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Original Article

Solanum dulcamara's response to eggs of an insect herbivore comprises ovicidal hydrogen peroxide production

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ABSTRACT

Plants can respond to insect oviposition, but little is known about which responses directly target the insect eggs and how. Here, we reveal a mechanism by which the bittersweet nightshade *Solanum dulcamara* kills the eggs of a generalist noctuid herbivore. The plant responded at the site of oviposition by *Spodoptera exigua* with formation of neoplasms and chlorotic tissue, accumulation of reactive oxygen species and induction of defence genes and proteins. Transcriptome analysis revealed that these responses were reflected in the transcriptional reprogramming of the egg-laden leaf. The plant-mediated egg mortality on *S. dulcamara* was not present on a genotype lacking chlorotic leaf tissue at the oviposition sites on which the eggs are exposed to less hydrogen peroxide. As exposure to hydrogen peroxide increased egg mortality, while catalase supplementation prevented the plants from killing the eggs, our results suggest that reactive oxygen species formation directly acts as an ovicidal plant response of *S. dulcamara*.

Key-words: egg-killing; herbivory; hypersensitive response; induced plant defence; microarray; phytohormones; plant–insect interactions; ROS.

INTRODUCTION

Plants deploy various defences against insect herbivory, and many are inducible by herbivore attack. Inducible plant defences are elicited by signals associated with the damage that the feeding herbivores inflict and by signals of the herbivore itself such as components of their oral secretions (Bonaventure 2012). Moreover, several plant species respond already to the oviposition of herbivorous insects (Hilker & Fatouros 2015). Whereas some of the plant responses to oviposition may result in dropping, crushing, desiccation or intoxication of the insect eggs, others can repel herbivorous insects or attract egg predators and parasitoids that kill the eggs (Hilker & Fatouros 2015). In addition to plant responses that directly or indirectly reduce egg survival, previous insect oviposition can affect development of the feeding larvae that hatch from these eggs (Beyaert *et al.* 2012; Pashalidou *et al.* 2012; Bandoly *et al.* 2015; Bandoly *et al.* 2016; Austel *et al.* 2016). Overall, plants likely evolved to perceive and respond to the oviposition of herbivorous insects to prevent feeding damage by the larvae hatching from these eggs (Hilker & Meiners 2006). However, our knowledge about the nature of most plant responses to insect oviposition that directly affects the insect eggs as well as the mechanisms by which plants can kill insect eggs is still restricted.

One of the best characterized plant responses that directly reduce egg survival is the release of ovicidal benzyl benzoate into watery lesions at the oviposition sites of the planthopper *Sogatella furcifera* on rice plants (Seino *et al.* 1996; Suzuki *et al.* 1996). One major and several minor quantitative trait loci associated with watery lesions and egg mortality are mapped (Yamasaki *et al.* 2003; Yang *et al.* 2014). Rice genotypes that do or do not exhibit this ovicidal response show global deviations in gene expression in response to *S. furcifera* infestation, but the biochemical pathways underlying this response and their regulation remain to be determined.

Other plants respond to insect oviposition with growth responses that physically affect the eggs. Egg deposition by the leaf beetle *Pyrrhalta viburni* on stems of *Viburnum* species elicits tissue production at the oviposition site that displaces the egg cap, partially crushes the eggs and encases egg masses, thereby reducing egg survival (Desurmont & Weston 2011). Pea and physalis plants also produce plant tissues in form of neoplasms underneath the eggs of pea weevils or of the lepidopteran herbivore *Heliothis subflexa*, respectively (Doss *et al.* 2000; Petzold-Maxwell *et al.* 2011). Neoplasm formation is characterized by limited non-meristematic growth and is associated with reduced pea weevil infestations of pea plants and reduced egg hatching rates of *H. subflexa* on physalis plants, but the mechanisms for these effects remain unknown. It has been suggested that neoplasm formation facilitates egg removal from the plant by physical ablation or predation (Doss *et al.* 2000; Petzold-Maxwell *et al.* 2011).

At the sites of insect oviposition, several plant species of the Brassicaceae and the Solanaceae exhibit chlorotic or necrotic responses, which are paralleling a hypersensitive response (HR) that is commonly described for pathogen infections of plant tissue (Shapiro & DeVay 1987; Balbyshev & Lorenzen 1997; Petzold-Maxwell *et al.* 2011; Fatouros *et al.* 2014; Bittner *et al.* 2017). In most of these plant species, this response is associated with reduced egg survival, although the mechanism

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remains unclear. Shapiro and DeVay (1987), who initially described HR-like necrosis at oviposition sites on *Brassica nigra*, suggested a lethal desiccation of the eggs as cause of humidity-dependent egg mortality on necrotic tissues. In accordance with this hypothesis, the mortality of sawfly eggs was suggested to increase on pine foliage that was either desiccated (Codella & Raffa 2002) or showed HR-like responses (Bittner *et al.* 2017). However, egg desiccation does not occur on potato leaves exhibiting HR-like necrosis at the oviposition sites of the Colorado potato beetle, which is generally not affected in its egg hatching rate (Balbyshev & Lorenzen 1997). Instead, the HR-like necrosis reduces egg attachment to the leaf surface and the reduced larval infestation of plants exhibiting the HR-like response in a field trial is attributed to predation of dropped eggs by ground predators. The response of physalis plants to *H. subflexa* eggs also involves necrosis, and even chlorotic neoplasms are reported, but the contribution of each of these responses to the reduction of egg hatching rates on responding plants and to the effect that more eggs vanish on these plants under field conditions remains elusive (Doss *et al.* 2000; Petzold-Maxwell *et al.* 2011).

The HR-like response to eggs of pierid butterflies on *Arabidopsis thaliana* leaves is further associated with the accumulation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), increased levels of the phytohormone salicylic acid (SA) and the induction of SA-responsive genes beneath the eggs (Little *et al.* 2007; Bruessow *et al.* 2010; Gouhier-Darimont *et al.* 2013). Therefore, *B. nigra* plants that exhibit the HR-like response to oviposition by pierid butterflies show enhanced expression of the SA-responsive marker gene *PR1* (*pathogenesis-related protein 1*; Fatouros *et al.* 2014). Comprehensive analyses of transcriptome regulation reveal large overlaps in *A. thaliana*'s response to *Pieris brassicae* eggs and infection by the bacterial pathogen *Pseudomonas syringae*, particularly in defence-related and stress-related genes (Little *et al.* 2007; Gouhier-Darimont *et al.* 2013), suggesting similarities in the HRs elicited by insect oviposition and biotrophic pathogens. Yet, *Pieris* eggs are not affected by this response of *A. thaliana* (Gouhier-Darimont *et al.* 2013), which has been mainly evaluated for its effects on the feeding larvae hatching from the eggs (Bruessow *et al.* 2010) and on *P. syringae* infection (Hilfiker *et al.* 2014). Considering the parallels in the response to oviposition between *A. thaliana* and plant species that exhibit negative effects on the insect eggs, a contribution of accumulation of ROS or SA-mediated defences, to egg mortality may be presumed (Reymond 2013).

Oviposition by some herbivore species involves wounding of plant tissue such as that of the conifer saw flies and *P. viburni* leaf beetles. Ovipositional wounding may induce the wound hormone jasmonic acid (JA) that is mediating many defences induced by herbivory (Campos *et al.* 2014). That JA may be involved in egg-killing plant responses is indicated by an increased hatching rate of spider mite eggs on JA-deficient tomato mutants (Ament *et al.* 2004). Moreover, JA-biosynthesis genes are induced upon oviposition by an omnivorous pirate bug that involves wounding of plant tissue and also the formation of ROS (Puysseleyr *et al.* 2011). Although the response of *A. thaliana* to oviposition is not affected on JA-deficient mutants, the transcriptional analyses of its responses also revealed the regulation of JA-responsive genes (Little *et al.* 2007).

In this study, we investigated the responses of *Solanum dulcamara*, a wild relative of potato and tomato, to oviposition. We discovered that the plant kills the eggs of the noctuid moth, *Spodoptera exigua*, and asked for the mechanism underlying the negative effect on this generalist herbivore. Therefore, we (1) characterized the plant's response to the moth's oviposition on physiological and transcriptional levels and (2) we examined which of the responsive plant traits is functionally linked to the egg killing.

MATERIALS AND METHODS

Plant and insects

We used *S. dulcamara* L. (Solanaceae) plants originating from different populations in the vicinity of Berlin (Erkner: 52°41′88.8″N; 13°77′34.1″E, Grunewald: 52°27′44.4″N; 13°11′24.6″E, Mehrow: 52°34′06.4″N; 13°38′04.0″E and Siethen 52°16′53.7″N; 13°11′18.7″E) and from the Netherlands (Friesland: 52°58′36.2″N 5°30′59.4″E). Except for one experiment, plants were grown from stem cuttings of 6 to 7-week-old plants. Stem segments that included two nodes were planted into 0.75 L pots with one node within and one above the soil. The microarray experiment was performed with plants grown from seeds and thus reflected the transcriptomic response of several genotypes from three *S. dulcamara* populations. The seeds were incubated in darkness on sterilized wet sand (2–4 mm grain size) in plastic containers (20 × 20 × 6.5 cm; Gerda, Schwelm, Germany) sealed with cling film at 4 °C. After 12 d, the containers were transferred to the greenhouse, and 10 d later, individual seedlings were transferred to 0.75 L pots with soil. The soil (Einheits Erde®, type: Profi Substrat Classic, Sinntal-Jossa, Germany) of plants for all experiments was covered with about 1 cm of sand (2–4 mm grain size) to prevent fungus gnats infestation. The plants were grown in the greenhouse with a 16/8 h light/dark cycle and a photon irradiance between 190 and 250 μmol m^{-2} s^{-1} for 3 weeks before they were used in the experiment.

Spodoptera exigua HÜBNER (Noctuidae) larvae were reared in vented plastic boxes (14 × 21 × 5 cm) on a bean flour-based artificial diet (Lortzing *et al.* 2016). Boxes were kept in a climate chamber (24 °C, 70% r.h., 16/8 h light/dark with 50% dimming for 1 h). The moths were kept in flight cages supplied with 20% honey solution and paper tissue as substrate for oviposition.

Experimental overview

Overall, we performed 11 experiments with *S. dulcamara* plants that were size-matched and genotype-matched between the treatments within each replicate. First, we investigated *S. exigua*-oviposited leaves of *S. dulcamara* plants originating from four different populations around Berlin for chlorosis,

neoplasm and H_2O_2 formation (exp. I) as well as transcript accumulation of two defence-related genes (exp. II) and the effect of *S. dulcamara* on egg survival (exp. III). As the plants of different populations responded consistently, we then assessed in plants of one population (Grunewald) the spatial induction pattern of oviposition-induced defence traits (exp. IV). We examined the local transcriptomic response of *S. dulcamara* to oviposition by microarray analysis in an oviposition experiment with three genotypes (exp. V: Erkner, Siethen, Friesland). As egg-dropping barely occurred, we tested whether egg mortality on *S. dulcamara* is connected to desiccation and/or the oviposition-induced response associated with chlorosis. Under divergent humidity conditions, we compared the hatching rates of eggs developing on two plant genotypes that strongly differ in their chlorosis response (exp. VII: Siethen, Friesland). In these two genotypes, we measured phytohormone levels in the leaf tissue at oviposition sites to test for a differential activation of defence pathways (exp. VI: Siethen, Friesland). We further tested whether egg mortality on the genotype deficient for the chlorosis response can be provoked by the application of the known HR-elicitor chitosan, a molecular pattern associated with fungal attack that is inducing necrosis (exp. VIII). We then compared the eggs developing on *S. dulcamara* genotypes exhibiting no or strong chlorosis for levels of H_2O_2 on the egg shells (exp. IX). As the eggs were differentially exposed to plant-produced H_2O_2, we then determined the effect of H_2O_2 exposure on egg viability (exp. X). Finally, we tested whether the mortality of eggs developing on the genotype exhibiting chlorotic tissue can be diminished by repeated applications of the antioxidant enzyme catalase (exp. XI).

Oviposition treatment

In experiments I–VIII, we exposed defined leaf positions (the first fully developed leaf in exp. I and II and the third fully developed leaf in exp. III–VIII) to oviposition by *S. exigua* moths by inserting the leaves through slits in a flight cage (40 × 71 × 40 cm) with 40 female and 40 male moths overnight. The leaves of the control plants were exposed to 80 male moths, respectively. In experiments IX and XI, we exposed the third fully developed leaf to 4–5 *S. exigua* moths of both sexes in mesh bags (15 × 11.5 cm) overnight. The egg load per plant ranged from 10 to 200 eggs in all experiments.

Leaf tissue sampling

To determine the transcriptional and phytohormonal regulation in oviposited leaves (exp. II and IV–VI), we harvested leaf tissue samples immediately after the eggs were carefully removed by using a fine paintbrush. Shortly before the larvae hatch (after 3–4 d), the eggs turn dark (Bandoly & Steppuhn 2016) and they were removed. In exp. II, one third of the local leaf was harvested by using a scalpel including the previously oviposited tissue. For analysis of the spatial induction pattern of oviposition induced defences (exp. IV), we separately harvested the leaf tissue directly beneath the eggs (13 mm Ø leaf disc) and surrounding the eggs (tissue ring of 5 mm width) by using a cork borer as well as the remaining leaf tissue of the oviposited leaf, the next upper leaf and the leaf five positions higher, which has a full vascular connection to the oviposited leaf (Viswanathan & Thaler 2004). The tissue directly beneath the eggs was also harvested for the transcriptome (exp. V: 13 mm Ø leaf disc) and phytohormone (exp. VI: 15 mm Ø leaf disc) analysis by using a cork borer. The samples were immediately flash frozen in liquid nitrogen and stored at −80 °C.

Egg hatching with and without leaf contact

In exp. III, we compared hatching rates of eggs developing on *S. dulcamara* leaves with and without leaf contact. At the same time that the leaves were exposed through slits in the flight cages to oviposition, stripes of Parafilm were offered for oviposition to obtain egg clutches without leaf contact. Oviposited eggs on the leaves were counted, and egg clutches of comparable size on Parafilm were attached next to the clutches on the leaves (Fig. 1f). Shortly before the larvae hatch, we transferred all egg clutches to Petri dishes with moistened filter paper and kept them in a climate chamber (24 °C, 70% r.h., 16/8 h light/dark with 50% dimming for 1 h). Egg clutches with leaf contact were isolated by cutting out leaf discs (Ø 13 mm) with a cork borer. For the next 3 d, the number of hatching larvae was recorded twice a day to assess the overall hatching rate.

Egg hatching under altered humidity

In a full-factorial experiment (exp. VII), we compared the hatching rates of eggs developing with and without leaf contact on plant genotypes that express particularly strong chlorosis (Siethen) or no chlorosis (Friesland) and under ambient and saturated air humidity. After counting the numbers of oviposited eggs, leaves with egg clutches and a comparable egg clutch on Parafilm next to it were enclosed in clip cages. The clip cages were either vented through gauze integrated in the top and the bottom part of the cage or closed, resulting in ambient and saturated relative humidity respectively (Fig. 3b). Egg hatching rates were determined as described in the previous paragraph.

Chitosan treatment

To elicit the formation of HR-like necrosis on the plant genotype (Friesland) exhibiting no chlorosis after egg deposition (exp. VIII), half of the plants were treated with a chitosan solution (1 mg/mL in 0.3% *v/v* acetic acid and 0.01% *v/v* TWEEN 20) at the upper leaf surface at the same location that *S. exigua* had deposited its eggs on the lower leaf surface immediately after removal from the oviposition flight cages. The amount of applied chitosan solution was adjusted to the leaf area covered by the egg clutches (between 3 and 8 μL) to ensure that the leaf area with necrotic tissue matches the surface area covered by the eggs. The treatment was repeated twice, after 24 and

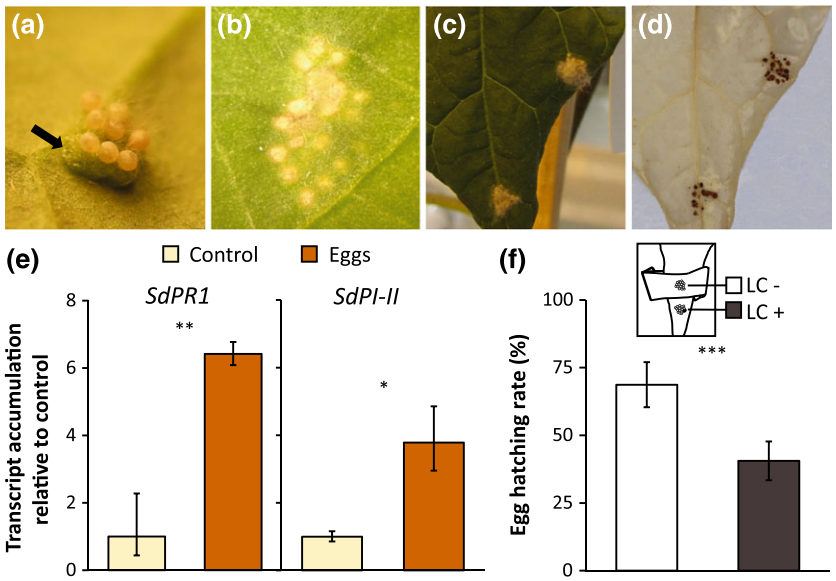

Figure 1. *Solanum dulcamara* responds to *Spodoptera exigua* eggs. (a) Formation of neoplasms (arrow) and (b) of chlorotic spots beneath the *S. exigua* eggs 4 d after oviposition on *S. dulcamara* leaves. (c) Egg clutches deposited on a leaf and (d) the same leaf after 3,3-diaminobenzidine (DAB) staining without the egg clutches revealing hydrogen peroxide (H_2O_2) formation in the leaf tissue with direct contact to the eggs (representative images of eight independent plants). (e) Relative transcript accumulation ($n = 5$) of the *SdPR1* and *SdPI-II* genes in oviposited leaves (eggs) and leaves of non-oviposited plants (control) normalized to the reference gene *SdELF1*. (f) Egg hatching rate ($n = 16$) of *S. exigua* eggs with (+) or without (−) leaf contact (LC). In the morning after oviposition, egg clutches deposited on Parafilm were placed next to egg clutches of similar size on *S. dulcamara* leaves (see inset). The bars represent means ± SE, and the asterisks indicate significant differences according to (e) Mann–Whitney *U* tests of log_2-normalized transcript accumulation or (f) a generalized linear mixed model(GLMM) at $P < 0.001$ (***).

48 h. Egg hatching rates were determined as described in the preceding texts.

Egg exposure to exogenous hydrogen peroxide

To test the effect of egg exposure to exogenous H_2O_2 on egg survival (exp. X), filter paper strips deposited with *S. exigua* eggs were constantly supplied with H_2O_2 solution of different concentrations during egg development. The filter paper stripes were attached to Petri dishes (Fig. 5b) containing 5 mL of a 0, 1, 10 or 100 mM H_2O_2 solution, prepared with 30% phosphate stabilized H_2O_2 (Carl Roth, Karlsruhe, Germany). The day before the larvae would hatch, the filter paper strips were transferred to fresh Petri dishes and kept in a climate chamber (24 °C, 70% r.h., 16/8 h light/dark with 50% dimming for 1 h). The number of hatching larvae was recorded twice a day for 2 d, and the hatching rates were calculated.

Catalase treatment

In experiment XI, we supplied eggs deposited on a plant genotype exhibiting chlorosis (Siethen) with the H_2O_2 scavenging enzyme catalase during development. Three times per day, the egg clutches were moistened with catalase (Sigma-Aldrich, St. Louis, Missouri, USA) solution (2000–5000 U/mL in 50 mM potassium phosphate buffer pH 7.0) or only with buffer by using a paint brush. Egg hatching rates were determined as described in the preceding texts.

Histochemical staining

To investigate H_2O_2 accumulation at oviposition sites (exp. I), the eggs were removed shortly before hatching and the excised leaves that were carrying the eggs were submerged in a 1 mg/mL 3,3-diaminobenzidine (DAB) solution (Carl Roth, Karlsruhe, Germany), gently vacuum infiltrated for 10 min and incubated for 6 h while shaking at 100 r.p.m. The leaves were destained by boiling in ethanol/acetic acid/glycerol (3:1:1) for 15 min. The leaves were transferred to fresh destaining solution, and the samples were stored in darkness at 4 °C overnight before photographs were taken.

To visualize plant-derived H_2O_2 on eggs that were deposited on leaves of *S. dulcamara* genotypes exhibiting strong or no chlorosis (Siethen, Friesland; exp. IX), excised leaves with the eggs still on were DAB-stained 3 d after *S. exigua* egg deposition. On eggs developing on gauze, no H_2O_2 was determined in DAB stainings of the eggs (Supporting Information Fig. S2b). Staining was conducted as described in the preceding texts, except that the leaves were incubated in ethanol/acetic acid/glycerol (5:1:1) at 60 °C overnight for destaining to prevent egg detachment. To verify that colouration results only from H_2O_2 formation, the DAB solution for five biological replicates per plant genotype was supplemented with 15 mM ascorbic acid, a scavenger of H_2O_2. Destained leaves were placed on a white

light panel, and photographs were taken with fixed camera settings. Further processing was carried out with Photoshop CS5 (Adobe Systems, San Jose, United States): After pictures were white point corrected by using a defined area (51 × 51 pixel average) on the white light panel, the eggs were selected with the quick selection tool and the average grey value (a measurement of brightness) was determined. We expressed staining intensity (the reciprocal of brightness) relative to the mean of the ascorbate controls, which showed no DAB staining.

Quantitative RT-PCR analysis

Leaf samples that were harvested shortly before the eggs would hatch (96 h after oviposition) were used to determine transcript accumulation of selected defence related genes (exp. II and IV). In the experiment with a separated harvest of the oviposited tissue and the tissues of different distances to it (exp. IV), tissue samples of two to three plants were pooled resulting in 7–8 biological replicates to receive sufficient leaf material (in exp. II, one third of the oviposited leaf was harvested). RNA extraction and analysis of transcript accumulation were performed as described by Bandoly *et al.* 2015. In brief, total RNA was extracted from powdered tissue samples with a TRIzol® Reagent-based RNA extraction followed by a DNase digestion with TURBO DNA-free™ (both Ambion® Life Technologies: http://tools.lifetechnologies.com/content/sfs/manuals). From 200 ng total RNA, cDNA was synthesized with the Reverse Transcriptase Core kit and subjected to SYBR®Green-based real-time PCR with the qPCR kit (both kits: Eurogentec, Seraing, Belgium, http://www.eurogentec.com) and gene specific primers for *S. dulcamara* genes (Supporting Information Table S2) on a Stratagene™ Mx3005P® instrument (Agilent Technologies, Santa Clara, California, USA, http://www.agilent.com).

Microarray analysis

Total RNA was extracted as in the preceding texts from 50 mg of leaf tissue harvested from the oviposition sites with a cork borer (13 mm Ø) shortly before the eggs would hatch (72 h after oviposition). For hybridization, pooled RNA representing at least four plants was additionally cleaned up by using the NucleoSpin® RNA Plant kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). All RNA samples had a RNA integrity index between 7.3 and 8.0 in an electrophoretic analysis with the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California, USA, http://www.agilent.com).

Fluorescent cRNA was generated by using the Low Input QuickAmp Labelling Kit (Agilent Technologies) using oligo-dT primer following the manufacture's protocol. Of the cyanine 3-CTP-labelled cRNA, 600 ng was hybridized by using the Agilent Gene Expression Hybridization Kit (Agilent Technologies) following the manufacturer's protocol at 65 °C for 17 h on an 8 × 60 K Agilent custom microarray that was based on a *S. dulcamara* transcriptome assembly (D'Agostino *et al.* 2013) containing 32 157 contigs and that was validated and described in detail elsewhere (Lortzing *et al.* 2017). After the microarray was washed twice, the fluorescence signals were detected by the SureScan Microarray Scanner (Agilent Technologies) at a resolution of 3 μm per pixel. RNA labelling, hybridization and scanning of the array were performed by Oaklabs (Henningsdorf, Germany).

We analysed microarray data with the 'LIMMA' software packages from Bioconductor in 'R' (R Core Team 2015; Ritchie *et al.* 2015) and set the detection limit according to the fluorescence values of the dark corners (non-labelled hairpin DNA oligos) at twice of the 90% percentile of each array. The data for expressed oligos (exceeding the detection limit in at least two replicates for at least one treatment) were background-corrected by using the 'normexp' method and normalized between arrays by using the 'quantile' method. Multiple oligos matching the same target sequence were averaged, and oligos matching several target sequences with large sequence similarity were assigned to the longest contig in the transcriptome assembly. Oligos that expressed in both strand directions were treated as individual targets but were assigned the same GO annotation. Average fluorescence values of the final 20 365 targets were $\log_2$-transformed and fit to a linear model by using the 'lmFit' function. Targets differing at least twofold between treatments and with a *P*-value below 0.1 (after adjustment for false discovery rate) were considered significantly different.

Analysis of PI and PPO activities

Trypsin proteinase inhibitor (TPI) and polyphenol oxidase activities (PPO) were analysed (exp. II) with a photometric assay in microwell plates as previously described by Bandoly *et al.* (2015). Leaf tissue from two to three plants was pooled for the local samples (leaf disc 13 mm Ø) to obtain 7–8 biological replicates.

Salicylic acid quantification

Leaf tissue samples of exp. VI were analysed for SA content by using a LC-MS/MS-based method after Wang *et al.* (2007) with minor modifications. About 50 mg powdered leaf material, pooled from two plants, was transferred to 2 mL screw-cap tubes containing 1.25 g homogenization matrix (Zirconox, 2.8–3.3 mm; Mühlmeier Mahltechnik, Bärnau, Germany) on liquid nitrogen. Ethyl acetate (1 mL) spiked with 20 ng of D4-SA (Purity Compounds Standards GmbH, Cunnersdorf, Germany) was added to each sample and afterwards homogenized on a FastPrep homogenizer (MP Biomedicals, Solon, USA). After centrifugation for 10 min at 17 000*g*, the supernatants were transferred to fresh 2 mL reaction tubes and pellets were re-extracted with 1 mL ethyl acetate as described in the preceding texts. The supernatants were combined and dried in a vacuum concentrator (concentrator 5301, Eppendorf, Hamburg, Germany). The residue was re-eluted in 300 μL 70% methanol containing 0.1% formic acid (*v*/*v*) by vortexing for 10 min, centrifuged at 17 000*g* for 10 min, and the supernatants were analysed by UPLC-ESI-MS/MS (Synapt G2-S HDMS; Waters®, Milford, Massachusetts, USA). Separation was conducted by injecting 7 μL sample on a C 18 column (Acquinity UPLC BEH-C18, ø 2.1 × 50 mm, particle size 1.7 μm) at a flow rate of 250 μL/min. The binary solvent system

composed of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in methanol) was used in a gradient mode (eluent B: 0 min: 30%; 1 min: 30%; 4.5 min: 90%; 8 min: 90%; 9 min: 30%; 3 min equilibration time between runs). Compounds were detected in ESI negative mode with parent ion/daughter ion selections of 137/93 for SA and 141/97 for D4-SA. SA was quantified by using MASSLYNX™ software (version 4.1; Waters) according to peak areas of the respective fragment ions relative to the internal standards.

Statistical analysis

All statistics were performed with R software, version 3.2.3 (R Core Team 2015). Data were graphically checked for normal distribution by using Q-Q plots. Log_2-normalized relative transcript accumulation (RQ), PI and PPO activity data were analysed with Welch *t*-tests, except that log_2-normalized RQs in exp. II were analysed with Mann–Whitney *U* tests to account for the non-normal data distribution. The effect of egg deposition and plant genotype on log-transformed leaf SA content was assessed with two-way ANOVA. We used generalized linear mixed models (function lmer in package lme4; Bates *et al.* 2015) with binomial error distribution and a logit link function to assess the effect of leaf contact (fixed factor in exp. III) or of leaf contact, plant genotype and humidity (fixed factors in exp. VII) or of catalase treatment (fixed factor in exp. XI) on the hatching rates of deposited eggs (ratios of not hatched eggs to hatched eggs) and included the number of deposited eggs as well as the individual plant (exp. III and VII) or the replicate block (exp. XI) as random factors (function glmer in library lme4; Bates *et al.* 2015). The ratios of not hatched eggs to hatched eggs developing on the Friesland genotype with and without a chitosan-induced HR at the oviposition site or no leaf contact were compared with a generalized linear mixed model with binomial error distribution and a logit link function including the number of deposited eggs and the individual plant as random factors. DAB staining intensity of eggs developing on the leaf surface of either the Siethen or the Friesland genotype and of ascorbate-treated eggs (exp. IX) were compared by one-way ANOVA followed by Tukey comparisons (function ghlt in package multcomp; Hothorn *et al.* 2008) between groups. A generalized linear model with quasi-binomial error distribution (to account for overdispersion) and a logit link function was used to test for a dose effect of exogenous H_2O_2 application on egg hatching rates (exp. X).

RESULTS

S. dulcamara strongly responds to insect eggs and increases egg mortality

When we exposed the youngest fully developed leaf of *S. dulcamara* plants to egg deposition by *S. exigua* moths, we frequently observed neoplasm formation and chlorotic tissue beneath the eggs (Fig. 1a,b). Staining of oviposited leaves with DAB revealed massive accumulation of H_2O_2 in the leaf tissue with egg contact (Fig. 1c,d). Additionally, transcript accumulations of the SA-responsive gene *PR1* (*SdPR1*) and the JA-responsive gene *protease inhibitor II* (*SdPI-II*) were increased in oviposited leaves compared with leaves of control plants (Fig. 1e).

As we observed that less *S. exigua* larvae hatched from eggs clutches laid on *S. dulcamara* plants than from clutches laid on paper tissue in our insect rearing, we tested whether leaf contact affects hatching rates of the eggs. The eggs were kept in the same conditions by attaching egg clutches deposited on a synthetic surface in the immediate vicinity of egg clutches laid on the leaf surface. Larval hatching rate from the egg clutches that had leaf contact was reduced (Fig. 1f).

JA-related and SA-related plant responses to oviposition are locally restricted

To elucidate the spatial pattern of *S. dulcamara*'s response to oviposition, we analysed leaf tissue in different distances to the eggs for transcript accumulation of prominent defence-related genes and activity of defence-related proteins. Again, transcript accumulation of *SdPR1* was increased in response to oviposition, but this induction was restricted to the leaf tissue directly beneath the eggs (Fig. 2a). Similarly, transcript accumulation of *SA-methyl transferase* (Supporting Information Fig. S1a) and of two genes under control of JA signalling, *SdPI-I* and *polyphenol oxidase* (*SdPPO*), was increased very locally (Fig. 2b,c). The protein activities corresponding to the latter two genes were induced likewise by the deposition of *S. exigua* eggs (Fig. 2d,e). The induction of all these responses was constrained to the site of egg deposition, and we were not able to detect any systemic response.

Transcriptional responses to oviposition

To comprehensively characterize *S. dulcamara*'s response to insect oviposition, we performed an untargeted transcriptome analysis by using a *S. dulcamara* microarray on RNA from leaf tissue beneath the egg clutches and corresponding tissue of control plants. Overall, 295 contigs were differentially expressed 72 h after *S. exigua* oviposition, 244 of which were induced and 51 repressed (Supporting Information Table S1). To confirm the reproducibility of our microarray data, the induction of six genes by oviposition was verified by qPCR (Supporting Information Fig. S1b). If possible, we assigned oviposition-regulated genes to functional groups (Table 1). Most genes in the functional groups were up-regulated (94%). Many up-regulated genes were related to plant defence responses against herbivores and fungal pathogens, for example, *PIs*, *polyphenol oxidases* and *chitinases*. Genes involved in positive and negative regulations of phytohormonal pathways were represented as well, for example, *lipoxygenase*, *allene oxide cyclase* and *jasmonate ZIM-domain protein 3* (JA-related genes); *suppressor of NPR1-1* and *SAMT* (SA-related genes); *auxin-responsive GH3 product*; and *IAA-amino acid hydrolases* (auxin-related genes). Other components of the plant's signalling network that responded to oviposition included different transcription factors such as ethylene responsive transcription factors, MYB-related transcription factors,

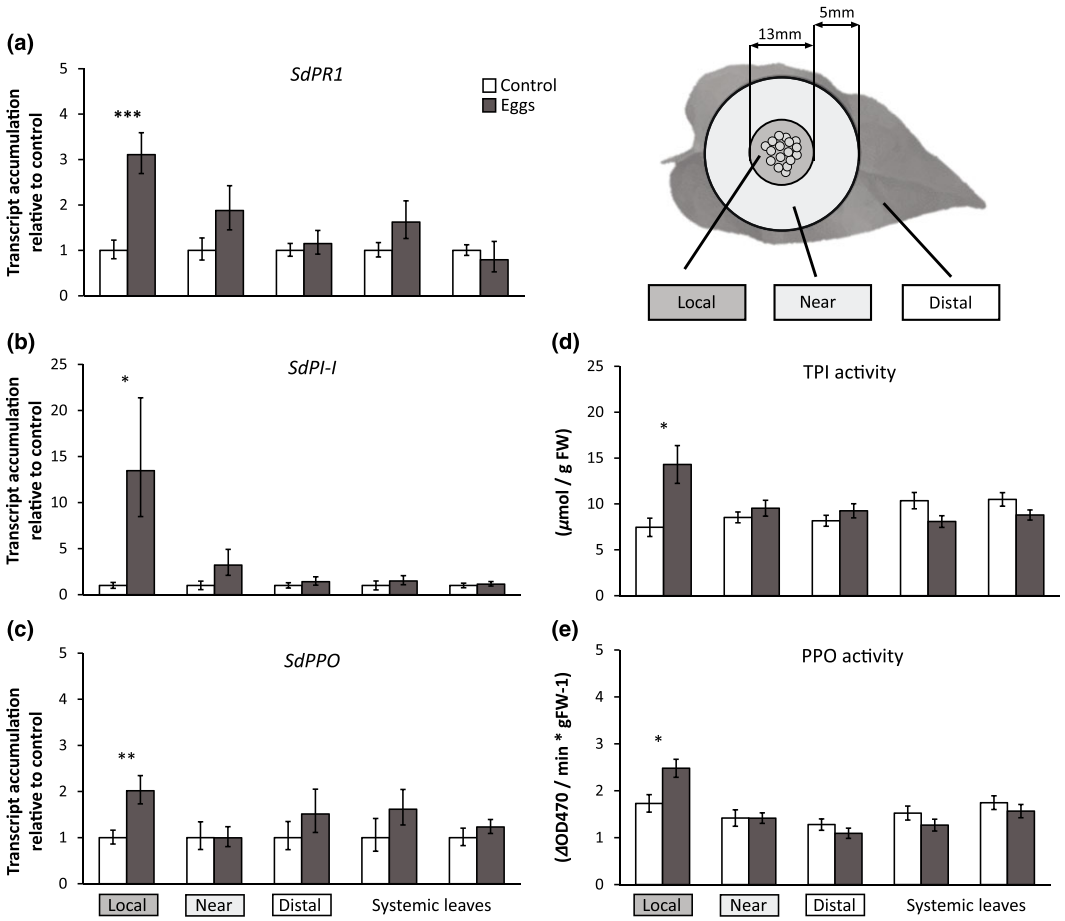

Figure 2. Spatial pattern of jasmonic acid (JA)-related and salicylic acid (SA)-related responses of *Solanum dulcamara* to insect oviposition. (a–c) Relative transcript accumulation (n = 7–8) of the *S. dulcamara* genes *SdPR1*, *SdPI-I* and *SdPPO* normalized to the reference gene *SdELF1*. (d) Activities of trypsin protease inhibitors (TPI) and (e) polyphenol oxidase (PPO; n = 7–16) in leaf tissues at different distances to the *Spodoptera exigua* eggs. Four days after oviposition, the eggs were removed and a leaf disc (Ø 13 mm) from the oviposition site (local), the surrounding tissue (within the next 5 mm; near), the remaining leaf tissue (distal) and the younger leaves 1 and 5 positions above (systemic leaves), and corresponding tissues of control plants were harvested. The bars represent means ± SE, and the asterisks indicate significant differences according to Welch t-tests on either $\log_2$-normalized transcript accumulation or enzyme activities at $P < 0.05/0.01/0.001$ ($^{*}/^{**}/^{***}$).

a WRKY transcription factor, a bHLH transcription factor and different protein kinases. The microarray data further suggested the involvement of peroxidases and phenylpropanoids in *S. dulcamara*'s response to *S. exigua* oviposition. Moreover, genes related to cell growth and cell wall modification were induced and, finally, genes of several primary metabolic processes such as carbohydrate, amino acid and lipid metabolism (Supporting Information Table S1).

Egg hatching rate does not depend on humidity but differs between *S. dulcamara* genotypes with and without chlorosis response to eggs

Of all genotypes we collected from different populations in Germany and the Netherlands, genotypes from Friesland (the Netherlands) did not respond with chlorotic tissue formation at the oviposition site, while genotypes from Siethen (near Berlin, Germany) expressed particularly strong chlorosis (Fig. 3a). To test whether the egg-killing effect of *S. dulcamara* is associated with the chlorosis response and/or mediated by a desiccation of the eggs as suggested for black mustard (Shapiro & DeVay 1987), we tested the effect of leaf contact to both of these genotypes under divergent humidity conditions (Fig. 3b). The hatching rate of egg clutches deposited on the genotype exhibiting strong chlorosis (Siethen) was reduced by 30% compared with adjacent egg clutches without leaf contact (Fig. 3c). This effect was independent of the humidity conditions, which had no effect on hatching rates. Yet, on the genotype without chlorosis (Friesland), the hatching rate of eggs was not affected by leaf contact. However, both genotypes exhibit neoplasms and phytohormone analysis revealed that both genotypes increase SA levels about fivefold in the leaf tissue beneath the eggs (Fig. 3b,d). This suggests that the plant response associated with the formation of chlorotic tissue, which is often termed as an HR-like response, is likely connected to the increased egg mortality and that neoplasm formation and induction of SA alone is not sufficient to kill eggs.

Table 1. List of selected genes regulated after *Spodoptera exigua* oviposition in *Solanum dulcamara*

Gene description	Assignment (AHRD V1)	Accession (comp)	ITAG 2.3 ID (Solyc)	Log2 FC	*P*-value	adj. *P*-value
Peroxidases						
Peroxidase	***-_B9VRK9_CAPAN	5470_c0_seq1	05g052280.2.1	4.70	0.002	0.082
Peroxidase 57	****_D7LXN0_ARALY	213_c0_seq1	09g072700.2.1	4.68	0.002	0.080
Peroxidase	***-_B9VRK9_CAPAN	5470_c0_seq1	05g052280.2.1	3.99	0.004	0.094
Peroxidase	***-_Q58GF4_9ROSI	16346_c0_seq1	03g025380.2.1	3.67	0.000	0.029
Peroxidase	***-_Q58GF4_9ROSI	25595_c0_seq1	03g025380.2.1	3.45	0.000	0.025
Peroxidase	***-_Q9XIV9_TOBAC	12126_c0_seq1	06g050440.2.1	3.18	0.001	0.070
Peroxidase	****_A0S5Z4_SESIN	1631_c0_seq1	02g092580.2.1	2.53	0.000	0.048
Peroxidase	****_Q43499_SOLLC	1005_c0_seq1	01g105070.2.1	2.06	0.000	0.051
Peroxidase 57	****_B6E500_LITCN	2185_c0_seq1	03g044100.2.1	1.53	0.002	0.085
Protease inhibitors (PIs)						
PI-I	***-_Q3S492_SOLTU	460_c0_seq1	09g089510.2.1	5.03	0.000	0.038
Kunitz-type PI-like	***-_Q2XPY0_SOLTU	11494_c0_seq1	03g098760.1.1	4.85	0.000	0.012
Kunitz trypsin inhibitor	****_B8Y888_TOBAC	7122_c0_seq1	03g098740.1.1	3.47	0.005	0.100
PI-II	***-_B3F0C1_TOBAC	10_c0_seq1	11g020990.1.1	1.80	0.004	0.097
Polyphenol oxidases						
Polyphenol oxidase	* *-_Q41428_SOLTU	2630_c0_seq3	08g074680.2.1	6.93	0.000	0.063
Polyphenol oxidase	* *-_Q41428_SOLTU	2630_c0_seq2	08g074680.2.1	2.69	0.004	0.094
Rnases						
Ribonuclease T2	***-_Q6A3R1_SOLLC	21083_c0_seq1	05g007950.2.1	6.92	0.000	0.009
S8-Rnase (fragment)	**--_Q5XPJ2_MALDO	521_c0_seq1	07g006570.2.1	5.92	0.001	0.073
S8-Rnase (fragment)	**--_Q5XPJ2_MALDO	1480_c0_seq1	07g006570.2.1	1.52	0.004	0.094
Transcription factors (TFs)						
WRKY TF 16	* *-_Q7X7E3_ORYSJ	13702_c0_seq1	07g056280.2.1	2.22	0.004	0.094
AP2-like ethylene-responsive TF	* *-_AP2L1_ARATH	15864_c0_seq1	11g008560.1.1	2.03	0.000	0.059
bHLH TF	* *-_B6TXR4_MAIZE	21837_c0_seq1	03g115540.1.1	1.46	0.000	0.055
Ethylene-responsive TF 2b	* *-_C0J9I6_9ROSA	17235_c0_seq4	08g082210.2.1	1.44	0.001	0.076
Ethylene-responsive TF 1A	* **-_A9P6A4_MEDTR	11003_c0_seq1	05g051200.1.1	1.42	0.002	0.085
bHLH TF	* **-_Q401N4_9LILI	10316_c0_seq1	01g096370.2.1	1.40	0.002	0.082
Myb-related TF	** *-_B2CZJ1_CAPAN	3643_c0_seq1	06g083900.2.1	1.36	0.000	0.023
GATA TF 1	* *-_Q0WTQ5_ARATH	4883_c0_seq1	05g056120.2.1	1.30	0.000	0.018
Ethylene-responsive TF	* *-_ERF78_ARATH	20363_c0_seq1	12g008350.1.1	1.24	0.000	0.037
Ethylene-responsive TF 4	* *-_B6THY5_MAIZE	3232_c0_seq1	07g053740.1.1	1.19	0.002	0.085
Ethylene-responsive TF 5	* *-_ERF4_NICSY	17799_c0_seq1	08g081960.1.1	1.12	0.001	0.077
bZIP TF	****_C0LQL1_9CARY	21568_c0_seq1	01g100460.2.1	1.05	0.000	0.054
MYB TF (fragment)	* *--_A1DR85_CATRO	4869_c0_seq1	03g098320.2.1	−1.07	0.002	0.083
Cys2/His2 zinc-finger TF	****_Q4U318_SILLA	18299_c0_seq1	06g074800.1.1	−1.38	0.000	0.055
Ccr4-not complex subunit 7	***-_B4FG48_MAIZE	2456_c0_seq1	06g074030.1.1	−1.44	0.001	0.076
MYB family TF	****_D7KSM0_ARALY	27080_c0_seq1	10g052470.1.1	−1.65	0.005	0.100
Salicylic acid (SA)-related						
SA carboxylmethyltransferase	****_C3VIX6_9ASTR	19930_c0_seq1	09g091550.2.1	4.99	0.001	0.067
Suppressor of NPR1 (SNI1)	***-_Q0ZFU7_SOLTU	14560_c0_seq1	02g077320.2.1	2.98	0.000	0.059
Jasmonic acid (JA)-related						
U-box domain-containing protein 10	**--_PUB10_ARATH	5580_c0_seq7	04g007640.2.1	2.05	0.000	0.012
Protein TIFY3B	*---_TIF3B_ARATH	12355_c0_seq1	12g049400.1.1	1.67	0.002	0.082
Lipoxygenase	****_Q96573_SOLLC	1092_c0_seq1	01g006540.2.1	1.42	0.004	0.097
Alleneoxidecyclase	****_Q9LEG5_SOLLC	955_c0_seq1	02g085730.2.1	1.41	0.000	0.048
JAZIM-domain protein 3	**--_B2XVS2_SOLLC	1508_c0_seq1	01g005440.2.1	1.28	0.000	0.059
Auxin-related						
Auxin-responsive GH3 product	***-_Q05680_SOYBN	19531_c0_seq1	01g107390.2.1	2.06	0.001	0.072
IAA-amino acid hydrolase 9	***-_B9GU29_POPTR	12562_c0_seq1	10g079640.1.1	2.02	0.002	0.083
IAA-amino acid hydrolase	****_D5FTH2_POPTO	2528_c0_seq1	03g121270.2.1	1.06	0.001	0.068
Glycine-rich protein	*---_D7M9Z2_ARALY	28020_c0_seq1	01g099980.2.1	1.97	0.005	0.100
Nodulin-like protein	**--_B6TIX8_MAIZE	11942_c0_seq1	00g052940.2.1	1.30	0.003	0.089
At1g69160/F4N2 9	***-_Q93Z37_ARATH	20148_c0_seq1	05g012030.1.1	−1.57	0.001	0.067
Phenyl-propanoid pathway						
Anthocyanidin synthase	**--_Q2QCX4_GOSHI	10758_c0_seq1	10g076660.1.1	4.03	0.000	0.041
Anthocyanidin synthase	**--_Q2QCX4_GOSHI	10758_c0_seq2	10g076660.1.1	2.89	0.001	0.073
Anthocyanidin 3-*O*-glucosyltransferase	***-_B6SU01_MAIZE	20978_c0_seq1	04g010110.2.1	2.83	0.000	0.056
HCT	**--_B9GF60_POPTR	15183_c0_seq1	11g071470.1.1	2.62	0.000	0.038

(*Continues*)

Table 1. (Continued)

Gene description	Assignment (AHRD V1)	Accession (comp)	ITAG 2.3 ID (Solyc)	Log2 FC	*P*-value	adj. *P*-value
Anthocyanidin synthase	**--_Q2EGB7_MALDO	17100_c0_seq1	10g085190.1.1	2.42	0.000	0.055
Chalcone isomerase	* *- -_B6TFR7_MAIZE	11575_c0_seq1	08g061480.2.1	2.05	0.005	0.100
HCT	*---_Q70G32_SOLLC	1110_c0_seq1	12g096790.1.1	1.33	0.001	0.070
UDP-glucosyltransferase family 1	****_C6KI44_CITSI	10215_c0_seq1	09g092500.1.1	−1.12	0.004	0.099
Cytochrome P450s						
Cytochrome P450		25705_c0_seq1	04g078340.2.1	3.71	0.000	0.048
Cytochrome P450		24670_c0_seq2	03g111300.1.1	3.55	0.000	0.048
Cytochrome P450		27958_c0_seq1	04g078340.2.1	3.29	0.000	0.065
Cytochrome P450		4927_c0_seq1	11g069800.1.1	1.64	0.000	0.021
Cytochrome P450		15490_c0_seq1	04g079730.1.1	1.58	0.000	0.056
Cytochrome P450		15191_c0_seq2	04g079660.2.1	1.47	0.003	0.094
Cytochrome P450		18576_c0_seq1	04g083150.1.1	1.42	0.000	0.021
Cytochrome P450		9977_c0_seq1	07g014670.2.1	1.24	0.000	0.059
Lipid metabolism/transport (FA, fatty acid)						
Diacylglycerol *O*-acyltransferase	***-_A5UW30_ROSS1	21413_c0_seq1	01g095960.2.1	5.16	0.000	0.056
GDSL esterase/lipase At5g03980	***-_GDL74_ARATH	12048_c0_seq2	01g099020.2.1	3.65	0.001	0.070
Fatty acyl coA reductase	***-_Q8L4M0_WHEAT	5067_c0_seq1	11g067190.1.1	3.24	0.005	0.099
GDSL esterase/lipase At1g28590	***-_GDL8_ARATH	702_c0_seq1	12g017460.1.1	2.85	0.000	0.048
Lipase-like protein	***-_Q8LF19_ARATH	21268_c0_seq1	08g022240.1.1	2.63	0.003	0.089
3-oxoacyl-reductase	**--_B6UEX0_MAIZE	18168_c0_seq1	10g078360.1.1	2.57	0.002	0.086
GDSL esterase/lipase At5g42170	***-_GDL90_ARATH	14825_c0_seq1	04g081800.1.1	2.56	0.003	0.089
Lipid transfer protein	***-_A5JUZ7_SESIN	13961_c0_seq1	06g069070.1.1	1.88	0.003	0.090
FA elongase 3-ketoacyl-CoA synthase	****_Q6DUV6_BRANA	5642_c0_seq1	10g009240.2.1	1.38	0.000	0.045
FA oxidation complex subunit alpha	***-_FADJ_ECOHS	6260_c0_seq1	08g068390.2.1	1.32	0.004	0.094
GDSL esterase/lipase At2g04570	***-_GDL34_ARATH	3630_c0_seq1	07g049440.2.1	1.32	0.001	0.073
Tafazzin	***-_C1BN32_9MAXI	12068_c0_seq2	02g068360.2.1	1.30	0.002	0.085
FA elongase 3-ketoacyl-CoA synthase	****_Q6DUV6_BRANA	5762_c0_seq1	10g009240.2.1	1.24	0.000	0.055
Phosphoinositide-spec. phospholipase c	* **_O49950_SOLTU	15819_c0_seq1	06g051620.2.1	1.04	0.000	0.056
Lipid A export ATP-binding/permease	*---_MSBA_CHRVO	10914_c0_seq1	02g087410.2.1	−1.63	0.000	0.059
Protein kinases						
Cys-rich receptor-like protein kinase	***-_C6ZRS1_SOYBN	19515_c0_seq1	12g005720.1.1	2.66	0.002	0.082
Ribosomal protein S6 kinase alpha-6	* **_KS6A6_HUMAN	8569_c0_seq1	07g056400.1.1	2.62	0.001	0.074
Receptor-like kinase		6000_c0_seq1	02g078780.2.1	1.66	0.001	0.076
Serine/threonine/tyrosine kinase	* **_Q9AWA6_ARAHY	19781_c0_seq1	06g082190.2.1	1.32	0.003	0.092
Calcium-dependent protein kinase 3	****_Q7Y050_CAPAN	1823_c0_seq2	01g112250.2.1	1.28	0.005	0.099
Receptor-like protein kinase	****_C6ZRS0_SOYBN	13036_c0_seq3	02g080040.2.1	−1.04	0.004	0.099
Serine/threonine/tyrosine kinase	****_Q9AWA6_ARAHY	22659_c0_seq1	10g045270.1.1	−1.04	0.003	0.092
Serine/threonine kinase receptor	****_Q7DMS5_BRANA	21980_c0_seq1	05g008310.2.1	−1.05	0.001	0.076
Receptor like kinase		16970_c0_seq3	02g071820.2.1	−1.77	0.004	0.099
Aquaporins						
Aquaporin 2	***-_O65357_SAMSA	400_c0_seq1	09g007760.2.1	2.93	0.000	0.055
Aquaporin	***-_Q39956_HELAN	12827_c0_seq1	06g060760.2.1	2.38	0.001	0.076
Aquaporin	***-_D6BRE1_9ROSI	14506_c0_seq1	06g075650.2.1	1.46	0.001	0.077
Aquaporin-like protein	***-_Q8W1A9_PETHY	92_c0_seq2	03g096290.2.1	1.39	0.002	0.080
Defence to fungi						
Polygalacturonase inhibitor protein	***-_Q40160_SOLLC	12914_c0_seq1	09g014480.1.1	4.61	0.000	0.029
Xylanase inhibitor	**--_Q53IQ4_WHEAT	4781_c0_seq1	01g079940.2.1	4.10	0.000	0.048
Chitinase	***-_D0QU15_LACSA	17963_c0_seq1	04g072000.2.1	3.05	0.000	0.048
Xylanase inhibitor	**--_Q53IQ3_WHEAT	6107_c0_seq1	01g079980.2.1	2.44	0.001	0.067
Acidic chitinase	**--_Q71HN4_FICAW	3194_c0_seq1	05g050130.2.1	1.04	0.004	0.094
Cell growth/cell wall metabolism						
Actin	***-_P93775_STRAF	31026_c0_seq1	00g017210.1.1	5.23	0.000	0.018
Cysteine-rich extensin-like protein-2	* *- -_Q08195_TOBAC	4767_c0_seq1	05g053960.2.1	3.80	0.000	0.018
α-1,4-Glucan-protein synthase	** *- _Q2HV87_MEDTR	25120_c0_seq1	02g065740.2.1	1.94	0.001	0.076
Pistil extensin-like protein	* *- -_Q40552_TOBAC	22_c0_seq2	12g098780.1.1	1.66	0.003	0.089
Expansin-like protein	***-_A7X331_SOLLC	4356_c0_seq1	01g112000.2.1	1.60	0.000	0.018
Glucan endo-1,3-beta-glucosidase A6	***-_B6TIF7_MAIZE	3909_c0_seq1	12g014420.1.1	1.54	0.004	0.094
Beta-glucosidase	****_D7L7Z3_ARALY	7005_c0_seq6	09g075060.2.1	1.54	0.000	0.018

(*Continues*)

Table 1. (Continued)

Gene description	Assignment (AHRD V1)	Accession (comp)	ITAG 2.3 ID (Solyc)	Log2 FC	*P*-value	adj. *P*-value
Beta-galactosidase	***-_Q9LLS9_SOLLC	1162_c0_seq1	02g084720.2.1	1.53	0.000	0.047
Laccase-22	**--_LAC22_ORYSJ	1666_c0_seq1	04g082140.2.1	1.36	0.001	0.076
Polygalacturonase	***-_Q9M7D3_SOLLC	10365_c0_seq1	03g116500.2.1	1.25	0.002	0.082
UDP-glucose 6-dehydrogenase	****_B6TBY8_MAIZE	1072_c0_seq3	02g088690.2.1	1.22	0.000	0.056
Os06g0207500 protein	**--_Q0DDQ9_ORYSJ	17972_c0_seq1	12g014360.1.1	1.14	0.004	0.098
Pectinacetylesterase-like protein	*---_Q56WP8_ARATH	2865_c0_seq3	01g102350.2.1	1.12	0.000	0.055
UDP-D-glucose dehydrogenase	***-_D2WK25_GOSHI	1072_c0_seq1	02g067080.2.1	1.12	0.000	0.029
Pectinacetylesterase-like protein	*---_Q56WP8_ARATH	2865_c0_seq1	01g102350.2.1	1.08	0.000	0.021
UDP-D-glucuronate 4-epimerase 1	****_D7MBW0_ARALY	2448_c0_seq1	07g006220.1.1	1.02	0.002	0.085
Pectinesterase	***-_B9T3X5_RICCO	138_c0_seq1	06g034370.1.1	1.01	0.000	0.018
Cell redox homeostasis						
Nucleoredoxin 2	*---_A8I9J4_CHLRE	1019_c0_seq1	05g005460.2.1	1.42	0.000	0.055
Thioredoxin 2	***-_Q5ZF47_PLAMJ	11756_c0_seq1	03g115870.2.1	1.64	0.000	0.040
Protein disulfide isomerase	****_Q6IV17_IPOBA	512_c0_seq1	06g005940.2.1	1.05	0.001	0.076
Thioredoxin/protein disulfide isomerase	** *- _D3TLX2_GLOMM	4244_c0_seq1	07g049450.2.1	1.01	0.001	0.073

Differentially regulated genes [log2-fold change (FC) = 1, $P_{\text{FDR adj.}} < 0.1$] in a microarray analysis ($n = 3$) of leaf tissue beneath *S. exigua* eggs and corresponding tissue of control plants 3 d after oviposition were assigned to functional groups (microarray raw data are available at NCBI Gene Expression Omnibus (GEO accession: GSE100221).
HCT, hydroxycinnamoyl-coenzyme A shikimate/quinate hydroxycinnamoyltransferase.

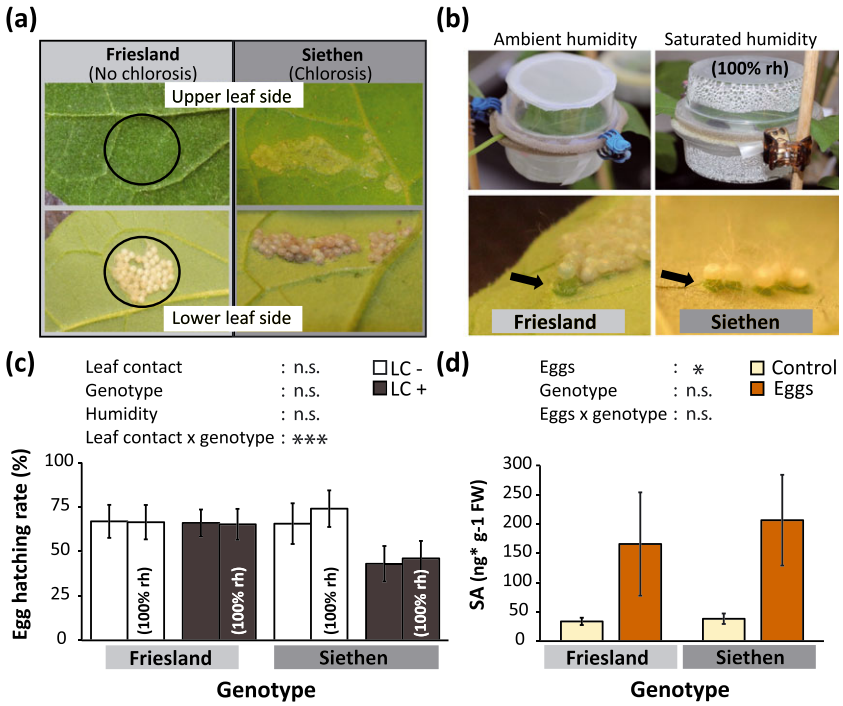

Figure 3. Hatching rates of *Spodoptera exigua* eggs are independent of humidity but differ between *Solanum dulcamara* genotypes. (a) Representative images of the eggs on the lower leaf side and of the respective area of the upper leaf side of *S. dulcamara* genotypes from Friesland and from Siethen, exhibiting no or strong chlorosis at the oviposition sites, respectively. (b) Experimental setup to expose eggs deposited on leaves to either ambient humidity by using vented clip cages or saturated humidity by using closed clip cages and neoplasm formation (arrows) on both genotypes below deposited eggs. (c) Hatching rates of *S. exigua* eggs ($n = 24$) that developed with (+) or without (−) leaf contact (LC) to *S. dulcamara* genotypes from Friesland or Siethen under ambient or saturated humidity (100% r.h.). (d) Levels of SA ($n = 4$–6) in leaves of *S. dulcamara* genotypes from Friesland and Siethen 3 d after *S. exigua* oviposition. The bars represent mean ± SE, and the asterisks indicate significant differences according to generalized linear mixed models (GLMMs) at $P < 0.05/0.001$ ($^{*}/^{***}$).

A chitosan-elicited HR does not affect egg hatching

Consistent with studies on *A. thaliana*'s response to insect eggs (Little *et al.* 2007; Hilfiker *et al.* 2014; Gouhier-Darimont *et al.* 2013), we determined also for *S. dulcamara* an overlap of the plant's response to *S. exigua* eggs and plant responses to pathogens, which raises the question whether similar traits as induced by pathogens may also affect insect eggs. A well-known elicitor of pathogen-induced HR is chitosan that is associated with fungal attack. Therefore, by eliciting pathogen-associated molecular pattern-triggered HR with chitosan at the sites of oviposition, we examined whether an increased mortality of *S. exigua* eggs can be provoked on the Friesland genotype exhibiting no chlorosis in response to oviposition. We applied chitosan to the upper surface of leaves directly opposite the location where *S. exigua* had deposited its eggs on the lower leaf surface, which led to necrotic tissue at the oviposition sites within about 2 d (Fig. 4a). The eggs survived equally well when developing with or without leaf contact on chitosan-treated and untreated leaves (Fig. 4b). Thus, despite overlaps in the plant's response to pathogens and insect eggs, the pathogen-induced HR elicited by chitosan is not ovicidal whereas the egg-induced HR-like response is. Both responses are associated with the formation of ROS but affect the *S. dulcamara* tissue differently. Whereas the tissue at oviposition sites became chlorotic but stayed alive, chitosan treatment resulted in necrosis within less than 2 d. As the necrotic tissue does not produce ROS (Supporting Information Fig. S2a), eggs may only shortly be exposed to ROS on chitosan-treated leaves.

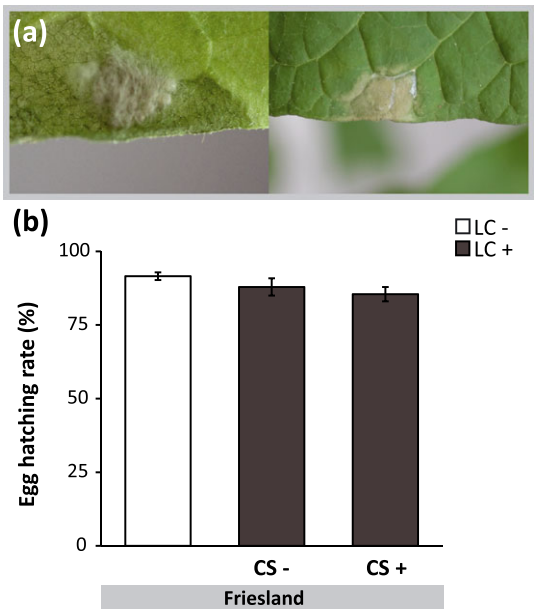

Figure 4. A hypersensitive response elicited by chitosan does not affect hatching rates of *Spodoptera exigua* eggs. (a) At the locations where *S. exigua* eggs were deposited on the lower leaf surface of *Solanum dulcamara* genotypes from Friesland [lacking the hypersensitive response (HR)-like response to insect eggs], chitosan solution was applied to the upper leaf surface and led to necrosis at the oviposition site. (b) Hatching rates of *S. exigua* eggs (mean ± SE, $n = 15$), which developed with (+) and without (−) contact to leaves (LCs), which where elicited by chitosan (CS+) or not (CS−). The hatching rates did not differ between any of the treatments according to a generalized linear mixed model (GLMM; CS: $P = 0.66$, leaf contact: $P = 0.20$).

S. exigua eggs are exposed to ovicidal H_2O_2 produced by *S. dulcamara*

3,3-Diaminobenzidine-staining was used to indicate whether *S. exigua* eggs were exposed to plant-produced ROS. Indeed, eggs on *S. dulcamara* showed H_2O_2 accumulation in contrast to eggs that developed on artificial substrates (Supporting Information Fig. S2b,c). Therefore, we tested whether egg exposure to plant produced H_2O_2 differs between the genotypes, exhibiting no or strong chlorosis in response to oviposition (Friesland, Siethen) by quantifying egg staining intensity. Eggs developing on the Siethen genotype showed stronger staining intensity than eggs developing on the Friesland genotype, while eggs treated with the H_2O_2 scavenger ascorbate showed no staining (Fig. 5a). We then tested whether H_2O_2 can affect egg survival. When eggs were exposed to exogenous H_2O_2, ranging from 1 to 100 mM, egg hatching rates decreased with increasing H_2O_2 concentration from 80 to 60% (Fig. 5b). To make evident that ROS released from the chlorotic tissue at the oviposition sites can reduce egg survival, we regularly added the H_2O_2-decomposing enzyme catalase to egg clutches deposited on the Siethen genotype exhibiting strong chlorosis. The catalase-supplementation was able to significantly increase the egg hatching rate (Fig. 5c).

DISCUSSION

Plant responses to insect oviposition

Solanum dulcamara exhibits various responses to oviposition by *S. exigua*, including visible morphological alterations of the leaf tissue beneath the eggs and the activation of many stress-related or defence-related physiological processes. The plant forms neoplasms in response to the generalist herbivore (Fig. 1), which was previously known for plant responses to specialized herbivores such as in pea and physalis (Doss *et al.* 2000; Petzold-Maxwell *et al.* 2011). Moreover, *S. dulcamara* exhibits chlorosis beneath the egg clutches. Chlorotic and necrotic tissues at the site of egg deposition have been described for several brassicaceous and solanaceous species in their interaction with specialist herbivores (Shapiro & DeVay 1987; Balbyshev & Lorenzen 1997; Little *et al.* 2007; Petzold-Maxwell *et al.* 2011; Fatouros *et al.* 2014). These responses are often assumed to be involved in direct defence against the eggs, and desiccation as well as a reduced attachment of the eggs have been suggested as the mechanisms involved (Balbyshev & Lorenzen 1997; Hilker & Fatouros 2015). Because plants that defend themselves against infections also exhibit chlorosis and necrosis, such plant reactions to insect oviposition are often termed as HR-like responses, but their function in this interaction remains speculative (Hilker & Fatouros 2015).

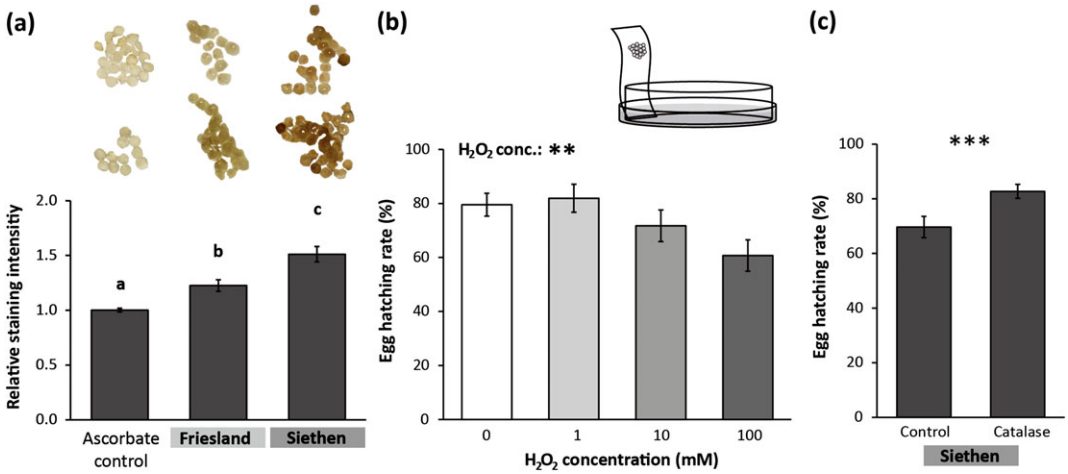

Figure 5. Exposure of *Spodoptera exigua* eggs to hydrogen peroxide depends on the *Solanum dulcamara* genotype and reduces egg hatching rate. (a) 3,3-Diaminobenzidine (DAB) staining intensity of eggs that developed on *S. dulcamara* genotypes from Friesland and Siethen (n = 11 and 15) relative to eggs treated with ascorbate as a hydrogen peroxide scavenger (n = 5, clutches from both genotypes). Images on top of the bars exemplarily depict the stained egg clutches of the first two replicates. (b) Hatching rates of *S. exigua* eggs on filter paper (n = 16–18) that were constantly exposed to hydrogen peroxide (H_2O_2) solutions of different concentrations (0, 1, 10 and 100 mM) during development (for the setup see inset). (c) Hatching rates of *S. exigua* eggs deposited on *S. dulcamara* genotypes from Siethen (n = 13) that were supplemented 3 times a day during egg development with a hydrogen peroxide decomposing enzyme in buffer (catalase) or the buffer only (control). The bars represent mean ± SE, and the significant differences between groups are indicated either by different letters according to Tukey contrasts following an ANOVA or by asterisks according to a generalized linear model (GLM) or generalized linear mixed model (GLMM) at $P < 0.01/0.001$ ($^{**}/^{***}$).

The establishment of pathogen-elicited HR is coupled to the accumulation of H_2O_2 and other ROS. They are components of the plant signalling network and can as such trigger programmed cell death, but they can also directly kill cells when accumulating at high levels (Shetty *et al.* 2008). At the oviposition sites, *S. dulcamara* strongly accumulates H_2O_2 (Fig. 1), which has been previously described for *A. thaliana*, tomato and pine, but only the last species also showed obvious HR-like response to insect oviposition (Little *et al.* 2007; Kim *et al.* 2012; Gouhier-Darimont *et al.* 2013; Bittner *et al.* 2017). Moreover, like *A. thaliana*, *S. dulcamara* accumulated SA and transcripts of SA-related genes such as *PR1* in the tissue beneath the eggs (Figs 2 & 3; Little *et al.* 2007; Bruessow *et al.* 2010). In *A. thaliana*, accumulation of H_2O_2 in response to treatments with egg extract depends on SA biosynthesis *via isochorismate synthase 1* (*ICS1*/*SID2*) under control of *EDS1* activity (*enhanced disease susceptibility 1*; Gouhier-Darimont *et al.* 2013). As the same study found that the egg-induced transcript accumulation of *PR1* was impaired in *sid2* and *eds1* mutants and when Col-0 plants were treated with a ROS scavenger, ROS signalling has been suggested to be involved in mediating plant responses to insect oviposition (Reymond 2013). Yet, *sid2* mutants of *A. thaliana* show no changes of the general transcriptional regulation in response to the eggs of *P. brassicae*, but a similarly strong induction of several defence-related genes as the wild type (e.g. *chitinase*, *trypsin protease inhibitor* and *thioredoxin H-type 5*), suggesting that a large portion of the plant's response to eggs does not require SA-dependent ROS signalling (Little *et al.* 2007).

Besides defence-related responses mediated by SA and ROS, oviposition on *S. dulcamara* locally induced JA-responsive defence genes and the corresponding protein activities (Fig. 2). In *A. thaliana*, JA biosynthesis and JA-responsive genes such as *13-lipoxygenease* (*LOX4*), *terpene synthase* (*TPS4*) and *12-oxophytodienoate reductase* (*OPR3*) are also induced in response to oviposition or treatments with egg extract (Little *et al.* 2007). Yet, the induction of JA-responsive genes by the subsequently feeding larvae is lower on plants that had been previously treated with egg-extract (Bruessow *et al.* 2010). Contrastingly, oviposition by *Helicoverpa zea* on tomato enhanced the JA accumulation in response to subsequently simulated herbivory, and, like oviposition by *S. exigua* on *S. dulcamara*, it directly induced transcript accumulation of a JA-responsive PI gene, which was also locally restricted (Kim *et al.* 2012). Similar to the study in tomato, we found no JA induction by oviposition (data not shown as levels varied around the detection limit of 1 ng/g FW). Parallel to the induction of protease inhibitors, oviposition by *S. exigua* on *S. dulcamara* induced PPO activity. Both protein activities have been associated with anti-nutritive defence against feeding herbivores and also with anti-microbial defence (Constabel & Barbehenn 2008; War *et al.* 2012), however, the role of their induction by insect eggs remains to be determined.

Plant-mediated effects on the insect eggs

We further revealed that *S. exigua* eggs suffered a higher mortality in contact with *S. dulcamara* leaves signifying that the plant expresses ovicidal traits. A plant-derived negative impact on egg performance has been previously described for several plant species, each of which showed at least some of the responses to insect oviposition that we also determined in

S. dulcamara (Shapiro & DeVay 1987; Balbyshev & Lorenzen 1997; Little *et al.* 2007; Petzold-Maxwell *et al.* 2011; Fatouros *et al.* 2014; Bittner *et al.* 2017). Similar to physalis and black mustard, the response strength in *S. dulcamara* underlies genotypic variability, which is related to the negative impact on the herbivore's egg performance. Eggs deposited on *S. dulcamara* genotypes that do not exhibit chlorosis did not suffer increased mortality (Fig. 3). This suggests that the chlorotic response is associated with the plant-mediated egg killing. Our data could not support the common assumption that HR-like necrosis underneath the eggs leads to lethal egg desiccation. The negative effect of leaf contact on egg survival was independent of humidity, and the eggs did not show increased mortality when developing on necrotic tissue elicited by chitosan (Figs 3 and 4). The response of *S. dulcamara* to natural egg deposition does not involve obvious necrosis, but the formation of chlorotic tissue, which is not dead but even shows neoplastic growth. As an HR in the context of plant infections with pathogens is characterized by rapid cell death, the response to eggs may not reflect a 'classic' HR even if both share commonalities such as H_2O_2 formation.

Although H_2O_2 is involved in the establishment of HR (Iriti & Faoro 2009), its formation inevitably stops upon the death of the cells, and accordingly, we did not detect H_2O_2 in the necrotic tissue induced with a pathogen elicitor (Supporting Information Fig S2). We further showed that the *S. exigua* eggs on *S. dulcamara* are exposed to H_2O_2 and that this exposure diverges between *S. dulcamara* genotypes with and without formation of chlorosis underneath the eggs (Fig. 5), which suggests that H_2O_2 is associated with the egg-killing effect. ROS, especially H_2O_2, were suggested as antimicrobial agents during plant defence responses (Legendre *et al.* 1993; Walters 2003; Custers *et al.* 2004) and can be harmful for insect larvae (Bi & Felton 1995; Liu *et al.* 2010), but whether they are toxic to insect eggs was not investigated until now. We showed that exogenous H_2O_2 released from the substrate that eggs are deposited on can reduce egg hatching rates (Fig. 5). However, whether the H_2O_2 concentrations we used resulted in an exposure of the eggs to ROS that is comparable to that of eggs that develop on the leaf surface remains elusive. Because the H_2O_2 applied in the solution undergoes temperature and light-dependent decomposition, we do not know the actual concentration at the egg-substrate interface over time. Moreover, we do not know the H_2O_2 levels on the leaf surface, as those were not determined in plants induced for HR-related responses and the reports on constitutive H_2O_2 levels are wide-ranging (<0.1 mM to >5 mM; Queval *et al.* 2008). Nonetheless, we could support the hypothesis of a negative effect by the plant-derived H_2O_2 on the eggs, as we significantly increased egg survival by supplementing egg clutches laid on genotypes exhibiting strong chlorosis with the H_2O_2-decomposing enzyme catalase. Thus, egg-induced ROS, in particular H_2O_2, may play an important role as a direct defence against herbivore eggs, which fits well together with the suggested role of an egg-protecting catalase activity in the oviduct secretion of the pine sawfly (Bittner *et al.* 2017).

Regulation of the plant transcriptome upon oviposition

More than 80% of the genes with a significantly altered expression in *S. dulcamara* plants that had been oviposited by *S. exigua* moths 3 d earlier were up-regulated (Table 1). A prominent group among these genes are peroxidases, raising the question whether their expression is linked to the H_2O_2 production underneath the eggs. In *A. thaliana*, oviposition of *P. brassicae* induced several peroxidase genes as well (Little *et al.* 2007). Whereas the pathogen-induced H_2O_2 burst that is involved in defence-related signalling is often mediated by NADPH oxidases, namely, respiratory burst oxidase homologues (RBOHs; Kadota *et al.* 2015), none of the four *RBOH* genes annotated on the *S. dulcamara* array responded to *S. exigua* oviposition (Supporting Information Table S1). However, in some plant-pathogen interactions, H_2O_2 production is independent of RBOHs, suggesting a different source of H_2O_2, which has been also previously suggested for the H_2O_2 produced beneath insect eggs (Gouhier-Darimont *et al.* 2013). Alternatively, a reduced catalase activity in the leaf tissue beneath the eggs could also result in H_2O_2 accumulation as has been proposed for pine (Bittner *et al.* 2017). Apoplastic class III peroxidases were identified as alternative sources of H_2O_2 in several plant species (O'Brien *et al.* 2012), and peroxidase-derived H_2O_2 production has been suggested as the major source of ROS during the oxidative burst in *A. thaliana* (Bindschedler *et al.* 2006; Daudi *et al.* 2012). Therefore, peroxidase activity may contribute to H_2O_2 accumulation in leaf tissue beneath the eggs. However, peroxidase expression in *A. thaliana* is independent of SA (Little *et al.* 2007), despite that H_2O_2 formation depends on SA biosynthesis under control of *SID2* and *EDS1* (Gouhier-Darimont *et al.* 2013).

In *S. dulcamara*, SA signalling may not explain the differential response strength to oviposition that we observed between different genotypes because SA levels were equally induced (Fig. 3). Unlike in *A. thaliana*, genes involved in the establishment of systemic acquired resistance like *ALD1*, *FMO1* and *NPR1* were not induced in *S. dulcamara*. Instead, a suppressor of *NPR1*, which is a negative regulator of systemic acquired resistance, was strongly induced (eightfold) upon oviposition. A large group of the oviposition-induced genes in *S. dulcamara* is related to cell growth/cell wall metabolism. Their up-regulation is also contrasting the regulation of such genes in *A. thaliana*. However, opposite to *A. thaliana*, *S. dulcamara* forms neoplasms beneath the eggs, which may be related to the expression of genes related to cell growth/cell wall metabolism as this response requires cell growth. This interpretation would be in line with the fact that auxin dependent non-meristematic cell growth is required for neoplastic growth in *A. thaliana* (Hu *et al.* 2003), and many of these genes are auxin responsive (e.g. *ACT1*, *EXLA1*, *UGD3* and *extensins*). Corresponding to that, several genes coding for enzymes that can hydrolyse Indole-3-acetic acid (IAA)-amino acid conjugates to free IAA were induced by oviposition, while an IAA-transporter was suppressed (Table 1).

Furthermore, our data reveal the transcriptional regulation of other phytohormonal pathways and different signalling

components such as transcription factors and protein kinases in response to the insect eggs. The strongly oviposition induced-expression (>30-fold) of *SAMT*, for example, may be related to the oviposition-induced SA-levels. In *A. thaliana*, its product methyl salicylate was identified as oviposition deterrence for female *P. brassicae* (Groux *et al.* 2014). However, this gene was not found to be egg-induced in *A. thaliana*. Several genes related to JA signalling were induced in oviposited plants, including JA-biosynthesis genes such as *lipoxygenase* and an *allene oxide cyclase* but also JA-responsive repressors of this pathway (e.g. *JAZ* and *PUB10*). However, with the exception of only one *LOX* gene, none of such genes responded to oviposition in *A. thaliana* (Little *et al.* 2007).

Overall, our data suggest that *S. dulcamara*'*s* response to *S. exigua* eggs shows more overlap with the responses of other solanaceous species to oviposition than with that of *A. thaliana*.

CONCLUSION

In this study, we comprehensively characterized *S. dulcamara*'s response to oviposition by a generalist noctuid herbivore and revealed a direct defence mechanism that reduces the hatching rate of the insect's eggs. The plant responded – localized at the site of egg deposition – with formation of neoplasms and chlorotic tissue, H_2O_2 accumulation and with the induction of defence related genes and proteins. We showed that the ovicidal effect of *S. dulcamara* is not mediated by a desiccation of the insect eggs and cannot be elicited by a pathogen-related elicitor of HR. Rather, the insect eggs are exposed to plant-produced H_2O_2, and this ROS directly diminishes egg survival. Thus, in the context of plant responses to insect oviposition, the function of H_2O_2 formation is exceeding a role as signalling component. To what extent ROS other than H_2O_2 may be involved in this direct defence mechanism remains to be determined.

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CONFLICT OF INTEREST

We have no conflict of interests.

AUTHOR CONTRIBUTIONS

AS and DG designed the study. DG performed the experiments, and DG, TL and SS performed chemical and data analysis. DG and AS wrote the first draft of the manuscript, and all authors revised it.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Figure S1. Spatial pattern of *SdSAMT* transcript accumulation in *Solanum dulcamara* after oviposition and qPCR validation for selected genes on the microarray.
Figure S2. Chitosan-elicited necrotic tissue of *Solanum dulcamara* leaves shows minor H_2O_2 accumulation and *Spodoptera exigua* eggs on artificial substrate are not exposed to H_2O_2.
Table S1. Complete list of all differentially regulated contigs in the microarray (separate excel file).
Table S2. Primers used for qPCR of *Solanum dulcamara* genes.
Table S3. Statistical comparison of leaf parameters of oviposited and egg-free *Solanum dulcamara* plants.
Table S4. Statistical models assessing the effects of plant parameters on hatching rates of *Spodoptera exigua* eggs.
Table S5. ANOVAs on data from quantification of salicylic acid in *Solanum dulcamara* leaves and DAB staining intensity of *Spodoptera exigua* eggs.

