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ORIGINAL ARTICLE

Understanding the genetic mechanism of resistance in aphid-treated alfalfa (*Medicago sativa* L.) through proteomic analysis

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Abstract

To minimize dependency on chemical pesticides, plant breeders are trying to emphasize on important agricultural pests for the development of pest resistant cultivars. However, the molecular approach and associated genetic tools conferring resistance have not been widely studied. In the current study, proteomic analysis of two of the alfalfa cultivars viz. a resistant (R) (Zhongmu-1) and a susceptible (S) (WL343), with (+ A) and without (− A) aphids rearing were carried out. Results indicated that 325 differentially expressed proteins (DEPs) up-regulated while 319 down-regulated with a pattern of R + A/R − A plants, whereas 371 up- and 583 down-regulated DEPs were identified in the S + A/S − A plants. Total number of DEPs found in (S + A/S – A) was around 19.7% greater than that of (R + A/R – A), whereas, the down-regulated DEPs of susceptible variety was 11.6% higher than the resistant cultivar. Applying the KEGG analysis, 96 and 142 DEPs were portrayed to 15 and 10 substantively augmented pathways for Zhongmu-1 and WL343, respectively. We also found that two of the shared pathways (carbon metabolism and pyruvate metabolism) are linking to important traits conferring resistance in alfalfa. Most importantly, the specific role of linoleic acid metabolism was found to be associated with jasmonic acid, flavonoid biosynthesis, and terpenoid backbone biosynthesis that might have been associated with the insect-resistant material synthesis in the resistant alfalfa cultivar. Our study suggested that both alfalfa cultivars (R, S) could govern protein expression through carbon and pyruvate metabolism. But only the resistant alfalfa cultivar (Zhongmu-1) can tune protein expression via linoleic acid metabolism and terpenoid backbone biosynthesis to induce the defensive protein expressions (e.g., jasmonic acid and flavonoid biosynthesis along with terpenoid backbone biosynthesis), to enhance plant defense capacity.

Keywords Alfalfa cultivar · Aphid resistance · Molecular mechanism · Proteomic analysis

Introduction

Medicago sativa L. (alfalfa or lucerne) is among the highly nutritive and digestible autotetraploid perennial legume fodders, known as the 'Queen of the Fodders' (Conrad and Klopfenstein 1988; Greene et al. 2015), whereas major portion of livestock industry of China is dependent on this crop (Ohshima et al. 1997). Required quality source of magnesium, calcium, phosphorus, vitamin D and for β-caratene has made it a perfect and complete diet for deprived people in the shape of sprouts (Tao et al. 2017; Mao et al. 2018). Besides, its versatility as a feed and food, alfalfa has the potential ability to improve soil structure like other legumes to fix the aerial nitrogen (Summers 1998). Being the oldest fodder crop, alfalfa is grown worldwide on variable soil under different climatic conditions and hence encounters varieties of field insects and danger pests (Erice et al. 2010). Aphids are the most serious pests of alfalfa (Hooks

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and Fereres 2006; Gutierrez and Ponti 2013) causing severe economic losses worldwide besides, the fact that this crop has thousands of arthropod species that survive in alfalfa grown field with no serious impact on crop (Smith and Clement 2012). Integrated pest supervision of most of the field pests including other biotic stresses and weeds in alfalfa crop is still a major acceptable strategy to overcome these yield problems in developing countries. Host plant interaction has also been studied intensively in this crop although it has a very complex inheritance of traits and show high inbreeding depression (Summers 1998). Meanwhile, variety of pesticides has been used for controlling field insect pests, which though have reduced the economic losses but affected the natural flora and fauna and have developed resistance in pests against crops.

Transgenic alfalfa having insect resistance has been developed successfully against the spotted alfalfa aphid, with the potential to cope against various environmental and abiotic stresses (Chekol and Vough 2001). Development of stable resistant cultivars in alfalfa against aphids and other insect pests is the hot target area of research for majority of plant breeders and entomologist in the next decade to understand the molecular basis of resistance and loss of resistance with the passage of time as majority of cultivars maintain their resistance against aphids (Perring et al. 1999). Spotted alfalfa aphid along with other aphids are the main vectors in crop plants to transmit more than 50% of the known viral diseases (Hull 2002; Hooks and Fereres 2006; Shah et al. 2015). Aphids survive on plant sap containing nutrients extracted from phloem component by damaging plant nutrients, which ultimately affect crop yield as the aphids move fast from plant to plant and colonise effectively (Tu et al. 2018b). Hence, it would be worth to adopt non-chemical strategy such as development of resistant cultivars against aphid's control and understanding their molecular and genetic mechanism. Previously a tool named 'aphid quantity ratio' was used to correlate insect populations with host plants (alfalfa) for measuring cultivar resistance level of aphids (Tu et al. 2018a). Natural foreign enemies and other disasters affect pest population dynamics in a particular crop, but they are only a part of the inherent components of crop resistance to pests, and the relationship between natural enemies and pests, their mode and molecular basis of resistance is not entirely clear (Huang et al. 2008; Fang et al. 2010) such as, host plant resistance is known to be an inherited quality that might reduce the yield loss due to pests (Dedryver et al. 2010).

In the past, many traits of alfalfa has been improved through phenotypic selection including disease resistance, insect resistance, winter survival, however, the success in overall yield has not been properly improved due to lack of knowledge of molecular mechanisms (Riday and Brummer 2002; Lamb et al. 2006; Qiang et al. 2015) and inserting exotic genes of resistance by applying genetic engineering (Lu et al. 2012). Plant showing resistance against insect pest could be a cheap, sustainable, and environmentally safe control management (Kozjak and Meglič 2012). Various advanced molecular and genomic approaches have recently been introduced to quantify aphid resistance in field plants by targeting specific genes, proteins or sequences in aphids (Goggin 2007). Insect resistance in legumes has been characterized to be associated with structural defenses, available necessary metabolites and compounds of anti-nutritional nature (Edwards and Singh 2006; Sylwia et al. 2006).

Defense mechanism to foliar resistance of *Phytophthora infestans* is found in roots and has been verified at transcriptomic level (Orłowska et al. 2012; Nalam et al. 2013; Soler et al. 2013) such as foliar resistance to *Phytophthora infestans*. Besides, number of plant hormones including jasmonic acid, ethylene, salicylic acid and abscisic acid (ABA) have been identified key essential elements that play significant role in insect–plant interactions (Conrath 2011), however, its hard to rely on specific hormones and present a unique or global model for all plants as different species have different hormonal balance and types (Denancé et al. 2013). Recently, *Xanthomonas* is used as a perfect model to study toxification caused by certain bacterial strains. Plant defense responses show hundreds of genome sequences associated with plant-pathogenic *Xanthomonas* that has opened the door for high-scale precise and accurate 'omics' application in other crops (da Silva et al. 2002; Yu et al. 2002; Lee et al. 2005; Thieme et al. 2005). These transcriptomic studies have provided valuable information for understanding the complex genetic mechanism and association between hormonal networks in certain plants, however, less work has been dedicated to proteomic studies of plants upon inoculation with pathogenic species or insects (Mahmood et al. 2006; Dogimont et al. 2010; Kumar et al. 2015). Keeping in view these diversified beneficial attributes the proteomic analysis of Zhongmu-1 and WL343 alfalfa cultivars with different level of immunity (resistance, susceptibility) was carried out, each with and without aphids, to obtain further insight into the genetic and molecular mechanisms underlying alfalfa resistance.

Materials and methods

Sample preparation

Two alfalfa cultivars, i.e., Zhongmu-1 (resistant) and WL343 (susceptible) were selected on the basis of previous field performance and key physiological traits associated with aphid resistance (Tu et al. 2016). Zhongmu-1 has been previously classified bearing aphid-resistant, whereas WL343 (America) as aphid-susceptible variety. Seeds of selected

cultivars were planted in small pots filled with soil collected from experimental field, and were allowed to grow under ambient conditions. Plants were watered with an interval of 2–3 times as when required. Fine mesh clothing was applied to alfalfa plants to avoid any possible infestation by aphids. At 50% budding stage just after 35 days of blooming, a single representative plant from both cultivars were taken and 30 spotted alfalfa aphids [*Therioaphis trifolii* (Monell)] were allowed to feed on young leaves for a period of 72 h under ambient conditions. No aphid feeding was made to the control treatment, however, the conditions were kept uniform. Hence we had a total of four treatments comprising resistant cultivar Zhongmu-1, i.e., (R + A; R − A) and susceptible cultivar WL343 (S + A; S − A) with only one replicate per treatment. Thus the two selected cultivars were either subjected to aphid feeding (+) or not (−). After 3 days, a well-established cluster of aphid population (~ 22 aphids) was observed on WL343 (susceptible) as compared to Zhongmu-1 (resistant) which had only nine aphids. After 72 h of aphid exposure the young leaves (3–4) of alfalfa were excised from each treatment with no aphids, and were retained in isolated plastic bags. The selected samples were then frozen at − 80 °C for omics comparative analysis within 20 min (Tu et al. 2018c).

Protein extraction and digestion

Protein extraction and digestion were conducted by applying previously defined protocol with slight modifications (Han et al. 2013; Tu et al. 2018c). Lysis buffer with (LB, 8 M urea, 2 M thiourea, 4% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate), 20 mM Tris-base, 30 mM dithiothreitol (DTT) was used to homogenized the samples. The mixture was then homogenized for 30 min on ice followed by sonication for 20 s at every 5 min to fragment cell organelle. The mixture was then centrifuged at 12,000*g* for 10 min at 4 °C (Han et al. 2013). For protein precipitation and desalination, a ¾ volume of ice-cold acetone was mixed with the obtained supernatants and was kept on ice for 30 min. After 30 min treatment, the mixture was centrifuged twice at 15,000*g* for 10 min at 4 °C. Afterwards, the supernatant was discarded and the pellets were re-dissolved in 5 M urea. Mixture was then homogenized for 5 min on ice and sonicated for 2 min. Protein concentration was determined according to the Bradford method at an absorption of 595 nm (spectrophotometer DU800, Beckman Coulter, Los Angeles, CA) coupled with BSA (Bovine Serum Albumin) standards (Bradford 1976).

For protein digestion, 200 µg proteins of each sample were reduced with 10 mM DTT and then alkylated with 50 mM iodoacetamide followed by overnight protein sample digestion at 37 °C through trypsin [enzyme to substrate ratio (w/w) of 1:50] treatment. The peptides (compound of amino acids) were obtained by centrifugation of the filter units. After digestion, 1 µL of formic acid was added to the solution to cease further reaction and dried using a Speed-Vac system (RVC 2–18, Marin Christ) for liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis (Wiśniewski et al. 2009).

Liquid chromatography–mass spectrometry (LC–MS/MS) Analysis

Quantification and measurement of peptide concentration were made using Thermo Fischer Scientific Inc., Wilmington-USA Nanodrop-2000 spectrophotometer after dissolving the extracted peptide samples with 0.1% of formic acid in purified water. EasyLC 1000 (Thermo Fisher Scientific Inc., Bremen, Germany) and QExactive (Thermo Fisher Scientific, Inc. Wilmington, USA) mass spectrometer were used for LC–MS/MS analysis. Samples were loaded onto a reversed-phase trap column (2 cm long, 100 µm inner diameter fused silica column filling with 5.0 µm Aqua C18 beads, Thermo Fisher Scientific) at a flow rate of 5 µL/min (Han et al. 2017). Buffer A (0.1% formic acid dissolved in water) and buffer B (0.1% formic acid dissolved in acetonitrile) were used as major mobile phasing buffer solution. Peptides were separated on an analytical column (15 cm long with 75 µm inner diameter fused silica column filled with 3.0 µm Aqua C18 beads of Thermo Fisher Scientific Inc.) at a flow rate of 350 µL/min using the following steps: step-1 (3–8% buffer B in 5 min), step-2 (8–20% buffer B in 80 min), step-3 (20–30% buffer B in 20 min), step-4 (30–90% buffer B in 5 min), and final step-5 (90% buffer B for 10 min). Immediately following the above-described protocol of Han et al. (2017) the peptides were eluted from the analytical column and injected directly into the mass spectrometer through nano-ESI source. Ion signals were received in a data-dependent mode and run with the following settings: full scan resolution at 70,000, automatic gain control (AGC) target: 3 × 106, maximum inject time (MIT): 20 ms, scan range: *m*/*z* 300–1800; MS/MS scans resolution at 17,500, AGC target: 1 × 105, MIT: 60 ms, isolation window: 2 *m*/*z*, normalized collision energy: 27, loop count 10, and dynamic exclusion: charge exclusion: unassigned, 1, 8, > 8; peptide match: preferred; exclude isotopes: on; dynamic exclusion: 30 s. Raw data were retrieved using Xcalibur (version 2.2, Thermo Fisher Scientific Inc.). Each sample was run with technical injections in triplicate.

Protein identification and label-free abundance quantitation

Using in-house PEAKS software (version 8.0, Bioinformatics Solutions, Waterloo, Canada), RAW MS/MS data were searched in a composite database for protein identification.

The database contained 85,694 protein entries of *Medicago truncatula* L. and the other common repository of adventitious proteins (cRAP, from The Global Proteome Machine Organization, released on January 1, 2012). The search parameters for membrane proteome were conducted with carbamido methyl (C, + 57.02) as fixed modification and oxidation as a variable modification (M, + 15.99). For membrane phosphoprotein identifications, carbamido methyl (C, + 57.02) was selected as a fixed modification while oxidation (M, + 15.99) was selected as variable modifications (Han et al. 2017). Parent ion mass tolerance, 15.0 ppm; fragment ion mass tolerance, 0.05 Da; enzyme, trypsin; max missed cleavages, two; maximum allowed variable post translational modification (PTM) per peptide, three were used as other parameters. A decoy fusion method, an enhanced strategy for conservative estimate of false discovery rate (FDR), was used to control FDR at ≤ 1.0% at both protein and peptide levels (Silva et al. 2006; Lin et al. 2013). As described previously, matched spectra of at least two with the chosen peptide sequence were considered for peptide identification.

Label-free approach in PEAKS Q module was applied for quantification of relative abundance level of proteins. Each sample was treated thrice as technical repeats and loaded to the software, whereas the PEAKS software automatically selected one of the samples as reference for alignment purpose. Similarly, expectation–maximization algorithm was used for feature detection of each sample independently (Beausoleil et al. 2006; Han et al. 2017), whereas the same peptide was effectively aligned from other samples using a high-performance retention time alignment algorithm. Finally, the average MS signal responses of the three most concentrated unique peptides from each protein were plotted against their calculated protein concentrations. During the process of sample or group ratios calculation, a normalization factor generated by dividing the total ion current (TIC) of the samples by the TIC of the automatically selected reference sample was used.

Bioinformatics and statistical analysis

Fold change of ≥ 3 along with an FDR value of ≤ 1% generated with PEAKS Q were labeled as significantly different for peptide and protein features (Cox et al. 2014; Han et al. 2017). Differential expression ratios for proteins were obtained from Mascot software (http://www.matrixscience.com), which calculates protein ratios using only ratios from the spectra that are distinct for each protein and excluding the shared peptides of protein isoforms. To calculate differential expression ratios, all identified spectra from a protein were used to obtain an average protein ratio relative to the control label (i.e., fold change). Student's *t* test was used to analyze the differential expression of proteins between the resistant and susceptible cultivars of alfalfa, with (+ A) and without (− A) aphids rearing. The *p* value was calculated using the confidence intervals from the error factor generated in Mascot. We also used $p \leq 0.05$ and fold change > 1.2 or < 0.8 as the thresholds to judge the significance level of differentiated protein expression (Gan et al. 2007).

Results

Annotation of all non-redundant proteins in selected cultivars of *Medicago sativa* L.

Selected resistant (Zhongmu-1) and susceptible (WL343) cultivars of alfalfa were screened for similarity, annotated by aligning within seven publicly available databases (Nr, Nt, Swiss-Prot, KEGG, GO, COG, Pfam) with (+) and without (−) aphid infestation. Repeated and short-length sequences were initially eliminated. Analysis showed that out of the seven searched databases, five databases matched for similarity of unigenes of more than 50%. The annotation results showed that 2512 proteins (58.42%) in Nr, followed by 2395 proteins (55.7%) in Pfam, 2340 proteins (54.42%) in Swiss-Prot, 2250 proteins (52.33%) in GO and 2191 proteins (51.07%) in Nt databases had significant match for similarity. Searching against the KO database, least similarity match of 34.51% (1484 proteins) was also noted. However, we obtained a total of 4300 unigenes that were successfully annotated by one of the database from both of the selected resistant and susceptible cultivars of alfalfa (Table 1).

Table 1 Annotation summary of protein sequence metrics from alfalfa [Zhongmu-1 (R) and WL343 (S)] cultivars proteomic exposed (+) or unexposed (−) to aphids in 7 databases

Public database	Annotation number	Annotation percentage (%)
Annotated in Nr	2512	58.42
Annotated in Nt	2196	51.07
Annotated in KO	1484	34.51
Annotated in Swiss-Prot	2340	54.42
Annotated in Pfam	2395	55.70
Annotated in GO	2250	52.33
Annotated in COG/KOG	1722	40.05
Total unigenes	4300	100.00

Nr NCBI non-redundant protein sequences, *Nt* NCBI non-redundant nucleotide sequences, *KO* KEGG Ortholog database ,*Swiss-Prot* Manually annotated and reviewed protein sequence database, *Pfam* Protein family, *GO* gene ontology, *COG/KOG* clusters of orthologous groups of proteins

Analysis of differentially expressed proteins (DEPs) of two alfalfa cultivars

Differentially expressed proteins (DEPs) between aphid-resistant (Zhongmu-1) and susceptible (WL343) alfalfa accessions following aphid treatment (+) and un-treated (−) with fold change have been given in Table 2. Observed DEPs were significantly different within two groups of resistant and susceptible cultivars of alfalfa, i.e., group 1: (Zhongmu-1 + Aphid = R + A) vs. Zhongmu-1 – Aphid = R − A); group 2: (WL343 + Aphid = S + A) vs. (WL343 – Aphid = S − A). A total of 644 including 319 down and 325 up-regulated DEPs were obtained when resistant cultivar (Zhongmu-1) was either exposed or devoid from aphids (R + A/R − A). On the other hand, the susceptible cultivar (WL343) gave 954 DEPs of which 583 down-regulated while 371 up-regulated with and without aphid treatments (S + A/S − A). Fold change for DEPs in the susceptible cultivar was comparatively higher than the DEPs expressed in resistant cultivar (FDR ≤ 0.001 and |log2Ratio| ≥ 1). Majority of proteins expressed within one- to threefold change (Table 2). Similarly, the total number of DEPs found in (S + A/S − A) was around 19.7% greater than that expressed and observed in (R + A/R − A), while the down-regulated DEPs of susceptible variety was about 11.6% greater than the resistant cultivar. However, for up-regulated DEPs, the increase of S + A/S − A was around 0.7% as compared to that of R + A/R − A. This indicated that the cellular metabolic activity in the susceptible (S) cultivar of alfalfa (WL343) was absolutely active in comparison to the resistant (R) cultivar (Zhongmu-1).

Analysis of KEGG pathways for aphid-resistant and susceptible cultivars of alfalfa

KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis predicted 96 DEPs to 15 substantially enriched pathways (Carbon metabolism, pyruvate metabolism, fatty acid degradation, lysine degradation, 2-oxocarboxylic acid metabolism, biosynthesis of amino acids, fatty acid metabolism, histidine metabolism, tryptophan metabolism, synthesis and degradation of ketone bodies, selenocompound metabolism, linoleic acid metabolism, terpenoid backbone biosynthesis, C5-branched dibasic acid metabolism, and ascorbate and aldarate metabolism) of resistant Zhongmu-1 variety (R + A/R − A). Similarly, KEGG revealed 142 DEPs to 10 substantially enriched pathways (Carbon metabolism, pyruvate metabolism, photosynthesis, photosynthesis—antenna proteins, carbon fixation in photosynthetic organisms, propanoate metabolism, glyoxylate and dicarboxylate metabolism, valine, leucine and isoleucine degradation, metabolic pathways, and glycolysis/gluconeogenesis) of susceptible WL343 variety (S + A/S − A). The pathways involving the highest numbers of unique DEPs were carbon metabolism (16), biosynthesis of amino acids (15) and pyruvate metabolism (10) in resistant Zhongmu-1 variety (R + A/R − A) of alfalfa. In susceptible WL343 variety (S + A/S − A) of alfalfa the highest numbers of unique DEPs were predicted in metabolic pathways (73), followed by carbon metabolism (15) and photosynthesis (13). KEGG analysis also identified carbon metabolism (mtr01200) and pyruvate metabolism (mtr0620) as the key shared pathways associated with plant resistance (Table 3). Results also exposed the significant contribution of linoleic acid metabolism along with jasmonic acid and flavonoid biosynthesis (downstream) and terpenoid backbone biosynthesis towards insect-resistant material synthesis in resistant (Zhongmu-1) alfalfa cultivar.

Discussion

Plants respond differently to the attack of insect pests through various means including morphological features, activating different hormones or through inherent genetic mechanism. Compounds conferring resistance or providing defense to plants against insect pests are either found constitutively or activated upon insect attack (War et al. 2012). Study reveled that plant hard tissues, hairs, pubescence, toxins or chemicals improve inherent plant features (Paiva 2000). Study was conducted to analyze the resistant and susceptible accessions of alfalfa with and without aphid infestation at proteomic level to understand the genetic mechanism of insect resistance. Out of the seven public databases, more than 50% protein similarity was confirmed by five

Table 2 Comparative analysis of DEPs between two groups of aphid-resistant (Zhongmu-1) and susceptible (WL343) alfalfa cultivars with (+) and without (−) aphid treatments, whereas fold change equals the signal response ratio of aphid infested/un-infested

Treatment	Protein expression	Fold change					
		1–2	2–3	3–4	4–5	> 5	Total
Zhongmu-1 + Aphid (R + A) vs. Zhongmu-1 – Aphid (R − A)	Down-regulated	139	98	32	17	33	319
	Up-regulated	135	103	38	13	36	325
	Total	274	201	70	30	69	**644**
WL343 + Aphid (S + A) vs. WL343 – Aphid (S − A)	Down-regulated	218	158	69	45	93	583
	Up-regulated	142	113	40	27	49	371
	Total	360	271	109	72	142	**954**

Table 3 Significantly enriched KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways in response to aphid exposure for aphid-resistant cv. Zhongmu-1 and susceptible cv. WL343 of alfalfa

Group	Nos.	Term	ID	DEPs	*p* value
Zhongmu-1 + Aphid (R + A) vs. Zhongmu-1 – Aphid (R – A)	1	Carbon metabolism	mtr01200	16	0.016
	2	Pyruvate metabolism	mtr00620	10	< 0.0001
	3	Fatty acid degradation	mtr00071	7	0.001
	4	Lysine degradation	mtr00310	6	0.004
	5	2-Oxocarboxylic acid metabolism	mtr01210	6	0.01
	6	Biosynthesis of amino acids	mtr01230	15	0.01
	7	Fatty acid metabolism	mtr01212	7	0.014
	8	Histidine metabolism	mtr00340	4	0.018
	9	Tryptophan metabolism	mtr00380	5	0.024
	10	Synthesis and degradation of ketone bodies	mtr00072	2	0.026
	11	Selenocompound metabolism	mtr00450	3	0.028
	12	Linoleic acid metabolism	mtr00591	4	0.028
	13	Terpenoid backbone biosynthesis	mtr00900	5	0.035
	14	C5-Branched dibasic acid metabolism	mtr00660	2	0.032
	15	Ascorbate and aldarate metabolism	mtr00053	4	0.035
WL343 + Aphid (S + A) vs. WL343 – Aphid (S + A)	1	Carbon metabolism	mtr01200	15	0.004
	2	Pyruvate metabolism	mtr00620	7	0.009
	3	Photosynthesis	mtr00195	13	< 0.0001
	4	Photosynthesis—antenna proteins	mtr00196	5	< 0.0001
	5	Carbon fixation in photosynthetic organisms	mtr00710	8	0.002
	6	Propanoate metabolism	mtr00640	4	0.01
	7	Glyoxylate and dicarboxylate metabolism	mtr00630	6	0.021
	8	Valine, leucine and isoleucine degradation	mtr00280	4	0.034
	9	Metabolic pathways	mtr01100	73	0.038
	10	Glycolysis/gluconeogenesis	mtr00010	7	0.047

databases (Nr, Pfam, GO, Swiss-Prot, Nt) in R + A/R − A and S + A/S − A alfalfa varieties (Tu et al. 2016, 2018c).

A total of 644 including 319 down and 325 up-regulated DEPs were expressed in resistant genotype (Zhongmu-1) as compared to the susceptible cultivar (WL343) which gave 954 DEPs of which 583 down-regulated while 371 up-regulated. This showed that the DEPs expressed in susceptible cultivar is comparatively more than the resistant genotype. Proteomic analysis of waterlogging stressed rape and Arabidopsis genotypes had the same expression for DEPs (Hsu et al. 2011; Lee et al. 2014). Significantly different pattern in DEPs up-regulation in susceptible alfalfa infested with aphids indicated that induced defenses playing vital part to pledge aphid attack (Maleck and Dietrich 1999; Walling 2000). Besides the shared pathways (carbon metabolism and pyruvate metabolism) found in both cultivars, the other pathways with maximum number of DEPs expressed by KEGG analysis was biosysnthesis of amino acids (mtr01230) which was found in resistant cultivar while photosynthesis (mtr00195) and metabolic pathways (mtr01100) was enriched in susceptible cultivar (Tu et al. 2018b) which promotes glycolysis, involved in sugar transportation while reducing the gluconeogenesis and glycogen storage (Waligora et al. 2014). The concentration of the important amino acids including alanine, tyrosine, glutamic acid and leucine was found to be more than 40% of the variation in natural speed of increase in brassica (Cole 1997). Similarly, amino acid metabolism with down-regulation of genes responsible for serine degradation and up-regulation of aspartic acid degradation in *Taxodium* inclined in considerable amount (Qi et al. 2014). This showed that the protein conferring resistance is somehow different in both cultivars. Profiling translatomes of distinct cell population of Arabidopsis showed that the term 'response to stress' was enriched in the entire root while depleted in the shoot (Mustroph et al. 2009).

In this study we observed an up-regulation of DEPs associated with metabolic pathways in alfalfa susceptible cultivar to aphid attack, which is in confirmation to the findings of Schwachtje and Baldwin (2008). The number of up and down-regulated proteins comparatively expressed in higher number in the sensitive alfalfa variety as compared to the resistant cultivar. Similarly, the photosynthesis activities in susceptible cultivar affected the process of respiration and decreased water potential showing its involvement in insect defense mechanism (Nabity et al. 2008; Bilgin et al. 2010). Former research findings of plant response to aphids urged that ethylene, jasmonic acid, and salicylic acid tuned

signaling pathways were at least partly triggered off by aphid feeding whereas jasmonic acid synthesis was up-regulated in various plant–aphid interactions (Bell et al. 1995; León and Sánchez-Serrano 1999).

Conclusion

We concluded that both aphid-resistant and vulnerable alfalfa genotypes could govern protein revelation in carbon and pyruvate metabolism. But only the resistant alfalfa cultivar can dominate protein expression in linoleic acid metabolism and terpenoid backbone biosynthesis to induce defensive proteins expression (e.g., jasmonic acid and flavonoid biosynthesis, and terpenoid backbone biosynthesis), to enhance plant defense capacity. Meanwhile the maximum DEPs were expressed in metabolic pathways were only found in sensitive variety. In future, however, the specific rule of these DEPs and their possible association with resistance in specific pathways can be identified.

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Author contributions JC, XT and ZZ conceived and designed the study; JC, HU performed the experiment, collected the data and analyzed the findings; HU, XT and ZZ drafted the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no competing financial interests.

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