Oecologia (2006) 148: 280–292
DOI 10.1007/s00442-006-0365-8

PLANT ANIMAL INTERACTIONS

André Kessler · Rayko Halitschke · Celia Diezel
Ian T. Baldwin

Priming of plant defense responses in nature by airborne signaling between *Artemisia tridentata* and *Nicotiana attenuata*

Received: 25 April 2005 / Accepted: 11 January 2006 / Published online: 7 February 2006

Abstract Plants release volatile organic compounds (VOCs) in response to wounding and herbivore attack, some of which trigger responses in neighboring unattacked plants in the laboratory under conditions that are not likely to occur in the real world. Whether plants 'eavesdrop' on the volatile emissions of their neighbors in nature is not known. The best documented field study of between-species signaling via above-ground VOCs involves increases in fitness parameters of native tobacco (*Nicotiana attenuata*) transplanted adjacent to clipped sagebrush (*Artemesia tridentata tridentata*). Clipped sagebrush releases many biologically active VOCs, including methyl jasmonate (MeJA), methacrolein and a series of terpenoid and green leaf VOCs, of which MeJA, while active under laboratory conditions, is not released in sufficient quantities to directly elicit induced resistance in the field. Here we demonstrate, with laboratory and field-based experiments, that priming (rather than direct elicitation) of native *N. attenuata*'s induced chemical defenses by a sagebrush-released VOC bouquet can account for earlier findings. With microarrays enriched in *N. attenuata* herbivore-regulated genes, we found transcriptional responses in tobacco growing adjacent to clipped sagebrush foliage, but failed to detect the direct elicitation of defensive chemicals or proteins. However, we observed an accelerated production of trypsin proteinase inhibitors when *Manduca sexta* caterpillars fed on plants previously exposed to clipped sagebrush. This readying of a defense response, termed priming, results in lower total herbivore damage to plants exposed to clipped sagebrush and in a higher mortality rate of young *Manduca* caterpillars. Our study demonstrates priming of plant defense responses as a mechanism of plant–plant signaling in nature, and provides an example for the analysis of between-plant signaling under ecologically realistic conditions. Although we describe priming as a potential mechanism for signaling between plants in nature, we critically discuss the ecological relevance of the particular interaction.

Keywords Plant communication · Plant–insect interaction · Proteinase inhibitors · Volatile compounds

Electronic Supplementary Material Supplementary material is available for this article at http://dx.doi.org/10.1007/s00442-006-0365-8 and is accessible for authorized users.

Communicated by Judith Bronstein

A. Kessler · R. Halitschke
Department of Ecology and Evolutionary Biology,
Cornell University, 445 Corson Hall, Ithaca, NY 14853, USA

C. Diezel · I. T. Baldwin (✉) · A. Kessler · R. Halitschke
Department of Molecular Ecology, Max-Planck-Institute for Chemical Ecology, Hans-Knoell-Str. 8, 07745 Jena, Germany
E-mail: Baldwin@ice.mpg.de
Tel.: +49-3641-571101
Fax: +49-3641-571102

Introduction

Plants release volatile organic compounds (VOCs) into the atmosphere in response to biotic and abiotic stresses (De Moraes et al. 2004). These VOCs have the potential to play multiple roles for the plant including defense against pathogens (Croft et al. 1993), ozone (Lerdau and Gray 2003; Lerdau and Slobodkin 2002), and as indirect defenses against herbivores by attracting predators to damaged plants (Dicke and van Loon 2000). The possibility that VOCs function as airborne signals between plants has nurtured a lively discussion (Bruin et al. 1995; Shonle and Bergelson 1995; Agrawal 2000; Dicke and Bruin 2001; Baldwin et al. 2002), which hinges on two mechanistic- and functional-level questions: what are the physiological mechanisms that mediate plant–plant signaling by VOCs in natural environments, and does the signaling have consequences for the Darwinian fitness of the interacting plants?

The release of some of the potential VOC signals are known to be influenced by abiotic factors such as nutrient availability (Schmelz et al. 2003), temperature (Charron et al. 1996), wind, UV radiation (Harley et al. 1996; Zepp et al. 1998; De Moraes et al. 2004), and

ozone exposure (Heiden et al. 1999) in addition to wounding and herbivore attack. These contingencies, in addition to the ephemeral nature of the volatile signal, explain why most studies of plant–plant signaling have been performed in the laboratory (Arimura et al. 2000a, b, 2001; Dicke and Bruin 2001; Engelberth et al. 2004). For example, in experiments with detached lima bean leaves in airtight glass chambers, the expression of defense-related genes was shown to increase in response to exposure to VOCs from spider mite (*Tetranychus urticae*) infested leaves. A detailed analysis revealed a series of terpenoid compounds, such as β-ocimene, dimethyl nonatriene and trimethyl nonatriene, as well as green leaf volatiles (GLVs, C-6 alcohols and aldehydes) as potential signal molecules (Arimura et al. 2000a, b, 2001). In addition, similar experiments with tomato, *Solanum lycopersicum*, (Farmer and Ryan 1990) and black alder, *Alnus glutinosa*, (Tscharntke et al. 2001) suggested that methyl jasmonate (MeJA) and ethylene-mediated resistance in nearby non-attacked plants. More recently, small carbonyl compounds, such as acrolein and malondialdehyde (Almeras et al. 2003; Weber et al. 2004), were added to the list of VOCs that elicit resistance-related gene expression. These experiments demonstrate that exposure to a variety of plant-derived VOCs can elicit responses in neighboring plants. However, the functional and ecological relevance of airborne plant–plant signaling in the real world remain unknown because comparable experiments have not been conducted in the rough and tumble of the natural environment. Moreover, we do not even know how much of a plant's emission in fact reaches a neighboring plant in nature (Baldwin et al. 2002).

Two recent studies with black alder, *Alnus glutinosa*, and a tobacco (*Nicotiana attenuata*) native to the southwestern USA, have provided evidence consistent with airborne plant–plant signaling in nature (Tscharntke et al. 2001; Karban et al. 2000). In both studies, plants growing adjacent to defoliated/clipped plants had a higher resistance to herbivore attack and suffered less damage. Additionally, the studies with native tobacco provide evidence of signaling between plants of different species and the first evaluation of the fitness consequences of this signaling for the receiver plant (Karban and Maron 2002). In southwestern USA, *N. attenuata* occasionally grows in close proximity to mountain sagebrush (*Artemisia tridentata*) (Karban et al. 2000). Plants growing in close proximity to clipped sagebrush suffered significantly less herbivore damage than did plants next to unclipped sagebrush or unexposed plants (Karban 2001; Karban et al. 2000; Karban and Baxter 2001). However, the effect was only significant for tobacco growing within 0–15 cm of the damaged sagebrush (Karban and Maron 2002; Karban et al. 2003). In such close proximity, the negative effects of competitive interaction between the neighboring plants exceeded the positive fitness effects of induced herbivore resistance (Karban et al. 2003). Moreover, the increased production of polyphenol oxidases (PPOs) was the only metabolic response measured in tobacco plants exposed to clipped sagebrush (Karban et al. 2003).

The VOCs released from damaged sagebrush plants that elicit responses in the neighboring tobacco plants remain unknown. MeJA, which is produced in unusually high amounts and released as a VOC from several *Artemisia* species, has been thought to elicit the responses in the neighboring plants. Farmer and Ryan (1990) reported that tomato plants which were exposed to MeJA from *A. tridentata* plants in air-tight glass jars increased their accumulation of defensive proteinase inhibitor (PI) proteins in a dose-dependent manner. In addition, when sagebrush plants were clipped, they released more of the biologically active 3R,7S-epimer of MeJA, than did undamaged plants (Preston et al. 2001). However, recent research demonstrates that the quantities of MeJA released from damaged sagebrush plants in nature are not sufficient to elicit increases in herbivore resistance or the production of secondary metabolites known to mediate herbivore resistance in neighboring plants (Preston et al. 2001, 2004). Clipped sagebrush releases a complex bouquet of VOCs, which include many GLVs, terpenoids and methacrolein (Personius et al. 1987) that could potentially account for the VOC-associated resistance of native tobacco.

The recently described phenomena of defense priming by VOC exposure (Engelberth et al. 2004) may resolve the apparent contradiction between the lack of measurable VOC-elicited defense metabolites and the evidence for induced resistance in the field. Maize seedlings previously exposed to individual GLVs, or to the blend of VOCs released from damaged plants, responded to elicitation by wounding and treatment of the wounds with beet armyworm, *Spodoptera exigua*, regurgitant with higher endogenous jasmonic acid (JA) accumulations and increased VOC production than did unexposed plants. Hence, exposure to VOCs from neighboring attacked plants may allow the neighbors to ready their defenses and respond more rapidly or perhaps to a greater degree if they are subsequently attacked. Here, we determine if priming of induced responses can account for the induced herbivore resistance of native tobacco previously exposed to clipped sagebrush plants. We use a combination of field and laboratory experiments to address the following questions: (1) are there differences in the VOC emission from unclipped and clipped sagebrush, other than that of MeJA (Preston et al. 2001, 2004) that could account for the resistance responses of the tobacco plants; (2) do tobacco plants respond to the volatiles from sagebrush with an increased expression of defense-related genes; (3) is the production of defensive compounds increased as a direct response to exposure to volatiles from clipped sagebrush; and (4) does exposure to clipped sagebrush prime the tobacco plants for a faster and/or stronger response to herbivore attack? Finally, we monitored herbivore damage and performance on sagebrush-exposed tobacco plants growing in natural populations in the Great Basin desert in southwestern Utah to

determine whether the exposure influences herbivory as reported previously in research conducted in California (Karban et al. 2000; Karban and Baxter 2001).

Material and methods

Plant and insect material

For the laboratory experiments, *N. attenuata* plants were grown from an inbred line (11 generations), which derived from seeds collected from a native population (DI Ranch, Santa Clara, Utah). Seeds were germinated on growth media using a described procedure (Krügel et al. 2002) and the seedlings remained on the medium for 5 days. In the young seedling stage, plants were transplanted into root trainers with peat-based soil and watered with distilled water. After 14 days of acclimation to soil, the seedlings were transferred to 1-l pots in which the plants remained until the end of the experiment.

Artemisia tridentata seeds were previously collected from a population in the environs of Santa Clara, Utah (Motagua road: 37°16′38″ N, 113°53′43″ W, elevation: 1,203 m) and germinated on peat soil. After 4 weeks, the plants were transplanted into 4-l pots filled with a peat soil mixture (Krügel et al. 2002). We used native *N. attenuata* and *A. tridentata* plants or foliage for the field experiments at the two sites: Lytle preserve, Beaver Dam Wash (37°08′45″ N, 114°01′11″ W, elevation: 840 m) and Goldstrike mine (37° 22′15.766″ N, 113° 56′15.986″ W, elevation: 1279 m) both located in southwestern Utah, close to the town of Santa Clara, Utah. VOC emissions and herbivory were monitored in two sagebrush populations at Motagua (Motagua: 37°20′12″ N, 114°02′15″ W, elevation: 1,285 m; Motagua road: 37°16′38″ N, 113°53′43″ W, elevation: 1,203 m).

We used larvae of the tobacco hornworm *Manduca sexta*—a natural herbivore of *N. attenuata*—in the herbivore bioassays. The *Manduca* caterpillars for both field and laboratory experiments were from eggs, which were purchased from the insectary of the North Carolina State University (Raleigh, N.C.). Eggs were kept in 250-ml polyethylene containers until they hatched and were used in the experiments.

Sagebrush VOC emission

To determine differences in VOC emissions from damaged and undamaged sagebrush foliage, we haphazardly selected two branches from each of eight *A. tridentata* plants growing in the Motagua road population in May 2002. One of the two branches on each plant was left undamaged; on the other branches we cut all leaves in half orthogonally to the stem. As a result of the clipping, approximately 2 g of sagebrush foliage was removed and remained together with the enclosed clipped branch in a 400-ml polyethylene chamber fitted with holes on two sides. We collected VOCs by pulling air at approximately 300 ml min^{-1} through the chamber over ORBO-32 charcoal traps (SUPELCO). The vacuum was provided by a 12 V Gast vacuum pump connected to a 12 V deep-cycling car battery. After 3 h of VOC trapping, we changed the traps to collect VOCs for a subsequent 3 h. The traps were returned to the laboratory and eluted with 250 μl HPLC-grade dichloromethane. The resulting solution was analyzed with a Varian 2000 GC-MS, which was programmed as previously described (Halitschke et al. 2000). Compounds were identified by comparing retention time and mass spectra with those of authentic standards.

In addition to the GC-MS procedure, we used a calibrated version of the PTR-MS (IONICON, Innsbruck, Austria) equipped with a heated gas inlet capillary system to examine the damaged-induced changes in methacrolein emission from *A. tridentata* foliage in the laboratory. Seeds for plants grown in the laboratory were collected from the same population of plants described in the VOC trapping experiment. Excised but unclipped branches from plants that were 5 months old were placed in a 1-l glass chamber and air from the headspace was pulled from this chamber directly into the PTR-MS. The instrument was operated at a drift tube pressure of 2 mbar. The sample gas was continuously introduced into the chemical ionization cell. The inlet of the mass spectrometer was equipped with a teflon tube with a flow rate of ca. 35 ml min^{-1} and the instrument was operated in ion scan mode (21–161 m/z). After 5 min of headspace accumulation, the leaves of the branches were clipped as described in the field experiment and the headspace was trapped and analyzed. This paired comparative sampling of damaged and undamaged plant tissues was repeated with branches from three different plants. The relative methacrolein concentration was measured as the ion signal intensity of mass 71 after 5 min of sampling. All VOC results were compared with a paired Student's *t*-test.

Laboratory sagebrush exposure experiments

Rosette stage *N. attenuata* plants in 1-l pots were placed in insect cages (30 cm×30 cm×60 cm, LxWxH) with a chimney ventilation system (Fig. 1) to provide conditions that resembled the soaring airflow conditions in the field experiments.

In the first experiment, we exposed two plants per cage to approximately 17 g cut *A. tridentata* foliage for 24 h, while two plants in another cage remained unexposed. The experiment was repeated 5 times (total of 10 cages and 20 plants). After 24 h of exposure to clipped sagebrush foliage, one *N. attenuata* plant from each cage was cut at the base of the shoot and flash frozen in liquid nitrogen for RNA extraction. After 4 days (when induced nicotine accumulations and PI activity are known to reach their maximum in response to damage), we harvested the first stem leaf (source-sink transition leaf) of the second plant, bisected the lamina along the

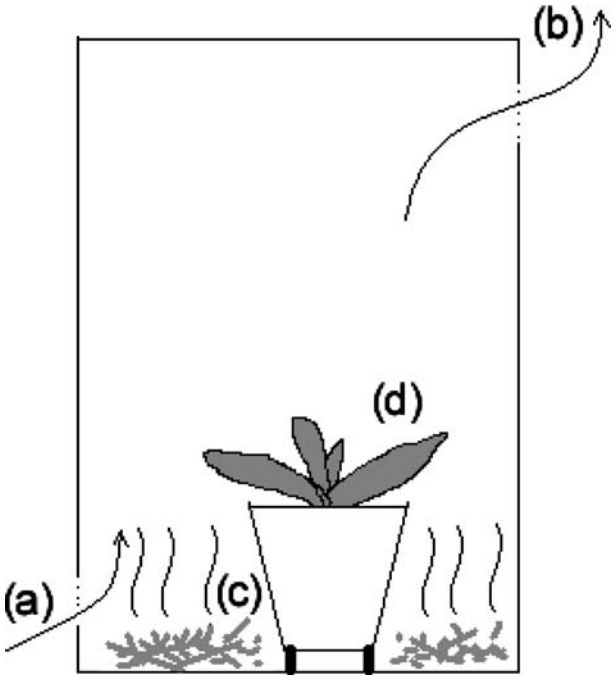

Fig. 1 Chimney ventilation system in the laboratory sagebrush exposure experiments. Ambient air flows into the chamber through a gauze inlet (**a**) and exhaust through an opening at the top of the chamber (**b**). Thereby VOCs from clipped sagebrush (**c**) flow bottom-up and pass the tobacco plant (**d**)

midvein, and separately flash froze (liquid nitrogen) each of the leaf halves (approximately 0.250 g) for secondary metabolite and protein analysis.

In the second experiment, we determined whether plants exposed to sagebrush are primed for more rapid or greater responses to subsequent elicitations. We used 24 cages and placed four *N. attenuata* rosette-stage plants in each cage. The plants in a cage were treated similarly and received one of the four following treatments: (1) no treatment; (2) exposure to 17 g cut *A. tridentata* foliage for 24 h beginning at time 0 h of the experiment; (3) two 2nd-instar caterpillars per plant at 24 h; and (4) exposure to 17 g cut *A. tridentata* foliage for 24 h beginning at time 0 h and the subsequent placing of two 2nd-instar caterpillars per plant at 24 h. We harvested one plant from each cage at 24 h, 48 h, 72 h and 96 h after the initial exposure, so that six replicates per treatment and time point were analyzed. We harvested the source-sink transition leaf of each plant for the analysis of secondary metabolites and proteins as described above and flash froze the rest of the plant for RNA extraction.

Field sagebrush exposure experiments

For the field exposures of *N. attenuata* plants to damaged sagebrush foliage, we used two populations in two consecutive years. For the first experiment in June 2002, four groups of rosette-stage *N. attenuata* plants were selected from a population at the Lytle Preserve, Santa Clara, Utah. One plant in each group remained undamaged (control), the other was exposed to 17 g of cut sagebrush foliage that was equally distributed around the circumference of the plant rosettes within 1 cm, avoiding direct contact with *N. attenuata* leaves. Before the exposure, we excised the source-sink transition leaf from each of the eight plants in the experiment for RNA extraction. After 24 h of exposure, we harvested the leaf at one position younger than the source-sink transition leaf also for RNA extraction.

To determine if the elicitation of secondary metabolites and PIs in plants is primed by exposure to volatiles from sagebrush foliage, we haphazardly selected four groups each with four similar-sized *N. attenuata* plants at the Goldstrike Mine site in June 2003. Each plant in a group received one of the following treatments: (1) no treatment; (2) exposure to 17 g cut *A. tridentata* foliage for 24 h beginning at 0 h; (3) two 2nd-instar caterpillars per plant at time 24 h; and (4) exposure to 17 g cut *A. tridentata* foliage for 24 h beginning at time 0 h and the subsequent placing of two 2nd-instar caterpillars per plant at 24 h. None of the caterpillars in the experiment was lost due to predation or abiotic mortality factors during the course of this experiment. Plants within a group grew at least 1 m from each other to avoid airborne interactions. The groups were selected to have at least 2 m distance between the closest plants of two neighboring groups. We harvested the first and the second stem leaf of all plants 72 h after the initial exposure to sagebrush volatiles. The leaf material was transported in aluminum foil on dry ice and stored at –20°C until analysis of secondary metabolites and trypsin PIs (TPIs).

Field exposure to single *A. tridentata* VOCs

To determine if the exposure to single VOCs from *A. tridentata* can prime metabolic responses in neighboring plants, we haphazardly selected 11 groups of *N. attenuata* plants each including four to eight similar sized plants along a transect across a population of about 100,000 plants. The population was situated on a burned site of the Great Basin habitat type on HI91 near Santa Clara, Utah, USA in July 2004. The plants were elongating but not yet flowering. While the plants within a group were of the same size, the size of plants among groups differed. Each group included an undamaged control plant and a plant that received one 2nd to 3rd instar *M. sexta* caterpillar 24 h (2 July 2004) after the start of the experiment. In addition, every plant group included one or more of the following treatment pairs: (1) exposure to sagebrush at time 0 (1 July 2004) of the experiment and exposure to sagebrush at time 0 plus one 2nd to 3rd instar caterpillar at 24 h (2 July 2004); (2) exposure to (*E*)-2-hexenal at time 0 and exposure to (*E*)-2-hexenal at time 0 plus one 2nd to 3rd instar caterpillar at 24 h; (3) exposure to methacrolein at time 0 and exposure to methacrolein at time 0 plus one 2nd to 3rd

instar caterpillar at 24 h, and (4) exposure to MeJA at time 0 and exposure to MeJA at time 0 plus one 2nd to 3rd instar caterpillar at 24 h. Every treatment was replicated 6 times and the undamaged control and the exclusive caterpillar treatments were replicated 11 times. We harvested three stem leaves from each of the 70 plants in the experiment 72 h after the initial exposure to the VOCs. The leaf samples were treated as described previously for the analysis of TPI activity. Several samples were excluded from the statistical analysis because they either received additional herbivore damage when undesired or no caterpillar damage when desired. Due to the heterogeneity of developmental stages across sample groups, TPI values of a particular treatment were expressed relative to the corresponding TPI value of the undamaged control in the respective experimental group.

Herbivory

We selected 40 similar-sized *N. attenuata* plants along a transect across a population of approximately 3,000 plants on a previously burned site of a montane Great Basin habitat type (Motagua). Total herbivore damage was monitored on plants that were either unexposed or exposed to 17 g of clipped sagebrush leaves in the field on 12 June 2003. The damage was estimated as percent total leaf tissue lost 1 week after the initial exposure. Three exposed and two unexposed experimental plants were excluded from the analysis because one or both of the plants disappeared for unknown reasons. The data were analyzed using ANOVA.

We used a similar experimental procedure on the same field site (Motagua, $n=20$) and two additional sites: Motagua road ($n=14$) and Goldstrike Mine ($n=32$) to study the performance of *M. sexta* caterpillars on unexposed and clipped sagebrush-exposed plants. One freshly hatched caterpillar was placed on the third stem leaf of each of the plants of the two different treatments 24 h after the initial exposure. After 4 days, we counted the surviving caterpillars to calculate mortality rates. The mortality rates at all three sites were compared with paired Student's *t*-tests.

To determine if predators are influenced by the clipped sagebrush VOC signal we selected 16 similar-sized plants of which 8 were haphazardly selected to remain unexposed and 8 were exposed to clipped sagebrush for 24 h. After the exposure, 5 *M. sexta* eggs were glued to the third stem leaf of each plant in the experiment as described in Kessler and Baldwin (2001) and the number of predator-attacked eggs was counted 1 day later. The predation rates were compared with Student's *t*-tests.

Secondary metabolite and protein analysis

Leaf samples were weighed, processed and analyzed for alkaloid and phenolic secondary metabolites by HPLC as described by Keinanen et al. (2001). We quantified nicotine and chlorogenic acid with external standards and expressed the contents as $\mu g\ g^{-1}$ fresh mass leaf material. Caffeoyl putrescine was expressed as chlorogenic acid equivalent. We used a Student's *t*-test for the first laboratory exposure experiment and an ANOVA with Bonferroni post hoc tests for the second laboratory and the field exposure experiment to compare differences among treatment groups.

Protein content and TPI activity of the other half of the flash frozen samples were determined with a radial diffusion assay as described by van Dam et al. (2001) and PPO activity was determined by spectrophotometry as described by Kessler and Baldwin (2002). Both TPI and PPO contents were expressed relative to the protein content as determined by the Bradford assay (van Dam et al. 2001). Student's *t*-test and ANOVAs were used to compare different treatment groups.

Nucleic acid analysis

The frozen plant material was ground in liquid nitrogen and RNA was extracted with TRI-Reagent (SIGMA) following the manufacturer's protocol. Fluorescently-labeled cDNA derived from differentially treated plant material was hybridized to a cDNA microarray (Halitschke et al. 2003) as described in the supplementary material (Table S1).

The transcript accumulation of NaTPI of treated and untreated plants was compared using quantitative real-time PCR. The isolated RNA of each treatment and each time harvest was quantified spectrophotometrically and was diluted to 100 ng μl^{-1}. Twenty nanogram of the diluted total RNA was reverse transcribed (Applied Biosystems, Foster City, CA), and 10 ng of the reverse-transcribed templates was used in a real time PCR as described by Halitschke et al. (2003). The 25 μl reaction mix contained 1× universal mix (Eurogentec, Brussels, Belgium), 300 nM forward (5′-TCAGGAGATAGT AAATATGGCTGTTCA-3′) and reverse primers (5′-ATCTGCATGTTCCACATTGCTTA-3′), 300 nM of fluorescence dye-labeled TaqMan® probe (FAM-5′-TCCTTGCTCTCCTCCTCTTATTTGGAATGTCT-3′-TAMRA) and 1× 18S RNA reagent kit (Applied Biosystems) to normalize TPI expression levels (Zavala et al. 2004b). Thermal cycling and detection was performed on a sequence detector (ABI Prism 7700; Applied Biosystems).

Results

VOC emission from clipped sagebrush

Clipped sagebrush released dramatically larger amounts of VOCs than did undamaged sagebrush in the first 3 h after damage. Most of the identified compounds were terpenoids, such as the monoterpenes

β-pinene, 1,8-cineole, (*E*)-ocimene, linalool, β-phellandrene, camphor and the sesquiterpene artemiseole and one unidentified sesquiterpene (UC7). Although all terpenoids tended to be emitted in higher amounts from clipped compared to unclipped sagebrush branches, only, 1,8-cineole, (*E*)-ocimene and *p*-cymene emissions were significantly higher as determined by a paired Student's *t* test (Fig. 2). In addition, we found a series of GLVs such as (*Z*)-3-hexen-1-ol, *cis*-3-hexenyl acetate, (*E*)-2-hexenal to be released in higher amounts after damage. Only the release of (*E*)-2-hexenal emission was significantly elevated by clipping (Fig. 2). A series of compounds, which resulted in some of the largest peaks in the chromatograms from damaged sagebrush, remain unidentified (Fig. 2).

The majority of the damage-induced emission occurs within the first 3 h after damage and we found equal amounts of VOCs emitted from damaged and undamaged plants in the second VOC trapping period, 3–6 h after the damage (Fig. 2a).

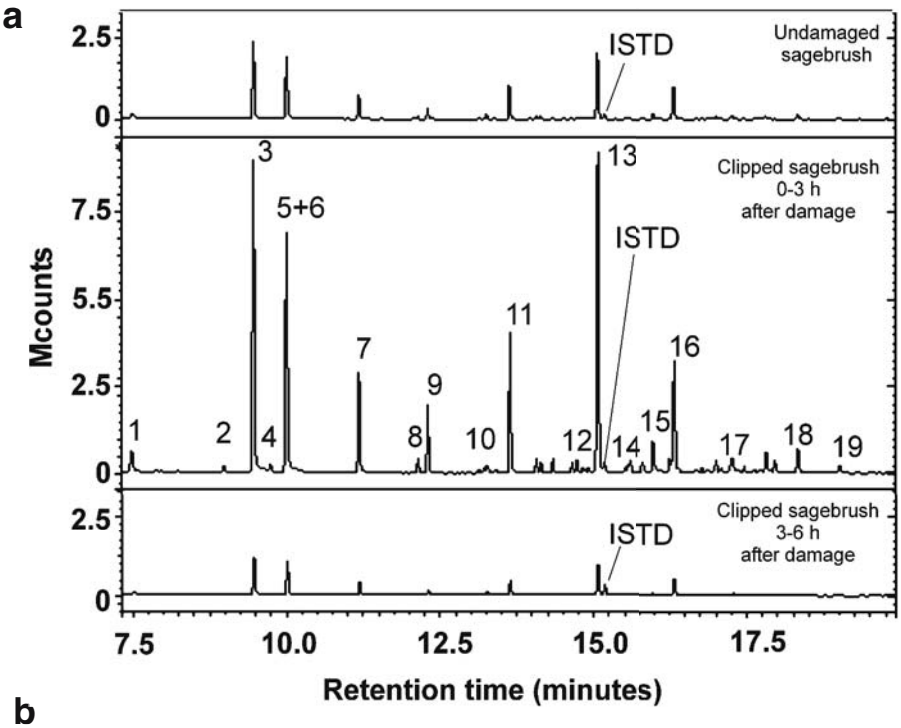

Fig. 2 Volatile organic compounds of sagebrush, *Artemisia tridentata*. **a** Representative Total Ion gas-chromatograms (*GC*) of headspace VOCs released from undamaged and clipped sagebrush in the first 3 h after damage and in the 3 h period 3–6 h after damage. **b** Mean (±SEM) VOC emission of the major compounds and compounds with the most significant increases in emission after clipping. Numbers (*No*) designate the compounds and their respective GC-peaks in **a**. The last column of the table provides the statistics of a paired Student's *t*-test as computed from VOC data of paired undamaged and clipped sagebrush branches of eight different plants in the field. Values are in ng per mg fresh mass of clipped plant material and h of VOC collection (ng mg^{-1} h^{-1}). Compound quantities were determined using tetraline as an internal standard (ISTD)

b

		Emission (ng mg^{-1} h^{-1})			
No	**Compound**	**Undamaged**	**Clipped sagebrush**	**Increase**	**Paired Student's *t*-test**
1	β-pinene	5.25±3.33	12.09±6.72	2.3x	*t*=-1.99, *P*=0.09
2	α-phellandrene	1.75±0.65	7.03±3.87	4.0x	*t*=-1.61, *P*=0.15
3	artemiseole	80.86±70.6	225.85±195.95	2.8x	*t*=-1.16, *P*=0.29
4	ocimene	1.55±0.94	3.26±1.59	2.1x	*t*=-2.54, *P*=0.04
5	1,8-cineole	53.47±23.65	127.20±48.25	2.4x	*t*=-2.94, *P*=0.02
6	(*E*)-2-hexenal	14.93±6.78	35.93±13.66	2.4x	*t*=-2.96, *P*=0.02
7	p-cymene	36.11±11.02	91.45±27.42	2.5x	*t*=-3.28, *P*=0.01
8	*cis*-3-hexenyl acetate	5.55±5.05	12.73±11.2	2.3x	*t*=-1.17, *P*=0.28
9	UC1	206.67±189.46	533.51±497.82	2.6x	*t*=-1.06, *P*=0.32
10	*cis*-3-hexenol	3.11±2.68	8.30±5.36	2.7x	*t*=-1.82, *P*=0.11
11	UC2	662.21±356.71	1697.46±992.57	2.6x	*t*=-1.62, *P*=0.15
12	UC3	1.35±0.48	14.20±4.83	10.5x	*t*=-2.92, *P*=0.02
13	(+/-) camphor	200.14±169.97	538.74±446.49	2.7x	*t*=-1.22, *P*=0.26
14	linalool	1.25±0.92	3.54±2.28	2.8x	*t*=-1.68, *P*=0.14
15	UC4	54.82±21.55	187.25±80.35	3.4x	*t*=-2.23, *P*=0.06
16	UC5	695.90±195.04	2161.01±769.63	3.1x	*t*=-2.48, *P*=0.04
17	UC6	93.64±27.20	279.03±85.93	3.0x	*t*=-3.08, *P*=0.02
18	UC7	213.39±68.75	660.18±210.82	3.0x	*t*=-2.83, *P*=0.03
19	UC8	60.21±42.68	213.73±114.63	3.5x	*t*=-2.10, *P*=0.07

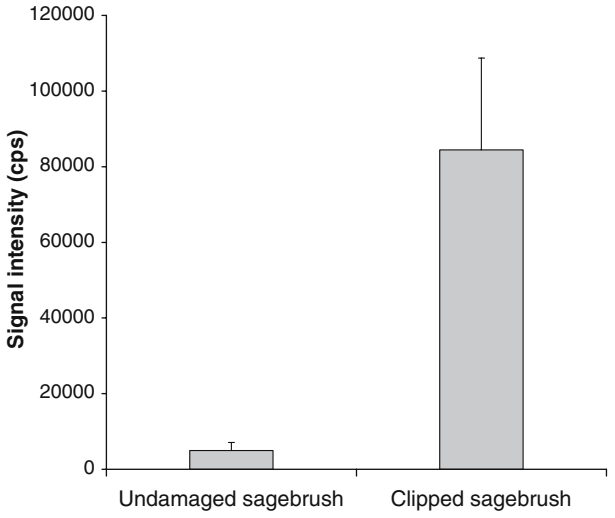

Fig. 3 Mean (±SEM) methacrolein emission from undamaged and clipped sagebrush foliage. Data are shown as signal intensity (counts per second, *cps*) of a PTR-MS analysis

The PTR-MS analysis revealed that 17 times more methacrolein was released from damaged compared to undamaged sagebrush plants (paired Student's *t*-test, $t = -4.66$, $P = 0.04$; Fig. 3).

N. attenuata gene expression in response to sagebrush exposure

The microarray analysis of glasshouse-grown plants revealed that the relative expression of very few of the genes on the microarray responded directly to the exposure of clipped sagebrush (Table S1). Interestingly, *N. attenuata's* xyloglucan endo-transglycosylase (NaXET) and NaTPI were among the few up-regulated mRNA species. Moreover, among the genes whose expression was down-regulated, we found genes coding for proteins involved in photosynthesis such as the small subunit of the tobacco ribulose-1,5-bisphosphate carboxylase and a photosystem II protein (NtPII10).

A similar analysis using cDNA produced from field-collected mRNA from the same plant before and after exposure to sagebrush and hybridized to the microarray also revealed only minor changes in expression as a result of direct exposure to sagebrush volatiles (Table S1). In contrast to the glasshouse study, other defense-related genes, such as *N. attenuata*'s threonine deaminase and pathogen-inducible α-dioxygenase (PIOX_NICAT), were found to be up-regulated. However, consistent with the glasshouse study, the expression of photosynthesis-related genes, such as tobacco ribulose-1,5-bisphosphate carboxylase small subunit and the photosystem II gene (NtPII10) were found to be down-regulated.

The RT-PCR analysis revealed that NaTPI reaches a maximum transcript accumulation 24 h after the beginning of the exposure to VOCs from clipped sagebrush (Fig. 4a) which declined to control levels by day 4. Caterpillar attack resulted in a similar increase in NaTPI

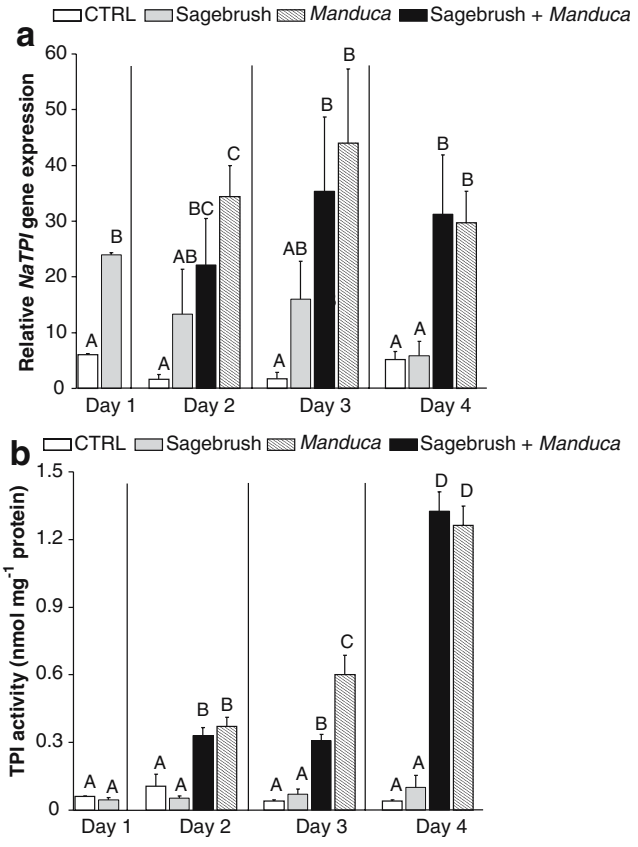

Fig. 4 Priming of the trypsin proteinase inhibitor (TPI) activity in *N. attenuata*. **a** *NaTPI* gene expression and **b** TPI activity in undamaged plants (CTRL) and *N. attenuata* plants that were exposed to *A. tridentata* VOCs for 24 h (sagebrush), damaged by *M. sexta* caterpillars (*Manduca*) or were first exposed to *A. tridentata* VOCs and subsequently attacked by *M. sexta* caterpillars (sagebrush + *Manduca*) on four consecutive days. On day 0, plants of the treatment groups "sagebrush" and "sagebrush + *Manduca*" were exposed to sagebrush for 24 h. On day 1, two 2nd instar caterpillars were released on each of the plants in the treatment groups "*Manduca*" and "sagebrush + *Manduca*". Different letters in the graphs designate significantly different relative *NaTPI* gene expression or TPI concentration as computed from a Bonferroni post hoc test ($P < 0.05$) of an ANOVA

transcripts after 24 h caterpillar feeding, which increased to higher levels as the caterpillars continued their feeding. Interestingly, in plants exposed to both sagebrush volatiles and 24 h of caterpillar attack, transcript levels were marginally, but not significantly, higher than those observed in attacked but unexposed plants (Fig. 4a; ANOVA, $F = 10.19$, $P = 0.0042$, Bonferroni post hoc test $P > 0.05$) suggesting that exposure to sagebrush volatiles may potentiate transcript accumulation.

TPI and secondary metabolite accumulation in response to sagebrush volatiles and caterpillar attack

Exposure to VOCs from clipped sagebrush did not significantly alter the accumulation of defensive compounds in *N. attenuata* (Table 1, Fig. 4b, Table S2).

However, caterpillar-attacked plants that were previously exposed to clipped sagebrush increased their TPI activity in leaf tissues more rapidly than did plants that had only been attacked by caterpillars, resulting in significantly higher TPI activity on day 3 (ANOVA, $F = 25.95$, $P < 0.0001$, Bonferroni post hoc test $P < 0.05$). By day 4, TPI activity had reached maximum values and were equal in both caterpillar-damaged plants and VOC-primed and caterpillar-damaged plants (Fig. 4b; ANOVA, $F = 114.96$, $P < 0.0001$, Bonferroni post hoc test $P > 0.05$).

This potentiation of herbivore-elicited TPI increases by exposure to clipped sagebrush was more pronounced in plants in the field experiments (Fig. 5; ANOVA, $F = 6.5$, $P = 0.012$). TPI concentrations of previously sagebrush-exposed plants were two-fold higher than those of exclusively caterpillar-damaged plants (Bonferroni post hoc test $P < 0.05$), which were not different from those of control and exclusively sagebrush-exposed plants (Bonferroni post hoc test $P > 0.05$). In the field experiment in Utah, TPI concentrations were in general higher than those of glasshouse-grown plants after 48 h (Day 3) of continuous caterpillar feeding.

The amount of diterpene glycosides (and to a lesser degree, chlorogenic acid) increased more rapidly in sagebrush VOC-exposed plants and reached higher total amounts compared to those of exclusively caterpillar-damaged plants (Table 1), and hence showed responses similar to those of the TPIs. Nicotine and caffeoylputrescine concentrations were significantly increased after caterpillar damage compared to undamaged controls but were not influenced by exposure to sagebrush volatiles (Table 1).

With a similar experimental design, we tested three different compounds [(*E*)-2 hexenal, methacrolein and MeJA] whose emissions from sagebrush are increased by mechanical damage for their ability to prime plants for more rapid and greater secondary metabolite responses to herbivore attack (Fig. 6). Plants that were exposed to these three compounds all increased their TPI production more rapidly when plants were attacked by *Manduca* caterpillars than did unexposed control plants [ANOVA, $F = 2.8$, $P = 0.012$, Fisher's PLSDs $P < 0.05$, for sagebrush, (*E*)-2 hexenal and methacrolein exposure]. However the priming effect in response to MeJA exposure was not statistically significant (Fisher's PLSD, $P = 0.08$).

Herbivory on sagebrush-exposed *N. attenuata* plants

N. attenuata plants growing in a natural population, which had been exposed to clipped sagebrush for 24 h, were not attacked by herbivores for a subsequent observation period of 7 days, while unexposed control plants received on average damage to 4.75% of their total leaf area (ANOVA, $F = 9.1$, $P = 0.02$, Fig. 7a). Consistent with these observations, *M. sexta* caterpillars feeding on previously sagebrush-exposed plants suffered on average higher mortality in three different native populations than did caterpillars feeding on unexposed control plants (Paired Student's *t*-test, $t = 0.64$, $P = 0.046$, Fig. 7b). As in earlier studies, the predatory bug *Geocoris pallens* was the only predator that occurred in notably high numbers (Kessler and Baldwin 2002, 2004). It preferentially attacks *Manduca*

Table 1 Mean (±SEM, $n = 6$) concentrations of *N. attenuata* secondary metabolites on four consecutive days in untreated plants (Control), after 24 h exposure to sagebrush (sagebrush exposure), after *Manduca* caterpillar damage starting on day 1 (*Manduca*), and after a combined treatment of 24 h sagebrush exposure and the continuous damage of the *Manduca* caterpillars starting on day 1 (Sagebrush + *Manduca*)

Compound[a]	Day	Control	Sagebrush exposure	*Manduca*	Sagebrush + *Manduca*	ANOVA
Nicotine	1	111.5 ± 7.2 (A)	95.7 ± 21.5 (A)	–	–	$F = 2.8$; $P = 0.125$
	2	173.3 ± 12.5 (AB)	142.3 ± 24.5 (A)	249.1 ± 27.0 (C)	227.7 ± 15.6 (BC)	$F = 5.5$; $P = 0.006$
	3	161.3 ± 10.8 (A)	140.0 ± 19.8 (A)	274.6 ± 21.7 (B)	242.3 ± 30.8 (B)	$F = 8.6$; $P = 0.0007$
	4	195.4 ± 6.1 (A)	187.9 ± 16.3 (A)	314.6 ± 30.2 (B)	276.1 ± 25.1 (B)	$F = 15.5$; $P < 0.0001$
Caffeoylputrescine	1	22.5 ± 7.5 (A)	21.4 ± 5.4 (A)	–	–	$F = 0.8$; $P = 0.39$
	2	21.9 ± 4.7 (A)	23.4 ± 1.0 (A)	25.5 ± 1.1 (A)	20.6 ± 5.4 (A)	$F = 0.8$; $P = 0.56$
	3	22.6 ± 1.0 (A)	23.7 ± 1.0 (A)	35.5 ± 11.4 (A)	19.5 ± 1.4 (A)	$F = 1.3$; $P = 0.3$
	4	20.0 ± 1.5 (A)	24.2 ± 1.3 (A)	41.2 ± 3.9 (B)	37.1 ± 2.9 (AB)	$F = 4.6$; $P = 0.011$
Chlorogenic acid	1	30.5 ± 0.2 (A)	30.5 ± 1.3 (A)	–	–	$F = 0.001$; $P = 0.97$
	2	26.3 ± 1.5 (A)	23.6 ± 0.9 (A)	24.3 ± 1.0 (A)	21.0 ± 4.3 (A)	$F = 0.9$; $P = 0.48$
	3	22.2 ± 0.8 (A)	22.2 ± 0.8 (A)	23.8 ± 0.8 (A)	21.2 ± 5.5 (A)	$F = 0.14$; $P = 0.94$
	4	24.1 ± 2.0 (A)	25.1 ± 4.9 (A)	38.9 ± 2.9 (B)	50.2 ± 2.2 (C)	$F = 10.2$; $P = 0.0001$
DTG total	1	7.2 ± 0.7 (A)	7.4 ± 1.2 (A)	–	–	$F = 2.1$; $P = 0.18$
	2	8.3 ± 1.8 (A)	7.0 ± 3.0 (A)	17.0 ± 3.3 (B)	26.1 ± 5.3 (B)	$F = 6.1$; $P = 0.004$
	3	6.7 ± 1.5 (A)	16.2 ± 4.3 (AB)	18.8 ± 3.8 (B)	25.3 ± 3.7 (B)	$F = 4.8$; $P = 0.01$
	4	7.7 ± 1.2 (A)	14.5 ± 3.5 (AB)	19.1 ± 4.8 (B)	29.6 ± 2.7 (C)	$F = 11.1$; $P < 0.0001$

Different letters in parenthesis designate significantly different relative concentration of the compounds as determined from a Bonferroni post hoc test ($P < 0.05$) of an ANOVA, whose statistics are given in the last column of the table

[a]Expressed as $\mu g\ g^{-1}$ fresh mass; caffeoylputrescine and cryptochlorogenic acid are expressed as chlorogenic acid equivalents. DTG total is expressed as peak areas at 210 nm μg^{-1} fresh mass

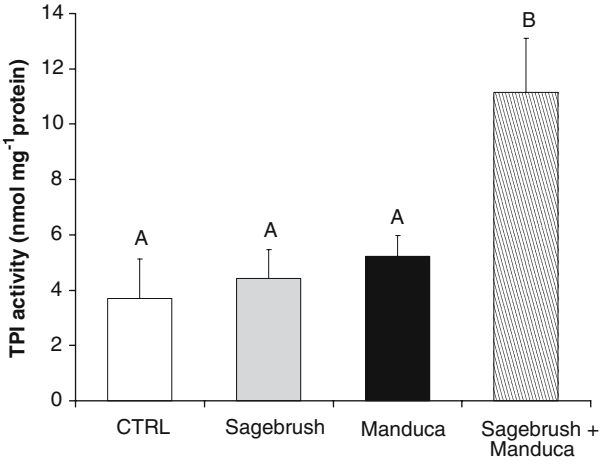

Fig. 5 Priming and induction of the TPI activity in nature. *N. attenuata* plants remained undamaged (CTRL), were exposed to *A. tridentata* VOCs for 24 h (sagebrush), damaged by *M. sexta* caterpillars (*Manduca*) or were first exposed to *A. tridentata* VOCs and subsequently attacked by *M. sexta* caterpillars (sagebrush + *Manduca*) on day 3 after initial exposure to sagebrush in the plant's native environment. On day 0, plants of the treatment groups "sagebrush" and "sagebrush + *Manduca*" were exposed to sagebrush for 24 h. On day 1, two 2nd instar caterpillars were released on each of the plants in the treatment groups "*Manduca*" and "sagebrush + *Manduca*". *Different letters* in the graph designate significantly different TPI activity as computed from a Bonferroni post hoc test ($P < 0.05$) of an ANOVA

eggs and caterpillars of the first two larval instars (Kessler and Baldwin 2002). In our experiment, eggs suffered similar predation rates when glued to untreated tobacco plants and plants that were exposed to clipped sagebrush (Student's *t*-test, $t = -0.47$, $P = 0.65$, Fig. 7c). Therefore, the higher mortality was not likely caused by an increased attraction of predators to clipped sagebrush material surrounding the caterpillar host plants.

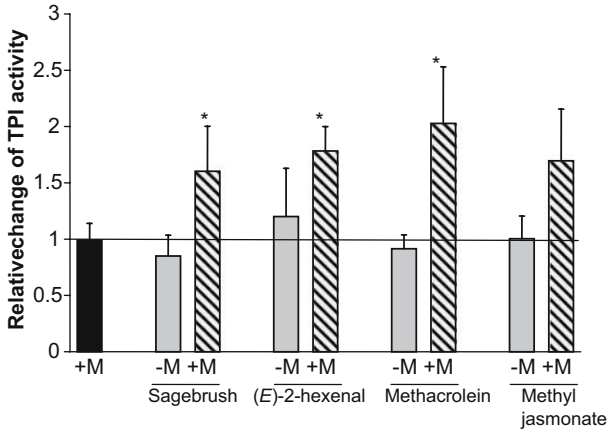

Fig. 6 Field exposure to single VOCs. Relative change in TPI activity (normalized to TPI levels of undamaged control plants: *solid line*) after sagebrush and single VOC [(*E*)-2-hexenal, methacrolein and methyl jasmonate] exposure and *Manduca* caterpillar damage. Results from treatments in which plants were exclusively exposed to clipped sagebrush or single compounds for 24 h are shown as *plain gray bars* (-M). Additional *Manduca sexta* caterpillar damage for 48 h after the exposure to the VOCs is shown in *gray bars with black lines* (+M) (exclusive *Manduca* damage *black bar*). *Stars* designate means of treatments which were significantly ($P < 0.05$) different from those of the undamaged control and the exclusive *Manduca* damage treatments as determined by a Fisher's PLSD of an ANOVA

Discussion

The clipping treatment increased the emission of a large number of sagebrush VOCs from different compound classes, all of which are candidates for signals that could trigger plant responses, because their emission is correlated with increased resistance in neighboring tobacco plants (Karban and Maron 2002; Karban et al. 2003; Fig. 7). A number of VOCs that we identified in the headspace of clipped sagebrush have been shown to elicit transcriptional responses in previous laboratory studies in other plant species (Baldwin et al. 2002) including (*E*)-2-hexenal, methacrolein and ocimene (Arimura et al. 2000a, 2001; Weber et al. 2004; Engelberth et al. 2004). Most of the previous studies had evaluated plant responses to the VOCs of neighboring herbivore-attacked plants on the transcriptional level and rarely considered the induced production of herbivore-defensive secondary metabolites and proteins (Engelberth et al. 2004). To better understand the mechanisms responsible for the airborne interaction between sagebrush and native tobacco, we measured the tobacco's responses to clipped sagebrush VOCs on the transcriptional, translational, metabolic, and functional level.

We used cDNA microarrays to detect transcriptional changes in the tobacco plants, which were exposed to volatiles from clipped sagebrush. Such transcriptional changes can function as rapid indicators of responses that eventually mature into a resistant phenotype, or of signals which are detected by the plant but later do not result in metabolic responses (Baldwin et al. 2002). Unattacked tobacco plants, growing adjacent to clipped sagebrush, accumulated defense-related transcripts in similar ways to that previous research had demonstrated in herbivore-attacked plants (Halitschke et al. 2003; Voelckel and Baldwin 2004). However, in comparison to previous studies of herbivore-attacked plants with the same microarrays (Halitschke et al. 2003; Voelckel and Baldwin 2004), the expression ratios as well as the number of genes that were differentially regulated were consistently lower in both the field and the glasshouse experiments. Moreover, some of the wound- and VOC-responsive genes, such as NaTPI and threonine deaminase, were differently regulated in the field and the laboratory experiments (Table S1). Two explanations can account for the low expression ratios that we observed: First, the native tobacco plants may respond in a dose dependent manner to clipped sagebrush VOCs and the exposures to VOCs that we thought were realistic doses in this study were not sufficient to elicit a significant transcriptional response. Second, we used custom cDNA

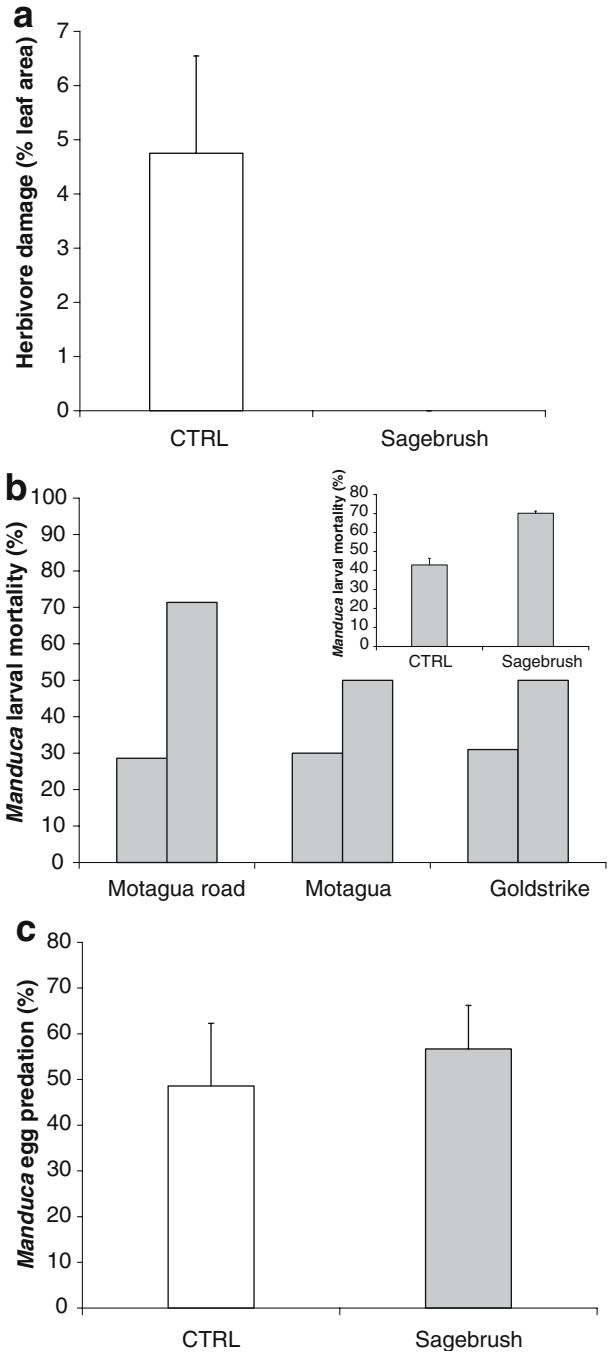

Fig. 7 Herbivore performance on untreated control plants (*white bars*) and plants exposed to clipped sagebrush (*gray bars*) in nature. **a** Mean (+SEM) total herbivore damage after 1 week of exposure to clipped sagebrush. **b** *Manduca* mortality after 4 days at three different field sites and mean (+SEM) *Manduca* caterpillar mortality (inset graph) and **c** mean (+SEM) *Manduca* egg predation after 48 h on untreated control plants and plants exposed to clipped sagebrush

microarrays that were enriched in *N. attenuata* herbivore-regulated genes (Halitschke et al. 2003) and these arrays may not have been able to detect the specific responses to sagebrush VOCs.

The differences in the transcriptional responses of the tobacco plants in the field and in the glasshouse could be a consequence of the two different experimental methods that we used. In the field experiment, the control and treatment leaves of sagebrush-exposed plants were taken from the same plant, with the control leaf harvested prior to the sagebrush exposure and the treatment leaf being removed after 24 h exposure. Hence the treated plants of the field experiment experienced a small amount of wounding before being exposed to sagebrush VOCs (B in Table S1). This apparent harvesting effect is indicated by a minor increase in the expression of certain genes in unexposed control plants (A in Table S1). However, more genes were regulated to a greater extent in the sagebrush-exposed plants in the field compared to the glasshouse experiments. If we subtracted the genes that are regulated in control plants from those regulated in sagebrush-exposed plants, the transcriptional response to sagebrush VOCs still appears to be stronger in the field. The additional wound signal may amplify the response to the sagebrush VOCs, a mechanism that has been suggested in the lima bean system (Arimura et al. 2001). Alternatively, the additional environmental signals that field-grown plants are exposed to may influence the signal perception and the response to sagebrush VOCs. However, both field and glasshouse experiments demonstrated that *N. attenuata* plants can receive and respond to VOCs from clipped sagebrush in natural settings, which extends earlier laboratory studies with lima bean (Arimura et al. 2000a, 2001), tomato (Farmer and Ryan 1990) and maize (Engelberth et al. 2004).

Despite the transcriptional responses, exposure to sagebrush VOCs did not directly increase the accumulation of defensive secondary metabolites or proteins. As with previous research that exposed native tobacco to amounts of MeJA that are released from clipped sagebrush (Preston et al. 2001, 2004), we found no evidence that exposure to clipped sagebrush VOCs or MeJA, directly influenced the accumulation of any defense metabolite (Tables 1, S2, Figs. 4b, 6). We did not detect a VOC-induced increase in PPO activity, as previously reported by Karban et al. (2003). In numerous glasshouse experiments we could not elicit increases in PPO activity in plants damaged mechanically and by *M. sexta* feeding or in plants exposed to MeJA in a wide range of doses (A. Kessler, U. Schittko and I. T. Baldwin, unpublished results). However, PPO activity varied strongly within the plant, depending on the leaf age (Kessler and Baldwin 2002). Therefore ontogenetic differences rather than elicitation may account for the patterns of PPO expression observed by Karban et al. (2003).

The defensive function of TPIs has been extensively demonstrated (Glawe et al. 2003; Zavala et al. 2004a, b) and their production increased in herbivore-damaged tobacco plants and tomato plants that had been exposed to MeJA extracted from sagebrush tissue in sealed chambers (Farmer and Ryan 1990). Our experiments were not able to confirm the elicitation of TPI activity by

exposure to sagebrush VOCs (Figs. 4, 5, 6, Table S2), which is congruent with previous research (Preston et al. 2001, 2004). We propose that exposure of plants to VOCs in sealed chambers subjects them to higher doses of VOCs than they experience under natural circumstances and these differences in elicitor doses may explain the differences in plant responses.

Despite the lack of direct defense responses to sagebrush exposure, we found lower herbivore damage and higher mortality rates of *Manduca* caterpillars on plants that had been previously exposed to clipped sagebrush, which is consistent with earlier findings on the same system (Karban et al. 2000; Karban and Maron 2002). The higher *Manduca* caterpillar mortality was most likely not a result of a predator attraction to the clipped sagebrush VOCs. *Manduca* eggs glued to the leaves of unexposed and sagebrush-exposed plants suffered from similar predation rates (Fig. 7c). This suggested that activation of direct plant defenses accounted for the increased mortality of *Manduca* caterpillars, which do not usually leave the plant on which the eggs have been laid before they reach the third or fourth larval instar (Kessler and Baldwin 2002).

The damage- or herbivore-induced production of defensive proteins (e.g. TPIs) and secondary metabolites (e.g. nicotine) causes significant fitness costs for tobacco plants, particularly when they are grown with competitors not producing these defenses (Baldwin 1998; Zavala et al. 2004a). A direct induction of defense compounds in response to the VOCs from damaged neighboring plants would cause similar fitness costs without an herbivore actually feeding on the plant. Hence, plants that avoid investing fitness-limiting resources in the production of costly defenses before an herbivore arrives, but are able to prime defense metabolism so as to launch faster or stronger defense responses when the herbivores attack, could realize a fitness benefit over plants that "ignored" the information provided by the VOCs emanating from the damaged neighbors. Consistent with this scenario, NaTPI transcripts significantly accumulated in response to sagebrush volatiles, but the plants did not increase TPI activity until the herbivores attacked the plant. Attacked plants that had been exposed to clipped sagebrush increased their TPI activity earlier than plants that had not been exposed, in both the glasshouse and field experiments (Figs. 4, 5). In addition, the elicitation of some secondary metabolites with potential herbivore-defensive function was also primed by exposure to clipped sagebrush VOCs (Table 1). Being able to eavesdrop on environmental signals and prime plastic responses without incurring the fitness-costs of bringing the response to maturity may be a significant component of the complicated transcriptional responses observed in plants growing in natural environments.

A similar priming of defense responses has recently been reported in laboratory experiments with maize seedlings (Engelberth et al. 2004). In these experiments, prior exposure to GLVs resulted in higher endogenous JA and sesquiterpene production in response to damage and caterpillar regurgitant treatment than in plants that had been similarly damaged and treated but not exposed to the VOCs. Interestingly, in these experiments exposure to GLVs alone without damage was sufficient to increase production of sesquiterpene emission and JA. This difference may reflect a particular sensitivity of maize to VOC exposure, but it may again have resulted from the lower amounts of VOCs that were experienced by the tobacco plants in our field and glasshouse experiments. Although we mimicked natural emissions, it remains unclear whether we mimicked exposures because it is unknown how much VOCs from herbivore-damaged plants are actually adsorbed and perceived by their neighbors (Baldwin et al. 2002).

In addition, the identity and characteristics of the signaling compounds remain elusive. Therefore we tested three compounds [(*E*)-2-hexenal, methacrolein and MeJA] of the clipped sagebrush bouquet for their ability to prime the tobacco TPI response (Fig. 6). We found priming effects with all three compounds, although the response to MeJA exposure was not statistically significant. All three compounds in addition to others (e.g. ocimene; Arimura et al. 2000a) have been shown to elicit responses in prior studies (Farmer and Ryan 1990; Arimura et al. 2001; Weber et al. 2004). Our results demonstrate that these compounds can also prime responses in nature. The fact that all three compounds were active and that many others with potential signaling function occur in the VOC bouquet of clipped sagebrush suggests an unspecific signal perception mechanism in the receiver plant. If this was the case, airborne plant–plant interactions would be far more common than currently expected.

Our results demonstrate that the priming of plant defense responses can be a response to the perception of VOC from damaged neighboring plants and a mechanism by which plants may increase their resistance in natural environments. Such a behavior is likely advantageous when the fitness of plants is strongly reduced by herbivore damage (as it is in *N. attenuata* plants in nature: Kessler and Baldwin 2004) and when induced defenses are associated with significant fitness costs when herbivores are absent (as it is in *N. attenuata*: Baldwin 1998; Zavala et al. 2004a). As a consequence, we would predict *N. attenuata* to be under strong selection to perceive signals from neighbors that are damaged by a shared herbivore community. However, when drawing these conclusions, we should keep the particular natural history of the players in mind. We doubt that the interaction between sagebrush *A. tridentata* and the wild tobacco *N. attenuata* is of ecological relevance. The herbivory-reducing effect of the interaction can only be observed if the plants grow within 15 cm of each other (Karban and Maron 2002; Karban et al. 2003), which is rarely observed in nature, and where the negative fitness effects of competition exceed the positive effects of VOC-induced herbivore resistance (Karban et al. 2003). In our study area in the southwest of Utah, these two plant species rarely co-occur. The two

species are signature species of plant communities of completely different successional stages with no temporal overlap. If tobacco plants are to gain significant fitness benefits from growing next to sagebrush, we would expect the co-occurrence of these two species to be more common. Moreover, the most common and damaging herbivores on wild tobacco plants are solanaceous specialists, such as the two sphingid species *M. sexta* and *M. quinquemaculata*, the mirid bugs (*Tupicoris notatus*) and the flea beetle (*Epitrix hirtipennis*). Grasshoppers (*Trimerotropis* spp.), which had been identified as common herbivores on both *A. tridentata* and *N. attenuata* (Karban and Baxter 2001), play a relatively minor role as herbivores on *N. attenuata* in our study sites. Therefore, the conclusion that tobacco plants, which are exposed to the VOCs of clipped sagebrush may receive information about their future herbivory, has to be drawn with caution. Future studies should consider the priming of herbivore responses by VOCs as a potential mechanism in plant–plant signaling but must consider the natural history of the interacting species to evaluate the ecological role of plant–plant interactions in nature.

Acknowledgements This research was supported by the Max-Planck-Gesellschaft. We thank Michael Haevecker and Robert Schlögl from the Fritz-Haber-Institut of the Max-Planck-Gesellschaft for the generous use of their PTR-MS and patient instructions for its use and the Brigham Young University for the use of their awesome field station, the Lytle Preserve.

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