Responses of *Pinus sylvestris* to different stages of infestation by the sawfly *Diprion pini*

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DECLARATION OF AUTHORSHIP

I hereby declare that I alone am responsible for the content of my doctoral dissertation titled "Responses of *Pinus sylvestris* to different stages of infestation by the sawfly *Diprion pini*" and that I have only used the sources or references cited in the dissertation.

Lügde, March 21, 2024

Janik Hundacker

LIST AND DECLARATION OF PUBLICATIONS

This cumulative dissertation is based on the following publications

1.) Bittner, N., Hundacker, J., Achotegui-Castells, A., Anderbrant, O., & Hilker, M. (2019)

Defense of Scots pine against sawfly eggs (Diprion pini) is primed by exposure to sawfly sex pheromones

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NB, AA and MH designed the study. JH (egg-survival rate, hydrogen peroxide- and water content, gene expression, EAG analysis), NB (gene expression), and AA (pheromone emission rate) ran the experiments and analyses; they evaluated the data and were supported by MH. OA provided the insect sex pheromones. NB and MH wrote a first draft of the manuscript, JH created the figures, and all authors read, edited and approved the final draft.

2.) Hundacker, J., Bittner, N., Weise, C., Bröhan, G., Varama, M., & Hilker, M. (2022)

Pine defense against eggs of an herbivorous sawfly is elicited by an annexin-like protein present in egg-associated secretion

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NB, JH and MH designed the study, supported by CW with respect to protein isolation and identification and by GB with respect to recombinant protein expression. The peptide mass fingerprinting measurements were done by CW. All other experiments and analyses were done by JH (gene expression) and NB (isolation and identification, elicitor activity assay, GC-MS analysis). MV contributed by collection of egg parasitoids in the field. NB, JH and CW analyzed the data, supported by MH. NB and JH wrote a first draft of the paper, MH revised the paper, and all authors read, edited and approved the final manuscript.

3.) Hundacker, J., Linda, T., Hilker, M., Lortzing, V., & Bittner, N. (2024)

The impact of insect egg deposition on Pinus sylvestris transcriptomic and phytohormonal responses to larval herbivory

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JH and MH designed the study and planned the experiments. JH conducted the experiments for RNA sequencing and prepared the RNA samples for this analysis. NB conducted the transcriptome assembly and annotation and prepared the DEG data set. TL prepared the samples for the qPCR experiments and phytohormone analysis. He conducted the qPCR analysis for validating the results obtained by the RNA sequencing analysis. JH analysed the DEG data set and conducted a GO term enrichment and KEGG pathway enrichment analysis. JH conducted the phytohormone analysis. VL provided advice for the GO term and KEGG analysis and prepared Figure 3. JH wrote a first draft of the manuscript. MH revised the manuscript. All authors contributed to the writing of the manuscript and approved the submitted version.

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SUMMARY

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SUMMARY

Plants can mobilize efficient defenses against eggs laid by herbivorous insects on their leaves as well as against the feeding insects. Various studies showed that angiosperm plants able respond to environmental cues that indicate impending insect infestation. Thus, the plants prepare and improve their defenses against the actual infestation. The mechanisms of this phenomenon – termed "priming" of inducible defense – were hardly investigated in gymnosperm species in the beginning of my doctorate studies.

For the gymnosperm species *Pinus sylvestris* (Scots pine)*,* it was known that exposure of this tree to the sex pheromone of the herbivorous sawfly *Diprion pini* results in improved defense against sawfly eggs. Furthermore, previous studies showed that Scots pine responds to eggs of *D. pini* with improved defense against later hatching sawfly larvae. However, prior to this thesis little information was available about the mechanisms of these responses. Furthermore, a proteinaceous elicitor of pine defenses against sawfly eggs had been identified prior to this thesis; however, it was unknown so far whether this elicitor can induce similar transcriptional changes as the natural egg deposition does.

To address these gaps in knowledge (outlined in detail in the general introduction of this thesis), I investigated the interactions between the gymnosperm pine *Pinus sylvestris* and the herbivorous sawfly *Diprion pini*.

In a first study, pine trees were exposed to the sawfly's sex pheromone and subsequently to egg deposition. The survival rate of eggs laid on pheromone-exposed pine was 20 % lower than the survival rate of eggs laid on control (untreated) pine. My analyses revealed that pheromone-exposed and subsequently egg laden pine needles accumulated significantly higher concentrations of hydrogen peroxide than non-exposed, egg laden pine needles. The strong accumulation of hydrogen peroxide might initiate the amplification of defense-relevant further responses, but might also directly harm the eggs. Furthermore, my chemical analysis by coupled gas chromatography – mass spectrometry revealed that pine trees exposed to the pheromone for 24 h did not emit pheromone components anymore already six hours later. My electrophysiological study of the antennal response of *D. pini* to the tested pheromone components revealed that males – as expected - showed clear responses, whereas females did not. Thus, in contrast to some lepidopteran females, *D. pini* females cannot perceive their pheromones and exploit them as abundance sensor for abundance-adjusted regulation of egg deposition.

The second study of this thesis addressed the transcriptomic and phytohormonal response of *P. sylvestris* to (a) egg deposition, (b) larval feeding and (c) egg deposition and subsequent larval feeding of *D. pini*. The results revealed comparably strong pine transcriptomic responses to both sawfly eggs

and larval feeding. Interestingly, the regulated genes in response to sawfly oviposition and larval feeding were of remarkably similar types and putative functions. These commonly regulated genes were mostly related to mechanisms like cell wall modification, cell death, jasmonic acid signaling and other defense related groups. However, pine trees laden with sawfly eggs showed a weaker transcriptomic response to subsequent feeding damage than control trees without prior egg deposition. This finding differs from so far known responses in angiosperm species; the transcriptomes of egg-laden angiosperms are known to show stronger or earlier responses to feeding. The very strong transcriptomic pine response to *D. pini* oviposition might already prepare the plant well for increased defense against hatching larvae, thus rendering an amplified response of egg-laden pine to later feeding damage by the larvae would be redundant. However, some results showed similar and thus possibly phylogenetically conserved responses to insect eggs and larvae in angiosperm species and the here analyzed gymnosperm species. For example, larval feeding damage induced a significant increase in the SA concentration in previously egg laden, but not in egg-fee pine needles, which was also observed in leaves of several angiosperm species.

The third study addressed the question how the known egg-associated elicitor of pine defenses against sawfly eggs affects the expression of defense-related pine genes. The proteinaceous, annexin-like elicitor – called diprionin – was first heterologously expressed and subsequently applied onto pine needles that had been slit to mimic the ovipositional wounding, which sawfly females inflict to pine needles when laying their eggs. Expression levels of various, potentially defense-related genes in egg laden and diprionin treated needles were determined by qPCR analyses. These were genes involved in the regulation of reactive oxygen species and calcium mediated signal transduction, as well as genes relevant for the biosynthesis of phenylpropanoid and terpenoid secondary metabolites. These genes showed similar expression patterns in egg laden and diprionin treated needles, thus corroborating the relevance of diprionin for defense against sawfly eggs. However, pathogenesis related (*PR)* genes were differentially expressed when comparing diprionin treated trees and pine with natural egg deposition, suggesting further compounds and/or conditions to be involved in the defense response.

The general discussion of this thesis focuses on a comparison of pheromone-mediated and egg-mediated pine defense and priming mechanisms and the (dis)similarities of defense responses against different stages of the sawfly infestation. Furthermore, it classifies diprionin in comparison to other known insect-associated plant defense elicitors and tries to elucidate possible ways of plant tissue interaction.

In a nutshell, this thesis on the interactions between *P. sylvestris* and the sawfly *D. pini* demonstrated that…

… the improved defense of pheromone-exposed pine against sawfly eggs is linked with enhanced accumulation of hydrogen peroxide

… the analyzed pine transcriptome shows a very strong response to sawfly egg deposition; this egg-induced response largely overlaps with the transcriptomic response to sawfly larval feeding damage

…in contrast to angiosperms, egg laden pine shows a weaker transcriptomic response to larval feeding instead of a stronger response

… diprionin, the elicitor of pine defense associated with *D. pini* eggs, affects the expression of several defense-related pine genes to a similar extent as *D. pini* egg deposition does.

ZUSAMMENFASSUNG

Pflanzen können sich nicht nur gegen pflanzenfressende Insekten wehren, sondern bereits gegen Insekteneiablagen auf ihren Blättern. Verschiedene Untersuchungen an Angiospermen haben gezeigt, dass Pflanzen auf solche Umweltreize reagieren, die auf bevorstehenden Insektenbefall hinweisen, um daraufhin ihre Verteidigung gegen den tatsächlichen Befall vorzubereiten und zu verbessern. Die Mechanismen dieses Phänomens der "Vorbereitung" (engl. *Priming*) auf befallsinduzierte Verteidigung war zu Beginn dieser Doktorarbeit bei Gymnospermen wenig untersucht.

Für die Gymnospermen-Art *Pinus sylvestris* war bekannt, dass sie auf das Sexualpheromon der herbivoren Blattwespe *Diprion pini* mit verbesserter Abwehr gegen die Eiablagen dieses Insekts reagiert. Darüber hinaus war bekannt, dass diese Waldkiefer auf Eiablagen von *D. pini* mit verbesserter Abwehr gegen schlüpfende Larven reagiert. Es lagen aber zu Beginn dieser Dissertation nur wenige Informationen über die Mechanismen dieser Reaktionen vor. Weiterhin war ein mit der Eiablage von *D. pini* identifizierter proteinöser Elicitor der Verteidigung von *P. sylvestris* gegen die Eier kurz vor Beginn dieser Arbeit beschrieben worden. Es war aber noch unklar, ob eine synthetische Version dieses Elicitors in *P. sylvestris* ähnliche transkriptionelle Änderungen auslöst wie die Eiablage selbst.

Um diese in der Einleitung dieser Dissertation näher vorgestellten Wissenslücken zu füllen, wurde als Untersuchungssystem für diese Doktorarbeit die Waldkiefer *P. sylvestris* und dessen Interaktion mit der herbivoren Blattwespe *D. pini* gewählt.

In einer ersten Studie wurden Waldkiefern dem Sexualpheromon und anschließend der Eiablage der Blattwespen ausgesetzt. Aus Eiern an Sexualpheromon-exponierten Kiefern schlüpften 20% weniger Larven als aus den Eiern an Kontroll-Kiefern, die nicht den Pheromonen ausgesetzt waren. Meine Analysen zeigten, dass Pheromon-exponierte, eierbelegte Kiefernnadeln signifikant mehr Wasserstoffperoxid anreicherten als eierbelegte Nadeln der Kontrollbäume. Die Akkumulation von Wasserstoffperoxid könnte zum einen eine Verstärkung der verteidigungsrelevanten Mechanismen initiieren, aber zum anderen auch direkt die Eier stark schädigen. Weiterhin zeigten meine chemischen Analysen mittels gekoppelter Gaschromatographie-Massenspektrometrie, dass die Waldkiefer nach 24-stündiger Pheromonexposition bereits nach sechs weiteren Stunden selbst keine Pheromone "ausdünstete". Meine elektrophysiologischen Analysen der Antennenreaktion von *D. pini* auf die getesteten Pheromonsubstanzen ergaben, dass die Männchen – wie erwartet – sehr gut auf die Substanzen reagierten, die Weibchen jedoch nicht. Somit können *D. pini* Weibchen im Gegensatz zu einigen Schmetterlingsarten ihre eigenen Pheromone nicht wahrnehmen und daher auch nicht entsprechend als "Abundanzfühler" für eine Abundanz-justierte Eiablageregulation nutzen.

Die zweite Studie dieser Dissertation befasste sich mit der Transkriptom- und Phytohormonreaktion von *P. sylvestris* auf (a) die Eiablage, (b) Larval-Fraß und (c) die Eiablage und anschließendem Larval-Fraß durch *D. pini*. Die Ergebnisse zeigten vergleichbar starke Reaktionen des Transkriptoms sowohl auf die Blattwespeneier als auch auf den Larval-Fraß. Interessanterweise waren die Gene, welche als Antwort auf die Eiablage der Blattwespen und den Larval-Fraß reguliert wurden, von erstaunlich ähnlicher Natur und ähnlicher möglicher Funktion. Diese Gene standen hauptsächlich im Zusammenhang mit Mechanismen wie Zellwandmodifikationen, Zelltod, Jasmonsäure-Signalisierung und anderen verteidigungsbezogenen Gruppen. Allerdings zeigten eierbelegte Kiefern eine schwächere Reaktion des Transkriptoms auf nachfolgende Fraßschäden im Vergleich zu Kontrollbäumen ohne vorherige Eiablage. Dieses Ergebnis stellt einen bemerkenswerten Unterschied zu den bisher bekannten Reaktionen bei Angiospermen dar, bei denen das Transkriptom eierbelegter Pflanzen meist stärker oder eher auf Larval-Fraß reagierte. Möglicherweise ist die Waldkiefer bereits durch die sehr starke Transkriptomreaktion nach *D. pini* Eiablagen so gut auf den Larval-Fraß vorbereitet, dass bei Beginn des Larval Fraßes eine verstärkte Transkriptomreaktion für die verbesserte Verteidigung eierbelegter Kiefern gegen die Larven überflüssig wird. Einige Ergebnisse zeigten jedoch, dass Angiospermen und die hier analysierte Gymnospermen-Art auch ähnliche und somit vermutlich phylogenetisch konservierte Reaktionen auf Insekteneiablagen und Fraß zeigen. So stieg beispielsweise nach Larval-Fraß nur in eierbelegten, nicht aber in eifreien Nadeln die Salicylsäurekonzentration deutlich an, was auch in Blättern verschiedener Angiospermen beobachtet wurde.

In einer dritten Studie wurde untersucht, welche Effekte der mit der Eiablage von *D. pini* assoziierte Elicitor der pflanzlichen Verteidigung gegen die Blattwespeneier auf die Expression von verteidigungsrelevanten Genen der Kiefer ausübt. Dazu wurde der proteinöse, Annexin-ähnliche Elicitor – genannt Diprionin – zunächst heterolog exprimiert und anschließend auf Kiefernnadeln appliziert. Diese wurden zuvor angeritzt, um damit die Verwundung bei der natürlichen Eiablage der Blattwespen nachzuahmen. Es wurden vergleichende qPCR Analysen verschiedener, potentiell verteidigungsrelevanter Gene in eierbelegten und Diprionin-behandelten Nadeln durchgeführt. Dies waren Gene, die in die Regulation von reaktiven Sauerstoffspezies und in die Kalzium-vermittelte Signaltransduktion involviert sind, sowie Gene, die für die Biosynthese verteidigungsrelevanter, phenylpropanoider und terpenoider Sekundärmetabolite wichtig sind. Diese Gene zeigten in eierbelegten und Diprionin-behandelten Nadeln ähnliche Expressionsmuster, was die Bedeutung von Diprionin für die Ei-assoziierte Abwehr von *P. sylvestris* unterstreicht. Pathogenese-bezogene *PR* Gene wurden jedoch beim Vergleich von Diprionin-behandelten und eierbelegten Kiefernnadeln unterschiedlich exprimiert, was darauf schließen lässt, dass weitere Ei-assoziierte Verbindungen und/oder Bedingungen an der Abwehrreaktion beteiligt sind.

Die allgemeine Diskussion dieser Dissertation konzentriert sich auf einen Vergleich der Pheromon-bedingten und Eiablage-bedingten Abwehr- und Vorbereitungs-(*Priming*-)Mechanismen von Kiefern sowie auf die (Un-)Ähnlichkeiten der Abwehrreaktionen gegen verschiedene Stadien des Blattwespenbefalls. Darüber hinaus wird Diprionin im Vergleich zu anderen bekannten Insekten-assoziierten Pflanzenabwehr Elicitoren klassifiziert und versucht, mögliche Wege der Interaktion mit Pflanzengewebe zu beschreiben.

Insgesamt zeigte diese Arbeit zur Interaktion von *P. sylvestris* und der Blattwespe *D. pini*, dass…

… die verbesserte Verteidigung von Pheromon-exponierter Kiefer gegen Blattwespeneier mit einer verstärkten Akkumulation von Wasserstoffperoxid einhergeht

… das Transkriptom der Kiefer bereits sehr stark auf die Eiablagen von *D. pini* reagiert und stark mit dem fraß-induzierten Transkriptom überlappt

...eierbelegte Kiefernadeln – im Gegensatz zu eierbelegten Blättern von Angiospermen – schwächer statt stärker auf Larval-Fraß reagieren

…Diprionin, der mit den Eiern von *D. pini* assoziierte Elicitor der Verteidigung der Kiefer gegen die Eier, die Expression verschiedener verteidigungsrelevanter Gene in ähnlicher Weise induziert, wie dies auch bei der natürlichen *D. pini* Eiablage zu beobachten ist.

CHAPTER 1

GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

Plants need to cope with a huge variety of herbivorous insect species (Lewinsohn et al., 2005; Bruce, 2015), which in turn face a plethora of plant anti-herbivore defenses. Plant infestation by many herbivorous insects starts with the egg deposition on their leaves. Defense mechanisms targeting this very early stage of infestation help preventing or limiting subsequent feeding damage. During the last decades, evidence is accumulating that plants show efficient defense responses to insect egg deposition, and can even improve their defenses against hatching larvae when having received eggs prior to larval feeding damage.

This thesis addresses mechanisms of plant defenses against insect eggs. It focusses on studies of a gymnosperm species, i.e. Scots pine (*Pinus sylvestris*), and its responses to infestation by the sawfly *Diprion pini,* whose larvae are feeding upon pine needles. The following introductory outline on plant defenses against insect infestation in general and on *P. sylvestris* defenses against *D. pini* in particular will highlight some gaps in knowledge and lead to the research question addressed in this thesis.

1.1 Plant defenses against infestation by herbivorous insects

Plant defenses are usually classified as direct and indirect defenses with constitutive and inducible mechanisms (Schoonhoven et al., 2005; War et al., 2012; Fürstenberg-Hägg et al., 2013; Aljbory & Chen, 2018). While direct defenses directly harm the insect, the indirect ones involve attraction or arrestment of predators or parasitoids, which kill the herbivores. Constitutive defenses are available independent of any infestation, while induced defenses are mobilized in response to the infestation.

The targets of these types of defenses can be insect eggs and the feeding insect stages.

Ample knowledge is available about the chemical and molecular basis of induced plant defenses against feeding insects, as has been excellently addressed in numerous review articles by e.g. Kessler & Baldwin (2002), Farmer et al. (2003), Arimura et al. (2005), Howe & Jander (2008), Heil (2009), Wu & Baldwin (2010), Wasternack & Feussner (2018), Erb & Reymond (2019) and Kloth & Dicke (2022); these aspects will not be addressed further here. This knowledge is mainly and solidly based on studies of model plant species, which belong to the angiosperms. Many gymnosperm species have been demonstrated to show defense mechanisms similar to those of the angiosperms. Hence, plant defenses against angio- and gymnosperm species are well known to show phylogenetically conserved defense traits against the feeding stages of insects. For example, conifers show – like angiosperms - accumulation of the phytohormone jasmonic acid (JA) (Hudgins et al., 2004; Ralph et al., 2006; Schmidt et al., 2011) and reactive oxygen species (ROS) (Franceschi et al., 2005) as a first response to damage by chewing insects,

followed by enhanced production of secondary metabolites (Ralph et al., 2006; Zulak & Bohlmann, 2010; Schmidt et al., 2011) that are harmful to the feeding insects. However, most studies on gymnosperm defense responses focus on stem borers or feeders (Krokene, 2015). So far, our knowledge about the mechanisms of gymnosperm defenses against leaf- or needle-chewing insects is limited. Some general (chemo)ecological aspects of plant defenses against feeding insects will be outlined in subchapter 1.1.1.

Knowledge about the chemical and molecular basis of insect egg-induced plant defenses increased considerably during the last two decades (Hilker & Meiners, 2006; Reymond, 2013; Hilker & Fatouros, 2015; T. Lortzing et al., 2020; Reymond, 2022). Again, these studies focus on angiosperm species, while only little knowledge is available on the mechanisms by which a gymnosperm species responds to insect eggs. The state of the art on plant responses to insect eggs and their effect on defenses against subsequent larval feeding will be summarized here in subchapter 1.1.2 to 1.1.4.

Current knowledge on how plants perceive insect infestation and on insect-associated elicitors of plant defenses will be addressed in subchapter 1.2.

Finally, general information about the biology of *P. sylvestris* and *D. pini* (subchapter 1.3) and the available knowledge about those pine interactions with *D. pini* eggs (subchapter 1.4) that served as basis for my research questions are addressed in the end of this introductory chapter.

1.1.1 Plant defenses against feeding insects

Numerous studies focused on plant defenses against the feeding insect stages. The most obvious plant defense traits are physical ones (Fürstenberg-Hägg et al., 2013), like thorns on twigs, trichomes or waxes on leaves (Riederer & Muller, 2008; Wang et al., 2021) or lignified plant tissue (Raupp, 1985; Nichols-Orians & Schultz, 1990; Schoonhoven et al., 2005). All these traits provide physical barriers that impede access to nutritious plant tissue. In several plant species, especially in conifers, sticky resin that flows out of feeding-damaged sites, may agglutinate mouthparts and legs of insects, thus physically killing them (Phillips & Croteau, 1999). Although these traits are constitutively present in most cases, insect attack can also induce their further formation. They may harm the herbivores directly, or serve indirect defense (Aljbory & Chen, 2018). Hollow thorns of acacia, for example, are well known to host predatory ants that feed upon acacia-infesting herbivorous insects (Ward & Branstetter, 2017).

In addition to these physical defenses against feeding insects, plants evolved numerous chemical defenses ranging from repellents over compounds impairing digestion to toxic secondary plant metabolites. As for the physical defenses, chemical ones may be available constitutively or act upon

induction by infestation. Furthermore, they may act directly on the herbivore or indirectly by attraction of predators or parasitoids. Examples for repellents are the so-called herbivory-induced plant volatiles (HIPVs), which may be blends of e.g. green leaf volatiles (C6-alcolhols, -aldehydes or -esters), terpenes, aromatic compounds, and nitrogen- and sulfur-containing compounds (e.g. indole, isothiocyanates). These HIPVs often also attract carnivorous insects, thus serving direct and indirect defenses. Examples for digestion-impairing plant compounds are high concentrations of cellulose, phenolic compounds like tannins, or proteinase inhibitors. The diversity of toxic secondary plant metabolites is amazing and raised the question of their "raison d'être" (Fraenkel, 1959) already many years ago. More recently, Erb & Kliebenstein (2020) discussed the multifunctionality of plant secondary metabolites. Many specialized insect species succeeded in adapting to them by evolving efficient detoxification mechanisms. Further modes of insect adaptation to plant secondary metabolites include the evolution of receptors and behaviours that enable them to tolerate toxic plant metabolites or to use the secondary metabolites for host plant location or even for their own defenses (Zhu-Salzman et al., 2005; Alba et al., 2011; War et al., 2018; Erb & Reymond, 2019). Such adaptations are expected to result into an evolutionary arms race, where each novel trait evolved by one antagonist requires a novel adaptation by the other, thereby driving the evolution of a further novel trait.

Moreover, feeding-damaged plants can enhance growth of leaves, thus compensating the loss of tissue due to insect feeding (Li et al., 2021). In addition, they often reallocate their resources from shoot to root (Schwachtje et al., 2006), thereby rendering feeding on leaves less nutritious for the insects and saving resources for later regrowth.

1.1.2 Plant defenses against insect eggs

Plants are known to defend themselves against insect egg deposition constitutively, but also via induction by a first egg deposition, thereby preventing further egg depositions. Constitutively present physical plant traits like thorns, trichomes, slippery waxy surfaces may impair egg depositions. Constitutive lack of oviposition stimulating or presence of oviposition deterring plant compounds may also prevent egg depositions on a plant (Städler, 2003).

Furthermore, induced plant defenses against insect eggs - once laid onto the leaves - attracted increasing attention during the last two decades (Hilker & Meiners, 2002; Hilker et al., 2003; Hilker & Meiners, 2006; Reymond, 2013; Hilker & Fatouros, 2015, 2016; Reymond, 2022; Hilker et al., 2023). For a wide range of plant species, including herbaceous and perennial ones, it has been shown that egg-laying by herbivorous insects induces the emission of plant volatiles – so-called oviposition-induced plant volatiles (OIPVs) - that attract egg parasitoids (Hilker & Fatouros, 2015) or deter further egg

depositions on the egg-laden plant (Fatouros et al., 2012). The first study showing such egg-induced indirect defense was the one by Meiners & Hilker (2000), who demonstrated that eggs laid by the elm leaf beetle onto elm leaves results in emission of elm leaf volatiles, which attract an egg parasitoid specialized on elm leaf beetle eggs.

Egg-induced plant defenses are not only indirect ones, but also direct ones. Some plants form neoplasms underneath insect eggs, thereby causing egg detachment from the plant or egg desiccation (Doss et al., 2000). The growth of neoplasms in certain lines of pea pods is elicited by egg deposition of bruchid beetles; their eggs trigger a very strong upregulation of a gene showing high sequence similarity with *Mt19,* i.e. a gene expressed in root nodules of *Medicago trunculata* (Doss, 2005). Additionally, the production of egg-induced ovicidal compounds was shown in rice plants (Seino et al., 1996). Egg-induced direct plant defenses killing the eggs may also be provided by growth of new tissue around the eggs, which finally crushes the eggs (Desurmont & Weston, 2011). Egg-induced accumulation of ROS at the site of egg deposition may result in formation of necrotic tissue, resulting in detachment of the eggs from the leaf or egg desiccation. Such formation of hypersensitive response (HR)-like symptoms was shown in several angiosperm species, among them several Brassicaceae and a solanaceous species (Shapiro & DeVay, 1987; Balbyshev & Lorenzen, 1997; Gouhier-Darimont et al., 2013; Fatouros et al., 2016). The accumulation of ROS and hypersensitive responses are well known in plants responding to phytopathogens, thereby isolating (hemi)biotrophic pathogens (e.g. Mur et al., 2008; McCombe et al., 2022); these plant responses are also often linked with the upregulation of pathogenesis-related (*PR)* genes, especially *PR1* and *PR5* in *Arabidopsis thaliana* (e.g. Balint-Kurti, 2019). Interestingly, these *PR* genes are also upregulated in response to egg deposition by *Pieris brassicae* on *Arabidopsis thaliana* (Little et al., 2007; V. Lortzing et al., 2019) and *Brassica nigra* (Fatouros et al., 2014; Bonnet et al., 2017), in response to application of egg washes of several lepidopteran species on *B. nigra* (Griese et al., 2021), in response to egg deposition by the stink bug *Halyomorpha halys* on *Vicia faba* plants (Rondoni et al., 2018), and in response to eggs of *Spodoptera exigua* on bittersweet nightshade *Solanum dulcamara* (Geuss et al., 2017). Microarray studies and RNAseq analyses of plant responses to insect eggs showed that insect egg deposition affects expression of a broad set of genes relevant for numerous functions of the primary and secondary plant metabolism, e.g. Little et al. (2007), Bonnet et al. (2017), Altmann et al. (2018), (V. Lortzing et al. (2019), Valsamakis et al. (2022).

A gymnosperm species, *P. sylvestris,* is also known to accumulate ROS in response to egg deposition by *D. pini* on pine needles (Bittner et al., 2017). However, prior to this thesis, other mechanisms of defense responses to insect oviposition on gymnosperm needles beyond ROS accumulation and volatile

terpene emission were – to the best of my knowledge – not analyzed (Mumm & Hilker, 2006). Research focused on defenses against insect infestation on and into stems of conifer trees (Mageroy et al., 2020).

1.1.3 Preparation for improved plant defenses against insect feeding damage

Plants are well known to respond to cues indicating an impending infestation by the feeding stages of insects, thereby improving their defenses against the actually feeding insects. Such cues may be a first event of herbivory (e.g. Haukioja, 1991), HIPVs or OIPVs released from neighboring plants (e.g. Frost et al., 2008; Pashalidou et al., 2020), or insect egg deposition indicating impending larval feeding on the egg-laden plant (Hilker & Fatouros, 2016). The response to the "warning" cue can lead to a faster, stronger, or faster and stronger defense response to the actual infestation (Hilker et al., 2016; Martinez-Medina et al., 2016). Mechanisms by which plants can store the information about a cue "warning" of impending stress have been addressed in numerous review articles, among them recent ones by Wilkinson et al. (2023) and Auge et al. (2023).

Several plant species ranging from herbaceous to perennial ones are known to take insect egg depositions as "warning" cue of impending larval herbivory. These "warned" plants improve their defenses against the larvae when having received eggs prior to the feeding damage. Various *Brassicaceae* fortify their defense response to feeding larvae after prior egg deposition by *P. brassicae*; as a consequence, the larvae perform worse on previously egg-laden plants (Pashalidou et al., 2015; Valsamakis et al., 2020; Valsamakis et al., 2022). Similar effects of plant responses to insect egg deposition on subsequent defense against hatching larvae were detected in *Nicotiana attenuata;* larvae of the moth *S. exigua* performed worse on previously egg-laden tobacco plants (Bandoly et al., 2016). Tomato plants (*Solanum lycopersicum)* infested with eggs of the moth *Helicoverpa zea* showed much higher induction of a gene encoding a protease inhibitor when exposed to simulated herbivory than egg-free plants when wounded (Kim & Felton, 2013). In *Ulmus minor* laden with eggs of the elm leaf beetle *Xanthogaleruca luteola,* larvae performed worse than on egg-free plants (Austel et al., 2016).

Common chemical and molecular patterns of these egg-mediated improved defense against feeding larvae are the following ones: Higher concentrations of different phenylpropanoid derivatives were detected in egg-laden, feeding-damaged *A. thaliana, N. attenuata,* and *U. minor* than in egg-free, feeding-damaged plants of these species (Bandoly et al., 2015; Austel et al., 2016; V. Lortzing et al., 2019; Schott et al., 2022). Several phenylpropanoids are well known for their detrimental effects on insects (e.g. Lattanzio et al., 2008). Interestingly, higher concentrations of the phytohormone salicylic acid (SA) were found in egg-laden, feeding-damaged plants of *A. thaliana* (V. Lortzing et al., 2019), *B. nigra* (Bonnet et al., 2017), and *U. minor* (Schott et al., 2022) than in egg-free, feeding-damaged plants

of these species. These findings do not agree with the often reported antagonistic effects of SA and JA on herbivore performance (Pieterse et al., 2012; Thaler et al., 2012). The latter phytohormone is strongly induced by larval feeding damage. In spite of high levels of SA and JA, the egg-laden plants showed improved defenses against the infesting larvae. Hence, here the phytohormones SA and JA do not act antagonistically. The effects of interactions between SA and JA may depend on the concentrations of the phytohormones (Mur et al., 2006), on the sequence and kinetics of concentration increases of JA and SA (Moreira et al., 2018), and on the spatial separation of the sites of activation of JA and SA (Betsuyaku et al., 2018; Tsuda, 2018). With respect to transcriptomic changes, egg-laden *U. minor* and *A. thaliana* showed more differentially expressed genes than egg-free ones in response to the early phase of larval feeding (Altmann et al., 2018; Valsamakis et al., 2022). The transcriptomic responses of several angiosperm species (*A. thaliana, N. attenuata, U. minor,* and *S. dulcamara*) to insect egg deposition allowed a generally applicable gene set enrichment (GAGE) analysis on gene ontology (GO) terms (T. Lortzing et al., 2020). This analysis revealed that the transcriptomic responses of these angiosperm plants to insect egg deposition have about a third of the differentially expressed gene sets in common. These gene sets comprise upregulated genes involved in transcriptional regulation of biological processes like cell death, ROS accumulation, systemic acquired resistance (SAR), endoplasmic reticulum stress, unfolded protein responses, SA signaling, but also signaling by other phytohormones (JA, abscisic acid and ethylene). Sets with downregulated genes related to plant development and cell cycle processes. Notably, the insects that laid eggs on these angiosperms have very different oviposition modes, including deposition of egg clusters and single eggs, oviposition with and without associated leaf damage. Hence, in spite of very different insect species with different oviposition modes, the studied angiosperm species showed a considerable phylogenetically conserved core response to insect eggs (T. Lortzing et al., 2020).

Beyaert et al. (2012) provided first evidence for improved defense against larval feeding damage in *P. sylvestris* after prior egg deposition by *D. pini*. However, no studies addressed so far the phytohormonal and transcriptomic changes of a gymnosperm species in response to insect eggs and subsequent larval feeding on needles.

1.1.4 Preparation for improved plant defenses against insect egg deposition

Since plants can defend themselves not only against the feeding stages of insects, but also against the eggs, the question arises whether there are also cues "warning" of impending egg deposition and preparing the defenses against insect eggs.

Mating occurs prior to oviposition and is in many insect species known to be associated with sex pheromones (Yew & Chung, 2015; Rizvi et al., 2021). Hence, insect sex pheromones might be a reliable "warning" cue of impending egg deposition. Many sex pheromones are volatile compounds, attracting mates over some distance.

The first proof that a plant can indeed respond to a volatile released from an insect has been provided by a study of (Helms et al., 2013). Their study showed that goldenrod plants (*Solidago altissima*) that had been exposed to a blend of volatiles released from males of the gallfly *Eurosta solidaginis* changed their defenses. Volatile-exposed goldenrod plants received fewer ovipunctures by female *E. solidaginis* females, showed less feeding damage by a goldenrod leaf beetle, and accumulated higher concentrations of JA when damaged by the leaf beetle. A later study (Helms et al., 2017) identified a spiroacetal (*E,S*-conophthorin) as the active compound in the male gallfly's volatile blend. Goldenrod plants exposed to this volatile compound showed a stronger increase in JA concentration and a reduction in tissue loss upon feeding by the goldenrod leaf beetle than plants that had not been exposed to this spiroacetal. The male gallflies, which emerge prior to their females from the pupal stage, release this compound after emergence when sitting on the upper leaves of a goldenrod plant (Helms et al., 2013).

Plants are known to respond to a wide range of volatile compounds, among them the volatile phytohormone ethylene and the above-mentioned OIVPs and HIPVs (Ali et al., 2013; Binder, 2020; Pashalidou et al., 2020; Sugimoto et al., 2021). This plant sensitivity to volatile organic compounds suggests the hypothesis that other plant species than goldenrod have also evolved the ability to respond to volatile sex pheromones of other insect species than of the goldenrod gall fly.

Pinus sylvestris was shown to respond to exposure of the sex pheromone of *D. pini* females by improved defense against *D. pini* eggs (Bittner, 2018). Since ROS accumulation is known as a defense response of pine to *D. pini* eggs (Bittner et al., 2017), the question arised whether the sex pheromone-mediated improved pine defense is associated with enhanced ROS accumulation.

1.2 Elicitors of plant defense against insect infestation and their perception

In order to activate defense responses specifically targeting insect eggs or the feeding stages, plants need to perceive compounds, which reveal the type of infestation and elicit defensive responses (Hilker & Meiners, 2010). Once deposited on the plant surface, the insect-derived elicitors of plant defense can initiate plant responses in various ways, in addition to damage-associated molecular patterns (DAMPs) of the plant and endogenous, damage-induced plant peptides (Reymond, 2021) .

Insect-derived elicitors are also referred to as defense-eliciting, herbivory-associated molecular patterns (HAMPS) and oviposition-associated molecular patterns (OVAMPS or EAMPs, egg-associated molecular patterns). Such molecular patterns, which have also intensively been studied especially in phytopathogens, bind to pattern recognition receptors (PRR) on the plant cell surface, thereby changing the plasma transmembrane potential and initiating a Ca^{2+}-mediated response cascade (J. D. G. Jones & Dangl, 2006). The elucidation and identification of PRRs relevant for plant perception of feeding insects has made progress during the last decades, especially in recent years (e.g. Truitt et al., 2004; Gilardoni et al., 2011; Hu et al., 2018; Steinbrenner et al., 2020; Sun et al., 2020; Uemura et al., 2020); these studies point to or identify PRRs responding to feeding insects or their HAMPs as leucine-rich repeat (LRR) receptor-like proteins (RLPs), LRR receptor(-like) kinases (RKs, RLKs), or lectin RKs/RLKs (Sun et al., 2020). Similarly, research on how plants detect insect eggs is progressing in recent years. A study by Tamiru et al. (2020) suggests that an LRR-RLK is also relevant for perception of insect egg deposition, in this case for triggering indirect defenses of *Zea mays* against egg depositions by the moth *Chilo* (Tamiru et al., 2020). Other studies on receptor kinases responding to insect eggs show that lectin-receptor kinases (LecRk1.8, LECRk-I.1) control the formation of HR-like symptoms and SA accumulation in *A. thaliana* in response to *P. brassicae* eggs (Gouhier-Darimont et al., 2019; Stahl et al., 2020; Groux et al., 2021). A study by Bassetti et al. (2022) also points to a lectin-receptor kinase involved in formation of HR-like symptoms in *B. nigra* plants responding to *P. brassicae* eggs.

Other ways to elicit a plant defense response are PRR independent. For example, glucose oxidase, an elicitor isolated from lepidopteran larval regurgitate, is oxidizing glucose, thereby producing hydrogen peroxide, which initiates a response cascade (Louis et al., 2013). Insect-associated compounds with amphiphilic character, as e.g. the fatty acid – amino acid conjugates isolated as elicitors in the regurgitate of several caterpillars, may disturb the plasmamembrane architecture and integrity, thereby initiating a change in the plasmamembrane potential (Spiteller et al., 2000; Erb & Reymond, 2019).

Once having entered the plant cell, plant responses to insect infestation may be triggered by intracellularly perceived effectors (Kourelis & van der Hoorn, 2018; Shih et al., 2023). A recent study by Bassetti et al. (2023) identified a cluster of *B. nigra* genes encoding intracellular receptor proteins, a so-called TIR-NBS-LRR (TNLs) gene cluster. TNLs have been described as plant receptors of effectors released by phytopathogens (DeYoung & Innes, 2006). A model by Bassetti et al. (2023) suggests in analogy to effector triggered immunity (ETI) against phytopathogens that candidate genes of this cluster might intracellularly detect effectors associated with *P. brassicae* eggs, which then initiate the formation of HR-like symptoms (for ETI, see e.g. Ngou et al., 2021; Yuan et al., 2021). Hence, both plant

surface receptors, i.e. the above-mentioned LecRks, and intracellular receptors might be involved in formation of HR-like symptoms in Brassicaceae in response to *P. brassicae* eggs.

Elicitors of plant defenses against the feeding insect stages were detected mainly in the oral secretion or regurgitate, which a feeding insect inevitably releases into the plant wound. But insect frass was also found to contain defense elicitors. These defense-eliciting compounds are especially fatty acid – amino acid conjugates and various insect enzymes, but also peptide fragments of plant enzymes (Acevedo et al., 2015; A. C. Jones et al., 2022).

Elicitors associated with insect eggs and their deposition belong to various substance classes and are of different origin (Hilker & Fatouros, 2015; Stahl et al., 2023). This diversity might help the plant to identify the attacking insect species. For example, females of *P*. *rapae* release indole to the leaf during oviposition; this compound is produced by male *P. rapae* and transferred to the female as an anti-aphrodisiac pheromone; the female stores this compound in their accessory reproductive gland and releases it with the egg deposition (Fatouros et al., 2009). Similarly, benzyl cyanide is the anti-aphrodisiac pheromone of *P. brassicae* and is also transferred from males to females; also this pierid species releases the pheromone to the site of egg deposition (Fatouros et al., 2008). Both indole and benzyl cyanide induce indirect plant defenses, which involve changes in plant volatile emissions or plant surface chemistry that render the plant attractive to egg parasitoids. But plants do not necessarily rely on a single elicitor compound. Phosphatidylcholine derivatives isolated from *P. brassicae* eggs activate plant immune responses when applied onto leaves (Stahl et al., 2020). Phosphatidylcholine derivatives were also shown to elicit the production of an ovicidal compound (benzyl benzoate) in rice plants when *Sogatella furcifera* lays its eggs onto rice leaves; in this case, the phosphatidylcholines were isolated from gravid *S. furcifera* females (Yang et al., 2013). Other elicitors of plant defenses against insect eggs, the so-called bruchins, were also obtained by extraction of gravid insect females; *Bruchus pisorum* and *Callosobruchus maculatus* produce these bruchins, which are long-chain C_{22}-C_{24} diols mono- or diesterified with hydroxypropanoic acid (Doss et al., 2000); these compounds induce the formation of neoplasms in response to egg deposition on pea pods. Furthermore, also proteinaceous compounds associated with insect egg deposition can elicit plant defenses against insect eggs. An N-terminal subunit of vitellogenin present on the surface of *Nilaparvata lugens* eggs was shown to induce defenses of *Oryza sativa;* fewer *N. lugens* eggs hatched when they were laid on plants treated with the vitellogenin elicitor; furthermore, rice plants treated with the vitellogenin elicitor changed their emission of volatiles, thus rendering the rice plant odor attractive to egg parasitoids (Zeng et al., 2023). Other plant defense-eliciting, proteinaceous compounds were found in the oviduct secretions of the elm leaf beetle *Xanthogaleruca luteola* (Meiners & Hilker, 2000) and *D. pini* (Hilker et al., 2005). These oviduct secretions are surrounding the eggs when laid on plant tissue (Hilker & Fatouros, 2015).

An elicitor of *P. sylvestris* defense against *D. pini* eggs is known to be located in the oviduct secretion, which is released with the eggs onto pine needles (Hilker et al., 2005). Bittner (2018) identified the sequence of the proteinaceous elicitor and named it diprionin. However, it remained unknown prior to this thesis whether heterologously expressed diprionin can indeed elicit similar transcriptional responses of defense-related genes as natural egg deposition by *D. pini* does.

1.3 Biology of the studied species *Pinus sylvestris* and *Diprion pini*

As outlined above, knowledge about the chemical and molecular basis of defense responses of gymnosperms to insect oviposition is limited. Therefore, my doctoral thesis focuses on studying interactions between *P. sylvestris* and the pine sawfly *D. pini.* The chemoecological knowledge that was available about these species provided the basis for the research questions addressed in my thesis (compare section 1.4). General aspects of the biology of the studied "players" of my thesis are outlined in the following.

The gymnosperm *P. sylvestris* is the most distributed *Pinus* genus in the world and the second most distributed conifer tree in the northern hemisphere (Carlisle & Brown, 1968; Judd et al., 2004; Durrant et al., 2016). It grows in central and eastern Europe, in almost all regions of Scandinavia and even occurs in Russia and other areas of Asia, especially Siberia. Systematically, Scots pine is a member of the conifer lineage among the gymnosperms, which comprise in addition to the conifers the following three further living lineages: cycads, gnetophytes, and Gingko (Mathews, 2009). In contrast to *A. thaliana, P. sylvestris* cells harbor a high number of chromosomes (2n=24; *A. thaliana:* 2n = 10). Transcriptomic data of several pine species have been made available during the last decade (e.g. Fox et al., 2018; Visser et al., 2018; Gao et al., 2022). Niu et al. (2022) recently decoded the genome of Chinese pine (*P. tabuliformis*); the presented chromosome-level assembly revealed that the large genome size of this species is due to huge intergenic regions and long introns with many transposable elements. This perennial evergreen tree (Carlisle & Brown, 1968) is also economically important for the timber industry because of the suitable properties of its wood (Kozakiewicz et al., 2020). Various herbivorous insect species are specialized to use Scots pine as their host plant (Mumm & Hilker, 2006). Pine trees are especially known for producing a huge variety of volatile and non-volatile terpenes (Kopaczyk et al., 2020), which may serve the specialized herbivores for host location, but can also act as defensive devices (Mumm & Hilker, 2006).

The hymenopteran species *D. pini* is one of the specialists on *P. sylvestris* and is rarely found on other *Pinus* trees (CABI, 2022). This species has been found all over in Europe, Asia (Russia and Turkey), and northern Africa. Systematically, *D. pini* is one of the about 140 species of the Diprionidae (Taeger et al.,

2018), a family belonging to the paraphyletic taxon "Symphyta" among the Hymenoptera; modern taxonomy refers to the Symphyta also as "Non-Apocrita", thereby opposing this taxon to the other large hymenopteran taxon Apocrita (e.g. ants, bees, wasps). A recent phylogenetic tree of Hymenoptera (Apocrita and "Symphyta") has been provided by Peters et al. (2017). Knowledge about the chemoecology of "Symphyta" was recently reviewed by Guignard et al. (2022). The larvae of *D. pini* feed gregariously on pine needles (Barre et al., 2003); adult males and females do not feed. During mass outbreaks, the larvae can defoliate huge areas (Lyytikäinen-Saarenmaa & Tomppo, 2002). Larvae defend themselves against antagonists by releasing foregut contents upon attack. This foregut content is stored in large foregut pouches, which have been described in detail for a close relative of *D. pini,* i.e. for *Neodiprion abietis* (Lucarotti et al., 2011). The pouches contain pine terpenes, which are very odorous and sticky, thus repelling antagonists (Eisner et al., 1974). The larvae also release their foregut content when disturbed by conspecific adults, thereby repelling the adults. Thus, the release of oral fluid also serves prevention of intraspecific competition (Eisner et al., 1974; Hilker & Weitzel, 1991). Mass outbreaks of *D. pini* are due to high numbers of eggs laid by females, the annual early larval hatching and the ability to have more than one generation per year under optimal conditions (CABI, 2022). Additionally, *D. pini* is able to reproduce parthenogenetically. Prior to mating, females release sex pheromones to attract the males. The sex pheromone components are *(*2*S*,3*R*,7*R)*-3,7-dimethyl-2-tridecanyl acetate and propionate (Bergström et al., 1995; Anderbrant et al., 2005). After mating, females lay their eggs in rows into pine needles. Therefore, they use their saw-like ovipositor to slit the needle open; they lay two to twenty eggs into a needle. The eggs are embedded in secretion from the oviduct; the slit needle with the egg row is covered by a secretion from the female's accessory reproductive gland; this secretion hardens (Hilker et al., 2002; CABI, 2022). The severe damaging associated with the oviposition of *D. pini* differs from the oviposition mode of many other insect species, which either only slightly harm the plant by removing the epidermal cell layer (Meiners & Hilker, 2000) or just glue their eggs on the plant surface by a secretion, as is known for e.g. many lepidopteran species.

1.4 Interactions between *P. sylvestris* and *D. pini* eggs: Gaps in knowledge and research questions addressed in this thesis

Responses of *P. sylvestris* to *D. pini* eggs have so far mainly been studied from a chemoecological perspective, leaving biochemical and molecular questions open, as will be outlined here.

Chemoecological studies revealed that the *D. pini* sex pheromone components act as kairomones for an egg parasitoid that is specialized on *D. pini* eggs. The eulophid parasitic wasp *Closterocerus ruforum*

is attracted by the sex pheromone components; only those stereoisomers of the sex pheromone are attractive to this egg parasitoid, which are also attractive to *D. pini* males (Hilker et al., 2000). While the egg parasitoid and *D. pini* males are known to respond to the sex pheromones, it has been unknown prior to this thesis whether *P. sylvestris* responds to these compounds and subsequent egg deposition by enhanced accumulation of hydrogen peroxide. Pine shows accumulation of hydrogen peroxide and hypersensitive-like symptoms in response to egg deposition (Bittner et al., 2017), which is a typical defense response to insect eggs (compare section 1.2). My first project (**Chapter 2**) aimed to elucidate whether *P. sylvestris* amplifies this hydrogen peroxide accumulation for improved defenses against *D. pini* eggs after prior exposure to the *D. pini* sex pheromones. In a laboratory set-up, I exposed small pine trees to the synthetic sex pheromone components of *D. pini,* i.e. to *(*2*S*,3*R*,7*R)*-3,7-dimethyl-2-tridecanyl acetate and propionate (Bergström et al., 1995; Anderbrant et al., 2005), which were kindly provided by Ole Anderbrant, Lund University, Sweden. The egg survival rates, water, and hydrogen peroxide contents were determined in pheromone-exposed and unexposed plants. A previous study determined the number of larvae hatching per egg row laid on pheromone-exposed and non-exposed pine trees (Bittner et al., 2018). In addition to this known egg survival rate per egg row, I recorded the egg survival rate from the total actual number of laid eggs on pheromone-exposed and non-exposed pine trees. The egg survival rate on non-exposed trees provided information about how efficiently a previously untreated pine can directly defend against the eggs. Recording the survival rate of all eggs laid on the pheromone-exposed trees was done to address the question whether the exposure of pine to the pheromones prior to oviposition can even improve (prime) the pine's direct defense against the eggs. Additionally, expression of further defense-related genes beyond the already investigated ones by Bittner (2018) were analyzed. A comparison of these data was supposed to further elucidate how pine exposure to *D. pini* sex pheromones leads to improved defense against *D. pini* eggs in the tree. Furthermore, I tested the electrophysiological response of male and female *D. pini* to the synthetic sex pheromone, thus testing their physiological pheromone responsiveness.

It has been shown prior to this thesis that *P. sylvestris* can take *D. pini* eggs as "warning" of impending larval feeding. Egg-laden pine can intensify its defenses against *D. pini* larvae (Beyaert et al., 2012). Performance of larvae on egg-laden pine is considerably lower than on egg-free pine. This defense effect on the herbivore's larval stage is even traceable in the adult stage of the sawfly. Females, which spent their juvenile development on egg-laden pine, show a lower fecundity than those, which developed on egg-free pine (Beyaert et al., 2012). The phytohormonal and molecular mechanisms of this egg-mediated pine defense against *D. pini* were unknown prior to this thesis. Therefore, my second project (**Chapter 3**) was conducted to reveal defense- and signaling pathways of pine in response to *D. pini* eggs and subsequent larval feeding. I conducted a transcriptome analysis of pine trees, which experienced (i) *D. pini* oviposition, (ii) larval feeding, or (iii) oviposition and subsequent larval feeding.

Additionally, gene-ontology term- and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analyses of the obtained transcriptomes of the differently treated trees were expected to provide a deeper insight into how a gymnosperm species responds to eggs and subsequent larval feeding. Furthermore, a phytohormone analysis elucidated which phytohormones are involved in egg-mediated effects on pine defenses against feeding larvae. The obtained results were compared with current knowledge about the phytohormonal and transcriptomic responses of angiosperm plants to insect eggs and subsequent larval feeding.

The studies on pine interactions with *D. pini* eggs clearly showed that pine can respond to these eggs by direct defenses targeting the eggs and by improved direct defenses targeting the larvae (see chapter 3). In addition, pine can respond to *D. pini* eggs by mobilizing indirect defenses. The above-mentioned egg parasitoid *C. ruforum* is not only attracted by *D. pini* sex pheromones, but also by egg-induced plant volatiles. Egg deposition by *D. pini* induces the emission of enhanced quantities of (*E*)-β-farnesene (Hilker et al., 2002). When the parasitoid perceives the enhanced quantities of this sesquiterpene in combination with the (non-induced) quantities of four other pine terpenes, it is attracted to this odor (Beyaert et al., 2010). The parasitoid-attracting odor of pine is inducible by treatment of pine needles with just the oviduct secretion that surrounds *D. pini* eggs and is thus in immediate contact with the pine cells in the slit needle (Hilker et al., 2002). Further studies indicated that a small protein in the oviduct secretion elicits the attractive odor (Hilker et al., 2005). Bittner (2018) identified the sequence of this protein and named it diprionin, as mentioned above. In the framework of the third project (**Chapter 4**), I heterologously expressed it in insect cells and applied it into slits of pine needles. Thereafter, I determined how selected pine defense genes respond to this treatment and compared this response with the one of pine defense genes to natural egg deposition.

In summary, by studying interactions between *P. sylvestris* and the sawfly *D. pini,* my thesis addressed the following main questions (Figure 1):

- Does exposure of *P. sylvestris* to the sex pheromone of *D. pini* improve pine defenses against the sawfly's eggs? Does exposure of pine to the pheromone amplify the hydrogen peroxide accumulation that pine shows after *D. pini* egg deposition? Does it furthermore amplify the expression of defense-related genes? (**Chapter 2**)
- How does the transcriptome of *P. sylvestris* respond to *D. pini* oviposition and subsequent larval feeding? How do phytohormones of a gymnosperm species change their concentrations when responding to insect oviposition and subsequent larval feeding? (**Chapter 3)**

- Does heterologously expressed diprionin, the sawfly egg-associated elicitor of pine defenses against eggs, induce similar changes in transcript levels of defense-related pine genes as *D. pini* egg deposition does? (**Chapter 4**)

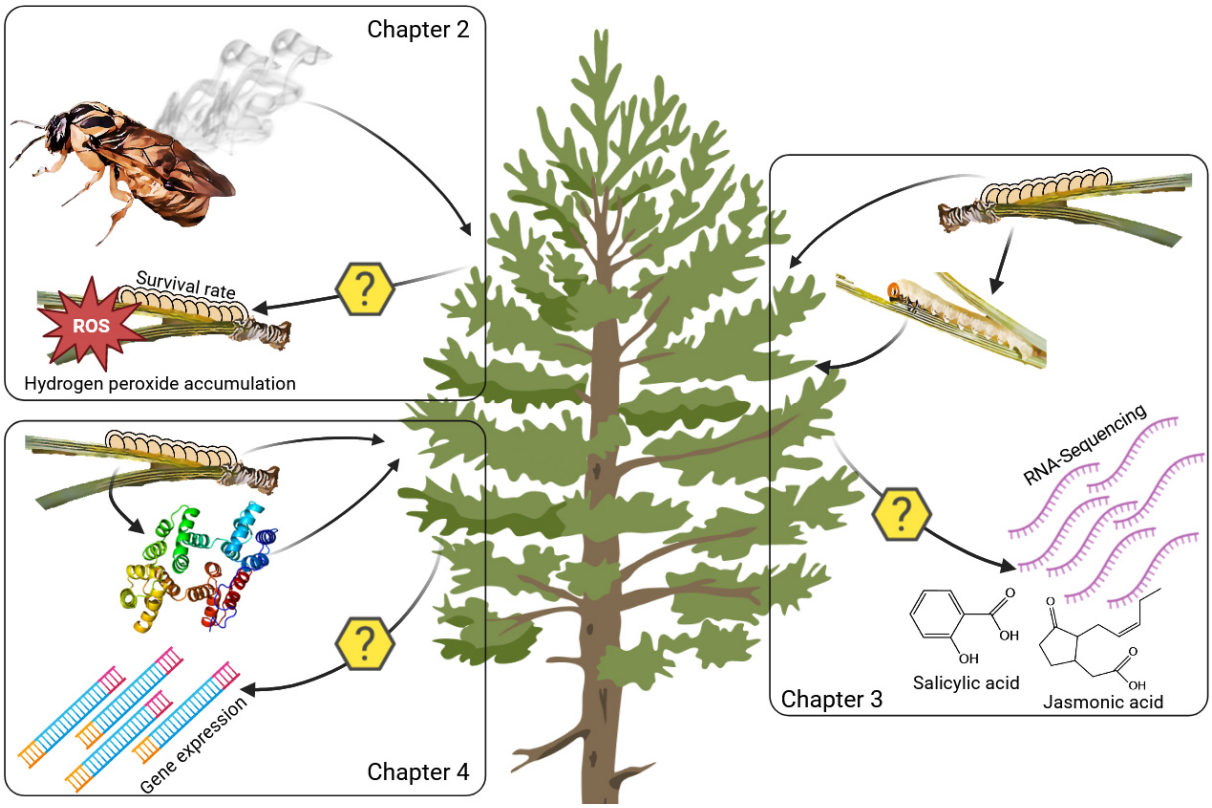

Figure 1. Schematic visualization of the main questions addressed in this thesis. Chapter 2 (upper left frame) addresses the question if pine exposure to sawfly sex pheromones affects the needle ROS concentration and the egg survival rates. Chapter 3 (right frame) aims to elucidate if and how oviposition, larval feeding and the combination of both affect the pine transcriptome and pine phytohormone concentrations. Chapter 4 (lower left frame) addresses the question how application of the egg-associated defense elicitor affects the expression of pine defense-related genes. Created with BioRender.com.

A summarizing discussion of my results is presented in **Chapter 5.** Special emphasis is paid to a comparison of the two types of defenses described in Chapter 2 and 3 (pheromone-mediated pine defense against the eggs and egg-mediated pine defense against the larvae). Furthermore, the role of the phytohormones JA and SA will be considered with respect to pine defenses against sawfly eggs and larvae. Moreover, the plant's perception of the sawfly egg-associated elicitor diprionin studied in Chapter 4 will be discussed in context with plant responses to other known insect-associated elicitors of plant defenses.

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CHAPTER 2

Defense of Scots pine against sawfly eggs (*Diprion pini*) is primed by exposure to sawfly sex pheromones

Defense of Scots pine against sawfly eggs (*Diprion pini*) is primed by exposure to sawfly sex pheromones

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Plants respond to insect infestation with defenses targeting insect eggs on their leaves and the feeding insects. Upon perceiving cues indicating imminent herbivory, such as damage-induced leaf odors emitted by neighboring plants, they are able to prime their defenses against feeding insects. Yet it remains unknown whether plants can amplify their defenses against insect eggs by responding to cues indicating imminent egg deposition. Here, we tested the hypothesis that a plant strengthens its defenses against insect eggs by responding to insect sex pheromones. Our study shows that preexposure of *Pinus sylvestris* to pine sawfly sex pheromones reduces the survival rate of subsequently laid sawfly eggs. Exposure to pheromones does not significantly affect the pine needle water content, but results in increased needle hydrogen peroxide concentrations and increased expression of defense-related pine genes such as *SOD* (superoxide dismutase), *LOX* (lipoxygenase), *PAL* (phenylalanine ammonia lyase), and *PR-1* (pathogenesis related protein 1) after egg deposition. These results support our hypothesis that plant responses to sex pheromones emitted by an herbivorous insect can boost plant defensive responses to insect egg deposition, thus highlighting the ability of a plant to mobilize its defenses very early against an initial phase of insect attack, the egg deposition.

priming | induced plant defense | insect oviposition | *Diprion pini* | hydrogen peroxide

Plants can respond to a wide array of volatile compounds released from microbes, plants, and insects (1–4). Plant responses to odors indicative of biotic stress (pathogens, herbivores) enable them to improve their stress management (5).

Volatile compounds released from damaged plants provide cues indicating herbivory. The perception of herbivory-induced leaf volatiles primes the defensive responses of undamaged plants to imminent herbivory, thus rendering their antiherbivore defense more potent (6–9). Priming of plant defense is an effective way to improve infestation-inducible defense against herbivores (10, 11).

Priming of inducible plant defenses against herbivory is not only mediated by plant volatiles. Plants can also take insect-released volatile compounds as an indicator of impending herbivory, as demonstrated in an exciting study of goldenrod plants exposed to a putative male gall fly sex pheromone, (*E*,*S*)-conophthorin, a spiroacetal (3, 4). Exposure of goldenrod to conophthorin primes the plant's defenses against herbivory by insects specialized on goldenrod plants, thus suggesting a coevolved signal–response pattern.

Priming of inducible plant defenses against insect eggs has thus far not been studied, although insect egg depositions can induce changes in the plant´s primary metabolism (12) as well as defensive plant responses capable of killing those same eggs (13). For example, several plant species form necrotic tissue at the site of egg deposition; this response may result in desiccation of the eggs and/or their detachment from leaves (13, 14). Egg-induced growth of novel plant tissue can squeeze and thus kill the eggs (15). Plants can also produce ovicidal compounds in response to egg deposition (16). In addition, many plant species have been shown to change their leaf odor in response to insect egg deposition; the egg-induced leaf odor attracts parasitic wasps that kill the eggs (17). Since insect mating precedes egg deposition, cues like insect sex pheromones might serve as reliable stimuli indicative of imminent egg deposition, thus eliciting plant responses harming the eggs.

Here we present a study testing the hypothesis that exposure of a plant to insect sex pheromones primes the plant's defensive response to insect eggs. We used young *Pinus sylvestris* trees and the pine sawfly *Diprion pini* to test this hypothesis in the laboratory. These plant and insect species are well suited as a model for several reasons. Conifer forests in the Northern Hemisphere are frequently heavily damaged by sawfly larvae feeding gregariously upon pine needles. Scots pine defends itself against *D. pini* eggs by accumulating reactive oxygen species (ROS) (18) and by releasing egg-induced needle volatiles that attract egg parasitoids (13). The male-attracting sex pheromone components of *D. pini* females, (2*S*,3*R*,7*R*)-3,7-dimethyl-2-tridecanyl acetate and propionate, have been intensively studied and are synthetically available (19).

Significance

Plant defenses against herbivorous insects can target the feeding stages and the egg stage. Feeding-induced plant defenses are known to be primed by cues indicating imminent infestation, including sex pheromones. However, priming of egg-induced plant defenses has been unknown so far. Therefore, we studied whether a plant's response to insect sex pheromones, which might indicate imminent egg depositions, primes defenses against the eggs. Indeed, exposure of pine to the sex pheromones of an herbivorous sawfly primes the tree's defense against sawfly eggs. The priming effect results in enhanced egg mortality, enhanced accumulation of hydrogen peroxide in egg-laden needles, and differential expression of several defense-related pine genes. These findings open up exciting research perspectives in plant protection from insect infestation.

Author contributions: N.B., A.A.-C., and M.H. designed research; N.B., J.H., and A.A.-C. performed research; O.A. contributed new reagents/analytic tools; N.B., J.H., A.A.-C., and M.H. analyzed data; and N.B., J.H., A.A.-C., O.A., and M.H. wrote the paper.

The authors declare no competing interest.

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Data deposition: Sequences of *Pinus sylvestris* PCR products as well as the template accession numbers in Genbank for the primer design and the annotation information referred to in this paper have been deposited at the repository of the Max-Planck-Institute for Molecular Plant Physiology with open access at https://primedb.mpimp-golm.mpg.de/index.html?sid=reviewer&pid=4afbcf5cea03476a56b57e44eb58e261.

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Our results show that exposure of pine to the sex pheromones of a female sawfly primes the tree's defenses against sawfly eggs and results in enhanced egg mortality, enhanced accumulation of hydrogen peroxide in pine needles, and differential regulation of defense-related pine genes. These results provide evidence that plants are capable of strengthening their defense against a very early step of insect infestation, the egg deposition, by responding to cues preceding egg depositions.

Results and Discussion

Survival Rates of Sawfly Eggs Are Lower on Pine Previously Exposed to Sawfly Sex Pheromones. We compared survival rates of *D. pini* eggs that have been deposited on small, 3-y-old *P. sylvestris* trees previously exposed for 1 d to *D. pini* sex pheromones or, as a control, to the pheromone solvent hexane. After 24 h of pheromone (or hexane) exposure, *D. pini* females were allowed to oviposit for 1 d on the needles of these trees. A *D. pini* female inserts her eggs in a row (about 15 eggs per row) into a pine needle. After 12 to 14 d (egg incubation time), the larvae hatch from surviving eggs. We exposed the trees to a pheromone dose comparable to that which pine trees are exposed to during a mass *D. pini* outbreak (*SI Appendix*, Table S1). Exposure of pine trees to the pheromones significantly affected the pines' resistance against sawfly eggs. The mean (±SE) survival rate of eggs on trees previously exposed to the pheromone (40.07 ± 2.89%) was significantly lower than the survival rate of eggs on untreated controls (60.37 ± 10.25%) and on trees exposed to the solvent hexane (59.65 ± 4.35%) (Fig. 1 and *SI Appendix*, Tables S2 and S3). The hexane treatment had no impact on the egg survival rate. This may be due to the high volatility of this solvent. Prior to treatment, the dispensers with hexane and the dispensers with pheromone dissolved in hexane were kept for 30 min in a fume cupboard, where the solvent evaporated; thereafter, pine trees were exposed to the dispensers for 24 h. The low survival rate of *D. pini* eggs on untreated trees in the absence of natural enemies and at favorable abiotic conditions indicates that *P. sylvestris* can directly defend itself against the eggs, as also suggested by an earlier study (18). The results here show that preexposure of pine to *D. pini* sex pheromones results in further reduction of the sawfly´s egg survival rate.

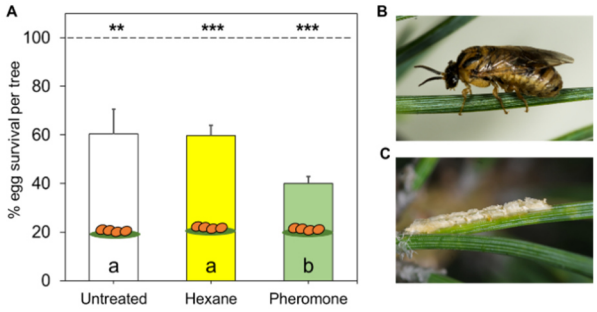

Fig. 1. Impact of exposure of *P. sylvestris* to sex pheromones of pine sawflies (*D. pini*) on sawfly egg survival rates. (*A*) Percentage (mean + SE) survival of *D. pini* eggs on untreated pine trees ($n = 6$), pine trees exposed to hexane ($n = 8$), and pine trees exposed to the pheromones (dissolved in hexane) ($n = 8$) for 24 h prior to egg deposition by 2 females per tree. Total number of eggs on untreated trees is 100% = 915 (mean number of eggs per tree ± SE: 152.5 ± 20.81), on hexane-treated trees is 100% = 1170 (mean ± SE: 146.3 ± 11.48), and on pheromone-treated trees is 100% = 858 (mean ± SE: 107.3 ± 11.76). Difference between numbers of eggs laid on the differently treated trees is not significant (n.s.) (ANOVA). Difference between numbers of laid eggs and hatched eggs within a treatment: $^{**}P < 0.01$; $^{***}P < 0.001$ (paired *t* tests). Different letters in bars indicate significant differences ($P < 0.05$) in survival rates among treatments (ANOVA followed by multiple pairwise *t* tests and a Benjamini–Hochberg *P* value correction) (compare *SI Appendix*, Tables S2 and S3). (*B*) *D. pini* female on *P. sylvestris*. (*C*) Egg row of *D. pini* on a pine needle.

Pheromone Exposure Promotes Hydrogen Peroxide Accumulation in Egg-Laden Pine Needles. That preexposure of pines to sawfly sex pheromones significantly reduced survival of *D. pini* eggs raised the question of what causes this ecological effect. At the immediate interface between insect egg and plant, environmental humidity and leaf hydrogen peroxide concentrations are known to affect development of insect eggs and their survival (18, 20, 21). The humidity to which an insect egg is exposed is not only determined by air humidity but also by leaf water content. An increase in leaf hydrogen peroxide concentration and accumulation of other ROS in response to insect eggs may result in formation of necrotic plant tissue (22). This plant response provides an environment in which eggs of several insect species have been shown to suffer increased mortality (20, 23). Formation of necrotic tissue has been described for pines in response to *D. pini* egg deposition (18), but whether ROS accumulation in response to *D. pini* eggs is amplified by prior exposure of pines to pheromones is unknown.

Therefore, we investigated whether exposure of pine trees to pheromones 1) reduces the pine needle water content, thus possibly resulting in desiccation of the eggs, or 2) enhances the concentration of pine needle hydrogen peroxide concentrations, thus directly harming the eggs or resulting in amplified plant defense signaling (24). The needle water and hydrogen peroxide contents were analyzed 2 and 12 d after pheromone exposure, that is, 1 and 11 d, respectively, after egg deposition (Fig. 2 and *SI Appendix*, Fig. S1).

The water content of pine needles exposed to the different treatments was similar, and no significant differences between treatments were detected at any of the 2 measurement time points after pheromone exposure (Fig. 2*A* and *SI Appendix*, Fig. S1*A* and Table S2).

Hydrogen peroxide accumulated in egg-laden needles at the end of egg incubation time (i.e., 11 d after egg deposition) (Fig. 2*B*). This egg-induced accumulation of hydrogen peroxide was significantly enhanced by the pheromone treatment 12 d earlier. In contrast, the pheromone treatment had no effect on the needle hydrogen peroxide concentration of the egg-free pines. Nor did exposure of the pines to hexane affect the needle hydrogen peroxide concentration (Fig. 2*B* and *SI Appendix*, Table S2). No induction of hydrogen peroxide accumulation was detectable shortly (1 d) after egg deposition. Nor did a preceding pheromone exposure affect the hydrogen peroxide concentration of pine needles shortly after egg deposition (*SI Appendix*, Fig. S1*B* and Table S2).

Thus, the pheromone-mediated strengthening of pine resistance against sawfly eggs is associated with enhanced accumulation of hydrogen peroxide in the pine needles, which becomes evident at the end of the egg incubation time. The enhanced hydrogen peroxide concentration might directly exert a detrimental effect on the eggs (20) and/or serve as an intensified early defense signal (24, 25). Several studies have shown an increase in plant hydrogen peroxide concentrations in response to wounding or herbivory (26–30) and to insect egg deposition (18, 20, 22). While a wound-induced increase in hydrogen peroxide concentration is known to be detectable almost immediately in response to herbivory (e.g., refs. 27 and 28), egg-induced increases have been observed only several days (22) after the egg treatment or at the end of the egg incubation time (18, 20). Here we show that exposure of a plant to a female insect sex pheromone (Fig. 2*C*), that is, an environmental cue indicating impending insect egg deposition, can even further promote the (egg) infestation-induced hydrogen peroxide accumulation.

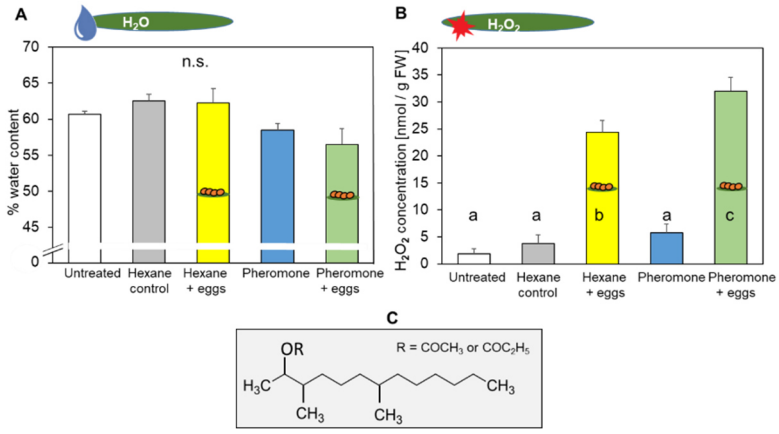

Fig. 2. (*A*) Water contents and (*B*) hydrogen peroxide concentrations of *P. sylvestris* after exposure to sawfly sex pheromones and subsequent egg deposition. Measurements were conducted 12 d after pheromone exposure, that is, 11 d after egg deposition, at the end of the egg incubation period. Water concentrations and hydrogen peroxide concentrations were determined in pine needles from untreated trees, from trees exposed to the solvent hexane (without eggs: hexane control; with eggs: hexane + eggs), and from trees exposed to the pheromones (dissolved in hexane) (without eggs: pheromone; with eggs: pheromone + eggs). Means + SE of water contents and hydrogen peroxide concentrations are given ($n = 5$ for water content untreated; $n = 8$ for all other treatments). All data were evaluated by ANOVA and, for the hydrogen peroxide concentrations, by multiple pairwise *t* tests and a Benjamini–Hochberg *P* value correction (different lowercase letters in bars indicate significant differences at $P < 0.01$) (compare *SI Appendix*, Table S2). (*C*) Chemical structure of *D. pini* sex pheromone components.

Pheromone Exposure Results in Changes of Expression of Defense-Related Pine Genes. To figure out whether, and if so how, exposure of pines to sawfly sex pheromones affects expression of defense-related pine genes, we ran qPCR analyses of needles from trees treated in different ways (Table 1 and *SI Appendix*, Table S4). Samples were harvested 2 and 12 d after pheromone or hexane exposure to differentiate between early and late treatment effects. We selected the following genes (for information on sequences, see *SI Appendix*, Tables S5 and S6): *PsRboh* (sequence homolog to a respiratory burst oxidase—plant NADPH oxidase), involved in ROS production; *PsSOD* (superoxide dismutase) encoding an enzyme catalyzing hydrogen peroxide formation; and *PsCAT* (sequence homolog to catalase) and *PsAPX* (sequence homolog to ascorbate peroxidase), both of which are involved in ROS degradation (25). We tested expression levels of a putative lipoxygenase encoding gene (*PsLOX*) initiating the jasmonic acid (JA) pathway (31) and of *PsPDF* putatively encoding a plant defensin, which is inducible by early JA- and ethylene-mediated defense signaling (32). Additionally, we determined transcript levels of *PsPR-1* (sequence homolog to pathogenesis-related protein 1), which is inducible by insect egg depositions on *Arabidopsis thaliana* (22, 33). Because accumulation of phenylpropanoid derivatives is involved in egg-mediated strengthening of antiherbivore defenses in several plant species (33–35), we also determined transcript levels of *PsPAL* encoding a putative phenylalanine ammonia lyase, an enzyme at the entrance of the phenylpropanoid pathway (36). As the hexane treatment did not affect expression of the genes tested (*SI Appendix*, Table S7), we normalized the gene expression levels of all other treatments to those determined for hexane-treated pines.

The pheromone exposure per se affected expression of only 2 of the 8 genes investigated. Shortly (2 d) after sawfly pheromone exposure, expression levels of *PsRboh* were significantly higher. Priming plants for improved resistance against phytopathogens by preexposure to pathogens or to priming chemicals such as β-aminobutyric acid also results in enhanced expression of *RbohD* in *A. thaliana* (37). In contrast to *PsRboh*, none of the other genes showed significantly altered transcript levels at this early time point after treatment. Twelve days after pheromone exposure, expression levels of *PsPR-1* were significantly reduced in pheromone-treated trees.

Sawfly egg deposition without prior exposure of the pines to pheromones affected expression of pine catalase *PsCAT*, whose transcript levels were significantly higher only shortly after egg deposition, but not later. Expression levels of *PsRboh* coding for a putative ROS-generating enzyme and of *PsAPX* coding for a putative hydrogen peroxide detoxifying enzyme were lower at the later sampling time. When trying to relate these data to the hydrogen peroxide concentrations shown in Fig. 2*B*, these findings suggest that the high hydrogen peroxide levels in needles of egg-deposited pine trees are not due to Rboh-mediated production of ROS. This interpretation is in line with a previous study (22), which found no indication that Rboh is involved in hydrogen peroxide accumulation induced by application of butterfly egg extracts on *A. thaliana*. However, reduced degradation of ROS because of reduced availability of the ROS-degrading enzyme PsAPX at the end of the egg incubation period (Table 1) might at least contribute to the high hydrogen peroxide concentrations in egg-laden pine needles.

Interestingly, pheromone treatment followed by egg deposition resulted in enhanced expression of *PsSOD*-encoding superoxide dismutase, which catalyzes the formation of hydrogen peroxide. This result is in line with the higher hydrogen peroxide concentrations in pheromone-treated, egg-deposited needles at the end of the egg incubation period (i.e., 12 d after pheromone treatment). In contrast, expression levels of *PsRboh*, producing superoxide radicals as substrate for SOD, were low at this time point in pheromone-treated, egg-deposited pine needles. Regulation of hydrogen peroxide concentrations may not only be mediated by the expression of genes encoding ROS generating and degrading enzymes. Also, the activation of these enzymes and other factors like a change in the abundance of ROS scavenging secondary compounds might have contributed to hydrogen peroxide accumulation in pheromone-exposed, egg-deposited needles. In *A. thaliana*, ROS accumulation is important for egg-induced up-regulation of *PR-1* (22). In pines, expression of *PsPR-1* was significantly up-regulated in the pheromone-exposed, egg-deposited needles with the highest hydrogen peroxide concentrations. These results suggest that

Table 1. Expression of selected genes of *P. sylvestris* after exposure to sawfly sex pheromones and egg deposition

Time*	Hexane control†	Hexane + eggs	Pheromone	Pheromone + eggs	Significance‡ (*P* values)
PsRboh (Respiratory burst oxidase homolog – plant NADPH oxidase)					
2d	**1.00 ± 0.12^{a}**	**2.27 ± 0.71^{ab}**	**3.07 ± 0.31^{b}**	**1.21 ± 0.13^{a}**	**0.001**
12d	**1.00 ± 0.13^{a}**	**0.19 ± 0.04^{b}**	**0.67 ± 0.20^{ab}**	**0.43 ± 0.16^{b}**	**0.023**
PsSOD (Superoxide dismutase)					
2d	1.00 ± 0.20	1.51 ± 0.32	1.01 ± 0.11	0.85 ± 0.09	0.254
12d	**1.00 ± 0.10^{a}**	**0.70 ± 0.18^{a}**	**1.04 ± 0.14^{a}**	**1.62 ± 0.29^{b}**	**0.026**
PsCAT (Catalase)					
2d	**1.00 ± 0.24^{a}**	**1.77 ± 0.33^{b}**	**1.23 ± 0.16^{ab}**	**0.72 ± 0.12^{a}**	**0.048**
12d	1.00 ± 0.20	0.60 ± 0.13	1.12 ± 0.26	1.36 ± 0.58	0.423
PsAPX (Ascorbate peroxidase)					
2d	1.00 ± 0.09	1.18 ± 0.18	0.89 ± 0.08	0.75 ± 0.06	0.112
12d	**1.00 ± 0.16^{a}**	**0.40 ± 0.06^{b}**	**0.86 ± 0.10^{a}**	**1.26 ± 0.38^{a}**	**0.026**
PsLOX (Lipoxygenase)					
2d	1.00 ± 0.29	0.80 ± 0.18	0.53 ± 0.19	1.00 ± 0.15	0.205
12d	**1.00 ± 0.17^{a}**	**1.30 ± 0.24^{a}**	**0.92 ± 0.16^{a}**	**2.19 ± 0.31^{b}**	**0.015**
PsPDF (Plant defensin)					
2d	1.00 ± 0.42	0.49 ± 0.23	0.52 ± 0.21	0.47 ± 0.08	0.559
12d	1.00 ± 0.27	0.93 ± 0.19	1.13 ± 0.19	1.22 ± 0.20	0.729
PsPR-1 (Pathogenesis related protein 1)					
2d	1.00 ± 0.57	0.27 ± 0.10	0.43 ± 0.24	1.59 ± 1.08	0.384
12d	**1.00 ± 0.29^{a}**	**3.29 ± 1.58^{a}**	**0.27 ± 0.19^{b}**	**6.13 ± 2.40^{c}**	**0.001**
PsPAL (Phenylalanine ammonia lyase)					
2d	1.00 ± 0.30	0.90 ± 0.41	0.73 ± 0.23	0.92 ± 0.18	0.654
12d	**1.00 ± 0.23^{a}**	**1.66 ± 0.67^{a}**	**0.90 ± 0.22^{a}**	**3.43 ± 0.72^{b}**	**0.017**

Relative transcript abundance (mean ± SE) after treatment with pure hexane (the pheromone solvent; hexane control), with hexane and subsequent egg depositions (hexane + eggs), with pheromones dissolved in hexane only (pheromone), or with pheromone and subsequent egg deposition (pheromone + eggs); $n = 5$ to 8 trees for each treatment. Green highlights: transcript abundance significantly decreased as compared to hexane control. Yellow highlights: transcript abundance significantly increased as compared to hexane control. Within a line: numbers in bold with different lowercase letters denote statistical differences ($P \leq 0.05$).

*Days after start of pheromone exposure for 24 h; 2d = directly after 1 d of egg deposition.

†Expression values determined in untreated control trees did not differ from those in the "hexane control" (*SI Appendix*, Table S7).

‡Significance values (*P*) were calculated by Kruskal–Wallis *H* tests (compare *SI Appendix*, Table S4). Significant differences between 2 treatments were evaluated by a post hoc Conover–Iman test with a Benjamini–Hochberg correction for multiple comparisons.

PsPR-1 needs a high ROS level to be significantly up-regulated in response to sawfly egg deposition. In addition to expression of *PsSOD* and *PsPR-1*, expression levels of *PsLOX* were also enhanced in trees preexposed to pheromones and subsequently to egg deposition. Hence, the pheromone preexposure resulted in significant up-regulation of both a salicylic acid (SA)-responsive gene (*PR-1*) (32) and *PsLOX*, a gene involved in JA signaling (31), suggesting that both JA and SA signaling are involved in pheromone-mediated priming of plant defense against insect eggs. Despite numerous studies showing antagonistic interactions between JA- and SA-mediated plant defenses (38), our finding supports the growing evidence that these hormones can also act synergistically in a dose- and kinetics-dependent manner (33, 39). Expression of *PsPDF* was not affected by either treatment. However, *PsPAL* was significantly up-regulated in pheromone-exposed, egg-laden needles when sampled 12 d after pheromone treatment. Phenylalanine ammonia lyase catalyzes the biosynthesis of cinnamic acid, which is a precursor of numerous compounds formed along the phenylpropanoid pathway, among them compounds that contribute to plant cell wall lignification (36), which might impair larval hatching from *D. pini* eggs inserted into needle tissue.

Altogether, exposure of pine trees to sawfly sex pheromones affected the expression of several defense-related genes in a time-dependent manner after egg deposition (Table 1 and *SI Appendix*, Table S4). The combinatory effects of pheromone exposure and subsequent egg deposition on the expression of *PsSOD*, *PsLOX*, *PsPR-1*, and *PsPAL* are striking. Hence, the pheromone exposure primes the enhanced expression of these genes in response to the sawfly's egg deposition.

Sawfly Females Show No Electrophysiological Response to Their Pheromones. Because pines and pine sawflies share an evolutionary history of about 50 million years (40), we asked whether the sawflies have developed a counteractive strategy to cope with the pheromone-mediated defenses of pines against their eggs. If *D. pini* females are able to detect their own pheromones, they might disperse away from sites with high pheromone concentrations, thus avoiding competition for resources, as has been observed in females of some lepidopteran species, which are capable of autodetecting their own male-attracting sex pheromones (41). However, our electroantennogram (EAG) studies did not support this hypothesis. While *D. pini* male antennae clearly responded to both pheromone components, *D. pini* female antennae did not show these responses (Fig. 3 and *SI Appendix*, Table S8). We checked by gas chromatography–mass spectrometry (GC-MS) analyses whether pheromone traces were still left on pheromone-exposed pine needles when the trees were exposed to *D. pini* females for oviposition and, thus, might be perceivable by contact. However, no pheromone traces were detectable on pine needles at the time when females were exposed to the trees. The ability of a *D. pini* female to lay numerous

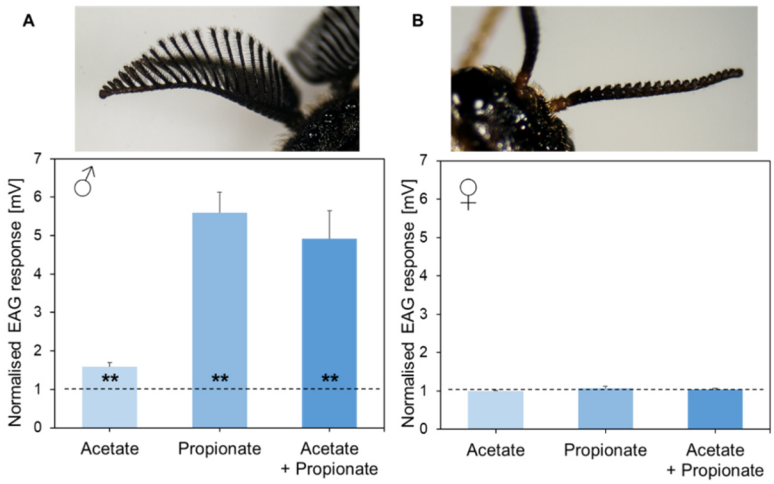

Fig. 3. Electrophysiological response of (*A*) male and (*B*) female antennae of *D. pini* to sex pheromone components. Acetate: (2*S*,3*R*,7*R*)-3,7-dimethyl-2-tridecanyl acetate. Propionate: (2*S*,3*R*,7*R*)-3,7-dimethyl-2-tridecanyl propionate. Data show the responses to 500 ng of each pheromone component tested separately (acetate, propionate), or to a blend of both components (acetate + propionate) with 500 ng of each component, that is, 1,000 ng of pheromone in total. Each test odor (acetate, propionate, or the blend) was offered to $n = 8$ antennae of each sex. Data show means + SE of responses normalized to responses to ambient air and hexane, which were set to value 1 (dashed line). The antennal response to air was almost the same as the one to hexane. Statistical difference of the response to the pheromone from the response to air/hexane was evaluated by the Wilcoxon matched pairs test (**$P < 0.01$) (compare *SI Appendix*, Table S8).

eggs—a hundred eggs or more—might be a means to maintain a critical population density despite the pine's effective defense against them.

Another possibility of counteradaptation to the pheromone-mediated defenses of pine against *D. pini* eggs could be avoidance of oviposition on pheromone-exposed pine because of pheromone-induced oviposition-deterring changes in the needles. Further studies are necessary to investigate this possible counteradaptation. Such a counteradaptation of an herbivorous insect to pheromone-primed defense against herbivory is suggested by results of the study of goldenrod plants exposed to male gall fly emissions; fewer oviposition punctures were detected in male-exposed plants than in control plants (3); however, in this study, the survival of gall fly eggs and gall fly larval feeding upon the previously male-exposed plants could not be recorded. Nevertheless, these gall fly performance parameters are expected to be reduced because exposure of goldenrod plants to male gall fly emissions and their major component, conophthorin, primed the plants for improved defense against feeding damage by other goldenrod-specialized insects than the gall fly (3, 4).

Conclusion. Our study highlights that plant defense against eggs can be primed by an insect's sex pheromone, which reliably indicates an impending very first step of plant infestation, the egg deposition. Hence, these findings show that a plant cannot only be primed for improved defense against impending feeding damage (3–11) but can even prepare its defense against insect eggs, which indicate impending larval feeding damage. Thus, the ability to respond to insect pheromones allows a plant to resist even the very beginnings of insect infestation, the eggs, in a more efficient way. These results suggest that such an early and enhanced defensive response to the eggs might save costs of investment in later feeding-induced defense against hatching larvae, because the greater egg mortality results in reduced abundance of hungry larvae that will hatch from surviving eggs. While constitutive defenses of pine have been shown to trade off with inducible ones and growth rates, possible costs of priming have not been studied yet in pine (42). The costs of priming of plant antiherbivore defenses—measurable by, for example, reduced seed set, aboveground or belowground growth rate, and resistance against other biotic threats like phytopathogens—are considered to depend on various factors, among them the reliability of the priming cue, the presence of priming-sensitive targets, and resource availability and competition (7, 10, 43–45).

Scots pine is shown here to improve its defense against insect eggs by responding to the insect's sex pheromones with changes in the expression of its own defense-related genes and increased accumulation of egg-induced hydrogen peroxide. Our results provide the basis for further research addressing the questions arising here, such as about the specificity of the pine's response to sawfly pheromones, the specificity of the response effects, and the perception of these pheromones. Components similar to the *D. pini* pheromonal components are released by closely related sawfly species. Females of other diprionid genera than *Diprion* emit esters similar to the *D. pini* pheromonal esters, for example, esters with an alcohol component having a longer or shorter chain length than tridecanol or with other methylation patterns of tridecanol than in the *D. pini* pheromonal compounds (46). The sawfly *Diprion jingyuanensis*, a pest of Chinese pine (*Pinus tabulaeformis*), has been shown to be attracted by the *D. pini* propionate pheromonal compound, suggesting that this is also a pheromone of *D. jingyuanensis* (47). Whether the Chinese pine species responds similarly to the pheromone and whether the eggs of *D. jingyuanensis* react similarly to the tree´s defense remains to be addressed in future studies. The lipophilic character of *D. pini* sex pheromones might facilitate direct interactions with the plant's plasma membrane, and thus change transmembrane ion fluxes and initiate early defense signaling (2). In addition to these proximate questions on the mechanisms involved, it will be interesting to address evolutionary ecology aspects of this pheromone-mediated plant defense strategy. If the ability to respond to insect sex pheromones by priming defenses against insect eggs is widespread among plants, this might place some selective pressure on pheromone communication among insects and on their oviposition behaviors. Furthermore, if the priming effect shown by our study is not limited to the species studied here, but extends to other ones relevant in, for example,

agriculture and viticulture, application of the pheromone-mediated mating disruption technology in integrated insect pest management not only will cause negative effects on the fertilization of females due to olfactory insect disorientation (48) but will also reduce survival of insect eggs due to pheromone-primed plant defense.

Materials and Methods

Experimental Organisms. Three-year-old pine trees (*P. sylvestris*) were obtained from a tree nursery (Schlegel & Co.) and used for the experiments. The small trees (45 to 55 cm high) were kept in a greenhouse under long-day conditions (18:6 h light:dark, average temperature 20 °C) until the experiment started.

The pine sawfly *D. pini* was reared in the laboratory on *P. sylvestris*. The sawfly rearing was based on specimens collected in the surroundings of Goettingen, Germany, and in the Berlin–Brandenburg area, Germany. Branches of *P. sylvestris* were obtained from a forest northwest of Berlin and offered to *D. pini* females for oviposition and to larvae for feeding. The sawflies were reared according to established protocols (49, 50). The development of *D. pini* from egg to adult takes from 50 to 55 d under the given laboratory conditions (18:6 h light:dark, 20 °C, 70% relative humidity). The adults mate and start egg depositions within several days after emergence from cocoons. No distinct mate calling behavior has been described for *D. pini* females, nor has it been observed by us. When we observed mating couples, they were sitting on the pine needles.

Plant Treatments. Prior to each experiment, trees from the greenhouse were acclimatized for 3 d in a climate chamber at 20 °C, 18:6 h light:dark, 70% relative humidity, 155 μmol photons per square meter per second. To avoid cross-contamination with volatiles from plants that had been treated differently, the small trees were placed in Plexiglas cylinders (60 cm height, 9.5 litre), which were ventilated by charcoal-filtered air (inflowing and outflowing air: ~200 mL·min^{-1}). As described above, the *D. pini* sex pheromones were dissolved in hexane; therefore, we also treated the trees with hexane only. Specifically, we used the following types of pine treatments (n = 5 to 8 trees each) for later analysis of needle water content, hydrogen peroxide concentrations and gene expression levels: treatment a, untreated pines; treatment b, exposure of pines to hexane for 24 h; treatment c, exposure of pines to hexane for 24 h and subsequent egg deposition for 24 h; treatment d, exposure of pines to *D. pini* sex pheromones (dissolved in hexane) for 24 h; and treatment e, exposure of pines to *D. pini* sex pheromones for 24 h and subsequent egg deposition for 24 h.

For treatment b, we applied 100 μL of hexane to a cotton wool pad (diameter: 5.6 cm, thickness: 0.4 cm) as the dispenser. To allow evaporation of the hexane, pads were kept for 30 min under the fume hood prior to exposure to the trees. Following hexane evaporation, a pad was placed into the aforementioned Plexiglas cylinder, along with a pine tree, for 24 h.

For treatment c, the plants were treated as in b, and, thereafter, 2 *D. pini* females were allowed to oviposit on the tree for 24 h. Only trees with at least 4 egg rows were used for the experiments.

Treatments d and e were conducted as described for b and c except that 100 μL of a pheromone solution was applied to the cotton wool pad instead of only hexane. The trees were exposed to the pheromone components for 24 h, because we expect a high pheromone concentration to be present for at least a day in a pine forest, where a mass outbreak of *D. pini* takes place (*SI Appendix, SI Material and Methods*).

Pheromones. Previous field and electrophysiological studies showed that the acetate and propionate esters of (2*S*,3*R*,7*R*)-3,7-dimethyl-2-tridecanol are the active, male-attracting components of the sex pheromone released by female *D. pini* (51, 52). Synthesized esters dissolved in hexane were obtained from the laboratory of Olle Anderbrant (Lund University, Sweden). The pheromones were synthesized by Helen Edlund and Erik Hedenström at Mid Sweden University (52, 53). We controlled the pheromone purity and concentration using GC-MS (Agilent 7890 A GC model coupled to an Agilent 5975 C MS unit).

To determine the concentration of the pheromone components, 10 ng·μL^{-1} methyl undecanoate (Sigma Aldrich) was used as an internal standard. We injected 1 μL of a 1/100 and of a 1/1,000 dilution of the obtained pheromone solution in hexane (including the internal standard) in splitless mode (injector temperature 300 °C; J&W DB-5-ms capillary column: length: 30 m; inner diameter: 0.25 mm; film thickness: 0.25 μm). Helium was used as carrier gas, with an inlet pressure of 0.1 bar and an outlet pressure of 50 kPa. The following program was used for analysis: 4-min hold at 40 °C followed by a temperature increase of 10 °C·min^{-1} until 180 °C, followed by a temperature increase of 20 °C·min^{-1} until 280 °C, and a 5-min hold at the end of the program. The column effluent was ionized by electron impact ionization at 70 eV (mass range from 35 to 600 *m/z*).

The pheromone solution, which the plants were exposed to, contained both pheromone components, each at a concentration of 50 ng·μL^{-1} hexane. We determined the release rate of the pheromones from the cotton pads by GC-MS analyses as described in *SI Appendix, SI Material and Methods*. The results confirmed that the release rate was equivalent to the release rate of a high abundance of *D. pini* females, that is, 270 to 450 females. The upper end of this range is similar to the number (around 400 females per tree) counted during a mass outbreak in the surroundings of Berlin (*SI Appendix, SI Material and Methods*).

To determine whether pheromone residues were left on pine needles when the trees were exposed to *D. pini* egg deposition, we extracted pine needles 6 h after the end of a 24-h pheromone exposure time and analyzed the extract by GC-MS as described in *SI Appendix, SI Material and Methods*.

Determination of Egg Survival. To determine the effect of pheromone exposure on the survival rate of *D. pini* eggs, 2 sawfly females were offered an untreated pine tree, a hexane-exposed tree, or a pheromone-exposed tree for a period of 24 h. We counted the number of eggs and larvae hatching on each tree. The egg survival rate was calculated by relating the number of eggs laid to the number of larvae hatching from the eggs per tree. Egg survival rates were determined on n = 6 untreated trees, n = 8 hexane-exposed trees, and n = 8 pheromone-exposed trees.

Determination of Pine Needle Water Content and Hydrogen Peroxide Concentration. To determine the water content of needles from the differently treated trees, we harvested 3 to 4 needles that were adjacent to the oviposition site. The needles were sampled 1) 2 d after pheromone or hexane exposure (i.e., 1 d after egg deposition) and 2) at the end of the egg incubation period, shortly before larvae would hatch, that is, 12 d after pheromone or hexane exposure and 11 d after egg deposition (egg incubation is around 12 to 14 d in the abiotic conditions used). Needles from equivalent positions and in comparable quantities were harvested from egg-free trees. Immediately after harvesting, the needles were weighed. The needles were then dried for 72 h in an oven (60 °C) and weighed once again. Based on these weights, the relative water content (percent) was calculated. Drying for more than 72 h showed no further weight loss. We determined the water content of needles taken from n = 5 untreated trees and n = 8 trees subjected to the aforementioned treatments.

To determine the hydrogen peroxide concentrations of needles from the differently treated trees, we used the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes by Invitrogen), which provides a fluorescing product with hydrogen peroxide. Our protocol followed the manufacturer's recommendations modified after Chakraborty et al. (54). Needles were harvested from similar tree positions and at the same time points as described above for determining the water content. The needles were immediately transferred to liquid nitrogen after being detached from the experimental trees and were ground to a powder. A sample of 30 mg of powdered needle tissue per tree was mixed with 250 μL (0.05 M; pH 7.4) of sodium phosphate buffer and placed on a shaker with 50 rpm at 25 °C for 30 min. Thereafter, the needle sample was centrifuged at 15,000 × g for 15 min, and the supernatant was centrifuged again at 15,000 × g for 2 min. A sample (50 μL) was taken from the final supernatant and incubated with 50 μL of a solution consisting of 100 μM Amplex Red reagent and 0.2 U·mL^{-1} horseradish peroxidase. The incubation took 30 min at 30 °C in dark conditions. To prepare samples with distinct hydrogen peroxide concentrations for recording a reference standard curve, samples with hydrogen peroxide concentrations ranging from 0 to 30 μM H_2O_2 were prepared according to the protocol provided with the kit. These samples were incubated with the Amplex Red reagent and horseradish peroxidase as described for the needle samples. After incubation and centrifugation, the fluorescence of each sample (50 μL; 3 technical replicates) was determined by using an Infinite 200 PRO plate reader (Tecan Life Science) (excitation: 560 nm; emission: 590 nm). The hydrogen peroxide concentrations were calculated based on the standard curve value and then divided by 30 mg (needle sample weight). The hydrogen peroxide concentration was determined in needles taken from n = 8 trees of each treatment, as well as from n = 8 untreated trees.

Gene Expression Analysis. Needles were collected from sites adjacent to the oviposition site (about 1 g per tree) and from equivalent positions and in comparable quantities from egg-free trees. We harvested the needles at the same time points after pheromone exposure and egg deposition as described

above for determining the water content. Needles that had been immediately frozen in liquid nitrogen after sampling were powdered. A powdered needle sample (50 mg) was used for RNA extraction with the InviTrap Spin Plant RNA Mini Kit (Stratec). RNA was eluted in 50 µL of nuclease-free H_2O, and contaminating DNA remains were digested with the TURBO DNA *free* kit (ThermoFisher Scientific). RNA integrity and purity were checked by analysis on a 1.1% agarose gel in 1× TAE buffer with 0.006% EtBr. A volume of 10 µL of the sample was diluted 1:1 with 2× RNA loading dye (ThermoFisher Scientific), heated for 10 min to 70 °C, and placed on ice immediately afterward. A volume of 4 µL of the RiboRuler High Range RNA Ladder (ThermoFisher Scientific) was treated likewise. After loading samples, the gel was run for 90 min at 120 V. Spectrophotometric determination of the RNA concentration was performed on a Multiscan GO microplate spectrophotometer (ThermoFisher Scientific) by measuring absorbance at 260 nm.

For synthesis of cDNA, 500 ng of extracted RNA was used as a template for reverse transcription utilizing the AMV-RT (avian myeloblastosis virus reverse transkriptase) native enzyme (Roboklon). The RNA was mixed with 1 µL of Oligo dT_{20} (50 µM) and 2 µL of dNTPs (10 mM) and filled up to a reaction volume of 14 µL with nuclease-free H_2O. The mixture was incubated for 5 min at 65 °C, followed by 5 min incubation at 4 °C. To start the reaction, 4 µL of 5× RT buffer (Roboklon), 0.5 µL of RNase inhibitor (Roboklon; 30 U·µL^{-1}), 1 µL of 100 mM DTT (dithiothreitol), and 1 µL of AMV-RT native (Roboklon; 10 U·µL^{-1}) were added and heated to 42 °C for 15 min and to 50 °C for 45 min. To inactivate the AMV-RT enzyme, the mixture was finally heated to 80 °C for 10 min and thereafter cooled on ice.

Primers (*SI Appendix*, Table S5) for the selected genes and for the housekeeping genes ubiquitin (*PsUBI*), cytochrome subunit 6 (*PsPETB*), and chloroplast ATPase beta subunit (*PscATP*) were designed and evaluated according to the MIQE guidelines (minimum information for publication of quantitative real-time PCR experiments) (55, 56) with the online tool named PRIMER-BLAST (57). For genes for which no annotated template sequences have been published for *P. sylvestris* (*LOX*; *PR-1*; *PETB*; *cATP*; *UBI*), we searched in BLAST (basic local alignment search tool), EST (expressed sequence tags), and nr databases for *Pinus* sequences, which showed high homology with annotated sequences from other plant species. Primers were designed based on sequences with the lowest *E* value, and the identity of the PCR products was evaluated by Sanger sequencing at Seqlab and BLAST analysis (*SI Appendix*, Table S6) (58).

We performed qPCR analyses using the qPCRBIO SyGreen Mix Lo-Rox kit (Nippon Genetics Europe) on an MX3005P (Stratagene) cycler. For the qPCR reactions, 12.5 ng of cDNA was mixed with 5 µL of qPCRBIO SyGreen Mix Lo-Rox Master Mix (Nippon Genetics Europe) and 0.17 µL of each primer (10 pmol·µL^{-1}) and filled up to a 10-µL reaction volume with nuclease-free H_2O. To control for primer dimerization, H_2O controls were run, and, to control for genomic DNA contamination, DNase-treated RNA from each sample was used. Each reaction was performed with 3 technical replicates under the following running conditions: after an initial heating step of 2 min at 95 °C, 40 cycles of 5 s at 95 °C, followed by 30 s at 60 °C, were performed. At the end of each cycle, the fluorescence was measured twice. Following the 40 cycles of PCR amplification, a dissociation curve ranging from 55 °C to 95 °C in 1 °C steps was measured to check for primer dimer reaction products. C_q (cycle quantification value) values and PCR efficiency of all reactions were calculated with LinRegPCR version 2015.2 (59). Normalization of response genes to the reference genes *PsUBI*, *PsPETB*, and *PsCATP* was performed as described by Vandesompele et al. (60). Gene expression analyses were conducted with samples taken from $n = 5$ to 8 trees of each treatment.

Sawfly Antennal Responses to Pheromones. Electrophysiological antennal responses of *D. pini* adult males and females to their sex pheromones [(2*S*,3*R*,7*R*)-3,7-dimethyl-2-tridecanyl acetate and propionate] were recorded by EAG. We chilled the sawflies by each sawfly at 4 °C for several minutes and then cut off the antenna at its base, where we inserted the reference electrode, that is, a glass electrode filled with Ringer solution (NaCl 128.3 mmol/L, KCl 4.7 mmol/L, $CaCl_2$ 2.6 mmol/L) and linked with a grounded Ag wire. The tip of the antenna was connected to the recording glass electrode filled with Ringer as well and linked via an interface (IDAC 2; Syntech) to a PC for signal recording. To record the electrophysiological response of an antenna to the pheromones, we applied 500 ng of the acetate pheromone component, or 500 ng of the propionate pheromone component, or 500 ng of each of the components as a blend on a filter paper (28 mm^2) (5 µL of pheromone solution in hexane; these quantities are equivalent to that released by about 27 to 45 *D. pini* females). For control measurements, 5 µL of hexane was applied to a filter paper. Prior to exposure to the antenna, the solvent was allowed to evaporate for 15 min. Thereafter, the filter paper with the test odor was inserted into a Pasteur pipette, which was connected to a stimulus controller (CS-05; Syntech), which allows puffing the test odor in a standardized manner to the antenna (flow: 20 mL/s; stimulus time: 0.5 s). Each antenna was first exposed to ambient air and then to one of the test odors or to the control solvent. The EAG signals (millivolts) were amplified 100-fold by a microelectrode amplifier and recorded by EAG software (Syntech). The EAG signals were evaluated by normalizing the responses to test odors (R-t) to the responses to ambient air (R-a) by dividing the signals (R-t/R-a). Likewise, the responses to the solvent hexane (R-h) were normalized to those to air (R-h/R-a). Thereafter, the air-normalized response to the solvent hexane was set to value 1.0 (R-h/R-a divided by R-h/R-a = 1), and the air-normalized responses to the test odors were adjusted accordingly (R-t/R-a divided by R-h/R-a). The signals recorded in response to the solvent were almost the same as those in response to ambient air. We determined the responses of $n = 8$ antennae (taken from 8 individuals) of each sex.

Data Analysis. The gene expression data were evaluated with the statistical software R version 3.4.1 (61) using the packages car, lawstat, and PMCMR. All other data were evaluated with the statistical software SigmaPlot version 11.0 (Systat Software GmbH, 2008). All datasets were tested for normal distribution by the Shapiro–Wilk test. Variance homogeneity was measured with Levene's test. Normally distributed data (with variance homogeneity) were subjected to parametric tests, and nonnormally distributed data were subjected to nonparametric tests. All tests (and respective *P* values) were run 2-sided with confidence intervals of 95%. To analyze the difference between the recorded egg survival rates per pine treatment and the theoretically possible survival rate (100% survival of all deposited eggs), we used the paired *t* test. To analyze whether the survival rates, the water content, and hydrogen peroxide concentrations differed among treatments, we used an ANOVA, and, in the case of statistical significance, we further analyzed the data by multiple pairwise *t* tests and a Benjamini–Hochberg *P* value correction. Statistical details are given in *SI Appendix*, Tables S2 and S3. The qPCR data were normalized to the expression values recorded in the treatment "hexane control." The expression values in the hexane control treatment did not differ from those in the untreated samples, as analyzed by the Mann–Whitney *U* test in the case of nonnormally distributed data and by the paired *t* test in the case of normally distributed data (*SI Appendix*, Table S7). Differences in expression values between the hexane control and the other treatments were evaluated by Kruskal–Wallis *H* test followed by a pairwise comparison with the Conover–Iman test, with a Benjamini–Hochberg correction for multiple comparisons (Table 1 and *SI Appendix*, Table S4). To analyze the difference in electrophysiological antennal responses to the hexane solvent and the pheromone components, we used the Wilcoxon matched pairs test (Fig. 3 and *SI Appendix*, Table S8).

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Supplementary Information

Defense of Scots pine against sawfly eggs (*Diprion pini)* is primed by exposure to sawfly sex pheromones

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- **Supplementary References**

Supplementary Material and Methods

Pine exposure to pheromones for 24 h. Male and female *D. pini* adults are spending their lives in the pine trees. Since no distinct mate calling behavior has been observed in *D. pini* females, no information is available on their active pheromone release. Interestingly, pheromonal components have also been detected in extracts of *D. pini* cuticle (1), suggesting some continuous, passive pheromone release. Regardless of the exposure to pheromones released from *D. pini* females sitting in a tree, the tree might also perceive pheromones *via* (gusts of) wind transferring diprionid pheromones over some distance, as indicated by studies showing that attraction of diprionids to traps baited with female sex pheromones are affected by wind conditions (2). Thus, depending on the distance of a tree from a pheromone source and on the speed of wind carrying a pheromone plume to a tree in a pine forest subjected to a mass outbreak of *D. pini,* an individual pine tree might be exposed to diprionid pheromones at any daytime. During a mass outbreak of *D. pini* with successive emergence of adults*,* high concentrations of pheromones might be around even for longer than 24 h.

Determination of pheromone release rate. To calculate the release rate of *D. pini* pheromones from cotton pads placed into the cylinders with the pine trees, we determined the initial quantity of pheromones applied to the pads and the remaining pheromone quantity after a 24 h exposure to pine trees. The pheromones were supplied by Olle Anderbrant from Lund University in Sweden; they were synthesized by Helen Edlund and Erik Hedenström at Mid Sweden University, with a GC purityof 99%.

More specifically, we applied 100 µl of a pheromone solution in hexane (50 ng μl^{-1} mixture of each of the pheromone esters (2*S*,3*R*,7*R*)-3,7-dimethyl-2-tridecanyl acetate and propionate) to a cotton pad (as described in the main text). After pheromone application, the pads were placed for 30 min in a fume hood, thus allowing the hexane to evaporate. Thereafter, we extracted the pheromone with hexane from the dispenser cotton pads (n = 5). Analysis of the extracts by GC-MS (conditions as described in the main text, Material and Methods) provided data on the initial amount of pheromone per pad (and tree). After being used in the experiments (i.e. after the 24 h treatment of plants), we also extracted the cotton pads and analyzed the quantity of pheromone remaining on them (n = 48). Based on our data and assuming a continuous release rate, we calculated the proportion of the pheromone released and the release rate in ng h^{-1} (*SI Appendix,* Table S1). Approximately half of the propionate of (2*S*,3*R*,7*R*)-3,7-dimethyl-2-tridecanol, and two-thirds of the acetate were released during the 24 h incubation period.

The amounts of pheromone components per *D. pini* female were found to vary within a wide range (1). Small amounts of the acetate and propionate component were detected in a similar ratio, but the maximum amount of the acetate component detected in a female was 1000 pg, and of the propionate component 500 pg (1). Hence, the ratio of the two pheromone components may range from about 1:1 to 2:1. When comparing the release rate determined here in our study (see Table S1) with that of the maximum amount of pheromonal compounds determined by Anderbrant *et al.* per *D. pini* female (1), the quantity of pheromones released from a cotton pad in our study was equivalent to the possible emission by 270 to 450 females. This number of females is very similar to the numbers per tree that were previously observed during mass outbreaks of *D. pini* by us and others (3).

Pheromone residues on plants. To examine whether residues of the pheromone were left on pine needles, pine was exposed to both pheromone esters for 24 h following the method described in the main text. After exposure to the pheromones, pine was exposed to clean, charcoal-filtered air for additional 6 h. We exposed three *P. sylvestris* trees to the pheromones and harvested 1g needles of each tree. The three needle samples were extracted each with 1 ml hexane. The extracts were analyzed (i) directly and (ii) after concentration to 50 µl under N_2. A volume of 1 µl of the extracts was injected into a GC-MS (Agilent 7890 A GC model coupled to an Agilent 5975 C MS unit) in splitless mode (injector temperature 250 °C; Zebron ZB-5HT capillary column; 30 m x 0.25 mm i.d.; film thickness: 0.25 µm). Helium was used as carrier gas with a flow rate of 1 ml min^{-1}. The following program was used for analysis: 4 min hold at 40 °C, ramp of 10 °C min^{-1} to 180 °C, followed by a ramp of 20 °C min^{-1} to 280 °C and a 5 min hold of 280° C. A solvent delay of 4 min was added. The column effluent was exposed to electron impact ionization at 70 eV. We recorded a total ion current chromatogram (TIC) with a mass range of 25 to 300 m/z and additionally analyzed samples in the single ion mode (SIM) in search for characteristic ions of the pheromone esters: 87 *m/z*, 101 *m/z*, 210 *m/z.*

No (traces of) pheromone esters were detected in neither type of extract.

Supplementary Table S1. Determination of release rate of *Diprion pini* sex pheromones from cotton pads used in the experiments. Emission rate and percentage of emitted total proportion of the acetate and propionate esters of *D. pini* sex pheromone ((2*S*,3*R*,7*R*)-3,7-dimethyl 2-tridecanyl acetate and propionate) are given (means ± SE).

Pheromone ester	Emission rate in ng h^{-1}	Percentage emitted during 24 h
Acetate	270±16.3	64.7±3.9
Propionate	225±16.6	53.9±4.0

Supplementary Table S2. Details of evaluations by ANOVA. For data on egg survival and number of eggs laid: please compare Fig. 1, main text. For measurements of water content and H_2O_2 concentrations, which were conducted 2 and 12 days after pheromone exposure (i.e. 1 or 11 days after egg deposition): please compare Fig. 2, main text and Fig. S1, *SI Appendix*.

Analysis	Degrees of Freedom*	Sum of Squares*	Mean Square*	*F* value	*P value*
Egg survival	2 / 19	2015.47 / 4679.54	1007.74 / 246.29	4.092	= 0.033
Number of eggs laid	2 / 19	8977.36 / 28112.50	4488.68 / 1479.61	3.034	= 0.072
Water content, day 2	4 / 32	29.07 / 87.71	7.27 / 2.92	2.486	= 0.065
Water content, day 12	4 / 32	210.60 / 661.60	52.65 / 20.68	2.547	= 0.058
H2O2 conc., day 2	4 / 35	158.18 / 1159.24	39.54 / 33.12	1.194	= 0.331
H2O2 conc., day 12	4 / 35	544.93 / 90.12	136.23 / 2.58	52.911	< 0.001

*Source of variation: between groups / within groups

Supplementary Table S3. Details of paired *t*-test evaluations of comparison of numbers of laid eggs with numbers of hatched eggs per treatment. Compare Fig. 1, main text.

Treatment	Degrees of Freedom	*t* value	*P value*
Untreated	5	4.584	= 0.006
Hexane	7	6.200	< 0.001
Pheromone	7	8.233	< 0.001

Supplementary Table S4. Details of statistical evaluations of differences in gene expression by Kruskal-Wallis *H* test. Compare Table 1, main text.

Gene	Day*	Degrees of Freedom	*H* value	*P value*
ROS mediating genes				
PsRboh	2	3	15.763	0.001
PsRboh	12	3	9.568	0.023
PsSOD	2	3	4.071	0.254
PsSOD	12	3	7.937	0.026
PsCAT	2	3	7.910	0.048
PsCAT	12	3	2.895	0.423
PsAPX	2	3	5.993	0.112
PsAPX	12	3	9.260	0.026
Genes involved in SA- and JA-mediated responses				
PsLOX	2	3	4.588	0.205
PsLOX	12	3	10.513	0.015
PsPDF	2	3	2.065	0.559
PsPDF	12	3	1.215	0.729
PsPR-1	2	3	3.048	0.384
PsPR-1	12	3	16.682	0.001
PsPAL	2	3	1.622	0.654
PsPAL	12	3	10.177	0.017

* days after pheromone exposure

Supplementary Table S5. Sequences of primers used in this study for qPCR and related search information. Compare Table 1, main text.

Gene	Primer sequence (5‘ -> 3‘)	Pine template for primer design	Species for primer design	Species for BLAST search for pine primer template design
		Housekeeping genes		
PsUBIF	ACTTTACCAGAGTCATCAACC	HE629096	*Pinus sylvestris*	*Picea abies* (EF681766)
PsUBIR	GGTTCTTCGTCTGAGAGGTG			
PscATPF	GGGTCGGTCAAGTCGTCAGC	GW765967	*Pinus banksiana*	*Ginkgo biloba* (EU071049)
PscATPR	GCACGGAAATGGGTTCTTTGC			
PsPETBF	ACCATCATACTTGCCGACCATC	CV035597	*Pinus taeda*	*Populus euphratica* (XM011050173)
PsPETBR	TCGTCCGACCGTTACAGAAGC			
		ROS-mediating genes		
PsRbohF	GATGTACCTGGCAGTTCC	MF389973	*Pinus sylvestris*	*Picea abies* (KT192592)
PsRbohR	GCCACTCTTGTATCTGAACC			
PsSODF	GCTGATGTCAAGGGGGTTGT	X58578	*Pinus sylvestris*	-
PsSODR	ACCATGCTCCTTGCCTAACG			
PsCATF	AAGGGCTTTTTCGAGGTGAC	AL751103	*Pinus pinaster*	-
PsCATR	GGAATTACCTGCATGGCATC			
PsAPXF	TCTGGTTTTGAAGGACCATG	AY485994	*Pinus pinaster*	-
PsAPXR	AAACTAGGATCAGCCAGCAG			
		Genes involved in SA- and JA-mediated responses		
PsLOXF	TGGACTAATGATGGAAGAGCAC	DR169048	*Pinus taeda*	*Picea sitchensis* (CO218750)
PsLOXR	TGATGTTGGCAGCAATAACTCG			
PsPDFF	GGCAAGGGAGTTGGCAGTCG	EF455616	*Pinus sylvestris*	-
PsPDFR	TGGTGCTGTTCACACAATACCC			
PsPR-1F	TCGTCAACGTACACAGATGTTG	HE627106	*Pinus sylvestris*	*Arabidopsis thaliana* (NM127025)
PsPR-1R	ACTACGATCCGCCTGGGAAC			
PsPALF	CTGGCAGCGATCCACTGAAC	AF353967	*Pinus sylvestris*	-
PsPALR	CTTCGAGCAACGGCAGCAAC			

Supplementary Table S6. Nucleotide sequences of PCR products obtained from primers used in thisstudy (if based on published sequences, the references are given here in the *SI Appendix,* section "References"). Compare Table 1, main text.

Name	Nucleotide sequence 5'-3'
	Housekeeping genes
PsUBI	ACTTTACCAGAGTCATCAACCTTGTAGTACTGCAGAACAGCCAATTTTACCTTCTTCTTCTTGT GCTTGAGCTTCTTAGGCTTAGTGTAAGTCTTCTTCTTTCTCTTCTTGGCACCACCTCTCAGACG AAGAACCAA
PscATP	GGGTCAAGTCGTCAGCAGGTACATAAACTGCTTGAATCGAGGTTATGGATCCCTTTTTTGTGG AGTAATTCTCGTGCCTTTACCCAAGAAACGTT
PsPETB	ACCATCGATGAATTGATCGGATTAACCAACCAAAGTTAACTTCGGTCATTAGGTATTGAACAG AGGCAAAAGCTTCTGTAACGGTCGGACGA
	ROS-mediating genes
PsRboh	GATGTACCTGGCAGTTCCCGTATTATTATATGGAGGAGAACGAACACTGAGAGCTTTCAG ATCAGGTTCAAAACCCGTGCAAATACTCAAGGTTTGTCTTTACATCAATTTTCATTTTTT GTGATTCTAGCTTTGCATCTGCAATCCTTGATGTGACCAATAGAATCTGTTGCATTTTTG GGGGATTTTGCTCTTAACATCTACACGGTCCACATTTGCAGGTAGCAATCTATCCTGGTA ATGTCTTGACATTTCACATGTCCAAACCTCAAGGGTTCAGATACAAGAGTGGC
PsSOD (4)	GCTGATGTCAAGGGGGTTGTTCAATTCACCCAGGAAGGAGATGGGCCAACAACTGTAACT GGGAAGATCAGTGGTCTGAGCCCTGGTCTCCATGGTTTCCATGTTCATGCACTAGGTGAC ACAACAAATGGGTGCATGTCAACTGGACCACATTTTAATCCGTTAGGCAAGGAGCATGGT
PsCAT (5)	TAAGGGCTTTTTCGAGGTGACCCACTATGTCTCCGATCTCACCTGTGCAGATTTCATGAG GGCACCTGGCGTTCAGACCCCAGTGATTGTTCGGTTTTCTACTGTCATACATGAACGTGG GAGCCCGGAGACTATGAGAGACCCCAGGGGTTTCGCTGTCAAGTTTTACACGAGAGAAGG GAACTTCGACATTGTTGGAAACAATATTCCCGTTTTCTTCACTCGTGATGCCATGCAGGT AATTCC
PsAPX (6)	TCTGGTTTTGAAGGACCATGGACCTCTAACCCTCTTATCTTTGACAACTCTTACTTCACA GAGCTTGTGACTGGAGAGAAGGAAGGCCTGCTTCAGCTGCCATCTGATAAGGCACTGCTG GCTGATCCTAGTTTA
	Genes involved in SA- and JA-mediated responses
PsLOX	TGGACTAATGATGGAAGAGCACTGGAGGCCTTTCAAAGGTTTTCTACCACAGTTCAGGGGGT AGAGGAAATCATACATCAGAGAAATGAAGATTCGAGTAAGAAGAACAGGAATGGGGCSGG CGTACTTCCTTACGAGTTATTGCTGCCAACATCAACC
PsPDF	GGCAAGGGAGTTGGCAGTCGACTCAGCACTCTTTTTCTGCTCGTGCTGCTTGTTATAACC ATTGGGATGATGCAGGTTCAAGTTGCAGAGGGCCGAATGTGCAAAACCCCGAGCGGCAAG TTCAAAGGGTATTGTGTGAACAGCACCA
PsPAL (7)	CTGGCAGCGATCCACTGAACTGGGTTCGAGCAGCCAAGGCCATGGAAGGAAGTCACTTTG AAGAAGTGAAAGCGATGGTGGATTCGTATTTGGGAGTCAAGGAGATTTTCATTGAAGGGA AATCTCTGACAATCTCAGACGTTGCTGCCGTTGCTCGAAG
PsPR-1	TCGTCAACGTACACAGATGTTGAAGATTTACAGTAACACGGAATATTAGAAGGAAATTAACG AAAACTAATACGATATGATAGGTCGGGATATCAGAATTCAGTATGGTTTCTGCCCTACATAGT TCCCAGGCGGATCGTAGT

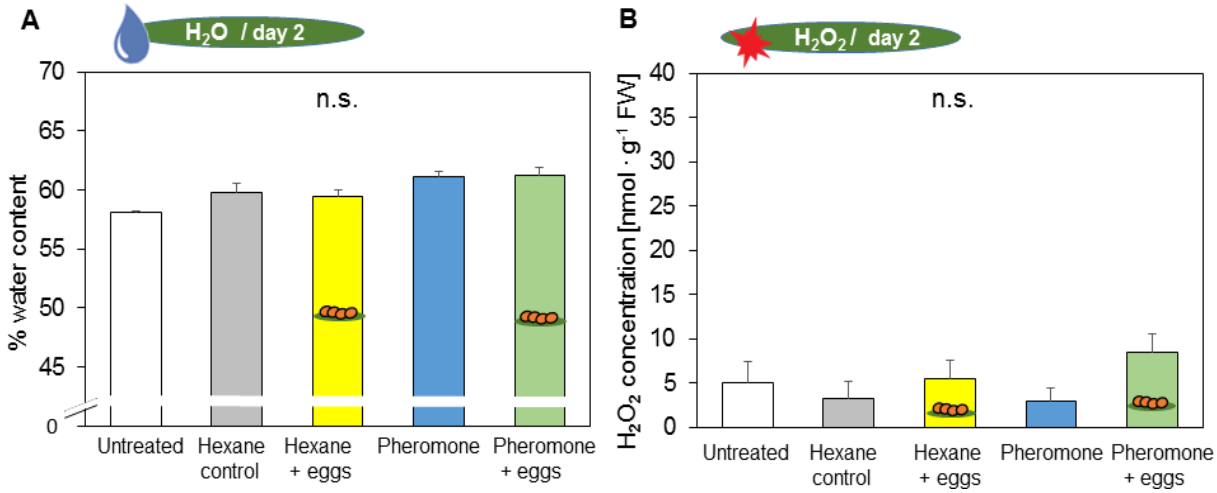
Supplementary Table S7. Transcript levels of genes in untreated pine trees and in trees exposed to hexane. Gene expression in untreated trees was normalized to the expression of the housekeeping genes (see main text, Material and Methods) and set to value 1. Gene expression in hexane-treated trees expressed as fold-change to expression levels in untreated controls. Data show means ± SE. *n* = 8 untreated and *n* = 5-8 hexane-treated trees. Expression levels were determined 2 and 12 days after treatment. *P* values: pairwise comparison of untreated and "hexane control" by **t*-test or †Mann-Whitney *U* test. Compare Table 1, main text.

Time	Hexane control	Untreated	*P* value
***PsRboh* - Respiratory burst oxidase homolog (plant NADPH oxidase)**			
2d*	0.76±0.09	1.00±0.20	0.311
12d†	2.06±0.28	1.00±0.16	0.126
***PsSOD* - Superoxide dismutase**			
2d*	1.21±0.24	1.00±0.17	0.498
12d*	1.46±0.15	1.00±0.13	0.720
***PsCAT* – Catalase**			
2d†	0.56±0.13	1.00±0.25	0.222
12d†	0.56±0.10	1.00±0.21	0.228
***PsAPX* - Ascorbate peroxidase**			
2d*	0.98±0.10	1.00±0.20	0.924
12d†	1.44±0.24	1.00±0.07	0.081
***PsLOX* - Lipoxygenase**			
2d†	1.59±0.46	1.00±0.17	0.442
12d*	0.68±0.12	1.00±0.09	0.055
***PsPDF* - Plant defensin**			
2d†	1.27±0.54	1.00±0.34	0.878
12d*	0.55±0.15	1.00±0.22	0.128
***PsPR-1* - Pathogenesis related 1**			
2d†	4.11±2.36	1.00±0.48	0.442
12d*	0.56±0.16	1.00±0.41	0.382
***PsPAL* - Phenylalanine ammonia lyase**			
2d*	1.48±0.44	1.00±0.18	0.335
12d*	0.75±0.17	1.00±0.16	0.294

Supplementary Table S8. Details of statistical evaluations of the EAG responses by *Diprion pini* to the acetate / propionate sex pheromonal components. Responses to test substance compared to responses to controls; Wilcoxon matched pairs test**.** Compare Fig. 3, main text.

Test substance	Sex	*Z* value	*P value*
Acetate pheromone component	male	2.521	0.008
Propionate pheromone component	male	2.521	0.008
Acetate + Propionate pheromone components	male	2.521	0.008
Acetate pheromone component	female	0.840	0.461
Propionate pheromone component	female	1.540	0.148
Acetate + Propionate pheromone components	female	1.183	0.297

Supplementary Fig. S1. (*A*) Water contents and (*B*) hydrogen peroxide concentrations of *Pinus sylvestris* after exposure to sawfly sex pheromones and subsequent egg deposition. Measurements were conducted 2 days after pheromone exposure, i.e. 1 day after egg deposition, and at equivalent time points in controls. Water concentrations and hydrogen peroxide concentrations were determined in pine needles from untreated trees, from trees exposed to the solvent hexane (withouteggs: hexane control; with eggs: hexane + eggs), from trees exposed to the pheromones (dissolved in hexane) (without eggs: pheromone; with eggs: pheromone + eggs). Means + SE of water contents and hydrogen peroxide concentrations are given (n = 5 for water content untreated; n = 8 for all other treatments). All data evaluated by ANOVA (n.s., not significant) (compare *SI Appendix,* Table S2).



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CHAPTER 3

The impact of insect egg deposition on Pinus sylvestris transcriptomic and phytohormonal responses to larval herbivory

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The impact of insect egg deposition on *Pinus sylvestris* transcriptomic and phytohormonal responses to larval herbivory

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Plants can improve their resistance to feeding damage by insects if they have perceived insect egg deposition prior to larval feeding. Molecular analyses of these egg-mediated defence mechanisms have until now focused on angiosperm species. It is unknown how the transcriptome of a gymnosperm species responds to insect eggs and subsequent larval feeding. Scots pine (*Pinus sylvestris* L.) is known to improve its defences against larvae of the herbivorous sawfly *Diprion pini* L. if it has previously received sawfly eggs. Here, we analysed the transcriptomic and phytohormonal responses of Scots pine needles to *D. pini* eggs (E-pine), larval feeding (F-pine) and to both eggs and larval feeding (EF-pine). Pine showed strong transcriptomic responses to sawfly eggs and—as expected—to larval feeding. Many egg-responsive genes were also differentially expressed in response to feeding damage, and these genes play an important role in biological processes related to cell wall modification, cell death and jasmonic acid signalling. EF-pine showed fewer transcriptomic changes than F-pine, whereas EF-treated angiosperm species studied so far showed more transcriptional changes to the initial phase of larval feeding than only feeding-damaged F-angiosperms. However, as with responses of EF-angiosperms, EF-pine showed higher salicylic acid concentrations than F-pine. Based on the considerable overlap of the transcriptomes of E- and F-pine, we suggest that the weaker transcriptomic response of EF-pine than F-pine to larval feeding damage is compensated by the strong, egg-induced response, which might result in maintained pine defences against larval feeding.

Keywords: biotic interactions, defence, gymnosperm, phytohormone, pine, transcriptome.

Introduction

Forests are often challenged by mass outbreaks of herbivorous insects. In addition to constitutively available resistance traits, trees have evolved multiple inducible defences to insects (Haukioja 2006, Büchel et al. 2016, Celedon and Bohlmann 2019, Whitehill et al. 2023). For example, feeding-damaged trees can enhance their levels of secondary plant compounds and the activities of enzymes that are harmful to attackers (Lämke and Unsicker 2018, Whitehill and Bohlmann 2019). Furthermore, trees are known to release damage-induced volatiles that repel herbivores or attract antagonists of feeding larvae (e.g., Mumm and Hilker 2006, Holopainen 2011, Suckling et al. 2012, Fabisch et al. 2019).

Trees do not need to 'wait' until they are exposed to larval feeding damage; they can defend themselves beforehand against the initial egg deposition on their leaves (Hilker and Fatouros 2015, Reymond 2022). These egg-induced tree defences act, for instance, by releasing leaf volatiles that attract egg parasitoids or by changes of leaf chemistry that are harmful to the eggs (Meiners and Hilker 2000, Hilker et al. 2005, Bittner et al. 2017). Thus, tree responses to insect eggs can reduce the number of surviving eggs.

In addition, there is increasing evidence that plant responses to insect eggs significantly improve plant defences against the impending feeding damage by hatching larvae. Larvae developing on previously egg-laden plants have been shown to gain less weight and suffer higher mortality (Hilker and Fatouros 2016, Lortzing et al. 2020). This egg-mediated, improved defence against herbivory may benefit the plant, as has been shown for *Arabidopsis thaliana* L. Heynh. Egg-laden and subsequently feeding-damaged *A. thaliana* plants produce a significantly higher seed weight when they regrow and flower after herbivory than egg-free, feeding-damaged *A. thaliana* (Valsamakis et al. 2022).

The transcriptomic and phytohormonal plant responses to insect egg deposition, and their effects on responses to subsequent insect larval feeding, have been well studied in angiosperm species, especially in herbaceous plants (Brassicaceae and Solanaceae), but also in a tree species, *Ulmus minor* L. (overview: Lortzing et al. 2020). These angiosperm species show some conserved, common transcriptomic and phytohormonal core responses to insect eggs and larval feeding (Lortzing et al. 2019, 2020, Valsamakis et al. 2020). According to De La Torre et al. (2020), gymnosperms show a 58–61% sequence similarity of expressed genes with those of angiosperms. The Coniferales, a well-studied major group of the Gymnospermae, show strong constitutive and also damage-inducible defences (Schmidt et al. 2005, Krokene 2015, Celedon and Bohlmann 2019, Whitehill and Bohlmann 2019, López-Goldar et al. 2020, Vázquez-González et al. 2020).

In the gymnosperm *Pinus sylvestris* L., several previous studies addressed the tree's responses to egg deposition and larval feeding damage by the common pine sawfly *Diprion pini* L. (Hilker et al. 2002, Beyaert et al. 2012, Bittner et al. 2017, Blomqvist et al. 2022). The tree mounts its defences against infestation by this sawfly already after egg deposition on the needles. The egg phase takes about 2 weeks until the larvae hatch. Egg deposition by this sawfly is linked with pine needle damage. During oviposition, the female saws a longitudinal slit into the needle with its chitinous ovipositor valves and releases the eggs in a row into the slit. The mechanical slitting by the sawfly's ovipositor alone does not induce the release of needle volatiles that attract egg parasitoids. However, the sawfly's subsequent insertion of the eggs, which are covered with an egg secretion, induces the emission of terpenoids, which then attract egg parasitoids that kill the sawfly eggs (Hilker et al. 2002). A recent study showed that the elicitor of this indirect pine defence is an annexin-like protein, which is associated with the egg secretion that the sawfly female releases with her eggs into the needle pouch (Hundacker et al. 2022). In addition to this indirect defence, egg-laden Scots pine needles accumulate greater quantities of hydrogen peroxide, which might either directly harm the sawfly eggs or induce further pine reactions (such as lignification of needle tissue), which ultimately hinder egg survival (Bittner et al. 2017, 2019).

In addition to these pine defences targeting sawfly eggs, pine responses to *D. pini* eggs have also been shown to significantly impair the performance of sawfly larvae. When *D. pini* larvae feed upon pine with prior sawfly egg deposition, they suffer higher mortality and gain less weight than larvae feeding upon egg-free pine (Beyaert et al. 2012). These findings suggest that pine takes the egg deposition by *D. pini* as a 'warning' of impending larval herbivory and subsequently improves its anti-herbivore defences against the larvae.

However, the molecular mechanisms resulting in this ecological effect, especially the transcriptomic and phytohormonal responses of pine as a gymnosperm species are currently unknown. Here, we asked whether and how these responses differ from those of angiosperm species to insect eggs and subsequent larval feeding. Therefore, we studied the transcriptomic and phytohormonal changes of *P. sylvestris* exposed to *D. pini* eggs only, to larvae only, or to both eggs and subsequent larval feeding. With respect to the phytohormone analyses, we focused on salicylic acid (SA), jasmonic acid (JA), JA-isoleucine (JA-Ile) and abscisic acid (ABA). Quantitative analyses of the transcriptomes, especially Gene Ontology (GO) term analyses, provided insights into possible biological processes that might be involved in pine responses to eggs and larvae. Analyses of samples exposed to the same treatment and harvested after different lengths of time helped us to elucidate the dynamics of pine responses. Analyses of samples exposed to different treatments allowed us to detect similarities and differences between pine responses to sawfly eggs and larvae, as well as to uncover the effects that pine responses to eggs had on subsequent responses to feeding damage.

Materials and methods

Plants and insects

For the transcriptomic analysis, 3-year-old *P. sylvestris* trees (not taller than 50 cm) were acquired from a tree nursery (Schlegel & Co., Riedlingen, Germany). For the phytohormone and qPCR analysis, 3-year-old *P. sylvestris* trees were obtained from a forest northeast of Berlin, Germany (53°08′36.0″N 13°33′56.2″E). Trees of this age are known to show defensive responses to *D. pini* eggs (Bittner et al. 2019). In European forests, young trees as well as older ones up to 140 years were found to be infested by *D. pini* (Brauns 1991). Needles from both the nursery trees and the forest trees were of the Δ-3-carene chemotype (Thoss et al. 2007), as tested by gas chromatography–mass spectrometry analyses of the needles (data not shown).

Our experimental trees grew in pots filled with potting soil Classic T (Einheitserde, Uetersen, Germany). When potting the trees, we very gently placed the roots of the young trees into the pots, thus paying attention to avoid damage of the roots. Prior to the experiments, all trees were first kept in a greenhouse under long-day conditions (18 h:6 h light:dark, average temperature 20 °C) for at least 2 months. At least 3 days prior to treatments with eggs and/or larvae, the potted trees were transferred to a climate chamber for acclimation to the experimental abiotic conditions (20 °C, 18 h:6 h light:dark, 70% relative humidity, 100-μmol photons m^{-2} s^{-1}).

Diprion pini was reared according to established protocols of Bombosch and Ramakers (1976) and Eichhorn (1976) with minor changes. Branches from *P. sylvestris* trees (at least 10 years old) were cut in forests in the surroundings of Berlin. Prior to offering them to *D. pini*, they were kept in water and stored in a cool climate chamber (10 °C, 18 h:6 h light:dark, 70% relative humidity, 100-μmol photons m^{-2} s^{-1}). For *D. pini* rearing, the branches were transferred into a warm climate chamber (20 °C, 18 h:6 h light:dark, 70% relative humidity, 100-μmol photons m^{-2} s^{-1}). Here, the branches were offered to *D. pini* adults for mating and egg deposition. The egg incubation time until hatching of larvae takes 10–14 days under the abiotic conditions used here. *Diprion pini* larvae fed upon the needles of these pine branches. They progress through five (male) to six (female) larval stages until pupation. Each pupa was placed individually in a small glass vial (5 ml) that was closed with a perforated lid. The pupae were kept in darkness at 7 °C until needed for further rearing or for the experiments.

To obtain adults for further rearing, the pupae were transferred to a warm climate chamber (20 °C, 18 h:6 h light:dark, 70% relative humidity, 100-μmol photons m^{-2} s^{-1}). Adults emerging from the pupae were exposed to pine branches again for further rearing.

To obtain age-synchronized adults for starting the treatment of experimental trees, we also transferred a set of the individually kept pupae from the cool climate chamber to the warm chamber. Since the adults emerged in the small vials, males and females could not mate prior to their exposure to experimental trees. We only used adults that were not older than 5 days for the experiments.

Plant treatments

All plant treatments were conducted in a climate chamber at 20 °C, 18 h:6 h light:dark, 70% relative humidity, 100-μmol photons m^{-2} s^{-1}. For the treatment, an acclimatized, potted tree was placed in a PLEXIGLAS cylinder (60-cm height, 9.5 L). The cylinder was closed at the bottom and the top with a PLEXIGLAS lid. The lids had small openings for insertion of a tube through which charcoal-filtered air was introduced

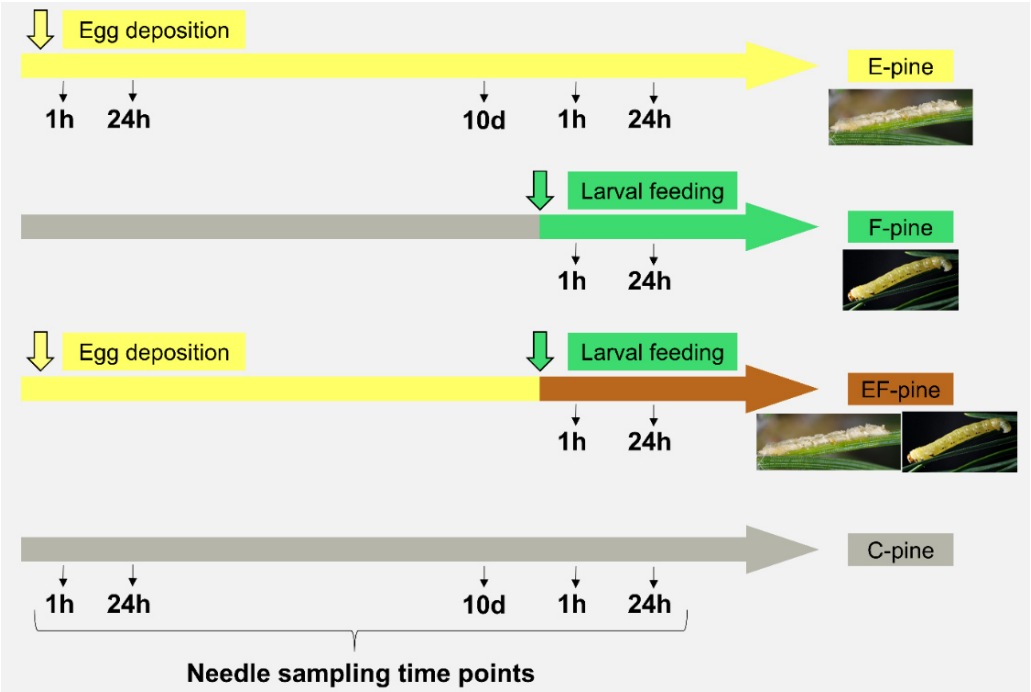

Figure 1. Scheme of *P. sylvestris* treatments and sampling time points. Needles of 3-year-old *P. sylvestris* trees were treated with natural egg deposition by *D. pini* (E-pine), larval feeding (F-pine) or natural egg deposition with subsequent feeding (EF-pine). Untreated control (C-pine) trees (grey arrow, no treatment) were included into the experiments. Needles were harvested from E-, F-, EF- and C-pine at different time points after treatments. A new set of trees was used for each sampling time point, thereby avoiding the possibility that sampling at an early time point affects the tree's response at a later time point. Needles were harvested at 1 h, 24 h and 10 days after egg deposition (yellow arrow). Eleven days after egg deposition, which is an early possible hatching time point after development of *D. pini* eggs under the abiotic conditions used, 10 *D. pini* larvae were placed each on egg-free and previously egg-laden pine trees (green arrow and brown arrow, respectively). Needles were harvested after a 1- and 24-h larval feeding period. At equivalent time points, we also harvested needles from egg-laden E-pine trees that had not received any larvae. Needles from control pine trees were harvested at all above-mentioned sampling time points. For the RNA sequencing and phytohormone analysis, n = 4–5 trees were used for each treatment and time point. For the qPCR, we used n = 3–5 trees per treatment and time point.

into the cylinder from the bottom and allowed to leave the cylinder from the top (airflow about 200 mL × min^{-1}).

Each tree was exposed to *D. pini* egg deposition (E), to *D. pini* larval feeding (F) or to both egg deposition and subsequent larval feeding (EF). We also kept trees untreated for control (C) in PLEXIGLAS cylinders. We simultaneously placed E-, F-, EF- and C-trees (n = 5 of each type) in the climate chamber and collected their needles after a certain treatment period (Figure 1). For each treatment period, a new set of trees was treated, and new control trees were included. Two experiments were conducted, one for harvesting needles for the RNA sequencing analysis and another one for the qPCR and phytohormone analysis. The schedule for needle harvesting after different treatment periods is outlined below (Figure 1, section 'sampling of needle material').

To obtain egg-treated (E) pine, two virgin male and two virgin female adults were placed on a tree and left there for 24 h to allow mating and egg deposition on the pine needles. Thereafter, the adults were removed, and the egg-laden pine was left in the cylinder for the treatment periods outlined in Figure 1. The natural egg incubation time of *D. pini* takes about 11–14 days under the abiotic condition used here.

To obtain pine exposed to larval feeding damage (F-treatment), 10 young larvae (L2 to L3) were taken from 'provider' trees and placed on the needles of egg-free pine. No first instar larvae (L1) were transferred to the experimental trees; these larvae are too vulnerable and mortality was always high after transfer. Pine needles with larvae were in a position equivalent to those where females had deposited their eggs on trees in the E-treatment setup.

To obtain pine exposed to eggs and standardized larval feeding (EF-treatment), trees were first exactly treated as E-pine. On Day 11 after experimental start, we placed 10 young larvae (L2 to L3) on the trees. Thus, the larvae were placed here briefly before the egg incubation time ended and before larvae would hatch naturally (Figure 1). If larvae had already hatched naturally from the eggs laid on a tree, this tree was excluded from the experiment.

This experimental procedure allowed us to standardize the onset of larval feeding as well as the number of feeding larvae in the F- and EF-treatment.

Sampling of needle material

We harvested locally treated needles from E-, F- and EF-pine trees and from the respective control C-pine trees after

different treatment periods (Figure 1). The entire treatment period lasted 12 days. Needles were always harvested during daytime (9:00–12:00 h).

Egg-laden needles were harvested 1 and 24 h after egg deposition to analyse early responses to eggs. Furthermore, egg-laden needles were harvested toward the end of the egg phase, i.e., 10 days after egg deposition, to determine transcriptomic pine responses just prior to larval hatching.

On Day 11, larvae were transferred to the plants and could feed there for either 1 or 24 h. Feeding-damaged needles were harvested 1 and 24 h after the onset of feeding damage from F-trees and EF-trees. Additionally, we sampled needles from E-trees at time points equivalent to those at which needles were sampled from F- and EF-trees; we collected needles only from those E-trees from which no larvae had hatched yet.

Needles from the untreated control (C) trees were harvested at the same time points and from equivalent positions as needles that were taken from E-, F- and EF-trees.

The harvested needles were frozen in liquid nitrogen and stored at −80 °C. Frozen needles were ground to a fine powder under liquid nitrogen with heat-sterilized mortars. We ground the entire intact needles from C-trees and the entire locally treated needles from F- and EF-trees. The egg-laden needles from E-trees were processed by cutting out the egg row and grinding only needle parts with a length of 2 cm maximum directly next to both sides of the egg rows. Needles were kept frozen during this process to exclude responses to the mechanical removal of the egg rows from the needles.

For the RNA sequencing and phytohormone analyses, we obtained n = 4–5 samples, and for the qPCR analyses, we had n = 3–5 samples of each treatment (E, F, EF and C) and each sampling time point. For the vast majority of treatments and time points, we obtained n = 5 samples as expected from the number of trees used. The irregular number of replicates is due to the rare exclusion of trees from sampling because (i) larvae hatched earlier than 10 days after egg deposition, (ii) larvae escaped from treated needles or died for unknown reasons, (iii) the number of available sawfly females was limited or (iv) the extraction of RNA or phytohormones was unsuccessful.

RNA extraction

RNA was extracted from ground frozen pine needles with the InviTrap Spin Plant RNA Mini Kit (Stratec, Berlin, Germany) according to the manual. Further details about extraction, purification and quality control are provided in Method S1 available as Supplementary data at *Tree Physiology* Online.

RNA sequencing

A volume of 25-μl RNA (dissolved in nuclease-free H_2O) of each sample was sent on dry ice for sequencing (Novogene Co., Ltd, Beijing, China). From this volume, 1-μg RNA per sample was used. The company conducted the following steps for sequencing. In short, first the RNA purity was checked using the NanoPhotometer spectrophotometer (IMPLEN, CA, USA). Thereafter, RNA integrity and quantitation were checked using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). Finally, sequencing libraries were generated using NEBNext UltraTM RNA Library Prep Kit for Illumina (NEB, USA) following the manufacturer's recommendations, and index adapters were added to attribute sequences to each sample. The library preparations were sequenced on an Illumina platform with a 150-bp paired end sequencing protocol. Further details of the company's purification, sequencing and library preparation are provided in Method S2 available as Supplementary data at *Tree Physiology* Online.

Transcriptome de novo assembly and annotation

Quality control of RNA sequencing raw reads and transcriptome de novo assembly from RNA sequencing was performed at Novogene.

For QC, reads containing adapter sequences, reads with more than 10% of uncertain nucleotides (labelled 'N' from the Illumina sequencing machine) and reads with more than 50% low-quality bases ($\leq$5) were removed. In total, 18.5×10^6 to 27.3×10^6 clean reads for each sample were obtained after QC and used for analysis.

For transcriptome de novo assembly, Trinity version 2.6.6 (Grabherr et al. 2011) was used, followed by hierarchical contig clustering with Corset version 4.6 (Davidson and Oshlack 2014) to remove redundant contigs. Reads from all samples were used to generate the assembly. The longest transcript of each cluster was then assigned as a unigene.

For annotation of the resulting unigene transcripts, we performed blast analysis on the Galaxy Europe platform (The Galaxy Community 2022) with its built-in tools. We constructed a blast database with the makeblastdb tool using release 55 of the TAIR10 Arabidopsis peptide annotation file from ENSEMBL plants (Yates et al. 2022). Unigene transcripts were annotated with blast against this database with a threshold of 10^{-5}. The highest-ranked hit was used for further analysis. In total, 60,295 (35.5%) of the pine unigene transcripts could be annotated to *A. thaliana* transcripts.

We used the built-in analysis tools of the BLAST2GO version 6.0.3 suite (Conesa and Götz 2008) to retrieve functional GO terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways from the annotated transcripts.

Differential gene expression analysis

For read counting, we used kallisto version 0.46.0 (Bray et al. 2016) with 100 bootstraps. The index file was created with the unigene transcript file from the de novo assembly pipeline. For the (putative) transcripts detected here in treated pine needles, we refer to differentially expressed genes (DEGs) as standard terminology while keeping in mind that the number of genes does not necessarily match the number of transcripts (Niu et al. 2022). The DEG analysis was conducted in R (version 3.6.1) (R Development Core Team 2015) with the DESeq2 package (Bioconductor version 3.9) (Love et al. 2014). R basic syntax was extended with the tidyverse package (version 1.3.0) (Wickham et al. 2019). Prior to importing the kallisto count files to DESeq2, tximport (Bioconductor version 3.9) (Soneson et al. 2015) was used to convert count files to the DESeq data format.

All genes with a read count sum greater than five in each sample were considered valid for further DEG analyses. In addition, we excluded all transcripts from statistical analysis that were not considered to be related to plant species. To identify the taxonomy of the unigene transcripts, we performed a Diamond Blast analysis with a rigid threshold of 10^{-9} against the complete ncbi_nr_2021_01 database included in the Galaxy server. To identify the taxonomic relationship between the blast results identified, we used the R package taxonomizr version 0.9.2 (https://cran.r-project.org/web/packages/taxonomizr/index.html).

cDNA synthesis and qPCR

To validate the RNA sequencing data, we conducted qPCR expression analyses of selected genes in needles from untreated trees and trees exposed to egg deposition, to larval feeding or to both egg deposition and larval feeding. In total, gene expression levels in control trees were compared with those in the seven following sample types: egg-treated needles 1 h, 24 h and 10 days after egg deposition; feeding-treated needles 1 and 24 h after the onset of feeding and egg-treated plus subsequently feeding-damaged needles 1 and 24 h after the onset of feeding (Figure 1). Samples for the qPCR analyses were collected from trees (n = 3–5) treated in an experimental setup independent of the setup used for RNA sequencing. For each sampling time point, new trees were used, thus avoiding the possibility that sampling at an early time point affected the tree's responses at a later time point.

We normalized the C(t) values of E-, F- and EF-samples to those of untreated C-samples and to the three housekeeping genes ubiquitin (*PsUBI*), cytochrome subunit 6 (*PsPetB*) and chloroplast ATPase beta subunit (*PsC-ATP*) according to Pfaffl (2001) and Vandesompele et al. (2002). Further details about the methods of the qPCR analyses are provided in Method S3 available as Supplementary data at *Tree Physiology* Online.

To validate the RNA sequencing data, we focused (i) on genes that might be involved in defence against insects, i.e., genes involved in cell wall modification, in phenylpropanoid and terpenoid biosynthesis, chitinase activity, Ca^{2+} signalling and phytohormone biosynthesis/signalling, and (ii) on genes that, according to the results of the RNA sequencing analysis, were significantly differentially expressed due to the treatment in at least three of the seven aforementioned sample types (Table S1 available as Supplementary data at *Tree Physiology* Online). The primer sequences of these genes and of the three selected housekeeping genes are presented in Table S2 available as Supplementary data at *Tree Physiology* Online.

Phytohormone analyses

In order to elucidate the phytohormonal responses of pine to *D. pini* eggs and larvae, we analysed concentrations of salicylic acid (SA), jasmonic acid (JA), jasmonic acid isoleucine (JA-Ile) and abscisic acid (ABA) in needles from untreated control trees and trees exposed to the E-, F- and EF-treatments (Figure 1). Samples for the phytohormone analyses were collected from trees that were also used for the qPCR analysis, i.e., from an experiment independent of that used for the RNA sequencing analysis. Phytohormone extraction and analyses were conducted following the methods described by Bandoly et al. (2016) and Drok et al. (2018). In short, ethyl acetate (spiked with deuterated phytohormones as internal standards) was used as extraction buffer. Extracted phytohormones were dried and resolved in 70% methanol. Phytohormones were analysed by UPLC-MS/MS (Q-ToF-ESI) and normalized to the respective internal standards and the weight of the extracted plant material. Further details are provided in Method S4 available as Supplementary data at *Tree Physiology* Online.

Data visualization and statistical analysis

Statistical analyses of RNA sequencing data were performed with DESeq2 (Wald test) for comparison of gene expression in control needles to those subjected to different treatments. Genes were considered to be DEGs at a significance level of $P \leq 0.05$ after Benjamini–Hochberg correction for multiple testing. The total number of DEGs per treatment and time point is given in Table S3 available as Supplementary data at *Tree Physiology* Online.

The GO term enrichment analysis and KEGG pathway enrichment analysis were performed on biological processes with DAVID version 2021 (https://david.ncifcrf.gov) (Da Huang et al. 2009). All GO terms and KEGG pathways containing at least three genes were considered enriched at P-value < 0.05 after using Fisher's exact test. The GO terms used in the enrichment analysis are given in Table S4 available as Supplementary data at *Tree Physiology* Online.

Calculation, visualization and statistical analyses of the qPCR and phytohormone data were performed using the software R version 3.6.1 (R Development Core Team 2015), SigmaPlot version 11.0 (Systat Software GmbH 2008) and Excel version 16.0 (Microsoft Corporation 2019). Data were tested for normal distribution with the Shapiro–Wilk test and for homogeneity of variances with Levene's test. Since a new set of trees was used for each sampling time point, samples taken at different time points were independent from each other. Pairwise comparisons of phytohormone and qPCR data obtained from treated needles with those of their respective controls were analysed using the Mann–Whitney U test. Multiple comparisons of phytohormone data obtained from feeding-damaged F- and EF-trees, as well as from E-pine trees and controls at equivalent times points, were analysed using the Kruskal–Wallis test followed by a Tukey post hoc test.

Results

RNA sequencing: transcript abundance, validation and overview of DEG analyses

The de novo assembly of the transcriptomes of untreated *P. sylvestris* needles (C), egg-treated needles (E), feeding-damaged needles (F) and those exposed to both eggs and subsequent larval feeding (EF) resulted in 169,750 putative transcripts (here referred to as DEGs) with a mean length of 1036 bp and an N50 length of 1511 bp (Table S5 and Item SI1 available as Supplementary data at *Tree Physiology* Online). Completeness of the transcriptome was assessed with BUSCO version 3.0.2 (Simão et al. 2015) and resulted in 69.9% complete matches, 7.4% duplicate matches, 6.0% fragmented matches and 16.7% missing matches with the pine unigene transcripts.

Overall, 13,344 genes were differentially expressed in treated trees when compared with control plants. Of these, 7510 were upregulated and 5834 downregulated (Figure 2, Table S3 available as Supplementary data at *Tree Physiology* Online).

The differential expression detected by the RNA sequencing analysis was validated by performing a qPCR analysis of 13 DEGs detected in differently treated samples harvested at different time points after treatment (seven sample types in total, see 'Materials and methods', section 'cDNA synthesis and qPCR'). These 91 comparisons of qPCR and RNA sequencing data resulted in about 87% of DEGs being regulated in the same direction, and about 69% that did not differ by more than 50% in their expression levels, while still being regulated in the same direction (Table S1 available as Supplementary data at *Tree Physiology* Online).

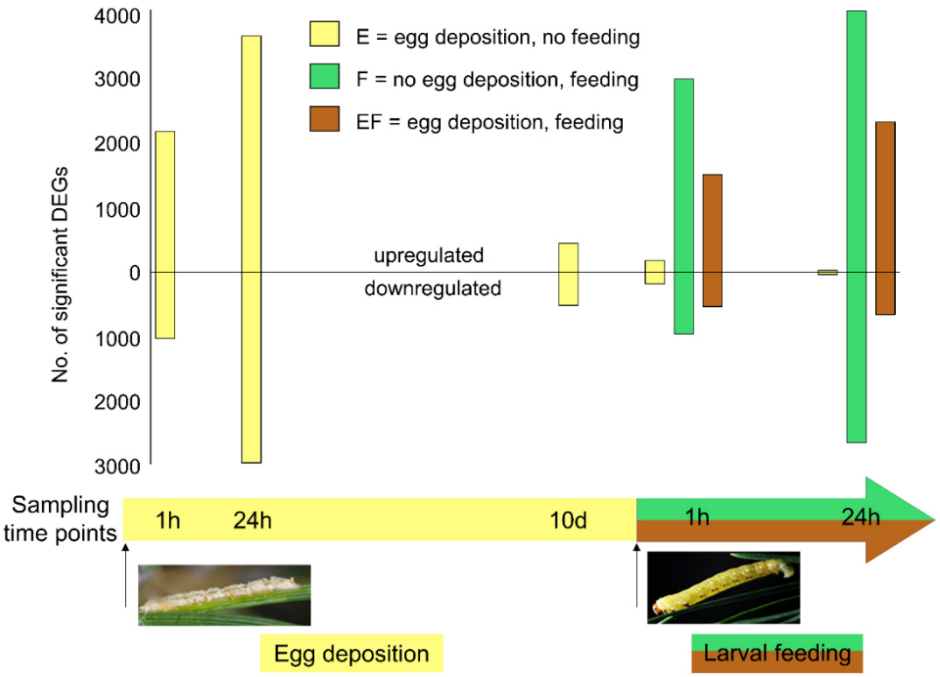

Figure 2. Number of DEGs. Needles of *P. sylvestris* were treated with *D. pini* egg deposition (E; yellow bars), larval feeding on previously egg-free pine (F; green bars) or natural egg deposition with subsequent feeding (EF; brown bars). Needles were sampled 1 h, 24 h and 10 days after egg deposition, as well as 1 and 24 h after the onset of larval feeding. The DEGs were differentially expressed to a significant degree when compared with untreated controls (C; Wald test; corrected *P*-value ≤ 0.05). Bars above (below) the zero *x*-axis show the number of upregulated (downregulated) DEGs. Number of replicates: $N = 4–5$ for each treatment and time point.

In the following sections, the transcriptomic responses of Scots pine are considered separately according to the different treatments applied and as compared with the untreated control. In addition, we subjected all genes that were differentially expressed in treated trees as compared with untreated control trees to a GO term analysis, as well as to a KEGG pathway analysis. We further analysed how the transcriptomes of the differently treated trees overlap.

Scots pine responds to sawfly egg deposition with strong transcriptomic changes and higher JA concentrations

To determine how the transcriptome and concentrations of phytohormones of a gymnosperm species change in response to sawfly egg deposition, we analysed the transcriptome and phytohormone levels of *P. sylvestris* at early and late time points after egg deposition. When analysing how many of the DEGs detected in all of the treatments were already regulated during the egg treatment of pine needles, we found that about 66% of all upregulated and about 69% of all downregulated DEGs were egg-responsive (Table S3 available as Supplementary data at *Tree Physiology* Online).

More than 3200 genes were significantly differentially expressed 1 h after egg deposition (Figure 2). This number more than doubled (to more than 6600 genes) 24 h after egg deposition. Following this strong, rapid transcriptomic response, the number of DEGs decreased to almost the control level during the egg incubation phase.

Overall, the pine trees showed a strong transcriptomic response especially in the 24 h following egg deposition. Thereafter, gene expression levels returned to almost the control level at the end of the egg phase.

A qualitative analysis of the egg-responsive genes (E vs C) by GO term enrichment analysis (Figure 3, Tables S4 and S6 available as Supplementary data at *Tree Physiology* Online) revealed that photosynthesis-related GO terms were enriched with downregulated genes 24 h after egg deposition. The GO terms involved in cell wall modification, lignin biosynthesis and cell death—including hypersensitive response (HR)—were mostly enriched with upregulated genes. Many GO terms related to secondary metabolites such as terpenes, flavonoids and other phenylpropanoids were enriched with upregulated genes at the first three time points during egg treatment. The GO terms involved in responses to chitin were also enriched in egg-treated pine. Among the phytohormone-related GO terms, those that were auxin-related were mostly enriched with upregulated genes, but only 1 h and 10 days after egg deposition. Ethylene-related terms were enriched with upregulated genes at all three time points during egg treatment. Among the ABA-related GO terms, some were enriched with upregulated DEGs (see Figure 3, top, ABA slot), but several were also enriched with downregulated DEGs 1 and 24 h after egg deposition (see Figure 3, bottom, ABA slot). Jasmonic acid-related terms were only enriched with upregulated genes; the number of enriched JA-related terms decreased during the egg phase. Salicylic acid-related GO

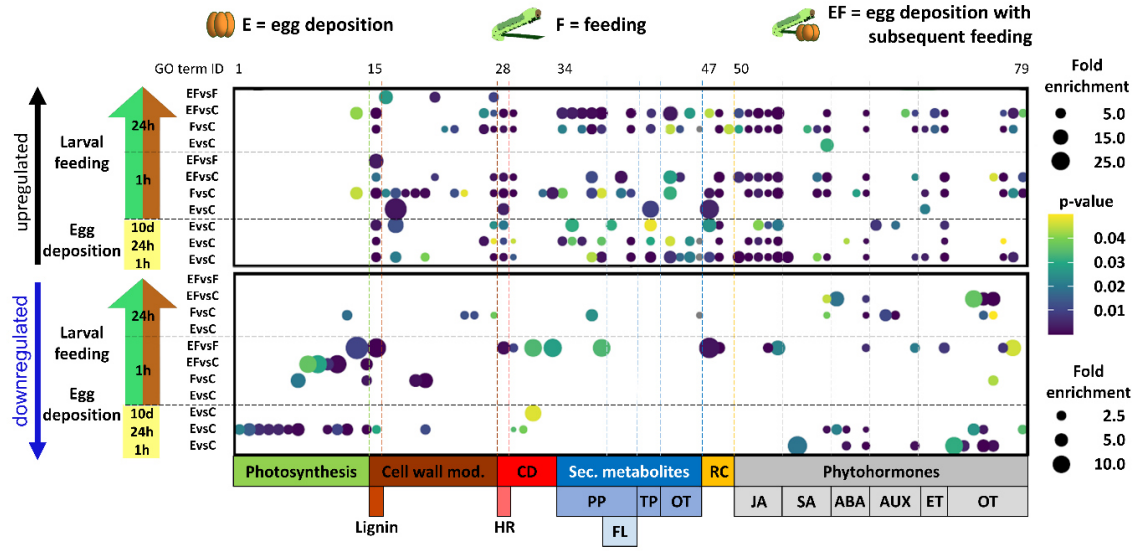

Figure 3. Gene Ontology term enrichment. Shown are significantly DEGs in needles of *P. sylvestris* 1 h, 24 h and 10 days after *D. pini* egg deposition, and 1 and 24 h after the onset of larval feeding. Top figure: enrichment with upregulated genes; bottom figure: enrichment with downregulated genes. Differently coloured horizontal bars below the figure show groups of GO terms related to similar biological processes, i.e., GO terms related to 'photosynthesis', 'cell wall modification' (cell wall mod.) (including lignin), 'cell death' (CD) (including 'hypersensitive response' (HR)), 'secondary metabolites' (sec. Metabolites) (including 'phenylpropanoids' (PP), 'flavonoids' (FL), 'terpenes' (TP) and 'others' (OT)), 'response to chitin' (RC) and those related to 'phytohormones' (including 'jasmonic acid' (JA), 'salicylic acid' (SA), 'abscisic acid' (ABA), 'auxin' (AUX), 'ethylene' (ET) and 'others' (OT)) are grouped here. The GO term identities included in these groups are listed in Table S4 available as Supplementary data at *Tree Physiology* Online (compare GO term ID numbers given above the figure with numbers in Table S4 available as available as Supplementary data at *Tree Physiology* Online). The enrichment of each GO term is shown by different circles for each treatment and sampling time point. The fold enrichment is illustrated by the size of each circle (highest enrichment = 25 in top figure; highest enrichment = 10 in bottom figure). The *P*-value (modified Fisher's exact test; $P < 0.05$) is visualized by the colour of each circle. Numbers in the yellow (egg deposition)/green/brown (feeding) arrows on the left side of the figure indicate the different sampling time points. The enrichments of GO terms for the treatments of egg deposition (E), larval feeding (F) and natural egg deposition with subsequent feeding (EF) were all compared to the respective, untreated control (C). Additionally, EF was compared with F. Horizontal, dashed lines separate data from E samples from those of EF and F samples, and data from EF and F samples at the 1 and 24 h sampling time points. Vertical, dashed lines separate the different GO term groups. A list of all significantly enriched GO terms is provided in Table S6 available as Supplementary data at *Tree Physiology* Online.

terms were enriched with both up- and downregulated genes mostly 1 h after egg deposition.

The KEGG pathway analysis supported the results obtained by the GO term analysis and revealed highly significant enrichment of downregulated genes involved in 'carbon fixation in photosynthetic organisms' and highly significant enrichment of upregulated genes involved in 'linolenic acid metabolism' and 'phenylpropanoid biosynthesis'. 'Biosynthesis of secondary metabolites' was strongly enriched with upregulated genes 1 h after egg deposition; however, 24 h after egg deposition, this category was strongly enriched with downregulated genes (Table S7 available as Supplementary data at *Tree Physiology* Online).

The phytohormone measurements (Figure 4) revealed a clear trend for an enhanced SA concentration 10 days after egg deposition. The JA concentration increased significantly 1 h after egg deposition; at later time points, JA levels no longer significantly differed between egg-laden and egg-free control needles. Concentrations of JA-Ile increased significantly 1 and 24 h after egg deposition. The ABA concentration was significantly higher 10 days after egg deposition. In contrast, just 1 h after egg deposition ABA levels were significantly lower than in the control needles.

Taken together, pine showed strong transcriptomic changes in response to sawfly egg deposition. Gene Ontology terms related to photosynthesis were enriched with downregulated genes, while GO terms related to cell wall modification, phenylpropanoids, terpenes and JA signalling were especially enriched with upregulated genes. The changes in phytohormone concentrations in response to the egg treatment were moderate, but significant, for JA, JA-Ile and ABA.

Pine transcriptomic responses to sawfly larval feeding largely overlap with responses to sawfly egg deposition

To address the question of how insect egg deposition on a gymnosperm species affects the plant's responses to subsequent larval feeding, we first analysed the transcriptomic and phytohormonal responses of pine to larval feeding on egg-free pine and compared them with the responses to egg deposition.

Feeding by sawfly larvae on egg-free pine needles caused the differential expression of 71% of all upregulated, and 55% of all downregulated, DEGs (Table S3 available as Supplementary data at *Tree Physiology* Online). Almost 4000 genes were differentially expressed 1 h after feeding upon egg-free needles, i.e., just a few more than the number of DEGs briefly after egg deposition (Figure 2). More than 6700 genes were differentially expressed 24 h after larval feeding. This was about the same number as was detected in response to a 24-h egg phase.

The GO term analysis of feeding-responsive genes in F-pine (F vs C) revealed enrichment at both sampling time points after the onset of larval feeding; these GO terms are related to photosynthesis, lignin, cell wall modification, HR, cell death, several classes of secondary metabolites, response to chitin and to phytohormones, especially JA (Figure 3, Tables S4

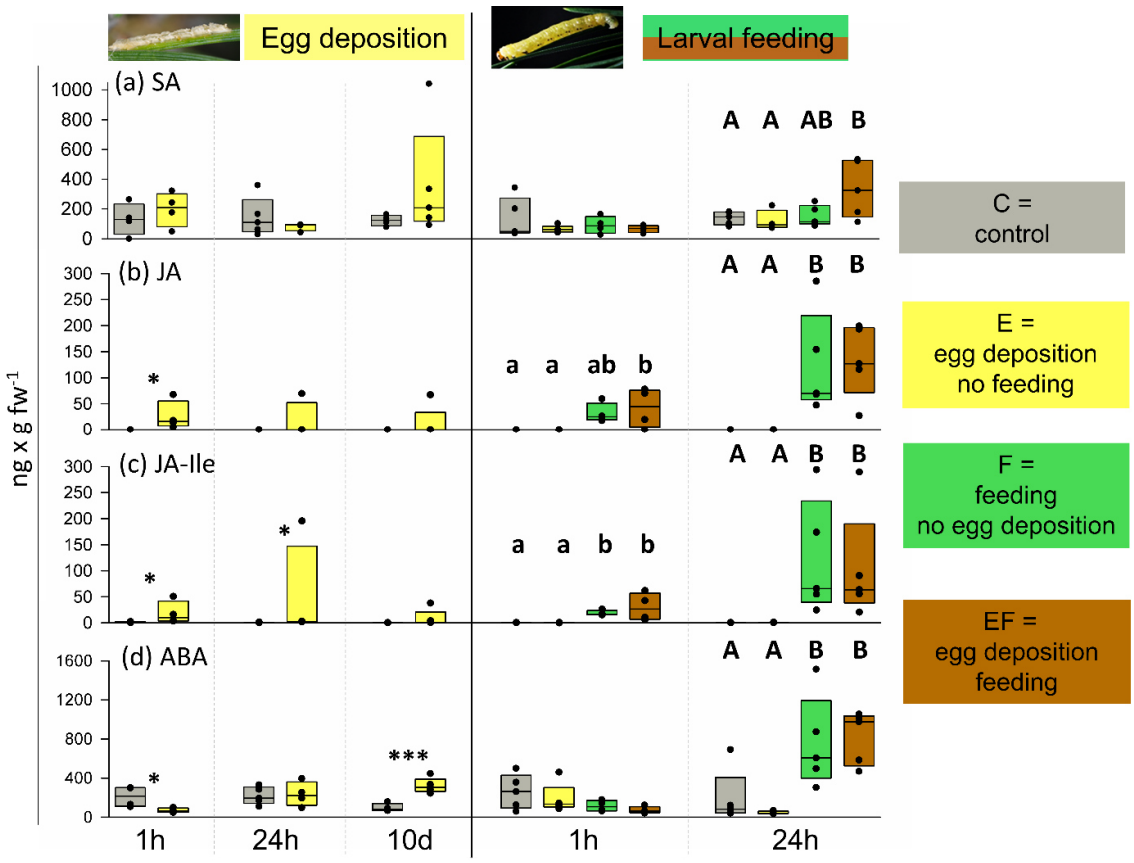

Figure 4. Phytohormone concentrations in needles of *P. sylvestris*. Shown are the results 1 h, 24 h and 10 days after *D. pini* egg deposition, as well as 1 and 24 h after the onset of larval feeding. The non-normally distributed data are visualized as boxplots with the median as centre and all data points as dots. (a) Salicylic acid (SA), (b) jasmonic acid (JA), (c) jasmonic acid-isoleucine (JA-Ile) and (d) abscisic acid (ABA). Treatments were: natural egg deposition (E; yellow), larval feeding (F; green), egg deposition with subsequent feeding (EF; brown) and an untreated control (C; grey). Significant differences between concentrations in C-pine and E-pine 1 h, 24 h and 10 days after egg deposition are indicated by asterisks (Mann–Whitney U test; $*P < 0.05$, $***P < 0.001$). Significant differences between control, E-, F- and EF-pine at the 1 and 24 h time points of larval feeding (and equivalent time points in C- and E-pine) are indicated by different letters ($P < 0.05$; Kruskal–Wallis test with Tukey post hoc test). For each treatment and time point: $N = 4$–5 replicates. In some cases, fewer dots than four are visible per treatment; these dots (data) are overlapping.

and S6 available as Supplementary data at *Tree Physiology* Online). In contrast, few GO terms (photosynthesis, cell wall modification, phenylpropanoids, SA, ABA and auxin) were enriched with downregulated genes in F-pine (Figure 3, Tables S4 and S6 available as Supplementary data at *Tree Physiology* Online).

According to the KEGG pathway analysis, the categories most significantly enriched with upregulated genes in response to feeding damage were 'phenylpropanoid biosynthesis', 'biosynthesis of secondary metabolites' and 'plant–pathogen interaction'. 'Zeatin biosynthesis' was strongly enriched with downregulated genes after a 1-h feeding period, but after a 24-h feeding period, this pathway was significantly enriched with upregulated genes (Table S7 available as Supplementary data at *Tree Physiology* Online).

The phytohormone analysis revealed that SA levels did not significantly change in response to larval feeding. Jasmonic acid and JA-Ile levels slightly increased already after a 1-h feeding period and strongly increased after a 24-h feeding period. ABA levels were significantly enhanced in needles of F-trees 24 h after the onset of larval feeding (Figure 4).

When comparing the pine responses to larval feeding (F vs C) with those to egg deposition (E vs C), our transcriptomic data revealed that many of the egg-responsive genes were also differentially expressed in response to feeding damage (Figure 5a). The upregulated DEGs in E- and F-pine overlap by 40.2% of the total number of upregulated DEGs, while the downregulated DEGs overlap by 28.1% of the total downregulated DEG number (Table S3 available as Supplementary data at *Tree Physiology* Online). Both E-pine and F-pine showed especially strong transcriptomic responses to genes involved in cell wall modification and JA signalling. Accordingly, both E- and F-pine showed increases in JA and JA-Ile concentrations (Figure 4).

Egg-laden, feeding-damaged pine shows weaker transcriptomic responses, but higher SA levels, than egg-free, feeding-damaged pine

To elucidate the impact of insect egg deposition on the transcriptomic and phytohormonal responses of a gymnosperm species to larval feeding damage, we analysed the

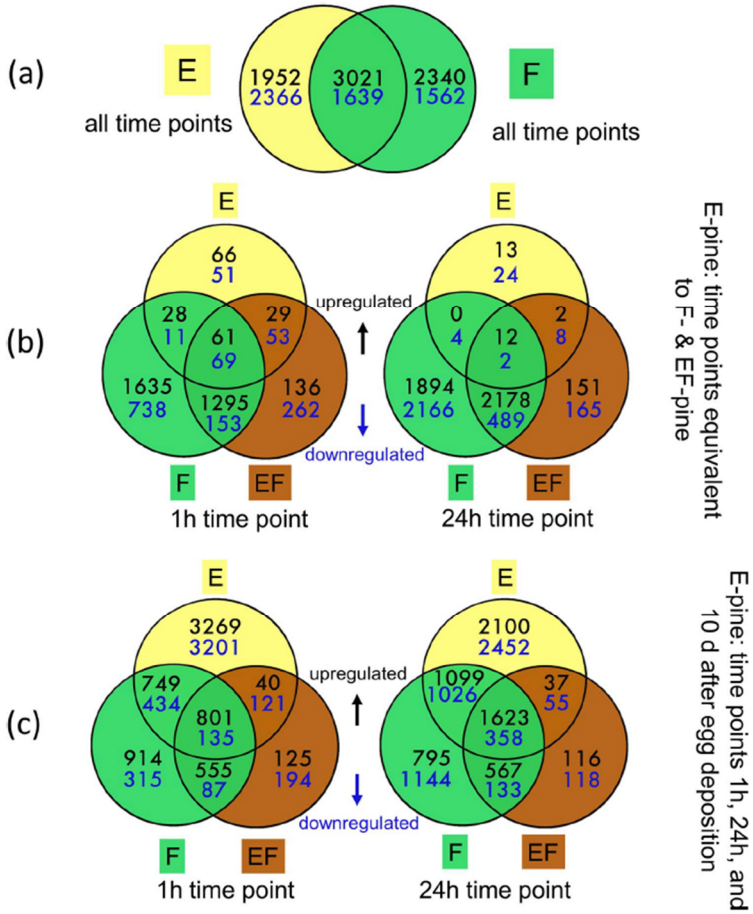

Figure 5. Overlapping DEGs in differently treated *P. sylvestris* trees. Venn diagrams are showing the number of pine genes uniquely and commonly (overlapping) differentially expressed in trees that were treated with natural egg deposition (E; yellow), larval feeding (F; green) or natural egg deposition with subsequent feeding (EF; brown). (a) Differentially expressed genes of E-trees and F-trees at all sampling time points; (b) DEGs in F- and EF-pine 1 and 24 h after the onset of larval feeding and in E-trees at equivalent time points; (c) DEGs in F- and EF-pine 1 h and 24 h after the onset of larval feeding and in E-trees 1 h, 24 h and 10 days after egg deposition. Black numbers show upregulated, and blue numbers downregulated, genes, all normalized to untreated controls (C).

transcriptome and phytohormone concentrations of previously egg-laden pine after a 1- and 24-h larval feeding period. In a further step, we compared the responses of these EF-pine trees to those of egg-free, feeding-damaged F-pines.

In total, 43% of all detected DEGs were upregulated, and 20% downregulated, in previously egg-laden and subsequently feeding-damaged (EF) trees (Table S3 available as Supplementary data at *Tree Physiology* Online). When combining the number of DEGs in EF- and F-trees over the two sampling time points during feeding damage, they made up 75% of the upregulated DEGs and 63% of the downregulated DEGs detected overall.

The number of DEGs in EF-pine after 1 h of feeding damage was about 2000, which was almost half the number of DEGs in egg-free, feeding-damaged F-pine (Figure 2). In summary, the number of DEGs was surprisingly much higher in feeding-damaged F-pine without prior egg deposition than in feeding-damaged EF-pine with prior egg deposition.

To address the question of how many of the DEGs in EF-pine are uniquely expressed in these trees and how many are also differentially expressed in F-pine, we conducted two overlap analyses.

First, we conducted an overlap analysis that included the DEGs in E-pine sampled at equivalent time points as in F- and EF-pine (compare Figures 1 and 5b). This comparison allowed us to detect how many of the genes that were still regulated by the egg treatment at these time points overlap with those in EF- and F-pine. One hour after the onset of larval feeding, there were fewer than 400 genes uniquely expressed in EF-treated plants, while almost 1600 genes were expressed in both F- and EF-pine. About 2400 genes were additionally uniquely expressed in F-pine. This pattern was even clearer

after 24 h, with about 300 genes uniquely expressed in EF-pine, but around 2600 expressed in both treatments and about 4000 uniquely expressed genes in F-treated pine. Therefore, while F- and EF-pine had many DEGs in common, F-pine had more uniquely expressed DEGs. There was minimal overlap of DEGs in F- and EF-pines with those in E-pines because of the low number of DEGs that were detected at these time points (see Figure 2, sampling time points for E-pine equivalent to 1 and 24 h after the onset of feeding upon F- and EF-pine).

In a second overlap analysis, we compared the DEGs in F- and EF-pine and additionally included the DEGs in E-pine detected 1 h, 24 h and 10 days after egg deposition. This comparison allowed us to determine the number of DEGs that were uniquely expressed only during the feeding phase, but not during the egg phase (Figure 5c). When comparing the overlap of these DEGs just between E-pine and F-pine after a 1-h feeding period (~1200) with the overlap of DEGs just between E- and EF-pine after a 1-h feeding period (~160), the number of overlapping DEGs in E- and F-pine was almost 10-fold higher. When doing the same analysis after 24 h of feeding, the difference was even stronger, with more than a 20-fold higher number of DEGs in the E- and F-pine overlap (~2100 vs ~90). In spite of this huge overlap of DEGs in E- and F-pine, there were still many genes uniquely expressed during the egg phase when comparing them to the feeding-damaged plants at both time points. This analysis again revealed a substantial overlap of DEGs in F- and EF-pine. It further showed that the number of common DEGs in E- and EF-pine was much smaller than in E- and F-pine.

A qualitative comparison of the DEGs in EF-pine and controls (EF vs C) revealed many GO terms enriched with upregulated genes at both sampling time points (Figure 3, Tables S4 and S6 available as Supplementary data at *Tree Physiology* Online). These GO terms include all those mentioned in Figure 3. For GO terms enriched with downregulated genes, we found a conspicuous enrichment of photosynthesis-related GO terms early (1 h) after the onset of feeding. This finding is supported by the KEGG analysis, which also showed significant enrichment with downregulated genes, such as in the category 'carbon fixation of photosynthetic organisms' at this time point (Table S7 available as Supplementary data at *Tree Physiology* Online).

When directly comparing the GO term enrichment in EF- and F-pine (EF vs F, Figure 3), the most prominent differences were detected for GO terms related to photosynthesis, lignin, HR and cell death, secondary metabolites, responses to chitin and to JA. These GO terms were significantly more enriched with downregulated genes in EF- than F-pine after 1 h of feeding. These differences vanished after 24 h of feeding. At this time point, three GO terms related to cell wall modification were significantly more enriched with upregulated genes in EF-pine than F-pine (GO terms 'xyloglucan metabolic process', 'plant epidermis development' and 'cell wall organization'; Figure 3, Table S4 available as Supplementary data at *Tree Physiology* Online).

The KEGG pathway enrichment analysis also showed highly significant enrichment with upregulated genes involved in 'biosynthesis of secondary metabolites' as well as in 'phenylpropanoid biosynthesis' for EF-pine, which were also enriched in F-pine (Table S7 available as Supplementary data at *Tree Physiology* Online). A further pathway highly enriched with upregulated genes in EF-pine was 'alpha-linolenic acid metabolism' in EF-pine after a 24-h feeding period.

With respect to phytohormone concentrations, JA concentrations were significantly higher 1 h after larval feeding in EF-pine needles than in C-pine needles, whereas the concentrations in F-pine were only tentatively higher. At the same time point, JA-Ile concentrations were higher in both EF-pine and F-pine compared with C-pine, but EF-pine and F-pine did not differ from each other. After 24 h of larval feeding, all phytohormone concentrations were significantly higher in EF-pine and F-pine compared with C-pine, except for SA which was only significantly higher in EF-pine. However, none of the phytohormone concentrations differed between EF-pine and F-pine needles.

Overall, sawfly egg deposition changed the transcriptomic responses to feeding damage by attenuating the feeding-induced transcriptomic response and by enriching especially GO terms related to cell wall modification with upregulated genes. With respect to phytohormonal changes in response to feeding damage, egg deposition affected only the SA concentrations in feeding-damaged pine, but none of the other analysed phytohormones.

Discussion

Our study demonstrated that *P. sylvestris* showed strong and rapid transcriptomic responses to egg deposition of the sawfly *D. pini*. The differential expression of genes in response to egg deposition almost reverted to control levels toward the end of the egg phase. Feeding by young larvae upon egg-free pine needles induced a strong transcriptomic response that largely overlapped with the response to egg deposition. The transcriptomic response to larval feeding was much weaker when needles had been previously exposed to egg deposition. While both EF-pine and F-pine showed significantly enhanced levels of JA, JA-Ile and ABA, only EF-pine had significantly enhanced SA levels when compared with untreated control pine. We found the enrichment of phenylpropanoid-related GO terms and of the KEGG pathway 'phenylpropanoid biosynthesis' with upregulated genes after egg deposition in E-pine, but also in feeding-damaged F- and EF-pine.

To highlight the responses of a gymnosperm species to insect egg deposition and feeding compared with the known responses of angiosperm species, we will first contrast the transcriptomic and phytohormonal responses of *P. sylvestris* to sawfly egg deposition with the known responses of angiosperms to egg deposition. Then, we will compare the effects of insect egg deposition on pine responses to larval feeding damage with the impact of insect eggs on responses of angiosperm plants to larval feeding.

Pine responses to insect egg deposition: a comparison with angiosperm plant responses

When considering the dynamics of pine transcriptomic responses to *D. pini* egg deposition, the intense gene expression observed 24 h after egg deposition then declined until it had almost vanished by the end of the egg phase (Figure 2). Similarly, in elm (*U. minor*) leaves, the highest number of egg-responsive genes has been detected 1 h after elm leaf beetle egg deposition (Altmann et al. 2018). Other angiosperm plants such as *A. thaliana* (Little et al. 2007, Valsamakis et al. 2022), bittersweet nightshade *Solanum dulcamara* L. (Geuss et al. 2017) and tobacco plants (*Nicotiana attenuata* Torr.

ex S. Watson) (Drok et al. 2018) have shown a considerable number of DEGs 1–3 days after egg deposition. Similar to the response of pine to sawfly eggs, egg-induced differential expression of genes in elm had almost reverted to the control level by the end of the egg phase (Altmann et al. 2018). Thus, these perennial wooden plant species of pine and elm show similar dynamics of transcriptomic responses to insect egg deposition.

In pine, more than half of all DEGs in E-pine were also regulated in F-pine. A similar overlap was found in elm trees infested by elm leaf beetle eggs or elm leaf beetle larvae (Altmann et al. 2018). The overlaps may be due to the oviposition mode of these two herbivorous insect species. *Diprion pini* slits a needle longitudinally, cutting the parenchymatic tissue, and inserts its eggs in a row into the slit needle (Hilker et al. 2002). The egg deposition of the elm leaf beetle is also associated with leaf wounding; the beetle removes the leaf epidermis at the oviposition site and lays its eggs on parenchymatic tissue (Hilker and Meiners 2006). The leaf wounding associated with egg deposition by *D. pini* and the elm leaf beetle might explain (i) that the egg deposition process induces a similar set of genes in the host plants of these insect species as larval feeding does and (ii) that the dynamics of the transcriptomic responses to insect egg deposition are similar in pine and elm. However, an overlap of egg- and feeding-responsive genes was also found in *A. thaliana* (Valsamakis et al. 2022), in black mustard plants (*Brassica nigra* L. W. D. J. Koch) (Bonnet et al. 2017) and in tobacco plants (*N. attenuata*) (Drok et al. 2018) infested with eggs or larvae of lepidopteran species, which do not damage the leaf tissue during oviposition. Furthermore, a Generally Applicable Gene set Enrichment analysis of four angiosperm species treated with insect eggs and larval feeding also revealed a large overlap of insect egg- and feeding-induced responses (Lortzing et al. 2020). Thus, regardless of ovipositional wounding, plant transcriptomic responses to eggs and to larvae obviously share a common and conserved core response.

The type of GO terms and KEGG pathways enriched with DEGs in response to sawfly egg deposition on pine suggests that this gymnosperm species shares several similarities with angiosperm species in its response to insect eggs (Figure 3, Table S7 available as Supplementary data at *Tree Physiology* Online). In the following, we will focus on GO terms related to photosynthesis, hypersensitive responses (HR), response to chitin, phenylpropanoid biosynthesis and terpenoid biosynthesis.

The enrichment of photosynthesis-related GO terms and of the KEGG pathway 'carbon fixation in photosynthetic organisms' with downregulated pine genes supports previous studies that have shown reduced photosynthetic activity in egg-laden *P. sylvestris* (Schröder et al. 2005). When considering that pine increased its JA levels briefly after sawfly egg deposition, it is an interesting parallel that the downregulation of photosynthesis-related genes was also found in other conifers (e.g., *Pinus albicaulis* Engelm., *Picea abies* L. H. Karst.) treated with methyl jasmonate (Liu et al. 2017, Wilkinson et al. 2022). Downregulation of photosynthetic activity or related genes was found as well in angiosperm species responding to methyl jasmonate (Lee and Zwiazek 2019) or insect egg deposition (Little et al. 2007, Valsamakis et al. 2022). The downregulation of photosynthetic activity may be considered a trade-off of defence against the eggs (Schröder et al. 2005).

Enrichment of the GO term 'plant type hypersensitive response' with upregulated genes in egg-laden pine corroborates previous studies that have shown the accumulation of ROS and necrotic plant tissue in *P. sylvestris* laden with *D. pini* eggs (Bittner et al. 2017, 2019). Accumulation of ROS and the formation of HR-like symptoms have also been shown in *A. thaliana* (Little et al. 2007, Gouhier-Darimont et al. 2013), several brassicacean species other than *A. thaliana* (Bruessow and Reymond 2007, Griese et al. 2021, Caarls et al. 2023) and a solanaceous species (*S. dulcamara*) (Geuss et al. 2017). Responses such as these might result in desiccation of the eggs (Hilker and Fatouros 2015, Griese et al. 2021). Furthermore, the oviposition mode of the sawfly results in considerable disruption of cell wall integrity. Such a change in cell wall architecture induced by stress is well known to be linked with hydrogen peroxide accumulation—which might lead to HR and lignin deposition (Rui and Dinneny 2020, Baez et al. 2022). Enrichment of the GO term 'chitin response' in egg-laden pine may be caused by the chitinous ovipositor valves of the sawfly female, and possibly abrased, minute particles of the saw teeth. A previous study by Davis et al. (2002) revealed that chitinases are inducible by exogenous application of JA onto slash pine, a finding that is interesting in light of the induction of JA in *P. sylvestris* early after sawfly egg deposition.

One early response of pine to egg deposition showed a clear enrichment of phenylpropanoid-related GO terms and the KEGG pathway 'phenylpropanoid biosynthesis' with upregulated genes. Egg-induced concentrations of phenylpropanoids or egg-induced expression of genes involved in phenylpropanoid synthesis have been observed in angiosperm species, e.g., *A. thaliana* (Little et al. 2007, Lortzing et al. 2019) and bittersweet nightshade (Geuss et al. 2017). The defensive function of these increased concentrations of phenylpropanoids, such as flavonoids, against eggs remains unclear. However, if hatching larvae encounter enhanced the concentrations of phenylpropanoids produced during the egg phase, these compounds might harm those larvae. A defensive function of phenylpropanoids against the feeding stages of insects has been shown in numerous studies (War et al. 2018, Singh et al. 2021).

Our data show egg-induced expression of a sesquiterpene synthase (Table S1 available as Supplementary data at *Tree Physiology* Online) and enrichment of the GO term 'diterpenoid biosynthetic process' with upregulated genes (Figure 3, Table S4 available as Supplementary data at *Tree Physiology* Online). Gymnosperms are rich in terpenes, which serve as defensive compounds against many herbivorous insects (Mumm and Hilker 2006). So far, we do not know whether the enrichment of the GO term 'diterpenoid biosynthetic process' in the pine trees used here would result in an enhanced production of viscous, sticky diterpenes (Keeling and Bohlmann 2006) that might harm the gas exchange of developing sawfly eggs. It has been shown that egg-induced changes in the emission of mono- and sesquiterpenes in some angiosperm species, e.g., elm (Büchel et al. 2011) and black mustard (Fatouros et al. 2012), serve as an attraction of egg parasitoids to host eggs.

The pine phytohormonal responses to *D. pini* eggs show some parallels to the responses of *A. thaliana* to *Pieris brassicae* eggs. Egg-induced increase in JA and JA-Ile levels was found in both plant species (Valsamakis et al. 2020), although *A. thaliana* leaves are not wounded by *P. brassicae* egg

deposition. Levels of SA were enhanced by trend in egg-laden pine, whereas *A. thaliana* and *S. dulcamara* laden with eggs showed significantly higher SA levels (Bruessow et al. 2010, Geuss et al. 2017). While the ABA concentrations in *A. thaliana* did not change in response to egg deposition, *P. sylvestris* did have increased ABA levels by the end of the egg incubation phase. Future studies need to quantify whether this increase in ABA concentrations is related to increased abscission of egg-laden needles. We observed very little abscission of egg-laden needles in our experiments and the abiotic laboratory conditions used here.

Impact of egg deposition on pine responses to feeding damage

When comparing the effect of egg deposition on the transcriptomic response of pine and angiosperms to the one of feeding damage, one difference is immediately apparent. While EF-pine showed strikingly less differential expression of genes than F-pine, previously egg-laden angiosperm plants have been found to respond to larval feeding damage with more transcriptomic activity than egg-free plants, at least when the feeding damage began (Bonnet et al. 2017, Altmann et al. 2018, Drok et al. 2018, Lortzing et al. 2019). The higher number of DEGs in EF- than F-angiosperm plants at the onset of larval feeding was found regardless of whether the oviposition was associated with leaf wounding or not. This suggests that the attenuated transcriptional response of EF-pine to larval feeding when compared with F-pine is not only due to the particular *D. pini* oviposition mode.

The considerable overlap of DEGs in E- and F-pine, in combination with the initially high but subsequently diminishing response to egg deposition, suggests that EF-pine can afford a less powerful transcriptional response to larval feeding damage because many genes have already been expressed during the egg phase and might need regulation only in F-pine, i.e., plants that have not experienced egg deposition prior to larval feeding. This implies that processes induced by the differential expression of genes in the egg phase remain active or can be quickly reactivated, in response to feeding damage. If indeed processes induced by gene expression early after egg deposition remained in a 'stand-by' mode until the end of the egg phase but were activated upon feeding more sensitively and efficiently in EF-pine than feeding-inducible processes in F-pine, this would fit into the concept of priming, which here would occur on the posttranscriptional level (Conrath et al. 2015, Hilker et al. 2016, Martinez-Medina et al. 2016, Wilkinson et al. 2019). At the beginning of larval feeding (within 1 h), fewer 'cell wall modification'- and 'cell death'-related GO terms enriched with upregulated genes were detected in EF-pine than in F-pine when compared with C-pine. These GO terms were also found to be enriched with upregulated genes in egg-laden pine. Changes triggered by the differential expression of these genes might still be effective against feeding larvae. However, when considering GO terms related to 'secondary metabolites', and especially to 'phenylpropanoids' and 'terpenes', after 24 h of feeding, more GO terms were more strongly enriched with upregulated genes in EF-pine than F-pine. If these transcriptomic responses of EF-pine trees result in enhanced concentrations of phenylpropanoids, it would parallel the metabolic responses of egg-laden angiosperms to larval feeding damage. Several angiosperm plants increase their concentrations of distinct phenylpropanoids when exposed to insect egg deposition prior to larval feeding damage, e.g., caffeoyl putrescine in tobacco plants (Bandoly et al. 2015, 2016) and quercetin and kaempferol derivatives in elm and *A. thaliana* (Altmann et al. 2018, Lortzing et al. 2019).

Sawfly egg deposition on pine significantly affected the SA concentration in EF-pine after 24 h of larval feeding, whereas no such effect was found in F-pine. None of the other phytohormonal responses to larval feeding in pine was affected by prior egg deposition. The high concentration of SA in EF-pine seems not to be based on a maintained (high) egg-induced SA level concentration lasting into the end of the egg phase. Several EF-treated angiosperm plants have also shown higher levels of SA than controls (Bonnet et al. 2017, Lortzing et al. 2019, Schott et al. 2022). No antagonistic ecological effects of (feeding-induced) high JA and SA levels were detected in these EF-plants, as might be expected based on other studies of the interaction between JA and SA (Erb et al. 2012, Pieterse et al. 2012, Thaler et al. 2012, Caarls et al. 2015). However, the dynamics of concentration changes and the ratio of SA and JA(-Ile) might play a role in determining the ecological effects of JA and SA interactions. Rather than leading to the antagonistic interactions often observed, the elevated levels of SA and JA in EF-plants might result instead in coordinated interactions, thus contributing to improved plant defences. Several other studies addressing the interactions of JA and SA have also found neutral or positive interactions between JA and SA, both in angiosperms (e.g., Schenk et al. 2000, Mur et al. 2006, Lortzing et al. 2019, Zhang et al. 2020, Aerts et al. 2021, Ullah et al. 2022) and gymnosperms (Arnerup et al. 2013).

Conclusions

Our study revealed that *P. sylvestris* responds to *D. pini* egg deposition by remarkable changes in the expression of numerous genes. These responses affected later transcriptional responses to larval feeding damage. Pine transcriptional responses to both the egg deposition and larval feeding damage showed considerable overlaps and occurred rapidly, indicating a fast and sensitive perception of infestation-associated molecular patterns, which might be important to limit the infestation already in its initial phase.

A comparison of pine responses with those of angiosperms to insect egg deposition and subsequent larval feeding highlights several common features, among them the downregulation of photosynthesis and changes in cell wall structure in E-plants as well as a stronger upregulation of phenylpropanoid biosynthesis and a stronger increase in SA levels in EF-plants compared with F-plants. A striking difference between the transcriptomic responses of EF-pine and EF-angiosperms is the clearly attenuated response of EF-pine to larval feeding, while EF-angiosperms studied until now have shown stronger transcriptomic responses to the onset of larval feeding. A strong transcriptomic response of EF-pine to feeding damage might be redundant. Processes rapidly triggered by expression of the numerous genes induced by the sawfly's severe ovipositional wounding might still be active, or easily be reactivated, when larvae start feeding. Thus, a more 'relaxed' transcriptomic response of egg-laden pine to feeding damage might help to avoid 'hyper-immunity' and benefit the 'maintenance of signal homeostasis', as recently discussed by Pontiggia et al. (2020) with regards to plant responses to stress. Future studies of gymnosperms infested by other insect species, which do not inflict severe ovipositional wounding to the needles, need to clarify whether the attenuated transcriptomic responses of *P.*

sylvestris laden with *D. pini* eggs to larval feeding damage are characteristic of gymnosperm species, or whether this is due in pine to the severe ovipositional damage inflicted by this sawfly species.

Furthermore, since *D. pini* shares a long evolutionary history with its host plant species like many other herbivorous insects (Kergoat et al. 2017), more research is needed to elucidate the counteradaptations of these insects to the plant's egg-mediated defences. Studies of possible suppressive effects of insect egg deposition on plant defences against larvae (Bruessow et al. 2010) as well as of avoidance behaviour of the insects (e.g., egg deposition on the bark or larval movements to egg-free needles) will shed further light on how insects can counteract egg-mediated plant defences.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

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Conflict of interest

None declared.

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Authors' contributions

J.H. and M.H. designed the study and planned the experiments. J.H. conducted the experiments for RNA sequencing and prepared the RNA samples for this analysis. N.B. conducted the transcriptome assembly and annotation and prepared the DEG data set. T.L. prepared the samples for the qPCR experiments and phytohormone analysis. He conducted the qPCR analysis for validating the results obtained by the RNA sequencing analysis. J.H. analysed the DEG data set and conducted a GO term enrichment and KEGG pathway enrichment analysis. J.H. conducted the phytohormone analysis. V.L. provided advice for the GO term and KEGG analysis and prepared Figure 3. J.H. wrote a first draft of the manuscript. M.H. revised the manuscript. All authors contributed to the writing of the manuscript and approved the submitted version.

Data availability statement

The transcriptomic data set is available at the BioStudies database under the accession no. S-BSST1074. Further data are available within the manuscript and/or its supplementary materials.

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Supplementary Data

The impact of insect egg deposition on *Pinus sylvestris* transcriptomic and phytohormonal responses to larval herbivory

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Overview Supplementary Data

Online available as pdf (see below):

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Table S2: Primers used for validation of RNA sequencing data by qPCR analysis**Table S3:** Number of differentially expressed genes (DEGs) per treatment **Table S4:** GO-term ID given in Figure 3, main text

Online available at https://www.ebi.ac.uk/biostudies/studies/S-BSST1074

Table S5: Gene expression in differently treated pine needles compared to the control
Table S6: Enriched gene ontology (GO) terms per treatment
Table S7: Enriched Kyoto Encyclopedia of Genes and Genome (KEGG) pathways per treatment
Item SI1: Nucleotide sequences obtained from the translated RNA sequencing data

Method S1: RNA extraction, purification and quality control

For RNA sequencing and qPCR analyses, RNA was extracted from 50 mg of frozen, powdered *Pinus sylvestris* needles with the InviTrap Spin Plant RNA Mini Kit (Stratec, Berlin, Germany) according to the manual. After the RNA was eluted in 50 µl nuclease-free H_2O, remaining traces of DNA were digested with the TURBO DNA free™ kit (ThermoFisher Scientific) according to the manual. Integrity and purity of the RNA was checked by using a 1.1% agarose gel in 1X TAE buffer with 0.00005% ROTI®GelStain (Carl Roth). RNA loading dye 2x (ThermoFisher Scientific) was mixed 1:1 v/v with 10 µl of sample. The mixture was heated to 70°C for 10 min, then placed on ice for a short cooling period, and loaded on the gel. In addition, 4 µl of RiboRuler High Range RNA Ladder (ThermoFisher Scientific), which was previously treated likewise, was loaded on the gel. The gel ran for 90 min at 120 V. RNA concentrations were measured using the nanodrop method by determining the absorbance of 2 µl sample volume at 230 nm with an Infinite® M Nano+ plate reader (Tecan Trading).

Method S2: RNA sequencing

To analyse the pine transcriptome response to *Diprion pini* egg deposition and larval herbivory, 25 µl RNA (dissolved in nuclease-free H2O) of each sample was sent on dry ice to Novogene Co., Ltd. for sequencing. RNA purity was checked again by the sequencing company using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). RNA integrity and quantitation were also checked again by using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

In total, 1 µg RNA per sample was used for the RNA sequencing library preparations. Sequencing libraries were generated using NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations, and index adapters were added to attribute sequences to each sample. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X). First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNase H-). Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3' ends of DNA fragments, NEBNext Adaptor with hairpin loop structure were ligated to prepare for hybridization. In order to select cDNA fragments of preferentially 150~200 bp, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). Then 3 µl USER Enzyme (NEB, USA) was used with size- selected, adaptor-ligated cDNA at 37°C for 15 min followed by 5 min at 95 °C before PCR. Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. Finally, PCR products were purified (AMPure XP system), and library quality was assessed on the Agilent Bioanalyzer 2100 system.

The clustering of the index-coded samples was performed on a cBot Cluster Generation System using PE Cluster Kit cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina platform and paired-end reads were generated.

Method S3: cDNA synthesis and qPCR

To validate the RNA sequencing results, a qPCR of selected genes was performed, and the results were compared to those of the RNA sequencing analysis. Synthesis of cDNA was conducted using 1 µg RNA

as template for reverse transcription with the AMV-RT native protein (Roboklon, Berlin, Germany) according to the manual. In an initial step, nuclease-free H_2O was added to RNA, 2 µl Oligo dT20 (50 µM), 4 µl dNTPs (10 mM) until a total volume of 28 µl was reached. This mixture was incubated for 5 min at 65°C followed by 5 min incubation on 4 °C. In the second step, 8 µl 5x RT buffer (Roboklon, Berlin, Germany), 1 µl RNASE inhibitor (Roboklon; 30 U μl^{-1}), 2 µl 100 mM DTT, and 2 µl AMV-RT native(Roboklon; 10 U μl^{-1}) were added and heated to 42°C for 15 min to start the reaction followed by 45 min of 50°C. Finally, the mixture was heated to 80°C for 10 min to inactivate the reaction and thereafterimmediately cooled on ice.

Primers of the selected genes were designed according to the MIQE guidelines (Bustin et al. 2009, Taylor et al. 2010) with the online tool Primer3 version 4.1.0 (Untergasser et al. 2012) using FASTA sequences of the RNA sequencing results as templates. The three genes ubiquitin (*PsUBI*), cytochrome subunit 6 (*PsPetB*), and chloroplast ATPase beta subunit (*PsC-ATP*) were selected as housekeeping genes.

The qPCRBIO SyGreen Mix Lo-Rox kit (Nippon Genetics Europe, Düren, Germany) was used according to the manual. The qPCR was conducted in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories). The reaction volume of 10 µl contained 12.5 ng cDNA, 5 µl qPCRBIO SyGreen Mix Lo-Rox Master Mix, 0.17 µl of each primer (10 pmol μl^{-1}) and was filled up with nuclease-free H_2O. Each reaction was performed in triplicates and ran through a temperature profile of 2 min at 95°C and 40 cycles of 5 sec at 95°C followed by 30 sec at 60°C with a fluorescence measurement after each cycle. To exclude the formation of untargeted side products or primer dimerization, a dissociation curve ranging from 55°C to 95°C in 1°C steps followed the 40 cycles.

C(t) values were calculated with Bio-Rad CFX Maestro 1.1 version 4.1.2433.1219 (Bio-Rad Laboratories) using the identical threshold for all reactions. In order to normalize the data, transcript levels of all treated samples relative to untreated controls were calculated following a second normalization to the above-mentioned housekeeping genes as described by Pfaffl (2001) and Vandesompele et al. (2002). For the second normalization, the geometric mean of the expression level of the three housekeeping genes (housekeeping-gene-index) was calculated and the results of the first normalization were divided by the housekeeping-gene-index. The control of primer efficiency allowed application of the perfect PCR amplification value for expression calculations of each gene. Finally, the log_2fold change of transcript levels relative to the untreated controls was calculated.

Method S4: Phytohormone analyses

Phytohormones (salicylic acid (SA), jasmonic acid (JA), jasmonic acid-isoleucine (JA-Ile), and abscisic acid (ABA)) were extracted from 100 mg frozen, powdered plant material per sample. For extraction, we used 1 ml ethyl acetate per sample. To facilitate homogenization of the extract, a homogenization matrix (Zirconox, 2.8-3.3 mm, Mühlmeier Mahltechnik, Bärnau, Germany) was added. For quantification of the phytohormones, 2 µl of an internal standard were added to the extraction solvent. The internal standard is composed of deuterated 10 ng μl^{-1} D4-SA, 30.2 ng μl^{-1} D6-JA, 10 ng μl^{-1}, D6-JA-Ile conjugate, and 10 ng μl^{-1} D6-ABA (HPC Standards GmbH, Cunnersdorf, Germany). All samples were homogenized for 3 x 30 sec at 5000 rpm in a FastPrep homogenizer (Bertin technologies Precellys® Evolution, Montigny-le-Bretonneux, France) following a centrifugation step with 18,213 g for 10 min at 4°C (Eppendorf® centrifuge 5427R, Eppendorf AG, Hamburg, Germany). The supernatants were transferred to new tubes, and another 1 ml of ethyl acetate without the internal standard was added to each pellet, which also still contains the homogenization matrix. After another homogenization and centrifugation step, the supernatants were combined with the first ones

and concentrated using a centrifugal vacuum concentrator (Eppendorf® Concentrator 5301, Eppendorf AG, Hamburg, Germany). The original pellet was extracted two more times with just ethyl acetate as described above, and the supernatants were again combined in the tube with the extracts from the first two extraction steps. Finally, the whole extraction was concentrated to dryness in the centrifugal vacuum concentrator. We added 400 µl of the re-elution buffer (70% methanol and 0.1% formic acid (v/v) to each concentrated sample. Samples were then vortexed for 10 min and centrifuged with 18,213 g for 10 min at 4°C. A volume of 200 µl of the particle-free supernatant was transferred to an HPLC vial, which was stored at -20°C until measurement.

For phytohormone analysis, a UPLC-MS/MS (Q-ToF-ESI) (Synapt G2-S HDMS; Waters®, Milford, Massachusetts, USA) equipped with a UPLC system (AQUITY™, Waters, Milford, Massachusetts, United States) and a C_{18} column (Acquity UPLC Waters, BEH-C18, Ø 2.1 mm × 50 mm, particle size 1.7 µm) was used as described by Bandoly et al. (2016) and Drok et al. (2018), and references therein. A sample volume of 7 µl was injected. Water and methanol, each with 0.1% formic acid (v/v), were used as eluents A and B in a gradient mode with a constant flow of 250 µl min^{-1} at 30°C (eluent B: 0 min: 30%; 1 min: 30%; 4.5 min: 90%; 8 min: 90%; 9 min: 30%; 3 min equilibration time between the runs). Compounds were negatively ionized by electrospraying (ESI) under the following conditions: capillary voltage 2.5 kV, nebulizer 6 bar, N_2 as desolvation gas with a flow rate of 500 l h^{-1}, 80°C source temperature, and 150°C desolvation temperature. The analysis was conducted by tandem mass spectrometry, which scanned the full compound mass spectrum between 50–600 *m/z*. Annotation was based on the characteristic parent $[M–H]^-$-ion, a diagnostic daughter ion, and on co-elution with the respective deuterated derivative in the internal standard (SA (*m/z* 137 and 93), JA (*m/z* 209 and 59), JA-Ile (*m/z* 322 and 130), ABA (*m/z* 263 and 153), and their deuterated derivatives: D4-SA (m/z 141 and 97), for D6-JA (*m/z* 215 and 59), D6-JA-Ile (*m/z* 328 and 130), D6-ABA (*m/z* 269 and 159). MassLynxTM Software (version 4.1; Waters) was used to quantify the peak areas of each compound and the respective internal standard. Data were then normalized to the internal standard and the weight of the extracted plant material by dividing the peak area of the compound by the peak area of the respective internal standard, multiplying this result with a thousandfold of the used amount of the internal standard, and dividing this latter result by the weight of the extracted plant material.

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Table S1 Comparison of RNA sequencing data and qPCR data of 13 selected genes. Shown are the mean log_2 fold changes ± SE of relative expression (normalized to the control and the housekeeping genes). Expression levels obtained by qPCR and RNA sequencing were compared for seven different types of samples obtained after different treatments and harvested after different treatment durations. Treatments (E – Egg deposition; F – Feeding; EF – Egg deposition and subsequent feeding; 1hae, 24hae, 10dae = different time periods after egg deposition; 1haf and 24haf = different time periods after onset of feeding). In total, seven different sample types were analysed by qPCR and compared to RNA sequencing data. We calculated for each gene: **(match[1])** in how many of the seven sample types (total) does the respective gene differ in its expression level by no more than 50% in the qPCR and RNA-seq analysis and **(match[2])** in how many of the seven sample types (total) is the respective gene analysed by qPCR and RNA-seq regulated in the same direction (up / down). From these data, percentages of match were calculated for each gene. The **similarity** gives the mean percentage ± SE over all the listed genes based on the match[1] or match[2] percentages of each gene. Statistically significant differences in expression (compared to control = untreated pine) are marked grey ($p < 0.05$; Wald test for RNA sequencing, Mann-Whitney-*U*-test for qPCR).

Genes related to…		**Cell wall modification**	**Secondary metabolites**		**Response to chitin**		**Ca^{2+} signalling**	**Phytohormone**						
Sample types	Source of data	*CAD9*	*PAL*	*AFS*	*Chit3*	*Chit7*	*CALM*	*LOX1*	*LOX4*	*SA-CAMT*	*PR-4B*	*ARP*	*Tify-45504*	*Tify-65221*
E 1h ae	qPCR	2.55 ±0.79	1.50 ±0.14	1.71 ±0.30	3.03 ±0.30	4.55 ±0.44	1.23 ±0.41	8.02 ±1.03	3.90 ±0.31	1.85 ±0.71	5.72 ±0.57	2.06 ±0.40	5.74 ±0.53	6.63 ±0.67
	RNAseq	3.69 ±0.50	6.18 ±0.68	3.43 ±0.67	1.85 ±0.90	3.93 ±0.63	4.42 ±1.13	9.34 ±1.23	3.54 ±0.27	7.03 ±1.34	6.58 ±1.60	2.26 ±0.36	3.12 ±0.58	7.95 ±1.29
E 24h ae	qPCR	0.20 ±0.68	3.25 ±0.56	1.57 ±0.71	3.81 ±0.55	6.73 ±1.13	1.24 ±0.45	5.32 ±1.13	2.86 ±0.49	4.60 ±1.08	5.97 ±1.02	2.38 ±0.31	4.71 ±1.10	5.37 ±1.24
	RNAseq	3.30 ±0.29	2.71 ±0.62	1.59 ±0.47	3.55 ±1.58	4.19 ±0.43	2.32 ±1.25	8.42 ±0.73	4.03 ±0.29	3.44 ±1.62	3.91 ±1.58	2.07 ±0.28	5.59 ±0.94	7.97 ±1.22
E 10d ae	qPCR	0.94 ±0.76	1.38 ±0.85	1.11 ±0.56	3.36 ±0.76	5.42 ±1.66	-0.97 ±0.30	3.59 ±1.38	1.62 ±0.32	3.48 ±1.54	5.51 ±1.05	1.31 ±0.32	5.07 ±0.85	3.55 ±1.42
	RNAseq	1.75 ±0.50	2.38 ±0.77	1.29 ±0.85	5.48 ±1.18	3.02 ±0.42	0.81 ±1.40	no data	1.32 ±0.29	no data	6.56 ±1.01	0.89 ±0.34	4.58 ±0.77	no data
F 1h af	qPCR	-2.48 ±0.44	4.37 ±0.21	no data	-0.41 ±0.55	1.62 ±1.08	8.96 ±0.53	0.99 ±1.51	0.05 ±0.24	1.16 ±0.63	5.89 ±1.08	8.00 ±0.43	-0.41 ±0.00	-0.22 ±0.78
	RNAseq	3.84 ±0.44	8.63 ±0.83	6.11 ±0.99	-0.59 ±1.34	4.14 ±0.72	7.20 ±1.25	5.62 ±1.98	2.50 ±0.40	6.80 ±2.62	3.20 ±1.45	3.15 ±0.41	9.11 ±1.08	9.11 ±1.29
EF 1h af	qPCR	1.79 ±0.86	3.69 ±0.23	8.30 ±0.20	0.62 ±0.59	1.46 ±0.42	7.18 ±0.36	1.08 ±1.15	0.72 ±0.22	2.06 ±1.21	9.19 ±0.60	7.35 ±0.22	-2.31 ±0.29	-7.83 ±0.44
	RNAseq	1.42 ±0.44	6.45 ±0.83	2.00 ±0.99	-2.36 ±1.34	1.90 ±0.73	6.34 ±1.25	5.81 ±1.98	1.54 ±0.40	2.69 ±2.67	1.44 ±1.45	1.67 ±0.41	4.90 ±1.08	4.90 ±1.31
F 24h af	qPCR	4.12 ±1.02	5.59 ±0.48	5.47 ±0.73	10.60 ±0.45	5.98 ±0.68	5.46 ±0.50	13.49 ±0.94	6.07 ±0.41	4.07 ±1.09	11.57 ±0.45	3.80 ±0.21	4.08 ±1.51	6.97 ±0.82
	RNAseq	5.08 ±0.47	7.23 ±0.77	5.34 ±0.96	8.79 ±1.09	4.45 ±0.65	3.66 ±0.94	11.63 ±1.24	3.60 ±0.57	8.30 ±1.10	6.56 ±1.33	2.69 ±0.42	11.80 ±0.88	11.81 ±1.21
EF 24h af	qPCR	3.60 ±1.09	5.16 ±0.47	4.80 ±0.33	9.44 ±0.33	4.72 ±0.49	5.22 ±0.40	13.27 ±0.57	6.29 ±0.24	4.09 ±0.88	10.61 ±0.45	4.07 ±0.11	6.90 ±0.64	7.85 ±0.68
	RNAseq	4.03 ±0.50	5.49 ±0.82	3.10 ±1.02	8.70 ±1.16	4.51 ±0.69	2.57 ±0.99	11.06 ±1.28	2.43 ±0.60	6.78 ±1.14	6.76 ±1.41	2.33 ±0.44	4.47 ±0.93	10.22 ±1.24
Match[1] / total		5 / 7	5/7	5 / 7	6 / 7	6 / 7	5/7	4/7	4/7	4/7	6/7	5/7	4/7	4/7
% match[1] per gene		**71.43**	**71.43**	**71.43**	**85.71**	**85.71**	**71.43**	**57.14**	**57.14**	**57.14**	**85.71**	**71.43**	**57.14**	**57.14**
Similarity[1] over all genes:		**69.23 ±3.17**												
Match[2] / total		6 / 7	7/7	6 / 7	6 / 7	6 / 7	6/7	6/7	7/7	6/7	7/7	7/7	5/7	4/7
% match[2] per gene		**85.71**	**100.00**	**85.71**	**85.71**	**85.71**	**85.71**	**85.71**	**100.00**	**85.71**	**100.00**	**100.00**	**71.43**	**57.14**
Similarity[2] over all genes:		**86.81 ±3.42**												

Table S2 Primers used for validation of RNA sequencing data by qPCR analysis (for design see Method S3). Shown are the primers of the target genes (compare Table S1), the housekeeping genes, the cluster number of the RNA sequencing data and the forward and reverse primer sequences (5' to 3' direction). For the gene sequence, which the primer design is based on, see the respective cluster number in Supplementary Information Item SI1. *Ps Pinus sylvestris.*

Gene abbreviation	Gene name	Cluster no. of RNA-Seq	Forward primer sequence	Reverse primer sequence	Amplicon size (bp)
Housekeeping genes					
PsUBI	Ubiquitin	Cluster-61924.34956	ACTTTACCAGAGTCATCAACC	GGTTCTTCGTCTGAGAGGTG	135
PsPetB	Cytochrome subunit 6	Cluster-61924.32053	GGGTCGGTCAAGTCGTCAGC	GCACGGAAATGGGTTCTTTGC	103
PsC-ATP	Chloroplast ATPase beta subunit	Cluster-61924.25426	ACCATCATACTTGCCGACCATC	TCGTCCGACCGTTACAGAAGC	108
Cell wall modification related					
CAD9	Cinnamyl alcohol dehydrogenase 9	Cluster-61924.40501	TCTGCCTGAGGTGTGGATTC	AGCCCCTCCTTAATTTGCTGA	196
Secondary metabolite related					
PAL	Phenylalanine ammonia lyase	Cluster-61924.21329	GCTCGTTGCAGAGGGTGATTTG	CCCCGGCGAAGATATAGAAAAGG	196
AFS	α-Farnesene synthase	Cluster-61924.43320	GGAGCGAGAAAGGCATTGGA	GCCTCTGTCTGAGCATTGGCA	178
Chitinase related					
Chit3	Chitinase 3	Cluster-61924.17276	ATGGCATAAAGAACCCCGACA	CCGTTAATGATGTTGGTCACC	196
Chit7	Chitinase 7	Cluster-61924.17274	ATAGATGCAACTCCCTGTCCAC	GCTTACAATGGCGACGATGAAT	216
Calcium signalling related					
CALM	Calmodulin	Cluster-61924.57202	CCTCACACCCATCCCCATTG	CCCTGTGTCGGATCGGAATC	174
Phytohormone related					
LOX1	Lipoxygenase 1	Cluster-61924.33026	ACACCCTATGAGACCAGCAAA	AGGGTTGCCAAGATCGTTGT	192
LOX4	Lipoxygenase 2	Cluster-61924.28006	CACGATGAGCCTCCACTTGA	TCTTCTTGTGTTGGCCAGCT	196
SA-CAMT	SA-Carboxyl methyltransferase	Cluster-61924.60889	CGTACTGTGTTTCTGCTGGAAGC	CAATGCAAACTGTCTTGCATTCCA	203
PR-4B	Pathogenesis-related protein 4B	Cluster-61924.56483	GCAGGCGTCTAATGTGCGAT	CGCAAGCACTTTCCACAGGA	195
ARP	Auxin responsive protein	Cluster-61924.16342	GGAACGCCAGAGGATCTGTG	GGTCCCATTCTTGGAGGGTC	183
Tify-45504	Tify domain (45504)	Cluster-61924.45504	CCCTATACGTGCTCGCCTTC	GGGGCACGGTTAATACTGGA	189
Tify-65221	Tify domain (65221)	Cluster-61924.65221	CGCAAGCTGTGCCTACTTTT	CCTCTCGTTGGCTTGTCCAT	172

Table S3 Number of differentially expressed genes (DEGs) per treatment. Sample codes: E vs C – Egg deposition vs. control; F vs C – Feeding vs. control; EF vs C – Egg deposition and subsequent feeding vs. control; time points: 1h ae, 24h ae, 10d ae = different time periods after egg deposition; 1h af and 24h af = different time periods after onset of feeding. Full response per treatment: number and percentage of treatment-responsive DEGs when taking all sampling time points together. Overlap: DEGs that two (or all) treatments have in common. Unique response: DEGs detected only in the respective treatment. All percentages were calculated relative to the total number of up- and downregulated DEGs. Downregulated DEGs: blue.

Sample code / time point		No. of Regulated DEGs	up-regulated DEGs	down-regulated DEGs	full E response	full F response	full EF response	full EF & F response	overlap E & F response	overlap E & EF response	overlap F & EF response	overlap all	E unique response	F unique response	EF unique response	F & EF unique response
E vs C	*1h ae*	*3226*	2212	1014												
	24h ae	*6670*	3693	2977	*4973 (66.2%)*					*1989 (26.5%)*			*1873 (24.9%)*			
	10d ae	*969*	451	518	*4005 (68.7%)*				*3021 (40.2%)*	*681 (11.7%)*			*2179 (37.4%)*			
	1h af	*368*	184	184					*1639 (28.1%)*							
	24h af	*65*	27	38								*3100 (41.3%)*				
F vs C	*1h af*	*3990*	3019	971		*5361 (71.4%)*						*1826 (31.3%)*		*1337 (17.8%)*		
	24h af	*6745*	4084	2661		*3201 (54.9%)*		*5637 (75.1%)*			*2913 (38.8%)*			*1365 (23.4%)*		2537 (33.8%)
EF vs C	*1h af*	*2058*	1521	537			*3189 (42.5%)*	*3655 (62.6%)*			*692 (11.9%)*				*197 (2.6%)*	*1829 (31.4%)*
	24h af	*3007*	2343	664			*1146 (19.6%)*								*267 (4.6%)*	
Total DEGs	-	*13344*	7510	5834												

Table S4 GO term ID given in Figure 3 with the respective GO term name and number.

GO term ID	Term-No.	GO term name
1	GO:0009767	Photosynthetic electron transport chain
2	GO:0010304	PSII associated light harvesting complex II catabolic process
3	GO:0010380	Regulation of chlorophyll biosynthetic process
4	GO:0009768	Photosynthesis light harvesting in photosystem I
5	GO:0009765	Photosynthesis light harvesting
6	GO:0015994	Chlorophyll metabolic process
7	GO:0048564	Photosystem I assembly
8	GO:0009773	Photosynthetic electron transport in photosystem I
9	GO:0009643	Photosynthetic acclimation
10	GO:0015995	Chlorophyll biosynthetic process
11	GO:0010206	Photosystem II repair
12	GO:0010207	Photosystem II assembly
13	GO:0045333	Cellular respiration
14	GO:0015979	Photosynthesis
15	GO:0009809	Lignin biosynthetic process
16	GO:0010411	Xyloglucan metabolic process
17	GO:0016998	Cell wall macromolecule catabolic process
18	GO:0009832	Plant type cell wall biogenesis
19	GO:0030244	Cellulose biosynthetic process
20	GO:0009833	plant type primary cell wall biogenesis
21	GO:0090558	Plant epidermis development
22	GO:0042546	Cell wall biogenesis
23	GO:0045489	Pectin biosynthetic process
24	GO:0009834	Plant type secondary cell wall biogenesis
25	GO:0071554	Cell wall organization or biogenesis
26	GO:0009969	Xyloglucan biosynthetic process
27	GO:0071555	Cell wall organization
28	GO:0009626	Plant type hypersensitive response
29	GO:0006979	Response to oxidative stress
30	GO:0042744	Hydrogen peroxide catabolic process
31	GO:0019430	Removal of superoxide radicals
32	GO:0042542	Response to hydrogen peroxide
33	GO:0010941	Regulation of cell death
34	GO:0009094	L-phenylalanine biosynthetic process
35	GO:0006559	L-phenylalanine catabolic process
36	GO:0009698	Phenylpropanoid metabolic process
37	GO:2000762	Regulation of phenylpropanoid metabolic process
38	GO:0009699	Phenylpropanoid biosynthetic process
39	GO:0009812	Flavonoid metabolic process
40	GO:0051555	Flavonol biosynthetic process
41	GO:0009813	Flavonoid biosynthetic process
42	GO:0016102	Diterpenoid biosynthetic process
43	GO:0009800	Cinnamic acid biosynthetic process

44	GO:0042430	Indole containing compound metabolic process
45	GO:0009308	Amine metabolic process
46	GO:0019748	Secondary metabolic process
47	GO:0006032	Chitin catabolic process
48	GO:0010200	Response to chitin
49	GO:0071323	Cellular response to chitin
50	GO:0009694	Jasmonic acid metabolic process
51	GO:0009867	Jasmonic acid mediated signalling pathway
52	GO:2000022	Regulation of jasmonic acid mediated signalling pathway
53	GO:0009753	Response to jasmonic acid
54	GO:0009695	Jasmonic acid biosynthetic process
55	GO:0009696	Salicylic acid metabolic process
56	GO:0046244	Salicylic acid catabolic process
57	GO:2000031	Regulation of salicylic acid mediated signalling pathway
58	GO:0009863	Salicylic acid mediated signalling pathway
59	GO:0009751	Response to salicylic acid
60	GO:0009787	Regulation of abscisic acid activated signalling pathway
61	GO:0009738	Abscisic acid activated signalling pathway
62	GO:0071215	Cellular response to abscisic acid stimulus
63	GO:0009737	Response to abscisic acid
64	GO:0009926	Auxin polar transport
65	GO:0010315	Auxin efflux
66	GO:0009734	Auxin activated signalling pathway
67	GO:0010252	Auxin homeostasis
68	GO:0009733	Response to auxin
69	GO:0009723	Response to ethylene
70	GO:0009693	Ethylene biosynthetic process
71	GO:0009873	Ethylene activated signalling pathway
72	GO:0080037	Negative regulation of cytokinin activated signalling pathway
73	GO:0009736	Cytokinin activated signalling pathway
74	GO:0009938	Negative regulation of gibberellic acid mediated signalling pathway
75	GO:0009739	Response to gibberellin
76	GO:0009740	Gibberellic acid mediated signalling pathway
77	GO:0009755	Hormone mediated signalling pathway
78	GO:0010817	Regulation of hormone levels
79	GO:0009741	Response to brassinosteroid

CHAPTER 4

Pine defense against eggs of an herbivorous sawfly is elicited by an annexin-like protein present in egg-associated secretion

ORIGINAL ARTICLE

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Pine defense against eggs of an herbivorous sawfly is elicited by an annexin-like protein present in egg-associated secretion

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Abstract

Known elicitors of plant defenses against eggs of herbivorous insects are low-molecular-weight organic compounds associated with the eggs. However, previous studies provided evidence that also proteinaceous compounds present in secretion associated with eggs of the herbivorous sawfly *Diprion pini* can elicit defensive responses in *Pinus sylvestris*. Pine responses induced by the proteinaceous secretion are known to result in enhanced emission of (*E*)-β-farnesene, which attracts egg parasitoids killing the eggs. Here, we aimed to identify the defense-eliciting protein and elucidate its function. After isolating the defense-eliciting protein from *D. pini* egg-associated secretion by ultrafiltration and gel electrophoresis, we identified it by MALDI-TOF mass spectrometry as an annexin-like protein, which we named 'diprionin'. Further GC-MS analyses showed that pine needles treated with heterologously expressed diprionin released enhanced quantities of (*E*)-β-farnesene. Our bioassays confirmed attractiveness of diprionin-treated pine to egg parasitoids. Expression of several pine candidate genes involved in terpene biosynthesis and regulation of ROS homeostasis was similarly affected by diprionin and natural sawfly egg deposition. However, the two treatments had different effects on expression of pathogenesis-related genes (*PR1*, *PR5*). Diprionin is the first egg-associated proteinaceous elicitor of indirect plant defense against insect eggs described so far.

KEYWORDS
annexin, elicitor, herbivory, insect eggs, pine, plant defense

1 | INTRODUCTION

Plants can effectively protect themselves against an initial step of infestation by herbivorous insects, the egg deposition on their leaves (Hilker & Meiners, 2010). They can avoid receiving insect eggs by a wide range of constitutive traits, such as constitutive production of oviposition-deterring compounds or physical structures (e.g., Braccini et al., 2015; Schoonhoven et al., 2005). Additionally, they can respond to deposited insect eggs by various countermeasures (Hilker & Fatouros, 2015, 2016). Egg-induced direct defenses range from biosynthesis of ovicidal compounds to formation of neoplasms or necrotic leaf tissue resulting in, for example, detachment of eggs from leaves or desiccation of eggs. Egg-induced indirect defenses comprise changes in leaf surface chemistry and leaf odor composition, thereby informing egg parasitoids about the location of their hosts (Bertea et al., 2020; Fatouros et al., 2016; Hilker & Fatouros, 2015; Reymond, 2013).

Elicitors of egg-induced plant defenses have been isolated from gravid females, from the eggs or from secretion associated with eggs and attaching eggs to the oviposition site. The currently identified elicitors are low molecular weight organic compounds (Hilker & Fatouros, 2015). Elicitors isolated from females are, for example, 3-hydroxypropanoic acid esterified with long-chain alcohols, identified from bruchid beetles. These so-called bruchins elicit growth of plant neoplasms, thus detaching eggs from the oviposition site (Doss et al., 2000). Another group of amphiphilic egg-associated elicitors are phospholipids. Various phospholipids including phosphatidylcholine (PC) derivatives have been identified in extracts of planthopper females infesting rice plants; these phospholipids elicit the production of an ovicidal compound (benzyl benzoate) in rice plants (Seino et al., 1996; Yang et al., 2014). A recent study isolated PC derivatives from *Pieris brassicae* eggs eliciting hypersensitive-response (HR)-like symptoms in *Arabidopsis thaliana* plants (Stahl et al., 2020). Egg-induced HR-like formation of leaf necrosis can significantly contribute to insect egg mortality (e.g., Griese et al., 2021; Shapiro & DeVay, 1987). In pierid butterflies, elicitors of plant defensive responses have also been isolated from egg-associated secretion (Fatouros et al., 2008, 2009). The pierid elicitors isolated from egg-associated secretion, that is, benzyl cyanide in *P. brassicae* and indole in *Pieris rapae*, induce indirect plant defense by attracting egg parasitoids (Fatouros et al., 2008, 2009). Egg deposition by insects onto leaves results in a complex signalling cascade mediated by Ca^{2+}, ROS, and phytohormones (Reymond, 2013).

The amphiphilic character of some elicitors of plant defenses against insect eggs is a trait shared with several elicitors known to be released by feeding insects into leaf wounds. Several fatty acid–amino acid conjugates (FACs) have been isolated from regurgitate of lepidopteran larva. Application of these compounds onto wounded plants elicits the release of a distinct pattern of leaf volatiles attracting larval parasitoids (Acevedo et al., 2015; Alborn et al., 1997; Erb & Reymond, 2019; Felton & Tumlinson, 2008; Mithöfer & Boland, 2008; Schmelz, 2015; Schmelz et al., 2009; Wu & Baldwin, 2010). Orthopteran nymphs and adults release disulfooxy fatty acids (caeliferins) into plant wounds, thus also inducing a change in plant odor (Alborn et al., 2007). In addition, several other compounds are known to be released by feeding insects into plant wounds and eliciting plant defense, among them also proteins (enzymes; e.g., Mattiacci et al., 1995) or their derivatives, as, for example, an ATP synthase fragment, the so-called inceptin (Schmelz et al., 2006). Especially the amphiphilic FACs have been suggested to directly interact with the plant plasma membrane (Spiteller et al., 2000). They are involved in initiating plant defenses by plasma membrane depolarization and changing transmembrane ion fluxes (Maffei et al., 2004; Maischak et al., 2007). In addition to these elicitors released with the regurgitate of feeding insects, several wound-induced plant endogenous elicitors are known, which are formed in response to damage of plant tissue (e.g., Duran-Flores & Heil, 2016). For example, the peptide systemin is a classic, well-studied plant endogenous elicitor (Orozco-Cardenas et al., 1993; Pearce et al., 1991; Wang et al., 2018).

In contrast to plant defense elicitors released by feeding insects, no proteinaceous elicitor of plant defense against eggs has been identified so far. However, indirect defense of *Pinus sylvestris* (Coniferales, Pinaceae) against eggs of the sawfly *Diprion pini* (Hymenoptera, Diprionidae) is known to be elicited by a proteinaceous secretion, which the sawfly female releases from her oviduct onto the eggs (Hilker et al., 2002). The needles respond to sawfly egg deposition or application of the egg-associated oviduct secretion by emitting enhanced quantities of the sesquiterpene (*E*)-β-farnesene (Mumm et al., 2003). The egg- or secretion-induced pine odor attracts parasitic wasps (*Closterocerus ruforum*, Hymenoptera, Eulophidae), which kill the eggs. The parasitoid *C. ruforum* shows highest olfactory sensitivity towards (*E*)-β-farnesene when compared to other pine terpenes. This egg parasitoid is highly attracted by a synthetic blend of (*E*)-β-farnesene and four other terpenes (two mono- and two sesquiterpenes), which showed no egg-induced emission rates in contrast to (*E*)-β-farnesene (Beyaert et al., 2010).

Oviposition by *D. pini* is associated with wounding of a pine needle. A sawfly female slits a needle longitudinally with her sclerotized ovipositor valves and inserts several eggs in a row into the slit needle. Each egg inside the needle is encased by a secretion released from the oviduct. While this secretion elicited indirect defense when experimentally applied into slit, egg-free pine needles, just slitting of pine needles did not result in emission of pine odor, which attracts the egg parasitoids (Hilker et al., 2002). The slit pine needle with the egg row is covered on top with a secretion released from the female's accessory reproductive gland in the abdomen. Our previous studies showed that this covering secretion has no defense-elicitor activity when applied onto slit needles without eggs (Hilker et al., 2002). The pine defense-eliciting *D. pini* oviduct secretion treated with a proteinase lost its activity and did no longer induce a parasitoid-attracting odor, when applied onto slit pine needles. Hemolymph of *D. pini* females is always co-extracted when dissecting oviducts for isolation of oviduct secretion. The protein pattern of hemolymph is almost similar to the one of the oviduct secretion except for a small protein fraction of ~12 kDa in the secretion. Application of hemolymph to pine needles did not result in a change of plant odor that attracts parasitoids, suggesting that the elicitor is a small protein present in the egg-associated oviduct secretion (Hilker et al., 2005).

This study aimed to identify the pine defense-eliciting protein(s) from egg-associated oviduct secretion of the sawfly *D. pini* and to elucidate its effects on *P. sylvestris*. To identify the indirect defense-eliciting protein, we fractionated the oviduct secretion and tested the fractions for their elicitor activity. We analyzed the active protein by tandem mass spectrometry and expressed it heterologously. We hypothesized that the recombinant protein elicits pine indirect defense similar to natural egg deposition. We tested this hypothesis by treating pine with the recombinant protein and investigated the emission of (*E*)-β-farnesene from treated pine as well as the attractiveness of treated pine to egg parasitoids. Since egg deposition by insects onto leaves is well-known to affect expression of a broad set of genes (Altmann et al., 2018; Little et al., 2007; Lortzing et al., 2019; Reymond, 2013), we also addressed the question whether treatment of pine with diprionin induces similar changes in gene expression as *D. pini* egg deposition does. So far, no large-scale study of transcriptional responses of Scots pine to sawfly eggs has been conducted. Based on the available knowledge of plant transcriptional responses to insect eggs and of Ca^{2+}-dependent activity of annexins, we selected a small set of candidate genes and investigated the effect of diprionin on their expression levels.

2 | MATERIALS AND METHODS

2.1 | Plants and insects

We used *P. sylvestris* for all experiments and insect rearing. The plant material was collected in forests in northwestern Berlin, Germany. We used pine branches of trees, which were at least 10 years old because in forests *D. pini* has so far not been observed to infest younger trees (Brauns, 1991). The branches were kept in the laboratory under conditions as described for rearing of *D. pini* (Bombosch & Ramakers, 1976; Eichhorn, 1976) and as applied in our previous studies on pine responses to sawfly eggs (Bittner et al., 2017; Hilker et al., 2002, 2005; Mumm et al., 2003, 2005).

The sawfly *D. pini* was reared in the laboratory on pine branches according to established protocols for sawfly rearings (Bombosch & Ramakers, 1976; Eichhorn, 1976). The egg parasitoid *C. ruforum* was collected in the field in southern Finland in the regions of Hanko and Puumala by picking pine needles with parasitoid-infested sawfly eggs. They were kept in the laboratory until emergence as previously described by Mumm et al. (2005). The emerged adult female parasitoids used for bioassays were about 5–10 days old. To obtain parasitoids experienced with host eggs, *C. ruforum* females were exposed to *D. pini* eggs on a *P. sylvestris* twig for 24 h at 20°C, 18:6 h, L:D, 70% humidity, 100 µmol photons m^{-2} s^{-1}. Thereafter, they were kept a further day at the same abiotic conditions, but deprived from host eggs. This lag phase was expected to enhance the parasitoid's motivation to search for host eggs. Parasitoids with these pre-treatments were used for the bioassays as described by Mumm et al. (2005).

2.2 | Collection and fractionation of oviduct secretion

Oviduct secretion samples were taken from at maximum 5-day-old sawfly females. Oviduct secretion was collected from the *oviductus communis* as described earlier by Hilker et al. (2005) and transferred to a protein storage buffer (pH 7.2; 70 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 1 mM $NaHCO_3$; or 150 mM Tris-HCl, 50 mM NaCl; 2 µl per oviduct of a female). Freshly dissected secretion was used for the bioassays testing its pine defense-eliciting activity. The secretion dissected from 16 females was pooled and represented a sample. The sample was fractionated by ultrafiltration as described in Supporting Information Method S1. A prefiltrate with proteins smaller than 100 kDa was centrifuged with 30 kDa MWCO (molecular weight cut-off) centrifugator tubes. The final sample was concentrated to a volume of ~20 µl and used for further processing and analyses.

2.3 | Blue Native-PAGE (BN-PAGE)

We used BN-PAGE analyses to check (1) the molecular weight of proteins isolated from the oviduct secretion after ultrafiltration and (2) the molecular weight of the candidate protein, which we had heterologously expressed and referred to as diprionin (see below, and Figure S1). Furthermore, (3) proteins were isolated from the gels by electro-elution for mass spectrometric analyses (see below). All BN-PAGE analyses were performed as described by Wittig et al. (2006) with minor modifications. Further details are provided by the description in Supporting Information Method S2.

2.4 | Electro-elution and concentration of target proteins from oviduct secretion

To isolate BN-PAGE separated target protein fractions for bioactivity assays and for peptide mass fingerprinting, we adapted the electro-elution protocol described by Wittig et al. (2006). Further details are provided in Supporting Information Method S3.

The BN-PAGE analyses of proteins from the oviduct secretion was initially loaded with a secretion equivalent of 20 females. The electro-eluted sample was estimated to contain oviduct secretion from about ~12 female equivalents (recovery of 91% after each centrifugal concentration step with MWCO 100, 50 and 5 kDa; Greening & Simpson, 2010; recovery of 90% by electroelution; Dunn, 2004). Hence, an electro-eluted sample of 25 µl contained oviduct secretion proteins from about 12 females.

2.5 | Elicitor activity assay: Olfactory response of egg parasitoids to differently treated pine

To test whether odor of pine twigs treated with different types of samples (see below) is attractive to the egg parasitoid *C. ruforum*, bioassays were carried out in a four-field olfactometer as described previously (e.g., Schröder et al., 2008). The test field was ventilated with odor of a treated test twig. The three other fields of the four-field olfactometer were ventilated with clean, charcoal filtered air. Two of these fields were adjacent to the test field and considered as buffer zone, while the field opposite of the test field was considered the control field (Schröder et al., 2008).

For treatment of pine twigs, small *P. sylvestris* twigs were detached from field-collected pine branches for experimental treatments and acclimatized for 72 h at 20°C, 18:6 h, L:D, 70% relative humidity, 100 µmol photons m^{-2} s^{-1}. Test pine twigs were treated with (1) sawfly oviduct secretion, (2) candidate protein fraction obtained from oviduct secretion by ultrafiltration and BN-PAGE, and electro-eluted from the gel, or (3) recombinant protein (diprionin) that had been separated from recombinant protein tag cleavage reactions by BN-PAGE and electro-eluted from the gel. The latter two types of samples were always taken from unstained BN-PAGE lanes that had run in parallel to the stained ones.

Pine twigs subjected to the above-mentioned treatments were used for olfactometer biosassays with the egg parasitoid *C. ruforum*, while pine twigs treated with the recombinant protein were also used for chemical analysis of pine odor. All samples were applied into artificially wounded (slit) pine needles, thus mimicking the ovipositional wounding, by which an egg-laying sawfly female damages a pine needle

(compare Hilker et al., 2002). We treated eight needles per small twig (with in total about 100 needles). An equivalent of proteins from four *D. pini* females was used for each twig subjected to treatments with the oviduct secretion and the candidate protein fraction. Twigs treated with electro-eluted recombinant protein (diprioinin) received 250 ng of protein per needle (2 µg per twig). The protein concentration of the oviduct secretion of a *D. pini* female is about 5.8 µg μl^{-1} as determined by the Bradford assay (unpublished data).

For control of the effects of test samples treated with either candidate protein fractions or recombinant protein, we investigated whether the artificially wounded pine twig itself emits attractive odor when treated with protein storage buffer. We treated $n = 9$ twigs each with test and control samples. Further details of the assay and the treatments are provided in Supporting Information Method S4.

2.6 | Chemical analysis of odor of pine treated with recombinant protein (diprionin)

Egg deposition by *D. pini* on pine needles and treatment of pine needles with the sawfly's oviduct secretion is known to result in enhanced emission of the sesquiterpene (*E*)-β-farnesene (Mumm et al., 2003). To determine whether treatment of *P. sylvestris* with recombinant annexin B9 (diprionin) also induces this effect, we treated pine with recombinant protein that had been separated from recombinant protein tag cleavage reactions by BN-PAGE and electro-eluted from the gel. Hence, we treated pine twigs with the recombinant protein as described above for the olfactometer assay and also used the respective reference (control) sample. We treated $n = 12$ test and 12 control twigs this way.

Odor of treated test and control twigs was collected 72 h after treatment for a period of 5 h as described by Mumm et al. (2003) (for details see Supporting Information Method S5). (*E*)-β-farnesene was identified by comparing its mass spectrum and retention index (RI:1460) to NIST library spectra (Viña & Murillo, 2003). The peak areas of (*E*)-β-farnesene in odor of test and control pine were determined and normalized by dividing them by the peak area of the internal standard (IS, 10 ng μl^{-1} methyl nonanoate). The IS-normalized peak areas were statistically compared.

2.7 | Peptide mass fingerprinting

For protein identification, two types of samples were subjected to peptide mass fingerprinting: (1) the protein(s) of the pine defense-eliciting secretion sample fractionated by ultrafiltration and BN-PAGE from *D. pini* oviduct secretion (referred to as 'candidate protein fraction', Figure 1) and (2) the recombinant protein electro-eluted from BN-PAGE analysis (referred to as 'diprionin fresh', Figure 1). Peptides were obtained from these two types of samples by trypsin (Roche, recombinant, sequencing grade) in-gel digestion as described previously (Shevchenko et al., 1996).

Peptide masses were analyzed by matrix-assisted laser desorption ionization–time of flight–mass spectrometry (MALDI-TOF-MS) using an Ultraflex-II TOF/TOF instrument (Bruker Daltonics) equipped with a 200 Hz solid-state Smart beam™ laser. The mass spectrometer was operated in the positive reflector mode. Mass spectra were acquired over an *m/z* range of 600–4000. Alpha-cyano-4-hydroxycinnamic acid (CHCA) was used as matrix, and protein digest samples were spotted using the dried-droplet technique (Vorm et al., 1994). MS/MS spectra of the peptides listed in Table 1 were acquired in LIFT mode (Suckau et al., 2003). For identification of peptide fragments, spectra were compared with entries in the MASCOT database (Perkins et al., 1999) against all entries of NCBInr and Swiss-Prot databases. The following parameters were applied: trypsin digestion, up to one missed cleavage; fixed modifications: carbamidomethyl cysteine; variable modifications: oxidation (M); peptide tolerance: was typically set at 75 ppm and MS/MS tolerance at ±0.7 Da; peptide charge: +1. Only proteins with a MASCOT score greater than or equal to the significance threshold ($p < 0.05$) were accounted as valid. BLAST analysis of identified amino-acid sequences and MASCOT protein matches was performed with the blastp program against the non-redundant protein database (NCBInr prot) restricted to Diprionidae (Altschul et al., 1990; Altschul et al., 1997).

2.8 | RNA extraction from female *D. pini* sawflies and complementary DNA (cDNA) synthesis

To elucidate the coding sequence of the pine defense-eliciting sawfly protein for recombinant expression, we extracted RNA from the abdomen of three *D. pini* females according to the protocol of the RNeasy® Mini kit (QIAGEN GmbH). The extracted RNA was pooled in one sample (for further details see Supporting Information Method S6).

For cDNA synthesis, 200 ng RNA was used, and we followed the protocol of the AMV-RT native enzyme by Roboklon applying the optional pre-heating step at 65°C. Additionally, we included an enzyme inactivation step of 80°C for 10 min at the end of the protocol.

2.9 | Identification of *D. pini* annexin B9 like coding sequence (diprionin)

We aimed to identify a nucleotide sequence coding for *D. pini* annexin (diprionin) in RNA extracted from *D. pini* females. Primers (Table S1) were designed based on the sequence of an annexin B9 of the sawfly *Neodiprion lecontei*, which showed the highest BLAST score with the annexin peptide sequences identified from the *D. pini* active candidate fraction by peptide mass fingerprinting (Table 1). Primers for all PCRs were designed with PRIMER-BLAST (Ye et al., 2012).

To account for possible mismatching nucleotides in the designed primers due to species differences between *D. pini* and *N. lecontei*, a gradient PCR was performed (for details see Supporting Information Method S7). PCR products were gel-extracted following the protocol of the peqGOLD gel extraction kit (Peqlab) and eluted in 30 µl nuclease-free H_2O. Sanger sequencing was performed at Seqlab.

To obtain the full-length cDNA coding sequence we followed the small reaction volumes protocol of the FirstChoice RLM RACE kit (Thermo Fisher Scientific). Only 3′ RACE-PCR was necessary as the 5′-end of the coding sequence was already captured with the preceding PCR. After adapter ligation and reverse transcription reactions as described in the protocol, cDNA was cleaned from enzymes and reagents with the Invisorb® Fragment Clean Up kit (STRATEC Biomedical AG) and eluted in 30 µl nuclease-free H_2O.

A primer for 3′ RACE PCR (Table S1) was designed based on the sequence obtained by the gradient PCR reaction mentioned above (Figure S2). The PCR conditions are described in Supporting Information Method S7. PCR products were analyzed and sequenced as described above.

The obtained sequences were aligned and translated to an amino acid sequence with Clone Manager Suite 7 (SciEd Central). Possible signal peptide sequences were analyzed online with SignalP 4.1 (Petersen et al., 2011).

2.10 | Recombinant expression of *D. pini* annexin (diprionin)

The full coding sequence obtained by RACE-PCR was introduced into vector plasmids, which were further processed in *Escherichia coli* and insect (Sf21 and *Hi*-5) cells. For sequence isolation from the plasmids and later purification of the heterologously expressed protein, we introduced nucleotide sequence restriction sites, maltose-binding protein (MBP) tags and a factor X_A cleavage site to the target sequence. A detailed protocol is described in Supporting Information Method S8. The resulting cleavage products after recombinant protein expression, protein extraction and MBP tag cleavage were analyzed by BN-PAGE. The heterologously expressed *D. pini* annexin provided a band with a molecular weight of 20 kDa (Figure S1). We electro-eluted the 20 kDa band as described for the protein fractions of oviduct secretion. We measured the obtained protein concentration by Pierce BCA protein assay kit (Thermo Fisher Scientific). We obtained sufficient protein to treat pine twigs each with 2 µg recombinant protein for the elicitor activity bioassays and chemical analysis.

For control, we further analyzed the electro-eluted 20 kDa band from the BN-PAGE gel (Figure S1), which we had obtained by loading the gel with the heterologously expressed protein (diprionin). We analyzed this electro-eluted protein by sodium dodecyl sulphate polyacrylamide gel electrophoresis on a 4% – 20% gradient gel (Carl Roth) according to the manufacturer's protocol, and stained according to the Coomassie staining protocol by Dyballa and Metzger (2009). Here, the recombinant protein revealed a band at ~35 kDa, thus matching the calculated weight of the respective sequence (Figure S3). Shortcomings of protein mass estimation by BN-PAGE due to differing interactions of the native protein with the gel and Coomassie G-250 are known from several other studies (e.g., Braz & Howard, 2009; Wittig et al., 2006).

2.11 | Impact of diprionin on expression of defense-related pine genes

To investigate the impact of diprionin on expression of defense-related pine genes, we conducted qPCR analyses of (1) artificially wounded pine needles treated with diprionin. The determined transcript levels were compared with those from (2) naturally egg-laden pine needles. For control, we also determined expression of the candidate genes in (3) untreated pine needles and (4) artificially wounded needles treated with only the buffer used for protein storage, thus testing the impact of the ovipositional wounding per se on gene expression.

The needles were taken from small pine twigs (each with about 100 needles). Pine twigs were treated as described for the twigs used for the olfactometer bioassays and gas chromatography–mass spectrometry (GC-MS) analyses of pine odor. Before and post treatments, the twigs were collected and acclimatized as described for the olfactometer bioassays (compare Supporting Information Method S4, S9). We used $n = 7–8$ twigs for each treatment. The methods applied for RNA extraction from pine needles, primer design, cDNA synthesis, qPCR analyses of pine sequences and data evaluation are described in Supporting Information Method S9.

We determined pine transcript levels of genes assigned to the following enzymes based on homology alignments (Table S2):

(a) geranyl pyrophosphate synthases (GPP2, GPP3) and farnesyl pyrophosphate synthase as well as a (*E*)-β-farnesene synthase (FPP, TPS5). Expression of the respective genes was tested because they catalyze the formation of typical *P. sylvestris* volatiles (Mumm et al., 2003); GPP2 and GPP3 are enzymes of the methylerythritol phosphate (MEP) path leading to volatile monoterpenes, FPP and TPS5 are enzymes of the mevalonate path (MVA) leading to volatile sesquiterpenes (Dudareva et al., 2013), which are known to be involved in indirect defense of pine against *D. pini* eggs (Beyaert et al., 2010; Köpke et al., 2008).

(b) enzymes involved in generation and turnover of reactive oxygen species (ROS), that is, respiratory burst oxidase homolog protein A (RbohA) and superoxide dismutase (SOD), and enzymes acting as ROS scavengers, that is, ascorbate peroxidase (APX) and catalase (CAT). Transcript levels of genes encoding these enzymes were tested because ROS are well known to be involved in plant responses induced by insect eggs (e.g., Geuss et al., 2017; Gouhier-Darimont et al., 2013; Griese et al., 2021); furthermore, pine needles accumulate ROS in response to sawfly egg deposition (Bittner et al., 2017; Bittner et al., 2019).

(c) pathogenesis-related proteins (PR1, PR2 and PR5) and phenylalanine ammonia lyase (PAL). Expression of the respective genes was analyzed because we hypothesized that *P. sylvestris* shows similar transcriptional changes in response to insect egg deposition as those known from other plant species. *Arabidopsis thaliana* is well-known to respond to insect eggs by enhanced accumulation of salicylic acid (SA) and enhanced transcription of the SA-responsive genes *PR1*, *PR2* and *PR5* (Hilfiker et al., 2014; Little

et al., 2007; Valsamakis et al., 2020). Furthermore, several plant species (tobacco, elm, *A. thaliana*) are known to show enhanced levels of phenylpropanoid derivatives in response to egg deposition when combined with leaf wounding (Austel et al., 2016; Bandoly et al., 2015; Lortzing et al., 2019); a key enzyme for biosynthesis of a great variety of phenylpropanoids is PAL.

(d) enzymes involved in Ca^{2+} signalling. Expression of these genes was analyzed because annexin-like proteins and their functions are Ca^{2+}-dependent (Davies, 2014; Gerke & Moss, 2002). We determined transcript levels of a calcium exchanger (*CAX3*), which is strongly induced by insect egg deposition in leaves of *A. thaliana* (Valsamakis et al., 2020). We also determined expression levels of the calcium-dependent protein kinase *CDPK1*; CDPKs are well known to be involved in stress responses and regulation of ROS accumulation (Asano et al., 2012).

2.12 | Statistics

Data of the elicitor activity assays with parasitoids were statistically evaluated by the two-sided Wilcoxon signed-rank test. We compared the time periods, which the parasitoids spent actively walking in the olfactometer test field and the control field (=opposite field) (Ninkovic et al., 2001; Schröder et al., 2008).

For statistical comparison of (*E*)-β-farnesene emission from diprionin-treated pine samples and control pine samples, we first normalized the peak areas to the internal standard. After $\log_{10}$ transformation, data were checked for their normal distribution by the Shapiro–Wilk test and then analyzed by a two-sided paired *t*-test.

For statistical analysis of the pine gene expression data, we used the nonparametric Mann–Whitney *U*-test since the data did neither show normal distribution (determined by Shapiro–Wilk test) nor variance homogeneity (checked by Levene's test). We statistically compared transcript levels of genes (1) in egg- and diprionin-treated pine samples versus those in artificially wounded ones treated with buffer for protein storage and (2) in egg-treated versus diprionin-treated samples. Furthermore, the nonparametric Mann–Whitney *U*-test was applied to statistically compare expression levels of transcripts in untreated controls with those in artificially wounded, buffer-treated pine.

All statistical calculations were performed with the statistical software R version 3.6.0 (R Development Core Team, 2020) using the packages car, lawstat and PMCMR.

3 | RESULTS

3.1 | A ~20 kDa protein fraction of the sawfly's oviduct secretion shows pine defense-eliciting activity

Our previous studies revealed that elicitor-inactive hemolymph of *D. pini* females and elicitor-active oviduct secretion differ in their protein profile especially with respect to the presence of a small protein, not detectable in the hemolymph (Hilker et al., 2005). Therefore, we focused on the isolation of proteins of about 30 kDa or smaller and isolated them by ultrafiltration. The ultrafiltrate was analyzed by BN-PAGE and revealed a protein fraction of about 20 kDa (Figure S1). The fraction was isolated by gel electro-elution and applied onto slit pine needles. As a control, a gel piece at the same position as the candidate protein fraction of a gel lane loaded with protein storage buffer only was electro-eluted and used for treatment of pine needles.

Elicitor activity assays testing the parasitoid's response to odor of artificially wounded (slit) pine needles treated with the isolated candidate protein fraction showed a significantly positive response of the parasitoids to odor from pine treated with this protein fraction (Figure 1a). The parasitoids were not attracted by odor of slit pine needles treated for control with protein storage buffer (Figure 1b).

3.2 | The candidate protein shows similarities to an annexin B9-like protein

Analysis of the bioactive candidate protein fraction by MALDI TOF-TOF tandem mass spectrometry revealed several signals, which were annotated to peptide sequences matching well to sequences known from a close relative of *D. pini*, the redheaded pine sawfly *N. lecontei*. We could assign most of these sequences to three annexin B9-like protein isoforms (Figure 2a, Table 1). Tandem mass spectrometry could not disentangle, which of the three annexin isoforms is present in *D. pini* female oviduct secretion. The peptide sequence of one peak (peptide mass 1231.52) matched with a protein of *N. lecontei*, of which no function is known as yet (Figure 2a, Table 1).

3.3 | Odor of pine treated with recombinant annexin-like protein–diprionin–attracts egg parasitoids

To figure out whether an annexin B9-like protein induces a pine odor, which is attractive to egg parasitoids, we determined the full coding sequence of the candidate protein for heterologous expression in insect cell culture (see Supporting Information Method S7, Table S1, Figure S2). The MALDI-TOF spectra of the recombinantly expressed protein and the active fraction of the oviduct secretion resembled each other, except for some oxidized methionine and tryptophan residues in the recombinant protein (Figure 2a,b).

The heterologously expressed protein was named 'diprionin'. Its calculated 3D structure shows the annexin-typical core domain with four repeats, each with 63–65 amino acids per repeat and made up of five α-helices (Figure 3a).

We applied the recombinant *D. pini* protein to artificially wounded pine needles and tested the parasitoid's behavioral response to odor of these needles. The olfactometer bioassays revealed that the parasitoids were significantly attracted to odor of pine treated with diprionin (Figure 1c), although some amino acids were oxidized during

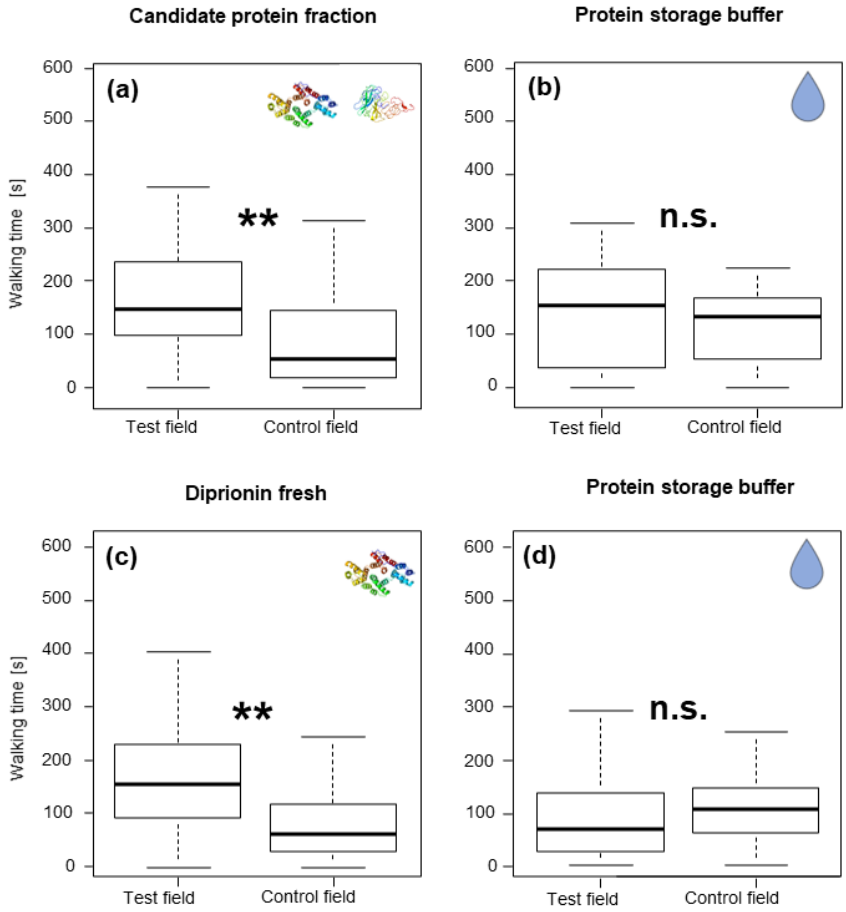

FIGURE 1 Elicitor activity assay: Olfactory response of egg parasitoids to odor of differently treated pine. Slit *Pinus sylvestris* needles were treated with (a) a candidate protein fraction (~20 kDa), obtained by ultrafiltration of oviduct secretion of *Diprion pini* females, separation of ultrafiltrate by Blue Native (BN)-PAGE, and electroelution of candidate band from gel; (b) protein storage buffer as control for assay (a); (c) electro-eluted recombinant annexin (diprionin) after affinity tag removal and BN-PAGE separation and (d) protein storage buffer as control for (c). Recombinant annexin (diprionin) was expressed in *Hi*-5 insect cell culture, and for each slit needle 250 ng protein was used. We treated eight needles per pine sample. Time (median, interquartile range, minimum, maximum), which parasitoid females spent walking in the test and opposite control field of a four-arm olfactometer during a 10 min (=600 s) observation period, is shown. The test field was provided with volatiles from pine twigs 72 h after treatment, the control field contained just charcoal-filtered air. (a) $n = 43$ parasitoids; $n = 9$ pine samples, (b) $n = 25$ parasitoids; $n = 9$ pine samples, (c) $n = 35$ parasitoids; $n = 9$ pine samples and (d) $n = 29$ parasitoids; $n = 9$ pine samples. Statistical differences were evaluated by a two-sided Wilcoxon signed ranks test and indicated by asterisks. Significant difference: $^{**}p \leq 0.01$; n.s. not significant ($p > 0.05$)

the purification process of the recombinant protein (Table 1). In contrast, odor released from control-(buffer)-treated needles was not attractive to the parasitoids (Figure 1d).

The oxidation of some amino acids already in freshly generated, bioassayed and chemically analyzed diprionin indicates high susceptibility of this protein to further oxidation. This susceptibility might be an explanation for the loss of eliciting activity of the protein after keeping it in protein storage buffer at 4°C temperature for 24 h (Figure S4). Previous studies on the activity of the oviduct secretion also showed that the pine defense-eliciting activity is very labile already shortly after dissection (Hilker et al., 2005).

3.4 | Diprionin induces enhanced emission of (*E*)-β-farnesene from pine needles

We further studied whether treatment of pine needles with freshly generated diprionin exerts similar effects on pine needle odor emission as treatment with *D. pini* eggs or oviduct secretion.

Our GC-MS analyses revealed that artificially wounded pine needles treated with diprionin showed a higher emission rate of (*E*)-β-farnesene than control-(buffer)-treated needles. The (*E*)-β-farnesene emission rate from diprionin-treated needles was about twice as high as from control-treated pine needles (Figure 3b). Hence, like *D. pini*

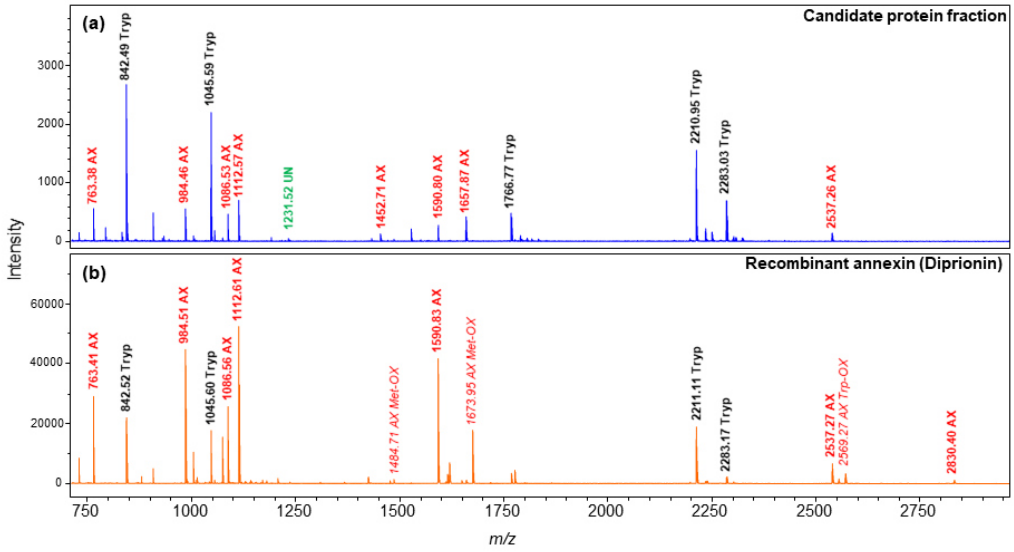

FIGURE 2 MALDI–TOF peptide mass fingerprints of pine defense-eliciting protein fractions obtained from *Diprion pini* oviduct secretion. Spectra of (a) an oviduct secretion fraction (after ultrafiltration and BN-PAGE; Figure S1a; ~20 kDa protein fraction) and (b) annexin (diprionin) recombinantly expressed in *Hi*-5 insect cell culture. Amino acid sequences of peaks labelled with an *m/z* value could be assigned to *Neodiprion lecontei* annexin B9-like protein (AX), to a protein from *N. lecontei* with yet unknown function (UN) and to the recombinant trypsin used for digestion of proteins for mass spectrometry (Tryp). Numbers in italics are for peptides with an oxidized methionine (Met-OX) or tryptophan (Trp-OX) residue. For detailed sequence information see Table 1

TABLE 1 Peptide sequences identified from the candidate protein fraction of *Diprion pini* oviduct secretion by mass spectrometry

Peptide mass[a]	Peptide sequence	BLAST result	Organism	Accession	Theoretical mass
763.38(41)	SYP(Q/K)LR	Annexin B9-like (all isoforms)	*Neodiprion lecontei*	XP_015522930	763.41
984.46(51)	(I/L)F(Q/K)EYER	Annexin B9-like (all isoforms)	*N. lecontei*	XP_015522930	984.48
1086.53(56)	RD(Q/K)TGYFAER	Annexin B9-like (all isoforms)	*N. lecontei*	XP_015522930	1086.48
1112.57(61)	(Q/K)(I/L)F(Q/K)EYER	Annexin B9-like (all isoforms)	*N. lecontei*	XP_015522930	1152.54
1231.52	VYC(cam)FEEGDGR	Uncharacterized protein	*N. lecontei*	XP_015513784	1231.50
1452.71	AMAGMGTDDTT(I/L)(I/L)R	Annexin B9-like (all isoforms)	*N. lecontei*	XP_015522930	1452.68
1484.71	AM(ox)AGM(ox)GTDDTTLIR	Annexin B9-like (all isoforms)	*N. lecontei*	XP_015522930	1484.67
1590.80(83)	GFGTDE(Q/K)A(I/L)(I/L)DV(I/L)GR	Annexin B9-like (all isoforms)	*N. lecontei*	XP_015522930	1590.81
1657.87	A(I/L)VA(I/L)MTP(I/L)PE(I/L)YAR	Annexin B9-like (all isoforms)	*N. lecontei*	XP_015522930	1657.93
1673.95	A(I/L)VA(I/L)M(ox)TP(I/L)LPE(I/L)YAR	Annexin B9-like (all isoforms)	*N. lecontei*	XP_015522930	1673.92
2537.26(27)	LLEAGEGQWGTDESTFNSILITR	Annexin B9-like (all isoforms)	*N. lecontei*	XP_015522930	2537.25
2569.27	LLEAGEGQW(ox/ox)GTDESTFNSILITR	Annexin B9-like (all isoforms)	*N. lecontei*	XP_015522930	2569.24
2830.40	LLVSLSTANRDESPDVDVDAATADAER	Annexin B9-like (all isoforms)	*N. lecontei*	XP_015522930	2830.37

[a]Experimental and theoretical peptide masses are given as mono-isotopic values $[M+H]^+$. Numbers in parentheses are different decimal values from different measurements of the same peptide. Peptide sequence annotations were performed with a MASCOT search against the NCBIprot database. Small letters in parentheses denote amino acid modifications by carbamidomethylation (cam) and oxidation (ox). Capital letters in parentheses denote ambiguous amino-acid annotation (mass accuracy insufficient to discriminate between Leu/Ile and Lys/Gln; theoretical values were calculated for Gln). Proteins were annotated by a protein BLAST search of peptide sequences against the NCBInr database restricted to Diprionidae. For annexin B9-like protein only the accession number of isoform X1 is shown. Accession numbers of isoforms X2 and X3 end with 31 and 32.

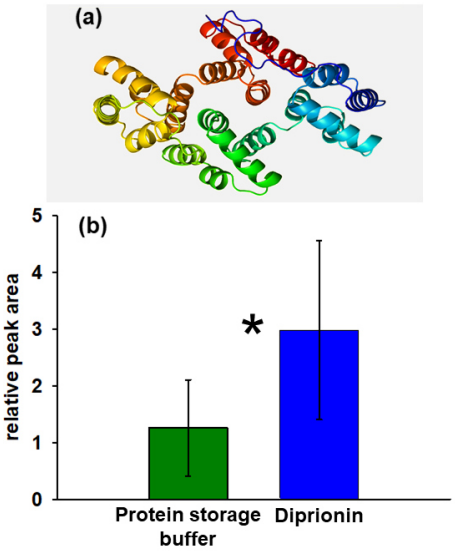

FIGURE 3 Diprionin (3D structure) and relative amount of (*E*)-β-farnesene in odor released from differently treated *Pinus sylvestris*. (a) Diprionin structure was calculated with the online tool Phyre2 (Kelley et al., 2015). The different colors show each of the 4 core domains of the 63–65 amino acids containing 5 α-helices common to all annexins. (b) Relative peak areas of (*E*)-β-farnesene (EBF) normalized to an internal standard (IS; 10 ng μl^{-1} methyl nonanoate); EBF emission from artificially wounded (slit) pine needles treated with either protein storage buffer or heterologously expressed diprionin; pine odor collection 72 h post treatment. Recombinant diprionin was expressed in *Hi*-5 insect cell culture, and for each slit needle 250 ng protein was used for the treatment. We treated 8 needles per twig. Shown are the mean (±SE) $\log_{10}$ transformed relative peak areas (peak area EBF/peak area IS) of each $n = 12$ test and control twigs. Statistical differences were evaluated by a two-sided paired *t*-test ($p = 0.045$, $t = 2.2602$, $df = 11$) and indicated by an asterisk. Significant difference: $^*p \leq 0.05$

egg deposition (Mumm et al., 2003), also diprionin elicits enhanced emission of (*E*)-β-farnesene from pine needles.

3.5 | Diprionin induces changes in expression of some defense-related genes

To further elucidate pine responses to the elicitor of indirect pine defense against sawfly eggs, we studied the impact of diprionin on expression levels of a set of selected defense-related pine genes (Table S2). The rationale for the selection of the investigated genes is explained in Section 2.11. The following two comparisons were made: (1) transcript levels of genes in egg- or diprionin-treated samples versus those in artificially wounded ones treated with the buffer; this comparison allowed us to detect the impact of sawfly eggs and diprionin per se apart from the impact of ovipositional wounding. Furthermore, we compared (2) transcript levels in egg-treated versus diprionin-treated samples; this comparison allowed us to elucidate whether compounds other than diprionin overwrite or synergize the effect of diprionin on gene expression. Supporting Information Table S3 shows how gene expression was affected by the treatment of artificially wounded (slit) needles with the protein storage buffer when compared to expression levels in untreated control needles.

Overall, expression of genes involved in terpene biosynthesis and in ROS homeostasis was similarly affected by egg deposition and diprionin treatment (Figure 4a,b). In response to these two treatments, transcript levels of terpene synthases showed moderate upregulation, which was significantly different from the artificially wounded control for *GPP3* (both in egg- and diprionin-treated samples) and for *GPP2* and *FPP* (only in egg-treated samples). Expression levels of terpene synthases did not significantly differ between egg- and diprionin-treated samples, except for *GPP2*, which was induced by the egg deposition, but not by diprionin (Figure 4a). Expression of *TPS5* was neither significantly affected by egg deposition nor by the diprionin treatment (Figure 4a). Transcript levels of *APX* and *CAT* encoding ROS scavenging enzymes were slightly and significantly downregulated by both egg deposition and diprionin treatment when compared to the artificially wounded control. *RbohA* expression was not significantly affected by the two treatments. While *SOD* expression varied strongly in response to egg deposition and was slightly, but not significantly downregulated in the egg-treated samples, this gene was moderately, but significantly downregulated by the diprionin treatment when compared to the artificially wounded control (Figure 4b).

In contrast to the above-mentioned genes, responses of especially the tested *PR* genes to sawfly egg deposition and diprionin treatment showed a poorly consistent pattern. Expression of *PR1* and *PR5* was significantly upregulated by egg deposition, whereas diprionin had no significant effect on the expression of these genes when compared to the artificially wounded control. *PR2* was moderately, but significantly downregulated by egg deposition, but its expression was not affected by diprionin. However, both diprionin and egg deposition significantly downregulated expression of *PAL* (Figure 4c). When considering the two genes involved in Ca^{2+} signalling, *CAX3* expression was strongly downregulated in response to egg deposition, but diprionin had no significant impact on the expression of this gene when compared to artificially wounded control samples. In contrast, both the treatment of pine with sawfly eggs and diprionin led to significant downregulation of *CDPK1* (Figure 4d).

4 | DISCUSSION

We identified a novel type of insect egg-associated elicitor of plant defense different from the low molecular weight elicitors previously described (Hilker & Fatouros, 2015). The identified elicitor — an annexin-like protein named diprionin — is released with secretion associated with eggs of the diprionid sawfly *D. pini* into needles of *P. sylvestris*. Treatment of pine with heterologously expressed diprionin

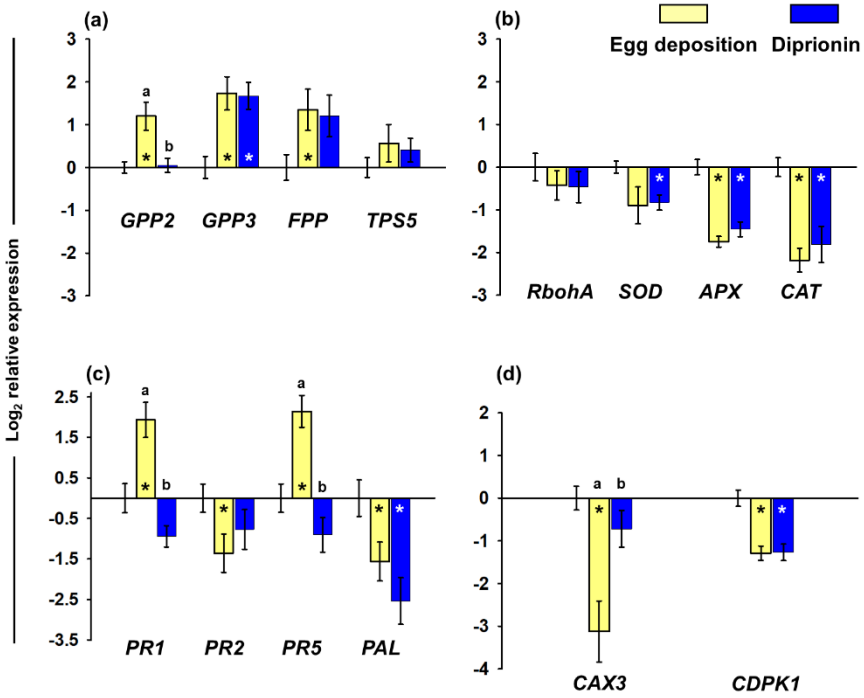

FIGURE 4 Effect of *Diprion pini* egg deposition and diprionin treatment on relative transcript levels in *Pinus sylvestris* needles. Recombinant diprionin was expressed in *Hi*-5 insect cell culture, and for each slit needle 250 ng protein was used for the treatment. We treated eight needles per pine sample. Transcript abundance (log_2, mean ± SE) 72 h after natural egg deposition (light yellow bars) or 72 h after treatment with recombinant diprionin (blue bars) relative to transcript abundance in wounded-plus-buffer-treated trees (zero ± SE on *y*-axes). (a) terpene synthases, *GPP*, geranyl pyrophosphatases; *FPP*, farnesyl pyrophosphatase; *TPS5*, *P. sylvestris* (*E*)-β-farnesene synthase; (b) genes involved in generation and degradation of reactive oxygen species (ROS); (c) pathogenesis-related *PR* genes and *PAL*, phenylalanine ammonia lyase; (d) genes involved in calcium signalling, *CAX*, cation exchanger; *CDPK1*, calcium-dependent protein kinase. Transcript quantity was first calculated relative to untreated control followed by normalization of the expression to the housekeeping genes as described by Pfaffl (2001) and Vandesompele et al. (2002). After normalization, log_2 was calculated for all data. Results for expression of wounded-plus-buffer-treated pine relative to untreated control are shown in Table S3. Normalized log_2 expression of genes in wounded-plus-protein-buffer-treated pine was then set to zero; relative to this, expression of the investigated genes is shown for needles treated by egg deposition and diprionin. Asterisks indicate significant differences ($p < 0.05$) between wounded-plus-buffer-treated pine versus either the egg deposition treatment or diprionin treatment; different letters above bars indicate significant differences ($p < 0.05$) between the egg treatment versus diprionin treatment. All statistical differences were calculated by Mann-Whitney *U*-test. Biological replicates (pine samples) per treatment: $n = 7–8$

results in effects, which were also observed when pine received *D. pini* egg deposition. Our analyses showed that diprionin-treated pine emits — like egg-laden pine — enhanced quantities of the sesquiterpene (*E*)-β-farnesene, which is crucial for attraction of egg parasitoids. A comparison of responses of a set of defense-relevant pine genes to diprionin and to egg deposition revealed similarities when considering genes involved in terpene biosynthesis and ROS homeostasis, but also dissimilarities, especially with respect to *PR* genes.

Annexins, the protein family to which diprionin belongs, are ubiquitously distributed proteins detected in all eukaryotic kingdoms (Gerke & Moss, 2002; Moss & Morgan, 2004). They are Ca^{2+}- and phospholipid-binding proteins with diverse cellular functions including membrane organization, mediation of exo- and endocytosis, regulation of redox processes at the plasma membrane and signal transduction in stress responses (Gerke & Moss, 2002; Konopka-Postupolska et al., 2011; Raynal & Pollard, 1994).

Plant annexins are involved in protection from oxidative stress (Gorecka et al., 2005; Konopka-Postupolska et al., 2009). They are well known to be involved in plant responses to various abiotic stresses (e.g., Clark et al., 2010; Dalal et al., 2014; Jami et al., 2010; Konopka-Postupolska et al., 2011; Laohavisit & Davies, 2011) and to phytopathogens (e.g., Jami et al., 2008; Mortimer et al., 2008). A recent study demonstrated that plant annexins are also relevant for plant defenses against chewing herbivores; expression of ANNEXIN1 (ANN1) of *A. thaliana* was shown to be induced by leaf wounding and insect feeding damage; experiments with mutant plants (ann1, ANN1) revealed that this annexin is clearly involved in damage-induced Ca^{2+} signalling and in conferring resistance against chewing insect larvae (Malabarba et al., 2021).

Insect annexins take on diverse functions, for example, in microapocrine secretion (Ferreira et al., 2007), apoptosis control during metamorphosis (Tsuzuki et al., 2001), or regulation of multivesicular

trafficking (Tjota et al., 2011). Furthermore, they have been suggested to play a role in maintaining integrity of tissues that are stretched due to, for example, food uptake in case of gut tissue (Kotsyfakis et al., 2005).

The *D. pini* sawflies might benefit from expressing diprionin because this protein could contribute to the necessary elasticity of the oviduct when eggs pass through. In several insect species, expression of annexin-encoding genes was found in different tissues including the salivary glands (Huang et al., 2016; Tsuzuki et al., 2001), the midgut and ovary (Kotsyfakis et al., 2005). The presence of *D. pini* annexin in the exocrine secretion of the oviduct raises the question how the protein reaches the extracellular space although it has — like other annexins (Moss & Morgan, 2004) — no signal peptide sequence for membrane trafficking (Petersen et al., 2011). Presence of annexins in insect exocrine secretion is not unique to *D. pini*. Proteomic analysis revealed the presence of annexins also in, for example, the secretion of saliva glands of a planthopper (Huang et al., 2016) or the Dufour gland of the honey bee (Teixeira et al., 2017). In animals, 'leaderless protein secretion' (Cheng & Williamson, 2010) is well known and may occur via transmembrane channels, endolysosomes, exosomes, or detachment of membrane protrusions (Cheng & Williamson, 2010). The question how annexins translocate into the extracellular space has especially been addressed in human medical studies focusing on the role of annexins in, for example, neurodegeneration (Valapala et al., 2014) or epithelial wound repair (Leoni et al., 2015). In plants, transmembrane trafficking of annexins has been discussed to occur via similar paths as in animals (Konopka-Postupolska & Clark, 2017) and has been shown by Rutter and Innes (2017) to take place via exosomes. Except for diprionin, no other insect annexin is known so far to be involved in plant defensive responses.

However, annexins of nematodes and phytopathogens have been suggested to play a role in interactions with plants. Constitutive expression of an annexin-encoding nematode gene in transgenic lines of *A. thaliana* resulted in enhanced infestation of the plant by the nematode. The nematode annexin was shown to interact with a plant enzyme (oxidoreductase), which promotes susceptibility to oomycete phytopathogens (Patel et al., 2010). Interestingly, oomycetes of the genus *Phytophthora* contain an annexin-like protein in their cell wall (Meijer et al., 2006; Savidor et al., 2008), which has been suggested to be involved in penetration of the phytopathogen into host plant tissue (Khalaj et al., 2015).

So far, it remains unknown how the internal pine needle tissue, which is in immediate contact with the *D. pini* egg-encasing oviduct secretion, interacts with diprionin (Hilker et al., 2002; Supporting Information Figure S5). Since diprionin was found to lose its elicitor activity already after a 24 h storage in buffer, the needle tissue is supposed to respond promptly to freshly generated diprionin and freshly released oviduct secretion. These immediate responses are expected to trigger further ones, thus mounting the indirect defense response, that is, the emission of increased quantities of (*E*)-β-farnesene 72 h after egg deposition or diprionin treatment. Like plant annexins, animal annexins have been shown to form Ca^{2+} channels in artificial membranes (Kourie & Wood, 2000). As suggested for the defense-eliciting FACs present in larval regurgitate, diprionin might induce a change in the membrane potential, thus initiating a pine defense cascade (Maffei et al., 2004; Maffei et al., 2007; Maischak et al., 2007; Spiteller et al., 2000), which results in changes in expression of genes with various functions and finally ecologically relevant chemical changes.

Extrapolation of diprionin-affected pine gene expression on the function of diprionin needs to be considered with the reservation that the tested sequences may represent just one member of a gene family and that their assignment is based on homologies. Nevertheless, our data cast a spotlight on the effects of diprionin on transcription of a subset of pine sequences.

Expression of genes involved in terpene biosynthesis was upregulated in the same direction when responding to diprionin and egg deposition, albeit differences in response intensities were detected. In contrast to the expectation that egg deposition induces expression of an (*E*)-β-farnesene synthase (TPS5) encoding gene, a study by Köpke et al. (2010) revealed that *D. pini* egg deposition does not regulate this gene. Our results here confirm this finding. Thus, the release of enhanced quantities of (*E*)-β-farnesene from egg-laden or diprionin-treated pine needles might be regulated on a level other than transcription. Although *D. pini* egg deposition does not induce significantly enhanced release of any other terpene than (*E*)-β-farnesene, our analyses showed that egg deposition significantly induced *FPP* encoding a farnesyl pyrophosphate synthase, and both diprionin and sawfly egg deposition induced a geranyl pyrophosphate synthase (*GPP3*). Since a previous study by Mumm et al. (2003) as well as the current one analyzed the headspace (released odor) of pine induced by sawfly eggs or diprionin, we cannot exclude that egg- or diprionin-treated pine biosynthesized enhanced quantities of terpenes, but stored them in, for example, resin ducts instead of releasing them. Alternatively, expression levels of *GPP*s and *FPP* might not correlate with the levels of their respective terpenoid products, as was also found by, for example, Laule et al. (2003).

Among the genes involved in regulating ROS homeostasis, expression of *RbohA*, a gene encoding an NADPH oxidase involved in hydrogen peroxide production, was neither significantly affected by egg deposition nor by diprionin treatment. Neither did a previous study find enhanced pine NADPH oxidase activity in response to *D. pini* egg deposition (Bittner et al., 2017). Nevertheless, pine shows direct defense against *D. pini* eggs and forms hypersensitive response (HR)-like symptoms, that is, necrotic leaf tissue at the oviposition site (Bittner et al., 2017); these HR-like symptoms are linked with accumulation of ROS in egg-laden pine (Bittner et al., 2019). This accumulation might be due to reduced ROS scavenging activity rather than to enhanced ROS production, as indicated by reduced activities of ROS scavenging enzymes in egg-laden pine needles (Bittner et al., 2017). The significant downregulation of *APX* and *CAT* in the current study further supports this assumption. In several annual plant species, ROS-generating NADPH oxidases are known to be activated by Ca^{2+}-dependent phosphorylation, which is mediated by CDPKs (e.g., Bredow & Monaghan, 2019; Dubiella et al., 2013;

Kobayashi et al., 2007; Pan et al., 2019). Here, a pine *CDPK1* sequence was downregulated in response to both egg deposition and diprionin treatment. It is unknown whether this pine *CDPK1* sequence encodes an enzyme involved in regulating NADPH activity and ROS production. The similar effects of insect egg deposition and diprionin on the tested genes involved in ROS homeostasis give rise to the assumption that diprionin might also contribute to the elicitation of direct pine defense against *D. pini* egg deposition.

This suggestion is opposed by the result that the diprionin treatment downregulated expression of the tested *PR* genes, while direct plant defense against eggs by formation of necrotic tissue is expected to involve upregulation of these *PR* genes. *PR1*, *PR2*, and *PR5* are known to be upregulated in leaf tissue showing HR-like symptoms in response to fungal infection (e.g., Stone et al., 2000). Upregulation of *PR1* expression is associated with direct defense of brassicaceous plants against butterfly eggs, that is, with formation of necrotic leaf tissue at the oviposition site, thus reducing egg survival rates (e.g., Griese et al., 2021). However, all tested *PR* genes — including *PR1* — were downregulated in response to diprionin treatment and not induced. Plant theory expects trade-offs between direct and indirect plant defense (Koricheva et al., 2004). Since egg deposition, but not diprionin treatment induces *PR1* and *PR5*, the question arises whether diprionin itself would attenuate pine direct defense by repressing transcription of these genes, while other compounds released with the eggs can compensate for such an effect.

Treatment of pine with diprionin did not regulate expression of all the tested pine genes in the same direction and with the same intensity as *D. pini* egg deposition did. Differences in responses to egg deposition and to diprionin are most probably due to the numerous further compounds, which are released in addition to diprionin with sawfly eggs. Even the active protein fraction of the defense-eliciting *D. pini* oviduct secretion contained an additional protein that could not be characterized as yet (Table 1).

Furthermore, several genes were regulated by both *D. pini* egg deposition or diprionin in another direction than expected from known responses of other plant species to insect eggs. For example, while *PR2* and *CAX3* are known to be upregulated in response to *P. brassicae* egg deposition on *A. thaliana* (e.g., Valsamakis et al., 2020), both *D. pini* egg deposition and diprionin treatment reduced transcription of these genes. This might be due to the different egg deposition modes of *P. brassicae* and *D. pini*. While no leaf damage is associated with *P. brassicae* egg deposition, the sawfly egg deposition comes along with wounding of a needle. CAX3 is encoding a Ca^{2+}/H^{+} exchanger, that is, a member of a group of enzymes extruding Ca^{2+} from the cytosol (Demidchik et al., 2018); the downregulation of this gene by *D. pini* egg deposition might help preventing Ca^{2+} efflux, thus contributing to keep a cytosolic Ca^{2+} level, which is important for defense signalling. However, in contrast to egg deposition, diprionin itself did not significantly repress expression of *CAX3*, thus indicating that other factors than diprionin released with the natural egg deposition are involved in regulating the cytosolic Ca^{2+} level.

The *PAL* sequence studied here was downregulated by *D. pini* egg deposition and diprionin application, although both treatments were applied to artificially wounded needles. The artificial wounding per se (control treatment; artificially wounded twigs treated with buffer only) induced the expression of this *PAL* sequence only by trend, but not significantly (Table S3). In contrast, leaf wounding per se has been long known to result in increased activity of PAL (e.g., Hartley & Firn, 1989), a central enzyme catalyzing an initial step of the phenylpropanoid path providing a broad set of plant secondary plant compounds with anti-herbivore activity (Lattanzio et al., 2008). Moreover, several studies revealed that angiosperm plants, which experience first insect egg depositions and subsequently leaf damage (by feeding larvae), accumulate higher concentrations of phenylpropanoid derivatives (Austel et al., 2016; Bandoly et al., 2015, 2016; Lortzing et al., 2019). In the interaction between pine and *D. pini*, the leaf damage precedes egg deposition; the *D. pini* female first slits a pine needle with her ovipositor and subsequently oviposits into the slit pine needle. Future studies need to elucidate whether levels of *PAL* transcripts and resulting phenolic compounds are dependent on the temporal sequence of egg deposition and leaf damage. Furthermore, gymnosperms have an especially diverse set of *PAL* genes (Bagal et al., 2012). Other members of the *PAL* gene family might show other responses to diprionin than the tested *PAL* sequence.

In summary, the oviduct secretion encasing sawfly eggs was shown here to contain an annexin-like protein named diprionin, which induces indirect pine defense against the eggs. While our study clearly demonstrated that pine treatment with diprionin results in attraction of egg parasitoids, future studies need to further elucidate whether diprionin is also involved in eliciting direct defense against the eggs. Furthermore, the question whether diprionin as an annexin-like protein facilitates transmembrane transport of Ca^{2+} and thus pushes Ca^{2+}-mediated stress signalling deserves future investigations. The discovery of diprionin as an insect egg-associated elicitor of plant defense shows that plants have evolved the ability to respond to a broad spectrum of elicitors indicating insect infestation. Our study highlights a novel type of elicitor of plant defense against insect eggs and points to new directions to study how plants respond to an early step of insect infestation, the egg deposition.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

Sequences of *Pinus sylvestris* PCR products and their respective accessible template accession numbers in Genbank for the primer design as well as the annotation information referred to in this paper have been deposited at the data repository of the Max-Planck-Institute for Molecular Plant Physiology, Potsdam-Golm, Germany, with open access at https://primedb.mpimp-golm.mpg.de/index.html?sid=reviewer%26pid=a544940db9f1d9e71e327cfe6d65b1f2.

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SUPPORTING INFORMATION

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SUPPORTING INFORMATION

Pine defense against eggs of an herbivorous sawfly is elicited by an annexin-like protein present in egg-associated secretion

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Supporting Information Method S1: Fractionation of *Diprion pini* oviduct secretion by ultrafiltration

Our earlier publications indicated that the indirect defense eliciting protein fraction of *D. pini* oviduct secretion has a low molecular weight (Hilker et al. 2005). To isolate this low molecular weight protein fraction from the various differently sized proteins (10 – 250 kDa) of *D. pini* oviduct secretion, we used Vivaspin2® (Sartorius) centrifugal concentrators with polyethersulfone membranes of different molecular weight cut-off (MWCO) sizes (100 kDa; 30 kDa; 5 kDa). In a first step, 100 kDa ultrafiltration tubes were used to pre-filter for large proteins > 100 kDa. The MWCO size of the ultrafiltration tubes is defined by the molecular weight, at which at least 90% of a globular protein is retained by the membrane. Depending on the specific protein characteristics, the membrane also allows proteins that are somewhat heavier or somewhat lighter to flow through (Schratter, 2004). Concentrator tubes were first cleaned by centrifugation of 200 µl ice-cold protein storage buffer (pH 7.2; 70 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 1 mM $NaHCO_3$, or 150 mM Tris-HCl, 50 mM NaCl) for 4 min at 4°C at 4000×*g*. The resulting filtrate was discarded. In order to dilute the oviduct secretion sample and to minimize potential membrane fouling effects (Huisman, Prádanos & Hernández, 2000), we added 1.8 ml of ice-cold protein storage buffer to an oviduct secretion sample. The diluted sample was centrifuged at 4°C, 4000×*g* with 100 kDa MWCO centrifugator tubes until the whole solution except a dead stop volume of ~ 8 µl passed through. This is followed by a second filtering step with a 30 kDa ultrafiltration tube at the same conditions. The remaining filtrate consisting of proteins smaller than 30 kDa was further concentrated in a 5 kDa ultrafiltration tube to a volume of ~ 20 µl. The supernatant of this filtration step consisting of proteins of a size between 5 – 30 kDa was loaded on the BN-PAGE gel (Supporting Information, Method S2) for further analysis.

Supporting Information Method S2: Blue Native-PAGE (BN-PAGE)

To analyze the proteins present in the oviduct secretion sample, an equivalent of ultrafiltrated oviduct secretion (dissected from 20 females) in protein storage buffer was initially loaded on the gel. A 12% acrylamide mini separation gel (4.5 ml) overlaid with a 4% acrylamide collection gel (1.5 ml) was casted and polymerized overnight at 4°C. A pooled secretion sample (35 µl) was mixed with 35 µl 2X loading buffer (0.02% Coomassie Blue G-250, 7.5 mM imidazol, 50 mM Tricine, 10 % glycerol; pH 7) and applied onto the gel. The loading buffer was stirred over night at room temperature to avoid aggregation of colloidal Coomassie particles. For better visualization of small sized proteins we omitted the Coomassie Blue G-250 in the cathode buffer as used in the original protocol and only added Coomassie dye to the loading buffer. For determination of protein masses, 5 µl of NativeMark™ unstained protein marker (ThermoFisher Scientific) was additionally loaded on each BN-PAGE gel. Gels were run at 180 V and 30 mA for 150 min at 10°C and stained with silver nitrate as described by Chevallet, Luche & Rabilloud. (2006). The analysis revealed that the remaining filtrate of the oviduct secretion after pre-

filtering with concentrators of MWCO sizes 100 kDa and 30 kDa contained a protein fraction of about 20 kDa (Supporting Information Figure S1a).

For analyses of successful tag cleavage and further purification of the recombinant protein, a 15% acrylamide mini separation gel was casted. Here we used the Coomassie Blue G-250 staining method by Dyballa & Metzger (2009) as not so high sensitivity was needed. Furthermore, staining with this dye minimized the staining time and thus, reduced the risk of potential protein inactivation during protein purification. The analysis revealed a protein band at 20 kDa (the recombinant protein) and four further bands > 20 kDa, which represented recombinant protein tag cleavage products (Supporting Information Figure S1b).

Supporting Information Method S3: Electro-elution and concentration of target proteins from *Diprion pini* oviduct secretion

Target proteins were isolated from electrophoresis gels by electro-elution.

For BN-PAGE analyses of proteins from the oviduct secretion we used silver nitrate staining. Because this staining might interfere with the electro-elution efficiency and activity of proteins, we only stained one lane of the ultrafiltration-fractionated samples and used it for determination of mass and migration distance, while another parallel lane was left unstained. The respective target protein fraction with a molecular weight of about 20 kDa was cut out from the unstained lane, minced into small pieces and mixed with 2 ml electro-elution buffer (25 mM Tricine, 3.75 mM imidazole, 5 mM 6-aminohexanoic acid; pH 7.0). The solution was pipetted in standard grade regenerated cellulose dialysis tubes with a MWCO membrane of 3.5 kDa (Spectrum Laboratories). Thus, loss of proteins larger than ~ 3.5 kDa (including our target protein) was prevented; these proteins were kept in the dialysis tube. The dialysis tubes were soaked in ddH_2O for 30 min before use. To gain maximum recovery, the gel pieces were electro-eluted overnight at 10°C with 100 V and 10 mA in a horizontal electrophoresis chamber. Finally, the polarity was reversed for 2 min to retrieve proteins bound to the dialysis membrane. The 2 ml protein solution from the dialysis tube was concentrated with 5 kDa MWCO size Vivaspin 2 ultrafiltration tubes with cellulose triacetate membranes to maximize protein recovery. For buffer exchange from electro-elution buffer to buffer for protein storage, 2 ml protein storage buffer were added to the protein solution supernatant concentrate after having been concentrated to ~ 25 µl volume and then concentrated to 25 µl again.

For electro-elution of the recombinant protein from the BN-PAGE gel, we also used the target protein of an unstained lane that had run in parallel to the stained lane. The electro-elution was done as described above for the proteins isolated from oviduct secretion.

Supporting Information Method S4: Elicitor activity assay: Olfactory response of egg parasitoids to differently treated pine

The response of the egg parasitoid *Closterocerus ruforum* to odor of differently treated pine was tested in a four-field olfactometer. The four fields are quarters of an arena (258 cm^2), where the parasitoid could move around. The fields were obtained by introducing air into each quarter (flow 155 ml s^{-1}); the air was sucked out in the center of the arena. One of the olfactometer fields was supplied with the odor of a pine twig. This field is here referred to as test field. The other three fields contained only charcoal-filtered air. Since a parasitoid that is attracted to the odor in the test field also easily reaches the neighbored fields when foraging at the edges of the test field, we labelled the fields adjacent to the test field as buffer fields and the field opposite of the test field as control field (Schröder, Wurm, Varama, Meiners, & Hilker, 2008). After release of a single parasitoid into the center of the olfactometer, we recorded the time that a foraging parasitoid spent moving around in each field for an observation period of 600 s. Parasitoids, which moved around for less than 300 s, were considered not actively foraging and thus, were not included in the statistical analysis. We used the Observer 3.0 (Noldus) for recording the data.

For each type of pine twig treatment, 25 to 43 parasitoids were tested on nine twigs. Twigs were replaced by another one after having tested three to five parasitoids. No more than three twigs (obtained from different trees) were tested on one day. For each test day, protein samples used for pine treatments were freshly prepared.

Prior to the bioassays and post pine treatment, twigs were kept at 20°C, 18:6 h, *L:D*, 70 % relative humidity, 100 µmol photons m^{-2} s^{-1} for 72 h and then used for the olfactometer bioassay. We chose this incubation time post treatment, since previous assays revealed that egg-laden and oviduct secretion-treated pine twigs release odor that is attractive to parasitoids 72 h after treatment (Hilker, Kobs, Varama, & Schrank, 2002; Hilker, Stein, Schröder, Varama, & Mumm, 2005).

Supporting Information Method S5: Chemical analysis of odor from pine treated with recombinant diprionin

The collected pine volatiles that had adsorbed onto 5-mg charcoal filters were eluted with 50 µl dichloromethane containing 10 ng μl^{-1} methyl nonanoate (Sigma Aldrich) as an internal standard (IS). The eluate (1 µl) was injected in splitless mode (injector temperature 300°C) into an Agilent 7890 A GC model coupled to an Agilent 5975 C MS unit. A J&W 30 m DB-5-ms capillary column was used (length: 30 m; inner diameter: 0.25 mm; film thickness: 0.25 µm). Helium was used as carrier gas with

an inlet pressure of 0.1 bar and an outlet pressure of 0.5 bar. The following program was used for analysis: 4 min hold at 40°C followed by a temperature increase of 10°C min^{-1} until 220°C. After a 1 min hold, the temperature rose to 300°C by 50°C min^{-1}. This final temperature was kept for 2 min. The column effluent was ionized by electron impact ionization at 70 eV (mass range from 35 to 300 *m/z*).

Supporting Information Method S6: RNA extraction from female *Diprion pini* sawflies and cDNA synthesis

Insect tissue was homogenized with a plastic pistil on ice in 1 ml of RLT cell-lysis buffer with β-mercaptoethanol. Further homogenization was performed by up and down pipetting and rigorous vortexing of the tissue solution. The RNA was DNAse-treated with the TURBO DNA *free*™ kit (ThermoFisher Scientific) to avoid any DNA contamination. RNA stability was visually checked on a 1.1 % agarose gel in 1X TAE buffer with 0.006% ethidium bromide. A volume of 10 µl of the sample was mixed with 10 µl of 2X RNA loading dye (ThermoFisher Scientific), heated to 70°C for 10 min and immediately placed on ice after heating. Four µl of the RiboRuler High Range RNA Ladder (ThermoFisher Scientific) were treated accordingly. After loading the samples, the gel run for 90 min at 120 V. The RNA concentration was determined spectrophotometrically on a Multiscan® GO microplate spectrophotometer (ThermoFisher Scientific) by measuring absorbance at 230 nm.

Supporting Information Method S7: Identification of *Diprion pini* annexin B9 like coding sequence (diprionin)

The following PCR conditions for identifying a *D. pini* annexin B9-like coding sequence were applied:

We performed a gradient PCR of cDNA generated from RNA of *D. pini* females (compare Supporting Method S6). We used the primers designed for the *D. pini* annexin B9 like protein and applied the following protocol: 15 ng cDNA was mixed with 0.5 µl of forward (DPAnnexin1F), 0.5 µl reverse primer (DPAnnexin1R) (each 10 pmol $µl^{-1}$), 10 µl 2X Perpetual Taq PCR Master Mix (Roboklon), and 2 µl 10X color load (Roboklon). The mixture was filled up to 20 µl reaction volume with nuclease-free H_2O. After an initial denaturation period of 5 min at 95°C, 40 cycles of 60 sec denaturation at 95°C; 30 sec of annealing at 52 to 62°C and 80 sec of extension at 72°C were followed by a final extension of 7 min at 72°C. PCR products were analyzed on a 1.5% agarose gel at the conditions described above for RNA, except that 7 µl of the 100 bp extended ladder (Carl Roth) were used as marker, and no pre-heating of samples was required.

To obtain the full-length cDNA coding sequence of the elicitor candidate, a 3'RACE PCR was performed as follows: 10 µl of 5X Phusion Green HF buffer (ThermoFisher Scientific) was mixed with 1 U of Phusion HSII enzyme (ThermoFisher Scientific), 2 µl of dNTPs (10 mM), 5 µl of forward (DPAnnexRaceF1), and 5 µl of reverse primer (3'RACE Outer Primer) (each 10 pmol μl^{-1}), 3 µl DMSO and 50 ng of 3' RACE modified cDNA. The mixture was filled up to 50 µl reaction volume with nuclease-free H_2O. After an initial denaturation of 30 sec at 98°C, 35 cycles of 10 sec denaturation at 98°C, 30 sec of annealing at 53°C, and 60 sec of extension at 72°C were followed by a final extension of 10 min at 72°C.

Supporting Information Method S8: Recombinant expression of *Diprion pini* annexin (diprionin)

For heterologous expression of the *Diprion pini* elicitor candidate protein, the full length nucleotide coding sequence of the *D. pini* annexin was needed. To determine this sequence, we first designed primers (position bp 81 to bp 564 bp) based on the *Neodiprion lecontei* nucleotide sequence coding for annexin (B9-like isoform X1; XP_015522930) that showed best matches (BlastP E-value 0.39 – $4\times e^{-21}$) with the respective *D. pini* sequences detected by mass spectrometry peptide fingerprinting (Table 1, main text). In a further step, the primers were used to identify a partial coding sequence from *D. pini* cDNA generated from RNA isolated from the abdomen of sawfly females (Supporting Information Method S7 for PCR conditions). We could amplify a partial *D. pini* cDNA sequence with highest similarity to the sequence coding for the *N. lecontei* annexin B9 like protein isoform X1 (XP_015522930) (BlastX E-value $4\times e^{-84}$). In a third step, the identified partial cDNA sequence was used for primer design for subsequent RACE-PCR to elucidate the full coding sequence (Supporting Information Figure S2, Supporting Information Method S7 for RACE PCR conditions).

For recombinant expression of *D. pini* annexin (diprionin), the full coding sequence without any regulatory 5' or 3' sequences was amplified by PCR with primers starting at the beginning and the end of the coding sequence (Supporting Information Table S1). For the PCR analyses, we mixed 10 ng of *D. pini* abdominal cDNA with 25 µl 2X OptiTaq PCR Master Mix (Roboklon), 2 µl of forward- (Ann-ORF-F), and 2 µl of reverse primer (Ann-ORF-R) (each 10 pmol μl^{-1}). The mixture was filled up to 50 µl reaction volume with nuclease-free H_2O. After an initial denaturation of 5 min at 95°C, 40 cycles of 20 sec denaturation at 95°C; 30 sec of annealing at 50°C, and 80 sec of extension at 72°C were followed by a final extension of 7 min at 72°C.

PCR products were analyzed by agarose gel electrophoresis and cloned into a pGEM®-T vector (Promega), which was amplified in chemically competent *E. coli* DH-5α cells. To verify correct insertion of the target sequence into DH-5α cells, plasmids extracted with the peqGOLD Plasmid MiniPrep Kit I (Peqlab) were Sanger-sequenced at Seqlab (Goettingen, Germany).

To enable isolation of the sequence from the vector plasmid and later purification of the expressed protein, we further introduced restriction sites, maltose binding protein (MBP) tags, a factor X_A cleavage site, and overlapping sites for Megaprimer PCR (Ke & Madison, 1997). After factor X_a affinity tag cleavage of the heterologously expressed *D. pini* annexin, a BN-PAGE analysis revealed the presence of a band of about 20 kDa and four further bands > 20 kDa (Supporting Information Figure S1). All bands were isolated and electro-eluted. We conducted a peptide mass fingerprinting analysis of the bands electro-eluted from this gel to check whether one of the bands represents the expected native recombinant protein. The bands > 20 kDa could all be assigned to the fusion protein MBP.

Primers were designed for PCRs with the annexin-ORF vector plasmid and the pMALp2x *E. coli* plasmid cloning vector (Promega) as a template (Supporting Information Table S1). PCRs with the annexin-ORF vector plasmid and the pMALp2x *E. coli* plasmid cloning vector (Promega) as a template (Supporting Information Table S1) were performed as follows (in brackets are the primers and templates for the pMALp2x PCR): 50 ng of annexin-ORF vector plasmid (pMALp2x) was mixed with 10 µl 5x Phusion HF buffer, 1 µl of dNTPs (10 mM), 2 µl of the forward primer MBP-ANN-F (MBP-EcoRI-F) (10 pmol μl^{-1}), 2 µl of the reverse primer ANN-HindIII-R (MBP-ANN-R) (10 pmol μl^{-1}), and 1U of Phusion HS II. The mixture was filled up to a 50 µl reaction volume with nuclease-free H_2O. After an initial denaturation period of 30 sec at 98°C, 40 cycles of 20 sec denaturation at 98°C, 20 sec of annealing at 50°C, and 40 sec of extension at 72°C were followed by a final extension of 5 min at 72°C.

Resulting PCR products were gel-extracted following the protocol of the peqGOLD gel extraction kit (Peqlab) and eluted in 30 µl nuclease-free H_2O. To fuse the resulting products, a Megaprimer PCR was performed. A volume of 2 µl of each gel-extracted PCR product was mixed with 10 µl 5x Phusion HF buffer, 4 µl dNTPs (10 mM), 1U Phusion HSII, and filled up to 45.5 µl reaction volume with nuclease-free H_2O. The PCR conditions were as follows: initial denaturation period of 30 sec at 98°C, 30 cycles of 30 sec for denaturation at 98°C, 30 sec for annealing at 55°C with a temperature decrease of 0.5°C per cycle, and 90 sec for extension at 72°C, addition of 2µl of MBP-EcoRI-F primer (10 pmol μl^{-1}), 2 µl of ANN-HindIII-R primer (10 pmol μl^{-1}), and 1 U of Phusion HSII. Thereafter, the PCR reaction was continued with again 25 cycles of 98°C for 30 sec, 55°C for 30 sec and 72°C for 90 sec, and a final extension period of 5 min at 72°C.

The obtained sequence was cleaved at the 5´-end with EcoRI and at the 3´end with HindIII for directed insertion into a pFast-Bac-Dual vector (pFBD). This vector was cleaved with the same enzymes. The sequence was ligated into the vector, which contained an additional sequence of enhanced green fluorescence protein (EGFP). The vector plasmid was amplified in DH-5α cells and extracted with the peqGOLD Plasmid MiniPrep Kit I (Peqlab). The plasmid (100 ng) was transformed in *E. coli* DH-10-

BAC cells, and the resulting high molecular weight bacmid shuttle vector was extracted following the protocol of the Bac to Bac Baculovirus expression system kit (ThermoFisher Scientific). The correct insertion of the bacmid construct was verified with the M13 PCR protocol of the kit.

After bacmid amplification in *Sf21* insect cells through two cell culture passages to generate a high virus titer supernatant, *Hi-5* insect cells were infected for efficient recombinant protein expression. Successful infection of the insect cells with the shuttle vector was inspected by checking green fluorescence with an inverse fluorescence microscope (Zeiss AXIO observer) at all stages.

Hi-5 insect cells were harvested three days after bacmid infection by centrifugation at 5000×*g* for 20 min at 4°C. The resulting pellet was resuspended in 8 ml of protein extraction buffer (150 mM Tris-HCl, 50 mM NaCl; pH 7.2) with EDTA-free proteinase inhibitor cocktail (Sigma-Aldrich). Cells were sonificated on ice for 2 min at level 2 and 20 % power with a Branson sonifier 250 (Branson Ultrasonics, St.Louis, USA). Cell fragments were pelleted as above, and the resulting supernatant was particle-filtered. Recombinant proteins were purified by affinity chromatography on an amylose resin column, which is applicable for the isolation of proteins fused to MBP. We followed the protocol provided for the amylose resin kit by New England Biosystems. Ten fractions of 1 ml were collected, analyzed on a 4-20 % gradient SDS-PAGE gel (Carl Roth) according to the manufacturer's protocol, and stained with the Coomassie staining protocol by Dyballa & Metzger (2009). For estimation of protein masses, 6 µl of PageRuler™ Protein ladder Plus (ThermoFisher Scientific) was loaded on the gel (Supporting Information Figure S3).

After measurement of protein concentration with the Pierce BCA protein assay kit (ThermoFisher Scientific), the MBP tag was cleaved with a sufficient amount of factor X_A for 24 h according to the kit protocol (New England Biosystems).

Supporting Information Method S9: Impact of diprionin on expression of defense-related pine genes

The diprionin treatment of pine needles was conducted with the same method as the one described for treatment of pine used for olfactometer biossays by using freshly generated recombinant protein. We slit eight needles of each twig to mimic the wounding, which a sawfly female inflicts to a pine needle prior to egg deposition. Then we applied 1 µl with 250 ng recombinant protein solved in protein storage buffer on each slit needle.

We compared transcript levels of genes in diprionin-treated pine samples with those in naturally egg-laden pine. To obtain egg-laden pine needles, we used the method as described previously by Hilker et

al. (2002) and Schröder et al. (2008) since pine twigs treated according to this method released pine odor attractive to egg parasitoids.

For control, we also determined transcript levels of pine genes in untreated pine and artificially wounded pine needles treated with the buffer for protein storage. For the latter treatment, we slit eight needles of each twig and applied 1 µl pure protein storage buffer onto each slit needle.

Post treatments, the samples were kept for 72 h at the same abiotic conditions as described for treatments of pine used in the olfactometer assays. Thereafter, the needles were frozen in liquid nitrogen and stored at -80 °C.

Primers for the selected genes and for the housekeeping genes ubiquitin (*PsUBI*), cytochrome subunit 6 (*PsPetB*) and chloroplast ATPase beta subunit (*PsCATP*) were designed and evaluated according to the MIQE guidelines (Bustin et al., 2009; Taylor, Wakem, Dijkman, Alsarraj, & Nguyen, 2010) with the online tools PRIMER-BLAST (Ye, Coulouris, Zaretskaya, Cutcutache & Rozen, 2012) and Primer3 (v. 0.4.0) (Untergasser et al., 2012). For some of the analyzed *P. sylvestris* sequences, no published annotation was available. Therefore, we searched in BLAST EST and nr databases (Altschul, Gish, Miller, Myers, & Lipman, 1990) for those sequences restricted to the taxon *Pinus,* which showed highest homology with annotated sequences from different plant species. Primers were designed based on sequences with the lowest E-value (Supporting Information Table S2).

Frozen *P. sylvestris* needles were powdered in liquid nitrogen, and 50 mg of needle powder were used for RNA extraction with the InviTrap Spin Plant RNA Mini Kit (Stratec, Berlin, Germany). RNA was eluted in 50 µl nuclease-free H_2O, and contaminating DNA remains were digested with the TURBO DNA free™ kit (ThermoFisher Scientific). RNA integrity and purity were checked by analysis on a 1.1 % agarose gel in 1X TAE buffer with 0.00005 % ROTI®GelStain (Carl Roth). A volume of 10 µl of the sample was diluted 1:1 with 2X RNA loading dye (ThermoFisher Scientific), heated for 10 min to 70°C and immediately placed on ice afterwards. 4 µl of the RiboRuler High Range RNA Ladder (ThermoFisher Scientific) were treated likewise. After loading of the samples, the gel run was performed for 90 min at 120 V. Spectrophotometric determination of RNA concentration was performed on an Infinite® M Nano+ plate reader (Tecan Trading) by measuring absorbance at 230 nm.

For synthesis of cDNA, 500 ng of the extracted RNA was used as a template for reverse transcription utilizing the AMV-RT native protein (Roboklon, Berlin, Germany). The RNA was mixed with 1 µl Oligo dT20 (50 µM) and 2 µl dNTPs (10 mM) and filled up to a reaction volume of 14 µl with nuclease-free H_2O. The mixture was incubated for 5 min at 65°C followed by 5 min incubation on 4°C. To start the reaction, 4 µl 5x RT Puffer (Roboklon, Berlin, Germany), 0.5 µl RNASE inhibitor (Roboklon; 30 U

μl^{-1}), 1 µl 100 mM DTT and 1 µl AMV-RT native (Roboklon; 10 U μl^{-1}) were added and heated to 42°C for 15 min and to 50°C for 45 min. To inactivate the AMV-RT enzyme, the mixture was finally heated to 80°C for 10 min and immediately cooled on ice.

The qPCR analysis was performed by using the qPCRBIO SyGreen Mix Lo-Rox kit (Nippon Genetics Europe, Düren, Germany) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories). For the qPCR reactions 12.5 ng of cDNA was mixed with 5 µl of qPCRBIO SyGreen Mix Lo-Rox Master Mix (Nippon Genetics Europe, Düren, Germany), 0.17 µl of each primer (10 pmol μl^{-1}) and filled up to 10 µl reaction volume with nuclease-free H_2O. As controls for primer dimerization, H_2O controls were run, and as a control for DNA contamination, DNAse treated RNA from each sample was used. Each reaction was performed with three technical replicates at the following running conditions: After an initial heating step of 2 min at 95 °C, 40 cycles of 5 sec at 95 °C followed by 30 sec at 60 °C were performed. At the end of each cycle the fluorescence was measured twice. Following the 40 cycles of PCR amplification a dissociation curve ranging from 55 °C to 95 °C in 1 °C steps was measured to check for primer dimer reaction products. C(t) values of all reactions were calculated with Bio-Rad CFX Maestro 1.1 version 4.1.2433.1219 (Bio-Rad Laboratories).

For data evaluation, we determined the transcript level of the candidate genes in treated samples relative to those in the untreated control. Thereafter, we first normalized the expression level of the candidate genes in untreated controls to those of three housekeeping genes (cATP, UBI, PETB) as described by Pfaffl (2001) and Vandesompele et al. (2002) (see Supporting Information Table S2 for full names of housekeeping genes). To focus on the effect of egg deposition and diprionin treatment on gene expression rather than on any effect caused by the wounding coming along with the egg deposition and diprionin treatment, we set the expression of the analyzed genes in the artificially wounded samples treated with protein storage buffer to zero. Thereafter, $\log_2$ fold change of expression of genes in egg- and diprionin-treated samples relative to the artificially wounded samples was calculated. To separately evaluate the effect of wounding, gene expression in artificially wounded and protein storage buffer-treated pine samples was expressed as $\log_2$ fold-change to expression levels in untreated controls (see Supporting Information Table S3).

Supporting Information Figure S1

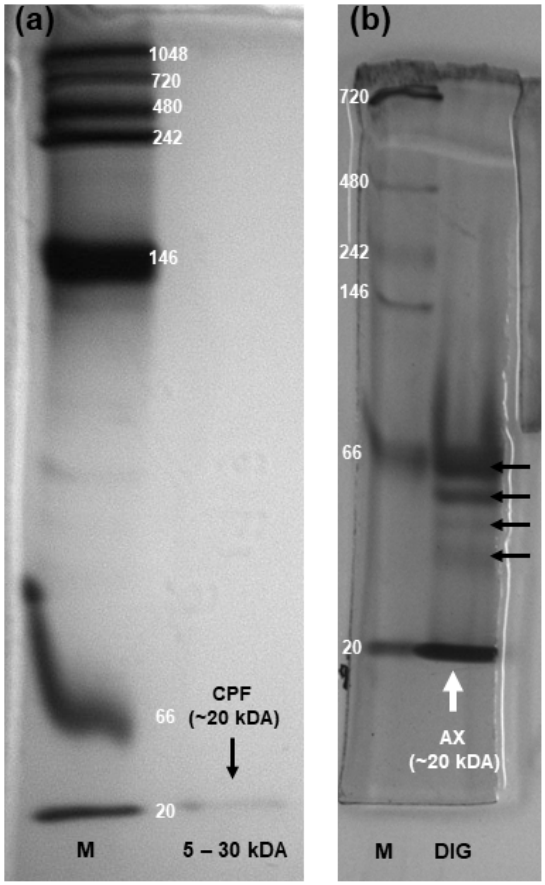

FIGURE S1 Blue Native PAGE of tested candidate proteins from sawfly oviduct secretion and of heterologous protein expression in Hi-5 insect cell culture**. (a**) Silver nitrate stained, 12 % Blue Native (BN)-PAGE gel of the low-molecular weight fraction after ultrafiltration of the oviduct secretion of *Diprion pini* females. Lanes: M, marker; 5 – 30 kDa, ultrafiltration fraction of sawfly oviduct secretion proteins. The black arrow shows the band of the small candidate protein fraction (CPF) used for the olfactometer bioassay and for peptide mass fingerprinting. **(b)** Coomassie Brilliant Blue-stained 15% BN-PAGE after cleavage of the maltose binding protein (MBP) affinity tag with factor XA; band with heterologously expressed annexin was electro-eluted from a corresponding unstained gel and used for treatment of pine twigs subjected to bioassays with parasitoids (compare Figure 1c, main text) and GC-MS analyses (compare Figure 3b, main text). Lanes: M, marker; DIG, Factor XA-digested recombinantly expressed annexin (AX – white arrow) and fusion protein / maltose binding protein (black arrows) after 24 h digestion. White numbers refer to the molecular weight in kDa of marker proteins

Supporting Information Figure S2

Diprionin - *Diprion pini* – Annexin B9 like isoform X1 (969 bp)

```
  1  ATGGCACCGC AATATTACCA CGTACAGTGC ACCCCAACCG TGTACCCCGC CGATCCGTTT GACGCGGAGG CAGATGCGAC
 81  TCTCTTGAGA ACCGCGATGA AAGGTTTCGG AACAGACGAA CAGGCTATAA TCGATGTTCT GGGTCGCCGT GGGATAGTCC
161  AGCGTTTAGA AATTGCCGAA AAATTCAAGA CGATGTACGG AAAAGATTTG ATATCCGAAT TGAAGTCCGA GCTTGGGGGA
241  CATTTCGAAA AGGCCATCGT AGCCCTAATG ACTCCTCTGC CAGAGTTGTA CGCCCGTGAA ATACACGACG CGATTTCTGG
321  AATCGGTACA GACGAAGGTG CCCTTGTCGA GGTCCTGGCA TCTCTCAGCA ATTACGGCAT CAAGACTATT TCTGCCGTTT
401  ACAAGGATCT GTACGGCAAC GAACTTGAAG ATGACCTGAA GAGTGATACG TCGGGCCACT TTAAGAGACT TCTGGTCTCC
481  CTTAGCACAG CTAACAGAGA CGAGTCACCC GACGTCGACG TTGACGCAGC AACCGCTGAT GCAGAGAGGC TCCTCGAGGC
561  TGGTGAGGGG CAATGGGGAA CCGATGAAAG TACATTTAAC TCTATCTTGA TAACCAGAAG CTACCCTCAG CTTCGTAAGA
641  TATTCCAAGA GTACGAGCGA CTTTCAGGAT CCGACTTGGA AGATACCATC AAGAAAGAAT TTTCTGGCTC CATCGAGGAT
721  GGCTACCTTG CCGTTGTCAA GTGCGCCCGG GACAAGACTG GTTATTTCGC TGAAAGATTA CACAAAGCAA TGGCTGGTAT
801  GGGAACAGAC GACACTACCC TGATCCGTAT TATTGTCTTG CGCTCTGAAA TTGATCTGGG TGATATCAAG GAAGCGTATG
881  AACAGATATA TGGCCAATCG CTGGCTGGAG ACATTGATGG TGACTGTTCG GGAGACTACA AGAGACTGTT GCTTAGTCTA
961  CTCGGCTAA
```

Diprionin - *Diprion pini* – Annexin B9 like isoform X1 (322 aa)

```
  1  MAPQYYHVQC TPTVYPADPF DAEADATLLR TAMKGFGTDE QAIIDVLGRR GIVQRLEIAE KFKTMYGKDL ISELKSELGG
 81  HFEKAIVALM TPLPELYARE IHDAISGIGT DEGALVEVLA SLSNYGIKTI SAVYKDLYGN ELEDDLKSDT SGHFKRLLVS
161  LSTANRDESP DVDVDAATAD AERLLEAGEG QWGTDESTFN SILITRSYPQ LRKIFQEYER LSGSDLEDTI KKEFSGSIED
241  GYLAVVKCAR DKTGYFAERL HKAMAGMGTD DTTLIRIIVL RSEIDLGDIK EAYEQIYGQS LAGDIDGDCS GDYKRLLLSL
321  LG
```

FIGURE S2 Dipronin full-length nucleotide and amino acid sequence. Sequences from the protein-coding part of the *Diprion pini* annexin B9 like isoform X1 (diprionin) protein identified in this study

Supporting Information Figure S3

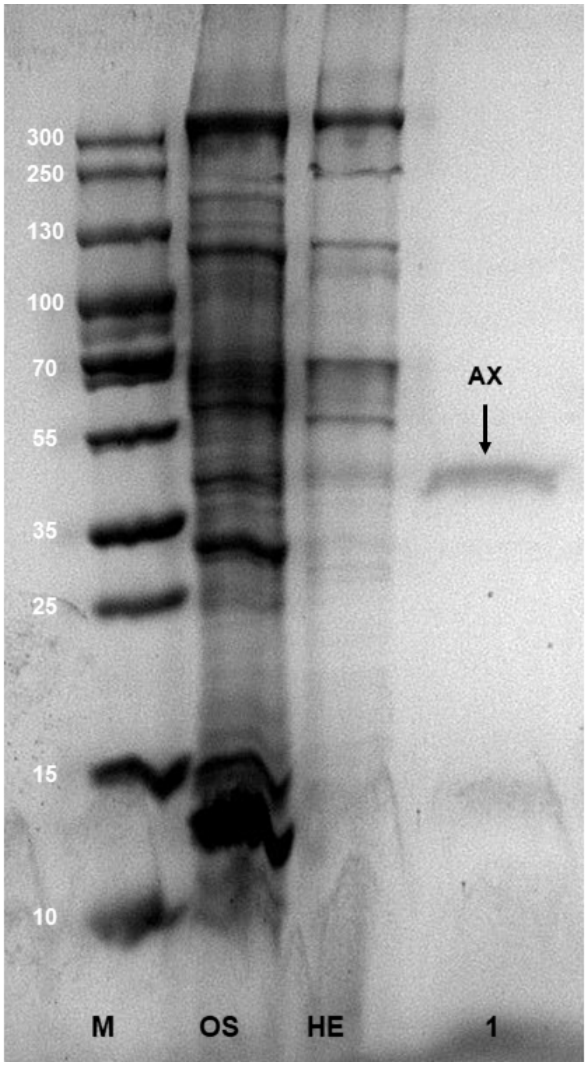

FIGURE S3 SDS-PAGE of purified recombinant annexin (AX) (= diprionin) expressed in Hi-5 insect cell culture. SDS PAGE (4 – 20%) after affinity tag removal and electro-elution from BN-PAGE, which separated diprionin from maltose-binding proteins (= bioassayed heterologously expressed annexin) (compare Fig. 1c and 2b, main text). Coomassie Brilliant Blue staining. Lanes: M, marker; OS, *Diprion pini* oviduct secretion from two females; HE, *D. pini* hemolymph from two females; 1, BN-PAGE analyzed and electro-eluted recombinant protein (AX). White numbers: molecular weight in kDa of marker proteins

Supporting Information Figure S4

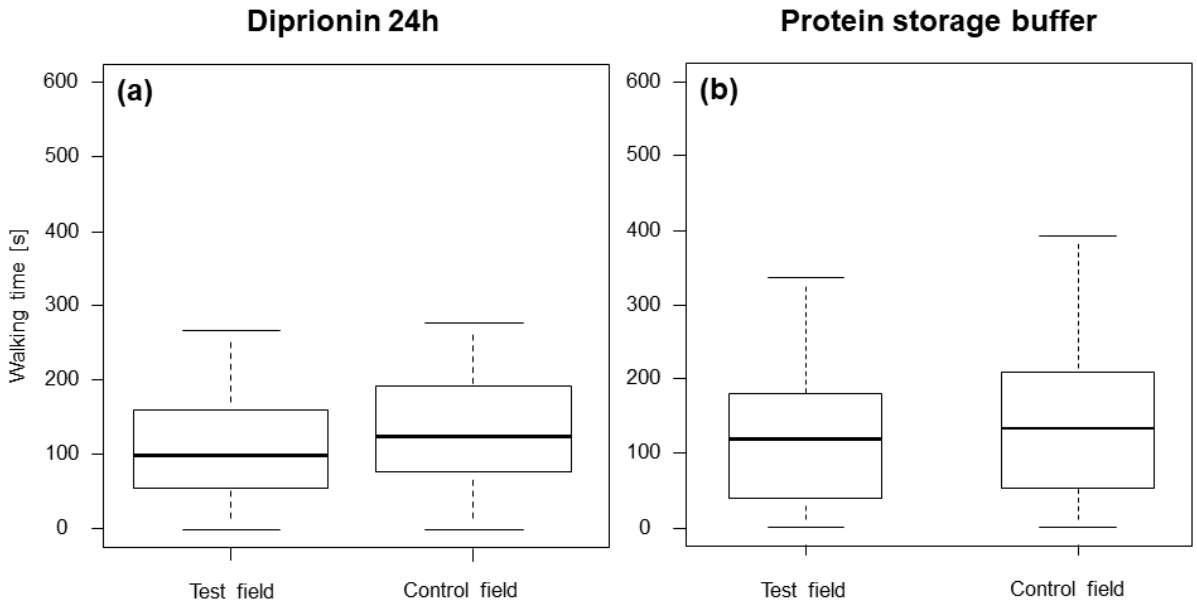

FIGURE S4 Test of elicitor activity of recombinant annexin (diprionin) after storage for 24 h. Behavioral response of egg parasitoids to odor of pine after treatment with stored diprionin. Recombinant diprionin was expressed in Hi-5 insect cell culture, and for each slit needle 250 ng protein was used for the treatment. We treated 8 needles per pine sample. Analysis of behavioral responses of egg parasitoids to pine odors after treatment with stored diprionin. Slit *Pinus sylvestris* needles were treated with **(a)** electro-eluted recombinant diprionin protein after affinity tag removal and BN-PAGE separation and after storage of the sample for 24 h at 4°C in the dark and **(b)** protein storage buffer for control. Time (median, interquartile range, minimum/maximum), which parasitoid females spent in the test field and opposite control field of a four-arm olfactometer during a 10 min (= 600 s) observation period, is shown. The test field was provided with volatiles from pine twigs 72 h after the treatment, the control field contained just charcoal-filtered air. **(a)** n = 28 parasitoids; n = 9 pine samples; **(b)** n = 26 parasitoids; n = 9 pine samples. Statistical differences were evaluated by a two-sided Wilcoxon signed ranks test. No significant differences were detected between walking times in test and opposite control field

Supporting Information Figure S5

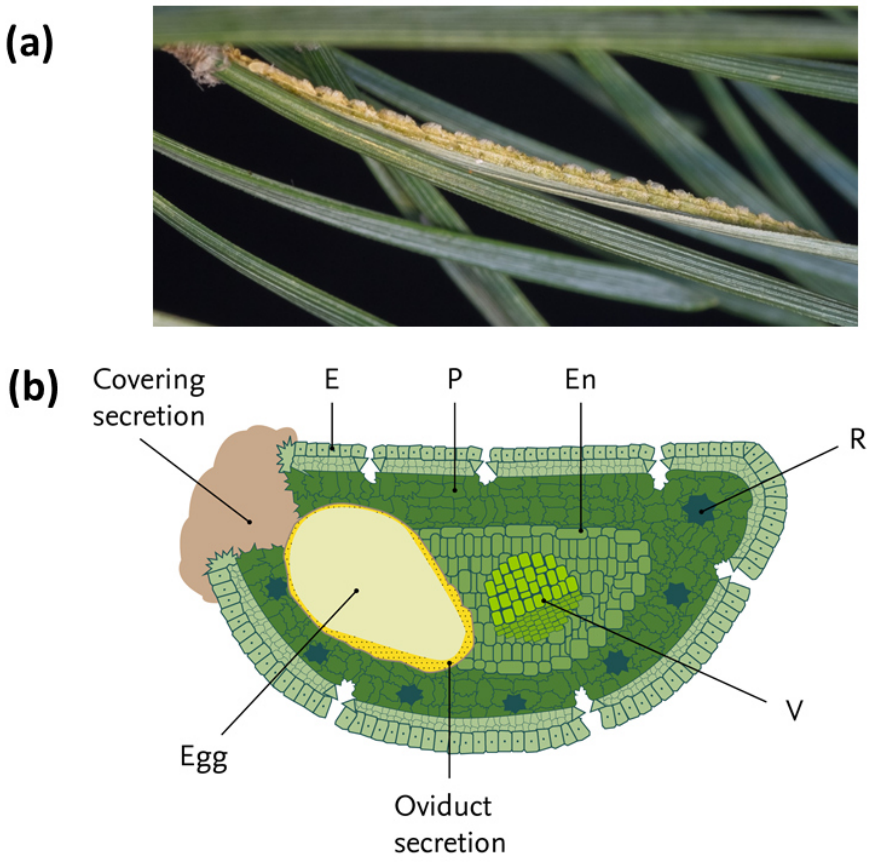

FIGURE S5 (a) Picture of *Diprion pini* egg row on a pine needle and **(b)** schematic illustration of a cross section through a pine needle with *D. pini* egg deposition. The *D. pini* female slits a pine needle longitudinally with her sclerotized ovipositor valves, thereby tearing parts of the needle epidermis, parenchyma and endodermis. Eggs are inserted in a row into the slit needle. Each egg is encased by a secretion, which comes from the *D. pini* oviduct. This egg-encasing oviduct secretion is in immediate contact with the internal pine needle tissue. The oviduct secretion contains diprionin with pine defense elicitor activity. The slit pine needle is covered on top of the slit by a further secretion, the so-called "covering secretion", which comes from an abdominal gland of the *D. pini* female, i.e. the accessory reproductive gland. In contrast to the oviduct secretion, this covering secretion does not elicit emission of parasitoid attracting volatiles (Hilker, Kobs, Varama & Schrank, 2002). Pine needle: E, epidermis; P, parenchyma; En, endodermis; R, resin canal; V, vascular bundle

Supporting Information Table S1

TABLE S1 Primers and their sequences used throughout this study for identification and expression of *Diprion pini* annexin (diprionin)

Primer name	Primer sequence (5‘ -> 3‘)
DPAnnexin1F	TTTCGTGTCTGTCATTCGA
DPAnnexin1R	TCGTTGCCGTAGTTGATGAG
DPAnnexRaceF1	GAGGCAGATGCGACTCTCTTG
3’RACE Outer Primer (ThermoFisher Scientific)	GCGAGCACAGAATTAATACGAC
Ann – ORF – F	ATGGCACCGCAATATTACCA
Ann – ORF – R	TTAGCCGAGTAGACTAAGCA
MBP-EcoRI-F	TACTCAGAATTCATGAAAATAAAAACAGGTGC
Ann-HindIII-R	TACTCAAAGCTTTTAGCCGAGTAGACTAAGCA
MBP-Ann-R	ATATTGCGGTGCCATCCTTCCCTCGATCCCGAGGT
MBP-Ann-F	GGGATCGAGGGAAGGATGGCACCGCAATATTACCAC

Supporting Information Table S2

TABLE S2 Pine gene expression analysis: Search information and sequences of primers used for qPCR analyses

Gene	Template for primer design	Species	Species for BLAST search		Primer sequence (5‘ >3‘)	Name and function
cATP	GW765967 [1]	*Pinus banksiana*	*Ginkgo biloba* (EU071049)	F R	GGGTCGGTCAAGTCGTCAGC GCACGGAAATGGGTTCTTTGC	Chloroplast ATPase beta subunit Housekeeping gene
PETB	CV035597 [1]	*Pinus taeda*	*Populus euphratica* (XM011050173)	F R	ACCATCATACTTGCCGACCATC TCGTCCGACCGTTACAGAAGC	Cytochrome subunit 6 Housekeeping gene
UBI	HE629096 [1]	*Pinus sylvestris*	*Picea abies* (EF681766)	F R	ACTTTACCAGAGTCATCAACC GGTTCTTCGTCTGAGAGGTG	Ubiquitin Housekeeping gene
GPP2	Schmidt & Gershenzon, 2008 *	*Picea abies*		F R	GTTGTTGTCACATAGACTTCTGC CTGTTCAGACAGATCAGCTAG	Geranyl pyrophosphate 2 Monoterpene synthase
GPP3	Schmidt & Gershenzon, 2008 *	*Picea abies*		F R	GATGATTCTTACCGAGATTCC CTTTTGCCCTCCACTCC	Geranyl pyrophosphate 3 Monoterpene synthase
FPP	Schmidt & Gershenzon, 2008 *	*Picea abies*		F R	GTCTGTAATAGACAGCTACAGG CCAGCCAAGCACACATCC	Farnesyl pyrophosphate Sesquiterpene synthase
TPS5	GU248335 [2]	*Pinus sylvestris*		F R	GAAGGCGTGTTCTCACAGAGC TGGACGCCAATTCTCCACGAG	Terpene synthase 5 (*E*)-β-farnesene synthase
RbohA	GILN010446779 [3]	*Pinus. sylvestris*	*Arabidopsis thaliana* (O81209)	F R	CACTCGATTTCACTCGCAAA GCAACCCAAACACATGACAG	Respiratory burst oxidase homolog A ROS production
SOD	X58578 [2]	*Pinus sylvestris*		F R	GCTGATGTCAAGGGGGTTGT ACCATGCTCCTTGCCTAACG	Superoxide dismutase
APX	AY485994 [2]	*Pinus pinaster*		F R	TCTGGTTTTGAAGGACCATG AAACTAGGATCAGCCAGCAG	Ascorbate peroxidase ROS scavenger

CAT	AL751103 [1]	*Pinus pinaster*		F R	AAGGGCTTTTTCGAGGTGAC GGAATTACCTGCATGGCATC	Catalase ROS scavenger
PR1	HE627106 [1]	*Pinus sylvestris*	*Arabidopsis thaliana* (NM127025)	F R	TCGTCAACGTACACAGATGTTG ACTACGATCCGCCTGGGAAC	Pathogenesis related 1 SA signaling
PR2	GHKY01019355 [3]	*Pinus sylvestris*	*Arabidopsis thaliana* (P33157)	F R	ATCTTGTTCCTGCCATGAGG GGGAGACCCGTGATCTAACA	Pathogenesis related 2 SA signaling
PR5	GILN010589346 [3]	*Pinus sylvestris*	*Arabidopsis thaliana* (P28493)	F R	CAACGGCAACAAGGATTTCT AAACTTGAACGCATCACACG	Pathogenesis related 5 SA signaling
PAL	AF353967 [2]	*Pinus sylvestris*		F R	CTGGCAGCGATCCACTGAAC CTTCGAGCAACGGCAGCAAC	Phenylalanine ammonia lyase Phenylpropanoid pathway
CDPK1	Hu *et al.,* 2014 *	*Pinus massoniana*		F R	GGAAGTCGTTCAGCTCTGCCACAAA GCGATCCCCAGGTTTGAAGAATACA	Calcium dependent protein kinase 1 Ca^{2+} signaling
CAX3	GILP01417777 [3]	*Pinus sylvestris*	*Arabidopsis thaliana* (Q93Z81)	F R	TATGGGTTCTGCCACACAGA GCAGCAGCACTAAACCCTTC	Cation exchanger 3 Ca^{2+} signaling

[1] Nucleotide [mRNA]. Database: Expressed Sequence Tags (EST).

[2] Nucleotide [mRNA]. Database: Plant and Fungal sequences (PLN).

[3] Nucleotide [mRNA]. Database: Transcriptome Shotgun Assembly (TSA).

All available from: National Library of Medicine (US). National Center for Biotechnology Information. – https://www.ncbi.nlm.nih.gov/

* please see section “References” here in the Supporting Information

Supporting Information Table S3

Table S3 Transcript levels of genes in untreated control pine and in artificially wounded and protein buffer-treated pine. Transcript quantity in both treatments was first calculated relative to untreated control followed by normalization of the expression to the housekeeping genes by ΔΔ C(t) method as described by Pfaffl (2001) and Vandesompele et al. (2002). Shown is the gene expression in artificially wounded (slit), buffer-treated pine needles relative to expression levels in untreated controls as $\log_2$ fold-change. Data show means ± SE. *n* = 7-8 pine samples. Expression levels were determined 72 h after treatment. *P*-values: pairwise comparison of untreated and buffer-treated pine samples by Mann-Whitney *U*-test. Bold: significantly increased transcript abundance compared to untreated control

Gene	Untreated control	Artificially wounded + protein storage buffer	*P*-value
GPP2	0.00 ± 0.47	-0.43 ± 0.13	0.495
GPP3	0.00 ± 0.33	-0.32 ± 0.26	0.372
FPP	0.00 ± 0.48	-0.93 ± 0.30	0.270
TPS5	0.00 ± 0.33	-0.44 ± 0.23	0.875
RbohA	0.00 ± 0.42	-0.82 ± 0.32	0.104
SOD	0.00 ± 0.09	0.89 ± 0.14	**0.003**
APX	0.00 ± 0.23	1.91 ± 0.18	**0.001**
CAT	0.00 ± 0.18	0.38 ± 0.22	0.270
PR1	0.00 ± 0.60	2.04 ± 0.36	**0.041**
PR2	0.00 ± 0.95	3.86 ± 0.35	**0.031**
PR5	0.00 ± 0.90	6.08 ± 0.35	**< 0.001**
PAL	0.00 ± 0.75	2.30 ± 0.45	0.066
CAX3	0.00 ± 0.43	1.42 ± 0.28	**0.041**
CDPK1	0.00 ± 0.21	1.12 ± 0.19	**0.007**

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CHAPTER 5

GENERAL DISCUSSION

5. GENERAL DISCUSSION

This thesis expands and deepens the knowledge about defense responses of Scots pine (*Pinus sylvestris*) against insect eggs of the hymenopteran sawfly *Diprion pini.* While the mechanisms of plant responses to insect eggs have so far mainly been studied in angiosperm species responding to lepidopteran and coleopteran eggs, this thesis contributes to our understanding of how a gymnosperm species responds to the egg deposition by a hymenopteran species, and how these responses to the initial step of infestation shape further responses to larval feeding.

Three major questions were addressed in this thesis:

- First, the study described in chapter 2 investigated whether *P. sylvestris* amplifies its hydrogen peroxide accumulation as an improved defense response to insect eggs after previous exposure to a "warning" of impending infestation, i.e. to the sex pheromones of *D. pini*.
- The second main question addressed in chapter 3 was about how the transcriptome of Scots pine responds to oviposition and subsequent larval feeding of the sawfly; furthermore, it was studied how pine changes its phytohormone concentrations when responding to insect oviposition and subsequent larval feeding.
- The third main question addressed in chapter 4 was about Scots pine perception of the sawfly eggs. Previous studies pointed to a proteinaceous elicitor associated with the sawfly eggs that induces pine defense responses (Hilker et al., 2005). This elicitor was identified as an annexin-like protein named diprionin (Bittner, 2018). The question was if a heterologously expressed version of diprionin induces similar changes in transcript levels of defense-related pine genes as *D. pini* egg deposition does.

The results described in chapter 2 showed that pine trees, which had previously been exposed to *D. pini* sex pheromones, enhanced their defensive responses to the eggs of this sawfly; the lower egg survival rate on pheromone-exposed pine than on non-exposed pine was associated with amplified hydrogen peroxide accumulation in pheromone-exposed, egg-laden pine. This accumulation might elicit a direct hypersensitive-like defense response which is killing the eggs, as shown before (Bittner et al., 2017). In addition, it might indicate a ROS-mediated signaling cascade leading to improved defense of pheromone-exposed pine against the eggs. However, pheromone-mediated, enhanced ROS accumulation in egg-laden pine needles was not rapidly activated, but at a very late stage of egg development, i.e. shortly prior to larval hatching. Hence, the exposure of pine to sawfly pheromones might not only improve pine defenses against the eggs, but might also act against hatching larvae. This pheromone-mediated priming effect may be of high importance to the plant since this very early initiated defense reduces the egg survival rate and might thereby lead to significantly less feeding damage caused by hatching larvae. Gene expression analyses supported the involvement of ROS in the

enhanced defense response, but also suggested the involvement of phenylpropanoids in the response of pheromone-exposed pine to sawfly eggs, as indicated by a significantly increased expression level of *PsPAL (phenylalanine ammonia lyase)*. It is well known that plant responses to cues indicating impending insect infestation can prime plant responses to insect herbivory. Exposure of plants to herbivory-induced leaf volatiles (Arimura et al., 2000; War et al., 2011; Pérez-Hedo et al., 2021; Qian et al., 2024), to oviposition-induced leaf volatiles (Hilker & Meiners, 2002; Reymond, 2013; Hilker & Fatouros, 2015) and even to insect volatiles (Helms et al., 2013; Helms et al., 2017) is known to prime plant defenses against feeding insects. The study described in chapter 2 study is the first demonstrating that exposure of a plant to insect sex pheromones improves a plant's direct defenses against insect eggs.

Chapter 3 shows that there are several similarities of pine defense responses to sawfly oviposition with those to larval feeding. When comparing pine responses to sawfly eggs and larval feeding with the responses of angiosperms, many similarities were detected (compare references chapter 3). Both pine and the so far studied angiosperms show reduced photosynthesis activity in response to insect egg deposition. Both egg-laden pine and egg-laden angiosperm species show a modification of their cell wall, accumulation of secondary metabolites (especially phenylpropanoids) and significant changes in concentrations of salicylic acid in response to larval feeding when compared to egg-free, feeding-damaged plants. Although these species separated phylogenetically a long time ago, they still have a common origin, leading to the suggestion of phylogenetically conserved defense traits against insect eggs in gymnosperm and angiosperm species. Additionally, both in pine and angiosperms, the responses to insect eggs show similarities to responses to larval feeding. Again, these results support the suggestion of a phylogenetically conserved defense response. However, when considering the number of differentially expressed genes in response to insect eggs and subsequent larval feeding, egg-laden pine showed a weaker transcriptomic response to larval feeding than egg-free pine, whereas all comparable studies with angiosperms showed the opposite. Thus, the findings of chapter 3 indicate for the first time that also an insect egg-mediated attenuation of the plant’s transcriptomic response to larval feeding can result in improved defense against insect larval herbivory.

Chapter 4 describes the isolation, sequence and structure analysis of diprionin, which was identified as the proteinaceous defense elicitor compound from the secretion associated with *D. pini* eggs (Bittner, 2018). Diprionin was the first identified egg-associated proteinaceous plant defense elicitor mediating plant defense responses against insect eggs. Only one more proteinaceous egg-associated plant defense elicitor has been identified so far, which is an N-terminal subunit of vitellogenin coming from planthopper eggs and egg fluids, but also from the planthopper's saliva (Zeng et al., 2023). This elicitor also induces plant responses that reduce egg survival. The chapter 4 studies here showed that the

heterologously expressed version of diprionin elicited several pine defense responses similar to the responses to natural egg deposition. For example, application of the heterologously expressed diprionin on pine needles caused a significantly enhanced emission of (*E*)-β-farnesene, a terpene that is induced also by *D. pini* egg deposition (Mumm et al., 2003). The egg-induced emission of (*E*)-β-farnesene is known to attract the egg parasitoid *Closterocerus ruforum* (Hilker et al., 2002). Similarly, odor of diprionin treated pine also attracted this parasitoid species, which is killing the sawfly eggs (Bittner, 2018). Besides this indirect defense, diprionin treated pine trees showed a change in the expression of some ROS related and terpene biosynthesis related genes, i.e. genes that may be involved in direct pine defense responses. In most cases, the change in gene expressions was similar after natural egg deposition and treatment with heterologously expressed diprionin, which is another indicator for diprionin to be an important egg-associated defense elicitor. However, not all tested genes responded similarly to the diprionin treatment and to natural egg deposition, suggesting that further factors, such as environmental cues, interaction with other egg-associated compounds or additional elicitors, are necessary to induce the plant's full defense response against the insect eggs.

Here in this chapter 5, I will discuss the plant's advantage of defending against insect eggs as an early stage of infestation. Furthermore, in search for general plant responses to environmental cues warning of impending infestation, I will compare the sex pheromone-mediated pine defense against the insect eggs with the egg-mediated defense of pine against insect larvae. In addition, I will provide an in-depth discussion of the role of jasmonic acid (JA) and salicylic acid (SA) in pine responses to insect eggs and larvae. Moreover, I will discuss how the elicitor associated with *D. pini* eggs – diprionin – might be perceived by pine needles and will embed this discussion in the current knowledge about insect-associated elicitors of plant defenses. In a final subchapter, I will address similarities of pine transcriptional responses to *D. pini* eggs and to larval feeding, thereby highlighting the common differentially expressed genes in response to these two infestation stages.

5.1 The advantages of plant defense responses to early stages of insect infestation

Most studies focus on plant defenses against the feeding stages of insect herbivores (Bonaventure, 2012; War et al., 2012; Fürstenberg-Hägg et al., 2013; War et al., 2018; Erb & Reymond, 2019; Wang et al., 2023). But plants can raise their defenses against an earlier stage of infestation, the insects egg deposition, as shown in numerous studies (Hilker & Meiners, 2006, 2011; Hilker & Fatouros, 2015).

Chapter 3 of this thesis demonstrated that the intensity of pine responses to insect eggs is comparable to the response to feeding, at least on the transcript level. Many genes related to known defense mechanisms against feeding insects, i.e. cell wall modification, cell death and accumulation of

secondary defense metabolites like phenylpropanoids, were clearly regulated also in response to insect egg deposition. Defense against the early stage of infestation – the insect eggs – offers the chance to reduce the number of hatching larvae, thereby possibly lowering the upcoming feeding damage.

As shown in chapter 2, the survival rate of *D. pini* eggs on the *P. sylvestris* trees tested here was just 60%, although egg parasitoids and predators were excluded and the abiotic conditions were most convenient. This low survival rate may be due to the defense responses of the plant, but also insect intrinsic factors (e.g. quality of egg deposition, immune state) might have contributed to this result. A 60% egg survival rate is expected to result in less plant tissue loss caused by larval feeding than a 100% survival rate. Since the results described in chapter 2 showed that even fewer insect eggs than 60% survived on previously pheromone-exposed trees, cues "warning" of impending egg deposition obviously can improve pine defenses against eggs. The gene expression analyses described in chapter 2 showed a significant upregulation of *PsRboh,* a sequence homologue to a respiratory burst oxidase – plant NADPH oxidase, just in response to the pheromone exposure without actual egg deposition. This upregulation might contribute to intensified ROS signaling and an upregulated direct defense state already prior to the egg deposition. Thus, the eggs probably face intensified pine defense as soon as having been laid into the pine needle. However, it remains unclear so far how this response to the pheromone exposure might contribute to enhanced direct defense against the eggs because *PsRboh* was no longer upregulated two and twelve days after egg deposition on pheromone-exposed trees. Here, the question comes up whether the eggs are associated with compounds that can tune down upregulation of *PsRboh.*

It would be interesting to address in future studies the question whether the exposure of plants to insect pheromones also affects the oviposition behavior of insect females. It is well known that plants can respond to volatiles released by other plants (stressed and unstressed) (Das et al., 2013; Kalske et al., 2019; Ninkovic et al., 2021). Helms et al. (2013) were able to show that *Solidago altissima* plants received less ovipunctures by a gallfly when having been previously exposed to a volatile released by male gallflies and attractive to gallfly females. If *P. sylvestris* is capable of repelling females of *D. pini* from egg deposition after exposure of the trees to the sex pheromones, the tree could reduce the number of sawfly egg depositions; thereby, the plant would not only reduce later larval feeding damage, but also save resources that might be needed for more efficient defense against those egg depositions that could not be prevented. A plant's response to insect sex pheromones that results in fewer insect egg deposition on the pheromone-exposed plant would be a very early preventive defense strategy. Future studies on the insect's oviposition behavior towards pheromone-exposed plants as well as further gene expression and metabolite analyses of pine responses to insect pheromones could give a deeper insight into this phenomenon.

5.2 Similarities and differences in pheromone-mediated and egg-mediated effects on pine defenses against sawfly infestation

The study described in chapter 3 compares the transcriptomic responses of egg-laden pine to sawfly larval feeding damage with those of egg-free pine to feeding damage. This transcriptome analyses revealed that pine defenses against larval feeding were clearly shaped by the plant's responses to preceding egg deposition. The study described in chapter 2 showed that pine defenses against insect eggs were significantly affected by the plant's responses to preceding exposure of the plant to the insect's pheromones.

These findings give rise to the question whether pine responses to a cue indicating impending stress show similarities independent of the type of the cue (here: pheromone or eggs) and whether defensive responses that have been prepared by a response to a "warning" cue show similarities independent of the defense target (here: sawfly eggs or larvae). Thus, this subchapter addresses the questions:

(i) Do the pheromone-induced pine responses show similarities to egg-induced responses?

(ii) How (dis)similar are the pheromone-mediated responses to egg deposition when compared to egg-mediated responses to larval feeding?

Both questions will be considered with a focus on phenylpropanoids, ROS, and the phytohormones JA and SA.

Question (i) with respect to phenylpropanoids. Phenylalanine ammonia lyase encodes an important enzyme that catalyzes the biosynthesis of a precursor of a wide range of phenylpropanoids that can act as defensive compounds against herbivorous insects (Dixon et al., 2002; Rehman et al., 2012; Ramaroson et al., 2022). When comparing pine transcriptional responses to pheromone exposure and those to egg deposition, expression of *PsPAL (phenylalanine ammonia lyase)* was not induced by the pheromone exposure (chapter 2). Egg deposition induced the expression of the analyzed *PsPAL* sequence by trend (chapter 2). Furthermore, the RNAseq analysis described in chapter 3 revealed that egg deposition induced the expression of several phenylalanine ammonia lyase homologues even significantly at different stages of egg development (chapter 3, Suppl. Table 5). Thus, the results of this thesis indicate that the "warning" cues "pheromone" and "eggs" exert different effects on the expression of phenylalanine ammonia lyases.

Question (ii) with respect to phenylpropanoids. Interestingly, expression of *PsPAL* was found to be significantly enhanced in pheromone-exposed, egg-laden pine trees compared to just egg-laden trees without prior pheromone exposure. This finding indicates that the pine response to sawfly pheromones shapes the *PsPAL* expression in response to subsequent egg deposition, although the pheromone

exposure *per se* had no effect on *PsPAL* expression. It remains an open question how this works. The phenylpropanoid pathway of a plant that is responding to insect infestation is not only affected by prior pheromone exposure. In several plant species (*Arabidopsis,* tobacco, bittersweet nightshade, elm, pine), also insect egg deposition was shown to affect the phenylpropanoid pathway in response to subsequent feeding damage (Bandoly et al., 2015; Geuss et al., 2018; Lortzing et al., 2019; Schott et al., 2022) as also found in chapter 3. Hence, regardless of the type of the "warning" cues (pheromone or eggs) and of the type of infestation (eggs or larvae), the phenylpropanoid pathway was found to be affected when considering the enhanced expression levels of *PAL* and/or enhanced levels of certain phenylpropanoid metabolites in previously "warned" and subsequently infested plants. While the defensive function of egg-mediated enhanced concentrations of phenylpropanoids against feeding insects is well known (e.g. Lattanzio et al., 2008; Austel et al., 2016), the role of eventually pheromone-mediated enhanced phenylpropanoid levels in plant responses to insect eggs remains to be studied. Lignification of pine needle tissue that is generated via the phenylpropanoid pathway might result in egg desiccation or affect the very young neonates with their soft and tiny mouthparts.

Question (i) with respect to ROS. Pine transcriptional responses of ROS related genes to pheromones and eggs differed, as was shown by the study described in chapter 2. Hence, ROS-mediated pine responses to these "warning" cues are dependent of the type of cue.

Question (ii) with respect to ROS. For enhanced pine defense against sawfly eggs after pheromone exposure, hydrogen peroxide seems to play a key role. This is corroborated by the enhanced expression of *SOD* (chapter 2), but also by the enhanced level of hydrogen peroxide itself in pheromone-exposed, egg-laden pine needles when compared to non-exposed, egg-laden needles (chapter 2). ROS in general is involved in mediation of programed cell death and hypersensitive-like responses to insect infestation (Lamb & Dixon, 1997; Bittner et al., 2017; Balint-Kurti, 2019; Noman et al., 2020; Caarls et al., 2023). These mechanisms also play a role in defense against feeding larvae, as shown by enriched ROS related GO terms and gene expressions in just feeding-damaged, but also egg laden and feeding-damaged needles (chapter 3, Suppl. Table 5 and 6). However, no enhanced enrichment of ROS related GO terms was found in feeding-damaged pine trees with prior egg deposition compared to just feeding-damaged trees. This suggests that the role of ROS is more relevant in pheromone-mediated, direct defense against the eggs than in egg-mediated defense against feeding larvae. It is unknown so far whether the pheromone-mediated, ROS related effects on pine responses to eggs also play a role in indirect pine defenses against the eggs. It is well known that attraction of an egg parasitoid by egg-induced needle volatiles is an important indirect defense mechanism of *P. sylvestris* (Hilker et al., 2002). Future studies need to show whether this egg-induced, indirect defense can even be improved and result in more efficient attraction of egg parasitoids by prior exposure of the plant to sawfly pheromones.

Question (i) with respect to JA. One of the initial steps of the biosynthesis of the phytohormone JA is catalyzed by lipoxygenases (LOX). According to the analyses described in chapter 2, expression of a *PsLOX* was not induced by pheromone exposure of pine and only very slightly, but not significantly by sawfly egg deposition. However, the RNAseq analyses described in chapter 3 revealed that the expression of several homologues of lipoxygenases increased considerably in response to sawfly egg deposition. These finding suggest that the "warning" cues "pheromone" and "eggs" elicit different responses in pine with respect to the expression of genes encoding lipoxygenases.

Question (ii) with respect to JA. However, the phytohormone JA seems to be involved in both, pheromone-mediated effects on pine defense against eggs and egg-mediated effects on pine defenses against larvae. The expression of *PsLOX* was significantly enhanced in egg-laden pine trees with prior pheromone exposure (chapter 2). The JA concentrations were not measured in pheromone-exposed, egg-laden pine. Feeding-damaged plants with prior egg deposition had slightly more JA related GO terms enriched with upregulated genes than just feeding-damaged plants (chapter 3). Furthermore, briefly (1 h) after the onset of larval feeding, JA concentrations in egg-laden, feeding-damaged pine were significantly higher than in untreated pine, whereas JA concentrations in egg-free, feeding-damaged pine increased only by trend when compared to untreated controls. Hence, the results suggest that the previous cues "pheromone" and "eggs" can both fortify the JA response to subsequent infestation steps, the eggs and larval feeding, respectively.

Question (i) with respect to SA. While the phytohormone SA is predominantly biosynthesized via the isochorismate pathway in *Arabidopsis,* biosynthesis of SA via *PAL* might be relevant as well in other plants (Chaman et al., 2003; Chen et al., 2009; Dempsey et al., 2011; Lefevere et al., 2020). Only one pine gene homologous to an isochorismate synthase (*ICS2*) responded to pine sawfly egg deposition by trend (not significantly) (chapter 3, Suppl. Table 5). The study described in chapter 2 revealed that the expression of the studied *PsPAL* sequence was neither affected by pheromone exposure *per se* nor by egg deposition *per se*. However, as mentioned above for phenylalanine ammonia lyases, the chapter 3 study revealed that several homologues of *PAL* showed increased expression in egg-laden pine, while the SA concentrations in egg-laden pine were enhanced only by trend. Thus, although the "warning" cues "pheromone" and "eggs" exert different effects on the expression of phenylalanine ammonia lyases, hints on their different effects on pine SA concentrations are lacking so far.

Question (ii) with respect to SA. When focusing on SA and comparing the (dis)similarity of pheromone-mediated responses to egg deposition with the egg-mediated responses to larval feeding, egg-laden pine trees with prior pheromone exposure showed significantly enhanced expression of *PAL* when compared to non-exposed, egg-laden pine (chapter 2). Feeding-damaged pine trees with prior egg deposition produced a significantly enhanced SA concentration when compared to untreated control

pine, whereas egg-free, feeding-damaged pine did not show such an increase. Furthermore, feeding-damaged pine with prior egg deposition showed enhanced expression of some phenylalanine ammonia lyase homologues when compared to control pine (chapter 3, Suppl. Table 5). Interestingly, the expression of an *ICS2* gene was significantly reduced in 24 h feeding-damaged pine with prior egg deposition. If the enhanced expression of *PsPAL* in pheromone-exposed, egg-laden pine is indeed linked with enhanced SA biosynthesis, the "warned" responses of pine to eggs and larvae involve SA signaling independent from the warning cue and defense target. Measurements of SA concentrations are needed to investigate this suggestion.

Taken together, pine responses to the warning cues "sawfly pheromone" and "sawfly eggs" differ with respect to the phenylpropanoid pathway, ROS signaling and phytohormonal responses. However, the studies in chapter 2 and 3 provide some hints that pheromone-mediated pine responses to eggs and egg-mediated responses to larvae show some similarities, indicating that "warning" of impending infestation might rely on some general mechanisms that are independent of the "warning" cue and the actual defense target (eggs or larvae).

5.3 The role of jasmonic acid and salicylic acid in pine defense against insect infestation

The phytohormones JA and SA are well known to play a significant role in plant defenses against insect herbivores (Smith et al., 2009; War et al., 2012; Fürstenberg-Hägg et al., 2013). The results shown in chapter 3 provide for the first time information on how *P. sylvestris* changes its JA and SA concentrations in response to sawfly egg deposition and larval feeding damage. Here, I will first compare the detected pine JA and SA responses to *D. pini* eggs and larvae with the known responses of other plants to these steps of insect infestation. Finally, pine egg-mediated JA and SA responses to larval feeding damage will be compared to those of other plant species to this sequence of insect infestation.

JA plays a special role in the defense responses of *P. sylvestris* to *D. pini* egg deposition and to larval (chapter 3). Especially when looking at the GO terms of the pine transcriptome analysis, none of the other phytohormone related genes showed a response intensity comparable to the response of JA related genes to both stages of infestation, the eggs and the larvae. A clear JA related response to feeding larvae was expected since the importance of JA signaling in response to plant wounding has been shown in many other plant species (Farmer et al., 2003; Dicke & Baldwin, 2010; Frago et al., 2012; Meldau et al., 2012; Wasternack, 2015). However, JA concentrations of pine needles also increased significantly in response to *D. pini* egg deposition. This seems surprising at a first glance because most plants have been demonstrated to respond to egg deposition with an SA burst instead of JA (Bruessow et al., 2010; Hilker & Fatouros, 2015; Bonnet et al., 2017; Geuss et al., 2017; Lortzing et al., 2019). A JA burst is often just a simple wound response in plants (Howe, 2004; Wasternack et al., 2006; Ikeuchi et

al., 2020). The severe wounding that *D. pini* inflicts to pine needles during oviposition probably contributes to the strong pine JA response to egg deposition of this sawfly. However, an increase in the concentration of JA isoleucine has also been detected in response to egg deposition by *Pieris brassicae* on *A. thaliana* (Valsamakis et al., 2020). Like other lepidopteran species, *P. brassicae* does not damage the leaf when sticking the eggs to the leaf surface. Since plants are well known to respond just to touch by JA signaling (e.g. (Chehab et al., 2012; T.-H. Yang et al., 2023), the leaf response to insect eggs might also be due to such mechanostimulation. In *D. pini,* a proteinaceous compound associated with the eggs was identified and shown to induce indirect pine defense against the eggs (chapter 4). The question arises whether wounding alone would cause a JA accumulation similar to the natural egg deposition by *D. pini* (chapter 3), or whether the elicitor would be necessary to cause a comparable response. A comparative phytohormone analysis of only wounded and wounded plus diprionin treated needles could unravel if this JA accumulation is just a general wound response, or if it is a specific plant response to *D. pini* egg deposition.

SA concentrations increased only by trend in egg-laden pine in the end of the egg incubation time. A significant increase in SA concentrations was detected in *A. thaliana* in response to *P. brassicae* egg deposition (Bruessow et al., 2010; Lortzing et al., 2019; Valsamakis et al., 2020). Upregulation of SA related defense genes in response to *P. brassicae* egg deposition was also found in *Brassica nigra* (Caarls et al., 2023); this brassicaceous plant species also showed an increase in SA concentration in response to an egg extract of *P. brassicae* (Bonnet et al., 2017). Pine did not show an increase in SA concentration in response to larval feeding. However, SA responses to larval feeding damage have been described so far at least for a few plant species responding to herbivory. For example, cotton (Bi et al., 1997), tomato (Peng et al., 2004), and tobacco plants (Heidel & Baldwin, 2004) showed SA accumulation in response to feeding damage by lepidopteran larvae. Microbes released from the insect's mouth into the plant wound might contribute to such SA related plant responses to larval feeding (Yamasaki et al., 2021).

When looking at the egg-mediated pine defense response to larvae after 24 h of feeding, the difference in JA concentrations between egg-laden and subsequently feeding-damaged trees and egg-free, feeding-damaged trees vanished. The strong JA response of pine to the *D. pini* larval feeding damage seems to overwrite the previous, moderate JA response to the eggs. However, the egg-laden, feeding-damaged trees showed a higher SA content after a 24 h larval feeding period than the egg-free, feeding-damaged pine trees. Such an SA accumulation that is higher in egg-laden, feeding-damaged plants than in egg-free, feeding-damaged ones was also shown for other plant species than pine. For example, *A. thaliana* laden with *P. brassicae* eggs and damaged by larvae of this species (Lortzing et al., 2019; Valsamakis et al., 2020), *B. nigra* treated with *P. brassicae* egg extract and larval feeding damage, elm laden with elm leaf beetle eggs and larval feeding (Schott et al., 2022), all these angiosperm species

also showed SA accumulation in response to these sequential insect infestation steps. Furthermore, they also all showed – like pine – improved defense against feeding larvae if they had received egg depositions prior to the feeding damage (Beyaert et al., 2012; Geiselhardt et al., 2013; Austel et al., 2016; Bonnet et al., 2017).

Taken together, both JA and SA signaling seem to be relevant for efficient plant defenses against eggs, larvae and the infestation sequence eggs and subsequent larval feeding. In addition, other phytohormones like ethylene, abscisic acid, auxin, cytokinins may interact with the JA and SA signaling paths when a plant is exposed to insect infestation (Wu & Baldwin, 2010; Erb et al., 2012; Nguyen et al., 2016). While JA and SA signaling has often been shown to result in an antagonistic interaction (Niki et al., 1998; Cipollini et al., 2004; Mur et al., 2006; Smith et al., 2009), the JA- and SA-mediated responses of both angiosperms and gymnosperms to insect eggs and subsequent larval feeding obviously can result in a positive ecological effect, i.e. the improved defense of an egg-laden plant against larval feeding damage. This positive interaction might be dependent on the phytohormone concentrations and the dynamics of changes in phytohormone concentrations (Lortzing et al., 2019; Zhang et al., 2020; Aerts et al., 2021; Ullah et al., 2022).

5.4 Insect-associated elicitors of plant defenses and the *Diprion pini* egg-associated elicitor diprionin

Plant responses to insect eggs and feeding insect herbivores are elicited by several cues, among them by touch by the attacker (e.g. (T.-H. Yang et al., 2023), by the insect's footsteps (Bown et al., 2002), by wounding- and damage-associated molecular patterns (Howe & Schaller, 2008; Tanaka & Heil, 2021) as well as by compounds – so called elicitors – released or associated with the infesting insect stage (Hilker & Meiners, 2010; Jones et al., 2022). Various chemical structures of such elicitors are known. Thus, the chemical identity and possibly also the released quantity of the elicitor provide information to the plant about the type of attacker (Mithöfer & Boland, 2008; Bonaventure et al., 2011; Snoeck et al., 2022).

Most of the known elicitors of plant antiherbivore defenses are associated to insect feeding and were found in oral secretion, regurgitates, feces or honeydew of the feeding insect (Acevedo et al., 2015; Wari et al., 2019). The known elicitors of plant defense against leaf chewing insects belong to e.g., fatty acid - amino acid conjugates (FACs), sulfated alpha-hydroxy fatty acids like caeliferins, enzymes like β-glucosidase and peptides like inceptin (Hilker & Meiners, 2010; Jones et al., 2022). In piercing or sucking insects, a mucin-like salivary protein as well as compounds from honeydew (possibly microbially produced ones) were detected as elicitors of plant defenses (Shangguan et al., 2018; Wari et al., 2019).

In a spider mite, peptides belonging to the tetranins were found to be putative plant defense elicitors (Iida et al., 2019).

Known oviposition-associated elicitors of plant defenses against insect eggs are benzyl cyanide and indole released with the eggs of pierid butterflies (Fatouros et al., 2009), amphiphilic compounds like the so called bruchins isolated from bruchid beetle females (long-chain α,ω-diols mono- or di-esterified with 3-hydroxypropanoic acid) (Doss et al., 2000), and various phospholipids associated with oviposition of lepidopteran, hymenopteran, coleopteran and hemipteran insect species (J.-O. Yang et al., 2014; Hilker & Fatouros, 2015; Stahl et al., 2020). It remains an open question so far whether the composition of the different phospholipids in the different insect species provide species specificity and enables a plant to respond specifically to the eggs of the attacking insect species.

Proteinaceous oviposition-associated elicitors were only found very recently. An N-terminal subunit of vitellogenin (VgN) associated with the eggs, egg fluids and saliva of *Nilaparvata lugens,* a plant hopper that infests rice plants, was found to be both a feeding and oviposition-associated elicitor of plant defense (Zeng et al., 2023). Thus, this compound is not specific for the stage of infestation. Since VgN of other planthoppers was found to also elicit rice defense responses, this elicitor is not species specific.

Prior to the discovery of the oviposition asscociated, proteinaceous elicitor VgN, we identified the only other known proteinaceous oviposition-associated defense elicitor (chapter 4). Diprionin was isolated from *D. pini* oviduct secretion, which is encasing the eggs. It belongs to the protein superfamily of annexins, which are calcium dependent, phospholipid binding proteins. In insects, annexins can maintain the integrity of tissue upon stretching (Kotsyfakis et al., 2005), which might be an explanation for the diprionin involvement during oviposition and why this protein is present on the egg surface.

The question remains how exactly diprionin elicits the plant's defense when it contacts pine needle tissue. In general, elicitor - receptor interactions and disturbance of plasmamembrane architecture by surfactant activity of amphiphilic elicitors is considered. Steinbrenner et al. (2020) identified in maize plants a receptor of the elicitor peptide inceptin that is released into leaf wounds with the regurgitate of moth larvae. Phosphatidylcholine derivatives associated with insect eggs (Stahl et al., 2020) and insect secretions (Lortzing et al., 2024) were shown to induce *A. thaliana* defense genes; this induction is reduced in a T-DNA knock-out *lecrk-I.8* mutant of *A. thaliana,* suggesting that the lectin receptor-like kinase LecRK-I.8 is involved in perception of these oviposition-associated elicitors (Stahl et al., 2020). Maffei et al. (2004) and later also Maffei et al. (2012) suggested that FACs released by feeding moth larvae disturb the architecture of the leaf cell plasma membranes by acting like a detergent. Ion fluxes that are induced thereby, may initiate depolarization of the membrane potential and opening of voltage-dependent Ca^{2+} channels. Since annexins can mediate Ca^{2+} transport (Laohavisit et al., 2010),

it is tempting to speculate that diprionin affects the plant's Ca^{2+} fluxes and ROS signaling. However, if this would be proven by measuring Ca^{2+} fluxes and ROS concentrations of diprionin treated pine needles, the question remains how the insect's diprionin is "recognized" by the plant. Among other functions, the plant's own annexins are involved in stress tolerance, especially when it comes to abiotic stress (Saad et al., 2020). Since other plants than pine showed accumulation of plant annexin induced by insect infestation (Fernández et al., 2012), the question arised if diprionin also induces plant annexins as part of the defense mechanism. But neither the qPCR analyses described in chapter 4 nor the RNAseq analysis described in chapter 3 showed any hints for an induction of pine annexins in response to *D. pini* egg deposition.

Diprionin is likely not the only elicitor associated with the *D. pini* oviduct secretion and sawfly egg deposition. Recently, it was shown that that the oviduct secretion of *D. pini* elicits defense responses also in *A. thaliana* (Lortzing et al., 2024). These responses induced by *D. pini* oviduct secretion are also known to be inducible by phosphatidylcholine (PC) derivatives (Stahl et al., 2020). Interestingly, PC derivatives are also present in the oviduct secretion of *D. pini* (Lortzing et al., 2024). Future studies need to elucidate how PCs and diprionin interact when released with sawfly eggs into pine needles.

5.5 Similarities of transcriptional pine responses to insect egg deposition and to larval feeding

Many pine transcriptionally responses to sawfly eggs were no specific responses to the eggs. More than half of the pine DEGs responding to egg deposition were also differentially expressed in response to feeding (chapter 3). This suggests an induction of common defense mechanisms in response to these very different stages of infestation. These mechanisms are related – amongst others - to cell death, cell wall modification, chitinases, Ca^{2+} and phytohormonal signaling, and accumulation of secondary metabolites (Figure 1). In the following, I will address the question how induction of the same genes by eggs and feeding larvae can act as defense response against both of these different stages of infestation.

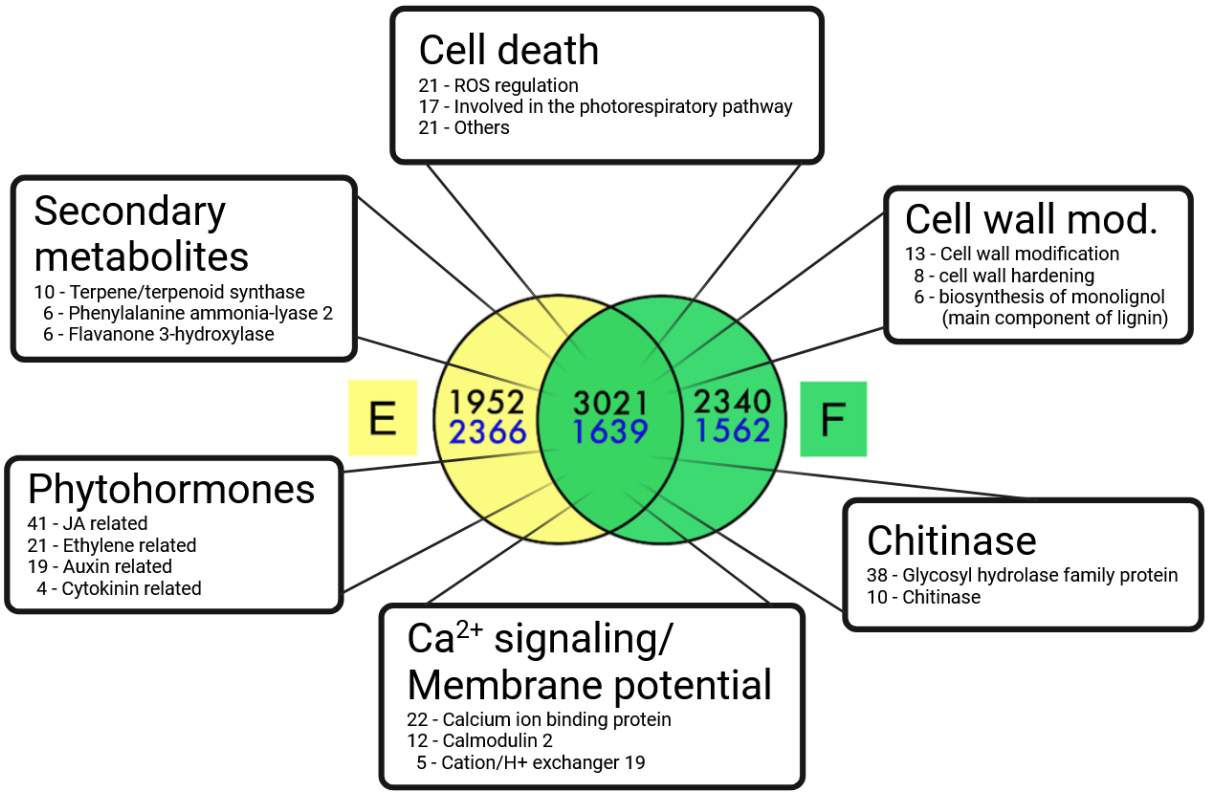

Figure 1. Differentially expressed genes in egg-laden *Pinus sylvestris* (versus untreated pine) and feeding-damaged *P. sylvestris* (versus untreated pine). Circles in the center of the figure: Number of genes upregulated (black numbers) and downregulated (blue numbers); uniquely regulated in egg-laden needles (E; yellow circle), uniquely regulated in feeding-damaged needles (F; green circle), and commonly regulated by both treatments (circles overlap). Black framed windows around the circles: Groups of upregulated genes shown in the overlap of the two circles, i.e. genes induced by both sawfly egg deposition and larval feeding. Information inside the windows: name of gene group, number of genes and the type of gene or the putative function in which it is involved. Created with BioRender.com.

Cell death of plant tissue surrounding infested parts of the plant is a well known defense response against insect eggs (Little et al., 2007; Fatouros et al., 2015; Hilker & Fatouros, 2016). This hypersensitive-like response is mostly related to egg-induced changes in ROS concentrations in the targeted tissue, as was also shown for the interaction of *P. sylvestris* and *D. pini* (Bittner et al., 2017). ROS that accumulates in leaf tissue at the site of egg deposition may directly harm the eggs (Geuss et al., 2017). Furthermore, cell death at the oviposition site may harm the eggs by resulting in egg desiccation (e.g. Griese et al., 2020) or detachment from leaves (e.g. Balbyshev & Lorenzen, 1997). However, when plants respond to insect feeding damage, it is likely that accumulation of ROS does not lead to cell death, but serves as a signaling pathway to induce other defense mechanisms targeting the feeding larvae (Maffei et al., 2007; Kerchev et al., 2012). Hence, induction of the same DEGs by different

infestation stages may finally trigger different defense traits, probably because the overlapping DEGs act in concert with infestation stage specifically induced genes.

Cell wall modification and integrity plays an important role in pathogen resistance of plants (Swaminathan et al., 2022). Most of the pine DEGs related to this group and detected by the RNAseq analysis described in chapter 3 are involved in cell wall hardening. Biosynthesis of lignin and the process of lignification may be of importance for pine defenses against both the sawfly eggs and larvae. Lignification can result in strengthening of the cell wall (Barros et al., 2015), thus rendering it more difficult for larvae to feed on the harder tissue and to process the fed tissue. Lignification in response to sawfly egg deposition might harm the egg, which might be jammed together between lignified, though cells and finally crushed. Egg crushing by egg-induced plant tissue has been shown for e.g. eggs of the chrysomelid species *Pyrrhalta viburni* on *Viburnum* twigs (Hilker & Fatouros, 2015; Desurmont et al., 2021). Overall, a harder cell wall serves as a physical barrier against both stages of infestation.

Chitin is an important component of all insect exoskeletons, but this polymer also lines the insect fore- and hindgut (Marks & Ward, 1987). Furthermore, it is a component of the peritrophic membrane of the midgut in most insect taxa (Terra, 2001). Both sawfly feeding and egg deposition induced pine genes homologous to known plant chitinase genes. The activity of plant chitinases ingested by insects might be impaired by unfavorable pH values in the insect gut. However, there is some evidence that uptake of high plant chitinase concentrations with the food may harm insects (e.g. Gomes et al., 1996; Lawrence & Novak, 2006). In addition, plant chitinases might harm the insect already prior to food uptake by attacking the insect's chitinous insect mouthparts. However, pine chitinase genes were also differentially regulated in response to egg deposition. Plants are well known to perceive fungal infections by responding to chitin fragments released from fungal cell walls during infection (Wan et al., 2008; Sánchez-Vallet et al., 2015). The chitin oligomers elicit plant immune responses to the fungal invader. Insect eggshells also contain chitin, but in their inner layers (Farnesi et al., 2015; Battampara et al., 2020). Therefore, it seems unlikely that chitinases directly harm the eggs, unless other compounds disintegrate the outer layers first. Pine chitinases (constitutive and those induced upon the egg deposition) might attack the chitinous female’s ovipositor. Future studies need to investigate whether and how pine chitinase can affect sawfly eggs or the egg laying female.

A remarkable number of pine DEGs involved in Ca^{2+} and phytohormonal signaling overlapped when considering the tree's response to sawfly egg deposition and to larval feeding. This is not surprising for Ca^{2+} signaling related genes because a Ca^{2+} burst is well known to occur in response to wounding (Hilleary & Gilroy, 2018; Mostafa et al., 2022), and pine needles are wounded by sawfly egg deposition as well as by larval feeding. When considering the overlap of phytohormone related DEGs that responded to eggs and to larval feeding, pine showed a considerable overlap with respect to JA related

genes, which are well known known to be responsive to wounding and feeding damage (Howe, 2004; Wasternack et al., 2006; Ikeuchi et al., 2020). The severe wounding of pine needles that is associated with sawfly egg deposition explains the induction of JA related genes. When considering SA related pine DEGs, both feeding-damaged pine needles and egg-laden pine needles showed significant upregulation of *PAL* genes, which might contribute to SA biosynthesis. However, an *ICS2* homologue was significantly downregulated in feeding-damaged pine and in egg-laden, feeding-damaged pine, but not in egg-laden pine without feeding damage. It will be interesting to figure out by future studies how the SA related biosynthesis genes (*ICS2* and *PAL* genes) contribute in pine to the biosynthesis of SA.

Another interesting group of overlapping pine DEGs in response to eggs and to larval feeding were related to secondary metabolites. Genes relevant for the biosynthesis of terpenoids like e.g. a *farnesyl diphosphate synthase* were induced by both the sawfly egg deposition and larval feeding (chapter 3, Suppl. Table 5). Egg-induced terpene biosynthesis genes might serve indirect defense against the eggs by egg parasitoids, which are attracted to *D. pini* eggs by an egg-induced pattern of pine needle volatiles that is attractive to the parasitoids (Mumm & Hilker, 2005; Beyaert et al., 2010). Whether *D. pini* larval feeding induces terpenes that are attractive to larval parasitoids of this species is unknown. Sawfly larvae can even use pine terpenes for their own defense by accumulating them in foregut pouches and releasing them upon disturbance by enemies (e.g. Eisner et al., 1974). Genes involved in phenylpropanoid biosynthesis like the now often mentioned *PAL* genes were also induced by sawfly egg deposition and by larval feeding (chapter 3, Suppl. Table 5). The expression of these genes that initiate the phenylpropanoid pathway (Dixon et al., 2002; Yadav et al., 2020) might result in numerous different phenylpropanoid derivatives. A study by Bohman et al. (2008) investigated the antifeedant activity of pine phenylpropanoids against the pine weevil and referred to ethyl cinnamate as one of the feeding deterrent compounds isolated from pine bark. A *cinnamate-4-hydroxylase* homologue*,* which (putatively) encodes a pine enzyme catalyzing the formation of coumaric acid by hydroxylating cinnamic acid, was found to be induced by *D. pini* egg deposition and larval feeding in *P. sylvestris* needles (chapter 3, Suppl. Table 5). Coumaric acid might serve as precursor for lignification (Heuschele et al., 2020), thereby harming both eggs and larvae. Future studies need to compare the concentration of coumaric acid and its derivatives in egg-laden pine needles with those in feeding-damaged ones in order to elucidate whether these two infestation stages both can trigger the accumulation of these pine metabolites. It will also be interesting to address in future studies whether esters of this phenylpropanoid act as antifeedant against *D. pini* larvae (Anyanga et al., 2021).

5.6 Outlook

As outlined above in this chapter 5, the results of this thesis open several new questions. In addition to above-mentioned questions, it needs to be considered that all studies described in chapters 2 to 4 were conducted with very young trees, which could be kept in our climate chambers. However, in nature, *D. pini* predominantly infests older trees (Brauns, 1991). Plant defenses are well known to depend on the developmental stage of the plant (Quintero & Bowers, 2011, and references therein). The inducibility of defenses is expected to be greater in young plants than in mature ones (Karban & Baldwin, 1997), whereas older plants seem to be more tolerant towards insect infestation (Haukioja & Koricheva, 2000). Therefore, it will be interesting to investigate in future studies the plant age dependency of the results.

In addition, to gain further insight into common and differing defense responses of angiosperms and gymnosperms to insect egg deposition, it is suggested to study the responses of gymnosperm species to an insect species that does not wound the needles during oviposition. For example, the moth *Panolis flammea,* which is also a major pest in pine forests (Schwenke, 1978), also lays eggs in a line onto pine needles, but does not wound them, as *D. pini* does (Hicks et al., 2001). Studies of pine needle responses to eggs of this species might further elucidate commonalities with angiosperm responses to eggs laid by lepidopteran species, thereby further contributing to our understanding of general, conserved plant responses to the first step of plant infestation by numerous herbivorous insect species, the egg deposition.

While this thesis focused on plant defenses against insect eggs and larvae of herbivorous insects, we still know only little about how insects can cope with egg-induced plant responses. Since herbivorous insects make up a major part of all known eukaryotic species, and since the majority of these insects is oviparous, knowledge about how the vulnerable egg stage of this rich source of biodiversity interacts with plants and how the eggs and/or the egg-laying females can cope with egg-inducible plant defenses will be interesting as well from an entomological perspective.

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