BMC Genomics

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Tandem mass tag (TMT)-based quantitative proteomics analysis reveals the different responses of contrasting alfalfa varieties to drought stress

Wenli Quan[1] and Xun Liu[1*]

Abstract

Background Drought stress restricts the growth, distribution and productivity of alfalfa (*Medicago sativa* L.). In order to study the response differences of alfalfa cultivars to drought stress, we previously carried out physiological and molecular comparative analysis on two alfalfa varieties with contrasting drought resistance (relatively drought-tolerant Longdong and drought-sensitive Algonquin). However, the differences in proteomic factors of the two varieties in response to drought stress still need to be further studied. Therefore, TMT-based quantitative proteomic analysis was performed using leaf tissues of the two alfalfa cultivars to identify and uncover differentially abundant proteins (DAPs).

Results In total, 677 DAPs were identified in Algonquin and 277 in Longdong under drought stress. Subsequently, we conducted various bioinformatics analysis on these DAPs, including subcellular location, functional classification and biological pathway enrichment. The first two main COG functional categories of DAPs in both alfalfa varieties after drought stress were 'Translation, ribosomal structure and biogenesis' and 'Posttranslational modification, protein turnover, chaperones'. According to KEGG database, the DAPs of the two alfalfa cultivars after drought treatment were differentially enriched in different biological pathways. The DAPs from Algonquin were enriched in 'photosynthesis' and 'ribosome'. The pathways of 'linoleic acid metabolism', 'protein processing in endoplasmic reticulum' and 'RNA transport' in Longdong were significantly enriched. Finally, we found significant differences in DAP enrichment and expression patterns between Longdong and Algonquin in glycolysis/glycogenesis, TCA cycle, photosynthesis, protein biosynthesis, flavonoid and isoflavonoid biosynthesis, and plant-pathogen interaction pathway after drought treatment.

Conclusions The differences of DAPs involved in various metabolic pathways may explain the differences in the resistance of the two varieties to drought stress. These DAPs can be used as candidate proteins for molecular breeding of alfalfa to cultivate new germplasm with more drought tolerance to adapt to unfavorable environments.

Keywords Drought stress, Alfalfa, Proteomic analysis, Functional classification, Biological pathway, Differential enrichment

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Background

Alfalfa (*Medicago sativa*) is a perennial leguminous crop with abundant crude protein and amino acids, which is an excellent forage grass for livestock [1]. It has been widely grown around the world because of its great

agronomical traits and prominent economic values, such as high biomass yield and low production cost [2, 3]. The deep root system of alfalfa plays important roles in preventing soil erosion [4] and improving soil infiltrability [5] in arid areas.

Drought is a major abiotic stress that limits plant growth, development and yield by causing various physiological and biochemical changes [6, 7]. Although alfalfa is a relatively drought-tolerant crop [8], water deficit still inhibits its photosynthesis [9], decreases amino acid content in nodules of alfalfa [10] and affects its distribution and productivity [11]. With the intensification of global warming and human activities, drought is becoming more and more severe around the world. Therefore, improving the drought resistance of alfalfa has become the main focus of improving its yield and quality [12, 13].

A multitude of studies have shown that cultivar-specific morphological, physiological and molecular responses to drought stress exist in alfalfa, which are responsible for significant differences in drought resistance among diverse alfalfa varieties [9, 14, 15]. For example, the plant height, number of shoots, stomatal resistance and biomass yield in drought tolerant alfalfa genotypes are different from that in sensitive ones under drought stress [16]. Under PEG-induced osmotic stress, *M. sativa* cv. Yazdi exhibits higher reducing sugar contents, higher antioxidant enzyme activities (such as SOD, POD, CAT and APX), and less electrolyte leakage and MDA contents, compared with *M. sativa* cv. Gharayonje [17]. It has been reported that two alfalfa cultivars, Zhong Mu and San Isidro, exhibit different drought resistance mechanisms at physiological, hormonal and metabolic levels [15]. In addition, the expression level of (iso)flavonoid biosynthesis-related genes in Wisfal (*Medicago sativa* ssp. *falcate* var. Wisfal) shoots was significantly higher than that in Chilean (*M. sativa* ssp. *sativa* var. Chilean), indicating that this variety may have stronger antioxidant capacity under the drought condition [9]. Ma et al. [18] revealed genotype-specific drought response mechanisms in two contrasting alfalfa seedlings using transcriptomic analysis and identified several candidate genes for engineering alfalfa plants with higher drought resistance. So far, lots of drought-responsive genes in alfalfa have been identified and characterized, and some of them are proved to be beneficial to enhance drought tolerance of transgenic plants [19–26]. For instance, the expression of *MsNTF2L* can be highly triggered by drought stress, and overexpression of *MsNTF2L* confers drought tolerance to transgenic alfalfa plants by modulating leaf water loss and promoting ROS removal [27]. However, the molecular mechanisms of plant response to drought stress are complex and interconnected [28]. The unknown molecular mechanisms of alfalfa in response to drought stress and the response differences of contrasting alfalfa varieties need to be further studied.

Proteomic methods have become a powerful tool to comprehensively understand plant physiological adaptation to stress [29–33]. Furthermore, comparative proteomics can better elucidate the molecular mechanisms of stress response in plants with different stress sensitivities under stress conditions [34–37]. Previously, the two-dimensional gel electrophoresis (2-DE)-based proteomic approaches were mainly employed to identify the differentially abundant proteins (DAPs) from alfalfa leaves or roots after drought [35, 38, 39], salt [40] or heat [41] stress treatment. However, the 2-DE-based proteomic methods have limitations in efficient identification and accurate quantification of proteins [4]. With the development of proteomics, quantitative proteomics based on tandem mass tags (TMTs) can identify more proteins and quantify them more accurately [31, 42]. TMTs are a set of stable isotope-coded chemical labels used to label peptides for simultaneous quantitation of the relative abundance of proteins in two different samples [43]. Li et al. [44] quantified a total of 6,704 proteins from two alfalfa varieties, MS30 (heat-tolerant) and MS37 (heat-sensitive), after high temperature stress through the TMT-based proteomic analysis. In total, 213 and 160 DAPs were identified from MS37 and MS30, respectively.

In our previous research, the adaptability of two alfalfa varieties, relatively drought-tolerant Longdong and drought-sensitive Algonquin, to long-term drought stress was compared at morphological, physiological and transcriptional levels [11]. To further explore the differential responses of different alfalfa varieties to drought stress at proteomic level, the above-mentioned alfalfa cultivars were used as experimental materials in this study. The TMT-based quantitative proteomic analysis, coupled with liquid chromatography-tandem mass spectrometry/mass spectrometry (LC–MS/MS), was employed to identify and quantify DAPs from the leaf tissues of two alfalfa cultivars under drought stress. Meanwhile, we performed comparative analysis of these DAPs using various bioinformatics software or database, including subcellular location, functional classification, biological pathway enrichment, etc.. These results will provide evidence to elucidate the tolerance differences of contrasting alfalfa varieties to drought stress.

Results

Statistical analysis of LC–MS/MS results

In this study, 12 samples (each genotype/treatment combination including three biological replicates) were taken for proteomic analysis after 8 days of

drought stress. Through LC–MS/MS analysis, a total of 842,765 spectra were obtained from Algonquin and Londong under control and drought conditions, and 123,115 matched spectra were available. Among them, 60,375 peptides were confirmed by spectrum analysis, including 53,742 unique peptides. Finally, 5,886 proteins were identified and 4,881 proteins were quantifiable in at least one of three biological replicates of different combinations (Fig. S1a, Table S1). Additionally, most of peptides were composed of 7 ~ 20 amino acids, which met the quality control requirements (Fig. S1b).

In order to evaluate the sample repeatability, relative standard deviation (RSD) and Person correlation coefficient were calculated using protein quantitative values. The RSD of each sample (the upper quartile) was less than 0.1 (Fig. S2a) and the Person correlation coefficients between the repeated samples were greater than 0.6 (Fig. S2b), which indicated that the protein quantitative repeatability of each sample is statistically consistent.

Identification of DAPs using TMT-based quantitative proteomics analysis

Using the criterion of fold change > 1.2 or < 1/1.2 (0.8333) and *P*-value < 0.05, a total of 518 DAPs were screened in A vs. B, with 258 up-regulated and 260 down-regulated proteins under control condition; 214 up-regulated and 147 down-regulated proteins were selected in C vs. D under drought stress (A, B represents untreated Longdong and Algonquin; C, D represents drought-treated Longdong and Algonquin, respectively). In Algonquin, drought treatment significantly affected the abundance of 677 proteins, of which 291 were up-regulated and 386 were down-regulated (D vs. B). However, fewer DAPs were modulated by drought stress in Longdong, including 145 up-regulated and 132 down-regulated (C vs. A) (Fig. 1a). The detailed description of DAPs in each comparison group was listed in Table S2. Overlapping analysis showed that 72 up-regulated and 40 down-regulated DAPs were jointly modulated by Longdong compared to Algonquin under control and drought conditions (A vs. B and C vs. D). After drought treatment, 57 up-regulated and

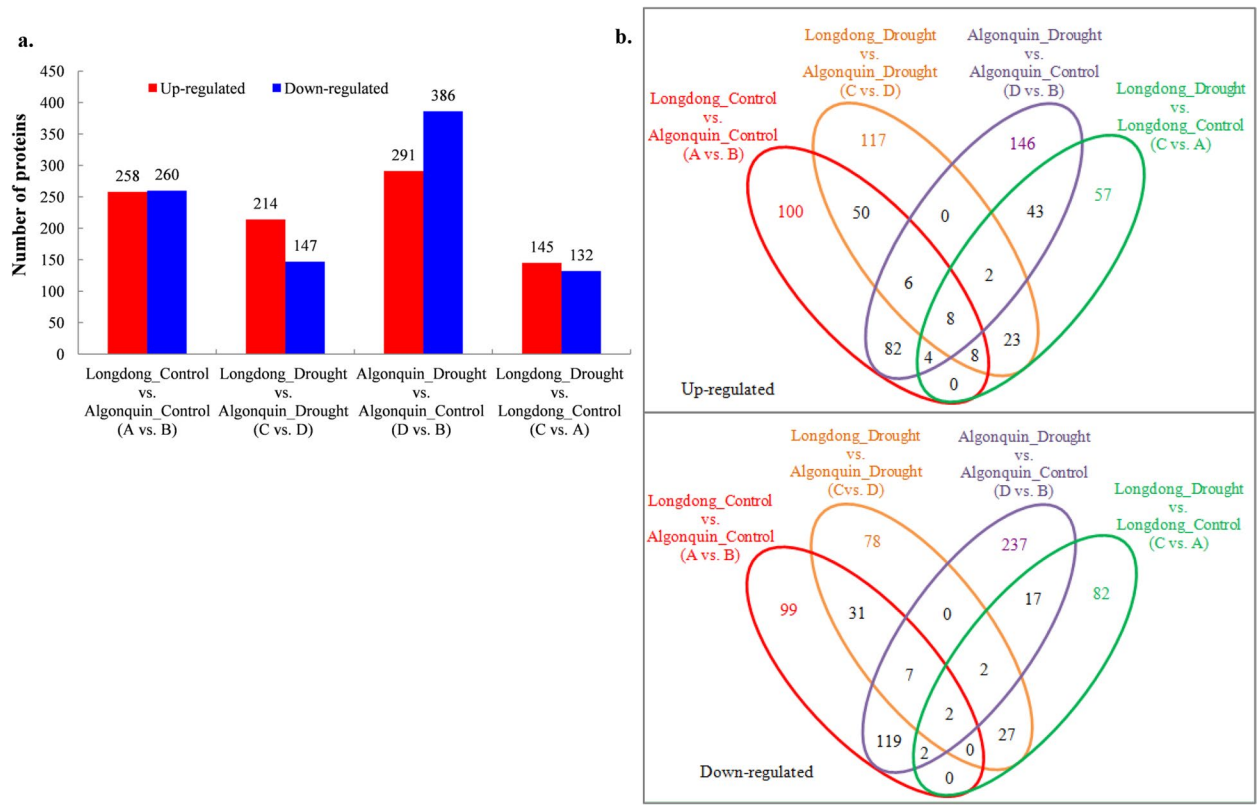


Fig. 1 Number of DAPs in the two alfalfa cultivars under drought stress. **a**: Number of up-and down-regulated DAPs in different comparison groups; **b**: Overlapping analysis of up- and down-regulated DAPs. The seedlings of relatively drought-tolerant Longdong and drought-sensitive Algonquin at 4-week-old were withheld water for 8 days. A: Longdong_Control; B: Algonquin_Control; C: Longdong_Drought; D: Algonquin_Drought

23 down-regulated DAPs were found in Longdong and Algonquin (D vs. B and C vs. A) (Fig. 1b), indicating that these DAPs are only induced by drought stress and might be independent of genotypes.

Subcellular location and COG functional classification of DAPs

The putative subcellular location of DAPs was predicted using WoLF PSORT database. The results showed that DAPs from the four comparison groups were mainly located in the chloroplast, cytoplasm and nucleus, accounting for more than 79% (Fig. 2). In addition, the DAPs were classified into various COG (Clusters of Orthologous Groups of proteins) functional categories in different comparison groups (Fig. 3). The top four COG categories in all the comparison groups were 'Translation, ribosomal structure and biogenesis', 'Posttranslational modification, protein turnover, chaperones', 'General function prediction only' and 'Carbohydrate transport and metabolism'. Interestingly, different from the other three comparison groups, the largest COG category in Algonquin under drought stress (D vs. B) group was 'Translation, ribosomal structure and biogenesis', containing 107 DAPs. Meanwhile, the DAPs in the functional category of 'Secondary metabolites biosynthesis, transport and catabolism' were also significantly enriched in Algonquin after drought treatment (D vs. B) (Fig. 3).

Gene Ontology (GO) enrichment of DAPs in the two alfalfa varieties

To determine the function of all the DAPs affected by drought stress in two alfalfa varieties, they were subjected to GO term enrichment-based clustering analysis in three sets of ontologies, including biological process (BP), cellular component (CC) and molecular function (MF). For BP GO terms, 'organic acid catabolic process', 'defense response to bacterium' and 'response to bacterium' were the most significantly enriched terms in A vs. B under control condition; 'inorganic ion homeostasis', 'mental ion transport', 'glyceraldehyde-3-phosphate metabolic process' and 'glucose 6-phosphate metabolic process' were the most significantly enriched terms in C vs. D under drought stress. In Algonquin, drought stress induced the most significant enrichment of 'peptide biosynthetic/metabolic process', 'cellular macromolecule biosynthetic process', 'amide biosynthetic process' and 'response to desiccation' (D vs. B). However, 'hydrogen peroxide catabolic process', 'regulation of cellular protein metabolic process' and 'regulation of proteolysis' were the mainly enriched terms induced by drought stress in Longdong (C vs. A) (Fig. 4a).

For CC GO terms, 'membrane protein complex', 'organelle subcompartment' and 'photosystem I' were the

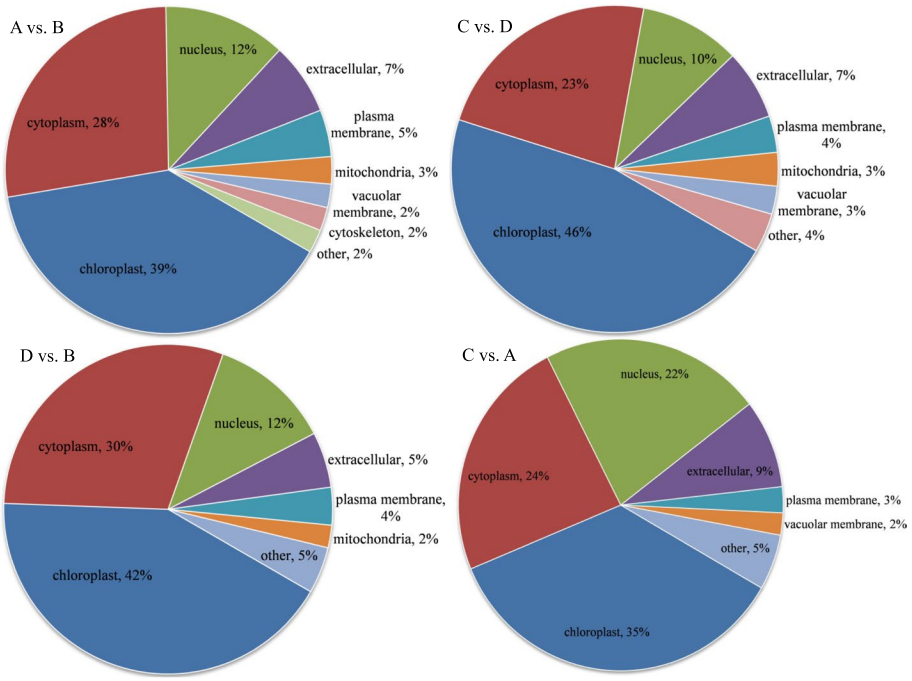
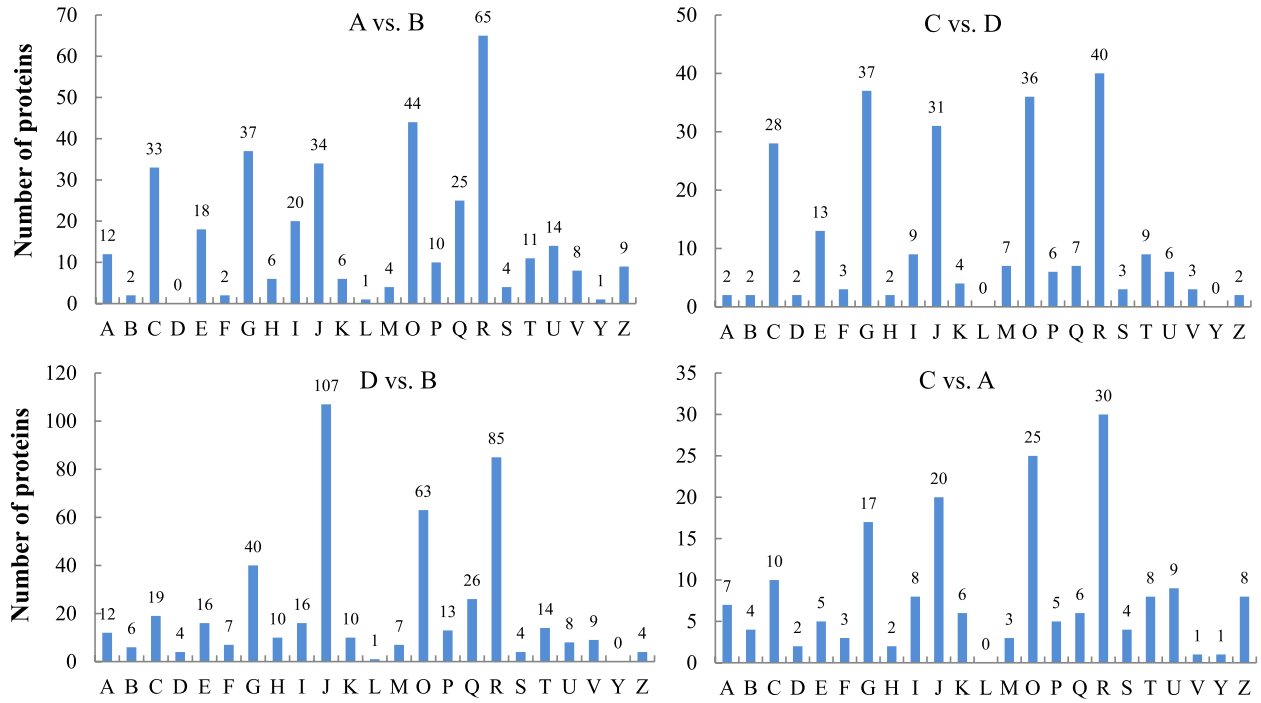


Fig. 2 Subcellular localization of DAPs in different comparison groups. The percentage represents the ratio of DAPs located in a specific subcellular structure to all DAPs in each comparison group. A: Longdong_Control; B: Algonquin_Control; C: Longdong_Drought; D: Algonquin_Drought



[A] RNA processing and modification
[B] Chromatin structure and dynamics
[C] Energy production and conversion
[D] Cell cycle control, cell division, chromosome partitioning
[E] Amino acid transport and metabolism
[F] Nucleotide transport and metabolism
[G] Carbohydrate transport and metabolism
[H] Coenzyme transport and metabolism
[I] Lipid transport and metabolism
[J] Translation, ribosomal structure and biogenesis
[K] Transcription
[L] Replication, recombination and repair
[M] Cell wall/membrane/envelope biogenesis
[O] Posttranslational modification, protein turnover, chaperones
[P] Inorganic ion transport and metabolism
[Q] Secondary metabolites biosynthesis, transport and catabolism
[R] General function prediction only
[S] Function unknown
[T] Signal transduction mechanisms
[U] Intracellular trafficking, secretion, and vesicular transport
[V] Defense mechanisms
[Y] Nuclear structure
[Z] Cytoskeleton

Fig. 3 COG functional categories of DAPs in different comparison groups. A: Longdong_Control; B: Algonquin_Control; C: Longdong_Drought; D: Algonquin_Drought

significantly enriched terms in A vs. B under control condition. Under drought stress, the most significantly enriched terms in C vs. D included 'organelle envelope', 'cytoplasmic part' and 'envelope'. A total of 12 significantly enriched terms were found in Algonquin after drought stress (D vs. B), including 'extrinsic component of membrane', 'photosystem I reaction center', 'photosystem II oxygen evolving complex' and 'ribosome'. By contrast, the DAPs from Longdong after drought treatment (C vs. A) were significantly enriched in 'extracelluar region', 'golgi membrane' and 'cytoskeleton' (Fig. 4b).

For MF GO terms, 'tetrapyrrole binding' and 'chlorophyll binding' were the most significantly enriched terms in A vs. B under control condition; however, seven terms were significantly enriched in C vs. D under drought stress, including 'calcium-dependent phospholipid binding', 'ferroxidase activity', '6-phosphogluconolactonase activity' and 'transporter activity'. The DAPs of Algonquin (D vs. B) were enriched in 'structural molecule activity', 'methyltransferase activity', 'structural constituent of ribosome' and 'rRNA binding'. Nevertheless, drought stress induced the enrichment of 'dioxygenase activity', 'protein dimerization activity', 'structural constituent of cytoskeleton' and 'peroxidase activity' in Longdong (C vs. A) (Fig. 4c).

Pathway enrichment of DAPs in the two alfalfa varieties

To uncover the biological mechanisms of DAPs, the cluster analysis of pathway enrichment was performed based on KEGG (Kyoto Encyclopedia of Genes and Genomes) database. As shown in Fig. 5, the prominently enriched pathways were different between up-regulated and down-regulated DAPs in four comparison groups. Compared with Algonquin, the enrichment of DAPs co-upregulated

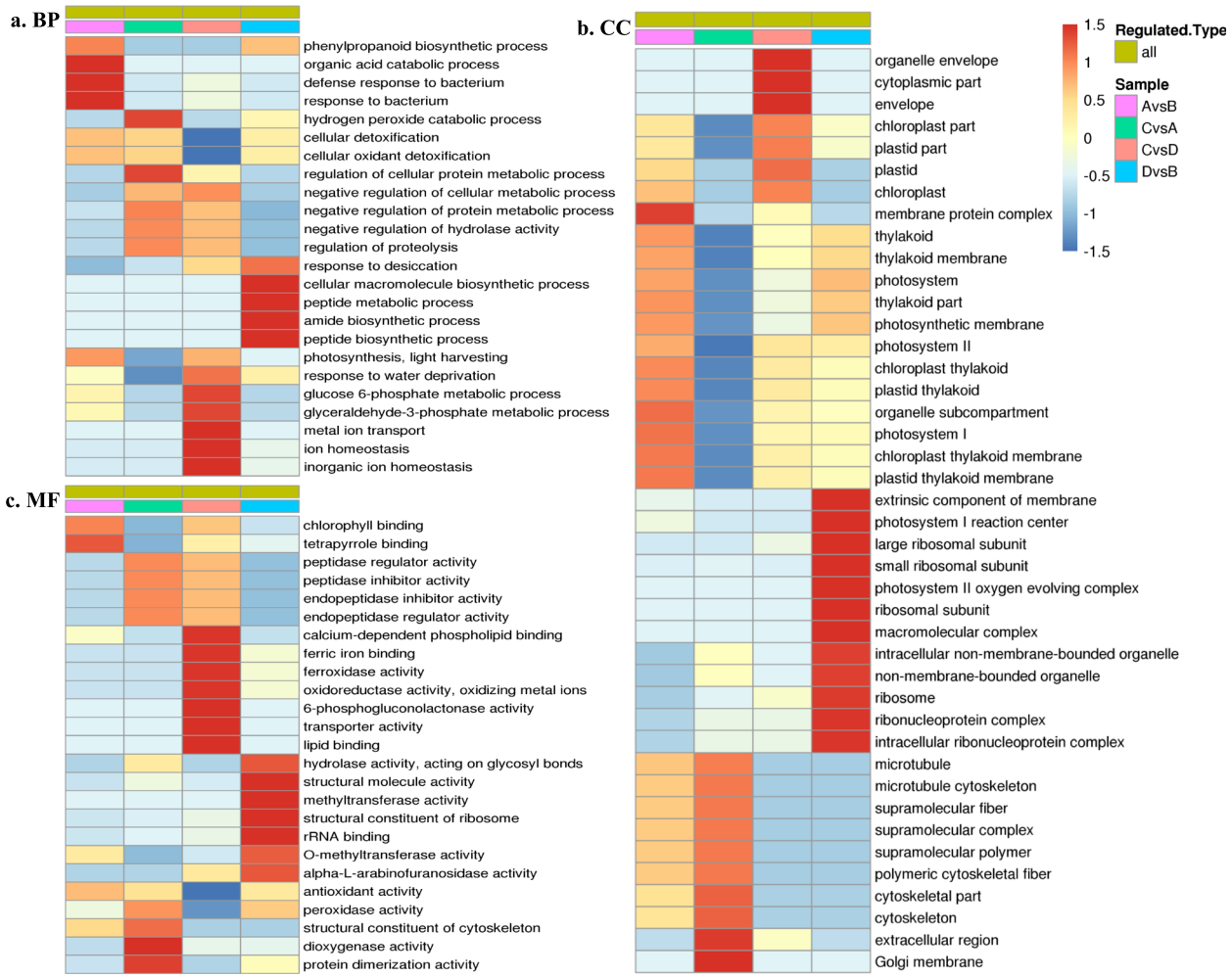


Fig. 4 GO enrichment-based clustering analysis of all DAPs in different comparison groups. Red indicates a strong degree of enrichment, and blue indicates a weak degree of enrichment. **a**: Biological process (BP); **b**: Cellular component (CC); **c**: Molecular function (MF). A: Longdong_Control; B: Algonquin_Control; C: Longdong_Drought; D: Algonquin_Drought

by Longdong under control and drought stress mainly occurred in the "photosynthesis-antenna protein" pathway (Fig. S3), and the co-downregulated DAPs were mainly enriched in the "carbon metabolism" pathway (A vs. B and C vs. D) (Fig. 5).

In Algonquin (D vs. B), the down-regulated DAPs were significantly enriched in 'ribosome' (Fig. S4) and 'flavonoid biosynthesis' pathways; while, the up-regulated DAPs were mainly involved in 'photosynthesis', 'phenylpropanoid biosynthesis', 'nitrogen metabolism', 'pyruvate metabolism' and 'alpha-linolenic acid metabolism' pathways. Additionally, in Longdong (C vs. A), the up-regulated DAPs were mainly associated with 'linoleic acid metabolism' and 'alpha-linolenic acid metabolism' pathways; the down-regulated DAPs were significantly enriched in 'phagosome', 'protein processing in endoplasmic reticulum' and 'RNA transport' pathways (Fig. 5).

To further investigate the effect of genotype or drought stress on enriched pathways of DAPs, the Classification SuperViewer Tool (http://bar.utoronto.ca/ntools/cgi-bin/ntools_classification_superviewer.cgi) was used to annotate these DAPs affected by Longdong (A vs. B and C vs. D) or drought stress (D vs. B and C vs. A) (Table S3). It was shown that 112 DAPs modulated by Longdong no matter under control or drought condition were enriched in 'PS', 'fermentation', 'OPP' and 'glycolysis' pathways. In addition, 'Gluconeogenesis/glyoxylate cycle', 'S-assimilation', 'major CHO metabolism' and 'metal handling' were the mainly enriched pathways for 80 DAPs regulated by drought stress no matter in Longdong or Algonquin (Table 1). In summary, the main enrichment pathways of DAPs in Longdong and Algonquin under drought stress were markedly different. The enriched pathways of DAPs

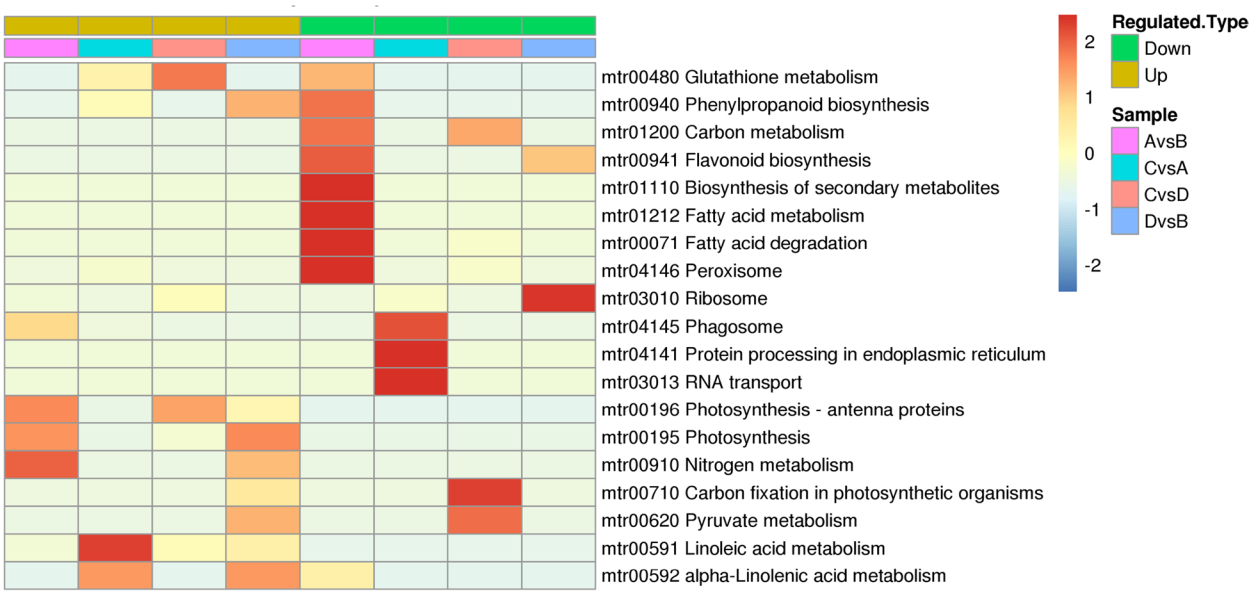


Fig. 5 Cluster analysis of KEGG pathway enrichment of up- and down-regulated DAPs in different comparison groups. Red means a strong degree of enrichment, and blue means a weak degree of enrichment. A: Longdong_Control; B: Algonquin_Control; C: Longdong_Drought; D: Algonquin_Drought

regulated by Longdong may play a role in explaining its stronger genotype-specific drought tolerance.

Protein–protein interaction (PPI) analysis of DAPs regulated by drought stress

To better study the regulatory mechanisms of DAPs and their roles in drought stress response, the top 50 DAPs with the closest interaction relationship and the proteins directly interacting with them were selected from D vs. B and C vs. A, respectively, based on STRING database (Table S4). In Algonquin, these DAPs were mainly associated with seven KEGG pathways, such as 'ribosome', 'photosynthesis', 'photosynthesis-antenna proteins', 'RNA transport' and 'purine metabolism' (Fig. 6a). For these DAPs in Longdong, the mainly enriched KEGG pathways included 'ribosome', 'phagosome', 'RNA transport', 'protein processing in endoplasmic reticulum' and 'alpha-linolenic acid metabolism' (Fig. 6b). Comparatively, in the two alfalfa varieties after drought stress, DAPs involved in 'ribosome' pathway had the closest interaction relationship with themselves or other proteins. Moreover, some DAPs might play a critical role in bridging the indirect interaction between other DAPs participating in different pathways, such as G717H7, G7IBJ3 (Fig. 6a) and B7FMD4 (Fig. 6b).

Validation of selected DAPs by quantitative real-time PCR

Proteins are the ultimate executors of various biological processes, and their expression changes can be reflected to some extent by gene expression changes. Quantitative real-time PCP (RT-qPCR) was used to detect the transcriptional expression of nine DAP-encoding genes to determine the correlation between mRNA and corresponding proteins. The selected DAPs involved in carbohydrate metabolism (AMY), protein metabolism (RP-L7 and HSPA1s), photosynthesis (LHCA3), stress and defense (SOD2, POD and Chitinase), and linoleic acid and alpha-linolenic acid metabolism (LOX1_5 and AOS) (Table S5). Compared to the control, the expression of four genes was increased by drought stress (Fig. 7a, f, h, i). The significant differences appeared in the expression of *AMY*, *AOS* and *Chitinase* between the control and drought conditions in the two alfalfa varieties (Fig. 7a, h, i). In addition, drought treatment caused significantly decrease in the expression of *HSPA1s* and *SOD2* in the both varieties (Fig. 7c, e). The results showed that the expression of most genes was consistent with the variation trend of corresponding proteins under drought stress (Fig. 7, Table S5).

Discussion

Drought is one of the main environmental factors that negatively affect plant growth, survival rate and productivity [11, 45]. As an excellent forage grass, alfalfa with deep root system is widely grown on dry and semi-dry lands due to its relatively high drought tolerance [46, 47]. However, different alfalfa varieties exhibit genotype-specific response to drought stress, leading to distinct

Table 1 MapMAN pathway enrichment of DAPs affected by genotypes or drought stress

MapMAN pathway	A vs. B and C vs. D		D vs. B and C vs. A	
	NF	*P*-value	NF	*P*-value
gluconeogenesis / glyoxylate cycle	/	/	35.40	**0.0280**
S-assimilation	/	/	35.40	**0.0280**
PS	27.45	**0.0000**	2.23	0.2880
fermentation	23.76	**0.0400**	/	/
OPP	21.46	**0.0038**	/	/
glycolysis	8.42	**0.0220**	/	/
mitochondrial electron transport / ATP synthesis	6.60	**0.0096**	/	/
redox	4.75	**0.0220**	2.19	0.2910
metal handling	4.00	0.1950	11.09	**0.0130**
secondary metabolism	3.74	**0.0088**	6.22	**0.0004**
major CHO metabolism	3.29	0.2250	13.67	**0.0013**
transport	2.60	**0.0083**	/	/
hormone metabolism	2.45	0.0570	8.50	**0.0000**
stress	2.43	**0.0078**	1.12	0.2240
misc	1.25	0.1420	2.31	**0.0140**
protein	0.61	**0.0340**	0.85	0.1230
not assigned	0.42	**0.0000**	0.31	**0.0000**
RNA	0.10	**0.0007**	0.60	0.1040
DNA	0.10	**0.0005**	0.44	0.0540

DAPs were annotated by the Classification SuperViewer Tool, and classified using MapMAN. 112 DAPs were commonly regulated by Longdong no matter under control or drought condition (A vs. B and C vs. D); and 80 DAPs were commonly regulated by drought stress no motter in Longdong or Algonquin (D vs. B and C vs. A). The original data were presented in Table S3. A: Longdong_Control; B: Algonquin_Control; C: Longdong_Drought; D: Algonquin_Drought. The scales of Normed Frequency (NF) are as follows: > 15 | 10-15 | 5-10 | 1-5 | < 1

resistance difference at morphological, physiological and molecular levels [13]. According to our previous results, the relatively drought-tolerant Longdong showed higher leaf water content, lower MDA and ROS contents, stronger antioxidant enzyme activities and higher transcription levels of some drought-related genes than the relatively drought-sensitive Algonquin [11]. In order to further elucidate the differential responses of the two alfalfa varieties to drought stress, comparative proteomic analysis based on TMT labeling technique was performed in the present research.

Effect of drought stress on protein profiling changes of the two alfalfa varieties

Using TMT-based quantitative analysis, 677 DAPs and 277 DAPs were respectively identified from Algonquin and Longdong under drought stress (Fig. 1a). Among them, 80 proteins were modulated by drought stress in both alfalfa varieties (Fig. 1b). The functions of some important DAPs and their major participation pathways (Table S6) were analyzed based on KEGG database in the following sections. Meanwhile, the differences between the two alfalfa varieties in response to drought stress at the proteomic level were compared and discussed.

Carbohydrate and energy metabolism-related proteins

Carbohydrates serve as storage products of energy in the form of starch and sucrose [48, 49], which are essential for biological processes and survival in plants.

Starch and sucrose metabolism

In this study, eight DAPs in Algonquin including six up-regulated and two down-regulated and six DAPs in Longdong including three up-regulated and three

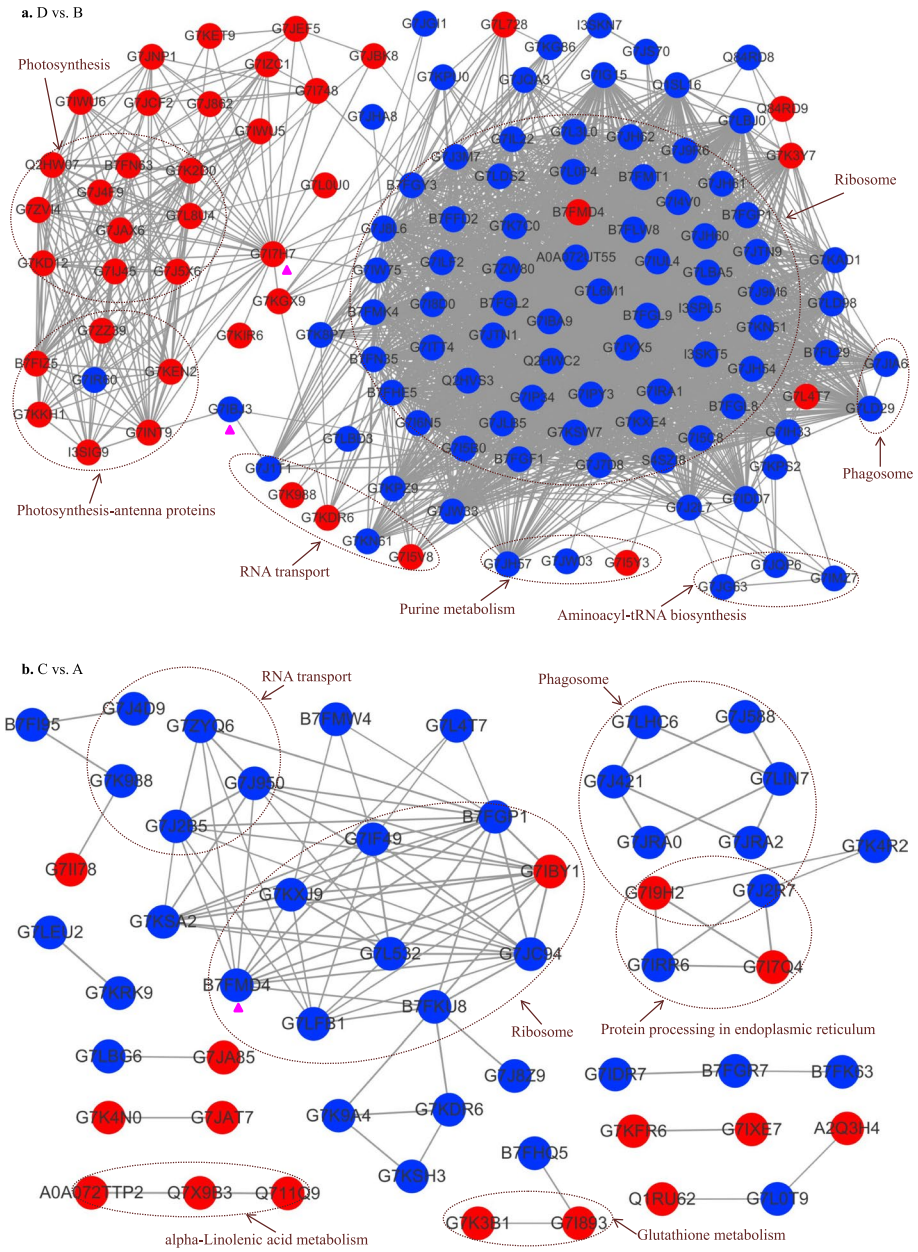


Fig. 6 Networks of DAPs regulated by drought stress in Algonquin (**a**) and Longdong (**b**). The red balls represent upregulated DAPs, and the blue balls represent downregulated DAPs. A: Longdong_Control; B: Algonquin_Control; C: Longdong_Drought; D: Algonquin_Drought

down-regulated were found to be associated with starch and sucrose metabolism. Starch metabolism plays critical roles in plant growth, development and stress responses and is restrained by a series of enzymes. Amylases, including α-amylase (AMY, EC 3.2.1.1) and β-amylase (BAM, EC 3.2.1.2), play key roles in the regulation of starch degradation. AMY and BAM hydrolyze starch to produce maltose, which is one of the important metabolites and osmotic substances. The activities and transcription of AMY and BAM could be modulated by certain stress factors, including drought, heat and cold stresses [48, 50]. Under drought stress, the expression levels of *CsAMY2* and *CsBAM1/3/4/5/9* were up-regulated at different points in tea (*Camellia sinensis*) plants after PEG-6000 treatment [48]. Similarly, our proteomic data showed that an AMY protein (A0A072TSJ2) was significantly enriched in the two alfalfa cultivars, and a BAM (A0A072UUL3) in Algonquin was also notably up-regulated under drought stress. The α-1,4 glucan phosphorylase (GP, EC 2.4.1.1) is commonly referred to as

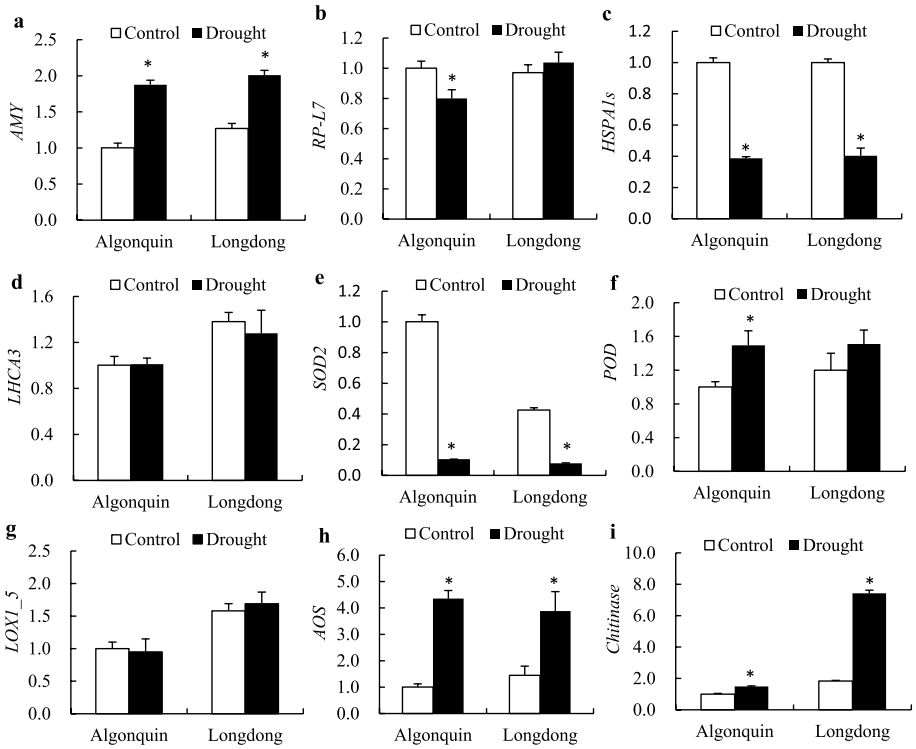


Fig. 7 Transcriptional expression of nine DAP encoding genes in leaves of two alfalfa varieties. (**a-i**) Expression changes of *AMY*, *RP-L7*, *HSPA1s*, *LHCA3*, *SOD2*, *POD*, *LOX1_5*, *AOS* and *Chitinase* genes in leaves of Algonquin and Longdong after 8 days of drought stress. The results shown are means ± SE ($n = 3$). Asterisk symbols above columns indicate that there were significant differences in gene expression at $P \leq 0.05$ (Student's *t*-test) after drought treatment compared with the control

starch phosphorylase in plants and is mainly known for the phosphorylated degradation of starch [51]. It plays a critical role in sustaining cellular and biological glucose homeostasis [52]. Pyeon et al. [53] confirmed that the *GP* gene was up-regulated in *Pleurotus ostreatus* accompanied with the increasing activity of glycogen phosphorylase under NaCl treatment. However, the expression of GP (G7JHG9) was up-regulated and down-regulated occurred in Algonquin and Longdong, respectively. Granule-bound starch synthase (GBSS, EC 2.4.1.242) is an important enzyme in starch biosynthesis, which is mainly responsible for the synthesis of amylose [54]. It was found that the accumulation of amylose, amylopection and total starch were reduced by drought stress in sorghum and wheat grains during grain filling stage in conjunction with the decreased starch synthase activities, including GBSS [55], especially in inferior spikelets in wheat [56]. The expression of *GBSS* gene was also down-regulated by high temperature and drought stress in wheat grains [57]. Here, the protein abundance of GBSS (A0A072TMC9) was significantly reduced in the two alfalfa varieties under drought stress, indicating that drought could affect alfalfa starch biosynthesis to a certain extent.

Sucrose content is a central factor controlling nutrient transport rate, which directly affects the crop growth, yield and quality [58]. Sucrose metabolism generally involves several enzymes, such as sucrose synthase (SuSy, EC 2.4.1.13) and sucrose phosphate synthase (SPS, EC 2.4.1.14). SPS plays a vital role in sucrose synthase from 6-phosphate fructose and uridine diphosphate-glucose (UDPG). Previously, it was shown that overexpression of *SPS* could increase sucrose synthesis in older leaves and promote plant growth and biomass accumulation [59–61]. Compared with the control condition, the abundance of SPS (G7JFF2) was significantly increased in Algonquin, which might be helpful for plant survival under drought stress. SuSy catalyzes reversible reactions of sucrose synthesis or cleavage (sucrose + NDP ↔ NDP-glucose + fructose), and it is generally believed that it mainly plays a role in sucrose degradation pathway [62]. The main function of SuSy is to provide substrate UDPG and indirect substrate adenosine diphosphate-glucose (ADPG) for the biosynthesis of cellulose, callose and starch. So it plays important roles in the regulation of carbon source allocation in plants, and affects related agronomic traits and abiotic stress responses. In a previous report, SuSy in soybean regulated sucrose metabolism and nitrogen

fixation ability, ultimately leading to improved drought resistance [63]. The expression of *SuSy* could be induced by cold, drought, salt or wounding stress in sugarbeet [64]. In this study, drought triggered the apparent accumulation of SuSy (A0A072V740) in Longdong, which may be beneficial to plant response to stress.

In plants, trehalose is a non-reducing disaccharide produced by a two-step pathway using trehalose-6-phosphate synthase (TPS, EC 2.4.1.15) and trehalose-6-phosphate phosphatase (TPP, EC 3.1.3.12) enzymes during the process of starch and sucrose metabolism. Briefly, TPS catalyzes UDPG and glucose 6-phosphate to synthesize trehalose-6-phosphate (T6P), and T6P is dephosphorylated by TPP to produce trehalose [65]. Trehalose and its intermediate metabolite, T6P, play key roles in plant growth and development and stress responses through affecting carbon allocation [66]. In addition, TPS and TPP also play important roles in plant response to adverse environments [67]. For instance, overexpression *OsTPS1* enhanced plant tolerance to cold, salt and drought stress by increasing proline and aglucon content and up-regulating some stress responsive genes in rice [68]. It was reported that the expression of *CqTPS4* was up-regulated, while the other three *CqTPS* genes were down-regulated in quinoa after saline-alkali treatment, which may be related to the different functional division of these genes and the complicated regulatory mechanisms of trehalose biosynthesis [66]. Similar to the above results, one TPS (G7JCN5) was up-regulated in Algonquin and one TPS (G7LBG6) was down-regulated in Longdong, which may be due to the different biological functions of these two TPS proteins in the two alfalfa varieties under drought stress.

Glycolysis/gluconeogenesis and TCA cycle

Glycolysis pathway converts glucose into pyruvate through a set of enzymes, accompanied by a small amount of energy production. In contrast, the gluconeogenic process is the synthesis of glucose from pyruvate, which shares some reversible reactions with the glycolysis pathway [69]. Pyruvate dehydrogenase complex (PDH) oxidizes pyruvate to acetyl-CoA, which subsequently enters into the tricarboxylic acid (TCA) cycle [70]. Therefore, glycolysis plays essential roles in the full utilization of carbohydrates and the production of energy required by plant cells. In the current study, 12 DAPs were found to be involved in the glycolysis/gluconeogenic process in the two alfalfa varieties after drought treatment. Among them, one glyceraldehyde 3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) protein (G7J2H2) and one enolase (ENO, EC 4.2.1.11) protein (A0A072U338) were down-regulated in Algonquin. Meanwhile, the accumulation of three pyruvate kinase (PK, EC 2.7.1.40) proteins (G7IDV6, A0A072VLB0 and A0A072U7N4) was also down-regulated in Algonquin under drought stress, which is similar to the findings of Bittencourt et al. [70] in saline-stressed oil palm leaves. PK plays a critical regulatory role in the last irreversible reaction of glycolysis. Dihydrolipoamide acetyltransferase (DLAT, EC 2.3.1.12) and dihydrolipoamide dehydrogenase (DLD, EC 1.8.1.4) are important components of PDH. The declined abundance of these two enzymes (A0A072VNF5 and G7JMT6) in Algonquin suggested that the formation of acetyl-CoA, which is a key substrate for TCA, might be inhibited by drought stress. Meanwhile, it was shown that two ATP citrate (pro-S)-lyase (ACLY, EC 2.3.3.8) proteins (A2Q2V1 and A0A072V822) and one aconitate hydratase (ACO, EC 4.2.1.3) protein (G7JDJ4) in Algonquin, and one ADP-malate dehydrogenase (ADP-MDH, EC 1.1.1.37) protein (G7LBJ9) in Longdong were down-regulated. These proteins are important enzymes involved in the TCA cycle. The results indicated that drought may cause more severe suppression on the glycolysis/ gluconeogenesis pathway and TCA cycle in Algonquin than in Longdong, potentially leading to the inhibition of energy production.

Amino sugar and nucleotide sugar metabolism

In the study, we identified nine DAPs involved in the amino sugar and nucleotide sugar metabolism in the two alfalfa varieties after drought treatment. In Algonquin, two UDP-glucose 4,6-dehydratase (RHM, EC 4.2.1.76) proteins (A0A072VAU0 and A0A072TI08) and two reversibly glycosylated polypeptide/UDP-arabinopyranose mutase (RGP, EC 2.4.1.- 5.4.99.30) proteins (A0A072TX38 and A0A072V8I1) were down-regulated. Meanwhile, one xylan 1, 4-beta-xylosidase (XYL4, EC 3.2.1.37) protein (G7IEW2) and one alpha-N-arabinofuranosidase (abfA, EC 3.2.1.55) protein (D7RIC7) were up-regulated, which can catalyze hydrolysis of belta-D-xylans and alpha-L-arabinosides, respectively. However, the abundance of one XYL4 (G7ILA8) was decreased, and one UDP-glucuronate 4-epinerase (EC 5.1.3.6) protein (G7JA85) was up-regulated in Longdong. The results showed that compared with Longdong, the amino sugar and nucleotide sugar metabolism-related proteins in Algonquin were more susceptible to drought.

Besides, it was shown that 13 DAPs, including five up-regulated and eight down-regulated, participating in fructose and mannose metabolism, galactose metabolism, N-glucan biosynthesis, and other glucan degradation were only found to be modulated in Algonquin, suggesting that these metabolic processes may also

contribute to the response of Algonquin against drought stress to some extent.

Protein metabolism-related proteins

Ribosome and ribosome biogenesis

Ribosomes are the site of protein biosynthesis. The accumulation of ribosomal proteins is helpful for plants to improve tolerance to cold stress [71]. It was shown that the accumulation of ribosomal proteins was improved in citrus under PEG stress [72]. However, Zhang and Shi [4] observed four ribosomal proteins were down-regulated in alfalfa Gannong No. 3 in response to PEG-induced osmotic stress. In this study, we discovered 97 ribosomal proteins (3 up-regulated and 94 down-regulated) and two down-regulated proteins involved in ribosome biogenesis in Algonquin. However, fewer ribosomal proteins were significantly modulated in Longdong, including 4 up-regulated and 14 down-regulated. These findings indicated that more ribosomal proteins are down-regulated in Algonquin compared to Longdong, which may lead to a greater reduction in protein biosynthesis in Algonquin under drought stress.

Protein processing in the endoplasmic reticulum

In addition, 13 and 11 DAPs involved in protein processing in endoplasmic reticulum were observed in Algonquin and Longdong, respectively. Heat shock proteins (HSPs) are important molecular chaperones involved in protein folding, assembly, transport and degradation, and have key roles in protecting plants from stress damage [4]. It has been reported that the expression of some HSPs was enhanced in alfalfa under salt stress [73] and PEG-induced osmotic stress [4]. Here, five HSPs were respectively found in Algonquin (two up-regulated and three down-regulated) and Longdong (one up-regulated and four down-regulated). Protein disulfide isomerase (PDI, EC 5.3.4.1) belongs to the thioredoxin subfamily of redox proteins and has the activities of thiol-disulfide oxidoreductase and disulfide isomerase, which can promote the accurate folding of target proteins by catalyzing the formation or rearrangement of disulfide bonds [74, 75]. Plant PDIs are multifunctional proteins due to their structural differences and diversities. PDIs could be up-regulated under abiotic stress and overexpression of *PDI* enhanced the stress tolerance of transgenic plants in *Arabidopsis thaliana* [76] and *Zoysia matrella* [77]. Nevertheless, the accumulation of protein disulfide isomerase A1 (PDIA1) (A0A072V0J2 in Algonquin and G7IRR6 in Longdong) was markedly repressed. The above results suggested that protein processing was negatively affected by drought stress in these two alfalfa varieties.

Biosynthesis of amino acids

Amino acids are the basic units of protein biosynthesis. In this study, nine enzymes participated in biosynthesis of amino acids, were notably decreased by drought stress, except one increased S-adenosylmethionine synthetase (SAMS, EC 2.5.1.6) protein (A0A072V8Q4) in Algonquin. Conversely, drought stress enhanced the accumulation of four enzymes related to biosynthesis of amino acids, including two SAMS proteins (A0A072V8Q4 and G7JQ29) in Longdong. SAMS plays an important role in catalyzing methionine to produce S-adenosyl-L-methionine (SAM). SAM is involved in various biochemical reactions and is a precursor of polyamines in plants [78]. As secondary metabolites, polyamines have critical roles in regulating plant growth and development, and improving plant stress tolerance [79]. Previous studies displayed that abiotic stress can induce the expression of *SAMS* genes, most of which make a key contribution to plant development and stress responses [80]. The up-regulated HvSAMS3 protein was identified in barley under combined drought and salt stress [81], which is similar to our results. This implies that the up-regulation of SAMS proteins may promote polyamine synthesis, thereby enhancing plant resistance to drought stress to a certain extent, especially in Longdong.

Photosynthesis-related proteins

Light reaction

Photosynthesis is an important physiological process, including light reaction and dark reaction. The light reaction occurs in photosystem (PS) II and I, involving the light capture and electron transport. Chlorophyll (Chl) a/b binding proteins are vital components of the light-harvesting complex (LHC), and are also known as photosynthesis-antenna proteins because they are located at the outside reaction center of photosystem and can capture the light energy like antennas. The LHC Chl a/b binding proteins (LHCPs) surrounding PS II are called as LHCBs, including LHCB1 ~ 6, and the LHCPs connected to PS I are known as LHCAs, including LHCA1 ~ 4 [82, 83]. It was shown that LHCPs play critical roles in plant response to abiotic stresses [84, 85], such as drought [86] and shading [87]. The expression of *Lhcb1* was up-regulated under drought stress in three *Apium graveolens* cultivars (Liuhe Huangxinqin, Jinnan Shiqin, and Ventura), however, a slight down-regulation appeared in *Oenanthe javanica* (cv. Baguazhou Shuiqin) [86]. The abundance of LHCB1, LHCB2 and LHCB6 was significantly up-regulated, while LHCB4 was down-regulated compared to control condition in soybean leaves after shading treatment [87]. In the present study, drought resulted in the prominent accumulation of seven LHCPs, including five LHCBs (LHCB1, LHCB2, LHCB4 and LHCB6) and two LHCAs (LHCA2 and LHCA3), while the expression of LHCB3 declined in Algonquin. For Longdong, only two LHCPs (LHCA3 and LHCB3) were up-regulated under drought treatment.

The process of water splitting happens in the oxygen-evolving complex (OEC), which consists of three extrinsic nuclear-encoded subunits, PsbO (33 kDa), PsbP (23 kDa) and PsbQ (17 kDa) in eukaryotic PS II [88]. In Algonquin, four oxygen-evolving enhancer proteins were up-regulated under drought stress, including PsbO (G7ZVI4), PsbP (B7FJ16 and G7KD12) and PsbQ (I3SSE5), and seven PS I reaction center subunit proteins were also significantly accumulated, including PsaD (G7K2D0), PsaE (G7KZJ5), PsaH (B7FN63), PsaL (Q2HW07), PsaN (G7JAX6) and PsaO (B7FN10). Additionally, we found that plastocyanin (Pc) (G7J5X6) and ferredoxin (Fd) (G7L8U4) involving in electron transport, were significantly up-regulated only in drought-treated Algonquin. The results indicated that the light reaction of Algonquin may be enhanced, and more sunlight may be captured and converted into chemical energy during drought situation.

Dark reaction (carbon fixation in photosynthetic organisms)

In the dark reaction, the carbon assimilation is carried out by using ATP and NADPH generated by the light reaction to convert carbon dioxide (CO_2) into sugar. Ribulose-1,5-bisphosphate (RuBP) is a receptor for CO_2, which is catalyzed by ribulose-diphosphate carboxylase/oxygenase (Rubisco, RBCS, EC 4.1.1.39) to produce two 3-phosphoglyceric acids (PGA) in Calvin cycle (C_3 pathway). Hence, RBCS plays a crucial role in carbon fixation and is decisive for net photosynthetic rate [89]. One RBCS small chain was down-regulated after light and medium drought stress in roots of Liquorice [42]. Similarly, the abundance of one RBCS small chain (G7KMR3) also showed a visible decrease in Algonquin, suggesting that the efficiency of carbon fixation in the C_3 pathway may be affected by drought.

In order to reduce water loss through transpiration, plants close their stomata to retain water content under drought stress, but this leads to a decrease in intracellular CO_2 concentration and a weakening of photosynthesis [90]. In many C_3 plants, not only RBCS, but also C_4 photosynthetic enzymes are present, such as phosphoenolpyruvate (PEP) carboxylase (PPC, EC 4.1.1.31), NADP-malate dehydrogenase (NADP-MDH, EC 1.1.1.82), NADP-malic enzyme (NADP-ME, EC 1.1.1.40) and pyruvate orthophosphate dikinase (PPDK, EC 2.7.9.1) [91]. These enzymes play an important role in the efficient utilization and transfer of low concentration CO_2 in C_4 photosynthesis [92]. Zhang et al. [42] found that with the intensification of drought stress, the enzyme activities of PPC, PPDK and NADP-MDH first increased and then decreased. Meanwhile, two NADP-MDH and three PPC proteins were remarkably up-regulated in liquorice roots after medium and severe stresses, respectively. In this study, we identified that five C_4 photosynthetic enzymes were prominently up-regulated in Algonquin after drought stress, including one NADP-ME (G7L7H0), two PPDK (G7JHV6 and A0A072URD9) and two PPC proteins (G7IU25 and A0A072TLJ0). Up-regulation of these enzymes improved CO_2 transfer and fixation to compensate for the adverse effects of RBCS downregulation on photosynthesis [42, 93]. This may be a stress strategy of Algonquin to alleviate the metabolic loss of the C_3 pathway due to drought. In addition, only one up-regulated PPDK protein (G7JHV6) was identified in Longdong. The above results show that photosynthesis in Longdong was less affected by drought stress.

Stress- and defense-related proteins

Antioxidant defense system

Facing with water scarcity, unused excess energy in photosynthesis may be transferred to the production of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), singlet oxygen, hydroxyl radical and superoxide anion (O_2^-), leading to oxidative stress in chloroplasts [94]. Furthermore, ROS can also be excessively accumulated in other organelles, primarily due to changes of metabolism during drought [95]. Subsequently, ROS acts as a signaling molecule to regulate programmed cell death, pathogen defense, and abiotic stress responses in plants [96]. In order to mitigate the ROS damage, the antioxidant defense system is triggered to scavenge excess ROS, including enzymatic antioxidants, for example superoxide dismutase (SOD, EC 1.15.1.1), peroxidase (POD, EC 1.11.1.7), catalase (CAT, EC 1.11.1.6), glutathione peroxidase (GPX, EC 1.11.1.9) and ascorbate peroxidase (APX, EC 1.11.1.11), and non-enzymatic antioxidants, such as glutathione, flavonoids, carotenoids, and ascorbic acid.

SOD plays a key role in catalyzing O_2^- into H_2O_2; POD, CAT, GPX and APX are responsible for converting H_2O_2 to H_2O and modulating H_2O_2 content [97, 98]. Xiao et al. [31] reported that the enzymatic activities of SOD and CAT of fine roots significantly increased in cotton (*Gossypium hirsutum*) after drought treatment for 30 days compared to control plants. However, the expression of corresponding proteins was not notably regulated [31]. Previously, we found that the enzymatic activities of total SOD, POD and CAT in Longdong were higher than that in Algonquin under drought stress [11]. In this study, one SOD2-Fe–Mn protein (A0A072VH43) was up-regulated in Longdong, while it was down-regulated in Algonquin. Another SOD1-Cu–Zn protein (B7FHQ5) was down-regulated in Longdong, while two SOD1-Cu–Zn proteins were significantly up-regulated in Algonquin, including B7FHQ5 and G7KPK9. Except for one POD protein (A0A072V2Y0) down-regulated in both alfalfa

cultivars, five and four POD proteins were up-regulated in Algonguin (G7IJU0, A0A072VLG5, A0A072UXA0, etc.) and Longdong (G7IJU0, G7IKK4, G7IM82, etc.), respectively. In addition, one GPX protein (G7I893) was up-regulated in Longdong, and two GPX proteins were pronouncedly modulated in Algonquin, including the up-regulated G7I893 and the down-regulated G7LBF8. Meanwhile, one APX protein (G7JB12) was significantly up-regulated in Algonquin. Glutathione S-transferases (GSTs, EC 2.5.1.18) consist of 14 subclasses encoded by a supergene family with diverse functions, including cellular detoxification by scavenging ROS under stress conditions [99–102]. Overexpression of a GST gene enhanced drought tolerance of transgenic plants [103] by improving activities of antioxidant enzymes, such as GPX, SOD and POD, to maintain ROS homeostasis [104]. Here, the expression of four GST proteins was visibly up-regulated in Longdong, including G7L6H6, G7K3B1, I3T9M4 and A0A072VLL6. In Algonquin, G7L6H6 was down-regulated with the other two GST proteins (A0A072V9B8 and A0A072UGV0), and two GST proteins (G7K3B1 and G7LB15) were up-regulated.

Flavonoids are one of the main families of plant secondary metabolites and have a basic structure with three phenolic rings (C_6-C_3-C_6), called A (6 carbon) and B (6 carbon) connected with the intermediate C (3 carbon) ring [105]. It was reported that flavonoids have mitigating effects on abiotic stresses by detoxifying ROS [106, 107]. The accumulation of flavonoids increased under drought stress, which improved the ROS scavenging ability and drought tolerance of peppermint [108] and tea tree [109]. Many genes involved in flavonoid biosynthesis were reported to be modulated by drought stress, such as phenyl ammonium lyase (*PAL*), chalcone synthase (*CHS*), chalcone isomerase (*CHI*), flavone synthase (*FNS*), flavonol synthases (*FLS*) and anthocyanidin synthase (*ANS*) [107]. For example, it was found that the transcription levels of *TaCHS*, *TaCHI*, *TaFLS*, *TaFNS* and *TaANS* rapidly enhanced after drought stress in wheat [110]. Similarly, one CHI (EC 5.5.1.6) (G7IEZ8) was significantly up-regulated in Longdong, while six enzymes participated in flavonoid and isoflavonoid biosynthesis were down-regulated in Algonquin under drought stress, including one CHI (G7IEZ6), one vestitone reductase (VR, EC 1.1.1.348) (A2Q3W4) and two caffeoyl-CoA O-methyltransferases (EC 2.1.1.104) (G7KJ71 and G7JK14). The 9-*cis*-epoxycarotenoid dioxygenase (NCED, EC 1.13.11.51) is a key enzyme in the biosynthesis pathway of abscisic acid (ABA), which plays a critical role in plant growth, development, seed dormancy and stress resistance [111]. Abiotic stresses such as high temperature, low temperature and drought stress could strongly induce the up-regulation of *MpNCED2* in apple (*Malus prunifolia*) [112]. The expression of *SiNCED1* gene in *Setaria italica* was induced by ABA, salt and osmotic stress. Ectopic overexpression of *SiNCED1* improved the drought resistance of transgenic Arabidopsis plants by increasing endogenous ABA content and promoting stomatal closure [113]. Likewise, two NCED proteins (G7K2Q5 and G7K3Y7) involved in carotenoid biosynthesis pathway were significantly up-regulated under drought stress in Algonquin. In summary, the above results indicated that the types, amounts and expression patterns of enzymatic and non-enzymatic antioxidants mobilized by Longdong and Algonquin under drought stress are somewhat different, which may eventually lead to the difference in ROS scavenging capacity of these two alfalfa cultivars.

Linoleic acid and α-linolenic acid metabolic pathway

Plant lipoxygenases (LOXs) catalyze the oxidation of polyunsaturated fatty acids. For instance, linoleic acids and linolenic acids can be transferred into fatty acid hydroperoxides, which can be converted to oxylipins later on [114]. The α-linolenic acid can be converted into 12-oxo-phytodienoic acid (12-OPDA) through the oxygenation of LOX, allene oxide synthase (AOS) and allene oxide cyclase (AOC) in turn, which is an intermediate for jasmonic acid (JA) biosynthesis [115]. LOX-derived oxylipins play crucial roles in plant growth, senescence, signal transduction, homeostasis and other physiological functions [116]. JA is a class of oxylipins and makes important contributions to plant abiotic stress responses, including cold, salt, drought and heavy metal stress, through physiological and molecular mechanisms [117, 118]. Biotic and abiotic stresses such as pathogen attacks, wounding, oxidative stress and water deficit can induce the expression of LOX genes. Up-regulated transcriptional level of the *AOS* gene and increased AOC activity were detected in wounded leaves of plants [116]. In this study, 10 and 13 DAPs involved in linoleic acid and α-linolenic acid metabolic pathways were identified in Algonquin and Longdong, respectively, and all of them were significantly up-regulated under drought condition. Specifically, five linoleate 9S-lipoxygenase (LOX1_5, EC 1.13.11.58), two lipoxygenase (LOX2_S, EC 1.13.11.12), one AOS (EC 4.2.1.92), one hydroperoxide lyase (HPL, EC 4.1.2.-) and one chloroplastic oxoene reductase (EC 1.3.1.-) were found in Algonquin; and nine LOX1_5, two LOX2_S, one AOS and one AOC (EC 5.3.99.6) were found in Longdong. It was shown that drought stress enhanced the expression of key genes participated in the metabolic pathways of linoleic acid and α-linolenic acid, especially the LOX genes, indicating that drought may increase the biosynthesis of JA, especially in Longdong cultivar.

Plant-pathogen interaction pathway

Pathogenesis-related (PR) proteins are key components of plant defenses against pests and pathogens [119]. Based on the protein sequence similarity and the enzyme activity, PR proteins have been classified into 17 families, namely PR1 to PR17 [120]. PR1 proteins play an important regulatory role in plant growth, development, aging and response to abiotic stresses [121]. It has been reported that 10 *PR1* genes were discovered in mango (*Mangifera indica* L.), and their expression profiles showed significant differences under PEG and NaCl treatments. For example, after 48 h of PEG treatment, seven *MiPR1* genes were up-regulated and two *MiPR1* genes were down-regulated [122]. Overexpression of PR 1a gene *OsSCP* enhanced the tolerance of rice plants to NaCl and mannitol treatments [123]. Chitinases catalyze the hydrolysis of chitin to *N*-acetylglucosamines, including endochitinases (EC 3.2.1.14) and exochitinases (EC 3.2.1.200/201). Chitinases are divided into seven classes (Class I-VII) [124, 125], which are considered to fall under the category of PR proteins (PR3, PR4, PR8 and PR11) [126]. Chitinases are not only involved in response to biotic stresses but also in response to abiotic stresses such as heavy metal pollution, UV light, wounding, salt and drought [127]. The knockdown of chitinase gene *CaChiIV1* increased the relative electrolyte leakage and reduced the total chlorophyll content in the leaves of pepper (*Capsicum annuum* L.) treated with mannitol [128]. After 8 days of drought stress, 14 of 23 *ClChi* genes were significantly up-regulated and seven were down-regulated in watermelon (*Citrullus lanatus* [Thunb.]) [129]. Our data showed that one PR1 (G7ILE4) and two chitinases (EC 3.2.1.14) (G7ID31 and A0A072V210) were prominently up-regulated in Longdong after 8 days of drought treatment, while four chitinases (EC 3.2.1.14) were notably down-regulated in Algonquin, including G7LA76, G7IS19, G7KNL2 and A0A072TT91. These results suggested that the accumulation patterns of some DAPs associated with plant-pathogen interaction pathway are completely opposite in the two different alfalfa cultivars, which may be one of the reasons for the difference in drought resistance.

Conclusions

In this study, we mainly compared and analyzed the difference of response to drought stress between Longdong and Algonquin based on proteomic analysis. Drought led to changes in various metabolic pathways in alfalfa plants, and differences in expression patterns of key proteins involved in important metabolic pathways could partly explain the difference in drought resistance between the two alfalfa varieties (Fig. 8). In short, compared with Algonquin, the number of down-regulated glycolysis/gluconeogenesis and TCA cycle-related proteins as well as ribosomal proteins was lower in Longdong, so the energy production and protein biosynthesis of Longdong were less negatively affected by drought stress. Interestingly, the abundance of many proteins involved in light reaction and C_4 pathway was significantly increased in Algonquin, which may be an adaptation strategy of Algonquin to drought stress. In addition, drought induced the significant up-regulation of some stress- and defense-related proteins in both varieties, especially in Longdong. Some DAPs associated with flavonoid and isoflavonoid biosynthesis and plant-pathogen interaction pathway were up-regulated in Longdong, while related DAPs were down-regulated in Algonquin. These differences may cause stronger antioxidant capacity in Longdong. Some of the above results from the proteomic level echo the results of our previous comparative analysis of the two alfalfa varieties at the physiological and molecular levels [11]. Moreover, these DAPs can be used as candidate proteins in molecular breeding of alfalfa to breed new germplasm with better drought tolerance to adapt to increasingly harsh environmental changes.

Methods

Plant materials and drought treatment

In the experiment, we used two alfalfa varieties with different drought tolerance (relatively drought-tolerant Longdong and drought-sensitive Algonquin) as experiment materials based on our previous research [11]. Seeds of the two cultivars were kindly provided by Dr. Haiqing Wang (Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining, China).

After surface-sterilization with 5% NaClO solution for 10 min, alfalfa seeds were vernalized at 4 ℃ for 2 days in the dark. The seeds were germinated on half-strength Murashige and Skoog medium (pH 5.7) under a 16 h light/8 h dark cycle at 22 ~ 25 ℃. After 7 d germination, the seedlings with the same size were transferred to plastic pots (10 cm diameter at top, 7.5 cm diameter at bottom, and 8.5 cm height) filled with pre-autoclaved vermiculite. Nine seedlings were planted in each pot and irrigated with nutrient solution twice every week for 3 weeks.

For some 4-week-old plants, water was withheld as the drought treatment group, while other plants were normally watered as the control group. Five pots of seedlings from each treatment/variety were served as replicates, and all potted plants were designed in a completely random block. Throughout the growing period, we randomly changed the position of pots every day to reduce the impact of environmental factors. After 8 days of drought treatment, leaf samples were collected and quickly frozen

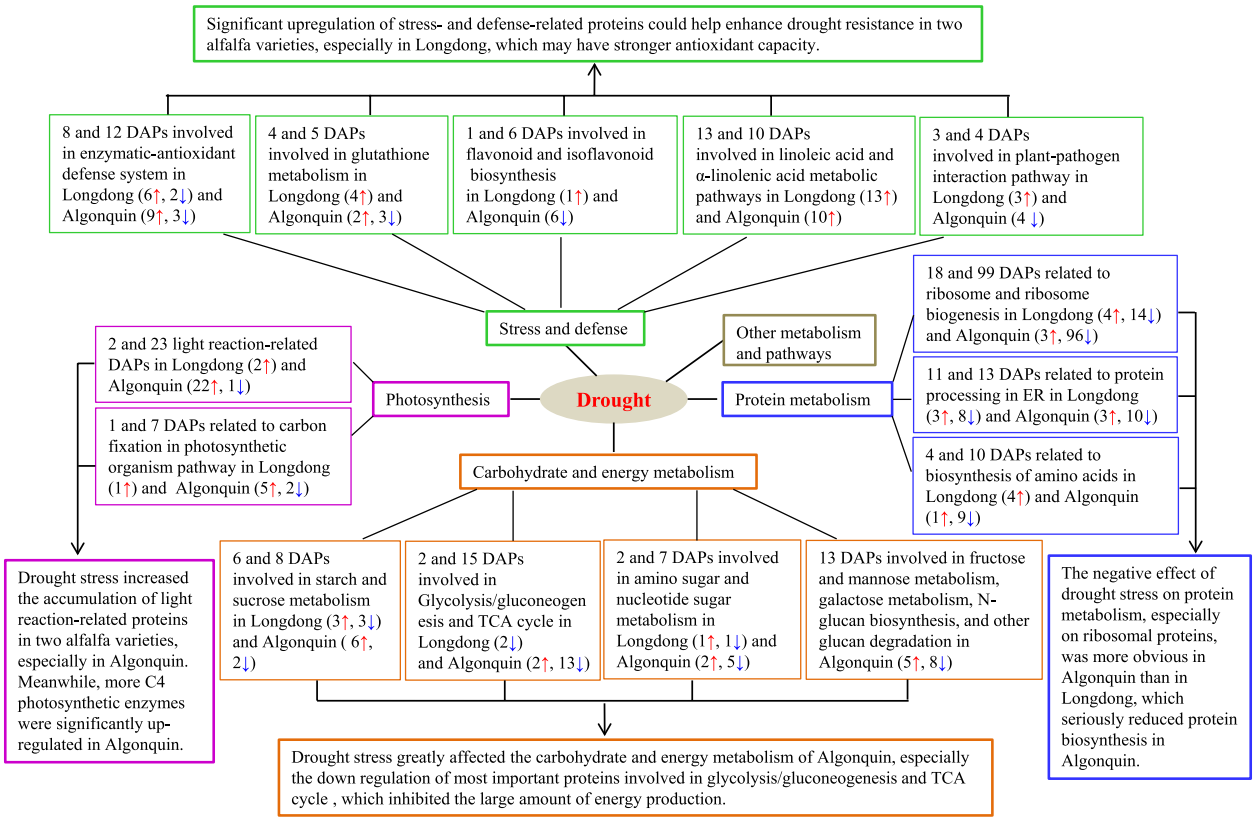


Fig. 8 Effects of drought on protein accumulation in the two different alfalfa varieties

in liquid nitrogen for subsequent quantitative proteomic analysis.

Protein extraction, digestion and TMT labeling

TMT based-quantitative proteomic analysis was executed by PTM BioLab, Inc. (Hangzhou, China). The experimental samples came from four genotype/treatment combinations, and each of them contained three biological replicates. The leaves were ground in liquid nitrogen. A 4×volume of phenol extraction buffer (containing 10 mM dithiothreitol, Sigma, Germany, 1% protease inhibitor, Calbiochem, Germany, and 2 mM EDTA, Sigma, Germany) was added to the sample, followed by ultrasonication three times on ice. Then, an equal volume of Tris-saturated phenol was added, and the mixture was centrifugated for 10 min at 4 ℃ and 5, 500 g. The supernatant was mixed with 5×volume of 0.1 M ammonium sulfate-saturated methanol, and incubated at -20 ℃ for at least 6 h. After centrifugation, the precipitate was washed with ice-cold methanol once, followed by ice-cold acetone for three times. Finally, the protein precipitate was redissolved in 8 M urea (Sigma, Germany), and the concentration was determined with bicinchoninic acid assay (BCA) kit (Beyotime, China) according to the manufacturer's instructions [31]. The protein quality was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using an equal amount of protein for each sample.

For digestion, the protein solution was reduced with 5 mM dithiothreitol for 30 min at 56 ℃, and alkylated with 11 mM iodoacetamide (Sigma, Germany) for 15 min at room temperature in darkness [31]. The protein solution was diluted by adding 100 mM tetraethyl-ammonium bromide (TEAB, Sigma, Germany) until to urea concentration less than 2 M. Finally, trypsin (Promega, USA) was added into the protein samples at a mass ratio of 1:50 (trypsin: protein) for the first digestion overnight and 1:100 for the second digestion for 4 h. After digestion, peptides were desalted by Strata X C18 column (Phenomenex, USA) and freeze-dried in a vacuum. The peptides were dissolved in 0.5 M TEAB and labeled with TMT kit (Thermo, USA) according to the manufacturer's instructions. Then, the labeled peptide mixtures were combined, desalted and vacuum-freeze-dried again.

HPLC fractionation and LC–MS/MS analysis

The labeled peptides were fractionated using high pH reversed-phase high performance liquid chromatography

(HPLC) with Agilent 300Extend C18 column (5 μm particles, 4.6 mm ID, 250 mm length; Aligent, USA). Briefly, peptides were first separated with a gradient of 8% to 32% acetonitrile (pH 9.0) over 60 min into 60 fractions. The peptides were then combined into nine fractions and freeze-dried in a vacuum.

The fractions were redissolved in 0.1% formic acid (Fluka, USA) and loaded onto a reversed-phase analytical column at a constant flow rate of 400 nL/min on an EASY-nLC 1000 UPLC system (Thermo, USA). The peptides were subjected to NSI source followed by tandem mass spectrometry (MS/MS) in Q Exactive™ Plus (Thermo, USA) coupled online to the UPLC. The electrospray voltage applied was 2.0 kV. The MS scan spectra range was 350 to 1800 m/z, and intact peptides were detected in the Orbitrap at a resolution of 70,000. The MS/MS scan spectra was selected in the Orbitrap at a resolution of 17,500.

Protein identification and quantification

The resulting MS/MS data were processed using Maxquant search engine (v.1.5.2.8) against the *Medicago truncatula*_UniProt database (57, 065 sequences). The mass tolerance for precursor ions was set as 20 ppm in the first search and 5 ppm in the main search. The mass tolerance for fragment ions was set as 0.02 Da. Carbamidomethyl on cysteine was specified as a fixed modification and oxidation on methionine was specified as a variable modification. The criterion of false discovery rate (FDR) was adjusted to > 1%. Only proteins with at least one unique peptide were used to further analysis. Proteins in different comparison groups with fold change > 1.2 or < 1/1.2 (0.8333) [3, 130] and *P*-value < 0.05 were considered to be differentially abundant.

Subcellular localization and COG functional classification

WoLF PSORT (https://www.genscript.com/psort/wolf_psort.html) was employed to predict subcellular localization for eukaryotic proteins. COG database was used for the functional classification of DAPs.

Annotation and enrichment analysis of GO and KEGG pathway

GO annotation was performed using the UniProt-GOA database (https://www.ebi.ac.uk/GOA/). If some identified proteins were not annotated by the UniProt-GOA database, the InterProScan (http://www.ebi.ac.uk/interpro/) was used to predict the protein's GO function according to protein sequence alignment method. KEGG database (https://www.kegg.jp/kegg/mapper.html) was employed to annotate protein pathways. A two-tailed Fisher's exact test was used to analyze the enrichment of GO function and KEGG pathway of DAPs against all identified proteins. The enrichment of GO or pathway with a corrected *P*-value < 0.05 was considered significant. Based on the enrichment analysis, the categories which were at least enriched significantly in one of different comparison groups were selected and then clustered by one-way hierarchical clustering (Euclidean distance, average linkage clustering). Finally, cluster heat maps were visualized using the "heatmap.2" function from the "gplots" R-package (https://cran.r-project.org/web/packages/cluster).

PPI network analysis

All the DAP database accession from different comparison groups were searched respectively using STRING database (https://www.string-db.org/) for protein–protein interactions. Based on the confidence score > 0.7 (high confidence), the interaction network of DAPs was obtained. In order to display the network clearly, the top 50 proteins with the closest interaction relationship and the proteins directly interacting with them were screened. Finally, the relatively clear network was mapped by STRING and was visualized using Cytoscape software.

RNA isolation and RT-qPCR

After drought treatment for 8 days, the leaf tissues of two alfalfa varieties from control and stress conditions with three biological replicates were sampled for corresponding gene expression analysis. Total RNA was extracted using plant total RNA extraction kit (TSP412, Tsingke, Beijing, China). DNA-free RNA was reverse-transcribed into cDNA with reverse transcriptase (TSK314S, Tsingke, Beijing, China) [4].

RT-qPCR was performed using the ABI QuantStudio StepOnePlus Real-Time PCR system (Applied Biosystems, USA) with SYBR-green fluorescence. The specific primers for RT-qPCR were listed in Table S7. The amplification reaction condition was 95° for 5 min, 40 cycles of 95° for 15 s, 60° for 20 s, and 72° for 20 s. The relative expression levels were analyzed by the $\Delta\Delta Ct$ method using the β-actin gene as internal control [11, 35].

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-024-10702-7.

Supplementary Material 1.
Supplementary Material 2.
Supplementary Material 3.
Supplementary Material 4.
Supplementary Material 5.
Supplementary Material 6.
Supplementary Material 7.
Supplementary Material 8.

Acknowledgements
We sincerely thank the editor and reviewers for their comments and suggestions. We are grateful to Dr. Haiqing Wang for providing alfalfa seeds of Longdong and Algonquin.

Authors' contributions
WQ designed the project. WQ performed the experiment and analyzed the data. WQ and XL wrote and revised the manuscript.

Funding
This work was supported by the National Natural Science Foundation of China (32202727) to Wenli Quan.

Availability of data and materials
All data generated or analyzed during this study are included in this published article and its supplementary information files.

Data availability
Data is provided within the manuscript or supplementary information files.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 30 April 2024 Accepted: 12 August 2024
Published online: 27 August 2024

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