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Proteomic analysis of the similarities and differences of soil drought and polyethylene glycol stress responses in wheat (*Triticum aestivum* L.)

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Abstract

Key message **Our results reveal both soil drought and PEG can enhance malate, glutathione and ascorbate metabolism, and proline biosynthesis, whereas soil drought induced these metabolic pathways to a greater degree than PEG.**

Abstract Polyethylene glycol (PEG) is widely used to simulate osmotic stress, but little is known about the different responses of wheat to PEG stress and soil drought. In this study, isobaric tags for relative quantification (iTRAQ)-based proteomic techniques were used to determine both the proteomic and physiological responses of wheat seedlings to soil drought and PEG. The results showed that photosynthetic rate, stomatal conductance, intercellular CO_2 concentration, transpiration rate, maximum potential efficiency of PS II, leaf water content, relative electrolyte leakage, MDA content, and free proline content exhibited similar responses to soil drought and PEG. Approximately 15.8% of differential proteins were induced both by soil drought and PEG. Moreover, both soil drought and PEG inhibited carbon metabolism and the biosynthesis of some amino acids by altering the accumulation of glyceraldehyde-3-phosphate dehydrogenase, ribulose-bisphosphate carboxylase, and phosphoglycerate kinase, but they both enhanced the metabolism of malate, proline, glutathione, and ascorbate by increasing the accumulation of key enzymes including malate dehydrogenase, monodehydroascorbate reductase, pyrroline-5-carboxylate dehydrogenase, pyrroline-5-carboxylate synthetase, ascorbate peroxidase, glutathione peroxidase, and glutathione *S*-transferase. Notably, the latter five of these enzymes were found to be more sensitive to soil drought. In addition, polyamine biosynthesis was specifically induced by increased gene expression and protein accumulation of polyamine oxidase and spermidine synthase under PEG stress, whereas fructose-bisphosphate aldolase and arginase were induced by soil drought. Therefore, present results suggest that PEG is an effective method to simulate drought stress, but the key proteins related to the metabolism of malate, glutathione, ascorbate, proline, and polyamine need to be confirmed under soil drought.

Keywords Water deficit · Osmotic stress · Proline · Glutathione · Ascorbate · Polyamine

Abbreviations

ADH	Alcohol dehydrogenase
APX	Ascorbate peroxidase
DP	Differentially accumulated protein
G6PD	Glucose-6-phosphate dehydrogenase
GO	Gene ontology annotation
GPX	Glutathione peroxidase
GST	Glutathione *S*-transferase
MDH	Malate dehydrogenase
P5CD	Pyrroline-5-carboxylate dehydrogenase
PDHB	Pyruvate dehydrogenase
PGK	Phosphoglycerate kinase
ROS	Reactive oxygen species
ALDO	Fructose-bisphosphate aldolase
AsA	Ascorbate

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FC	Field capacity
GAPA	Glyceraldehyde-3-phosphate dehydrogenase
GPI	Glucose-6-phosphate isomerase
GSH	Glutathione
IDH	Isocitrate dehydrogenase
MDHAR	Monodehydroascorbate reductase
P5CS	Pyrroline-5-carboxylate synthetase
PEG	Polyethylene glycol
POD	Peroxidase

Introduction

Soil drought is known to have serious detrimental effects on plant growth and development, and limits crop production worldwide. As such, major research efforts have focused on understanding the mechanisms driving plant response and adaptation to water deficit in soil at morphological, physiological, and molecular levels (Albert et al. 2014; Bechtold and Field 2018). Plants have evolved a multitude of strategies to overcome drought stress, including drought escape via developmental plasticity and drought tolerance via enhanced osmotic adjustment, water absorption, antioxidant capacity, and stomatal adjustment (Farooq et al. 2009). A wide range of transcriptomic, proteomic, and metabolomic studies have also been carried out to analyze the mechanisms of plant response to water deficit at the molecular level (Hamanishi et al. 2015; Kosová et al. 2016), and numerous proteins involved in many vital metabolic pathways, including carbohydrate and amino acid metabolism, redox homeostasis, stress response, photosynthesis, signal transduction, and protein processing, have been identified via proteomic techniques over the past several years (Faghani et al. 2015; Ford et al. 2011; Gietler et al. 2017).

Water deficit stress is associated with the accumulation of reactive oxygen species (ROS), which causes oxidative damage to proteins, DNA, RNA, and enzymes (Mittler 2002). Plants have evolved diverse mechanisms to maintain ROS homeostasis, such as antioxidative enzyme and non-enzymatic antioxidants (Apel and Hirt 2004). Several proteomic studies have shown that the abundance of proteins associated with antioxidative enzymes and non-enzymatic antioxidants increased in plants subjected to water deficit, including peroxidase (POD), GSH-AsA pathway, and glutathione peroxidase/glutathione *S*-transferase (GPX/GST) pathway (Deng et al. 2018; Echevarria-Zomeno et al. 2009; Hajheidari et al. 2007; Plumb et al. 2018).

Furthermore, proteomic analyses have revealed that numerous proteins involved in carbohydrate and energy metabolism (e.g., glycolysis, tricarboxylic acid cycle, electron transport chain, ATP synthesis) are activated in plants experiencing water-deficit conditions (Kosova et al. 2014; Peremarti et al. 2014; Sharma et al. 2017). These proteins include glyceraldehyde-3-phosphate dehydrogenase (GAPA) (Faghani et al. 2015) and fructose-bisphosphate aldolase (ALDO) (Pandey et al. 2010) associated with glycolysis and gluconeogenesis; aconitate hydratase of the tricarboxylic acid cycle (Xu and Huang 2010); and Rubisco, sedoheptulose bisphosphatase, carbonic anhydrase, phosphoenolpyruvate carboxykinase, and malate dehydrogenase (MDH) associated with carbon fixation (Hu et al. 2015; Xu and Huang 2010; Zhou et al. 2015). Moreover, concentrations of proteins involved in energy metabolism and the mitochondrial electron transport chain were altered in plants under water-deficit stress, including ATP synthesis (Cheng et al. 2016; Kosova et al. 2014), NADH dehydrogenase (Koh et al. 2015), quinone oxidoreductase (Ford et al. 2011), and cytochrome C oxidase (Budak et al. 2013).

Osmotic regulation is crucial for drought resistance in plants. Some important osmotic-related proteins, such as dehydrin, late embryogenesis abundant proteins, aldehyde dehydrogenase, and pyrroline-5-carboxylate synthetase (P5CS), significantly increased in plants under water deficit (Ashoub et al. 2015; Ford et al. 2011; Li et al. 2017); these proteins play important roles in protecting cells from dehydration stress (Samarah 2016). In addition, the accumulation of proteins related to soluble carbohydrate and amino acid metabolism were shown to reduce the osmotic potential of cells and enhance plant drought tolerance (Farooq et al. 2009). However, concentrations of some proteins associated with the synthesis of amino acids (i.e., aspartate aminotransferase and alanine aminotransferase) decreased under water-deficit conditions, an indication that amino acid metabolism and biosynthesis was inhibited by drought stress (Xu and Huang 2010).

Polyethylene glycol (PEG) causes osmotic stress and is widely used to induce drought-like stress responses in plants (Skriver and Mundy 1990). The physiological responses to soil drought and PEG stress are similar in wheat, including inhibited photosynthesis and photosystem II (PSII) efficiency, lower leaf relative water content, increased osmotic regulation, and enhanced antioxidant capacity (Cui et al. 2017, 2018; Tambussi et al. 2000; Zhou et al. 2015). In addition, as the stress level progresses, both PEG stress and soil drought can further induce the abovementioned plant responses. However, these physiological responses differ depending on the stress time and stress type, and stress responses—including phytohormonal balance, proline content, and leaf water content—can change within a few hours under PEG stress, whereas soil drought treatments must last for days or longer because control of soil moisture levels cannot be altered in a short period of time (Budak et al. 2013; Tambussi et al. 2000). Furthermore, the molecular characteristics of plants differ between soil drought and PEG stresses (Bray 2004; Fan and Blake 1997; Forner-Giner et al. 2011); for example, the differentially accumulated proteins (DPs)

that occur in plants under PEG stress may not be important given that they remain largely unchanged or exhibit opposing tendencies in plants exposed to soil drought. Although many proteomic analyses have been conducted in wheat seedlings to determine responses to soil drought (Cheng et al. 2016; Faghani et al. 2015) or PEG stress (Kang et al. 2012; Zhang et al. 2014), few studies have focused on the differences and similarities of proteomics in wheat seedlings in response to these two stresses; that is, what responses in wheat that are shared between the two different types of stress or that are unique to one or the other have not yet been elucidated. In the present study, we hypothesized that the physiological and proteomic responses of wheat seedlings to PEG solution and soil drought are similar, and that these similarities included repressed photosynthesis, increased osmotic regulation, and enhanced antioxidation capabilities, among other factors. Essentially, we conducted this study to compare and contrast rates of protein production and the physiological responses induced by soil drought and PEG as a means of assessing the effectiveness of drought simulation experiments using PEG.

Materials and methods

Materials and treatments

A winter wheat (*Triticum aestivum* L. 'Yan995') from Yantai Academy of Agricultural Science (Yantai, China) was selected for this study. A hydroponic system was used for the PEG treatment. After sterilization in 5% (v/v) sodium hypochlorite solution for 10 min and washing with distilled water, seeds were first germinated in 9 cm diameter Petri dishes with three layers of wet filter and then planted in black plastic pots (9 × 9 × 15 cm) containing 700 mL 1/2 Hoagland's nutrient solution. Pots were placed in a light growth chamber (14/10 h light/dark cycle; temperature 20/15 °C; light intensity 500 ± 50 μmol m^{-2} s^{-1}; relative humidity 70 ± 5%) and seedlings were transferred to the same solution with (PEG group) or without (CP group) 25% PEG-6000 for 72 h following the emergence of three fully expanded leaves. For the soil drought treatment, the same seeds were planted in black plastic pots (15 × 20 cm) containing a 1.7 kg mixture of farmland topsoil/sand/grass peat (1:1:2, v:v:v; pH 7.65; organic matter 43.97 g kg^{-1}; available N, P, and K 55.22, 31.67, 73.87 g kg^{-1}, respectively; maximum field capacity [FC] 29.3%). These pots were placed in the same light growth chamber as mentioned above. Soil moisture in the pots was maintained at 80% FC by adding the lost water, and maintained until seedlings had three fully expanded leaves, following which watering was controlled until soil moisture was reduced to 40% FC in the stressed group (SD), whereas the control group was maintained at 80% FC (CS). The second true leaf of the wheat seedlings were harvested. Seven biological replicates (a mixed sample of nine seedlings composing a replicate) were used in the analysis of physiological parameters and enzyme activity.

Photosynthetic and stress parameters

The second true leaf of seedling was used to measure the photosynthetic rate, stomatal conductance, intercellular CO_2 concentration, and transpiration rate. The experiment was initiated at the third hour of the light cycle by using an LI-6400XT lamp (LI-COR Biosciences, Nebraska, USA) with an intensity of 500 μmol m^{-2} s^{-1}. The maximum potential efficiency of photosystem II was measured using a Dual-PAM-100 (Heinz Walz GmbH, Effeltrich, Germany) after the leaves were kept in the dark for 30 min. Leaf relative water content was determined following the procedures described by Flexas et al. (2006); relative electrolyte leakage and MDA content were measured following the procedures described by Dionisio-Sese and Tobita (1998); and free proline levels were determined (using 0.2 g of leaf samples) following the procedures described by Bates et al. (1973).

Measurements of enzyme activities

Leaf samples (0.12 g) were homogenized in a 1.2 mL of pre-cooled extraction buffer containing 50 mM KH_2PO_4–KOH (pH 7.5), 0.1 mM EDTA, 20% (v/v) glycerin, and 2% (w/v) polyvinyl pyrrolidone. The homogenate was incubated at 4 °C for 10 min, then centrifuged at 4 °C and 12,000×*g* for 15 min, with the resulting supernatant used for enzyme activity analysis.

Ascorbate peroxidase (APX; EC, 1.11.1.11) activity was measured by monitoring the decrease in absorbance at 290 nm, in accordance with Hossain and Asada (1984). The 3 mL reaction system included 50 mM Hepes–KOH (pH 7.6), 0.5 mM AsA, and 1 mM H_2O_2, and 50 μL of enzyme extraction buffer and H_2O_2 was used to start the reaction at 25 °C. Monodehydroascorbate reductase (MDHAR; EC, 1.6.5.4) activity was measured at 340 nm, in accordance with Miyake and Asada (1992). The 3 mL reaction system included 0.1 mM NADH, 0.25 mM AsA, and 100 μL of crude enzyme, and the reaction was initiated by the addition of 0.3 units of ascorbate oxidase at 25 °C.

The activities of glutathione peroxidase (GPX, EC, 1.11.1.9) and glutathione *S*-transferase (GST, EC, 2.5.1.18) were tested following the procedures described by Nagalakshmi and Prasad (2001), with modifications. Levels of GPX activity were measured using a 3 mL of reaction solution containing 100 mM phosphate buffer (pH 7.0), 2 mM EDTA, 200 mM NaCl, 2 mM GSH, 0.4 mM NADPH, 0.5 mM H_2O_2, 1 unit of GR, and 50 μL of enzyme extraction; GR solution was used to initiate the reaction. GST activity

was determined using a 3 mL reaction system containing 100 mM phosphate buffer (pH 6.5), 1 mM GSH, and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB), with a 0.1 mL of enzyme extraction and CDNB used to initiate the reaction. Changes in absorbance at 340 nm were used to estimate GPX and GST activities.

Protein extraction, trypsin digestion, and iTRAQ labelling

Protein was extracted following the procedures described by Chu et al. (2013). Fresh leaf powder (0.05–0.1 g) was digested with 500 µL of lysis buffer (20 mM Tris–HCl, pH 7.5, 250 mM sucrose, 10 mM EGTA, 1% Triton X-100, 1 mM PI, and 1 mM DTT) in an ice-bath for 20 min and centrifuged at 15,000×*g* at 4 °C for 15 min. Three volumes of pre-cooled acetone were added to the supernatants, and the protein was precipitated at − 20 °C for ≥ 2 h. The precipitate was separated by centrifugation and rinsed three times with cold acetone. Protein concentration was determined using Bradford reagent (B6916) (Sigma-Aldrich, St. Louis, MO, USA), in accordance with the manufacturer's instructions. The protein quality was checked using SDS-PAGE and the samples that are not degraded and have clear protein bands can be further processed.

Approximately 200 µg of protein from each sample was digested with 4 µg of 50:1 trypsin (Promega Corp., Madison, WI, USA) for ≥ 12 h at 37 °C. Digested proteins were labelled with two iTRAQ Reagent-8plex Multiplex Kits (AB SCIEX, USA), in accordance with the manufacturer's instructions. For the soil drought treatment, the control groups (CS-1 and CS-2) were labelled with isobaric tags 113 and 114 in the first kit, and the stressed groups (SD-1, SD-2, and SD-3) were labelled with isobaric tags 115, 116, and 117 in the same kit. For the PEG stress treatment, the control groups (CP-1 and CP-2) were labelled with isobaric tags 113 and 114 in the second kit, and the stressed groups (PEG-1, PEG-2, and PEG-3) were labelled with isobaric tags 115, 116, and 117 in the same kit. The labelling process is shown in Fig. 1. After labelling, all samples were pooled and vacuum dried.

Protein isolation, identification, and quantification

Dried samples were re-dissolved in 100 µL of mobile phase A (2% acetonitrile, 98% H_2O pH 10) and separated using high-pH reverse-phase high-performance liquid chromatography (RP-HPLC) with a Gemini-NX 3µ C18 110A column (150 mm × 2.00 mm) and a linear gradient of 5% to 35% of mobile phase B (98% acetonitrile, 2% H_2O, pH 10) for 30 min, 35% to 95% of B for 30–32 min, 95% of B for 32–37 min, 95% to 5% of B for 37–39 min, and 5% of B for 39–45 min. The UA detector was set at 214 nm, and flow rate was 700 µL/min. Fractions were collected every 1.5 min from 5 to 45 min and dried by vacuum centrifugation.

The reconstituted peptides were analyzed with a Q-Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) coupled with a capillary HPLC system (UltiMate 3000 LC Dionex; Thermo Fisher Scientific). Peptides were re-dissolved in 0.5% v/v formic acid and loaded

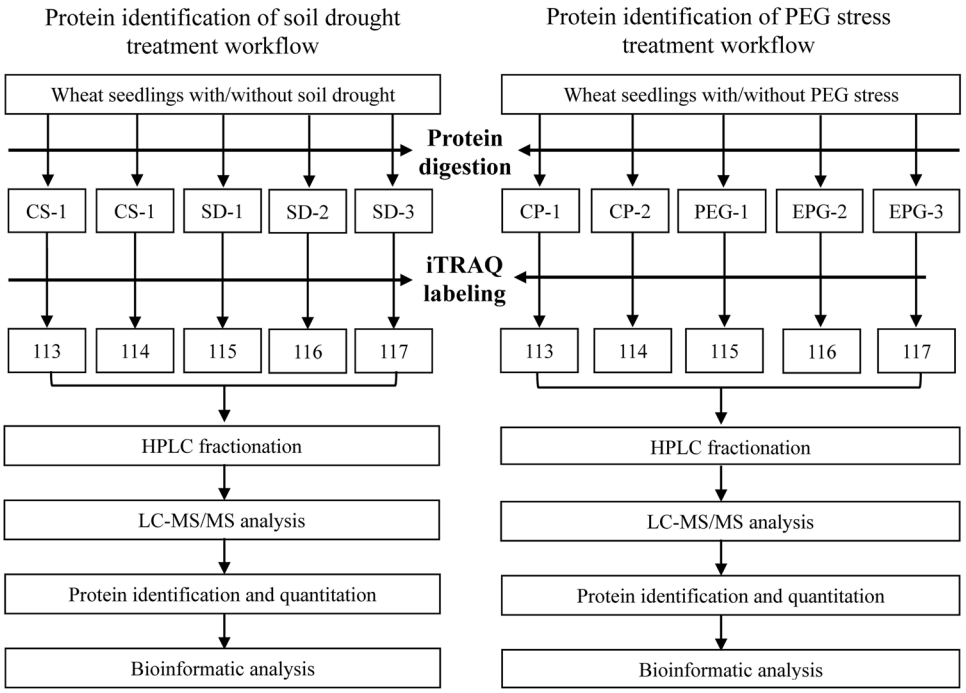


Fig. 1 Experimental process of protein accumulation induced by soil drought and PEG stress in wheat seedlings. CS-1, CS-2, CP-1, and CP-2 are the control groups (i.e., not exposed to soil drought or PEG stress); SD-1, SD-2, and SD-3 are the groups subjected to soil drought; and PEG-1, PEG-2, and PEG-3 are the groups subjected to PEG stress

onto a C18 trap column (C18 3 μm 0.10 × 20 mm) and an analytical column (C18 1.9 μm 0.15 × 120 mm), with mobile phase A (0.1% formic acid in water) and B (0.1% formic acid in 80% acetonitrile). Peptides were separated using a linear gradient as follows: 6% to 9% of B for 0–8 min, 9% to 14% of B for 8–24 min, 14% to 30% of B for 24–60 min, 30% to 40% of B for 60–75 min, 40% to 95% of B for 75–78 min, 95% of B for 78–85 min, 95% to 6% of B for 85–86 min, and 5% of B for 86–90 min, with a flow rate of 600 nL/min. MS/MS analysis was conducted using a full-mass spectrometry scan (300–1400 m/z) in the positive ion mode at a resolution of 120,000, an AGC value of 3e6, a maximum IT of 80 ms, 1 scan range, and a dynamic exclusion of 12.0 s. The dd-MS^2 was acquired at a resolution of 15,000, an AGC value of 5e4, a maximum IT of 45 ms, an isolation window of 1.6 m/z, and a normalized collision energy of 30 eV.

Tandem mass spectra were searched using Mascot 2.1 (Matrix Science, Boston, MA, USA) equipped with Proteome Discover 1.4 (Thermo Fisher Scientific, Waltham, MA, USA). Peptide identification was performed with the SEQUST search engine using wheat proteome databases containing reviewed sequences downloaded from UniProt (*Triticum aestivum*, 101003 sequences, ftp://ftp.uniprot.org/pub/databases/uniprot). Software parameters were as follows: peptide mass tolerance = ± 15 ppm; fragment mass tolerance = ± 0.05 Da; enzyme = trypsin; max missed cleavages = 2; fixed modification was iTRAQ8plex (K) and iTRAQ8plex (N-term), carbamidomethyl(C); variable modification was oxidation (M); and database pattern = decoy. A unique protein with at least two unique peptides and a false discovery rate (FDR) set at 0.01 was used as the criteria for further protein quantification data analysis. The mass spectrometry proteomics data has been deposited in the ProteomeXchange Consortium via the PRIDE (Vizcaíno et al. 2016) partner repository under the dataset identifier PXD012216.

Protein quantification was based on the total intensity of the assigned peptides, and the average of proteins in the control and stressed groups were calculated (Supplementary Table S3). A *t* test was performed to assess whether protein abundance differed significantly between the control and stressed groups under soil drought and PEG stresses (Supplementary Table S2). The final ratios of proteins were normalized between the stressed and control groups. The protein confidence threshold was set to 1.500, and fold-changes of > 1.500 and < 0.667 ($P < 0.05$) were set as the cutoff values indicating statistically significant changes in protein accumulation.

Quantitative real-time PCR

The experiment was conducted using four biological replicates, with each biological replicate composed of a mix of the second true leaves from nine plants. Frozen leaf samples (50–100 mg) were ground into powder in liquid nitrogen, and total RNA was extracted using TRIzol Reagent (Thermo Fisher Scientific), following which 2 μg total RNA of each sample was reverse transcribed using a PrimeScript™ RT Reagent Kit (TaKaRa BIO, Dalian, China), in accordance with the manufacturer's instructions. The gene-specific primers were designed using Primer Premier 5.0; these primers, along with their PCR amplification efficiency, correlation coefficient (R^2), and regression equation, are listed in Supplementary Table S1. Quantitative real-time PCR was performed using 20 μL SYBR® Advantage® qPCR Premix (TaKaRa BIO), in accordance with the manufacturer's instructions. A two-step PCR approach was used under the following conditions: pre-denaturation at 95 °C for 5 min, and 40 cycles of 95 °C for 15 s and 60 °C for 30 s. For normalization of gene expression, *Actin* (gene ID: AB181991) was used as an internal reference.

Bioinformatic analysis and other data processing

The DPs were annotated using AgriGO for gene ontology annotation (GO) and KEGG Automatic Annotation Server (KAAS, http://www.genome.jp/kaas-bin/kaas_main) for metabolic pathways enrichment analysis (Kanehisa et al. 2012). For physiological and gene expression data statistics and analyses, Microsoft Excel 2013 (Microsoft Corp., Redmond, WA, USA) and IBM SPSS Statistics 19.0 (IBM Corp., Armonk, NY, USA) were used in this study. In IBM SPSS Statistics 19.0, Duncan's Multiple Range Test ($P < 0.05$) was used for all physiological data analysis and ANOVA ($P < 0.05$ and $P < 0.01$) was used for gene expression data analysis.

Results

Physiological responses of wheat seedlings under soil drought and PEG stress

Photosynthetic rate, stomatal conductance, transpiration rate, maximum potential efficiency of PS II (Fv/Fm), and relative water content were all lower in wheat seedlings exposed to soil drought conditions and treated with PEG (Fig. 2), whereas intercellular CO_2 concentration, relative conductivity, and MDA content were higher in seedlings exposed to the two stressors (Fig. 2). Furthermore, none of the parameters differed significantly between soil drought and PEG stress, indicating a similar degree of stress response (Fig. 2).

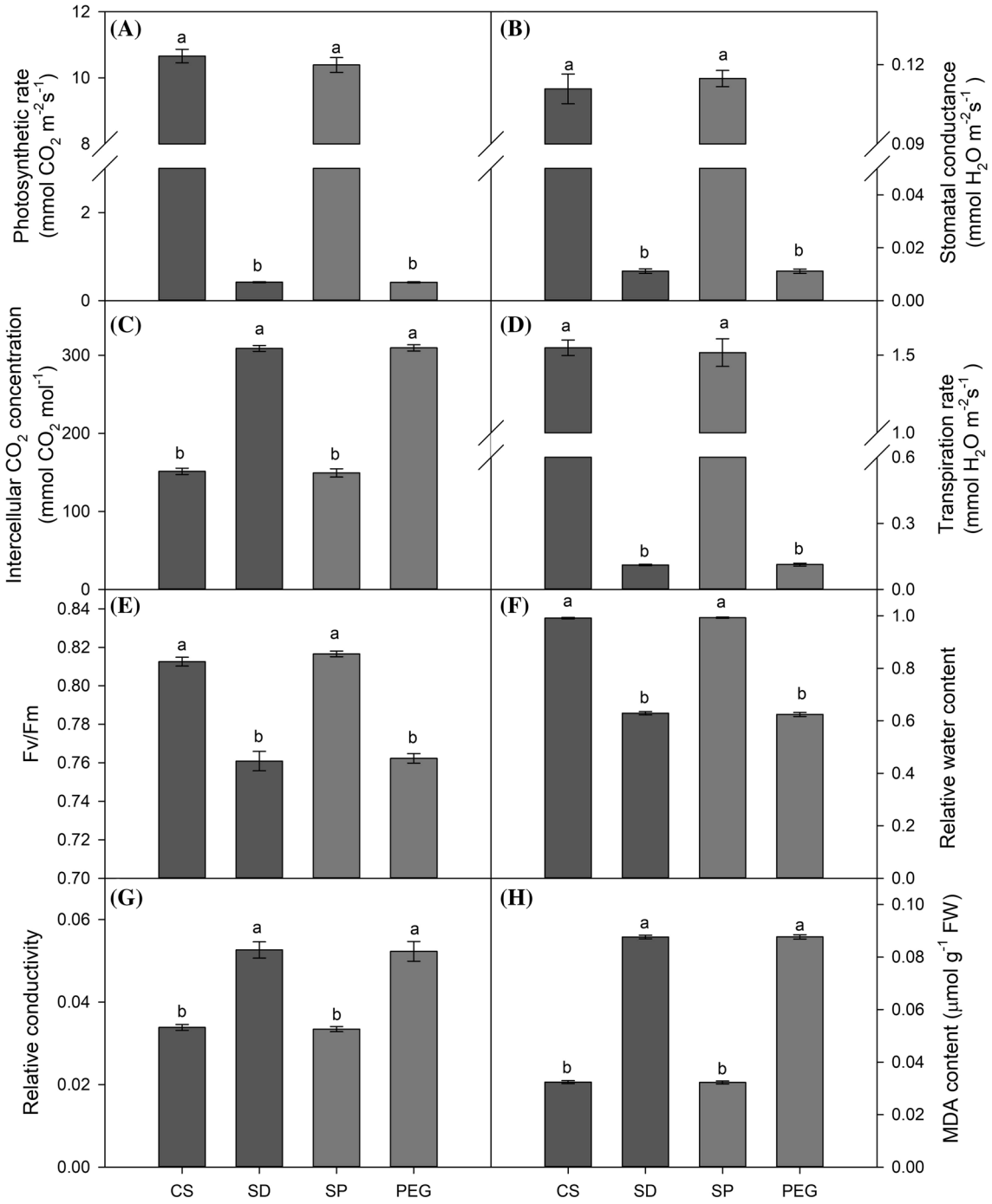


Fig. 2 Photosynthetic gas exchange parameters, maximum potential efficiency of photosystem II (Fv/Fm), relative water content, relative conductivity, and MDA content of wheat seedlings under soil drought and PEG stress. CS and CP indicate that the wheat seedlings were treated without stress condition; SD and PEG indicate that the wheat seedlings were treated with soil drought and PEG stress, respectively. Different lowercase letter indicates significant differences (Duncan's multiple range test, n = 7, $P < 0.05$). Bar = mean ± SE

Protein profiling of wheat seedlings under soil drought and PEG stress

All identified peptides and proteins are listed in Supplementary Tables S2 and S3. In total, 3926 proteins were identified in soil drought stress and PEG-simulated osmotic stress among the control and stressed groups (Fig. 3a). Furthermore 1643 proteins (41.8%) were expressed in both soil-drought- and PEG-stressed plants (Fig. 3a). In all, 590 DPs were identified under soil drought and PEG-induced

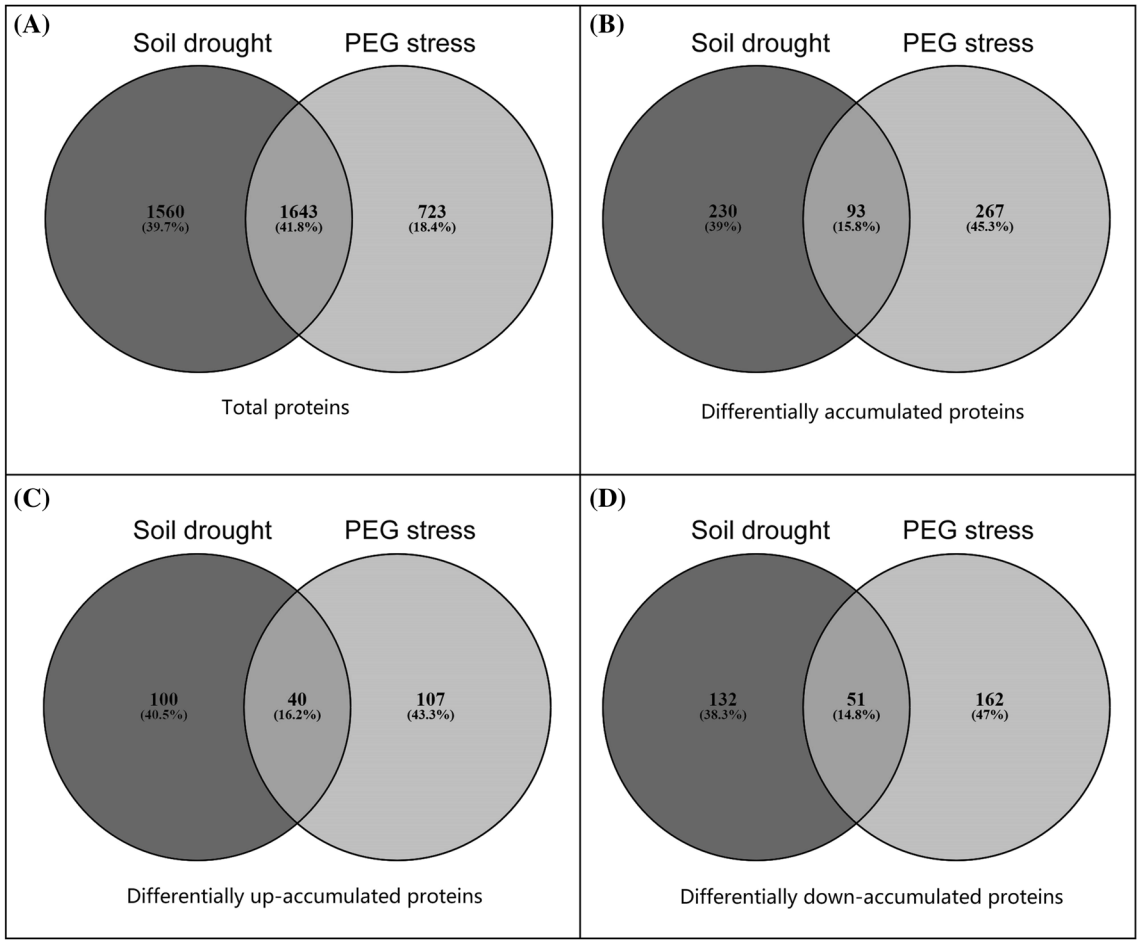


Fig. 3 Venn diagram of protein accumulation induced by soil drought and PEG stress in wheat seedlings. The protein confidence threshold cutoff was set to 1.500, and the fold-changes of > 1.500 or < 0.667 ($P < 0.05$) were set as cutoff values for significant alterations in protein accumulation

stress conditions (Fig. 3b). Under soil drought, a total of 323 DPs was identified in the control and stressed groups (Fig. 3b), of which 140 were up-regulated (Fig. 3c) and 183 were down-regulated (Fig. 3d), whereas under PEG stress, a total of 360 DPs was identified (Fig. 3b), of which 147 were up-regulated (Fig. 3c) and 213 were down-regulated (Fig. 3d). Only 93 proteins (15.8%) were common to and differentially regulated in both stressors, but two of these exhibited differing accumulation tendencies under the two stresses; thus only 91 proteins were common to and differentially accumulated in, and showed uniform accumulation tendencies in both stressors. Furthermore, 232 DPs were exclusively in wheat seedlings exposed to soil drought, of which 100 were up-regulated and 132 were down-regulated (Fig. 3c, d), and 269 DPs were more common in plants exposed to PEG stress, of which 107 were up-regulated and 162 were down-regulated (Fig. 3c, d).

GO and KEGG enrichment analysis of common DPs induced by soil drought and PEG stress

The DPs common to both soil drought and PEG-induced osmotic stress were searched in the GO and KEGG databases; 80 DPs were found to be present in the GO enrichment database and 53 presents in the KEGG database (Supplementary Table S4). The DPs found in the GO enrichment database were distributed into the categories "biological process", "cellular component", and "molecular function" (Supplementary Fig. S1). The biological processes consisted of metabolic process (33.53%), cellular process (25.88%), single-organism process (17.65%), biological regulation (5.29%), response to stimulus (3.53%), cellular component organization or biogenesis (3.53%), multicellular organismal process (1.76%), developmental process (1.76%), multi-organism process (1.76%),

reproduction (1.18%), immune system process (1.18%), reproductive process (1.18%), localization (1.18%), and signaling (1.18%). Most DPs were classified as cell (21.05%), cell part (19.08%), organelle (17.11%), macromolecular complex (14.47%), organelle part (10.53%), and membrane (7.89%); other DPs were classified as extracellular region (3.95%), membrane part (2.63%), membrane-enclosed lumen (1.32%), nucleoid (0.66%), cell junction (0.66%), and symplast (0.66%). Moreover, the DPs were classified into several groups according to their molecular function; these consisted of catalytic activity (40.59%), binding (35.64%), structural molecule activity (14.85%), electron carrier activity (3.96%), molecular function regulator (3.96%), and nutrient reservoir activity (0.99%). The first 20 pathways of the common DPs are shown in Fig. 4. The preferential pathways of DPs were concentrated in ribosome, porphyrin, and chlorophyll metabolism; plant-pathogen interaction; one carbon pool by folate; carbon fixation in photosynthetic organisms; phenylalanine metabolism; sulfur metabolism; and tyrosine metabolism. The significantly enriched DPs were concentrated in the first four pathways.

GO pathway enrichment of unique DPs induced by soil drought and PEG stress

The unique DPs induced by soil drought and PEG stress were searched in the GO database, and 174 DPs induced by soil drought and 226 DPs induced by PEG stress were annotated in the GO enrichment (Supplementary Tables S5 and S6). In general, unique DPs induced by soil drought and PEG were concentrated in similar GO pathways of molecular function, cellular component, and biological process. The molecular functions of those DPs induced by both stressors included catalytic activity, binding protein, structural molecule activity, transporter activity, antioxidant activity, electron carrier activity, and molecular function regulator. In contrast, the nutrient reservoir activity was unique to DPs induced by PEG stress (Supplementary Fig. S2). In terms of the cellular components, most DPs induced by soil drought and PEG stress were categorized as cell, cell part, organelle, macromolecular complex, organelle part, membrane, membrane part, and extracellular region; however, among the DPs induced by soil drought, two were located in the membrane-enclosed lumen and one was located in

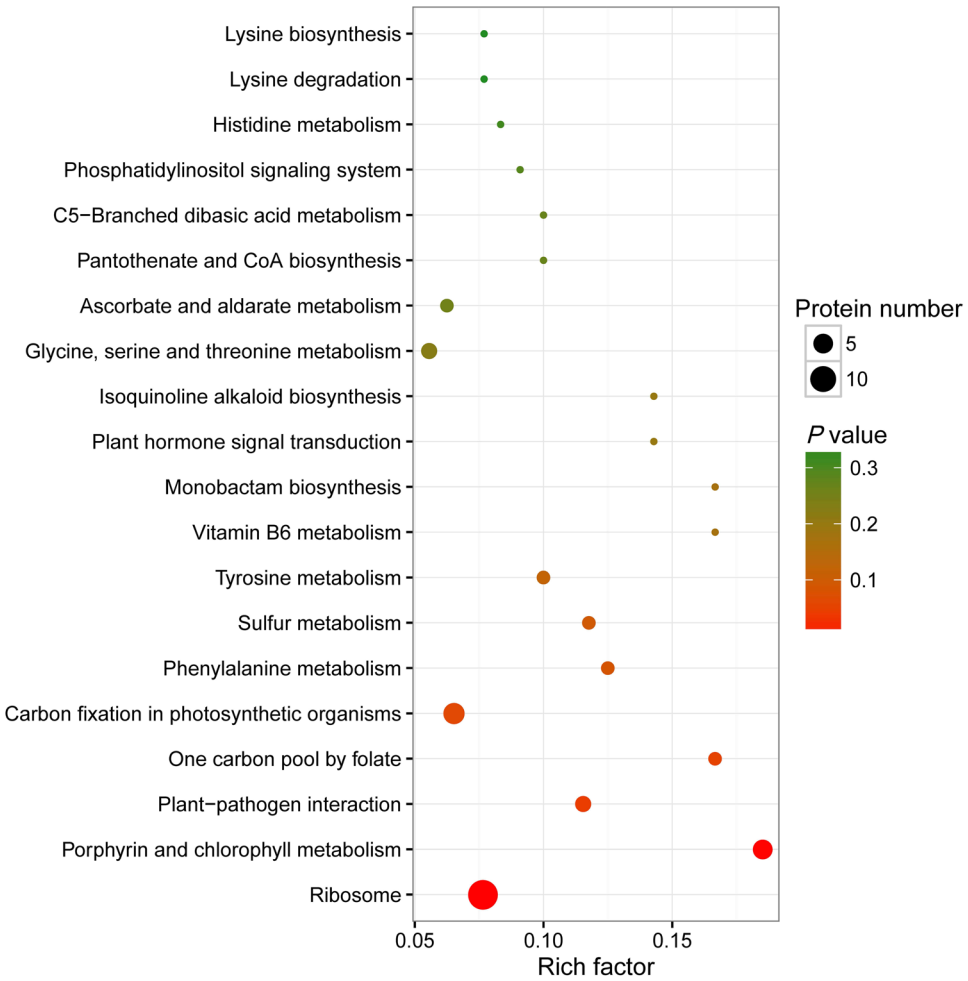


Fig. 4 The first 20 pathways of common differentially accumulated proteins induced by soil drought and PEG stress identified using KEGG analysis

the supramolecular fibre (Supplementary Fig. S2). Similarly, among the PEG-induced proteins, five were located in the symplast, cell junction, and nucleoid (Supplementary Fig. S2). The DPs induced by soil drought and PEG stress were primarily associated with the categories of metabolic process, cellular process, single-organism process, response to stimulus, localization, biological regulation, cellular component organization or biogenesis, multicellular organismal process, developmental process, reproduction, reproduction processes, signaling, and multi-organism process. However, one unique soil drought DP and three unique PEG stress DPs were determined to be associated with the growth and immune system process category (Supplementary Fig. S2).

KEGG pathway enrichment of unique DPs induced by soil drought and PEG stress

All unique DPs were annotated in KAAS. In total, 101 unique DPs induced by soil drought and 158 unique DPs induced by PEG stress were annotated in KAAS (Supplementary Tables S5 and S6). The first 20 enriched pathways of unique soil drought and PEG stress DPs are shown in Fig. 5. Among the proteins induced by soil drought, 15 DPs were significantly associated with porphyrin and chlorophyll metabolism, GSH metabolism, and lysine biosynthesis, and most of the other DPs were associated with amino acid biosynthesis, carbon metabolism, phenylpropanoid biosynthesis, cysteine and methionine metabolism, starch and sucrose metabolism, and oxidative phosphorylation pathways (Fig. 5a). Among the PEG-induced DPs, three pathways were significantly activated, including the ribosome, arginine and proline metabolism, and plant hormone signal transduction pathways. There are 58 unique DPs that were concentrated in the ribosome, with the remaining DPs concentrated in the carbon metabolism, glutathione metabolism, photosynthesis, amino acid biosynthesis, arginine and proline metabolism, starch and sucrose metabolism, protein processing in the endoplasmic reticulum, pyruvate metabolism, and carbon fixation in photosynthesis, among others (Fig. 5b). Proteins mainly differed in the ribosome metabolism, amino acid biosynthesis, GSH metabolism, photosynthesis, phenylpropanoid biosynthesis, arginine and proline metabolism, starch and sucrose metabolism, cysteine and methionine metabolism, porphyrin and chlorophyll metabolism, protein processing in endoplasmic reticulum, glyoxylate and dicarboxylate metabolism, purine metabolism, pyruvate metabolism, oxidative phosphorylation, alanine, aspartate and glutamate metabolism, fructose and mannose metabolism, and peroxisome pathways (Fig. 5c). No common DPs were found in the GSH metabolism, photosynthesis, cysteine and methionine metabolism, protein processing in the endoplasmic reticulum, oxidative phosphorylation, peroxisome pathways, and the metabolism of alanine, aspartate, and glutamate (Fig. 5c).

Carbon metabolism

Carbon metabolism was inhibited under both soil drought and PEG stress conditions, although the proteins MDH, ALDO, pyruvate dehydrogenase (PDHB), and isocitrate dehydrogenase (IDH) were enhanced under soil drought or PEG stress (Fig. 6a). Concentrations of five key enzymes involved in carbon metabolism were significantly reduced in plants exposed to both soil drought and PEG-induced stresses, including Rubisco, phosphoglycerate kinase (PGK), GAPA, serine hydroxymethyl-transferase (SHMT), formate-tetrahydrofolate ligase (FHS), and MDH2. In addition, concentrations of two proteins (A0A096ULI5 and W4ZWI7) homologous to *S*-2-hydroxy-acid oxidase (HAO) and glucose-6-phosphate isomerase (GPI) were decreased under soil drought only, whereas three others (W5H4R3, W5GIB3, and W5DCF1) related to glucose-6-phosphate dehydrogenase (G6PD), glutathione dehydrogenase/alcohol dehydrogenase (ADH), and glycine dehydrogenase (GLDC) were only decreased under PEG (Fig. 6a). However, several DPs were up-regulated under soil drought and PEG stress conditions, including two ALDO-related DPs (W5DTC2 and W5FL86) under soil drought and three DPs (W5C4H2, W5CC40, and W5FLK6) associated with the MDH, isocitrate dehydrogenase (IDH), and pyruvate dehydrogenase (PDHB) under PEG, respectively.

Reduced expression of the genes coding for W5E659, W5EN32, W5ATV6, W5BAB9, W5ETI9, and G8D5C5 was consistent with the observed patterns of protein accumulation under soil drought conditions; likewise, reduced expression of the genes coding for W5ATV6, W5BAB9, W5ETI9, and W5C4H2 was observed in wheat seedlings under PEG stress (Fig. 6). However, major differences were detected between gene expression levels and protein accumulation under both stressors. Expression of the ALDO (W5DTC2 and W5FL86) genes moved in the opposite direction to protein accumulation under soil drought, and W5DTC2 gene expression was increased by PEG stress but the protein level did not change (Fig. 6). Expression of the genes coding for W5E659 and W5EN32 did not significantly differ in seedlings exposed to PEG stress, but protein levels declined (Fig. 6). Moreover, among wheat seedlings in the soil drought treatment, concentrations of MDH and MDH2 did not change under soil drought, yet their gene expression increased significantly (Fig. 6), whereas gene expression of PDHB significantly decreased, but no changes in protein level were observed.

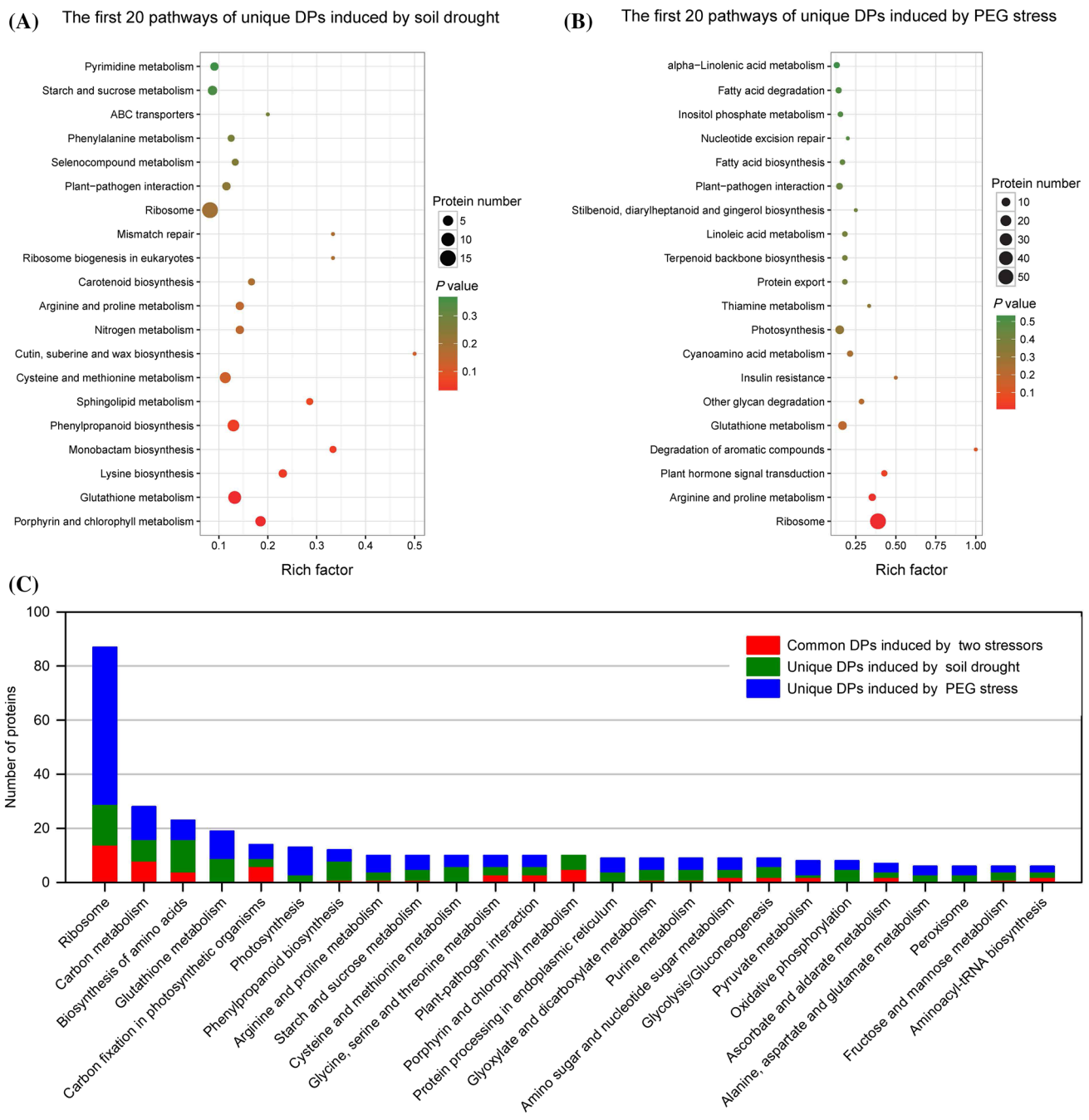


Fig. 5 KEGG analysis of unique differentially accumulated proteins induced by different water deficit stresses. (**a**) The first 20 pathways of unique differentially accumulated proteins induced by soil drought. (**b**) The first 20 pathways of unique differentially accumulated proteins induced by PEG stress. (**c**) Comparative analysis of differentially accumulated proteins participating in different pathways induced by soil drought and PEG stress

Differences in the biosynthesis of amino acid

Of the DPs induced by soil drought and PEG, more than 20 proteins were involved in amino acid biosynthesis (Fig. 7). Soil drought affected synthesis of the amino acids leucine, lysine, homoserine, methionine, tyrosine, and phenylalanine, and activity levels of some key enzymes, including isopropylmalate synthase (IMS), aspartokinase (APK), hydroxy-tetrahydrodipicolinate synthase (DAPA), diaminopimelate aminotransferase (DPAT), homocysteine methyltransferase (HMET), and dehydroquinate dehydratase/shikimate dehydrogenase (DHQ/SDH), were altered by soil drought, but not by PEG stress (Fig. 7). Furthermore, concentrations of enzymes involved in the biosynthesis of

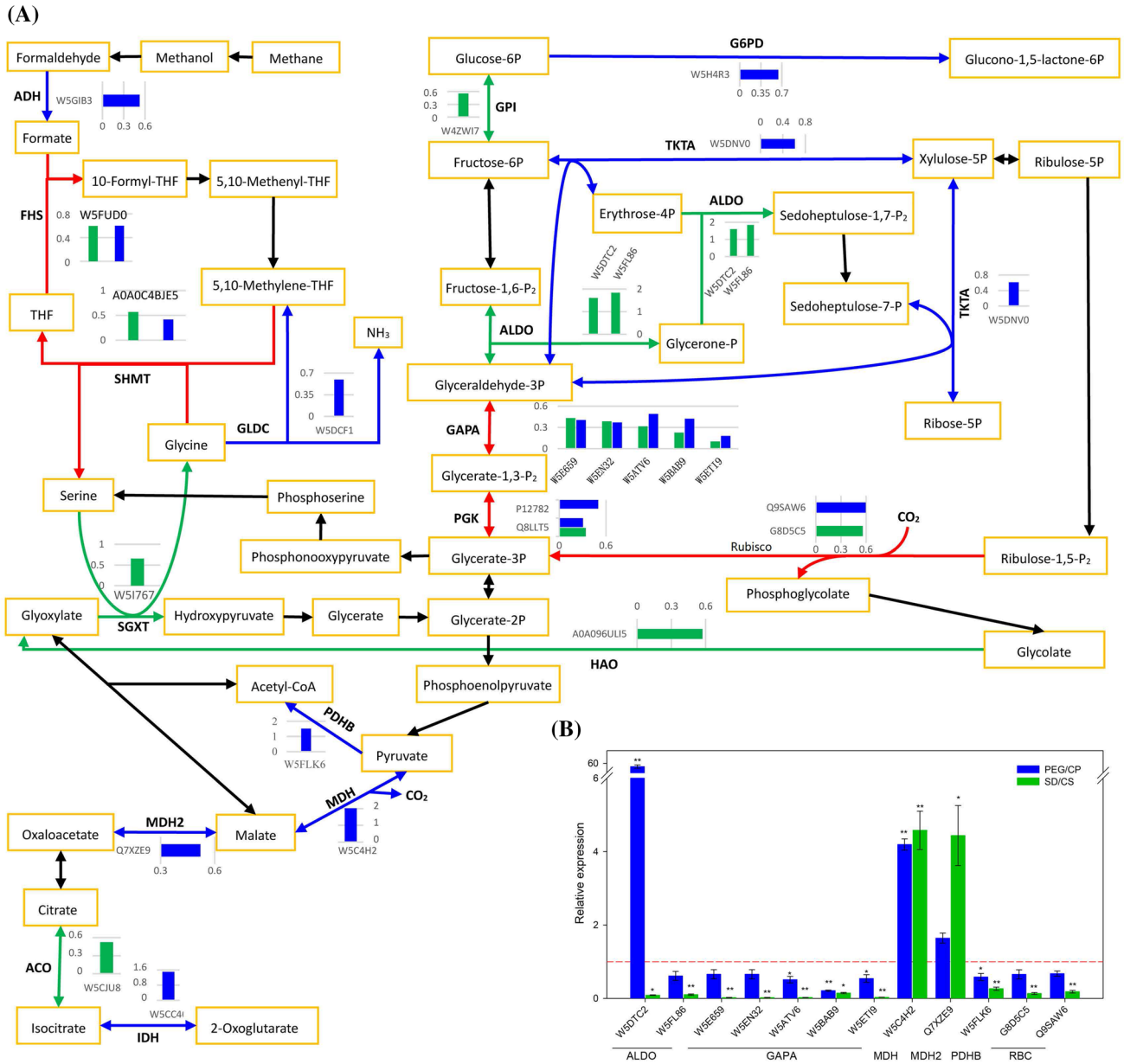


Fig. 6 Differentially accumulated proteins participating in carbon metabolism under soil drought and PEG stress. The abbreviations of enzyme names are shown in Supplementary Table S7. **a** The differentially accumulated proteins in carbon metabolism: blue and green bars indicate the fold change of PEG/CP and SD/CS, respectively; blue and green arrows indicate the enzymes that were differentially accumulated by PEG stress and soil drought (fold change > 1.500 or < 0.667, $P < 0.05$), respectively; the red arrow indicates that the enzymes were commonly differentially accumulated under soil drought and PEG stress. **b** Gene expression of some core proteins in carbon metabolism: * and ** indicate that the differences of PEG vs. CP and SD vs. CS were significant at the level of $P < 0.05$ and $P < 0.01$ (ANOVA, n = 4). Bar = mean ± SE; red dashed line indicates that the ratio of PEG/CP or SD/CS is 1

valine, isoleucine, glycine, serine, and cysteine were all affected by soil drought conditions, but were unaffected by PEG stress (Fig. 7). In contrast, concentrations of enzymes associated with the synthesis of histidine and tryptophan, including TKTA and tryptophan synthase (TRPA), were affected by PEG stress (Fig. 7), and three of these enzymes—cysteine synthase (CS), HMET, and diaminopimelate aminotransferase (DPAT)—were up-regulated by exposure to soil drought but were not induced by PEG.

Proline and polyamine metabolism

Metabolism of proline and polyamine were both affected by soil drought and PEG, with proline content significantly

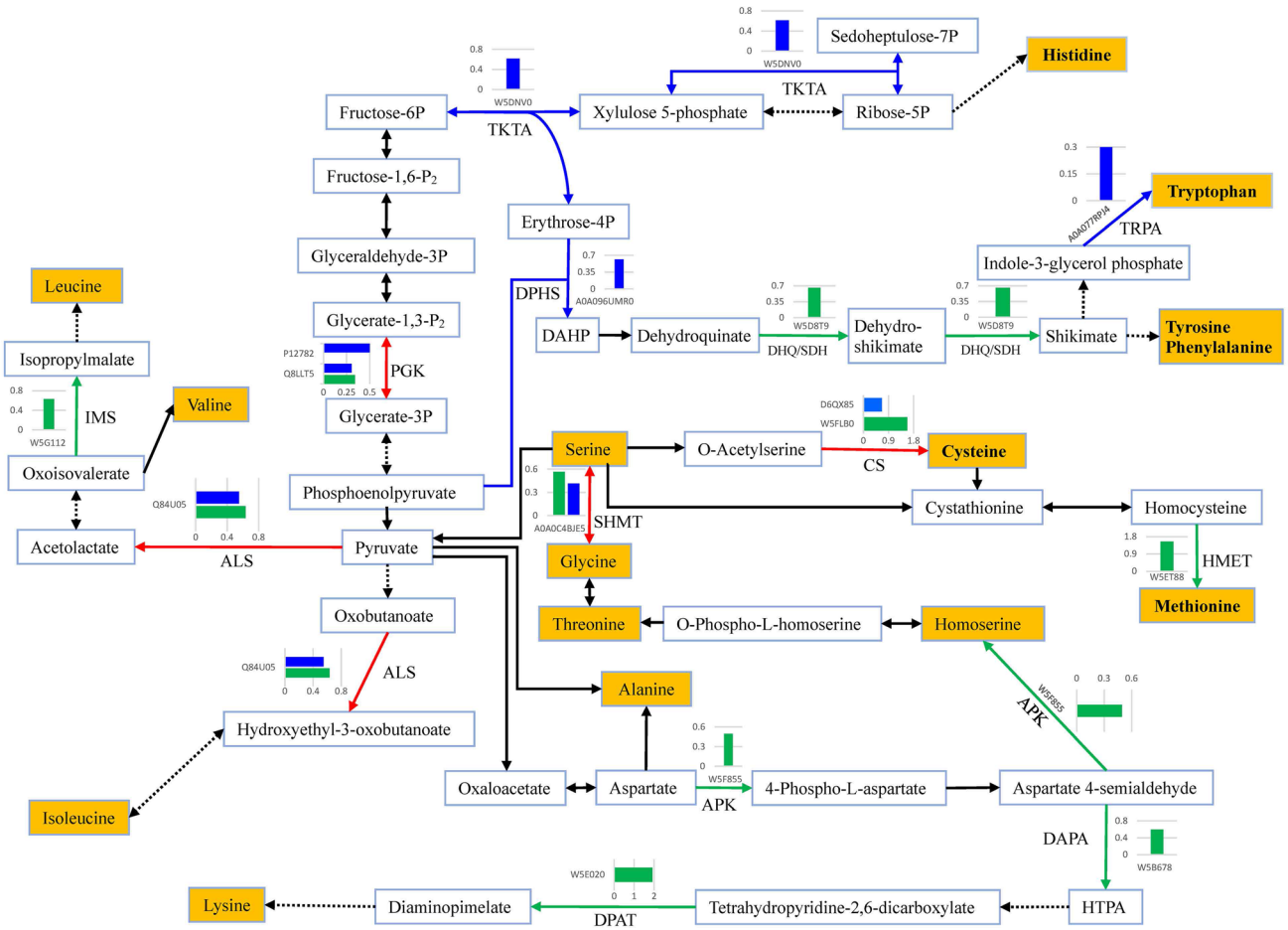


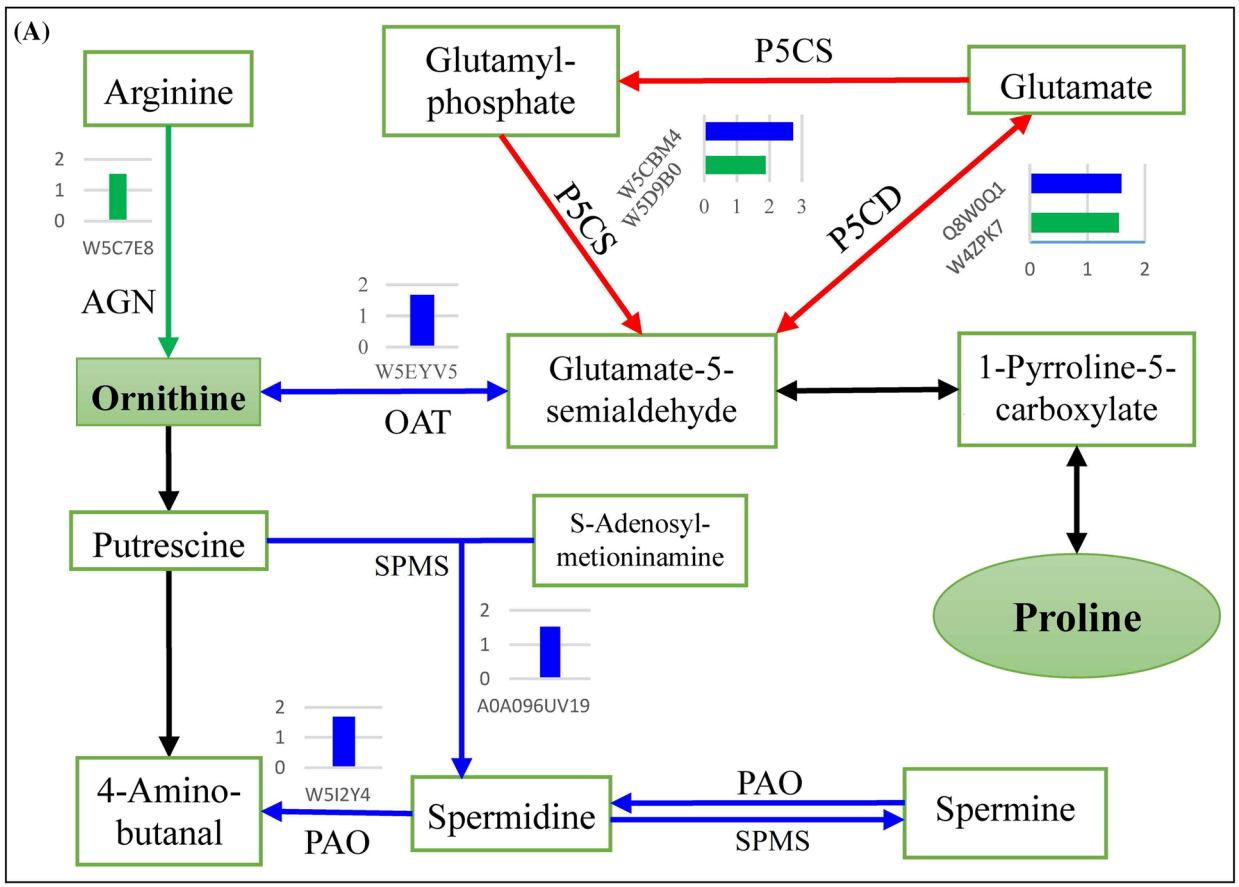
Fig. 7 Differentially accumulated proteins participating in amino acid biosynthesis under soil drought and PEG stress. The differentially accumulated proteins in carbon metabolism: blue and green bars indicate the fold change of PEG/CP and SD/CS, respectively; blue and green arrows indicate the enzymes that were differentially accumulated by PEG stress and soil drought (fold change > 1.500 or < 0.667, $P < 0.05$), respectively; the red arrow indicates that the enzymes were commonly differentially accumulated under soil drought and PEG stress. The dotted arrow indicates that the process has more than one step. Enzyme name abbreviations are presented in Supplementary Table S7

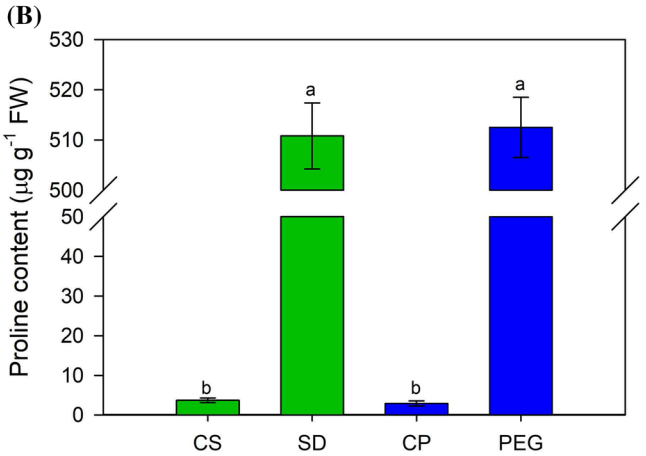
increased in both treatments (Fig. 8b). Concentrations of key enzymes in the ornithine and glutamate pathways, including ornithine aminotransferase (OAT), arginase (AGN), and P5CS, were higher in both PEG and soil drought conditions (Fig. 8a), with concentrations of the multifunctional enzyme pyrroline-5-carboxylate dehydrogenase (P5CD) significantly increased in wheat seedlings exposed to both treatments (Fig. 8a). Furthermore, soil drought directly induced synthesis of ornithine by stimulating AGN production, whereas PEG indirectly ornithine synthesis through an increase in concentrations of polyamine oxidase (PAO) and spermidine synthase (SPMS) (Fig. 8a).

In addition, expression levels of genes coding for AGN, P5CD, and P5CS significantly increased under soil drought, whereas only expression of P5CS significantly increased under PEG stress (Fig. 8c), and expression of the genes regulating PAO and SPMS production increased in a manner consistent with increases in concentrations of the two proteins under PEG stress (Fig. 8c). In contrast, although expression levels of the genes for W5I2Y4 (PAO), A0A096UV19 (SPMS), and Q8W0Q1 (P5CD) were modified by soil drought, the protein concentrations remained the same as in controls (Fig. 8a, c). Moreover, although gene expression of W5CBM4 (P5CS) and W5D9B0 (P5CS) increased under both stressors, concentrations of W5CBM4 increased in PEG-stressed but not soil-drought seedlings, whereas concentrations of W5D9B0 increased in soil-drought but not PEG-stressed seedlings (Fig. 8a, c).

Glutathione and ascorbate metabolism

Glutathione and ascorbate metabolism were both affected by both stressors, and more than 20 DPs were found to be involved in GSH and AsA metabolism (Fig. 9a).





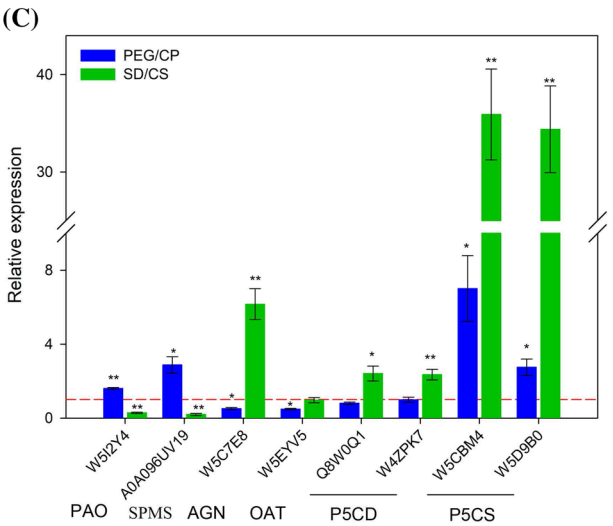


Fig. 8 Differentially accumulated proteins induced by soil drought and PEG stress participating in proline and polyamine biosynthesis. Enzyme name abbreviations are presented in Supplementary Table S7. **a** The differentially accumulated proteins in proline and polyamine biosynthesis (fold change > 1.500 or < 0.667, $P < 0.05$). **b** Proline content of wheat seedlings under soil drought and PEG stress: different lowercase letters indicate that the differences are significant (Duncan's multiple range test, n = 7, $P < 0.05$); bar = mean ± SE. **c** Gene expression of some core proteins in proline and polyamine biosynthesis: * and ** indicate that the differences of PEG vs. CP and SD vs. CS are significant at the level of $P < 0.05$ and $P < 0.01$, respectively (ANOVA, n = 4). Bar = mean ± SE; red dashed line indicates that the ratio of PEG/CP or SD/CS is 1

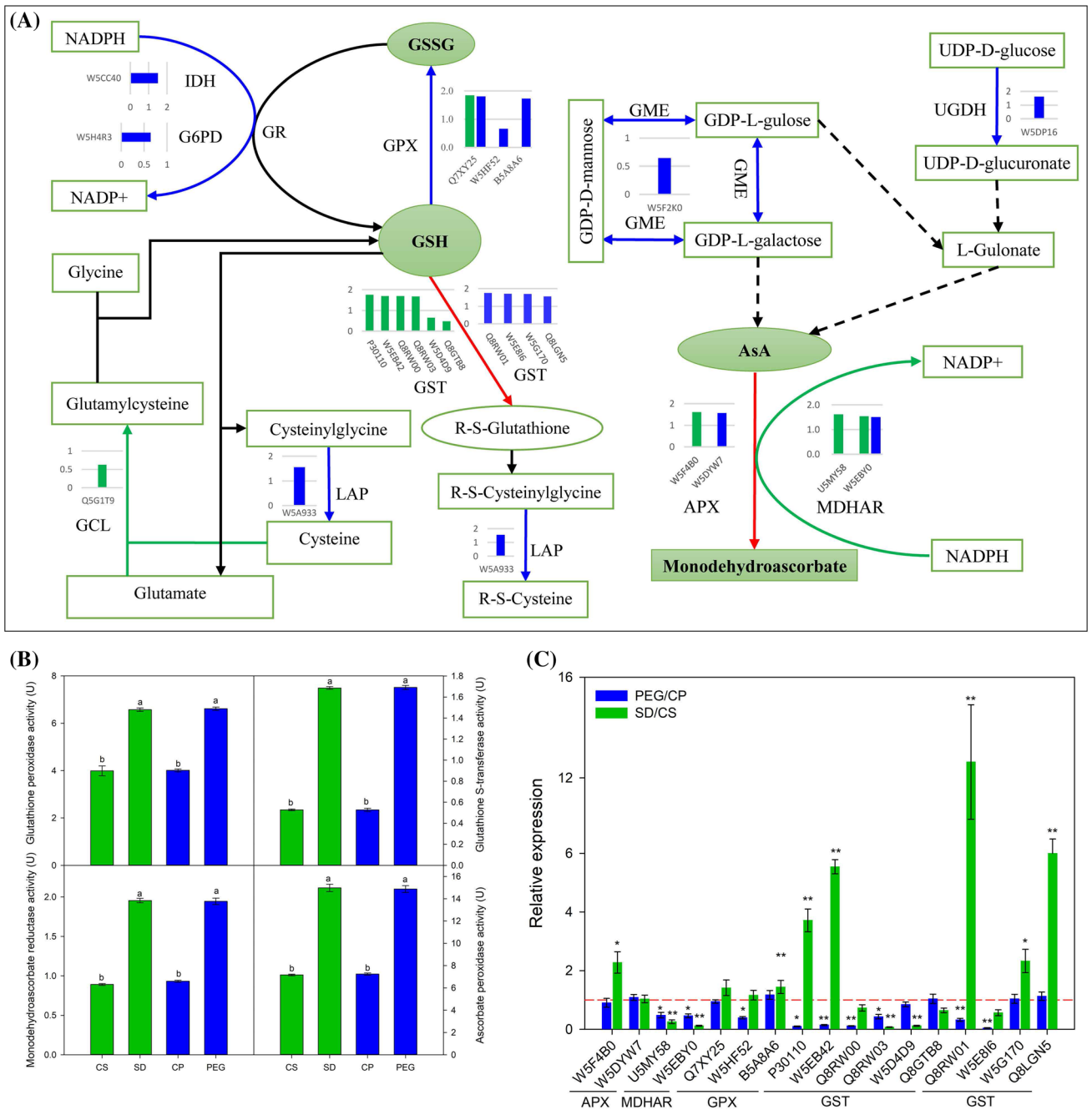


Fig. 9 Differentially accumulated proteins induced by soil drought and PEG stress participating in glutathione and ascorbate metabolism. Enzyme name abbreviations are presented in Supplementary Table S7. **a** The differentially accumulated proteins in glutathione and ascorbate metabolism (fold change > 1.500 or < 0.667, $P < 0.05$). **b** The enzymatic activity of glutathione peroxidase, glutathione *S*-transferase, monodehydroascorbate reductase, and ascorbate peroxidase: different lowercase letters indicate that the differences are significant (Duncan's multiple range test, n = 7, $P < 0.05$); bar = mean ± SE. **c** Gene expression of some core proteins in glutathione and ascorbate metabolism: * and ** indicate that the differences of PEG vs. CP and SD vs. CS are significant at the level of $P < 0.05$ and $P < 0.01$, respectively (ANOVA, n = 4). Bar = mean ± SE; red dashed line indicates that the ratio of PEG/CP or SD/CS is 1

Additionally, GPX, GST, MDHAR, and APX activity all increased in response to both soil drought and PEG (Fig. 9b). At the protein level, all GST and GPX proteins significantly increased in seedlings under both soil drought and PEG stress, with the exception of W5HF52, W5D4D9, and Q8GTB8 (Fig. 9a). Concentrations of glutamate-cysteine ligase (GCL) were inhibited by soil drought (Fig. 9a), but more enzymes related to GSH metabolism, including IDH,

G6PD, and leucyl aminopeptidase (LAP), were affected by PEG stress. In AsA metabolism, concentrations of MDHAR and APX proteins increased in response to both soil drought and PEG treatments (Fig. 9a), and PEG induced changes in concentrations of UDP glucose 6-dehydrogenase (UGDH) and GDP-D-mannose epimerase (GME), two enzymes involved in AsA biosynthesis (Fig. 9a).

At the gene level, of the ten GST proteins, transcripts of P30110, W5EB42, Q8RW01, W5G170, and Q8LGN5 were significantly increased by soil drought (Fig. 9c), whereas transcripts of P30110, W5EB42, Q8RW00, Q8RW03, Q8RW01, and W5E8I6 were significantly lower in seedlings treated with PEG, and the proteins Q8RW03 and W5D4D9 were reduced by soil drought (Fig. 9c). Transcripts of the GPX protein B5A8A6 were significantly increased in soil-drought-treated seedlings, whereas W5HF52 was decreased in seedlings treated with PEG (Fig. 9c). Transcripts of the APX protein W5F4B0 were significantly higher by soil drought, but those of W5DYW7 were unchanged under both stressors (Fig. 9c). In addition, transcripts of MDHAR proteins, including U5MY58 and W5EBY0, were significantly reduced in both soil drought and PEG treatments (Fig. 9c).

Lignin, starch, and sucrose biosynthesis

Differences in the DPs induced by soil drought and PEG were also observed in lignin, starch, and sucrose biosynthesis (Supplementary Figs. S3 and S4). Specifically, treatment with PEG resulted in altered concentrations of shikimate *O*-hydroxy-cinnamoyl-transferase (HCT) in lignin synthesis, as well as the levels of starch synthase and glycogen phosphorylase involved in starch and sucrose synthesis (Supplementary Fig. S3). Soil drought suppressed production of glucose-1-phosphate adenylyl-transferase (GLGC) and GPI in starch and sucrose synthesis (Supplementary Fig. S3). Furthermore, concentrations of cinnamyl-alcohol dehydrogenase (CAD) and 4-coumarate-CoA ligase (CL) involved in lignin synthesis were affected by soil drought but unchanged by PEG treatment (Supplementary Fig. S4). However, both water deficit stressors induced changes in several enzymes that participate in starch and sucrose biosynthesis (e.g., glucan branching enzyme [GBE] and β-glucosidase [GCD]), and in lignin synthesis (e.g., phenylalanine/tyrosine ammonialyase [PTAL], GCD, and POD).

Discussion

Drought is one of the environmental factors that most affects the growth and yield production of wheat, and artificial application of PEG to plants is an effective method of simulating water-deficit stress. In this study, the differences and similarities of how wheat responds to water-deficit stress in different culture media (soil drought and PEG solution) were examined, providing physiological and molecular evidence for PEG-simulated osmotic stress and revealing the differences between soil drought and PEG-simulated osmotic stress. The results showed that in wheat, several physiological responses were induced by both PEG and soil drought, including alterations in the concentrations of several key enzymes such as GAPA, Rubisco, and TKTA in carbon metabolism; moreover, both PEG and soil drought promoted malate metabolism, proline and polyamine biosynthesis, and GSH and AsA metabolism. Furthermore, the two water-deficit stressors had similar effects on the same pathways, primarily by altering activity levels or concentrations of different enzymes involved in those pathways, as well as the same key enzyme by altering concentrations of different proteins homologous to that key enzyme.

Differences and similarities of physiological responses and protein profiling

Because both PEG stress and soil drought can induce osmotic stress, PEG is often used to simulate drought conditions in laboratory and experimental settings (Skriver and Mundy 1990), and the similar physiological and molecular responses of wheat seedlings to both soil drought and PEG support the use of this alternative (Fig. 2). Photosynthetic rate, stomatal conductance, intercellular CO_2 concentration, transpiration rate, maximum potential efficiency of PS II, and relative water content were lower under soil drought and PEG stress, whereas relative conductivity and MDA content increased (Fig. 2). These findings are consistent with those reported previously for soil drought and PEG stress (Cui et al. 2017, 2018; Fan and Blake 1997; Forner-Giner et al. 2011). At the protein level, the protein accumulation pattern was similar, and 41.8% of proteins were induced by both soil drought and PEG stress (Fig. 3a). Furthermore, the two stressors also induced 15.8% of DPs, 97.8% of which exhibited identical concentration trends in both control and stressed groups (Fig. 3). This consistency was higher in wheat than that reported for *Arabidopsis thaliana*, which was attributed to similarities in growth conditions and genetic backgrounds (Bray 2004).

Proteomic analysis revealed marked differences between soil-drought- and PEG-induced stresses, however. First, the proportion of identified DPs in total proteins differed between the two stresses, with 39% of DPs exclusive to soil drought and 45.3% exclusive to PEG stress (Fig. 3b). It suggested that these proteins are induced by water deficit and other culture conditions, but are not limited to culture medium. Nonetheless, the unique proteins induced by soil drought (39% of DPs, as shown in Fig. 3b) was necessary for effective response to drought stress, but were not induced by PEG stress because of differences in the experimental

conditions. Similarly, of the PEG-induced DPs, three-quarters were induced only by PEG stress (Fig. 3b) and were not differentially accumulated after soil drought. Although PEG has long been used to simulate osmotic stress, present results suggest that there are conspicuous differences between different culture conditions, and are consistent with the findings of previous studies (Bray 2004; Forner-Giner et al. 2011). These differences were attributed to factors such as stress time, osmotic pressure, and culture medium composition. However, eliminating such potential error in drought simulation experiments associated with the use of PEG is difficult, thus requiring the development of more reliable methods.

Carbon metabolism and amino acid biosynthesis

Carbon metabolism and amino acid biosynthesis pathways are activated in response to water deficit and play important roles in signal transduction (Smeekens and Rook 1997) and osmoregulation (Nahar et al. 2016). In present study, most of the responses common to both sources of water deficit were not positive, and adversely affected carbon metabolism and protein biosynthesis (He et al. 1999; Tambussi et al. 2000), including reductions in such DPs as GAPA, Rubisco, PGK, SHMT, FHS, ALS, and CS (Figs. 6a, 7). These results were in at least partial agreement with those of previous studies (Table 1). GAPA and Rubisco either increased or decreased under both soil drought and PEG stress, and concentrations of PGK varied under soil drought (Table 1) (Budak et al. 2013; Caruso et al. 2009; Zeng et al. 2016), suggesting similar inhibitory effects on responses to drought in wheat seedlings regardless of stress source (i.e., soil drought or PEG) and was consistent with decreased photosynthetic rate and maximum potential efficiency of PS II (Fig. 2a, e). However, the ways in which soil drought and PEG stress inhibited these two metabolic pathways differed; for example, the two stressors clearly targeted different enzymes, given that some DPs decreased under soil drought but were unaffected by PEG stress, whereas some proteins were only affected by PEG stress (Figs. 6a, 7). These findings are consistent with previous studies of plants exposed to PEG stress (Cheng et al. 2015; Kang et al. 2012; Zhang et al. 2014).

Notably, several DPs that were induced by both stressors responded effectively to water deficit in carbon metabolism and amino acid biosynthesis, including ALDO, MDH, IDH, PDHB, CS, HMET, and DPAT (Figs. 6, 7). MDH provides CO_2 during stomatal limitation in order to enhance photosynthetic performance (Nunes-Nesi et al. 2005) and were found to increase under PEG stress, as was reported in an earlier study (Kang et al. 2012), suggesting it plays an important role in plant response to PEG stress. In addition, in the present study, IDH and PDHB were up-regulated, indicating an enhancement of malate metabolism and consequently CO_2 fixation (Fig. 6). Furthermore, the higher concentrations of ALDO under soil drought led to greater production of ribulose-1, 5-bisphosphate, which participates in CO_2 fixation and plays an important role in water deficit (Lu et al. 2012). Thus, one common response to both soil drought and PEG treatment in wheat seedlings was enhancement of the pathway related to ribulose-1,5-bisphosphate and malate synthesis during CO_2 fixation.

Gene expression of most of the proteins was inhibited under both soil drought and PEG stress (e.g., GAPA, PDHB, Rubisco), and provided limited support for the results of our protein quantification analysis (Fig. 6). Regardless of stress type, although gene expression of these proteins was not always consistent with protein concentrations, W5DTC2, W5C4H2, and Q7XZE9 responded positively to water stress at both the gene and protein level (Fig. 6), suggesting that

Table 1 Summary of some core proteins in wheat proteome analyses in response to soil drought and PEG stress

ALDO	APX	CS	GAPA	GST	MDH	PAO	PGK	Rubisco	TKTA	Stressed condition for wheat seedlings	References
↑↓	↑		↑↓	↑				↓		Drought tolerant, soil drought for 48 h	Cheng et al. (2016)
	↓				↑↓			↑↓	↓	Drought sensitive, soil drought for 48 h	
↑↓	↑	↓	↑	↓	↓		↑↓	↑↓		Drought sensitive, 20% field capacity	Faghani et al. (2015)
↑	↑	↓	↑	↑	↑		↑↓	↑↓		Drought tolerant, 20% field capacity	
				↑		↑		↑		Soil drought for 9 days	Budak et al. (2013)
↓	↓						↑	↑		Soil drought for 7 days	Caruso et al. (2009)
↑	↑	↑	↓	↑↓			↓	↓	↓	Soil drought, 40% field capacity, in this study	
↓						↑↓				Drought sensitive, 25% PEG-6000 for 48 h	Cheng et al. (2015)
↓	↓		↓			↓				Drought tolerant, 25% PEG-6000 for 48 h	
↓		↑	↑↓	↑				↑↓	↓	20% PEG-6000 for 48 h	Liu et al. (2015)
↓	↑			↑					↓	-0.5 MPa PEG-6000 for 48 h	Zhang et al. (2014)
↑	↑		↓	↑	↑			↑	↑	15% PEG-6000 for 3 days	Kang et al. (2012)
↑	↑	↓	↓	↑	↑	↑	↓	↓	↓	25% PEG for 72 h, in this study	

↑ and ↓ mean up- and down- accumulation of the enzyme in the reported study

ALDO, MDH, and MDH2 play important roles in water-deficit responses in wheat, results that accord with those of previous studies (Kang et al. 2012; Lu et al. 2012). Thus, both soil drought and PEG stress inhibited carbon metabolism and amino acid biosynthesis by affecting key enzymes in these pathways, of which some are induced by both stressors, but the remedial measures were also induced by wheat seedlings under water deficit, including enhanced malate metabolism and increased ALDO accumulation.

Proline and polyamine metabolism

Proline, an osmotic solute, plays a crucial role in plant response to drought stress (Delauney and Verma 1993; Nahar et al. 2016). The observed increase in proline content was consistent with the increases in concentrations of key enzymes and elevated levels of gene expression under both soil drought and PEG stress (Fig. 8), indicating that both stressors induce proline biosynthesis by stimulating the production of key enzymes (Fig. 8) (Cheng et al. 2015; Manivannan et al. 2007). However, differences between soil drought and PEG stress in gene expression and in enzyme accumulation were noted; protein accumulation of OAT increased only in response to PEG stress but was unchanged under soil-drought stress (Parida et al. 2008) and increased activity under PEG (Hsu et al. 2003). In addition, gene expression of AGN, P5CD, and P5CS were higher under soil drought than under PEG stress (Fig. 8c), suggesting that soil drought induces expression of P5CS, P5CD, and AGN to a greater degree than does PEG stress. Spermine and spermidine are known to be useful osmotic solutes and important regulation signals (Li et al. 2016), and we found that concentrations of PAO and SPMS proteins were higher in wheat exposed to PEG stress but were more or less unaffected by soil drought. These results were consistent with the pattern of gene expression (Fig. 8c) and those of a previous study (Budak et al. 2013; Cheng et al. 2015), and strongly suggest that concentrations of PAO and SPMS are induced to a greater degree by PEG stress than by soil drought.

GSH and AsA metabolism

The GSH and AsA metabolic pathways are important antioxidative response pathways to water-deficit stress (Apel and Hirt 2004). In these pathways examined in this study, the concentrations of only a few proteins were found to decrease under soil drought or PEG stress, none of which were key enzymes (Fig. 9a). Most of the vital enzymes in this pathway (e.g., GPX, GST, APX, MDHAR) increased in wheat seedlings exposed to both soil drought and PEG stress (Fig. 9a), in a manner consistent with their enhanced enzymatic activity (Fig. 9a, b), an indication that the higher concentrations of these proteins promoted increased enzymatic activity, which accorded with the findings of other studies (Cheng et al. 2016; Faghani et al. 2015; Kang et al. 2012; Zhang et al. 2014). At the gene level, however, expression levels were often inconsistent with trends in protein concentrations, and in some cases—such as MDHAR—even moved in the opposite direction regardless of stress source (Fig. 9c). Gene expression of these proteins did not significantly increase following PEG treatment, and in some instances expression levels actually declined (Fig. 9c); notably, gene expression of key enzymes was more effectively induced by soil drought. The inconsistencies observed between gene expression and protein accumulation under the two stressors was attributed to post-transcriptional regulation and different stress time, given that soil-drought stress can be sustained over a longer time period than PEG-induced stress (Bray 2004; Mazzucotelli et al. 2008). In summary, although both soil drought and PEG stress induced the accumulation of GST, GPX, APX, and MDHAR, soil drought induced the gene expression of these enzymes more efficiently.

Although the differences and similarities of protein accumulation between soil drought and PEG stress were described here, our study has certain limitations. The differences in protein accumulation was attributed to the experimental conditions, including stress degree (80% FC to 40% FC vs. 25% PEG for 72 h), and the composition of the soil drought and PEG stress culture mediums. However, these problems can be overcome via the use of more accurate methods of controlling water deficit and culture medium composition. Despite the limitations of our analysis, we found that PEG stress did not completely simulate soil drought, and furthermore that malate metabolism, proline and polyamine biosynthesis, and GSH and AsA metabolism play key roles in wheat seedling response to water deficit (Fig. 10). The concentrations of several key enzymes (e.g., MDH, P5CS, P5CD, GPX, GST, APX, MDHAR) significantly increased under soil drought and PEG stress. The similarities of these pathways in wheat seedlings under either PEG stress and soil drought is one reason why PEG was often used to simulate water deficit in plants in previous studies (Farooq et al. 2009). Interestingly, gene expression of some functional enzymes involved in proline, GSH, and AsA metabolism, including AGN, P5CS, P5CD, GPX, GST, and APX, was higher in plants under soil drought than in plants under PEG-induced stress. In addition, the increased protein accumulation and gene expression of spermidine and spermine in response to PEG stress indicated that PEG differentially induced their synthesis given that the levels of these proteins were largely unaffected by soil drought.

Conclusion

Here, we explored the differences and similarities in wheat seedling response to soil-drought-induced and PEG-induced stress at the protein level. Several pathways were activated

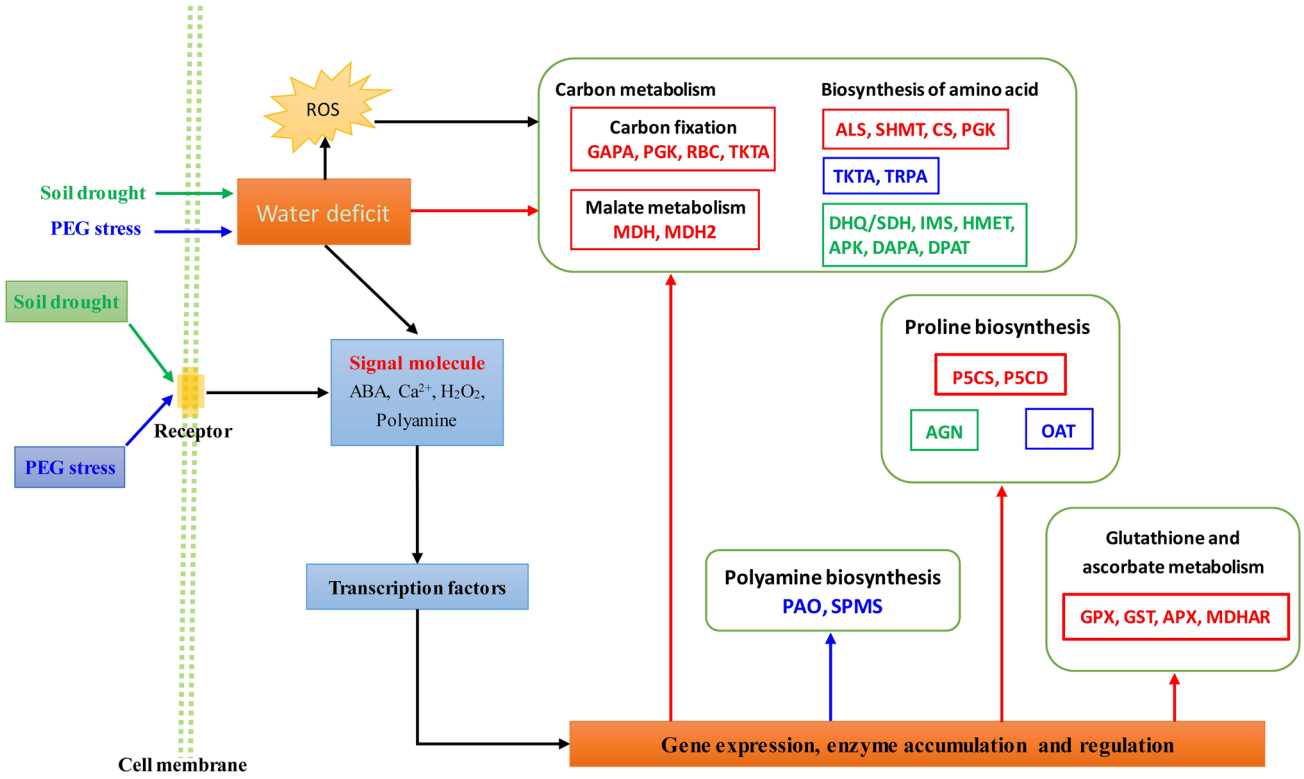


Fig. 10 A model showing the differences and similarities of the response of wheat seedlings to soil drought and PEG stress. Enzyme name abbreviations are presented in Supplementary Table S7. Enzymes marked in blue were induced solely by PEG; enzymes marked in green were induced solely soil drought; enzymes marked in red were induced by both soil drought and PEG

in wheat seedlings under both soil drought and PEG stress, including malate metabolism, glutathione and ascorbate metabolism, and proline biosynthesis, although soil drought induced these metabolic pathways to a greater degree than PEG, whereas polyamine biosynthesis was induced solely by PEG treatment. Moreover, both soil drought and PEG exposure inhibited carbon metabolism and the biosynthesis of some amino acids, and consequently stunted the growth of wheat seedlings. Because PEG stress and soil drought both induced the production of several key proteins, additional research at the genetic level is required to identify the mechanisms regulating wheat response to water deficit.

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Author contributions YX designed and directed this study as well as drafted and revised the manuscript. GC and YZ performed the experiments and analyzed the data as well as drafted and revised the manuscript. MC and JZ conducted the physiological and stress parameters determination. KX measured the gene expression of all selected proteins. FS, CZ and SL improved the data analysis and revised the manuscript.

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