Research article

Metabolomics response to drought stress in *Morus notabilis* variety Yu-711

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Abstract: Mulberry is an economically significant crop for the sericulture industry worldwide. Stresses such as drought exposure have a significant influence on plant survival. Metabolome directly reflects plant physiological status; thus, a way to assess this impact is to perform a global metabolomic analysis. This study investigated the effect of drought stress on mulberry Yu-711 metabolic balance using a liquid chromatography-mass spectrometry (LC-MS) based on an untargeted metabolomic approach. For this objective, Yu-711 leaves were subjected to two weeks of drought stress treatment and control without drought stress. Multivariate and univariate statistical analyses highlighted numerous differentially-accumulated metabolic elements as a function of time and treatment. Drought stress led to a more differentiated metabolites response than the control. We found that the levels of total lipids and galactolipids, and phospholipids (PC, PA, PE) were significantly altered, producing 48% of the total differentially expressed metabolites. Fatty acyls were the most abundant lipids expressed and decreased considerably by 73.6%. Prenol lipids class of lipids increased in drought leaves. Other classes of metabolites, including polyphenols(flavonoids and cinnamic acid), organic acid (amino acids), carbohydrates, benzenoids, and organoheterocyclic, all had a dynamic trend in response to the drought stress. However, their levels under drought stress generally decreased significantly compared to the control. These results provide an overview of the metabolic profile of the mulberry plant through differentially-accumulated compounds and provide a better understanding of global plant metabolic changes in defense mechanisms.

Keywords: untargeted approach; metabolites; drought stress; mulberry, LC-MS.

1 Introduction

Crop losses have increased in recent decades due to changing weather patterns linked to climate change, and climate models forecast that droughts, floods, and severe temperatures will become more prevalent[1]. Combined with rising food demand, this condition is expected to threaten global food security. Drought is one of the most damaging environmental stresses, as it inhibits plant growth and decreases productivity[2]. Drought stress has severe morphological, physiological, and biochemical consequences for plants, including reduced photosynthesis, disrupted cell elongation and division, and loss of cell turgor[3]. It also affects crop gene expression, distribution, yield, and quality by preventing plants from absorbing additional nutrients[4, 5]. Therefore, a deeper understanding of tree crop responses to drought stress is critical to screen drought-tolerant varieties, cultivars, or genotypes that can respond to future climate scenarios in other to combat this effect.

Mulberry (*Morus alba L.*, Moraceae) is cultivated for its leaves and fruits in most provinces of China[6]. In addition, mulberry leaves are the only food source for domestic silkworms (Bombyx mori L.), making them a valuable tree species in the sericulture industry[7]. The Morus genus contains 68 species that grow in a wide range of climates from temperate to tropical, mainly in Asia, with one-third (24) of the species naturally occurring in China[8, 9]. Thus, mulberry, in addition to its traditional use in silkworm rearing, is a promising pioneer tree species on marginal lands[8, 10].

Mulberry trees need much water to have rapid growth, which is one reason why large-scale mulberry plantations have historically been developed in humid areas of China. Conversely, new mulberry plantations spread to areas where water is scarce to meet the economic and ecological demands[8]. As a result, drought events occurred in these plantations on a seasonal or regional basis, resulting in lower yield and deterioration of mulberry tree quality[11]. Abiotic stress tolerance is a polygenic trait in plants that includes signal transduction pathways and interactions between multiple genes[12]. Nonetheless, drought or soil moisture deficit stress is a negative abiotic factor that significantly reduces mulberry foliage yield. It is estimated that about 20% of the world's land surface is expected to be in drought at any given time[13]. Therefore, drought adaptation traits, including effective water conservation, broader and deeper root systems, increased photosynthetic yield, water use efficiency, and cellular

tolerance, must be introgressed into mulberry to combat drought stress[9].

Drought, alkalinity, salinity, and frost-tolerant viable mulberry varieties have been developed using conventional breeding practices based on morphological and physiological phenotyping[14]. Wild mulberry species, including *M. serrata* and *M. laveigata*, and germplasm resources, have a robust growth pattern, greater adaptability to harsh environmental conditions, and silkworm palatability[15]. On the other hand, abiotic stress adaptive traits from wild species have yet to be introduced into cultivated mulberry varieties for commercial use.

Increasing food supply significantly in an unstable environment and passing this information to farmers in the required timeframe is essential [16]. Achieving this has been done gradually through breeding[17] and biotechnology[18], employing novel methods to develop more resilient plants to abiotic stress. In recent years, a lot of progress has been achieved in plant metabolomics applied to abiotic stress research[19-21]. Many plants have responded to various abiotic challenges using their extensive metabolic homeostasis by creating a large spectrum of primary and secondary metabolites. Plant metabolites and associated pathways may be affected by abiotic stresses[3, 22]. Plant responses to drought stress could involve vital metabolic pathways, including photosynthesis, sugar synthesis, tricarboxylic acid (TCA) cycle, glycolysis, amino acids, and hormone synthesis[3].

Metabolomics has primarily focused on the organic molecular compounds (metabolites) found in or formed by organisms, tissues, and cells[23, 24]. Metabolomics is now widely used in plant science as an essential biotechnological method for various molecular biology studies. It encompasses a wide range of analytical techniques for identifying organic molecular metabolomic materials. These include but are not limited to metabolic fingerprinting, metabolite profiling, and targeted analysis, as well as gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), and nuclear magnetic resonance (NMR)[25]. In addition, these technologies may be used to precisely classify metabolomic components. Metabolomics technologies have been used previously to investigate plant drought responses and tolerance. For instance, Bowne et al. used a targeted GC-MS method to classify compounds that differ in three genotypes of bread wheat with varying levels of drought tolerance[26].

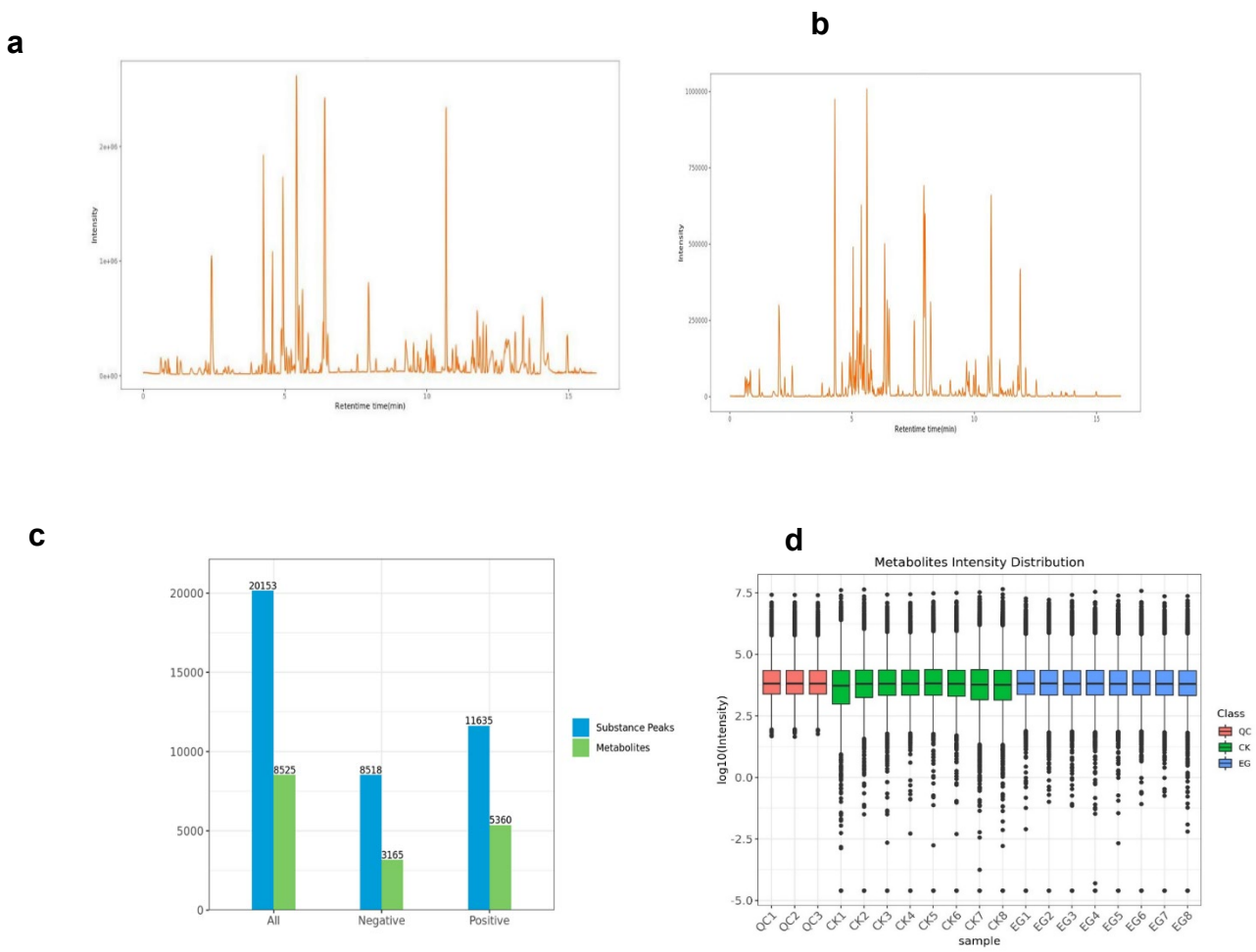
Even though extensive research has been done on the mulberry plant under drought stress using transcriptomics[9], little is known on the metabolomics approach in revealing the

mulberry's metabolites under drought stress. Hence, this study aimed to use non-targeted liquid chromatography-mass spectrometry (LC-MS) approach to investigate the metabolites of mulberry plants under water deficit using leaves from two experimental groups (control and drought stress) at a global level. In addition, this study will help to understand the drought-tolerant metabolites involved in the mulberry variety Yu-711.

2 Results

2.1 Material peak and metabolite statistics

A total of 20153 sample peaks and 8525 metabolites at both the ESI (-) and ESI (+) modes were detected from both the experimental and control treatments. Out of the total peaks and metabolites, 8518 and 3165 were obtained from the negative ion mode. In the positive ion mode, 11635 peaks and 5360 metabolites were detected (**Figure. 1; Supplementary Table S1**)



126 **Figure 1.** Sample peaks and metabolites. (A) peaks at the positive ion mode. (B) peaks at the
127 negative ion mode. (C) bar chart of all sample peaks and the corresponding metabolites in both
128 negative and positive mode. (D) metabolites intensity distribution between EG, CK, and QC.
129 EG; drought stress treatment, CK; control treatment and QC; quality control

130

131 ## 2.2 Metabonomic changes in the mulberry leaves between EG_CK

132 The metabolites in the EG were compared with those in the CK using LC-MS to identify
133 the overall metabolomic changes in the mulberry leaves. Clean data were used for further
134 analyses after the data had been filtered. The repeatability and stability of the metabolic profiles
135 from the LC-MS were evaluated using PCA, PLS-DA, and OPLS-DA score plots, which were
136 used to analyze the similarities and differences between all samples associated with drought
137 stress and controls(**Figure 2**). Using metabolite data from EG and CK, three main principal
138 components (PCs) were created (Figure 2a), indicating that samples were classified into three
139 groups for both the electron spray ionization (ESI) models, ESI (-) and ESI (+). Furthermore,
140 samples from the same treatments were clustered together, indicating the data's quality. Here, the
141 first two PCs show a total variation of 61.8%, with the principal component (PC1) explained
142 48.8% of the total variation. In contrast, the second principal component (PC2) explained 13%
143 variation across the data set. This indicates changes in the metabolite profiles caused by the
144 drought stress, and the differences in control samples were significant.

145 In the ESI (+) or ESI (-) model, partial least square discriminant analysis (PLS-DA) was
146 used to detect and assess the differences between drought stress and the control group EG _CK.
147 Similar classification results with PCA analysis were observed (**Figure 2b)**. Samples assembled
148 in a specific area according to the treatment. All EG and CK data validated the predictive
149 accuracy of the model.

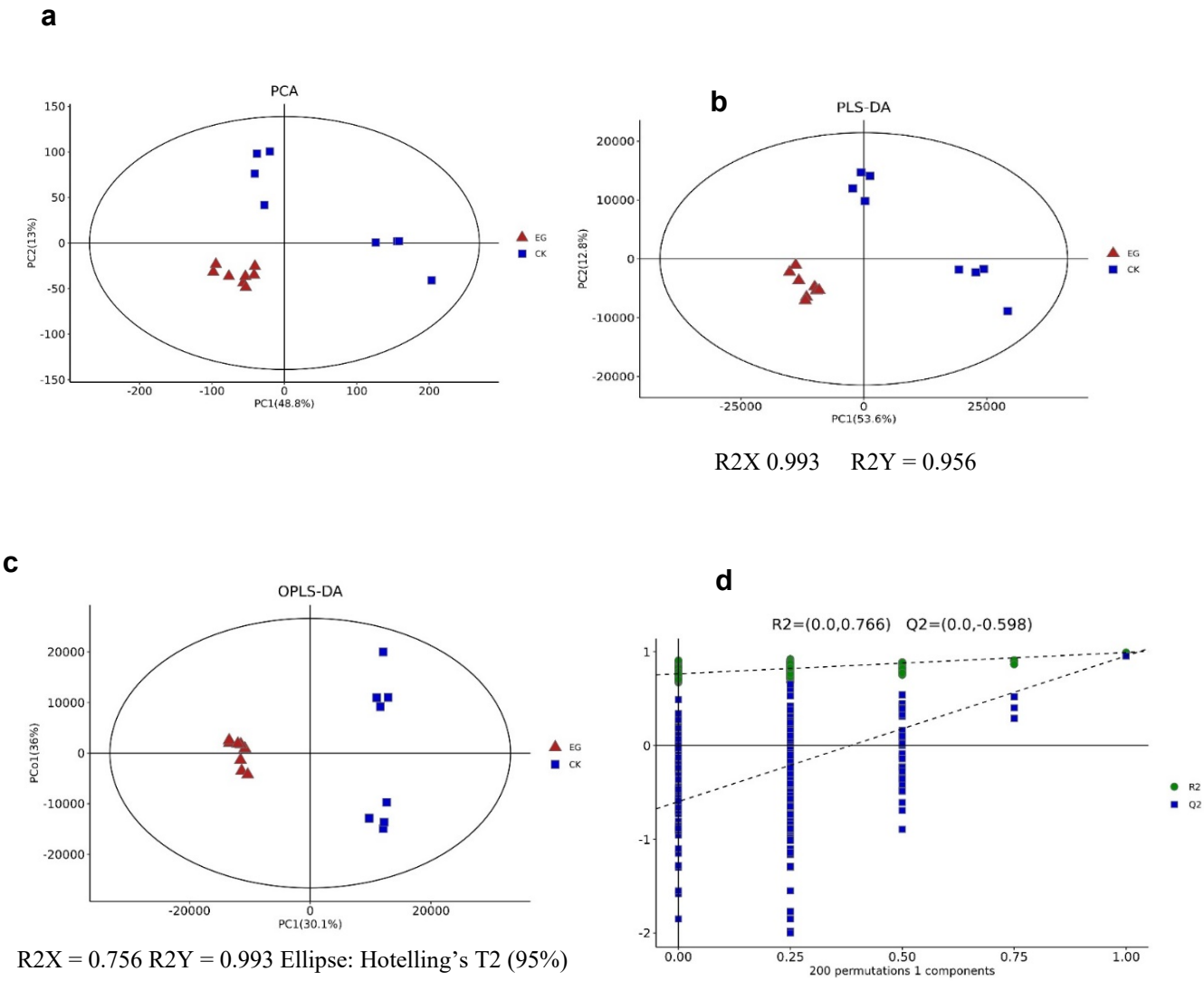


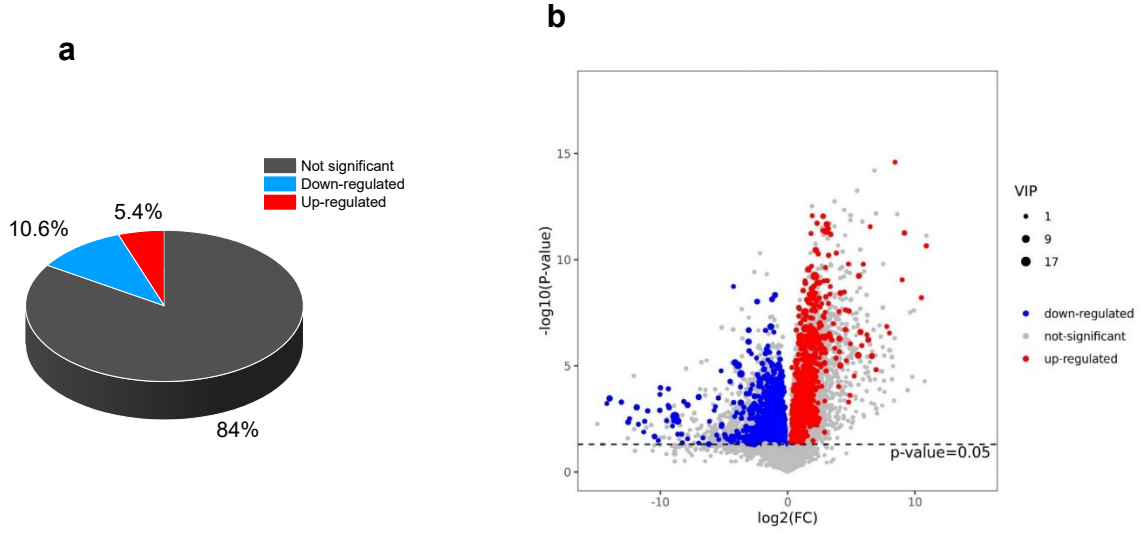
Figure 2. Multivariate statistical analyses of the differences in metabolites between EG and CK in mulberry (Yu-711) leaves. (a) Principal component analysis (PCA). (b) Partial least-squares-discriminant analysis (PLS-DA). (c) Orthogonal partial least-squares-discriminant analysis (OPLS-DA). (d) A 200 times permutation test of OPLS-DA mode. R^2 = (0.0, 0.766); Q^2 = (0.0, -0.598).

A total of 8523 metabolites were identified from 20153 material peaks (**Supplementary Table S1**). Amongst the metabolites are lipids and lipid-like molecules, phenylpropanoids and polyketides, Organic acids and derivatives, Organoheterocyclic compounds, Nucleosides, nucleotides, and analogs. The lipid and lipid-like molecules include prenol lipids, sphingolipids, glycerophospholipids, glycerolipids, fatty acyls and steroids, and steroid derivatives were detected. In addition, phenylpropanoids and polyketides include flavonoids, cinnamic acids, and derivatives, were also detected. Also, organoheterocyclic compounds such as tetrapyrroles and

derivatives and pyridines and derivatives were determined. Carbohydrates and carbohydrate conjugates in the class of the organooxygen compound and benzenoids compounds were expressed in response to the water deficit.

2.3 Analysis of the main differential metabolites

A total of 945 metabolites were obtained from EG_CK via LC-MS that met the VIP criterion(VIP > 1) of the OPLS-DA model and had a significant t-test ($p < 0.05$). A total of 794 metabolites (84%) had no change. However, 100 (10.6%), 51 (5.3%) metabolites were down-regulated and up-regulated, respectively (**Figure 3a, Supplementary Table S2**). Further analyses are revealed in **Figure 3b**, which indicates drought stress or the control led to the differences in the metabolites. The heat map pattern of metabolites between samples and consistency among biological replicates is shown in Figure 3c. As a result, the 945 differentially expressed metabolites were classified based on their abundance (fold change) and weight (VIP) **(Table 1, Supplementary Table S2).**



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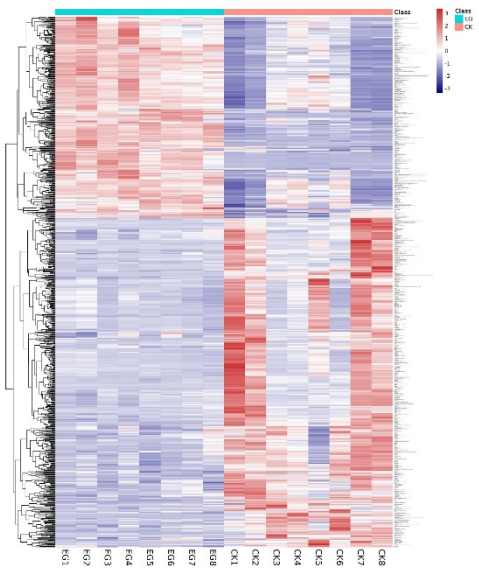

Figure 3 Fold change (FC) arrangement and pattern of metabolites that were different in EG_CK in Yu-711 leaves. (a) The proportion of differential metabolites in EG_CK. (b) Volcano plot metabolites that were significantly different in EG_CK. Each dot represents an individual metabolite. The red color indicates metabolites with high concentrations. The blue color is metabolites with low concentration. The grey color indicates no change in different metabolites. (c) Pattern heat map between samples. The color legend from blue to red indicates the abundance of metabolites from low to high, respectively.

Table 1. Top 50 different metabolites with VIP values of all significantly different metabolites in EG_CK of Yu-711 leaves.

RT (min)	Ion mode	Metabolites	VIP	P-value	adj. P-value	log2(FC)
13.030	pos	2E,13Z-Octadecadienal	19.33	0.02	0.05	-0.44
5.538	pos	Kaempferol 3-(6"-malonylgalactoside)	14.84	0.00	0.01	-8.86
0.712	pos	1-Deoxynojirimycin	14.68	0.00	0.00	-1.69
2.717	pos	3,4-Dihydro-2H-1-benzopyran-2-one	10.43	0.03	0.07	-3.00
11.472	pos	1-heptadecanoyl-sn-glycero-3-phosphocholine	10.02	0.01	0.04	-0.40
13.987	pos	PG(16:1(9Z)/0:0)	9.07	0.02	0.05	-1.45

0.824	neg	Quinic acid	8.83	0.04	0.08	-1.67
4.052	pos	3-amino-2-naphthoic acid	8.79	0.00	0.01	-2.91
12.609	pos	Annosquamosin B	8.77	0.01	0.02	-2.12
11.065	pos	TOFA	8.71	0.01	0.04	-1.18
4.704	pos	Kaempferol 3-(6''-malonylglucoside)-7-glucoside	8.59	0.00	0.00	-3.89
11.267	pos	beta-kamlolenic acid	8.22	0.00	0.01	-1.13
12.304	pos	Farnesyl acetone	8.05	0.03	0.06	-0.51
13.527	pos	PI(18:4(6Z,9Z,12Z,15Z)/0:0)	7.68	0.01	0.04	-1.12
5.595	pos	Tetramethylquercetin 3-rutinoside	7.44	0.00	0.01	1.19
0.841	neg	D-Maltose	7.43	0.02	0.05	0.37
8.883	pos	16-J1-PhytoP	7.42	0.01	0.04	-0.34
4.145	pos	p-CHLOROPHENYLALANINE	7.34	0.02	0.05	-0.30
12.115	pos	Methyl 8-hydroxy-11E,17-Octadecadien-9-ynoate	7.32	0.01	0.02	-1.45
5.528	neg	6''-Malonylastragalin	7.19	0.00	0.02	-8.64
5.772	pos	Neryl rhamnosyl-glucoside	7.17	0.00	0.00	3.04
5.557	pos	4-Hydroxy-5-(phenyl)-valeric acid-O-glucuronide	7.16	0.00	0.02	-1.23
4.546	pos	1-O-p-Coumaroyl-beta-D-glucose	7.06	0.02	0.04	-1.28
12.490	pos	PG(a-13:0/i-12:0)	6.96	0.03	0.06	-0.40
0.831	pos	Galactosylglycerol	6.70	0.01	0.04	-0.43
3.092	pos	4-Hydroxy-5-(3',5'-dihydroxyphenyl)-valeric acid-O-methyl-O-glucuronide	6.65	0.00	0.00	-1.78
15.079	pos	Araliacerebroside	6.38	0.04	0.07	-0.66
11.513	pos	(3b,7b,22x)-Cucurbita-5,24-diene-3,7,23-triol 7-glucoside	6.36	0.00	0.00	-0.94
14.449	pos	1-(O-alpha-D-mannopyranosyl)-3-keto-(1,27R)-octacosanediol	6.33	0.01	0.04	-1.90
14.007	pos	Nonadecandioic acid	6.25	0.02	0.05	-1.23
5.480	pos	4,8 dimethylnonanoyl carnitine	6.15	0.00	0.00	-1.29

14.002	neg	LysoPA(0:0/18:1(9Z))	6.00	0.02	0.04	-1.61
5.195	pos	L-Olivosyl-oleandolide	6.00	0.00	0.01	-1.00
4.585	pos	Kaempferol 3-rhamnosyl-(1->6)-glucosyl-(1->6)-galactoside	5.97	0.00	0.00	1.33
10.195	pos	Scillirosidin	5.83	0.02	0.05	-0.41
2.403	pos	(S)-Oleuropeic acid	5.76	0.00	0.00	-1.32
0.712	pos	L-Asparagine	5.64	0.00	0.00	-2.19
5.933	pos	Epothilone A	5.55	0.00	0.00	1.47
12.629	pos	PA(0:0/18:2(9Z,12Z))	5.48	0.04	0.08	-2.88
4.706	neg	Quercetin 3-(6"-malonylneohesperidoside)	5.36	0.00	0.00	-4.14
10.157	pos	(S)-Nerolidol 3-O-[a-L-Rhamnopyranosyl-(1->4)-a-L-rhamnopyranosyl-(1->2)-b-D-glucopyranoside]	5.33	0.05	0.09	-2.89
11.886	pos	3alpha,6alpha,7alpha,12alpha-Tetrahydroxy-5beta-cholest-24-en-26-oic acid	5.24	0.00	0.00	-0.99
12.493	neg	2-hydroxyhexadecanoic acid	5.23	0.00	0.00	5.55
5.195	pos	Pederin	5.20	0.00	0.01	-2.44
14.071	pos	DG(9D3/9D3/0:0)	5.11	0.03	0.07	-0.99
10.724	pos	8-hydroxy-13Z-octadecene-9,11-diynoic acid	5.11	0.00	0.01	-0.26
2.738	pos	4-Hydroxy-5-(3',4'-dihydroxyphenyl)-valeric acid-O-methyl-O-glucuronide	5.09	0.00	0.00	1.66
12.778	pos	Palmitic amide	5.07	0.02	0.05	-0.46
5.270	pos	Quercetin 3-(6"-malonyl-glucoside)	5.06	0.00	0.02	-34.29
8.364	neg	Thioridazine	5.03	0.00	0.01	1.81

190 RT (min) represents retention time. pos/neg means the metabolite profiling obtained via ESI
191 positive or ESI negative ion modes. VIP is the variable important in projection.

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Figure 4a depicts the VIP values for metabolites classified by the superclass**.** Again, the lipids and lipid-like molecules categories had the highest weight. For example, the compound 2E,13Z-Octadecadienal of the lipids and lipid-like molecules recorded a VIP value of 19.331 even though the average mean of the exclusive VIP was 2.213. Figure 4b shows that the most abundant differentially expressed metabolites, lipids, and lipid-like molecules made up 47.7% of the total, with unclassified and organic oxygen compounds accounting for 19.2% and 10.2%, respectively..

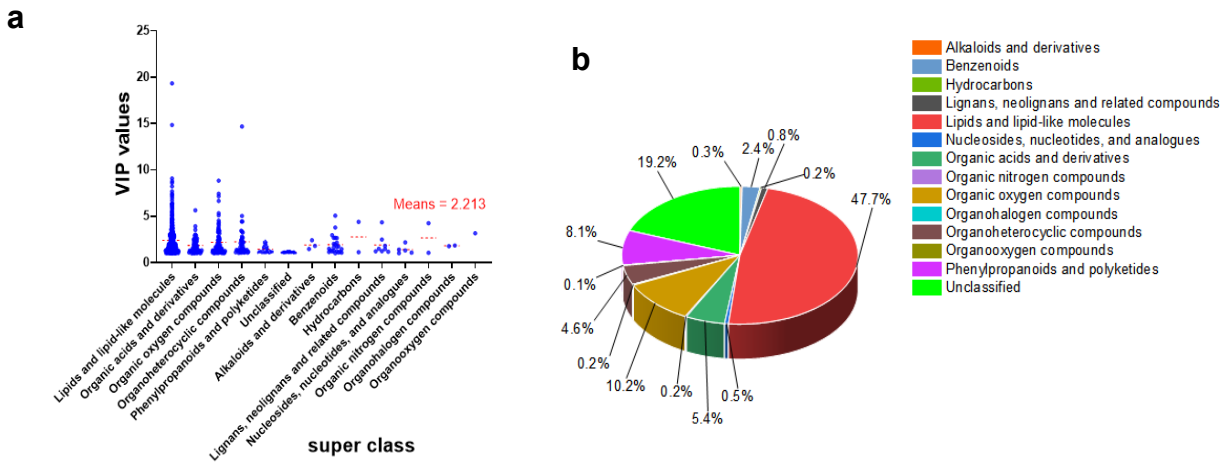

Figure 4. Distribution of variable importance in the projection (VIP) values and classification of the differentially expressed metabolites in EG_CK in mulberry Yu-711 leaves. **(a)** Scatter plot of the VIP distribution in each superclass of metabolite. The red dashed line represents the means with (2.213) average mean of the differentially expressed metabolites. **(b)** Pie chart of the proportion of each superclass of metabolite. Lipids and lipid-like molecules represent 47.7%, followed by unclassified at 19.2%, phenylpropanoid and polyketides at 8.1%, and organic oxygen compounds at 10.2%.

Hierarchical cluster analysis (HCA) was performed to demonstrate the relative contents of the significantly differentially abundant metabolites. Figure 5a shows the HCA heat map, whereas Figure 5b shows the histogram of the top 50 metabolites. Most of the altered metabolites were more abundant in EG. However, the majority had a low concentration in an abundance of the significantly expressed metabolites compared with those in CK, suggesting that long drought

stress led to more substantial fluctuations in the metabolites. Remarkably, Kaempferol 3-rhamnosyl-(1->6)-glucosyl-(1->6)-galactoside, epothilone A, thioridazine, 2-hydroxyhexadecanoic acid, tetramethylquercetin 3-rutinoside, D-maltose 2-hydroxyhexadecanoic acid, and neryl rhamnosyl-glucoside all increased significantly in EG, whiles 2E,13Z-octadecadienal, farnesyl acetone, and palmitic amide all decreased significantly in EG. The higher the VIP value, the greater the contribution. Figure 6 shows the 20 most differentially expressed metabolites with fold change values (Supplemental Table S3).

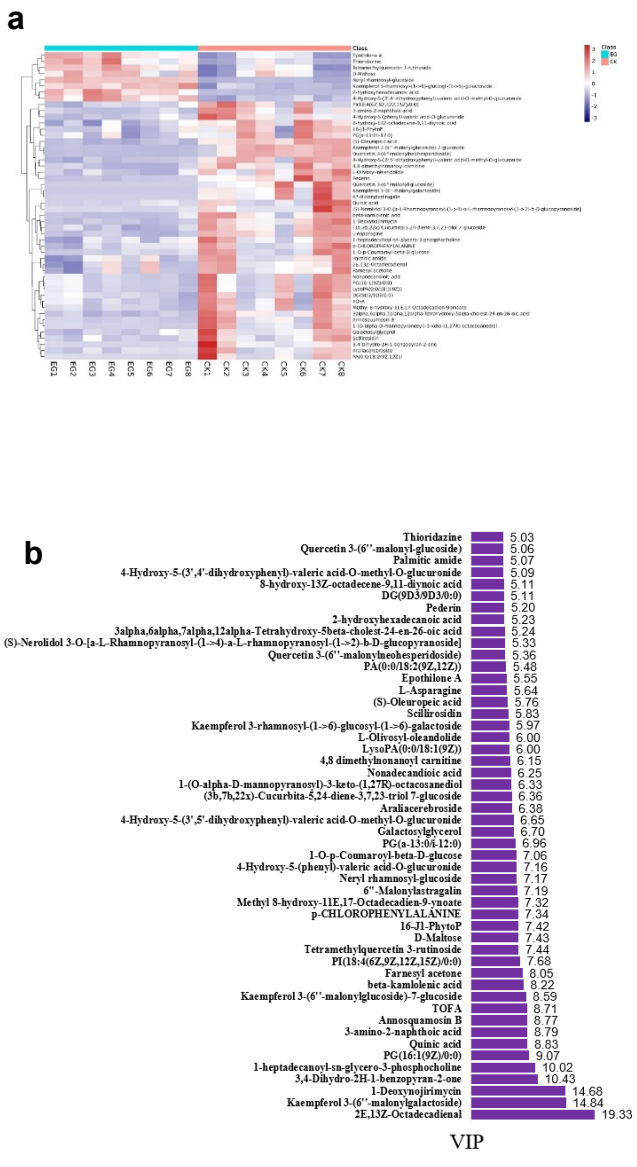

Figure 5. Heat map of the hierarchical clustering analysis of the top 50 differentially expressed metabolites in EG_CK in mulberry Yu-711 leaves. (**a**) Differentially expressed metabolites separated using hierarchical clustering. The x-axis has the eight (1–8) biological replicates of each type of treatment sample, and the y-axis represents the differentially expressed metabolites separated by hierarchical clustering. The color from blue to red indicates an increase in the abundance of metabolites from low to high. (**b**) VIP values of each differentially expressed metabolite.

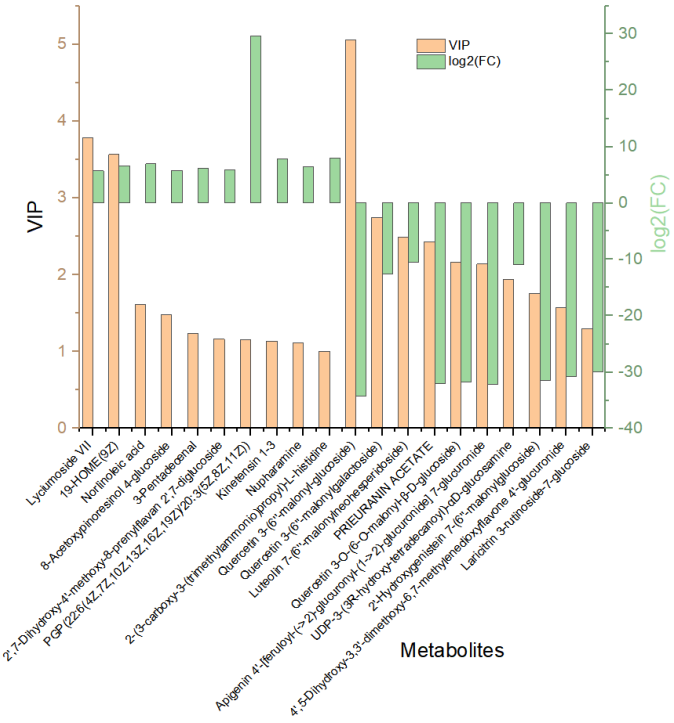

Figure 6. Bar graph of 20 differentially expressed metabolites in EG_ CK in mulberry Yu-711 leaves. The brown columns represent variable importance in the projection (VIP) values, and the green columns represent log2 (fold change, FC) values. The metabolites in the left half were high in concentration, while those in the right half were low concentration in EG_CK in Yu-711.

A correlations analysis was done to determine the correlations between EG and CK metabolites with significant differences. The Pearson correlation coefficients of the correlations utilizing the VIP values of the top 50 metabolites in EG and CK are shown in Figure 7a. The network interactions among metabolites are depicted in Figure 7b (**Supplementary Table S4**).

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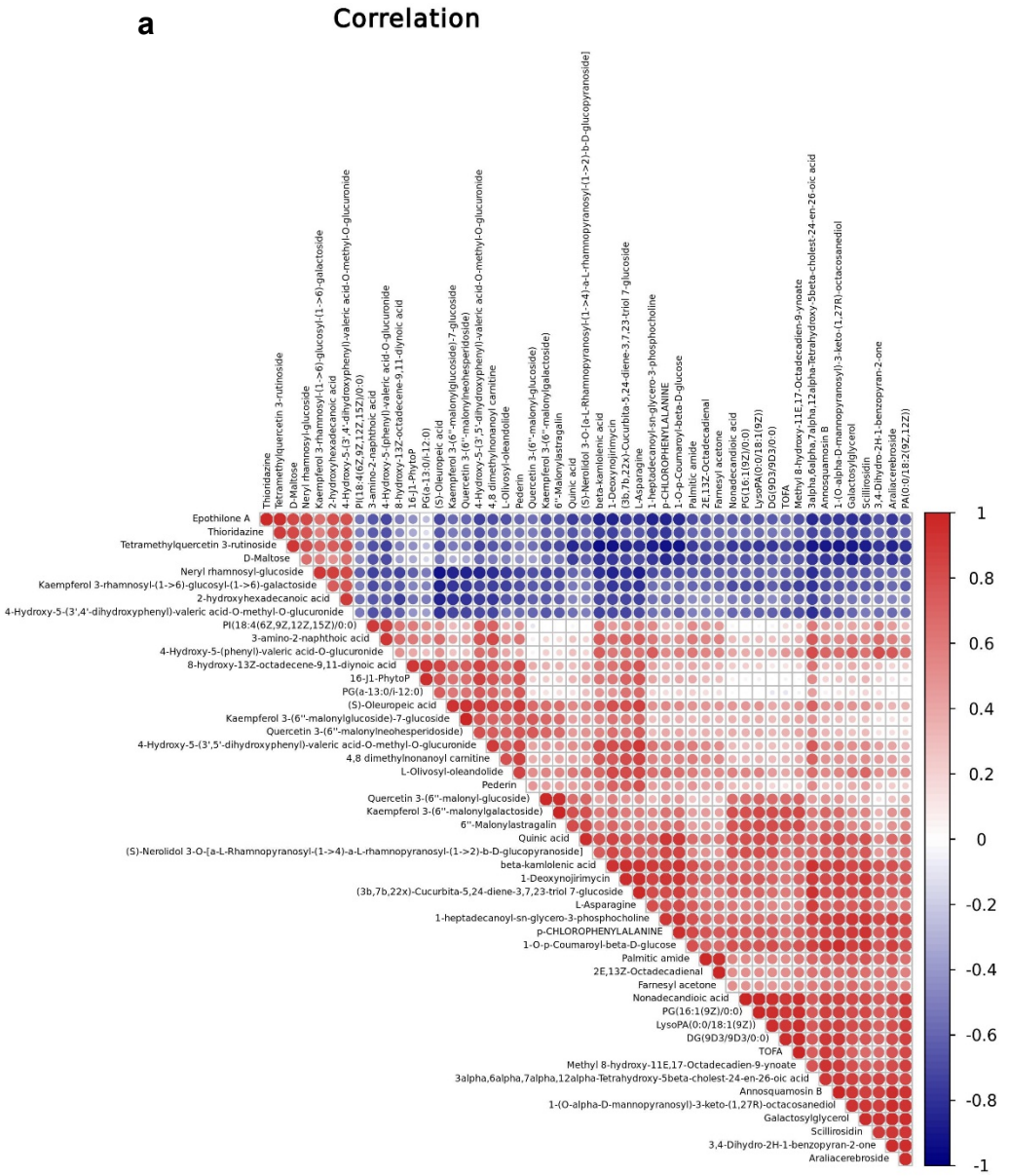

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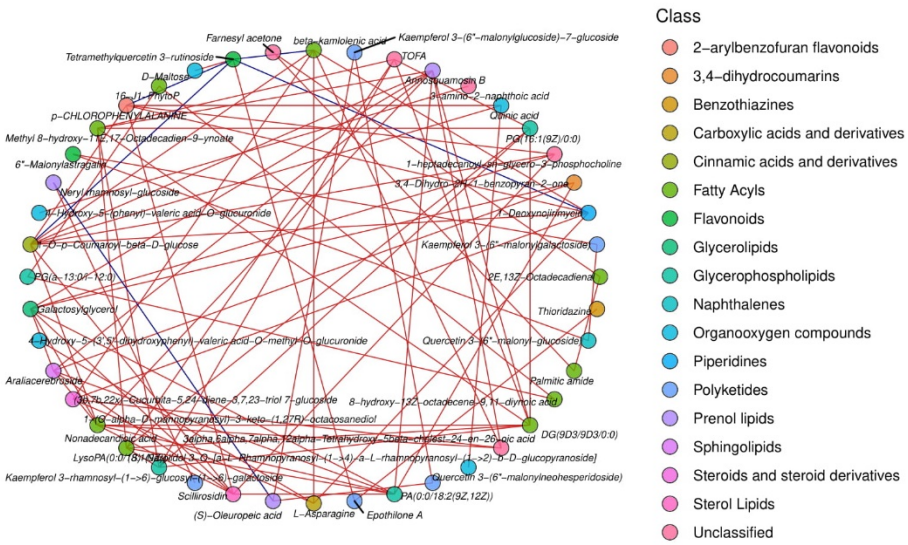

Figure 7. Correlation analysis and network of differentially expressed metabolites in EG_CK in the mulberry Yu-711 leaves. (a) Pearson's coefficients of correlation of VIP values were used to analyze the correlations among metabolites between EG and CK. Red represents a positive correlation; blue is the negative correlation. Different sizes of circles indicated the correlation of Pearson's coefficients. (b) Network interactions among classes of the top 50 differentially expressed metabolites. The Pearson correlation coefficient threshold was set at 0.9. Red and blue lines represent positive and negative correlations between substances, respectively.

2.4 metabolic pathways enrichment analysis

Pathway enrichment analysis was performed using the KEGG (https://www.kegg.jp/) pathways database to investigate biochemical changes better. Among the metabolic pathways that were significantly enriched with differentially expressed metabolites were arginine biosynthesis, galactose metabolism, alanine, aspartate, glutamate metabolism, ABC transporters, Linoleic acid metabolism, arachidonic acid metabolism, aminoacyl-tRNA biosynthesis, and glycerophospholipid metabolism. Figure 8a-b shows the top 20 metabolic pathways that were enriched by differentially expressed metabolites. Based on the enrichment analysis, 14 out of 64 pathways, including arginine biosynthesis, galactose metabolism, alanine, aspartate and

glutamate metabolism, ABC transporters, linoleic acid metabolism, and others, were significantly enriched with p-values less than 0.05, as shown in Figure 8c-d(**Table 2, Supplementary Table S5).** Those involved in arginine biosynthesis, galactose metabolism, starch and sucrose metabolism, and arachidonic acid metabolism increased, while those involved in alanine, aspartate, and glutamate metabolism, linoleic acid metabolism, glycerolipid metabolism, carbon fixation in photosynthetic organisms, and arachidonic acid metabolism decreased, which aid in their function as energy storage,

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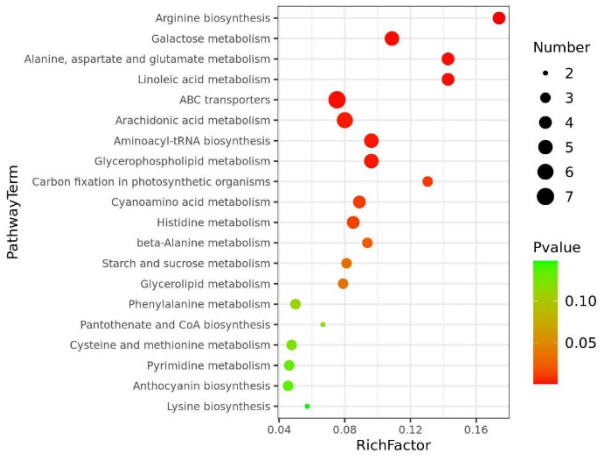

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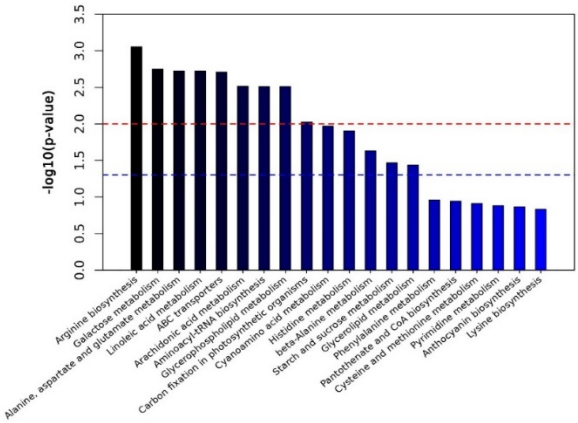

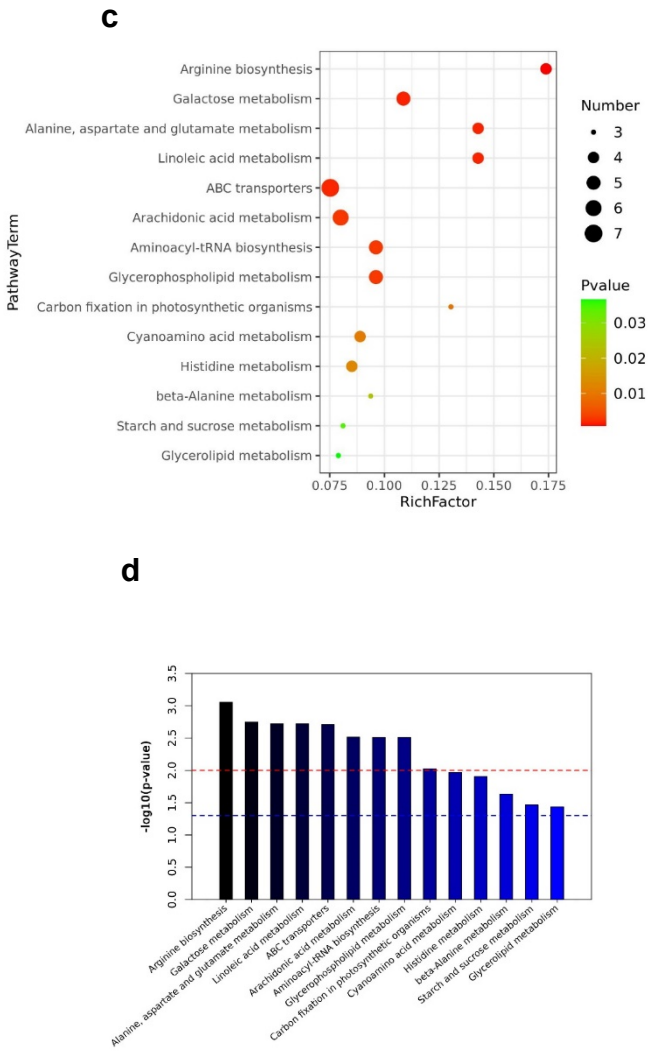

Figure 8. Pathway enrichment analysis of the differentially expressed metabolites of EG _ CK in mulberry Yu-711. (a) Heat map analysis of the top 20 metabolic pathways enriched by the differentially expressed metabolites. The analysis was based on a visualization analysis of metabolic pathways originated from KEGG (ttp://www.kegg.jp/). From green to red indicates p-value decreases sequentially; the point size indicates the number of metabolites enriched in each pathway. (b) Bar graph showing the p-value in the metabolic pathway is the significance of the enrichment of the metabolic pathway of the top 20 metabolites; The red dash line indicates the p-value = 0.01, and the blue dash line indicates the p-value = 0.05. The top of the bar above the blue line indicates the signal pathway represented by it is significant. (c) Heat map analysis of the top 20 metabolic pathways enriched by the differentially expressed metabolites with a p-value less than 0.05. From green to red indicates p-value decreases sequentially; the point size indicates the number of metabolites enriched in each pathway. (d) Is the bar graph showing the p-value in the metabolic pathway is the significance of the enrichment of the metabolic pathway of the

metabolites with a p-value not more than 0.05. The red dash line indicates the p-value = 0.01, and the blue dash line indicates the p-value = 0.05

Table 1. Metabolic pathway enrichment analysis based on p-value less than 0.05.

ID Annotation	Annotation	Match status	RichFactor	p-value	-lg(p-value)	FDR correction
ath00220	Arginine biosynthesis	4/23	0.173913043	0.00088	3.055582081	0.024688537
ath00052	Galactose metabolism	5/46	0.108695652	0.001781	2.749220415	0.024688537
ath00250	Alanine, aspartate, and glutamate metabolism	4/28	0.142857143	0.00189	2.723430403	0.024688537
ath00591	Linoleic acid metabolism	4/28	0.142857143	0.00189	2.723430403	0.024688537
ath02010	ABC transporters	7/93	0.075268817	0.001956	2.708576464	0.024688537
ath00590	Arachidonic acid metabolism	6/75	0.08	0.003057	2.514704648	0.024688537
ath00970	Aminoacyl-tRNA biosynthesis	5/52	0.096153846	0.003086	2.510594625	0.024688537
ath00564	Glycerophospholipid metabolism	5/52	0.096153846	0.003086	2.510594625	0.024688537
ath00710	Carbon fixation in photosynthetic organisms	3/23	0.130434783	0.009444	2.024862056	0.067154545
ath00460	Cyanoamino acid metabolism	4/45	0.088888889	0.010732	1.969316695	0.068685218
ath00340	Histidine metabolism	4/47	0.085106383	0.012479	1.90380436	0.072607742
ath00410	beta-Alanine metabolism	3/32	0.09375	0.023355	1.631620415	0.124559918
ath00500	Starch and sucrose metabolism	3/37	0.081081081	0.034158	1.466512782	0.167226148
ath00561	Glycerolipid metabolism	3/38	0.078947368	0.036581	1.436747753	0.167226148

Match Status = Hit/Total. The total is the total number of compounds in the pathway; the hit is the matched number. The p-value is originally calculated from the enrichment analysis; the -lg(p-value) is the p-value adjusted by logarithm operation based on 10; the FDR is the p-value adjusted using False Discovery Rate results from the pathway analysis.

3 Discussion

Drought is one of the most significant environmental factors limiting plant performance, development, and yield worldwide. The phenomenon causes many dramatic alterations in all plant organs on a morphological, physiological, and biochemical level, disrupting the sink and source plant organs[33]. Thus, a plant stress response is a complicated and dynamic process that aims to build new homeostasis in unfavorable growing conditions. More specifically, drought-responsive systems include hormone induction, kinase signaling, gene expression regulation, scavenging of reactive oxygen species, osmolyte production, cell structure modification, ion channel activation, carbohydrates and energy metabolism, nitrogen absorption, and amino acid metabolism, including fatty acid metabolism[34]. Thus, these processes involving genes, proteins, and small molecules (metabolites) all play a role in this dynamic process. However, metabolites are critical as they are directly involved in plant cell structure and metabolism, influencing the final phenotype[35]. Therefore, understanding the principles of stress adaption physiology and biochemistry requiring a precise and simultaneous investigation of the metabolome in drought-tolerant and susceptible plant cultivars is of great essence.

China faces diverse climatic conditions, including low and high temperatures and drought, affecting agriculture productivity[6]. Mulberry cultivar Yu-711 exhibits distinct anatomical, morphological, and agronomic characteristics under natural conditions such as water stress. It is still unclear the metabolomic mechanism under which mulberry Yu-711 adapts to water stress. Metabolites are implicated in numerous biological cascades through the regulation of phosphorylation, acetylation, and peroxidation, and recent advancements in the identification and measurement of metabolites have improved understanding of the complex process[31]

In this study, seedlings of mulberry variety, Yu-711, were subject to two weeks of drought stress to investigate the metabolomics response to the water deficit and control using a non-targeted approach via LC-MS. The results show that lipids, organic acids, phenylpropanoid, organic oxygen compounds, organoheterocyclic, and other metabolites (**Supplementary Table S3**), and their pathway metabolism may play a role in drought stress response.

3.1 Lipids and lipid-like metabolites change in response to drought stress

Lipids function as major energy storage compounds, as essential components of

membranes and as signaling molecules[36]. Data set from the LC-MS results in this study show that lipids and lipid-like metabolites were more prevalent after days of drought stress in mulberry Yu-711. Lipids constituted 451 of the total 945 differentially significant metabolites accounting for 48% of the total differentially significant metabolites (**Figure 4b**). Only 65 lipids metabolites, accounting for 14.4%, were significantly changed in the drought stress leaves compared to the control. However, 386 lipids constituting 85.6% were unchanged in the drought stress leaves compared to the control(**Supplementary Table S6**). Water deficiency strongly altered lipids composition.

Major classes of lipids levels reveal a significant decrease under drought stress leaves compared to the control. This trend was prevalent for lipid classes such as fatty acyl, steroids and steroid derivatives, polyketides, sphingolipids, glycerolipids, glycerophospholipids, and sterol lipids (**Additional file, figure S1**). Interestingly, a significant increasing trend was observed for prenol lipids. Fatty acyl (FA) molecules and their derivatives were the most abundant lipids fractions produced in response to drought stress after the two-week drought period. Further investigation of the FA molecules revealed that 129 of the 192 metabolites were of low concentration, with lower VIP values in EG than CK(**Supplementary Table S6, sheet 3**), implying that the FA concentrations fell after drought stress treatment. Interestingly, 19 FA metabolites were significantly changed in the drought stress leaves compared to the control **(Additional file, figure S2)**.

The significantly changed FA metabolites had a diverse trend producing a 73.6% decrease. The most affected metabolites belong to the subclass eicosanoids, linoleic acids, and fatty acids. The eicosanoids, including prostaglandin G2 (PGG2), and 6-keto-PGF1alpha, decreased by 42.8%. In addition, linoleic acids (9(S)-HODE, 9(S)-HPODE, gamma-linolenic acid) and as well as fatty acids ((4Z,7Z,10Z,13Z,16Z,19Z)-docosahexaenoate, 9,10,13-TriHOME, and traumatic acid) decreased by 21.4% each. On the other hand, a slight increase (23.4%) in FA content in the drought stress leaves compared to the control treatment. However, eicosanoid was the most significantly affected, increasing by 40%. The substantial decrease in FA observed in mulberry could be due to membrane damage. Our results agree with the finding report from oat plant leaves [36]. Similar results on FA have been reported(Moradi et al., 2017), which agrees with our results.

An increase in eicosanoid levels during drought stress in oat plants has been reported to

help maintain membrane stability[37], which also agrees with our result. FA, such as linolenic acid, is essential for membrane integrity and the functionality of important membrane proteins, such as those that make up the photosynthetic machinery; thus, a fall in its concentration significantly influences photosynthesis[36]. Furthermore, the degradation of linolenic acid and other polar lipids through hydrolytic processes could release free fatty acids (FFAs) and lipid hydroperoxides, which initiate the senescence process[38]. Prenol lipids (PR)content was the second most abundant after the fatty acyl after the drought stress period. Prenols are made from the five-carbon precursors' isopentenyl diphosphate and dimethylallyl diphosphate, synthesized mainly by the mevalonic acid pathway[39, 40].

Our results reveal that 97 of the 451 lipids metabolites (**Supplementary Table S6, sheet 4**), representing 21.5% (**Additional file 1, figure S1**), were PR. Only 17 prenol metabolites changed significantly. The significant change in PR metabolites exhibits a diverse trend with a 64.7% increase and 35.3% decrease in the drought stress leaves compared to the control. Interestingly, the most affected metabolites are a subclass of isoprenoids and terpenoids (**Additional fine, figure S3**). Changes in PR levels have been reported in soybean[41] when subjected to drought treatment. The study demonstrated PR levels elevated during drought stress

Similarly, our study also produces increased levels of PR in mulberry exposure to drought stress. Another study (Gupta et al., 2018) indicated that the PR levels were increased due to the accumulation of ABA levels. ABA signaling plays a crucial role in plant physiology and helps plants respond to stressful environmental conditions[42]. In addition, Prenol lipids play a key role in the transport of oligosaccharides across a membrane[39]. Here, the accumulated level of the PR in the drought stress mulberry leaves could suggest a high level of ABA accumulation in the membrane during the drought period.

Glycerophospholipids (GPL) and glycerolipids (GL) are widespread and are important components of a cell's lipid bilayer. These galactose lipids are a key component of biological membranes and provide intracellular and intercellular protein binding sites. Some glycerophospholipids in eukaryotic cells are either precursors of membrane-derived second messengers or are second messengers themselves[39]. Water deficiency reveals a significant change in GPL and GL content in mulberry leaves. In this study, 47 of the 451 were in the GPL class representing 10.4% of the total lipids, whiles 14 of the total lipids representing 3.1% were GL(**Supplementary Table S6, sheet 5&6**). Even though most of the GPL and GL content

411 decrease in the drought stress leaves, they were significantly not changed from the control.
412 Interestingly, 8 and 1 metabolite(s) changed significantly in the GPL and GL,
413 respectively, under drought stress (**Additional file Figure S4**). The GPL decrease by 75% and a
414 slight increase of 25%. However, the most affected metabolites were glycerophosphocholines
415 (PC) and glycerophosphates. On the other hand, the glycosylglycerols of GL decreased by 100%.
416 According to Nam et al. (2019), GPL metabolite 1-(sn-Glycero-3-phospho)-1D-myoinositol was
417 at a lower concentration when soybean was exposed to drought[41]. In this study, 1-(sn-glycero-
418 3-phospho)-1D-myoinositol level decreased under drought stress, consistent with those obtained
419 in soybean leaves[41].
420 The decrease in galactose lipids and phospholipids content in thyme plant drought
421 stress[43] is consistent with our results. Even though some of the GPL increased, they were not
422 significant from the control treatment, which agrees with the report [31, 44]. The increased PC
423 and glycerophosphoethanolamines (PE) in the current study agree with those obtained [45]. PC
424 and PE are part of major cell membrane lipids, which acts as an osmoprotective compound,
425 indicating that these metabolites act as a protective mechanism against osmotic stress in the
426 mulberry plant[46].
427 Exposure of the mulberry plant to drought stress reveals other lipids classes such as
428 steroid and sterol and their derivates, polyketides (PKs), sphingolipids, and saccharolipids
429 **(Supplementary Table S6)**. In this study, 35 PK metabolites consisting of a subclass of
430 flavonoid, aromatic polyketides, macrolides, and lactone polyketides were identified
431 **(Supplementary Table S6, sheet 8)**. Interestingly, only 4 PKs metabolites changed significantly
432 under water deficit. However, the PKs level increased by 75%, and the most affected compounds
433 are in the subclass of macrolides and lactone polyketides and aromatic polyketides(**Additional**
434 **file figure S4**). PKs are typically responsible for the biosynthesis of essential secondary
435 metabolites involved in plant defense and signal transduction[50].
436 Sphingolipids, which forms major structural components of the plasma membrane and
437 other endomembrane systems, are inevitable cellular constituents in signal transduction under
438 drought condition[51]. Plants maintain a stringent equilibrium between nonphosphorylated and
439 phosphorylated sphingolipids to govern the cell's destiny under stress. Sphingolipids (mainly free
440 LCBs) exist in cells at very low concentrations[52]. Our result shows 8 eight metabolites of the
441 sphingolipids class of lipids were detected with a decrease in content(**Supplementary Table S6,**

sheet 10); however, only one sphingolipid metabolite changed significantly under water deficit(**Additional file figure S4**). The amounts of the various sphingolipid classes in plants differ by species and tissue[53]. In this study, the level of GlcCer was the highest sphingolipids forming 37% but no significant change under the drought and control treatment. An increasing level of sphingolipid has been reported in plants under water deficit[54]. Their results gave evidence about the involvement of sphingolipids in drought-related signaling[54]. Here, our result supports those obtained in the thyme plant[55].

Our results indicate that 53 of the 451 lipids metabolites were steroids and sterols. However, only 15 of them had a significant change. The steroid and sterols content decreased significantly by 86.6%, and a slight increase of 13.3% under the drought stress leaves compared to the control(**Additional file figure S5**). Phytosterols are essential components of a plant's membrane lipid bilayer as they protect plants against stresses and control the membrane's fluidity, thus affecting its properties, functions, and structure[47]. Some studies have reported increased phytosterol during drought stress[43, 48]. However, our study reveals a decreased level of phytosterol. As an adaptive response to environmental conditions, high terpene synthase (TPS) activity levels increase sterol content[49]. The terpene level in this study was higher, and that TPS might have enhanced the sterol content. The reduced phytosterol level in this study indicates that the mulberry under stress conditions activates a distinct pathway as a defense to the stress treatments and may have led to forming other derivatives with a more substantial water holding capacity. The results here agree with the study reported by [49].

3.2 Organic oxygen metabolites change response to drought stress

Drought stress strongly affected the level of organic oxygen when the mulberry plant was exposed to drought stress accounting for 10.2% (**Figure 4b**) of the total differential metabolites. The metabolites were mainly carbohydrates and their conjugates and. An exciting trend of total carbohydrate metabolites was observed. Generally, higher (40) and lower (41) carbohydrate metabolites concentrations were detected under the drought leaves compared to the control (**Supplementary Table S7**). However, further analysis shows that most of the sugars were not significantly changed. Remarkably, only 21 organic oxygen metabolites changed significantly(**Additional file, figure S6**). The most affected compounds are carbohydrates, alcohols, and polyols, decreasing at 65%. Among these compounds include beta-cortol,

chlorogenic acid, fructose 6-phosphate, fucose 1-phosphate, glucose 6-phosphate, N-Acetyl-D-glucosamine, N-Acetyl-D-glucosamine 6-Phosphate, and quinic acid.

On the other hand, an increased level of organic oxygen, mainly carbohydrates, increasing at 35%, was detected under the drought stress leaves. This trend was evident in metabolites such as 3-methoxy-4-hydroxyphenylglycol glucuronide, alpha-Santalyl acetate, D-(+)-raffinose, D-maltose, levan, maltotriose, and octanoylglucuronide. Intercellular CO_2 (Ci) decreases due to stomatal closure during moderate water deficit while photosynthetic capacity is maintained. Reversible inhibition of some enzymes, such as sucrose-phosphate synthase(SPS), may occur due to the decrease in Ci. At the same time, starch content decreases, and reducing sugars are maintained or even increase. This change in the carbohydrate status can alter cellular processes such as gene expression[56]. Significant physiological and biochemical changes occur in sugar deprivation to maintain respiration and other metabolic activities[57]. However, sugar concentration and source-sink partitioning are not affected according to distinctive patterns in different organs and under different stresses[57-59].

Generally, under drought and other stresses such as salinity, soluble sugar concentrations increase. In contrast, high light irradiance (PAR, UVBR), heavy metals, nutrient shortage, and ozone decreased sugar concentrations[57]. Nonetheless, sugar changes vary with genotype and stress factors and do not follow a static model[60]. Our result is similar to those reported on the diverse trend of sugar changes under water deficit[61]. For example, maltose, an essential sugar that helps plant growth and development, was reported to increase after a water deficit[62]. Also, some reports have revealed that the accumulation of sugar metabolites such as glucose, fructose, and raffinose levels elevated and were the earliest metabolites accumulated after withholding water to induce water deficits[63, 64]. Remarkably, not all soluble sugars play similar roles in events associated with metabolites of stressed plants. For example, sucrose and glucose serve either as substrates for cellular respiration or osmolytes to maintain cell homeostasis. However, fructose is unrelated to osmoprotectant and synthesizes secondary metabolites[65].

According to Hilal et al. (2004), fructose may be linked to the synthesis of erythrose-4-P, which serves as a substrate to produce lignin and phenolic compounds. All this indicates soluble sugar metabolism is a dynamic process involving degrading and synthesizing reactions under stressful conditions[66]. The carbohydrate status of the leaf, which is affected by water deficit in quantity and quality, could function as a metabolic signal in the stress response[67], even though

the signaling role of sugars is still not fully understood. Plant sugar signaling is part of a complex network that also includes a plant-specific hormone signaling pathway. Glucose-6-phosphate (G-6-P) is reported to be involved in repression signals. However, a decrease in intracellular concentration after treatment with glucose reveals that G-6-P is involved in the direct signal(Brun et al., 1993). Thus, the low concentration level of G-6-P in the results may suggest the crucial signal pathway role of G-6-P in the mulberry under water deficit.

3.3 Phenylpropanoid and polyketide metabolites change response to drought stress

The phenylpropanoid and polyketides metabolites content was significantly altered, accounting for 8.1% (**Figure 4b**) of the total differentially metabolites when mulberry seedlings were exposed to drought stress. However, the metabolites were mainly involved in the class of flavonoids (38.9%), cinnamic acid derivatives (23%) (**Supplementary Table S8**). Generally, there was a dynamic trend in the polyphenols expressed on the mulberry plant under the water deficit. Interestingly 40 of the metabolites were of low concentration, whiles 38 of them had increase concentration. However, most of them did not change significantly. Polyphenols such as Quercetin 3-O-(6"-malonyl-glucoside) 7-O-glucoside, apigenin 4'-[feruloyl-(->2)-glucuronyl-(1->2)-glucuronide] 7-glucuronide, 2'-hydroxygenistein 7-(6"-malonylglucoside), 2',7-Dihydroxy-4'-methoxy-8-prenylflavan 2',7-diglucoside, Kaempferol 3-sophorotrioside were strongly altered under water deficit, however they were Significantly not changed. Interestingly, only 14 of the polyphenols changed significantly. These metabolites mainly involve flavonoids and cinnamic acids and derivatives subclass(**Additional file, figure S7**). In the present study, the polyphenols content increase at 57.2% as against a 42.8% decrease under the drought stress. Flavonoids are antioxidant phenolic compounds, an important class of secondary metabolites found in plants that protect them against various stressors. Secondary metabolites are synthesized from phenylalanine through a conserved pathway in plants[63]. There have been several reports on phenolic compound alteration in plants under drought stress. For example, an increase in the flavonoid compound in Arabidopsis under drought stress has been reported[61]. Our result is similar to those obtained by these authors. Flavonoids' responses in times of drought stress may serve as reactive oxygen species (ROS) scavengers in the vacuole.

Cinnamic compound, another important phenylpropanoid compound, was significantly altered. Thus, drought stress has a reducing effect on cinnamic content. According to Antunes et

al. (2019), a reduced cinnamic acid was recorded when strawberry plants were exposed to drought stress. Similarly, our results produced a decrease in cinnamic content. For instance, 2-hydroxycinnamic acid and m-Coumaric acid were mostly affected with lower concentrations. Cinnamic acid may play a role by regulating the flux into the phenylpropanoid pathway[49]. Sun et al. (2012) exposed cucumber to water deficit and found that the level of cinnamic acid was reduced[68].

Further downstream from cinnamic acid has been found in mulberry fruits, described as a potential antioxidant compound[69]. Plants under stress have been observed to accumulate phenylpropanoids. Their role in combatting this stress may be linked to scavenging free radicals in their function as a stress response marker.

3.4 Organic acids metabolic changes to drought stress

The exposure of mulberry plants to drought stress altered the organic acid metabolites contents, constituting 5.4% of the total differential metabolites. The metabolites involved were mainly in the class of carboxylic acid and their derivatives (90%), comprising a subclass of amino acids, peptides, and their analogs (**Supplementary Table S9**). The accumulation of amino acids under drought stress has been described in many plant species[61]. In this study, 51 organic acid metabolites were expressed under drought stress. Further analysis reveals that 39 metabolites of the organic acids altered, but they were not changed significantly under the drought stress compared to the control. Among them include, 13E-tetranor-16-carboxy-LTE4, arginyl-phenylalanine, ceanothine D, 2-(3-carboxy-3(trimethylammonio)propyl)-L-histidine, and kinetensin 1-3. Conversely, only 12 metabolites of organic acid changed significantly (**Additional file, figure S8**).

Accumulation of amino acids under drought stress has been described in many plant species[61]. The amino acid altered decreased by 81% and the most affected include branch chain amino acids (BCAA) and aspartate family. Amino acids play a crucial role in drought stress by acting as an osmolyte, regulating ion transport, modulating stomatal opening, enzyme synthesis, and affecting gene expression and redox-homeostasis [26, 64].

Branch chain amino acids (BCAA) such as valine, leucine, isoleucine, and other related amino acids are reported to generally increase in response to drought stress[61, 64]. An increased

isoleucine level has been reported in rice under drought stress[70]. Conversely, the isoleucine content in the present study decreases significantly under the drought stress compared to the control. BCAAs are known to accumulate in relatively large concentrations under stress; therefore, it is uncertain if they could act as suitable solutes[71]. However, under water-deficit stress, alternate respiration pathways have been demonstrated to boost the utilization of BCAAs. Importantly, in Arabidopsis, the alternate pathway of respiration and enhanced BCAA catabolism, but not their accumulation, contributed to drought tolerance[64]. The significantly decreased level of isoleucine could suggest the involvement of the compound in the scavenging of ROS.

Free amino acid such as arginine has been shown to provide osmotic adjustment in plants under drought stress by inhibiting stomatal opening[72]. An increased level of arginine has been reported in rice leaves after drought stress[73]. Also, Aninbon et al.(2017) reported high arginine content in peanuts after drought stress[74]. Conversely, in our results, arginine content significantly decreased drought leaves compared to the control. Nevertheless, drought stress does not always cause an increase in amino acid content. Moral and colleagues reported decreased arginine content when the wheat plant was grown under drought stress[75]. They argue that the level of amino acid accumulation could depend on genotype and duration of drought stress. Our result agrees with their findings as almost all the significantly changed amino acids decreased.

Histidine, an essential amino acid required by the plant for growth and development. It also acts as a metal-binding ligand and is a vital component of the metal hyperaccumulator molecule, mitigating heavy metal stress. Its role in drought stress has been implicated, including aiding in the stomatal opening[72, 76]. Under drought stress, histidine concentration is said to increase[76]. However, our result shows that histidine decreased under water stress leaves compared to the control. Water deficit increases the generation of reactive oxygen species (ROS). Maintaining or increasing the activity of enzymes involved in eliminating harmful ROS to minimize cellular damage is thought to be a key element in dehydration tolerance[76]. Thus, the low content of histidine may suggest the compound's active role in scavenging the ROS to maintain cellular integrity.

Aspartate family, comprising aspartic acid and asparagine, plays a crucial role in plants against drought stress. Accumulation of aspartate family, notably asparagine in plants during water deficit, has been reported[77]. The authors reported that the concentration of asparagine

increased throughout the drought period from 4 to 10 days in bread wheat. Interestingly, our result revealed that all the aspartate families decreased in concentration, and which agrees with the observation made in other plant species[33].

3.5 Other metabolic changes to drought stress

Other superclasses of metabolites, including organoheterocyclic compounds, benzenoids, nucleosides, nucleotides, analogs, lignans, neolignans, and related compounds, were notably detected. Remarkably, organoheterocyclic compounds and benzenoids were among the most differentially regulated compounds during drought stress. From the present study, metabolites in the superclass of organoheterocyclic compounds only formed 4.6%(**Figure 4b**) of the total differentially detected metabolites. However, this compound class was one of the most significantly changed metabolites in the drought leaves than the control (**Supplementary Table S10**).

Further analysis reveals that 18 metabolites of organoheterocyclic compounds significantly changed. Remarkably, adenine, aminophylline, guanine, milrinone, 1-Deoxynojirimycin and fagomine are among those decrease significantly(**Additional file, figure S9**). In addition, metabolites such as 2-Methyl-1-hydroxypropyl-ThPP, limonene-1,2-epoxide, riboflavin, mefloquine, topotecan increased significantly. Major purine bases such as adenine and guanine are essential compounds in activating tolerance mechanisms in protecting nucleic acids[33]. These compounds provide the ultimate energy source for synthesizing carbohydrates, lipids, peptides, and secondary metabolites[78]. An exposure of spring wheat to drought stress increased adenine and guanine content[33]. On the contrary, we observed a decrease in content which clearly shows that the purine and metabolites were significantly altered when the mulberry plant was exposed to drought stress. Interestingly, a down-regulated purine level has been reported in soybeans after exposure to drought and heat stress(Das et al., 2017), which agrees with our results.

The piperidines class of compounds, including fagomine and 1-Deoxynojirimycin (1-DNJ), are essential organoheterocyclic compounds. These iminosugars have been isolated from the mulberry plant, which plays a vital role in Chinese traditional medicine[79, 80]. Several reports on iminosugars have been on their biological function in treating various human diseases. In the present study, mulberry exposure to drought stress revealed a significant decrease in the

content of 1-DNJ and fagomine, with 1-DNJ having a fold change of -1.689 and a VIP value of 14.682 and fagomin having -1.909 and a VIP of 4.430. The content of 1-DNJ varies based on some factors such as environment, location, and mulberry variety.

High 1-DNJ and fagomine content have been determined in the mulberry plant[81]. According to Lou et al. (2011), 1-DNJ content in the mulberry (Yu-711) plant was lowest when different mulberry varieties from different locations and weather conditions were studied[79], which agrees with the current result. During drought stress, the sugar level is altered due to the limitation on CO_2 assimilation. Thus, the low level of iminosugars may suggest their active role in scavenging ROS during the drought period and may act as an osmoprotectant. The results show that drought stress may have a negative effect on iminosugar content in the mulberry plant.

Riboflavin (vitamin B_2) is an essential compound for plant growth and development. Riboflavin belongs to the pteridines class, and its role in drought stress has been reported[82]. Our result revealed that the content of riboflavin was up-regulated in the mulberry plant after drought stress. The fold change of the compound was 0.660, with a VIP value of 1.651. Thus, riboflavin at small and moderate quantities has been inferred to enhance plant drought tolerance, while very high content impaired drought tolerance in plants[82]. According to Deng and colleagues, high levels of riboflavin in the plant cause an accumulation of ROS. Interestingly, in our results, the content of riboflavin increases in the drought leaves compared to the control, and the magnitude was less. This may suggest that the lower extent of the compound in the drought leaves might have enhanced the plant tolerance to the drought period by scavenging the ROS.

Metabolites in the benzenoid superclass were significantly altered in the drought leaves compared to the control (**Supplementary Table S10**). From the results, 12 compounds in the class belonging to naphthalenes, phenols, and benzene substitute and derivatives under the benzenoid superclass were significantly altered in the drought leaves compared to the control(**Additional file, figure S10**). Remarkably, compounds such as hippuric acid, p-salicylic acid, quercetin 3-(6''-malonyl-glucoside), and isoproterenol are amongst the compounds significantly decreased(down-regulated) in the drought leaves; however, 2-hydroxy-2-[4-hydroxy-3-(3-methylbut-2-en-1-yl)phenyl] acetic acid, metoprolol, and 6-paradol significantly increased(up-regulated) under the drought leaves compared to the control.

Salicylic acid (SA), a plant hormone, is a promising compound crucial in plants' sensitivity to environmental stresses through regulating the antioxidant defense system,

transpiration rates, stomatal movement, and photosynthetic rate[83]. Furthermore, exogenous SA application has been reported to enhance plant tolerance to drought stress by reducing ROS activity, thereby improving membrane stability[84]. Interestingly, SA content in our results decreased in the drought stress leaves compare to the control. This may suggest that during the drought stress, the biosynthesis of SA was used to scavenge ROS activity to enable membrane stability for the plant to endure the drought conditions.

On the other hand, quercetin 3-(6"-malonyl-glucoside) content was far lower in concentration from the drought leaves than the control with a fold change (log2FC) of -34.294 and a VIP of 5.056. Quercetin is a crucial polyphenol compound that acts as a plant antioxidant during abiotic stress. Mulberry plants have been reported to contain many polyphenols(flavonoids)[85]. The low level of the quercetin 3-(6"-malonyl-glucoside) suggests it active against ROS, thus maintaining membrane integrity.

3.6 Metabolic Pathway Analysis

The differential metabolites pathway map indicates that some essential metabolites were significantly involved in the drought stress response. For example, in the arginine biosynthesis pathway, metabolites such as L-aspartic acid (C00049), L-arginine (C00062), argininosuccinic acid (C03406), and oxoglutaric acid (C00026) were significantly involved in the pathway (**Additional file, figure S11a**). These metabolites (Carboxylic acids and derivatives class) significantly decreased except oxoglutaric acid (Keto acids and derivatives), which increases in response to the drought stress. The decrease in amino acid and an increased oxoglutaric acid content involved in drought stress response has been reported[86], which agrees with our results suggesting the role of organic acid in drought stress management. Metabolites involving lipid and carbohydrates were significantly altered(**Additional file, figure S11b**). Drought stress has a significant influence on lipid and sugar content in plat response to such stress. The lipid and sugar metabolites involved in the pathway include galactosylglycerol, alpha-Santalyl acetate, D-(+)-Raffinose, and beta-cortol.

Alanine, aspartate, and glutamate metabolism pathway reveal significant changes in key metabolites involved in the pathway during drought stress. These metabolites comprise oxoglutaric acid, L-aspartic acid, L-asparagine, and argininosuccinic acid (**Additional file, figure S11c**). In the linoleic acid metabolism pathway, 9(S)-HODE, gamma-linolenic acid, 9(S)-

HPODE, and 9,10,13-TriHOME changed significantly in response to the water stress (**Additional file, figure S11d**). Similar results on lipids changes[43] on plants respond to drought stress effect has been reported. In the ABC transporters, key metabolites such as L-aspartic acid, L-arginine, D-glycerol 1-phosphate, L-histidine, N-acetyl-D-glucosamine (GlcNAc), D-maltose, and riboflavin altered significantly in response to drought stress (**Additional file, figure S12a**). The increased riboflavin content in this work in managing ROS agreed with a report on other plant species when exogenous riboflavin was studied under drought stress [82].

Some metabolites were enriched in multiple transporters. For instance, L-arginine (C00062) is involved in various transporters such as phosphate and amino acid transports. A significant change in metabolites affecting aminoacyl-tRNA biosynthesis during the drought stress in the plant response to the water deficit. These metabolites are L-aspartic acid, L-arginine, L-histidine, L-isoleucine, and L-asparagine (**Additional file, figure S12b**). The changes in these amino acids may suggest their enhancement role in the plant cell during drought stress[72]. They play various functions such as ion transport across the membrane, stomatal inhibition, and opening during drought stress.

The arachidonic acid metabolism pathway comprising 20-hydroxy-LTE4, 20-hydroxy-leukotriene B4, 6-keto-PGF1alpha, prostaglandin G2 (PGG2), traumatic acid, and 9S,11R,15S-trihydroxy-2,3-dinor-13E-prostaenoic acid-cyclo[8S,12R] metabolites changed significantly during the drought stress and enriched in this pathway(**Additional file, figure S12c**). In addition, key metabolites such as D-glycerol 1-phosphate, PA(0:0/18:2(9Z,12Z)), phosphocholine, glycerylphosphorylethanolamine(PA), and lysoPC(17:0) were significantly enriched in the glycerophospholipid metabolism pathway during the drought stress (**Additional file, figure S12d**). A study report revealed that stress-induced changes in the profile of lipid classes could cause remodeling of membrane lipids and activate plant defense responses against various biotic and abiotic stresses such as drought[87]. The significant changes of the membrane lipids in the present study agree with the research reported in other plant species[43]. Also, in the carbon fixation in photosynthetic organisms pathway, L-aspartic acid, fructose 6-phosphate, and D-sedoheptulose 7-phosphate were altered significantly in response to the water deficit (**Additional file, figure S13a**). The decrease level of these compounds in the pathway suggests their role in scavenging ROS to protect the plant from membrane damage.

In the cyanoamino acid metabolism pathway, L-asparagine, L-aspartic acid, L-isoleucine, and dhurrin were significantly involved during the water deficit. Interestingly, all the metabolites involved in the pathway significantly reduced in concentration **(Additional file, figure S13b)**. A decrease in the amino acid content in this pathway may suggest that these compounds protected the plant membrane from excess water loss by causing stomatal inhibition during the water stress[72]. On the other hand, the content of dhurrin, cyanogenic glucoside, which serves as a defensive compound in sorghum[88], was significantly altered in response to the water deficit, which may suggest its membrane defensive role in the mulberry plant. The histidine metabolism pathway was significantly enriched with oxoglutaric acid, L-aspartic acid, L-histidine, and 4-imidazolone-5-propionic acid **(Additional file, figure S13c)**.

In addition, the beta-alanine metabolism pathway comprises L-aspartic acid, L-histidine, and pantothenic acid, which were significantly enriched in the pathway during the drought stress**(Additional file, figure S13d)**. The starch and sucrose metabolism route significantly altered fructose 6-phosphate, glucose 6-phosphate, and D-maltose **(Additional file, figure S14a)**. Finally, mulberry exposure to water deficit, glycerolipid metabolism pathway also showed significant changes in D-glycerol 1-phosphate, PA(0:0/18:2(9Z,12Z)), and galactosylglycerol **(Additional file, figure S14b)**

4 Materials and methods

4.1 Source of mulberry plants and growth conditions

The National Mulberry Gene Bank in Zhenjiang, Jiangsu, China provided the mulberry species (Morus alba) 'Yu-711'. Plant growth was as per Adolf et al. (2020) and are described below. Mulberry plants were grown in a greenhouse with a 14 h light/10 h dark photoperiod, at 25°C Day/20°C night temperature, and relative humidity of 70–80%. The cuttings were grafted to the Rootstocks. The grafted nurseries, reaching the three-leaf stage, were planted in pots of 35 cm diameter containing loam soil with three seedlings per pot. The grafted plants were randomly grouped when new shoots had grown to 20 cm.

4.2 Drought stress treatment leaves sampling

Upon developing the seedlings in the pots when the fresh leaves have emerged, the drought stress experiment commenced. Water supply was withdrawn for 14 days in the experimental group. However, the control group was constantly supplied with water daily. Once the experimental seedlings reached the wilting point (symptoms apparent), leaves sampling was done. The first three-time point for sampling after 14 days of drought stress was the first day (1d), the third day (3 d), and the fifth day (5 d). To account for diurnal variations in metabolite levels, control and drought-treated plants were sampled simultaneously (midday). The primary leaf tissue samples were harvested and immediately frozen in liquid nitrogen and then store at -80°C. Leaves from experimental and control groups from the 3d time point (n = 16) were collected and pooled for metabolite extraction and analysis.

4.3 Metabolite Extraction

All chemicals and solvents used were analytical or HPLC grade. Water, methanol, acetonitrile, and formic acid were purchased from CNW Technologies GmbH (Germany). However, L -2-chlorophenylalanine was obtained from Shanghai Hengchuang Biotechnology Co. Ltd (China). The metabolites were extracted as described[27, 28]. Eight replicates (n = 16) were analyzed in each group of mulberry (YU-711). In brief, Briefly, samples were ground at 60 Hz for 2 min and ultrasonicated at ambient temperature (25–28°C) for 10 min. After centrifuging at 10,000 × g at 4 °C for 15 min, supernatants were dried using a freeze-concentration centrifugal dryer and then resuspended in methanol and water (1:4, v:v), vortexed for 30 s, incubated at 4°C for 2 min, and centrifuged at 10,000 × g at 4°C for 5 min. Finally, the supernatants were filtered through 0.22 µm microfilters, transferred to LC vials, and stored at t 80°C for LC-MS analysis.

4.4 Mass Spectrum Data Calling

An Acquity UHPLC (Waters Corporation, Milford, MA, United States) coupled with an AB SCIEX 5600 TripleTOF System (AB SCIEX, Framingham, MA, United States) (Q Exactive Orbitrap, Thermo Fisher Technologies, Waltham, MA, United States). The conditions for the LC-MS were followed as described previously[27]

4.5 Data Filtering and Analysis

The acquired LC-MS raw data were analyzed by the progenesis QI v2.3 software (Nonlinear Dynamics, Newcastle, UK) using the following parameters: Precursor tolerance was set at 5 ppm, fragment tolerance was 10 ppm, and retention time (RT) tolerance was set at 0.02 min. Internal standard detection parameters were deselected for peak RT alignment, isotopic

peaks were excluded for analysis, and noise elimination level was set at 10.00. The minimum intensity was set to 15% of base peak intensity. Any peaks with missing values (ion intensity = 0) in more than 50% of samples were removed, further reducing the resulting matrix. An internal standard was used for data quality control (QC). The QC samples were prepared by mixing aliquots of all samples into a pooled sample, injected at regular intervals (every 10 samples) throughout an analytical run to provide a set of data in which repeatability could be assessed. Progenesis QI v2.3 software (Nonlinear Dynamics, Newcastle, UK) for data processing was used to identify the metabolites using public databases from http://www.hmdb.ca/;http://www.lipidmaps.org/ and self-built databases (Lu-Ming Biotech Co., Ltd., Shanghai, China)

Principal component analysis (PCA) and (orthogonal) `partial least squares discriminant analysis (O)PLS-DA analysis[29] were carried out on the combined positive and negative ion mode data to visualize the metabolic alterations among experimental groups to identify the differentially expressed metabolites. In addition, the variable importance in the projection (VIP), which is a weighted sum of PLS loading that is widely used to find the most important characters in metabonomic data[30], was employed to rank the overall contribution of each variable to the OPLS-DA model and those variables with VIP >1 and $p < 0.05$ (two-tailed Student's t-test) were considered as differentially expressed metabolites. Also, the 7-fold cross-validation and response permutation test (n = 200) was adopted in validating the model (**Figure 2d**). The R^2 and Q^2 validation plot (**Figure 2d)** indicates that the model was reliable without overfitting.

The correlations between the VIP levels of metabolites in EG and CK were investigated using Pearson's correlation coefficients. In addition, metabolic pathway enrichment using metabolites mapped to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.kegg.jp/) was used further to investigate the relative abundance of differentially expressed metabolites. A hypergeometric test was used to determine the p-value of each metabolic pathway derived to find significantly enriched pathways[31] and false discovery rate (FDR) correction, and the metabolic pathways with $p < 0.05$ were retained. Furthermore, compound names were uploaded to pathway analysis through MetaboAnalys[32]. Finally, the precise pathway analysis algorithms of the hypergeometric test were used in mapping to the pathway library of the *Arabidopsis thaliana* KEGG database(in real-time).

5 Conclusion

Investigations of the effects of various abiotic stresses, such as drought, on relevant food and medicinal plants are educational and helpful. They will undoubtedly assist plant growers, and breeders in expanding their knowledge of these plants' stress tolerance mechanisms. As a result, they will be able to increase their yield under stressful situations. In this regard, we investigated metabolomics changes at the global level caused by drought stress in the mulberry variety Yu-711 plant for the first time. According to our findings, the leaves of the mulberry variety Yu-711 exposed to water deficiency displayed considerable alterations in numerous metabolite classes. In stressed plants, total lipids and galactolipids (MGDGs and DGDGs) as well as phospholipids (PG, PE, PA, and PS) decreased. Fatty acyl lipids were the most abundant lipids produce. Prenol lipids, on the other hand, increased significantly in the drought-stressed plant compared to the control. Organic oxygen, precisely carbohydrates, had dynamic changes with decreased and increased patterns under the water deficit. In addition, polyphenols (mainly antioxidant secondary metabolites including flavonoids and cinnamic acids) also produced a dynamic change leading to both increasing and decreasing patterns under the drought stress plant.

A significant change in organic acids, particularly amino acids, significantly decreases in content following the drought stress. In addition, other classes of metabolites, including benzenoid and organoheterocyclic, decreased significantly under water deficit. As a result, we can speculate that droughts drive the biosynthesis of structural membrane lipids and other metabolite types in mulberry Yu-711 plants to protect the cell and chloroplast membranes from severe drought damage and preserve their structure and function. Furthermore, increased antioxidant lipids and flavonoid levels in these plants would also help scavenge ROS generation under droughts.

Finally, other molecular approaches, such as integrated transcriptomics and metabolomics, will undoubtedly contribute to understanding the gene network behind the observed phenomena, thus providing new insight into mulberry plant adaptation mechanisms to drought stress conditions in the current global climate change situation.

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validation, C.Q and Q.L; supervision, M.A; writing-original draft preparation, M.A; writing-review and editing, W.Z; funding acquisition. All authors have read and agreed to the published version of the manuscript.

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