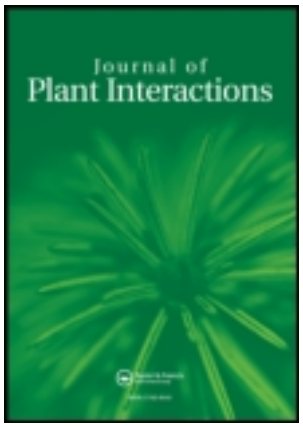

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RESEARCH ARTICLE

Induced defence in lima bean plants exposed to the volatiles from two-spotted spider mite-infested conspecifics is independent of the major protein expression

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Non-infested plants respond to volatiles from neighboring herbivore-infested plants, triggering induced defence responses against the threat. Information about protein expression in volatile-exposed plants after the 'plant–plant signalling' is scarce. We focused on the defence response and the protein expression of lima bean plants exposed to the volatiles from their conspecifics infested by the two-spotted spider mites (*Tetranychus urticae*) using a flow chamber. The number of eggs of spider mite was significantly reduced on the plants exposed to infested plant volatiles, suggesting defence induction in the exposed plants. A proteomic analysis on the volatile-exposed plants showed that two proteins associated with photosynthesis and an unknown protein showed a marginally significant decrease and increase, respectively. These results suggest that the induced defence caused by volatiles is essentially independent of changes in the major proteins.

Keywords: herbivore-infested plant volatiles; lima bean; two-spotted spider mite

1. Introduction

Plants emit various volatile compounds in response to herbivory (Takabayashi & Dicke 1996). One of the ecological functions of these volatiles is to attract carnivorous natural enemies of herbivores, such as predators and parasitoids (Arimura et al. 2009). These volatiles are also known to be involved in plant–plant signaling, in which uninfested receiver plants increase their defence against attacking herbivores (Arimura et al. 2009; Heil & Karban 2010). For example, Arimura et al. (2000a) reported that uninfested lima bean plants exposed to volatiles from conspecific plants infested by the two-spotted spider mite, *Tetranychus urticae*, showed an increased defence response against conspecific mites. In this system, it was assumed that the reduction in mite damage on the receiver plants was owing to the induction of a subset of defensive genes that overlapped those induced by the spider mite damage (Arimura et al. 2000a, 2000b).

Comprehensive changes in the defensive genes in lima bean plants exposed to the volatiles from herbivore-infested plants are well documented (Arimura et al. 2000b), illustrating the induced defence strategy. However, no study has reported on the comprehensive changes of protein expressions in plants exposed to the volatiles from herbivore-infested plants. We performed proteomic analysis to address the above issue in the model system of lima bean plants infested by the two-spotted spider mite and neighboring intact conspecific plants using a flow chamber. The nature of the defensive responses of lima bean plants exposed to plant volatiles is discussed.

2. Materials and methods

2.1. Plants and mites

Each lima bean plant (*Phaseolus lunatus* cv. Pole Sieva) was grown in a plastic pot with soil in a growth room at 25°C under a 14/10-h light/dark photoperiod (100 μmol photons m^{-2} s^{-1}). One- to two-week-old potted plants were used for the experiments. Colonies of the herbivorous spider mite (*Tetranychus urticae*) were cultured and maintained on lima bean leaves under identical conditions.

2.2. Air-flow setup

For volatile exposure, an airflow system was built using two glass cylinders (2 L) connected with Teflon tubes, a pressure-regulated air compressor and flow meters. To remove contaminated volatile compounds, a charcoal filter was used with the air pumped through the system. In the first cylinder a plant was infested with 200 adult female mites. The plant in the second chamber received no mites. Airflow was maintained at 500 mL min^{-1} for 3 days under the growth conditions previously described. As a control, uninfested plants were placed in the first cylinder as emitter plants. The receiver plants exposed to the

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infested plant volatiles (IV) and to the control (uninfested) plant volatiles (CV) were designated as the IV- and CV-exposed plants, respectively. We repeated the volatile exposure experiments five times in different experimental days.

2.3. Mite fecundity assay

To assess mite fecundity, an adult female mite was placed on a leaf-disc (8 mm diameter) on a wet paper towel in a glass Petri dish. After 4 days, the numbers of eggs were counted using a binocular microscope (SZ-PT, OLYMPUS, Tokyo, Japan).

2.4. 2D-PAGE and protein sequencing

Approximately 100 mg of lima bean leaves was extracted by same weight of extraction buffer [10 mM potassium phosphate (pH 7.8) containing 8.5 M urea, 0.05% w/v Tween 20, 1 mM phenylmethylsulfonyl fluoride, 1% w/v dithiothreitol, and 2% v/v Pharmalyte (pH 3–10, GE Healthcare, Buckinghamshire, UK)]. The extract was ultracentrifuged at $100{,}000 \times g$ for 1 h at 4°C to remove membranes and Rubisco complexes. Three hundred micro-grams of proteins was separated by 2D-PAGE (Fukao et al. 2009). A rehydrated IPG gel strip (GE Healthcare) was used for IEF with CoolPhoreStar IPG-IEF system (Anatech, Tokyo, Japan) according to the manufacturer's instructions, and the gel strip was subsequently used for SDS-PAGE. Protein spots were detected and quantified using Flamingo fluorescent gel stain (Bio-Rad, Hercules, CA, USA) and ProEXPRESS 2D Proteomic Imaging System (PerkinElmer Inc., Waltham, MA, USA) with Progenesis SameSpot software (Nonlinear Dynamics, Newcastle, UK). Protein spots of interest were excised from the gels and digested with trypsin gold (Promega, Madison, WI, USA) for LC-MS/MS analysis as described in Kley et al. (2010). The obtained sequences were analysed by Swiss-Port search (http://www.uniprot.org/) and determined as deduced protein in conserved results between control and exposed spots.

3. Results and discussion

3.1. Defensive property

We examined the performance of mites on the volatile-exposed lima bean leaves using the experimental airflow setup. After exposure, we detected no visible change in the appearance of leaves such as coloring or wilting. The fecundity of female mites on the treated plants was estimated by counting the number of eggs after 4 days on the excised leaf discs. There was a significant difference in the mean number of eggs on the IV- and CV-exposed lima bean plants (Figure 1, Welch t-test, $p = 0.0002$). The result suggests that plants exposed to volatile compounds from mite-infested conspecific plants become more resistant against the mites compared with the plants exposed to uninfested plant volatiles. The increased defence in the IV-exposed plants was consistent with a previous study performed by Arimura et al. (2000a), in which they employed detached lima bean leaves infested with mites as an emitter and uninfested detached leaves as a receiver in the closed chamber without airflow.

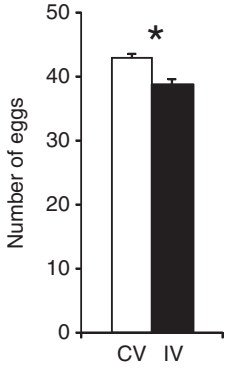

Figure 1. Number of spider mite eggs on lima bean leaves. Adult female spider mites were allowed to produce eggs for 4 days on each leaflet of lima bean plants exposed to control volatiles (white bar, CV) or from spider mite-infested volatiles (solid bar, IV). The number of eggs on IV plants was significantly reduced compared with those on the control plants. The average number of eggs produced per female on each plant is shown with standard errors (*: $P < 0.01$, $N = 146$ and 156).

3.2. Comparison of protein expression

We performed a proteomic analysis of IV- and CI-exposed lima bean plants. Soluble proteins were separated by isoelectronic focusing (pI = 3–10) and SDS-PAGE (12.5% acrylamide), and non-specifically stained by Flamingo fluorescent dye. We detected only three spots that showed marginal significance in changes in their amounts after volatile exposure. In the IV-exposed plants, two of them decreased (spot #1545, exposed/control $= -2.215$, $p = 0.094$; spot #1690, E/C $= -2.589$, $p = 0.057$) and one of them increased (spot #1843, E/C $= 2.29$, $p = 0.078$) (Figure 2). It is important to note that only slight changes in the protein expression were detected in IV-exposed plants, even though such plants showed higher direct defence against spider mites than CV-exposed plants. In an interaction between wild tobacco plants (*Nicotiana attenuata*) and the tobacco hornworms (*Manduca sexta*), it is reported that ca. 15% of the proteins changed in the herbivory mimicking leaves with artificial damage and oral secretion of the tobacco hornworms (Giri et al. 2006).

To identify the proteins, the spots were excised, then served to the in-gel trypsin digestion where the resultant peptides were sequenced (Table 1). The spots #1545 and #1690 had conserved similarity to OEC33 (oxygen evolving complex 33 kDa protein) and chloroplast ATPase α-subunit, respectively, which are essential for photosynthesis. Spot #1545

Table 1. Deduced amino acid sequence of scarcely changed spots in exposed plants.

Spot No.	Observed size	Deduced sequence of peptide fragment	Swiss-Prot search results
1545 (CV)	30 kDa	(-)TPENVELVFTDQVTQPSDTDLGAK(-) (-)TPENVEVLFTDQVTQPSDTDLGAK(-) (-)NRPTFDQPK(-) (-)RNPTFDGAPK(-) (-)RNPTFDQPK(-) (-)NVFGLCTTLGQEPFLVYSPR(-) (-)VNFGLCTTLGQEPFLVYSPR(-) (-)GLFTNVASPGTK(-) (-)GLFTNVASPSAK(-) (-)GLFTNVTGPSAK(-) (-)VPFLFLTK(-) (-)VPFLFTLK(-) (-)VPFLLTFK(-) (-)ASVELLTVNPVLGK(-) (-)SAVELLTVNPVLGK(-) (-)TGVELLTVNPVLGK(-) (-)LFSPGLNR(-) (-)LFSPGNLR(-) (-)LFSPNGLR(-) (-)KLCNKPTSFTVK(-) (-)KLCNKTPSFTVK(-) (-)LTDFELQSK(-) (-)LTFDELQSK(-) (-)AADLFSNPK(-) (-)AAEVFSNPK(-) (-)AAVEFSNPK(-) (-)NVFGLCTSGVREPFLVYSPR(-) (-)TPENVEVLFTDQVTKPSDTDLGAK(-) (-)NAPLEFAGNTK(-) (-)NAPLEFGANTK(-) (-)NAPLEFQNTK(-)	O49079; Oxygen-evolving enhancer protein 1, chloroplastic 35 kDa pI 6.2
1545 (IV)	30 kDa	(-)LCLEFGSEVLK(-) (-)VPFLFLTK(-) (-)VPFLFTLK(-) (-)VGESLDLVAVR(-) (-)VGETVDLVAVR(-) (-)VGETVDLVGLR(-) (-)LTFDELAGSK(-) (-)LTFDELQSK(-) (-)LCLETRVEFK(-) (-)LCLETRVFEK(-) (-)VAEPSNGTR(-) (-)VAEPSNSAR(-) (-)VAEPSNTGR(-)	A7LCN2 Chloroplast photosynthetic water oxidation complex 33kDa subunit 28 kDa pI 5.3 A9UIK9 Putative ammonia monooxygenase subunit A 29 kDa pI 4.4
1690 (CV)	28 kDa	(-)TMLLGDR(-) (-)ASPAALGAKPLDGR(-) (-)GTPAALGAKPLDGR(-) (-)SAPAALGAKPLDGR(-) (-)ADLSASESR(-) (-)DALSASESR(-) (-)EGLSASESR(-) (-)LELSPAPGLLSR(-) (-)LLESPAPGLLSR(-) (-)LLESPAPGLSLR(-) (-)AVLESPAPGLLSR(-) (-)GLLESPAPGLLSR(-)	A6H5B8 Putative ATP synthase CF1 alpha subunit 18 kDa pI 4.8 A6H5B9 Putative ATP synthase CF1 alpha subunit 18 kDa pI 4.8

Table 1. (*Continued*)

Spot No.	Observed size	Deduced sequence of peptide fragment	Swiss-Prot search results
		(-)LGLESPAPGLLSR(-)	
		(-)HTLLLDYDLSK(-)	
		(-)HTLLLYDDLSK(-)	
		(-)THLLLYDDLSK(-)	
		(-)GVLNALAQEPVGR(-)	
		(-)VGLNALAQEPVGR(-)	
		(-)VGLNALAKEPVGR(-)	
		(-)PFQASGEVSALLGR(-)	
		(-)ALQLPVSEAYLSR(-)	
		(-)LAQLPVSEAYLSR(-)	
		(-)QALLPVSEAYLSR(-)	
		(-)AVAQLPVSEAYLSR(-)	
		(-)GLAQLPVSEAYLSR(-)	
		(-)LGAQLPVSEAYLSR(-)	
1690 (IV)	28 kDa	(-)LVNALAGAPLDGR(-)	A6H5B8
		(-)VLNALAGAPLDGR(-)	Putative ATP synthase CF1 alpha subunit
		(-)LVNALAGAPRER(-)	18 kDa
		(-)ELLLDGR(-)	pI 4.8
		(-)ELLLGDR(-)	A6H5B9
		(-)LLESPAPGLSLR(-)	Putative ATP synthase CF1 alpha subunit
		(-)LLESPAPGLVTR(-)	18 kDa
		(-)LLESPLPGASLR(-)	pI 4.8
		(-)LGLESPALSPGLR(-)	
		(-)LGLESPAPNVGKK(-)	
		(-)LGLESPAPNVKGK(-)	
		(-)ADLSASESR(-)	
		(-)DALSASESR(-)	
		(-)EGLSASESR(-)	
		(-)ALQLPVSEAYLSR(-)	
		(-)LAQLPVSEAYLSR(-)	
		(-)QLALPVSEAYLSR(-)	
		(-)LEQYTNPMK(-)	
		(-)GVPVSLPR(-)	
		(-)VGPVSLPR(-)	
		(-)LEQYNTEVK(-)	
		(-)LEQYTNEVK(-)	
		(-)LLESPAPGLLSR(-)	
		(-)LLESPAPGLSLR(-)	
		(-)LLESPAPGLTVR(-)	
1843 (CV)	22 kDa	(-)SEDLLFAAR(-)	O64981
		(-)TDDLLAAFR(-)	Ribulose bisphosphate carboxylase/ oxygenase activase, chloroplastic
		(-)TDDLLFAAR(-)	48 kDa
		(-)LNDLDAGAGR(-)	pI 8.3
		(-)LNDLDAGQR(-)	
		(-)LNDLDQAGR(-)	
		(-)FGLNDLDAGAGR(-)	
		(-)FGLNDLDAQGR(-)	
		(-)GFLNDLDAGAGR(-)	
1843 (IV)	22 kDa	(-)TDDLLAAFR(-)	P08215
		(-)TDDLLAFAR(-)	ATP synthase subunit alpha, chloroplastic
		(-)TDDLLFAAR(-)	55kDa
		(-)FLENPTLGTK(-)	pI 5.6
		(-)FLENPTLSAK(-)	
		(-)FLENPTLTGK(-)	

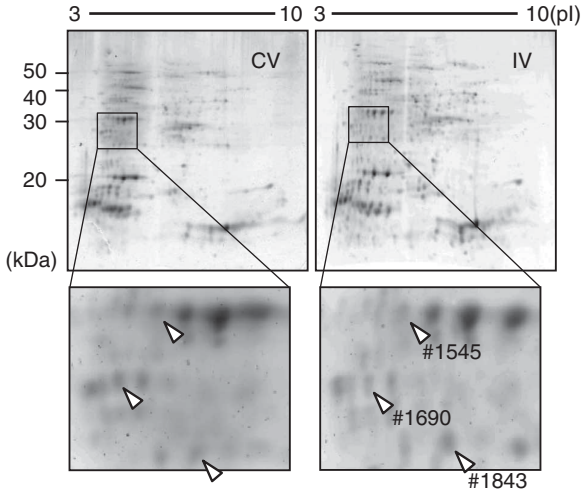

Figure 2. Two-dimensional PAGE analysis of protein expression. Total proteins were separated through IEF (pH3–10) and SDS-PAGE. Overview of the gel images of the protein profile from receiver plants exposed to control volatiles (CV) and spider mite-infested volatiles (IV) are shown in the upper panels. Three spots of marginally changed proteins are shown in the magnified panel with arrowhead.

showed a similar size to deduced proteins (OEC33, ca. 33 kDa), whereas spot #1690 was smaller than the deduced protein (ATPase α-subunit, ca. 55 kDa). The spot #1690 might correspond to a degraded protein. The sequences of peptides derived from Spot #1843 showed no significant similarity to any known proteins. Giri et al. (2006) reported that the amount of proteins involved in photosynthesis decreased in *N. attenuata* leaves when they were wounded and treated with oral secretions of *M. sexta*, which probably resulted in a reduced rate of photosynthesis. Such negative impacts from herbivory on photosynthetic activity have been reported (Zangerl et al. 2002). In contrast, little change in photosynthetic activity in the volatile exposed lima bean plants was detected in a previous study (Arimura et al. 2000a). We assumed that the detected changes in the two proteins involved in photosynthesis were too small to cause a significant change in photosynthetic activity.

In our comprehensive comparison, we detected no significant increase in protein content other than the unknown protein that showed a marginal increase. We previously reported that a subset of defence-related genes were upregulated after exposing intact lima bean plants to the volatiles emitted from mite-infested lima bean plants through a transcriptome analysis (Arimura et al. 2000b). Therefore, this minor change in protein profiles was not expected. One possible explanation for this is a regulation of translation of the defence-related genes. Farag et al. (2005) reported that (*Z*)-3-hexenol-exposed maize plants accumulated the transcripts of the gene for proteinase inhibitor, but its protein was not accumulated. Moreover, the defence response primed by volatiles has been reported in lima beans, poplar plants and maize (Choh et al. 2004; Engelberth et al. 2004; Ton et al. 2007; Frost et al. 2008). For example, Choh et al. (2004) demonstrated that volatiles from mite-infested lima bean plants primed intact conspecific plants for the production of herbivore-induced natural enemy attractants upon subsequent herbivory.

The defensive property of lima bean plants using slight changes of proteins in IV-exposed plants may be a defence strategy against impending herbivores. To understand the defensive response of volatile-exposed plants in plant–plant signaling, a comprehensive analysis that integrates transcriptome, proteome, and metabolome is essential (Giri et al. 2006). For proteomic analysis, a targeted and more sensitive strategy through fractionation and concentration of proteins with post-translational modifications and/or through isolation of proteins in a distinct localization in a cell is required.

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