Received: 4 January 2017 | Revised: 24 March 2017 | Accepted: 14 April 2017

DOI: 10.1111/1755-0998.12687

RESOURCE ARTICLE

WILEY MOLECULAR ECOLOGY RESOURCES

Transcriptomic responses of *Solanum dulcamara* to natural and simulated herbivory

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Funding information
Deutsche Forschungsgemeinschaft, Grant/Award Number: CRC 973/B2

Abstract

Plants are attacked by diverse herbivores and respond with manifold defence responses. To study transcriptional and other early regulation events of these plant responses, herbivory is often simulated to standardize the temporal and spatial dynamics that vary tremendously for natural herbivory. Yet, to what extent such simulations of herbivory are able to elicit the same plant response as real herbivory remains largely undetermined. We examined the transcriptional response of a wild model plant to herbivory by lepidopteran larvae and to a commonly used herbivory simulation by applying the larvae's oral secretions to standardized wounds. We designed a microarray for *Solanum dulcamara* and showed that the transcriptional responses to real and to simulated herbivory by *Spodoptera exigua* overlapped moderately by about 40%. Interestingly, certain responses were mimicked better than others; 60% of the genes upregulated but not even a quarter of the genes downregulated by herbivory were similarly affected by application of oral secretions to wounds. While the regulation of genes involved in signalling, defence and water stress was mimicked well by the simulated herbivory, most of the genes related to photosynthesis, carbohydrate- and lipid metabolism were exclusively regulated by real herbivory. Thus, wounding and application of oral secretions decently mimics herbivory-induced defence responses but likely not the reallocation of primary metabolites induced by real herbivory.

KEYWORDS
caterpillar oral secretions, elicitation, induced plant defence, microarray, photosynthesis, phytohormone signalling, plant–insect interactions, simulated herbivory

1 | INTRODUCTION

Plants defend themselves against a great variety of herbivorous insects with a range of traits that are constitutively expressed and/or inducible upon herbivore attack (Schaller, 2008). Lima bean for example increases its production of extrafloral nectar to attract ants which defend it from herbivores (Kost & Heil, 2008). Besides such indirect defences, plants produce repellents, antidigestives or toxins that directly deter, slowdown or kill herbivores. For example, tomato and many other plants respond to herbivory with the production of protease inhibitors that block proteolytic enzymes in the gut of herbivorous insects (Green & Ryan, 1972; Jongsma & Bolter, 1997). In addition to such physiological responses, plants can alter morphological parameters like thickness of the cuticle, the density of defensive

trichomes or toughness of their leaves and stems (Clauss, Dietel, Schubert, & Mitchell-Olds, 2006; War et al., 2012). To respond in such a way, plants have to perceive cues associated with the herbivore or with the damage it provoked (Bonaventure, VanDoorn, & Baldwin, 2011; Heil & Land, 2014) and they need to transduce this signal into a phenotypic response.

The key phytohormone mediating the induction of many plant defence traits against herbivores, such as protease inhibitors and extrafloral nectar, is jasmonic acid (JA; Wasternack, 2015). Biosynthesis of the so-called wound hormone JA is elicited upon tissue damage. The signalling function of JA depends on its conjugation to isoleucine (Ile). JA-Ile binds to the COI1 domain of a SFC ubiquitin ligase complex that tags repressor proteins of certain transcription factors for their proteolytic removal. As a consequence, it activates the transcription of JA-responsive genes regulated by these transcription factors.

Yet, plant defence signalling is not solely governed by JA. Herbivore attack commonly elicits various signalling cascades including some that are just beginning to be explored such as signalling via brassinosteroids or strigolactones (De Bruyne, Höfte, & De Vleesschauwer, 2014; Oh et al., 2010; Pandey, Sharma, & Pandey, 2016; Torres-Vera, García, Pozo, & López-Ráez, 2014; Yang, Baldwin, & Wu, 2013). The different phytohormone pathways interact with each other in a complex signalling network, and the activation strength of different hormonal pathways depends on the herbivore species (Lortzing & Steppuhn, 2016; Pieterse et al., 2012). This is because insects of different feeding guilds with distinct damage patterns trigger the plant signalling network in quite diverging ways. For example, phloem-sucking aphids predominantly induce salicylic acid (SA) signalling while leaf-chewing caterpillars mainly induce JA-mediated defences (Appel et al., 2014). But plants can also respond differently to herbivores of the same feeding guild as they perceive not only damage cues but also insect-derived elicitors (Schmelz, 2015). Such elicitors have been found in the regurgitates of several caterpillar species, in beetles and locusts and even in oviduct secretions that insects use to attach their eggs on host plants (Hilker, Kobs, Varama, & Schrank, 2002; Schmelz, 2015).

In addition to these specificities, plants in different environments react divergently to herbivory. The plant response depends on a multitude of abiotic factors like light conditions, water availability and temperature as well as on temporal and spatial factors like the time of the day and the type of tissue that is attacked (Arimura et al., 2008; Heidel-Fischer, Musser, & Vogel, 2014). On the one hand, this is due to the fact that plants regulate their phenotypic appearance in response to diverse environmental factors over the same regulatory network. On the other hand, herbivory imposes physiological stress, such as desiccation, that it shares with other environmental threats. This may explain, the overlapping transcriptomic and metabolomic responses of a plant to chewing herbivores and for example drought (Nguyen et al., 2016). Additionally, the risks of plant fitness losses imposed by herbivory as well as the availability of resources required for defence production depend on the physiological state of the plant that is shaped by diverse factors.

These functional relationships have likely shaped the evolution of the signalling network in plants. After decades of research on plant–herbivore interactions, some very general aspects of plant responses to herbivory, such as the JA signalling pathway, are well-resolved. Yet, we have barely started to untangle the complexity of functional and physiological interactions between the different plant signalling pathways involved in a plant's response to herbivory.

In the light of this complexity, it is obvious that investigating the physiological mechanisms of these interactions requires a high degree of standardization. Whereas it is comparatively simple to control for abiotic and developmental factors, the herbivory itself is more difficult to standardize. In particular, analysis of the early signalling response, which occurs within minutes to a few hours, requires a high degree of control over the spatial and temporal feeding pattern of an herbivore. However, feeding pattern and feeding motivation are not constant and consequently the timing and amount of feeding damage vary tremendously between individuals for many herbivore species. And because experiments with feeding herbivores are demanding in terms of space, time and experimental effort, the feasibility of a biological replication that can account for this variation is limited.

To cope with this dilemma, plant responses to herbivory are often investigated by using treatments that mimic herbivory. Many ecological studies on plant–insect interactions have used application of JA or its methyl ester (MeJA) to induce plant responses normally induced by herbivores (e.g., Thaler, Stout, Karban, & Duffey, 1996; Wu, Wang, & Baldwin, 2008). However, how well exogenous hormone applications match with the endogenous signal strength upon herbivory is usually not determined. Moreover, such treatments ignore the various other hormones that are elicited in concert with JA and modulate the plants response during attack by a real herbivore.

Another frequently used standardized simulation of herbivory is to damage plants mechanically, which elicits endogenous JA signalling and other phytohormonal pathways. Such treatments have been successfully used to study plant defence responses to herbivory, but the degree to which it mimics true herbivory depends on the type and the spatio-temporal pattern of the applied damage (Bricchi et al., 2010; Mithöfer, Wanner, & Boland, 2005). In many cases, the response to mechanical damage was found to be different from that to real herbivory and a range of herbivore-derived elicitors of plant defence responses were identified and characterized during the last decades (Bonaventure et al., 2011).

Two intensively studied elicitors are fatty acid–amino acid conjugates and glucose oxidase activity in the oral secretions (OS) of caterpillars. The application of these elicitors to mechanical wounds can elicit plant responses that are more similar to that of natural herbivory than mechanical wounding alone. This has for example been shown for the production of volatiles that function as indirect defence and for metabolites or proteins involved in direct defence (Alborn et al., 1997; Bonaventure et al., 2011; Giri et al., 2006; Halitschke, Gase, Hui, Schmidt, & Baldwin, 2003; Musser et al., 2002, 2005; Tian et al., 2012; VanDoorn, Kallenbach, Borquez, Baldwin, &

Bonaventure, 2010). Because this form of simulated herbivory allows to control the timing and amount of damage and elicitors, it is commonly used to study the early signalling responses of plants to herbivory (Bricchi et al., 2010; Consales et al., 2011; Ferrieri et al., 2015; Gilardoni et al., 2010; Mattiacci, Dicke, & Posthumus, 1995; Qi et al., 2016; Schäfer, Fischer, Baldwin, & Meldau, 2011). Studies on insect-derived elicitors have mainly focused on their role in modulating the wound response. Therefore, transcriptomes have been usually compared between plants that were either mechanically wounded and plants that were additionally treated with herbivore-derived elicitors, oral secretions (OS) or regurgitate (Consales et al., 2011; Halitschke et al., 2003; Lawrence, Novak, Ju, & Cooke, 2008). Alternatively, transcriptomes were compared between plants fed by herbivores with and without ablated salivary glands (Musser et al., 2012). Most of these studies were conducted on a handful of model species and none of them evaluated how well the simulation of herbivory by adding elicitors to mechanical damage resembles the plants' response to real herbivore attack.

Here, we examined the transcriptional response of the wild plant *Solanum dulcamara* to herbivory by lepidopteran larvae and tested how well the simulation of herbivory by OS application to wounds mimics this transcriptional response.

The bittersweet nightshade *S. dulcamara* is a wild perennial wine, native and widely distributed in Europe, North Africa and Asia but also present in the USA and Canada. As a close relative to tomato and potato, it is increasingly investigated as a model system for its phenotypic plasticity in response to various environmental factors (Visser, Zhang, De Gruyter, Martens, & Huber, 2016). It mostly occurs in wet habitats where it forms adventitious roots in response flooding (Dawood et al., 2014, 2016). It hosts the economically relevant pathogen *Phytophtora infestans* (Golas et al., 2009, 2012), a variety of different generalist and specialist herbivores (Calf & Van Dam, 2012; Viswanathan, Narwani, & Thaler, 2005), expresses inducible defence responses to herbivory such as the production of protease inhibitors (PIs; Nguyen et al., 2016; Viswanathan, Lifchits, & Thaler, 2007) and releases extrafloral nectar from herbivore-inflicted wounds (Lortzing et al., 2016). Studying wild model plants and their natural interactions with herbivores potentially allows to identify and to functionally explore successful traits that plants evolved to cope with herbivores. As *S. dulcamara* is predominantly attacked by specialists (Lortzing et al., 2016), it likely evolved effective defences against generalist herbivores and therefore we aimed to investigate the plant's response to a generalist. Native to Asia and now spread worldwide, the geographic range of the noctuid generalist *S. exigua* is overlapping with the native and invasive distribution of *S. dulcamara*. Its host plants include solanaceous plants and the elicitors in *S. exigua* OS are well characterized (Diezel, von Dahl, Gaquerel, & Baldwin, 2009; Tian et al., 2012).

In order to analyse the transcriptomic responses of *S. dulcamara*, we designed and validated a 60K custom microarray based on a recent transcriptome assembly with about 32,000 contigs representing about 24,000 unigenes (D'Agostino et al., 2013). In a greenhouse experiment, we exposed *S. dulcamara* plants originating from four different natural populations to herbivory by *Spodoptera exigua* caterpillars and compared their transcriptome profile with that of plants that were left undamaged or that were mechanically wounded and supplied with *S. exigua* OS (W + OS). Although the early transcriptional response to the W + OS treatment largely overlapped with the response of *S. dulcamara* to actual herbivory, the downregulation of genes was mainly specific for the response to feeding *S. exigua* larvae.

2 | MATERIALS AND METHODS

2.1 | Plants and insects

We grew *Solanum dulcamara* L. (Solanaceae) plants from stem cuttings of plants that we initially collected from four different populations on lakeshores in the vicinity of Berlin (Erkner: 52°41′88.77″N; 13°77′34.09″E, Grunewald: 52°27′44.37″N; 13°11′24.63″E, Mehrow: 52°34′06.38″N; 13°38′03.97″E and Siethen 52°16′53.65″N; 13°11′18.65″O). Stem segments with two nodes were planted in 0.75-L pots with one node above and one below the soil (Einheits Erde®, type: Profi Substrat Classic, Sinntal-Jossa Germany). About 1 cm of sand (2–3 mm grain size) on top of the soil prohibited propagation of fungus gnats. Plants were grown in the greenhouse under a 16/8 hr light/dark cycle, a photon irradiance between 190 and 250 μmol $m^{-2}s^{-1}$ and ample water supply.

Larvae of *Spodoptera exigua* HÜBNER (Noctuidae) cultured in vented plastic boxes in a climate chamber (24°C, 70% r.h., 16/8 hr light/dark cycle with 50% dimming for 1 h) were fed on a bean flour-based artificial diet (35 g agar-agar, 4 g 4-hydroxybenzoic acid methyl ester, 1 g Wesson salt mix, 1 g, L-(+)-ascorbic acid, 6 g sorbic acid, 1 g L-leucine, 64 g brewer's yeast, 23 g Alfalfa flour pellet, 213 g bean flour, 1 ml maize germ oil, 4 ml of 37% formaldehyde, 20 mg nicotine acid, 10 mg riboflavin, 4.7 mg thiamine, 4.7 mg pyroxidine, 4.7 mg folic acid, 0.4 mg biotin in 1.5 L water). The moths were kept in flight cages and were provided with 20% honey solution and paper tissue as substrate for oviposition.

2.2 | Experimental setup

To determine the transcriptional response of *S. dulcamara* to real and simulated herbivory by *S. exigua*, we used three-week-old plants of four genotypes (Erkner: e_09, Grunewald: x_11, Mehrow: m_04, Siethen s_10). We assigned most similar individuals of each genotype according to size and habitus to six replicate blocks of four plants that were randomly assigned to the treatment groups. The third leaf from the top was selected for the treatments. Treatments and harvest were performed blockwise. While plants of the first treatment group were left untreated, plants of the second received 2 *S. exigua* third-instar larvae that were confined on the leaf in a clip cage. Plants of the third and the fourth treatment group received two rows of puncture wounds on each site of the midvein using a tracing wheel. Immediately, 20 μl of OS was dispersed on these wounds with a pipette (W + OS). The OS was previously collected from third-instar *S. exigua* larvae that had fed on *S. dulcamara* leaf material

with a Teflon tube connected to 2-ml glass vials and a vacuum pump. The collected OS was centrifuged to remove solid particles, diluted twofold with water and was stored until usage at −20°C for a few days. Plants of one W + OS treatment group and half of the untreated control plants were harvested 1 h after treatment application whereas all other plants were harvested 24 hr after treatment application. The treated leaf or a corresponding leaf of control plants was harvested into 2-ml tubes and frozen in liquid N_2. The leaf material was stored at −80°C until extraction.

2.3 | RNA extraction

For the microarray analysis, we ground the leaves under liquid N_2 and pooled 25 mg leaf powder from each of the six treatment replicates per genotype (occasionally, a replicate was lost, e.g., if the larvae fed at the midvein at the leaf base and the leaf dried out, then we pooled 30 mg of five plans). From these pools, we extracted the total RNA with the NucleoSpin® RNA Plant kit (Macherey-Nagel GmbH & Co. KG) according to the manufacturer's instructions using double the amount of RAP lysis buffer. RNA was DNase-digested using TURBO DNA-free™ (AmbionTM) according to the manufacturer's instructions.

For quantitative real-time RT–PCR for a set of selected genes, we used leaf material of the individual plants from a subset of plants including all genotypes. RNA was extracted as described earlier (Oñate-Sánchez & Vicente-Carbajosa, 2008) with minor modifications. About 40 mg of ground leaf material was transferred to 2-ml screw-cap tubes containing 0.5 g Zirconox, 2.8- to 3.3-mm beads (Mühlmeier Mahltechnik, Bärnau, Germany) and homogenized in 600 μl of cell lysis buffer (2% SDS, 68 mM sodium citrate, 132 mM citric acid, 1 mM EDTA) on a FastPrep®-24 instrument (MP Biomedicals, Solon, USA) at 5 m/s for 20 seconds. After centrifugation, we added 200 μl of the protein–DNA precipitation solution (4 M NaCl, 16 mM sodium citrate, 32 mM citric acid) to the supernatant, in order to account for the high protein content of *S. dulcamara* leaves. After centrifugation, the RNA was precipitated by adding 600 μl of isopropanol. The pellet was washed in 70% ethanol, air-dried and resolved in 25 μl of water. RNA integrity was verified by gel electrophoresis, and samples were adjusted to 200 ng/μl according to spectrophotometric measurements (Multiskan™ GO Microwell plate reader).

2.4 | 2.4 cDNA labelling and microarray hybridization

The RNA samples were inspected for concentration, integrity and purity by electrophoretic analysis using the RNA 6000 Pico Kit with the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA, http://www.agilent.com), and all samples had an RNA integrity number between 6.8 and 7.8. Fluorescent cRNA was generated using the Low-Input QuickAmp Labelling Kit (Agilent Technologies) with oligo-dT primers following the manufacturer's protocol. Of the cy3-labelled cRNA, 600 ng were hybridized using the Agilent Gene Expression Hybridization Kit (Agilent Technologies) following the manufacturer's protocol at 65°C for 17 hr on the custom 8 × 60K microarray described below. After the microarray was washed twice, the fluorescence signals on microarrays were detected by the SureScan Microarray Scanner (Agilent Technologies) at a resolution of 3 μm per pixel.

2.5 | Microarray design and validation

In order to design the 60mer oligonucleotides (probes) that represent *S. dulcamara*'s transcriptome on a custom microarray, we first categorized all 32,157 contigs in the *S. dulcamara* transcriptome assembly (D'Agostino et al., 2013) according to their sequence variation into A) unique contigs (less than 90% identity with other sequences), B) contigs with large sequence identity (overlapping sequences of minimal 500 bp and maximal 1.5% mismatches) and C) contigs with moderate sequence identity (overlapping sequences cover less than 70% of the contigs). We generated probe sequences based on DNA nearest-neighbour thermodynamics (SantaLucia, 1998). In a second step, we selected 10–15 probes with homogeneous melting temperatures. These probes were specific for unique contigs (A) and contigs with moderate sequence identity (C) or they were targeting the consensus sequences of contigs with large sequence identity (B). This resulted in 483,851 probes targeting 32,157 target sequences of which 90% were displayed in both orientations on a 1M Agilent array.

The 1M microarray was hybridized with fluorescently labelled samples from total RNA, mRNA and gDNA. For RNA extraction, a pooled sample of various tissues (seedlings, roots, young and senescing leaves, flowers, buds) of 21 *S. dulcamara* accessions (15 Dutch, 6 German) and from differently treated plants (standard conditions, treatments with MeJA, salicylic acid, ethephon and W + OS) was generated. The total RNA was extracted from approximately 100 mg of the different sample tissues as described above for the microarray analysis and then pooled. From 75 μg of the total RNA, mRNA was purified using the Dynabeads™ mRNA Purification Kit (Invitrogen, Thermo Fisher Scientific Inc.) according to the manufacturer's instruction. DNA was extracted from 100 mg ground leaf material (of different *S. dulcamara* accessions) with 800 μl extraction buffer (100 mM Tris/HCL [pH 8.5], 100 mM NaCl, 10 mM EDTA [pH 8.0]) and 800 μl PCI (phenol/chloroform/isoamylalcohol 25:24:1), and after centrifugation, the aqueous phase was treated twice with 800 μl chloroform/isoamylalcohol (24:1) before it was RNase-digested. The DNA was precipitated with 1/10 volume sodium acetate (3M, pH 5.2) and 1 volume isopropanol, washed with 1 ml 70% ethanol and resolved in 50 μl TE buffer.

The hybridizations of the 1M Agilent array with samples from both, total RNA and mRNA, similarly showed higher signals for probes binding towards the 3′ prime end of the mRNA. The sample from mRNA resulted in about 20% less targets with significant signals, and thus, we continued to use total RNA in the experiments. For each target, we selected one to two probes (preferentially in the 1.5 kb 3′ prime end) that provided the strongest signals in the hybridization with the sample from total RNA, or for targets without significant detection in the total RNA sample, in the hybridization with DNA. These probes were represented in the final microarray

design in one orientation if the signals in the RNA hybridization matched the strand prediction for the contigs in the transcriptome assembly (D'Agostino et al., 2013). Nonexpressed targets were represented in both orientations, as were targets that had a lower than twofold difference in the mean signals of probes in both orientations, indicating contradiction in determined and predicted expression. The final custom 8 × 60K Agilent microarray (design ID 048820) consisted of 1319 structural control probes mainly for quality assurance and 60,432 probes targeting 27,504 target sequences of which 5344 target sequences were represented in both orientations resulting in 32,848 targets on the microarray.

2.6 | Microarray data analysis

Data were analysed using the "limma" software packages from Bioconductor in "R" (R Core Team, 2015; Ritchie et al., 2015). For each microarray, twice the fluorescence value of the 90% percentile of nonlabelled hairpin DNA probes (dark-corner spots) was set as limit of detection. About 40% of the probes showed fluorescence values below this threshold in at least one microarray within each treatment group. These probes were considered nonexpressed and removed from further analysis. The remaining data were background-corrected using the "normexp" method and normalized between microarrays using the "quantile" method. Multiple probes matching the same target sequence were averaged. In cases where a probe matched several target sequences with large sequence similarity (B and C categories), the probes were assigned to the longest target sequence. Probes that were spotted and expressed in both orientations (for/rev) were treated as individual targets and not averaged for gene expression analysis, but they were assigned the same GO annotation. Average fluorescence values of the final 18,608 targets were $\log_2$ transformed and fit to a linear model using the "lmFit" function with dual contrasts (control versus each of the three treatments: *S. exigua* herbivory, W + OS 1 h, W + OS 24 hr, and *S. exigua* herbivory versus W + OS after 1 h and after 24 hr). Targets are from here on referred to as genes which were considered significantly differentially expressed if they showed a $\log_2$-fold change of at least 1 (twofold expression change) and p-value <.05 after correction for false discovery rate according to the Benjamini–Hochberg method (Appendix S1).

Gene ontology enrichment in biological processes was performed using a previously described annotation (Nguyen et al., 2016) and the package "TOPGO" (Alexa & Rahnenfuhrer, 2010). The GO distribution in the set of targets that were differentially expressed after *S. exigua* herbivory was compared to the GO distribution of all targets included in the data analysis using the "elim" algorithm at a minimum node size of 20. p-values for the enrichment of each GO term are based on Fisher's exact tests.

2.7 | Confirmation of microarray results via real-time qRT–PCR

The Reverse Transcriptase Core kit (Eurogentec, Seraing, Belgium) was used to synthesize first-strand cDNA from 200 ng RNA in 10 μl reactions according to manufacturer's instructions. Real-time quantitative PCR was performed on a Stratagene™ Mx3005P instrument (Agilent Technologies) in three technical replicates per sample using 1 μl of the cDNA in 10 μl reactions with a SYBR®Green I-based q-PCR kit (Eurogentec, Seraing, Belgium). Melting curves and gel electrophoresis of the PCR product confirmed specificity of the used primers. Measured CT values were normalized to two reference genes (comp28_c0_seq4/EF1a and comp141_c0_seq1/GAPDH see (Nguyen et al., 2016) supplemental material) and to the untreated control. Data were $\log_2$-transformed for statistical analysis.

3 | RESULTS

3.1 | Magnitude of the transcriptional regulation by real and simulated *S. exigua* herbivory

Overall, about 19% of the expressed *S. dulcamara* transcripts were regulated in response to *S. exigua* herbivory (24 hr after onset) or application of its oral secretion to puncture wounds (W + OS, either 1 or 24 hr after the treatment) compared to untreated control plants. Of the 3,512 genes that were differentially expressed in plants exposed to *S. exigua* herbivory compared to untreated controls (full herbivory response), 40% (1,410) also responded in the same direction when comparing the W + OS treatment to the untreated controls, at least at one of the time points ("common response," Figure 1a, Appendix S2). The vast majority of these genes (94%) responded to the W + OS treatment after 1 hr and therefore constituted an early response. But another 29% of the genes (1,005) that responded to *S. exigua* herbivory compared to the untreated control differed also significantly in their expression when comparing *S. exigua*-fed and W + OS-treated plants at both time points (Figure 1a, Appendix S2). The expression of genes in this "*S. exigua*-specific response" did not differ between control and W + OS-treated plants. The expression of almost a third of the genes regulated in response to *S. exigua* herbivory (1,097) differed neither significantly between W + OS-treated plants and *S. exigua*-fed plants nor between W + OS-treated plants and untreated control plants, and thus, it was not clear whether they are specifically regulated by *S. exigua* herbivory or were just not elicited strong enough by the W + OS treatment ("unclear response"). In addition, the W + OS treatment significantly altered the expression of 592 genes compared to untreated control plants that were also different from the expression in plants with *S. exigua* herbivory ("W + OS specific response").

Taken together, the W + OS treatment reproduced at least 40% of the plants' transcriptomic response to *S. exigua* herbivory but clearly failed to induce 29% of the response.

Whereas *S. exigua* herbivory on *S. dulcamara* in general regulated slightly more genes down than up, two-thirds of the genes within the common response were upregulated in comparison with the untreated control (Figure 1b). On the other side, only a third of the genes responding exclusively to *S. exigua* herbivory were upregulated and thus two-thirds downregulated. Consequently, W + OS treatment was able to elicit 63% of the herbivory-induced but only 23% of the herbivory-repressed gene expression, while only 20% of the

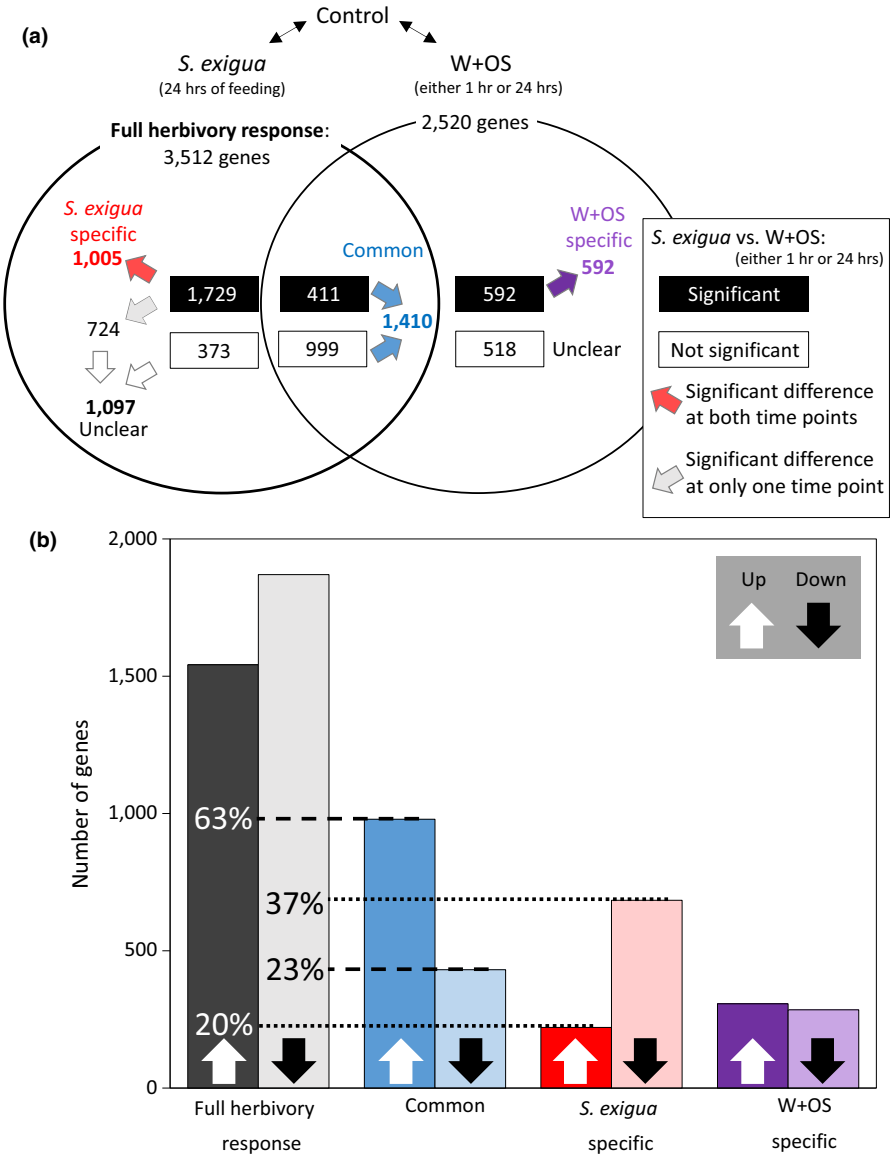

FIGURE 1 Number of differentially regulated genes in response to real and simulated herbivory. (a) Numbers of genes expressed differentially (FC > 2 and p_{adjust} < .05, n = 4) between untreated control plants of *Solanum dulcamara* and either plants exposed to *Spodoptera exigua* feeding for 24 hr (full herbivory response) or plants that had been treated with *S. exigua* oral secretion added to puncture wounds (W + OS) 1 or 24 hr before. Genes that were differentially regulated when comparing *S. exigua* feeding to untreated controls (full herbivory response) were further compared to the W + OS treatments and classified into i) genes that also showed an altered expression after the W + OS treatment at least at one of the time points in the same direction as found after *S. exigua* feeding (common), ii) genes that were expressed significantly different in *S. exigua*-fed and W + OS-treated plants at both time points (*S. exigua* specific). The W + OS treatment also regulated genes that significantly differed from the real herbivory response (W + OS specific). The specificity of genes that did respond to either of the treatments but did not significantly differ in their expression between W + OS-treated and *S. exigua*-fed plants is uncertain (unclear). For the Venn diagram displaying all of the summarized comparisons and more details on the classification, see Appendix S2. (b) Numbers of up- and downregulated genes in these response categories and the percentage of the coverage by the common and *S. exigua*-specific responses of the full herbivory response

upregulated but 37% of the downregulated genes of the response to *S. exigua* herbivory were specific for real larval feeding (Figure 1b).

3.2 | Functional annotation of the transcriptional response to *S. exigua* herbivory

Gene Ontology (GO) term enrichment analysis revealed 90 highly enriched GO terms (p < .0001) for the set of genes that responded to real *S. exigua* herbivory. These 90 terms cover 60% of all the genes that responded to *S. exigua* herbivory. We could classify 81 of these terms into major functional groups (Table 1), and most (25%) belonged to a variety of defence responses, like defence to bacteria, fungi and insects while ~14% are involved in more general stress responses like those to wounding and water and oxidative stress. Moreover, a large part of the terms (15%) was related to the activation of phytohormone pathways, mostly to JA but also to SA,

TABLE 1 GO enrichment in response to *Spodoptera exigua* herbivory

#	GO.ID	GO term description	Genes in the term	Enrichment *p*-value	Number of genes regulated			
					By *S. exigua* herbivory	In common	*S. exigua* specific	W + OS specific
Phytohormonal responses								
1	GO:0009737	Response to ABA	1,720	4.50E−05	437	258	87	81
2	GO:0009753	Response to JA	1,079	4.50E−19	354	215	66	56
3	GO:0009751	Response to SA	1,045	6.30E−10	317	191	68	53
4	GO:0009723	Response to ethylene	880	5.80E−06	239	150	39	40
5	GO:0009863	SA-mediated signalling pathway	787	7.30E−05	227	136	46	36
6	GO:0009738	ABA-activated signalling pathway	698	2.70E−08	194	130	29	32
7	GO:0009867	JA-mediated signalling pathway	622	6.10E−10	188	120	33	32
8	GO:0080167	Response to karrikin	498	3.00E−18	178	97	51	21
9	GO:0009694	JA metabolic process	406	5.20E−12	184	114	34	17
10	GO:0009697	SA biosynthetic process	380	7.00E−07	113	54	38	18
11	GO:0009695	JA biosynthetic process	316	2.30E−23	138	82	29	12
12	GO:0010583	Response to cyclopentenone	225	1.10E−06	74	46	16	12
Defence responses								
13	GO:0019748	Secondary metabolic process	1,367	2.60E−05	413	201	102	50
14	GO:0009620	Response to fungus	1,344	3.20E−10	396	213	90	70
15	GO:0042742	Defence response to bacterium	1,225	3.10E−10	329	158	96	52
16	GO:0009611	Response to wounding	1,165	1.00E−30	386	239	69	48
17	GO:0050832	Defence response to fungus	949	1.40E−12	271	138	64	53
18	GO:0009627	Systemic acquired resistance	914	9.50E−09	277	137	78	35
19	GO:0010200	Response to chitin	846	1.70E−14	254	168	40	50
20	GO:0010363	Regulation of plant hypersensitive response	794	1.40E−09	222	131	46	37
21	GO:0031348	Negative regulation of defence response	528	8.60E−06	143	87	29	28
22	GO:0009862	Systemic acquired resistance, SA-mediated	459	6.60E−08	136	79	33	21
23	GO:0002831	Regulation of response to biotic stimulus	282	3.50E−06	91	54	19	11
24	GO:0002679	Respiratory burst involved in defence response	229	2.40E−07	77	51	12	15
25	GO:0009595	Detection of biotic stimulus	227	1.10E−07	84	50	20	13
26	GO:0009612	Response to mechanical stimulus	196	3.00E−05	62	36	16	11
27	GO:0002237	Response to molecule of bacterial origin	148	5.50E−05	49	33	5	14
28	GO:0046482	para-aminobenzoic acid metabolic process	101	1.60E−05	38	27	5	6
29	GO:0002213	Defence response to insect	91	2.30E−08	41	27	7	1
30	GO:0018874	Benzoate metabolic process	81	2.50E−07	36	22	5	6
31	GO:0009821	Alkaloid biosynthetic process	61	8.40E−06	27	13	9	2
32	GO:0080027	Response to herbivore	41	3.70E−08	24	9	7	2
33	GO:0002215	Defence response to nematode	22	8.80E−07	15	6	6	5
Response to oxidative stress								
34	GO:0055114	Oxidation–reduction process	1,890	4.90E−05	441	167	128	53
35	GO:0010310	Regulation of hydrogen peroxide metabolism	316	9.10E−07	97	52	26	14
36	GO:0042744	Hydrogen peroxide catabolic process	228	1.80E−06	74	20	31	7
37	GO:0071456	Cellular response to hypoxia	97	5.10E−06	38	22	9	6
Response to abiotic stimuli								
38	GO:0009409	Response to cold	1,561	1.90E−05	375	176	115	72
39	GO:0010167	Response to nitrate	347	2.00E−07	107	52	19	20
40	GO:0010114	Response to red light	318	3.40E−07	99	36	35	16

(Continues)

TABLE 1 (Continued)

#	GO.ID	GO term description	Genes in the term	Enrichment *p*-value	Number of genes regulated			
					By *S. exigua* herbivory	In common	*S. exigua* specific	W + OS specific
41	GO:0071497	Cellular response to freezing	31	1.20E−05	17	12	2	2
Response to water stress								
42	GO:0009414	Response to water deprivation	1,283	2.20E−12	370	206	79	66
43	GO:0042538	Hyperosmotic salinity response	474	2.10E−11	152	94	33	33
44	GO:0009269	Response to desiccation	127	1.60E−05	45	26	9	6
Photosynthesis								
45	GO:0015979	Photosynthesis	737	1.80E−09	257	34	130	4
46	GO:0046148	Pigment biosynthetic process	661	6.10E−05	263	83	106	21
47	GO:0009658	Chloroplast organization	545	7.50E−08	186	44	94	11
48	GO:0019684	Photosynthesis, light reaction	539	3.70E−07	184	28	94	3
49	GO:0019288	Isopentenyl diphosph. biosynth. process	395	1.20E−24	164	21	99	6
50	GO:0010027	Thylakoid membrane organization	344	2.80E−26	152	18	88	4
51	GO:0015995	Chlorophyll biosynthetic process	311	1.50E−20	131	33	60	10
52	GO:0010103	Stomatal complex morphogenesis	257	6.60E−13	99	24	49	9
53	GO:0016117	Carotenoid biosynthetic process	240	3.60E−14	97	12	49	3
54	GO:0010207	Photosystem II assembly	239	1.20E−09	86	12	43	1
55	GO:0009902	Chloroplast relocation	211	3.70E−11	82	14	44	4
56	GO:0016226	Iron–sulphur cluster assembly	150	1.40E−06	54	5	37	2
57	GO:0042793	Transcription from plastid promoter	96	3.70E−09	44	4	29	1
Amino acid metabolism								
58	GO:0019344	Cysteine biosynthetic process	449	5.30E−07	130	39	48	10
59	GO:0009073	Aromatic amino acid biosynthetic process	208	8.60E−09	83	29	30	9
60	GO:0015824	Proline transport	134	6.20E−06	48	33	5	4
61	GO:0000162	Tryptophan biosynthetic process	104	3.30E−05	38	21	8	7
Carbohydrate metabolism								
62	GO:0009744	Response to sucrose	525	6.20E−06	143	62	42	18
63	GO:0006098	Pentose phosphate shunt	347	1.90E−18	137	19	68	2
64	GO:0019252	Starch biosynthetic process	298	3.60E−13	111	20	43	6
65	GO:0000023	Maltose metabolic process	273	6.70E−12	101	22	40	6
66	GO:0015976	Carbon utilization	94	1.60E−05	36	9	12	3
67	GO:0016998	Cell wall macromolecule catabolic process	36	3.70E−05	18	5	8	1
68	GO:0010143	Cutin biosynthetic process	26	9.60E−05	14	5	3	1
Lipid metabolism								
69	GO:0006636	Unsaturated fatty acid biosynthetic process	185	3.90E−06	62	17	27	6
70	GO:0000038	Very long-chain fatty acid metabolic process	117	2.20E−05	42	13	19	4
71	GO:0009106	Lipoate metabolic process	116	1.70E−05	42	15	17	6
72	GO:0006655	Phosphatidylglycerol biosynthetic process	108	3.60E−05	39	2	26	1
Phenylpropanoid metabolism								
73	GO:0009813	Flavonoid biosynthetic process	543	6.50E−06	180	105	36	27
74	GO:0009805	Coumarin biosynthetic process	265	5.00E−10	94	48	20	8
75	GO:0009963	Positive regulation of flavonoid biosynthesis	237	5.20E−08	81	53	12	9
76	GO:0009809	Lignin biosynthetic process	200	6.30E−08	71	32	19	6
77	GO:0009718	Anthocyanin-containing compound biosynth.	132	2.20E−07	51	30	11	9
78	GO:0010023	Proanthocyanidin biosynthetic process	38	9.10E−05	18	12	2	1

(Continues)

TABLE 1 (Continued)

#	GO.ID	GO term description	Genes in the term	Enrichment *p*-value	Number of genes regulated			
					By *S. exigua* herbivory	In common	*S. exigua* specific	W + OS specific
RNA/DNA modification								
79	GO:0045893	Positive regulation of transcription	1,116	3.50E−06	276	120	91	35
80	GO:0016556	mRNA modification	180	1.10E−19	93	26	43	1
81	GO:0006598	Polyamine catabolic process	116	4.10E−05	41	21	8	5
Not classified								
82	GO:0019761	Glucosinolate biosynthetic process	386	3.90E−06	119	49	32	14
83	GO:0015706	Nitrate transport	317	2.20E−07	105	51	19	19
84	GO:0045036	Protein targeting to chloroplast	121	9.60E−06	44	10	28	0
85	GO:0042343	Indole glucosinolate metabolic process	68	5.20E−05	35	24	4	2
86	GO:0042939	Tripeptide transport	44	7.80E−05	20	13	3	3
87	GO:0042938	Dipeptide transport	42	3.40E−05	20	12	3	3
88	GO:0009901	Anther dehiscence	38	2.20E−05	19	9	4	1
89	GO:0009759	Indole glucosinolate biosynthetic process	26	9.60E−05	14	10	0	1
90	GO:0006032	Chitin catabolic process	22	7.00E−06	14	2	8	0

Significantly enriched GO terms ($p < .0001$, # refers to the numbers on top of Figure 3) in the set of differentially regulated genes after *Spodoptera exigua* herbivory were classified into major functional groups. The number of genes annotated to each term as well as the number of genes that responded to real *S. exigua* herbivory are given and can be compared to the number of genes that responded to real herbivory and to wounding and the application of *S. exigua* oral secretions (W + OS) in common as well as the ones that responded specifically to *S. exigua* feeding and specifically to W + OS treatment.

abscisic acid, ethylene and strigolactones (karrikin). Another large proportion of the terms (17%) referred to processes involved in photosynthesis and to other primary metabolic pathways such as carbohydrate (9%), amino acid (5%), lipid (5%) and phenylpropanoid (8%) metabolism.

3.3 | Contribution of the W + OS-simulated and the herbivory-specific response to the regulated biological processes

To investigate whether the W + OS treatment is able to elicit certain herbivory-induced biological processes better than others, we calculated for each of the GO terms regulated in response to *S. exigua* herbivory the fraction of the genes that fell either into the common or into the *S. exigua*-specific response (Figure 2). Although, over the whole transcriptome, only 40% of the *S. exigua*-regulated genes were similarly regulated by the W + OS treatment, this proportion of commonly regulated genes was much higher (mostly 50%–70%) within the majority of enriched GO terms related to phytohormonal pathways and defence responses (Figure 2a). Within the enriched GO terms related to photosynthesis, the majority (~40%–70%) of the *S. exigua*-regulated genes responded exclusively to real *S. exigua* feeding, although only 27% of the whole transcriptomic response was *S. exigua* specific. The enrichment in GO terms linked to water stress was also mainly explained by "common response" genes that responded to real herbivory and W + OS treatment alike, while GO terms related to other abiotic stimuli and to oxidative stress did not show such a clear pattern (Figure 2b). GO terms involved in amino acid metabolism could also not generally be assigned to common or *S. exigua*-specific responding genes. However, carbohydrate and lipid metabolism seemed to be affected mainly in a *S. exigua*-specific manner, while the regulation of herbivory-responsive genes associated with phenylpropanoid metabolism was largely explained by genes that were commonly affected by *S. exigua* feeding and by W + OS treatment (Figure 2C).

When considering the direction of the regulation of the herbivory-responsive genes of different functional groups, our data show that genes within the GO terms related to phytohormonal and defence responses were largely upregulated after *S. exigua* herbivory (Figure 3). This upregulation was mirrored quite well by elicitation with W + OS the only exception being GO term 33 "defence response to nematodes," in which a group of four highly homologous peroxidases was downregulated specifically after the W + OS treatment. Genes in GO terms related to photosynthesis, on the other hand, were mostly downregulated, and this downregulation occurred almost exclusively after real *S. exigua* feeding. Genes involved in responses to water stress were mostly upregulated, but genes in GO terms related to other abiotic or oxidative stress showed no uniform regulatory direction (Figure 3). Within the GO terms related to metabolism, the genes involved in phenylpropanoid metabolism were mostly upregulated, but within the amino acid, carbohydrate and lipid metabolism, the direction of the regulation varied between the GO terms. With only few exceptions, gene regulation within predominantly upregulated GO terms could be mimicked by the W + OS treatment but that of downregulated GO terms was rather *S. exigua* specific.

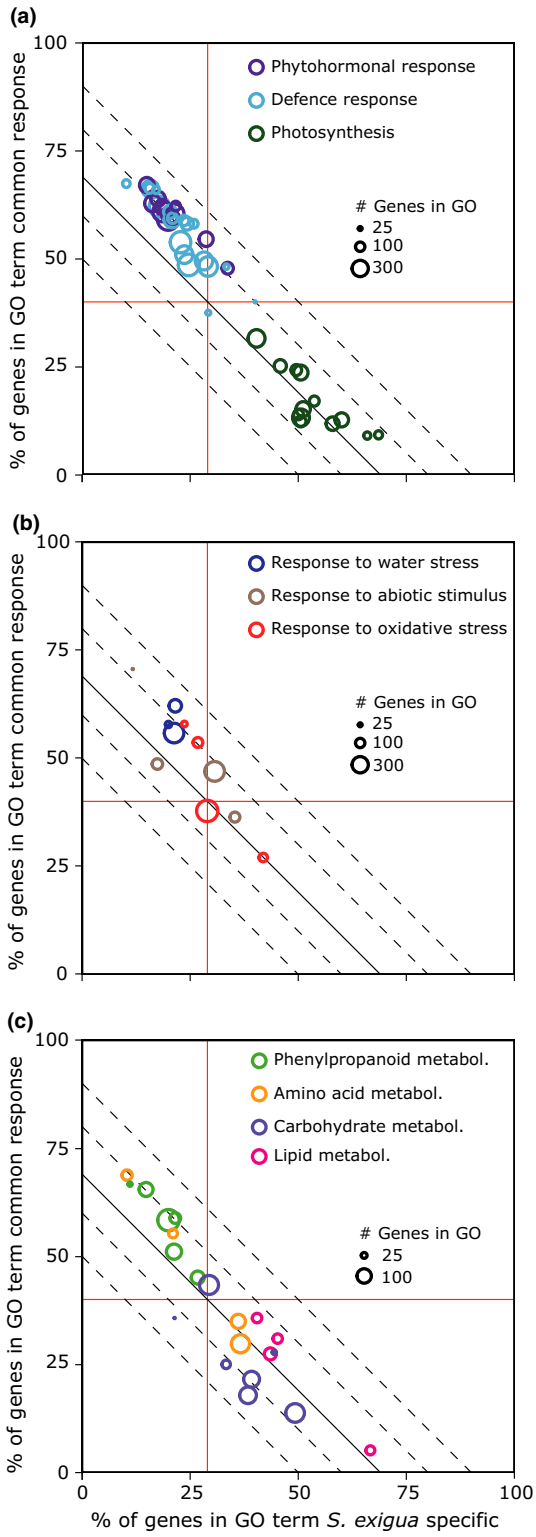

FIGURE 2 Proportion of genes regulated by real and simulated herbivory within herbivory-enriched GO terms. GO terms that are significantly enriched ($p < .0001$) in *Solanum dulcamara* plants fed by *Spodoptera exigua* larvae for 24 hr are depicted as circles of different sizes representing the number of genes regulated by *S. exigua* in each GO term. The GO terms assigned to major functional groups (circle colour) are displayed in three separate sets (a–c) and their positions in the coordinate system indicate the proportion of commonly regulated genes (*y*-axis, responding similar to *S. exigua* feeding as to wounding and application of *S. exigua* oral secretions) and the proportion of *S. exigua*-specific genes (*x*-axis, exclusively regulated by *S. exigua* herbivory) of the herbivory-regulated genes in each GO term (see Figure 1 for the response classification of genes). Orange lines represent these proportions for all herbivory-regulated genes over the whole transcriptome, and GO terms are expected around their intersection if the response specificity is independent from the gene function. Diagonal lines indicate the total amount of the GO enrichment that is explained by the two responses

3.4 | Protease inhibitor gene regulation after real and simulated *S. exigua* herbivory

We directly compared our microarray data with a recent RNA-seq analysis on *S. dulcamara* plants from an independent experiment with *S. dulcamara* genotypes from Dutch populations performed in a different laboratory and harvested after 48 hr instead of 24 hr of herbivory (Nguyen et al., 2016). Our microarray analysis reproduced the enrichment of 57% of the GO terms that were found enriched after *S. exigua* herbivory in the previous study under normal watering conditions despite all differences in the experimental approach and data analysis methodology. However, our analysis found about three times more genes to be significantly induced by *S. exigua* herbivory.

Focusing on genes encoding for protease inhibitors that are involved in antiherbivore defence, a large set of them were found to be inducible by *S. exigua* feeding in the RNA-seq study. In direct comparison of these genes, our microarray data confirmed the inducibility through *S. exigua* herbivory of 10 of 13 of these genes (Table 2). Half of them were affected by the W + OS treatment as well.

4 | DISCUSSION

This study thoroughly examines to what extent a plant's transcriptomic response to herbivory can be mimicked by a common herbivory simulation. Our data show that *S. dulcamara*'s transcriptional responses to *S. exigua* feeding and to a W + OS treatment have a rather large overlap in comparison with studies that contrasted herbivory to wounding (Appel et al., 2014; Ralph, Yueh et al., 2006), but at the same time, it shows that there are still limitations in simulating herbivory by W + OS treatment. Whereas the W + OS treatment mirrored the upregulation of early responsive genes involved in phytohormone signalling, defence, phenylpropanoid metabolism and water stress reasonably well, it clearly failed to elicit the downregulation of genes related to photosynthesis and lipid metabolism as well as the changes in expression of genes related to carbohydrate metabolism.

In general, the transcriptomic response of *S. dulcamara* to leaf-chewing herbivory determined in our microarray analysis is in line with that of other plant species (Heidel-Fischer et al., 2014; Ralph, Oddy et al., 2006; Rodriguez-Saona, Musser, Vogel, Hum-Musser, & Thaler, 2010; Zhou, Lou, Tzin, & Jander, 2015). Considering that the reproducibility of transcriptomics studies even under very controlled conditions and using the same methodology can be rather low

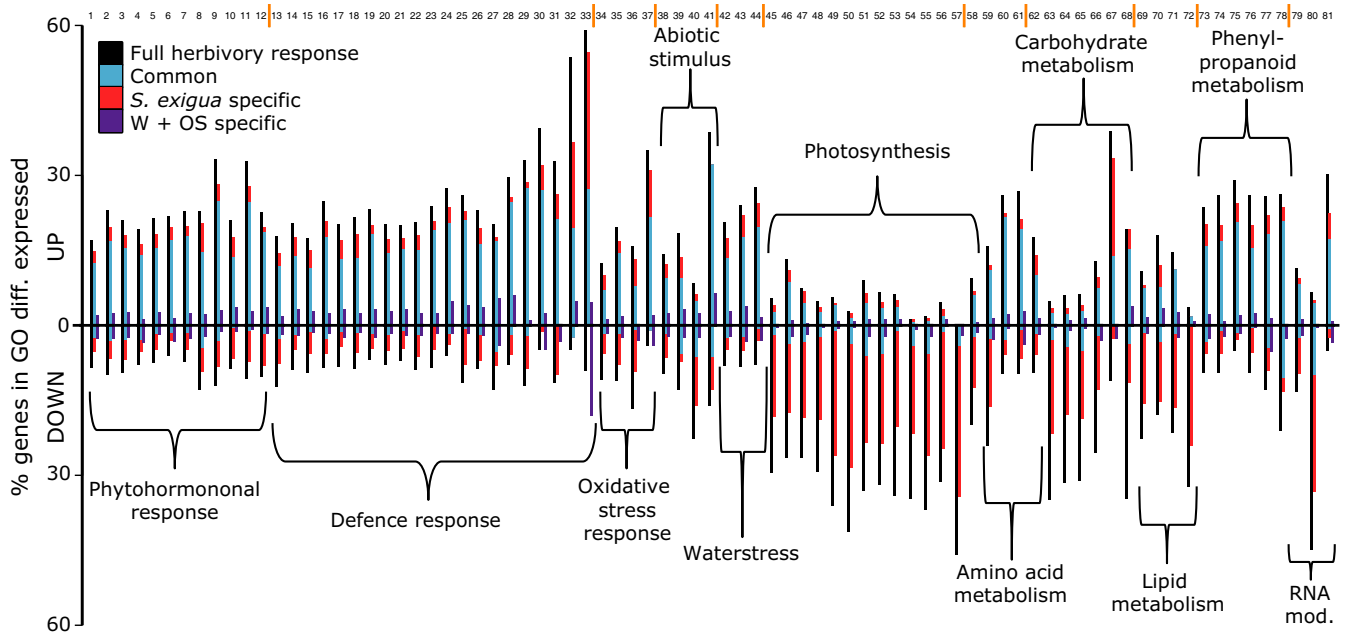

FIGURE 3 Simulated herbivory leads to an up- but not downregulation of herbivory-responsive genes within herbivory-enriched GO terms. Black bars represent the percentage of all *Solanum dulcamara* genes that were significantly up- and downregulated in response to 24 hr of *Spodoptera exigua* feeding in each of 81 GO terms, which were enriched strongest in response to *S. exigua* herbivory (the numbers on top code for the GO-term identity and description given in Table 1). Blue/red staked bars represent the same percentage for either commonly regulated genes (blue) responding similar to *S. exigua* feeding as to wounding and application of *S. exigua* oral secretions (W + OS) or *S. exigua*-specific genes (red), exclusively regulated by *S. exigua* herbivory (see Figure 1 for the response classification of genes). Within each GO term, herbivory regulated only few genes that could not be confidently categorized into either of these two responses (compare black and blue/red bars). Violet bars show fraction of genes in each GO term that is specifically regulated in response to W + OS

TABLE 2 Response of protease inhibitor genes to real and simulated herbivory

Gene ID	FC *S. exigua* 24 hr	FC W + OS 1 hr	FC W + OS 24 hr	ITAG 2.3 ID
comp10_c0_seq1	**40.2**	**2.9**	**9.4**	**Solyc11 g020990.1.1**
comp11494_c0_seq1	**81.3**	7.2	14.2	Solyc03 g098760.1.1
comp4199_c0_seq1	**10.1**	1.5	1.4	Solyc03 g098700.1.1
comp673_c0_seq1	**104.8**	**11.3**	7.2	**Solyc11 g022590.1.1**
comp1295_c0_seq1	**9.6**	0.1	0.1	Solyc07 g007240.2.1
comp255_c0_seq1	**10.0**	0.0	1.1	Solyc03 g098720.2.1
comp1119_c0_seq1	**19.5**	0.0	**5.7**	**Solyc11 g022590.1.1**
comp458_c0_seq1	**n.e.**	**n.e**	**n.e**	**Solyc03 g098710.1.1**
comp460_c0_seq1	**69.1**	**8.9**	**21.3**	**Solyc09 g089510.2.1**
comp251_c0_seq1	0.2	0.0	0.4	Solyc07 g007760.2.1
comp978_c0_seq1	**4.4**	0.1	0.2	Solyc06 g072230.1.1
comp14010_c0_seq1	**1.5**	**1.3**	0.0	**Solyc02 g069470.2.1**
comp1799_c0_seq1	0.1	0.0	0.2	Solyc09 g097850.1.1

All genes listed were inducible in *Solanum dulcamara* in response to *Spodoptera exigua* herbivory in an earlier study (Nguyen et al., 2016). Values represent fold changes (FC) compared to undamaged control plants in plants fed by *S. exigua* for 24 hr or plants 1 and 24 hr after mechanical wounding combined with application of *S. exigua* oral secretions (W + OS). Statistically significant FC values >2 ($p_{adjust} < .05$, $n = 4$) are signified in bold, and Gene IDs and ITAG IDs of genes that were significantly induced by *S. exigua* herbivory and W + OS treatment are highlighted.

(Sanchez, Szymanski, Erban, Udvardi, & Kopka, 2010), our microarray data match well with results from Nguyen et al. (2016), a recent RNA-seq analysis that investigated the transcriptional response of Dutch *S. dulcamara* plants to 48 hr of *S. exigua* herbivory using a very different methodology. Together with the analysis of the expression levels of candidate genes by real-time PCR that confirmed their regulation (Appendices S3 and S4), this provides good validation of the microarray design we developed.

4.1 | Herbivory-induced signalling pathways in *S. dulcamara*

Many of the genes that are mirrored well by the W + OS treatment are involved in signalling (Figures 2, 3). As expected, many genes related to JA signalling and the plant wound response but also those involved in responses to SA, ABA and ethylene were elicited in common by real and simulated *S. exigua* herbivory (Table 1, #1-11). This is largely in line with recent phytohormone measurements in *S. dulcamara* in response to *S. exigua* herbivory, which show an induction of ethylene emission immediately after the onset of feeding as well as elevated levels of JA and ABA two days after continuous *S. exigua* herbivory, although at that time point SA levels were reduced (Nguyen et al., 2016). The OS of *S. exigua* contains at least two classes of elicitors, fatty acid–amino acid conjugates and glucose oxidase (GOX), and GOX activity mediates the induction of SA in the wild tobacco *Nicotiana attenuata* (Diezel et al., 2009). Overall, the responses of *S. dulcamara* and *N. attenuata* to *S. exigua* herbivory seem to parallel each other, as the latter also involves the activation of JA, ethylene and SA signalling.

In addition to phytohormone pathways, we found GO terms related to the respiratory burst that results in the release of reactive oxygen species (ROS) as well as responses to oxidative stress to be enriched in *S. dulcamara* plants fed on by *S. exigua* larvae (#20, #24, #34-37). ROS are involved in many stress responses and are known to act as signalling molecules (Scheler, Durner, & Astier, 2013; Sharma, Jha, Dubey, & Pessarakli, 2012). They are likely also involved in the regulation of plant responses to larval feeding (Kerchev, Fenton, Foyer, & Hancock, 2012; Maffei et al., 2006). The mentioned GOX activity in the OS of *S. exigua* produces hydrogen peroxide, which likely is the active signal of the GOX-mediated effects on defence induction, which can involve the suppression but also the increase of defence responses in tobacco and tomato, respectively (Bede, Musser, Felton, & Korth, 2006; Diezel et al., 2009; Tian et al., 2012). Accordingly, the differential expression of the many genes involved in ROS metabolism may result from both, ROS produced by an endogenous oxidative burst and by the hydrogen peroxide introduced by the caterpillar OS. However, *S. dulcamara*'s regulation of genes related to oxidative stress in response to *S. exigua* feeding was only partially mimicked by the W + OS treatment (Figures 2, 3).

4.2 | Defence-related responses to real and simulated *S. exigua* herbivory

Besides genes involved in signalling, W + OS treatment resembled well the upregulation of many genes related to defence responses after *S. exigua* herbivory. Many genes in the enriched GO terms related to responses to wounding and herbivores may be directly linked to the regulation of the wound-related phytohormone pathways JA and ABA, whereas other GO terms related to responses to other biotic stresses such as defence against fungi, bacteria and nematodes are also enriched in response to *S. exigua* herbivory as well as GO terms related to general or abiotic stress responses (#14-33). This is likely due to the multifunctionality of the phytohormone pathways that are elicited in concert by *S. exigua* herbivory but also of the secondary metabolite pathways they govern. The genes within the enriched GO terms secondary metabolism, alkaloid and phenylpropanoid metabolism (#13, #31, #73-78) are also largely upregulated by herbivory and the W + OS treatment and likely encompass genes that are involved in the production of antiherbivore defence in *S. dulcamara* plants. The defensive functions of alkaloids, phenylpropanoids and other secondary metabolites against insect herbivores are well established in other plants (Mithöfer & Boland, 2012) including other solanaceous plants defending against *S. exigua* larvae (Bandoly, Hilker, & Steppuhn, 2015; Hartl, Giri, Kaur, & Baldwin, 2010; Jassbi, Zamanizadehnajari, & Baldwin, 2010; Steppuhn & Baldwin, 2007). In *S. dulcamara*, 27 genes annotated to alkaloid biosynthesis were regulated by *S. exigua* herbivory and 20 of these were upregulated, suggesting that *S. exigua* in general induces alkaloid production. Also in response to the W + OS treatment, 13 of these 20 genes were upregulated. Saponins and steroid alkaloids are considered as the main chemical defence compounds in the genus *Solanum* (Eich, 2008).

Genes involved in biosynthesis of terpenes were induced by herbivory ($p < .01$, Appendix S5) as well, which is in line with the RNA-seq data of a previous study (Nguyen et al., 2016). Like for alkaloid biosynthesis genes, from 203 terpene biosynthesis genes regulated after *S. exigua* herbivory, a third was part of the common response while another third responded *S. exigua* specific. Here again, 75% of the genes within the common response were upregulated while 75% of genes that responded exclusively to herbivory were downregulated (Appendix S5).

Our data further confirmed the induction of a set of *S. dulcamara* PI genes after *S. exigua* feeding that was reported before (Nguyen et al., 2016). Several of these genes were also induced by W + OS treatment (Table 2). Herbivory-induced PI activity is also well established as a direct defence mechanism (Hartl et al., 2010; Zavala, Giri, Jongsma, & Baldwin, 2008; Zhu-Salzman, Luthe, & Felton, 2008), which is regularly induced by mechanical wounding and by application of OS from various herbivores to plant wounds (Bode, Halitschke, & Kessler, 2013; Green & Ryan, 1972; Orians, Pomerleau, & Ricco, 2000; Yang, Hettenhausen, Baldwin, & Wu, 2011). The W + OS treatment did not fully mimic the induction of all *S. dulcamara* PI genes elicited by real *S. exigua* feeding; however, the contribution to plant defence of the different PI genes remains to be determined as PIs may serve other functions such as the regulation of the plant's own proteases (Hartl, Giri, Kaur, & Baldwin, 2011; Schaller, 2004; Solomon, Belenghi, Delledonne, Menachem, & Levine, 1999).

4.3 | Photosynthesis-related genes responded to real *S. exigua* herbivory

The most striking difference between the W + OS treatment and real *S. exigua* herbivory is the large number of genes that are downregulated after S. *exigua* herbivory but not regulated through the W + OS treatment, neither after 1 hr nor after 24 hr. Most of these genes are related to photosynthesis (#45-57) and one of the largest groups of those genes downregulated exclusively after herbivory belonged to

the nonmevalonate (MEP) pathway (#49). Together with the mevalonate pathway, it is the major source for isopentenyl diphosphate (IPP) which is essential for the biosynthesis of organic pigments such as chlorophyll A (Kim et al., 2013) and carotenoids (Rodríguez-Concepción, 2010) but also serves as the main building block for various compounds involved in plant defence such as sterols and alkaloids. Whereas the secondary metabolite pathways connected to the MEP pathway were for the majority of the genes upregulated, genes in carotenoid and chlorophyll biosynthesis were principally downregulated. Matching the regulation pattern of genes in the MEP pathway, this downregulation occurred only in response to real herbivory but not in response to W + OS treatment (Table 1, #46, #51, #53). These biosynthetic processes take place in the chloroplasts as these pigments are essential for photosynthesis. In fact, multiple genes involved in chloroplast and thylakoid membrane organization and other photosynthesis-related genes were downregulated exclusively after *S. exigua* feeding (Figure 3). A reduced photosynthesis in response to herbivory is consistent with an array of previous studies on the effects of herbivory, but it was also found after mechanical wounding and other biotic stresses on different plants (Bilgin et al., 2010; Heidel-Fischer et al., 2014; Ralph, Yueh et al., 2006; Rodriguez-Saona et al., 2010; Tang et al., 2006). In a previous study on *S. dulcamara*, drought stress dominated the downregulation of photosynthesis genes, but it was also observed after *S. exigua* herbivory (Nguyen et al., 2016). The particularly strong effect of drought stress on photosynthesis is likely related to the accompanying stomatal closure which reduces availability of CO_2. The repression of photosynthesis by herbivory has been suggested to partially result from a water stress response of the plant due to local desiccations at the wound sites (Tang et al., 2006). Also in *S. dulcamara*, we found responses to water stress enriched after herbivory. Opposite to the downregulation of photosynthesis genes, this was well mirrored by the W + OS treatment which contradicts the idea that a reduction in photosynthesis is just a secondary effect of desiccation of the wounded tissue.

In concert with the downregulation of the MEP pathway, expression of genes in the pentose phosphate pathway (#63) was reduced in *S. dulcamara* exclusively after *S. exigua* feeding. This pathway metabolizes assimilates and is as such connected to the MEP pathway, as it provides the building blocks for the IPP biosynthesis. But it is also connected to other pathways like starch biosynthesis and maltose metabolism (#64, #65) that are downregulated specifically by *S. exigua* herbivory. A matching downregulation of many genes involved fatty acid and lipid metabolism (#69-72) suggests that the downregulation of genes related to photosynthesis is likely part of a global reprogramming of the plants' primary metabolism in the attacked leaf.

It is frequently assumed that the energy and resources that are invested in the production of plant defence are reallocated at the expense of the photosynthetic machinery and other primary plant metabolism, which are therefore downregulated (Zhou et al., 2015). However, as W + OS treatment reasonably mimicked the herbivory-induced defence response but barely the herbivory-induced downregulation of photosynthesis, a reduction of photosynthesis may not be a prerequisite to produce defence. Instead, it could be a supplementary strategy of the plant to optimize its response to the herbivore. For example, plants might shift their resource investment in the photosynthetic machinery towards noninfested plant parts and thus optimize their overall photosynthetic capacity (Heidel-Fischer et al., 2014; Schwachtje & Baldwin, 2008; Zhou et al., 2015). In addition, plants may benefit from the reduced nutritional value of the attacked leaf when less photosynthetic proteins are available (Mitra & Baldwin, 2008), which could even act synergistic with the production of toxic and antidigestive metabolites.

4.4 | What differentiates W + OS treatments from real herbivory?

Similar to our data that clearly suggest that the downregulation of photosynthesis in *S. dulcamara* is specific for real *S. exigua* herbivory and cannot be mimicked by W + OS, a proteome analysis in potato revealed a lower expression of photosystem proteins, which was specific for the response to herbivory by the colorado potato beetle and could not be mimicked by wounding (Duceppe, Cloutier, & Michaud, 2012). However, application of these beetles regurgitate to wounded leaves of potato facilitated the repression of several photosynthesis-related genes (Lawrence et al., 2008). Yet, how this regulation compares to real herbivory was not assessed and also in *S. dulcamara*, a small set of the genes in photosynthesis-related GO terms were downregulated by W + OS though this accounted only for a minority of herbivory-repressed genes. In tomato, feeding by *Helicoverpa zea* caterpillars with functioning salivary glands resulted in stronger downregulation of photosynthesis-related genes than feeding of caterpillars with ablated salivary glands suggesting elicitors of this response in the saliva (Musser et al., 2012). Yet, fewer genes related to photosynthesis were downregulated by wounding and this was not clearly changed by the application of salivary gland extract suggesting that this mimic treatment was also not sufficient to elicit the photosynthesis-related response of tomato to herbivory. Altogether, it seems like the downregulation of photosynthesis may be a response to herbivore-derived cues that is not fully mimicked by application of elicitor containing secretions to artificial wounds.

The reasons why such simulations of herbivory fail to elicit most of the downregulation of genes after herbivory may be based in different temporal and spatial patterns in which the plants are exposed to the damage and elicitors. Whereas we elicited the plant with a one-time wounding using a pattern wheel leaving small puncture wounds spread over the leaf blade, *S. exigua* larvae repeatedly take small bites of the leaf before pausing for a while, resulting in a series of small holes fed into the leaf blade. This inevitably leads to increasing amounts of leaf tissue loss over time and repeated induction of plant responses due to the release of damage and herbivore-associated molecular patterns with every caterpillar bite. While the amount of lost leaf tissue after 24 hr of feeding by two *S. exigua* larvae was still rather small and quite comparable to the leaf damage inflicted by our puncture wheel, it is well known that the spatial and temporal pattern of inflicted damage influences a plant's response (Mithöfer et al., 2005). In addition, the amount and composition of the applied OS may not reflect the

combination of elicitors a plant is exposed to during feeding. Plants can perceive more elicitors from insects than the above-mentioned fatty acid–amino acid conjugates and GOX that are well known for *S. exigua*. Wild tobacco, for example, perceives the product of its own herbivory-induced enzyme αDOX-1 from the OS of a caterpillar feeding on the plant (Gaquerel, Steppuhn, & Baldwin, 2012). As this oxylipin-forming enzyme is not active in the leaf tissue but instead in the insect's gut, its product is only formed during feeding and it elicits a modulation of the plant's defence response. Considering the multitude of not completely resembled influential factors, it is astonishing that the W + OS treatment successfully mimicked between 50% and 70% of the enriched GO terms related to phytohormones and defence.

5 | CONCLUSION

Taken together, our results suggest that a one-time W + OS treatment is a suitable method to induce plant defence responses in a standardized manner. But since this treatment failed to elicit the downregulation of photosynthesis and other related pathways in primary metabolism that are associated with real herbivory, it is likely not adequate to elucidate ecological consequences of herbivore attack in a natural setting. On the one hand, this stresses the need for more comprehensive investigations of methodologies we use to standardize our experiments as well as for other tools that allow us to standardize the induction of plant responses by herbivory. On the other hand, the characteristics of the W + OS treatment could provide the opportunity to study the consequences of plant defence responses uncoupled from the large-scale reprogramming of the plants primary metabolism that is associated with a real herbivore attack, which may be especially useful for example when elucidating costs and benefits of induced defence responses.

ACKNOWLEDGEMENTS

We thank Steffi Parlitz for RNA and DNA extractions for the initial 1M array; Daniel Geuß, Michele Bandoly and Sylvia Drok for help during the experiment; Norbert Bittner for valuable advice; and the DFG for funding (CRC 973/B2).

AUTHOR CONTRIBUTIONS

T.L. and A.S. designed and performed the experiment; T.L. and V.F. conducted the microarray data analysis; D.N. and I.R. annotated the *S. dulcamara* transcriptome; M.S., J.K. and A.S. designed the microarray; S.S. and T.L. processed samples and performed real-time qRT–PCR; T.L. and A.S. wrote the first draft of the manuscript which was revised by all authors.

DATA ACCESSIBILITY

The design and the experimental data of the microarray are available at NCBI Gene Expression Omnibus (GEO Accession: GSE97043).

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SUPPORTING INFORMATION

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

How to cite this article: Lortzing T, Firtzlaff V, Nguyen D, et al. Transcriptomic responses of *Solanum dulcamara* to natural and simulated herbivory. *Mol Ecol Resour*. 2017;17: e196–e211. https://doi.org/10.1111/1755-0998.12687