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Research Paper

Aphid Acceptance of Barley Exposed to Volatile Phytochemicals Differs Between Plants Exposed in Daylight and Darkness

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KEY WORDS

methyl jasmonate, methyl salicylate, allelobiosis, barley, PR1, allene oxide synthase, Rhopalosiphum padi, light

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ABSTRACT

It is well known that volatile cues from damaged plants may induce resistance in neighboring plants. Much less is known about the effects of volatile interaction between undamaged plants. In this study, barley plants, *Hordeum vulgare* cv. Kara, were exposed to volatiles from undamaged plants of barley cv. Alva or thistle *Cirsium vulgare,* and to the volatile phytochemicals, methyl salicylate or methyl jasmonate. Exposures were made either during natural daylight or darkness. Acceptance of exposed plants by the aphid *Rhopalosiphum padi* was assessed, as well as the expression of putative marker genes for the different treatments. Aphid acceptance of plants exposed to either barley or *C. vulgare* was significantly reduced, and an effect of the volatiles from undamaged plants was confirmed by the induction of pathogenesis-related protein, *PR1a* in exposed plants. However the effect on aphid acceptance was seen only when plants were exposed during darkness, whereas *PR1a* was induced only after treatment during daylight. Aphid acceptance of plants exposed to either methyl salicylate or methyl jasmonate was significantly reduced, but only when plants were exposed to the chemicals during daylight. *AOS2* (allene oxide synthase) was induced by methyl jasmonate and *BCI-4* (barley chemical inducible gene-4) by methyl salicylate in both daylight and darkness. It is concluded that (a) the effects on aphids of exposing barley to volatile phytochemicals was influenced by the presence or absence of light and (b) the response of barley to methyl salicylate/methyl jasmonate and to volatiles from undamaged plants differed at the gene and herbivore level.

INTRODUCTION

Volatile phytochemicals, such as those released by herbivore-infested plants, can induce a range of responses in plants, including activation of defences against pathogens and herbivores, modification of volatile profile and increased attractiveness to herbivore natural enemies.[1,2] In some cases, release of plant volatiles has been found to differ between day and night. For example, caterpillar feeding-induced emissions from tobacco contain certain compounds released only at night.[3] In other cases, volatile emissions have been found to peak during daylight hours.[4-6] However influence of light on plant responsiveness to phytochemicals has not been reported.

Barley plants exposed to volatiles released by undamaged plant neighbors become less acceptable to aphids,[7,8] a process that has been named allelobiosis.[9] Aphids are sucking herbivores that feed directly on plant phloem and use a variety of chemical cues to assess host quality. They are therefore excellent model herbivores to assess changes in host plant status. The changes in plant chemistry and physiology that are induced by allelobiosis are unknown, as are the nature of volatiles causing these effects.

Methyl salicylate and methyl jasmonate are components of the blend emitted by some herbivore or pathogen-damaged plants and, along with their nonvolatile acids, are known as plant defence inducers and regulators of a range of other processes.[10,11] There are several examples of methyl salicylate or jasmonate-mediated affects on plant-aphid interactions. Exposure to methyl salicylate reduced acceptance and population development of *Rhopalosiphum padi* on barley.[12] In Arabidopsis, constitutive activation of jasmonate signalling was correlated with enhanced resistance against *Myzus persicae*,[13] and sorghum became less acceptable to *Schizaphis graminum* after treatment with methyl jasmonate.[14]

The aims of this study were (a) to investigate the influence of the presence or absence of light on the response of barley to phytochemicals and (b) to compare the effects of exposing barley to phytochemicals from undamaged plant neighbors and the stress-related

cues methyl salicylate and methyl jasmonate. Plant acceptance by the aphid *R. padi* was used as an indirect measure of herbivore-detectable changes in plant status after exposure to volatile phytochemicals during either natural daylight or darkness. To obtain more direct evidence of changes in plant status, the expression of genes known to be induced by salicylates, jasmonates or by other stress treatments were examined.

MATERIALS AND METHODS

Plants and Insects. Barley plants, *H. vulgare* (cv. Kara), used for experiments and as food plants for aphid rearing were grown in plastic pots (8.5 cm wide x 7 cm high) with six plants per pot, and were at the two-leaf stage at the beginning of exposure to phytochemicals. The cultivar Kara was selected for the experiments because it has previously been shown to respond with reduction in acceptability to aphids after exposure to other barley plants,[7,15] *C. vulgare*[8] and methyl salicylate and methyl jasmonate [Glinwood R, Ninkovic V, unpublished], but is unresponsive to exposure to volatiles from plants of the same cultivar (self-exposure).[15] In these cited studies, barley plants were exposed to volatiles during complete light-dark cycles. Plants were grown in a glasshouse at 18–22°C, with a L16:D8 light cycle. Barley plants of cv. Alva were grown in the same way as cv. Kara (six seeds per pot), and were kept in the same glasshouse chamber, but on a separate bench about 3 m away from Kara plants.

Cirsium vulgare seeds were obtained from Lund University Botanical Garden, Sweden, collected from the Skåne region of Sweden. Several seeds were sown in pots (8.5 cm wide x 7 cm high) with potting soil (Hasselfors Garden, Sweden) and sand (approx 30% sand in the mixture) and kept in a glasshouse at 18–22°C, with a L16:D8 light cycle. Seedlings were separated individually into new pots, and grown for several months prior to the experiments. *C. vulgare* and barley plants were kept in separate glasshouse chambers. Bird cherry-oat aphid *R. padi* was reared on barley in multi-clonal cultures in a glasshouse at 18–22°C, with a L16:D8 light cycle. Aphids used in the experiments were wingless, mixed-instar individuals, and were collected from the cultures immediately prior to the bioassays. Aphid cultures were kept in a separate glasshouse chamber to barley and Cirsium plants.

Exposure of barley plants to volatiles from plants and chemical sources. Barley plants were exposed to barley cv. Alva and *C. vulgare* inside clear Perspex cages,[8,15] divided into two separate chambers (each 10 x 10 x 40 cm), connected by an opening (7 cm diameter) in the dividing wall. Air entered the forward chamber through an opening in the cage wall (7 cm diameter) and passed over a pot containing barley cv. Alva or a *C. vulgare* plant before entering the rear chamber containing the barley cv. Kara to be exposed. Air was extracted through a tube attached to a vacuum tank and vented outside the room by an electric fan. Airflow through the cages was 1.3 l/min. Pots containing plants were placed in Petri dishes to prevent interaction via roots, and watered via an automated water drop system, with two separate two minute deliveries repeated daily, 2 hours after sunrise and 2 hours after sunset. The total amount of water delivered daily to each pot of plants was 45 ml. Control treatments consisted of two-chamber cages with a pot of barley plants in the rear chamber and an empty front chamber.

Methyl salicylate (98%, CAS no. 119-36-8) and methyl jasmonate (95%, CAS no. 1211-29-6) were purchased from Sigma-Aldrich, Sweden. Barley plants were exposed to methyl salicylate and methyl jasmonate in the two-chamber cages described above. A pot containing six barley plants was placed into the rear chamber, and into the front chamber was placed a Petri dish with a filter paper, onto which was dispensed 10 µl of either methyl salicylate or methyl jasmonate. Chemicals were dispensed at the start of each exposure period (i.e., at sunrise and sunset), at which time a clean Petri dish and filter paper were introduced into each cage to receive the chemical. The concentration of chemicals in the air that plants were exposed to was measured using entrainment onto molecular absorbent (Tenax) followed by gas chromatography analysis on an Agilent 6890 GC with a HP-1 column and OPTIC-3 direct thermal desorption inlet. The release of substances from the filter paper fell steadily during the first four hours of exposure and was then lower and stable during the following eight hours. Mean aerial concentrations in the exposure cages were for methyl salicylate: first 4 hours 300 ng/l (1.9 nM) (peak 625 ng/l, 4.5 nM), following 8 hours 50 ng/l (0.3 nM) and for methyl jasmonate: first 4 hours 30 ng/l (0.1 nM) (peak 50 ng/l, 0.3 nM), following 8 hours 15 ng/l (0.05 nM). Control treatments consisted of two-chamber cages with a pot of barley plants in the rear chamber and a Petri dish with a clean filter paper in the front chamber.

Table 1 **Sunrise and sunset times during the experiments in 2004 and 2005***

Date	Sunrise	Sunset
March 10	06:19	17:39
March 17	05:59	17:55
March 30	06:20	19:26
April 6	06:00	19:43

*Source: www.timeanddate.com.

Experimental design. The experiment was performed on two occasions; between March 30 and April 6 2004, and between March 10 and March 17, 2005. On both occasions the period of natural daylight was approximately 12–13 h (clocks are put back one hour at the end of March when official summertime begins). The exact sunrise and sunset times during the experimental period are given in Table 1. No artificial light sources were used anywhere within the experimental glasshouse.

Two separate, self-contained but neighboring glasshouse chambers were used, one for exposure of barley to living plants the other for exposure of barley to chemicals. Each chamber had two opposing benches, along which were placed the two-chamber cages. Treatments were assigned to alternating cages along each bench. One bench housed cages containing the exposure treatment in the front chamber, while the other housed cages with front chambers that were either empty or contained a Petri dish with clean filter paper, depending on the experiment. At sunrise on the first day, one set of barley plants to be exposed was placed inside the cages containing the exposure stimuli, and a second set was placed in the otherwise empty cages. At each sunset and sunrise during the experiment, the two entire sets of plants were transferred from one bench to the other i.e., switched between empty cages and those containing the exposure stimuli. Thus, one set of plants was exposed to the stimuli only during daylight hours, and the other set exposed only during darkness. Plants were always transferred between the same cages, thus, in treatments with barley and *C. vulgare*, each experimental plant was always exposed to the same stimulus plant. Each set of plants (each bench) had its own set of control plants (i.e., never exposed to any exposure stimulus).

Each treatment was replicated six times (represented by six pots of barley plants, in six cages). The experiment ran for seven days, meaning

that plants were actually exposed to stimuli for a total period of about 3.5 days. Previous experiments have shown this period to be sufficient for induction of effects in Kara by all the exposure stimuli used[7,8,15] [Ninkovic V, Glinwood R, unpublished]. On day seven, aphid acceptance of plants from the different treatments was assessed by means of a settling test (see below). The test was started two hours after sunrise and on this final day, although the two sets of plants were switched between cages at sunrise as usual, the daylight-exposed set of plants was not exposed to the stimuli for the two hour period between sunrise and the settling test.

The temperature in the glasshouse was regulated by a combination of heating and ventilation, controlled by a computer. In all experiments, there was a mixture of cloudy and sunny days, with 4–5 sunny days and 2–3 cloudy days during the seven day exposure period. Light intensity (irradiance) was measured during daylight hours inside an exposure cage, at the same height as an exposed plant on two sunny days and two cloudy days. Mean irradiance during daylight hours on a sunny day was 152 µmol/m^2/s with a peak of 305 µmol/m^2/s. Mean irradiance during daylight hours on a cloudy day was 81 µmol/m^2/s with a peak of 125 µmol/m^2/s. Logged hourly temperatures were obtained for a sunny day and cloudy day. Mean daylight temperature for a sunny day was 21.2°c (maximum: 21.7°c, minimum: 20.4°c), and for a cloudy day 21.1°c (maximum: 21.2°c, minimum: 20.9°c). The mean night temperature was 20.7°c on both days and fluctuated very little.

Aphid plant acceptance. A no-choice settling test[7,8] was used to measure aphid acceptance of experimental plants. A 50 ml polystyrene tube was placed over the second leaf, which was the youngest fully developed leaf. The upper end of the tube was covered with a net and the lower end with a plastic sponge plug with a slit for the leaf. Ten mixed-instar, wingless *R. padi* were placed in the tube and after 3 hours the number of aphids settled (not walking) on the leaf was recorded. Three hours was selected as a delay since this is sufficient time for aphids to settle and reach the phloem with the stylet.[16] Four plants per pot (and therefore per cage since each cage held a single pot) were randomly selected for the test, and each pot was considered to be a block for the statistical analysis. Thus there were 24 replicates for each treatment. Data were found to be normally distributed, and were analyzed by two-way ANOVA in the SAS statistical package.[17] Small differences between treatments are probably harder to detect with this test than with a choice-test, since aphids must choose between settling on the plant or risking death or desiccation. It was employed in order to give a more reliable indication of aphid-detectable changes in plant quality.

Plant material for gene expression analysis. Between March 10–17, 2006 (i.e., the same daylight cycle as for the experiment in 2005, and almost the same as that in 2004), the exposures were repeated to obtain plant material for gene expression analysis. The exposures were performed in an identical way. For each treatment, three two-chamber cages were used, each containing a pot with six barley plants. At the end of the seven day exposure period, at the same time of day that aphid plant acceptance tests had been performed, whole plants were harvested by cutting just above the seed. The stem and leaves were frozen in liquid nitrogen and ground with mortar and pestle. Each sample consisted of six plant individuals, representing a pot of plants exposed in a separate cage to a separate stimulus i.e., a pot of six barley cv Alva plants, a *C. vulgare* individual or a chemical. Aphid settling tests were not performed on these plants.

AOS2 and *BCI-4* were used as molecular indicators of plant responses to methyl jasmonate and methyl salicylate respectively. *AOS* plays an important role in jasmonate-dependent stress responses. It is inducible by external application of jasmonates but not salicylic acid and its expression is correlated with elevated levels of jasmonates.[18] *BCI-4* is a putative Ca^{2+}-binding EF-hand protein that is upregulated by external application of benzo-(1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH), but not jasmonates, and it belongs to a group of barley chemically-induced genes that correlate with resistance to powdery mildew.[19]

Real time RT-PCR. From the plant material collected in 2006, total RNA was extracted from the tissue using NucleoSpin® RNA Plant kit which includes a DNase I digestion (Macherey-Nagel, Düren, Germany). Real time RT-PCR was performed and analysed with MyiQ™ Single-Color Real-Time PCR Detection System (Bio-Rad, California, USA) using the iScript™ One-Step RT-PCR Kit With SYBR® Green (Bio-Rad). Specific primers for BCI-4 (AJ250283: BCI-4.FOR; 5'-AAAGGAAGGTTTCTTCCCCAAA AC-3'; BCI-4.REV; 5'-GAATAATAGGCCCCACTCAACCTG-3'), AOS2 (AJ251304: AOS2.FOR; 5'-CTTCACCTCCTTCGAGTTC ATCGC-3'; AOS2.REV; 5'-GAGCTGGAATATGAGCCACTTG-3 ') and PR1a (X74939: HVPR1a.FOR; 5'-TGGACGAGAAGAAGG ACTACGAC-3'; HVPR1a.REV; 5'-ATGTACTGCGAAAAGCAAT CACA-3') were employed. The constitutively expressed gene actin2 (AY145451: ACT2.FOR; 5'-TTCTCGACTCTGGTGATGGTG T-3'; ACT2.REV; 5'-CAAGCTTCTCCTTGATGTCCCT-3') was used as a reference gene to ensure normalization of expression levels. Reactions contained 12.5 µl 2x SYBR® Green RT-PCR Reaction Mix (Bio-Rad), 0.75 µl of each gene-specific primer (final concentration 300 nM), 5.5 µl nuclease-free H_2O, 1 µl i Script Reverse Transcriptase for One-Step RT-PCR (Bio-Rad) and total RNA of 30 ng in a final volume of 25 µl. Three biological replicates (each consisting of six plant individuals) were used and all reactions were prepared in duplicates. A NTC (no template control) was run simultaneously with the other samples. PCR was performed at 50°C for 10 min, 95°C for 5 min, 45 cycles of 95°C for 10 s, 59/59/55°C respectively for 30 s. A melting curve from 55°C to 95°C over 400 s was performed after the PCR reaction. The PCR products were analysed on agarose gels to ensure only one end product (not shown). The formula:

$$[(\text{Etarget})^{\Delta\text{Cttarget(control-sample)}}] \div [(\text{Eref})^{\Delta\text{Ctref(control-sample)}}]$$

was used to calculate the relative transcription ratio between a sample and a control compared to a reference gene.[20] E is the primer efficiency of the reaction, target is the gene of interest, ref is the reference gene and ΔCt is the difference in cycle number between control and sample when they pass a certain fluorescence threshold. Results were correlated for primer efficiencies with the LinRegPCR software.[21]

RT-PCR. RT-PCR was performed using SuperScript™ III One-Step RT-PCR with Platinum® *Taq* (Invitrogen, California, USA). The same primers as for the real time RT-PCR were employed for BCI-4, AOS2 and actin2. For visualization of PR1 transcript levels, primers for PCR-products were designed that were longer than those for real time RT-PCR (X74939: PR1a.FOR; 5′- CCCAGAA TGGAGACGCCCAAG-3′; PR1a.REV; TAGCTAATTATAGATA CGAGCGTG-3′). Reactions contained 12.5 µL 2x Reaction Mix, 1 µl of each gene specific primer (final concentration 400 nM), 9 µl dH_2O, 0.5 µl SuperScript™ III RT / Platinum® *Taq* Mix and total RNA of 30 ng in a final volume of 25 µl. RT-PCR was performed at 45°C for 30 min, 94°C for 2 min, 33/25/25 cycles respectively of 94°C for 30 s, 59/57/57°C respectively for 30 s, 72°C for 1 min, 72°C for 5 min. Products were analysed on agarose gels for amplicons of expected sizes.

RESULTS

Aphid settling on barley cv. Kara exposed to volatiles. The results of the experiments in 2004 and 2005 are shown in Table 2. There were no significant differences between the number of aphids settling on unexposed barley cv. Kara and barley exposed to barley cv. Alva or *C. vulgare* during natural daylight in either 2004 (ANOVA, $F_{2,54} = 1.67$, $p = 0.2$) or 2005 (ANOVA, $F_{2,54} = 3.11$, $p = 0.06$). However, in both 2004 and 2005, aphid settling was significantly lower on barley cv. Kara exposed to barley cv. Alva or *C. vulgare* during natural darkness than on unexposed barley (2004: ANOVA, $F_{2,54} = 4.53$, $p = 0.01$, 2005: ANOVA, $F_{2,54} = 10.75$, $p = 0.0001$). Thus only exposure of plants during darkness resulted in changes in plant status that affected aphid plant acceptance.

In both 2004 and 2005, aphid settling was significantly lower on barley cv. Kara exposed to methyl salicylate or methyl jasmonate during natural daylight than on unexposed barley (2004: ANOVA, $F_{2,54} = 8.2$, $p < 0.0008$, 2005: ANOVA, $F_{2,54} = 12.62$, $p < 0.0001$). However, there were no significant differences between the number of aphids settling on unexposed barley cv. Kara and barley exposed to methyl salicylate or methyl jasmonate during natural darkness in either 2004 (ANOVA, $F_{2,54} = 0.13$, $p = 0.98$) or 2005 (ANOVA, $F_{2,54} = 1.17$, $p = 0.31$). Thus only exposure of plants during daylight resulted in changes in plant status that affected aphid plant acceptance.

Table 2 **Mean number of *R. padi* settling on barley cv. Kara plants previously exposed to phytochemicals from barley v. Alva or *C. vulgare*, or artificial sources of methyl salicylate or methyl jasmonate either during natural daylight or natural darkness in experiments in 2004 and 2005**

Phytochemical Source/ Exposure Period[1]	Mean Number Aphids Settling (± s.e.) 2004[2]	Mean Number Aphids Settling (± s.e.) 2005[2]
Control	7.38 (0.32)[a]	8.33 (0.28)[a]
Barley cv. Alva/ light	6.75 (0.31)[a]	7.50 (0.28)[a]
C. vulgare/ light	6.58 (0.39)[a]	8.13 (0.21)[a]
Control	7.67 (0.25)[a]	8.25 (0.22)[a]
Barley cv. Alva/ dark	6.63 (0.32)[b]	7.16 (0.27)[b]
C. vulgare/ dark	6.63 (0.27)[b]	6.67 (0.25)[b]
Control	7.63 (0.37)[a]	8.42 (0.24)[a]
Methyl salicylate/ light	6.54 (0.28)[b]	6.79 (0.26)[b]
Methyl jasmonate/ light	5.83 (0.49)[b]	6.87 (0.24)[b]
Control	7.33 (0.40)[a]	8.17 (0.21)[a]
Methyl salicylate/ dark	7.29 (0.42)[a]	8.12 (0.24)[a]
Methyl jasmonate/ dark	7.25 (0.35)[a]	8.50 (0.26)[a]

[1]N, six pots of barley exposed to each treatment, with aphid settling tests performed on four plants from each pot (24 settling tests per treatment), and ten aphids in each settling test. [2]ANOVA followed by Tukey test. In each separate experiment, means followed by different letters (a or b) are significantly different.

Gene expression in barley cv. Kara exposed to volatiles. Expression of pathogenesis-related protein *PR1a* was examined as a possible indicator of plant volatile interaction. Expression of *PR1a* was increased in barley cv. Kara exposed to volatiles from barley cv. Alva or *C. vulgare* (Fig. 1A). This increase occurred only in plants that had been exposed during daylight. None of the other treatments caused induction of *PR1a* expression.

The expression of *AOS2* (allene oxide synthase), a marker for jasmonic acid-induced transcripts[18] was increased in plants exposed to methyl jasmonate during either darkness or daylight (Fig. 1B). Expression of *AOS2* in other treatments was similar to that in unexposed plants, apart from in plants exposed to cv. Alva during darkness or to *C. vulgare* during either darkness or daylight, which had lower expression of *AOS2*.

Barley chemically induced gene-4 (*BCI-4)* has been shown to be induced in barley by BTH, a chemical thought to mimic salicylic acid.[19] *BCI-4* expression increased in barley exposed to methyl salicylate (Fig. 1C). Although there was variation in the level of expression, the overall pattern was the same for all three replicates. Expression was higher in plants exposed during darkness than in plants exposed during daylight.

DISCUSSION

When barley cv. Kara was exposed to methyl salicylate or methyl jasmonate, or to volatiles from cv. Alva or *C. vulgare*, settling by *R. padi* was significantly reduced. This is in line with previous studies in which plants were exposed continuously throughout the light-dark cycle.[7,8,12,23] However, the current experiment showed that effects on aphid behavior are dependent on the presence or absence of light during the exposure. Plants exposed to volatiles from cv. Alva or *C. vulgare* only became less acceptable to the aphid if exposed during darkness. Conversely, plants exposed to methyl salicylate or methyl jasmonate only became less acceptable if exposed during daylight.

PR1a was induced in barley exposed to cv Alva or *C. vulgare* during daylight. To our knowledge, this is the first report of volatile interaction between undamaged plants expressed at the molecular level. However, *PR1a* was induced in daylight-exposed plants whereas aphid settling was reduced in darkness-exposed plants, suggesting that the PR1a protein itself did not contribute to the reduced aphid acceptance. That the induction was only evident in plants exposed during daylight might indicate that the active volatiles were released only during daylight, or that activity of the inducer and/or later transduction pathway components are light-dependent. The latter explanation is supported by reports from rice and *Arabidopsis*[24-26] indicating that *PR1* induction in these species requires light. Exposure to methyl salicylate did not affect the *PR1a* transcript level, and this is in accordance with earlier studies in barley and wheat.[27,28] Thus, the current results support the idea that salicylates do not induce *PR1a* in barley. However other plant volatiles may do so. For example, the ethylene precursor ethephon, at relatively high concentrations, induced PR-proteins in rice seedlings.[24] The terpenoids (*E*)-β-ocimene, (*E*)-4,8-dimethyl-1,3-7-nonatriene and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene induced PR-proteins in lima beans,[29] and (Z)-3-hexenol has been shown to induce a number of defence-related genes in maize.[30]

AOS2 was upregulated by methyl jasmonate and *BCI-4* by methyl salicylate in the current study. Both genes were upregulated after daylight and darkness exposure, suggesting that methyl jasmonate and methyl salicylate are able to enter the tissue of the receiving plants during the night, when stomata are generally closed. However, since the dark treatments were followed by three hours in the light before samples were taken, this is not an indication that the signal

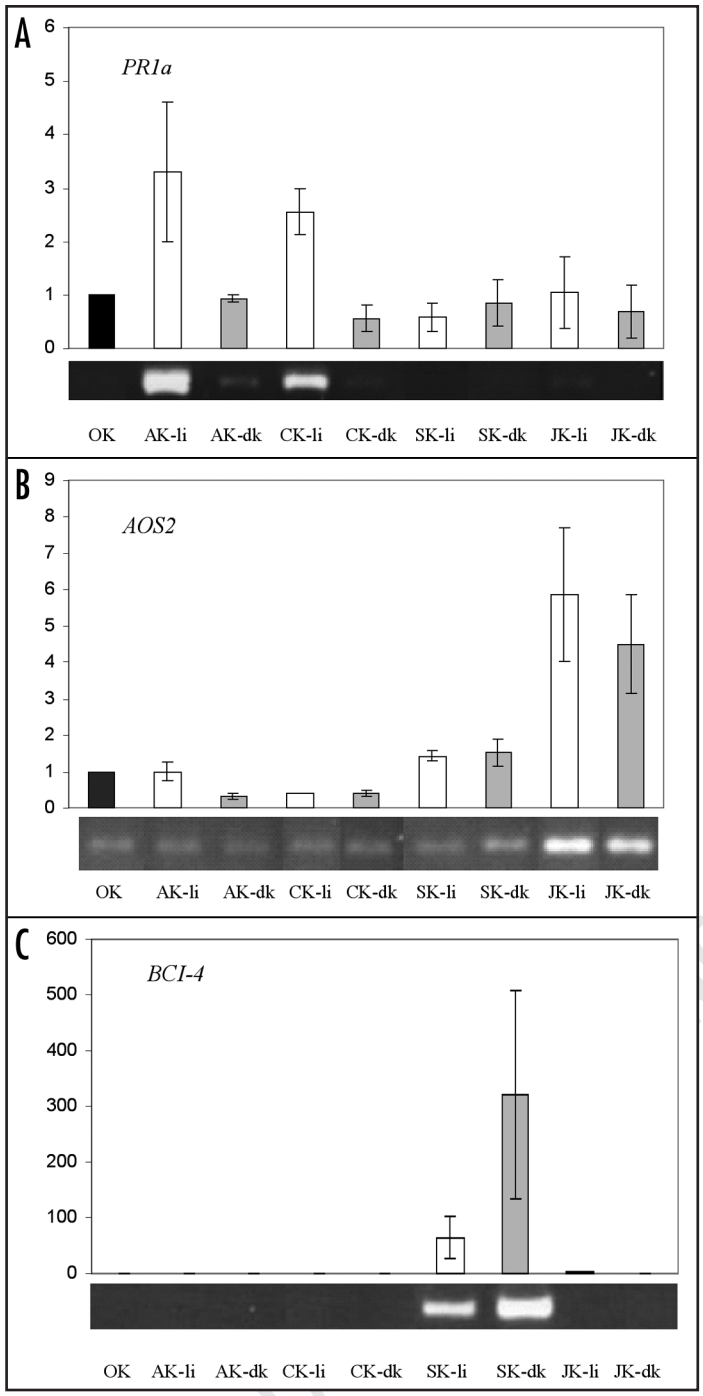

Figure 1. Relative transcript levels of (A) *PR1a* (B) *AOS2* (C) *BCI-4* in barley plants by real time RT-PCR. Plants were exposed to volatiles from barley plants of a different cultivar or thistle *Cirsium vulgare*, or to chemical sources of methyl salicylate or methyl jasmonate during either daylight or darkness with a cumulative exposure time of four days. The transcript levels were normalized to actin and are presented in comparison to the control, Kara exposed to air (OK =1). A, *Hordeum vulgare*, cv. Alva; C, *Cirsium vulgare*; S, Methyl salicylate; J, Methyl jasmonate; K, *Hordeum vulgare*, cv. Kara; li, daylight exposed plants; dk, darkness exposed plants. The results for each sample is presented as the mean ± SE of three biological replicates (each consisting of 4–6 plant individuals) performed in duplicate. Gels from RT-PCR are shown underneath the graphs.

transduction pathways are independent of light. Furthermore, since daylight treatments were followed by 12 hours in the dark and then three hours in the light before samples were taken, it is difficult to directly deduce the influence of light on the expression levels. Nevertheless, the fact that only plants exposed to methyl salicylate or methyl jasmonate during daylight had reduced aphid acceptance suggests that the signal transduction pathways or metabolic changes responsible for the effects on aphids are light dependent. Light-dependence in a plant defensive pathway has been demonstrated previously.[26]

This study provides further evidence that volatile interaction between undamaged plants affects host acceptance by aphids.[7,8,15] In the case of barley exposed to volatiles from barley or *C. vulgare*, plants may have responded only when exposed during darkness. However, it is also possible that the volatiles responsible for triggering the response were only emitted during darkness. Diurnal periodicity of plant volatile release has been reported previously and, in almost all cases, peak emissions were found to occur during the photophase, often with very low emissions during darkness. However, the majority of studies have focussed on the release of volatiles induced by herbivory,[4] pathogen attack[5] or exposure to inducing chemicals,[6,31] rather than from undamaged plants. In one case, certain herbivore-induced volatiles were detected only during darkness.[3] Plant physiology, for example stomatal opening, may substantially affect volatile emissions.[32] In the current study, exposure of plants to cv. Alva and *C. vulgare* began at sunset, a time of major changes in plant physiology that may be linked to changes in volatile emission in grasses.[33] However, initial investigations of volatile profiles of Cv. Alva and *C. vulgare* did not reveal major differences between dark and light-exposed plants (Glinwood R, unpublished).

Previous studies in which plants were exposed during full light-dark cycles (Glinwood R, Ninkovic V, unpublished) show that aphid settling remains significantly reduced on Kara exposed to Alva, *C. vulgare* or either chemical for 72 hours after the end of the exposure. Thus it is unlikely that the detection of a response in barley exposed to phytochemicals from living plants during darkness was due to the fact that the aphid behavioral assay was carried out shortly after the end of the exposure period, in contrast to daylight-exposed plants in which the test was carried out at least 12 hours after the end of the exposure period.

Quantification of methyl salicylate and methyl jasmonate in the headspace of plants exposed to the chemicals during daylight and darkness (data not shown) did not suggest an influence of passive adsorption/rerelease of the substances on aphid plant acceptance. Previous experiments have shown that *R. padi* does not respond behaviorally to volatiles from *C. vulgare*, nor does it discriminate between the odour of cv. Alva and cv. Kara (Glinwood R, Ninkovic V, unpublished).

It is concluded that plant acceptance by *R. padi* on barley exposed to volatile phytochemicals as well as expression of *PR1a* is affected by the presence or absence of light during the exposure period. It is further concluded that the investigated marker genes are regulated differently to the mechanisms leading to the changes in aphid behavior. There are several mechanisms that could be examined to explain the effects reported in this study; for example availability of volatile phytochemicals (in the case of living plant sources), stomatal conductance, and light-dependence of genes involved in processes putatively leading to reduced aphid acceptance. The presence of light had an opposing influence on the outcome of exposing barley to methyl salicylate and methyl jasmonate or volatiles from undamaged plants. Considering also that methyl jasmonate- or methyl salicylate-responsive genes were not induced by volatiles from barley or *C. vulgare*, it is concluded that barley responds differently to these two types of phytochemical.

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