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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).

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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

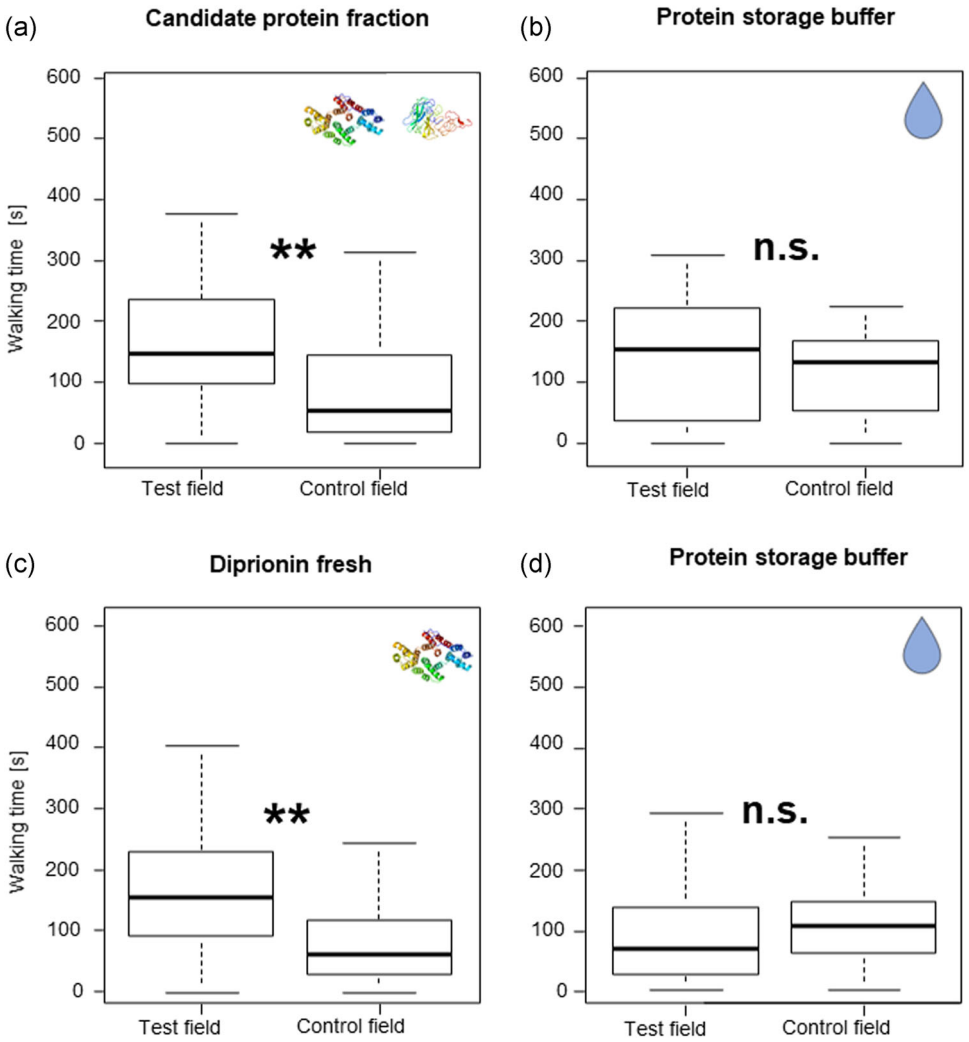

FIGURE 1 Elicitor activity assay: Olfactory response of egg parasitoids to odor of differently treated pine. Slit *Pinus sylvestris* needles were treated with (a) a candidate protein fraction (~20 kDa), obtained by ultrafiltration of oviduct secretion of *Diprion pini* females, separation of ultrafiltrate 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*

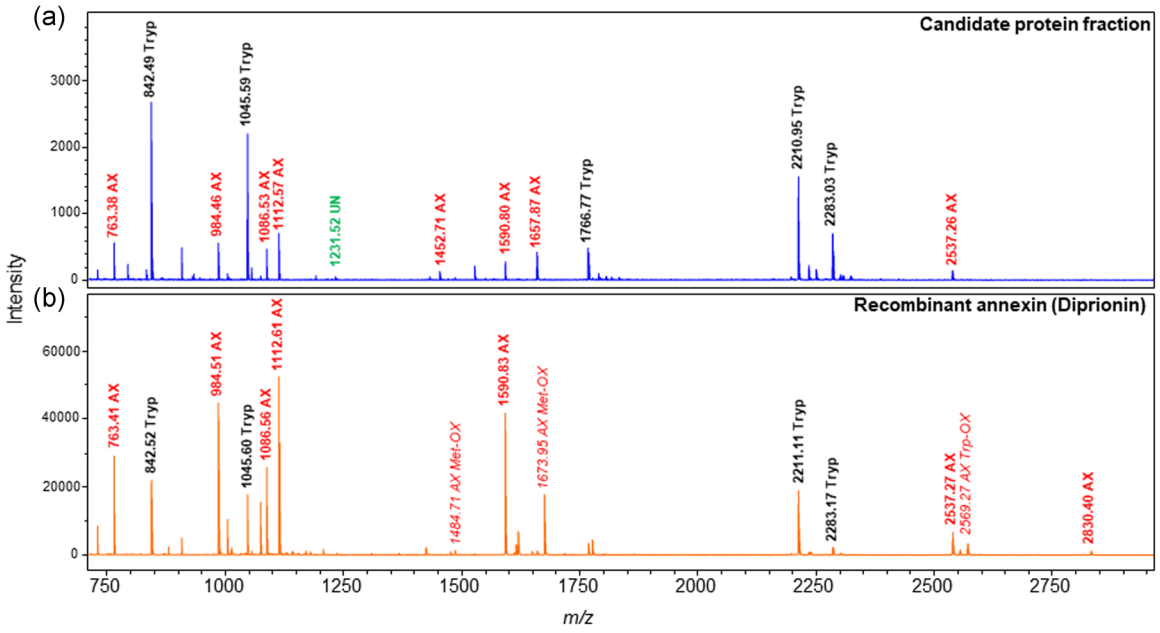

FIGURE 2 MALDI–TOF peptide mass fingerprints of pine defense-eliciting protein fractions obtained from *Diprion pini* oviduct secretion. Spectra of (a) an oviduct secretion fraction (after ultrafiltration and BN-PAGE; Figure S1a; ~20 kDa protein fraction) and (b) annexin (diprionin) recombinantly expressed 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.

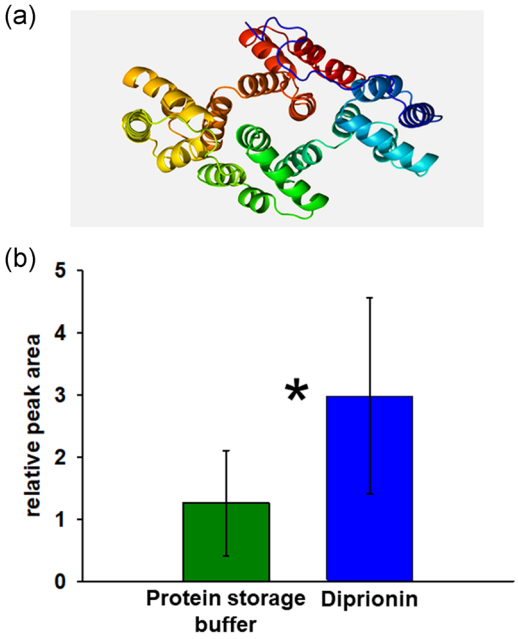

FIGURE 3 Diprionin (3D structure) and relative amount of (*E*)-β-farnesene in odor released from differently treated *Pinus sylvestris*. (a) Diprionin structure was calculated with the online tool Phyre2 (Kelley et al., 2015). The different colors show each of the 4 core domains of the 63–65 amino acids 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

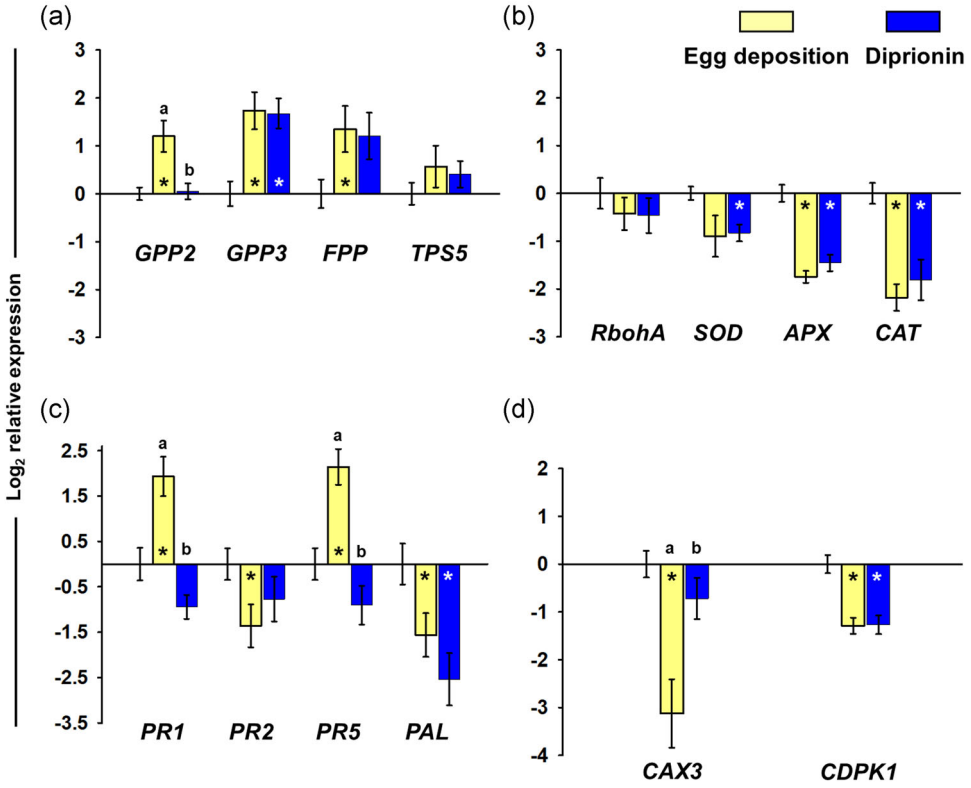

FIGURE 4 Effect of *Diprion pini* egg deposition and diprionin treatment on relative transcript levels in *Pinus sylvestris* needles. Recombinant diprionin was expressed in *Hi*-5 insect cell culture, and for each slit needle 250 ng protein was used for the treatment. We treated eight needles per pine 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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REFERENCES

Acevedo, F.E., Rivera-Vega, L.J., Chung, S.H., Ray, S. & Felton, G.W. (2015) Cues from chewing insects — the intersection of DAMPs, HAMPs, MAMPs and effectors. *Current Opinion in Plant Biology*, 26, 80–86.

Alborn, H.T., Hansen, T.V., Jones, T.H., Bennett, D.C., Tumlinson, J.H., Schmelz, E.A. et al. (2007) Disulfooxy fatty acids from the American bird grasshopper *Schistocerca americana*, elicitors of plant volatiles. *Proceedings of the National Academy of Sciences of the United States Of America*, 104, 12976–12981.

Alborn, H.T., Turlings, T.C.J., Jones, T.H., Stenhagen, G., Loughrin, J.H. & Tumlinson, J.H. (1997) An elicitor of plant volatiles from beet armyworm oral secretion. *Science*, 276, 945–949.

Altmann, S., Muino, J.M., Lortzing, V., Brandt, R., Himmelbach, A., Altschmied, L. et al. (2018) Transcriptomic basis for reinforcement of elm antiherbivore defence mediated by insect egg deposition. *Molecular Ecology*, 27, 4901–4915.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990) Basic local alignment search tool. *Journal of Molecular Biology*, 215, 403–410.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. et al. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, 25, 3389–3402.

Asano, T., Hayashi, N., Kikuchi, S. & Ohsugi, R. (2012) CDPK-mediated abiotic stress signaling. *Plant Signaling & Behavior*, 7, 817–821.

Austel, N., Eilers, E.J., Meiners, T. & Hilker, M. (2016) Elm leaves 'warned' by insect egg deposition reduce survival of hatching larvae by a shift in their quantitative leaf metabolite pattern. *Plant, Cell & Environment*, 39, 366–376.

Bagal, U.R., Leebens-Mack, J.H., Lorenz, W.W. & Dean, J.F. (2012) The phenylalanine ammonia lyase (*PAL*) gene family shows a gymnosperm-specific lineage. *BMC Genomics*, 13, S1.

Bandoly, M., Grichnik, R., Hilker, M. & Steppuhn, A. (2016) Priming of anti-herbivore defence in *Nicotiana attenuata* by insect oviposition: herbivore-specific effects. *Plant, Cell & Environment*, 39, 848–859.

Bandoly, M., Hilker, M. & Steppuhn, A. (2015) Oviposition by *Spodoptera exigua* on *Nicotiana attenuata* primes induced plant defence against larval herbivory. *The Plant Journal*, 83, 661–672.

Bertea, C.M., Casacci, L.P., Bonelli, S., Zampollo, A. & Barbero, F. (2020) Chemical, physiological and molecular responses of host plants to lepidopteran egg-laying. *Frontiers in Plant Science*, 10, 1768.

Beyaert, I., Wäschke, N., Scholz, A., Varama, M., Reinecke, A. & Hilker, M. (2010) Relevance of resource-indicating key volatiles and habitat odour for insect orientation. *Animal Behaviour*, 79, 1077–1086.

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. *Proceedings of the National Academy of Sciences of the United States of America*, 116, 24668–24675.

Bittner, N., Trauer-Kizilelma, U. & Hilker, M. (2017) Early plant defence against insect attack: involvement of reactive oxygen species in plant responses to insect egg deposition. *Planta*, 245, 993–1007.

Bombosch, S. & Ramakers, P.M.J. (1976) Zur Dauerzucht von *Gilpinia hercyniae* Htg. *Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz*, 83, 40–44.

Braccini, C.L., Vega, A.S., Aráoz, M.V.C., Teal, P.E., Cerrillo, T., Zavala, J.A. et al. (2015) Both volatiles and cuticular plant compounds determine oviposition of the willow sawfly *Nematus oligospilus* on leaves of *Salix* spp. (Salicaceae). *Journal of Chemical Ecology*, 41, 985–996.

Brauns, A. (1991) *Taschenbuch der Waldinsekten*. Stuttgart: Gustav Fischer Verlag.

Braz, V.A. & Howard, K.J. (2009) Separation of protein oligomers by blue native gel electrophoresis. *Analytical Biochemistry*, 388, 170–172.

Bredow, M. & Monaghan, J. (2019) Regulation of plant immune signaling by calcium-dependent protein kinases. *Molecular Plant Microbe Interactions*, 32, 6–19.

Cheng, F. & Williamson, J.D. (2010) Is there leaderless protein secretion in plants? *Plant Signaling & Behavior*, 5, 129–131.

Clark, G., Konopka-Postupolska, D., Hennig, J. & Roux, S. (2010) Is annexin 1 a multifunctional protein during stress responses? *Plant Signaling & Behavior*, 5, 303–307.

Dalal, A., Vishwakarma, A., Singh, N.K., Gudla, T., Bhattacharyya, M.K., Padmasree, K. et al. (2014) Attenuation of hydrogen peroxide-mediated oxidative stress by Brassica juncea annexin-3 counteracts thiol-specific antioxidant (TSA1) deficiency in Saccharomyces cerevisiae. *FEBS Letters*, 588, 584–593.

Davies, J.M. (2014) Annexin-mediated calcium signalling in plants. *Plants*, 3, 128–140.

Demidchik, V., Shabala, S., Isayenkov, S., Cuin, T.A. & Pottosin, I. (2018) Calcium transport across plant membranes: mechanisms and functions. *New Phytologist*, 220, 49–69.

Doss, R.P., Oliver, J.E., Proebsting, W.M., Potter, S.W., Kuy, S., Clement, R. et al. (2000) Bruchins: insect-derived plant regulators that stimulate neoplasm formation. *Proceedings of the National Academy of Sciences of the United States of America*, 97, 6218–6223.

Dubiella, U., Seybold, H., Durian, G., Komander, E., Lassig, R., Witte, C.-P. et al. (2013) Calcium-dependent protein kinase/NADPH oxidase activation circuit is required for rapid defense signal propagation. *Proceedings of the National Academy of Sciences of the United States of America*, 110, 8744–8749.

Dudareva, N., Klempien, A., Muhlemann, J.K. & Kaplan, I. (2013) Biosynthesis, function and metabolic engineering of plant volatile organic compounds. *New Phytologist*, 198, 16–32.

Dunn, M.J. (2004) Electroelution of proteins from polyacrylamide gels. In: Cutler, P (Ed.) *Methods in molecular biology*, 244, pp. 339–343.

Duran-Flores, D. & Heil, M. (2016) Sources of specificity in plant damaged-self recognition. *Current Opinion in Plant Biology*, 32, 77–87.

Dyballa, N. & Metzger, S. (2009) Fast and sensitive colloidal coomassie G-250 staining for proteins in polyacrylamide gels. *Journal of Visualized Experiments*, 30, 1431.

Eichhorn, O. (1976) Dauerzucht von *Diprion pini* L. (Hym.: Diprionidae) im Laboratorium unter Berücksichtigung der Fotoperiode. *Anzeiger für Schädlingskunde Pflanzenschutz Umweltschutz*, 49, 38–41.

Erb, M. & Reymond, P. (2019) Molecular interactions between plants and insect herbivores. *Annual Review of Plant Biology*, 70, 527–557.

Fatouros, N.E., Broekgaarden, C., Bukovinszkine'Kiss, G., van Loon, J.J.A., Mumm, R., Huigens M.E. et al. (2008) Male-derived butterfly anti-aphrodisiac mediates induced indirect plant defense. *Proceedings of the National Academy of Sciences of the United States of America*, 105, 10033–10038.

Fatouros, N.E., Cusumano, A., Danchin, E.G.J. & Colazza, S. (2016) Prospects of herbivore egg-killing plant defenses for sustainable crop protection. *Ecology and Evolution*, 6, 6906–6918.

Fatouros, N.E., Pashalidou, F.G., Aponte Cordero, W.V., van Loon, J.J.A., Mumm, R., Huigens, M.E. et al. (2009) Anti-aphrodisiac compounds of male butterflies increase the risk of egg parasitoid attack by inducing plant synomone production. *Journal of Chemical Ecology*, 35, 1373–1381.

Felton, G.W. & Tumlinson, J.H. (2008) Plant-insect dialogs: complex interactions at the plant-insect interface. *Current Opinion in Plant Biology*, 11, 457–463.

Ferreira, A.H.P., Cristofoletti, P.T., Lorenzini, D.M., Guerra, L.O., Paiva, P.B., Briones, M.R.S. et al. (2007) Identification of midgut microvillar proteins from *Tenebrio molitor* and *Spodoptera frugiperda* by cDNA library screenings with antibodies. *Journal of Insect Physiology*, 53, 1112–1124.

Gerke, V. & Moss, S.E. (2002) Annexins: from structure to function. *Physiological Reviews*, 82, 331–371.

Geuss, D., Stelzer, S., Lortzing, T. & Steppuhn, A. (2017) *Solanum dulcamara's* response to eggs of an insect herbivore comprises ovicidal hydrogen peroxide production. *Plant, Cell & Environment*, 40, 2663–2677.

Gorecka, K.M., Konopka-Postupolska, D., Hennig, J., Buchet, R. & Pikula, S. (2005) Peroxidase activity of annexin 1 from *Arabidopsis thaliana*. *Biochemical and Biophysical Research Communications*, 336, 868–875.

Gouhier-Darimont, C., Schmiesing, A., Bonnet, C., Lassueur, S. & Reymond, P. (2013) Signalling of *Arabidopsis thaliana* response to *Pieris brassicae* eggs shares similarities with PAMP-triggered immunity. *Journal of Experimental Botany*, 64, 665–674.

Greening, D.W. & Simpson, R.J. (2010) A centrifugal ultrafiltration strategy for isolating the low-molecular weight (≤25 K) component of human plasma proteome. *Journal of Proteomics*, 73, 637–648.

Griese, E., Caarls, L., Bassetti, N., Mohammadin, S., Verbaarschot, P., Bukovinszkine'Kiss, G. et al. (2021) Insect egg-killing: a new front on the evolutionary arms-race between brassicaceous plants and pierid butterflies. *New Phytologist*, 230, 341–353.

Hartley, S.E. & Firn, R.D. (1989) Phenolic biosynthesis, leaf damage, and insect herbivory in birch (*Betula pendula*). *Journal of Chemical Ecology*, 15, 275–283.

Hilfiker, O., Groux, R., Bruessow, F., Kiefer, K., Zeier, J. & Reymond, P. (2014) Insect eggs induce a systemic acquired resistance in Arabidopsis. *The Plant Journal*, 80, 1085–1094.

Hilker, M. & Fatouros, N.E. (2015) Plant responses to insect egg deposition. *Annual Review of Entomology*, 60, 493–515.

Hilker, M. & Fatouros, N.E. (2016) Resisting the onset of herbivore attack: plants perceive and respond to insect eggs. *Current Opinion in Plant Biology*, 32, 9–16.

Hilker, M., Kobs, C., Varama, M. & Schrank, K. (2002) Insect egg deposition induces *Pinus sylvestris* to attract egg parasitoids. *The Journal of Experimental Biology*, 205, 455–461.

Hilker, M. & Meiners, T. (2010) How do plants 'notice' attack by herbivorous arthropods? *Biological Reviews*, 85, 267–280.

Hilker, M., Stein, C., Schröder, R., Varama, M. & Mumm, R. (2005) Insect egg deposition induces defence responses in *Pinus sylvestris*: characterisation of the elicitor. *Journal of Experimental Biology*, 208, 1849–1854.

Huang, H.-J.J., Liu, C.-W.W., Huang, X.-H.H., Zhou, X., Zhuo, J.-C.C., Zhang, C.-X.X. et al. (2016) Screening and functional analyses of *Nilaparvata lugens* salivary proteome. *Journal of Proteome Research*, 15, 1883–1896.

Jami, S.K., Clark, G.B., Turlapati, S.A., Handley, C., Roux, S.J. & Kirti, P.B. (2008) Ectopic expression of an annexin from *Brassica juncea* confers tolerance to abiotic and biotic stress treatments in transgenic tobacco. *Plant Physiology and Biochemistry*, 46, 1019–1030.

Jami, S.K., Hill, R.D. & Kirti, P.B. (2010) Transcriptional regulation of annexins in Indian mustard, *Brassica juncea* and detoxification of ROS in transgenic tobacco plants constitutively expressing AnnBj1. *Plant Signaling & Behavior*, 5, 618–621.

Kelley, L.A., Mezulis, S., Yates, C.M., Wass, M.N. & Sternberg, M.J.E. (2015) The Phyre2 web portal for protein modeling, prediction and analysis. *Nature Protocols*, 10, 845–858.

Khalaj, K., Aminollahi, E., Bordbar, A. & Khalaj, V. (2015) Fungal annexins: a mini review. *SpringerPlus*, 4, 721.

Kobayashi, M., Ohura, I., Kawakita, K., Yokota, N., Fujiwara, M., Shimamoto, K. et al. (2007) Calcium-dependent protein kinases regulate the production of reactive oxygen species by potato NADPH oxidase. *Plant Cell*, 19, 1065–1080.

Konopka-Postupolska, D. & Clark, G. (2017) Annexins as overlooked regulators of membrane trafficking in plant cells. *International Journal of Molecular Sciences*, 18, 1–34.

Konopka-Postupolska, D., Clark, G., Goch, G., Debski, J., Floras, K., Cantero, A. et al. (2009) The role of annexin 1 in drought stress in Arabidopsis. *Plant Physiology*, 150, 1394–1410.

Konopka-Postupolska, D., Clark, G. & Hofmann, A. (2011) Structure, function and membrane interactions of plant annexins: an update. *Plant Science*, 181, 230–241.

Köpke, D., Beyaert, I., Gershenzon, J., Hilker, M. & Schmidt, A. (2010) Species-specific responses of pine sesquiterpene synthases to sawfly oviposition. *Phytochemistry*, 71, 909–917.

Köpke, D., Schröder, R., Fischer, H.M., Gershenzon, J., Hilker, M. & Schmidt, A. (2008) Does egg deposition by herbivorous pine sawflies affect transcription of sesquiterpene synthases in pine? *Planta*, 228, 427–438.

Koricheva, J., Nykänen, H. & Gianoli, E. (2004) Meta-analysis of trade-offs among plant antiherbivore defences: are plants jacks-of-all-trades, masters of all? *American Naturalist*, 163, E64–E75.

Kotsyfakis, M., Vontas, J., Siden-Kiamos, I. & Louis, C. (2005) The annexin gene family in the malaria mosquito *Anopheles gambiae*. *Insect Molecular Biology*, 14, 555–562.

Kourie, J.I. & Wood, H.B. (2000) Biophysical and molecular properties of annexin–formed channels. *Progress in Biophysical and Molecular Biology*, 73, 91–134.

Laohavisit, A. & Davies, J.M. (2011) Annexins. *New Phytologist*, 189, 40–53.

Lattanzio, V., Kroon, P.A., Quideau, S. & Treutter, D. (2008) Plant phenolics — secondary metabolites with diverse functions. *Recent Advances in Polyphenol Research*, 1, 1–35.

Laule, O., Fürholz, A., Chang, H.S., Zhu, T., Wang, X., Heifetz, P.B. et al. (2003) Crosstalk between cytosolic and plastidial pathways of isoprenoid biosynthesis in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences*, 100, 6866–6871.

Leoni, G., Neumann, P., Kamaly, N., Quiros, M., Nishio, H., Jones, H.R. et al. (2015) Annexin A1-containing extracellular vesicles and polymeric nanoparticles promote epithelial wound repair. *Journal of Clinical Investigation*, 125, 1215–1227.

Little, D., Gouhier-Darimont, C., Bruessow, F. & Reymond, P. (2007) Oviposition by pierid butterflies triggers defense responses in Arabidopsis. *Plant Physiology*, 143, 784–800.

Lortzing, V., Oberländer, J., Lortzing, T., Tohge, T., Steppuhn, A., Kunze, R. et al. (2019) Insect egg deposition renders plant defence against hatching larvae more effective in a salicylic acid-dependent manner. *Plant, Cell & Environment*, 42, 1019–1032.

Maffei, M.E., Bossi, S., Spiteller, D., Mithöfer, A. & Boland, W. (2004) Effects of feeding *Spodoptera littoralis* on lima bean leaves. I. Membrane potentials, intracellular calcium variations, oral secretions, and regurgitate components. *Plant Physiology*, 134, 1752–1762.

Maffei, M.E., Mithöfer, A. & Boland, W. (2007) Before gene expression: early events in plant-insect interaction. *Trends in Plant Science*, 12, 310–316.

Maischak, H., Grigoriev, P.A., Vogel, H., Boland, W. & Mithöfer, A. (2007) Oral secretions from herbivorous lepidopteran larvae exhibit ion channel-forming activities. *FEBS Letters*, 581, 898–904.

Malabarba, J., Meents, A.K., Reichelt, M., Scholz, S.S., Peiter, E., Rachowka, J. et al. (2021) ANNEXIN1 mediates calcium-dependent systemic defense in Arabidopsis plants upon herbivory and wounding. *New Phytologist*, 231, 243–254.

Mattiacci, L., Dicke, M. & Posthumus, M.A. (1995) beta-Glucosidase: an elicitor of herbivore-induced plant odor that attracts host-searching parasitic wasps. *Proceedings of the National Academy of Sciences of the United States of America*, 92, 2036–2040.

Meijer, H.J.G., van de Vondervoort, P.J.I., Yin, Q.Y., de Koster, C.G., Klis, F.M., Govers, F. et al. (2006) Identification of cell wall-associated proteins from *Phytophthora ramorum*. *Molecular Plant-Microbe Interactions*, 19, 1348–1358.

Mithöfer, A. & Boland, W. (2008) Recognition of herbivory-associated molecular patterns. *Plant Physiology*, 146, 825–831.

Mortimer, J.C., Laohavisit, A., Macpherson, N., Webb, A., Brownlee, C., Battey, N.H. et al. (2008) Annexins: multifunctional components of growth and adaptation. *Journal of Experimental Botany*, 59, 533–544.

Moss, S.E. & Morgan, R.O. (2004) The annexins. *Genome Biology*, 5, 219.

Mumm, R., Schrank, K., Wegener, R., Schulz, S. & Hilker, M. (2003) Chemical analysis of volatiles emitted by *Pinus svlvestris* after induction by insect oviposition. *Journal of Chemical Ecology*, 29, 1235–1252.

Mumm, R., Tiemann, T., Varama, M. & Hilker, M. (2005) Choosy egg parasitoids: specificity of oviposition-induced pine volatiles exploited by an egg parasitoid of pine sawflies. *Entomologia Experimentalis et Applicata*, 115, 217–225.

Ninkovic, V., Al Abassi, S. & Pettersson, J. (2001) The influence of aphid-induced plant volatiles on ladybird beetle searching behavior. *Biological Control*, 21, 191–195.

Orozco-Cardenas, M., McGurl, B. & Ryan, C.A. (1993) Expression of an antisense prosystemin gene in tomato plants reduces resistance toward *Manduca sexta* larvae. *Proceedings of the National Academy of Sciences of the United States of America*, 90, 8273–8276.

Pan, G., Zhang, H., Chen, B., Gao, S., Yang, B. & Jiang, Y.-Q. (2019) Rapeseed calcium-dependent protein kinase CPK6L modulates reactive oxygen species and cell death through interacting and phosphorylating RBOHD. *Biochemical and Biophysical Research Communications*, 518, 719–725.

Patel, N., Hamamouch, N., Li, C., Hewezi, T., Hussey, R.S., Baum, T.J. et al. (2010) A nematode effector protein similar to annexins in host plants. *Journal of Experimental Botany*, 61, 235–248.

Pearce, G., Strydom, D., Johnson, S. & Ryan, C.A. (1991) A polypeptide from tomato leaves induces wound-inducible proteinase inhibitor proteins. *Science*, 253, 895–897.

Perkins, D.N., Pappin, D.J., Creasy, D.M. & Cottrell, J.S. (1999) Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis*, 20, 3551–3567.

Petersen, T.N., Brunak, S., von Heijne, G. & Nielsen, H. (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nature Methods*, 8, 785–786.

Pfaffl, M.W. (2001) A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Research*, 29, e45.

R Development Core Team. (2020) *R: A language and environment for statistical computing*. R Development Core Team.

Raynal, P. & Pollard, H.B. (1994) Annexins: the problem of assessing the biological role for a gene family of multifunctional calcium- and phospholipid-binding proteins. *Biochimica et Biophysica Acta*, 1197, 63–93.

Reymond, P. (2013) Perception, signaling and molecular basis of oviposition-mediated plant responses. *Planta*, 238, 247–258.

Rutter, B.D. & Innes, R.W. (2017) Extracellular vesicles isolated from the leaf apoplast carry stress-response proteins. *Plant Physiology*, 173, 728–741.

Savidor, A., Donahoo, R.S., Hurtado-Gonzales, O., Land, M.L., Shah, M.B., Lamour, K.H. et al. (2008) Cross-species global proteomics reveals conserved and unique processes in *Phytophthora sojae* and *Phytophthora ramorum*. *Molecular & Cellular Proteomics*, 7, 1501–1516.

Schmelz, E.A. (2015) Impacts of insect oral secretions on defoliation-induced plant defense. *Current Opinion in Insect Science*, 9, 7–15.

Schmelz, E.A., Carroll, M.J., LeClere, S., Phipps, S.M., Meredith, J., Chourey, P.S. et al. (2006) Fragments of ATP synthase mediate plant perception of insect attack. *Proceedings of the National Academy of Sciences of the United States of America*, 103, 8894–8899.

Schmelz, E.A., Engelberth, J., Alborn, H.T., Tumlinson, J.H. & Teal, P.E.A. (2009) Phytohormone-based activity mapping of insect herbivore-produced elicitors. *Proceedings of the National Academy of Sciences of the United States of America*, 106, 653–657.

Schoonhoven, L.M., Van Loon, J.J.A. & Dicke, M. (2005) *Insect-plant biology*. Oxford, UK: Oxford University Press.

Schröder, R., Wurm, L., Varama, M., Meiners, T. & Hilker, M. (2008) Unusual mechanisms involved in learning of oviposition-induced host plant odours in an egg parasitoid? *Animal Behaviour*, 75, 1423–1430.

Seino, Y., Suzuki, Y. & Sogawa, K. (1996) An ovicidal substance produced by rice plants in response to oviposition by the whitebacked planthopper, *Sogatella furcifera* (Horvath) (Homoptera: Delphacidae). *Applied Entomology and Zoology*, 31, 467–473.

Shapiro, A.M. & DeVay, J.E. (1987) Hypersensitivity reaction of *Brassica nigra* L. (Cruciferae) kills eggs of *Pieris* butterflies (Lepidoptera, Pieridae). *Oecologia*, 71, 631–632.

Shevchenko, A., Wilm, M., Vorm, O. & Mann, M. (1996) Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. *Analytical Chemistry*, 68, 850–858.

Spiteller, D., Dettner, K. & Boland, W. (2000) Gut bacteria may be involved in interactions between plants, herbivores and their predators: Microbial biosynthesis of *N*-acylglutamine surfactants as elicitors of plant volatiles. *Biological Chemistry*, 381, 755–762.

Stahl, E., Brillatz, T., Queiroz, E.F., Marcourt, L., Schmiesing, A., Hilfiker, O. et al. (2020) Phosphatidylcholines from *Pieris brassicae* eggs activate an immune response in Arabidopsis. *eLife*, 9, e60293.

Stone, J.M., Heard, J.E., Asai, T. & Ausubel, F.M. (2000) Simulation of fungal-mediated cell death by fumonisin B1 and selection of fumonisin B1–resistant (*fbr*) Arabidopsis mutants. *Plant Cell*, 12, 1811–1822.

Suckau, D., Resemann, A., Schuerenberg, M., Hufnagel, P., Franzen, J. & Holle, A. (2003) A novel MALDI LIFT-TOF/TOF mass spectrometer for proteomics. *Analytical and Bioanalytical Chemistry*, 376, 952–965.

Teixeira, A.dD., Games, P.D., Katz, B.B., Tomich, J.M., Zanuncio, J.C. & Serrão, J.E. (2017) Proteomic analysis in the Dufour's gland of africanized *Apis mellifera* workers (Hymenoptera: Apidae). *PLOS One*, 12, e0177415.

Tjota, M., Lee, S.K., Wu, J., Williams, J.A., Khanna, M.R. & Thomas, G.H. (2011) Annexin B9 binds to βH-spectrin and is required for multivesicular body function in *Drosophila*. *Journal of Cell Science*, 124, 2914–2926.

Tsuzuki, S., Iwami, M. & Sakurai, S. (2001) Ecdysteroid-inducible genes in the programmed cell death during insect metamorphosis. *Insect Biochemistry and Molecular Biology*, 31, 321–331.

Valapala, M., Maji, S., Borejdo, J. & Vishwanatha, J.K. (2014) Cell surface translocation of annexin A2 facilitates glutamate-induced extracellular proteolysis. *Journal of Biological Chemistry*, 289, 15915–15926.

Valsamakis, G., Bittner, N., Fatouros, N.E., Kunze, R., Hilker, M. & Lortzing, V. (2020) Priming by timing: *Arabidopsis thaliana* adjusts its priming response to Lepidoptera eggs to the time of larval hatching. *Frontiers in Plant Science*, 11, 1969.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. et al. (2002) Accurate normalization of real-time

quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology*, 3, 1–12.

Viña, A. & Murillo, E. (2003) Essential oil composition from twelve varieties of basil (*Ocimum* spp) grown in Colombia. *Journal of the Brazilian Chemical Society*, 14, 744–749.

Vorm, O., Roepstorff, P. & Mann, M. (1994) Improved resolution and very high sensitivity in MALDI TOF of matrix surfaces made by fast evaporation. *Analytical Chemistry*, 66, 3281–3287.

Wang, L., Einig, E., Almeida-Trapp, M., Albert, M., Fliegmann, J., Mithoefer, A. et al. (2018) The systemin receptor SYR1 enhances resistance of tomato against herbivorous insects. *Nature Plants*, 4, 152–156.

Wittig, I., Braun, H.-P. & Schägger, H. (2006) Blue native PAGE. *Nature Protocols*, 1, 418–428.

Wu, J. & Baldwin, I.T. (2010) New insights into plant responses to the attack from insect herbivores. *Annual Review of Genetics*, 44, 1–24.

Yang, J.-O., Nakayama, N., Toda, K., Tebayashi, S. & Kim, C.S. (2014) Structural determination of elicitors in *Sogatella furcifera* (Horvath) that induce Japonica rice plant varieties (*Oryza sativa* L.) to produce an ovicidal substance against *S. furcifera* eggs. *Bioscience, Biotechnology, and Biochemistry*, 78, 937–942.

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S. & Madden, T.L. (2012) Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics*, 13, 134.

SUPPORTING INFORMATION

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