Proteomic analysis of *Arabidopsis thaliana* (L.) Heynh responses related to a generalist sucking pest (*Myzus persicae* Sulzer) colony set up

Dieu-Hien Truong[1*], Julien Bauwens[2], Pierre Delaplace[3], Gabriel Mazzucchelli[4], Georges Lognay[5], Frédéric Francis[2]

[1]Faculty of Applied Sciences, Ton Duc Thang University, Vietnam

[2]Functional & Evolutionary Entomology, University of Liège, Gembloux Agro-Bio Tech, Belgium

[3]Plant Biology, University of Liège, Gembloux Agro-Bio Tech, Belgium

[4]Mass Spectrometry Laboratory, University of Liège, Belgium

[5]Analytical Chemistry Laboratory, University of Liège, Gembloux Agro-Bio Tech, Belgium

*truongthidieuhien@tdt.edu.vn

Tel.: 0084(0)837755058; Fax: 0084(0)837755055

Abbreviation

2-DE	: Two-dimensional gel electrophoresis
AlaAT	: Alanine aminotransferase
DIGE	: Difference gel electrophoresis
DTT	: Dithiothreitol
GPA	: Green peach aphid
IEF	: Isoelectric focusing
IPG	: Immobilized pH gradient
ISR	: Induced systemic resistance
LC-ESI-MS/MS	: Liquid chromatography-electrospray ionization-ion-trap tandem mass spectrometry
MALDI-TOF MS	: Matrix-assisted laser desorption time-of-flight mass spectrometry
MW	: Molecular weights
NCBI	: National Center for Biotechnology Information
PR proteins	: pathogenesis related proteins
RH	: Relative humidity

RLK : Receptor-like protein kinase

ROS : Reactive oxygen species

RuBisCo : Ribulose bisphosphate carboxylase

SA : Salicylic acid

SAR : Systemic acquired resistance

SDS : Sodium dodecyl sulfate

VOC : Volatile organic compound

Abstracts

Herbivorous insects can cause deep cellular changes to plant foliage following infestations depending on feeding behavior. Here, a proteomic study was conducted to investigate green peach aphid (*Myzus persicae* Sulzer) influence as a polyphagous pest on the defense response of *Arabidopsis thaliana* (L.) Heynh after aphid colony set up on host plant (3 days). Analysis of about 574 protein spots on 2-DE gel revealed 31 differentially expressed protein spots. Twenty out of 31 differential proteins were selected to be analyzed by mass spectrometry. From 12 out of the 20 analyzed spots, we identified 7 and 9 proteins by MALDI-TOF-MS and LC-ESI-MS/MS, respectively. Twenty five percents of the analyzed spots contain a couple of proteins. Different metabolic pathways were modulated in *Arabidopsis* leaves according to aphid feeding: most of them corresponded to carbohydrate, amino acid and energy metabolism, photosynthesis, defense response and translation. This paper has established a survey of early alterations induced in the proteome of *Arabidopsis* plants by the *M. persicae* aphids. It provides valuable insights to uncover the complex response of plants to biological stress, particularly with herbivorous insects with sucking feeding behavior.

Keywords. *Arabidopsis thaliana*, *Myzus persicae*, proteomic expression, 2-DE, MALDI-TOF MS, LC-ESI-MS/MS.

Introduction

In order to ensure their growth and survival in natural habitats, plants have triggered a wide range of physiological and biochemical defenses against a lot of stress conditions (e.g., temperature, herbivorous insects,

and pathogens) (Francis et al., 2010; Holopainen and Gershenzon, 2010; Loreto and Schnitzler, 2010; Kosova et al., 2011; Atkinson and Urwin, 2012). Similarly, *Arabidopsis* plants respond adaptively to their environmental stresses by mediating a range of defense mechanisms, including both forms of induced resistance (systemic acquired resistance (SAR) and induced systemic resistance (ISR)) (Van Poecke, 2007; Snoeren et al., 2010; Truong et al., 2014b). Consequently, these defense responses lead to changes in the host cells at the level of either gene or proteomic expression (De Vos et al., 2005; Amme et al., 2006; De Vos et al., 2007; Kempema et al., 2007; Kosova et al., 2011; Maserti et al., 2011; Rocco et al., 2013; Appel et al., 2014; Ghosh and Xu, 2014). Existing data indicate that aphid feeding on *Arabidopsis* plants results in SAR induction through salicylic acid (SA) defense signaling (Vallad and Goodman, 2004; De Vos and Jander, 2010; Louis et al., 2012; Truong et al., 2014b).

Among the insect species that have been described, green peach aphid (GPA, *Myzus persicae* Sulzer) is a very polyphagous species able to infest 400 host plants including *Brassica* (De Vos et al., 2007; Kettles et al., 2013; Louis and Shah, 2013). Although aphids are well-known to establish a prolonged interaction with the damaged plant tissue, the mechanisms by which plants respond to hemiptera pests to protect themselves and how aphids can induce plant processes are not fully understood (Coppola et al., 2013; Kettles et al., 2013; Louis and Shah, 2013) even if *M. persicae* saliva was described and known to include potential plant defense elicitors (Harmel et al., 2008; Vandermoten et al., 2014).

Being a model for studying plant-aphid interactions (Van Poecke, 2007), *Arabidopsis thaliana* (L.) Heynh has been used to investigate the mechanism of inducible defense of host plants against pests (Kliebenstein et al., 2002; De Vos et al., 2007; Van Poecke, 2007). Published studies revealed that aphid *M. persicae* feeding on *Arabidopsis* leads to altered gene expressions in the host plants (Ellis et al., 2002; Moran et al., 2002; De Vos et al., 2005; Thompson and Goggin, 2006; De Vos et al., 2007; Nalam et al., 2012; Appel et al., 2014). It is investigated that the infestation of *M. persicae* leads to the largest number of transcriptome changes in *Arabidopsis* plants compared to the other biotic factors (i.e., pathogenic leaf bacterium (*Pseudomonas syringae* pv. Tomato), pathogen leaf fungus (*Alternaria brassicicola*), tissue chewing caterpillars (*Pieris rapae*), cell-content feeding thrips (*Frankliniella occidentalis*)) (De Vos et al., 2005). Appel et al. (2014) recently observed the down-regulation of amino acid catabolism in *M. persicae*-infested *Arabidopsis* plants after 6 and 24 h treatments. In general, a wealth of information about the expression of genes in plants response to herbivores was determined through microarray experiments (Seki et al., 2001; Moran et al., 2002; Seki et al., 2002; De Vos et al., 2007). Recently, the developments of proteomic methods have provided complementary functional

evidences about the physiological and biochemical modifications in plants response to herbivorous insects (Francis et al., 2006; Liu et al., 2010b; Zhang and Li, 2010; Kryvych et al., 2011). Compared to the studies on gene expression in *Arabidopsis* response to *M. persicae* infestation, the use of proteomic approaches using two-dimensional gel electrophoresis (2-DE) coupled with mass spectrometry is still limited.

Here, a proteomic study was conducted using 2-DE coupled with mass spectrometry (i.e., matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS) and liquid chromatography-electrospray ionization-ion-trap tandem mass spectrometry (LC-ESI-MS/MS)) to explore the alteration of protein expression in *A. thaliana* leaves infested by adults *M. persicae* (Sulzer). This approach promises to gain valuable new insights on the physiological and molecular adaptations of plants toward pest infestation in more functional approach.

Materials and methods

Plants and insects

A. thaliana, ecotype Columbia (Col-0), were grown from seeds (Lehle Company, TX, USA) in a growth chamber at 22 ± 0.6 °C, light/dark cycle 16/8 h (LED lighting: 43 µmol m^{-2} s^{-1} photosynthetically active radiation during the light period), and relative humidity (RH) 64.5 ± 2.6%. Plants were cultivated in plastic pots (0.2 l) with potting soil, and watered twice a week (tap water, 10-20 ml/pot) for five weeks. The phloem-feeding insects (*M. persicae*) were reared on broad bean plants (*Vicia faba* L.), under controlled environment in a room at 20 ± 2 °C with a 16L: 8D photoperiod.

Plant treatment

Five-week old *A. thaliana* plants, including root balls (9-10 leaves, 0.25-0.40 g/plant) were removed from the plastic pots, and wrapped in aluminum foil around the root balls. Three plants were carefully placed together in a reaction vessel (100 ml, Duran Group, Germany) and subjected to each stress treatment. At the end of each experiment, harvested leaves were immediately frozen in liquid nitrogen and kept at -80 °C until use.

Aphid infestation

In order to consider the impact of an aphid whole colony, infestation by *M. persicae* aphids was carried out by placing 70 adults on each randomly *Arabidopsis* plant at 20 ± 2°C within 72 h. The aphid density used in this study was selected according to the results of our previous research (Truong et al., 2014a). Plants considered as controls were not infested. Aphids and their honeydew were then removed carefully by using a soft paint brush and plant leaves were harvested after 72 h infestation. Three biological replicates were conducted for each experimental condition.

Protein extraction and assay

Proteins were extracted using a phenol-based extraction method for *Arabidopsis* plants as described by Huang et al. (2011), with some modifications. The frozen *A. thaliana* leaves (0.3-0.4 g fresh mass) were finely powered in liquid nitrogen using a pestle and a mortar, and suspended in 2.5 volumes of extraction buffer (0.9 M sucrose, 0.5 M Tris-HCl, 5 mM ethylenediamine tetraacetic acid, 0.1 M KCl, and 1% w/v dithiothreitol (DTT)) by vortexing until obtaining a thick paste. Prior to the addition of an equal volume of Tris-saturated phenol, at pH 8.0, the suspension was sonicated in the ice-cold sonication bath (4 °C) for 5 min in duplicates. The mixture was vortexed, agitated 10 min in a thermomixer at 1000 RPM, and then centrifuged at 5000 x 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. The proteins comprised in the phenol phase were precipitated by the addition of 5 volumes of 0.1 M ammonium acetate in methanol (precipitation solution), and kept overnight at -80 °C. Precipitated proteins were centrifuged again at 5000 x g for 10 min at 4 °C and the supernatant was discarded. The pellet was washed with 5 volumes of precipitation solution, and then by 5 volumes of ice-cold (4 °C) 80% acetone, centrifuged at 5000 x g for 10 min at 4 °C. Two replicates were conducted for each step. The protein suspension was stored at -80 °C until 2-DE analysis.

The concentration of the protein extracts was assayed using “RC/DC Protein Assay” from Bio-Rad (Hercules, CA, USA), using bovine serum albumin as a standard. All the steps were conducted according to the instructions of manufacturer at room temperature.

2-D gel electrophoresis and gel image acquisition

The protein extracts were labeled with Cydyes (GE Healthcare, Little Chalfont, UK) according to the standard difference gel electrophoresis (DIGE) protocol (50 µg protein for each Cydye). Two samples corresponding to two different groups (uninfested or infested leaves) were labelled either with Cy3 or Cy5 and were mixed with an internal reference (pool from equal protein amount aliquots from all of the experimental samples) labeled with Cy2. A conventional dye swap for DIGE was performed by labelling 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 labelled proteins was adjusted to a final volume of 450 µl and loaded onto a 24 cm, pH 3-10, immobilized pH gradient (IPG) strips (pH3–10NL, GE Healthcare) for 12 h at 20 °C and at constant voltage of 50 V. Isoelectric focusing (IEF) was carried out at 9700 V at 20 °C (200 V for 200 Vh, 500 V for 500 Vh, 1000 V for 1000 Vh and 8000 V for 60.000 Vh) and a maximum current setting of 50 mA per IPG strip from BioRad. Following IEF, the IPG strips were equilibrated for 15 min in 375 mM Tris (pH 8.8) containing 6 M urea, 130 mM DTT, 20% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate (SDS), and then for a further 15 min in the same buffer except that DTT was replaced 135 mM iodoacetamide. The IPG strips were then sealed with 0.5% agarose in SDS running buffer at the top of slab gels (240 mm x 200 mm x 1 mm) polymerized from 12% (w/v) acrylamide and 0.1% N,N'-methylenebisacrylamide. The 2-DE was carried out on the 12% polyacrylamide gels (180 x 240 x 1 mm) using a Protean apparatus (Bio-Rad), using electrophoresis buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine and 0.1% w/v SDS), with 120 V applied for 12 h, until the dye front reached the bottom of the gel.

Gel scanning, image analyses, and protein digestion

Gels were scanned with an Ettan Dige imager (GE Healthcare) at wavelengths corresponding to each dye (Cy2, Cy3 or Cy5). Images were analyzed with Progenesis samespots software (Nonlinear Dynamics Ltd, Newcastle, UK) according to the manufacturer's instructions. Protein spots were excised (based on their significant expression changes among the treatments) from one preparative gel (500 µg of a non labeled protein mix related to both treatments were added before first dimension on one of the strip) 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 1:1 mix of 50% acetonitrile and 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 MS

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).

LC-ESI-MS/MS

Peptide separation by reversed-phase liquid chromatography was performed on an Ultimate LC system (Dionex) complete with Famos autosampler and Switchos II microcolumn switching device for sample clean-up and preconcentration. Sample (30 ml) was loaded at a flow rate of 200 nl/min on a micro-precolumn cartridge (300 mm i.d. x 5mm length, packed with 5 mm C18 100A PepMap). After 5 min, the precolumn was connected with the separating nano-column (75 µm x 15 cm, packed with C18 PepMap100, 3 µm, 100 Å) and the gradient started. Elution gradient varied from 0% to 30% buffer B over 30min, 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 nano electrospray 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-dependant scheme picked the 4

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

The database used was the public National Center for Biotechnology Information (NCBI) non-redundant database with parameters set for plants. A mass tolerance of 80 ppm with close calibration and one missing cleavage site were allowed. Partial oxidation of methionine residues and complete carbamylation of cystein 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 (data not shown).

More criteria used to consider the identified organism (mainly *A. thaliana*) and protein nature and function in the studied biological matrix were considered to confirm identified significances. Proteins were classified based on the literature and information available in the uniform resource locators (i.e., Swiss-Prot/TrEMBL, Kegg pathways and Gene Ontology databases).

Results

Diverse 2-DE protein patterns of Arabidopsis leaves uninfested and infested by M. persicae

To detect the variation of proteins expressed in healthy and aphid infested *Arabidopsis* plants, the gel images of 2-D DIGE patterns were compared and revealed 574 protein spots from plant leaves following 3 days of aphids feeding (identified by Progenesis samespots software software) (Fig. **1**). Of these, 31 protein spots showed significant different expression ($p < 0.05$) according to aphid infestation status, among which 9 proteins were up-regulated by the infestation of aphids on *Arabidopsis* in comparison with uninfested plants (Fig. **2**). From the 31 differentially expressed protein spots, 5 varied significantly in abundance in uninfested and infested samples ($p < 0.05$; $n = 4$). Twenty out of 31 differential proteins were picked and analyzed by MALDI-TOF MS and LC-ESI-MS/MS (Fig. **1**).

Spot identification by MS

241

242 Twelve out of the 20 differentially expressed protein spots were successfully identified by MALDI-TOF MS (7
243 spots) and LC-ESI-MS/MS (5 spots) (see Tables **1** & Fig. **2)**. The results indicated that 25% of the analyzed
244 spots contain a couple of proteins. Most of the differentially regulated proteins appeared to be of *A. thaliana*
245 origin, only one appeared to be derived from another plant species, namely *Morus notabilis* (spot 1263).
246 Most of the identified proteins were classified according to the literature and information available in the Swiss-
247 Prot/TrEMBL, Kegg pathways and Gene Ontology databases. Functional classification analysis indicated that
248 two differentially regulated proteins observed in this study were associated with carbohydrate (i.e., bifunctional
249 enolase 2/transcriptional activator (spot 829) and probable ribose-5-phosphate isomerase 3 (spot 1087)), energy
250 metabolism (i.e., ATP synthase subunit beta (spot 829) and ADP-ribosylation factor (spot 1263)), defense
251 response (i.e., receptor-like protein kinase (spot 765) and hydroxyproline-rich glycoprotein (spot 777)). Eight
252 proteins were associated with photosynthesis, namely chlorophyll a/b binding protein (spot 661), core-2/I-
253 branching beta-1,6-N-acetylglucosaminyltransferase (spot 1012), chlorophyll a-b binding protein 2 (spot 1087),
254 photosystem II light harvesting complex protein B1B2 (spot 1097), oxygen-evolving enhancer protein 2-1,
255 carbonic anhydrase and carbonic anhydrase (spot 1115), ribulose bisphosphate carboxylase (RuBisCo) small
256 chain 2B and ruBisCo small chain 1A (spot 1259). In addition, one protein related to translation, namely
257 elongation factor Tu (spot 862) and another protein potentially involved in amino acid metabolism and transport,
258 was also identified (alanine aminotransferase; spot 985) (Table **1** & Fig. **2**).
259

260 **Discussion**

261

262 Recent studies found that the impact of temperature, insects, pathogens, or the combination of temperature and
263 water stress lead to the up-regulated or down-regulated expression of proteins in *Arabidopsis* plants (Amme et
264 al., 2006; Koussevitzky et al., 2008; Collins et al., 2010; Liu et al., 2010b; Mukherjee et al., 2010; Nalam et al.,
265 2012; Rocco et al., 2013). In the present study, the variation in the expressed proteome of *A. thaliana* leaves due
266 to aphid feeding was analyzed after 3 days exposure to identify potential impact of plant defense on aphid
267 biology. Indeed, whether early plant defensive responses could happen from first few hours of pest infestation,
268 real impact on aphid development and reproduction will only be induced after a 2 to 72 h of feeding period on
269 host plant. The time of infestation used in this study was selected according to the comparison of changes in
270 proportions of *A. thaliana* volatile organic compound (VOC) due to aphid infestation during different periods (1,

2, and 3 day periods) in the previous research (Truong et al., 2014a). The results showed that the infestation of *M. persicae* over a 3 day period leads to the largest number of VOC changes in *Arabidopsis* plants compared to the other time periods.

Quantitative variations induced by aphid infestation were observed according to comparison of proteomic gel based approach that remains useful for descriptive works such as the one proposed here. Thirty-one protein spots exhibited significant quantitative alterations under experimental treatments. The alteration in gene expression of *Arabidopsis* leaves challenged with aphid feeding have been found in previous studies (Moran and Thompson, 2001; Moran et al., 2002; De Vos et al., 2007; Barah et al., 2013; Appel et al., 2014). For example, Moran and Thompson (2001) found the infestation of *M. persicae* on *Arabidopsis* leaves leads to induced transcription of two genes related to SA-dependent responses to pathogens (*PR-1* and *BGL2*). By using microarray and macroarray gene expression analysis technologies, Moran et al. (2002) observed genes involved in calcium-dependent signaling, oxidative stress, pathogenesis-related proteins, and signaling in the profile of *A. thaliana* infested by aphids over 72 and 96 h. It was investigated that aphid feeding (*Brevicoryne brassicae* L.) on *A. thaliana* led to altered significantly differential regulation of 4.979 genes (i.e., 2.803 up-regulated and 2.176 down-regulated) (Barah et al., 2013). De Vos et al. (2007) documented that on the plant side in *Arabidopsis-M. persicae* interaction, the expression of plant proteins can be induced in response to pest attack.

Feeding by phloem-feeders can lead to the alteration of expression of genes required for photosynthesis (Zhu-Salzman et al., 2004; Yuan et al., 2005). Sangha et al. (2013) observed large changes in photosynthesis-related proteins in rice attacked by brown planthopper (*Nilaparvata lugens* Stal). Here, the up-regulation of several photosynthesis-related proteins in aphid-infested *A. thaliana* leaves were observed (i.e., spots 1012, 1097, and 1115). This is in line with Maserti et al. (2011) who found up-regulated photosynthesis-related proteins in citrus plant proteomic profiles (*Citrus clementina* cv Tomatera) infested by the spider mite *Tetranychus urticae* C. L. Koch. These authors suggested that the infestation duration and intensity of insects can maintain the activity of photosynthesis during feeding. However, the down-regulation of RuBisCo small chain (2B and 1A) (spot 1259) occurred in *Arabidopsis* leaves infested by aphids. Several proteomic studies observed the down-regulated RuBisCo expression in insect-infested leaves (Hermsmeier et al., 2001; Giri et al., 2006; Wei et al., 2009; Maserti et al., 2011). It is hypothesized that the phenomenon could occur in terms of metabolic strategies to sustain fitness of plants or insects. However, a number of authors proposed that the degradation of RuBisCo is necessary for reallocation of carbon resources towards defense response of the host plants to insects (Hermsmeier et al., 2001; Schwachtje and Baldwin, 2008). Therefore, the down-regulation of RuBisCo proteins

in insect-infested plants is still difficult to interpret. Existing data indicate that aphid *M. persicae* feeding on *Arabidopsis* leaves could induce production of reactive oxygen species (ROS) in guard cells (Louis et al., 2012). Additionally, ROS can accelerate the degradation of RuBisCo (Feller et al., 2008). It may be one possible reason to explain the down-regulation of RuBisCo proteins in aphid-infested *Arabidopsis* leaves.

In contrast to RuBisCo protein in response to aphid feeding, protein related to carbohydrate metabolism, bifunctional enolase 2/transcriptional activator protein involved in glycolysis was up-regulated (spot 829). Nevertheless, protein in pentose biosynthesis, probable ribose-5-phosphate isomerase 3 (spot 1087), was down-regulated. Louis et al. (2012) reported that aphid infestation influence carbohydrate metabolism in *Arabidopsis* plants. Indeed, it has been shown that the expression of genes involved in the transport and metabolism of sugar were changed in aphid-infested *Arabidopsis* (Moran and Thompson, 2001; Moran et al., 2002; Louis et al., 2012). Therefore, changes in these enzymes observed in this study may reflect the alteration of sugar metabolisms after herbivorous insect infestation on plants. Indeed, salivary secretions of aphid *M. persicae* contains glucose-oxidase that is associated with glycolysis (Giordanengo et al., 2010). Additionally, the salivary chemicals and/or proteins of pests can trigger plant defensive reactions by inserting the stylet into the phloem (Walling, 2008; De Vos and Jander, 2009). Recently, Carrillo et al. (2014) found the induction of glycolysis expression in pea aphid (*Acyrthosiphon pisum* Harris) feeding on plants (*Pisum sativum* ssp. sativum cv. Messire and *P. s.* ssp. syriacum).

Amino acids are well-targetted as indicators of plants response to aphids (Cole, 1997; Sandström and Moran, 1999; Sempruch et al., 2012). According to the previous study of Sempruch et al. (2012) reporting an increased induction of alanine aminotransferase (AlaAT) in winter triticale plants infested by rain aphid (*Sitobion avenae* F.), the up-regulation of alanine aminotransferase (spot 985) was here observed in aphid-infested *Arabidopsis* leaves. Miyashita et al. (2007) investigated that the increase of AlaAT within *A. thaliana* roots during hypoxia is associated with the induction of *AlaAT1* and *AlaAT2* genes.

Similarly to AlaAt expression, the regulation of protein related to energy metabolism, namely ATP synthase subunit beta (spot 829), also increased in *Arabidopsis* leaves infested by *M. persicae*, but the regulation of ADP-ribosylation factor (spot 1263) was down-regulated. This is in line with Chen et al. (2011) who observed the up-regulation of ATP-synthase subunit in methyl jasmonate-treated *Arabidopsis* leaves.

The down-regulation of two defense-related proteins was found in the present work (spots 765 and 777). It is documented that the receptor-like protein kinase (RLK) and hydroxyproline-rich glycoprotein take substantial role in plants response to abiotic and biotic stress factors (Morris and Walker, 2003; Deepak et al.,

2007). Stintzi et al. (1993) reviewed that the induction of defense-related proteins (mainly pathogenesis-related (PR-) proteins) could be involved in plants response to fungi, bacteria, viruses, or herbivorous insects. Maserti et al. (2011) noted that the role of a PR protein in plants is still unclear under piercing/sucking insect attack. However, it is noteworthy to mention that most PR proteins are involved by microbes and not by insect attack (Stintzi et al., 1993; Almagro et al., 2009; Liu et al., 2010a). To the best of the authors' knowledge, it is not clear why all of the important defense-related proteins (i.e., receptor-like protein kinase and hydroxyproline-rich glycoprotein) are down-regulated in *Arabidopsis* in response to aphid feeding. In previous study, Nwugo et al. (2013) observed the reduction of defense-related proteins in lemon plants in response to '*Candidatus* Liberibacter asiaticus' (Las) infection. These authors suggested that the reduction of these proteins may be a part of energy conservation mechanism for an efficient utilization of resources by lemon plants under Las infection. Taken together, maybe the defense-related proteins found in our study are not specific for *M. persicae*, thus the mechanism of energy conservation could also be induced in aphid-infested *Arabidopsis* plants.

In contrast, one translation-related protein, elongation factor Tu (spot 862), was significantly up-regulated in its abundance from aphid-infested leaves. The expression of translation-related proteins is a crucial part of the plant defense response to environmental stresses (Ristic et al., 2007; Lefebvre et al., 2010; Chen et al., 2011).

In conclusion, the proteome changes induced in the leaves of *A. thaliana* during aphid infestation were investigated after 3 days by 2-DE coupled with MALDI-TOF MS and LC-ESI-MS/MS in this study. A total of 12 differentially expressed proteins were successfully identified. The classification of identified proteins based on their biochemical functions indicated that the majority of these proteins were associated with photosynthesis (8 proteins were situated within 6 spots). In addition, two proteins related to amino acid, carbohydrate, defense response and energy metabolisms were also observed. One protein was associated with amino acid metabolism and translation. Results suggested that the different metabolic pathways could be involved in *Arabidopsis* leaves response to aphid feeding. This paper has established a survey of global alterations induced in the proteome of *Arabidopsis* plants by 70 *M. persicae* aphids during a 3 day period corresponding to the conditions of colony setting on host plant. The high pest density per plant and the few day duration did not induce stronger proteome changes then for shorter time and reduced damages such as shown in other works. This lead to focus a bit more on the role and effectiveness of aphid saliva elicitors: more than the amount, the occurrence (or not) of elthe latters seems to be the most important aspect on plant – aphid interactions. Availability of functional tests to use

artificially collected saliva with a natural application mimicry and to identify the active elicitors is the next challenge to understand plant – aphid interactions.

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Table legend

Table 1. List of identified proteins and related metabolic pathways in *Arabidopsis* leaves uninfested and infested by *M. persicae* at 22 °C over 3 days.

Figure legends

Fig. 1. A 2D-PAGE gel separation from *A. thaliana* uninfested and infested by *M. persicae* at 22 °C over 3 days. Numbered spots correspond to differentially expressed proteins between uninfested and infested *Arabidopsis* leaves by aphids that were picked to be analyzed by MS. Data of protein identification for each particular spot number are given in Table **1** when they are available. MW, molecular weight (kDa).

Fig. 2. Comparison of protein expression between *A. thaliana* leaves infested and uninfested by *M. persicae* for 0–72 h at 22 °C. Data of protein identification for each particular spot number are given in Table 1 when they are available. Black and grey bars represented spots up-regulated and down-regulated, respectively. Labels on the right show the functional categories to which the proteins are assigned. *; identified by MALDI-TOF MS; and **; identified by LC-ESI-MS/MS.