**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 larva’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

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**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, & Duffy, 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; Halitsche, 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; Hallitschke 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 to flooding (Dawood et al., 2014, 2016). It hosts the economically relevant pathogen Phytophthora 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, Litschis, & Thaler, 2007) and releases extraloral 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″E; 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 (Einheitspfannen, 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⁻²s⁻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 pyridoxine, 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 N2. The leaf material was stored at –80°C until extraction.

2.3 | RNA extraction

For the microarray analysis, we ground the leaves under liquid N2 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 (Onate-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 Maltechnik, 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 the RNA. DNA was extracted from 100 mg ground leaf material (of different S. dulcamara accessions) with 800 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 instructions. 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/isooamylic alcohol 25:24:1), and after centrifugation, the aqueous phase was treated twice with 800 µl chloroform/ isooamylic alcohol (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 probe 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 log2 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 log2-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 genes responding exclusively to S. exigua herbivory compared to untreated controls (full herbivory OS treatment) (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
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,
| # | 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 |
|---|---------|--------------------------------------------|-------------------|--------------------|----------------------------|------------------------|------------|-------------------|-----------------|
| 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                |                 |
| 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:009611 | Response to wounding                      | 1,165             | 1.00E⁻³⁰           | 386                        | 239                    | 69          | 48                |                 |
| 17| GO:0005032 | Defence response to fungus                | 949               | 1.40E⁻¹²           | 271                        | 138                    | 64          | 53                |                 |
| 18| GO:009627 | 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:009862 | Systemic acquired resistance, SA-mediated | 459               | 6.60E⁻⁰⁸           | 136                        | 79                     | 33          | 21                |                 |
| 23| GO:002831 | 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:009595 | Detection of biotic stimulus              | 227               | 1.10E⁻⁰⁷           | 84                         | 50                     | 20          | 13                |                 |
| 26| GO:009612 | Response to mechanical stimulus            | 196               | 3.00E⁻⁰⁵           | 62                         | 36                     | 16          | 11                |                 |
| 27| GO:0002337 | 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:008027 | 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                 |                 |
| 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                 |                 |
| 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)
| #  | 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                 |                 |
| 42 | GO:0009414 | Response to water deprivation      | 1,283             | 2.20E −12          | 370                       | 206                    | 79         | 66                |                 |
| 43 | GO:0042538 | Hypersomatic salinity response     | 474               | 2.10E −11          | 152                       | 94                     | 33         | 33                |                 |
| 44 | GO:009269  | Response to desiccation            | 127               | 1.60E −05          | 45                        | 26                     | 9          | 6                 |                 |
| 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:009902  | 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                 |                 |
| 58 | GO:0019344 | Cysteine biosynthetic process     | 449               | 5.30E −07          | 130                       | 39                     | 48         | 10                |                 |
| 59 | GO:009073  | 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                 |                 |
| 62 | GO:0009744 | Response to sucrose               | 525               | 6.20E −06          | 143                       | 62                     | 42         | 18                |                 |
| 63 | GO:006098  | 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:000023  | 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                 |                 |
| 69 | GO:006636  | Unsaturated fatty acid biosynthetic process | 185        | 3.90E −06          | 62                        | 17                     | 27         | 6                 |                 |
| 70 | GO:000038  | Very long-chain fatty acid metabolic process | 117        | 2.20E −05          | 42                        | 13                     | 19         | 4                 |                 |
| 71 | GO:009106  | Lipoate metabolic process         | 116               | 1.70E −05          | 42                        | 15                     | 17         | 6                 |                 |
| 72 | GO:006655  | Phosphatidyglycerol biosynthetic process | 108        | 3.60E −05          | 39                        | 2                      | 26         | 1                 |                 |
| 73 | GO:0009813 | Flavonoid biosynthetic process    | 543               | 6.50E −06          | 180                       | 105                    | 36         | 27                |                 |
| 74 | GO:009805  | Coumarin biosynthetic process     | 265               | 5.00E −10          | 94                        | 48                     | 20         | 8                 |                 |
| 75 | GO:009963  | Positive regulation of flavonoid biosynthesis | 237        | 5.20E −08          | 81                        | 53                     | 12         | 9                 |                 |
| 76 | GO:009809  | Lignin biosynthetic process       | 200               | 6.30E −08          | 71                        | 32                     | 19         | 6                 |                 |
| 77 | GO:009718  | 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                 |                 |
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         |
| 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 |
| 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:0007959 | Indole glucosinolate biosynthetic process | 26 | 9.60E–05 | 14 | 10 | 0 | 1 |
| 90 | GO:006032 | Chitin catabolic process      | 22               | 7.00E–06            | 16 | 4 | 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.
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...
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.

**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-α-mono 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 (Schler, 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-Conception, 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 (Bliggin 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 CO2. 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 down-regulation 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 (Duceptpe, 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 (Misser 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 \textit{xDOX-1} from the OS of a caterpillar feeding on the plant (Gaquerel, Steppuhn, & Baldwin, 2012). As this oxylin-pin-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 standard-ized 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.

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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 \textit{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.

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