenoid biosynthesis including prenyltransferases and terpene synthases were found, but low EST numbers made a statistical analysis between treatments impossible (data not shown). Putative enzymes with increased transcript abundances in the EF versus MeJA, F, E, and C treatments with significant Rstat values (highlighted in the map) are lipoxygenase (A = EC:1.13.11.12; oxylipin [octadecanoid] metabolism), catalase (B = EC:1.11.1.6; hydrogen peroxide catabolic process), glyceraldehyde-3-phosphate dehydrogenase (C = EC:1.2.1.13; glycolysis), cobalamin-independent methionine synthase (D = EC:2.1.1.14; methionine metabolism), and sucrose synthase (E = EC:2.4.1.13; sucrose metabolism). The EC numbers used for generating maps are listed in Additional file 10, showing the normalized counts for Unitrans and R values for the different cross-comparisons between treatments.

The Unitrans associated with the GO category "defense response" included genes for pathogen related proteins (PR), phytohormone signaling, plant innate immunity, and other regulatory processes (Table 2). Cross-comparison of the different treatments revealed genes with increased transcript abundances in egg- and feeding-treated plants. Ten putative genes were specifically enhanced in all the insect egg-treatments (libraries EF, E and EF + F) in comparison to the other treatments. These were annotated as: a class I chitinase, a glucan endo-1,3-beta-glucosidase, a MLP-like protein, a jasmonate ZIM-domain protein, an auxin signaling F-box protein, the regulatory protein NPR1, a peroxisomal acyl-coenzyme A oxidase, a patatin-like protein, heat shock protein 81, and a cyclic nucleotide-gated ion channel) (bold numbers Table 2). The most abundant transcripts in this group were the class I chitinase (2111 ESTs), the heat shock protein 81 (309 ESTs), and the glucan endo-1,3-beta-glucosidase (190 ESTs). Interestingly five of these transcripts showed simultaneous increases in the MeJA–treated plants, again suggesting a role for MeJA

Table 2 Relative abundance of Unitrans annotated as having a predicted function in defense response in six libraries representing different elm leaf treatments

Gene description based on homology	# of Unitrans	# of ESTs	Best database match	E-value	Treatment (pptt)					
					EF	EF + F	E	F	MeJA	C
PR Proteins										
Class I chitinase	12	2111	*Brassica napus*	8e-58	**161**	70	28	13	133	33
Disease resistance response protein	5	192	*Arabidopsis thaliana*	4e-25	-	1	-	-	1	-
Glucan endo-1,3-beta-glucosidase	7	190	*Prunus persica*	2e-115	10	2	**19**	-	7	-
Cysteine proteinase inhibitor	3	189	*Vigna unguiculata*	1e-26	5	5	-	-	6	5
MLP-like protein	3	86	*Arabidopsis thaliana*	3e-42	**86**	10	2	3	8	3
Pathogenesis-related protein	3	34	*Medicago truncatula*	5e-22	-	0.3	-	2	-	-
MLO-like protein	2	4	*Arabidopsis thaliana*	4e-32	-	0.1	-	-	-	-
Phytohormone signaling										
Jasmonate ZIM-domain protein 3	1	111	*Arabidopsis thaliana*	3e-24	**21**	3	-	2	10	-
Ethylene-responsive transcription factor	3	97	*Arabidopsis thaliana*	1e-41	-	3	5	2	4	9
Auxin signaling F-box 2	2	33	*Arabidopsis thaliana*	3e-79	**21**	1	5	2	1	-
ABC transporter G family member 40-	1	10	*Arabidopsis thaliana*	1e-62	-	0.1	-	0.1	1	-
Regulatory protein NPR1	1	7	*Arabidopsis thaliana*	1e-25	**9**	-	0.2	0.3	-	-
Coronatine-insensitive protein 1	1	8	*Arabidopsis thaliana*	9e-54	-	0.1	-	-	-	-
Probable WRKY transcription factor 33	1	4	*Arabidopsis thaliana*	7e-28	16	-	-	-	-	-
Ethylene-insensitive protein 2	1	2	*Arabidopsis thaliana*	9e-23	-	0.1	-	-	-	-
Jasmonic acid synthesis										
Allene oxide synthase	4	391	*Linum usitatissimum*	3e-39	10	12	9	13	30	5
Peroxisomal acyl-coenzyme A oxidase 1	2	8	*Arabidopsis thaliana*	6e-35	**5**	0.1	-	-	-	-
Innate immunity										
Pre-mRNA-splicing factor SPF27	1	13	*Arabidopsis thaliana*	3e-52	-	0.5	-	-	-	-
Pre-mRNA-processing factor 19	4	11	*Arabidopsis thaliana*	2e-29	-	0.3	-	-	1	-
Cell division cycle 5-like protein	2	11	*Arabidopsis thaliana*	5e-50	-	0.3	-	-	-	-
Protein pleiotropic regulatory locus 1	2	7	*Arabidopsis thaliana*	5e-75	-	0.3	-	-	-	-
Serine / threonine-protein kinase PBS1	1	2	*Arabidopsis thaliana*	7e-42	-	0.1	-	-	-	-
Regulatory role in defense response										
Patatin-like protein	1	557	*Solanum tuberosum*	7e-83	**47**	13	9	15	48	-
Heat shock protein 81	4	309	*Arabidopsis thaliana*	0	-	**5**	-	2	3	-
Ankyrin repeat domain-containing protein 2	4	136	*Arabidopsis thaliana*	5e-59	-	3	9	19	4	-
(+)-neomenthol dehydrogenase	3	29	*Arabidopsis thaliana*	2e-23	-	1	-	2	1	-
Cyclic nucleotide-gated ion channel	3	15	*Arabidopsis thaliana*	2e-45	**10**	0.3	-	0.1	-	-
Two pore calcium channel protein 1	2	5	*Nicotiana tabacum*	6e-31	-	0.2	-	-	-	-
Cell wall metabolism										
Cellulose synthase A catalytic subunit 3	3	17	*Arabidopsis thaliana*	5e-63	-	1	-	-	-	-

Libraries: C (untreated control), E (artificial scratching & eggs transferred), EF (egg laying & feeding), F (feeding), MeJA (methyl jasmonate), mixed library EF + F. Relative Unitrans abundance calculated on library counts by parts per ten thousand (pptt) based on the annotation to Plant Swiss Prot (BLASTx, E-value ≤ 1e-20). Annotated transcripts filtered depending on their predicted function to the category "GO:0006952 defense response" of the Gene Ontology term. **Bold** = increased relative Unitrans abundance in egg-induced plants.

in response to egg laying. Ten putative genes were present at low transcript abundances (2-17 ESTs) exclusively in those plants that were induced by egg laying, and almost all of these were from the large EF + F library. These were annotated as: MLO-like protein 6, coronatine-insensitive protein, WRKY transcription factor 33, ethylene-insensitive protein, pre-mRNA-splicing factor, cell division cycle 5-like protein, protein pleiotropic regulatory locus, a serine / threonine-protein kinase, two pore calcium channel proteins, and cellulose

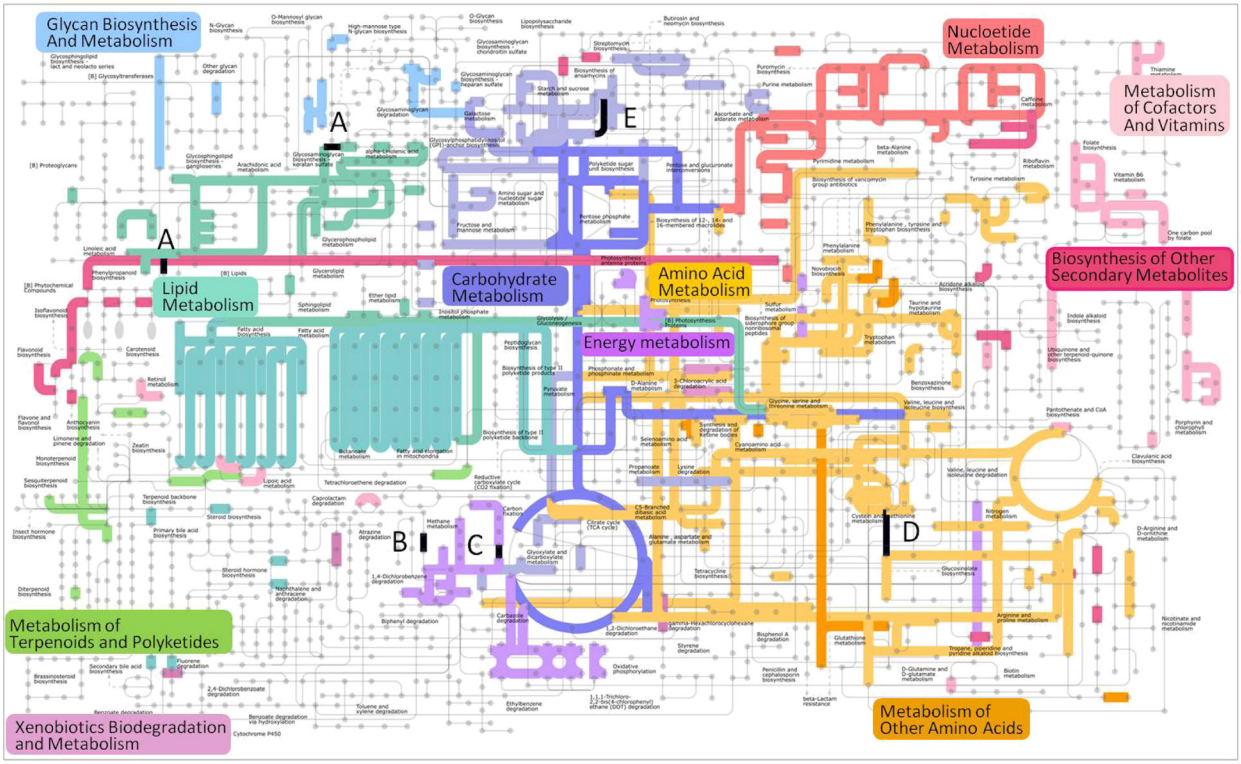

Figure 4 Metabolic pathways expressed in *Ulmus minor* after egg laying and feeding by the elm leaf beetle *Xanthogaleruca luteola*. 451 enzymes (based on EC numbers, shown as bold colored lines) identified *via* Blast searches against the UniProt database (E-value ≤1e-40) were used to generate the map with iPath [43], a web-based tool for the visualization of metabolic pathways. Enzymes A–E highlighted in **black** are referentially expressed in egg-induced plants (see Results).

synthase A catalytic subunit 3. Three genes (ABC transporter, allene oxide synthase, and pre-mRNA-processing factor) showed apparent increases in MeJA induced plants (10-391 ESTs). Two additional gene transcripts (pathogenesis-related protein, and ankyrin repeat domain-containing protein) showed increased abundance in feeding-induced plants (34-136 ESTs). Transcripts annotated as an ethylene-responsive transcription factor were enhanced in untreated plants (97 ESTs).

From the 15 most abundant protein transcripts in egg- and feeding-treated plants, the three with EST counts >1000 were (a) lipoxygenase which is involved in JA biosynthesis, (b) a sieve element-occluding protein preventing the loss of photoassimilates after wounding [44] and (c) catalases which are known to serve as common antioxidant enzymes and to induce suberization and other protective mechanisms after wounding [45] (Table 3). Four proteins with EST counts >100 were (d) peptidyl-prolyl cis-trans isomerases which are also known as cyclophilins and accelerate the folding of proteins [46], (e) proteasome subunits responsible for protein degradation and turnover [47], (f) auxin-repressed proteins known to affect auxin signaling as negative regulators [48] and (g) methionine synthase (cobalamin-independent), which catalyses the last step in the production of the amino acid L-methionine used by plants for many essential direct or indirect cellular processes [49]. Two further proteins almost unique to the EF library in these elms were (h) the enzyme methionine sulfoxide reductase, which functions in plant defense *via* the regulation of the cell redox status and is known to be involved as an antioxidant in repairing proteins damaged by oxidative stress [50], and the transport protein SFT2, which in yeast is involved in traffic to the Golgi complex and vesicle-associated membrane fusion [51,52]. The R statistic was applied in order to detect differences in relative transcript abundances between the elm treatments [42]. Transcripts with $R > 3$ (~99% true positive rate for our libraries) were considered to be differentially expressed between the libraries. For all these protein types, the R statistic revealed a significant difference in transcript abundances between the treatments.

Discussion

The large-scale EST sequencing results shown here represent the first step in studying the defensive responses of field elms to egg laying by the specialist elm leaf beetle *Xanthogaleruca luteola*, at a molecular level. 361,196

Table 3 Identification of putative regulatory proteins with high occurrence in egg laying- and feeding-induced *Ulmus minor* leaves

Pfam accession	Gene description	# of ESTs	GO Biological process	Treatment (pptt)						
				EF	EF+	E	F	MeJA	C	R
PF00305	Lipoxygenase	1602	lipid biosynthetic process	110	43	33	38	162	38	30.9
No family	Sieve element-occluding protein	1545	-	245	40	66	19	86	33	27
PF00199	Catalase	1159	response to stress	73	24	28	25	54	19	6.7
PF00160	Peptidyl-prolyl cis-trans isomerase	773	protein folding	52	15	9	19	34	33	5.3
PF00227	Proteasome subunit	341	response to stress	21	8	5	4	24	9	3.9
PF05564	Auxin-repressed protein	207	signal transduction	26	3	-	2	-	-	4.3
PF01717	Methionine synthase	200	methionine biosynthetic process	42	4	-	2	16	9	8.7
PF01641	Methionine sulfoxide reductase	58	catalytic activity	34	1	-	-	-	5	8.3
No family	Protein transport protein SFT2	16	vesicle-mediated transport	78	-	-	-	-	-	29.9

Treatments: C (untreated control), E (artificial scratching & eggs transferred), EF (egg deposition & feeding), F (feeding), MeJA (methyl jasmonate), mixed library EF + F. Relative Unitrans abundance calculated on counts by parts per ten thousand (pptt) based on the annotation to Plant UniProt (BLASTx, E-value ≤1e-20). Transcripts correlated on their predicted function to the Pfam = protein family database. R–values >3 were considered as significantly differentially expressed for the respective treatment against C (true positive rate of ~99%) by Test Statistics R [42].

expressed sequence tags (ESTs) were assembled into 52,823 unique transcripts (Unitrans). Although the gene discovery rate among the transcripts was low due to the low number of *Ulmus* genes in public databases, we were nevertheless able to identify a large number of candidate genes with possible roles in the response of elm to egg laying by the elm leaf beetle. Normalization based on sequence sample size and analysis using R statistics provided the basis for comparative gene expression analysis using EST frequencies across five different biological treatments: egg laying and feeding by *X. luteola* (EF), feeding (F), transfer of egg clutches (E), methyl jasmonate spraying (MeJA) and an untreated control (C). The function of these candidate genes must now be confirmed in further studies. Despite a similar sample size and the fact that clonal plant material, identical sequencing technologies, and sequence assembly were used, the EST frequencies of the five treatments showed astonishingly small intersections as can be seen in the Venn diagrams and visualization of metabolic pathways (Figures 3 and 4). Therefore, although the influence of *X. luteola* feeding on transcripts cannot be ruled out, the ten-fold larger library EF + F is still capable of being used for detecting the less abundant transcripts induced by egg laying, as it represents a broad snapshot of the transcriptome and of the activity in the different biochemical pathways in elm. We compared Unitrans distributions and gene ontology (GO) terms and identified enzyme differences among the treatments especially with regard to egg-induced changes in transcript abundances.

Leaf beetle egg laying increases defense gene transcripts and decreases transcripts for photosynthesis

Gene ontology analysis indicated a decrease in the transcription level for those genes involved in photosynthesis in the egg- and MeJA-induced plants. Egg laying by herbivorous insects can cause a reduction in photosynthetic activity, as has been shown for a tree species (*Pinus sylvestris*) and a crop plant (*Brassica oleracea* L.) [53,54]. Whether transcription of photosynthesis genes in egg-free leaf parts is affected by eggs has not been studied so far. There has been only one previous study showing a reduction of transcription of photosynthesis-related genes after egg laying; however, in this study tissue situated directly underneath the egg masses without full access to light had been sampled [31]. In our study, the material sampled for sequencing included leaf tissue immediately adjacent to the egg laying site as well as that some distance away. The analyzed tissue was not covered by eggs and had full access to light, and thus the response seen in photosynthesis-related genes is not just a response to low light. Our results are consistent with that of other studies showing the reduction of photosynthesis-related genes after MeJA treatment [55,56].

Further it appears that MeJA affected transcript levels in a manner similar to the insect treatments, which has also been observed in several other studies of plant responses to insect feeding damage [57-60]. The transcripts of MeJA treated plants showed GO term distributions similar to the transcripts of EF treated plants. Both egg laying (represented by the two libraries EF and EF + F) and JA (or MeJA) treatments induce the indirect defenses of elms by stimulating the emission of volatiles that attract egg parasitoids. Nevertheless, these different experimental treatments induce volatile patterns that differ qualitatively and quantitatively ([8,39], Meiners T. unpublished data). In contrast, only minor differences in the overall transcript levels were detected between untreated plants and plants with transferred eggs, indicating that the experimental imitation of the egg laying

event does not cause any wholesale change in transcriptional levels.

The GO analysis indicated an increase in the number and quantity of expressed genes involved in defense responses for egg-induced plants. In a similar way, an inverse correlation between photosynthesis- and defense-related genes was observed in *Arabidopsis thaliana* after egg laying by *Pieris brassicae* [31], which might indicate a reallocation of resources from primary to secondary metabolism. However, in *Brassica oleracea* var. *gemmifera,* only a few defense genes were found to respond to treatment of leaves with pierid eggs [32].

Induced defense genes encode PR proteins, chitinases, WRKY transcription factors and other proteins

In this study, special attention was paid to the detection of expressed genes associated with plant defense against insect eggs, as indicated by enhanced transcript abundances after egg laying in comparison to the other treatments. In egg-induced plants, we observed an increase in transcripts annotated as chitinases, glucan endo-1,3-ß-glucosidases, pathogenesis-related protein (PR), major latex protein (MLP), heat shock protein 81, patatin-like protein, NPR1, and WRKY transcription factor 33. In *Ulmus americana* similar upregulation of chitinase and PR-1 transcripts were induced after inoculation with the fungus *Ophiostoma novo-ulmi* at a similar time point (48–72 h) after treatment [30]. Almost all of the 53 upregulated transcripts reported in this study with sequence similarities to defense related proteins were also found in our much larger *U. minor* database. PR proteins are well known to be involved in defense responses after herbivore attack [61]. Our results suggest the potential importance of *de novo* PR protein expression by *U. minor* in response to attack by *X. luteola*. Transcripts detected with high expression in egg-treated elms show sequence similarities to genes belonging to different PR protein families (PR-1, PR-2, PR-3, and PR-10). Chitinases (PR-2) play a direct role in plant defense by degrading microbial cell wall components, often coordinated with the induction of glucan endo-1,3-ß-glucosidases (PR-3), and seem to be a prominent feature of the inducible defense profile after pathogen attack [4,30,62]. Our data suggest that this is also true after insect attack in trees. Chitinases and glucan endo-1,3-ß-glucosidase are also known to be induced at and near the egg laying site in *A. thaliana* by pierid eggs and could play a defensive role against newly hatched larvae [31]. Chitin is an important structural component of the exoskeleton and the midgut in all insects [63,64]. Chitinases might also be effective defenses against the egg stage even though chitin-like components are not known from egg shells except in mosquitoes [65]. But, if chitinases were to penetrate the eggs they could prevent larvae from hatching, and might serve as a direct defense against the beetle eggs.

MLP-like proteins belong to the PR-10 protein family, which are induced by both biotic and abiotic stress conditions in various plant tissues [61]. The biological function of these proteins remains to be elucidated, but they very likely participate in binding of ligands, such as plant hormones and secondary metabolites [66]. Many PR genes are regulated by WRKY transcription factors, and WRKYs are known to fine-tune stress responses, including defense responses [67]. WRKY 33 initiates the positive regulation of JA-induced defense genes and negative regulation of SA-related defense genes [68]. WRKY factors allow binding to the W-box motif, which is found in promoters of PR defense genes such as PR-10 [69] and chitinase [70]. W-boxes have also been identified in the promoter region of *NPR1,* an important receptor which helps to regulate SA/ JA-phytohormone signaling [71].

Two proteins which also showed increased expression in egg-induced elms are patatin-like protein and heat shock protein (HSP) 81. Patatin proteins are related to the major storage protein known from potato tubers and have the enzymatic activity of phospholipases and release fatty acids from membrane lipids. These proteins have been identified in many plant species and were shown to be involved *inter alia* in pathogen-triggered cell death and to be induced by wound stimuli [72]. They might also be associated with the herbivore-induced defense pathway *via* the mobilization of linolenic acid from the cell membrane, which activates the octadecanoid pathway and finally leads to the synthesis of JA and other oxylipins [73,74]. HSPs meanwhile, are molecular chaperones which can modulate the folding of a variety of other specific target proteins involved, for instance, in cell cycle control and signal transduction [75]. HSP 81 belongs to the HSP 90 family of stress proteins, which are known to influence several resistance-gene signaling pathways, the inhibition of which lead to decreased resistance to pathogens and increased resistance to insect herbivores [76,77]. Thus, a suite of defense response genes, that work together to protect the plant from insect attack appears to be coordinately activated by egg laying on elm.

Transcripts of jasmonic acid biosynthesis genes are present in high abundance

JA has been determined to be an integral part of the plant signal transduction pathway, which leads to the activation of direct- and indirect defenses against herbivorous insects [36,78,79]. Decreased resistance to herbivores and enhanced egg laying activity has been observed in tomato mutants with impaired JA biosynthesis [80]. Moreover, transcriptome analyses using

microarrays indicated that a large portion of herbivory-induced responses are mediated through the JA pathway [58,81].

In egg-induced elms, we found high levels of transcripts of genes encoding key enzymes involved in the biosynthesis of JA including lipoxygenase and allene oxide synthase. Our findings support the expected involvement of the octadecanoid signal transduction pathway in egg-induced plant defense, as the treatment of elms with MeJA leads to the release of volatiles that are attractive to egg parasitoids. Genes involved in JA biosynthesis were also upregulated after pierid eggs laying on *A. thaliana* [31]. However, we also found enhanced transcript abundances after egg laying in comparison to the other treatments for jasmonate ZIM-domain proteins, which are known to repress JA responsive genes [38]. Auxin might be another phytohormone involved in elm responses to eggs, and transcripts of both positive and negative regulators of auxin signal transduction, an auxin receptor (Auxin signaling F-box 2) and an auxin-repressed protein, were also found [48,82]. After JA treatment of poplar, down regulation of genes involved in auxin signaling was observed [83]. Auxin interferes with JA and SA signaling, and the negative regulation of auxin is supposed to mediate adaptive response to biotic stress [84,85]. Another hormone, salicylic acid, may also be involved in plant responses to eggs since SA-deficient mutants of *A. thaliana* showed different responses to pierid eggs than wild type plants [86]. Further studies are necessary to understand the role of JA in concert with other phytohormones in signaling in order to regulate egg-induced defenses.

Gene transcripts for terpenoid biosynthesis were detected at only low levels

There is strong evidence that damage-dependent JA levels activate distinct sets of defense genes leading to terpenoid formation [87]. To elucidate the molecular basis underlying volatile biosynthesis associated with the indirect defenses of elm in response to egg laying, we compared the different treatments with reference to transcripts involved in terpenoid metabolism. Although it has been established previously that a volatile blend with an enhanced fraction of terpenoids that is attractive to egg parasitoids is produced by these elms 2–3 d after egg laying [22], we detected only a few transcripts involved in terpenoid metabolism in the elm leaves following egg treatment. The respective genes may be differentially expressed, but below the detection threshold of our analysis or else possibly the expression is not controlled at the transcript level. In general it is supposed that herbivore-induced *de novo* production of terpenoids takes place several hours following the activation of terpene synthase genes [87]. Enhanced abundance of transcripts for terpene synthases were also found in samples taken from the needles of *Pinus sylvestris*, that were laden with eggs of the herbivorous sawfly *Diprion pini*; these egg-laden pine needles emit a volatile terpenoid blend that attracts egg parasitoids. However, transcript levels for a sesquiterpene synthase from *P. sylvestris* which produces (*E*)-*β*-farnesene, the compound responsible for the attraction of an egg parasitoid of sawfly eggs, were not enhanced by *D. pini* egg laying [41].

The time window in which egg-induced elm leaf material was harvested for sequencing and the large size of our database should have enabled the detection of even relatively rare transcripts associated with the early and late direct and indirect defense responses against the leaf beetle. In *A. thaliana* the number of up- or down-regulated genes increased as time elapsed from 1–3 d after pierid eggs have been laid on plants [31]. Because transcripts for terpenoid metabolism are under-represented in our database, we can only speculate about the molecular basis of egg-induced volatile production for indirect defense in elm. We hypothesize that egg-enhanced JA levels increase transcript abundances for JA biosynthesis genes, thereby activating so far unidentified genes which stimulate the emission of a volatile blend of terpenoids from elms, but by a mechanism that does not involve an increase in the transcript levels for the genes associated with the formation of these compounds, as has been demonstrated for other plants [41,88,89].

Since plant defense signaling mechanisms may well be selected to respond as rapidly as possible to the presence of herbivores, their initial response is probably modulated by physiological means in the first instance, rather than by changes in expression levels. To confirm this hypothesis further studies are needed to measure the levels and activities of terpenoid biosynthetic enzymes participating in volatile formation.

Transcripts were induced encoding other protein types

In addition to transcripts for proteins known to be involved in defense responses, we found enhanced transcript abundances of proteins (and protein families) in egg-induced plants for which little knowledge is available on their possible role in defense responses towards insect eggs. These proteins are assigned to general functions, such as stress response, protein metabolism, signaling and transport. They probably represent a critical link between defense and developmental processes in these plants. Next to the up-regulation of lipoxygenase especially high EST numbers and a strong significant difference between the treatments were found for transcripts associated with sieve element-occluding proteins, which supposedly play a role under stress conditions

after insect attack [90]. Among the enhanced transcript abundances in egg-induced plants high EST numbers were found for transcripts of catalases, which protect cells from the toxic effects of reactive oxygen species (ROS) such as hydrogen peroxide, which are often found in stressed tissues [45]. Herbivory has been found to elicit the production of ROS that are involved in further downstream transduction cascades, leading to the induction of defense-response genes [35], as well as in localized cell death [91]. We hypothesize that enhanced ROS levels caused by injury during egg laying are most likely responsible for the increased expression of related classes of catalases in elm, where localized cell death has been observed under the egg clutches [13].

Interestingly high EST numbers of trancripts associated with methionine metabolism were found in egg-induced plants. An increase of methionine synthase after MeJA treatment was also reported for *A. thaliana* [55]. The proteinogenic amino acid L-methionine has many essential direct and indirect functions in cellular metabolism, including ethylene biosynthesis [49], as well as the biosynthesis of defense compounds [92]. High EST numbers were also found for transcripts involved in protein folding (cyclophilins) and degradation (proteasome subunits), possibly indicating that turning over and re-configuring the proteome might be a critical step in the defensive responses of plants, as well possibly having an important role in signal transduction [93], including the fine-tuning of JA signaling [94]. Among those gene trancripts that were enhanced by elm beetle egg laying, we also identified transcripts associated with proteins involved in the transport of ions and other compounds, such as cyclic nucleotide-gated ion channels [95], and the transport protein SFT2, albeit with lower EST number. Especially interesting among these is the transport protein SFT2, as this was exclusively present in leaf samples after egg laying treatment. SFT2 is a member of the SNARE protein family, which is known to function in vesicle-associated membrane fusion events during transport processes in plants. Plant SNARE proteins are thought to be involved in developmental processes and pathogen defense, but it remains unproven whether SFT2 functions like their yeast counterpart [52,96].

Conclusions

While insect feeding is known to trigger major changes of the transcriptome in herbaceous and woody plants (e. g. [58,83,97,98]), insect egg laying has so far only been shown to elicit large scale changes in the transcriptome of herbaceous plants [31,32]. Our elm EST database shows for the first time that insect eggs can induce similarly transcriptional changes in a woody plant, a deciduous tree. There was a pronounced shift towards transcripts involved in general stress responses such as oxidative stress (catalases, methionine sulfoxide reductase), and defense responses (PR proteins), phytohormone signaling (in particular JA), and transport processes (cyclic nucleotide-gated ion channels and transport protein SFT2). Further changes were observed in primary metabolism (sucrose synthase, glyceraldehyde-3-phosphate dehydrogenase, methionine synthase, and cyclophilins), and a possible downregulation of photosynthesis suggests a metabolic shift from growth and development to defense. As such, this work presents a large data set from a well established, ecological natural plant – insect system which will be important for further studies of the mechanisms of direct and indirect plant defenses against insects and other serious pests such as the Dutch elm disease fungi.

Methods

Plants

All plants originated by propagating a single genotype of the European field elm, *U. campestris*, referred to as *U. campestris* cv. 'Dahlem', that originated from a forest 50 km east of Berlin, Germany. Shoots were maintained by monthly subculture on DKW propagation medium, which contained 1 mg dm^{-3} 6-benzylaminopurine (BAP; Sigma) and 0.01 mg dm^{-3} indole-3-butyric acid (IBA; Sigma) [99,100]. Rooted shoots were produced by transferring 3–5 cm shoots from the propagation medium (above) on DKW media containing 3 mg dm^{-3} IBA hormone and no BAP. After 3–5 days shoots were transferred into soil and grown in a climate chamber (22°C, 55% relative humidity (RH), 150–200 μmol m^{-2} s^{-1} PAR) under a 16 h /8 h light:dark (LD) photoperiod. To rear mature plants, shoots were transferred individually in plastic pots (11 × 11 × 12 cm) filled with potting soil (type T, Kausek GmbH, Germany). All experiments were conducted with 3–4-month-old elm plants with 15–20 leaves and a height of about 50 cm. Elms generated from this culture were found to retain their responses to the beetles [22].

Insects

Adults of *Xanthogaleruca luteola* (Coleoptera: Chrysomelidae) were collected in the environs of Montpellier and Perpignan (France) and in Palava (Spain). Adult beetles and hatching larvae were reared in the laboratory in cages (40 × 40 × 70 cm) on 'Dahlem' elm plants in the greenhouse (20–40°C, 40–50% RH, 150 μmol m^{-2} s^{-1} PAR) under a 16 / 8 h LD photoperiod. Pupae were transferred in transparent plastic boxes (20 × 20 × 6 cm) for hatching in the climate chamber (see above).

Treatments

Elm leaf samples were taken at three time points (3 h, 48 h and 72 h) after applying five different treatments (see below) since elms are known to respond to elm leaf

beetle infestation by releasing synomones attractive to egg parasitoids in this time scale [21,40]. For each time point and treatment, six replicate plants were harvested. For induction with *X. luteola*, 7–15 beetles were kept within micro perforate plastic bags (180 × 350 mm, Weber Packaging GmbH, Germany) on each treated elm plant. *Egg laying & feeding:* Female beetles were allowed to lay eggs and to feed (leaf material sampled at 48 h and 72 h after egg deposition). *Feeding*: Male beetles were used for feeding experiments (sampling at all time points), in order to exclude any possibility of egg laying in these samples. *Artificial scratching & eggs transferred:* To experimentally mimic the egg laying event by the beetle, leaves were scratched with a scalpel (thus mimicking removal of leaf epidermis by female beetles prior to egg deposition), and eggs were glued with oviduct secretion (which attaches the eggs to the leaves) to the wound (sampled at all time points). *Untreated control:* Intact elm plants with micro perforate plastic bags (sampled at all time points). *Methyl jasmonate:* Elm plants with undamaged leaves were sprayed with 50 ml each plant of an aqueous solution of methyl jasmonate (1 μmol / ml; Sigma, Germany; 95% pure) with 0.05% Tween 20 (for adhesion on leaves) to simulate insect attack (sampled at 24 h). To reduce contaminations by insect material all visible contaminations (eggs and feces) from the insects were removed thoroughly from the leaves with a fine brush.

RNA isolation and quality control

For isolation of total RNA, elm leaves were removed from stems of variously treated plants, flash frozen in liquid nitrogen and stored at -80°C. RNA was extracted by using a modified method developed for polysaccharide rich plant tissue [101] that employs repeated steps of phenol: chloroform:isoamyl alcohol (PCI; 25:24:1) extraction, and lithium chloride (LiCl) and ethanol precipitations over night. All glassware was treated with RNase AWAY® (Roth, Germany) and RNAse-free water. Plant material (0.5 g) was mixed with 10 ml lysis buffer (0.2 M Tris-HCL, 0.1 M LiCl, 5 mM Na_2EDTA adjusted to pH 8.2) to which 1% SDS, 0.01% ß-mercaptoethanol, 9% sodium acetate (2 M, pH 4) 10 ml phenol, 2 ml chloroform and 2% polyvinylpolypyrrolidone (PVPP) were added. The tubes were shaken (15 min, 250 rpm), then centrifuged (15,557 ×g, 4°C, 20 min), and the RNA was extracted three times with PCI. RNA was precipitated with LiCl (2 M final concentration) and collected in high speed 30 ml KIMBLE glass tubes (Kimble, Glass Inc., Vineland, NJ, USA) by centrifugation at 15,557 ×g for 60 min and finally precipitated with three volumes ethanol and 1/10 vol sodium acetate (3 M, pH 5.8) in 1.5 ml plastic tubes. For final purification and removal of genomic DNA, the RNeasy plant mini kit (Qiagen, Germany) including the on-column DNaseI treatment step was used. Aliquots of each purified RNA extract sample were prepared, and RNA concentration was determined spectrophotometrically at 280 and 260 nm. For final quality control and quantification, the total RNA samples were analyzed with an Agilent 2100 Bioanalyzer and Nano RNA 6000 chips (Agilent Technologies, Palo Alto, CA, USA) using the Expert Software (Agilent, version B.02.02.SI258). Total RNA extract samples were immediately frozen for long term storage as ethanol precipitates at −80°C.

cDNA library construction and 454 sequencing

For cDNA preparation, total RNA from six plant replicates and different time points of each of the respective treatments was pooled together. cDNA was synthesized using the SMART cDNA library construction kit (Clontech, Mountain View, CA, USA). First-strand cDNA was synthesized for each library from 0.5–1.0 μg of total RNA in a 10-μl reaction as described in the kit protocol using the SMART IV primer (AAGCAGTGGTATCAACGCA GAGTGGCCATTACGGCCGGG), a modified oligo(dT) primer (TAGAGACCGAGGCGGCCGACATGTTTTGT TTTTTTTTCTTTTTTTTTTVN), where V = A, G, or C and N = A, G, C, or T), and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Double-stranded cDNA was synthesized using the modified oligo (dT) primer and the SMART 5′ PCR primer (AAG CAGTGGTATCAACGCAGAGT) followed by a SfiI digestion as described in the SMART kit protocol. Amplified cDNA was purified using the QIAquick purification kit (Qiagen, Hilden, Germany). All column elutions for a specific library were pooled, and the relative cDNA concentration was estimated by running a 1% agarose gel electrophoresis with ethidium bromide staining and comparison to a standard molecular weight ladder. The first round of sequencing involved the use of equal amounts of all five libraries (EF, F, E, MeJA, and C) and ligating them to the 454 adapters as described in the original 454 paper [102]. The second round involved an individual mix containing 3.0 μg of each of the F and EF libraries. Sequencing was done using the GS 20 sequencer (454 Life Sciences, Branford, CT, USA) at the Michigan State University Research Technology Support Facility.

Bioinformatics: EST processing, assembling, and annotation

The 454 sequencing reads were processed and trimmed to remove low-quality sequence and primer sequences. The trimmed 361,196 high-quality ESTs were used for assembly by the PAVE (Program for Assembling and Viewing ESTs) software package, which incrementally builds unique transcripts (Unitrans) using Megablast for clustering and CAP3 for assembling ESTs [103]. For

annotation, sequences were blasted against the plant taxonomic database of UniProt, the full UniProt database (Swiss-Prot and TrEMBL) [104], and the non-redundant NCBI nucleotide database with an e-value threshold of 1e-20. The GO (gene ontology) trees were built using only UniProt annotations that were the best match for a Unitrans (E-value ≤ 1e-40) where at least 60% of the individual ESTs in the Unitrans also matched that protein with an E-Value ≤ 1e-10.

In silico analysis and comparisons of EST libraries

Cross-comparisons between the different libraries were done on the basis of EC numbers, GO categories, and UniProt identifiers. The library counts were normalized based on the library size and displayed as parts per 10,000 (pptt) and parts per 1,000 (ppt). ESTs used in the library counts were required to match the UniProt ID with an E-Value ≤ 1e-10, while their Unitrans were required to match with ≤ 1e-20. This ensures that UniProt IDs identified with high representation in a library are truly representative (i.e., that they align not just to Unitrans from the library, but to parts of the Unitrans containing reads from the library). Significant differences in relative transcript abundances between the GO categories were determined using Fisher's exact test. The R statistic (a log-likelihood ratio) was applied in order to detect differences in relative transcript abundances between the elm libraries. Thresholds with believability greater than 99% (i.e., false positive rate below 1%) were estimated for each library pair individually, using simulations as described in the original reference [42].

Enzymes (EC numbers) identified via Blast searches against the UniProt database (E-value ≤ 1e-40) over queries on the PAVE system were used to reconstruct pictorially biochemical pathway maps using the iPATH software, which can be accessed at http://pathways.embl.de.

Database web interface

The PAVE elm assembly is accessible through a web interface. It is possible to query the different elm libraries based on ESTs, Unitrans, UniProt IDs / descriptions [104], Protein Families (Pfam) [105], Enzyme Commission numbers (EC) [106] and Gene Ontology terms (GO) [107] without programming knowledge. BLAST searches [108] allow users to blast any sequence (nucleotide or protein) against the elm database. Individually calculated R values are part of the web database display. For further detailed descriptions see "PAVE Information" on the webpage (www.agcol.arizona.edu/pave/elm).

Sequence submission

The 361,196 EST sequences reported in this paper will be submitted to GenBank's Short Read Archive (http://trace.ncbi.nlm.nih.gov/Traces/sra/sra.cgi) under accession number SRA045857.

Additional files

Additional file 1: Figure A1. Size distribution of the unique transcripts (≥2 EST) (=contigs) derived from *Ulmus minor* assemblies.

Additional file 2: Figure A2. Number of unique transcripts (≥2 EST) (= contigs) derived from *Ulmus minor* assemblies by EST count.

Additional file 3: Figure A3. Number of ESTs derived from *Ulmus minor* assemblies sorted by open reading frame length (ORF; complete bases); contigs = unique transcripts (≥2 EST).

Additional file 4: Table A1: Most abundant gene products in *Ulmus minor* leaf EST database.

Additional file 5: Table A2. Distribution of annotated *Ulmus minor* unique transcripts according to the plant genus.

Additional file 6: Excel file of the library comparisons E vs EF vs C of GO category "defense response", sorted by R stat.

Additional file 7: Excel file of the library comparisons F vs C vs EF +F of GO category "defense response", sorted by R stat.

Additional file 8: Excel file of the library comparisons F vs EF vs MeJA of GO category "defense response", sorted by R stat.

Additional file 9: Ipath maps.

Additional file 10: Excel file of the Ipath EC query.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TM and TF conceived and designed the experiments in cooperation with KB, JG, and MH. TF and KB generated elm clonal material. KB carried out RNA extractions, plant treatments and provided the RNA samples. cDNA libraries were developed by EMD. DG coordinated sequencing. CS, AD and WN performed sequence alignment, assembling, annotation and database construction. Data were analyzed by KB with assistance of TM and DG. KB drafted the manuscript, and all authors contributed and approved the final manuscript.

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Additional file 1: Figure A1. Size distribution of the unique transcripts (≥2 EST) (=contigs) derived from *Ulmus minor* assemblies.

Additional file 2: Figure A2. Number of unique transcripts (≥2 EST) (=contigs) derived from *Ulmus minor* assemblies by EST count.

Additional file 3: Figure A3. Number of ESTs derived from *Ulmus minor* assemblies sorted by open reading frame length (ORF; complete bases); contigs = unique transcripts (≥2 EST).

Additional file 4: Table A1: Most abundant gene products in *Ulmus minor* leaf EST database.

Additional file 5: Table A2. Distribution of annotated *Ulmus minor* unique transcripts according to the plant genus.

Additional file 6: Excel file of the library comparisons E vs EF vs C of GO category "defense response", sorted by R stat.

Additional file 7: Excel file of the library comparisons F vs C vs EF +F of GO category "defense response", sorted by R stat.

Additional file 8: Excel file of the library comparisons F vs EF vs MeJA of GO category "defense response", sorted by R stat.

Additional file 9: Ipath maps.

Additional file 10: Excel file of the Ipath EC query.

Chapter 5

Elm defence: From a morphological over a chemical to a genomic perspective

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Abstract

Elms (*Ulmus* spp.) have long been appreciated for their environmental tolerance, landscape and ornamental value, as well as for the quality of their wood. Although elm trees are extremely hardy against abiotic stresses such as wind and pollution, they are still susceptible to attacks of biotic stressors. Over 100 phytopathogens and invertebrate pests are associated with elms: fungi, bacteria and insects like beetles and moths, and to a lesser extent aphids, mites, viruses and nematodes. While the biology of the pathogen and insect vector of the Dutch elm disease has been intensively studied, less attention has been paid so far to the defence mechanisms of elms to other biotic stressors. However, an in-depth understanding of the morphological, chemical and molecular mechanisms of the defence of elms to biotic stressors is essential for the development of sustainable integrated pest management strategies for these trees. This review highlights knowledge of direct and indirect elm defences against biotic stressors focusing on morphological, chemical and molecular aspects. Induced defence mechanisms are orchestrated through the interaction of a huge variety of genes and pathways. Future investigations should attempt to elucidate how molecular processes are regulated in elm defence.

Abbreviations: (DED) Dutch elm disease, (ELB) elm leaf beetle, (EY) elm yellows, (IR) induced resistance, (JA) jasmonic acid, (MeJA) methyl jasmonate, (PAL) phenylalanine-ammonia-lyase, (ROS) reactive oxygen species, (SA) salicylic acid

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1. Introduction

In many respects, elms are a large and important group of trees that have been closely associated with humans for at least 5000 years. Their timber has been used for agricultural equipment, ship building and furniture making, their leaves for fodder, and their crown as support for grape vines among many other uses of elm wood and bark (Richens 1983). As hardwood trees with rapid growth and large size, elms are greatly valued for their timber qualities and interesting wood grain and still used by the furniture industry for unique prints. Elms survive stressful conditions such as prolonged flooding even by sea water and exposure to most air pollutants. Because of their remarkable tolerance to a broad range of climates and soils and their majestic architecture, they were among the most widely planted urban ornamental and shade trees in Europe and North America until the mid 20th century. As very famous ornamental trees with an intricate relationship with human settlement, elms regularly feature in literature and visual arts (Richens 1983). In Germanic myths, Odin, Lodur and Hunir created the first man out of an ash and the first woman out of an elm (Heybroek 2002). The outbreak of the Dutch elm disease (DED) in the early 1900s, one of the most devastating tree diseases ever, decimated millions of elm trees worldwide. Huge international efforts have been undertaken since for elm conservation and breeding. Today, there is a revival of interest in the elm because of newly bred elm cultivars that may be resistant to the disease (Dunn 2000).

Elms are deciduous and semi-deciduous trees of the genus *Ulmus* L. (Ulmaceae). The genus originated in Asia and became abundant in Europe in Oligocene times about 40 million years ago. The genus distributed and established itself primarily in the northern temperate regions across North America, Europe and Asia, but also extended into subtropical parts of Central America and Southeast Asia. However, through cultivation elms now occur throughout the whole temperate world (Richens 1983). With approximately 45 species (Wiegrefe et al. 1994), elms are one of the world's major groups of tree species. Most elms naturally occur in Asia, where about 30 species are present. In North America about 10 species are distributed, and four or five species are distributed across Europe (Richens 1983). The taxonomic status of a few species of the European elms, among them in particular species within the group of the European field elm *U. minor* Mill. (also known as *U. carpinifolia* or *U. campestris*), has been the subject of many debates. The weak crossability barriers between different species result in the natural formation of so many hybrids and sub-hybrids that the conventional definition of a species in this context may not be sufficient. All elms are woody perennials with small

hermaphrodite flowers. Most species can grow up to 30 or 40 m, and life spans up to 400 years are known. Elm species are often distributed near riparian ecosystems, while others prefer mountainous areas. They usually co-occur with ash, oak and maple. In common with other riparian species, elms reproduce either asexually (from root suckers and the spontaneous rooting of broken branches) or sexually, resulting in the production of small winged seeds about 1cm across. Self-sterility is common in elms, and outcrossing is the rule. Most members of the genus are wind-pollinated, although bees do visit flowers (Dunn 2000; Schütt et al. 1995).

Fungi, bacteria, and insects like beetles and moths are the major biotic stressors of elms, with aphids, mites, viruses, nematodes and parasitic plants such as mistletoe, also having an effect (Stipes and Campana 1981; Richens 1983). Among hundreds of insect pests and diseases, three important elm-specific ones are known: (i) DED caused by two ascomycete fungi (*Ophiostoma* ssp.) which are vectored by elm bark beetles (*Scolytus* ssp.), (ii) the elm yellows (EY) caused by phytoplasms (Sticklen and Sherald 1993; Mittempergher 2000) and (ii) the elm leaf beetle (ELB). Elm species show a great variability in their morphological and physiological characteristics which render several species resistant to diseases like DED and EY and pest insects like ELB (Miller 2000).

Most of the current knowledge on the molecular mechanisms of plant defence against biotic stressors is based on studies of herbaceous plants. Although trees differ from herbaceous plants in their longer life times, larger sizes and their development of bark and wood caused by secondary growth, similar defence mechanisms have been found in trees and herbaceous plants such as the model plant species *Arabidopsis* (Eyles et al. 2009; Germain and Seguin 2011; Fineschi and Loreto 2012). A recent study showed that transcript levels of genes associated with essential defence functions hardly differ between herbaceous annuals and woody perennial plants (Quesada et al. 2008). Defence mechanisms unique to trees include all processes which involve the living cambium. One example is barrier zone formation as a non-specific defence mechanism against vascular pathogens, such as those described in detail for elms by Bonsen *et al.* (1985) and Rioux and Ouellette (1991a, b).

Current knowledge of tree defence against biotic stressors is dominated by the economically valuable pines and spruces used in forestry plantation and by fast-growing angiosperm trees including birch and the closely related poplars (Eyles et al. 2009; Haukioja 1990) (Ralph 2009; Novriyanti et al. 2010; Kolosova and Bohlmann 2012; Tuzun and Bent 2006). Elms are listed in previous reviews about tree defence in particular in the

context of morphological and chemical defence mechanisms against DED (Blanchette and Biggs 1992; Veluthakkal and Dasgupta 2010; Pearce 1996; Shigo 1984).

During the last decades, elm research concentrated in particular on the breeding of DED resistant elm hybrids, on elm conservation, and on DED pest management. Many international elm resistance breeding programs have developed disease-resistant elms with the most successful strategy using Asian elm species as the source of resistance genes (Mittempergher and Santini 2004). Now, a changing trend of pest and disease management in elms can be observed that leads away from sanitation and pesticide application towards enhancing induced resistance and biological control efforts (Scheffer et al. 2008; Hubbes 2004).

Many plant pathologists cited in this review generally use the term ‘resistance’ to refer to the protection from disease caused by biotic agents that activate the host plant`s physical or chemical barriers (Kloepper et al. 1992). In the context of this article the term resistance and inducible resistance (IR) refers to any mechanism that negatively affects the preference for (or performance on) the plant of an herbivore or pathogen (van Dam and Heil 2011; Karban and Baldwin 1997).

Knowledge about elm defences mainly arises from investigations of DED, EY and ELB resistant and susceptible elm species. Resistance to DED varies in elm species among continents from the highest level in species from Asia (*U. pumila*) to lower levels in species from North America (*U. americana*) and Europe (*U. minor*). Resistance against one pest can enhance susceptibility to another. Some of the Asian elm species with high levels of resistance to DED, EY, or ELB are susceptible to attack by the Japanese beetle and the gypsy moth (Bosu et al. 2007; Miller 2000; Mittempergher 2000; Paluch et al. 2006).

Distinguishing between morphological, chemical, molecular and genomic aspects of elm defence, this review focuses on biotic stressors of elm outside of DED research. We conclude with a summary and analysis of the recent advances in research on elm defence against biotic stressors and their role in pest and disease management.

2. Biotic stressors: Major pests and diseases of elms

Among hundreds of insect pest species and diseases associated with elms (reviewed in detail by Stipes and Campana 1981), DED, EY and ELB are the most serious elm specific ones. Further species and diseases mentioned in this review here are specified in this section (Fig. 1).

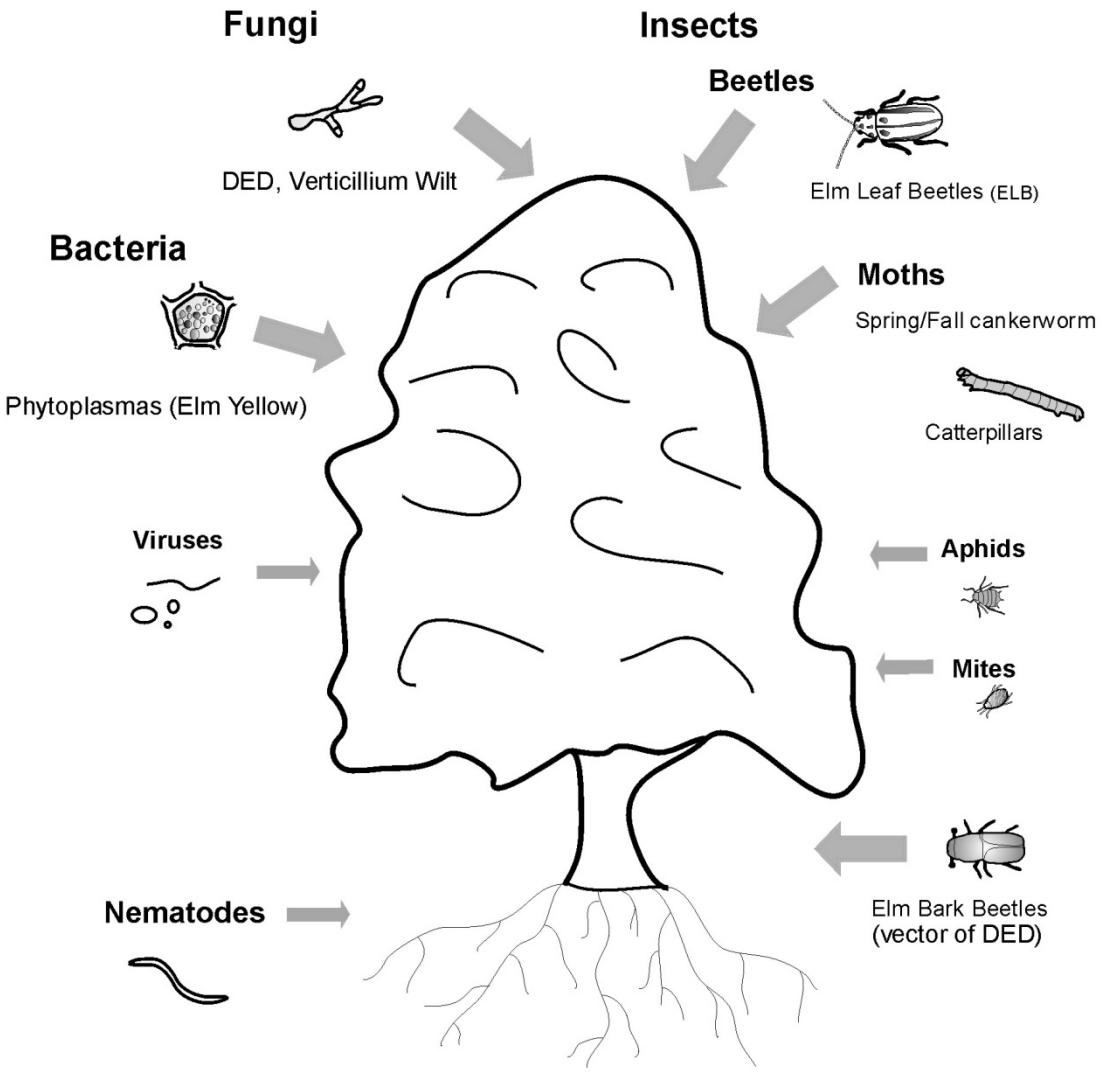

Figure 1. Overview of biotic stressors of *Ulmus* trees.

The DED pandemics, named after the first discovery in Holland, developed in the second half of the 20th century into one of the most devastating tree diseases ever. The DED caused by two ascomycete fungi (*Ophiostoma ulmi* (Buisman) Nannf. and the more aggressive strain *O. novo-ulmi* (Brasier) massively reduced European and North American elm populations during the past century. The vascular fungi, transmitted from diseased to healthy trees by *Scolytus* and *Hylurgopinus* bark beetle-vectors block the vascular system of the tree. The leaves wilt and cause death of the trees sometimes within as little as a few weeks (Sticklen and Sherald 1993).

Verticillium wilt is another fungus-caused wilt disease which is a common problem on elms in North America. The soilborn fungi *V. albo-atrum* or *V. dahlia* that are

responsible for this disease may not only affect elm, but numerous other herbaceous and woody plant species (Rauscher et al. 1974). Further infectious and worldwide distributed elm diseases include cankers caused by several fungi and the elm black leaf spot disease caused by the fungus *Gnomonia ulmea* (Stipes and Campana 1981).

Phytoplasms, which are parasitic phloem-restricted bacteria, cause the elm phloem necrosis, better known as EY, which is a very aggressive disease. Phytoplasms are spread by insect-vectors such as phloem-feeding Hemiptera, among them leafhopper, planthopper and psyllid species. Infection and death of the phloem result in an undersupply of water and nutrients and so kill the tree. EY is epidemic and lethal only to elms native to North America and is much less severe in the European elms (Mittempergher 2000, Sinclair et al. 2000).

Many chewing defoliator insects (e.g. beetles such as Chrysomelidae, Scarabaeidae or caterpillars of moths, and sawflies), leaf sap-sucking insects (bugs, leafhoppers, cicadas and aphids), and wood-boring insects (caterpillars of moths, beetles such as Scolytidae, Curculionidae or Cerambycidae) feed on elms worldwide. According to a list compiled in 1942, worldwide 585 insect species are associated with elm through feeding, breeding, ovipositing and hibernating (Stipes and Campana 1981). In the European forests, 106 insect pests are associated with the genus *Ulmus* L. Two-third of the pest species are beetles and moths (Klimetzek 1993). Elms can survive heavy infestation of beetles and moth caterpillars during one or even more seasons. However, biotic attacks such as these can weaken the elm's defence and render them more susceptible to other diseases.

Among beetle species specialised on elm, the most serious one - in addition to the DED-transmitting bark beetle vector species - is *Xanthogaleruca luteola* (Müller) (Coleoptera: Chrysomelidae), the ELB. The ELB was accidentally introduced to the USA and Australia and is there responsible for fatal defoliation of elms owing to the absence of any specialist predators and parasitoids. ELB adults feed holes into leaves of the same twig where they oviposit. In Europe the indigenous ELB are often heavily predated by the chalcidoid egg parasitoid wasp *Oomyzus gallerucae*, a species which can parasitise 50 to 90% of the eggs of an ELB population (Kielbaso and Kennedy 1983; Kwong and Field 1994; Dahlsten et al. 1994), so enabling elms to survive ELB infestation. The Japanese beetle, *Popillia japonica* (Newman) (Coleoptera: Scarabaeidae) is a general feeder on about 250 host plants, including elm. In Japan, where the beetle is native, it is controlled by natural predators, whereas in America it is a serious pest.

Among moth species that are pests of elm, larvae of the spring and fall cankerworm (*Paleacrita vernata* (Peck) and *Alsophila pometaria* (Harris); Lepidoptera: Geometridae) may attack elm, but can also feed on a variety of other trees. In North America cankerworms commonly appear as destructive populations. Similarly, the gypsy moth (*Lymantria dispar* (L.); Lepidoptera: Lymantriidae) is a major forest defoliator in North America and Europe. The caterpillars can completely defoliate an entire elm tree in one season (Stipes and Campana 1981).

Larvae of the sawfly *Fenusa ulmi* Sundevall (Hymenoptera: Tenthredinidae) are mining elm leaves; this elm leaf miner species is a common pest in America and Canada. The only other known elm leaf-mining sawfly is *Anafenusa shinoharai* (Smith and Altenhofer 2011).

In addition to elm infesting insect species, also mites can cause severe damage of elms. According to Weidhaas (1979), several spider mite species (at least eight) attack elm leaves and suck upon leaf cell contents. Leaf injury caused by spider mites usually leads to premature leaf fall.

Elms do not only need to cope with pest species living aboveground, but also need to defend against root feeders. More than 15 genera of nematodes are known to suck endo- or ectoparasiticly cell contents out of elm root tissue and thus reduce tree growth (Stipes and Campana 1981).

3. Morphological defence of elm

3.1 Constitutive morphological defence

Elms have evolved, like most plant species, a combination of constitutive and induced defence mechanisms. Physical barriers including bark, tough leaves and trichomes represent the first effective constitutive barrier of elms against insects and fungal pathogens (Lucas et al. 2000, Bosu and Wagner 2008).

The outer bark consists of mostly lignified and suberised cells. Furthermore the tendency of Ulmaceae to accumulate calcium carbonate and silicic acid crystals results in characteristic membrane incrustation of cell lumina (parenchyma) in the affected wood (cited in Hegnauer 1973 p. 545, 553).

Leaves of most elm species have bulbous glandular trichomes and hairlike non-glandular trichomes, similar to many other vascular plants (Bosu and Wagner 2007; 2008). Trichomes can contribute to plant defence in different ways. Non-glandular trichomes can

physically obstruct the movements of herbivorous arthropods over the plant surface or prevent herbivores from reaching the surface with their mouthparts. Glandular trichomes function as important chemical barriers against biotic factors like herbivory by the production and accumulation of terpenoids, flavonoids, sugars and defensive proteins (Glas et al. 2012; Tian et al. 2012). Nothing is known about chemicals in elm trichomes, whereas skin irritation (personal observation K. Büchel) and the taxonomic relationship of elm to other families in the *Urticales* such as the *Urticacae* strongly hint at the presence of secondary compounds in elm leaf trichomes. Future studies are recommended to investigate secondary compounds produced by elm leaf trichomes, and to evaluate their role in elm resistance against biotic stressors.

Higher trichome density on the foliage of elm species but not leaf toughness may be associated with reduced herbivory of the ELB (Miller and Ware 1999; Bosu and Wagner 2008). Dix *et al*. (1996) evaluated spring cankerworm (Lepidoptera: Geometridae) preferences for elm leaves with low trichome density. Leaf trichome density has been correlated with insect avoidance also in other trees (Soetens et al. 1991; Gange 1995). Further studies focused on the variation in leaf traits such as leaf water content, leaf protein content (Young and Hall 1986), (water stress induced changes in) trichome density and leaf nutritional quality (Bosu and Wagner 2007); all these parameters implicated to play a role in the resistance to ELB.

DED resistant trees differ from susceptible ones especially in the anatomical structure of the vascular system. Narrow vessels with small lumina restrict the pathogen dispersal and are more easily and quickly occluded by gums and tyloses which cause an early isolation of the infection (Sinclair et al. 1975; Solla and Gil 2002). A recent study on a Dutch elm hybrid species with a better tolerance to DED observed higher values in leaf traits including leaf dimensions, net photosynthetic rate and other vascular traits (Durkovic et al. 2013).

3.2 Inducible morphological defence

In general, the success of induced resistance (IR) in protecting a tree against pathogen attack depends on the genetic constitution of the tree, its health and environmental conditions (Hubbes 2004). The effectiveness of IR is dependent on the timely expression of the morphological and chemical resistance mechanisms causing incompatibility in host-pathogen interactions and isolating the pathogen in rapid time. Therefore the regulation of IR becomes a critical determinant of the effectiveness of plant defence.

Barrier zone formation is an important non-specific and inducible morphological defence mechanism that can prevent colonisation by most wood- and bark-inhabiting fungi and bacteria. This compartmentalisation of unique cells separates infected xylem tissue (sapwood) from healthy living cambium allowing formation of new healthy tissue. The zone is thus distinct from heartwood which mainly consists of dead xylem cells and protects living tissue from damage by the pathogen or diffusion of fungal toxins (Tippett and Shigo 1981; Shigo 1984). In *U. americana* barrier zones were formed of parenchyma cells and fibers in contrast to *Populus balmifera* (only fibers) and *Prunus pensylvanica* (only parenchyma cells).

When elm species resistant to DED are exposed to the DED-eliciting fungus, they form more axial parenchyma which is full of starch grains and enriched with polyphenolic compounds including lignin and suberin. This response has previously been described as tissue browning (Bonsen et al. 1985; Martin et al. 2005). During the maturation of cells in barrier zones much of the starch is replaced by the accumulation of polyphenolic compounds; these cells persist up to several years after their formation (Tippett and Shigo 1981). Both lignin and suberin represent efficient barriers against pathogens. Phenylpropanoids are known to re-enforce cell walls (Tuncel and Nergiz 1993; Mandal and Mitra 2007). They may act as protectors against cell wall degradation, as it was shown for *U. americana* (Jones et al. 2012). The fact that barrier zones produced after *O. ulmi* inoculation form later (after 22 days) in elms than in non-host species like *P. balmifera* and *P. pensylvanica* (after 10 days) and the fact that barrier zones form discontinuously in elms and continuously in the non-hosts may contribute to the elm's susceptibility to DED (Rioux and Ouellette 1991a; Rioux et al. 1995).

Infected elms form suberised tyloses that are distributed within or very near the barrier zones. DED-resistant elms are able to quickly and efficiently induce more tyloses than susceptible elms, so preventing the spread of *O. ulmi* by filling xylem vessels (Elgersma 1973). The structures of tyloses and their walls are well characterised and often include thick, inner suberised walls (mature tyloses) and pectic external layers (Rioux et al. 1995).

Vessel occlusion as a common response in plant defence represents a further mechanism of compartmentalisation against vascular pathogens and has been studied in DED infested elms in detail (Sticklen et al. 1991; Ouellette et al. 2004). Vessel occlusion is caused by pectic substances within the xylem of elm trees invaded by vascular pathogens, whereas several types of occlusion by tyloses and/ or deposition of mucilage in gels/gums

occur (Gardner et al. 1983; Rioux et al. 1998; Beckman 2000; Eynck et al. 2009; Rajput et al. 2009). In earlier studies no general consensus was reached as to the origin of the occlusion products. Often deposition of pectic substances, singly or mixed with further compounds such as lignins and suberin, proceeds occlusion.

The xylem vessel diameter plays an important role in the spread of the DED pathogens, the transport of toxins, and the tree´s ability to prevent colonisation by the fungal disease. The smaller diameters of xylem vessels are, the more resistant are elm species to DED. The rapidity with which compartmentalisation occurs, probably determines resistance. Vascular blocking slows down and becomes more difficult in large diameter vessels than in small diameter vessels (Elgersma 1970; Sinclair et al. 1975; Solla and Gil 2002; Venturas et al. 2013).

With respect to morphological defences induced by biotic stressors, pathogen-induced H_2O_2 production in plants is thought to play a role in cell wall reinforcing processes (lignification). Furthermore, infection-induced H_2O_2 production is involved in killing invading pathogens, in triggering programmed plant cell death during the hypersensitive response that restricts the spread of infection, and in inducing defence genes (Kuzniak and Urbanek 2000). *In vitro* bioassays demonstrate that H_2O_2 inhibits the growth of *O. novo-ulmi*, but the further role that H_2O_2 production plays in inducible elm defence responses is presently unknown (De Rafael et al. 2001). Oliviera et al. (2012) demonstrated *in vitro U. minor* plants that H_2O_2 production, membrane degradation in leaves and activity of major reactive oxygen species (ROS) and scavenging enzymes (catalase, peroxidase, superoxide dismutase) increased after inoculation with *O. novo-ulmi* subsp. *americana*. Peroxidases are known to be involved in the cell wall reinforcement during plant responses to pathogens; they are involved in polymerisation of saccharides and phenols which leads to stable vascular-occluding gels (Crews et al. 2003). The cambium region of both healthy and diseased elm trees shows very strong peroxidase activity, but in infected trees the activity was also found in fibers and vessels (Gagnon 1968). H_2O_2 is produced in elm mostly during the first day after infection, suggesting that the oxidative burst occurs early after infection as already described for other plant species. The function of ROS in plant defence against pathogens has been intensively studied (reviewed by Lamb and Dixon 1997).

Additional extraneous substances in vessel lumina have received much attention in later studies on DED infested elms and other plant species affected by other fungal wilt diseases. The so-called alveolar network with associated coating layers accumulating on

vessel walls was observed to be connected with fungal cells and to occasionally contain opaque matter. The compact coating and bands of opaque matter were clearly shown to be different from tyloses, did not label for chitin, cellulose or pectin, but DNA, which most likely originates from the pathogen. Therefore, recent studies have suggested a role for these coatings and opaque matter in pathogenesis rather than in plant defence, where it might play a role in the initial infection stages but also in recurrent infections at a time when host resistance mechanisms are ineffective. The fact that alveolar network rarely occurs in *U. pumila*, which is very resistant to DED, supports this suggestion (Ouellette et al. 2004; Ouellette et al. 2011).

To sum up knowledge on inducible morphological defence of elm against diseases, resistance to DED is associated with compartmentalisation of the fungal pathogen through barrier zone formation, vessel occlusion and H_2O_2 production. Yet studies demonstrating a direct effect of these inducible responses on disease development in DED infested elms are still needed.

4. Chemical defence of elm

The chemistry of the elm has been studied to a limited extent (Tab. 1). Most knowledge of biological activity of secondary compounds in elms originates from medicinal research. Yet, in comparison with other plant species, elms are remarkably poor in their content of medically important substances, and their leaves can be eaten without harm. The bark and leaf extracts of elms (e.g. *U. wallichiana* and *U. davidiana*) have long been used in oriental medicine to treat inflammation, edema, mastitis, and to accelerate fracture repair (Richens 1983; Schütt et al. 1995). The mucilaginous inner bark of slippery elm (*U. rubra*) has been used as a remedy in North America for centuries. It is the only elm pharmaceutical that has survived modern scrutiny and is produced commercially to treat throat irritation (Watts and Rousseau 2012). Recent studies have shown that elm glycoproteins may have anti-cancer and anti-aging properties, and that flavonoid-C-glucoside compounds display osteoprotective effects (Jung et al. 2007; Sharan et al. 2011; Hartmann et al. 2011; Kim et al. 2012).

Many secondary compounds are produced by elm, mainly including terpenes, phenolics, and alkaloids in the leaves, and triterpenes, phytosterols, free fatty acids and suberins with smaller amounts of glycoproteins in the bark. Furthermore, elm trees produce polysaccharide-containing mucilage in the bark (Beveridge et al. 1971; Paluch et al. 2006; Hartmann et al. 2011). However, there is a lack of knowledge as to which role these

compounds play in the defence of elms against biotic stressors. Prominent groups of chemicals known to be involved in elm defence are terpenoids (volatile terpenoids, mansonones and triterpenoids) and phenolics (lignans, scopoletin, flavonoids). These chemical defence metabolites can be constitutively synthesised in the bark and leaves, or can be induced by biotic stressors. Many elm secondary compounds act directly as toxins, repellents or anti-nutrients for herbivores, or as inhibitory substances against microbial infections, whereas others act indirectly as anti-herbivore devices *via* the attraction of predators or parasitoids of herbivorous insects.

4.1 Terpenoids

Terpenoids synthesised by the isoprenoid pathway are the most abundant and structurally diverse group of plant secondary metabolites (Cheng et al. 2007; Gershenzon and Dudareva 2007). In elms their ecological function was demonstrated in induced direct and indirect defence (see below).

Volatile terpenoids

Terpenoids are constitutively present in small amounts in the volatile bouquet of undamaged elm leaves, and the blend qualitatively and quantitatively differs from that of ELB-infested plants as shown for the field elm *U. minor*. Feeding-damaged elms are known to emit more than 40 compounds (Wegener et al. 2001) with a six-fold increase in the total amount of terpenoids (mono- and sesquiterpenes) and up to a 58-fold increase in the amount of the sesquiterpenoid (*E*)-*ß*-caryophyllene (Büchel et al. 2011). Little is known about the role of constitutively emitted terpenoids in elm, but the role of herbivore-induced terpenoids as volatile signal in indirect defence in elms is well-investigated (see section 5). Volatile terpenoids of *U. americana* wood including (-)-*ß*-pinene, (-)-*α*-cubebene, (+)-spiroaxa-5,7-diene and (+)-*δ*-cadinene were up-regulated after inoculation with *O. novo-ulmi* and are simultaneously attractive to the elm bark beetle *Hylurgopinus rufipes* (McLeod 2005; Byers et al. 1980). While nothing is known about the protective role of these volatile bark terpenoids, emitting these semiochemicals is detrimental to the tree, as they are attractive to the beetle vector of DED and so ultimately increase fungal infection (McLeod 2005).

Sesquiterpenoid phytoalexins

Different sesquiterpenes in elm are classed as phytoalexins, antimicrobial compounds which biosynthesis is induced in plants upon infection by phytopathogens. In common with other species of the Mavales, in *Ulmus* quinone sesquiterpenes were detected constitutively in the root and heartwood of *Ulmus*, and accumulated after stress induction in young wood.

The accumulation of mansonones as an integral component of IR against DED was first reported by Elgersma and Overeem (1970) in *U. hollandica*. Mansonones are a group of highly oxidised sesquiterpenoids, mainly *o*-quinones, that were originally isolated from the West African tree *Mansonia altissima* and have since been identified in many other plant species (Bettòlo et al. 1965; Chen et al. 1990). In elms different mansonones (A,C-I) were isolated from the sapwood of *U. americana* and *U. glabra* (Dumas et al. 1983; Burden and Kemp 1984), and other elm species in response to infection by *O. ulmi* (Elgersma and Overeem 1971; Duchesne et al. 1986). Their accumulation is correlated with resistance to aggressive strains of the fungus *O. ulmi* in susceptible *U. americana* L. after seedlings were first inoculated with a non-aggressive isolate of *O. ulmi* (Jeng et al. 1983; Duchesne et al. 1985; 1990). The effect of manosonones on the fungi includes inhibition of growth, ion leakage, cell wall disruption, aggregation of ribosomes, and the accumulation of electron-dense material in the mitochondria (Dumas et al. 1986; Wu et al. 1989). The antioxidative activities of elm mansonones in root bark of *U. davidiana* evaluated by measuring their inhibitory effect on lipid peroxidation of rat liver microsomes (Kim et al. 1996) may protect elm cells from the toxic effects of ROS which are often found in stressed tissues (Kuzniak and Urbanek 2000). However, treatment of elm cell suspension culture with exogenous H_2O_2 did not induce accumulation of mansonones (De Rafael et al. 2001). Mansonone accumulation is also associated with the formation of barrier zones (see section 3). Mansonone turnover rates in elms indicate theirrapid degradation and formation (Duchesne et al. 1986) with accumulation reaching its maximum mostly two weeks after fungal inoculation. Mansonone F accumulation was detected prior to fungal colonisation suggesting a remote signal induction (Nasmith et al. 2008a). The long term contribution of these phytoalexins to DED resistance was once a matter of controversy, presumably because of different experimental conditions. However, considerable evidence suggests that mansonones in elm act as phytoalexins and play a role in disease resistance (reviewed in detail Duchesne 1993).

Further quinone sesquiterpenes including cadalene- and 1,2,3,4-tretrahydrocadalene derivatives and lacinilene were also detected constitutively in elm heartwood (cited in Hegnauer 1990 p.658). Cadalene derivatives and lacinilene are characteristic wood components in the Section Madocarpus (*U. laciniata*, *U. glabra*, *U. carpinifolia*, *U. rubra*) (cited in Hegnauer 1973 p.584; Rowe et al. 1972). Inoculation of Wych elm (*U. glabra*) with the fungus *O. ulmi* induces accumulation of a series of antifungal cadalene derivatives like (-)-7-hydroxycalamenene and 7-hydroxycadalene (Burden and Kemp 1984). It is known that mansonones can easily be produced through oxidation of these compounds (Strunz et al. 1989). However, interestingly DED infested young twigs of *U. glabra* first produced the mansonones and later the related cadalene derivatives (cited in Hegnauer 1990 p.658). Cadalene derivatives very likely play a role as phytoalexins (or precursors) in elm defence, as demonstrated up to date only for *Gossypium* (cotton) spp. defence against phytophagous insects and phytopathogens (Essenberg et al. 1990; cited in Hegnauer 1989 p.146; Dubery and Slater 1997).

Triterpenes and sterols

Elm bark extracts are mainly composed of triterpenes and sterols (up to 60%), and biological activity was demonstrated in medicine where elm bark extracts had anticancer effects (Hartmann et al. 2011). Sterols play important roles in all plants as membrane components and hormones. One type of steroid with much more restricted taxonomic distribution, the phytoecdysteroids, mimics arthropod hormones and play a defensive role by disrupting moulting and other developmental and physiological processes with lethal consequences (Slama 1979). In elm, sterols including *ß*-sitosterol (Baker and Norris 1967; Dumas et al. 1983), stigmasterol and stigmastenone (Martin-Benito et al. 2005) have been identified in elm bark extracts, but few studies have related the sterol metabolism to plant - microbe interactions. The pathogen-inducible conversion of the known membrane sterol *ß*-sitosterol to stigmasterol has been shown to promote plant disease susceptibility (Griebel and Zeier 2010). The involvement of phytosterols in plant innate immunity against bacterial infections by restricting nutrient efflux into the apoplast has recently been demonstrated for *Nicotiana benthamiana* by Wang *et al*. (2012). In general, terpenoids in elms are considered to be major defence compounds against pathogens and herbivores even if knowledge about their role in direct defence is limited.

Many triterpenoids were detected in root or bark extracts of several elm species, among them the recently identified lupenol, alnulin, ilexol, moretenol and betulin (Martin

et al. 2004; Wang et al. 2006). Wegener (2002) identified several triterpenoids including *β*-amyrin, friedelin, and epifriedelinol in *U. minor* leaf extracts of leaves that had experienced ELB feeding or egg deposition, or treatment by jasmonic acid (JA). These substances were constitutively present and not enhanced by the treatments. They seem to play a role as toxins against herbivores as shown for *β*-amyrin and other triterpenes, and may act by compromising the digestion of essential sterols by herbivore insects (Gershenzon and Croteau 1991). In elms their significance as feeding stimulants or deterrents for the elm bark beetle *S. multistriatus* remains controversial. In *U. americana,* a pentacyclic triterpene serves as feeding stimulant for the elm bark beetle (Baker and Norris 1967). Martin-Benito *et al.* (2005) indicated an inverse relationship between the total triterpene content in the bark of elms and elm suitability for bark beetles. They identified various triterpenes and sterols among elm species. *ß*-Amyrin which showed high concentrations in some elm species including *U. laevis* and *U. glabra* (less preferred by bark beetles) was absent or present in only low concentrations in *U. minor* and *U. pumila* (preferred by bark beetles); it may be involved in deterring *Scolytus* beetles. Interestingly current year bark contains mainly aliphatic hydrocarbons, whereas 2-4-year-old bark contains mainly triterpenoids, which may result from adaptation of different stages of the tree to different attackers (Martin et al. 2004). Both compound groups are characteristic constituents of plant epicuticular waxes with important water repellent and protection functions (Baker 1982). The high triterpenoid content in birch (*Betula* sp) bark was implicated in resistance to mountain hare (*Lepus timidus*) feeding (Laitinen et al. 2004).

4.2 Phenolics

Phenolic compounds are synthesised *via* the phenylpropanoid pathway, and many compounds including flavonoids, lignans, tannins, and coumarins are a ubiquitous feature of inducible defence in woody species, although their exact role in plant defence remains unclear. The fact that the phenylpropanoid pathway is involved in elm resistance to DED is demonstrated by the increasing activity of the pathway`s key enzyme phenylalanine-ammonia-lyase (PAL) 42h - 72h after infection by the DED pathogen. In DED resistant *U. pumila*, but not in susceptible *U. campestris* suspension cultures, the pathogen induces a large increase in PAL activity (Corchete et al. 1993). Similarly, Nasmith *et al*. (2008b) reported higher PAL expression in leaf midribs of *U. americana* after inoculation with *O. novo-ulmi*. Expression of PAL was correlated with the accumulation of suberin, lignin and other phenolic compounds in *O. novo-ulmi* infected callus cultures of *U. americana*.

Inhibition of PAL reduces flavonoid content and decreases tissue browning in cultured elm tissue (Jones et al. 2012). Phenolics in *U. americana* accumulating after DED infection are mainly composed of catechins, the individual units that make up condensed tannins. Condensed tannins detected at the later stages of infection in callus tissues were proposed to possibly serve as building blocks in the synthesis of lignin-like molecules (Aoun et al. 2009). The phenolic polymer lignin plays a long-recognised role in disease resistance in higher plants (Vance et al. 1980). The importance of lignification and suberization in resistance against DED has also been noted for elms (see section 3). In addition, PAL participates in the production of phenylpropanoid-derived phytoalexins (see above) produced in plants in response to infection.

Scopoletin, a coumarin phenolic, is known as another phytoalexin in elms. Its induced accumulation in response to pathogen infection has been mainly investigated in several members of the Solanaceae family, but scopoletin has also been shown to possess antibacterial and antifungal properties in many other plant species (Gnonlonfin et al. 2012). DED resistant *U. pumila* cell cultures accumulate more scopoletin than DED susceptible *U. campestris* cultures. In *in-vitro* bioassays, scopoletin shows a direct antifungal activity against *O. ulmi* spore germination, but the role for scopoletin in limiting the spread of the pathogen in elm has yet to be demonstrated (Valle et al. 1997). De Rafael et al. (2001) detected differentially elicited scopoletin accumulation among various elm cultures. In *U. minor* leaves, scopoletin was found to be induced by ELB feeding and egg deposition, as well as by treatment with JA. In contrast to furanocumarins, scopoletin does not have the ability to intercalate into double stranded DNA and is considered to be more effective against generalist herbivorous insects than against specialists (Wegener 2002, Gnonlonfin et al. 2012).

Further phenolic compounds were isolated from wood of *U. thomasii* including the lignan thomasic acid with a content of 0.2%, but nothing is known about their role in plant defence (Seikel et al. 1968). In contrast to lignin, the structurally diverse lignans are not ubiquitously distributed in all higher terrestrial plants. Nevertheless, the wood of many tree species contains lignans, and those have been reported to be involved in constitutive and

Flavonoids

Numerous flavonoids including the flavonols, quercetin, kaempferol, rutin and myricetin, the anthocyanidins delphinidin and cyanidin, and various leucoanthocyanidins, catechins and condensed tannins were identified in elm species worldwide (Bate-Smith and Richens

1973; Hegnauer 1973). Flavonoid identification in *Ulmus* was proposed for chemosystematic classification of the genus to distinguish artificial or natural interspecific hybrids. Early investigations identified glycosides of quercetin as major compounds (Santamour 1972). Subsequent investigations identified more than 30 foliar flavonoids in six North American elm species, whereby American elms comprise two distinct groups, one that produces the two flavonols, kaempferol and quercetin, and one that produces myricetin in addition (Sherman and Giannasi 1988).

In elms (and in other plants) the most investigated and abundant flavonol is quercetin. The role of quercetin in plant defence ranges from a beneficial antioxidant scavenging ROS to a damaging prooxidant depending on concentration and free radical source. The pro-oxidant quercetin develops its toxicity after its metabolic activation to quinoidal radicals and contributes to pathogen resistance *via* H_2O_2 burst (Metodiewa et al. 1999; Jia et al. 2010). In rapid IR of silver birch (*Betula pendula*) lipophilic flavonoids increase after feeding by gypsy moth larvae, while several glycosides of quercetin decrease (Martemyanov et al. 2012).

Flavonoids are generally considered to contribute to resistance against pathogens. In Scots pine (*Pinus sylvestris*) flavonoids occur constitutively in phloem tissues and have been related to reaction efficiency against the bark beetle associated fungi. The low molecular phenolic flavonoid (+)-catechin and the phenolic chlorogenic acid were demonstrated as constitutively present compounds of *Salix* spp. and *Picea* ssp. with a suggested role in resistance against pathogens (Witzell and Martin 2008). Despite the identification of many flavonoids, there is a lack of studies on elms demonstrating a direct role of flavonoids in plant defence.

Among the flavonoids the class of condensed tannins represents the most abundant secondary metabolites typically found in woody plants. Tannins can defend leaves against insectsby deterrence and toxicity, and their induction by herbivory has been reported for several tree species. Tannins are often referred to as anti-digestive protein-binding agents, but there is a lack of studies on herbivorous insects demonstrating the ability of tannins to decrease protein utilization. More recent studies supposed that the deterring and toxic activity of tannins towards insects is due to oxidative stress caused by auto-oxidation or enzymatic oxidation of tannins. Such effects depend especially on the interaction between the plant-specific tannin and specific pH conditions in different parts of the digestive tract of the herbivore species (Salminen and Karonen 2011; Barbehenn and Constabel 2011). Osier and Lindroth (2001) showed that in *P. tremuloides* phenolic glycosides rather than

condensed tannins act as constitutively present defensive compounds against the gypsy moth. *Ulmus* species contain mainly condensed tannins instead of hydrolysable tannins. European elm leaves contain more tannins than the twigs, and tannins may constitute over 4% of their dry mass (cited in Hegnauer 1973 p.547). In Alaska paper birch (*Betula resinifera*) condensed tannins were shown to significantly contribute to delayed induced resistance. Previous defoliation of these birch trees prepares the plant for future attack; defence mechanisms of previously defoliated trees were induced more rapidly and more strongly by subsequent herbivore attack (Bryant et al. 1993).

4.3 Mucilage

Mucilage is one important biochemical component typically present in the inner bark of slippery elm *U. fulva* (*U. rubra*), but is also present in leaves and bark of other elm species (Anderson 1934; Gill et al. 1946; Hough et al. 1950; citations in Hegnauer 1973 p.546). The inner bark of *U. fulva* contains around 7% mucilage, mainly composed of galactose, rhamnose, galacturonic acid and 3-O-methylgalactose (Beveridge et al. 1971). The polymeric nature of mucilage is composed of polar glycoprotein and dense polysaccharide coatings, which provides its characteristic viscosity and gelling properties (Watts and Rousseau 2012). These pectic polysaccharides are produced by many plants in different organs such as roots, seeds, foliar and inner bark in high concentrations and are assumed to play a role in water and food storage and seed germination (Yang et al. 2012; Malviya et al. 2011). Its role in wound responses and plant defence against pathogens and parasitic plants has also been demonstrated for *Zea mays* and *Vicia sativa* (Crews et al. 2003; Pérez-de-Luque et al. 2006). However, in elm species, vessel occlusion by such pectic substrates is a common response and seems to improve elm resistance to wilt disease by limiting their spread through the tree´s vascular system (see section 3).

4.4 Alkaloids

Alkaloids are toxic defensive compounds to herbivorous vertebrates as well as to arthropods, having no role in primary plant metabolism. However, the Ulmaceae do not belong to the alkaloid-rich plant families such as the Solanaceae or Papaveraceae where the alkaloid synthesis is a central part of the chemical defence (Mithöfer and Boland 2012). There are no confirmed reports of alkaloids in Ulmaceae although alkaloids were mentioned for *U. pumila* (cited in Hegnauer 1973 p.552). Nevertheless, there may be other yet-to-be discovered classes of defence compounds in elms. Paluch *et al*. (2006) analysed

leaf extracts of Asian elm species for differences in lipid, phenolic, and terpene diversity to link with susceptibility to Japanese beetle feeding damage. Asian elm species (closely associated to the *U. davidiana* complex), which are known to be more resistant to DED, EY and the elm leaf miner (Miller 2000), show a larger diversity of leaf chemicals than other elm species. However, compound diversity may not necessarily be an advantage, because elms with greater levels of leaf lipids are more susceptible to infestation by Japanese beetles and gypsy moths (Paluch 2006).

5. Chemical ecology: Indirect defence of elm

Elms have played a prominent role in research on indirect defence against insect eggs. The first study demonstrating indirect defence against insect eggs was on the European field elm (*U. minor*), where egg deposition by the ELB induced volatiles that attract the egg parasitoid *O. gallerucae,* a wasp specialised on ELB eggs (Meiners and Hilker 1997). During the past two decades knowledge about indirect defence strategies of plants has grown continuously. Most studies concentrated on indirect plant defence *via* the emission of plant volatiles - so-called synomones - that are induced by feeding activity of herbivorous arthropods and attract predators or parasitoids of the herbivores (Dicke and Sabelis 1988, Arimura et al. 2009; Dicke and Baldwin 2010). Yet, more and more studies have revealed that also egg deposition by herbivorous insects induces indirect plant defence. This has been shown both for trees (elm, pine) and herbaceous crops (e.g. bean, cabbage) (reviewed by Hilker and Meiners 2010; 2011). It is supposed that the elm – ELB – *O. gallerucae* tritrophic system co-evolved during a long time period because of high species specificity of the elm´s defence response and the close relationship of the leaf beetle, its egg parasitoid and the tree. Neither ELB egg deposition on the leaves of the mountain elm (*U. glabra*), nor egg deposition by the related leaf beetle *Galeruca tanaceti L*. on field elm resulted in the emission of synomones that were attractive to *O. gallerucae*. Only ELB eggs laid on field elm induced the emission of leaf volatiles that were attractive to the egg parasitic (Meiners et al. 2000). Further indication of the plant and herbivore specificity of these interactions is the strong feeding and egg laying preference of ELB for *Ulmus* spp. and their hybrids (Miller and Ware 1994). Prior to egg deposition, female beetles scratch the lower leaf surface by gnawing shallow grooves in the epidermis and then glue eggs in place with an oviduct secretion into those grooves. It is important for the induction of the indirect defence reaction that the elicitor which induces the elm´s response to eggs contacts the cells exposed by the epidermal scratching. The elicitor itself is most

likely a proteinaceous compound present in the oviduct secretion. The ovipositional wounding of the leaf surface prior to egg deposition or artificial application of oviduct secretion onto an undamaged leaf *per se* do not cause the release of the attractive volatiles from elm leaves (Meiners et al. 2000; Hilker and Meiners 2011). Treatment with JA or methyl jasmonate (MeJA) also elicited the emission of attractive volatiles in field elms, but the volatile patterns differed quantitatively and qualitatively from those of elms induced by egg deposition and beetle feeding activity (Wegener et al. 2001; Meiners T. unpublished data).

The induction of elm leaf volatiles attractive to egg parasitoids was demonstrated on a time scale of a few hours after egg deposition for up to five days later. This time period exactly matches the development time of the eggs (Hilker and Meiners 2006; and unpublished data). Furthermore, induction of leaf volatiles mediated by ELB egg deposition was shown to occur locally at leaves with eggs and systemically atleaves that were egg-free, but adjacent to the leaves with eggs. The systemic signal extended acropetally along the elm tree to a height of at least 2m above the egg-infested leaves (Meiners and Hilker 2000; Tillmann and Meiners unpublished data).

The blend of egg-induced elm leaf volatiles attracting egg parasitoids consisted mainly of GLVs including (*Z*)-3-hexenyl acetate and terpenoids like (*E*,*E*)-*α*-farnesene, (*E*)-*β*-caryophyllene and (*E*)-4,8-dimethyl-1,3,7-nonatriene. These substances were attractive to the egg parasitoids both in lab and in field studies and therefore probably play a crucial role in indirect elm defence responses (Wegener et al. 2001; Büchel et al. 2011; Büchel et al. 2012). These terpenoids are ubiquitous compounds in most blends of higher plants and have been found to play a significant role in indirect defence also in other tritrophic systems (Colazza et al. 2004; Vet and Dicke 1992).

In the USA and Australia where no *O. gallerucae* are present, elms are sometimes almost completely defoliated by the ELB indicating how strongly these trees can benefit from indirect defence *via* infestation-induced volatile emissions. Attraction of the egg parasitoids reduces the future number of larvae that would further damage the plant.

Little is known in elms about the costs of defence. The production of volatile terpenoids for indirect defence of field elms against ELB egg deposition seems to proceed without major photosynthetic costs since no difference in photosynthetic activity was observed when field elms were induced by egg deposition (Austel and Meiners unpublished data). However, in other tree and crop species it was shown that egg-laden plants showed reduced photosynthetic activity (Schroeder et al. 2005; Velikova et al.

2010). Interestingly transcription of photosynthesis-related genes in elm was also reduced after insect egg deposition (Büchel et al. 2011). However, the expression of defence traits in response to herbivore attack requires major changes both in primary and secondary metabolism, and plants invest a large amount of resources to produce volatile isoprenoids for defence against biotic stressors (Schwachtje and Baldwin 2008; Fineschi and Loreto 2012; Gershenzon 1994).

6. Molecular and genomic aspects of elm defence

During the last decade a rapid advancement in our understanding of the "molecular aspects" of plant defences has taken place. Numerous studies have addressed the molecular and physiological processes that trigger plant responses to biotic stressors such as herbivorous insects or pathogens (reveiwed e.g. by Schaller 2008; Smith and Clement 2012; Mithöfer and Boland 2012; van Loon 2009; Robert-Seilaniantz et al. 2011). Most molecular research on elm has been performed in relation to the DED pathogen, including the elicitors that induce host defence, the characteristics of the fungal strain and its population dynamics (Sticklen and Sherald 1993). Induction of resistance by the injection of fungal elicitors is viewed as a new biological approach against DED, although there is little knowledge about the molecular processes behind it.

While genetic transformation of elms was first developed more than 20 years ago (Bolyard et al. 1991; Fenning et al. 1996; Gartland et al. 2000a), molecular investigations of the defence mechanisms of elms, the genes and pathways involved, started only a few years ago (Tab. 2). There are only two studies of transgenic elms encoding antimicrobial peptides for enhanced resistance against DED (Gartland et al. 2005; Newhouse et al. 2007). The time consuming method of genetic transformation could be the reason why genetically modified elms have not yet been employed in research on elm defence. Many studies have noted that elms have proved to be problematic for molecular work, due to the release of mucilaginous compounds that impede DNA or RNA isolation and downstream analysis (noted in Loureiro et al. 2007; Büchel et al. 2011; Nasmith et al. 2008a; b).

The first transcript expression analysis of *Ulmus* stress-related genes showed increased expression of PAL, chitinase, and polygalacturonase inhibiting protein (PGIP) during DED disease development in leaf midrib, root and bark of DED-resistant *U. pumila* in comparison to DED-susceptible *U. americana*. These three genes are supposed to act in DED resistance (Nasmith et al. 2008a, b). PAL is involved in phytoalexin, lignin and

flavonoid synthesis, while PGIPs inhibit fungal polygalacturonases and as a consequence reduce fungal damage to the cell wall (De Lorenzo et al. 2001).

The availability of the first tree genome to be sequenced (*Populus trichocarpa*) has enabled efforts to identify genes and pathways involved in angiosperm tree defence (Tuskan et al. 2006; Muchero et al. 2013 in press). In the meantime "next generation sequencing" has allowed the publication of an increasing number of other tree genomic sequences, mainly those of commercial fruit trees, including apple, *Eucalyptus*, peach, papaya, cacao, citrus (Xu et al. 2013; Argout et al. 2011; Ming et al. 2012; Arus et al. 2012; Myburg et al. 2011; Velasco et al. 2010).

Despite their high economic importance prior to DED and the massive reduction of elms by DED, only two large scale gene expression studies are known for elm. In one study of elms, using tissue cultures of *U. americana* inoculated with *O. novo-ulmi*, 314 unique transcripts were identified. After differential screening and RT-qPRC analyses, transcripts connected to the phenylpropanoid pathway, the compartmentalisation process, and phytoalexin production were shown to be up-regulated in response to fungal infection (Aoun et al. 2010). Another, much larger EST database containing information on 52,823 unique transcripts from the leaves of the field elm (*U. minor*), represents the largest genome resource for the elms to date. Comparative *in silicio* analysis among different treatments including MeJA treatment, ELB feeding and egg laying, ELB feeding only and ELB eggs only (by artificial transfer of egg clutches), revealed increased abundance of defence- and stress-related elm gene transcripts after egg laying and feeding of the ELB. Many further transcripts with a potential relevance in egg-induced defences involved in processes like signal transduction, transport, and primary metabolism were detected (Büchel et al. 2012).

General and classic assumptions that plant resistance to herbivore attack is principally determined by its secondary metabolism have already been overtaken through newer transcriptomic and proteomic studies. Of the hundreds of genes regulated during the plant - herbivore or plant - pathogen interaction substantial involvement of the primary metabolism was demonstrated for several plant species (Schwachtje and Baldwin 2008). A preliminary first proteomic study on field elms demonstrated that levels of elm leaf proteins involved in primary metabolism increase after ELB feeding or after egg deposition accompanied by feeding activity. Putative proteins with increased quantities in *U. minor* leaves after these treatments were involved in energy metabolism (succinyl CoA-ligase), sugar- and amino acid metabolism (UDP-glucose-dehydrogenase (UGDH), arginase), and

synthesis of the phytohormone ethylene (S-adenosylmethionin synthase) (Büchel unpublished data). All these proteins are closely associated with defence mechanisms, e.g. through enhanced cell wall biosynthesis (UGDH,Karkonen et al. 2005) and enhanced amino acid metabolism activity, which was demonstrated to enhance resistance against necrotrophic fungal pathogens (arginase, Brauc et al. 2012).

Here, we highlight those sequences of the above-mentioned two large-scale elm gene expression studies which were upregulated in high abundance in response to ELB infestation or DED, i.e. elevated levels of transcripts encoding enzymes belonging to different branches of the phenylpropanoid and shikimate pathways, and to different classes of pathogen-related (PR) proteins, proteinase inhibitors (PI), and proteins involved in phytohormone signalling (Tab. 2). In particular, PR proteins seem to be a prominent feature of the defence profile of elms inducible by ELB and DED, among them PR 1-3, 6, peroxidases (PR9) and PR 10 proteins. PR genes and proteins are known to be involved in host – pathogen interactions in many tree species. PR 1 genes were induced by DED (and by other pathogens or salicylic acid (SA) treatment), but the mode of action of PR 1 proteins towards DED is unknown (Veluthakkal and Dasgupta 2010). Chitinases (PR 3) transcripts were among the most up-regulated transcripts in field elm after ELB feeding and were induced at a similar point in time (48 to 72 h) after inoculation with the DED fungus. Chitinases play a direct role in plant defence by hydrolyzing chitin and degrading microbial cell wall components, often coordinated with the induction of glucan endo-1,3-*ß*-glucosidases (PR 2). Transformation of an elm chitinase gene of resistant *U. americana* into bentgrass (*Agrostis palustris*) causes disease resistance in the transformed plant against the brown patch fungus *Rhizoctonia solani* (Chai et al. 2002). Transcripts encoding genes of a Kunitz-like proteinase inhibitor (PR 6) were strongly induced in DED infected elm calli. Further upregulated sequences had sequence similarity to genes coding for proteinase inhibitor I (PR 6). This protease inhibitor participates in defence mechanisms of plants against herbivorous insects and pathogens. Further DED upregulated sequences showed sequence similarity to genes coding for S-norcoclaurine synthase (PR 10), which catalyses the first committed step in the biosynthesis of benzylisoquinoline alkaloids, a large and diverse group of secondary metabolites found in several plant families. Transcript abundance of major latex protein proteins (PR 10) were strongly induced by ELB egg-laying. Although PR 10 proteins were induced by both biotic stressors (ELB and DED) in various plant tissues, the biological function remains to be elucidated.

Increased transcripts of compartmentalisation-associated proteins are consistent with the high accumulation of the respective proteins in compartmentalisation processes in DED infected elms (see section 3). Sieve element occluding proteins upregulated in ELB infested elm are possibly involved in sieve cell occlusion after wounding. In DED infested elms, transcripts had sequence similarity to genes coding for proteins that may be involved in the production of isoflavonoids (isoflavone reductase-like protein), anthocyanin pigments (O-methyltransferase), and lignans (phenylcoumaran benzylic ether reductase), which could also be associated with the compartmentalisation process against pathogens. Phenylcoumaran benzylic ether reductase is the most abundant protein in the secondary xylem of *P. trichocarpa*, strongly associated with phenylpropanoid biosynthesis in lignifying cells (Vander Mijnsbrugge et al. 2000). Yet, genes directly involved in lignin and suberin biosynthesis were neither up-regulated in response to DED nor to ELB infestation (Aoun et al. 2010)

Further defence- and stress-related transcripts which were present in high abundance in leaves after ELB egg laying coded for a key enzyme involved in JA synthesis (LOX = lipoxygenase), and proteins involved in JA, SA and auxin signalling (JAZ = Jasmonate ZIM-domain protein; NPR1 = non-expresser of PR genes; auxin signalling F-box 2). These proteins are intimately associated with plant defence or disease development. A phospholipase protease (patatin-like protein) known to be involved in oxylipin biosynthesis contributed in *Arabidopsis* mutants to plant cell death and pathogen resistance (La Camera et al. 2009). Almost all of the elm transcripts that were upregulated in response to DED and reported to have sequence similarities to defence related proteins (Aoun *et al.* 2012) were also found in the much larger database on *U. minor* induced by ELB activity (Büchel *et al*. 2012). It is a challenging future task to elucidate how the expression of genes encoding these defence related proteins is regulated and how protein activity is mediated in response to DED and ELB attack.

7. Use of knowledge about induced resistance for control of elm diseases

IR in elms was shown for the first time in 1980 in *U. hollandica.* Mixed inoculation with a non-aggressive pathogen strain followed by the aggressive one results in fewer symptoms than inoculation with the aggressive strain alone (Scheffer et al. 1980). Efforts to use IR for DED control by inoculating trees first with a strain of *O. ulmi*, (earlier by using a glycoprotein elicitor of the pathogen, later by isolating the vascular wilt pathogen *Verticillium albo-atrum*) are well documented and not part of this review (Scheffer et al.

2008, Sutherland et al. 1995; Jeng et al. 1983). IR has been studied more and more in woody plant species (Hubbes 2004; Haukioja 1990; Eyles et al. 2009; Haukioja 2006).

Application of plant hormones as elicitors of IR signalling has been tested in several tree species, with JA and MeJA being the most widely used. For elms it was demonstrated that such phytohormone treatment induces defence against insect attack. Treatment of ELB-infested elms with JA or MeJA elicited indirect defence responses in field elms by stimulating the emission of parasitoid-attracting elm leaf odour (Meiners and Hilker 2000; Wegener et al. 2001; Meiners T. unpublished data). These results indicate that the octadecanoid pathway is also involved in IR. How JA-mediated signalling leads to the activation of specific genes involved in induced leaf odour production is still unknown for elms. After ELB infestation, transcripts encoding JA biosynthesis enzymes were expressed (see section 6), and the endogenous content of JA increased from 0.2 nmol per gram fresh weight (gFG) in untreated leaves to 1.5 nmol/gFG in leaves which had been fed on by beetles and to 5 nmol/gFG in leaves which have been fed upon and had eggs laid upon them by ELB (Meiners T. unpublished data).

Phytohormone treatment of elms also induces resistance against DED. A recent study has evaluated the role of MeJA applied by spraying for inducing resistance in young *U. minor* against *O. novo-ulmi*. No significant effect of this MeJA treatment on DED development was found. Two possible reasons are discussed for this finding: (i) the spread of the pathogen within the tree tissues was faster than the formation of effective defence responses; (2) the plants were too young to activate sufficiently effective resistance mechanisms (Vivas et al. 2012).

SA plays a crucial role in the induction of systemic acquired resistance, a type of IR against microorganisms which is associated with the accumulation of pathogenesis-related proteins that contribute to resistance (Beckers and Spoel 2006). Exogenous applications of SA to *U. minor* successfully enhanced the resistance to the fungal pathogen *O. novo-ulmi* (Martin et al. 2010) and altered the chemical composition of the xylem tissues in elms by accumulation of sinapyl alcohol, a precursor of lignin and other phenylpropanoid-derived products (Martín et al. 2012).

The effectiveness of IR is dependent on the timely expression of the morphological, chemical and molecular resistance mechanisms causing incompatibility in host-pathogen interactions. Therefore the regulation of IR mechanisms becomes a critical determinant of the effectiveness of plant defence.

8. Outlook for further research on elm defence against biotic stressors

Overall, the susceptibility or resistance of elms to major biotic stressors (including fungi, bacteria and herbivorous insects like beetles and moths) has been ascribed to morphological, chemical and molecular traits. Most research concentrated on morphological defence mechanisms, such as barrier formation and vessel occlusion, which prevent colonisation by wood- and bark-inhabiting fungi and bacteria. Chemical defensive metabolites of the classes of terpenes and phenolics are also known to be involved both in constitutive and induced defence mechanisms of elms. However, there is a lack of knowledge on the role of other compounds in the defence of elms against biotic stressors. IR mechanisms of elm species, which have been documented to include morphological and chemical changes, are orchestrated through the interaction of a variety of genes and phytohormone pathways.

Although elm indirect defences against insect eggs *via* egg-induced leaf odour that attracts egg parasitoids have been studied in detail on the chemical and ecological side, little is known on the molecular part of this tritrophic interaction between elm, herbivorous insect and its egg parasitoid. Molecular investigations of defence mechanisms of elms, the genes and the pathways involved only started a few years ago. A multitude of defence- and stress-related transcripts are associated with defence mechanism of elms against DED or ELB, and it remains to be examined how the different genes are regulated in concert in elm defence.

Today, there is a revival of interest in elms. More elms than previously assumed survived DED, and new DED resistant elm hybrid cultivars, which are the result of crosses with resistant Asian species, have been released on the market. However, their value as a replacement for native elm species has yet to be proved. In recent decades, scientists working on plant - pathogen interactions have become increasingly interested in elm species. The enormous damage caused by the DED pandemics has led to the development of diverse international initiatives for conservation of the genetic elm resource and breeding programs in Spain, Italy, The Netherlands and USA. However, no effective means have been developed so far that can successfully prevent or control DED on a practical scale. Genetic transformation is considered as an alternative to conventional breeding by enabling the introduction of genes that might confer resistance to elm clones (Gartland et al. 2000b). Due to cost-intensive implementation and a strong public opinion against genetically modified trees in Europe and other parts of the world, it is unlikely that transformed elms will be released in the next few years. IR could be a valuable tool in

sustainable pest management and biological control. Whereas injection of pathogens into single trees for formation of IR is already in use, this is not a practical approach for dealing with such a widespread tree disease such as DED (Scheffer et al. 2008; Hubbes 2004).

For many people the beauty of elms is the first reason why they are motivated to improve elm defence against pests. The revival of interest in elms may also increase their economic importance allowing more resources to be devoted to molecular elm research or combining molecular genetics with traditional breeding (Warren 2000; Heybroek 1993; Guries and Smalley 2000). Before the devastation wrought against elm species worldwide by DED, elms were ideal trees for our urban environments with remarkably few pest and disease problems. It is therefore a worthwhile objective to attempt to bring back such a magnificent group of trees into our cities and their surroundings by focusing on tree defence research.

Table 1. Compounds involved in elm´s (*Ulmus ssp.*) chemical defence or susceptibility to biotic stressors. (For details, see text Section 4).

Compound(s)	Effect of stressor and proposed role in defence	Biotic stressor	Elm species[1]	Source	References[1]
Terpenoids and derivatives					
Mansonones	Inhibitory effect on fungal growth, increased amounts after infection	DED[2]	*U. hollandica, U. americana*	Sapwood	Elgersma & Overeem 1970; Duchesne et al. 1985; Wu et al. 1989
Cadalene derivates	Induced antimicrobial activity	DED	*U. glabra*	Sapwood	Burden & Kemp 1984
Triterpenoids	Proposed activity as constitutive feeding deterrents against ELB[3]	ELB	*U. laevis*, *U. glabra*	Root, bark, leaves	Martin et al. 2004; Martin-Benito 2005
Sterols[4] (e.g. stigmasterol)	Induced accumulation promotes plant disease susceptibility	Pathogen	*U. americana*	Bark	Baker & Norris 1967; Wang et al. 2012
Volatile terpenoids (e.g. (*E*)-ß-caryophyllene)	Induced emission attracts egg parasitoids	Eggs of ELB	*U. minor*	Leaves	Meiners & Hilker 1997; Büchel et al. 2011
Phenolics and derivatives					
Lignin	Barrier zone formation, induced after infection	DED	*U. minor*	Parenchyma cells, twigs	Martin et al. 2005
Scopoletin	Induced and antimicrobial activity	DED	*U. pumila*, *U. minor*	Cell cultures, leaves	Valle et al. 1997; Wegener 2002

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Table 1. Compounds involved in elm´s (*Ulmus ssp.*) chemical defence or susceptibility to biotic stressors. (continued)

Flavonoids and other phenolics (e.g. quercetin, chlorogenic acid, (+)-catechin) [4]	Induced and constitutive antioxidants, deterrents and toxins in plant-pathogen/ herbivore interactions	Pathogen, herbivore	*Ulmus ssp.*	Leaves	Sherman & Giannasi 1988; Witzell & Martin 2008; Barbehenn & Constabel 2011
Other					
Polysaccharides[4] (Mucilage, pectic substrates)	Induction prevents the spread of the fungi by triggering the formation of vessel occlusions	DED, injuries, or infections	*U. americana*	Twigs	Rioux et al. 1998

[1] Examples; [2] DED, Dutch Elm Disease (*Ophiostoma ulmi* or *O. novo-ulmi*), [3] ELB, elm leaf beetle (*Xanthogaleruca luteola*), [4] Compound present in the described elm species, effect described in other plant species

Table 2. Genes with an indicated function in the defence response of elms (*Ulmus ssp.*) against biotic stressors. (For details, see text Section 6).

Gene description	Biological activity	Proposed role in defence	Biotic stressor	Elm species	Plant organ	References
PAL	Phenylpropanoid pathway	Phytoalexin, lignin and flavonoid synthesis	DED	*U. pumila, U. americana*	Midrib, root, bark	Nasmith et al. 2008 a,b
PR-proteins (1,2,3,6,10)	hydrolysis of pathogen cell walls, proteinase inhibitors	Disease resistance Defence against insect eggs	DED Eggs of ELB	*U. pumila, U. americana U. minor*	Midrib, root, bark, callus or leaf	Nasmith et al. 2008 a,b; Aoun et al. 2010[1]; Büchel et al. 2011[1]
Polygalacturonase inhibiting protein	Blocks cell wall cleavage	Inhibiting fungal damage of the cell wall [2]	DED	*U. pumila, U. americana*	Midrib, root, bark	Nasmith et al. 2008 a,b; De Lorenzo 2001[2]
Kunitz inhibitor-like	”	Anti-nutritive effects	”	*U. americana*	Callus	Aoun et al. 2010
Isoflavone reductase	Isoflavonoid biosynthesis	Involved in compartment-alisation process	”	”	”	”
O-methyltransferase	Anthocyanin biosynthesis	”	”	”	”	”
Phenylcoumaran benzylic ether reductase	Phenylpropanoid biosynthesis	Cells lignification [2]	”	”	”	Aoun et al. 2010; Vander Mijnsbrugge et al. 2000[2]

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Table 2. Genes with an indicated function in the defence response of elms (*Ulmus ssp.*) against biotic stressors. (continued).

JAZ protein	Repressor of JA signaling	JA-regulated transcription	Eggs of ELB	*U. minor*	Leaf	Büchel et al. 2011
NPR1	Receptor in SA/ JA signalling	Defence gene expression	”	”	”	La Camera et al. 2009[2]; Büchel et al. 2011
Auxin signaling F-box 2	Auxin signaling	Defence gene expression	”	”	”	Büchel et al. 2011
Patatin-like protein	Phospholipase	Oxylipin biosynthesis/ resistance to pathogens [2]	”	”	”	”
LOX	Octadecanoid pathway	JA signaling	”	”	”	”
Sieve element-occluding protein	Occlusion of sieve elements	Compartmentalisation process	”	”	”	”
Catalase	Scavenging enzyme	Prevents for oxidative stress	”	”	”	”

[1] Gene description based on database homology; [2] demonstrated for other plant species, see reference; for abbreviations see text section 6

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Chapter 6 Summary

Plants can defend themselves against insect herbivory prior to larval feeding damage through response to egg deposition on their leaves. Egg deposition by the herbivorous leaf beetle *Xanthogaleruca luteola* (Müller) (Coleoptera: Chrysomelidae) induces the emission of leaf volatiles in the European field elm *Ulmus minor* Mill. (Ulmaceae). This volatile blend attracts the egg parasitoid wasp *Oomyzus gallerucae* (Hymenoptera: Eulophidae), which destroys the insect eggs by parasitising, thus protecting indirectly the plant.

The main goal of this doctoral thesis is to improve our knowledge of induced plant defence against insect egg deposition. Studies have been carried out by using a co-evolved, naturally occurring tritrophic system consisting of *U. minor* - *X. luteola* - *O. gallerucae*. The thesis focused on four questions, three of them were addressed experimentally by combining chemical and molecular analyses, and one implies a theoretical review.

Do terpenoids emitted from oviposition-induced elm leaves play a role in mediating indirect elm defence? Only a few studies have addressed the question which of the plant volatiles induced by insect egg deposition are relevant for egg parasitoid attraction. Previous studies showed that the oviposition-induced elm odour consists mainly of terpenoids ((*E*)-*β*-farnesene, (*E*)-*ß*-caryophyllene, (*E*)-4,8-dimethyl-1,3,7-nonatriene) and the green leaf volatile (*Z*)-3-hexenyl acetate. To elucidate the relevance of terpenoids for parasitoid attraction, elms were treated with inhibitors of terpenoid biosynthesis, and attractiveness of odour of these elms to the parasitoid was tested. Quantitative analysis by coupled gaschromatography – mass spectrometry (GC-MS) demonstrated that inhibition of terpenoid biosynthesis in leaves reduced the emission of (*E*)-*ß*-caryophyllene, (*E*)-4,8-dimethyl-1,3,7-nonatriene and a yet unidentified oxygenated sesquiterpenoid. Unexpectedly, also the emission of green leaf volatiles such as 1-hexanol was reduced. Laboratory olfactometer assays revealed that inhibitor treatment of elm leaves rendered oviposition-induced elm odour unattractive for the egg parasitoid. Further bioassays showed that single terpenoids such as (*E*)-*ß*-caryophyllene and (*E*)-4,8-dimethyl-1,3,7-nonatriene were *per se* attractive to *O. gallerucae*. However, 1-hexanol as single volatile compound was not attractive to parasitoids in bioassays. Field studies corroborated the findings of the ability of *O. gallerucae* to orientate towards single terpenoid volatiles. *O. gallerucae* were attracted to (*E*)-4,8-dimethyl-1,3,7-nonatriene-baited traps in the presence of natural surrounding odours of an elm stand. These results strongly suggest that field

elms alert the egg parasitoid after insect egg deposition by means of one or more terpenoid volatiles.

How does habitat background odour affect the orientation of egg parasitoids to oviposition-induced elm leaf terpenoids? Knowledge of induced plant defence *via* the emission of volatile compounds attracting parasitoid "helpers" against herbivore pests can be used for developing biological control strategies. However, in natural habitats a parasitoid will never be exposed only to odour of induced (attacked) plants, but will also experience the surrounding odour („habitat odour") released by non-attacked plants. In forestry – and agricultural monocultures non-attacked plants of the same species occur also next to the attacked ones. Little knowledge has been available on how habitat odour affects orientation of egg parasitoids towards oviposition-induced plant volatiles. Our lab and field studies indicated how host location in a plant - herbivore -parasitoid interaction might proceed under natural conditions. The sesquiterpenoid (*E*)-*ß*-caryophyllene is utilised by *O. gallerucae* as chemical signal for host location (demonstrated in this thesis). Hence, the behavioural response of the parasitoid to this sesquiterpenoid was investigated when it was offered in combination with different elm background odours of differently leaf beetle infested and non-infested elms. The olfactory bioassays revealed that addition of (*E*)-*ß*-caryophyllene renders odorous background of non-attractive undamaged elms attractive to *O. gallerucae*. Yet, an odorous background of non-attractive feeding-damaged elms or of the green leaf volatile (*Z*)-3-hexenyl acetate masks the attractive effect of (*E*)-*ß*-caryophyllene. Analyses of the different elm odours by GC-MS revealed decreased concentrations of (*Z*)-3-hexenyl acetate, accompanied by highly increased concentration of (*E*)-*ß*-caryophyllene in the headspace of oviposition- and feeding-infested elms when compared to odours of undamaged elms. In a field study *O. gallerucae* parasitised more host egg masses when elms, exposed to the natural surrounding background odours of an elm stand, were elicited to release an increase blend of sesquiterpenoids by applying methyl jasmonate onto their leaves. It was concluded that *O. gallerucae* locates eggs of its host on elms by orientation towards key sesquiterpenoids emitted in enhanced quantities by induced elm and received in the presence of background odour released from non-attacked elm. In contrast, odorous backgrounds consisting of high quantities of green leaf volatiles - as released from feeding-attacked elm leaves - negatively affect the host location behaviour of this parasitoid species.

How does insect egg deposition affect the elm´s transcriptome? Insect egg deposition on leaves of herbaceous plants has been shown to induce changes in the plant's transcriptome, but knowledge on the molecular-genetic responses of tree species to insect egg deposition on their leaves is scarce. High-throughput sequencing technology of the first generation was used for sequencing the transcriptomes of elm leaves after infestation (eggs, feeding) with the elm leaf beetle *X. luteola*. This approach enables a snapshot of the processes in the field elm *U. minor* after infestation with insect eggs. An EST (expressed sequence tags) database was generated from leaves of non-infested elms, of elms with eggs and damaged by feeding and elms damaged only by feeding by adult *X. luteola*. Additionally elm leaves were used to which egg clutches were experimentally transferred (only egg deposition) and which were treated with methyl jasmonate (known elicitor of oviposition-induced defence). This elm database with 361,196 expressed sequence tags that clustered into 52,823 unique transcripts represents the largest genome resource of any elms. Comparative *in silico* analysis of hundreds of transcripts of genes revealed differences in the transcript signature of differently treated elm leaves in comparison to untreated elms. For the first time it was shown for a tree species that insect eggs can induce changes in transcript levels of genes involved in the primary as well as secondary metabolism. There was a pronounced shift in egg-induced elms towards more transcripts of genes involved in general stress and defence responses, of genes encoding pathogenesis-related proteins, and of genes involved in jasmonic acid biosynthesis and activation of jasmonic acid responsive genes, oxidative stress, signal transduction, transport processes, and protein folding or degradation. The simultaneous down-regulation of transcripts involved in photosynthesis suggests a shift from plant growth to development of defence. Furthermore, transcripts of genes involved in terpenoid biosynthesis were detected at only low levels. It has been demonstrated that release of terpenoids - as crucial part of the field elm´s indirect defence against elm leaf beetle eggs - does not involve increase in transcript levels of the genes involved in terpenoid biosynthesis.

How do elm trees defend against biotic stressors? Morphological, chemical and molecular aspects of defence mechanisms of elms against biotic stressors were reviewed by referring to the results of this PhD thesis. Knowledge about elm defences mainly arises from investigations of elm species resistant and susceptible towards the most serious specific elm pest and diseases: the Dutch elm disease (caused by a fungus which is transmitted by a bark beetle into the elm trunk), the elm yellow (caused by phytoplasms

which are transmitted by phloem-sucking insects) and the elm leaf beetle (responsible for fatal defoliation by its feeding activity). Morphological defence mechanisms *via* barrier formation and vessel occlusion prevent colonisation and spread of wood- and bark-inhabiting fungi and bacteria. Secondary metabolites in leaves and bark are involved in constitutive and induced chemical defence mechanisms of elms. These metabolites belong to the group of terpenoids (volatile terpenoids, mansonones and triterpenoids) and phenolics (lignans, cumarins, flavonoids). Induced defence mechanisms are orchestrated through the interaction of a huge variety of stress- and defence-related genes. However, this review demonstrates the lack of knowledge on compounds and genes playing a role in the defence of elms against pests and diseases. After the massive reduction of elm abundance worldwide by especially the Dutch elm disease, there is a revival of interest in elms.

Further research is required to understand the complex mechanisms of elm defence against biotic stressors and to use this knowledge as valuable tool in sustainable pest management; the acquired knowledge might help bringing back the magnificent group of elm species into our cities and landscapes.

Zusammenfassung

Pflanzen können auf Eiablagen von Insekten auf ihren Blättern reagieren und sich dadurch gegen herbivore Insekten verteidigen, bevor deren Larven mit dem Fraß beginnen. Die Eiablage des herbivoren Ulmenblattkäfers *Xanthogaleruca luteola* (Müller) (Coleoptera: Chrysomelidae) induziert die Freisetzung von Blattdüften in der Europäischen Feldulme *Ulmus minor* Mill. (Ulmaceae). Dieser eiablageinduzierte Duft lockt den Eiparasitoiden *Oomyzus gallerucae* (Hymenoptera: Eulophidae) an, welcher die Käfereier durch Parasitierung zerstört und dadurch indirekt die Pflanze schützt.

Das Ziel der vorliegenden Promotionsarbeit ist es, die Erkenntnisse über induzierte Pflanzenverteidigung gegen Insekteneier zu vertiefen. Die Studien wurden unter Verwendung des natürlich vorkommenden tritrophischen Systems *U. minor -X. luteola - O. gallerucae* durchgeführt, das sich koevolutiv entwickelt hat. Es wurden insgesamt vier Fragestellungen untersucht, davon drei experimentell mittels chemischer und molekularer Methoden und eine theoretisch in Form eines Übersichtsartikels.

Spielen eiablageinduzierte Terpenoide, welche von Ulmenblättern abgegeben werden, eine Rolle bei der indirekten Pflanzenverteidigung? Nur wenige Studien haben sich bisher der Frage gewidmet, welche der Verbindungen der eiablageinduzierten Pflanzenduftmuster für die Anlockung von Parasitoiden relevant sind. Vorausgegangene Arbeiten zeigten, dass der eiinduzierte Ulmenduft hauptsächlich aus den Terpenoiden ((*E*)-*ß*-Farnesen, (*E*)-*ß*-Caryophyllen, (*E*)-4,8-Dimethyl-1,3,7-nonatrien) und dem grünem Blattduftstoff (*Z*)-3-Hexenylacetat besteht. Um die Relevanz von eiablageinduzierten Terpenoiden bei der Anlockung von Parasitoiden aufzuklären, wurden Ulmen mit Inhibitoren für die Terpenoidbiosynthese behandelt und der resultierende Ulmenduft auf seine Attraktivität für den Parasitoiden untersucht. Quantitative Gaschromatographie-Massenspektrometrie (GC-MS) Analysen zeigten, dass eine Hemmung der Terpenbiosynthese in Blättern die Emission von (*E*)-*ß*-Caryophyllen, (*E*)-4,8-Dimethyl-1,3,7-nonatrien, einem nicht identifizierten oxygenierten Sesquiterpoid und - unerwartet - auch von einigen grünen Blattdüften wie 1-Hexanol reduzierte. Wie Olfaktometertests im Labor zeigten, hatte der eiablageinduzierte Ulmenduft seine Attraktivität für den Eiparasitoiden nach Inhibitorbehandlung der Ulmenpflanzen verloren. In weiteren Tests lockten einzelne Terpenoide wie (*E*)-*ß*-Caryophyllen und (*E*)-4,8-Dimethyl-1,3,7-nonatrien *per se O. gallerucae* an. Der grüne Blattduftstoff 1-Hexanol hingegen war als einzelne

angebotene Verbindung im Olfaktometertest nicht attraktiv für den Parasitoiden. Freilanduntersuchungen bestätigten die Annahme, dass einzelne Terpenoide den Eiparasitoiden anlocken können. So ließ sich *O. gallerucae* in Gegenwart des Duftes eines natürlichen Ulmenbestandes in Fallen locken, die mit (*E*)-4,8-Dimethyl-1,3,7-nonatrien bestückt waren. Diese Ergebnisse zeigen, dass Ulmen den Eiparasitoiden nach Insekteneiablage durch ein oder mehrere flüchtige terpenoide Verbindungen gezielt anlocken können.

Wie beeinflusst der Hintergrundduft des Habitats die Orientierung des Eiparasitoiden hin zu dem Duft eiablageinduzierter Terpenoide aus Ulmenblättern? Kenntnisse über induzierte Pflanzenverteidigung mittels Emission von Düften, welche Eiparasitoide als “Helfer” gegen Insektenbefall alarmieren, können bei der Entwicklung von biologischen Pflanzenschutzmaßnahmen verwendet werden. In natürlichen Habitaten ist ein Parasitoid jedoch niemals nur dem Duft von induzierten (befallenen) Pflanzen ausgesetzt, sondern erfährt auch den Umgebungsduft („Habitatduft“), an dem auch nicht befallene Pflanzen ihren Anteil haben. In Monokulturen der Land- und Forstwirtschaft kommen ebenfalls befallene und nicht befallene Pflanzen von derselben Spezies dicht beieinander vor. Es ist wenig darüber bekannt, wie der Habitatduft die Orientierung von Eiparasitoiden zu eiablageinduzierten Pflanzendüften hin beeinflusst. Unsere Labor- und Freilandstudien zeigten, wie die Wirtsfindung in einer solchen Pflanzen-Herbivor-Parasitoid Interaktion unter natürlichen Bedingungen ablaufen könnte. Das Sesquiterpenoid (*E*)-ß-Caryophyllen wird von *O. gallerucae*, wie in dieser Arbeit beschrieben, als chemisches Signal zur Wirtssuche verwendet. Basierend auf diesem Befund wurde die Verhaltensreaktion des Parasitoiden auf dieses Sesquiterpenoid hin weitergehend untersucht. (*E*)-ß-Caryophyllen wurde dazu dem Parasitoiden in Kombination mit Hintergrunddüften von unterschiedlich durch den Ulmenblattkäfer befallenen und unbefallenen Ulmen angeboten. Die Olfaktometerversuche zeigten, dass eine Beimischung von (*E*)-ß-Caryophyllen den nicht attraktiven Hintergrundduft von unbefallenen Ulmen attraktiv für *O. gallerucae* werden lässt. Zugleich maskiert ein Hintergrundduft bestehend aus dem grünen Blattduftstoff (*Z*)-3-Hexenylacetat den attraktiven Effekt von (*E*)-ß-Caryophyllen. Die Analysen der verschiedenen Ulmenhintergrunddüfte mittels GC-MS zeigten eine verminderte Konzentration von (*Z*)-3-Hexenylacetat und eine erhöhte Konzentration von (*E*)-ß-Caryophyllen im Duft von eiablage- und fraßinduzierter Ulmen im Vergleich zum Duft unverletzter Ulmen. In einer

Freilandstudie parasitierte *O. gallerucae* mehr Wirtseigelege auf Ulmen, welche durch die Behandlung ihrer Blätter mit Methyljasmonat zusätzliche Sesquiterpenoide vor dem natürlich umgebenden Hintergrundduft eines Ulmenbestandes abgaben. Daraus ergab sich die Schlussfolgerung, dass *O. gallerucae* die Wirtseier auf Ulmen lokalisiert, indem sie sich zu Sequiterpenoiden hin orientiert, die eine Schlüsselfunktion bei der Anlockung dieser Parasitoide haben und in größerer Menge von induzierten Ulmen abgegeben werden. Diese Sesquiterpenoide können auch in Anwesenheit eines Hintergrundduftes von unbefallenen Ulmen wahrgenommen werden. Im Gegensatz dazu beeinflusst ein Hintergrundduft, welcher zu größeren Anteilen aus grünen Blattdüften besteht, die Wirtssuche dieser Parasitoidenart negativ.

Wie beeinflusst die Eiablage das Transkriptom der Ulmen? Für krautige Pflanzen wurde bereits eine Veränderung des Transkriptoms als Folge von Insekteneiablage auf deren Blätter demonstriert. Der Wissensstand über die molekular-genetischen Reaktionen von Bäumen auf die Eiablage auf ihren Blättern ist jedoch sehr gering. Zur Sequenzierung des Transkriptoms von Ulmenblättern nach Befall (Fraß, Eier) durch den Ulmenblattkäfer *X. luteola* wurde die Hochdurchsatz-Sequenzierungstechnik der ersten Generation verwendet. Dies ermöglichte eine breite Momentaufnahme der molekular-genetischen Prozesse, welche innerhalb einer Feldulme nach Befall durch Insekteneier ablaufen. Es wurde eine EST (Expressed Sequence Tag) Datenbank aus verschiedenem Blattmaterial generiert: zum einen von unverletzten Ulmen, zum anderen von Ulmen, welche durch Fraß und Eiablage behandelt wurden, sowie von Ulmen, welche nur durch Fraß durch adulte *X. luteola* beschädigt wurden. Zusätzlich wurde Blattmaterial von Ulmen verwendet, auf welche Eigelege experimentell übertragen worden waren (nur Eiablage) und Blattmaterial von Ulmen, die mit Methyljasmonat (bekannter Auslöser eiinduzierter Verteidigung) behandelt worden waren. Diese Datenbank, bestehend aus 361.196 ESTs, die zu 52.823 spezifischen Transkripten zusammengefasst sind, repräsentiert die größte genomische Ressource, welche bisher für jegliche Art von Ulmen bekannt ist. Durch vergleichende *in silico* Analysen von hunderten von Gentranskripten wurden Unterschiede im Transkriptommuster von Ulmenblättern nach den verschiedenen Behandlungen im Vergleich zu unbehandelten Ulmen aufgedeckt. Zum ersten Mal wurde hier für eine Baumart demonstriert, dass Insekteneier Veränderungen in Transkriptraten von Genen aus dem Primär- sowie Sekundärstoffwechsel auslösen können. In eiinduzierten Ulmen wurde eine ausgeprägte Verschiebung von Transkriptraten bei solchen Genen festgestellt, welche

verstärkt in generellen Stress- und Verteidigungsantworten involviert waren, welche pathogenesebezogene Proteine codieren und welche bei der Jasmonatbiosynthese und Aktivierung von Jasmonat-abhängiger Genregulation, oxidativem Stress, Signaltransduktion, Transportprozessen und Proteinfaltung und –abbau eine Rolle spielen. Die gleichzeitig reduzierten Transkriptraten von Genen, die für die Photosynthese relevant sind, lässt eine Verschiebung der Investition in Wachstum hin zur verstärkten Investition in Verteidigung vermuten. Darüber hinaus wurde nur ein sehr geringes Level an Transkripten detektiert, die in Terpenoidbiosynthese involviert sind. Es konnte gezeigt werden, dass die Terpenoidproduktion als essentieller Bestandteil der indirekten Verteidigung von *U. minor* gegen die Eier des Ulmenblattkäfers nicht mit einem Anstieg von Transkriptraten einhergeht.

Wie verteidigt sich die Ulme gegen biotische Stressoren? In einem Übersichtsartikel wurden, unter Bezugnahme auf die Ergebnisse aus dieser Doktorarbeit, morphologische, chemische und molekulare Aspekte der Ulmenverteidigung zusammengefasst. Bisherige Erkenntnisse über Verteidigungsmechanismen der Ulme stammten vor allem aus Untersuchungen von resistenten bzw. anfälligen Ulmenspezies gegenüber den ärgsten ulmenspezifischen Schädlingen und Krankheiten; diese Schädlinge und Krankheiten sind die Holländische Ulmenkrankheit (verursacht durch einen Pilz, welcher durch Borkenkäfer in den Ulmenstamm übertragen wird), die Phloemnekrose (verursacht durch Phytoplasmen, welche durch phloem-saugende Insekten übertragen werden) und der Ulmenblattkäfer (welcher durch seine Fraßtätigkeit ganze Bäume entlauben kann). Morphologische Verteidigungsmechanismen verhindern mit Hilfe von Barriereformation und Gefäßokklusion die Kolonisierung und Ausbreitung von Holz und Borke bewohnenden Pilzen und Bakterien. Die Sekundärmetabolite in Blättern und Borke von Ulmen, welche an konstitutiver und induzierter chemischer Abwehr beteiligt sind, gehören vor allem der Gruppe der Terpenoide (flüchtige Terpenoide, Mansonone und Triterpenoide) und der Phenole (Lignane, Cumarine, Flavonoide) an. Induzierte Verteidigungsmechanismen werden durch das Zusammenspiel einer Vielzahl von stress- und verteidigungsrelevanten Genen orchestriert. Dieser Übersichtsartikel demonstriert, wie wenig bisher über Metabolite und Gene bekannt ist, welche bei der Verteidigung von Ulmen eine Rolle spielen, obwohl das Interesse an Ulmen nach ihrer massiven Vernichtung durch die Holländische Ulmenkrankheit wieder zunimmt.

Es besteht weiterer Forschungsbedarf, um tiefere Einblicke in die komplexen Verteidigungsmechanismen von Ulmen gegen biotische Stressoren zu erhalten und um diese als ein wertvolles Werkzeug bei der nachhaltigen Schädlingsbekämpfung verwenden zu können. Die erworbenen Erkenntnisse können so zur Rückkehr solch einer bedeutenden Baumspezies in unser Landschaftsbild beitragen.

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Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Hilfsmittel angefertigt habe. Alle Stellen der Arbeit, die anderen Werken dem Wortlaut oder dem Sinn nach entnommen wurden, sind entsprechend kenntlich gemacht. Diese Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Berlin, den 28.11.2013

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(Kerstin Büchel)

The CV is not included in the online version for reasons of data security.