

Proteomic signature of fenugreek treated by methyl jasmonate and cholesterol

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Abstract

Main conclusion Changes in proteome level as a result of methyl jasmonate and cholesterol treatment were investigated. The identified proteins were often involved in response to stress caused by various treatments. Furthermore, 18 proteins were expressed in treatment-specific manner.

In this study, the fenugreek plants were treated with methyl jasmonate (as an elicitor) and cholesterol (as a precursor of steroids and steroidal saponins) to check reaction at the level of the proteome to stress and to investigate steroidal saponin (diosgenin) biosynthesis. Proteins were separated by two-dimensional electrophoresis (2-DE) and identified by MALDI-ToF/ToF followed by database searches using Mascot search engine. Totally, 63 and 41 protein spots were differentially expressed after methyl jasmonate and cholesterol treatment, respectively. These proteins were classified into seven groups: photosynthesis, energy, metabolism, protein metabolism, secondary metabolism, stress and defense, and other. We found that 9 proteins were responsive to all treatments, and 18 proteins expressed in treatment-specific manner. Higher level of photosynthetic proteins sensitive to both biotic and abiotic stimuli was detected. In addition, proteins related to the

stress (especially oxidative) and defense, protein, and secondary metabolism were overexpressed. The results indicate that methyl jasmonate and cholesterol elicited a defense reaction at the proteome level as a response to stress. The usefulness of 2-DE method for identification of proteins related with species-specific metabolic pathways is restricted. Integration of transcriptome data with proteomic analysis improved annotation process.

Keywords *Trigonella foenum-graecum* L. · 2-D electrophoresis · Plant proteomics · Stress and defense

Introduction

Fenugreek (*Trigonella foenum-graecum* L.) is an annual plant of family Fabaceae widely distributed throughout the world. This wild or cultivated crop is commonly used as a traditional food or medicine due to anti-diabetic, hypcholesterolaemic, and anti-microbial effects (Mehrafarin et al. 2011). Fenugreek is a source of saponins (i.e., diosgenin, gitogenin, and tigogenin), alkaloids (i.e., trigonelline, gentianine, and choline), flavonoids (i.e., apigenin, luteolin, quercetin, and vitexin), steroids (i.e., cholesterol and sitosterol) and volatile oils (Snehlata and Payal 2012). One of the most important compounds of fenugreek is diosgenin that controls metabolism of cholesterol, and shows anticancer and anti-inflammatory activities. Diosgenin also serves as intermediate for synthesis of sex hormones and oral contraceptives (Raju and Rao 2012; Patel et al. 2012).

The changes in a content of secondary metabolites (i.e., saponins and flavonoids) may result from a range of abiotic and biotic stimuli including herbivorous attack or pathogenic infection (Szakiel et al. 2011; Falcone Ferreyra et al.

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2012). Receptors, which are situated in plasma membrane, recognized an elicitor and activated signal transduction pathway. Next, the secondary messengers, e.g., jasmonates, ethylene, and salicylic acid, activated the expression of defense genes including the genes which are involved in synthesis of secondary metabolites (Lambert et al. 2011). Methyl jasmonate was used to induce production of diosgenin in the seedlings of fenugreek (De and De 2011).

Saponins also play role in plant growth regulation. It has been reported that production of α -galactosidase activity, which is needed to germinate, was inhibited by diffusible saponin substances (Faizal and Geelen 2013).

Proteomic studies have appeared as leading tools providing answers in field of plant physiology and development. Procedures based on two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) have become one of the most powerful techniques of plant proteomics (Nordhoff et al. 2001; Weiss and Görg 2007; Bocian et al. 2011; Correia et al. 2012). Combination relationships of protein populations, global protein identification along with internal patterns analysis, have ensured new insights into the connection of biochemical processes of different proteomes (Correia et al. 2012). The current status of proteomic research in legumes steps over the border of generating proteome reference maps, and now allows for understanding of key proteins answering for adaptation under the countless stress environments of abiotic and biotic nature (Rathi et al. 2016).

Plant has to contend with multiple abiotic and biotic factors. During the evolution, they have developed specific mechanisms enabling them to adapt and survive stressful events. After exposure to stress factor, plants activate specific ion channels and kinase cascades, phytohormones (salicylic acid, jasmonic acid, abscisic acid, and ethylene), and accumulate reactive oxygen species (ROS). To minimize the biological damage which is caused by stress, plants reprogrammed the genetic machinery, resulting in increase in plant tolerance and suitable defense reactions (Rejeb et al. 2014). Phytohormones play central role in abiotic and biotic stress signaling (Fraire-Velázquez et al. 2011). Jasmonic acid is involved in many different plant processes including storage, growth, reproduction, and senescence. It is activated in response to herbivory and plays important role in plant defense (Karban and Kuć 1999).

Within the current study, we provide the first proteomic data on reaction of fenugreek plants induced with methyl jasmonate and cholesterol. The aim of the study was to identify proteins differentially expressed after treatment of plants with (a) methyl jasmonate as the elicitor of diosgenin synthesis and plant defense response and (b) cholesterol as the precursor of steroidal saponins biosynthesis. To achieve this purpose, we induced in vitro cultures of

fenugreek by elicitors listed above, and we identified and quantified a diosgenin as steroidal saponin representative. Plant producing extreme amounts of diosgenin was used to comparison protein using 2-DE and differentially expressed proteins were identified by the use of mass spectrometry MALDI-ToF/ToF. This approach allowed knowing reaction in fenugreek, a non-model plant, in response to stress and secondary biosynthesis.

Materials and methods

Plant material

Seeds of *Trigonella foenum-graecum* (Accession No. 19271) were obtained from Botanic Garden in Bonn (Germany). Seeds were sterilized by soaking in 70% ethanol for 2 min and then in a 30% v/v Domestos[®] solution for 30 min. They were rinsed several times in sterile water before being germinated on a Murashige and Skoog (1962) agar medium containing 3% sucrose. After germination, the plantlets were transferred onto fresh Murashige–Skoog agar medium and maintained at 24 °C with a 16-h light photoperiod. After 6 months, plants were treated with methyl jasmonate (MeJ) (100 $\mu\text{L L}^{-1}$) and cholesterol (CHL) (100 $\mu\text{g L}^{-1}$) to induce accumulation of diosgenin. Plants were sprayed with 10%-ethanol solutions of induction factors and control plants were treated only by 10% ethanol. Then, plants were harvested on 1st, 3rd, and 6th days after spray, frozen in liquid nitrogen, and stored at -80°C .

Extraction and identification of diosgenin

Diosgenin was extracted with a liquid phase according to Savikin-Fodulovic et al. (1998) with modifications. Plant material was hydrolyzed with 1 M sulfuric acid in 70% isopropanol for 6 h under reflux. In the next step, water was added and obtained solution was extracted three times with hexane and finally washed with 1 M NaOH and distilled water. Organic phase was evaporated to dryness in vacuum concentrator.

Diosgenin standard was purchased from Sigma-Aldrich. Unless specified otherwise, all chemicals were of analytical reagent grade and solvents were of HPLC grade. Water was purified using Milli-Q system (Merck Millipore).

Diosgenin was identified by ultra-performance liquid chromatography coupled with mass spectrometry with triple quadrupole. UPLC analyses were performed using a Shimadzu apparatus on a Kinetex C-18 RP column (50 mm \times 2.1 mm I.D., 1.7 μm). The isocratic solvent system was 85% methanol, the flow rate was set at 0.6 mL min^{-1} , and the column temperature was

maintained at 35 °C. The injection volume was 1 µL. All samples were dissolved in 1 mL of methanol. AB Sciex QTRAP 4500 was equipped with an electrospray ion source operating in positive ion. For targeted metabolites, we used very sensitive multiple reaction monitoring (MRM) method. For diosgenin transitions, m/z 415.3 → 271.20 and m/z 415.3 → 253.19 were detected. For calculation of the concentration of diosgenin, calibration curve was used. The correlation coefficient of calibration curve was $r^2 = 0.997$. Measurements were taken in three replicates. ANOVA analysis was performed to test significance of differences between means in STATISTICA (Statistica 2010).

Protein extraction, quantification, and 2-DE separation

Samples with the highest and the lowest (control) contents of diosgenin were selected for protein extraction according to Hurkman and Tanaka (1986). The protein concentration in extracts diluted in thiourea sample solution [7 M urea, 2 M thiourea, 2% Nonidet P-40 substitute, 2% IPG buffer pH range 3–10, and 40 mM dithiothreitol (DTT)] was determined by 2D Quant kit (GE Healthcare) with using bovine serum albumin as the standard.

The extracted protein (550 µg) samples were individually loaded onto 24 cm Immobiline DryStrep Gel (pH 3–10). Isoelectric focusing was performed at 50 µA per strip at 20 °C using the Ettan IPGphor II (GE Healthcare) programmed as follows: 12 h of passive rehydration and 10 h of focusing at 1 h with 500 V, 2 h with 1000 V, 3 h with 8000 V, and 4 h with 10,000 V. The strips were then equilibrated twice successively for 15 min in an equilibration solution [75 mM Tris-HCl, 6 M urea, 29.3% (v/v) glycerol, 2% (w/v) SDS, and 0.002% bromofenol blue], supplemented with 1% (w/v) DTT (the first equilibration) and 2.5% (w/v) iodoacetamide (the second equilibration). The second dimension was performed as SDS-PAGE using 12% (v/v) resolving gel (EttanDALTSix, GE Healthcare). Gels were stained with Coomassie Brilliant Blue G250 and images were acquired on ImageScannerIII. All gels were prepared in three independent technical replications. Bioinformatic analyses of the gels were performed in ImageMaster 2D Platinum program. Spots were detected, normalized, and matched through the entire matchset. Spots presenting more than 1.5-fold changes of %Vol in effector-treated samples against control samples and p values lower than 0.05 were considered to be differentially expressed. Exclusively spots detected in treated or control gels as well as spots detected in both treated and control gels but with accumulation more than 1.5-fold change of %Vol, were selected to identification.

Protein identification using MALDI-ToF

The spots were successively subjected to a series of washes in solutions: 50 mM ammonium carbonate, acetonitrile, ammonium carbonate:acetonitrile (1:1), and acetonitrile. Spots were dried and subjected to reduction (45 min at 56 °C, 0.15% of DTT in ammonium carbonate) and alkylated (30 min in the darkness, 1% solution of iodoacetamide in ammonium carbonate). The gels were washed again with a mixture of ammonium carbonate and acetonitrile (1:1), acetonitrile, and dried. The dried gel was digested overnight at 37 °C after adding 0.2 µg of trypsin. After the digestion reaction was quenched by the addition of acetonitrile, the samples were sonicated and the liquid fraction was transferred to new tubes.

For analysis with MALDI-ToF (matrix-assisted laser-desorbed ionization-time-of-flight), 0.5 µL protein sample was directly imposed on MALDI target, mixed with 1 µL α -cyano-4-hydroxycinnamic acid matrix, and dried out. The samples were analyzed using MALDI-ToF/ToF (Bruker Daltonics) and mass spectra were calibrated using the peptide standard calibration as external patterns (Peptide Calibration Standards II, Bruker Daltonics). The sample ionization was carried out with laser beam at 337 nm. The analyzer operated in the reflective mode and positive ions were recorded in the mass range between 700 and 3500 Da. The received monoisotopic peptide mass maps were compared to protein databases NCBI nr, SwissProt, and MSDB using the Mascot search engine (Matrix Science Inc.) with criteria: a peptide mass tolerance of MS 0.1–0.5 Da, and allowance for up to one trypsin miscleavage, constant amino-acid modifications—cysteine carbamidomethylation and variable modification—methionine oxidation, and confidence level 95%. Individual peptides selected from mass spectrum were sequenced using LIFT ion source by laser-induced dissociation (LID). Tandem mass spectrum was analyzed as described above. The search parameters for MS/MS data were identical as those used for MALDI-ToF analyses. The only exception was mass tolerance which was set to 0.4 Da for MS mode and 0.2 Da for MS/MS mode.

Protein identification using tBLAST

To get information on fenugreek-specific proteins not annotated using Mascot, we exploited database of species-specific transcripts. Combinations of peptides corresponding to fragment masses were queried against four databases of translated transcripts (developed upon treatment with cholesterol, methyl jasmonate, squalene, and control). BLOSUM62 and PAM30 matrixes were used in local BLAST with tblastx module in CLC Main Workbench v.6.9.2. DNA sequences corresponding to the best hits were

retrieved from databases and further used to extract contigs, and consensus sequences. For each spot, best hits were treated as random and independent thus corresponding E-values were multiplied for sequences targeting single contig. Consensus sequences with the lowest cumulative E-value were functionally annotated at Blast2GO v.3.2.7.

Results

Change in diosgenin content

Fenugreek plants were treated with methyl jasmonate (MeJ) and cholesterol (CHL) to activate genes and proteins involved in biosynthesis of diosgenin. Changes of the levels of this steroidal saponin were measured in fresh weight of plants after the treatments vs control. Concentrations of diosgenin were not significantly different in subsequent days of culture, but depended on treatment ($p = 0.036$) and interaction of treatment with time ($p = 0.015$). The highest differences in content of diosgenin were observed on 6th day. Average contents of diosgenin in MeJ and CHL-treated plants (310.3 and 120.3 $\mu\text{g g}^{-1}$ fresh weight, respectively) were enhanced about five, and twofold when compared to control (Fig. 1).

Identification of protein using 2-DE and MALDI-ToF

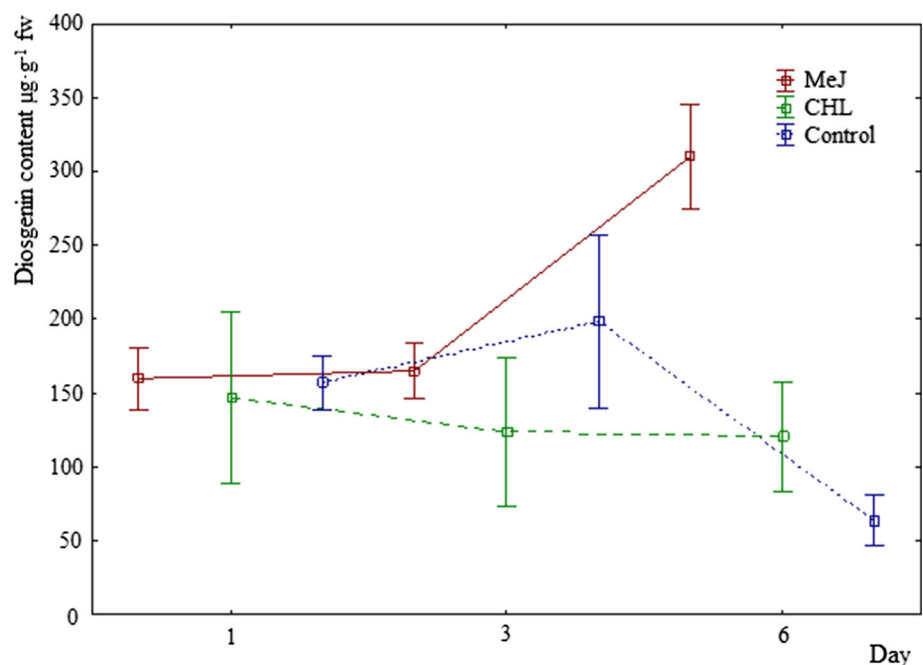
The proteome of *T. foenum-graecum* was profiled using 2-DE, and samples from 6th day after treatment methyl

jasmonate and cholesterol were used. Gel after treatment: MeJ contains 222 spots and CHL 236 spots. Control gel includes 226 spots. The majority of spots are within the range $\text{pI} = 4\text{--}8$ and possessed molecular masses between 20 and 120 kDa.

Proteins from effector-treated and control were analyzed with using ImageMaster 2D Platinum software. The protein spots that revealed repetitive changes under MeJ (Fig. 2) and CHL treatment (Fig. 3) were selected and used for further analysis. A number of 41 spots for cholesterol and 63 spots for methyl jasmonate were differentially expressed including 19 and 34 proteins that presented higher %Vol (blue frames in Figs. 2, 3) after treatment with cholesterol and MeJ, respectively. Moreover, five spots were detected only in treated gels (green frames in Figs. 2, 3) and 14 spots solely in control gel (red frames in Figs. 2, 3). The differentially expressed protein spots were cut out, in-gel digested by trypsin, analyzed using tandem MS, and then identified after searching the MS/MS spectra using Mascot (Table 1). In case of MeJ and cholesterol treatments, 46 and 26 spots were successfully identified, respectively. A 27–37% of proteins, both in control and in treated plants, have no hits in Mascot database.

Among protein spots obtained after MeJ and CHL treatments, 19 and 9, respectively, were localized in chloroplast. Spots identified as the same protein were considered to be isoform, like ribulose biphosphate carboxylase small chain C (spots 265 and 266) in control gel to MeJ treatment, or translation from alternatively spliced mRNAs and post-translational modifications (chlorophyll *a-b* binding protein AB80 (spots 184 and 198) in MeJ gel,

Fig. 1 Content of diosgenin in methyl jasmonate (MeJ) and cholesterol (CHL) treated plants vs control



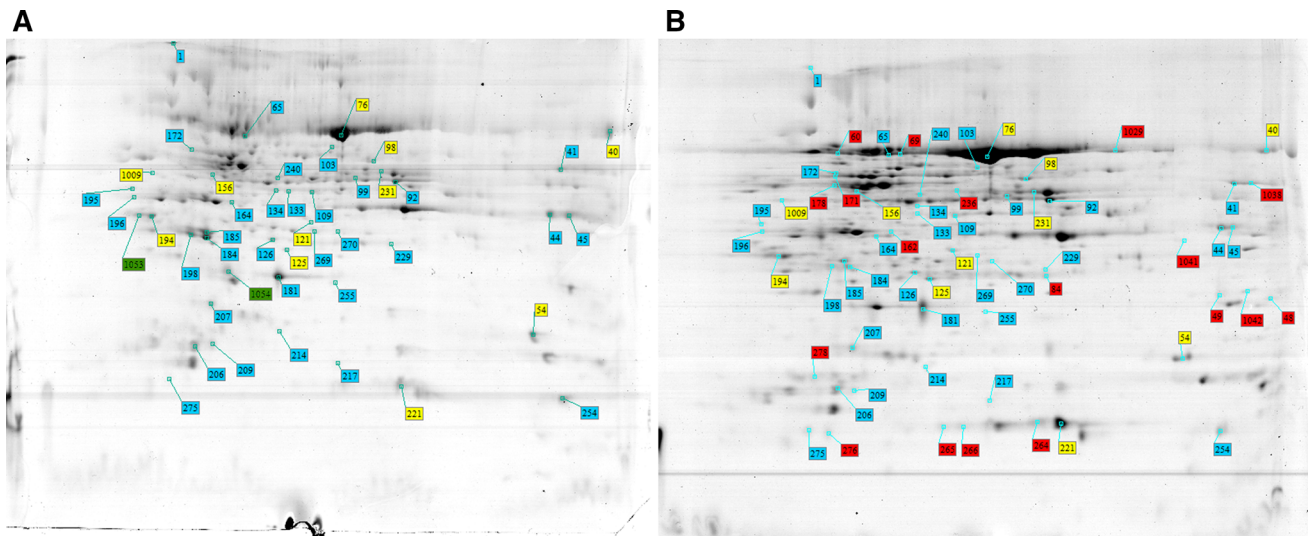


Fig. 2 2D-gel images of protein spots obtained after treatment of methyl jasmonate (a) and comparison with control gel (b). The numbers in the frames represent the numbers of spots. Colors of the

frames denote: red occur only in control gel; yellow higher accumulation in control gel; blue higher accumulation in tested gel; green occur only in tested gel

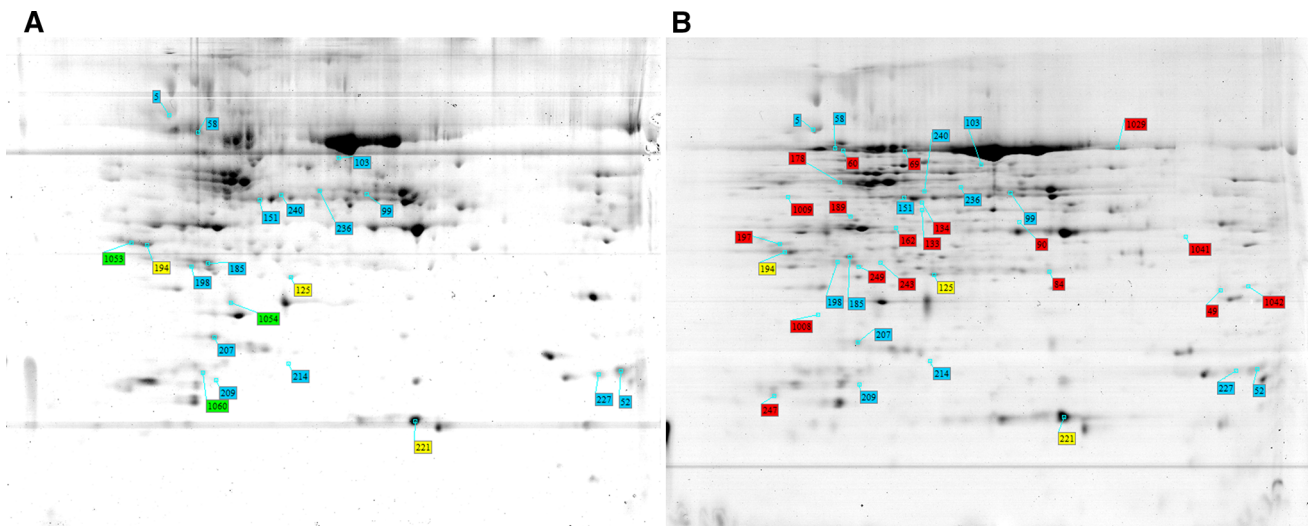


Fig. 3 2D-gel images of protein spots obtained after treatment of cholesterol (a) and comparison with control gel (b). The numbers in the frames represent the numbers of spots. Colors of the frames

denote: red occur only in control gel; yellow higher accumulation in control gel; blue higher accumulation in tested gel; green occur only in tested gel

fructose-bisphosphate aldolase 1 (spots 236 and 240) in CHL gel).

To subsequent investigate, the identified differentially expressed proteins were classified according to main function based on UniProt database (Fig. 4). The MeJ-treated proteins were involved in photosynthesis (40%), metabolism (29%), stress and defense (20%), protein metabolism (12%), energy (8%), and secondary metabolism (4%). Besides, 8% were represented by membrane and transport, and transcription-related. In CHL treatment gel, we classified protein to photosynthesis (20%), protein metabolism (20%), stress and defense (20%), metabolism

(7%), secondary metabolism (7%), and transcription-related (7%) (other in the diagram). Analysis of control gels reveals that proteins belong in the largest group to photosynthesis, followed by stress and defense, energy, metabolism, and protein metabolism.

Interestingly, that after MeJ treatment level of proteins involved in stress and defense, metabolism, and protein metabolism clearly increased when compared to the control. Moreover, the relative amount of proteins associated with energy production and process of photosynthesis decreased. Similarly, in cholesterol-treated plants, the number of proteins involved in energy and smaller extent

Table 1 List of proteins identified in treated and control gels using Mascot

Spot no.	Accession number	Protein name	Plant species	Group	Mass theor/exp	MS score	Sequence coverage/parent ion mass	Sequence/number of hits	Fold MeJ	Fold CHL
40	RCA0_HORVU	Ribulose biphosphate carboxylase/oxygenase activase A, chloroplastic	<i>Hordeum vulgare</i>	P	51/50	88	1882.8779			
76	RBL_MEDSA	IVDFPGQSIDFFGALR	3.90↓ <i>Medicago sativa</i>	P	53/60	94	25%	12	2.25↓	
221	RBS2_PETHY	Ribulose biphosphate carboxylase large chain	<i>Petunia hybrid</i>	P	20/20	64	25%	5	2.92↓	1.58↓
264	RBS0_SOLTU	Ribulose biphosphate carboxylase small chain SSU11A	<i>Solanum tuberosum</i>	P	20/20	58	933.5347	IIGFDNVR	(c)	
265	RBS0_SOLTU	Ribulose biphosphate carboxylase small chain C	<i>S. tuberosum</i>	P	20/20	59	933.5341	IIGFDNVR	(c)	
266	RBS0_SOLTU	Ribulose biphosphate carboxylase small chain C	<i>S. tuberosum</i>	P	20/20	57	933.5607	IIGFDNVR	(c)	
52	gil657371802	Oxygen-evolving enhancer protein	<i>Medicago truncatula</i>	P	28/20	109	1577.8495	FYQLPLTPTEAAR		1.56↑
54	gil22165105	Putative oxygen-evolving complex protein	<i>Oryza sativa</i>	P	19/25	66	1272.6799	FYTLTTGANER		1.68↓
181	PSBP_PEA	Oxygen-evolving enhancer protein 2	<i>Pisum sativum</i>	P	28/35	60	945.5726	EFPQVLR		1.73↑
189	PSBO_PEA	Oxygen-evolving enhancer protein 1	<i>P. sativum</i>	P	35/40	76	32%	8	(c)	
49	PSAD_NICSY	Photosystem I reaction center, subunit II	<i>Nicotiana sylvestris</i>	P	22/30	60	22%	7	(c)	
184	gil115788	Chlorophyll <i>a-b</i> binding protein AB80	<i>P. sativum</i>	P	28/30	74	1378.7021	VASSGSPWYGPDPR		7.64↑
198	CB22_PEA	Chlorophyll <i>a-b</i> binding protein AB80	<i>P. sativum</i>	P	28/30	42	1378.7046	VASSGSPWYGPDPR		4.04↑
156	gil350538149	Sedoheptulose-1,7-bisphosphatase	<i>Solanum lycopersicum</i>	P	43/45	62	1188.6526	FEETLYGSSR		1.90↓
5	RUB1_BRANA	RuBisCO large subunit-binding protein, subunit alpha, chloroplast	<i>B. napus</i>	P	57/75	74	26%	14		2.76↑
254	gil470136703	Photosystem II repair protein PSB27-H1	<i>Fragaria vesca</i>	P	18/20	101	1382.7794	NDPNVANVAELR		2.38↑
92	G3PC_PEA	Glyceraldehyde-3-phosphate dehydrogenase	<i>P. sativum</i>	P/G	36/40	108	1702.7740	LVSWDNELGYSTR		1.37↑
99	gil166702	Glyceraldehyde 3-phosphate dehydrogenase A subunit	<i>A. thaliana</i>	P/G	37/45	61	1786.8816	VIAWYDNEWGYSQR		2.03↑
231	G3PA_PEA	Glyceraldehyde-3-phosphate dehydrogenase A	<i>P. sativum</i>	P/G	43/40	63	1312.6769	TFAEVNEAFR		2.96↓
151	gil357473561	Fructose-bisphosphate aldolase	<i>M. truncatula</i>	P/G	43/45	130	1587.9275	VENVKAQAQALLFR		1.62↑
152	ALFC_SPIOL	Fructose-bisphosphate aldolase	<i>Spinacia olerace</i>	P/G	42/45	66	1387.7715	LASIGLENTEANR		1.98↓
236	gil399024	Fructose-bisphosphate aldolase I	<i>P. sativum</i>	P/G	38/40	70	1415.8252	LASIGLENTEVNR		1.78↑
240	ALFC1_PEA	Fructose-bisphosphate aldolase I	<i>P. sativum</i>	P/G	38/40	54	1018.6229	AAQEALLFR		3.39↑
121	gil57283985	Triose-phosphate isomerase	<i>Phaseolus vulgaris</i>	P/G	27/35	63	1388.7789	VIACIGETLEQR		1.52↓

Table 1 continued

Spot no.	Accession number	Protein name	Plant species	Group	Mass theor/exp	MS score	Sequence coverage/parent ion mass	Sequence/number of hits	Fold MeJ	Fold CHL
69	ENO_RICCO	Enolase	<i>Ricinus communis</i>	E	48/60	69	1601.9432	VNQIGSVTESIEAVR	(c)	
60	VATB2_ARATH	V-type proton ATP-ase, subunit B2	<i>A. thaliana</i>	E	54/60	76	21%	10	(c)	
65	ATPB_MEDSA	ATP-synthase, subunit beta	<i>Medicago sativa</i>	E	52/60	114	43%	16	2.16↑	
217	gi219674017	ATP-synthase CFI, subunit epsilon	<i>Trifolium subterraneum</i>	E	14/20	92	1574.9568	NHAPIATALDIGILR	2.41↑	
125	gi15235889	20S proteasome subunit PBA1	<i>A. thaliana</i>	PM	25/30	71	1540.8032			
109	gi435816	Cysteine-proteinase inhibitor LVLPEVYDQDGNPLR	2.32↓ <i>S. tuberosum</i>	PM	20/40	76	1727.9999			
207	gi357498529	Kunitz-type trypsin inhibitor-like 2 protein	<i>M. truncatula</i>	PM	22/25	69	1187.6459	LVPTENDPFR	1.43↑	1.33↑
58	gi657389745	ATP-dependent zinc metalloprotease FTSH protein	<i>M. truncatula</i>	PM	74/70	98	1621.8737	LSDEAYEIALTQIR		2.56↑
1053	OST3A_ARATH	Doli chyl-diphosphooligosaccharide-protein glycosyltransferase subunit 3A	<i>A. thaliana</i>	PM	39/35	43	1475.7526	SDSDLKNELVSLR	(s)	(s)
1029	CATA_PEA	Catalase, chloroplast	<i>P. sativum</i>	S&D	57/60	41	1255.5735	DEEVNYFPSR	(c)	
1041	gi357513969	Germin-like protein	<i>M. truncatula</i>	S&D	21/35	72	1132.6564	TTFLDPVLVK	(c)	
90	gi357448997	Glucan endo-1,3-beta-glucosidase, cytosol	<i>M. truncatula</i>	S&D	40/40	99	1473.7573	LYDPNQAALEALR	(c)	
162	CDSP_ARATH	Thioredoxin-like protein CDSP32, chloroplast	<i>A. thaliana</i>	S&D	33/35	42	999.657	GELIGEILR	(c)	
164	CDSP_ARATH	Thioredoxin-like protein CDSP32, chloroplast	<i>A. thaliana</i>	S&D	33/35	46	999.6689	GELIGEILR	1.40↑	
103	MDARS_CUCSA	Monodehydroascorbate reductase	<i>Cucumis sativus</i>	S&D	47/50	46	1150.6536	AYLFPESPAR	2.44↑	2.02↑
185	gi357441103	Harpin-binding protein	<i>M. truncatula</i>	S&D	29/30	125	1645.8302			
255	gi357481689	GSGDFEVTYLDADTR	2.62↑ <i>M. truncatula</i>	1.96↑ S&D	50/25	91	1847.8904			
45	NOA1_ARATH	Pathogenesis-related thaumatin-like protein ISCTADINGQCPNELR	5.46↑ <i>A. thaliana</i>	S&D	62/40	55	20%	10		2.63↑
44	AB7A_ARATH	NO-associated protein 1, chloroplastic/mitochondrial ABC transporter A family member 7	<i>A. thaliana</i>	M&T	106/40	39	1419.7954			
1054	gi4586568	Chalkone isomerase	<i>Cicer rictetium</i>	SM	15/25	64	1467.7125	NVNFFPGASVFYR	(s)	(s)
171	C7263_MEDTR	11-oxo-beta-amylin 30-oxidase	<i>M. truncatula</i>	SM	59/50	65	18%	10	(c)	
195	ADH2_SOLLC	Alcohol dehydrogenase 2	<i>S. lycopersicum</i>	M	41/40	62	17%	8	1.62↑	
172	GLNA2_MEDSA	Glutamine synthetase leaf isozyme	<i>M. truncatula</i>	M	47/45	45	1373.5544	VHIEAYGEGNER	1.67↑	

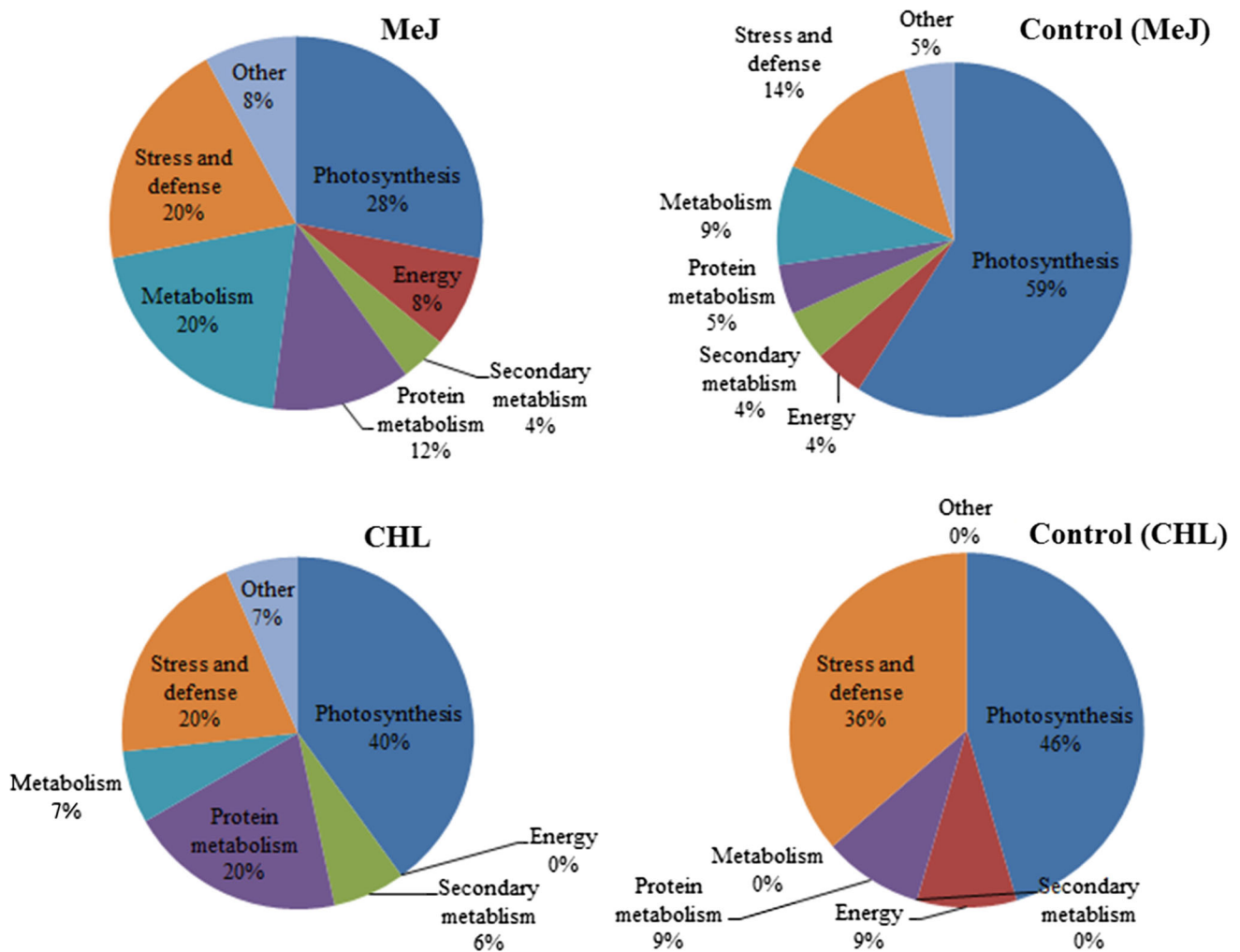


Fig. 4 Classification of identified proteins in treated plants and control

control plants, while higher amounts of ribulose biphosphate carboxylase small and large chains were found in control. In turn, chlorophyll *a-b* binding protein AB80 was overexpressed in treated plants. Photosynthesis cascades are strongly interdependent with the accumulation of RuBisCO and other photosynthesis-related proteins (Nouri et al. 2015). In our study, also RuBisCO large subunit-binding protein subunit alpha (spot 5) which belongs to chaperon family, and photosystem II repair protein PSB27-H1 (spot 254) had elevated expression in treated plants. It indicated their possible role in response to stress and protection of important photosynthetic proteins.

We identified proteins involved in ATP production processes. In control, V-type proton ATP-ase subunit B2 (spot 60) was identified, while two ATP-synthases: subunit beta (spot 65) and CF1 subunit epsilon (spot 217) were overexpressed in greater amount in MeJ-treated plants. Response to stress demands comparatively high energy

costs as indicated by thorough changes in energy metabolism, including β subunit of CF1 complex of mitochondrial ATP-synthase, indicating an increased requirement for energy (Kosová et al. 2014).

Glycolysis is important stage in the manufacture of energy and carbon skeletons of primary and secondary metabolites. Some proteins active in glycolytic pathway were differentially regulated under our treatment conditions. Glyceraldehyde-3-phosphate dehydrogenase and fructose-biphosphate aldolase of the Calvin cycle had strong responses to the effectors. Glyceraldehyde-3-phosphate dehydrogenase has been proposed to play role in ROS-mediated signaling in plants (Chattopadhyay et al. 2011). Sedoheptulose-1,7-bisphosphatase, which was overexpressed in MeJ-treated and control plants, is an essential regulator of the Calvin cycle, and has directly influences on the rate of photosynthesis. Expression of this gene is affected by various factors as development, light,

Table 2 List of differentially accumulated proteins classified as ‘unknown’ and identified in result of comparisons with transcriptome

Spot no.	Accession number	Protein name	Plant species	GO terms	Parent ion mass	Number of hits/contigs ^a	E-score	Transcript database	Fold
126	922375682	Transmembrane 9 superfamily protein	<i>M. truncatula</i>	Integral component of membrane	1238.6731; 1322.7298; 1618.8567; 1832.9422	54/18	6.87E−13	cms	1.53↑
	922400062	Serine/threonine kinase	<i>M. truncatula</i>	Chloroplast, protein kinase activity, ATP binding, protein phosphorylation, response to sucrose, flavonoid biosynthetic process, response to UV-B			6.34E−10	k	
134	357520217	Membrane attack complex/perforin (MACPF) domain protein	<i>M. truncatula</i>	–	1557.8051	28/17	2.68E−06	cms	4.24↑
84	922380315	ARM repeat RING/U-box protein	<i>M. truncatula</i>	Ubiquitin-protein transferase activity, ligase activity, protein ubiquitination	1475.7372; 1489.7282; 900.5120	26/16	1.54E−06	cms	(c)
194	125662829	Beta-tubulin	<i>Spinacia oleracea</i>	Cytoplasm, microtubule, GTPase activity, metabolic process	1069.6420; 1560.8533	1/1	2.31E−01	m	2.15↓
197	922334060	AT-hook motif nuclear-localized 7-like	<i>M. truncatula</i>	DNA binding	1193.7069; 132.6790; 1493.8356	43/23	1.62E−12	cmsk	(c)
243	922334060	Tiosephosphate isomerase, chloroplastic	<i>Cicer arietinum</i>	Gluconeogenesis, response to salt stress, indoleacetic acid biosynthetic process, calcium ion transport	1096.6158; 1647.9230	28/16	4.52E−12	cmsk	(c)
249	357436839	Transcriptional corepressor leunig-like protein	<i>M. truncatula</i>	Transferring glycosyl groups, metabolic process	1188.7079; 1309.7895; 1521.8715; 879.4592; 967.5285	77/47	4.08E−09	cmsk	(c)

^a Number of transcripts matched in four databases of transcripts: methyl jasmonate (m), cholesterol (c), squalene (s), and control (k), Fold, change in accumulation of proteins: ↑, increased protein accumulation in sample, ↓, decreased protein accumulation in sample, (s), protein detected only in sample, (c), protein detected only in control

and levels of hexose sugars (Raines et al. 1999; Raines and Paul 2006).

Photosynthesis, glycolysis, and synthesis of energy are closely associated processes that affect functioning of cell. Changes in light reactions are linked with energy production and sugar level, and are recorded in different expression of respective proteins. Obtained results indicate that differentially expressed proteins involved in photosynthesis and carbon metabolism were significant part of a defense response induced by MeJ.

Protein metabolism

In group of protein metabolism, we identified proteasome subunit beta type-6 (spot 125) in control plants. Higher accumulation in MeJ and cholesterol-treated plants was found for cysteine and/or aspartic-protease inhibitor (spot 109), Kunitz-type trypsin inhibitor-like 2 protein (spot 207), ATP-dependent zinc metalloprotease FTSH protein (spot 58), and dolichyl-diphosphooligosaccharide-protein glycosyltransferase 3A (spot 1053). Proteasomes are

multicatalytic complexes (14 α - and 14 β -subunits) engaged in processes like protein degradation in processes induced by environmental stresses, senescence, cell death, or wounding (Etienne et al. 2000; Vítámvás et al. 2015; Nam et al. 2012). Increased level of this protein in control may reflect protein degradation processes associated with stress induced by 10% ethanol. Increased level of proteinase inhibitors (PIs) was detected in MeJ-treated samples. Proteinase inhibitors play a meaningful defensive role against pathogens and predators, and are induced in plants in response to injury or attack by pathogens or insects, and induced by jasmonic acid (Habib and Fazili 2007; Farmer et al. 1992; Kang et al. 2002). Kunitz-type inhibitors are produced under stress in leguminous plants, and inhibit serine proteases, trypsin, chymotrypsin, subtilisin, aspartic-protease, and cathepsin D (Habib and Fazili 2007). ATP-dependent zinc metalloprotease FTSH protein accumulated in potato leaf tissues in a response to wounding (Ryan 1990), and was also identified in cholesterol-treated fenugreek samples.

Stress and defense-related proteins

Besides the proteins involved in process of photosynthesis, stress- and defense-related proteins formed the second most active group. In response to abiotic and biotic stresses, plants generate reactive oxygen species (ROS) that, in elevated levels, cause oxidative stress (Caverzan et al. 2016). Scavenging or detoxification of overabundance ROS is reached by an effective antioxidative system consisting of both non-enzymatic as well as enzymatic antioxidants. The enzymatic antioxidants include catalase, superoxide dismutase, guaiacol peroxidase, and enzymes of ascorbate glutathione cycle such as monodehydroascorbate reductase, dehydroascorbate reductase, ascorbate peroxidase, and glutathione reductase (Sharma et al. 2012).

A number of stress related proteins were overexpressed in control fenugreek plants including chloroplast catalase (spot 1029), germin-like protein (spot 1041), glucan endo-1,3-beta-glucosidase (spot 90), and thioredoxin-like protein CDSP32 (spot 162). Thioredoxin CDSP32 was also found in MeJ-treated sample (spot 164). Catalase is crucial during the oxidative stress for maintaining the redox balance and scavenges H_2O_2 produced in peroxisomes during β -oxidation of fatty acids, photorespiratory oxidation, and other enzyme systems (Sharma et al. 2012; Willekens et al. 1997). Germin-like proteins play critical roles in plant defense response to fungal, bacteria and virus infections, insect attack, chemical toxicities, and stress caused by salinity and drought (Wang et al. 2013; Komatsu et al. 2014). Glucan endo-1,3-beta-glucosidase degrades the beta-glucan, component of hemicelluloses which builds cell walls, and is accumulated in response to ABA, salt stress, and involved

in the defense against pathogens (Li et al. 2010, 2015). CDSP32 thioredoxin takes part in plastidic responses during oxidative stress (Rey et al. 2005).

Expression of monodehydroascorbate reductase (spot 103) and harpin-binding protein (spot 185) increased both in MeJ and CHL-treated plants. Monodehydroascorbate reductase is involved in elimination of H_2O_2 in ascorbate-glutathione cycle (Noctor and Foyer 1998), and plays a significant role in fighting oxidative stress induced by environmental factors (Sharma et al. 2012; Caverzan et al. 2016). Harpin-binding proteins are well known as elicitors that induce numerous responses in plants, such as hypersensitive response, systemic acquired resistance, reinforcement of growth, and tolerance to drought, and induce defense mechanism in response to nitrogen stress (Song et al. 2010; Zhang et al. 2011).

In MeJ-treated plants, we found enhanced expression of pathogenesis-related thaumatin-like protein (spot 255), NO-associated protein 1 (spot 45), and ABC transporter A family member 7 (spot 44). Pathogenesis-related (PR) thaumatin-like proteins are induced and accumulate locally and often systemically in plant tissues in response to infection and various abiotic and biotic stresses (Hammer-schmidt and Nicholson 1999). Beside stress stimuli, proteins of the PR5 family, also named thaumatin-like proteins, are induced by phytohormones (methyl jasmonate, salicylic acid, and abscisic acid) (Wang et al. 2010).

Proteins involved in secondary metabolism

The positive effect of MeJ on diosgenin production has been reported (De and De 2011). Similarly, cholesterol was applied for induction of steroidal saponins previously (Aasim et al. 2014). Two proteins involved in secondary metabolism were overexpressed in the fenugreek plants. Chalcone isomerase (spot 1054) was found exclusively in plants treated by methyl jasmonate and cholesterol. Chalcone isomerase catalyzes the cyclization of chalcone and 6'-deoxychalcone into (2S)-naringenin, and (2S)-5-deoxyflavanone, respectively (Gensheimer and Mushegian 2004). The activity of chalcone isomerase is substantial for the biosynthesis of phenylpropanoid compounds which participate in plant defense and flavanone precursors of floral pigments (Jez and Noel 2002).

Interestingly, 11-oxo-beta-amyrin 30-oxidase (spot 171) was up-regulated in control plants. The enzyme belongs to cytochrome CYP72A subfamily, is involved in the biosynthesis of triterpenoid saponins, and catalyzes three sequential oxidation or hydroxylation steps at C-30 of 11-oxo-beta-amyrin. In legumes like medic barrel (*Medicago truncatula* Gaertn.) or licorice (*Glycyrrhiza glabra* L.), this protein may be also responsible for the oxidation of triterpenes at positions C-22 and C-29 (Seki et al. 2011).

In fenugreek, however, steroidal saponins (like diosgenin, tigogenin, and gitogenin) are synthesized instead of triterpene saponins (beta-amyrin, ursolic acid, and their derivatives) and this protein similar to 11-oxo-beta-amyrin 30-oxidase, may be involved in steroidal saponin pathway. As biosynthesis of diosgenin, a main representative of steroidal saponins in fenugreek is still not explained, the possible participation of identified protein in biosynthesis of diosgenin should be tested and verified.

Comparison of MALDI-ToF with other methods

MALDI coupled with time-of-flight (ToF) mass analyzer is popular, simple, robust, and sensitive method to proteome analysis. Mass spectra obtained from this method are simple to interpret due to mainly singly charged ions that are generated (Aebersold and Goodlett 2001). However, in our study, a part of spots could not be identified using Mascot database. We made an effort to compare proteomic data with results obtained from fenugreek transcriptome analysis. This approach resulted in the identification of seven spots. Integration of proteomic data with transcriptome sequencing could be new pathway to enhance the efficiency of the identification process.

Besides MALDI, the LC-MS is the second important and routinely used method of proteomic analysis. It provides high sensitivity and reliability, and allows to reproducibly and to effectively match data to the predicted proteins (Lim et al. 2003). The possibility of using LC-MS could be alternative to obtained better results.

Our results indicate that treatment by methyl jasmonate and cholesterol has affected stress, defense, and biosynthesis of secondary metabolites in plants. Identified proteins were connected with alternations in photosynthesis or protein metabolism caused by oxidative stress or pathogen attack. Methyl jasmonate as an elicitor influenced up-regulation of proteins involved in stress and defense. In addition, proteins with overexpression induced by cholesterol were joined with acclimation to biotic and abiotic stresses. In both cases, chalcone isomerase was detected as one of the enzymes of phenylpropanoid pathway. This indicates overexpression of secondary metabolites pathway and structural-functional relationship between phenylpropanoid and isoprenoid pathways in plants with induced diosgenin synthesis. Biosynthesis of diosgenin originates from integration of mevalonate, non-mevalonate, and steroids pathways.

Conclusions

Proteome changes in response to methyl jasmonate and cholesterol treatment were recorded for 65 and 41 proteins, respectively. The identified proteins were most often

involved in direct and indirect responses to stress caused by various treatments. Upon MeJ treatment, elevated levels of proteinase inhibitors, thioredoxin, pathogenesis-related protein, monodehydroascorbate reductase, harpin-binding proteins, lectins, and chalcone isomerase were observed. These proteins are involved in response to abiotic and biotic stresses and confirm the role of methyl jasmonate as elicitor. Proteins with altered expression induced with cholesterol were also generally related with acclimation to stress. Level of diosgenin was elevated in MeJ-treated plants. In opposite to MeJ, treatment with cholesterol not resulted in increase the level of diosgenin in fenugreek plants. Using standard PMF method, actually no new proteins for species-specific unique pathways can be identified. To identify these genes, sequencing of peptides with subsequent matching with available predicted protein databases can put more light on alternations in diosgenin metabolism in fenugreek. These findings provide new insights into the response of fenugreek proteome to a methyl jasmonate and cholesterol treatment.

Author contribution statement JC prepared plant treatment, extraction, and identification of diosgenin, interpreted scientific information, and wrote the manuscript; AB performed 2D electrophoresis and identification of protein and helped with manuscript preparation; AK performed 2D electrophoresis; MS prepared plant treatment and helped in diosgenin identification; MJ performed statistical analysis; MT coordinated experiment.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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References

- Aasim M, Khawar KM, Yalcin G, Bakhsh A (2014) Current trends in fenugreek biotechnology and approaches towards its improvement. *Am J Soc Issues Hum. Fenugreek Special Issue Mar/Apr*, pp 128–136
- Aebersold R, Goodlett DR (2001) Mass spectrometry in proteomics. *Chem Rev* 101(2):269–295

- Allakhverdiev SI, Kreslavski VD, Klimov VV, Los DA, Carpentier R, Mohanty P (2008) Heat stress: an overview of molecular responses in photosynthesis. *Photosynth Res* 98:541–550
- Ashraf M, Harris PJC (2013) Photosynthesis under stressful environments: an overview. *Photosynthetica* 51(2):163–190
- Bocian A, Kosmala A, Rapacz M, Jurczyk B, Marczak Ł, Zwierzykowski Z (2011) Differences in leaf proteome response to cold acclimation between *Lolium perenne* plants with distinct levels of frost tolerance. *J Plant Physiol* 168:1271–1279
- Caverzan A, Casassola A, Patussi Brammer SP (2016) Reactive oxygen species and antioxidant enzymes involved in plant tolerance to stress. In: Shanker A (ed) *Abiotic and biotic stress in plants—recent advances and future perspectives*. InTech, Rijeka, pp 463–480. doi:10.5772/61368. ISBN 978-953-51-2250-0
- Chattopadhyay A, Subba P, Pandey A, Bhushan D, Kumar R, Datta A, Chakraborty S, Chakraborty N (2011) Analysis of the grasspea proteome and identification of stress-responsive proteins upon exposure to high salinity, low temperature, and abscisic acid treatment. *Phytochemistry* 72(10):1293–1307
- Correia S, Vinhas R, Manadas B, Lourenco AS, Verissimo P, Canhoto JM (2012) Comparative proteomic analysis of auxin-induced embryogenic and nonembryogenic tissues of the solanaceous tree *Cyphomandra betacea* (tamarillo). *J Proteome Res* 11:1666–1675
- De D, De B (2011) Elicitation of diosgenin production in *Trigonella foenum-graecum* L. seedlings by heavy metals and signaling molecules. *Acta Physiol Plant* 33:1585–1590
- Etienne P, Petitot AS, Houot V, Blein JP, Suty L (2000) Induction of tcl 7, a gene encoding a β -subunit of proteasome, in tobacco plants treated with elicitors, salicylic acid or hydrogen peroxide. *FEBS Lett* 466:1873–3468
- Faizal A, Geelen D (2013) Saponins and their role in biological processes in plants. *Phytochem Rev* 12:877–893
- Falcone Ferreyra ML, Rius SP, Casati P (2012) Flavonoids: biosynthesis, biological functions, and biotechnological applications. *Front Plant Sci* 3:222–237
- Farmer EE, Johnson RR, Ryan CA (1992) Regulation of expression of proteinase inhibitor genes by methyl jasmonate and jasmonic acid. *Plant Physiol* 98:995–1002
- Fraire-Velázquez S, Rodríguez-Guerra R, Sánchez-Calderón L (2011) Abiotic and biotic stress response crosstalk in plants. In: Shanker A (ed) *Abiotic stress response in plants - physiological, biochemical and genetic perspectives*. InTech, Rijeka. doi:10.5772/23217. ISBN 978-953-307-672-0
- Gensheimer M, Mushegian A (2004) Chalcone isomerase family and fold: no longer unique to plants. *Protein Sci* 13(2):540–544
- Habib H, Fazili KM (2007) Plant protease inhibitors: a defense strategy in plants. *Biotechnol Mol Biol Rev* 2(3):068–085
- Hammerschmidt R, Nicholson RL (1999) A survey of plant defense responses to pathogens. In: Agrawal AA, Tuzun S, Bent E (eds) *Inducible plant defenses against pathogens and herbivores: biochemistry, ecology, and agriculture*. American Phytopathological Society Press, St. Paul, pp 55–71
- Hurkman WJ, Tanaka CK (1986) Solubilization of plant membrane proteins for analysis by two-dimensional gel electrophoresis. *Plant Physiol* 81(3):802–806
- Jez JM, Noel JP (2002) Reaction mechanism of chalcone isomerase. pH dependence, diffusion control, and product binding differences. *J Biol Chem* 277(2):1361–1369
- Kang SG, Choi JH, Suh SG (2002) A leaf-specific 27 kDa protein of potato Kunitz-type proteinase inhibitor is induced in response to abscisic acid, ethylene, methyl jasmonate, and water deficit. *Mol Cells* 13(1):144–147
- Karban R, Kuć J (1999) Induced resistance against pathogens and herbivores: an overview. In: Agrawal AA, Tuzun S, Bent E (eds) *Inducible plant defenses against pathogens and herbivores: biochemistry, ecology, and agriculture*. American Phytopathological Society Press, St. Paul, pp 1–16
- Komatsu S, Kamal AHM, Hossain Z (2014) Wheat proteomics: proteome modulation and abiotic stress acclimation. *Front Plant Sci* 5:684–703
- Kosová K, Vítámvás P, Prášil IT (2014) Proteomics of stress responses in wheat and barley—search for potential protein markers of stress tolerance. *Front Plant Sci* 5:711–725
- Lambert E, Faizal A, Geelen D (2011) Modulation of triterpene saponin production: in vitro cultures, elicitation, and metabolic engineering. *Appl Biochem Biotechnol* 164:220–237
- Li XJ, Yang MF, Chen H, Qu LQ, Chen F, Shen SH (2010) Abscisic acid pretreatment enhances salt tolerance of rice seedlings: proteomic evidence. *Biochim Biophys Acta* 1804:929–940
- Li W, Zhao F, Fang W, Xie D, Hou J, Yang X, Zhao Y, Tang Z, Nie L, Lv S (2015) Identification of early salt stress responsive proteins in seedling roots of upland cotton (*Gossypium hirsutum* L.) employing iTRAQ-based proteomic technique. *Front Plant Sci* 6:732–746
- Lim H, Eng Yates JR, Tollaksen SL, Giometti CS, Holden JF, Adams MW, Reich CI, Olsen GJ, Hays LG (2003) Identification of 2D-gel proteins: a comparison of MALDI/TOF peptide mass mapping to mu LC-ESI tandem mass spectrometry. *J Am Soc Mass Spectrom* 14(9):957–970
- Mehrafarin A, Rezazadeh Sh, Naghdi Badi H, Noormohammadi Gh, Zand E, Qaderi A (2011) A review on biology, cultivation and biotechnology of fenugreek (*Trigonella foenum-graecum* L.) as a valuable medicinal plant and multipurpose. *J Med Plants* 10:6–24
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15:473–497
- Nam MH, Huh SM, Kim KM, Park WJ, Seo JB, Cho K, Kim DY, Kim BG, Yoon IS (2012) Comparative proteomic analysis of early salt stress-responsive proteins in roots of SnRK2 transgenic rice. *Proteome Sci* 10:25–44
- Noctor G, Foyer CH (1998) Ascorbate and glutathione: keeping active oxygen under control. *Annu Rev Plant Physiol Plant Mol Biol* 49:249–279
- Nordhoff E, Egelhofer V, Giavalisco P, Eickhoff H, Horn M, Gobom J et al (2001) Large-gel two-dimensional electrophoresis-matrix assisted laser desorption/ionization-time of flight-mass spectrometry: an analytical challenge for studying complex protein mixtures. *Electrophoresis* 22:2844–2855
- Nouri MZ, Moumeni A, Komatsu S (2015) Abiotic stresses: insight into gene regulation and protein expression in photosynthetic pathways of plants. *Int J Mol Sci* 16:20392–20416. doi:10.3390/ijms160920392
- Patel K, Gadewar M, Tahilyani V, Patel DK (2012) A review on pharmacological and analytical aspects of diosgenin: a concise report. *Nat Prod Bioprospect* 2:46–52
- Raines CA, Paul MJ (2006) Products of leaf primary carbon metabolism modulate the developmental programme determining plant morphology. *J Exp Bot* 57:1857–1862
- Raines C, Lloyd J, Dyer T (1999) New insights into the structure and function of sedoheptulose-1,7-bisphosphatase an important but neglected calvin cycle enzyme. *J Exp Bot* 50:1–8
- Raju J, Rao CV (2012) Diosgenin, a steroid saponin constituent of yams and fenugreek: emerging evidence for applications in medicine. In: Rasooli I (ed) *Bioactive compounds in phyto-medicine*. InTech, Rijeka, pp 125–142. doi:10.5772/26700
- Rathi D, Gayen D, Gayali S, Chakraborty S, Chakraborty N (2016) Legume proteomics: progress, prospects, and challenges. *Proteomics* 16:310–327
- Rejeb IB, Pastor V, Mauch-Mani B (2014) Plant responses to simultaneous biotic and abiotic stress: molecular mechanisms. *Plants* 3:458–475

- Rey P, Cui  Eymery F, Garin J, Court M, Jacquot JP, Rouhier N, Broin M (2005) Analysis of the proteins targeted by CDSP32, a plastidic thioredoxin participating in oxidative stress responses. *Plant J* 41(1):31–42
- Ryan CA (1990) Proteinase inhibitors in plants: genes for improving defenses against insects and pathogens. *Annu Rev Phytopathol* 28:425–449
- Savikin-Fodulovic K, Grubisic D, Culafic L, Menkovic N, Ristic M (1998) Diosgenin and phytosterols content in five callus lines of *Dioscorea balcanica*. *Plant Sci* 135:63–67
- Seki H, Sawai S, Ohshima K, Mizutani M, Muranaka T et al (2011) Triterpene functional genomics in licorice for identification of CYP72A154 involved in the biosynthesis of glycyrrhizin. *Plant Cell* 23:4112–4123
- Sharma P, Jha AB, Dubey RS, Pessarakli M (2012) Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. *J Bot* 2012:1–16
- Snehlata HS, Payal DR (2012) Fenugreek (*Trigonella foenum-graecum* L.): an overview. *Int J Curr Pharm Res* 2(4):169–187
- Song C, Zeng F, Feibo W, Ma W, Zhang G (2010) Proteomic analysis of nitrogen stress-responsive proteins in two rice cultivars differing in N utilization efficiency. *J Integr Omics* 1(1):78–87
- StatSoft, Inc. (2010) STATISTICA (data analysis software system), version 9.1. <http://www.statsoft.com>
- Szakiel A, P czkowski C, Henry M (2011) Influence of environmental biotic factors on the content of saponins in plants. *Phytochem Rev* 10:493–502
- Vit mv s P, Urban MO,  kod cek Z, Kosov  K, Pitelkov  I, Vit mv s J, Renaut J, Pr sil IT (2015) Quantitative analysis of proteome extracted from barley crowns grown under different drought conditions. *Front Plant Sci* 6:479–497
- Wang X, Tang C, Deng L, Cai G, Liu X, Liu B, Kang et al (2010) Characterization of a pathogenesis-related thaumatin-like protein gene TaPR5 from wheat induced by stripe rust fungus. *Physiol Plant* 139(1):27–38
- Wang T, Chen X, Zhu F, Li H, Li L, Yang Q, Chi X, Yu S, Liang X (2013) Characterization of peanut germin-like proteins, AhGLPs in plant development and defense. *PLoS One* 8(4):e61722. doi:10.1371/journal.pone.0061722
- Weiss W, G rg A (2007) Two-dimensional electrophoresis for plant proteomics. *Methods Mol Biol* 355:121–143
- Willekens H, Chamnongpol S, Davey M, Schraudner M, Van Camp W et al (1997) Catalase is a sink for H₂O₂ and is indispensable for stress defence in C-3 plants. *EMBO J* 16(16):4806–4816
- Zhang L, Xiao S, Li W, Feng W, Li J, Wu Z, Gao X, Liu F, Shao M (2011) Overexpression of a harpin-encoding gene hrf1 in rice enhances drought tolerance. *J Exp Bot* 62(12):4229–4238