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Plant defense in response to chewing insects: proteome analysis of *Arabidopsis thaliana* damaged by *Plutella xylostella*

Dieu-Hien Truong[a], Hoang Chinh Nguyen[a], Julien Bauwens[b], Gabriel Mazzucchelli[c], Georges Lognay[d] and Frédéric Francis[b]

[a]Faculty of Applied Sciences, Ton Duc Thang University, Ho Chi Minh city, Vietnam; [b]Functional & Evolutionary Entomology, University of Liège, Gembloux Agro-Bio Tech, Wallonia, Belgium; [c]Mass Spectrometry Laboratory, University of Liège, Liège, Belgium; [d]Analytical Chemistry Laboratory, University of Liège, Gembloux Agro-Bio Tech, Wallonia, Belgium

ABSTRACT

The interactions between *Arabidopsis thaliana* and *Plutella xylostella* have been considered as a model system to unravel the responses of plants to herbivorous insects. Here, we use a 2-DE proteome approach to detect protein expression changes in the leaves of *Arabidopsis* plants exposed to *P. xylostella* larval infestation at 27°C within 8 h. Approximately 450 protein spots were reproducibly detected on gels. Of these, comparing healthy and infested leaves, we identified 18 differentially expressed protein spots. Thirteen proteins were successfully identified by MALDI-TOF/MS and LC-ESI-MS/MS. Functional classification analysis indicated that the differentially identified proteins were associated with amino acid, carbohydrate, energy, lipid metabolism, and photosynthesis. In addition, their relative abundances were assessed according to larval pest feeding on *Arabidopsis* leaves. These data provide valuable new insights for further works in plant-biotic and environmental stress interaction.

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Arabidopsis thaliana; *Plutella xylostella*; proteomic expression; 2-DE; MALDI-TOF MS; LC-ESI-MS/MS

Introduction

Throughout the growing season, plants are often challenged by voracious insects, which triggers a broad range of molecular defense mechanisms, including profound alterations in gene/protein expression (Lippert et al. 2007; Atkinson and Urwin 2012; Duceppe et al. 2012; Kilian et al. 2012; Louis and Shah 2013; Chuang et al. 2014). It is well established that the infestation of chewing insects leads to extensive damage to plant cells, resulting in the upregulation or downregulation of gene/protein expression (Collins et al. 2010; Liu et al. 2010; Ali and Agrawal 2012; Silva and Furlong 2012; Pineda et al. 2017). Among the chewing insects, *Plutella xylostella* (L.) (diamondback moth) specializes in the Brassicaceae family, including *Arabidopsis thaliana*, and induces differential expression of host genes/proteins (Sarfraz et al. 2006; Barker et al. 2007; Ehlting et al. 2008; Collins et al. 2010; Liu et al. 2010; Silva and Furlong 2012).

The *A. thaliana*–insect interaction is a model system used to demonstrate the defense resistance of plants to leaf chewers, particularly the analysis of cellular changes at gene/proteomic levels (Kliebenstein et al. 2002; Collins et al. 2010; Truong et al. 2015; Kroes et al. 2017). Recently, Kroes et al. (2017) observed the upregulation of various JA-responsive genes in response to feeding by *P. xylostella* caterpillar, by a microarray analysis. The protein expression changes in *A. thaliana* leaves due to *P. xylostella* infestation were identified by two-dimensional gel electrophoresis (2-DE) coupled with mass spectrometry in several studies (Collins et al. 2010; Liu et al. 2010). However, such studies were only conducted at the optimum temperature for *Arabidopsis* growth (22°C) (Herde et al. 2008; Antoun and Ouellet 2013), even if changes in temperature in the environment can lead to altered protein profiles in plants (Amme et al. 2006; Loreto and Schnitzler 2010; Rocco et al. 2013; ČErnÝ et al. 2014). Therefore, here we aimed to detect changes in the proteins expressed in *Arabidopsis* leaves infested by larvae at a temperature higher than 22°C. To do this, we used a 2-DE approach coupled with mass spectrometry (MALDI-TOF MS or LC-ESI-MS/MS). *P. xylostella* larvae were placed on *A. thaliana* leaves at 27°C during 8 h. The gathered data provide valuable new insights into the complex response of plants to chewing insects at different temperatures.

Materials and methods

Plant material and growth condition

All of the experimental procedures were performed with 5-week-old *A. thaliana* (L.) Heynh (Col-0). Seeds (Lehle Company, TX, USA) were individually sown in plastic pots (0.2 l) with potting soil and were stored for three days at 4°C in the dark before being transferred to a growth chamber (21.8 ± 0.5°C). After germination, the seedlings were grown at 21.8 ± 0.5°C, 16L:8D (LED lighting: 43 µmol m^{-2} s^{-1} photosynthetically active radiation during the light period) and 66.2 ± 1.7% relative humidity (RH). The plants were watered twice a week (tap water, 10–20 ml/pot) for 5 weeks.

Insect rearing

Diamondback moth, *P. xylostella* (L.) larvae (2nd–3rd instar) were used in this study. Insects were kept in net cages in a controlled temperature room at 22 ± 2°C under a 16 h light regime and 50–70% RH. To maintain *P. xylostella* population,

CONTACT Dieu-Hien Truong truongthidieuhien@tdt.edu.vn

pupae were collected, transferred to a gauze cage, and provided with 15% sugar solution as a food source in a climate room. Eggs laid by the adults obtained from the field were grown on cabbage plants (*Brassica oleracea* L.) until hatching into larvae.

Plant treatment

For feeding experiments, nine larvae (2nd–3rd instar) were applied per *Arabidopsis* plant at 27°C within 8 h according to Truong et al. (2014). Non-infested plants at 27°C were used as controls. Infestation by 9 larvae on *Arabidopsis* plant at 22°C over 8 h was also considered as controls. Larvae were removed, and all plant leaves were carefully frozen in liquid nitrogen and stored at −80°C until used. Three biological replicates were used for the control and larval pest-infested plants.

Protein extraction

Proteins were extracted according to the phenol-based extraction method for *Arabidopsis* plants of Huang et al. (2011), with some modifications. Independent, frozen *Arabidopsis* leaves (0.3–0.4 g fresh mass) were finely powdered in liquid nitrogen using a pestle and mortar, and suspended in 2.5-volumes of extraction buffer (0.9 M sucrose, 0.5 M Tris, 0.005 M EDTA, 0.1 M KCl, and 1% W/V DTT) by vortexing until obtaining a thick paste. Prior to the addition of an equal volume of Tris-saturated phenol, pH 8.0, the suspension was sonicated in the ice-cold sonication bath (4°C) for 5 min two times. The mixture was vortexed, agitated for 10 min at room temperature in a Thermomixer (1000 RPM), and then centrifuged at 5000*g* for 10 min at 4°C. After removing the upper phenol phase (dark-green phase), the mixture was extracted again with the extraction buffer, vortexed, and centrifuged in the same conditions. The proteins contained in the phenol phase were precipitated by the addition of five volumes of saturated ammonium acetate in methanol (0.1 M; precipitation solution), and kept overnight at −80°C. Precipitated proteins were centrifuged again at 5000*g* for 10 min at 4°C and supernatants were discarded. The pellet was washed with five volumes of precipitation solution, and then by five volumes of ice-cold (4°C) 80% acetone, centrifuged at 5000*g* for 10 min at 4°C. Two replicates were conducted for each step. The protein suspensions were stored at −80°C until a 2-DE analysis.

Protein-cyanine dye labeling and 2-D gel electrophoresis

The protein extracts were labeled with one of three Cydyes (GE Healthcare) according to the standard DIGE protocol (50 µg protein for each Cydye). Two samples corresponding to two different groups (*Arabidopsis* plants were subjected or not to larval pest feeding at 27°C within 8 h) labeled either with Cy3 or Cy5 and were mixed with an internal reference protein standard (pooled from equal aliquots from all of the experimental samples) labeled with Cy2. A conventional dye swap for DIGE was performed by labeling two replicates from each treatment group with one dye (Cy3 or Cy5) and the third replicate with the other of the two Cydyes. This mix of labeled proteins was adjusted to a final volume of 250 µl and loaded onto a 24 cm, pH 3–10, IPG strips (pH3–10NL, GE Healthcare, Little Chalfont, UK) for 12 h at 20°C and a constant voltage of 50 V. Isoelectric focusing (IEF) was carried out at 200 V for 200 Vh, 500 V for 5 00 Vh, 1000 V for 1000 Vh, and 8000 V for 60.000 Vh at 20°C and a maximum current setting of 50 mA/strip in an IEF unit from GE Healthcare. Following IEF, the IPG strips were equilibrated for 15 min in 375 mM Tris (pH 8.8) containing 6 M urea, 20% (v/v) glycerol, 2% (w/v) SDS, and 130 mM DTT and then for a further 15 min in the same buffer except that DTT was replaced with 135 mM iodoacetamide. Active rehydration was carried out on the IPGphor (GE Healthcare) under the following conditions: 30 V during 1 h, 300 V during 3 h, gradient to 1000 V in 6 h, gradient to 8000 V in 3 h, 8000 V until 100,000 Vh. The IEF was conducted at 15°C. The 2-DE was performed at 20°C in Ettan Dalt-six electrophoresis unit (GE Healthcare) at 25 W/gel for 5 h.

Gel scanning, image analyses, and protein digestion

The 2-DE gel images were scanned with an Ettan DALT-six System (GE Healthcare) at wavelengths corresponding to each Cydye. Images were then analyzed with SameSpots software version 3.2 (Nonlinear Ltd, Newcastle) according to the manufacturer's instructions. Protein spots were excised (based on their significant expression changes among the treatments) from the gel using an Ettan spot picker robot (GE Healthcare).

Selected gel pieces were collected in 96-well plates designed for the Proteineer dp automated Digester (Bruker, Bremen, Germany). Briefly, gel pieces were washed with three incubations in 100% of 50 mM ammonium bicarbonate, and a mix of 50% acetonitrile plus 50% of 50 mM ammonium bicarbonate. Two additional washes were performed with 100% acetonitrile to dehydrate the gel. Freshly activated trypsin (Roche, porcine, proteomics grade) was used to rehydrate the gel pieces at 8 C for 30 min. Trypsin digestions were performed for 3 h at 30°C. Peptide extractions were performed with 10 µl of 1% formic acid for 30 min at 20°C.

Protein identification by mass spectrometry

MALDI-TOF/MS

Protein digests (3 µl) were adsorbed for 3 min on pre-spotted Anchorchips (R) using the Proteineer dp automaton. Spots were washed on-target using 10 mM ammonium dihydrogen phosphate in 0.1% TFA and MilliQ water (Millipore) to remove salts. High throughput spectra were acquired using an Ultraflex II MALDI mass spectrometer (Bruker) in positive reflectron mode with close calibration enabled. The Smartbeam laser focus was set to medium, and the laser fluency setting was 65–72% of the maximum. Delayed extractions were set to 30 ns. Spectra in the range of 860–3800 Da were acquired at a 200 Hz laser shot frequency with automated evaluation of intensity, resolution, and mass range. Six hundred successful spectra per sample were summed, treated, and de-isotoped in line with an automated SNAP algorithm using Flex Analysis 2.4 software (Bruker), and subsequently submitted in batch mode to the Biotools 3.0 software suite (Bruker) with an in-house hosted MASCOT search engine (www.MatrixScience.com).

Liquid chromatography-electrospray ionization-ion-trap tandem mass spectrometry (LC-ESI-MS/MS)

Peptide separation by reversed-phase liquid chromatography was performed on an Ultimate LC system (LC Packings) complete with Famous autosampler and Switchos II micro-column switching device for sample clean-up and pre-concentration. The sample (30 ml) was loaded at a flow rate of 200 nl/min on a micro-pre-column cartridge (300 mm i.d. × 5 mm, packed with 5 mm C18 100A PepMap). After 5 min, the pre-column was connected with the separating nano-column (75 mm i.d × 150 mm, packed with C18 PepMap100, 3 mm, 100 Å) and the gradient started. Elution gradient varied from 0% to 30% buffer B over 30 min, buffer A is 0.1% formic acid in acetonitrile/water 2:98 (vol/vol) and buffer B is 0.1% formic acid in acetonitrile/water 20:80 (vol/vol). The outlet of the LC system was directly connected to the nanoelectrospray source of an Esquire HCT ion-trap mass spectrometer (Bruker Daltonics, Germany). Mass data acquisition was performed in the mass range of 50–1700 m/z using the Standard-Enhanced mode (8100 m/z/s). For each mass scan, a data-dependent scheme picked the four most intense doubly or triply charged ions to be selectively isolated and fragmented in the trap and the resulting fragments were mass analyzed using the Ultra-Scan mode (50–3000 m/z at 26,000 m/z/s).

Identification

For identification, we used the public National Center for Biotechnology Information (NCBI) non-redundant database with parameters set for Viridiplants. A mass tolerance of 80 ppm with close calibration and one missing cleavage site were allowed. Partial oxidation of methionine residues and complete carbamoylation of cysteine residues were considered. The probability score calculated by the software was used as one criterion for correct identification. In order to confirm the identifications, experimental molecular weights (MW) and pI were compared to the predicted values resulting from the MASCOT analysis.

The significant interpretation was also correlated with the identified organism (mainly *A. thaliana*) and protein nature and function in the studied biological matrix. Proteins were classified based on the literature and information available in the Swiss-Prot/TrEMBL, Kegg pathways and Gene Ontology databases.

Results

Diverse 2-D DIGE protein patterns of P. xylostella-*infested* A. thaliana

A proteomic work was conducted by 2-DE to monitor the global alterations in protein expression from two samples types: healthy *Arabidopsis* leaves and *Arabidopsis* leaves infected by pest larvae at 27°C within 8 h. Approximately 450 protein spots were reproducibly detected (Figure 1). Comparing healthy and infested leaves, our statistical evaluation of relative spot volumes identified 18 proteins differentially expressed. Of these, only six proteins were upregulated by insect attack (12 were downregulated). Thirteen of 18 picked spots were successfully identified by MALDI-TOF/MS and LC-ESI-MS/MS. Nine positive identification results were derived from MALDI-TOF/MS data, whereas six were by LC-ESI-MS/MS. The list of identified protein species, together with their quantitative alterations as a result of insect infestation at high temperature, is presented in Table 1. Most of the differentially regulated proteins appeared to be of *A. thaliana*, and several other proteins appeared to be derived from other plant species (i.e. *Citrus sinensis* [spot 392]; *Vitis vinifera* [spot 655]; *Cymbidium goeringii* [spot 295]; *Glycine max* [spot 495]; and *Aegilops tauschii* [spot 709]) (Table 1).

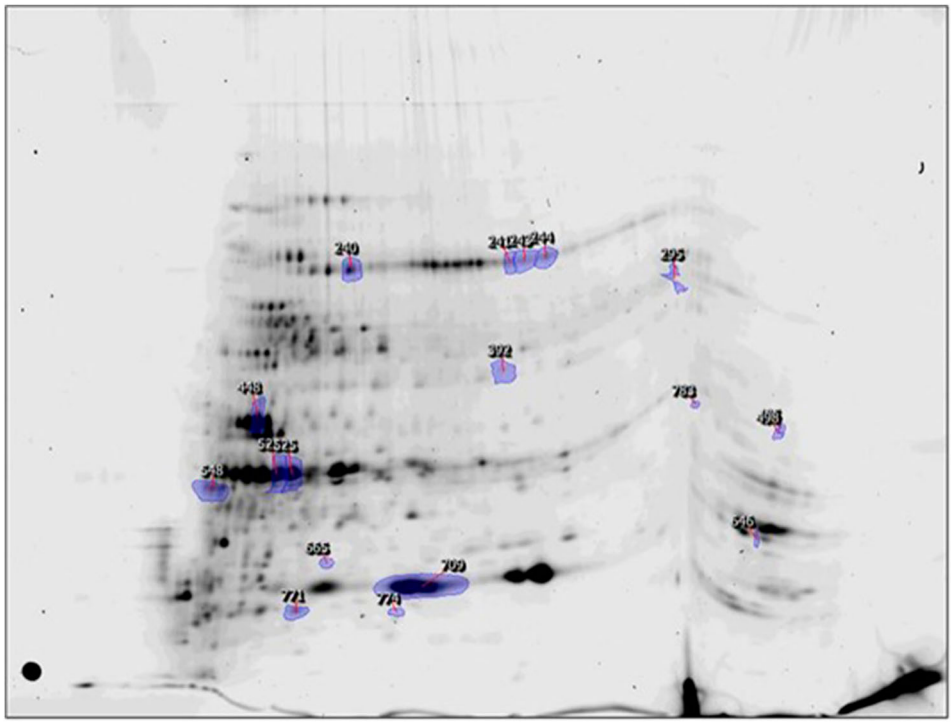

Figure 1. A 2D-PAGE gel separation from *A. thaliana* uninfested and infested by *P. xylostella* larvae at 27°C within 8 h. Numbered spots correspond to significantly expressed proteins between uninfested and infested *Arabidopsis* leaves by larvae that picked to analyzed by MS. Data of protein identification for each particular spot number are given in Table 1 when they are available.

Table 1. List of identified proteins and related metabolic pathways in *Arabidopsis* leaves uninfested and infested by *P. xylostella* larvae at 27°C within 8 h.

Spot number	Protein description	MW	pI	Mowse score	Score	MS coverage	Peptide numbers	Accession number	Organism	Identification
Amino acid metabolism and transport										
244	Hydroxymethyl transferase	65,040	8.89	42		9	4	gi|21537165	*Arabidopsis thaliana*	MALDI-TOF MS
392	Alpha/beta hydrolase	30,590	9.13	52		13	4	gi|568857313	*Citrus sinensis*	MALDI-TOF MS
522	GSTL3_ARATH Glutathione S-transferase L3	27,073	5.07			38	10		*A. thaliana*	LC-ESI-MS/MS
665	Metal-nicotianamine transporter YSL7	78,711	8.75	46		6	4	gi|225423773	*Vitis vinifera*	MALDI-TOF MS
774	Glutathione S-Transferase	23,983	5.89	111		34	9	gi|2554769	*A. thaliana*	MALDI-TOF MS
Carbohydrate metabolism										
243	Sedoheptulose-1,7-bisphosphatase	42,787	6.21	41		12	4	gi|15228194	*A. thaliana*	MALDI-TOF MS
522	CAHC_ARATH Carbonic anhydrase, chloroplastic	37,426	5.74		261	24	10		*A. thaliana*	LC-ESI-MS/MS
Energy metabolism										
241	ATPB_ARATH ATP synthase subunit beta, chloroplastic	53,900	5.38		458	35	17		*A. thaliana*	LC-ESI-MS/MS
295	ATP synthase beta subunit, partial (chloroplast)	52,041	5.34	51		14	4	gi|499069773	*Cymbidium goeringii*	MALDI-TOF MS
Lipid metabolism										
495	GDSL esterase/lipase	40,579	7.62	52		12	4	gi|356506014	*Glycine max*	MALDI-TOF MS
709	Sn1-specific diacylglycerol lipase alpha	57,160	8.08	46		4	3	gi|475509755	*Aegilops tauschii*	MALDI-TOF MS
Photosynthesis-related										
241	RBL_ARATH Ribulose bisphosphate carboxylase large chain	52,922	5.88			28	19		*A. thaliana*	LC-ESI-MS/MS
448	PSBO1_ARATH Oxygen-evolving enhancer protein 1–1, chloroplastic	35,121	5.55		268	43	16		*A. thaliana*	LC-ESI-MS/MS
548	PSBP1_ARATH Oxygen-evolving enhancer protein 2–1, chloroplastic	28,078	6.9		74	13	3		*A. thaliana*	LC-ESI-MS/MS
646	Ribulose bisphosphate carboxylase	20,588	7.59	105		41	7	gi|16194	*A. thaliana*	MALDI-TOF MS

Note: MW, molecular weight; pI, isoelectric point; Score, Mowse score according to Mascot search; Coverage, percentage of the protein sequence identified; Peptide number, number of peptide hits for each protein.

Five differentially regulated proteins were associated with amino acid metabolism and transport (spots 244, 392, 522, 665, and 774). Among these, four proteins (hydroxymethyltransferase, alpha/beta hydrolase, glutathione S-transferase L3, and metal-nicotianamine transporter YSL7) were downregulated in larvae-infested leaves, whereas glutathione S-Transferase was upregulated. In contrast, all of the identified proteins related to carbohydrate (i.e. sedoheptulose-1,7-bisphosphatase [spot 243] and carbonic anhydrase, chloroplastic [spot 522]) and energy (i.e. ATP synthase subunit beta, chloroplastic [spot 241] and ATP synthase beta subunit, partial [chloroplast] [spot 295]) metabolism were downregulated. Three proteins involved in photosynthesis (i.e. Ribulose bisphosphate [RuBis] carboxylase large chain [spot 241], oxygen-evolving enhancer protein 1–1, chloroplastic [spot 448], and Rubis carboxylase [spot 646]) and another protein related to lipid metabolism (Sn1-specific diacylglycerol lipase alpha [spot 709]) was downregulated due to insect feeding. However, in these classes, oxygen-evolving enhancer protein 2–1, chloroplastic (spot 548) and GDSL esterase/lipase (spot 495) expression were significantly increased in pest infested leaves (Table 1; Figure 2).

Discussion

Here, by 2-DE coupled with MALDI-TOF/MS and LC-ESI-MS/MS, we identified differentially expressed proteins of *A. thaliana* leaves comparing non-infested leaves with leaves infested with *P. xylostella* larval. Proteomic results suggested that larval feeding on *Arabidopsis* leaves at 27°C within 8 h had a significant effect on proteins related to different metabolic pathways. In fact, 18 proteins were differentially regulated by insect attacks at 27°C. This result was different in comparison with *Arabidopsis*-infested larvae at 22°C over 8 h. In agreement with Liu et al. (2010), we observed that 38 protein spots show a significant different expression ($p < .05$), among which 27 proteins were upregulated after the infestation of larvae on *Arabidopsis* in comparison with uninfested plants (data not shown).

More than half of the differentially expressed proteins obtained in the present study were those associated with photosynthesis (four proteins) and amino acid metabolism and transport (five proteins). The infestation of larval pests on *Arabidopsis* leaves led to the downregulation of such proteins, except for oxygen-evolving enhancer protein 2–1 (chloroplastic) and glutathione S-Transferase. Some of these proteins are known as either insect or abiotic responsive proteins (e.g. Rubisco, alpha/beta hydrolase, GST, and GST L3) (Fakae et al. 2000; Giri et al. 2006; Liu et al. 2010; Chen et al. 2011; Dubey et al. 2013).

Similar observations have been reported with herbivorous insects or temperature stress in *Arabidopsis* plants (Collins et al. 2010; Rocco et al. 2013). Photosynthesis-related proteins (RuBis carboxylase large chain, oxygen-evolving enhancer protein 1–1, chloroplastic, oxygen-evolving enhancer protein 2–1, chloroplastic, and RuBis carboxylase) were observed in

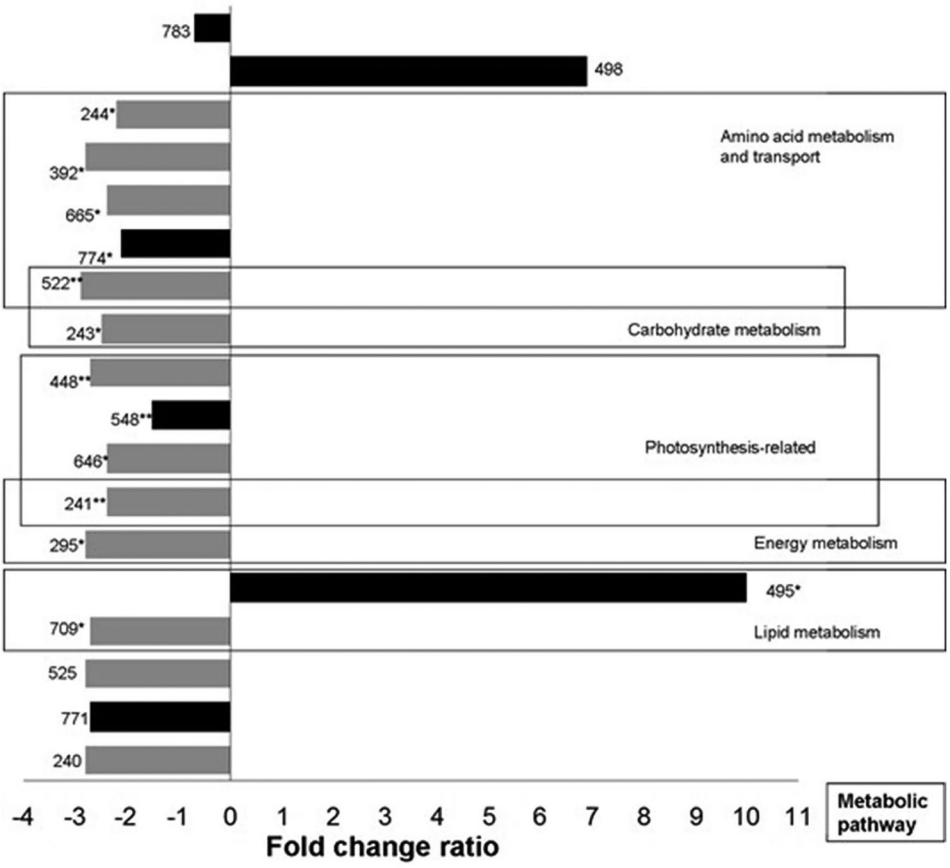

Figure 2. Comparison of protein expression between *A. thaliana* leaves infested and uninfested by *P. xylostella* larvae at 27°C within 8 h. Data of protein identification for each particular spot number are given in Table 1 when they are available. Black and gray bars represented proteins up-regulated and downregulated by the larval attack, respectively. Labels on the right show the functional categories to which the proteins are assigned. *identified by MALDI-TOF/MS; **identified by LC-ESI-MS/MS.

this study. Existing data demonstrated that chewing insects caused the reduction of photosynthesis in plants following their infestation (Zangerl et al. 1997; Tang et al. 2009; Liu et al. 2010; Halitschke et al. 2011; Nabity et al. 2013). The downregulation of RuBis expression in insect-challenged plant leaves was found in some previous studies (Hermsmeier et al. 2001; Giri et al. 2006; Wei et al. 2009). Moreover, it is indicated that heat stress causes downregulation of different key enzymes of the Calvin cycle in rice, including RuBis carboxylase (Han et al. 2009). The expression of RuBis carboxylase large chain was significantly downregulated in *Arabidopsis* leaves due to heat treatment (Rocco et al. 2013). Zou et al. (2011) indicated that RuBis carboxylase could be considered as a part of the plant adaptive response, to maintain CO_2 fixation under stress factors. Based on the existing data, the downregulation of RuBis proteins observed in the present study may be a result of larval feeding on *Arabidopsis* leaves at high temperature (27°C compared to 22°C of *Arabidopsis* growth temperature).

With respect to amino acid metabolism and transport, five proteins from this class were identified in the present study (hydroxymethyltransferase, alpha/beta hydrolase, glutathione S-transferase, metal-nicotinamide transporter YSL7 and Glutathione S-Transferase). The amino acid is well-addressed as indicators of plants response to herbivorous insects' attack on the plant (Schmelz et al. 2012; Sempruch et al. 2012). It is noted that glutathione plays a key role in detoxification of activated oxygen and can be up-regulated by jasmonates (JAs) (Sanchez-Sampedro et al. 2007; Chen, et al. 2011). In addition, *P. xylostella* caterpillars induce JA-signaling in the defense response of *A. thaliana* (Bidart-Bouzat and Kliebenstein 2011; Savchenko et al. 2013; Zhang et al. 2013). Therefore, a glutathione S-transferase was here observed to be more abundant in the larvae-infested *Arabidopsis* leaves compared to uninfested plant samples. That may be related to insect behavior. This is in agreement with Collins et al. (2010), who found the upregulation of this protein in *Arabidopsis* leaves attacked by *P. xylostella*.

For carbohydrate and energy metabolism, herbivorous insect and heat stress negatively influence the glycolytic pathway and decrease energy production in *Arabidopsis* plants (Liu et al. 2010; Rocco et al. 2013). In agreement with this scenario, we obtained a down-representation of sedoheptulose-1,7-bisphosphatase, carbonic anhydrase, chloroplastic, ATP synthase subunit beta, chloroplastic, and ATP synthase beta subunit, partial (chloroplast) in *Arabidopsis* leaves attacked by larvae. Nabity et al. (2013) suggested that the alterations of such proteins consequently led to a reduction of photorespiration rate of plant leaves under invasive insects. Hence, it was noted that plants must use their energy economically when challenging herbivorous insect infestation (Liu et al. 2010).

In contrast to proteins related to carbohydrate and energy metabolism, a strong increase in GDSL esterase/lipase expression appeared in the proteome profile of larvae-infested *Arabidopsis* leaves in comparison to healthy leaves (14-fold change in its abundance). However, the expression of another protein, Sn1-specific diacylglycerol lipase alpha, was significantly downregulated due to larval infestation on leaves of *Arabidopsis* leaves. It has been suggested that

GSDL esterase/lipase plays an important role in rice (*Oryza sativa* L.) response to various environmental stress factors (Jiang et al. 2012). GSDL esterase/lipase often appears in plant response to environmental factors like cold, insect, or bacteria stresses, and its expression can be induced through JA-signaling (Chepyshko et al. 2012).

Conclusions

This paper reports the characterization of the global proteome of *A. thaliana* leaves under larval pest infestation at high temperature for 8 h (27°C compared to 22°C of *Arabidopsis* growth temperature). By using 2-DE coupled with MALDI-TOF MS or LC-ESI-TRAP MS/MS, 13 of 18 differentially expressed protein spots were successfully identified. These proteins participate in multiple physiological processes. Functional classification analysis indicated that such proteins were associated with amino acid (5), carbohydrate (2), energy (2), and lipid (2) metabolism and photosynthesis (4). In addition, their relative abundance was up-regulated or downregulated according to larval pest feeding on *Arabidopsis* leaves. Our data demonstrate that combined temperature and larvae stresses can alter the proteome in plant leaves.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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