Label-free quantitative proteomic analysis of alfalfa in response to microRNA156 under high temperature

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Abstract

Background: Abiotic stress, including heat, is one of the major factors that affect alfalfa growth and forage yield. The small RNA, microRNA156 (miR156), regulates multiple traits in alfalfa during abiotic stress. The aim of this study was to explore the role of miR156 in regulating heat response in alfalfa at the protein level.

Results: In this study, we compared an empty vector control and miR156 overexpressing (miR156OE) alfalfa plants after exposing them to heat stress (40 °C) for 24 h. We measured physiological parameters of control and miR156OE plants under heat stress, and collected leaf samples for protein analysis. A higher proline and antioxidant contents were detected in miR156OE plants than in controls under heat stress. Protein samples were analyzed by label-free quantification proteomics. Across all samples, a total of 1878 protein groups were detected. Under heat stress, 45 protein groups in the empty vector plants were significantly altered (P < 0.05; |log2FC| > 2). Conversely, 105 protein groups were significantly altered when miR156OE alfalfa was subjected to heat stress, of which 91 were unique to miR156OE plants. The identified protein groups unique to miR156OE plants were related to diverse functions including metabolism, photosynthesis, stress-response and plant defenses. Furthermore, we identified transcription factors in miR156OE plants, which belonged to squamosa promoter binding-like protein, MYB, ethylene responsive factors, AP2 domain, ABA response element binding factor and bZIP families of transcription factors.

Conclusions: These results suggest a positive role for miR156 in heat stress response in alfalfa. They reveal a miR156-regulated network of mechanisms at the protein level to modulate heat responses in alfalfa.

Keywords: Alfalfa, Heat stress, miR156, Proteomic, LC-MS/MS

Background

Alfalfa (Medicago sativa L.) is an important leguminous crop that is grown worldwide as forage for livestock feed, and contributes to improved soil quality. Another important feature of this crop is the potential for multiple harvests throughout the growing season, allowing for abundant biomass yield. Perennial nature of alfalfa and its rapid biomass production make it a suitable source for bioenergy purpose. However, these benefits are offset by alfalfa’s susceptibility to environmental stresses including heat, which exerts adverse effects on its growth and productivity [1]. High temperature can negatively affect plant growth and development including reduced seed germination [2], damage to leaves and branches, increase in leaf senescence, discoloring of fruits, which ultimately leads to poor crop yield [3]. Climate models have predicted an increase in seasonal temperatures globally, which may have a negative impact on
crop growth, productivity and ultimately food security [4]. Developing alfalfa cultivars with improved heat stress tolerance could provide a sustainable solution to the unpredictable changes in the environmental conditions.

Molecular approaches have widely been used for dissecting the underlying biological and cellular processes under abiotic stress in plants [5–8]. Proteomic approaches have increasingly been used in plant research, and in particular to study abiotic stress responses as changes in protein abundance play a vital role in stress tolerance [9–12]. Proteomic analysis is a powerful technique to study gene products (proteins) at the molecular level [13, 14], and these have been used to study the underlying molecular and physiological processes for heat stress tolerance in different plant species. For example, a proteomic study showed that abundance of heat shock proteins (HSPs) and antioxidant enzymes were increased in heat-stressed leaves of rice [15]. Moreover, a heat stress-induced abundance of various protein groups involved in protein biosynthesis, degradation, and carbohydrate metabolism was reported in rice [16]. A similar study in grapevine showed that the abundance of HSPs and proteins involved in metabolism and signal transduction was significantly altered under heat stress [17]. Similarly, another study reported the differential abundance of 81 protein groups under heat stress in alfalfa. These proteins belonged to important functional categories such as metabolism, energy, protein synthesis, signal transduction and defense [6].

The microRNAs (miRNAs) are key regulators of gene expression at both the transcriptional and post-transcriptional levels [18, 19]. These miRNAs are approximately 18–24 nt long and are grouped based on the differences in their biogenesis and functional characteristics [20]. Recently, microRNA156 (miR156) has emerged as an effective molecular tool for trait improvement in different plant species including alfalfa. For example, miR156 overexpression increased alfalfa biomass and delayed flowering [21, 22]. Moreover, transcriptome analysis of miR156 overexpressing (miR156OE) alfalfa under drought revealed potential miR156 targets, and subsequent characterization confirmed its role in drought tolerance [23]. Major transcription factors regulated by miR156 belong to Squamosa Promoter Binding Protein-Like (SPL) family [24]. Previously, we identified miR156 target SPL genes, and characterized their functions in alfalfa, including their role in drought and salinity responses [25–27]. Despite a series of miR156-related studies in different plant species, there has been no reported proteome analysis on miR156OE alfalfa under heat stress. Proteome analysis of contrasting alfalfa genotypes under heat stress conditions could provide an insight into the underlying molecular mechanisms that control different physiological and molecular traits in alfalfa.

Accumulation of osmoprotectants, such as proline, is an important physiological mechanism that helps plants scavenge reactive oxygen species (ROS) to cope with heat-related oxidative stress [28, 29]. Proline helps plants keep a fully functional photosynthetic apparatus by stabilizing the photosynthetic complex II as well as membrane proteins such as rubisco [30]. Studies have shown that proline accumulation improves heat stress tolerance in a range of plant species such as tomato [31], rice [32], chickpea [33] and barley [34].

Recently, our group has shown that miR156 overexpression resulted in an improved physiological response of alfalfa to heat stress [35]. This finding triggered our interest in expanding our research to dissect the role of miR156 in modulating the proteome of alfalfa in response to heat. We employed a label-free quantification (LFQ) based quantitative proteomics approach to explore the effects of heat stress on protein levels in miR156OE alfalfa. Our major objective was to identify miR156-regulated gene products with differentially altered abundance under heat stress. In the current study, miR156OE plants showed enhanced levels of stress tolerance predictors (antioxidants and proline) under heat stress. Moreover, this study revealed that metabolism, photosynthesis and defense were the major processes affected by miR156 under heat. This combination of biochemical and proteomic analyses with miR156 influence provided additional knowledge of heat tolerance mechanisms, thereby shedding a light on the pathways mediated by miR156 for heat stress response in alfalfa.

**Results**

Findings from our previous study that miR156 modulated heat stress response in alfalfa [35], prompted us to further study the molecular mechanism for regulation of heat stress tolerance. We conducted this research with an aim to identify proteins with differentially altered abundance modulated by miR156 under high temperature.

**Biochemical characterization of miR156OE alfalfa**

Plants produce free radicals in response to stress, and these can be harmful to cellular membrane and lipids. To counter the negative effect of these free radicals, plants synthesize antioxidants as a defense mechanism, which prevent cellular damage by quenching free radicals [36]. To explore whether miR156 alters the ability of alfalfa to produce antioxidants for defense, we determined total antioxidant contents in EV and A8 under non-stressed control and heat stress conditions. Overall, A8 showed a mild increase in antioxidant content under non-stress control and stress conditions compared to EV (Fig. 1a).
As a defence mechanism, plants synthesize an array of metabolites under stress conditions including amino acids such as proline. Proline is a compatible solute that can help plants to increase water uptake from soil. Higher levels of proline are beneficial for plants under abiotic stress conditions [36]. In the current study, no significant difference in proline accumulation was observed between non-stressed and stressed plants for the EV control in either leaf or root (Fig. 1b). On the other hand, a significant increase in proline accumulation was
detected in both heat-stressed leaf and root of A8 compared to corresponding non-stressed control plants (Fig. 1b).

**Alfalfa proteome is affected by miR156 under heat stress**

Previously, our group showed that overexpression of miR156 improved multiple physiological traits and altered the transcriptome profile of alfalfa [37]. Further investigations revealed a positive role for miR156 in abiotic stress tolerance, including drought [26, 38], salinity [27] and heat [35]. In addition, transcriptomic analysis showed that miR156 affects a wide array of gene families under drought stress in alfalfa [23]. We, therefore, set out to identify miR156-regulated gene products (proteins) with differentially altered abundance under heat stress in alfalfa. Across all samples, a total of 1878 protein groups were detected (online repository). To assess the effect of heat on the protein profile, protein abundance was compared between non-stressed control and heat-stressed plants of EV and A8. Results illustrated that the abundance of 12 proteins was significantly increased ($P < 0.05; \text{log2FC} > 1$), and that of 33 proteins was significantly decreased ($P < 0.05; \text{log2FC} < -1$), in EV under heat stress relative to the corresponding non-stressed plants (Fig. 2a, b; Table 1). On the other hand, almost six-fold number of proteins (73) showed significantly enhanced abundance, and 32 proteins showed reduced abundance in A8 under heat stress relative to the corresponding A8 non-stressed plants (Fig. 2a, b; Table 2). While a small number of the differentially altered proteins (14) were common to both genotypes, a total of 91 proteins exhibited differential abundance uniquely in A8 under heat stress (68 proteins increased; 23 proteins reduced) (Fig. 2; Tables 1 and 2).

The abundance of some proteins belonging to heat shock family was altered in both EV and A8 genotypes. Glutamine synthetase, fructose-bisphosphate aldolase (FBA), Photosystem II proteins and Glucose-6-phosphate 1-dehydrogenase (G-6-PDH), Calnexin, lethal leaf-spot protein, $\alpha$-galactosidase, $\beta$-galactosidase and Chitinase were among the major protein groups with

![Fig. 2](image-url)
| Protein ID | Locus name | Log2 (fold change) | FDR | annotation |
|------------|------------|--------------------|-----|------------|
| G7JFK1     | MTR_4g130540 | -7.83              | 0.0161 | Heat shock 70 kDa protein |
| Q2HTU2     | MTR_4g091590 | -7.00              | 0.0191 | 17.6 kDa class I heat shock protein |
| A0A072TL89 | MTR_00046051 | -5.80              | 0.0035 | Putative small heat shock protein HSP20 |
| A0A072UP91 | MTR_4g084250 | -4.83              | 0.0191 | Calcylin-binding protein |
| G7LF61     | MTR_8g012340 | -4.56              | 0.0275 | Peptidylprolyl isomerase |
| G7L491     | MTR_7g012820 | -4.37              | 0.0191 | Casein lytic proteinase B3 |
| G7KD12     | MTR_5g099410 | -4.14              | 0.0002 | Oxygen-evolving enhancer protein 2–1 |
| G7KG40     | MTR_5g078040 | -3.56              | 0.0375 | Peroxisomal small heat shock protein |
| G7L1Y9     | MTR_7g010000 | -3.45              | 0.0161 | ATP-dependent zinc metalloprotease FTSH protein |
| G7KNT7     | MTR_6g061940 | -3.28              | 0.0436 | 17.6 kDa class I heat shock protein |
| G7J8C7     | MTR_3g104780 | -3.23              | 0.0462 | 17.1 kDa class II heat shock protein |
| G7K4W1     | MTR_5g096970 | -3.13              | 0.0035 | Carboxy-terminal TIM barrel domain enolase |
| G7JMP4     | MTR_4g104300 | -3.10              | 0.0345 | F-box/RING/FB-like domain protein |
| G7RL3      | MTR_2g089340 | -2.95              | 0.0127 | Dihydroxyacetyl dehydratase |
| G7KG90     | MTR_5g012030 | -2.86              | 0.0127 | Putative Heat shock chaperonin-binding |
| A0A072VBG9 | MTR_2g084715 | -2.84              | 0.0018 | Putative transcription factor C3H family |
| G7L4S2     | MTR_7g088490 | -2.77              | 0.0327 | Proteasome subunit beta |
| A0A072UGC6 | MTR_5g073235 | -2.50              | 0.0190 | Uncharacterized protein |
| G7KGT1     | MTR_5g080450 | -2.32              | 0.0115 | Ribulose bisphosphate carboxylase/oxygenase activase |
| G7KW94     | MTR_7g093500 | -2.30              | 0.0277 | Activator of 90 kDa heat shock ATPase-like protein |
| G7JL07     | MTR_4g072110 | -2.19              | 0.0191 | Amidophosphoribosyltransferase |
| B7FLU4     | MTR_4g103790 | -1.99              | 0.0375 | NOP56-like pre RNA processing ribonucleoprotein |
| A0A072TVH5 | MTR_0009s039 | -1.95              | 0.0434 | Heat shock protein 81–2 |
| G7KWU8     | MTR_7g024390 | -1.93              | 0.0126 | Heat shock cognate 70 kDa protein |
| A0A072UQ41 | MTR_4g105490 | -1.60              | 0.0375 | Synaptobrevin-like protein |
| G7J3Q2     | MTR_3g080703 | -1.50              | 0.0478 | Molecular chaperone Hsp40/DnaJ family protein |
| G7KEN6     | MTR_5g097320 | -1.50              | 0.0115 | Heat shock protein 81–2 |
| G7JNG4     | MTR_4g074480 | -1.49              | 0.0399 | Anamorsin homolog |
| G7HD7      | MTR_2g082590 | -1.46              | 0.0345 | Thioredoxin |
| B7FKA1     | MTR_4g021570 | -1.43              | 0.0191 | Armadillo/beta-catenin-like repeat protein |
| A0A072VEGB | MTR_1g017380 | -1.28              | 0.0035 | Putative chaperonin Cpn60/TCP-1 family |
| G7NZ6      | MTR_1g031650 | -1.26              | 0.0191 | Calcium-dependent lipid-binding (CaLB domain) family |
| A0A072UL44 | MTR_4g063710 | -1.23              | 0.0044 | Heat shock cognate 70 kDa protein |
| G7I6D7     | MTR_1g011800 | 1.07               | 0.0184 | Plant/F18G18–200 protein |
| A0A072TU5F8| MTR_8g090002 | 1.55               | 0.0269 | Haloacid dehalogenase-like hydrolase |
| G7ICF3     | MTR_1g018510 | 2.52               | 0.0184 | Subtilisin-like serine protease |
| G7LM0      | MTR_2g017730 | 2.52               | 0.0393 | Heat shock 70 kDa protein |
| A0A072UMH4 | MTR_4g066170 | 2.55               | 0.0191 | Lipoxigenase |
| G7JCT4     | MTR_4g095360 | 2.79               | 0.0351 | Putative tripeptidyl-peptidase II |
| A0A072VFH5 | MTR_2g023540 | 2.98               | 0.0253 | S-adenosylmethionine reductase |
| G7AX3      | MTR_1g116270 | 3.19               | 0.0359 | Glutathione S-transferase |
| A0A072UZVS | MTR_3g078633 | 3.63               | 0.0191 | Enhanced disease susceptibility protein |
| A0A072U496 | MTR_7g113480 | 4.25               | 0.0044 | Xaa-pro aminopeptidase P |
known functions in plant stress response, and their abundance was differentially altered in A8 under heat stress (Table 2). These protein groups with altered abundance uniquely in miR156OE plants under heat stress (Table 2) could be potentially regulated by miR156 specifically under stress conditions.

**Gene ontology (GO) enrichment analysis**

Gene ontology enrichment analysis was performed to identify pathways that may be affected in miR156OE plants under heat stress. We observed a large difference in GO function category representation between EV and miR156 genotypes. There were 76 GO terms that were assigned to protein with altered abundance in EV under stress, of which about half (49%) were represented by proteins belonging to the cellular component category (Fig. 3a). Only four, six and five GO functional categories were detected in the biological process, cellular component and molecular function categories, respectively. Biological process included response to heat, protein folding, response to stimulus and seed germination (Fig. 3b). Plastid, external encapsulating structure, cell, catalytic complex, organelle and extrinsic component of membrane were present in the cellular component category (Fig. 3c). Moreover, ATP binding, unfolded protein binding, enzyme activator activity, hydrolase activity and endopeptidase activity represented the molecular function category (Fig. 3d).

On the other hand, 227 GO terms were assigned to proteins with altered abundance in miR156 overexpressing genotype A8, of which a larger portion was represented by biological process (Fig. 4a). Many of these GO terms may reflect traits that miR156 overexpression modulates under stress conditions. Of the 21 GO terms in the biological process; response to temperature stimulus, single-organism carbohydrate metabolism, plastid organization and coenzyme metabolism (Fig. 4b) were unique to miR156 overexpression and may be of particular interest for stress response. The function chloroplast made up one of the largest portions in the cellular component category (Fig. 4c). In addition, extracellular region and apoplast were also represented by this category (Fig. 4c). Among the 11 functions classified as molecular function; purine ribonucleoside triphosphate binding, catalytic activity and fructose-bisphosphate adolase activity (Fig. 4d) were the main terms unique to miR156.

**Transcription factor enrichment**

Transcription factor (TF) enrichment analysis was carried out for the 91 proteins unique to miR156 to explore the functional mechanism of miR156 transcriptional regulatory systems. A total of 37 TFs were predicted (Table 3) that may be affected by miR156 under heat stress conditions. Major transcription factor families included heat shock transcription factors, MYB transcription factors, ethylene responsive factor, TCP family transcription factor, squamosa promoter-binding-like protein (SPL), ABA response element-binding factor and bZIP transcription factor (Table 3).

**Discussion**

Current climate change models predict an increase in average surface temperatures of 3 °C to 5 °C in the next 5 to 10 decades. This may have deleterious effects on crop plant growth and productivity [39]. High temperature can cause devastating effects on various aspects of plant function and physiology as well as disruption of cellular homeostasis [40]. Our group has recently shown that heat stress exerted negative impact on alfalfa plants where EV control leaves looked droopy and brownish whereas miR156 overexpression plants (miR156OE) including A8 maintained green and normal phenotype [35]. Moreover, miR156OE plants showed increased accumulation of antioxidants and water potential under heat stress compared to control plants. These results provided evidence that overexpression of miR156 enhances alfalfa tolerance to heat stress [35].

In the current study, MaxLFQ algorithm was used to assemble protein abundance profiles with maximum possible information from MS signals [41]. Heat stress response of miR156OE alfalfa was compared with that of the empty vector EV control genotype in an attempt to identify heat stress-related proteins regulated by miR156, as well as to further elucidate the biochemical and molecular mechanisms of heat tolerance in alfalfa, which are discussed below.

**Physiological response of miR156OE alfalfa to heat stress**

High temperature can cause an array of physiological and biochemical changes in plants that adversely affect growth, development, and yield [40]. Plants have, however, evolved mechanisms to cope with environmental stressors. In response to heat stress, plants produce reactive oxygen species (ROS), which can serve as stress...
Table 2: Identified proteins with differentially altered abundance in miR156 overexpressing genotype (A8) under heat stress relative to the non-stress control A8 plants.

| Protein IDs | Locus name | Log2 (fold change) | FDR   | annotation                                           |
|-------------|------------|---------------------|-------|------------------------------------------------------|
| aG7FK1      | MTR_4g130540 | −7.85               | 0.0064 | Heat shock 70 kDa protein                            |
| A0A072U9J1  | MTR_6g452990 | −6.82               | 0.0080 | Heat shock protein 81–2                              |
| *A0A072T8L89| MTR_00046051 | −6.52               | 0.0207 | Putative small heat shock protein HSP20              |
| aG7KG40     | MTR_5g078040 | −4.57               | 0.0050 | Peroxisomal small heat shock protein                 |
| G7JGX6      | MTR_4g010130 | −4.40               | 0.0157 | Sterol regulatory element-binding protein            |
| G7IF74      | MTR_1g088640 | −4.31               | 0.0048 | Putative universal stress protein A                  |
| aG7IRL3     | MTR_2g089340 | −3.60               | 0.0076 | Dihydroxyacid dehydratase                           |
| aG7JFX8     | MTR_8g095680 | −3.33               | 0.0173 | Calnexin 2                                          |
| aG7JFY8     | MTR_4g063710 | −2.91               | 0.0173 | Heat shock cognate 70 kDa protein                    |
| aG7KFW8     | MTR_4g021570 | −1.58               | 0.0350 | Armadillo/beta-catenin-like repeat protein           |
| aG7L491     | MTR_7g012820 | −1.76               | 0.0380 | Putative RIN4, pathogenic type III effector          |
| G7JL455     | MTR_8g077530 | −1.68               | 0.0422 | Tubulin alpha chain                                 |
| G7JNF8      | MTR_7g085800 | −1.55               | 0.0069 | Peroxisomal membrane PEX14-like protein             |
| G7JNF8      | MTR_7g085800 | −1.51               | 0.0076 | Importin subunit alpha                              |
| G7JNF8      | MTR_7g012820 | −1.38               | 0.0105 | Mevalonate/galactokinase family protein             |
| *A0A072UL44 | MTR_4g063710 | −1.29               | 0.0173 | Heat shock cognate 70 kDa protein                    |
| G7JL455     | MTR_8g077530 | −1.20               | 0.0258 | Prohibitin                                          |
| G7JL455     | MTR_7g085800 | −1.19               | 0.0202 | Quinone-oxidoreductase-like protein                  |
| G7JL455     | MTR_7g012820 | −1.18               | 0.0301 | Proteasome subunit alpha type                       |
| A0A072TUS4  | MTR_8g099795 | −1.16               | 0.0292 | Heat shock 70 kDa protein                            |
| G7JN14      | MTR_1g025430 | −1.15               | 0.0173 | Heat shock protein 81–2                              |
| G7JN14      | MTR_1g025430 | −1.10               | 0.0173 | Mitochondrial Rho GTPase                            |
| G7JN14      | MTR_1g088670 | −1.03               | 0.0341 | Dicarboxylate carrier protein                        |
| G7JN14      | MTR_1g077480 | −1.00               | 0.0392 | Alpha-galactosidase                                  |
| G7JN14      | MTR_7g022440 | −0.97               | 0.0329 | Glucose-6-phosphate 1-dehydrogenase                 |
| G7JN14      | MTR_5g022300 | −0.97               | 0.0155 | Ferredoxin--NADP reductase, chloroplastic           |
| G7JN14      | MTR_7g005380 | −0.97               | 0.0269 | Pyruvate dehydrogenase E1 component subunit         |
| G7JN14      | MTR_3g070100 | −0.97               | 0.0337 | Putative sedoheptulose-bisphosphatase               |
| G7JN14      | MTR_4g131760 | −0.97               | 0.0096 | Glucose-1-phosphate adenyllytransferase             |
| Q45FF2      | MTR_2g017520 | −0.97               | 0.0880 | Q45FF2_MEDTR Pyridoxal S-phosphate synthase         |
| A0A072V8Q4  | MTR_2g046710 | −0.97               | 0.0144 | S-adenosylmethionine synthase                       |
### Table 2

Identified proteins with differentially altered abundance in miR156 overexpressing genotype (A8) under heat stress relative to the non-stress control A8 plants (Continued)

| Protein IDs | Locus name | Log2 (fold change) | FDR       | annotation |
|-------------|------------|--------------------|-----------|------------|
| G7ED1       | MTR_1g072220 | 1.36               | 0.0221    | Putative NAD(P)-binding domain-containing |
| A0A072UUQ2  | MTR_4g045890 | 1.39               | 0.0173    | Photosystem II biogenesis protein |
| A0A072ULB0  | MTR_4g071880 | 1.39               | 0.0203    | Fructose-bisphosphate aldolase |
| A0A072UDY2  | MTR_5g004680 | 1.40               | 0.0173    | Presequence protease |
| A0A072VPS5  | MTR_1g023120 | 1.41               | 0.0389    | Beta-galactosidase |
| A0A072V4D0  | MTR_3g112420 | 1.42               | 0.0392    | ATP-dependent protease LA (Lon) domain protein |
| G7BJZ9      | MTR_3g092720 | 1.44               | 0.0329    | Putative ribosomal protein S30Ae/sigma |
| G7L028      | MTR_7g026340 | 1.46               | 0.0114    | Glucan endo-1,3-beta-glucosidase-like protein |
| A0A072VMH0  | MTR_1g076570 | 1.47               | 0.0173    | 2-methyl-6-phytylbenzoquinone methyltransferase |
| A0A072VZ02  | MTR_3g498725 | 1.49               | 0.0185    | ATP-dependent Clp protease ATP-binding subunit |
| A0A072U1Q8  | MTR_7g066120 | 1.50               | 0.0188    | Fructose-1,6-bisphosphatase |
| A0A072TGR0  | MTR_0151s003 | 1.55               | 0.0444    | Inositol-1-monophosphatase |
| A9YWS0      | MTR_5g030950 | 1.56               | 0.0294    | Serine hydroxymethyltransferase |
| I3S8V0      | MTR_7g111860 | 1.57               | 0.0050    | Putative NAD(P)-binding domain-containing protein |
| G7LJDS      | MTR_8g070530 | 1.62               | 0.0105    | Phototropin-2 protein |
| G7LE33      | MTR_8g093770 | 1.65               | 0.0105    | 40S ribosomal protein S12 |
| A0A072UYTS  | MTR_3g068030 | 1.68               | 0.0202    | Ribulose bisphosphate carboxylase/oxygenase activase |
| G7LXK6      | MTR_8g018510 | 1.69               | 0.0086    | Lipoygenase |
| A0A072TXS2  | MTR_8g012565 | 1.73               | 0.0050    | 1-deoxy-D-xylulose 5-phosphate reductoisomerase |
| aG7JCT4     | MTR_4g095360 | 1.76               | 0.0130    | Putative tripeptidyl-peptidase II |
| G7L4Q1      | MTR_7g077800 | 1.76               | 0.0258    | Putative HAD-like domain-containing protein |
| G7K1Y1      | MTR_5g079460 | 1.80               | 0.0173    | PfkB family carbohydrate kinase |
| G7KGB6      | MTR_5g011990 | 1.82               | 0.0155    | Uncharacterized protein |
| G7KET9      | MTR_5g011220 | 1.84               | 0.0080    | PGK-like protein 1A |
| G7N2N9      | MTR_1g073130 | 1.85               | 0.0479    | Carboxy-terminal processing peptidase-like protein |
| G7K4T4      | MTR_5g066700 | 1.87               | 0.0258    | Fructose-bisphosphate aldolase |
| G7LA76      | MTR_8g074330 | 1.88               | 0.0130    | Chitinase (Class Ib) / Hevein |
| A0A072VNF5  | MTR_1g096240 | 1.90               | 0.0405    | Dihydrodipamide acetyltransferase |
| A0A072UL99  | MTR_4g071190 | 1.91               | 0.0202    | Uncharacterized protein |
| G7JBK8      | MTR_3g096290 | 1.91               | 0.0317    | Cyanobacterial and plant NDH-1 subunit O |
| G7K999      | MTR_5g009010 | 1.92               | 0.0117    | Putative THUMP domain-containing protein |
| G7L3H7      | MTR_8g088210 | 1.95               | 0.0302    | Aspartokinase-homoserine dehydrogenase |
| A0A072TEN7  | MTR_03805004 | 1.98               | 0.0173    | Putative nucleoid-associated protein YbaB/EbfC |
| A0A072VAN9  | MTR_2g090200 | 1.99               | 0.0048    | Photosystem II Psb27 protein |
| A0A072VRL6  | MTR_1g107340 | 2.02               | 0.0156    | Limonoid UDP glucosyltransferase, putative |
| A0A072UYV0  | MTR_4g088615 | 2.03               | 0.0076    | Putative ribosomal protein S5 |
| I3SSE5      | MTR_8g005175 | 2.04               | 0.0072    | Oxygen-evolving enhancer protein |
| G7JK55      | MTR_4g101750 | 2.06               | 0.0290    | Elongation factor G, chloroplastic |
| G7KDR6      | MTR_5g030020 | 2.09               | 0.0301    | Putative nucleotide-binding alpha-beta protein |
| A0A072TTP2  | MTR_8g080230 | 2.12               | 0.0166    | Lipoygenase |
| A0A072TLC8  | MTR_0003056  | 2.16               | 0.0340    | Carboxypeptidase |
| G7BJQ7      | MTR_3g108040 | 2.19               | 0.0080    | PsBP domain protein |
| G7JEX7      | MTR_4g068280 | 2.21               | 0.0048    | Putative trigger factor |
signals to trigger defense responses; at the same time, 
ROS can cause cellular damage [42]. To neutralize ROS, 
plants synthesize antioxidants that protect the cellular 
machinery by scavenging ROS [36]. Enhanced accumula-
tion of antioxidants positively correlates with stress tol-
erance in several plant species [26, 27, 36]. Previously, 
our group showed that miR156OE alfalfa accumulated 
increased levels of antioxidants under drought and saline 
conditions, and the plants exhibited resilience to these 
stresses [26, 27]. In the current study, the miR156OE 
plants exhibited improved antioxidant capacity, which 
may suggest that miR156 can exert a defense response 
against ROS under heat stress conditions, and this could 
potentially improve heat stress tolerance in alfalfa.

Elevated proline levels help plants cope with stress, 
and accumulation of proline indicates improved cellular 
metabolism and enzymatic activity [43]. In line with the 
previous study that was conducted on non-transgenic 
control alfalfa plants [5], our results showed a mild in-
crease in proline accumulation in leaf and root of 
miR156OE alfalfa, suggesting that miR156 may regulate 
the biosynthesis of this osmolyte in response to heat 
stress. In addition to affecting heat stress responses, we 
previously showed that genotype A8 accumulated higher 
proline and had elevated relative water content (RWC) 
under drought stress, and this genotype also displayed 
improved tolerance under this stress [26]. Together, 
these results support our current results that miR156 
modulates a wide variety of abiotic stresses including 
heat [25–27, 44]. Increased proline accumulation corre-
lates with higher RWC. A wheat genotype sensitive to 
drought exhibited reduced RWC at 30% of soil moisture, 
whereas RWC was not reduced in a drought tolerant 
genotype [45]. Similarly, a reduction in leaf water poten-
tial, stomatal conductance and transpiration rate, and an 
increase in leaf temperature and abscisic acid (ABA) 
level were observed in two genotypes of soybean under 
heat stress [46]. ABA is a stress hormone that triggers 
proline synthesis and helps plants combat stress condi-
tions by altering physiological and molecular responses 
[47]. It will, therefore, be interesting to find out how 
miR156 modulates these physiological traits and hor-
mone biosynthesis particularly ABA under heat stress.

### Functional processes affected by miR156 under heat stress

MicroRNAs have emerged as a vital component of post-
transcriptional regulation of genes involved in numerous 
growth, development and stress responses in plants. The 
inhibitory effect of abiotic stress on photosynthesis is 
mainly linked to stomatal conductivity and metabolic 
limitations that have widely been described in several

| Protein IDs | Locus name | Log2 (fold change) | FDR | annotation |
|-------------|------------|---------------------|-----|------------|
| G7JZK0      | MTR_5g071360 | 2.25                | 0.0173 | Asparagine synthetase [glutamine-hydrolyzing] |
| A0A072VDJ3  | MTR_2g105490 | 2.38                | 0.0130 | Putative ATPase, AAA-type, P-loop |
| A0A072UF41  | MTR_5g084030 | 2.39                | 0.0144 | Indole-3-glycerol phosphate synthase |
| G7JFL4      | MTR_4g130680 | 2.39                | 0.0329 | ATP phosphoribosyltransferase catalytic subunit |
| G7KEK7      | MTR_5g020640 | 2.47                | 0.0050 | Glucose-6-phosphate 1-epimerase |
| G7LAE7      | MTR_8g091410 | 2.51                | 0.0189 | Peptidylprolyl isomerase |
| A0A072UMH4  | MTR_4g066170 | 2.53                | 0.0033 | Lipoygenase |
| G7J5S7      | MTR_3g100500 | 2.70                | 0.0144 | Aspartic proteinase nepenthesin-like protein |
| G7KPU0      | MTR_6g088270 | 2.70                | 0.0207 | Elongation factor Ts, mitochondrial |
| G8A394      | MTR_3g073860 | 2.74                | 0.0290 | Acetyl-CoA carboxylase |
| A0A072US15  | MTR_7g117430 | 3.20                | 0.0064 | Eukaryotic aspartyl protease family protein |
| G7L4R0      | MTR_7g088340 | 3.23                | 0.0155 | Magnesium-protoporphyrin IX monomethyl ester |
| A0A072U496  | MTR_7g113480 | 3.26                | 0.0050 | Xaa-pro aminopeptidase P |
| A0A072VDS5  | MTR_1g052535 | 3.27                | 0.0270 | GTP-binding protein TypA/BipA |
| A0A072UMH6  | MTR_6g085010 | 3.55                | 0.0096 | Aspartic protease in GUARD CELL-like protein |
| G7J6G6      | MTR_3g11610  | 3.98                | 0.0110 | Photosystem II reaction center PsbP family protein |
| G7LAD5      | MTR_8g091320 | 4.13                | 0.0050 | Myo-inositol 1-phosphate synthase |
| B7FKA0      | MTR_5g035010 | 5.32                | 0.0080 | Polyketide cyclase/dehydrase and lipid transporter |
| A0A072VIV9  | MTR_1g052165 | 7.39                | 0.0219 | Esterase D, putative |

*Proteins that were common to A8 and EV genotypes under heat shock. All other proteins were unique to A8

FDR False discovery rate

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**Table 2** Identified proteins with differentially altered abundance in miR156 overexpressing genotype (A8) under heat stress relative to the non-stress control A8 plants (Continued)
other studies, including studies on heat shock response [19, 48, 49]. In our current study, a number of proteins with altered abundance became prominent when heat stress was imposed on controls and miR156OE plants. Although, the number of proteins with reduced abundance in miR156 during heat stress was similar to that of the control, the number of proteins with increased abundance was six times more than the controls. This suggests that miR156 may be activating proteins for various physiological processes to cope with heat stress conditions. Interestingly, there were only 10% proteins common between control and miR156OE genotype whose abundance was altered under heat, indicating that miR156 may be modulating abundance of several unique proteins under the stress. In the current study, miR156OE alfalfa proteins responded to heat stress by modifying physiological processes that represent major protein groups under heat stress.

**Photosynthesis**

A large portion of cellular component GO term in miR156, but not in control, consists of chloroplast, indicating that photosynthetic processes are being modulated by miR156. Interestingly, our recent publication has shown that miR156OE alfalfa exhibited increased chlorophyll content under heat stress in alfalfa [35], which supports the proteomic response of miR156OE alfalfa in the current study. Photosynthesis is one of the major processes affected by abiotic stress [36], and energy deficit is a common indicator of photosynthetic plants under stress [50]. Overall, stress reduces photosynthesis and respiration, which leads to energy deprivation and ultimately growth retardation and cell death [50]. PSII is a sensitive protein complex and its structure is altered under abiotic stress [51]. Some heat shock proteins (HSPs) are involved in protecting PSII under heat stress [15, 16, 52]. A previous study in alfalfa showed 23 proteins with altered abundance under heat stress, and these proteins belonged to the PSII and HSPs [5].

An increased abundance of the photosynthetic enzyme fructose-bisphosphate aldolase (FBA) during stress maintains the CO2 assimilation rate in alfalfa [5]. Enhanced FBA abundance specifically in miR156 genotype under heat stress highlights the role of miR156 in altering the abundance of these proteins and maintaining photosynthesis under high temperature in alfalfa. Some other photosynthesis-related proteins with enhanced abundance were also detected in this study, including the oxygen evolving enhancer protein (OEE). Abiotic stress, such as cold and heat, alter the abundance of...
OEE family in plants [53]. In several plant species, this protein abundance was altered under abiotic stress [54], and in the current study OEE abundance was increased specifically in miR156OE genotype upon heat treatment. This suggests that OEE may directly or indirectly be regulated by miR156 and contributes to stress tolerance in alfalfa.

**Metabolism**

Plants allocate a significant supply of C and N resources to the synthesis of metabolites under stress conditions to maintain adequate growth [55]. Increased metabolic activity may be a vital response to elevated temperature. A reduction in photosynthesis results in energy shortage, which leads to the enhancement of carbohydrate metabolism. Previous studies have shown enhanced expression of glutamine synthetase (GS) under abiotic stress conditions [56]. In the current study, increased GS abundance specifically in miR156 genotype under heat stress may indicate that miR156 regulates GS expression. GS plays a crucial role in ammonia assimilation, and increased expression of cytosolic GS enhanced photorespiration and contributed to photosynthesis protection under stress condition [57].

Our results showed an increased abundance of other proteins (e.g., G-6-PDH, Calnexin, beta-galactosidase and Chitinase) that were previously reported to play a role in abiotic stress tolerance in various plant species. For example, transgenic tobacco overexpressing two chitinases (CHIT33 and CHIT42) conferred tolerance to salinity and heavy metals without any detrimental effect on plant growth and development [58]. Calnexin (CNX) maintains calcium homeostasis in plants and overexpression of CNX in tobacco improved tolerance to dehydration and osmotic stress [59]. Overexpression of beta-galactosidase enhanced stress tolerance in Arabidopsis by increasing leaf area and reducing senescence [60], and we also observed an increased abundance of beta-galactosidase in miR156OE plants under heat stress. Moreover, our study revealed a reduced alpha-galactosidase abundance in alfalfa under stress conditions, and these results are consistent with the previous research that showed down-regulation of alpha-galactosidase and ultimately improved tolerance to low temperature in petunia [61]. These observations suggest that miR156 modulates...
heat stress response in alfalfa by regulating some important proteins involved in physiological and metabolic processes.

**Defense**

Heat shock proteins (HSPs) are low molecular weight chaperones that play a vital role in providing plants with protection against stress by re-establishing normal protein conformation and cellular homeostasis, as well as assisting in protein refolding under stress. Li et al. (2013) detected 19 alfalfa proteins that belonged to the HSP group, most of which showed increased abundance in response to heat stress in alfalfa [5]. In contrast, a decrease in abundance of all HSPs (except one) and small

| Transcription factor | Annotation |
|----------------------|------------|
| Medtr6g086805        | heat shock transcription factor |
| Medtr7g091370        | heat shock transcription factor |
| Medtr4g022370        | Dof domain zinc finger protein |
| Medtr3g077750        | Dof domain zinc finger protein |
| Medtr8g005960        | squamosa promoter-binding-like protein |
| Medtr2g099610        | MYB transcription factor MYB91 |
| Medtr2g043050        | ethylene-responsive transcription factor ERF017-like protein |
| Medtr5g016750        | ethylene response factor |
| Medtr4g111975        | MYB-like transcription factor family protein |
| Medtr4g119270        | ethylene response factor |
| Medtr7g015010        | TCP family transcription factor |
| Medtr2g067420        | myb transcription factor |
| Medtr7g010210        | R2R3-myb transcription factor |
| Medtr4g100630        | MYB-like transcription factor family protein |
| Medtr6g092540        | MYB-like transcription factor family protein |
| Medtr7g067080        | MYB transcription factor MYBS1 |
| Medtr7g083700        | B3 domain transcription factor |
| Medtr7g080460        | AP2-like ethylene-responsive transcription factor |
| Medtr4g108370        | TCP family transcription factor |
| Medtr1g084980        | phytochrome-interacting factor 3.1 |
| Medtr6g017055        | TCP family transcription factor |
| Medtr8g033250        | MADS-box transcription factor |
| Medtr1g102860        | heat shock transcription factor A3 |
| Medtr3g101870        | heat shock transcription factor |
| Medtr5g010680        | heat shock transcription factor B2A |
| Medtr5g082950        | AP2 domain class transcription factor |
| Medtr1g101810        | TCP family transcription factor |
| Medtr7g028160        | TCP family transcription factor |
| Medtr8g033070        | TCP family transcription factor |
| Medtr5g026210        | beta-amylase-like protein |
| Medtr1g062940        | myb transcription factor |
| Medtr1g080920        | transcription factor bZIP88 |
| Medtr7g104480        | ABA response element-binding factor |
| Medtr8g070820        | bZIP transcription factor family protein |
| Medtr1g022495        | BZIP transcription factor bZIP124 |
| Medtr4g070860        | BZIP transcription factor bZIP124 |
| Medtr7g029400        | BZIP transcription factor |
heat shock protein (sHSP) was detected under heat stress in both control and miR156 genotypes. Plants induce expression of HSPs as an adaptive strategy for tolerance to heat stress. There are however substantial variations of HSP expression patterns in different plant species and even between genotypes of the same species [62]. Expression of four rice HSPs was rapidly increased under heat stress but two HSPs showed reduced expression after 3 h of heat stress in the same study, indicating that different HSPs were regulated by different time patterns or by different signals and may be affiliated with different functions in response to heat [62]. A repressive function of HSPs in different signals and may be affiliated with different functions were regulated by different time patterns or by different signals and may be affiliated with different functions in response to heat [62].

The small HSPs are of particular interest since they appear to protect PS II and thylakoid membranes under heat stress in plants [64]. Two studies have demonstrated the role of sHSPs in protecting the photosynthesis machinery. For example, sHSP interacts with proteins of the thermolabile oxygen-evolving complex (OEC) of PS II in Chenopodium album [65]. Similarly, an increase in sHSP26 abundance was found to improve the photochemical efficiency of PS II under heat stress in tall fescue [66]. These observations suggest that sHSPs can alter OEC proteins of PS II, pinpointing an important role for sHSPs in modulating plant response under high temperature. Although sHSPs may play a substantial role in protecting photosynthetic proteins against stress, more research is still needed to understand the underlying mechanisms governing the regulation of their biosynthesis and physiological functions, including their role in heat tolerance in plants under the influence of miR156.

Environmental stress, including high temperature, causes a rapid and excessive accumulation of reactive oxygen species (ROS) in plants. Excessive levels of stress-induced ROS are removed by enzymatic and non-enzymatic antioxidants [36]. This study showed an increased abundance of G-6-PHD and CNX in miR156OE plants under heat stress, and this is consistent with previous studies, which have shed light on the role of CNX in ROS signaling, scavenging ROS and improving oxidative stress response in plants [59, 67]. Similarly, Liu et al. (2007) revealed that G-6-PDH plays a crucial role in nitric oxide-dependent defense against oxidative stress, resulting in improved salt tolerance in red kidney beans [67].

MicroRNA156 affects various transcription factors under heat stress

Transcription factors (TFs) play a crucial role in regulating molecular response under abiotic stress in plants. In the current study, we detected TCP, bZIP, ethylene-responsive factor (ERF) and SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE (SPL) by TF enrichment analysis, and our previous study showed an altered expression of these TFs under drought stress in miR156OE alfalfa [23]. This may indicate that miR156 regulates these TFs not only under drought but also heat stress conditions. The SPLs are known targets of miR156, and our recent studies have shown that reduced SPL13 expression improved drought [25] and heat [35] stress tolerance in alfalfa. Given the diversity of important TFs targeted by miR156, and the physiological traits affected by miR156 in alfalfa, it is critical to identify and characterize these TFs and their downstream targets to further elucidate the role of miR156-regulated network in stress tolerance.

Conclusion

In this study, we conducted label-free quantitative proteomics analysis on miR156OE alfalfa under heat stress. Our biochemical data showed that miR156OE plants accumulated higher levels of proline and antioxidants when exposed to elevated temperature (Fig. 5). Furthermore, LC-MS/MS analysis revealed differential abundance of a range protein groups in miR156OE plants under heat stress. We detected 91 proteins that were unique to miR156OE (undetected in EV alfalfa) and belong to critical functional groups such as plant defence, photosynthesis and metabolism. These proteins and identified TFs showed differentially altered abundance only under heat stress, and could potentially be regulated directly or indirectly by miR156 (Fig. 5). In summary, the results from this study have increased our understanding of miR156 and miR156-mediated regulation that could result in potential tangible targets for practical applications in alfalfa and related legume species to address abiotic stress limitations to agricultural productivity. Transcription factors play an important role in regulating the molecular response of plants to stress. Detection of expression changes by transcriptome sequencing analysis in alfalfa could identify genes and transcription factors involved in heat stress tolerance. Therefore, future research should focus on combining physiology with the transcriptome, metabolome, and proteome under the influence of miR156 to provide better insights into the crosstalk between different functional pathways and the regulatory mechanisms controlled by miR156 for heat tolerance in plants.

Methods

Plant material, experimental design, and heat treatment

A miR156 overexpressing (miR156OE) alfalfa genotype (A8) was generated by our group in a previous study [21]. Rooted stem cuttings were made from A8 and
empty vector (EV) control plant. Stem cuttings were transferred to 5 1/2" standard pots containing homogenized PRO-MIX® BX soil. Emerging plants were then grown on the bench in the greenhouse under a 16-h light/ 8-h dark regime and watered twice weekly. A randomized experiment was designed and a heat stress trial was started on two-month-old plants of EV and A8, which were randomly assigned to non-stressed control or heat treatments. Heat stress treatment was set up as described in our published study [35]. A minimum of three plants from each genotype were completely randomized in one growth cabinet for heat treatment after watering them to field capacity, whereas the same number of plants were kept in the greenhouse for the non-stress control experiment. Growth cabinet temperature for the heat stress treatment was set to 40 °C and the same photoperiod and light intensity were used as in the greenhouse. Whole plant shoot (above-ground portion) tissues from at least three plants from each of A8 and EV under non-stressed control (22 °C), and after 24 h of heat treatment (40 °C), were collected in separate falcon tubes which were immediately frozen in liquid nitrogen and stored at −80 °C for further analysis. Frozen shoot tissues from each plant were ground separately into a fine powder with mortar and pestle in liquid nitrogen. Samples required for protein extraction,
and proline and antioxidant assays were obtained from this fine powder mixture.

Proline and antioxidant assays
Proline and antioxidant assays were performed on at least three plants (three biological replicates) from each genotype (EV and A8) obtained from each non-stressed control and heat stressed conditions. Proline measurements were conducted by following a previously described protocol [68]. Briefly, 0.5 g leaf samples were homogenized in 10 mL of 3% sulfosalicylic acid followed by filtration through Whatman #2 filter paper. Subsequently, 2 mL of filtrate was mixed with an equal volume of acid-ninhydrin and glacial acetic acid and reacted at 100 °C for 1 h. The reaction was terminated by cooling on ice, followed by extraction with toluene. The proline content was determined by measuring the absorbance of the supernatant at 520 nm using a plate reader (BioTek). Antioxidant content was determined by measuring the absorbance of the supernatant at 520 nm using a plate reader (BioTek) and comparing the values to a standard curve as described in previously published study [26].

For antioxidant measurements, approximately 100 mg of samples were used by following the protocol of an antioxidant assay kit (Sigma-Aldrich, Oakville ON; catalogue number CS0790). Trolox standards were prepared following the protocol provided in the kit. Assays were conducted in a 96-well plate by reading the endpoint absorbance at 405 nm in the plate reader (BioTek). Antioxidant concentration in the samples was calculated by comparing it to Trolox standard curve [26].

Protein extraction, lysis, and digestion
Protein extraction was performed as described in Marx et al. (2016) [62]. Briefly, leaf samples from three non-stressed control and three heat stressed plants from each of the EV and A8 genotypes were ground into a powder using a mortar and a pestle in liquid nitrogen. The extraction buffer (290 mM sucrose, 250 mM TRIS (pH 7.6), 25 mM EDTA (pH 8.0), 10 mM KCl, 25 mM NaF, 50 mM Na pyrophosphate, 1 mM ammonium molybdate, 1 mM EDTA (pH 8.0), 10 mM KCl, 25 mM NaF, 50 mM Tris-HCl (pH 8), 30 mM NaCl, 1 mM CaCl₂, 20 mM sodium butyrate, 10 mM nicotinamide, a mini EDTA-free protease inhibitor, and phosphoSTOP phosphatase inhibitor), followed by additional sonication. The protein content was measured using a bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Waltham, MA). Subsequently, 75 μg protein were reduced with dithiothreitol and alkylated with 15 mM iodoacetamide. Protein digestion was carried out in two steps. First, LysC (Thermo Fisher Scientific) was added followed by incubation at 37 °C for 2.5 h. Second, samples were diluted using 50 mM Tris and 5 mM CaCl₂. Mass Spec-grade trypsin protease (Thermo Fisher Scientific) was then added to the mixture, which was incubated overnight at ambient temperature. The digestion reaction was quenched by bringing pH ~ 2 using trifluoroacetic acid, immediately desalted using Waters Oasis HLB (1 mL, 30 mg sorbent), and the eluent was dried by vacuum centrifugation. Samples were reconstituted in 75 μL of 0.1% formic acid and transferred to an HPLC vial.

The peptide digests were separated on an Easy-nLC 1000 nano-flow HPLC system equipped with a 2 cm Acclaim C18 PepMap™ trap column and a 75 μm × 25 cm Acclaim C18 PepMap™ analytical column (Thermo Scientific). The flow rate was held at 300 nL min⁻¹ throughout the run and 10 μL of the digest was injected. The mobile phase A (97%) (LC/MS Optima water, 0.1% formic acid) was first decreased to 90% over 4 min. Peptides were then eluted with a linear gradient of 10 to 40% mobile phase B (LC/MS Optima acetonitrile, 0.1% formic acid) over 150 min, followed by 40–90% over 10 min, and maintained constant for an additional 10 min. Each sample was then analyzed using a top 10, data-dependent acquisition method in the mass range of m/z 300–2000 using a Thermo Q-Exact Orbitrap mass spectrometer coupled to an Agilent 1290 HPLC system. The nanospray voltage was set at 2.4 kV, capillary temperature at 275 °C, and the S-lens radio frequency (RF) level at 70. The full scan was acquired at 70,000 resolution with an automatic gain control (AGC) of 1 × 10⁵ and a maximum injection time (IT) of 250 msec. The MS/MS scans were acquired at 17,500 resolution, AGC of 5 × 10⁵, maximum IT of 110 msec, intensity threshold of 1 × 10⁵, normalized collision energy of 27 and an isolation window of 1.7 m/z. Unassigned, singly charged, and > 4 charged peptides were not selected for MS/MS, and a 30 s dynamic exclusion was used.

LC-MS/MS data analysis

Data analysis with MaxQuant
Alfalfa (Medicago sativa) genome has not been sequenced and therefore we used its close relative Medicago truncatula for analysis. The Thermo® raw files were searched against the related species Medicago truncatula protein sequence database (Uniprot UP000002051, accessed December 21st 2019) with MaxQuant software (1.6.1.0) [41]. Searches were conducted using default precursor mass tolerances (20 ppm for first search and 4.5 for the main search). Trypsin and LysC were selected
were used as input to carry out GO enrichment analysis. Identified proteins were searched from the PlantRegMap using GO and TF enrichment analysis [71, 72]. Venn diagrams were generated using the Venny tool [74]. The data was imported into Perseus software (http://www.perseus-framework.org) for LFQ comparisons and missing values were imputed with default settings. Only protein groups with measured LFQ values in two of the three sample replicates were retained. The raw mass spectrometry data and the MAXQUANT output files were deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD019560.

GO and TF enrichment analysis

Identified proteins were annotated using the Uniprot database [70]. M. truncatula GO terms for the selected proteins were searched from the PlantRegMap using GO Term enrichment tool [71, 72], and all the proteins identified with significant altered abundance in this study were used as input to carry out GO enrichment analysis. The enriched GO terms were summarized and plotted following the published REVIGO protocol [37, 73]. The ratios of molecular functions, cellular component and biological process were calculated based on the number of GO terms. TF enrichment was performed by blasting Uniprot IDs of the 91 proteins unique to miR156 against M. truncatula TF database. The TFs were identified based on the functional transcription factor binding site feature [72]. Venn diagrams were generated using the Venny tool [74].

Statistical analysis

GraphPad Prism software (https://www.graphpad.com/scientific-software/prism/) was used to statistically test significance of the data. For comparisons between two groups, the Student t-test was used, whereas Perseus software was used to compare protein groups obtained from MaxQuant data.

Abbreviations

miR156: microRNA156; miR1560E: microRNA overexpression; FBA: Fructose-bisphosphate aldolase; G-6-PDH: Glucose-6-phosphate 1-dehydrogenase; LFQ: Label-free quantification

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Authors’ contributions

MA designed the project, conducted experiments and analyzed data. MA drafted the manuscript and handled the authors/reviewers comments. AP helped in protein extraction, and AJ helped with proteomic data analysis. JR performed the LC-MS/MS and MaxQuant analysis, and edited the manuscript. MYG and FM edited the manuscript. AH secured funding, oversaw the project and edited manuscript. All authors have read and approved the manuscript.

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Availability of data and materials

The raw mass spectrometry data and the MAXQUANT output files were deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD019560.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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