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RESEARCH PAPERS

Changes in the Metabolome of Two Soybean Genotypes under Drought Stress

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Abstract—Soybean is the world's leading economic oilseed crop. Drought stress is a major constraint on the growth and yield stability of soybean. Here, wild soybean (*Glycine soja* Siebold & Zucc.) was found to be more drought tolerant than cultivated soybean (*Glycine max* (L.) Merr.) owing to morphological changes at the whole-plant level when subjected to 5% PEG-6000 treatment. Additionally, differential metabolites between two soybean genotypes seedlings leaves were analyzed at the cellular level using a gas chromatography-mass spectrometry-based metabolomics method. The root lengths of wild soybean increased and a high root/shoot ratio was maintained under drought stress conditions. In addition, the drought tolerance of wild soybean resulted from significantly greater levels of favorable metabolites, such as aromatic and serine family amino acids, as well as sugar and polylols involved in mannose and galactose metabolism, favorable secondary metabolites, and organic and fatty acids compared with cultivated soybean. At the same time, wild soybeans could maintain a stable TCA cycle and significantly enhance glycolysis to produce more energy and enhance the phosphate pentose pathway to create more reducing power. Our experimental results provide an important reference for the breeding of wild soybeans in arid environments, as well as methodological for utilizing wild soybeans and improving cultivated soybeans.

Keywords: Glycine soja, *Glycine max*, cultivated soybean, wild soybean, drought stress, GC-MS

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INTRODUCTION

In plants, drought is an important and complex abiotic stress, affecting plant growth and development. In the future, more regions are expected to be affected by severe droughts; therefore, they are predicted to become increasingly destructive to agriculture, and thus to agrarian-based societies [1]. Soybean, an important grain and legume, is essential for humans and animals, serving as an important source of micronutrients and minerals. It is an important cash crop, producing 30% of the world's edible oil and 69% of its dietary protein [2]. Wild soybean is the related ancestral species of cultivated soybean. Wild soybean has a stronger adaptability to various adverse environmental conditions. At present, to adapt to the serious problem of a global water deficit, the improvement and breeding of drought-tolerant cultivated crop varieties without introducing foreign species have attracted attention.

In recent years, there have been many important studies on drought tolerance mechanisms of soybean through analyses of proteomics [3] and other omics fields. Furthermore, in soybean, metabolite profiling has been performed mainly in response to water stress [4] and salt stress [5]. Nevertheless, investigating mechanisms of drought tolerance through comparisons between wild and cultivated soybeans is significantly hampered by the physiological and genetic complexity of the drought tolerance trait. Strengthening the understanding of the drought tolerance mechanism is a necessary condition for optimizing drought-tolerant crop varieties needed under water-deficit conditions.

In our study, sand-culture seedlings of wild and cultivated soybean genotypes were used as plant materials, and 5% PEG-6000 was used to simulate drought conditions. The growth parameters of different varieties were measured. Additionally, metabolite profiling analyses of seedling leaves of two soybean genotypes

Abbreviations: CK—control treatment; DS—drought stress; M—cultivated soybean; PC1—the first principal component; PC2—the second principal component; PPP—pentose phosphate pathway; W—wild soybean.

were determined using gas chromatography–mass spectrometry (GC-MS) analytical methods. A comparative study of the response mechanisms between wild and cultivated soybean under water-deficit conditions was performed and the mechanism of drought tolerance in wild soybean was determined to provide direction for the breeding and optimization of drought-tolerant new cultivars for arid regions.

MATERIALS AND METHODS

Plant materials and sand cultures. The plant materials were wild (*Glycine soja* Siebold & Zucc.) soybean (W; Huinan06116) and cultivated (*Glycine max* (L.) Merr.) soybean (M; Jinong24). Seeds were provided by the Jilin Academy of Agriculture Science, China.

Soybean seedlings were cultivated by sand-based culturing. Cleaned and sieved river sand was arranged in a pot with diameter of 14 cm and a hole in the bottom (2 cm in diameter). Each pot contained 2.5 kg of washed sand. We selected healthy, uniform M and W seeds, and removed the outer film of W with a blade in advance. Then, four seeds of a single material were planted per pot and one seedling in each pot was retained. They were grown at the outdoor experimental field of Northeast Normal University, Changchun, Jilin. During this experiment, the average nighttime temperature was 18.5 ± 1.5°C, the average daytime temperature was 26 ± 2°C, and the average relative humidity was 60 ± 5%.

Plant growth conditions and treatment. W and M plants were divided into two groups respectively, including drought stress group and the control group. Each group consisted of eight pots, each of which was regarded as a single replicate. Four pots were used to measure growth parameters; four pots were used for metabolomics analyses. These seeds in the pots were watered adequately every day. After the emergence of the seedlings, they were fully watered with 1× Hoagland's nutrient solution at 06:00–07:00 every morning.

The stress treatments began when the two soybean genotypes grew the third compound leaf stage. In stress group, the two soybean genotypes were exposed to the 5% PEG-6000 treatment to simulate drought stress for 14 days. In control group, soybeans were cultivated with 1× Hoagland's nutrient solution.

Sampling and growth indices' measurements. After 14 days, four biological replicates from each treatment of both soybean genotypes were selected randomly as test metabolic materials, and fully expanded functional leaves were harvested. Then, samples were immediately frozen in liquid nitrogen and stored at −80°C to extract metabolites. The other four biological replicates were used to measure growth parameters under each treatment condition.

The shoot heights and root lengths, as well as the fresh weights (FWs) and dry weights (DWs) of shoots and roots, were measured, and the relative growth rates (RGRs) of shoots and roots were determined using the following equation:

$$RGR = (\ln DW_1 - \ln DW_0)/(t_2 - t_1),$$

where W_0 represents the first DW, W_1 represents the last DW, and $(t_2 - t_1)$ represents the total treatment's duration [6].

Data statistical analysis was carried out by statistical product and service solutions (SPSS) software (ver. 18.0), using a one-way ANOVA for multiple comparison ($P < 0.05$).

Metabolite profiling analysis. Metabolites were extracted from W and M soybean leaves (100 ± 5 mg of plant material), then samples were transferred to 1.5 mL Eppendorf tubes. Then 60 μL of water containing ribitol (0.2 mg/mL stock in H_2O) as an internal standard and 0.1 mL of chloroform and 0.3 mL of methanol were added to each tube. The sample was homogenized in a ball mill for 3 min at 65 Hz. After mixtures were vortexed, a 70 Hz grinding mill system (Jinxin Biotech LTD, China) was used to grind the samples for 5 min, followed by incubation at 70°C for 10 min. Subsequently, the tubes were centrifuged at 12000 rpm at 4°C for 10 min (Tabletop Low-Speed Centrifuge L-500, Hunan Saite Xiangyi Centrifuge Instrument Co., Ltd., China). Then 0.35 mL of the supernatant was decanted into a 2 mL screw-top glass tube. At 30°C, dry with a vacuum concentrator for two hours. Each sample was dissolved in 80 μL of methoxamine hydrochloride (20 mg/mL in pyridine) and incubating at 37°C for 2 h [6]. Samples were further derivatized with *N*,*O*-bis(trimethylsilyl)trifluoroacetamid containing 1% trimethylchlorosilane (100 μL) at 70°C for 1 h. Finally, the derived samples were cooled to room temperature [7].

When the temperature of all the samples fell to room temperature, the GC-MS analysis was performed using an Agilent 7890 gas chromatograph system coupled to a Pegasus HT time-of-flight mass spectrometer (NYSE: A, China). The instrument is equipped with agilent db-5ms capillary column (30 m × 250 μm × 0.25 μm, J&W Scientific, Folsom, United States). The GC column temperature was programmed to rise from 50 to 330°C at a rate of 10°C/min. A 1 μL aliquot of the analyte was injected in splitless mode. Helium was used as the carrier gas with a flow rate of 20 mL/min, the front inlet purge flow was 3 mL/min, and the gas flow rate through the column was 1 mL/min. The column temperature was maintained at 50°C for the first 1 min and then was increased at a rate of 10°C/min until it reached 330°C. The temperature was kept at 330°C for 5 min. Ionization in the injection temperature was 280°C. Transfer line and ion source temperatures were 280 and 220°C, respectively. The energy was −70 eV in electron impact mode. Mass spectra data were recorded in the 85–650 m/z range at a rate of 20 spectra per s [7].

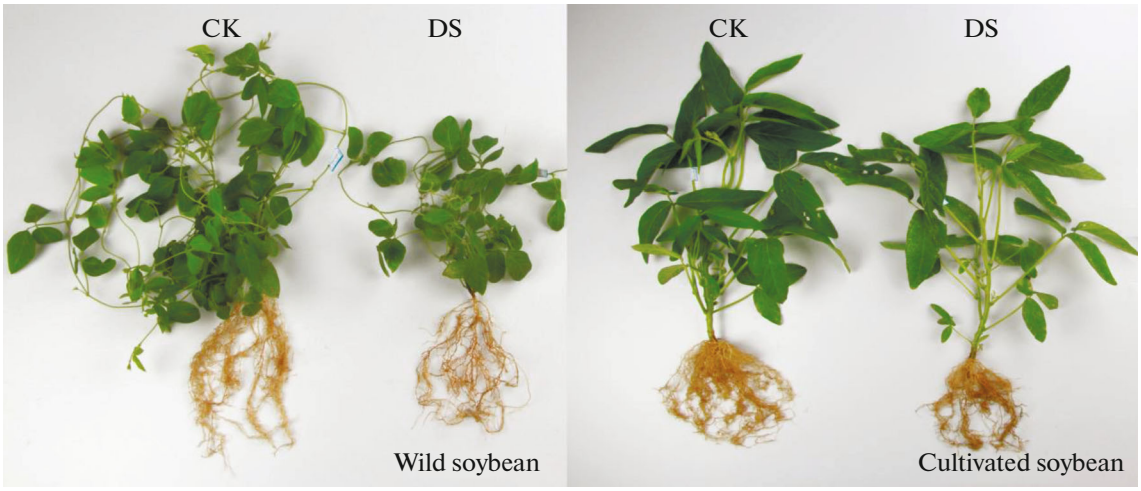

Fig. 1. The growth performances of the two soybean genotypes under control and drought stress conditions. CK—control treatment; DS—drought stress.

Data processing. The data was acquired and preprocessed using the manufacturer's ChromaTOF software (versions 2.12, 2.22, 3.34; LECO, United States). Metabolites were identified by searching the commercial EI-MS library, FiehnLib [8]. Then, at least 80% of missing values were removed and replaced with a small value, which was half of the minimum positive value in the original data. The data were filtered using the Interquartile Range (IQR) to remove metabolites with more missing values. Subsequently, the total mass of the signal integration area was normalized for each sample [5, 7, 9].

The resulting three-dimensional data involving the peak number, sample name and normalized peak area were inputted into the SIMCA-P 13.0 software package (Umetrics, Sweden) for the Principal Component Analysis (PCA), Partial Least Squares Discriminant Analysis (PLS-DA), Orthogonal Projection to Latent Structures-Discriminant Analysis (OPLS-DA) and loading plot. The variable importance projection (VIP) was obtained through PLS-DA and OPLS-DA. Then, differential metabolites were found using Student's *t*-test ($P < 0.05$) and VIP (VIP > 1), combined with Count Value > 20% and Similarity Value > 700 [9]. The metabolic pathway was constructed according to Kyoto Encyclopedia of Genes and Genomes (KEGG), which is a database resource for understanding high-level functions and utilities of biological systems (http://www.genome.jp/kegg/). The pathway was analyzed using MetaboAnalyst, which was based on the changes in metabolite concentrations compared with those of the corresponding controls [5].

RESULTS

Changes in Plant Growth Parameters under Control and Drought Stress Conditions

Under control group, the growth parameters of W, including root length, FWs of shoot and root, and DWs of shoot and root, were significantly less than those of M (Supplementary Table S1). In addition, compared with the control (CK), the growth of W and M were inhibited by drought stress (Fig. 1). The shoots of W and M were significantly inhibited under drought stress conditions, including shoot height, FW of shoots, DW of shoots and the RGR of shoots. However, the root length of M was significantly affected by drought stress, while that of W was not affected significantly. Under control and drought stress conditions, root/shoot ratio (dry weight) of W was 15 and 21% respectively, while that of M was 27 and 23% (Table 1).

Metabolic Profiling

We compared the metabolite profiles of W and M under control and drought stress conditions. We focused on 63 compounds ($P < 0.05$ and similarity value > 700; Supplementary Fig. S1), which were divided into the 9 major categories, including 10 amino acids, 9 sugars and polyols, 10 galactose metabolism-related substances, 10 organic acids, 6 secondary metabolites, 7 fatty acids, and 11 other TCA cycle intermediate-, glycolysis- and pentose phosphate pathway-related metabolites (Supplementary Table S2). The PCA revealed differences in the metabolites of W and M under drought stress conditions. Hence, the PCA presented a visual plot for the evaluation of the metabolite profiles of W and M based on differential metabolites. The first component (PC1) differed between W and M samples and explained 45.6% of the total variation in leaves. PC2 explained 24.0% of the variance in leaves and predominantly reflected the difference between the control and drought stress groups, indicating that the drought stress had a substantial effect on metabolites (Fig. 2a). Loading plot was obtained through PLS-DA, VIP was obtained through PLS-DA and OPLS-DA. From loadings plot, we noted that asparagine, fumaric acid, β-alanine, L-malic acid,

Table 1. The growth parameters of two soybean genotypes under drought stress conditions

Growth parameters	Treatments				Fold changes, $\log_2^{(DS/CK)}$	
	W		M			
	CK	DS	CK	DS	W	M
Shoot height, cm	73.60 ± 0.00	46.80 ± 0.01	56.65 ± 0.01	49.01 ± 0.02	–0.65*	–0.21*
Root length, cm	28.35 ± 0.02	29.42 ± 0.02	32.90 ± 0.02	26.18 ± 0.01	0.05	–0.33*
Fresh weight of shoots, g	20.75 ± 0.01	11.05 ± 0.02	38.52 ± 0.02	21.20 ± 0.02	–0.91*	–0.86*
Fresh weight of roots, g	4.95 ± 0.03	4.85 ± 0.01	15.20 ± 0.04	9.53 ± 0.04	–0.03	–0.67*
Dry weight of shoots, g	2.67 ± 0.01	2.03 ± 0.04	5.71 ± 0.02	4.31 ± 0.01	–0.39*	–0.41*
Dry weight of roots, g	0.42 ± 0.02	0.43 ± 0.05	1.47 ± 0.01	1.01 ± 0.04	0.05	–0.54*
RGR of shoots	73.60 ± 0.03	46.80 ± 0.02	56.65 ± 0.05	49.01 ± 0.01	–0.20*	–0.66*
RGR of roots	28.35 ± 0.01	29.42 ± 0.03	32.90 ± 0.01	26.18 ± 0.03	–0.01	–0.76*
Root/shoot ratio (DW)	0.15 ± 0.02	0.21 ± 0.04	0.27 ± 0.02	0.23 ± 0.03	0.49	–0.23

RGR were calculated using the formula $(\ln DW_1 - \ln DW_0)/(t_2 - t_1)$. DW—dry weight; W—wild soybean; M—cultivated soybean; CK—control treatment; DS—drought stress. The data are the means from four biological replicates. The fold changes were calculated using the formula $\log_2^{(DS/CK)}$. Values were presented as the mean ± standard deviation of our biological replicates. Significant differences are indicated: $^*P < 0.05$.

citric acid, tagatose, glycerol, D-glyceric acid, glycine, phenylalanine, proline, and valine contributed greatly to the PC1, while phenylalanine, tagatose, valine, proline, sorbitol, and mannose contributed greatly to the PC2 (Fig. 2b; Supplementary Table S3).

Metabolic Profiles Changes under Control and Drought Stress Conditions

We compared the metabolic profile of M with that of W under control conditions to investigate the metabolic changes between M and W. In total, 37 studied metabolites, 8 amino acids, 6 organic acids, 13 carbohydrates and polyols, 6 secondary metabolites and 4 other compounds, had greater accumulation levels in M than in W (Supplementary Table S4). In addition, 27 metabolites, 3 amino acids, 5 carbohydrates and polyols, 6 organic acids, 7 secondary metabolites and 6 other compounds, had greater accumulation levels in W than in M (Supplementary Table S4). A further comparative analysis indicated that the metabolite contents were different between W and M. The metabolites levels that had greater levels in W than in M consisted of mainly organic acids, including 5-aminovaleric, 4-aminobutyric, behenic acid, 4-hydroxyphenylacetic, glucuronic and mucic acids.

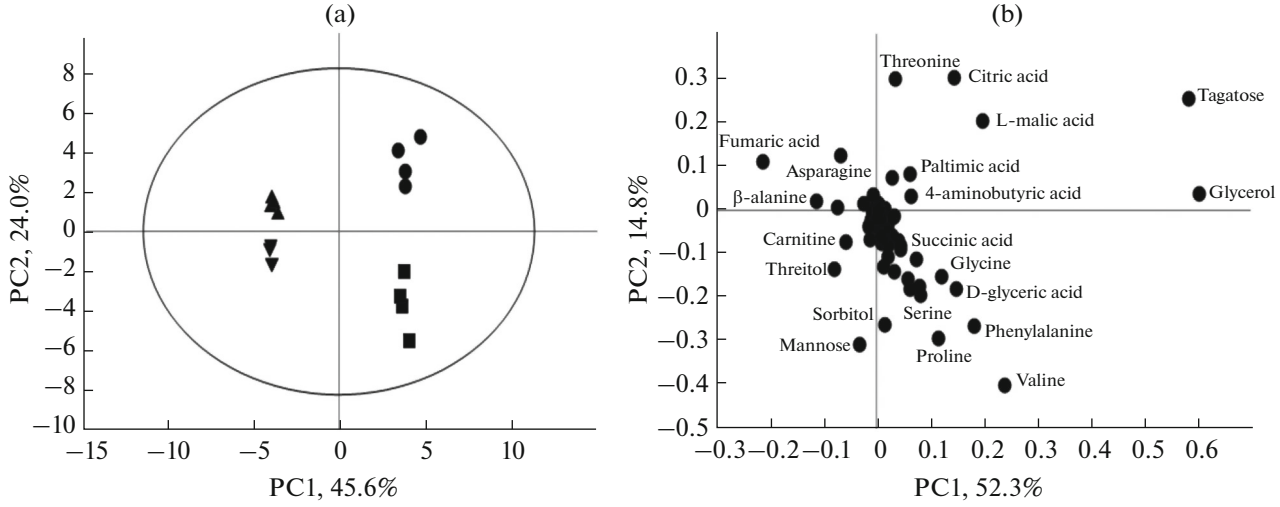

Fig. 2. Principal component analysis (PCA) of metabolic profiles and loading plots of metabolites showing the PC1 and PC2 in leaves of two soybean genotypes under control and drought stress conditions (four biological replicates). (a)—PCA in leaves; (b)—loading plot in leaves. ●—W-CK, ■—W-DS, ▲—M-CK, ▼—M-DS.

In addition, under control group, W had a greater content of secondary metabolites, including salicylic acid, 2-hydroxypyridine, ethanolamine, stigmasterol phosphate and neohesperidin than M. More importantly, the contents of 6-phosphogluconic acid and fructose1,6-diphosphate, which are intermediate metabolites of glycolysis, were greater in W. However, under control group, M had greater levels of carbohydrates, polyols and secondary metabolites, including dodecanol, myo-inositol, sorbitol, galactinol, mannose, mannitol, 1,5-anhydroglucitol, xylitol, maltotriose, ribitol, carnitine, catechol, squalene, caffeic and ferulic acids, than W.

The changes in the metabolites of W and M under drought stress conditions were different (Table 2). We investigated 10 amino acids with significant differences. Compared with the CK, valine, serine, glycine, proline and the aromatic amino acids tyrosine and phenylalanine were significantly increased in W under drought stress conditions. However, some amino acids, such as aspartic acid and asparagine, showed the opposite trend. Sugars and polyols, including xylitol, fucose, acetol and mannose increased in both W and M, with the latter two being significantly increased in W. The galactose metabolism-related metabolites melibiose, galactinol, tagatose and glycerol increased significantly in W and decreased significantly in M (Table 2).

In addition, 10 organic acids were further investigated. Compared with the CK, under drought stress conditions, pyrrole-2-carboxylic, glucoheptonic, allantoic, 2,6-diaminopimelic and jasmonic acids increased significantly in W, while glutaric, 5-aminovaleric, 4-aminobutyric and α-aminoadipic acids increased in W and decreased in M (Table 2). Under drought stress conditions, compared with the control group, secondary metabolites, including ferulic acid, squalene and gallic acid, increased significantly in W, while there was no significant change in M (Table 2). By the same token, 7 fatty acids were investigated. Compared with the CK, a number of fatty acids increased in M and decreased in W, including linoleic and oleic acids, under drought stress conditions. However, palmitic and stearic acids decreased in W and M, while myristic and arachidic acids increased in W (Fig. 3).

Under drought conditions, compared with the CK, the glycolysis-related metabolites glucose-1-phosphate and fructose 1,6-diphosphate in W and M were significantly increased (Table 2). Additionally, the intermediate metabolites of the TCA cycle, including fumaric acid and L-malic acid, decreased in both W and M (Table 2). The main metabolites associated with the pentose phosphate pathway, including 6-phosphogluconic acid, gluconic acid, D-glyceric acid and gluconic lactone, accumulated significantly in W. The energy synthesis and degradation of metabolites produced by W and M under drought stress conditions are presented in Fig. 3.

DISCUSSION

In this study, the growth rates of both W and M were inhibited under drought stress conditions. Compared with the CK, the root length of W increased, which indicated that increased root growth was a strategy to increase the water uptake from substrates under water-deficit conditions. This was consistent with previous studies on root system changes in soybean under water-deficit conditions [10]. The responses of plants to drought are crucial, and changes in root/shoot ratios of biomass have been frequently observed in response to drought [11]. In this study, under drought stress, the root/shoot ratio (DW) of W increased, while that of M decreased, and this indicated that the increased root/shoot ratio correlated with drought tolerance in W.

Free amino acids, as the basic units of protein synthesis, play important roles in plant responses to stress. Du et al. [12] showed that drought stress can significantly increase aromatic amino acid levels. In our study, compared with the CK group, aromatic amino acids, such as tyrosine, phenylalanine and serine family amino acids, including serine and glycine, increased significantly in W (Fig. 3). Under drought conditions, amino acid metabolites accumulated in W, suggesting that the accumulation of amino acids is an important strategy against drought stress. Proline can act as an osmotic adjustment substance to increase the concentrations of solutes in cells, reduce the water potential under adverse conditions and maintain photosynthesis and respiration [13]. In this experiment, compared with the CK, proline accumulated significantly in W and M under water-deficit conditions, indicating that proline plays an important role under drought stress conditions. Valine and threonine are used to form transamination products, which are glucogenic amino acids linked to pyruvate metabolism. In our study, the valine and threonine contents increased significantly in W, implying that pyruvate metabolism was enhanced, leading to the production of more energy to resist drought-related stress. Therefore, aromatic and serine family amino acids were significantly accumulated in W, significantly improving its drought resistance.

In plant-stress physiology, these small molecules play important roles in regulating the osmotic potential of leaves [14] and in maintaining the stability of biofilm structures. In our study, small-molecule metabolites related to sugar and polylol metabolism, such as fucose and xylose, were significantly increased in W (Table 2), which significantly enhanced the sugar and polylol metabolism of W (Fig. 3). Metabolites related to mannose metabolism, such as mannitol and mannose, increased significantly in W under drought stress conditions (Fig. 3). Mannitol is an important osmotic substance and compatible solute, and its accumulation in cytoplasm and vacuoles is required to balance the external water potential [15]. The signifi-

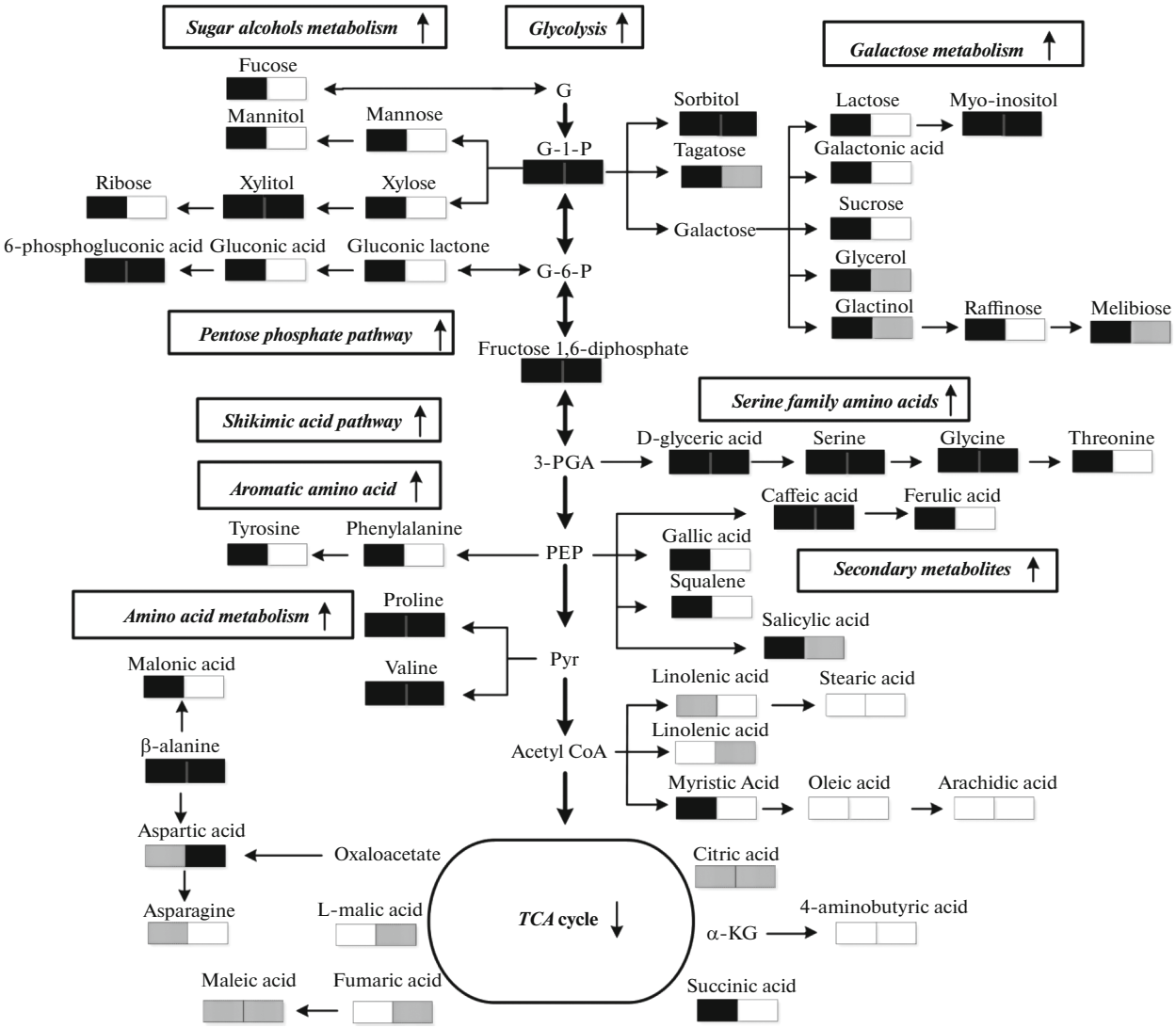

Fig. 3. Changes to metabolites in leaves metabolic pathways of the two soybean genotypes seedlings at 14 days after drought stress. The box on the left shows W and the box on the right shows M. White rectangle—no significant change; black rectangle—increased ($P < 0.05$); gray rectangle—decreased ($P < 0.05$).

cant accumulation of mannitol in W may increase its adaptability to an arid environment. In the present study, compared with the CK group, metabolites related to galactose metabolism, such as myo-inositol and sorbitol, increased significantly in W (Fig. 3). Myo-inositol is mainly involved in signal transduction and osmotic regulation. At the same time, myo-inositol and UDP-D-galactose can form galactinol, the galactosyl donor for raffinose biosynthesis [16]. Raffinose can be used as an osmotic protectant in the abiotic stress resistance of soybean. Moreover, sorbitol accumulates significantly under abiotic conditions [17]. Therefore, the accumulation of sugar and polylol metabolites and significant enhancements of mannose and galactose metabolism under drought stress conditions are closely correlated with the drought tolerance mechanism of W.

As small molecule osmotic regulators, organic acids help to balance the osmotic potential of vacuoles during drought resistance [18]. Thus, the organic acids in leaves of W played important roles in plant-stress-related physiology (Fig. 3). In addition, compared with the CK, fatty acids, such as myristic acid, increased significantly in W (Table 2). Gao et al. [19] reported that fatty acids and their derivatives are the main energy storage substances in organisms and are important components of cell membrane lipids. They play important roles in the plants' regulation of abiotic stress responses.

Plant secondary metabolites have protective effects that aid in the adaptation to stress [20], which are mainly reflected in compensating for the decreased biomass [21] and reducing damage to plant metabo-

Table 2. Changes of drought stress on relative metabolite content in leaves of two soybean genotypes

Metabolites pathways	Metabolites name	Relative concentration				Fold changes, $\log_2^{(DS/CK)}$	
		W		M			
		CK	DS	CK	DS	W	M
Amino acids	Aspartic acid	0.03 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.03 ± 0.00	−1.14*	1.65*
	Asparagine	46.17 ± 0.02	30.05 ± 0.03	42.31 ± 0.10	44.22 ± 0.07	−0.62*	0.06
	Valine	4.22 ± 0.04	73.15 ± 0.04	7.94 ± 0.11	15.71 ± 0.00	4.11*	0.98*
	Glycine	10.5 ± 0.10	22.71 ± 0.05	8.07 ± 0.03	11.79 ± 0.10	1.11*	0.55*
	Tyrosine	0.34 ± 0.07	1.21 ± 0.02	0.72 ± 0.02	0.89 ± 0.19	1.84*	0.30
	Phenylalanine	3.23 ± 0.04	37.90 ± 0.01	4.92 ± 0.04	5.63 ± 0.10	3.55*	0.19
	Threonine	3.33 ± 0.03	16.10 ± 0.02	5.96 ± 0.01	7.40 ± 0.02	2.27*	0.31
	Serine	15.09 ± 0.02	24.44 ± 0.01	13.77 ± 0.03	19.84 ± 0.01	0.70*	0.53*
	β-Alanine	3.60 ± 0.01	12.08 ± 0.00	3.84 ± 0.04	8.42 ± 0.04	1.75*	1.13*
	Proline	0.35 ± 0.08	21.90 ± 0.04	0.72 ± 0.04	11.02 ± 0.03	5.98*	3.94*
Sugars and polyols	Xylitol	0.91 ± 0.03	1.87 ± 0.12	1.15 ± 0.06	1.81 ± 0.07	1.04*	0.65*
	Acetol	0.12 ± 0.05	0.30 ± 0.01	0.20 ± 0.03	0.29 ± 0.02	1.38*	0.54*
	Fucose	2.26 ± 0.07	2.92 ± 0.07	2.03 ± 0.16	2.51 ± 0.10	0.37*	0.31
	Mannose	27.24 ± 0.05	45.40 ± 0.05	43.13 ± 0.05	48.73 ± 0.01	0.74*	0.18
	Allose	0.29 ± 0.04	0.42 ± 0.02	0.25 ± 0.01	0.11 ± 0.01	0.55*	−1.25*
	Mannitol	0.57 ± 0.07	0.85 ± 0.01	0.86 ± 0.20	0.74 ± 0.05	0.59*	−0.22
	Threitol	0.27 ± 0.02	0.31 ± 0.04	12.57 ± 0.09	0.41 ± 0.02	0.17	−4.95*
	Xylose	0.13 ± 0.01	0.15 ± 0.01	0.12 ± 0.02	0.11 ± 0.00	0.25*	−0.09
	Ribose	0.12 ± 0.01	0.16 ± 0.00	0.19 ± 0.03	0.19 ± 0.01	0.44*	−0.04
Galactose metabolism	Melibiose	0.22 ± 0.04	0.37 ± 0.07	0.27 ± 0.02	0.22 ± 0.03	0.72*	−0.31*
	Galactinol	0.29 ± 0.06	0.80 ± 0.09	0.57 ± 0.17	0.54 ± 0.02	1.47*	−0.07*
	Myo-inositol	0.21 ± 0.03	2.27 ± 0.09	0.61 ± 0.12	1.84 ± 0.00	3.46*	1.59*
	Galactonic acid	0.70 ± 0.05	1.42 ± 0.09	1.14 ± 0.12	1.25 ± 0.07	1.01*	0.13
	Lactose	0.02 ± 0.00	0.04 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	1.36 *	0.48
	Sucrose	0.09 ±0.02	0.87 ± 0.03	0.16 ± 0.03	0.51 ± 0.14	3.26*	1.64
	Tagatose	163.02 ± 0.05	219.57 ± 0.04	156.32 ± 0.01	145.23 ± 0.00	0.43*	−0.26*
	Sorbitol	3.75 ± 0.06	14.00 ± 0.10	8.18 ± 0.04	14.53 ± 0.02	1.90*	0.83*
	Raffinose	0.12 ± 0.04	0.30 ± 0.03	0.22 ± 0.00	0.29 ± 0.02	1.35*	0.42
	Glycerol	155.02 ± 0.02	284.97 ± 0.05	143.32 ± 0.07	113.32 ± 0.10	0.88*	−0.26*
Organic acids	Glutaric acid	0.80 ± 0.10	3.94 ± 0.48	1.48 ± 0.12	1.32 ± 0.11	2.30*	−0.17
	5-Aminovaleric acid	0.33 ± 0.06	0.56 ± 0.10	0.31 ± 0.05	0.20 ± 0.01	0.76	−0.63*
	4-Aminobutyric acid	62.54 ± 0.00	65.09 ± 3.08	57.24 ± 0.03	56.39 ± 0.01	0.06	−0.02
	α-Aminoadipic acid	1.21 ± 0.17	2.87 ± 0.25	1.79 ± 0.05	1.41 ± 0.08	1.25*	−0.34
	Pyrrole-2-Carboxylic acid	0.07 ± 0.01	1.43± 0.18	0.19 ± 0.04	1.08 ± 0.01	4.30*	2.49*
	Malonic acid	0.11 ± 0.01	0.33 ± 0.03	0.42 ± 0.07	0.51 ± 0.02	1.55*	0.29
	Glucoheptonic acid	9.19 ± 0.05	12.36 ± 0.62	10.31 ± 0.03	12.20 ± 0.12	0.43*	0.24*
	Allantoic acid	0.47 ± 0.07	1.27 ± 0.27	0.62 ± 0.13	0.76 ± 0.26	1.43*	0.29
	2,6-Diaminopimelic acid	0.17 ± 0.01	0.35 ± 0.07	0.13 ± 0.03	0.19 ± 0.02	1.07*	0.56
	Jasmonic acid	0.27 ± 0.02	0.35 ± 0.01	0.38 ± 0.00	0.59 ± 0.01	0.35*	0.62

Table 2. (Contd.)

Metabolites pathways	Metabolites name	Relative concentration				Fold changes, $\log_2^{(DS/CK)}$	
		W		M			
		CK	DS	CK	DS	W	M
Secondary metabolites	Caffeic acid	0.62 ± 0.02	1.20 ± 0.10	1.11 ± 0.02	1.61 ± 0.12	0.96*	0.54*
	Ferulic acid	0.28 ± 0.03	0.50 ± 0.03	0.44 ± 0.02	0.52 ± 0.04	0.85*	0.25
	Squalene	0.51 ± 0.12	1.43 ± 0.28	0.96 ± 0.16	1.07 ± 0.13	1.49*	0.16
	Carnitine	0.13 ± 0.00	0.43 ± 0.06	2.34 ± 0.13	2.99 ± 0.18	1.74*	0.35*
	Salicylic acid	2.21 ± 0.11	4.41 ± 0.15	3.00 ± 0.12	2.43 ± 0.13	1.00*	–0.30*
	Gallic acid	15.50 ± 0.01	19.70 ± 0.52	12.12 ± 0.03	12.74 ± 1.00	0.35*	0.07
Fatty acids	Linoleic acid	0.31 ± 0.07	0.25 ± 0.01	0.21 ± 0.04	0.21 ± 0.01	–0.31*	0.02
	Oleic acid	0.13 ± 0.02	0.12 ± 0.01	0.13 ± 0.01	0.15 ± 0.01	–0.19	0.17
	Palmitic acid	28.14 ± 0.00	27.53 ± 1.74	23.76 ± 0.05	22.74 ± 0.23	–0.03*	–0.06
	Stearic acid	10.86 ± 0.03	10.01 ± 0.84	10.44 ± 0.08	10.25 ± 0.22	–0.12	–0.03
	Linolenic acid	0.15 ± 0.05	0.15 ± 0.00	0.24 ± 0.00	0.18 ± 0.02	0.02	–0.41*
	Arachidic acid	0.03 ± 0.00	0.04 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	0.33	0.31
	Myristic acid	0.15 ± 0.02	0.26 ± 0.04	0.20 ± 0.01	0.21 ± 0.02	0.82*	0.08
Glycolysis	Glucose-1-phosphate	3.09 ± 0.02	7.13 ± 0.03	3.69 ± 0.12	6.04 ± 0.03	1.20*	0.71*
	Fructose1,6-diphosphate	0.65 ± 0.03	1.12 ± 0.07	0.22 ± 0.03	0.53 ± 0.02	0.79*	1.25*
TCA cycle	Fumaric acid	48.33 ± 0.02	38.53 ± 0.04	147.87 ± 0.05	29.18 ± 0.10	–0.33	–2.34*
	L-Malic acid	27.42 ± 0.06	25.70 ± 0.01	23.40 ± 0.00	22.55 ± 0.00	–0.09	–0.26*
	Citric acid	188.75 ± 0.03	168.69 ± 0.06	140.74 ± 0.02	119.13 ± 0.04	–0.16*	–0.24*
	Succinic acid	4.15 ± 0.23	10.87 ± 0.01	4.82 ± 0.06	4.71 ± 0.15	1.39*	–0.03
	Maleic acid	0.31 ± 0.02	0.03 ± 0.00	0.47 ± 0.07	0.41 ± 0.14	–3.32*	–0.18*
PPP	6-Phosphogluconic acid	0.03 ± 0.00	0.04 ± 0.01	0.02 ± 0.00	0.02 ± 0.00	0.47*	0.30*
	Gluconic acid	0.16 ± 0.04	3.29 ± 0.03	0.28 ± 0.05	0.83 ± 0.10	4.36*	1.57
	D-Glyceric acid	6.48 ± 0.05	12.89 ± 0.07	4.62 ± 0.02	11.43 ± 0.15	0.99*	1.31*
	Gluconic lactone	0.94 ± 0.01	1.10 ± 0.05	1.14 ± 0.04	1.63 ± 0.07	0.22*	0.51

The relative metabolite contents are the means of data from four biological replicates. Fold changes were calculated using the formula $\log_2^{(DS/CK)}$. Values were presented as the mean ± standard deviation of four biological replicates. W—wild soybean; M—cultivated soybean; CK—control treatment; DS—drought stress; PPP—pentose phosphate pathway. Relative concentrations and standard deviation were increased by a factor of ten times in each treatment. Significant differences are indicated: $*P < 0.05$.

lism and cell structures caused by ROS produced in plants. Here, there were significant accumulations of advantageous secondary metabolites, including caffeic and ferulic acids, in leaves of W, suggesting that the accumulation of these secondary metabolites is a response to drought conditions. Squalene can reduce the level of intracellular ROS [22], improve superoxide dismutase (SOD) activity, enhance plant tolerance, promote cell metabolism and increase plant resistance to hypoxia. Salicylic acid can be induced to enhance the antioxidant system, remove reactive oxygen species (ROS) in cells, reduce the level of cell membrane peroxidation [23] and act as a signal transducer or messenger for abiotic stresses to improve the resistance of plants. Here, the accumulation levels of organic acids, including glutaric, α-aminoadipic, salicylic and jasmonic acids, in W were significant under drought stress conditions (Table 2). Here, there were significant accumulations of squalene and salicylic acid in W, which can significantly improve W's drought tolerance (Fig. 3).

Furthermore, phenylalanine ammonialyase (PAL) is one of the most extensively studied enzymes related to plant responses to abiotic stresses [24]. PAL is also an important branch point controlling primary metabolism to secondary metabolism and key regulating enzyme for the synthesis of phenolic secondary metabolites [25]. Aromatic amino acids phenylalanine and tyrosine can be biosynthesized by the shikimic acid pathway, which is part of the primary metabolism of plants [26]. While gallic acid, as a phenolic secondary metabolite, had antioxidant and anti-free radical

effects in plants under abiotic stress conditions [27] and can be synthesized by shikimic acid pathway. In our study, under drought stress, W had higher primary metabolites such as phenylalanine and phenolic secondary metabolites such as gallic acid level than M compared with CK, respectively (Fig. 3). Therefore, under drought stress conditions, W can significantly improve drought tolerance by adjusting the activity of PAL and enhancing the shikimic acid pathway.

Respiratory pathways, such as TCA and glycolysis, are essential for maintaining energy transfer and various physiological functions in organelles [28]. The TCA cycle is an important energy-producing process in plants, and it plays an important role in the resistance to adverse environmental conditions [29]. In the present work, fumaric acid, L-malic acid and intermediate products of the TCA cycle in W and M leaves were inhibited under drought stress, and the inhibition was significant in M (Fig. 3). In addition, the decrease in malate may result from the suppression of the NAD-dependent malate dehydrogenase [18]. In this study, sugars related to the glycolysis of metabolites, including glucose-1-phosphate and fructose-1,6-diphosphate, significantly accumulated in W (Fig. 3), which significantly enhanced its energy-related metabolic reactions. This indicated that glycolysis metabolism is an important energy reaction for W's adaptability to drought conditions. In addition, some metabolites involved in the pentose phosphate pathway, such as 6-phosphogluconic and gluconic acids, increased significantly in W (Fig. 3). Hou et al. [30] showed that, in rice, 6-phosphogluconate dehydrogenase activity associated with the pentose phosphate pathway increased in response to abiotic stresses. Therefore, under drought stress conditions, W could generate more reductive power through the pentose phosphate pathway, allowing it to resist drought stress. The main fatty acid decomposition reaction, β-oxidation, provides a large amount of the energy required by the life activities of plants and plays an important role in responses to adverse reactions [7]. In our study, linoleic and oleic acids, which are involved in β-oxidation, declined significantly in W, suggesting that the main energy production pathway in W did not include the β-oxidation of fatty acids under drought stress conditions.

As a conclusion, the survival and growth of plants under drought stress conditions depend not only on the physiological level and growth-related morphology of plants, but also on metabolic changes at the cellular level. In this study, the growth phenotypes of W and M and metabolomics analyses of two soybean genotypes seedling leaves under drought stress conditions showed that W had a drought tolerance than M. Thus, we concluded that the drought tolerance mechanisms of W were mainly as follows: increasing the root length and the root/shoot ratio; increasing the synthesis of basic amino acids, such as serine, glycine and proline, and aromatic amino acids as much as possible; enhancing the production of small molecule organic acids, as well as sugar, polylol, mannoseand galactose metabolisms, to regulate the osmotic potential; synthesizing favorable secondary metabolites and fatty acids; maintaining a stable TCA cycle and significantly enhancing glycolysis to generate more energy; and accumulating significant levels of substances such as 6-phosphogluconic acid to enhance the pentose phosphate pathway, which produces more reducing power. This research provides a strong foundation for the development and utilization of effective W resources, and also provides a very important theoretical reference for the development of new soybean varieties resistant to drought under water-deficit conditions.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants as objects of research.

SUPPLEMENTARY MATERIALS

Supplementary materials are available for this article at https://doi.org/10.1134/S1021443720030085 and are accessible for authorized users.

AUTHOR CONTRIBUTIONS

H. Fu, R. Guo and L.X. Shi designed the research; H. Fu, R. Guo, M.X. Li and Y. Liu performed the research; M.L. Zhao and X.X. Wang provided experimental assistance to H. Fu; M.L. Zhao, X.X. Wang, S.Y. Wang and X.Y. Liu analyzed the data; H. Fu, R. Guo, M.X. Li, W.Y. Shen and L.X. Shi wrote the article. All authors reviewed the manuscript.

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