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Comparative physiological, biochemical and proteomic analyses reveal key proteins and crucial regulatory pathways related to drought stress tolerance in faba bean (*Vicia faba* L.) leaves

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ABSTRACT

Drought is one of the important abiotic factors that affect faba bean growth and productivity in the Mediterranean region. In order to study the response of faba bean plant to water-deficit stress, a physiological and proteomic analysis was carried out in leaf tissue. All physiological parameters were affected by drought. The physiological mechanism underlying the response of faba bean leaves to water-deficit was therefore attributed to the alleviation of oxidative stress via the accumulation of proline and to the synergistic action of the antioxidant enzyme system (CAT, SOD, APX and GPOX). Proteomic analysis identified 2000 proteins from faba bean leaves, of which were 81 differentially expressed. Of those, 36 were downregulated and 45 were upregulated under water-deficit treatment. KEGG and GO enrichments indicated differentially abundant proteins (DAPs) related to photosynthesis, antioxidants and ROS detoxifying enzymes, biosynthesis of amino acids and secondary metabolites, molecular chaperones, signal transduction, energy and carbohydrate metabolism and metabolic enzymes. The current results provide evidence for a complex synergetic pathway, in which ROS detoxification mechanisms and photoprotection constituted the major aspect of water-deficit tolerance in faba bean leaves. These results offer a foundational basis regarding the molecular mechanism involved in drought resistance within the faba bean species.

1. Introduction

Grain legume crops such as faba bean, chickpea and pea each fulfil critical roles within the farming system in Tunisia. Overall, a land area in excess of 85,000 ha is dedicated to the cultivation of these crops. The majority of that area is located in the Northern regions of the country, which receive more than 400 mm of precipitation per year [1]. The faba bean (large and small seeded) is the most important grain legume for Tunisia; the average area planted with this crop is just under 60,000 ha, about 70% of the total area dedicated to grain legume crops. Faba bean is a key source for human nutrition as well as for animal feed. In 2019, the average yield on a national level was 1.09 t/ha, though this is characterised by wide fluctuations, up to 52% below the global average (2.29 t/ha) (FAOSTAT database, http://www.fao.org/faostat, accessed on 10 December 2021). The reasons for this instability and low grain yield potential in faba bean cultivation are complex, but are likely driven by climate changes in the Mediterranean region and sensitivity to multiple environmental stresses [2]. Under rainfed conditions, faba bean plants are often subjected to terminal water scarcity, which has become the major limiting factor on faba bean crop growth and

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production in Tunisia [3]. Previous studies have shown that water deficit significantly affected quantitative and qualitative parameters in faba bean plants, such as seed germination, nutrient uptake, nodule formation, yield and yield components [4–6]. In addition, among grain legumes, faba bean plant is believed to be relatively more sensitive to water-deficit compared to pea, chickpea and common bean [7]. Improved yield under water-limited environment is therefore an important goal of faba bean plant breeding programmes in Tunisia and Mediterranean area. The identification of tolerant genotypes and traits linked to water-deficit tolerance may be useful in breeding programmes to improve drought resistance of faba bean.

Water deficiency is a complex abiotic stress factor causes significant changes in many morphological, physiological and biochemical plant parameters [8]. Plants avoid drought stress through physiological and molecular mechanisms [9]. Several classes of secondary metabolites such as flavonoids as well as transcription factors and various signalling molecules like phytohormones involved in plant responses to environmental stresses [10]. Because its one of the largest crop legume genomes (about 13 Gbp) faba bean genome assembly and map-based cloning have been delayed in comparison to that of *Medicago truncatula* as model plant of legumes or of other grain legumes with smaller genomes such as *Glycine max*. Consequently, combining phenotypic selection with marker assisted selection (MAS), which is the major tool used during conventional faba bean plant breeding programmes, has not been very successful in developing improved varieties, because some traits such as yield and environmental stress tolerance are complex and are greatly influenced by both environmental and genetic factors. Therefore, identification of genes associated with water-deficit stress tolerance by means of a candidate gene approach become one of the most effective successful tools and economical methods for the direct discovery of genes associated with complex traits [11].

The development and emergence of next-generation sequencing technologies for multiomics approaches such as high- throughput RNA sequencing (RNA-seq) approach has greatly increased the ability to discover novel and rare transcripts in plants associated with different biological processes involved in drought responses [12]. Recently, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) has emerged as an innovative and powerful analytical technique for identifying and quantifying a vast number of proteins that regulate abiotic stress in various plant species including maize [13], chickpea [14], sweet potatoes [15] and ginseng [16]. In this context, several differentially abundant proteins (DAPs) with characterised or uncharacterised function were found to be involved in drought response and tolerance, which enable plants to tolerate drought stress. A wide range of the identified DAPs were classified in a diversity of biological processes including photosynthesis, protein synthesis, oxidative stress and defence responses, carbon metabolism, respiration, signal transduction and multiple metabolic pathways [17]. In the faba bean, only 30 DAPs were identified in leaves of the “Gadadou” cultivar using a low-throughput proteomics approach by means of two dimensional-polyacrylamide gel electrophoresis (2-DE) coupled with liquid tandem mass spectrometry (MS/MS) method [18]. The regulatory mechanisms on a molecular level involved that enable faba bean plants to adapt to drought stress remain poorly understood and our knowledge is still very limited.

In the current study, a high-throughput quantitative analytical method based on liquid chromatography/tandem mass spectrometry (LC-MS/MS) analysis was used to quantitatively determine differentially abundant protein in leaves of drought-stressed faba bean plants. Understanding the molecular mechanisms underlying the drought-stress response in faba bean plants would therefore help accelerate the genetic improvement of this species.

2. Materials and methods

2.1. Plant materials, growth conditions and drought stress treatment

The commercial variety Bachar of faba bean (*Vicia faba* L. var. *minor*), which is largely cultivated in various semi-arid regions of Tunisia [19] was used in the current study. Surface sterilisation of seeds was performed in 5% sodium hypochlorite (NaOCl) for 3 min. Then, the seeds were washed with sterilized distilled water 3 times and soaked for 12 h and germinated on perlite. Two weeks after seed germination, faba bean seedlings were transferred into 5.0 L capacity plastic pots 20 cm in diameter and 30 cm deep at a rate of one seedling per pot. The soil had a sandy texture with 84.40% sand, 8.30% silt and 5.50% clay and contained the following chemical attributes: pH: 7.50; total organic carbon: 0.52%; total N: 0.28%; organic matter: 0.90%; P: 4.92; K: 292 ppm and EC: 4.02 dS m^{-1}. The experiment treatments consisted of two different treatments; plants under well-watered conditions and plants under drought stress. Three replicates and fifteen pots per replicate were designed for each treatment, making a total of 90 pots arranged in a simple randomised design. All seedlings were grown in a controlled growth chamber under photoperiod of 14 h day/10 h night (270 μmol of photons/m^2s light intensity), temperature throughout 23 ± 2 °C and a relative humidity ranged from 55%− 65%. Seedlings were irrigated daily with 200 ml to maintain well-watered conditions. Drought stress was thus applied to four weeks-old plants, by stopping all watering of the plants for 10 days. The plants were harvested, separated into leaves and roots, immediately frozen in liquid nitrogen and stored at at − 80 °C until analyses.

2.2. Measurement of leaf physiological parameters

Relative water content (RWC) was determined from the fully expanded third leaf from the top based on fresh (FW), turgid (TW), and dry weights (DW) using the following formula RWC (%) = [(FW-DW)/(TW-DW)] × 100 [20].

Chlorophyll content (Chla, Chlb and Chlt) of fresh leaf samples (0.1 g) was determined according to Lichtenthaler [21]. Photosynthetic parameters of leaves were determined between 9:00 and 11:00 a.m. using a portable gas exchange system LCpro+ (ADC BioScientific, Ltd., UK) at a photon flux density of 980 μmol m^{-2} s^{-1}. Five seedlings are randomly assigned to each treatment for photosynthesis measurements.

2.3. Determination of the contents of hydrogen peroxide (H_2O_2), free proline, malondialdehyde (MDA), soluble sugars and the level of membrane electrolyte leakage (EL)

Proline content was determined according to Bates et al. [22] from fresh leaf tissue (0.1 g). L-proline standard curve was used to determine proline content in the samples.

The concentration of soluble sugars was quantified in leaves by UV spectrophotometry at 490 nm according to phenol-sulfuric acid method [23] using glucose as standard.

Hydrogen peroxide content was determined according to Velikova et al. [24]. Briefly, fresh leaf samples (0.5 g) were finely grounded with 5 ml of 0.1% (w/v) trichloroacetic acid (TCA) in an ice bath with pestle and mortar. Homogenates were centrifuged at 10,000 g for 10 min and then 1 ml of the supernatant was added to 1 ml of 10 mM potassium phosphate buffer (pH 7.0) and 2 ml of 1 M KI. Finally, the homogenate was read at 390 nm. A standard calibration curve was prepared to determine H_2O_2 content.

The level of lipid peroxidation was measured in terms of malondialdehyde (MDA) content according to the method described by Dhindsa et al. [25]. The absorbance was read at 532 nm, and the value for the non-specific absorption at 600 nm was subtracted. The concentration of MDA was calculated using its extinction coefficient of 155 mM^{-1} cm^{-1}.

The electrolyte leakage was determined as described by Murray et al. [26] and was calculated using the formula: EL (%) = (Ci/Cf) × 100% after measuring the initial (Ci) and final (Cf) electrical conductivity.

2.4. Activities of antioxidant enzymes

Leaf freeze-powder samples (1 g) were ground in 1 ml of ice-cold50 mM phosphate buffer (pH 7.8) containing 1 mM of phenylmethylsulfonyl fluoride (PMSF), 2 mM EDTA, 1% (w/v) polyvinylpyrrolidone (PVP) and 0.1% (v/v) triton X-100. The mixture was centrifuged at 10,000 g for 30 min at 4 °C and the supernatant was used for enzymes assays. Protein concentration was determined based on the method of Bradford [27].

The activity of superoxide dismutase (SOD) was estimated adopting the nitroblue tetrazolium (NBT) method [28]. One unit of SOD was defined as the amount of enzyme which caused a 50% decrease in the SOD-inhibited NBT reduction at 25 °C. The reaction mixture contained 1 μM riboflavin, 10 mM L-methionine, 0.1 mM EDTA, 50 mM potassium phosphate buffer (pH 7.8), 75 μM NBT and crude enzyme extract with a final volume of 2 ml. The SOD activity was determined at 560 nm and expressed in Units SOD min^{-1} mg^{-1} protein.

The activity of catalase (CAT) was assayed by measuring the disappearance of H_2O_2 at 240 nm based on the method of Cakmak and Marschner [29]. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 15 mM H_2O_2 and 20 μl of crude enzyme extract. The CAT activity was expressed in μmol of H_2O_2 min^{-1} mg^{-1} protein.

The activity of ascorbate peroxidase (APX) was assessed by following the disappearance of ascorbate at 290 nm based on the method of Nakano and Asada [30]. The assay mixture contained 50 mM potassium phosphate buffer (pH 7.8), 1 mM EDTA-Na_2, 1 mM H_2O_2, 1 mM ascorbic acid and 30 μl of crude enzyme extract. The APX activity was expressed in μmol H_2O_2 min^{-1} mg^{-1} protein.

The activity of guaiacol peroxidase (GPOX) was assessed by determining the increase in absorbance due to the formation of tetra-guaiacol at 470 nm based on the method of Polle et al. [31]. The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.8), 10 mM guaiacol, 1 mM H_2O_2 and 50 μl of crude enzyme extract. The GPOX activity was expressed in μmol guaiacol min^{-1} mg^{-1} protein.

2.5. Protein extraction, digestion, and LC-MS/MS analyses

Leaf powder samples (100 mg) from drought-stressed and unstressed plants were homogenised using 1 ml of ice-cold Tris-Mg/NP-40 extraction buffer (50 mM Tris-Hcl, pH 8.3, 2% NP-40, 20 mM $MgCl_2$ and 1% protease inhibitor) followed by centrifugation at 12000 g for 10 min at 4 °C. Subsequently, for protein precipitation, the collected supernatant was mixed with 4 volumes of cold 10% trichloroacetic acid (TCA)/acetone/dithiothreitol (DTT) at − 20 °C for 2 h. The proteins were pelleted by centrifugation at 12,000 g for 10 min at 4 °C and the pellets were washed with 80%, 90% and 100% cold acetone/DTT. Finally, the proteins were dried and dissolved in solubilisation buffer (7 M urea, 2 M Thiourea, 4% 3[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and 30 mM Tris). Protein concentrations were determined by the Bradford protein assay and bovine serum albumin (BSA) as a standard. The total protein quality was assessed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie staining.

For protein digestion, Filter-Aided Sample Preparation (FASP) protocol using 30 kDa Vivacon 500 filters (Sartorius) was applied [32]. Twenty micrograms of protein from each sample were reduced by 20 mM of dithiothreitol for 15 min at room temperature and further alkylated using 0.05 M chloroacetamide for 30 min. Finally, samples were digested overnight at room temperature with endoproteinase rLys-C, Mass Spectrometry Grade (Promega, Madison, WI, USA) at an enzyme-to-protein ratio of 1:50, followed by a second digestion with Trypsin Gold, Mass Spectrometry grade (Promega, Madison, WI, USA) for 4 h at 37 °C at a trypsin-to-protein ratio of 1:100 in triethylammonium bicarbonate (TEAB) buffer. The digested peptides were recovered by centrifugation at 14, 000 g for 20 min and vacuum dried.

Mass spectrometry analysis was performed at the De Duve Institute (Brussels, Belgium) according to its in-house protocol. In brief, the dried peptides were resuspended in 3% (v/v) acetonitrile with 0.1% (v/v) trifluoroacetic acid (TFA). Peptides were directly loaded onto a reverse-phase pre-column (Acclaim PepMap 100, Thermo Scientific) and eluted in backflush mode. Peptide separation was performed using a reversed-phase analytical column (Pepmap RSLC C18, 250 ×0.075 mm, Thermo Scientific) with a linear gradient of 4%− 32% solvent B (0.1% formic acid in 80% acetonitrile) for 100 min, 32%− 60% solvent B for 10 min, 60%− 95% solvent B for 1 min and holding at 95% for the last 10 min at a constant flow rate of 300 nl/min on an Ultimate 3000 RSLC system. The peptides were analysed by an Orbitrap Exploris240 mass spectrometer (ThermoFisher Scientific). The peptides were subjected to Easyspray source ionisation followed by tandem mass spectrometry (MS/MS) in the Orbitrap analyser coupled online to the nano-LC. Intact peptides were detected in the Orbitrap at a resolution of 60,000. Peptides were selected for MS/MS using HCD setting at 30; ion fragments were detected in the Orbitrap at a resolution of 30.000. A data-dependent procedure that alternated between one MS scan followed by 40 MS/MS scans was applied for ions above a threshold ion count of 1.0E4 in the MS survey scan with 30.0 s dynamic exclusion. The electrospray voltage applied was 2.1 kV. MS1 spectra were obtained with an AGC target of 12E5 ions and a maximum injection time set to auto, MS2 spectra were acquired with an AGC target of 1E5 ions and a maximum injection time set to auto. For MS scans, the *m/z* scan range was 350 to 1800. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository [33] with the dataset identifier PXD041759.

2.6. Identification and quantification of proteins

The resulting MS/MS data were processed using Sequest HT search engine within a Proteome Discoverer 2.5 SP1 against a *Medicago truncatula* proteome downloaded from the UniProt database (https://www.uniprot.org/), with 90,553 entries accessed on 7 December 2021. Trypsin (RK) was specified as cleavage enzyme allowing up to 2 missed cleavages, 4 modifications per peptide and up to 5 charges. Mass error was set to 10 ppm for precursor ions and 0.02 Da for-fragment ions. Oxidation on Met (+15.995 Da), Carbamidomethyl on Cys (+57.021 Da), pyro-Glu formation from Gln or Glu (−17.027 Da or – 18.011 Da respectively), acetylation (+42.011 Da) and Met-loss (−131.040 Da) on protein-terminus were considered as variable modifications. The false discovery rate (FDR) was assessed using Percolator and thresholds for protein, peptide and modification site were specified at 1%. Label-free quantification was performed with Proteome Discoverer and the fold changes were calculated based on the AUC of each protein and statistical significance was calculated using ANOVA.

2.7. Bioinformatics and data analysis

In the current study, identified proteins were compared to UniProt (Universal Protein Resource) database to assess their function [34]. PANTHER (Protein Analysis Through Evolutionary Relationships) Classification System version 17.0 (http://www.pantherdb.org/) was used to GO (gene ontology) classification of identified DAPs (differentially abundant proteins). Then each protein was classified according to GO descriptions to 3 groups: protein class, biological process, and molecular function. Wolf-psort (http://www.genscript.com/wolf-psort.html) was used to predict subcellular localization of identified DAPs. Pathway enrichment analysis was performed using the KEGG (Kyoto Encyclopedia of Genes and Genomes) database (http://www.genome.ad

Table 1
Sequences of primers used for real time quantitative PCR (qRT-PCR).

Gene	Sequence (5'–3')	Product size (bp)	Tm (°C)
Calcium-transporting ATPase (*VfCaATPase*)	F: 5'-ATGGAGGCATTGACAAAAGG-3' R: 5'-TGACGACAACCCATGTGACT-3'	140	59
Protein-serine/threonine phosphatase (*VfPP2C*)	F: 5'-GAAGACAAACCGAAGCGAAG-3' R: 5'-TGCAGTTTGTGATGGGGATA-3'	94	60
Heat shock protein (*VfHSP*)	F: 5'-GGCTGCGTAAACCAGAAGAG-3' R: 5'-GCCTTGAACTCAAGCTGTCC-3'	127	61
Ascorbate peroxidase (*VfAPX*)	F: 5'-AGAGAGCTGTCCGATGCAAT-3' R: 5'-CCAAGCAGGGATGTCAAAAT-3'	146	59
Ras-related protein (*VfRAB*)	F: 5'-AGCGGTGTGGGTAAAAGTTG-3' R: 5'-CCATCAAGCTCAATGGTCCT-3'	113	60
Dehydrin (*VfDHN*)	F: 5'-TCCTCGGTACCTGTTGTTCC-3' R: 5'-CCGATCTGACAGCTCATCAA-3'	150	61
ATP synthase (*VfATPS*)	F: 5'-TAAGAAGGTAGCCGCAAGGA-3' R: 5'-TCACGCAGACTCGTATCGTC-3'	127	61
GATA type zinc finger transcription factor (*VfGATA*)	F: 5'-TAGGACAACCACCGTGTGAA-3' R: 5'-AAGCTGCTGGCATGTTTTCT-3'	138	59
Glutathione peroxidase (*VfGPx*)	F: 5'-CGCTTCAAAGCTGAGTTTCC-3' R: 5'-TTGATACCGTCCCCAAAGAG-3'	116	60
AP2/ERF transcription factor (*VfAP2/ERF*)	F: 5'-CTCCGACTTCATCCCACCTA-3' R: 5'-CTCGAAATCATCGTCAAGCA-3'	122	60
Delta-1-pyrroline-5-carboxylate synthase (*VfP5CS*)	F: 5'- TGCAGAGGGGTTAATTTTGG-3' R: 5'- AGAGAAGCCCATTCCCACTT-3'	128	60
Lipoxygenase (*VfLOX*)	F: 5'-CAGTGATGACCGTGTTGGAC-3' R: 5'-AGCTCCATCAACCTCCCTTT-3'	139	61
Thiamine thiazole synthase (*VfTHI*)	F: 5'-AACAGCTTCACGTTCGGTTT-3' R: 5'-GCAAACCAGCTCACCTCTTC-3'	137	60
S-adenosylmethionine synthase (*VfSAMS*)	F: 5'-GGGACTCCGATTGCATAAGA-3' R: 5'-GTTAGGCAAGCAGCCAAAAG-3'	83	60
Chalcone synthase (*VfCHS*)	F: 5'-ATGTGAATCGCAGGATGACA-3' R: 5'-TGGGATCAATCTGGAAAAGC-3'	104	58
Elongation factor 1 A (*VfELF1A*)	F: 5'-GACAACATGATTGAGAGGTCCACC-3' R: 5'-GGCTCCTTCTCAATCTCCTTACC-3'	542	58

.jp/kegg/).

2.8. Protein interaction

STRING (Search Tool for Retrieval of Interacting Genes) program (version 11.5) containing 70,883,737 proteins from 14,094 organisms and more than 20,000,000,000 interactions was use to visualize PPI (Protein-Protein Interaction) network. *Medicago truncatula* was applied as a reference organism. Network interactions of DAPs by STRING analysis was performed at confidence level 0.7. The network nodes represent proteins and the edges are the predicted functional associations. Presence of fusion was showed by red line and green line represents the presence of neighbourhood. Light blue line represents database evidence, while co-occurrence evidence was showed by blue line. Purple, yellow and black line represents experimental, text mining and co-expression evidence, respectively [35].

Table 2
Physiological and biochemical parameters in Bachar leaves subjected to well-watered and drought stress. Net photosynthetic rate (Pn), stomatal conductance (gs), transpiration rate (E), internal concentration of CO_2 (Ci), leaf relative water content (RWC), total chlorophyll (Chlt), proline (Pro), hydrogen peroxide (H_2O_2), soluble sugars (SS), electrolyte leakage (EL), malondialdehyde (MDA) and activities of catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX) and guaiacol peroxidase (GPOX). All data represent means ± standard deviation (SD) of three replicates. *Significant at $p \leq 0.05$.

Parameter	Control	Stressed	p
Pn (μmol CO_2 m^{-2} S^{-1})	8.11 ± 0.34	4.01 ± 0.14	$p < 0.004$ *
gs (mmol H_2O m^{-2} S^{-1})	176.05 ± 4.73	105.35 ± 3.74	$p < 0.004$ *
E (mmol H_2O m^{-2} S^{-1})	2.01 ± 0.16	1.31 ± 0.06	$p < 0.032$ *
Ci (μmol CO_2 mol^{-1})	319.25 ± 2.47	308.50 ± 2.12	$p < 0.043$ *
RWC (%)	77.88 ± 1.61	56.08 ± 2.48	$p < 0.009$ *
Chlt (mg g^{-1} FW)	126.33 ± 3.70	101.28 ± 5.09	$p < 0.030$ *
EL (%)	62.10 ± 3.25	83.56 ± 5.56	$p < 0.042$ *
Pro (μg mg^{-1} DW)	15.33 ± 2.19	44.16 ± 3.69	$p < 0.011$ *
SS (μg mg−1 DW)	30.56 ± 4.08	49.07 ± 3.17	$p < 0.037$ *
MDA (mmol g^{-1} FW)	2.39 ± 0.38	5.56 ± 0.24	$p < 0.010$ *
H_2O_2 (μmol g^{-1} FW)	26.64 ± 2.71	61.50 ± 3.47	$p < 0.008$ *
CAT activity (μmoles min^{-1} mg^{-1})	17.99 ± 1.89	40.52 ± 3.76	$p < 0.017$ *
APX activity (μmoles min^{-1} mg^{-1})	24.91 ± 3.59	73.47 ± 4.75	$p < 0.007$ *
SOD activity (unit mg^{-1} protein)	4.95 ± 1.06	12.98 ± 1.89	$p < 0.035$ *
GPOX activity (μmoles min^{-1} mg^{-1})	34.18 ± 3.71	65.42 ± 4.15	$p < 0.016$ *

2.9. Total RNA extraction, first strand cDNA synthesis and quantitative PCR (qPCR) assays

Using the NucleoSpin® RNA plant kit (MachereyNagel, Germany), total RNA was isolated from leaf tissues according to the manufacturer's protocol. NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific) was used to quantify and qualify extracted RNA samples. One microgram of total RNA was reverse transcribed using the GoScript™ Reverse Transcription System (Promega, Madison, WI, USA) according to the manufacturer's protocol.

The coding sequences (CDS) of faba bean genes were retrieved from nucleotide database of the National Center for Biotechnology Information (NCBI). Primers were designed with Primer 3 web version 4.1.0 (https://bioinfo.ut.ee/primer3/) and listed in Table 1. *Elongation factor 1 alpha* (*EF-1a*) gene was used as internal control. PCR reactions were performed with the 7300 Real-Time PCR Detection System (Applied Biosystems, Foster City, USA). The 30 μl reactions contained 2 μl diluted cDNA (50 ng), 0.5 μl of 10 μM each primer, 15 μl of Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Fisher Scientific) and 12 μl of nuclease free H_2O. A two-step qPCR protocol was carried out in triplicate for each sample: 95 °C for 10 min followed by 40 cycles of 95 °C for 30 s and 60 °C for 1 min. The cycle threshold (CT) value method [36] was used to analyze the relative changes in gene expression. Melting curve analyses were performed to confirm the presence of a single amplicon per reaction. The heatmap was constructed using the R package Heatmap3.

Table 3
Differentially abundant proteins (DAPs) in leaves of faba bean under drought stress measured by label-free LC-MS/MS.

No	Protein name	Function	Regulated type	Fold change	P-value	Protein accession	Unique peptides	MW [kDa]/ calc.pI	KEGG pathway
1	Glyoxalase I (GlyI)	Stress response	Up	14.94	1.47 E-15	A0A0C3VZJ3	1	39.10/ 7.81	Metabolic pathways
2	Photosystem I reaction center subunit III (PsaF)	Photosynthesis	Up	13.72	1.47 E-16	I3SN70	1	24.70/ 9.69	Energy metabolism
3	Helicase SWR1 (SWR1)	Chromatin organisation	Up	10.12	1.47 E-16	A0A072UUK6	1	25.80/ 8.90	Necroptosis
4	Calcium-transporting ATPase (CaATPase)	Solute transport	Up	10.00	1.47 E-16	G7L0M8	1	117.70/ 5.40	Calcium signaling pathway
5	AP2/ERF transcription factor (AP2/ERF)	Signal transduction	Up	5.07	3.44 E-9	G7JL93	1	26.20/ 4.93	Signaling pathway
6	Kunitz type trypsin inhibitor/ Alpha-fucosidase (AKPI)	Plant defense responses	Up	4.02	1.48 E-16	G7KMU3	1	23.80/ 5.36	Other glycan degradation
7	Outer envelope pore protein (OEP)	Solute transport	Up	3.75	3.56 E-6	I3SSC7	1	15.50/ 8.68	Transporters
8	Ankyrin repeat protein (ANK)	Stress response	Up	3.17	8.53 E-5	A0A072USU9	2	37.20/ 4.59	Metabolic pathways
9	Delta-1-pyrroline-5-carboxylate synthase (P5CS)	Amino acid metabolism	Up	3.01	1.47 E-16	G7J9Y9	1	77.70/ 6.87	Biosynthesis of amino acids
10	Arabinose kinase-like protein (ARA1)	Arabinose metabolic process	Up	3.00	0.22 E-3	G7I565	2	108.70/ 6.87	Carbohydrate metabolism
11	Early nodulin-like protein (ENODL)	Stress response	Up	2.93	0.34 E-3	G7JGC6	1	14.50/ 6.61	Metabolic pathways
12	Ras-related protein (RAB)	Hydrolase activity	Up	2.92	0.34 E-3	G7L0L9	1	23.90/ 7.83	Metabolic pathways
13	Ubiquitin-conjugating enzyme E2 (UBC2)	Protein homeostasis	Up	2.89	0.17 E-2	A0A072VRU4	1	121.50/ 4.79	Ubiquitin mediated proteolysis
14	Serine hydroxymethyltransferase (SHMT)	Glycine biosynthesis	Up	2.78	0.79 E-5	G7K5Z4	1	56.20/ 7.69	Biosynthesis of amino acids
15	Polygalacturonase-inhibiting protein (PGIP)	Plant defense responses	Up	2.77	0.85 E-5	G8A1C7	2	35.90/ 8.05	Metabolic pathways
16	Isocitrate dehydrogenase [NADP] (IDH1)	NADPH production	Up	2.53	0.01	G7IT36	1	46.70/ 7.99	Citrate cycle (TCA cycle)
17	60 S ribosomal protein L7a (60 S)	Protein biosynthesis	Up	2.51	0.38 E-2	A0A072TYM4	1	29.50/ 10.27	Ribosome
18	Histone H2A (H2A)	Chromatin organisation	Up	2.48	0.40 E-3	G7IHN1	1	16.30/ 10.86	Necroptosis
19	Dehydrin (DHN)	Stress response	Up	2.47	0.45 E-2	G7J7R2	3	23.50/ 5.80	Protein processing
20	Aldo/keto reductase family oxidoreductase (AKR)	Oxidoreductase activity	Up	2.40	0.66 E-2	G7JL33	1	36.20/ 6.73	Metabolic pathways
21	Pyruvate decarboxylase (PDC)	Aromatic compound biosynthetic process	Up	2.38	0.74 E-2	G7KSB5	1	65.40/ 6.13	Citrate cycle (TCA cycle)
22	Glutathione peroxidase (GPx)	Stress response	Up	2.35	0.02	G7LI01	2	25.90/ 9.19	Glutathione metabolism
23	L-ascorbate peroxidase (APX)	Redox homeostasis	Up	2.34	0.94 E-2	G7J4Y2	4	47.40/ 8.95	Glutathione metabolism
24	40 S ribosomal protein S14 (40 S)	Protein biosynthesis	Up	2.31	0.01	G7IPY3	1	16.30/ 10.59	Ribosome
25	ATP synthase subunit beta (ATPS)	Cellular respiration	Up	2.28	0.44 E-2	A0A072W1H5	1	59.90/ 6.15	Metabolic pathways
26	Peroxisomal membrane protein PEX14 (PEX14)	Protein transport	Up	2.23	0.01	G7JNV8	1	57.00/ 5.53	Peroxisome
27	Pyruvate dehydrogenase E1 component subunit alpha (PDHA1)	Aromatic compound biosynthetic process	Up	2.22	0.01	A0A072TYD9	3	47.30/ 7.11	Citrate cycle (TCA cycle)
28	Heat shock protein (HSP 90)	Protein homeostasis	Up	2.18	0.02	G7IDZ4	2	79.30/ 5.50	MAPK signaling pathway
29	UDP-arabinopyranose mutase (UAM)	Biosynthesis of polysaccharides	Up	2.18	0.02	A0A072V8I1	2	41.00/ 5.96	Metabolic pathways
30	Nascent polypeptide-associated complex subunit beta (NAC)	Signal transduction	Up	2.17	0.02	B7FMW7	2	17.50/ 7.40	Signaling pathway
31	Alpha-galactosidase (GLA)	Hydrolase activity	Up	2.16	0.02	A0A072VXV5	1	45.40/ 5.90	Carbohydrate metabolism
32	28 kDa heat-and acid-stable phosphoprotein-like protein (HASPP28)	Phosphorylation	Up	2.15	0.02	I3SYD0	1	18.90/ 8.72	Signaling pathway
33	Glycine cleavage system H protein (GCSH)	Glycine catabolic process	Up	2.15	0.02	G7JZW9	2	17.80/ 5.19	Metabolic pathways
34	Quinolinate synthase (NadA)	De novo biosynthesis of NAD+	Up	2.13	0.02	A0A072UQY1	1	75.30/ 5.94	Biosynthesis of cofactors

(*continued on next page*)

Table 3 (*continued*)

No	Protein name	Function	Regulated type	Fold change	P-value	Protein accession	Unique peptides	MW [kDa]/ calc.pI	KEGG pathway
35	Protein-serine/threonine phosphatase (PP2C)	Protein homeostasis	Up	2.11	0.03	B7FLN2	1	40.00/ 5.41	AMPK signaling pathway
36	50 S ribosomal protein L9 (50 S)	Protein biosynthesis	Up	2.10	0.03	B7FFD2	3	21.40/ 9.64	Ribosome
37	GATA type zinc finger transcription factor (GATA)	Signal transduction	Up	2.09	0.03	B7FME7	2	20.90/ 8.92	Signaling pathway
38	Thioredoxin-dependent peroxiredoxin (TPx-Q)	Redox homeostasis	Up	2.09	0.03	G7JS60	8	24.20/ 9.47	Metabolic pathways
39	Dihydroflavonol reductase (DFR)	Biosynthesis of anthocyanidins	Up	2.08	0.03	G7KZB6	1	36.40/ 7.11	Flavonoid biosynthesis
40	Xylose isomerase (XI)	Fructose and mannose metabolism	Up	2.08	0.03	G7JD38	2	54.20/ 6.44	Metabolic pathways
41	7-hydroxymethyl chlorophyll a reductase (HCAR)	Chlorophyll metabolism	Up	2.08	0.03	A0A072VIA1	1	49.90/ 7.96	Biosynthesis of secondary metabolites
42	Linker histone H1 and H5 family protein (H1/H5)	Chromatin organisation	Up	2.06	0.04	A0A072VL85	3	26.80/ 10.52	Necroptosis
43	Protein disulfide-isomerase (PDI)	Protein homeostasis	Up	2.04	0.04	G7IDU4	2	40.40/ 5.49	Protein processing
44	Light-harvesting Chlorophyll a-b binding protein (LHCB)	Chlorophyll metabolism	Up	2.04	0.04	A0A072U5R4	2	28.30/ 5.66	Biosynthesis of secondary metabolites
45	Photosystem I reaction center subunit II (PsaD)	Photosynthesis	Up	1.96	0.04	G7K2D0	7	23.00/ 9.57	Energy metabolism
46	Thiamine thiazole synthase (THI)	Thiamine (vitamin B1) biosynthesis	Down	0.48	0.03	A0A072W0W5	8	37.10/ 5.69	Metabolic pathways
47	Ribose-phosphate diphosphokinase (PRS1)	Phosphorylation	Down	0.47	0.02	G7K0V0	1	35.70/ 6.68	Biosynthesis of secondary metabolites
48	S-adenosylmethionine synthase (SAMS)	Phytohormone action	Down	0.46	0.02	G7L3W1	1	42.60/ 6.84	Biosynthesis of secondary metabolites
49	Aspartic proteinase nepenthesin-like protein (NEP)	Protein homeostasis	Down	0.45	0.01	G7J557	1	46.70/ 8.37	Protein processing
50	Valyl-tRNA synthetase (VARS1)	Protein homeostasis	Down	0.45	0.01	A0A072U849	3	111.70/ 6.55	Aminoacyl-tRNA biosynthesis
51	Lipoxygenase (LOX)	Phytohormone action	Down	0.44	0.01	A0A072UMH4	10	103.50/ 5.64	Metabolic pathways
52	Ferritin (FER)	Solute transport	Down	0.41	0.39 E-2	G7JLS7	3	28.10/ 6.04	Mineral absorption
53	Glycerophosphodiester phosphodiesterase GDPD)	Lipid metabolic process	Down	0.39	0.18 E-2	G7KW66	3	47.50/ 6.02	Metabolic pathways
54	Cytochrome P450 family fatty acid hydroperoxide lyase (HPL)	Oxidoreductase activity	Down	0.38	0.12 E-2	A0A072VD70	2	62.90/ 9.04	Biosynthesis of secondary metabolites
55	Epoxide hydrolase (EH)	Cutin synthesis	Down	0.11	1.47 E-16	A2Q321	1	36.20/ 6.09	Metabolic pathways
56	Chalcone synthase (CHS)	Flavonoid biosynthesis	Down	0.10	1.47 E-16	G7KXB8	2	43.00/ 6.70	Biosynthesis of secondary metabolites
57	Cinnamyl alcohol dehydrogenase-like protein (CAD)	Lignin biosynthetic process	Down	0.01	1.47 E-16	G7I8B8	1	39.00/ 6.70	Biosynthesis of secondary metabolites
58	Leucine aminopeptidase (LAP)	Protein homeostasis	Down	0.01	1.47 E-16	A0A072VIT3	1	26.20/ 5.02	Glutathione metabolism
59	Phosphoribosylamine–glycine ligase (purD)	Purine biosynthetic process	Down	0.01	1.47 E-16	G7K3Z1	1	55.00/ 5.43	Metabolic pathways
60	Protein-methionine-S-oxide reductase (Msr)	Amino acid metabolism	Down	0.01	1.47 E-16	G8A2F8	1	22.00/ 6.33	Biosynthesis of amino acids
61	DEAD-box ATP-dependent RNA helicase (DEAD-box RH)	Vesicle trafficking	Down	0.01	1.47 E-16	A0A072V6Z3	1	45.90/ 6.37	RNA degradation
62	Long-chain fatty acyl CoA ligase (ACSL)	Fatty acid biosynthesis	Down	0.01	1.47 E-16	G7I8F2	1	76.30/ 7.12	Biosynthesis of secondary metabolites
63	26 S proteasome non-ATPase regulatory subunit-like protein (26 S)	Protein homeostasis	Down	0.01	1.47 E-16	G7J2W0	1	75.20/ 7.93	Proteasome
64	Importin subunit alpha (IPOA)	Protein Transport	Down	0.01	1.47 E-16	G7IMW8	2	58.40/ 5.26	Nucleocytoplasmic transport
65	Acyl-coenzyme A oxidase (ACX)	Fatty acid degradation	Down	0.01	1.47 E-16	G7JXE0	2	74.40/ 7.36	Biosynthesis of secondary metabolites
66	Receptor-like kinase (RLK)	Phosphorylation	Down	0.01	1.47 E-16	G7IBS1	2	52.00/ 8.00	Protein processing
67	Glucose-1-phosphate adenylyltransferase (GlgC)	Starch biosynthesis	Down	0.01	1.47 E-16	G7J745	1	57.20/ 7.20	Amino sugar and nucleotide sugar metabolism
68	Formate dehydrogenase (FDH)	Formate degradation	Down	0.01	1.47 E-16	A0A072U694	1	36.70/ 6.33	Metabolic pathways
69	Inositol 2-dehydrogenase-like protein (idhA)	Oxidoreductase activity	Down	0.01	1.47 E-16	G7JIU8	2	41.00/ 6.80	Metabolic pathways

(*continued on next page*)

Table 3 (*continued*)

No	Protein name	Function	Regulated type	Fold change	P-value	Protein accession	Unique peptides	MW [kDa]/ calc.pI	KEGG pathway
70	2-carboxy-D-arabinitol 1-phosphate (CA1P) phosphatase	Rubisco regulation	Down	0.01	1.47 E-16	G7JKZ0	2	56.30/ 6.11	Metabolic pathways
71	CBS domain protein/transporter associated domain protein (CDCP)	Transcription regulation	Down	0.01	1.47 E-16	G7K4G4	1	91.70/ 6.21	ABC transporters
72	Farnesylcysteine lyase (FCLY)	Oxidoreductase activity	Down	0.01	1.47 E-16	A0A072V2M1	1	54.90/ 6.54	Biosynthesis of secondary metabolites
73	NAD(P)-binding rossmann-fold protein (MOD1)	Oxidoreductase activity	Down	0.01	1.47 E-16	A0A072UZ65	1	30.20/ 7.87	Biosynthesis of secondary metabolites
74	GDSL-like lipase/acylhydrolase (GELP)	Hydrolysis	Down	0.01	1.47 E-16	G7K7G4	1	39.50/ 6.77	Biosynthesis of secondary metabolites
75	GDP-L-fucose synthase (GFUS)	Oxidoreductase activity	Down	0.01	1.47 E-16	G7KRU3	1	35.30/ 7.28	Amino sugar and nucleotide sugar metabolism
76	Class I glutamine amidotransferase superfamily protein (GAT1)	Glutamine metabolic process	Down	0.01	1.47 E-16	A0A072V987	1	46.80/ 7.23	Metabolic pathways
77	Pti1-like kinase (Pti1)	Phosphorylation	Down	0.01	1.47 E-16	G7JQ35	1	43.50/ 8.95	Protein processing
78	Transmembrane 9 superfamily member (TM9SF)	Solute transport	Down	0.01	1.47 E-16	G7INP9	1	66.40/ 7.68	Transporters
79	Lung seven transmembrane receptor family protein (7TMR)	Receptor	Down	0.01	1.47 E-16	A0A072VJV6	1	49.80/ 6.44	Signaling pathway
80	Aspartyl aminopeptidase-like protein (DAP)	Protein homeostasis	Down	0.01	1.47 E-16	G7I369	1	58.20/ 7.90	Protein processing
81	Group 1 family glycosyltransferase (GT1)	Protein glycosylation	Down	0.01	1.47 E-16	G7JN01	1	57.50/ 8.47	Metabolic pathways

2.10. Statistical analysis

Statistical Package for the Social Sciences (SPSS 20.0, SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Data are shown as the mean ± standard deviation (SD). A one-way analysis of variance (ANOVA), followed by Tukey's Honestly Significant Difference (HSD) test ($P < 0.05$) were performed to estimate the effects of water-deficit on the analysed parameters.

3. Results

3.1. Physiological and biochemical parameters

Net photosynthesis (Pn), intercellular CO_2 concentration (Ci), transpiration rate (E), stomatal conductance (gs), total chlorophyll (Chlt) content and leaf relative water content (RWC) of faba bean plants declined under water deficit by 51%, 40%, 34%, 4%, 28% and 20%, respectively, compared to the control (Table 1). In contrast to photosynthetic parameters and RWC, drought stress increased the content of oxidative stress biomarkers such as electrolyte leakage (EL), malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) by 35%, 133% and 130%, respectively (Table 1). Moreover, drought increased the content of the osmoprotectants proline and soluble sugars by 188% and 61%, respectively (Table 2). Similarly, the application of drought stress increased the activity of guaiacol peroxidase (GPOX), catalase (CAT), ascorbate peroxidase (APX) and superoxide dismutase (SOD) by 91%, 125%, 162% and 195%, respectively (Table 1).

3.2. Protein identification under drought stress

A total of 2000 drought-induced proteins covered a wide range of biological processes were identified (Table S1). Of them, 81 differentially abundant proteins (DAPs) exhibited significant expression changes (a fold change of >1.5 or <0.5, $p < 0.05$), of which 45 proteins were upregulated and 36 proteins were downregulated (Table 3). In all DAPs, the most upregulated protein was glyoxalase (A0A0C3VZJ3) in plants subjected to drought stress, which was involved in methylglyoxal (MG) detoxification which was 14.94 times' that of the well-watered group. Compared to control condition, 7 DAPs showed increased levels at over 4 times, 37 showed increased levels between 2 and 4 times and 1 protein showed a less-than-2-fold increase. In the other hand, the 36 down-regulated proteins showed decreased levels from 0.48–0.01-fold compared to that of the unstressed condition and of those, 25 proteins were not detected in drought stress leaves proteome.

3.3. Subcellular localisation and functional classification of DAPs

Among the 4 categories of GO terms, molecular functions analysis (Fig. 1A) revealed that the DAPs related to other functions (40%), catalytic activity (37%), binding (16%), ATP-dependent activity (4%), transporter activity (2%) and structural molecule activity (1%). The 6 molecular functions (Fig. 1B) enriched with the most proteins were cellular process (42%), metabolic process (31%), localisation (11%), biological regulation (9%), response to stimulus (6%) and other biological processes (1%). Based on the protein class (Fig. 1C), DAPs were mainly grouped into metabolite interconversion enzyme (48%), unclassified proteins (23%), transporter (8%), translational protein (5%), protein modifying enzyme (5%), chaperone (2%), regulatory protein (2%), gene-specific transcriptional regulator (2%), RNA metabolism protein (1%), cytoskeletal protein (1%), protein-binding activity modulator (1%), storage protein (1%) and transmembrane signal receptor (1%). Moreover, the proteins induced by drought stress in faba bean leaves were mainly localized in the unknown cellular components (28%), chloroplast (15%), cytosol (14%), cytoplasm (11%), mitochondria (9%), membrane (7%), nucleus (6%), endoplasmic reticulum (4%), peroxisome (2%), Golgi apparatus (2%), cytoskeleton (1%) and extracellular region (1%) which suggests the critical role of chloroplast in response of faba bean to drought stress (Fig. 1D).

3.4. KEGG pathway enrichment analyses of DAPs

The results of KEGG enrichment analysis of the DAPs in faba bean leaves under drought stress are listed in Table 3. Pathway enrichment results revealed that biosynthesis of secondary metabolites, protein processing, signalling pathway and metabolic pathways are the most significantly enriched pathways for leaf drought-stressed plants (Fig. 2).

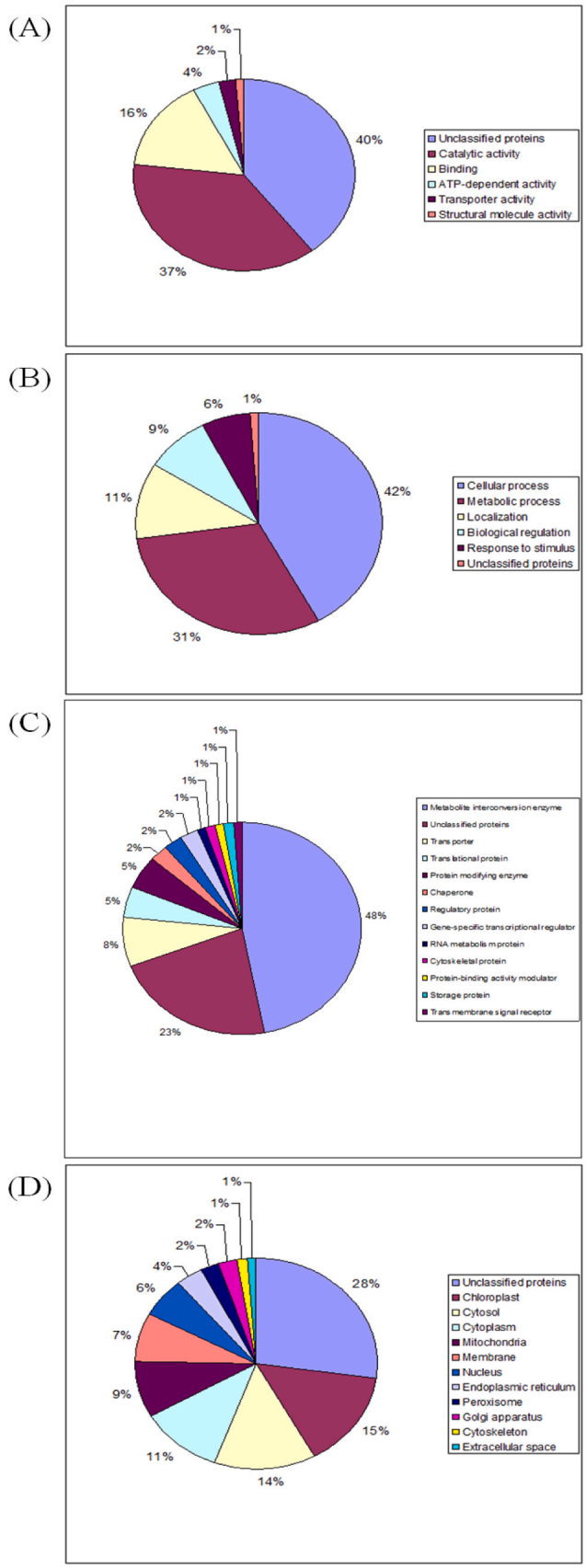

Fig. 1. A. molecular function. B. biological function. C. protein class. D. subcellular localization of DAPs.

Moreover, KEGG analysis showed that the glutathione metabolism, biosynthesis of amino acids, MAPK signalling pathway, TCA cycle and carbohydrate metabolism were enriched. Based on KEGG pathway enrichment analyses, the 81 DAPs were classified into 27 groups (Fig. 2), indicating that drought stress altered faba bean leaf protein profiles.

A total of 3 proteins related to the glutathione metabolism pathway. These play an important role in protecting plants from oxidative cellular damage caused by drought stress. L-ascorbate peroxidase (APX) and glutathione peroxidase (GPx) were upregulated, while leucine aminopeptidase (LAP) was downregulated. All identified proteins associated with the TCA cycle (6, 11 and 15) and carbohydrate metabolism (13 and 40) which are involved in plant carbon metabolism were upregulated. However, identified proteins associated with other carbohydrate metabolism such as amino sugar and nucleotide sugar metabolism (66 and 75) were downregulated. The expression patterns of photosystem I reaction centre subunit II (PsaD) and photosystem I reaction centre subunit III (PsaF) associated with photosynthesis, primarily represent a response to cell damage resulting from drought stress. Drought increased the abundance of proteins associated with photosynthesis (PsaD and PsaF). Six proteins that are involved in protein synthesis (40 S, 50 S and 60 S) and the regulation process (H2A, SWR1 and H1/H5) were detected that increased under drought stress. More than 25% of identified DAPs associated to metabolic pathways. Of those, 11 were induced, while 10 were repressed by drought. Moreover, 3 proteins were related to amino acid metabolism, with 2 of them (SHMT and P5CS) upregulated and one (Msr) downregulated. Twelve proteins related to biosynthesis and secondary metabolites pathway were identified; of those, 10 were downregulated and 2 upregulated. Interestingly, 8 proteins related to flavonoid biosynthesis (DFR), MAPK signalling pathway (HSP90), AMPK signalling pathway (PP2C), calcium signalling pathway (CaATPase) and signalling pathway (GATA, NAC, HASPP28 and AP2/ERF) were identified and their abundance significantly increased under drought treatment. In this context, the largest number of identified proteins was significantly differently expressed and grouped in other pathways associated with plant defence response to biotic and abiotic stresses such as PGIP, AKPI and DHN. These results therefore indicate that the upregulated proteins were significantly enriched in signalling pathway, energy metabolism, carbohydrate metabolism, citrate cycle (TCA cycle), ribosome, necroptosis, biosynthesis of amino acids and some proteins were enriched in protein processing and metabolic pathway. The downregulated proteins were significantly enriched in biosynthesis of secondary metabolites.

3.5. Validation of the abundance of DAPs in faba bean leaves by qPCR

A qPCR analysis was performed to confirm the correspondence between changes in gene transcription levels and changes in protein levels, as well as to confirm the authenticity of the LC-MS/MS analysis (Fig. 3A). Fifteen genes potentially involved in plant drought-stress tolerance encoding to *VfCaATPase*, *VfPP2C*, *VfHSP*, *VfAPX*, *VfRAB*, *VfDHN*, *VfATPS*, *VfGATA*, *VfGPx*, *VfAP2/ERF*, *VfP5CS*, *VfLOX*, *VfTHI*, *VfSAMS* and *VfCHS*, which were differentially expressed in faba bean leaves as revealed by LC-MS/MS analysis, were selected for qPCR assay (Fig. 3). In general, the results of the qPCR analysis confirmed data obtained by proteomic analysis. Only the transcript expression levels of *VfCaATPase*, *VfHSP*, *VfAP2/ERF* and *VfP5CS* differed slightly from the relative protein levels, which may be retated to the variation in protein post-translational modification. In order to support the reliability of our proteomic analysis, a correlation coefficient (R^2) (of the fold changes between qPCR and LC-MS/MS data) of 79.31% was obtained (Fig. 3B).

3.6. Protein-protein interaction (PPI) network analysis of DAPs

To discern the relationship of the 81 DAPs in the response to drought stress in faba bean plants, a PPI was generated using the STRING database (Fig. 4). Among the 81 DAPs, 38 comprise the interaction network;

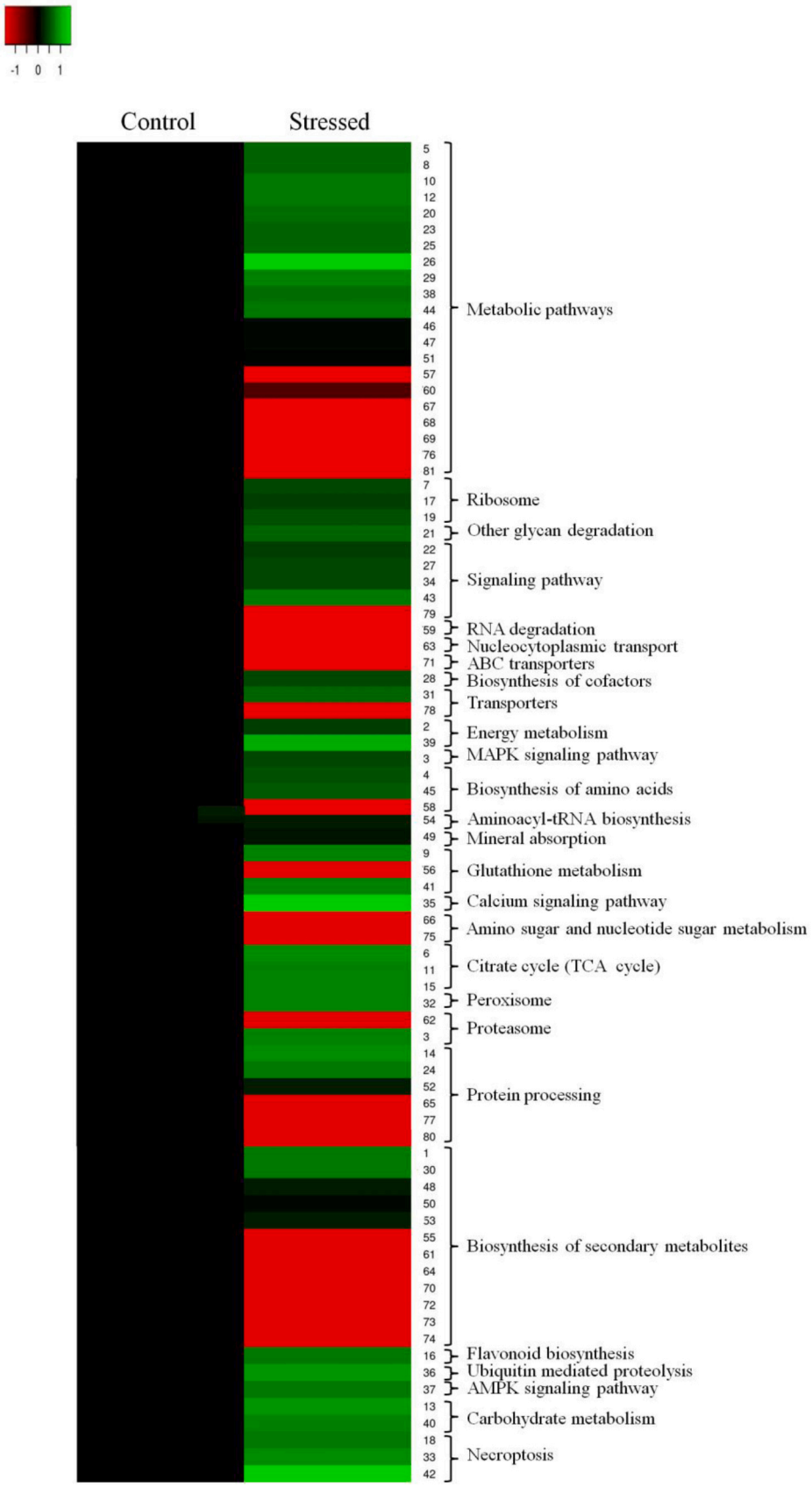

Fig. 2. KEGG pathway enrichment of DAPs induced by drought stress in faba bean leaves.

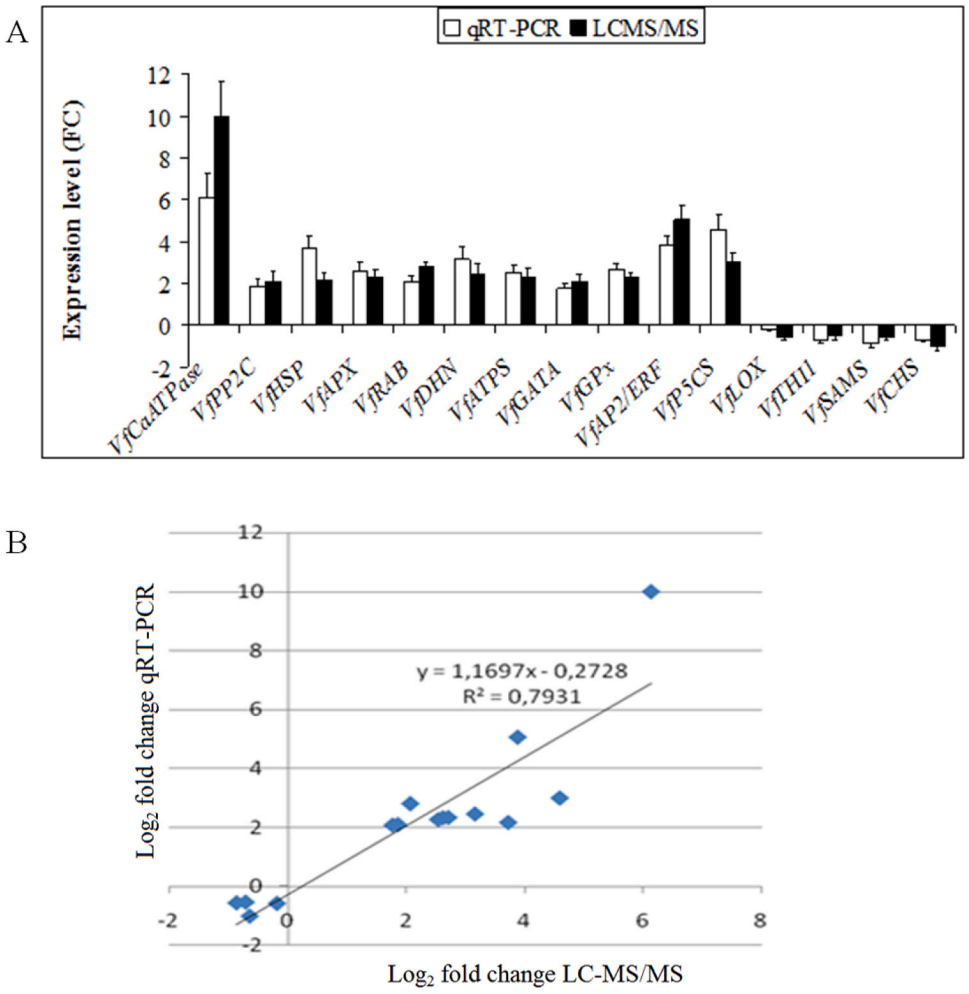

Fig. 3. A. qRT-PCR analysis of representative DAPs in faba bean leaves. B. Correlation coefficient (R^2) of the fold changes between qRT-PCR and LC-MS/MS seq.

while 40 proteins associated to drought-stress response including heat shock protein (HSP), lipoxygenase (LOX), AP2/ERF transcription factor, dehydrin (DHN) and calcium-transporting ATPase have no functional relationship to each other or with other proteins. Interestingly, the obtained data suggest that histone H2A, glutathione peroxidase, ubiquitin-conjugating enzyme E2, 26 S proteasome, thiamine thiazole synthase and photosystem I reaction centre could play a critical role in faba bean plant response to drought stress. These proteins play key roles in the response to oxidative stress, photosynthesis, gene expression regulation, protein synthesis and degradation. Furthermore, within the network of interactions, some enzymes like L-ascorbate peroxidase (APX), Delta-1-pyrroline-5-carboxylate synthase (P5CS), nascent polypeptide-associated complex subunit beta (NAC), DEAD-box ATP-dependent RNA helicase (DEAD-box RH) and thioredoxin-dependent peroxiredoxin (TPx-Q) were found to play crucial roles in faba bean response to abiotic stresses.

Within the protein interaction network, for antioxidant-defence-related glutathione peroxidase (GPx), interaction was observed with L-ascorbate peroxidase (APX), which in turn interacted with thioredoxin-dependent peroxiredoxin (TPx-Q), which further interacted with chlorophyll a-b binding protein (LHCB), photosystem I reaction centre subunit II (PsaD), 50 S ribosomal protein L9 (50 S) and aldo/keto reductase family oxidoreductase (AKR). These proteins are involved in ROS scavenging, photosynthesis and protein synthesis. P5CS had interactions with the glycine cleavage system H protein (GCSH), phosphoribosylamine–glycine ligase (Msr), NAD(P)-binding rossmann-fold protein (MOD1), isocitrate dehydrogenase [NADP] (IDH1), thiamine thiazole synthase (THI), protein disulfide-isomerase (PDI), group 1 family glycosyltransferase (GT1) and serine hydroxymethyltransferase (SHMT), which further interacted with valyl-tRNA synthetase (VARS1). These proteins involved in regulation of energy metabolism (NADPH production), amino acid metabolism, glycolysis and the tricarboxylic acid (TCA) cycle. S-adenosylmethionine synthase (SAMS) had interactions with cinnamyl alcohol dehydrogenase (CAD), dihydroflavonol reductase (DFR) and GDP-L-fucose synthase (GFUS), which further interacted with long-chain fatty acyl CoA ligase (ACSL). These proteins interact in polyamines synthesis and fatty acid metabolism. 26 S proteasome (26 S) had interactions with 40 S ribosomal protein S14 (40 S), ubiquitin-conjugating enzyme E2 (UBC2), leucine aminopeptidase (LAP), peroxisomal membrane protein PEX14 (PEX14), DEAD-box ATP-dependent RNA helicase (DEAD-box RH), which further interacted with histone H2A. All these proteins involved in energy metabolism, purine biosynthesis, glycine biosynthetic process, protein polyubiquitination, rRNA processing, protein synthesis and degradation interacted with nascent polypeptide-associated complex subunit beta (NAC). Among the 41 common proteins, 3 proteins (alpha-galactosidase, arabinose kinase-like protein and inositol 2-dehydrogenase-like protein) involved in carbon and energy metabolism were found to interact with each other forming a network, but were not found to interact with the other proteins.

4. Discussion

Water deficit is the main environmental stress that limits faba bean cultivation and production in semi-arid areas of Tunisia, where

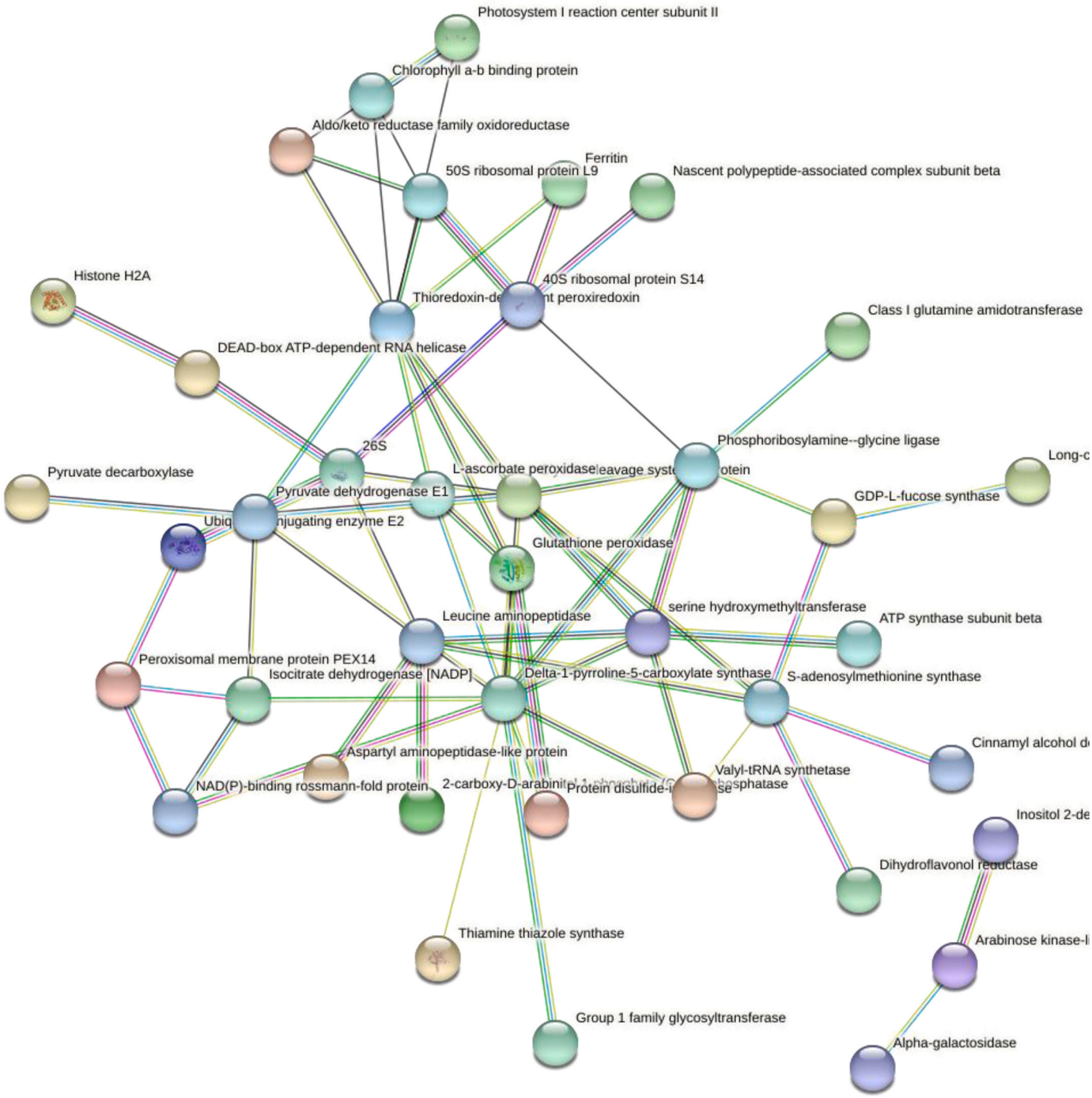

Fig. 4. Protein-protein interaction (PPI) network analysis among the significantly expressed proteins in faba bean leaves under drought stress using STRING database. The different line colours represent the types of evidence used in predicting the associations: neighbourhood (green), co-occurrence across genomes (blue), co-expression (black), experimental (purple), and association in curated databases (light blue) or texting (yellow). Disconnected nodes or proteins not connected to the main network were hidden in the network.

agriculture is predominantly rain-fed. Plants display a variety of physiological, biochemical, molecular and metabolic reactions to alleviate water-deficit stress effect, which differ from one species to another [37]. Gaining insights into drought response mechanisms is therefore a key step within faba bean breeding programmes. Agro-morphological and physiological traits associated with water-deficit stress tolerance have been demonstrated, while molecular mechanisms and regulatory networks related to drought stress adaptation and tolerance have so far been slightly studied and discussed.

In the current study, an integrative approach was used to determine the way in which proteins change and to investigate the molecular mechanisms of water-deficit stress response and resistance in faba bean, which could accelerate its genetic improvement.

Different physiological and biochemical characteristics were affected differentially in drought-stressed plants. Physiological analysis revealed that drought significantly reduced photosynthesis parameters including Pn, gs, E and Chlt. Other physiological traits and RWC were also affected by drought (Table 2). These physiological changes suggest that the water-deficit treatment was effective. Negative effects of water-deficit on the physiological performance of faba beans have been

documented by other authors [4,38]. In this study, 4 proteins (LHCB, HCAR, PsaD and PsaF) associated with photosynthesis and chlorophyll synthesis were identified and drought stress increased the abundance of these proteins in leaf tissues. These results were consistent with the previous studies of the soybean [39] and common bean [40], which showed up-regulation of LHCB and PsaD, respectively under drought stress and supported the hypothesis that these proteins may play a critical role in plant drought stress response and tolerance. In sugar cane [41] and foxtail millet [42], however, LHCB decreased in abundance under drought stress, which suggests the disruption and degradation of photosynthetic apparatus during stress. In the current study, an increased level of LHCB, HCAR, PsaD and PsaF proteins in leaf tissue indicated that the photosynthetic apparatus was still stable in faba bean plants exposed to drought stress by withholding water for 10 days. Decreases in Pn, gs, E and Ci parameters in drought-stressed plants compared to the control samples are in line with those of previous studies, in that drought stress induced rapid stomatal closure and impaired CO_2 assimilation, which lead to significant decreases in photosynthetic efficiency [43]. This could lead to a situation in which plants absorb more light than can be consumed by photosynthetic carbon fixation, resulting in a cellular oxidative state under drought stress by increasing reactive oxygen species (ROS) production including H_2O_2. In response to drought, an increased level of H_2O_2 was observed in leaf tissue and consequently membrane lipid peroxidation, which was monitored as MDA production increased (Table 2). The results agree with those in an earlier report on the faba bean [44]. Moreover, antioxidant enzymes (CAT, SOD, APX and GPOX) activity increased under drought stress, suggesting a response to increased ROS in leaf tissue and oxidative stress. Previous studies have noted that CAT, SOD and APX activity was increased in green pea plants [45] and faba bean [46], but decreased in chickpea plants [47] during drought stress, suggesting that the difference in drought-stress response may depend on stress severity, ROS production and plant species. Interestingly, in this study, abundance of proteins encoding ROS scavenging enzymes (APX, GPx and TPx-Q) has been identified and upregulated under drought stress. These data suggest the importance of the peroxiredoxin-thioredoxin pathway (PrxR-Trx cycle) and the ascorbate-glutathione cycle (AsA-GSH cycle) within the drought tolerance mechanism in the faba bean plant by maintaining the balance in the formation and elimination of ROS. Both PrxR-Trx and AsA-GSH cycle are efficient antioxidant systems for the detoxification of H_2O_2 [48]. However, the increased abundance of protein disulfide-isomerase (PDI), which plays a critical role in the formation and isomerisation of disulfide bonds during oxidative protein folding [49], indicates that leaf tissue may still be in an oxidative state.

The decreased photosynthetic efficiency which is contradicted by increased photosynthesis protein expression in faba bean plants under drought stress may be explained by a positive feedback response. Oxidative stress inactive the proteins involved in photosynthesis, but as enabled by the upregulated expression of translational machinery (60 S ribosomal protein L7a, 50 S ribosomal protein L9 and 40 S ribosomal protein S14), the faba bean plant enhanced the expression of photosynthesis-related proteins [50]. This strategy allows faba bean to maintain photosynthetic machinery and to acquire tolerance under drought by compensating for the decrease of photosynthetic efficiency.

Methylglyoxal (MG) is used as another marker of oxidative stress and considered as a potential indicator with which to evaluate plant abiotic stress tolerance. MG was produced in plant cells under drought stress as result of glycolysis, through conversion of glyceraldehyde 3-phosphate (G3P) from dihydroxyacetone phosphate (DHAP). At low concentration MG acts as a signalling molecule in stress responses, while the accumulation of MG at high levels is toxic to plant cells and results in several adverse effects such as promoting ROS generation, the degradation of proteins and inhibiting antioxidant enzyme system [51]. In order to avoid the toxic effects of MG, plants have evolved a MG-detoxifying glyoxalase (Gly) system, mainly consisting of two enzymes: glyoxalase I (Gly I) and glyoxalase II (Gly II). Gly I converts MG to S-D-lactoylglutathione (SLG) by using glutathione (GSH). Subsequently, Gly II converted SLG to D-lactate, regenerating GSH during this hydrolyse [52]. In this study, one glyoxalase I (Gly I) was significantly increased in drought-stressed faba bean plants (Supplementary Table S2) suggesting its potential role in MG detoxification in the faba bean plant. Overexpression of Gly I in transgenic rice improves abiotic stress tolerance in the presence of drought, salinity and heavy metals [53]. According to these authors, transgenic lines overexpressed OsGly I showed a decrease in MG levels compared to control after stress treatments.

Under drought stress, the accumulation of proline with osmoprotective effect increased in faba bean plants. This data was consistent with the previous study on faba beans that showed an accumulation of proline under drought stress [4,44]. Proline is mainly produced in a two-step process by pyrroline-5-carboxylate synthetase (P5CS), which reduces L-glutamic acid (Glu) to glutamate semialdehyde (GSA), which cyclises spontaneously to D1-pyrroline-5-carboxylate (P5C) and subsequently P5C gets reduced into proline by P5C reductase (P5CR). P5CS protein showed a significant increase in expression level in drought-stressed faba bean plants, resulting in increased proline content in leaf tissue. The ectopic overexpression of *Stipa purpurea* P5CS in *Arabidopsis thaliana* resulted in higher proline contents, may contribute to less membrane damage and a stabilisation of proteins and antioxidant enzymes, thereby improving the drought resistance of the plants [54].

Among the DAPs related to energy metabolism that were identified, ATP synthase subunit beta (ATPS), a key enzyme for ATP synthesis that is required for carbon assimilation in the light-reactions of photosystem (PS), was upregulated. This suggests that this protein may play a critical role in faba bean drought stress tolerance by improving the energy supply to protect the faba bean plant from injury in water deficit conditions. The result was consistent with previous studies showing that the abundance of the ATP synthase protein was greatly enhanced in sugar cane [55] and wild peach [56]. Moreover, an over-expression of the ATPS gene in transgenic Arabidopsis plants increased the resistance to cold, drought, salt and oxidative stresses [57].

Carbohydrate metabolism involves glycolysis, an important metabolic pathway through which glucose generates cellular energy. In this study, the expression of pyruvate dehydrogenase (PDHA1), which links the glycolysis metabolic pathway to the tricarboxylic acid cycle (TCA), was increased by drought. As for enzymes of the TCA, an increase in the abundance of pyruvate decarboxylase (PDC) and isocitrate dehydrogenase (IDH1) was detected. A similar increase in glycolysis and TCA-related enzymes under drought stress is reported for wild watermelon [58]. The upregulation of enzymes involved in carbon metabolism (TCA and glycolysis) and ATP-synthesis related enzymes (ATPS) may be explained by the additional energy required to protect the plant from drought stress and to maintain several cellular processes under water deficit conditions.

The proteomic analysis revealed a decreased abundance of S-adenosylmethionine synthetase (SAMS). This enzyme is involved in the biosynthesis of S-adenosylmethionine (SAM) from methionine and ATP, a precursor of polyamines (PAs), which are involved in plant response to biotic and abiotic stresses. Moreover, it was reported that ethylene production continues from SAM by two committed enzymes, 1-aminocyclopropane-1-carboxylic (ACC) synthase (ACS) and ACC-oxidase (ACO), indicating that ethylene biosynthesis is regulated by SAMS expression [59]. In the leaf tissue, SAMS protein abundance was significantly decreased, which suggests that SAMS is involved in the drought stress response in faba bean plants by limiting ethylene biosynthesis, which could result in controlled stomatal closure and retarded leaf senescence. Similarly, it was reported that 3 SAMS genes were downregulated in drought-tolerant soybean genotype roots under drought stress.

In this study, differentially expressed proteins were found to occupy a wide variety of secondary metabolic pathways. Here, cytochrome-P450-family fatty acid hydroperoxide lyase (HPL) involved in biosynthesis of volatile aldehydes and alcohols, ribose-phosphate

diphosphokinase (PRS1) involved in purine and pyrimidine synthesis, cinnamyl alcohol dehydrogenase-like protein (CAD) related to monolignol biosynthesis and acyl-coenzyme A oxidase (ACX), a key enzyme involved in jasmonic acid biosynthesis, were all found to be decreased in abundance in faba bean leaf tissues subjected to water deficit. Furthermore, two enzymes involved in biosynthesis of flavonoids, which dihydroflavonol reductase (DFR) was upregulated, while chalcone synthase (CHS) was downregulated, suggesting that drought stress promotes the increase in flavonoids, which is consistent which could aid in improves the ROS-scavenging capacity. This is consistent with the experimental results in herbaceous peony (*Paeonia lactiflora* Pall.) under drought stress [60].

Lipids are the main components of cell membrane and the precursor of some signalling molecules in plants. Under abiotic stress, lipid metabolism may help to maintain cell membrane integrity and stability [61]. Some proteins related to fatty acids metabolism were identified to be upregulated in foxtail millet [42]. However, three DAPs (Long-chain fatty acyl CoA ligase, GDSL-like lipase/acylhydrolase and glycerophosphodiester phosphodiesterase) related to the fatty acid and lipid metabolism were identified in this study and were downregulated in leaves of faba bean plants subjected to drought stress. These data suggest that faba bean plants could improve drought stress tolerance by reducing the accumulation of some enzymes associated with lipids metabolism.

Proteins involved in protein metabolism, generally including protein folding, protein synthesis and protein degradation, play a critical role in stress adaptation due to their role in post-transcriptional regulation. In this study, 3 DAPs (40 S, 50 S and 60 S), which are involved in synthesis and processing proteins, were upregulated, which suggests that these proteins each may play vital roles in the drought response by protecting the cellular process of translation and protein synthesis from negative effects of drought stress. Similar results were found in drought-treated maize leaves [62]. The abundance of repair, refolding and assembly proteins such as heat shock protein (HSP) and protein disulfide isomerase (PDI) increased in faba bean leaves under drought stress. Some members of the HSP family and PDI were found to be upregulated under drought stress in foxtail millet [42]. In response to drought, an increased abundance of proteins involved in protein degradation, including the ubiquitin/26 S proteasome system and E3 ubiquitin ligases, was observed in leaves of maize drought-stressed plants [63]. In this study, however, ubiquitin/26 S proteasome, which is involved in proteolysis mechanism for removing damaged/unnecessary proteins was downregulated. These results suggest that under long-term drought stress, complex changes to ubiquitin/26 S proteasome occurred in the leaf tissue, due to the increased sensitivity of leaves to severe drought stress [64]. Taken together, these results demonstrate that drought stress greatly influenced protein metabolism in faba bean leaves.

Epigenetic mechanisms, such as DNA methylation and histone modifications, are known to regulate the expression of stress-responsive genes by modifying the chromatin status of those genes. In faba bean leaves, proteins associated with histones (H2A, SWR1 and H1/H5) were upregulated in response to drought stress. This may be suggesting that DNA methylation and histone acetylation play a crucial role in the regulation of the expression of genes associated with drought stress responses in faba bean. Similarly, to our results, other studies into maize indicated that histones H2A and H1 accumulate during the response to drought only in drought-tolerant, but not in sensitive genotypes [65].

Abscisic acid (ABA) is a key plant stress-signalling hormone that regulates various physiological and biochemical signal transduction cascades in plants as they attempt to respond to drought stress. RCARs-PP2Cs-SnRK2s cascade constitute the ABA-signalling pathway [66]. In the presence of ABA, the ABA receptors PYR/PYL(PYR-Like)/RCAR deactivate type-2 C Ser/Thr protein phosphatases (PP2Cs), leading to the activation of SnRK2 kinases (Snf1-related protein kinase class 2), which subsequently phosphorylate downstream factors such as ABI5/ABFs transcription factors (ABA-Insensitive5/ABA-responsive element binding factors), resulting in initiate adaptive drought responses such as gene expression reprogramming and stomatal closure [67]. Moreover, for the regulation of ABA-signalling, pathway PP2C was shown to be inhibited by DEAD-box ATP-dependent RNA helicase protein, which is involved in various aspects of RNA metabolism such as RNA splicing [68]. These authors reported that a DEAD-box RNA helicase is critical, not only for the regulation of ABA signalling, but also to the drought stress response, by inhibiting PP2C activity.

A similar increase of DEAD-box RNA helicase protein was reported in drought-stressed roots of triticosecale [69] and Holm oak [70]. In this research, the expression of DEAD-box RNA helicase was downregulated, while PP2C upregulated in response to drought, which suggests that the signalling pathway in response to drought stress in faba bean plants is ABA-independent. This data is consistent with ectopic overexpression of rice PP2C (OsPP108), resulting in ABA-independent stomata movement in Arabidopsis transgenic lines, which showed high tolerance to drought [71].

In addition to enzymatic and non-enzymatic antioxidants that play a key role in ROS scavenger and in order to minimise the adverse effects of oxidative stress, plants produce different proteins that function in stress and defence response, such as dehydrins (DHNs), a group of hydrophilic proteins. This class of protein plays crucial roles in maintaining ROS homeostasis and dehydration tolerance [72]. Proteomic analysis revealed upregulation of dehydrin (DHNs) in maize [73] and the opium poppy [74] plants under drought stress. It has been reported that over-expression of the rice dehydrin gene (*OsDhn1*), improves drought stress tolerance via scavenging ROS [75]. The accumulation of DHN in leaves of drought-stressed plants therefore suggests that this protein plays a critical role in cell protection in faba bean plants.

Transcription factors (TFs) are regulatory proteins whose function is to regulate gene expression by binding to specific DNA sequences. TFs play important roles in plant growth and development. Moreover, TFs have been reported in the regulation of abiotic stress responses as imparters of drought tolerance, salt tolerance, heat tolerance and cold tolerance. The AP2/ERF superfamily is one of the largest groups of plant-specific TFs, which play important roles in plant growth and development and in the biosynthesis of various key metabolites, and also withstand biotic and abiotic stress such as heat, salinity and drought conditions [76]. The study revealed a significant increase in the abundance of the AP2/ERF protein, which suggests that this protein may be involved to a very large extent in drought-stress responses and drought tolerance in faba bean plants. Overexpression of an AP2/ERF-type transcription factor confers drought-stress tolerance in rice [77]. In this context, GATA transcription factors (GATA-TFs) are another class of transcriptional regulators identified in this study. These TFs consist of a family of zinc finger proteins that bind the consensus DNA sequence (T/A)GATA(A/G) and are involved in the regulation of growth processes and various environmental stresses. Proteomic analysis showed that the abundance of GATA-TF protein was upregulated by water deficit treatment, suggesting that it functions in response to drought in faba bean plants. Consistent with this data, the overexpression of *OsGATA8* in rice [78], *SlGATA17* in tomato plants [79] and *BdGATA13* in Arabidopsis [80] alleviated water-deficit stress in transgenic plants by increasing the abundance of drought stress-related genes.

5. Conclusion

This present investigation provides insights into physiological, biochemical and proteomic responses in the leaves of the faba bean plant to drought treatment. All physiological and biochemical parameters were affected by drought stress. Pn, Ci, gs, E, total chlorophyll content and RWC were significantly decreased, while electrolyte leakage level, proline, hydrogen peroxide and soluble sugars contents were increased under drought compared to the control plants. To restore the oxidation levels and sustain the plant during drought stress, faba bean plants increased activities of APX, SOD, CAT and GPOX. Proteomic analysis

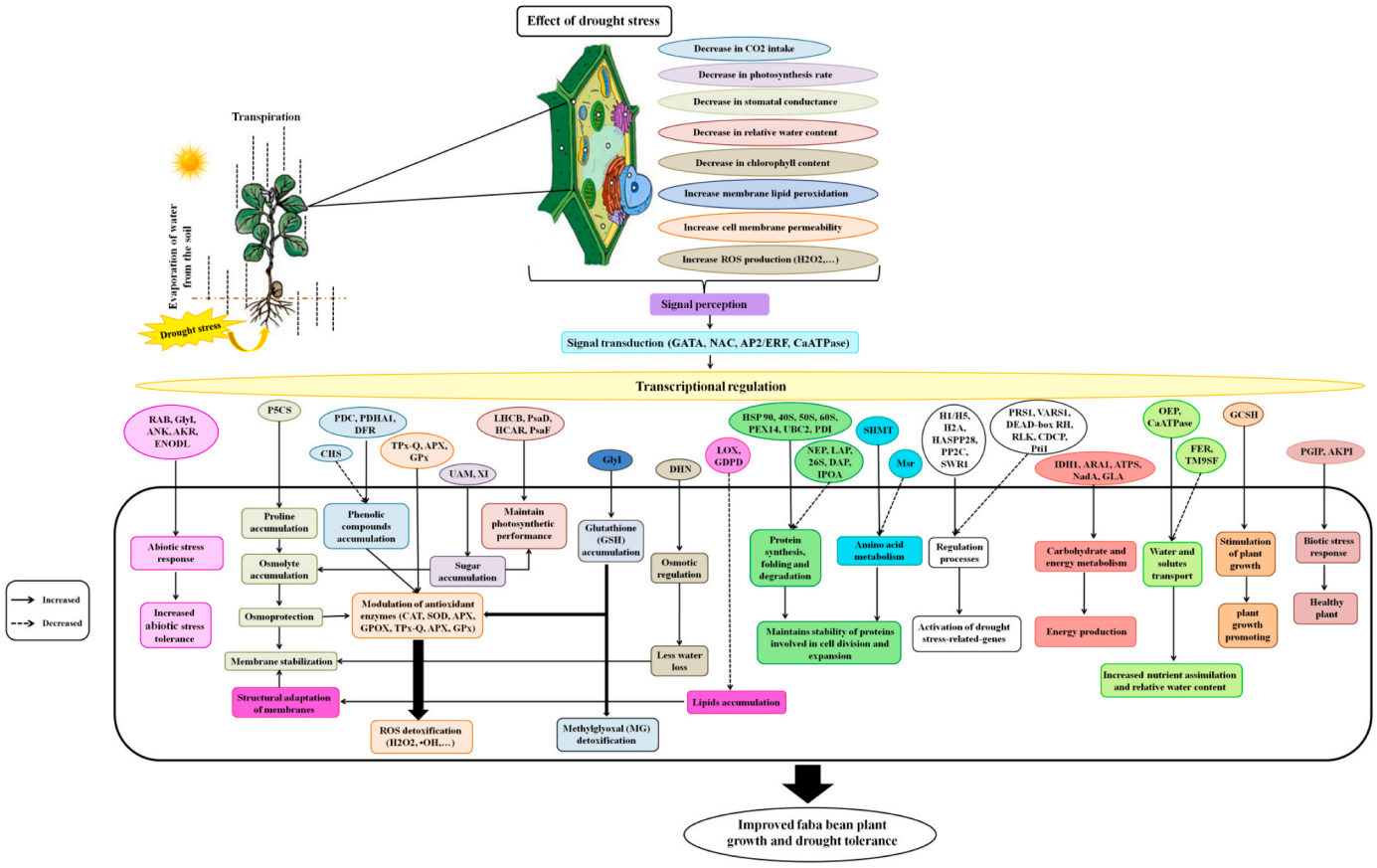

Fig. 5. An elucidated model of metabolic response to drought stress in faba bean.

revealed several DAPs with important roles in drought stress responses, suggesting that faba bean plants may use different mechanisms to cope with the adverse effects of drought. These mechanisms include proteins related relating to signal transduction, ROS detoxification, regulation process, carbohydrate and energy metabolism, photosynthesis, amino acid metabolism, protein synthesis, folding and degradation, abiotic stress response and plant defence (Fig. 5). Clearly, faba bean may use different mechanisms to adapt towards drought stress. Interestingly, protein analysis revealed that drought induced proteins involved in ROS scavenging and photosynthesis. Drought upregulated chlorophyll biosynthesis (LHCB and HCAR), which in turn increased photochemical reactions and photosynthesis electron transport (PsaD and PsaF). In addition, plants activated the ROS detoxifying system (TPx-Q, APX and GPx), augmented NADPH (IDH1), ATP production (ATPS), osmotic adjustment (P5CS), and proteins biosynthesis (40 S, 50 S and 60 S) and facilitated protein folding (HSP) in response to drought stress. However, additional analyses may be also necessary in order to characterise the potential mechanisms involved in faba bean drought stress response and tolerance in more detail.

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CRediT authorship contribution statement

Ghassen Abid and Yordan Muhovski conceptualized and designed the study; Ghassen Abid conducted the work; Ghassen Abid and Yordan Muhovski wrote the manuscript; Moez Jebara and Frédéric Debode revised and edited the manuscript; Didier Vertommenc, Sébastien Pyr dit Ruys, Emna Ghouili, Salwa Harzalli Jebara, Rim Nefissi Ouertani, Mohamed El Ayed and Ana Caroline de Oliveira provided help with the experiments.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.cpb.2024.100320.

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