TÜBİTAK

Turkish Journal of Botany

http://journals.tubitak.gov.tr/botany/

Research Article

Turk J Bot
(2017) 41: 234-243

doi:10.3906/bot-1608-10

Proteomic analysis of flowers at two developmental stages in *Thermopsis turcica* (Fabaceae)

Mustafa YILDIZ[1,*], Emre PEHLİVAN[1], Hakan TERZİ[2]

[1]Department of Molecular Biology and Genetics, Faculty of Science and Arts, Afyon Kocatepe University, Afyonkarahisar, Turkey
[2]Food Technology Program, Sultandağı Vocational School, Afyon Kocatepe University, Afyonkarahisar, Turkey

Received: 08.08.2016 • **Accepted/Published Online:** 09.02.2017 • **Final Version:** 24.05.2017

Abstract: Flower development is a complex physiological phenomenon that is affected by environmental stimuli and endogenous signals. Several molecular approaches have provided a comprehensive view of the physiological processes associated with flower development, but little is known about proteomic changes. Therefore, we investigated the proteomic alterations during flower development in endemic *Thermopsis turcica* (*Vuralia turcica*), an unusual legume species with 3–4 free carpels in a single flower. A comparative proteomic analysis to identify proteins involved in flower development was carried out in *T. turcica*. Alterations in proteomes of the flower buds and fully opened flowers were studied by two-dimensional gel electrophoresis. A total of 66 proteins were differentially expressed during flower development. Among them, 32 protein spots were identified by mass spectrometry. Functional annotation of these flower proteins revealed their involvement in transcription and protein metabolism, energy and carbohydrate metabolisms, plant defense, cell walls, photosynthesis, secondary metabolism, and amino acid metabolism. Moreover, most of these proteins were downregulated at the later stage of flower development. Proteins involved in protein metabolism, sugar metabolism, and stress defense are regulated during flower development, suggesting that they have possible roles in developmental regulation. These results have given new insights into the proteome alterations during flower development.

Key words: Endemic, flower development, proteomics, *Thermopsis turcica*

1. Introduction

Plant development is controlled by the interplay of multiple environmental cues and endogenous signals. High-throughput approaches such as proteomics, transcriptomics, and metabolomics have provided immense amounts of data, leading to a better understanding of biological processes (Hochholdinger et al., 2006). As the relationship between mRNA expression levels and protein abundance is poor, proteomics provides functional gene expression profiles during biological processes (Komatsu and Hossain, 2013). Flower development is controlled by a combination of genetics and environmental factors such as light and temperature affecting hormone levels (Davis, 2009; Domagalska et al., 2010). Studies in *Arabidopsis thaliana* and other angiosperm species including *Antirrhinum majus*, *Petunia hybrid*, and *Oryza sativa* have helped to elucidate the complex network regulated tightly during flower development (Ó'Maoiléidigh et al., 2014). Based on similarity searches, most of the flowering genes were found to be conserved in some legume species (Hecht et al., 2005). Transcription factors play a crucial role in flower development. The regulatory role of MADS-box transcription factors in regulating floral organ development is well demonstrated in soybean and chickpea (Jung et al., 2012; Singh et al., 2013). To date, several proteomic analyses have been employed to identify proteins involved in plant development. However, only a few proteomics studies on flower development are available (Dafny-Yelin et al., 2005; Ahsan and Komatsu, 2009). Analysis of rose petal proteomes showed that energy, cell rescue, and metabolism-related proteins were differentially regulated during development (Dafny-Yelin et al., 2005). The differential expression of several proteins involved in several biological processes such as energy metabolism, secondary metabolism, and signal transduction pathways provides a good example of a protein networks during flower development of soybean (Ahsan and Komatsu, 2009). Zhang et al. (2013) showed the differential expression of several proteins involved in metabolism, protein fate, signal transduction, cellular transport, and biogenesis during floral initiation in *Agapanthus praecox*. Recently, Zhang et al. (2016) demonstrated that the largest proportions of identified proteins were involved in metabolism, protein fate, and cell rescue during the flowering transition in *Crocus sativus*.

Fabaceae is a large family comprising three subfamilies including Caesalpinioideae, Mimosoideae,

* Correspondence: mustafa_yildizus@yahoo.com

and Papilionoideae, differing in floral symmetry (Tucker, 2003). The genus *Thermopsis* belongs to the subfamily Papilionoideae, in which the inflorescence is usually raceme. This genus has about 25 species, mostly distributed in montane regions of North America and Asia (Wojciechowski, 2003). The genus *Thermopsis* is only represented by endemic *T. turcica* Kit Tan, Vural & Küçük. in Turkey (Tan et al., 1983; Davis et al., 1988). Recently, based on nuclear internal transcribed spacer DNA sequences, this species was named as *Vuralia turcica* (Uysal et al., 2014). However, *T. turcica*, which is a common name, was preferred in this study. This endemic species is distributed around the southern part of Eber Lake and the southwestern part of Akşehir Lake. *T. turcica* is a perennial herb with a long rhizome. The inflorescence is racemose, and flowers are large, bracteate, zygomorphic, and hermaphroditic. The calyx is 9–13 mm in length. Petals are golden yellow in color. Flowers have 10 free stamens. The ovary has 3–4 free carpels. Fruit is a legume with 2–3 seeds (Özdemir et al., 2008). The unique trait of *T. turcica* is to have 3 or 4 free carpels per flower, which is the first such record in the subfamily Papilionoideae (Tan et al., 1983; Davis et al., 1988). This unique flower morphology makes *T. turcica* the most important species as a valuable gene source. The production of multiple free carpels from a single flower is very rare among flowering plants. For instance, the number of free carpels per flower ranged from 2 to 9 in *Paeonia cambessedesii* (Paeoniaceae), which is a herbaceous perennial plant endemic to the Balearic Islands (Méndez and Traveset, 2003). The identification of genes involved in reproductive development provides potential targets for crop improvement via breeding and reverse genetics approaches. The development of the flower is controlled by numerous genes that activate the expression of specific genes and thus the synthesis of specific proteins. Therefore, the priority of this study was to provide preliminary data on the proteome alterations during the flower development of *T. turcica*. In order to reveal the proteome alterations in the flowers at the two developmental stages, a comparative proteomic analysis was performed using the two-dimensional gel electrophoresis (2-DE) technique. The proteins that showed differences in abundance were identified by MALDI–TOF/TOF mass spectrometry to obtain new information about the molecular mechanisms involved in flower development.

2. Materials and methods

2.1. Sample collection

Inflorescences of *T. turcica* plants were collected in May 2013 from the natural population distributed at the southern part of Eber Lake (38°36′N, 31°09′E, and an altitude of 968 m above sea level) of Afyonkarahisar Province, Turkey (Figure 1). April and May are the flowering time, and the average ambient temperature is 10.4 and 15.0 °C, respectively. The average precipitation value of these months is about 49.3 kg/m^2, and the photoperiod is about 14 h. The lower ends of the aerial parts were immersed in water, and they were transferred immediately to the laboratory. Flower buds (approx. 1 cm in length) and fully opened flowers (approx. 2.5 cm in length) of equal size and shape were collected from five independent inflorescences, ensuring homogeneity of the sampling pool (Figure 1). The sampled flowers included all flower parts. After freezing in liquid nitrogen, flower samples were kept at –80 °C until protein extraction.

2.2. Protein isolation and 2-DE

For the proteomic study, three independent biological replicates were used throughout the experiment. Total proteins were extracted from flower buds and fully opened flowers according to Hurkman and Tanaka (1986) and Ahsan et al. (2008). Finely powdered samples (1 g) were mixed with 10 mL of extraction buffer [0.7 M sucrose, 500 mM Tris-HCl (pH 8.3), 20 mM $MgCl_2$, 2% NP-40, and 2% β-mercaptoethanol] (Kim et al., 2001). An equal volume of Tris-HCl-saturated phenol was added to the homogenates and then centrifuged at 3500 × *g* for 15 min. The resulting supernatants were transferred to new centrifuge tubes and mixed with 0.1 M ammonium acetate dissolved in methanol. After overnight protein precipitation at –20 °C, pellets were vacuum-dried. The protein concentration was determined according to Bradford (1976).

Total proteins were separated by 2-DE. For the first dimension, immobilized pH gradient (IPG) strips (pH 4–7; 17 cm) were passively rehydrated with 300 µL of rehydration buffer containing 80 µg and 500 µg proteins for analytical and preparative gels, respectively. Isoelectric focusing (IEF) was conducted with a Protean i12 IEF System (Bio-Rad). The IPG strips were applied on running gels using a Protean II XL Cell (Bio-Rad). The protein spots in analytical and preparative gels were visualized by silver staining (Sinha et al., 2001) and blue silver colloidal Coomassie staining (Candiano et al., 2004), respectively.

2.3. Image and data analysis

Images of the analytical gels were acquired using the ChemiDoc MP System (Bio-Rad). The gel images were analyzed with PDQuest software (Version 8.0; Bio-Rad). For quantitative analysis, the average volume values of each protein spot were calculated from three biological replicates. Comparisons and statistical analysis (Student's t-test) were performed using the calculated average values of each biological replicate. Only the spots showing significant changes of at least twofold were considered as differentially expressed proteins.

2.4. Proteolytic digestion and protein identification

Differentially expressed protein spots were manually excised from the preparative gels. Protein digestions with

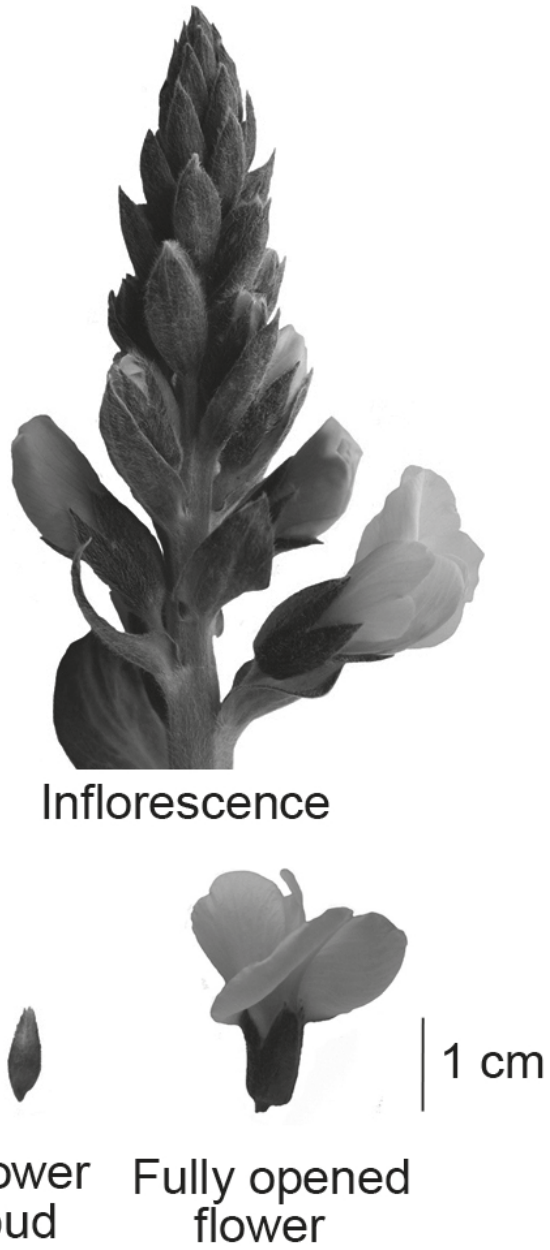

Figure 1. Inflorescence of *T. turcica* and its flower bud and fully opened flower used in proteomic analysis.

trypsin were done using an in-gel tryptic digestion kit (Thermo Fisher Scientific). The peptides were desalted with C18 ZipTips (Millipore) and mixed with 1 µL of 2 mg/mL α-cyano-hydroxy cinnamic acid. This mixture was spotted onto a stainless plate for analysis using AB Sciex TOF/TOF 5800 MS. The peptide MS/MS spectra were submitted to MASCOT. The search was performed using the following parameters: Swiss-Prot database; taxonomy of Viridiplantae; trypsin digest with one missing cleavage; fixed modification, carbamidomethylation of cysteine; mass accuracy, 50 ppm; MS/MS tolerance, ±0.4 Da; variable modifications, oxidation of methionine. Proteins with protein score confidence intervals above 95% were considered to be credibly identified.

3. Results and discussion

Flower development is tightly regulated by complex mechanisms triggered by internal and external signals (Rolland-Lagan et al., 2003). To date, comprehensive genetic and molecular analyses in *Arabidopsis thaliana*, *Antirrhinum majus*, *Petunia hybrida*, and *Oryza sativa* have identified numerous key floral regulatory genes such as members of the MADS-Box family and provided knowledge about their roles in morphogenesis (Ó'Maoiléidigh et al., 2014). The preferential expression of MADS-box genes in flowers of legumes suggested a similar regulatory network during flower development in legumes (Jung et al., 2012; Singh et al., 2013). Although several works have provided useful information for understanding the transcriptional regulation during flower development (Singh et al., 2013; Nakamura et al., 2014; Yang et al., 2014), only a few studies have been performed at the proteome level (Dafny-Yelin et al., 2005; Ahsan and Komatsu, 2009; Chen et al., 2016; Zhang et al., 2016). In our study, a proteomic approach was used to identify proteins that may have potential roles in regulatory networks.

To characterize the flower proteome, 2-DE was carried out with proteins extracted from flowers. More than 1000 protein spots were reproducibly detected in the 2-DE gels (Figure 2). The numbers of highly reproducible protein spots were determined to be 1.038±35 and 1.022±56 in the flower buds and fully opened flowers, respectively. Only spots that showed significantly and reproducible changes of at least 2-fold were considered as differentially expressed proteins. Most of these proteins showed no significant change in abundance, while the expression levels of 66 proteins were altered by at least twofold in fully opened flowers as compared to flower buds. To identify these proteins, corresponding protein spots were subjected to MS analysis followed by gel digestion. We successfully identified 32 protein spots by MALDI–TOF/TOF MS (Table). As there are limited data on the *T. turcica* genome, all of the proteins were identified as orthologous proteins from different plants. Two proteins showed strong homology with *Arabidopsis* proteins of unknown function. The stage-specific proteins could not be determined in the present study. According to their functional annotation and the literature, these proteins were classified into different functional categories: transcription and protein metabolism, plant defense, energy and carbohydrate metabolisms, photosynthesis, cell wall and cytoskeleton, secondary metabolism, and amino acid metabolism (Table; Figure 3). It has been reported that the 104 flower-specific

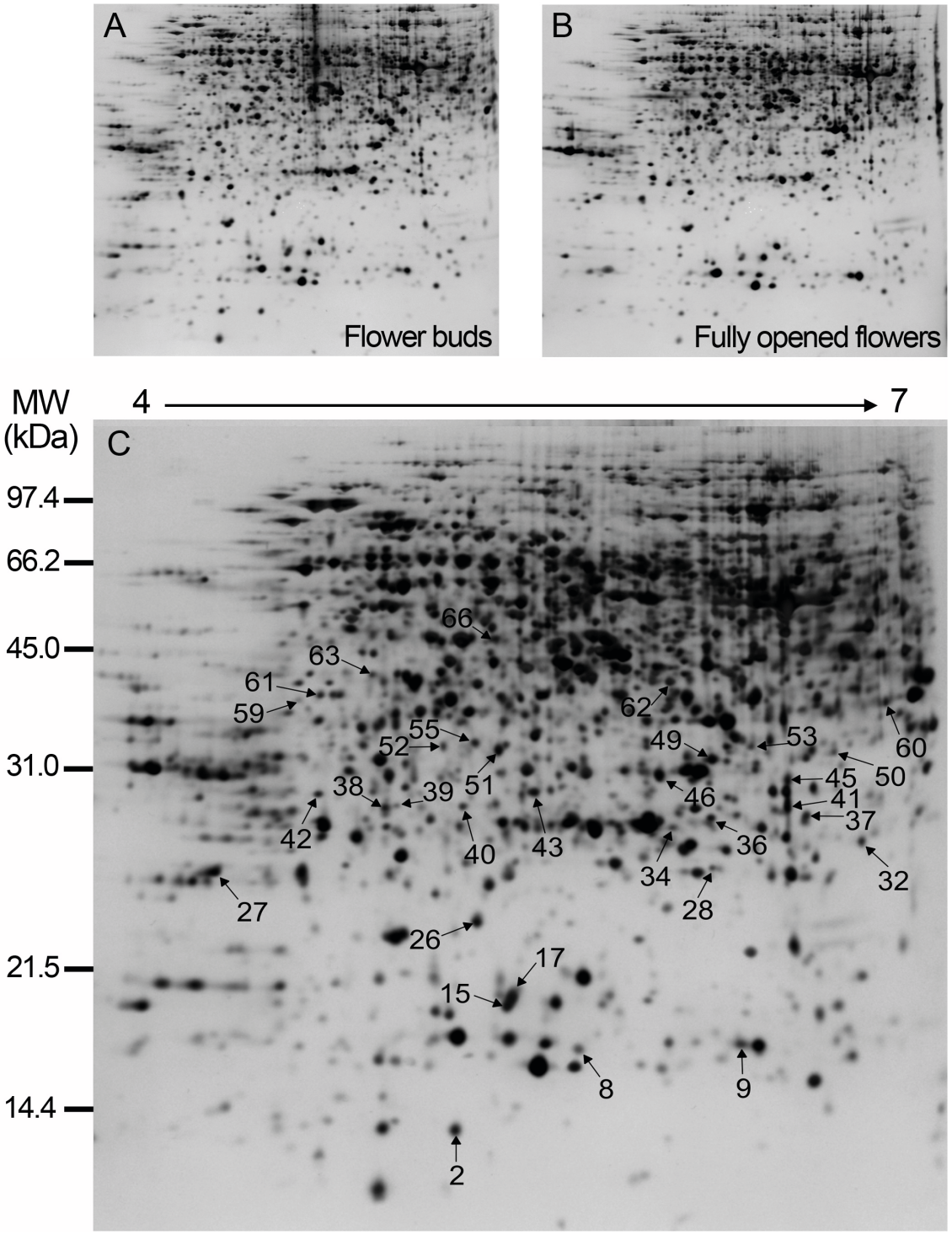

Figure 2. Representative 2-DE gel images of flower buds (A) and fully opened flowers (B) of *T. turcica*. (C) The reference 2-DE pattern of *T. turcica* flowers at two developmental stages. Isoelectric focusing was performed from 80 µg of proteins using 17-cm immobilized pH gradient strips (linear pH 4–7). In the second dimension, 12.5% SDS-PAGE gels were used. Arrows indicate the differentially expressed and identified proteins (Table).

proteins related to translation, glycolysis, photosynthesis, and amino acid metabolism were overrepresented in *Arabidopsis thaliana* (Baerenfaller et al., 2008). Although these flower-specific proteins function in similar metabolic pathways to those of our study, they are not identical to the proteins identified in our study.

Eight transcription and protein metabolism-related proteins including B3 domain-containing protein At5g60130 (spot 9), eukaryotic translation initiation factors eIF5A2 and eIF5A1 (spots 15 and 17), proteasome subunit beta type-1 and type-4 (spots 32, 36, and 37), coatomer subunit epsilon-1 (spot 38), and 26S proteasome non-ATPase regulatory subunit 14 (spot 50) showed significant changes in abundance during flower development. eIF5A1, eIF5A2, proteasome subunit beta type-1 and type-4, and 26S proteasome non-ATPase regulatory subunit 14 showed a significant decrease in abundance in fully opened flowers. ATP-dependent protease complexes are enzymes that degrade misfolded or damaged proteins and control the level of certain regulatory proteins involved in transcription, cell cycle progression, and metabolic regulation (Peters, 1994). The downregulation of these proteins in fully opened flowers suggests that they are important components of flower development. On the other hand, B3 domain-containing protein At5g60130 and coatomer subunit epsilon-1 were significantly upregulated in fully opened flowers. The coatomer is a vesicular trafficking regulatory protein functioning in the vesicle-mediated transport proteins between the Golgi complex and the endoplasmic reticulum (Shima et al., 1999). This finding suggests that Golgi-mediated protein transport may be activated in fully opened flowers, as the coatomer complex is required for budding from Golgi membranes (Béthune et al., 2006).

Seven stress defense-related proteins were identified. Most of these proteins were decreased in abundance during flower development. Thioredoxin H-type (spot 2), three thiazole biosynthetic enzymes (spots 46, 51, and 52), and pyridoxal biosynthesis protein PDX1.3 (spot 49) were decreased in abundance in fully opened flowers. On the other hand, other stress defense-related proteins, leucoagglutinating phytohemagglutinin (spot 42) and thioredoxin reductase 1 (spot 53), showed upregulation in fully opened flowers. Thioredoxin reductase is a key component of the mitochondrial thioredoxin system for H_2O_2 metabolism (Sweetlove et al., 2002). The abundance of NADPH thioredoxin reductase is enhanced obviously at the induction stage during *Agapanthus* flowering (Zhang et al., 2013). Cellular oxidative stress and the control of cell death are also relieved by thioredoxin, which regulates the activity of enzymes or transcription factors by altering the conformation of disulfide bonds within molecules (Zhang et al., 2016). Stress-related proteins such as superoxide dismutase, peroxidase, and catalase have also been shown to increase in abundance during rose petal development (Dafny-Yelin et al., 2005). As reactive oxygen species function as regulators and signaling molecules in biological processes, the control of their levels is crucial for maintaining cell metabolism (Mittler et al., 2004). Ye et al. (2000) suggested that downregulation of antioxidative enzymes would cause an accumulation of H_2O_2 to activate senescence-associated genes expression in leaves. Moreover, it has been proposed that H_2O_2 is one of the possible factors in flower induction (Lokhande et al., 2003). Differential regulation of stress-related proteins suggested that reactive oxygen species possibly regulated the maturation of flowers in *T. turcica*.

Some common metabolic reactions such as glycolysis, the tricarboxylic acid cycle, and amino acid synthesis exist globally in plant organs and tissues. The intensity of these metabolic reactions could directly reflect plant growth conditions (Carrari and Fernie, 2006; Fait et al., 2006). Six proteins involved in carbohydrate and energy metabolism were differentially regulated during flower development. Glyceraldehyde-3-phosphate dehydrogenase (spot 41), V-type proton ATPase subunit E (spot 45), and fructokinase-1 (spot 61) proteins were significantly downregulated in fully opened flowers. However, soluble inorganic pyrophosphatase (spot 55), another glyceraldehyde-3-phosphate dehydrogenase (spot 60), and one fructokinase-1 (spot 59) were significantly upregulated in fully opened flowers. Moreover, other studies have demonstrated the alterations in expression levels of these proteins during flower and fruit development (Dafny-Yelin et al., 2005; Rocco et al., 2006; Faurobert et al., 2007). These upregulated proteins related to glycolysis can probably facilitate energy generation during flower development. It has been demonstrated that glycolysis metabolism supplied the energy and nutrition for flower bud differentiation in *Crocus sativus* (Zhang et al., 2016). Chen et al. (2016) also reported that the upregulated proteins involved in carbohydrate and energy metabolisms may enhance successful pollination in *Medicago sativa* flowers. In light of this information, these upregulated proteins related to carbohydrate and energy metabolisms may be necessary for pollination in *T. turcica*. Vacuolar H^+-ATPase plays an important role in maintaining the pH of endomembrane compartments. It has also been suggested that V-H^+-ATPase plays essential roles in plant development (Dettmer et al., 2005; Strompen et al., 2005). It has been suggested that V-ATPase activity is needed for cell division and cell expansion (Padmanaban et al., 2004). The decreased V-ATPase activity showed that the cell expansion activity decreased in fully opened flowers. Further functional identification of these proteins would disclose their potential roles in flower development.

We identified three photosynthesis related proteins (Table). For light reaction-related proteins, oxygen-

Table. The 32 differentially expressed proteins between flower buds and fully opened flowers of *Thermopsis turcica*.

Spot[a]	Accession number[b]	Protein[c]	Theo. MW/pI[d]	Exp. MW/pI[e]	Score[f]	MP[g]	SC[h]	Fold change[i]
Transcription and protein metabolism								
9	Y5013_ARATH	B3 domain-containing protein At5g60130, *Arabidopsis thaliana*	37.2/4.39	15.2/6.37	56	5	9	+2.69
15	IF5A2_SOLLC	Eukaryotic translation initiation factor 5A-2, *Solanum lycopersicum*	17.5/5.78	18.1/5.59	216	5	24	−2.08
17	IF5A1_SOLLC	Eukaryotic translation initiation factor 5A-1, *Solanum lycopersicum*	17.3/5.71	18.7/5.75	261	8	23	−2.22
32	PSB1_ARATH	Proteasome subunit beta type-1, *Arabidopsis thaliana*	24.6/6.95	27.1/6.76	258	7	20	−3.70
36	PSA4_PETHY	Proteasome subunit alpha type-4, *Petunia hybrid*	27.2/5.60	28.1/6.27	274	11	14	−2.78
37	PSA4_PETHY	Proteasome subunit alpha type-4, *Petunia hybrid*	27.2/5.60	28.6/6.58	357	12	14	−3.85
38	COPE1_ORYSJ	Coatomer subunit epsilon-1, *Oryza sativa*	31.6/5.23	28.9/5.19	108	4	3	+2.22
50	PSDE_ARATH	26S proteasome non-ATPase regulatory subunit 14, *Arabidopsis thaliana*	34.3/6.31	33.0/6.67	326	15	24	−3.33
Plant defense								
2	TRXH_RICCO	Thioredoxin H-type, *Ricinus communis*	13.0/5.57	14.4//5.42	66	4	15	−3.33
42	PHAL_PHAVU	Leucoagglutinating phytohemagglutinin, *Phaseolus vulgaris*	29.6/4.90	29.6/4.97	93	3	10	+2.52
46	THI4_CITSI	Thiazole biosynthetic enzyme, chloroplastic, *Citrus sinensis*	37.6/5.40	30.7/6.09	353	14	33	−6.67
49	PDX13_ARATH	Pyridoxal biosynthesis protein PDX1.3, *Arabidopsis thaliana*	33.2/5.79	32.4/6.26	144	12	24	−3.57
51	THI4_CITSI	Thiazole biosynthetic enzyme, chloroplastic, *Citrus sinensis*	37.6/5.40	33.3/5.57	547	17	28	−5.88
52	THI4_CITSI	Thiazole biosynthetic enzyme, chloroplastic, *Citrus sinensis*	37.6/5.40	33.7/5.38	316	12	20	−3.33
53	TRXB1_ARATH	Thioredoxin reductase 1, *Arabidopsis thaliana*	35.3/5.82	33.7/6.60	195	6	15	+2.49
Energy and carbohydrate metabolism								
41	G3PC_HORVU	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic, *Hordeum vulgare*	33.2/6.20	29.1/6.52	98	7	25	−2.70
45	VATE_MESCR	V-type proton ATPase subunit E, *Mesembryanthemum crystallinum*	26.1/6.52	30.5/6.52	124	6	16	−2.17
55	IPYR_ORYSI	Soluble inorganic pyrophosphatase, *Oryza sativa*	24.1/5.56	33.9/5.49	350	13	33	+2.78
59	SCRK1_ORYSI	Fructokinase-1, *Oryza sativa*	34.7/5.14	39.5/4.91	190	9	18	+4.88
60	G3PC_ANTMA	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic, *Antirrhinum majus*	36.7/8.30	39.6/6.85	316	13	30	+2.13
61	SCRK1_ORYSI	Fructokinase-1, *Oryza sativa*	34.7/5.14	40.3/4.98	257	11	14	−2.17

Table. (Continued).

Photosynthesis								
26	PSBP_SOLTU	Oxygen-evolving enhancer protein 2, chloroplastic, *Solanum tuberosum*	27.9/8.27	23.2/5.50	308	9	21	−2.22
34	CB215_PEA	Chlorophyll a-b binding protein 215, chloroplastic, *Pisum sativum*	28.9/5.47	27.7/6.14	146	6	35	+2.76
63	CHLI_TOBAC	Magnesium-chelatase subunit ChlI, chloroplastic, *Nicotiana tabacum*	46.6/6.64	42.9/5.15	181	13	17	−2.17
Cell wall and cytoskeleton								
8	GRP1_SORBI	Glycine-rich RNA-binding protein 1, *Sorghum bicolor*	13.7/9.34	14.9/5.83	85	4	19	−2.17
27	TCTP_ORYSJ	Translationally-controlled tumor protein homolog, *Oryza sativa*	18.9/4.51	25.5/4.63	71	7	28	−2.13
43	CAMT2_POPTR	Caffeoyl-CoA O-methyltransferase, *Populus trichocarpa*	27.9/5.33	29.7/5.68	235	12	38	−4.00
66	ACT11_SOLTU	Actin-97, *Solanum tuberosum*	41.6/5.31	49.1/5.55	188	15	29	+2.04
Secondary metabolism								
28	CF1B1_SOYBN	Chalcone-flavonone isomerase 1B-1, *Glycine max*	25.0/5.26	25.8/6.26	71	2	4	−2.86
Amino acid metabolism								
62	GLNA2_HORVU	Glutamine synthetase leaf isozyme, chloroplastic, *Hordeum vulgare*	47.1/5.11	42.2/6.13	161	6	5	−3.03
Unknown								
39	Y2766_ARATH	Uncharacterized protein At2g37660, chloroplastic, *Arabidopsis thaliana*	34.9/8.37	29.0/5.23	207	6	9	+2.00
40	Y2766_ARATH	Uncharacterized protein At2g37660, chloroplastic, *Arabidopsis thaliana*	34.9/8.37	29.0/5.44	263	6	9	+2.00

[a] Spot number in the 2-DE gel as shown in Figure 2.
[b] Accession number according to the best hit of MASCOT search against SwissProt databases.
[c] The identified protein according to the best hit of MASCOT search against SwissProt databases.
[d] Theoretical molecular weight (MW) and pI, which were retrieved from the protein database.
[e] Experimental molecular weight and pI, which were calculated using Image Lab software (Bio-Rad).
[f] Probability score for protein identification based on MS analysis and MASCOT search.
[g] Number of matching peptides.
[h] The percentage of coverage of the sequence obtained by MS.
[i] Upregulated (+) or downregulated (−) protein in fully opened flowers compared to flower buds.

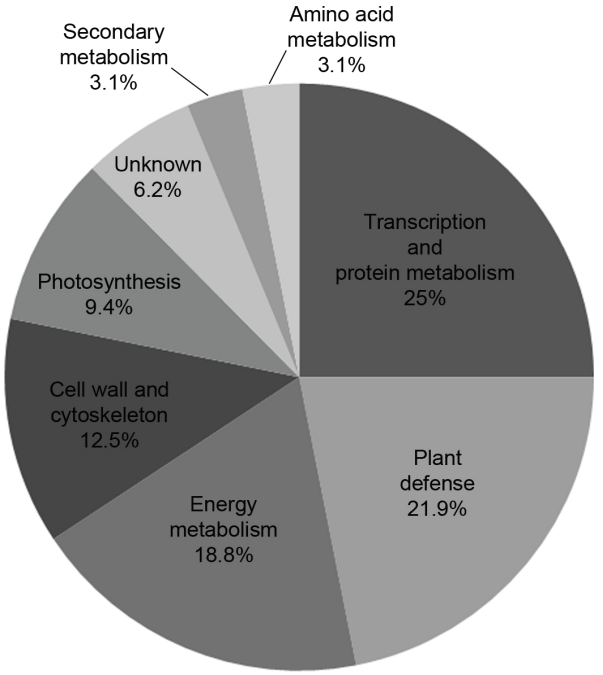

Figure 3. Distribution of 32 (out of 66) differentially expressed proteins based on their biological function (http://www.uniprot.org).

evolving enhancer protein 2 (spot 26) and magnesium chelatase subunit ChlI (spot 63) were downregulated while chlorophyll a/b binding protein 215 (spot 34) was upregulated in fully opened flowers. It has been suggested that a lower abundance of photosynthesis-related proteins might cause the low photosynthetic activity in mature flowers of soybean (Ahsan and Komatsu, 2009). Moreover, glutamine synthetase leaf isozyme (spot 62) showed a decrease in abundance in fully opened flowers. Since it catalyzes the ATP-dependent condensation of ammonium with glutamate, it is suggested that the levels of nitrogenous compounds decreased in the late stage of flower development. The decline in proteins related to photosynthesis and nitrogen metabolism might be beneficial for transportation and the reuse of nitrogen sources and nutrients in reproductive organs.

Furthermore, we also found some proteins functioning in the cytoskeleton and cell wall, secondary metabolism, amino acid metabolism, and unknown functions. Three of these four proteins decreased in abundance during flower development (Table). The expressions of glycine-rich RNA-binding protein 1 (spot 8), translationally controlled tumor protein (TCTP) homolog (spot 27), and caffeoyl-CoA O-methyltransferase (spot 43) were decreased during flower development. The downregulation of these proteins clearly showed that the cell expansion activity decreases in fully opened flowers. However, actin-97 protein (spot 66) showed an increase in abundance in fully opened flowers. In *Agapanthus praecox*, the abundances of actin, α-tubulin, and β-tubulin proteins showed a significant increase in the process of floral bud differentiation (Zhang et al., 2013). The expressed quantity of β-actin and tubulin continued to increase during the process of flower bud differentiation of *Crocus sativus* (Zhang et al., 2016).

It has been demonstrated that the production of secondary metabolites is activated during flower development (Ahsan and Komatsu, 2009). In our study, chalcone-flavanone isomerase 1B-1 (spot 28), one of the key enzymes in the flavonoid biosynthesis, showed a significant decrease during flower development. Previous studies showed that the biosynthetic pathway of secondary metabolites was regulated in floral organs (Laitinen et al., 2005; Sharma et al., 2012). However, the expression of this protein was decreased in off-crop flower buds of *Citrus* as compared to on-crop buds (Muñoz-Fambuena et al., 2013).

In conclusion, during flower development, the proteins linked to protein metabolism, energy metabolism, plant defense, photosynthesis, cell walls, secondary metabolism, and amino acid metabolism were mainly downregulated in fully opened flowers. As most of the regulatory proteins are low-abundance proteins, it was not possible to detect them in 2-DE gels. Therefore, it is necessary to combine proteomics with other omics technologies such as genomics, metabolomics, and transcriptomics to understand the complexities of flower development.

Acknowledgments

The authors wish to thank the Afyon Kocatepe University Research Fund (Project No: 15.FEN.BIL.20) for its financial support. The authors gratefully acknowledge the Biotechnology Unit of the Afyon Kocatepe University Central Research Laboratory and the DEKART Proteomics Laboratory of Kocaeli University for their technical help. The authors also wish to thank Afyon Kocatepe University's Foreign Language Support Unit for language editing.

References

Ahsan N, Komatsu S (2009). Comparative analyses of the proteomes of leaves and flowers at various stages of development reveal organ-specific functional differentiation of proteins in soybean. Proteomics 9: 4889-4907.

Ahsan N, Lee DG, Alam I, Kim P, Lee J, Ahn YO, Kwak SS, Lee IJ, Bahk JD, Kang KY et al. (2008). Comparative proteomic study of arsenic-induced differentially expressed proteins in rice roots reveals glutathione plays a central role during As stress. Proteomics 8: 3561-3576.

Baerenfaller K, Grossmann J, Grobei MA, Hull R, Hirsch-Hoffmann M, Yalovsky S, Zimmermann P, Grossniklaus U, Gruissem W, Baginsky S (2008). Genome-scale proteomics reveals *Arabidopsis thaliana* gene models and proteome dynamics. Science 320: 938-941.

Béthune J, Wieland F, Moelleken J (2006). COPI-mediated transport. J Membrane Biol 211: 65-79.

Bradford MM (1976). Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein–dye binding. Anal Biochem 72: 248-254.

Candiano G, Bruschi M, Musante L, Santucci L, Ghiggeri GM, Carnemolla B, Orecchia P, Zardi L, Righetti PG (2004). Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis. Electrophoresis 25: 1327-1333.

Carrari F, Fernie AR (2006). Metabolic regulation underlying tomato fruit development. J Exp Bot 57: 1883-1897.

Chen L, Chen Q, Zhu Y, Hou L, Mao P (2016). Proteomic identification of differentially expressed proteins during alfalfa (*Medicago sativa* L.) flower development. Frontiers in Plant Science 7: 1-13.

Dafny-Yelin M, Guterman I, Menda N, Ovadis M, Shalit M, Pichersky E, Zamir D, Lewinsohn E, Adam Z, Weiss D et al. (2005). Flower proteome: changes in protein spectrum during the advanced stages of rose petal development. Planta 222: 37-46.

Davis PH, Mill RR, Kit T (1988). Flora of Turkey and the East Aegean Islands. Edinburgh, UK: Edinburgh University.

Davis SJ (2009). Integrating hormones into the floral-transition pathway of *Arabidopsis thaliana*. Plant Cell Environ 32: 1201-1210.

Dettmer J, Schubert D, Calvo-Weimar O, Stierhof YD, Schmidt R, Schumacher K (2005). Essential role of the V-ATPase in male gametophyte development. Plant J 41: 117-124.

Domagalska MA, Sarnowska E, Nagy F, Davis SJ (2010). Genetic analyses of interactions among gibberellin, abscisic acid, and brassinosteroids in the control of flowering time in *Arabidopsis thaliana*. PLoS One 5: e14012.

Fait A, Angelovici R, Less H, Ohad I, Urbanczyk-Wochniak E, Fernie AR, Galili G (2006). *Arabidopsis* seed development and germination is associated with temporally distinct metabolic switches. Plant Physiol 142: 839-854.

Faurobert M, Mihr C, Bertin N, Pawlowski T, Negroni L, Sommerer N, Causse M (2007). Major proteome variations associated with cherry tomato pericarp development and ripening. Plant Physiol 143: 1327-1346.

Hecht V, Foucher F, Ferrándiz C, Macknight R, Navarro C, Morin J, Vardy ME, Ellis N, Beltrán JP, Rameau C et al. (2005). Conservation of *Arabidopsis* flowering genes in model legumes. Plant Physiol 137: 1420-1434.

Hochholdinger F, Sauer M, Dembinsky D, Hoecker N, Muthreich N, Saleem M, Liu Y (2006). Proteomic dissection of plant development. Proteomics 6: 4076-4083.

Hurkman WJ, Tanaka CK (1986). Solubilization of plant membrane proteins for analysis of two-dimensional gel electrophoresis. Plant Physiol 81: 802-806.

Jung CH, Wong CE, Singh MB, Bhalla PL (2012). Comparative genomic analysis of soybean flowering genes. PLoS One 7: e38250.

Kim ST, Cho KS, Jang YS, Kang KY (2001). Two-dimensional electrophoretic analysis of rice proteins by polyethylene glycol fractionation for protein arrays. Electrophoresis 22: 2103-2109.

Komatsu S, Hossain Z (2013). Organ-specific proteome analysis for identification of abiotic stress response mechanism in crop. Frontiers in Plant Science 4: 71.

Laitinen RA, Immanen J, Auvinen P, Rudd S, Alatalo E, Paulin L, Ainasoja M, Kotilainen M, Koskela S, Teeri TH et al. (2005). Analysis of the floral transcriptome uncovers new regulators of organ determination and gene families related to flower organ differentiation in *Gerbera hybrida* (Asteraceae). Genome Res 15: 475-486.

Lokhande SD, Ogawa K, Tanaka A, Hara T (2003). Effect of temperature on ascorbate peroxidase activity and flowering of *Arabidopsis thaliana* ecotypes under different light conditions. J Plant Physiol 160: 57-64.

Méndez M, Traveset A (2003). Sexual allocation in single-flowered hermaphroditic individuals in relation to plant and flower size. Oecologia 137: 69-75.

Mittler R, Vanderauwera S, Gollery M, Van Breusegem F (2004). Reactive oxygen gene network of plants. Trends Plant Sci 9: 490-498.

Muñoz-Fambuena N, Mesejo C, Reig C, Agustí M, Tárraga S, Lisón P, Iglesias DJ, Primo-Millo E, González-Mas MC (2013). Proteomic study of 'Moncada' mandarin buds from on- versus off-crop trees. Plant Physiol Bioch 73: 41-55.

Nakamura Y, Teo NZW, Shui G, Chua CHL, Cheong WF, Parameswaran S, Koizumi R, Ohta H, Wenk MR, Ito T (2014). Transcriptomic and lipidomic profiles of glycerolipids during *Arabidopsis* flower development. New Phytol 203: 310-322.

Ó'Maoiléidigh DS, Graciet E, Wellmer F (2014). Gene networks controlling *Arabidopsis thaliana* flower development. New Phytol 201: 16-30.

Özdemir C, Dural H, Ertuğrul K, Küçüködük M, Baran P, Şanda MA (2008). Morphology and anatomy of endemic *Thermopsis turcica* Kit Tan, Vural & Küçüködük. Bangladesh J Botany 37: 105-114.

Padmanaban S, Lin X, Perera I, Kawamura Y, Sze H (2004). Differential expression of vacuolar H^+-ATPase subunit c genes in tissues active in membrane trafficking and their roles in plant growth as revealed by RNAi. Plant Physiol 134: 1514-1526.

Peters JM (1994). Proteasomes: protein degradation machines of the cells. Trend Biochem Sci 19: 377-382.

Rocco M, D'Ambrosio C, Arena S, Faurobert M, Scaloni A, Marra M (2006). Proteomic analysis of tomato fruits from two ecotypes during ripening. Proteomics 6: 3781-3791.

Rolland-Lagan AG, Bangham JA, Coen E (2003). Growth dynamics underlying petal shape and asymmetry. Nature 422: 161-163.

Sharma R, Agarwal P, Ray S, Deveshwar P, Sharma P, Sharma N, Nijhawan A, Jain M, Singh AK, Singh VP et al. (2012). Expression dynamics of metabolic and regulatory components across stages of panicle and seed development in *indica* rice. Funct Integr Genomics 12: 229-248.

Shima DT, Scales SJ, Kreis TE, Pepperkok R (1999). Segregation of COPI-rich domains in endoplasmic-reticulum-to-Golgi transport complexes. Curr Biol 9: 821-824.

Singh VK, Garg R, Jain M (2013). A global view of transcriptome dynamics during flower development in chickpea by deep sequencing. Plant Biotechnol J 11: 691-701.

Sinha P, Poland J, Schnölzer M, Rabilloud T (2001). A new silver staining apparatus and procedure for matrix-assisted laser desorption/ionization-time of flight analysis of proteins after two-dimensional electrophoresis. Proteomics 1: 835-840.

Strompen G, Dettmer J, Stierhof YD, Schumacher K, Jürgens G, Mayer U (2005). Arabidopsis vacuolar H^+-ATPase subunit E isoform 1 is required for Golgi organization and vacuole function in embryogenesis. Plant J 41: 125-132.

Sweetlove LJ, Heazlewood JL, Herald V, Holtzapffel R, Day DA, Leaver CJ, Millar AH (2002). The impact of oxidative stress on *Arabidopsis* mitochondria. Plant J 32: 891-904.

Tan K, Vural M, Küçüködük M (1983). An unusual new *Thermopsis* from Turkey. Notes from the Royal Botanic Garden of Edinburgh 40: 515-518.

Tucker SC (2003). Floral development in legumes. Plant Physiol 131: 911-926.

Uysal T, Ertuğrul K, Bozkurt M (2014). A new genus segregated from *Thermopsis* (Fabaceae: Papili onoideae): *Vuralia*. Plant Syst Evol 300: 1627-1637.

Wojciechowski MF (2003). Reconstructing the phylogeny of legumes (Leguminosae): an early 21st century perspective. In: Klitgaard BB, Bruneau A, editors. Advances in Legume Systematics, Part 10, Higher Level Systematics. Kew, UK: Royal Botanic Gardens, pp. 5-35.

Yang M, Zhu L, Xu L, Pan C, Liu Y (2014). Comparative transcriptomic analysis of the regulation of flowering in temperate and tropical lotus (*Nelumbo nucifera*) by RNA-Seq. Ann Appl Biol 165: 73-95.

Ye ZZ, Rodriguez R, Tran A, Hoang H, de los Santos D, Brown S, Vellanoweth RL (2000). The developmental transition to flowering represses ascorbate peroxidase activity and induces enzymatic lipid peroxidation in leaf tissue in *Arabidopsis thaliana*. Plant Sci 158: 115-127.

Zhang D, Ren L, Yue JH (2013). A comprehensive analysis of flowering transition in *Agapanthus praecox* ssp. *orientalis* (Leighton) Leighton by using transcriptomic and proteomic techniques. J Proteom 80: 1-25.

Zhang H, Wei Q, Li C, Jiang C, Zhang H (2016). Comparative proteomic analysis provides insights into the regulation of flower bud differentiation in *Crocus sativus* L. J Food Biochem 40: 567-582.