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# An ecogenomic analysis of herbivore-induced plant volatiles in *Brassica juncea*

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## Abstract

Upon herbivore feeding, plants emit complex bouquets of induced volatiles that may repel insect herbivores as well as attract parasitoids or predators. Due to differences in the temporal dynamics of individual components, the composition of the herbivore-induced plant volatile (HIPV) blend changes with time. Consequently, the response of insects associated with plants is not constant either. Using *Brassica juncea* as the model plant and generalist *Spodoptera* spp. larvae as the inducing herbivore, we investigated herbivore and parasitoid preference as well as the molecular mechanisms behind the temporal dynamics in HIPV emissions at 24, 48 and 72 h after damage. In choice tests, *Spodoptera litura* moth preferred undamaged plants, whereas its parasitoid *Cotesia marginiventris* favoured plants induced for 48 h. In contrast, the specialist *Plutella xylostella* and its parasitoid *C. vestalis* preferred plants induced for 72 h. These preferences matched the dynamic changes in HIPV blends over time. Gene expression analysis suggested that the induced response after *Spodoptera* feeding is mainly controlled by the jasmonic acid pathway in both damaged and systemic leaves. Several genes involved in sulphide and green leaf volatile synthesis were clearly up-regulated. This study thus shows that HIPV blends vary considerably over a short period of time, and these changes are actively regulated at the gene expression level. Moreover, temporal changes in HIPVs elicit differential preferences of herbivores and their natural enemies. We argue that the temporal dynamics of HIPVs may play a key role in shaping the response of insects associated with plants.

**Keywords:** gene expression, green leaf volatiles, mustard, parasitoids, *Spodoptera*, sulphides

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## Introduction

Plant volatiles play an important role in a range of ecological processes in natural communities. These include

mediating interactions with neighbouring plants, herbivores, carnivores, mutualists and microbes (van Dam 2009). The composition of the emitted volatiles changes qualitatively and/or quantitatively upon insect herbivory (Dicke & Vet 1999; Paré & Tumlinson 1999; Arimura *et al.* 2009; Dicke & Baldwin 2010). These so-called herbivore-induced plant volatiles (HIPVs) attract the herbivores' natural enemies, deter other herbivores

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from ovipositing on the plant and prime neighbouring plants (Dicke & Baldwin 2010). Herbivores and their natural enemies rely on the specificity of the HIPV blend to increase their chances of finding a suitable host plant or prey (Vet 1999; Dicke & Baldwin 2010). For instance, they play an important role in enabling parasitoid wasps to locate their preferred host species and the most suitable larval stage for the development of their offspring (De Moraes *et al.* 2001; Holopainen 2004; Vuorinen *et al.* 2004; Gols *et al.* 2011). Many laboratory studies have demonstrated that HIPVs make plants more attractive to host-seeking natural enemies (van Poecke *et al.* 2003; Turlings & Wäckers 2004; de Boer *et al.* 2008; Gols *et al.* 2012), and field studies corroborate the role of HIPVs in the attraction of natural enemies towards induced plants (De Moraes *et al.* 1998; Kessler & Baldwin 2001; James & Grasswitz 2005).

Herbivore-induced plant volatile blends are chemically very diverse. Globally, the compounds in these blends can be categorized into three classes according to their biosynthetic origin (Arimura *et al.* 2009): (i) fatty acid-derivatives, called green leaf volatiles (GLVs), which are C<sub>6</sub> aldehydes, alcohols and their derivatives, as well as jasmonic acid (JA), which is produced by the lipoxygenase (LOX) pathway. GLVs are produced via the hydroperoxide lyase (HPL) pathway, which is a component of the LOX pathway; (ii) terpenoids, which are synthesized via the mevalonate and the nonmevalonate (also called as methylerythritol phosphate or MEP) pathways; (iii) aromatic volatiles derived from the shikimic acid pathway, such as indole and methyl salicylate (Holopainen 2004; Conti *et al.* 2008). Once the basic skeleton of these small-molecular-weight compounds is produced, their diversity is further increased by modifications such as acylation, methylation, oxidation/reduction and cyclic ring closure. Such modifications often result in increased volatility and changed olfactory properties (Pichersky *et al.* 2006).

One of the best studied plant families in terms of HIPV composition and function is Brassicaceae (Hopkins *et al.* 2009). This family includes many important crop species, such as various cultivars of mustard and cabbage, as well as a large diversity of wild species with a worldwide distribution (Warwick *et al.* 2006; Franzke *et al.* 2011). Brassicaceae are characterized by the production of secondary metabolites known as 'glucosinolates' (Hopkins *et al.* 2009). Upon herbivory, glucosinolates are hydrolysed by the enzyme myrosinase and yield volatile products such as nitriles and (iso) thiocyanates, depending on the reaction conditions (Wittstock *et al.* 2003). These hydrolysis products are associated with direct plant resistance strategy against various insects and pathogens (van Dam *et al.* 2009; Gols & Harvey 2009; Hopkins *et al.* 2009; Mathur *et al.*

2011), but can also serve as cues to natural enemies specialized on *Brassica* herbivores (Smid *et al.* 2002; Gols *et al.* 2008, 2011). The glucosinolate hydrolysis products can be further degraded, thus forming additional volatile sulphur compounds (Attieh *et al.* 2000a). These sulphur-containing compounds, along with other volatiles of the above-mentioned three volatile classes, contribute to the typical 'Brassica odour'.

Many studies have applied molecular techniques to unravel processes by which plants perceive insect attack and trigger HIPV emissions (Arimura *et al.* 2005; De Vos *et al.* 2007; Soler *et al.* 2012). Similar to other herbivore-induced responses, the emission of HIPVs is mainly controlled by the phytohormones JA, salicylic acid (SA) and ethylene (ET; Gatehouse 2002; Howe & Schaller 2008; Dicke & Baldwin 2010). Based on herbivore-derived external cues, such as salivary compounds and feeding strategies, a specific combination of these hormones is produced (De Vos *et al.* 2005; Ehrling *et al.* 2008). JA is mainly involved in the regulation of induced plant responses against wounding and herbivory (Koo & Howe 2009; Koo *et al.* 2009). Crosstalk between signalling pathways helps the plant to fine-tune its defence response to the invaders encountered (Pieterse & Dicke 2007; Koornneef & Pieterse 2008; Verhage *et al.* 2010; Pieterse 2012). In general, ET and JA act synergistically, while SA is known to act antagonistically on JA signalling (Adie *et al.* 2007; Koornneef & Pieterse 2008; Verhage *et al.* 2010). Hence, the functional outcome is determined by a complex regulatory network and is highly tissue and context specific (Pieterse 2012). Molecular tools such as qPCR now make it possible to directly link gene expression to the emission of HIPVs and insect behaviour (Halitschke & Baldwin 2003; Kessler & Baldwin 2004; Skibbe *et al.* 2008; Gosset *et al.* 2009; Zhang *et al.* 2013). These studies have shown that the production of HIPVs involves the activation of an intricate network of different genes in the hormonal signalling pathways above as well as biosynthetic genes involved in the production of various HIPV classes (Attieh *et al.* 2002; Zheng *et al.* 2011). The expression of many of these genes differ in time, illustrating the dynamic interplay between genes involved in signalling pathways and defence compound production (Koornneef & Pieterse 2008; Broekgaarden *et al.* 2010; Erb *et al.* 2012; Kerchev *et al.* 2012; Zhang *et al.* 2013).

Indeed, HIPV emissions are known to be highly dynamic (De Moraes *et al.* 2001). The blend of volatiles quickly changes within a short time span of days or even hours after onset of the damage. However, studies analysing ecological functions of HIPVs may not account for this dynamics. Usually one selects a time point for which the volatile emissions and the response of the insect of interest are maximized, thereby ignoring

the natural temporal dynamics of the induction process. Thus far, few studies have demonstrated temporal changes in HIPV emission and discussed their possible ecological role (Schmelz *et al.* 2003; Bruinsma *et al.* 2009). Moreover, different herbivores and their parasitoids may respond differently to temporal changes in the HIPV blend, as they may have different levels of specialization on the host plant. In this study, we correlate the temporal variation in HIPV blends and their corresponding molecular mechanisms with the preference of insects at different trophic levels to gain a more comprehensive insight into the exact role of HIPVs in the communication between plants and associated insects. This knowledge is essential to elucidate which ecological costs and benefits are associated with the production of HIPV in time and hence to better understand whether there is indeed a net value for the plant.

We explicitly link temporal patterns of HIPV emissions to their corresponding gene expression patterns and the preference of generalist and specialist herbivores as well as their natural enemies. We thereby hypothesize that herbivores will prefer undamaged plants as these provide an uninduced resource without competition by other herbivores. On the other hand, their natural enemies are expected to prefer plants that have been damaged for the longest time period, because we expect that volatile emissions will increase with time and amount of damage. To test these hypotheses, choice experiments were performed with the generalist herbivore *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae) and the specialist *Plutella xylostella* L. (Lepidoptera: Plutellidae) as well as their parasitoids *Cotesia marginiventris* Cresson and *C. vestalis* Kurdjumov (previously known as *C. plutellae*; Hymenoptera: Braconidae), respectively. These insects were offered plants that had been damaged by *Spodoptera* spp. for 24, 48 or 72 h or were undamaged. The expression of genes involved in hormone signalling (*MYC2*, *VSP2*, *ERF1*, *ETR1* and *PR1*), HIPV (*TPS10*, *TPS21*, *CYP82G1*, *HPL1*, *ChIADR*, *CHAT* and *TMT1*) and glucosinolate biosynthesis (*CYP79B2*, *CYP79F1*, *CYP83A1*), and HIPV profiles of similarly treated plants were analysed at corresponding time points. This combined approach provided us a comprehensive view on the temporal dynamics of mechanisms and ecological function of HIPVs in plants.

## Materials and methods

### Plants

Seeds of *Brassica juncea* var. *varuna* were obtained from the Division of Genetics, IARI, New Delhi, India, in 2008 and stored dry and in the dark at 10 °C. They were germinated on glass beads in water in 10 × 10 cm

plastic containers with a clear plastic lid. The greenhouse was kept at 21 °C during the day and 16 °C at night, under ambient light conditions that were supplied by sodium lamps to maintain the minimum PAR at 225 μmoles/m/s for at least 16 h. Seven days later, seedlings were transferred to 1.8-l pots, containing 1000 g peat soil-sand mixture [Lentse Potgrond no. 4, Lent, the Netherlands (NL)]. From third week onwards, plants were supplied with 0.5 Hoagland solution (Hoagland & Arnon 1950) once a week.

### Insects

Egg batches of beet armyworm (*Spodoptera exigua* Hübner; Lepidoptera: Noctuidae) and pupae of the parasitoids *Cotesia marginiventris* and *C. vestalis* were obtained from the Department of Entomology, Wageningen University, NL. They were maintained in a climate room at 27 °C, 50–70% RH and a 16-h light/–8 h dark photoperiod. A culture of *S. exigua* was maintained on an artificial diet as described by Vickerman & Trumble (1999). Unmated naive females of herbivores and parasitoids were used within 24 h of emergence.

### Induction of plants

All the experiments were performed when the plants were approximately 4 weeks old and in stage 63 according to BBCH scale (Lancashire *et al.* 1991). A single fourth instar of *S. exigua* larva was introduced in a 6-cm-diameter clip cage to the fourth leaf counted from the apex of the plant. Depending on the treatment group, larvae were allowed to feed for 24, 48 or 72 h. Undamaged plants received empty clip cages. To avoid positional bias, damaged and undamaged plants were randomly placed on the greenhouse tables.

### Insect preference

In contrast with all other experiments, which were performed in NL, the herbivore preference bioassay was performed in India at Sri Venkateswara College, University of Delhi, Delhi. There, plants were grown in earthen pots using garden soil in an insect-free enclosure. To obtain similar environmental conditions (light, temperature) as in NL, the experiments were performed from October until the beginning of December. *Spodoptera exigua* was not available in India, and transport of live insects between both countries is not allowed because of quarantine regulation. Therefore, the plants were induced using third instar larvae of *Spodoptera litura* as above in similar clip cages as in NL, after which the preference of adult *S. litura* and *Plutella xylostella* was tested. Earlier studies have revealed similar

extent and timing of induction of various traits due to herbivory by *S. litura* and *S. exigua* in *B. juncea* in both countries (Mathur *et al.* 2011). Larvae of *S. litura* and *P. xylostella* were obtained from laboratory cultures maintained at Sri Venkateswara College on castor bean and cabbage leaves, respectively. The adults of both *S. litura* and *P. xylostella* are nocturnal, and therefore herbivore orientation preference experiment was conducted at night. All other experimental procedures for the preference bioassays were performed in an identical way in India and NL.

The parasitoid preference experiments were performed in NL in full daylight when both *C. marginiventris* and *C. vestalis* are the most active.

To observe herbivore and parasitoid preference, an X-shape plexiglass olfactometer set-up was constructed that included a cylindrical releasing chamber of 20 cm diameter and 9 cm height (Fig. S1, Supplementary information). The floor of the chamber had an opening of 1.5 cm diameter for introducing the insects, which was closed with a rubber stopper. Four detachable cylindrical arms, each of 17 cm length and 5 cm diameter wide were fixed to the chamber at 90 degrees of angles. The other end of these arms was closed using a net.

For both herbivore and parasitoid preference experiments, plants were damaged 24, 48 or 72 h prior to the beginning of the experiment. In this way, these plant treatments groups, along with the undamaged plants, could be presented simultaneously. Feeding herbivores were removed from the leaves prior to the beginning of the experiment. Whole potted plants were placed on either arm of the olfactometer so that plant volatiles could diffuse through the arm closest to the plant. The experimental set-up was placed outside at temperatures ranging from 23 to 26 °C in a tent (100 × 70 × 70 cm) covered with fine mesh gauze.

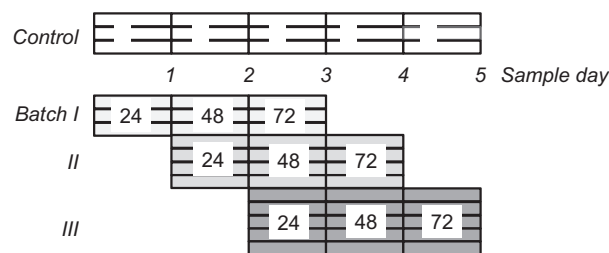
In total, 116, 48, 140 and 55 biological replicates of *S. litura*, *P. xylostella*, *C. marginiventris* and *C. vestalis*, respectively, were used. Cohorts of herbivore or parasitoid females were released in groups of five from the bottom of the chamber and observed after 15 min. If a female was found to have moved up to the end of an arm, it was recorded as making a choice for the corresponding plant. If a female did not make a choice, it was excluded from analysis. After testing five females, the plants were moved to another position to avoid any positional bias and the arms of the olfactometer were cleaned with ethanol. After four replicates of five females each, the test plants were replaced by new ones.

### Volatile analysis

Due to technical limitations of the volatile collection equipment, not all biological replicates at a certain time

point could be analysed simultaneously. Therefore, we divided the induced plants into three batches that were induced and analysed at different time points, over a total time period of 5 days according to the diagram shown in Fig. 1. Clip cages, along with the *S. exigua* larvae, were removed prior to volatile sampling and immediately put back thereafter. The damaged plants, 12 biological replicates in total, were repeatedly measured at 24, 48 and 72 h after damage in three partly overlapping series over 5 days. The undamaged plants, three biological replicates in total, were repeatedly measured on all 5 days of the experiment to control for temporal and ontogenetic variation.

Prior to the experiment, turkey roasting bags (Toppits, Melitta Nederlands BV, Gorinchem, NL), 25 × 40 cm, were heated at 120 °C for 2 h in an oven (as per Stewart-Jones & Poppy 2006). Bags were individually placed around a single leaf that was subjected to larval treatment in damaged plants or a leaf of similar age on undamaged plants. The bag was fitted with a steel trap that was placed just above the leaf. Volatiles were collected by pulling the headspace air with a vacuum pump over the trap filled with 150 mg Tenax TA and 150 mg Carbopack (Markes International Ltd., Llantrisant, UK). Flow rates over the traps were set to 100 mL/min using mass flow regulators (Sho rate TM, Brooks Instrument, Hatfield, PA, USA). After 60 min, the traps were removed, capped and stored at 4 °C until analysis. Four plants were sampled in parallel, and each day one background volatile profile from an empty bag was sampled. Volatiles were desorbed from the traps and analysed by GC-MS using the method and reference compounds described by van Dam *et al.* (2010). All integrated signals were generated from the MS chromatograms by the AMDIS software (NIST, USA). To correct for minor differences in sampling time and flow rates over individual traps, peak areas obtained in each sample were divided by the total



**Fig. 1** Experimental design for volatile collection. The plants were used in three batches: Batch I, II and III had 3, 4 and 5 damaged plants, respectively (indicated by small rectangles in boxes). The same three undamaged plants were used in the whole experimental period. The numbers inside the boxes indicate time for which damage has occurred (in hours), and the number outside the boxes represent the day of the sampling.



volume (in mL) collected over the trap. Peaks related to mixtures of volatiles and impurities were removed from the data set. Background volatiles were uniformly subtracted from the volatiles of both undamaged and damaged plants to obtain volatiles emitted only by the leaves. To remove day-to-day variation on the five experimental days, for each day of sampling the logarithmic fold changes in emission of individual volatile compounds were calculated by the formula:  $\ln [\text{treatment (peak area + 1)}/\text{control (peak area + 1)}]$ . Thereafter, the obtained fold changes for each sampling day were averaged (Table 1).

*Gene expression analysis*

We investigated the temporal expression of genes in local (fourth leaf from the apex where larva was introduced) as well as systemic (third leaf from the apex) leaves to match both volatile measurements performed on local leaves and insect preference experiments performed on the whole plant. Plants were damaged using a fourth-instar *S. exigua* larva in a clip cage. Control plants received empty clip cages. To analyse the hormonal pathway induced by herbivore feeding, an additional batch of plants were induced by ectopic

**Table 1** Mean ± SE of the fold changes of volatile emissions calculated as  $\ln [\text{treatment (X + 1)}/\text{control (X + 1)}]$  per day of sampling ( $n = 3$  per time point per day, in total  $n = 9$  per time point). X = peak area of the compound. Values in bold represent significant changes in volatile emissions over controls ( $P < 0.05$  after independent-sample *t*-test)

No.	Compound name	LRI (RTX-5 ms)*	Compound class	Mean ± SE fold changes $\ln (X + 1)$ 24 h	Mean ± SE fold changes $\ln (X + 1)$ 48 h	Mean ± SE fold changes $\ln (X + 1)$ 72 h
1	1-Butene-4-isothiocyanate	979	Isothiocyanate	<b>4.33 ± 1.02</b>	4.72 ± 1.02	<b>6.54 ± 0.59</b>
2	2-β-Pinene	971	Monoterpene	-0.31 ± 0.15	0.33 ± 1.33	-0.84 ± 0.46
3	α-Pinene	927	Monoterpene	-0.57 ± 1.35	0.48 ± 1.45	-0.46 ± 1.17
4	1-Monoterpene†	1007	Monoterpene	-1.73 ± 0.87	-0.89 ± 1.80	-2.00 ± 0.80
5	Limonene	1026.5	Monoterpene	0.51 ± 0.49	-0.38 ± 0.69	-0.67 ± 0.12
6	β-Ocimene	1048.8	Monoterpene	-2.57 ± 1.87	-3.01 ± 1.73	-0.27 ± 1.52
7	α-Copaene	1371	Sesquiterpene	0.25 ± 0.76	-0.48 ± 0.66	0.30 ± 0.69
8	E,E-alpha farnesene	1506.4	Sesquiterpene	1.67 ± 0.94	0.67 ± 0.53	0.10 ± 0.10
9	(E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT)	1577	Homoterpene	4.45 ± 1.30	2.00 ± 1.12	1.69 ± 1.03
10	<3E>-4,8-dimethyl-1,3,7-nonatriene (DMNT)	1117	Homoterpene	3.10 ± 1.59	0.48 ± 0.48	<b>1.93 ± 0.41</b>
11	3-Hexen-1-ol	851	GLV	<b>3.10 ± 1.58</b>	<b>4.22 ± 0.86</b>	4.89 ± 0.89
12	3-Hexen-1-ol-acetate	1008	GLV	<b>1.42 ± 0.43</b>	1.20 ± 0.19	<b>2.14 ± 0.38</b>
13	Acetaldehyde‡	498	GLV	-0.77 ± 0.36	<b>3.50 ± 1.88</b>	0.97 ± 1.88
14	Acetic acid ethyl ester	600	GLV	1.43 ± 1.26	0.35 ± 1.55	-0.56 ± 2.91
15	Acetic acid hexyl ester	1015.6	GLV	0.66 ± 2.25	1.03 ± 1.03	3.12 ± 1.73
16	Hexadecanoic acid methyl ester	1928.6	GLV	0.94 ± 0.62	0.31 ± 0.80	-0.94 ± 0.62
17	Dimethyldisulphide	738.8	Sulphide	0.33 ± 0.14	0.27 ± 0.29	<b>0.42 ± 0.18</b>
18	Dimethyltrisulphide	961	Sulphide	0.57 ± 1.43	2.35 ± 1.91	<b>2.60 ± 1.13</b>
19	Salicylic acid hexyl ester	1673.5	SA	-0.33 ± 0.86	-1.39 ± 0.65	-1.98 ± 0.29
20	1-Dodecanol	1474	Alcohol	-1.84 ± 1.90	-0.96 ± 1.64	-1.41 ± 1.35
21	1-Tetradecanol	1675	Alcohol	<b>1.91 ± 0.99</b>	0.31 ± 1.73	2.02 ± 1.46
22	Heptanal	901	Aldehyde	3.77 ± 2.42	-0.93 ± 1.01	1.00 ± 1.71
23	Hexadecanal	1818.5	Aldehyde	0.01 ± 0.13	-1.19 ± 2.68	-1.85 ± 1.02
24	Tetradecanal	1611.2	Aldehyde	1.73 ± 0.47	-1.41 ± 0.67	-0.60 ± 1.70
25	2-Butanone	576	Ketone	2.23 ± 1.15	-0.22 ± 1.81	-0.10 ± 1.09
26	2-Nonanone	1091.8	Ketone	2.27 ± 0.52	0.37 ± 0.63	0.83 ± 0.58
27	3-Methyl-2-pentanone	749.1	Ketone	<b>2.54 ± 1.25</b>	0.06 ± 2.71	-0.18 ± 1.16
28	3-Pentanone	700	Ketone	2.69 ± 2.62	<b>4.91 ± 0.89</b>	5.48 ± 2.09
29	Decanoic acid	1369	Fatty acid	0.02 ± 1.37	-2.80 ± 1.21	-1.27 ± 1.84
30	γ-Valerolactone	948.8	Lactone	-0.28 ± 0.80	-1.03 ± 0.60	0.14 ± 0.88
31	2-Acetyl furan	908.8	Furan	0.74 ± 1.43	0.09 ± 1.56	0.99 ± 1.12
32	Indane	1031	Indane	-1.76 ± 2.05	-1.53 ± 1.30	-2.63 ± 1.06

GLV, green leaf volatiles.

\*Linear retention index for rtx-5 ms column.

†Carene isomer or pseudolimonene.

‡Tentative.

application of 500 µg JA (pH 3.7; Sigma, St Louis, IL, USA) or received a mock treatment with acidic water on the fourth leaf from the apex of the plant (Supplementary information). Local and systemic leaves from the control plants as well as the herbivore- and JA-treated plants were harvested as separate sets at 6, 20, 24, 48 and 72 h after induction. Leaves from three plants were pooled together to obtain one sample, and three replicate pools were collected for each time point.

Gene expression of several enzymes involved in volatile synthesis and hormonal pathways controlling induced responses were analysed by RT-qPCR. The expression of three GLV biosynthesis genes, viz. hydroperoxide lyase1 (*HPL1*; Matsui *et al.* 1999; Matsui 2006), Chloroplastic Aldehyde Reductase (*ChlADR*; Yamauchi *et al.* 2011) and acetyl CoA:(Z)-3-hexen-1-ol acetyltransferase (*CHAT*; D'Auria *et al.* 2007), was measured. For the terpenoids biosynthetic pathway, gene expression of *TPS10* (Bohlmann *et al.* 2000), *TPS21* (Tholl *et al.* 2005) and *CYP82G1* (Lee *et al.* 2010), which are involved in the synthesis of mono-, sesqui- and homoterpenes respectively, were examined. For the glucosinolate-derived volatiles, expression of genes involved in the synthesis of aliphatic (*CYP79F1* and *CYP83A1*; Bak & Feyereisen 2001; Chen *et al.* 2003; Naur *et al.* 2003) and indole glucosinolates (*CYP79B2*; Glawischnig *et al.* 2004) was measured. Additionally, the expression of thiol methyltransferase1 (*TMT1*), a gene involved in the biosynthesis of sulphur volatiles (Attieh *et al.* 2002), was analysed. To investigate the hormonal pathways involved, the expression of marker genes for the JA pathway (*MYC2* and *VSP2*; Berger *et al.* 2002; Lorenzo *et al.* 2004; Dombrecht *et al.* 2007), the SA pathway

(*PR1*; Bowling *et al.* 1997) and the ethylene pathway (*ETR1* and *ERF1*; Lorenzo *et al.* 2003, 2004) was measured.

For primer design, orthologous sequences of the respective *Arabidopsis thaliana* gene were collected from all *Brassica spp.* sequences available in GenBank. Primers were designed on conserved stretches within the *Brassica* orthologous sequences, whereby cross-reactivity with paralogous *Brassica* sequences was avoided (Table 2). For each sample, 0.5 µg of total RNA was reverse-transcribed into cDNA with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories Inc., California, USA) according to the manufacturer's instructions. For each RNA sample, a negative control cDNA reaction was made by omitting the reverse transcriptase to verify that no samples were significantly contaminated with genomic DNA. Subsequently, all samples were diluted 20-fold with water. For each cDNA sample, qPCR amplification reactions were performed in triplicate.

The qPCR amplification mix was: 5 µL diluted 1st strand cDNA, 0.75 µL forward primer (10 µM), 0.75 µL reverse primer (10 µM), 12.5 µL iQ™ SYBR Green Supermix (Bio-Rad Laboratories Inc., CA, USA) and 6 µL H<sub>2</sub>O. The qPCR was performed on the MyIQ Single-Color Real-Time PCR Detection System (Bio-Rad Laboratories Inc., CA, USA) according to the following protocol: an initial denaturation for 5 min at 95 °C, followed by 45 cycles of 15 s at 95 °C, 15 s at 58 °C and 15 s at 72 °C. Thereafter, a melting curve analysis was performed to verify that only a single gene transcript had been amplified. To verify that primers were targeting the right gene, amplification fragments were cloned and sequenced.

**Table 2** Primers sequences for gene expression analysis

Gene	<i>Arabidopsis thaliana</i> locus	Forward Primer	Reverse Primer
<i>GAPC2</i>	AT1G13440	5'-AGTTGTTGACCTCACGGTTAGAC-3'	5'-TTCCTCCTTGATAGCCTTCTTG-3'
<i>PP2A</i>	AT1G13320	5'-CATGCTCCAAGCTCTTACCTG-3'	5'-AATTTGATGTTTGAAGCTCTGTCTT-3'
<i>MYC2</i>	AT1G32640	5'-AGGTTGATGTCGGCGTTG-3'	5'-CGTTAACCACCGACATACTCG-3'
<i>VSP2</i>	AT5G24770	5'-ATCTCGAAGCTGCTGGTTTC-3'	5'-TTTGTGTTCTGAACCCGTTG-3'
<i>ERF1</i>	AT3G23240	5'-CGGCCGAGAGAGTAAAGAG-3'	5'-AACACCCATCCTCGTAGCTG-3'
<i>ETR1</i>	AT1G66340	5'-CACCAAAGGCCACTGCTC-3'	5'-GTGGATTTGTCGGTGTTACCAG-3'
<i>PR1</i>	AT2G14610	5'-CTACGCCGACCGACTAAGAG-3'	5'-CTACTCCCGCCAAGTTCTC-3'
<i>HPL1</i>	AT4G15440	5'-TGGTGATGAGAGACGCTAACA-3'	5'-CCGATCCGGTTTAAATTCCT-3'
<i>ChlADR</i>	AT1G54870	5'-CCTGGCTTGTAACCAATTGCT-3'	5'-CACCTCCGTTAGGGTGAAGA
<i>CHAT</i>	AT3G03480	5'-TGTACGGTGAACCGCTAAG-3'	5'-GGCAGCTAGAAGCTCACTCCT-3'
<i>TPS10</i>	AT2G24210	5'-AACTCTTACTGCGCCTTTG-3'	5'-ACTCGGGGAGTTCATCGAGAC-3'
<i>TPS21</i>	AT5G23960	5'-GAGCACATTGTCTCTTTGCTCA-3'	5'-AATCTCCACAGTCCACCAC-3'
<i>CYP82G1</i>	AT3G25180	5'-TGTGGACATGTACGCGATG-3'	5'-GGTGATGGATGTGCTGTCTG-3'
<i>CYP79B2</i>	AT4G39950	5'-AAGAGGTTGTGCTGCTCCG-3'	5'-TCCAAGTGAACCTTGAAGAAGTC-3'
<i>CYP79F1</i>	AT1G16410	5'-TTGGAACATTGATGGTCAAGAG-3'	5'-TCTCGTCAATGATCGGATTG-3'
<i>CYP83A1</i>	AT4G13770	5'-CTCCTTATCCCTCGTGCTTG-3'	5'-TGTGCTAACCAGCGATCTTG-3'
<i>TMT1</i>	AT2G43920	5'-CGCCACTCGTAAGGGTAAAG-3'	5'-TGGATCAGTTGATCTTCTTCCA-3'

Out of several potential reference genes tested, two most stable, *GAPC2* and *PP2A*, were selected with the geNorm software (<http://medgen.ugent.be/genorm/>), and the relative expression levels of the target genes were calculated by normalization with the expression of the two reference genes (Vandesompele *et al.* 2002). Primer pair amplification efficiencies were determined with LinRegPCR software (Ruijter *et al.* 2009). Fold changes in gene expression levels were calculated by dividing the mean normalized expression of damaged plants group by the mean normalized expression of the undamaged plants group (Muller *et al.* 2002).

*Statistical analyses*

Insect preference was analysed using nonparametric replicated goodness of fit test with the null hypothesis of no preference (Sokal & Rohlf 1995). Females that did not make a choice were excluded from the analysis.

For volatile analysis, we constructed a separate model for each day of volatile sampling and compared the profiles of the damaged plants collected on that day to control plants of the same day. Orthogonal partial least squares-discriminant analysis (OPLS-DA) method was applied to these models (Bylesjo *et al.* 2006): the

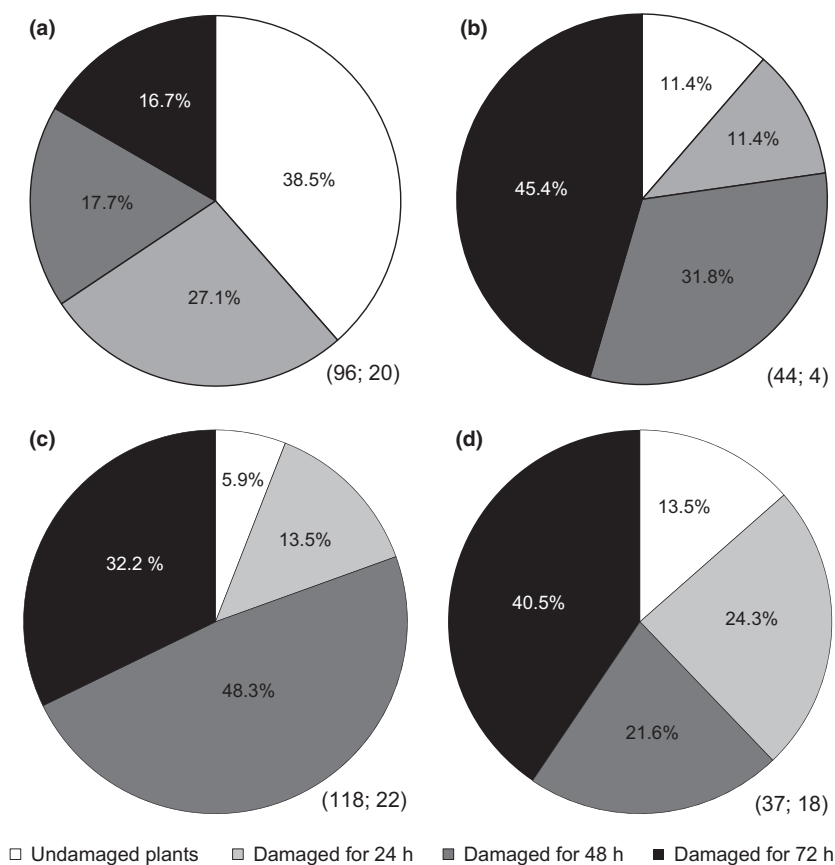
resulting weight vector values reflect the volatile blend emitted from damaged plants compared with that of untreated control plants on each day. The *P*-values were determined using a permutation test. Models based on randomly permuted class labels (treatment groups) and of identical complexity were evaluated for their classification potential compared to the null hypothesis of no response.

Data of individual volatiles and their gene expression were statistically analysed by independent-sample *t*-test assuming unequal variances following the procedure in Rieu & Powers (2009) using SPSS 17.0 (SPSS, Chicago, IL, USA).

**Results**

*Insect preference*

When offered a choice between undamaged plants and plants damaged for 24, 48 or 72 h, the generalist herbivore *Spodoptera litura* significantly preferred undamaged plants (Fig. 2a, replicated G-test, *P* < 0.01). Its parasitoid *Cotesia marginiventris*, however, favoured plants damaged for 48 h (Fig. 2c, replicated G-test, *P* < 0.001). In contrast, the specialist herbivore *Plutella xylostella*



**Fig. 2** Orientation response of naive adult females of (a) *Spodoptera litura* (*n* = 96), (b) *Plutella xylostella* (*n* = 44), (c) *Cotesia marginiventris* (*n* = 118), (d) *C. vestalis* (*n* = 37) when undamaged plants and plants damaged for 24, 48 or 72 h were offered as choice. The data were analysed using replicated G test goodness of fit and were found significant between treatments for (a; *P* < 0.01), (b; *P* < 0.005), (c; *P* < 0.001) and (d; *P* < 0.05). The pie charts represent only the percentage of females that made a choice. Numbers in parentheses represent the number of females that made a choice and females that did not make a choice and were excluded during the experiment, respectively.



(Fig. 2b; replicated G-test,  $P < 0.005$ ) and its parasitoid *C. vestalis* (Fig. 2d; replicated G-test,  $P < 0.05$ ) both preferred plants damaged for 72 h as compared to undamaged plants or plants damaged for 24 and 48 h.

### Volatile analysis

The volatile blends consisted of compounds of various classes including an isothiocyanate, GLVs, a salicylic acid ester, sulphides and terpenes along with other (unidentified) acids, ketones and aldehydes (Table 1). For each of the three time points, fold changes in the emission rates of individual compounds after *S. exigua* feeding compared with control values were calculated, and the complete blends were fitted in an OPLS-DA model, comparing the blends over three sampling days. The overall volatile blends of damaged plants were significantly different between days (Permutation test of the weight vector value;  $P < 0.01$ ). The three discriminant axes represent the contrast between the fold changes of plants damaged for 24, 48 and 72 h (Fig. 3a–c). The importance of each VOC in each contrast is represented by its position according to its weight vector value on each axis. Increased levels of a compound after 24, 48 or 72 h damage are indicated by a positive weight vector value, and reduced levels are shown by a negative value on the respective axis.

The only isothiocyanate emitted, 1-butene-4-isothiocyanate, was strongly induced at 24 h after damage and remained at higher levels throughout the entire study period (independent-sample *t*-test,  $P < 0.01$  at 24 h and  $P < 0.005$  at 72 h; compound 1 in Fig. 3a–c). The emission of GLV compounds, viz. 3-hexen-1-ol (compound 11;  $P < 0.05$  at 24 and 48 h) and 3-hexen-1-ol-acetate (compound 12;  $P < 0.05$  at 24 and 72 h), was also significantly increased following herbivory (Fig. 3a–c). We also observed a short burst in the emission of acetaldehyde (compound 13) at 48 h ( $P < 0.05$ ; Fig. 3a, c). In addition, acetic acid hexyl ester (compound 15) emissions showed a marginally significant increase at 72 h of damage only ( $P = 0.058$ ; Fig. 3b, c). The levels of acetic acid ethyl ester (compound 14) and hexadecanoic acid methyl ester (compound 16) showed no significant change in emission following herbivory (Table 1).

Among terpenes, which constituted the largest number of known compounds in the *B. juncea* volatile blend, the homoterpenes were the most prominently induced. Both homoterpenes, viz. (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT; compound 9) and <3E>-4,8-dimethyl-1,3,7-nonatriene (DMNT; compound 10), were only emitted from damaged plants within 24 h of damage and emitted at higher rates, but inconsistently so, throughout the study period (Fig. 3a–c). The emission of DMNT was found to be significantly elevated at 72 h

of damage ( $P < 0.05$ ), but TMTT did not show significantly increased emissions at any time point tested due to the large variation between replicates (Table 1). We did not find any major changes in mono- and sesquiterpene emissions (Table 1).

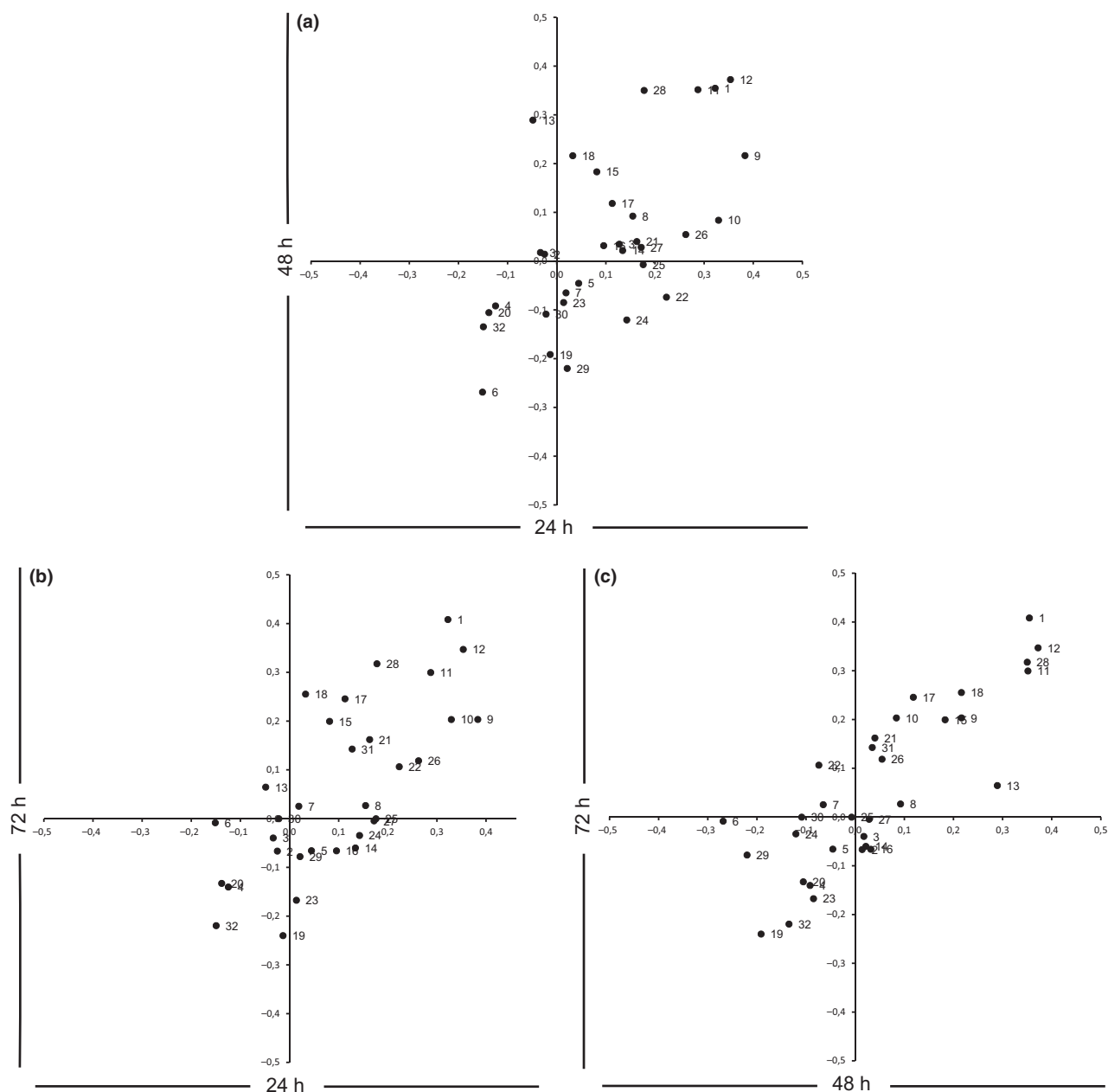
Both the sulphides detected, viz. dimethyldisulphide (DMDS) and dimethyltrisulphide (DMTS; compound 17 and 18, respectively), increased significantly over control levels at 72 h of damage ( $P < 0.05$ ; Table 1, Fig. 3b, c). The emission of salicylic acid hexyl ester (compound 19) showed a decreasing trend after 72 h of damage (Fig. 3b, c).

In addition, several other compounds were significantly affected by *S. exigua* feeding at different time points of the study. Among these, the most prominent compounds were 1-tetradecanol (compound 21;  $P < 0.05$ ) and 3-methyl-2-pentanone (compound 27, ketone;  $p < 0.05$ ) that increased at 24 h of damage, as well as 3-pentanone (compound 28, ketone;  $p < 0.01$ ) that increased at 48 h of damage (Fig. 3a–c). Other than the volatiles mentioned above, the OPLS-DA model shows several compounds belonging to various classes, such as alcohols, aldehydes and ketones that were different at various time points (e.g. compounds 26, 31, 32). However, the differences in their emission rates were not statistically significant due to high variation among the biological replicates (e.g. compound 9 at 24 h and compound 11 at 72 h in Table 1).

### Gene expression analysis

Changes in the expression levels of all the tested genes were essentially stronger in damaged (local) leaves than in systemic leaves. In the local leaves, we observed a significant induction of *MYC2* starting at 20 h after *S. exigua* damage (independent-sample *t*-test,  $P < 0.05$ , Fig. 4a). *VSP2* was repressed at 6 and 48 h, but induced at 20 h (Fig. 4b). In systemic leaves, no response was found for *MYC2*, while *VSP2* was significantly induced almost twofold at time points 6 and 20 h, but was also significantly repressed at 48 h (independent-sample *t*-test,  $p < 0.05$ ). *PR1*, *ETR1* and *ERF1* principally did not show significant changes in their expression levels in either local or systemic leaves, except for a significant repression of *PR1* at 72 h in systemic leaves (Fig. 4c–e). Similar gene expression patterns were found when we artificially induced plants using JA (Supporting information). We found that *MYC2* and *VSP2* were strongly induced in both local and systemic leaves, whereas no response was observed for *PR1*, *ETR1* and *ERF1* in the local as well as the systemic leaves (Fig. S2, Supporting information).

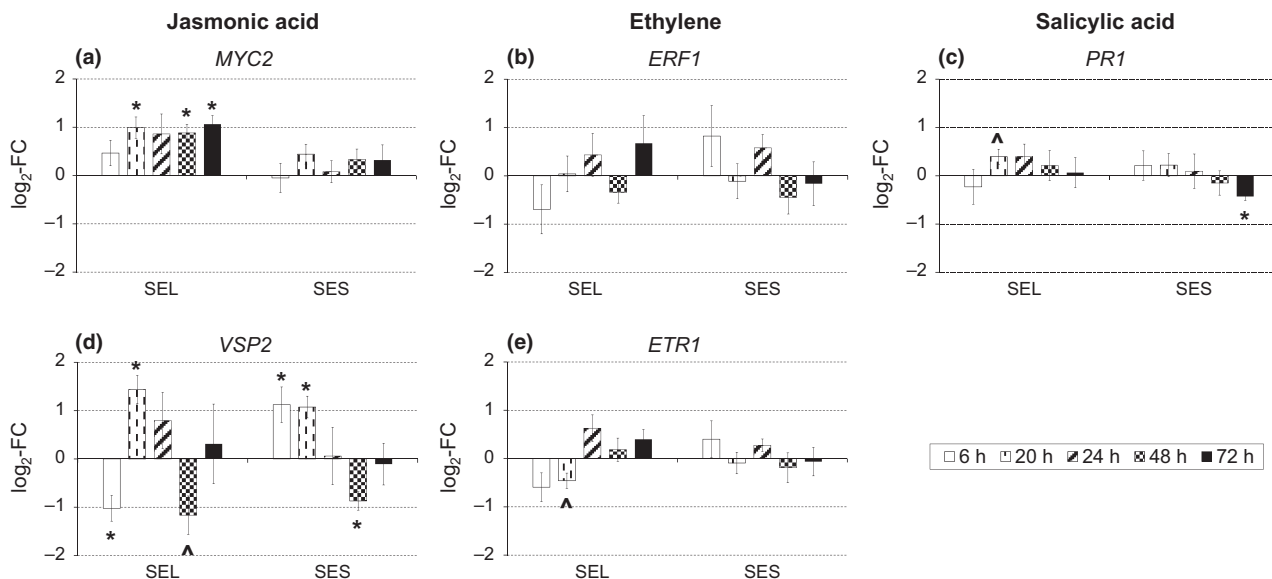
Temporal changes in the genes involved in GLV synthesis were quite pronounced, as was also observed in



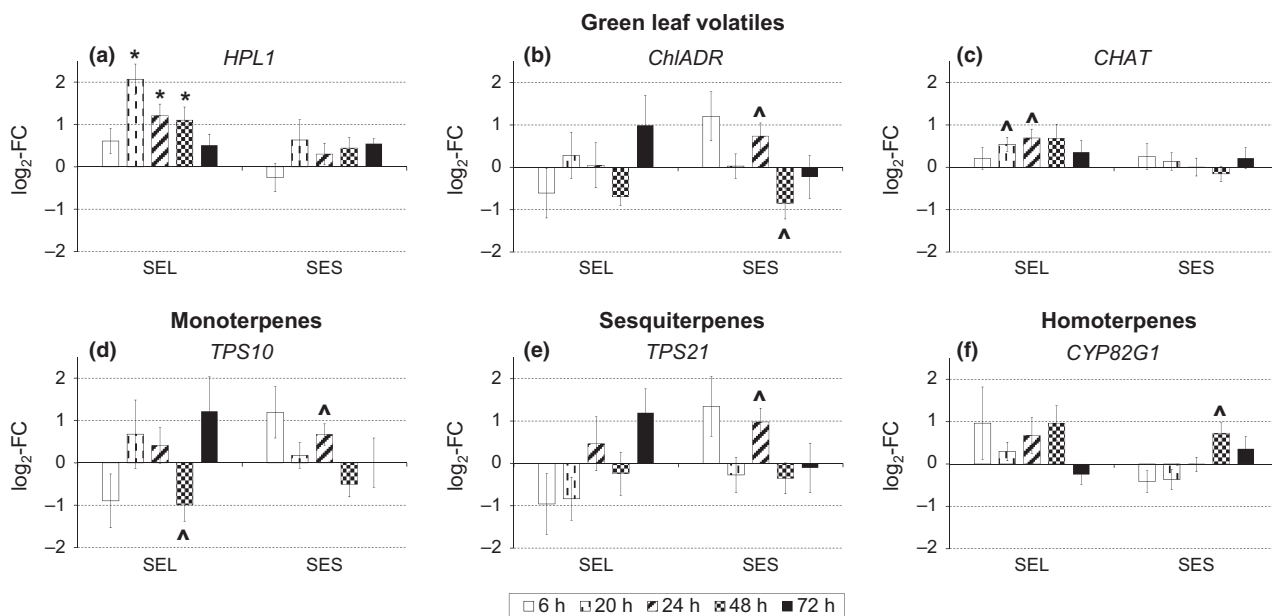
**Fig. 3** Two-dimensional OPLS-Discriminant plots for VOCs emitted by *Brassica juncea* plants due to damage by *Spodoptera exigua* larvae, represented as discriminant functions of damage for 24, 48 and 72 h plotted against each other. The two-dimensional plots show the contribution of each VOC for each treatment group on the x or the y axis: (a) 24 h vs. 48 h, (b) 24 h vs. 72 h and (c) 48 h vs. 72 h of damage. Numbers correspond to compounds listed in Table 1. The position of each point is determined by its weight vector value in the VOC blend of each treatment group plotted.

the volatile analysis. *HPL1* was significantly induced in the local leaves at time points 20, 24 and 48 h, while in systemic leaves this induction was delayed until 72 h after damage (independent-sample *t*-test,  $P < 0.05$ , Fig. 5a). In contrast, *ChIADR* was significantly repressed after 48 h of damage in the local leaves. In the systemic leaves, there was an increasing trend after 24 h followed by a decrease after 48 h of damage (Fig. 5b).

*CHAT* expression showed a trend for increase following herbivory in the local leaves. However, no significant induction or repression was observed in either local or systemic leaves at any of the analysed time points (Fig. 5c). The expression of genes involved in mono- (*TPS10*) and sesquiterpene (*TPS21*) biosynthesis showed a large biological variation (Fig. 5d, e). Following herbivory, both *TPS10* and *TPS21* were slightly induced in



**Fig. 4** RT-qPCR on marker genes for hormonal pathways. Relative fold changes in mean normalized expression at different time points of treatment vs. control for genes of the jasmonic acid, salicylic acid and ethylene hormonal pathways. SEL, local leaf after *Spodoptera exigua* induction; SES, systemic leaf after *S. exigua* induction. Error bars represent standard errors; statistically significant fold changes between control and treatment are marked by: ^,  $P < 0.1$ ; \*,  $P < 0.05$  ( $t$ -test assuming unequal variances).



**Fig. 5** RT-qPCR on volatile biosynthetic genes. Changes in gene expression levels are shown as relative fold changes in mean normalized expression at different time points of treatment vs. control. SEL, local leaf after *Spodoptera exigua* induction; SES, systemic leaf after *S. exigua* induction. Error bars represent standard errors; statistically significant fold changes between control and treatment are marked by: ^,  $P < 0.1$ ; \*,  $P < 0.05$  ( $t$ -test assuming unequal variances).

the local leaves at 72 h. In systemic leaves, these genes tended to increase at 24 h. The gene *CYP82G1*, which is involved in homoterpene synthesis, had an increasing trend in local leaves following herbivory (Fig. 5f). Plants induced with JA also showed similar patterns for these genes, but the expressions of *HPL1*, *CHAT* and *CYP82G1*

were more pronounced than in the herbivore-damaged plants, possibly because the application of excess JA provides a stronger induction signal than herbivore feeding (Fig. S3, Supporting information).

*CYP79F1* and *CYP83A1* are involved in the synthesis of the aliphatic glucosinolates that are the precursors of

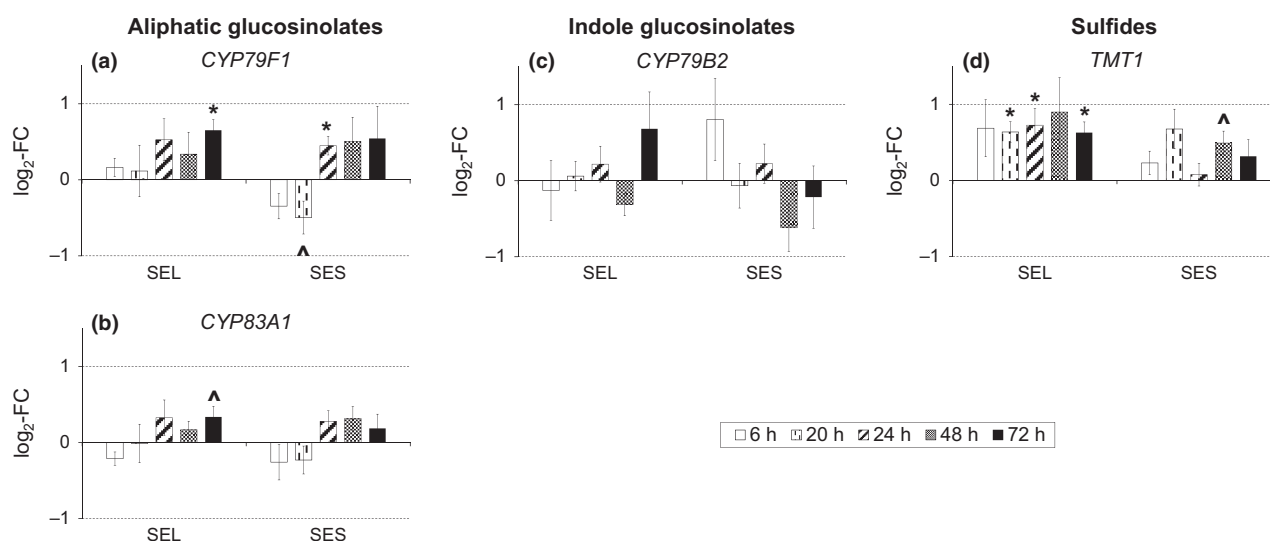
1-butene-4-isothiocyanate (compound 1, Table 1). They showed a comparable induction pattern in response to herbivory (Fig. 6a, b). Both genes were significantly up-regulated at 72 h of damage in local leaves (independent-sample *t*-test;  $P < 0.05$  for *CYP79F1*;  $P < 0.1$  for *CYP83A1*). In systemic leaves, *CYP79F1* was repressed at 20 h ( $P < 0.1$ ) and induced at 24 h ( $P < 0.05$ ). In contrast, no significant response due to herbivory was observed for *CYP79B2*, which is involved in the production of indole glucosinolates (Fig. 6c). The expression of *TMT1*, involved in the synthesis of sulphides, showed a marked induction in local leaves at 20, 24 and 72 h after damage (independent-sample *t*-test,  $P < 0.05$ ). The expression pattern of *TMT1* showed a similar trend in systemic leaves (Fig. 6d). In JA-induced plants, a largely similar response for the glucosinolate biosynthesis genes and *TMT1* was observed (Fig. S4, Supporting information).

## Discussion

In this study, we associated the preference of generalist and specialist herbivores and their parasitoids to the temporal dynamics of HIPV emissions and corresponding changes in their gene expression in *Brassica juncea*. We found that the generalist *Spodoptera litura* was most attracted to undamaged plants, whereas the specialist *Plutella xylostella* preferred plants that were damaged for 72 h. The parasitoids of both of these herbivores were attracted to damaged plants. However, *Cotesia marginiventris*, the parasitoid of *S. litura*, preferred plants

damaged for 48 h, whereas *C. vestalis*, corresponding to the preference of its host *P. xylostella*, preferred plants damaged for 72 h. A clear difference was found in the composition of volatile blends between damaged and control plants as well as between the induced plants measured at three time points (24, 48 or 72 h) following herbivory. OPLS-DA revealed that a few specific compounds characterized the odour blend of the induced plants at each time point. Prominent volatiles in the HIPV blends of *B. juncea* were 1-butene-4-isothiocyanate, several GLVs and ketones that increased, as well as the homoterpenes TMTT and DMNT that were emitted at higher levels following herbivory. Additionally, there was a conspicuous increase in sulphides at 72 h and decrease in the monoterpene  $\beta$ -ocimene, salicylic acid hexyl ester and decanoic acid at 48 h. HIPV emissions thus did not simply increase with time or amount of damage, but individual compounds within the blend showed specific temporal dynamics to which the herbivores and parasitoids may have responded. Gene expression patterns largely mirrored the observed temporal dynamics of the different volatile emissions, indicating that these volatiles were mainly newly synthesized and that the formation of these volatiles is an active and well-regulated process in the plants.

Following herbivory, we found that 1-butene-4-isothiocyanate increased consistently with time after damage. The emission of 1-butene-4-isothiocyanate results from the myrosinase-catalysed conversion of 3-butenyl glucosinolate (gluconapin), the main glucosinolate in



**Fig. 6** RT-qPCR on sulphur-containing volatile biosynthetic genes. Changes in gene expression levels are shown as relative fold changes in mean normalized expression at different time points of treatment vs. control. SEL, local leaf after *Spodoptera exigua* induction; SES, systemic leaf after *S. exigua* induction. Error bars represent standard errors; statistically significant fold changes between control and treatment are marked by: ^,  $P < 0.1$ ; \*,  $P < 0.05$  (*t*-test assuming unequal variances).

*B. juncea* leaves (Mathur *et al.* 2011, 2013). A previous study showed that herbivory increased leaf concentrations of gluconapin within 4 days after damage by *Spodoptera* spp. (Mathur *et al.* 2011). Indeed, we found that expression of alkenyl glucosinolate biosynthesis genes is induced within the same time frame, thereby fuelling the induction of both direct (glucosinolates) and indirect (break-down products) compounds.

Both herbivores and their natural enemies responded to damage-induced variation in the concentration of glucosinolate breakdown products as cues to localize their hosts. Our results are in line with the previous studies that show that their preference to induced volatiles differs with the degree of their specialization (Mumm *et al.* 2008a; Gols & Harvey 2009; Pierre *et al.* 2011). As expected, the generalist herbivore *S. litura* preferred undamaged plants, on which the performance of its offspring will be better, than damaged plants (Mathur *et al.* 2011). These studies are consistent with earlier studies demonstrating that the same HIPVs that attract specialist herbivores and parasitoids can repel generalist herbivores (De Moraes *et al.* 2001; Karban & Baxter 2001). *Plutella xylostella*, on the other hand, preferred plants damaged for 72 h, for which 1-butene-4-isothiocyanate emissions were found to be the highest. Earlier studies revealed that increased levels of isothiocyanates are highly attractive to *P. xylostella* (Pivnick *et al.* 1994). This suggests that 1-butene-4-isothiocyanate plays an important role in the attraction of this specialist to *B. juncea* as well. The question is why *P. xylostella* would prefer to oviposit on previously damaged plants on which their offspring may be confronted with a number of challenges, including competition for available food, elevated plant defences and enhanced attraction of natural enemies such as predators and parasitoids. The induction of glucosinolates may not pose a threat to *P. xylostella* because it is known to 'disarm' these compounds in several plant species with a specific sulphatase that prohibits the formation of isothiocyanates (Ratzka *et al.* 2002). Earlier, Shiojiri *et al.* (2002) found that *P. xylostella* preferred plants already infested with *Pieris rapae* L. over uninfested plants and that parasitism of *P. xylostella* by its parasitoid *C. vestalis* was lower when the plant was doubly infested than when it was infested by *P. xylostella* alone. Thus, the production of 'signal noise' by other herbivore species may represent a form of enemy-free space for the herbivore, and its preference for plants infested by other species of herbivores might be an adaptation to reduce the chances of being parasitized (Hare 2011).

Our results also showed, however, that the preference of *P. xylostella* for damaged *B. juncea* plants does not necessarily provide enemy free space, because its parasitoid, *C. vestalis* was also most attracted to the same

plants. Likely, *C. vestalis* uses the isothiocyanate as a cue as well, but we also observed that the emission of two other sulphur-containing compounds, viz. DMDS and DMTS, was the highest at 72 h of damage. Earlier studies by Reddy *et al.* (2002) have established that *C. vestalis* is attracted to these sulphides. Changes in the emissions of sulphides after shoot or root herbivore damage in *Brassica* species have been described before (Geervliet *et al.* 1998; Crespo *et al.* 2012; van Dam *et al.* 2012). Thus, elevated emissions of DMDS were found to be correlated with the deterrence of a specialist parasitoid (Soler *et al.* 2007). Our observation that *C. vestalis* females were attracted to plants damaged for 72 h, which also emitted the highest levels of sulphides, underscores the possible role of these HIPVs in attracting these specialized natural enemies to damaged plants. Moreover, the fact that *TMT1*, the gene responsible for the synthesis of DMDS and DMTS, was induced after *S. exigua* larval feeding, shows that the emission of sulphides is actively regulated by the plant. Earlier, several thiol methyltransferases were described that methylate glucosinolate hydrolysis products formed by myrosinases (Attieh *et al.* 2000a,b, 2002), which are further oxidized to form volatile compounds such as DMDS and DMTS. Eventually, the sum of all positive and negative effects will determine whether the attraction of *P. xylostella* to damaged plants is an example that 'mother does not always know best' or whether long-term fitness gains may play a role in this seemingly counterintuitive result (Mayhew 2001). Further research in the field is necessary to fully understand the costs and benefits for *P. xylostella* in preferring infested over uninfested plants for oviposition.

Green leaf volatiles and terpenoids together comprise a large and diverse portion of the volatile blends emitted by some species of intact as well as damaged brassicaceous plants (van Poecke *et al.* 2001; Mumm *et al.* 2008b). In *B. juncea*, most of the GLV compounds increased within 24 h of herbivore damage, and the expression profile of two of the three genes associated with their production showed a similar pattern. In contrast, the emission of  $\beta$ -ocimene, a monoterpene, had decreased within 48 h of initial damage. In *Arabidopsis thaliana*, *TPS10* is involved in the synthesis of this compound (Bohlmann *et al.* 2000) and the activity of this gene was indeed slightly repressed both in local and systemic leaves in damaged plants. Sesquiterpenes showed no reaction to damage either at the level of volatile emissions or at the level of gene expression. On the other hand, the two homoterpenes, viz., TMTT and DMNT, were produced *de novo* following herbivory. In other plants species, these compounds were also found to be newly produced or increased in quantity following damage or treatment with elicitors (Turlings *et al.*



1990; van Poecke *et al.* 2001; Herde *et al.* 2008; Pierre *et al.* 2011). Homoterpenes serve as very specific and reliable cues for parasitoids (Mumm *et al.* 2008b) and thus may have contributed to the enhanced attraction of the parasitoids in our study.

Both GLVs (Mattiacci *et al.* 1994; Halitschke *et al.* 2008) and terpenes (Degenhardt *et al.* 2003; de Boer *et al.* 2004) are well known to attract natural enemies of herbivores. McCormick *et al.* (2012) describe three possible means of odour discrimination: (i) species-specific odour recognition, in which the carnivore separates plant volatile compounds restricted to a single species or group of related species of herbivores; (ii) ratio-specific odour recognition, whereby a ratio of compounds in the volatile blend is recognized; (iii) whole-blend odour recognition, in which the entire blend or many of its components are perceived as a whole. The ability of a parasitoid to distinguish between volatile blends depends on the dietary specialization of both parasitoid and its herbivore host (Vet & Dicke 1992; Steidle & van Loon 2003). Specialist parasitoids cannot rely solely on induced terpenoids and GLVs for the detection of their specific host, because these compounds are not specific to any particular herbivore damage and hence do not give a reliable cue of the presence of their host (Vet *et al.* 1991; van Dam *et al.* 2010; Gols *et al.* 2011). Conversely, generalist parasitoids that attack several herbivores feeding on plants of different families may rely on more generalized cues (Gols *et al.* 2012). Earlier studies have demonstrated that *C. marginiventris* takes cues from both terpenoids and GLVs for its host location (Turlings *et al.* 1991). Electro-antennogram recordings of *C. marginiventris* confirm their ability to detect GLVs (Chen & Fadamiro 2007; Ngumbi *et al.* 2010). Based on our results, we speculate that this parasitoid uses changes in the amounts or ratios in terpenoids and GLVs after 48 h of damage as cues to locate their host, as they were most attracted to these plants.

To date, more than 25 species of natural enemies in the third trophic level are known to be attracted to HIPVs (Mumm & Dicke 2010; Reddy 2012). However, the chemical diversity of HIPVs makes it difficult to establish which of the blend components may evoke innate responses in naïve parasitoids. In fact, studies aimed at identifying the minimal blend showed that some compounds in the complete blend may mask the attractive components (Turlings & Fritzsche 1999). Therefore, the value of attraction is believed to be determined by the relative reliability and detectability of plant volatile signals (Vet *et al.* 1991). By showing different blends of volatiles at different time points, our study provides a fair explanation for the differential preference of the parasitoids and herbivores to these plants. Since we combine the behaviour of insect herbivores and their

parasitoids with the dynamics of HIPV blend, we can infer the ecological importance of these dynamics. However, as the most important odour cue for the generalist parasitoids may be determined by specific ratios of the emitted volatiles within the blend, it is challenging to pinpoint which compounds are the most important (Gols & Harvey 2009).

We also found that after the plants were subjected to *S. exigua* damage, *MYC2* and *VSP2* were both significantly induced indicating that this herbivore induced the JA pathway in *B. juncea*. In contrast, there were no significant changes in the expressions of genes involved in the SA or ET pathways; *PR1* was even repressed by 72 h of damage. Moreover, the expression profiles of the hormonal pathway marker genes and the volatile biosynthesis genes were largely comparable in the herbivore-damaged and ectopically JA-induced plants. This clearly indicates that the responses induced by *S. exigua* feeding are mainly controlled by the JA pathway in *B. juncea*. Many herbivore-induced responses regulated by the JA pathway are coregulated by the ET and SA pathway (Dicke *et al.* 1999; Genoud & Metraux 1999). For example, the JA and ET pathways converge in the transcriptional activation of *ERF1*, and hence increase in *ERF1* expression would indicate a synergistic response of JA and ET pathways (Lorenzo *et al.* 2003). Moreover, components in *S. exigua* oral secretions can suppress the JA pathway by activating the SA pathway in *N. attenuata* (Diezel *et al.* 2009). In *B. juncea*, however, we did not find any significant activity in the ET pathway, whereas the SA pathway was found to be repressed.

Herbivore-induced plant volatiles have been studied extensively in plant–herbivore–carnivore systems in crops, and this has greatly enhanced our understanding of the mechanisms involved in their expression. There is ample evidence that carnivores selectively exploit damage-induced plant volatiles in Brassicaceae for locating their herbivorous hosts or prey in greenhouse and in agro-ecosystems (Geervliet *et al.* 1996; Bukovinszky *et al.* 2005; Poelman *et al.* 2009; van Dam *et al.* 2010). Here, we tested whether the temporal dynamics of HIPV emissions could influence the plant's interactions with herbivores and their enemies. We demonstrated that HIPVs allow herbivores as well as their parasitoids to discriminate between herbivore-infested plants subjected to different time periods of damage. Furthermore, the responses of the insects are dependent on temporal variations in the emissions of volatiles after initial feeding damage to plant tissues. This temporal pattern is also reflected in the dynamics of the genes involved in the synthesis of the volatiles and their precursors. Our study thus provides a comprehensive analysis of the mechanisms underlying temporal patterns of HIPV emissions and their function in

natural environments. We suggest further studies to understand the ecological implication of temporal dynamics of HIPVs under field conditions in which multitrophic interactions in agro- and natural ecosystems are compared. In particular, little is known about the accuracy of agricultural systems as proxies for natural systems in which the insects originally evolved (Gols & Harvey 2009; Gols *et al.* 2011). Wild plants often possess traits, such as volatile blends, that are more attractive to natural enemies than cultivated plants which have undergone many generations of artificial selection to accentuate certain traits that may or may not be compatible with indirect defence (Gols & Harvey 2009; Hare 2011). A better knowledge of insect responses in wild plants under natural selection can thus contribute to the development of better biological control practices through selective breeding and hence more sustainable agricultural practices for *Brassica* crops.

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V.M. designed and carried out experiments and wrote the study, T.O.G. Tytgat designed and carried out experiments and co-authored the study, C.A.H. performed the volatile analyses, H.R.H. carried out part of the gene expression analysis, J.J.J. performed the multivariate analyses of the volatile data sets, A.S.R., J.A.H., L.E.M.V. and N.M.van D. supervised and advised the first author during the experiments and commented on earlier versions of the study.

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### **Data accessibility**

Data from the insect preference bioassay, and volatile and gene expression analyses are uploaded as online supplemental material.

### **Supporting information**

Additional supporting information may be found in the online version of this article.

**Fig. S1** Olfactometer set-up for the insect preference test.

**Fig. S2** RT-qPCR on marker genes for hormonal pathways.

**Fig. S3** RT-qPCR on volatile biosynthetic genes.

**Fig. S4** RT-qPCR on sulfur-containing volatile biosynthetic genes.

**Appendix 1** Data S1 Volatile Analysis.

**Appendix 2** Data S2 qPCR analysis.

**Appendix 3** Data S3 Insect preference experiments