Analysis of Differential Metabolites in Selenium-enriched Rice Based on Extensive Non-targeted Metabolome

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Abstract [**Objectives**] Selenium (Se)-enriched rice is the main type of Se-enriched agricultural product developed in China, and this study aimed to understand the impact of selenium application on the metabolites in rice. [**Methods**] Se-enriched rice was prepared by foliar application of a sodium selenite aqueous solution, and high-throughput analysis of differential metabolites in Se-enriched rice was conducted based on extensive non-targeted metabolome. [**Results**] There were significant differences in metabolites between Se-enriched rice and ordinary rice, and a total of 535 differential metabolites were identified. Among them, 420 metabolites in Se-enriched rice were upregulated, accounting for 78.5%, far higher than downregulated metabolites. The enrichment differences of three KEGG metabolic pathways, including cysteine and methionine metabolism, zeatin biosynthesis, and arachidonic acid metabolism, reached a significant level, indicating that selenium enrichment had a significant regulatory effect on the metabolism of sulfur-containing amino acids, the synthesis of natural cytokinin zeatin, and arachidonic acid bioactive components in rice. [**Conclusions**] The results can provide a theoretical basis for the production of Se-enriched rice.

Key words Rice; Selenium; Non-targeted metabolome; Differential metabolites

Selenium (Se) has a variety of physiological functions such as anti-aging, anti-cancer and detoxification[1-2]. It has been identified by the World Health Organization and the Chinese Medical Association as the third largest micronutrient health care element that must be supplemented in the 21st century after iodine and zinc. However, the intake of Se is generally insufficient among global residents[3]. Eating Se-enriched foods is an ideal way to increase people's Se intake. Since entering the new century, Se-enriched rice, poultry eggs, vegetables and other agricultural products have begun to enter the mass consumption level in China, and Se-enriched agricultural products are increasingly favored by consumers. Rice is the main food crop in China, and for this reason, various parts of the country are actively developing the Se-enriched rice industry.

There are two main ways to produce Se-enriched agricultural products, one of which is to increase the Se content in agricultural products through soil addition or foliar spraying of Se fertilizers, and the other is to plant crops in natural Se-enriched soil and produce natural Se-enriched agricultural products through natural absorption. The use of Se-enriched soil to produce Se-enriched agricultural products requires availabie Se-enriched soil resources, which limits the widespread development of Se-enriched agriculture. However, foliar spraying of Se fertilizers can break through regional limitations and is an important agronomic measure for producing Se-enriched agricultural products in many Se-deficient areas. Spraying Se-enriched foliar fertilizers has various physiological effects on rice, including increasing yield, improving milled rice ratio, amylose content, protein content and gel consistency, reducing chalkiness and the contents of elements such as cadmium, mercury, lead, chromium, and magnesium[4-9]. However, the impact on metabolic products in rice has not been reported yet. In this study, a Se-enriched foliar fertilizer was prepared with sodium selenite as the Se source, and sprayed to rice during heading to culture Se-enriched rice, and the differences in metabolites in rice were analyzed based on extensive non-targeted metabolome, providing a theoretical reference for the development of Se-enriched functional rice.

Materials and Methods

Experimental materials

Se-enriched rice and ordinary rice: Rice variety Gannuo 8 was adopted, and the rice seeds were provided by Rice Research Institute of Jiangxi Academy of Agricultural Sciences. It was planted as medium rice in Gao'an base of Jiangxi Academy of Agricultural Sciences. Analytically-pure sodium selenite was used to prepare a Se-enriched foliar fertilizer, which was sprayed on the leaves at the heading stage. The Se content of the foliar fertilizer was 2 mg/L, and 1 500 L was sprayed evenly every hectare. After yellow ripe, rice was harvested and milled into Se-enriched rice. Common rice raw materials were prepared by conventional cultivation without spraying foliar fertilizer as the control (CK). The rice was crushed and sieved through an 80-mesh sieve as analytical test samples. Before planting, three soil samples were taken from the field for testing the total Se content in the soil according to NY/T 1104-2006 *Determination of selenium in soils*, and the detection result was 0.35 mg/kg.

Main instruments

Hydride generation-atomic fluorescence spectrometer (AFS-230E, Beijing Kechuang Haiguang Company); ultra-high

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performance liquid chromatography (UPLC Acquire I-Class PLUS, Waters); high-resolution mass spectrometer (UPLC Xevo G2-XS QTof, Waters); chromatographic column (Acquire UPLC HSS T3 1.8 μm 2.1×100 mm, Waters).

Determination of Se in rice

The Se content in rice was determined by the first method, hydride generation-atomic fluorescence spectrometry according to GB 5009.93-2017 "*National food safety standard: Determination of selenium in foods*".

Metabolite extraction

First, a 50 mg of sample was weighed and added with 1 000 μl of extraction solution containing internal standard (1 000∶2) (volume ratio of methanol∶acetonitrile∶water=2∶2∶1, internal standard concentration 2 mg/L) to obtain a mixture, which was vortex-mixed for 30 s. Next, ceramic beads were added, and the mixture was treated with a 45 Hz grinder for 10 min, and then ultrasonically treated for 10 min (in an ice water bath). After standing at −20 ℃ for 1 h, the sample was centrifuged at 4 ℃ and 12 000 rpm for 15 min, and 500 μl of supernatant was carefully transferred to an EP tube and dried in a vacuum concentrator. Next, 160 μl of extraction solution (volume ratio of acetonitrile∶water=1∶1) was added to re-dissolve the dried metabolites, and the obtained solution was vortex-mixed for 30 s, and ultrasonically treated in an ice water bath for 10 min. The sample was then centrifuged at 4 ℃ and 12 000 rpm for 15 min. Finally, 120 μl of supernatant was carefully transferred to a 2 ml injection vial, and 10 μl of each sample was prepared into a QC sample to be detected on the instrument.

Detection on instruments

The liquid chromatography-mass spectrometry system used for metabolomics analysis was composed of Waters Acquisition I-Class PLUS ultra-high performance liquid chromatograph in series with Waters Xevo G2-XS QTof high-resolution mass spectrometer.

Liquid phase conditions: The chromatographic column was Waters Acquisition UPLC HSS T3 (1.8 μm 2.1×100 mm). Mobile phase A was ultra-pure aqueous solution (added with 0.1% formic acid), and mobile phase B was acetonitrile solution (added with 0.1% formic acid). Elution gradient: The proportion of phase B was 2% at 0.00 min, and it was linearly increased to 98% within 10.00 min and remained at 98% for 3.00 min, and then decreased to 2% within 13.00−13.10 min and equilibrated at 2% until 15 min. The HPLC separation was performed with a column temperature at 40 ℃, an injection volume of 1 μl, and a flow rate at 0.40 ml/min.

Mass spectrometry conditions: Primary and secondary mass spectrometry data were collected in MSe mode under the control of Waters Xevo G2-XS QTof high-resolution mass spectrometer acquisition software (MassLynx V4.2, Waters). In each data collection cycle, dual-channel data collection was conducted simultaneously for low collision energy and high collision energy under following conditions: a low collision energy of 2 V, a high collision energy range of 10−40 V, and a scanning frequency of 0.2 s per mass spectrum. The ESI ion source parameters were as follows: capillary voltage 2 000 V (positive ion mode) or −1 500 V (negative ion mode), cone voltage 30 V, ion source temperature 150 ℃, desolvation gas temperature 500 ℃, blowback gas flow rate: 50 L/h, and desolvation gas flow rate: 800 L/h.

Data processing

The original data collected by MassLynx V4.2 was used for peak extraction, peak alignment and other data processing operations through Progenisis QI software. Identification was carried out based on the online METLIN database of Progenisis QI software and the self-built database of Biomarker Technologies Co., Ltd., and the theoretical fragment identification was carried out simultaneously. The mass number deviation was within 100 ppm. Principal component analysis (PCA) and orthogonal partial least-squares discrimination analysis (OPLS-DA) were performed on the identified metabolites using R software (https://www.r-project.org/). Differential metabolites were screened according to variable importance in project (VIP) scores obtained from the OPLS-DA model with VIP>1 and $P<0.05$ as standards. Meanwhile, corresponding differential metabolites obtained were analyzed through the KEGG metabolic pathway database (www.genome.jp/kegg).

Results and Analysis

Analysis of Se content in rice

The Se-enriched rice and ordinary rice had a Se content of 103.6 and 35.2 μg/kg. The former was 2.94 times higher than the latter, with a significant difference. The Se content of Se-enriched rice met the requirement of 40−300 μg/kg in the national standard GB/T 22499-2008 *Rich selenium paddy*.

Multivariable statistics

According to principal component analysis (PCA) of the samples, the contribution rate of the first principal component (PC1) was 77.22%, and the contribution rate of the second principal component (PC2) was 13.18%. The two groups of samples showed a clear separation trend. The cumulative contribution rate of the principal components reached 90.40% (Fig. 1), so the PCA results could reflect the metabolic differences between Se-enriched rice and ordinary rice overall, indicating that the established PCA model was stable and could be used for subsequent analysis of metabolic product differences.

In order to more accurately display the metabolic differences between Se-enriched rice and ordinary rice, a supervised partial least square with discriminant analysis (OPLS-DA) model was used for further analysis. The samples of the Se-enriched rice group were concentrated on the positive axis of PC1, while the samples of ordinary rice group were concentrated on the negative axis of PC1, indicating that the model could effectively distinguish the rice samples of the two treatments. The model evaluation parameters R2Y=0.999 and Q2Y=0.971 were both close to 1 (Fig. 2), indicating that the model was stable and reliable. The permutation test of the OPLS-DA model showed that Q2 of the

right initial OPLS-DA model was far greater than Q2 of the left random model (Fig. 3), indicating that the OPLS-DA model was free of overfitting and had good reliability. Differential metabolites could be screened according to the variable projection importance (VIP) analysis.

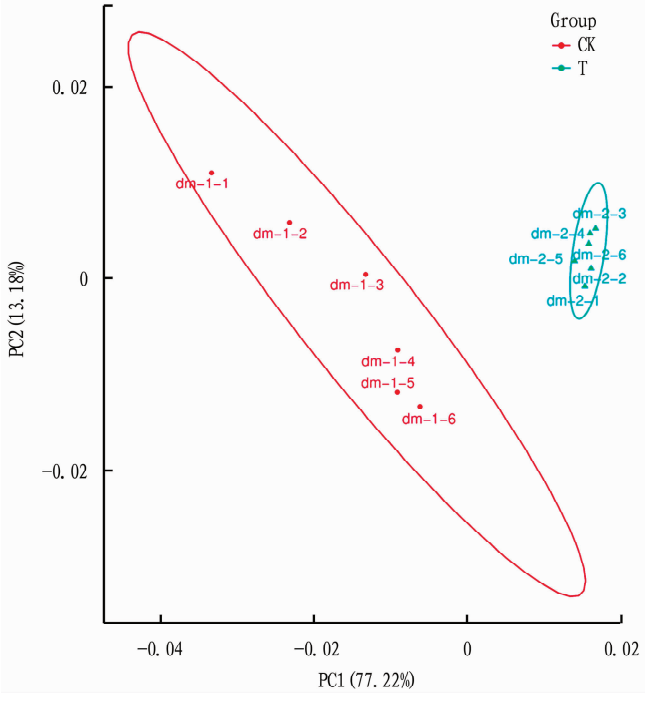

Fig. 1 Principal component analysis of metabolites of Se-enriched rice (T) and ordinary rice (CK)

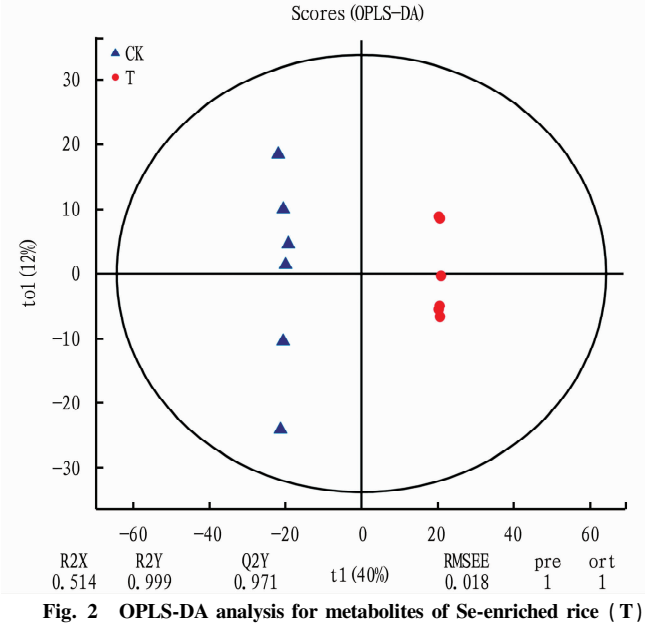

Fig. 2 OPLS-DA analysis for metabolites of Se-enriched rice (T) and ordinary rice (CK)

Identification and analysis of differential metabolites

In Se-enriched rice and ordinary rice, 1 441 metabolites were identified using metabolomics methods. Based on the OPLS-DA results, using $VIP > 1$ and $P < 0.05$ as the screening criteria for

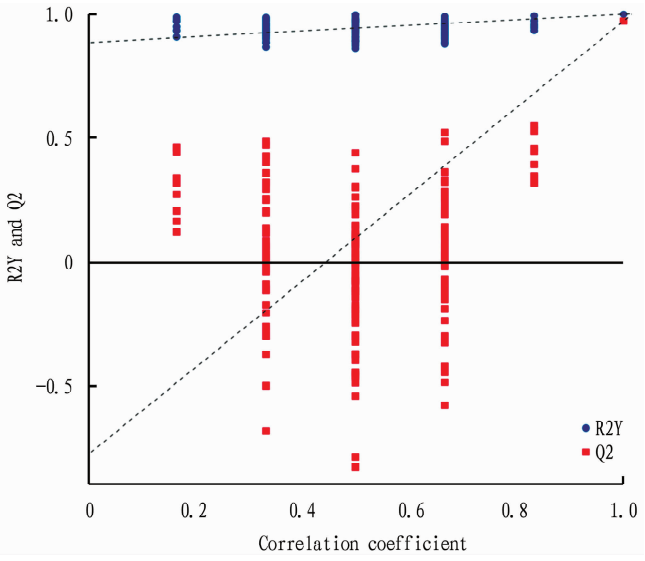

Fig. 3 Permutation test of OPLS-DA model

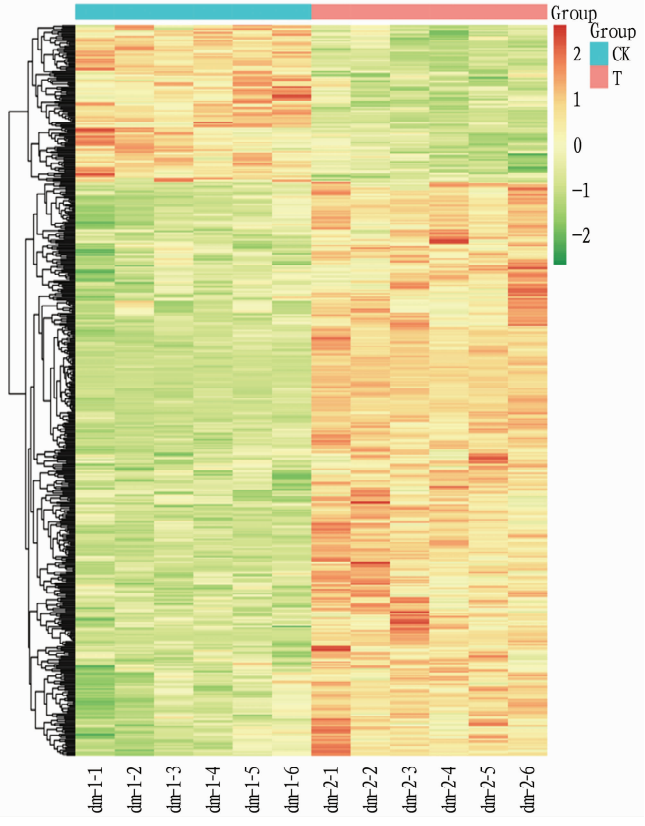

Fig. 4 Heat map of differential metabolites between Se-enriched rice (T) and ordinary rice (CK)

differential metabolites, a total of 535 differential metabolites were detected (Fig. 4), accounting for 37.1% of all detected metabolites, indicating that Se enrichment had a significant impact on the generation of rice metabolites. Among the 535 differential metabolic components, 420 components of Se-enriched rice were upregulated, with a significantly higher relative content than that of ordinary rice, accounting for 78.5%; and 115 components were downregulated, with a relative content significantly lower than that

of ordinary rice, accounting for 21.5%. The number of upregulated metabolites was much greater than that of downregulated metabolites (Fig. 5).

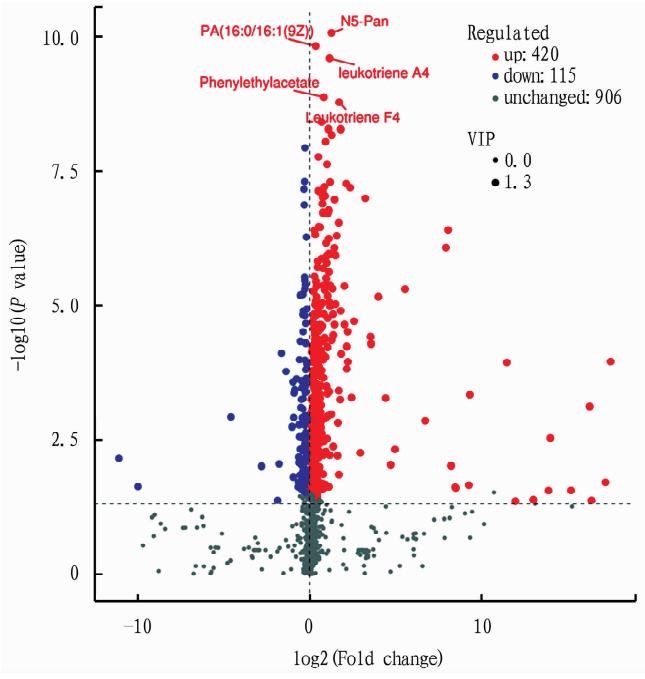

Fig. 5 Volcano diagram of differential metabolites between Se-enriched rice (T) and ordinary rice (CK)

According to the Human Metabolome Database (HMDB) classification, fatty acyls, carboxylic acids and their derivatives, glycerophospholipids, organooxygen compounds, and prenol lipids were the most diverse metabolites, accounting for 8.60%, 7.66%, 7.48%, 4.11%, and 3.93%, respectively (Fig. 6). Among the clearly classified differential metabolites, 42 fatty acyls, 29 carboxylic acids and their derivatives, and 22 organooxygen compounds had a higher relative content in Se-enriched rice, and 38 glycerophospholipids, 13 carboxylic acids and their derivatives, and 4 fatty acyls had a higher relative content in ordinary rice.

Analysis of main differential metabolites

Fold change (FC) was added as an important evaluation index, and 21 differential metabolites were obtained through screening metabolites with larger fold changes using log2FC > 5 or log2FC < −5, VIP > 1, $P < 0.05$ as the standards. Among them, 2 were significantly downregulated and 19 were significantly upregulated in Se-enriched rice (Table 2).

KEGG pathway enrichment analysis

Differential metabolites were subjected to pathway enrichment analysis through the KEGG database, and a total of 80 pathways were identified. Among the 535 identified differential metabolites, by KEGG were annotated 169 differential metabolites, of which 136 were significantly upregulated and 33 were significantly downregulated, and they were mainly distributed in 20 metabolic pathways (Fig. 7). Among them, there were 3 pathways that were significantly enriched ($P < 0.05$), namely: ① cysteine and methionine metabolism, annotated with 10 differential metabolites such as S-adenosylmethionine, methionine sulfoxide, S-adenosylhomocysteine, O-acetyl-L-homoserine, 5-methylthioribose, S-adenosylmethionine amine, 5'-methylthioadenosine, o-acetylserine, and homoserine, ② zeatin biosynthesis, annotated with 4 differential metabolites including 5'-methylthioadenosine, o-acetylserine, S-adenosylmethionine, and adenine, and ③ arachidonic acid metabolism, annotated with 14 different metabolites, including phosphatidylcholine, eicosatetraenoic acid, leukotriene, prostaglandins, and thromboxane B2.

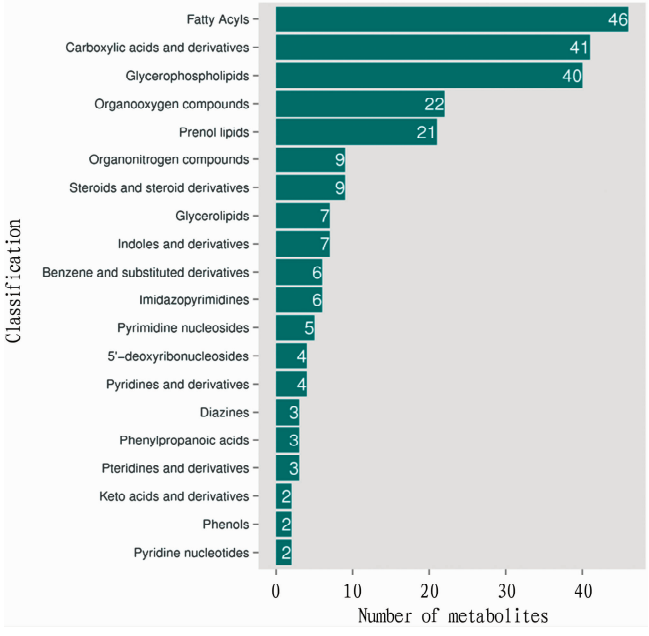

Fig. 6 HMDB classification of differential metabolites

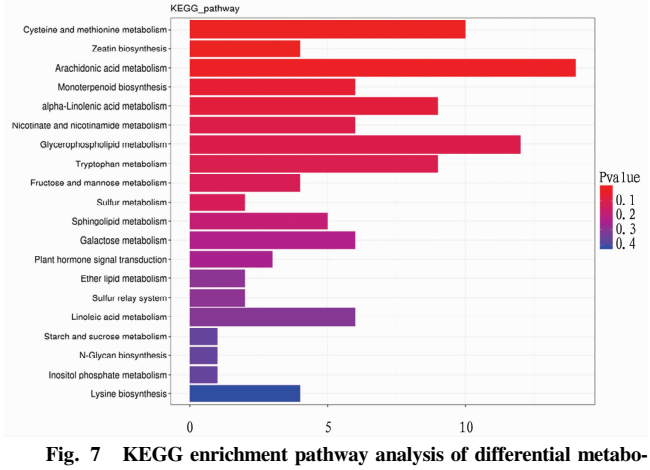

Fig. 7 KEGG enrichment pathway analysis of differential metabolites

Discussion and Conclusions

Se-enriched rice is currently the main type of Se-enriched agricultural product developed in China[10]. Metabolomics, as an emerging high-throughput identification technology, has unique advantages in the qualitative and quantitative analysis of small-molecule metabolic components. At present, there have been reports on the application of metabolomics to study the effects of Se on animal and plant metabolites. Se has a significant impact on

Table 2 Main differential metabolites with log2FC >5 or log2FC < −5, and *VIP* >1, *P* <0.05

No.	Metabolite	Class	*VIP*	*P*	log2FC
1	L-Dihydroorotic acid	Carboxylic acids and their derivatives	1.186 061 513	0.007 076 767	−11.089 344 490
2	L-Ala-gamma-D-Glu-Dap	Carboxylic acids and their derivatives	1.060 585 166	0.023 432 104	−9.977 429 695
3	Phosphatidyl ethanolamine (16:0/16:1); PE(12:0/16:1(9Z))	Glycerophospholipids	1.565 193 999	4.979 55E-06	5.548 673 828
4	Maltoheptaose	Organooxygen compounds	1.376 622 509	0.001 416 473	6.724 605 538
5	17 alpha-Hydroxyprogesterone	Phenylpropionic acid	1.575 770 011	8.437 67E-07	7.927 473 447
6	Geldanamycin	Macrolactam	1.577 814 170	3.920 59E-07	8.059 071 479
7	S-Adenosylmethionine	5'-Deoxyribonucleoside	1.191 883 882	0.009 658 305	8.228 957 471
8	L-Ala-gamma-D-Glu-DAP-D-Ala	Carboxylic acids and their derivatives	1.033 000 420	0.024 177 738	8.469 455 533
9	Octyl 2-acetamido-2-deoxy-alpha-D-glucopyranoside	Organooxygen compounds	1.118 052 359	0.024 908 723	8.505 085 725
10	Serinyl-Histidine	Carboxylic acids and their derivatives	1.041 603 671	0.021 978 026	9.264 826 422
11	Astilbin	Flavonoid	1.367 749 770	0.000 455 780	9.319 958 317
12	Guanine	Imidazopyrimidines	1.462 582 936	0.000 115 174	11.471 766 040
13	Fructoselysine-6-phosphate	Organooxygen compounds	1.021 939 679	0.044 685 287	11.966 834 360
14	Virginiamycin S1	Pyrrolidine	1.041 048 659	0.041 760 789	13.013 179 810
15	Threoninyl-proline	Carboxylic acids and their derivatives	1.012 938 438	0.028 024 675	13.895 270 580
16	L-Ala-D-Glu-meso-A2pm	Carboxylic acids and their derivatives	1.3117 962 87	0.002 897 953	14.000 910 560
17	Glutathionylspermidine	Carboxylic acids and their derivatives	1.103 242 901	0.027 820 023	15.204 626 400
18	Xanthine	Imidazopyrimidines	1.460 847 775	0.000 759 400	16.284 948 380
19	2-Amino-6-(hydroxymethyl)-7,8-dihydropteridin-4-ol	Pyridine and its derivatives	1.037 262 841	0.043 229 260	16.396 656 540
20	4-Hydroxyphenylacetaldehyde	Benzene and substituted derivatives	1.164 004 157	0.019 495 513	17.208 253 070
21	Cortisol 21-acetate	Steroids and steroid derivatives	1.470 070 180	0.000 110 577	17.506 285 330

metabolites of animals and plants such as *Ginkgo biloba*[11], *Brassica oleracea* L. var. *italica* Plenck[12], *Amaranthus caudatus* L.[13], cow[14], *Procapra przewalskii*[15], Rex rabbits[16], and pigs[17]. However, there have been no relevant reports on the effect of Se on the metabolic components of rice.

The results of this study showed that the number of upregulated expression products was much higher than that of downregulated expression products in Se-enriched rice in relative to regular rice. Fatty acyls, carboxylic acids and their derivatives, and organooxygen compounds were the main types of upregulated metabolites, and among further screened main differentially-expressed metabolites with a large fold change, the number of upregulated expression products was also much higher than that of downregulated expression products, so the Se application process mainly promoted the biosynthesis of metabolites in rice. The KEGG metabolic pathway analysis found that the enrichment differences of 3 pathways, including cysteine and methionine metabolism, zeatin biosynthesis, and arachidonic acid metabolism, reached a significant level. The metabolites associated with cysteine and methionine metabolism mainly involve the metabolism of sulfur-containing amino acids such as methionine and cysteine, and selenomethionine and selenocysteine are the main forms of Se present in rice[18]. Therefore, in the biosynthesis process of seleno-amino acids, it is possible to regulate related products in the synthesis pathway of sulfur-containing amino acids. Zeatin is a natural cytokinin in plants, which increases the content of zeatin in rice roots and grains, promotes grain filling, and improves grain plumpness[19], so it may be an important factor in Se application to increase rice yield. Arachidonic acid is a polyunsaturated fatty acid and a direct precursor of a variety of bioactive substances. The differential metabolites of leukotrienes, prostaglandins and thromboxane B2 in the metabolic pathway of arachidonic acid in this study are all functional components with good physiological regulation activity to the human body, and they were all up-regulated in Se-enriched rice, which might be one of the factors that Se-enriched rice plays a physiological role in anti-aging, anti-cancer and detoxification.

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treatment time and ultrasonic treatment temperature were selected to conduct single-factor experiments. Through data and line chart, it was determined that the best parameters were ammonium sulfate mass fraction 20% , ethanol mass fraction 25% , liquid-to-material ratio 50, ultrasonic extraction time 35 min, and ultrasonic extraction temperature 47 ℃.

On the basis of single-factor experiments, the surface response method was used to further optimize the extraction conditions, and the effects of ethanol concentration, liquid-to-material ratio and ultrasonic treatment time on the extraction efficiency of sweet potato leaf polysaccharides were studied. The experimental data results showed that the optimal parameters were ethanol concentration 25.68% , liquid-to-material ratio 55.83, and ultrasonic treatment time 38.33 min. Under these conditions, the yield of sweet potato leaf polysaccharides could reach 20.646 mg/g.

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