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BIOLOGY

Ploidy variation on gene differential expression in cowpea

Xuewen Qiu[1] Huiyun Kuang[2] Chuntao Zeng[1] Dan Li[1] Youxin Yang[1] Yudi Gan[1] Shuying Fan[1] Caijun Wu[1*]

[1]College of Agronomy, Jiangxi Agricultural University, 330045, Nanchang, Jiangxi, China. E-mail:wucj12@126.com. *Corresponding author.
[2]Crop Breeding and Cultivation Research Institute, Shanghai Academy of Agricultural Sciences, Shanghai, China.

ABSTRACT: This study investigated the differences in gene expression profiles of diploid and autotetraploid in cowpea, and provided theoretical basis for screening key genes of differential expression and ploidy breeding. The phenotypes and contents of chlorophyll, soluble sugar and soluble protein of diploid and autotetraploid of cowpea were compared and transcriptome sequencing was performed. The autotetraploid leaves of cowpea were thicker and darker green than diploid leaves, and the contents of chlorophyll, soluble sugar and soluble protein in leaves were higher. A total of 2678 differentially expressed genes (DEGs) were analyzed in the diploid and autotetraploid of cowpea. Among them, there were 421 genes with higher expression of tetraploid than diploid, and 2257 genes with lower expression of tetraploid than diploid. All 2678 DEGs were annotated into the Gene Ontology (GO) functional library. The DEGs were mainly concentrated in metabolism and cell composition. Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway analysis showed that cowpea diploid and autotetraploid have significant differences in flavonoid biosynthesis, degradation of other glycan, phenylpropane biosynthesis, starch sucrose metabolism, keratin, amber and wax biosynthesis, circadian rhythm and plant pathways.
Key words: *Vigna unguiculata*, polyploidy, phenotype and physiological characteristics, transcriptome sequencing, differentially expressed gene (DEG).

Efeitos da variação da ploidia na expressão diferencial de genes em feijão-caupi

RESUMO: O objetivo deste estudo foi investigar as diferenças nos perfis de expressão gênica de diplóides e autotetraplóides em feijão-caupi e fornecer base teórica para a triagem de genes-chave de expressão diferencial e melhoramento de ploidia. Os fenótipos e teores de clorofila, açúcar solúvel e proteína solúvel de diplóides e autotetraplóides de feijão-caupi foram comparados e o sequenciamento do transcriptoma foi realizado. As folhas autotetraploides do feijão-caupi apresentaram-se mais espessas e de cor verde mais escura que as folhas diplóides, e os teores de clorofila, açúcar solúvel e proteína solúvel nas folhas foram maiores. Um total de 2678 genes diferencialmente expressos (DEG) foram analisados no diplóide e autotetraploide do feijão-caupi. Entre eles, havia 421 genes com maior expressão de tetraploide do que diplóides, e 2257 genes com menor expressão de tetraploide do que diplóides. Todos os 2678 DEGs foram anotados na biblioteca funcional GO. Os DEGs foram concentrados principalmente no metabolismo e na composição celular. A análise da via KEGG mostrou que o diplóides e o autotetraplóide do feijão-caupi apresentam diferenças significativas na biossíntese de flavonoides, degradação de outros glicanos, biossíntese de fenilpropano, metabolismo da sacarose do amido, biossíntese de queratina, âmbar e cera, ritmo circadiano e vias vegetais.
Palavras-chave: *Vigna unguiculata*, poliploidia, fenótipo e características fisiológicas, sequenciamento do transcriptoma, gene diferencialmente expresso (DEG).

INTRODUCTION

As one of the most important grain legumes, cowpea (*Vigna unguiculata* (*Linn.*) *Walp.*) is an annual twine vine or nearly erect herb in the leguminous family and rich in nutritional value. Some scholars believe that the global cultivation area of cowpea will reach approximately 14 million hectares by 2025 (ALLUQMANI et al., 2023). In recent years, due to the change of environmental conditions and continuous cropping obstacles and other human factors, diseases and insect pests in the production process of cowpea increased, so that the yield and quality of cowpea is difficult to meet the needs of vegetable production (JIN et al., 2023). The heterosis of tetraploid cowpea was more stable than that of diploid cowpea, and the morphological characteristics of tetraploid cowpea showed great significance and the yield was significantly increased. However, there were few reports on tetraploid cowpea, so whether polyploidy would affect the physiological characteristics of cowpea is still unclear. Polyploidy in higher plants includes homopolyploidy and allopolyploidy (LI et al., 2018). In plant breeding studies, allopolyploidy has uncertainty and introgge hybridization compared with homopolyploidy, so homopolyploidy is more conducive to the study of chromosome doubling and its variation mechanism (SHEN et al., 2017; CORNEILLIE et al., 2019). At present, autologous polyploid has been reported in

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potato, safflower alfalfa, beet, watermelon, etc. (ZHU et al., 2018; MANZOOR et al., 2019).

Polyploidy enriches plant genotypes and phenotypes (WANG et al., 2016), and polyploid resynthesis is a significant feature of gene evolution in many plants, such as cassava (CHEN et al., 2021), sea-thorn, rice (WANG et al., 2021), etc. Polyploids usually exhibit new phenotypes, such as increased drought resistance, enlarged cells and organs, and improved yield and quality, etc. (SOLTIS et al., 2015). The emergence of new phenotypes may cause polyploids to enter new ecological niches or be used in production practice. More and more breeders in order to improve crop yield and quality of one of the effective means is to screen for tetraploid. CHEN et al. (2020) induced new tetraploids of asparagus, which increased heat resistance. HASSAN et al. (2020) used colchicine to induce tetraploid pointy melon, which is helpful for the production of seedless or less seeded fruits. LI et al. (2019) induced tetraploid *Actinidia sinensis* with increased yield.

The application of autopolyploid greatly shortened the breeding period and played an important role in the protection of germplasm resources (LIU et al., 2017). In order to understand the physiological, biochemical and molecular mechanisms of tetraploid cowpea, the phenotype and physiological indexes of diploid cowpea and autotraploid cowpea were compared and analyzed. Transcriptome sequencing has been widely used in the field of ploidy breeding. For example, transcriptome analysis on the leaves of *Solidago canadensis* polyploid and corresponding diploid has revealed that a large number of single genes are differentially expressed in the biosynthesis of secondary metabolites, carbohydrate metabolism, lipid metabolism and environmental adaptation pathways (XU et al., 2019). By combining leaf phenotype data of triploid poplar with transcriptional data of the 5th, 10th and 25th leaves of triploid and diploid poplar trees, we found that PPNGRF5-1 was strongly correlated with leaf development and net photosynthetic rate (Pn)(WU et al., 2021). In this study, high throughput sequencing technology (RNA-seq) (RYU et al., 2021) was used for transcriptome sequencing of the two, and the differentially expressed genes (DEGs) of the two were explored, which is of great significance for the production and polyploid breeding of tetraploid cowpea in the future.

MATERIALS AND METHODS

Plant materials

The diploid and autotetraploid tissue culture seedlings of cowpea (*Vigna unguiculata (Linn.) Walp.*, HN-56) were obtained from the laboratory of vegetable science, College of agriculture, Jiangxi Agricultural University. The temperature in the tissue culture room was maintained at 25 ºC, and the photoperiod was 14h light/10h dark. The diploid and tetraploid cowpea tissue culture plantlets which had been subcultured for 60 days were transferred to Murashige and Skoog (MS) medium for rooting. The physiological indexes and transcriptome sequencing were determined 20 days after rooting.

Five diploid and five tetraploid tissue culture seedlings were randomly selected. Fresh leaves were taken to determine the chlorophyll content, soluble sugar content, soluble protein content and repeated three times. Then six plantlets were obtained through the random selection of three individual plants each for sampling. After sampling, they were quickly ground into powder in liquid nitrogen and stored in refrigerator at -80 ºC for determination of transcriptome sequencing.

Formation, identification and chlorophyll content

Cotyledon nodes of diploid cowpea were induced in vitro and identified by flow cytometry (CyFlow® Ploidy Analyser,partec company, Germany), determination of physiological indexes.

Chlorophyll content was determined according to the method of hiscox (HISCOX & ISRAELSTAM, 1979). The content of soluble sugar was determined by Kjeldahl method of spring (ZHU et al., 2017). The content of soluble protein was determined by anthrone colorimetry of Qiang (SMITH et al., 1985).

Transcriptome sequencing

RNA was extracted from cowpea leaves by RNA Kit (Takara). The quality of the RNA was tested. RNA samples were quantified and evaluated for purity using a NanoDrop 2000 spectrophotometer. Those meeting the predefined criteria with absorbance ratios in the range of 1.80 to 2.1 for 260/280 were selected for further analytical steps. After passing the quality inspection, the cDNA library was constructed and sequenced. The transcriptome was sequenced by bgiseq-500 platform of Wuhan Huada gene Co., Ltd. In order to ensure the reliability of the results, the original data from sequencing contains the reads with low quality, joint contamination and high content of unknown base N, which need to be removed before data analysis. Using hisat, the filtered clean reads were aligned to the reference genome (asm411807v1) sequence (KIM et al., 2015), and the alignment results were obtained.

Gene Ontology (GO) classification and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of differential genes

The predicted new transcript with coding potential was added to the reference gene sequence to obtain a complete reference sequence information. Based on this reference sequence, gene expression level and gene function annotation were analyzed and calculated (SANSEVERINO et al., 2010; BUCHFINK et al., 2015; ZHOU et al., 2016). DEGs between different samples were detected (WANG et al., 2010), and differences were analyzed for all expressed genes. The DEGs were annotated by GO database and KEGG database, and then the annotated DEGs were further analyzed by GO, KEGG cluster analysis and functional enrichment analysis.

RESULTS

DNA content of diploid and autotetraploid cowpea

The results of DNA content analysis by flow cytometry showed that the relative DNA content of diploid cowpea was around 50, and that of Autotetraploid cowpea was around 100 (Figure 1).

Comparison of phenotypic and physiological indexes between diploid and autotetraploid cowpea

The stem diameter, leaf length and width, leaf thickness, pod diameter, pod length and fresh weight of tetraploid cowpea were significantly different from those of diploid cowpea. At the initial stage of induction, some leaves were deformed, grew slowly and had deep leaf color. After a period of culture, they gradually returned to normal. Compared with diploid, the stem diameter increased by 28.70%, leaf length and width increased by 7.03% and 21.98%, leaf thickness increased by 30.30%, pod transverse diameter, pod length and fresh weight increased by 21.96%, 15.21% and 36.90%, respectively (Table 1). The contents of chlorophyll, soluble sugar and soluble protein in leaves of tetraploid cowpea were significantly different from those of diploid cowpea ($P < 0.05$). The content of chlorophyll a, chlorophyll b, total chlorophyll, soluble sugar and soluble protein increased by 34.86%, 36.38%, 35.18%, 28.09% and 33.33% respectively (Table 2).

Sequencing results of diploid and autotetraploid of cowpea

Transcriptome sequencing showed that the maximum value of total clean reads was 46.38 m, the minimum value was 42.59 m, and the average value was 43.64 m (Table 3). The average output of each sample is 6.55 GB. The Q20 and q30 of clean reads in the sample are more than 97.18% and 89.5% respectively, which is highly reliable and can be used for all subsequent analysis.

Differential expression of diploid and autotetraploid genes in cowpea

The results showed that there were 2678 genes differentially expressed between diploid and tetraploid cowpea (fold change ≥ 2 and adjusted P value ≤ 0.001). The red and blue dots respectively symbolize up-regulated and down-regulated genes in tetraploids, with diploids serving as the control.

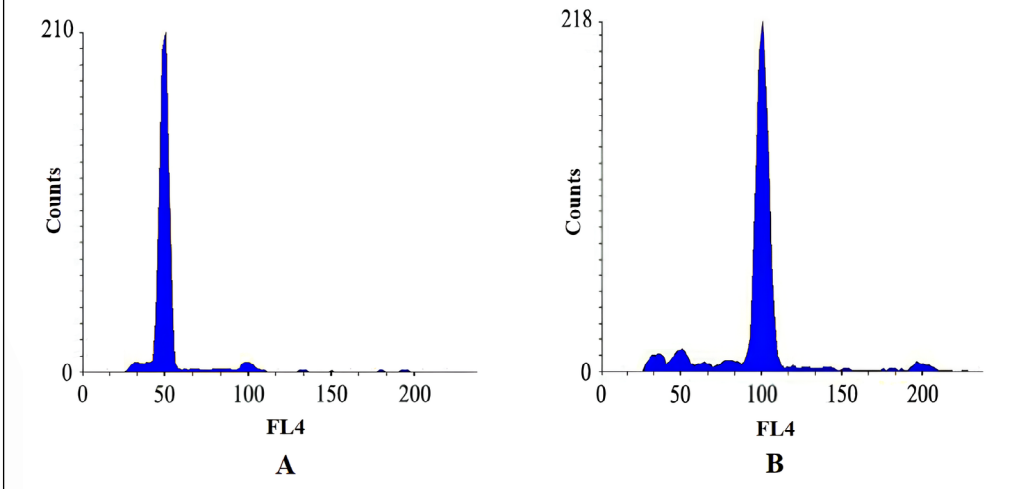

Figure 1 - The relative DNA content curve of cowpea leaves of diploid (A) and tetraploid (B).

Table 1 - Measurement results of main phenotypes of diploid and tetraploid cowpea.

Group	Thick stem/mm	----------Leaf----------		Thickness of leaf/mm	----------Pod----------		
		Length/cm	Width/cm		Diameter/mm	Pod length/cm	Fresh weight/g
Diploids	3.45±0.32b	9.53±0.21b	6.05±0.44b	0.33±0.01b	7.65±0.34b	43.40±1.40b	19.54±1.02b
Tetraploids	4.44±0.19a	10.2±0.28a	7.38±0.06a	0.43±0.02a	9.33±0.36a	50.00±1.70a	26.75±1.15a

Note: the statistical data are presented as the mean ± standard deviation. Differences were compared by Duncan's test with a significance level of $P < 0.05$.

Among them, 421 genes in tetraploid cowpea were higher than those in diploid cowpea, and the expression multiples ranged from 1.0 to 9.7, and 2257 genes in tetraploid cowpea were lower than those in diploid cowpea, the expression multiple ranged from 1.0 to 10.3 (Figure 2).

GO function analysis of DEGs

In order to further analyze the biological functions of tetraploid and diploid differential genes, according to go annotation results and official classification, 2236 differential genes annotated into gene ontology database were divided into 33 categories from molecular function, cellular component and biological process, there are 13 biological processes, 11 cellular components and 9 molecular functions (Table 4). 635 DEGs were involved in cellular process, followed by 571 in metabolic process; among the cell components, 882 DEGs were involved in membrane, followed by 825 in membrane part and 780 in cell; in the classification of molecular function, 1186 genes are involved in catalytic activity, 1095 genes are involved in binding.

KEGG analysis of DEGs

As shown in table 5, there are 2196 DEGs in cowpea diploid and autotetraploid annotated into five specific KEGG pathways, which are cell process accounting for 3.05%, environmental information processing accounting for 10.02%, genetic information processing accounting for 14.98%, metabolism accounting for 65.39%, and organic system accounting for 6.56% (Environmental adaptation). Among them, the number of DEGs annotated was the most, accounting for 65.39% of all annotated DEGs. A total of 11 subclasses were annotated, including amino acid metabolism, biosynthesis of other secondary metabolites, carbohydrate metabolism, energy metabolism, global pathway, carbohydrate biosynthesis and metabolism, lipid metabolism, cofactor and vitamin metabolism, other amino acids metabolism, terpenoids and polyketides metabolism, nucleotide metabolism.

Analysis of doubling related pathways in cowpea diploid plants

According to the annotation results of DEGs in KEGG, 6 pathways were significantly enriched in KEGG pathway (Q value < 0.05), 33 DEGs were related to flavonoid biosynthesis pathway, and 33 DEGs were related to other glycan degradation pathway. There are 112 DEGs related to phenylpropanoid biosynthesis pathway, which is the most gene enriched pathway, 89 DEGs related to starch and sucrose metabolism

Table 2 - Determination results of chlorophyll, soluble sugar and soluble protein of cowpea diploid and tetraploid.

Group	Chlorophyll a content/mg·g^{-1}	Chlorophyll b content/mg·g^{-1}	Chlorophyll content/mg·g^{-1}	Soluble sugar content/mg·g^{-1}	Soluble protein content/mg·g^{-1}
Diploids	1.959±0.003b	0.624±0.003b	2.584±0.001b	0.89±0.02b	0.78±0.01b
Tetraploids	2.642±0.145a	0.851±0.048a	3.493±0.192a	1.14±0.03a	1.04±0.01a

Notes: the statistical data are presented as the mean ± standard deviation. Differences were compared by Duncan's test with a significance level of $P < 0.05$.

Table 3 - Data filtering quality results.

Sample	Raw Reads (M)	Clean Reads (M)	Clean Bases (Gb)	Clean Reads Q20 (%)	Clean Reads Q30 (%)	Clean Reads Ratio (%)
Diploid 1	45.57	43.35	6.5	97.3	89.72	95.11
Diploid 2	45.57	43.15	6.47	97.28	89.65	94.68
Diploid 3	45.57	42.59	6.39	97.38	89.97	93.45
Tetraploid 1	45.57	43.12	6.47	97.31	89.75	94.61
Tetraploid 2	45.57	43.26	6.49	97.43	90.12	94.92
Tetraploid 3	49.08	46.38	6.96	97.18	89.5	94.5

Note: Q20: The ratio of bases with sequencing quality value greater than 20; Q30: The ratio of bases with sequencing quality value greater than 30.

pathway, and 20 DEGs related to cutin, amber and wax biosynthesis pathway, 39 DEGs were related to circadian rhythm plant pathway (Table 6). In addition, 97 DEGs were associated with MAPK signaling pathway plant pathway, but not significantly enriched. These results indicated that diploid and tetraploid cowpeas were different in biosynthesis, metabolism and signal transduction.

DISCUSSION

Analysis of phenotypic and physiological indexes of diploid and tetraploid

The cells and organs of tetraploid are usually larger than those of diploid, and the appearance of polyploid plants is huge (LIU & SUN, 2017). The results showed that the leaves of tetraploid cowpea

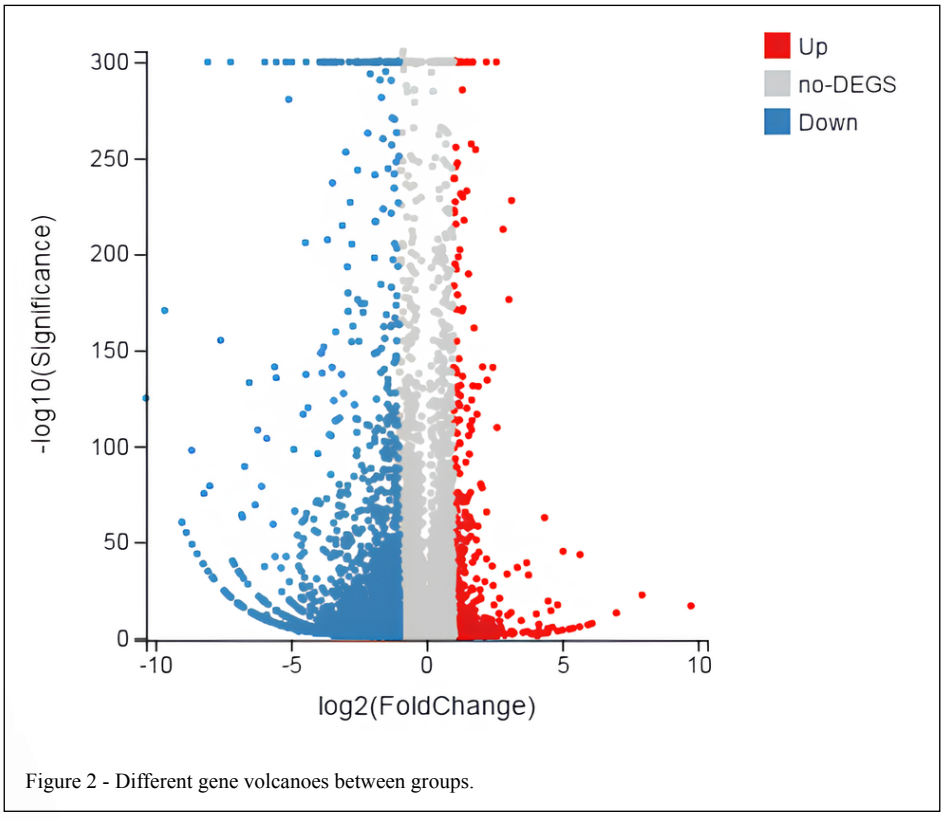

Figure 2 - Different gene volcanoes between groups.

Table 4 - Gene Ontology (GO) function distribution of differentially expressed genes (DEGs).

Classify 1	----------Classify 2----------	All gene number	--DEG number--	-----Ratio/%-----
Biological process	Cellular process	84978	635	23.72
	Metabolic process	52825	571	21.32
	Biological regulation	43322	252	9.41
	Response to stimulus	9552	239	8.92
	Cellular component organization or Biogenesis	12916	143	5.34
	Localization	18920	139	5.19
	Signaling	695	65	2.43
	Developmental process	1464	57	2.13
	Multicellular organismal process	3346	46	1.72
	Multi-organism process	1483	42	1.57
	Reproduction	347	38	1.42
	Reproductive process	531	38	1.42
	Immune system process	57	8	0.30
Cellular component	Membrane	7750	882	32.94
	Membrane part	20116	825	30.81
	Cell	46828	780	29.13
	Organelle	12844	526	19.64
	Organelle part	21332	157	5.86
	Extracellular region	556	131	4.89
	Protein-containing complex	5376	82	3.06
	Cell junction	316	26	0.97
	Symplast	316	26	0.97
	Supramolecular complex	340	22	0.82
	Membrane-enclosed lumen	484	20	0.75
Molecular function	Catalytic activity	57516	1186	44.29
	Binding	98052	1095	40.89
	Transporter activity	10249	151	5.64
	Transcription regulator activity	1491	117	4.37
	Molecular function regulator	1713	53	1.98
	Antioxidant activity	296	29	1.08
	Molecular transducer activity	641	29	1.08
	Structural molecule activity	909	17	0.63
	Nutrient reservoir activity	35	9	0.34

were bigger, deeper and thicker, and the contents of chlorophyll, soluble sugar and soluble protein were significantly higher than those of diploid cowpea. It shows that the cowpea plants with higher ploidy not only show huge appearance, but also have significant changes in physiological indexes, which is consistent with the research results of GAO ZHEN et al. (2020). The significant difference of physiological indexes between tetraploid and diploid may be the main reason for appearance difference. The deeper green of tetraploid leaves than diploid leaves may be due to higher chlorophyll content, stronger photosynthesis and thicker tetraploid leaves. The higher soluble sugar content and soluble protein can provide more energy materials and faster growth and metabolism of tetraploid leaves.

Differential gene analysis between diploid and tetraploid

Plant chromosome doubling can cause phenotypic and physiological variation, which is mainly caused by the change of gene expression after gene doubling. With the development of high-throughput technology (MANSOURI et al.,

Table 5 - Distribution of Kyoto Encyclopedia of Genes and Genomes (KEGG) functional annotation of differentially expressed genes (DEG).

Classify 1	----------Classify 2----------	All gene number	DEG number	--Ratio/%--
Cellular Processes	Transport and catabolism	801	67	2.50
Environmental Information Processing	Signal transduction	1529	187	6.99
	Membrane transport	245	33	1.23
Genetic Information Processing	Folding, sorting and degradation	1308	106	3.96
	Translation	1829	106	3.96
	Transcription	884	90	3.36
	Replication and repair	833	27	1.01
Metabolism	Global and overview maps	1182	540	20.16
	Carbohydrate metabolism	2724	235	8.78
	Biosynthesis of other secondary metabolites	1096	175	6.53
	Lipid metabolism	1204	138	5.15
	Amino acid metabolism	1260	82	3.06
	Metabolism of other amino acids	536	77	2.88
	Metabolism of terpenoids and polyketides	468	61	2.28
	Glycan biosynthesis and metabolism	504	46	1.72
	Metabolism of cofactors and vitamins	440	34	1.27
	Energy metabolism	629	29	1.08
	Nucleotide metabolism	287	19	0.71
Organismal Systems	Environmental adaptation	1026	144	5.38

2019), transcriptome sequencing provides abundant sequence resources for gene expression and functional analysis (VAATTOVAARA et al., 2019). The transcriptome and DEGs of polyploid have been studied in watermelon, *Zizyphus jujuba* and cabbage (ZHU et al., 2017; LI et al., 2019; BRAYNEN et al., 2021). In this study, RNA-seq technique was used to analyze 6 samples of diploid and autotetraploid cowpea. Gene differential expression was the molecular basis of variation of tetraploid cowpea. Further analysis of the differential expression genes between diploid and tetraploid cowpea showed that there were 2678 differential expression genes (DEG), among which 421 were higher expressed in tetraploid cowpea than in diploid cowpea. There are 2259 genes with lower expression in tetraploid than diploid, which confirms the previous research results that the number of up-regulated genes in tetraploid is lower than that in diploid (BRAYNEN et al., 2017). Through gene ontology annotation analysis, it was found that differential genes were mainly enriched in metabolism and cell composition, and metabolism and cell composition mainly affected plant growth and metabolism, which was consistent with the results of morphological and physiological differences between diploid and tetraploid cowpea. It confirmed the results of previous studies on populus tetraploid that differential genes were mainly enriched in cell process and metabolic process (ZHANG et al., 2020). KEGG pathway analysis showed that the DEGs were significantly enriched in six pathways: flavonoid biosynthesis, degradation of other glycans, phenylpropane biosynthesis, starch sucrose metabolism, cutin, amber and wax biosynthesis, circadian rhythm plant pathway. In this study, 112 and 33 DEGs were enriched in flavonoid metabolism and phenylpropane metabolism pathway. The expression of these differential genes is closely related to the enhancement of tetraploid resistance in cowpea. The expression of these genes could provide more energy and promote the growth and development of tetraploid. 89 and 39 differential genes were enriched in starch sucrose metabolism and circadian rhythm pathway. These differential genes were closely related to plant growth and photosynthesis, which was the main reason why tetraploid cowpea showed great diversity. This verified that there were differences in morphology,

Table 6 - The top 6 pathways were significantly enriched with differentially expressed genes (DEGs).

ID	----------Pathway name----------	----------Number----------	----------P value----------
ko00940	Phenylpropanoid biosynthesis	112	0.0195
ko00500	Starch and sucrose metabolism	89	0.0286
ko04712	Circadian rhythm-plant	39	0.049
ko00941	Flavonoid biosynthesis	33	0.0142
ko00511	Other glycan degradation	33	0.0195
ko00073	Cutin, suberine and wax biosynthesis	20	0.0377

physiology and photosynthesis between diploid and tetraploid cowpea. This is similar to previous studies on Chinese Cabbage that reported that differential genes were significantly enriched in biosynthesis pathway of secondary metabolites such as phenylpropanoid synthesis, and also enriched in circadian rhythm (KIM et al., 2019). The 20 DEGs were enriched in the biosynthesis pathway of cutin, amber and wax, which was closely related to the thickness of leaf and wax layer, making the leaves of tetraploid cowpea thicker. These different gene enrichment pathways may be responsible for the enhanced photosynthesis, better growth and development, dark green and thick leaves, and changes in resistance of tetraploid cowpea compared with diploid cowpea.

CONCLUSION

In this study, the morphological and physiological characteristics of diploid and autotetraploid cowpea were analyzed and compared. Combined with transcriptome sequencing technology, it was found that there were differences in phenotype and physiology between diploid cowpea and autotetraploid cowpea. This experiment laid a foundation for key gene screening and breeding.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

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