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Metabolomics and transcriptomics reveal the effect of hetero-chitooligosaccharides in promoting growth of Brassica napus

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Article

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Abstract

The hetero-chitooligosaccharide (HTCOS) are a naturally occurring biopolymer in the exoskeleton of crustaceans and insects. Although some studies have been carried out on HTCOS in inducing plant resistance and promoting growth, the molecular regulated of HTCOS in plant is not clear. In this study, an integrated analysis of metabolomics and transcriptomics was performed to analyze the response of *Brassica napus* to hetero-chitooligosaccharides treatment. The levels of 26 metabolites in *B. napus* were significantly changed under the HTCOS treatment. Amongst these metabolites, 9 metabolites were significantly up-regulated, such as pentonic acid, indole-3-acetate, and γ-aminobutyricacid. Transcriptome data showed that there were 817 significantly up-regulated genes and 1064 significantly down-regulated genes in *B. napus* under heterotypic chitosan treatment. Interestingly, the content of indole-3-acetate (IAA) under the HTCOS treatment was about five times higher than that under the control condition. Moreover, four genes related to plant hormone signal transduction, three AUX/IAA genes, and one ARF gene, were significantly up-regulated under the HTCOS treatment. Furthermore, the plant height, branching number, and biomass of *B. napus* under the HTCOS treatment were significantly increased compared to that in the control condition. This evidence indicated that the HTCOS treatment contributed to accumulating the content of plant hormone IAA in the *B. napus*, up-regulating the expression of key genes in the signaling pathway of plant growth, and improving the agronomic traits of *B. napus*.

Introduction

Hetero-chitooligosaccharides (HTCOS), chitooligosaccharides (COS), N-acetyl-chitooligosaccharides (NACOS), and other oligosaccharides derived from chitin, are obtained by enzymatic preparation of chitin, a naturally occurring biopolymer in the exoskeleton of crustaceans and insects. These oligosaccharides are all composed of N-acetylglucosamine and its deacetylation product, glucosamine, with a degree of polymerization < 20 and an average molecular weight < 3.9 kDa, differing only in the ratio of the glucosamine and GlcNAc (Degree of deacetylation)[1, 2].

Chitin-derivedoligosaccharides, such as COS and NACOS have good plant immune-inducing activity. A variety of bio-pesticide products containing COS as the major ingredient has been registered in China and widely used in agricultural production[3]. However, it is believed that the acetyl group on the monosaccharide is essential for the binding of the oligosaccharide to the receptor, and higher polymerization levels result in greater activity of Chitin-derived oligosaccharides. The COS had a degree of deacetylation at around 50 ~ 70% indicating strong plant immune-modulating activity[4].

HTCOS have become the latest research direction for chitin-derived oligosaccharides as they have a sufficient number of acetyl groups to participate in the binding of receptors on the plant surface, thus activating the plant immune system more effectively, and their positive charge can also play an active role as COS, while still having good water solubility at a high degree of polymerization (DP > 8). In addition, the degree of deacetylation of HTCOS is closer to the range of deacetylation of fungal cell wall

chitin/chitosan in nature, thus better mimicking such natural plant excitons, a feature that may also be the structural basis for their excellent plant immune-inducing activity.

Marine oligosaccharides are obtained by degrading polysaccharides derived from marine organisms. COS and HTCOS are marine oligosaccharides, which are low molecular weight products with good water solubility, easy absorption, high bioactivity, and environmental protection without pollution[5–7]. The degree of polymerization of COS is mostly 2 ~ 20 molecular weight 340 ~ 3500 Da. Numerous studies have shown that COS can not only induce an increase in the expression level of endogenous plant hormones such as indoleacetic acid[8, 9], gibberellin, and salicylic acid in plants but also cause an increase in the activity of defense enzymes such as polyphenol oxidase and peroxidase in plants[10, 11]. COS is an effective immune exciton in plants[12], enabling the induction of resistance to cold, disease, and insects[13]. HTCOS is an oligosaccharide with 50–80% deacetylation, which is more difficult to produce than COS with a lower degree of deacetylation and has a very promising application in the field of plant protection.

Oilseed rape is one of China's important oil crops. About 50% of edible vegetable oil is from oilseed rape, in the domestic edible vegetable oil occupies an important position, cultivating oilseed rape seedlings is to seize the key foundation of high and stable yields. In rape cultivation, COS is widely used as a seed coating[14] to promote the growth of rape seedlings, and has been shown to induce resistance to *Sclerotinia sclerotiorum*, a major disease of rape[15], as well as to improve photosynthesis, frost and drought resistance in rape[16], and to alleviate high salt damage[17]. HTCOS also promotes the growth and development of oilseed rape, improves agronomic traits such as branch number and biomass, and induces resistance to the small rape moth. This paper analyses the response of kale-type oilseed rape to HTCOS treatment through a combination of transcriptomic and metabolomic analyses to find and investigate changes in key genes in the growth signal transduction pathway in oilseed rape.

Results

Metabolic changes in Brassica napus under the HTCOS treatment

To assess the response of *B. napus* to the HTCOS treatment, we analyzed the difference of metabolites in *B. napus* leaves. The orthogonal partial least squares discriminant analysis showed significant differences in metabolite content between HTCOS treatment and control (Fig. 1A). The level of 26 metabolites (9.89% of total metabolites) was significantly changed (p-value < 0.05) in response to HTCOS treatment, including nine up-regulated metabolites and seventeen down-regulated ones (Fig. 1B and Supplementary Table 1). Among the 26 different metabolites, the difference in metabolite content between the treatment group and the control group was 1.87 times on average, ranging from 0.25 to 21.12 (Supplementary Table 1).

Functional annotation based on the KEGG database indicated that a total of 18 changed metabolites were enriched in 15 KEGG pathways, which were related to phenylpropanoid biosynthesis, pyrimidine

metabolism, beta-alanine metabolism, butanoate metabolism, biosynthesis of phenylpropanoids, hormones metabolic pathway, and so on (Fig. 1C and 1D). Interestingly, the contents of pentonic acid, indole-3-acetate (IAA), and gamma-aminobutyricacid under the HTCOS treatment were 21.1, 5.0, and 4.3 times than that in the control condition, respectively. IAA is an important plant hormone that regulates many processes of plant growth and development and is closely related to the response of plants to adversity stress. γ-aminobutyric acid is a non-protein free amino acid commonly found in animals, plants and microorganisms, and plays an important role in plant growth, development and resistance response. It indicated that metabolites related to plant growth were enriched under the HTCOS treatment, promoting the growth and development of *B. napus*.

Effects of gene expression in Brassica napus under the HTCOS treatment

A total of 95,225,228 (~ 13.8 Gb) and 94,206,911 (~ 13.6 Gb) transcriptome reads on average were generated under the HT-COS treatment and control conditions respectively (Table 1), in which have three biological replicates for each condition. About 95% of reads could align to the reference genome of *B. napus*, including 62.5% of multiple mapped and 33.2% of uniquely mapped. Approximately 67.6% and 67.7% of total genes were aligned by the transcriptome data under the two conditions, respectively.

Table 1
Data statistics after transcriptome sequencing filtered for *B. napus* experimental treatments

Sample	raw reads	raw bases	clean reads	clean bases	valid bases	Q30	GC
CK72-7	98.00M	14.70G	94.44M	13.72G	93.31%	93.09%	42.62%
CK72-8	99.03M	14.85G	95.03M	13.83G	93.13%	92.80%	42.73%
CK72-9	99.61M	14.94G	96.21M	13.97G	93.47%	93.39%	42.85%
Z272-7	99.81M	14.97G	94.14M	13.58G	90.72%	91.88%	43.18%
Z272-8	98.47M	14.77G	93.59M	13.50G	91.42%	92.43%	43.17%
Z272-9	99.49M	14.92G	94.89M	13.72G	91.96%	92.71%	42.94%

Based on the differential expression gene (DEG) analysis of *B. napus* RNA-seq between HT-COS treatment and control, a total of 1881 DEGs were identified in this study, including 817 significantly up-regulated genes and 1064 significantly down-regulated genes (Fig. 2A). Based on the analysis of gene function, there were 671 genes enriched in 23 KEGG pathways, such as carbohydrate metabolism, amino acid metabolism, biosynthesis of other secondary metabolites and signal transduction, and so on (Fig. 2B). Compared to the down-regulated genes, the up-regulated genes were significantly enriched in the pathway of lipid metabolism, energy metabolism, and biosynthesis of other secondary metabolites under the HT-COS treatment, indicating that it may provide energy for the growth and development of *B. napus* (Fig. 2C).

Enhanced plant hormone synthesis of Brassica napus under the HT-COS treatment

Auxins are mainly composed of indole-3-acetate (IAA), which are one type of plant hormone that is involved in many developmental processes, including cell division, cell differentiation, phototropism, root gravitropism, apical dominance, and vascular differentiation[18]. As a plant growth hormone, IAA plays an important role in regulating plant growth and development[19].

In this study, the content of indole-3-acetate (IAA) under the HT-COS treatment was significantly higher about 5.0 times than that under the control condition (Supplementary Table 1). Based on the analysis of transcriptome data, three AUX/IAA genes (IAA9, IAA12, and IAA3) and one auxin response factor (ARF18) of *B. napus* were related to plant hormone signal transduction were significantly up-regulated under the HT-COS treatment (Fig. 3). Furthermore, the plant height, fresh weight, dry weight, and the number of leaves of *B. napus* under the HT-COS treatment were 25.57 ± 0.61 cm, 15.72 ± 0.66 g, 2.18 ± 0.14 g, and 14.2 ± 0.7 respectively, which were significantly higher than that in the control condition (Table 2). This evidence indicated that the HT-COS treatment contributed accumulated content of plant hormone IAA in the *B. napus*, promoted the up-regulation expression of key genes in the signaling pathway of plant growth, and accelerated the agronomic traits of plant growth, such as plant height, branching number and biomass.

Table 2
Effects of HT-COS treatment on *B. napus* growth

Treatments	Plant height /cm	Fresh weight /g	Dry weight /g	Number of leaves
CK[a]	18.40 ± 0.98	11.68 ± 0.65	1.64 ± 0.04	10.57 ± 0.75
DA treatment[b]	25.57 ± 0.61	15.72 ± 0.66	2.18 ± 0.14	14.23 ± 0.70
Sig.	0.000	0.002	0.003	0.003

[a, b] Data are mean ± standard error. The results of the independent samples t-test showed that the difference between samples was significant when Sig. <0.05.

Verification Of The Transcriptome Reliability Using Qrt-pcr

To confirm the differential expression of the DEGs under the HT-COS treatment condition, the two IAA genes (IAA3 and IAA9) were selected for qRT-PCR analysis. Expression of the selected DEGs as determined by qRT-PCR fitted well with the expression as determined by RNA-Seq analysis (Fig. 4). The primers of DEG were shown in Table 3. These results further demonstrated that under HT-COS treatment, the IAA genes expression were significantly up-regulated more than 2 times.

Table 3
List of primers used for the Real-time RT-PCR

unigene ID	Primer sequence (5'-3')	Annealing temperature / ℃	Fragment size /bp
NC_027771.2: IAA3	F: TGGGACTACCAGGAACAG	55	306
	R: CGACCACCCTCACTATCA		
NC_027767.2: IAA9	F: GGCCCTTCTTACCTTTGG	55	163
	R: TTCCGTGGCACATCCTTC		
ACT7	F: GCTGACCGTATGAGCAAAG	55	182
	R: AAGATGGATGGACCCGAC		

Discussion And Conclusion

Chitosan, which is a good biogenic pesticide, not only induce disease resistance in plants to improve the efficacy of some other pesticides, but also enhance abiotic stress tolerance[20–23]. In addition, it was found that the net photosynthetic rate, stomatal conductance and intercellular CO_2 concentration of oilseed rape leaves were significantly increased after chitosan spraying on seedling leaves, which was achieved through NO and ABA pathways[24]. In this study, we found that seed dressing with hetero-chitooligosaccharides (HTCOS) can promote the growth of rapeseed, with increasing about 35% in plant height, fresh weight, dry weight and number of leaves compared with that without HTCOS treatment, which were corresponding to previously research[20–21, 23]. Interestingly, the contents of indole-3-acetate (IAA) and gamma-aminobutyricacid, which played important roles in regulating many processes of plant growth and development and response of plants to adversity stress, were significant increase of 5.0, and 4.3 times under the HTCOS treatment, respectively. Furthermore, four genes (IAA9, IAA12, IAA3 and ARF18) related to plant hormone signal transduction in *B. napus* were significantly up-regulated under the HT-COS treatment. In addition, HT-COS has sufficient number of acetyl groups involved in the binding of plant surface receptors. Those results suggested that the HT-COS could induce the biosynthesis and hormone signal of plant hormone in rapeseed, promoting the recovery of sugar metabolism level in leaves to provide the carbon source required for plant growth and development. Our results will provide not only new insights for chitin-derived oligosaccharides in promoting plant growth, but also not only theoretical guidance for future field application.

Materials And Methods

Experimental species and reagents

Kale-type oilseed rape Hua You No. 9, produced by GuchengShengguang Seed Industry Co. Hetero-chitooligosaccharides (HTCOS) were provided by the Institute of Process Engineering, Chinese Academy

of Sciences.

Experimental methods:

Select full oilseed rape seeds and place them in a 9 cm Petri dish lined with filter paper, add 8 ml of sterile water and leave overnight at 4 ℃ to allow the seeds to absorb the water. The seeds are then placed in an artificial incubator at 26±2 ℃ for 24 h, selected for consistent germination potential, and planted in a 161-hole floating tray with seedling substrate and allowed to grow to 2 leaves and 1 heart. The seedlings are then transferred to a 10 cm diameter, 8.5 cm high seedling bowl with the seedling substrate, and 1 seedling of rape is planted in each pot. When growth reached 4 leaves and 1 heart, potted oilseed rape seedlings of uniform growth were selected and sprayed with 80 mg/L of HTCOS (recorded as DA treatment group), with an additional clear water treatment as a blank control. Each pot was sprayed with 10 mL, and 42 plants were sprayed at each concentration, divided into two groups, 12 of which were used for the two determinations, and the remaining 30 plants were used for phenotypic data statistics. Treated rape seedlings were placed in an artificial climate chamber at a temperature of 26±2 ℃, humidity RH 70±10%, and light L:D=14:10.

Sample collection

After 72 h of HTCOS spray once treatment. The third and fourth leaves of each plant, counted from bottom to top, were packed in 50 ml centrifuge tubes and frozen rapidly in liquid nitrogen, with one part sent to Shanghai Ouyi Biomedical Co Ltd for determination of transcriptome (3 biological replicates) and metabolome (6 biological replicates), and one part kept in an ultra-low temperature refrigerator at -80 ℃.

Phenotypic measure

After 21 d of HTCOS spray once treatment, the growth of rape in pots seedling height, number of leaves, wet weight of above-ground parts and dry weight of above-ground parts were investigated uniformly in each treatment group and control group. Both treatment and control groups had 10 plants as one replication and were replicated three times, counting 30 plants each.

Metabolite extraction

360 μL of cold methanol and 40 μL of 2-chloro-l-phenylalanine (0.3 mg/mL) dissolved in methanol as internal standard was added to each sample, samples were placed at -80 ℃ for 2 min. Then ground at 60 Hz for 2 min. The mixtures were ultrasonicated at ambient temperature for 30 min. 200 μL of chloroform was added to the samples, and the mixtures were vortexed, 400 μL water was added. Samples were vortexed again, then ultrasonicated at ambient temperature for 30 min. The samples were centrifuged at 12000 rpm for 10 min at 4 ℃ QC sample was prepared by mixing aliquots of all samples to be a pooled sample. And 80 μL of 15 mg/mL methoxylamine hydrochloride in pyridine was subsequently added. The resultant mixture was vortexed vigorously for 2 min and incubated at 37 ℃ for 90 min. 80 μL of BSTFA (with 1% TMCS) and 20 μL n-hexane were added into the mixture, which was vortexed vigorously for 2

min and then derivatized at 70 ℃ for 60 min. The samples were placed at ambient temperature for 30 min before GC-MS analysis.

The derivative samples were analyzed on an Agilent 7890B gas chromatography system coupled to an Agilent 5977A MSD system (Agilent Technologies Inc., CA, USA). A DB-5MS fused-silica capillary column (30 m ×0.25 mm ×0.25 μm, Agilent J & W Scientific, Folsom, CA, USA) was utilized to separate the derivatives. Helium (>99.999%) was used as the carrier gas at a constant flow rate of 1 mL/min through the column. The injector temperature was maintained at 260 ℃. The initial oven temperature was 60 ℃, ramped to 125 ℃ at a rate of 8 ℃/min, to 210 ℃ at a rate of 4 ℃/min, to 270 ℃ at a rate of 5 ℃/min, to 305 ℃ at a rate of 10 ℃/min, and finally held at 305 ℃ for 3 min. The temperature of MS quadrupole and ion source (electron impact) was set to 150, and 230 ℃, respectively. The collision energy was 70 eV. Mass data were acquired in a full-scan mode (50-500 m/z).

Metabolite analysis

ChemStation (version E.02.02.1431, Agilent, USA) software was used to convert the raw data to CDF format, and then the CDF data were imported into the Chroma TOF software (version 4.34, LECO, St Joseph, MI) for data processing. Metabolites were annotated through Fiehn or NIST database. After alignment with the Statistic Compare component, the ‘raw data array’ (.cvs) was obtained from raw data with three-dimension data sets including sample information, peak names (or retention time and m/z), and peak intensities. In the ‘data array’, all internal standards and any known pseudo positive peaks (caused by background noise, column bleed, or BSTFA derivatization procedure) were removed. The data were normalized to the total peak area of each sample, and multiplied by 10000, and the peaks from the same metabolite were combined.

Data were transformed by log2 (use 0.000001 to replace 0 before transforming), and the resulting data matrix was then imported into the SIMCA software package (v14.0). Principle component analysis (PCA) and (orthogonal) partial least-squares-discriminant analysis (OPLS-DA) was performed to visualize the metabolic difference among experimental groups, after mean centring and unit variance scaling. The Hotelling’s T2 region, shown as an ellipse in score plots of the models, defines the 95% confidence interval of the modeled variation. Variable importance in the projection (VIP) ranks the overall contribution of each variable to the OPLS-DA model, and those variables with VIP >1 are considered relevant for group discrimination.

The differential metabolites were selected based on the combination of a statistically significant threshold of variable influence on projection (VIP) values obtained from the OPLS-DA model and p-values from a two-tailed Student’s t-test on the normalized peak areas from different groups, where metabolites with VIP values larger than 1.0 and p-values less than 0.05 were considered as differential metabolites.

RNA extraction and establishment of cDNA library

The total RNA of *B. napus* leaf under the HTCOS conditions was extracted using an RNA extraction kit and checking the quality of extracted RNA using Nanodrop 2000 spectrophotometer. The integrity of total RNA was checked using formamide denaturing gel electrophoresis, and mRNA isolated from total RNA using Dynabeads Oligo (dT) 25 isolation beads. The RNA of the extracted sample was used for cDNA synthesis using a reverse transcription kit based on the manufacturer's instruction (NEBNext Ultra^{TM} RNA Library PrepKit for Illumina), and the establishment library of cDNA. The insert size of the cDNA library was checked by Agilent 2100 bioanalyzer. The cDNA library was sequenced on the Illumina sequencing platform using the paired-end (PE) technology within a single run, in which 150 bp PE reads were obtained.

Sequencing and differentially expressed genes (DEGs) analysis

The reference genome sequence of *Brassica napus* was downloaded from NCBI (GenBank: GCA_000686985.2). The raw transcriptome data of all samples in this trial were uploaded to the NCBI database (BioProject: PRJNA781006). The cDNA library of high quality was sequenced on the Illumina sequencing platform based on the second technology of sequencing. To obtain localization information of reads in reference genomic, compare clean reads with reference genomic using HISAT2-2.0.5[25], and the expression level was calculated using the FPKM method (fragments per kilobase million). The difference expressed genes (DEGs) were analyzed using the DEseq2 package version 3.8.6[26]. The genes with $\log_2$ Fold Change ³1, and false discovery rate (FDR) <0.05 were considered as differentially expressed genes (DEGs). The KEGG enrichment analysis of functional significance terms based on KEGG (http://www.kegg.jp/kegg/pathway/html) database was conducted using a hypergeometric test to find significant KEGG terms in DEGs for comparison with the genome background.

Validation of gene expression by qRT-PCR

To verify genes that were differentially expressed in RM-challenged samples compared with unchallenged ones, qRT-PCR was performed, using an iQ SYBR Green SuperMix kit (Bio-Rad) on an iCycleriQ system (Bio-Rad, Hercules, CA, USA). Gene-specific primers of 3 candidate genes (Table 3) were designed using the Primer Premier 5.0 software. The ramie gene encoding actin, which displays a stable expression under different stress condition[27], was used as an internal control for data normalization. For each sample, first-strand cDNA was synthesized from 1 μg from the pooled RNA sample of the CK or HTCOS plants, using a Revert Aid First-Strand cDNA Synthesis Kit (ThermoScientific, Fermentas, Vilnius, Lithuania), according to the manufacturer's instructions. All reactions were performed in triplicate with six replicates. Expression levels of each gene are presented as the fold change relative to that of the control gene, calculated with the method[28].

Declarations

Author contributions:

Zeng L-B, Zhang D-Y, and Zhao Y developed the idea for the study and guided it all the way. Tang C, Zhai Y designed the study and completed the research with Wang Z, Zhao X, and Yang C. All authors analyzed the data and were involved in writing the manuscript. Zeng L-B and Zhai Y finished revising the English manuscript. The author(s) read and approved the final manuscript.

Data Availability Statement

Supplementary Table 1.xls contains the levels of 26 metabolites in *B. napus* were significantly changed under the hetero-chitooligosaccharide treatment. The reference genome sequence of *B. napus* was downloaded from NCBI (GenBank: GCA_000686985.2). The raw transcriptome data of all samples in this trial were uploaded to the NCBI database (BioProject: PRJNA781006). In addition to that, the authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables.

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Figures

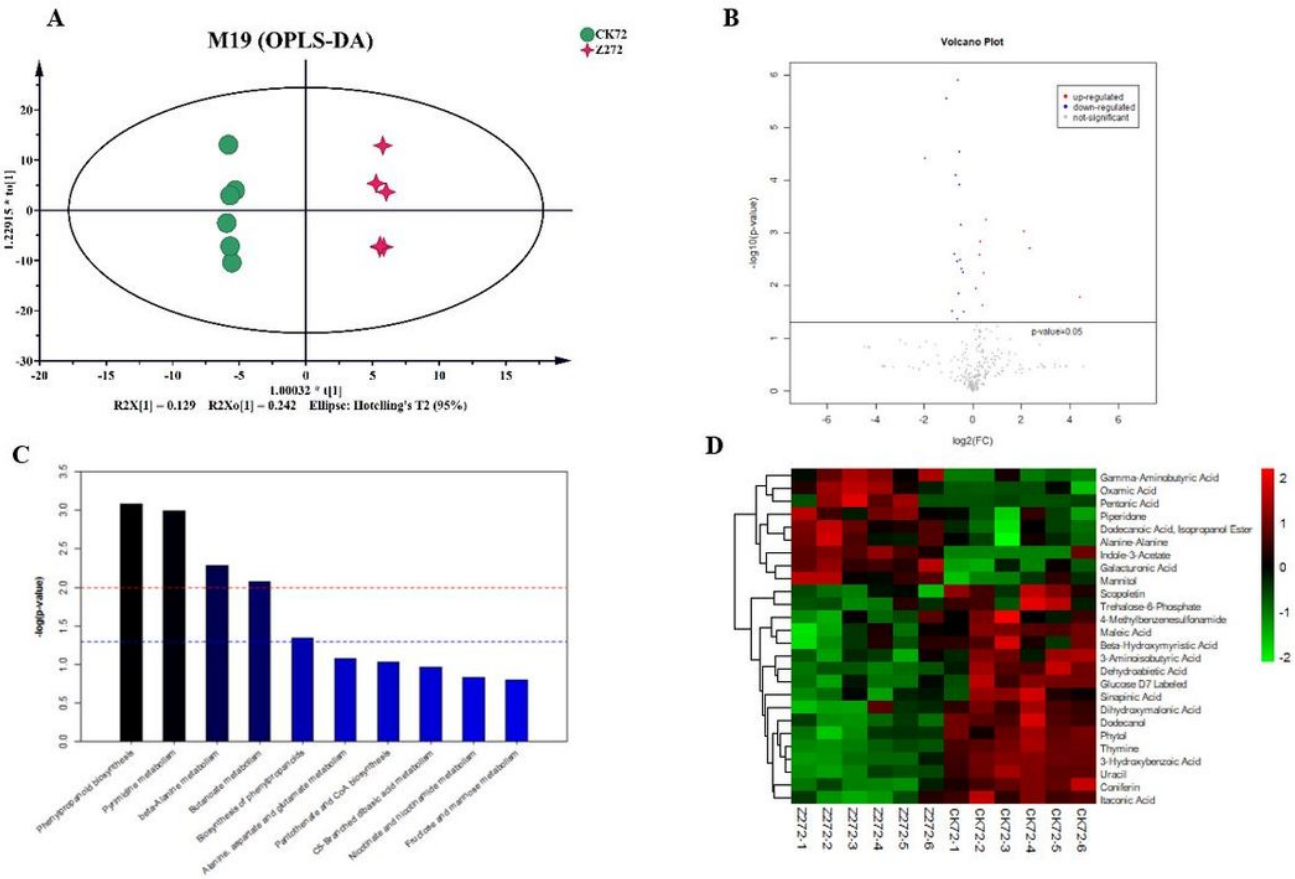

Figure 1

The changes of metabolites in *B. napus* between control (CK72) and HT-COS (Z272) treatments. A. The orthogonal partial least squares discriminant analysis in metabolite contents. B. The volcano plot of significantly differentiated metabolite contents. C. The gene functional enrichment of significantly up-regulated metabolites under the DA treatment. D. The heat map of metabolites changes.

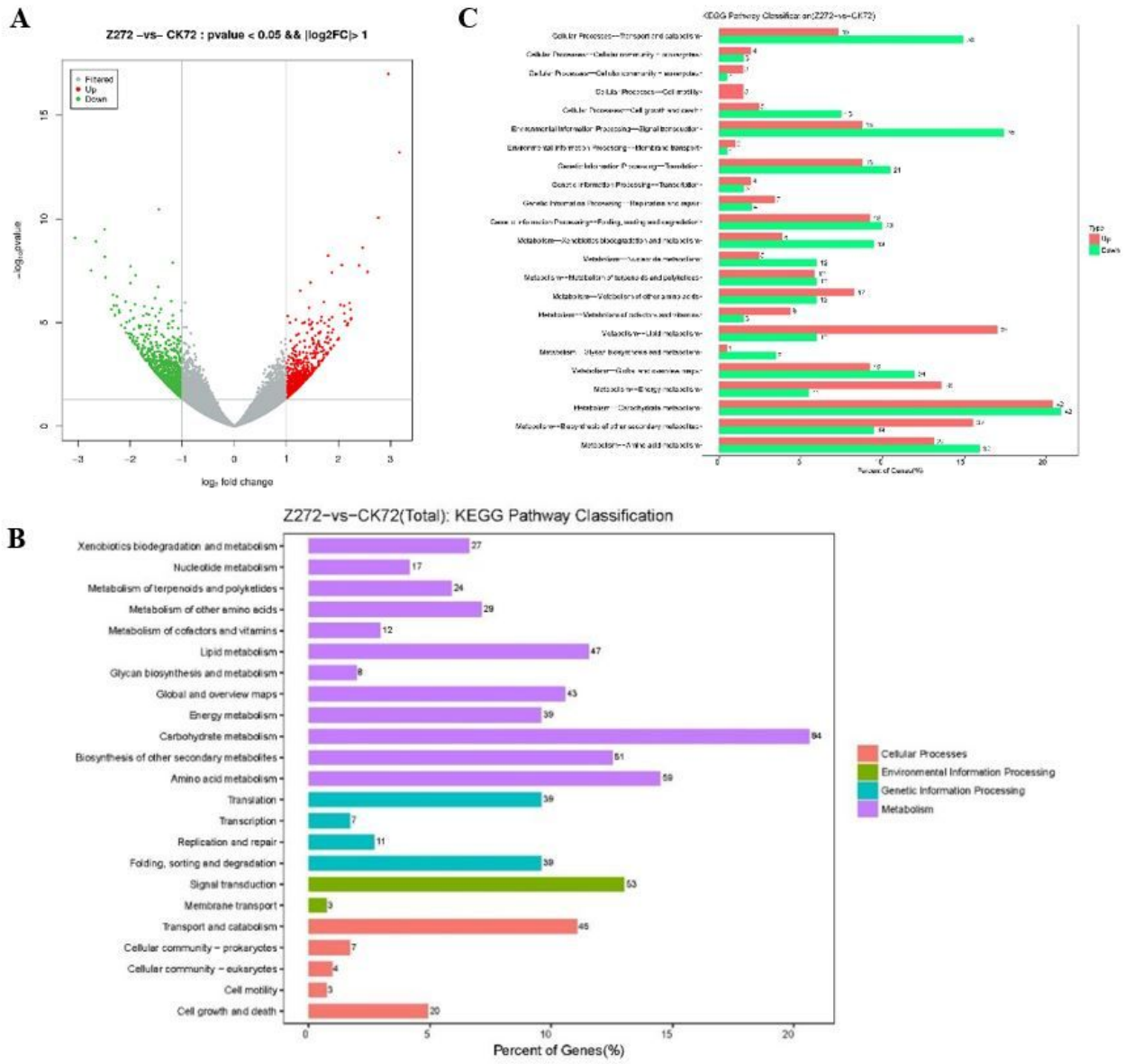

Figure 2

The gene expression of *B. napus* between control (CK72) and HT-COS (Z272) treatments. A. The volcano plot of differentiated expression genes. B. The KEGG pathway enrichment of DEGs. C. The KEGG pathway enrichment of significantly up-regulated genes.

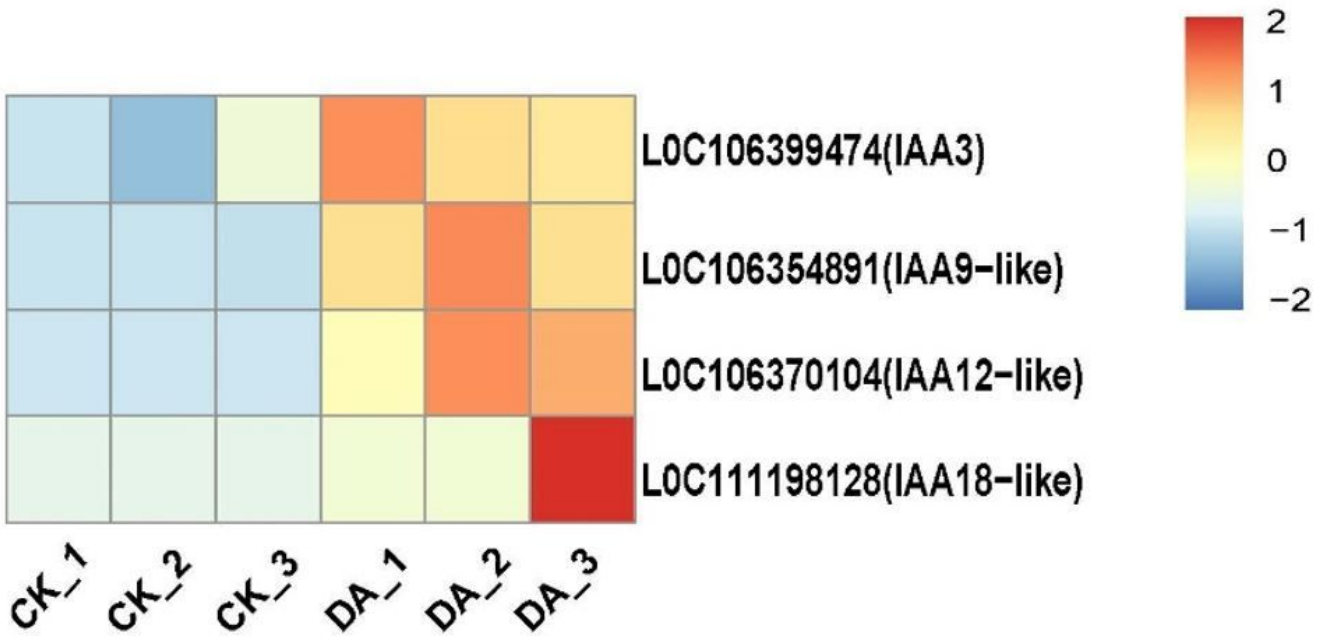

Figure 3

The heat map of plant hormone gene expression in *B. napus* between control (CK) and HT-COS (DA) treatments.

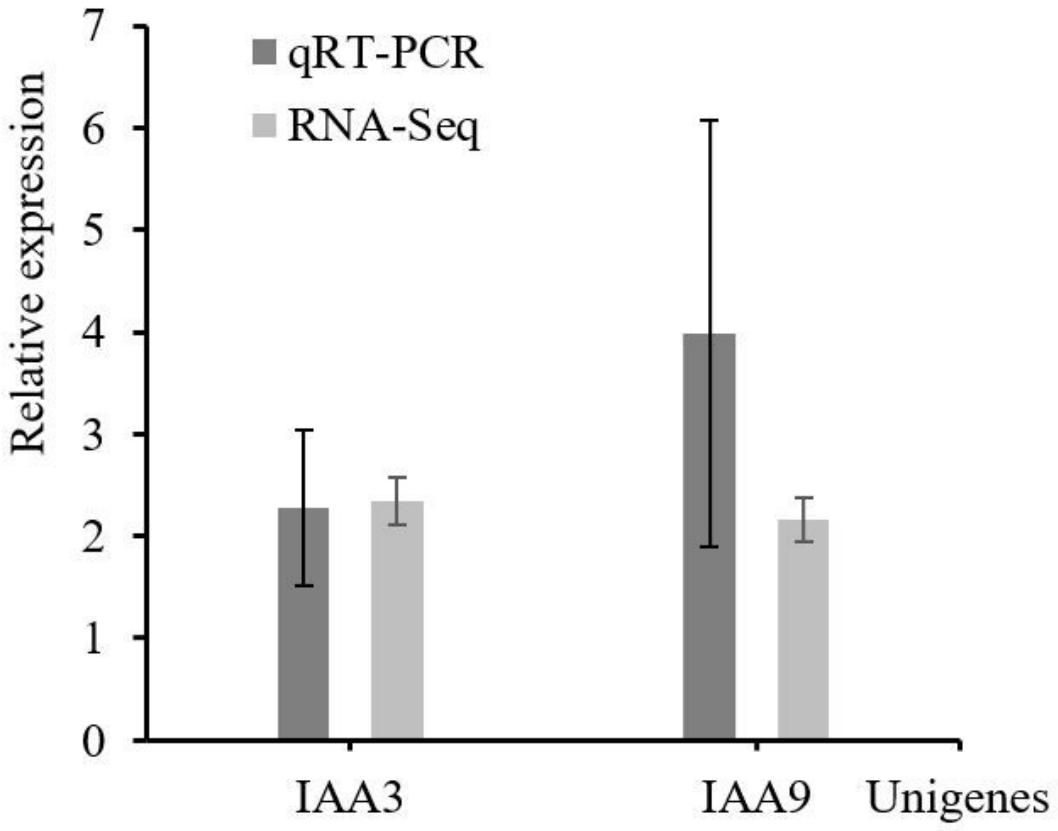

Figure 4

Expression of genes IAA3 and IAA9 relative to control (CK) at the transcriptome level in the HT-COS (DA) treated group and validation by qRT-PCR -ΔΔCt values.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaryfile.xls