Proteomic and Metabolomic Analyses of the Inflorescences Axis of Castor (*Ricinus communis* L.) at Different Developmental Stages

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Abstract

Background: Castor is a dicotyledon, *ricinus communis*, Euphorbiaceae, and is one

of world ten oil crops. Lm female line castor has three inflorescence types (female with marker, haplometrotic and monoecious). Female with marker castor was realized as a restorer line and as a maintainer line, which was applied to castor hybrid breeding. The developmental mechanism of the three inflorescences is not clear.

Results: Therefore, in this study, proteomic and metabolomic analyses of different inflorescences axis were performed. Seventy-three differentially abundant protein species (DAPS) were detected in the proteomics analysis and 86 differentially abundant metabolites (DAMs) were identified from the proteomic and metabolomic analyses of the inflorescences axis, respectively. These DAPS and DAMs are primarily mainly involved in carbon metabolism, comprising carbon fixation in the photosynthetic organism pathway, the glycolysis pathway, carbon metabolic pathways, and the biosynthesis of amino acids. Quantitative real-time PCR (qRT-PCR) results demonstrated that the proteomics and metabolomic data collected in this study were reliable.

Conclusions: The results of this study provides some valuable insights into the inflorescence development of castor. Specifically, the results showed that DAPS and DAMs are involved in photosynthesis and respiration to control the distribution of imported carbohydrates and exported photoassimilates and thus affect inflorescence development of castor. Furthermore, the dynamic balance of amino acids in different types of inflorescences was also shown to be essential in affecting inflorescence structure.

Background

Castor (*Ricinus communis*) is a renewable industrial oil crop that can be classified as annual or perennial depending on the climate and temperature [1]. Castor oil contains special fatty acids through cracking to form sebacic acid, which is a raw material for nylon, high-grade lubricants, cold-resistant plastics, and paints. [2]. Castor flowers are compound raceme, and most are monoecious [3].

Castor plants bear, separate male and female flowers on the same individual; a castor haplometrotic inflorescence bears only female flowers, and a castor monoecious inflorescence bears both female and male flowers [4–8]. The Lm female line castor, which is cultivated by the Tongliao Agricultural Science Institution, is used in the hybridization of species and strains as a male sterility line or maintainer line. It basically solves the technological problem of large area plantations of castors in China. The Lm female line castor includes three inflorescence types: female with marker, haplometrotic and monoecious. Huang Fenglan performed a microscopic comparison on terminal bud development of three Lm female line inflorescence types, finding that flower bud differentiation was in the 5 ~ 6-leaf stage and that willow-shaped functional leaves began to appear in the 7-leaf stage [9].

Proteomics technologies allow for systematic exploration of differences in the expression patterns of thousands of proteins [10]. Two-dimensional gel electrophoresis (2-DE) is one such method [11]. With the continuous improvement of 2-DE technology, many plant tissue sample preparation techniques have been developed in the past 30 years to enhance 2-DE proteomics analysis [12–14]. These methods are typically based on trichloroacetic acid (TCA) / acetone precipitation and phenol extraction. 2-DE is a quantitative method for precisely measuring multiple samples in one experiment. Previous studies have shown that 2-DE can effectively recognize protein changes during plant development. For example, in *Pinus nigra* Arn embryogenesis, more than 366 proteins in three developmental stages were identified and quantified by 2-DE, and 24 protein spots were altered between embryogenic and non-embryogenic tissues [15]. The development of the castor inflorescence is complex and ordered, though the proteins that show changes in expression during this process remain unknown. Therefore, we performed 2-DE analysis on castor inflorescences at different stages of development.

Metabolomics is a powerful tool for studying the metabolic dynamics of small molecules. With the development of mass spectrometry technology, metabolomics has been applied to the determination of the physiological and pathological factors of plants [16]. In general, metabolic conditions change as plants develop. In this review, we used metabolomics to explore the dynamics of the accumulation of metabolites in different castor inflorescences. However, comparative metabolomics methods have not been widely used in the study of castor inflorescence development. Therefore, the physiological and molecular mechanisms of the castor inflorescence are still unclear.

In this study, we conducted proteomics and metabolomic analyses of different castor inflorescences. The results revealed protein and metabolite changes during the development of castor inflorescences. This study lays the foundation for the determination of the mechanism of protein level in the development of castor inflorescence.

Results

Identification of differentially expressed proteins in different inflorescence types

Differential proteomics analysis in different inflorescence types in the same developmental period was performed. Seventy-three DAPS were identified in the different inflorescence types (Fig. 1, panel B, Supplemental Table S2), of which 16 DAPS were identified in the 4 euphylla stages, 14 DAPS were identified in the 5 euphylla stages, 25 DAPS were identified in the primary stem heading stage, and 17 DAPS were identified in the second-level branching stage.

Functional annotation of differentially expressed proteins

We carried out GO annotation on 73 DAPS and divided them into 22 functional groups (Fig. 3, Supplemental Table S3-1), including 8 GO terms for biological processes (the representative group is “cellular process”), 8 GO terms for cellular components (the representative group is “cell and cell part”) 6 GO terms for molecular functions (the representative group is “binding”).

73 DAPS were divided into 12 homologous groups of proteins clusters, of which the representative group was “energy production and conversion” (group G, 18 DAPS), followed by “posttranslational modification, protein turnover, chaperones” (group A, 13 DAPS) (Fig. 4, Supplemental Table S3-2).

KEGG pathway analysis showed that 41 DAPS were involved in 30 metabolic pathways (Fig. 5, Supplemental Table S3-3). These DAPS were significantly enriched by the following pathways: “carbon metabolism”, “carbon fixation in photosynthetic organisms”, “biosynthesis of amino acids”, “proteasome”, “glycolysis/ gluconeogenesis”, “cysteine and methionine metabolism” (Table 1).

Principal component analysis (PCA) and heat map construction

Analysis of total metabolite content by gas chromatography-mass spectrometry (GC-MS) was performed for samples collected at primary stem heading stage of castor inflorescences axis. PCA can reveal the internal structure of the data to better interpret data variables. PCA modeling could separate all samples into three distinct groups (Supplemental Fig. 1).

To gain more insights into the modulation of metabolites in inflorescences from the primary stem heading stage of castor, we visualized the metabolic data using a clustered heat map that charted the changes in metabolite concentrations between different inflorescences (Fig. 6 and Supplemental Table S4) The metabolites were divided into 3 categories. In Cluster I, differentially abundant metabolites were increased in the haplometrotic and monoecious inflorescences. In contrast, in Cluster II, differentially abundant metabolites were increased in female with marker inflorescences. In Cluster III, differentially abundant metabolites were increased in female with marker and haplometrotic inflorescences. These results suggest that metabolites affect the development of inflorescences through changes in concentration.

Identification of metabolites in different inflorescences

Eighty-six DAMs were identified in the three types of castor inflorescences axis at the primary stem heading stage, of which 69 DAMs were identified in ZB/ZD (ZD, primary stem heading stage haplometrotic inflorescences axis; ZB, primary stem heading stage female with marker inflorescences axis), 69 DAMs were found in ZL/ZB (ZL, primary stem heading stage monoecious inflorescences axis), and 35 DAMs were found in ZD/ZL (Supplemental Table S4). Eighty-six DAMs were classified into 7 categories of clusters of orthologous groups of metabolites, among which the most abundant was, “carbohydrates” (group k, 12 DAMs), followed by “amino acids” (group L, 14 DAMs) (Fig.7 and Supplemental Table S5-1)

Pathway mapping

The KEGG pathway analysis results showed that 33 DAPS mapped to 27 pathways (Fig. 8, Supplemental Table S5-2). To obtain a detailed and clear overview of the differences and/or similarities of the identified metabolites in the inflorescences, we generated a simplified schematic of the primary metabolic pathways (Fig.9). Fig. 6 shows the metabolic pathways leading to most primary metabolites involved in

the citrate cycle (TCA cycle, cis-aconitic acid, citric acid, L-malic acid, pyruvic acid, and fumaric acid); carbon fixation in photosynthetic organisms (L-malic acid and pyruvic acid); alanine, aspartate and glutamate metabolism; pyruvate metabolism and the valine, leucine and isoleucine biosynthetic pathways.

Validation of the differentially expressed proteins and metabolites

To assess the correlation between the abundance of DAPS or DAMs and the transcript levels of their corresponding genes, ten DAPS and ten DAMs were selected for qRT-PCR analyses. The results showed that the DAPS and DAMs could be clustered into three groups—including group I, up-regulated at both transcript and protein or metabolism level; group II, no change at transcript level but upregulated at protein or metabolic levels; group III, down-regulated at both transcript and protein or metabolic levels (Table 2). These differences in mRNA and protein expression profiles indicates that the abundances of proteins depends on the level of transcription, as well as mRNA stability, post-transcriptional regulation, post-translational modifications, and protein degradation.

Discussion

In agriculture, the shape of the inflorescence determines the yield [17, 18]. The development of inflorescence is a complex process with many interactions, including the establishment of meristems, and the differences in expansion and division modes [19]. The results of this study revealed protein and metabolomic changes during the development of castor inflorescences. Analysis of 2-DE and metabolomics data revealed that castor inflorescence DAMs and DAPS are primarily involved in carbon metabolism, amino acid metabolism, and protein biosynthesis. For example, DAPS are primarily involved in four carbon metabolic pathways (carbon metabolism, carbon fixation in photosynthetic organisms, the TCA cycle, and glycolysis/gluconeogenesis). For example, differentially expressed proteins were primarily involved in four carbon metabolic pathways (carbon metabolism, carbon fixation in photosynthetic organisms, the TCA, and glycolysis/gluconeogenesis), and DAMs primarily participated in three carbon metabolic pathways (carbon metabolism, pyruvate metabolism, the TCA cycle, and carbon fixation in photosynthetic organisms). The observed DAPS involved in carbon metabolism include S-adenosylmethionine synthase (spot 11), oxygen-evolving enhancer protein 1, (spot 14), enolase (spot 23), phosphoglycerate kinase (spot 42), triosephosphate isomerase (spot 58), isocitrate dehydrogenase (spot 71), fructose-bisphosphate aldolase (spot 74), malate dehydrogenase (spot 87); and the DAMs included 2-isopropylmalic acid, L-malic acid, pyruvic acid, citric acid, fumaric acid, with these DAPS and DAMs primarily being involved in photosynthesis and respiration (Supplemental Table S3 and Supplemental Table S5). Carbohydrate metabolism is involved in regulating the developmental process from embryonic development to aging [20, 21]; carbohydrate metabolism also plays an important role in plant sexual reproduction, as the sugar content determines flower formation and flower organ development [21, 22]. In herbaceous plants, flowering represents the most energy-consuming step crucial for reproductive success, and photosynthesis in the inflorescence provides the carbon needed for reproduction. It is well known that the photosynthesis of plants is mainly done by leaves, but the reproductive organs of some

plants can also photosynthesize and fix a large amount of carbon. In previous studies, some changes in inflorescence photosynthesis during plant development have been reported. Previous studies have shown that the inflorescence can absorb and distribute carbon mainly in the vegetative organs during flower development. [23]. However, the inflorescence is the primarily source of carbon uptake, and it has an important function in nutritional balance by promoting leaf development, which in turn will ensure the availability of carbohydrates for flower development. Previous studies have shown that carbohydrates not only ensure energy supply during flowering but also affect flower bud differentiation of inflorescence. For example, Walter noted that in the early inflorescence stages of oil palm, the vegetative to reproductive phase transition of the meristem is regulated by sugar balance [27]. We also observed that proteins and metabolites assigned to carbon metabolism were differentially expressed in the primary stem heading stage of inflorescence (the carbon metabolite contents in the three inflorescences followed the order of ZL, ZD and ZB from high to low). Therefore, different inflorescences may primarily affect growth and development through differences in the distribution of imported carbohydrates and exported photoassimilates.

The identified DAPS and DAMs participate in carbon metabolism as well as amino acid and protein biosynthesis. Specifically, the DAMs participated in the biosynthesis of amino acids, cysteine and methionine metabolism and 9 amino acid metabolic pathways (valine, leucine and isoleucine biosynthesis; tyrosine metabolism; biosynthesis of amino acids; glycine, serine and threonine metabolism; alanine, aspartate and glutamate metabolism; phenylalanine metabolism; phenylalanine, tyrosine and tryptophan biosynthesis; arginine and proline metabolism; and cyanoamino acid metabolism). Amino acids play a variety of important roles in plants. Amino acids are the building blocks of enzymes and proteins and are important for the metabolism and structure of plants. In addition, they can also be used as precursors or nitrogen donors to synthesize a variety of compounds that are vital to plant development, including nucleotides, chlorophyll, hormones and secondary metabolites. There is growing evidence that amino acids affect the development of plant inflorescences, for example, studies have shown that in addition to affecting the flowering of plants, proline also influences the configuration of inflorescences [24]. To a large extent, proline accumulation is considered as a specific response to overcome stress conditions [25, 26]. Studies have shown that proline metabolism plays an important role in plant reproductive development. Pyrroline-5-carboxylic acid (P5C) synthetase (P5CS) and P5C reductase (P5CR) catalyze the conversion of glutamate to proline [27]. Simultaneous silencing or co-suppression of two highly similar P5CS genes in *Arabidopsis* resulted in decreased proline levels, delayed flowering and reduced apical dominance [24, 28]. In our results, we observed that the three metabolites involved in proline 5-aminovaleric acid, pyruvic acid and L-Glu had very different abundances in the three inflorescences, with the lowest abundance observed in ZB and no significant difference was noted between ZD and ZL. Amino acids have been shown to affect spikelet development in the maize inbred line Oh43 in vitro, including isoleucine, tyrosine, serine, leucine, glycine, threonine, alanine, aspartic acid and glutamic acid [29]. Interestingly, these amino acids were differentially expressed in the proteomic and metabolic assays in our study, leading us to speculate that the development of the three inflorescences is

achieved through a dynamic balance of amino acids, although the mechanism involved in regulating this dynamic balance is not clear.

Conclusions

The structure of castor inflorescences directly affects the yield of castor, but until now, little research has been performed on the three types of castor inflorescences because of their complex structure. In this study, three types of inflorescences at different developmental stages were analyzed using proteomic and metabolomic approaches, resulting in the identification of 73 DAPS and 86 DAMs. The primary metabolic pathways involved in DAPS and DAMs include carbon metabolism (photosynthesis carbon fixation, and respiration) and amino acid metabolism. According to previous studies, different inflorescences appear to primarily affect growth and development through differences in the distribution of imported carbohydrates and exported photoassimilates, and the dynamic balance of amino acids in different types of inflorescences is also essential to affect their inflorescence structure. Future work will focus on assessing how the dynamic balance of amino acids affects the development of inflorescence structure. Do the dynamic changes in amino acids directly or indirectly affect the structure of inflorescence? If it is an indirect effect, these changes may alter the structure of inflorescence by affecting the metabolism of hormones.

Methods

Plant materials

The experiments were carried out at the Tongliao Experimental Inner Mongolia University for Nationalities in June 2016, and aLmAB2 castor seeds were provided by the Tongliao Agricultural Science Research Institute. aLmAB2 seeds were planted in the Tongliao Agricultural Science Research Institute experimental field.

At each period (4 euphylla stage, 5 euphylla stage, primary stem heading stage and the second-level branching stage), the inflorescence axis of haplometrotic lines, female lines with marker and monoecious lines were harvested. The inflorescence axis was then immediately frozen in liquid nitrogen and stored at -80 °C until analysis. Each group was conducted with three independent biological replicates.

Protein extraction

The proteins were extracted by the Tris saturated phenol method. The inflorescence (3 g) was ground to a fine powder in liquid nitrogen with a mortar and pestle and then added to 10 ml of Tris saturated phenol and 10 ml of extraction solution (0.1 M Tris-HCl, 10 mM EDTA, 0.4% 2-ME, 0.9 M sucrose). Liquid nitrogen was continuously added to the ground powder. The powder was placed in a 50 ml centrifuge tube and allowed to stand at room temperature until the solid in the tube became liquid. Oscillation was continued for 30 min, and for each 1 min oscillation, the samples was placed on ice for 1 min and cooled until the powder turned into liquid. The mixture was centrifuged at $10,000 \times g$ at 4 °C for 20 min, and the phenol

top phase was transferred to a 50 ml tube. For precipitation, 5 ml of Tris saturated phenol and 5 ml of extraction solution were added, and the samples underwent swirl oscillation for 30 min. The mixture was centrifuged at $10,000 \times g$ at 4 °C for 20 min, and the phenol top phase was transferred to a 50 ml tube. The pools from the first and second centrifuges were obtained by mixing the phenol. A 5-fold volume of 0.1 M ammonium acetate methanol solution was added to the phenol at -20 °C overnight. Subsequently, the mixture was centrifuged at $20,000 \times g$ at 4 °C for 20 min, and the supernatant was discarded. Then, 10 ml of a 0.1 M ammonium acetate methanol solution was added to the precipitate after which the mixture was stored at -20 °C for 10 min. Subsequently, the mixture was centrifuged at $20,000 \times g$ at 4 °C for 20 min, and the supernatant was discarded twice. The pellets were then freeze-dried and stored at -80 °C. The protein powder was solubilized in lysis buffer [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 40 mM DTT, 2% (v/v) pharmalyte (pH 4–7)], and 4% (v/v) protease inhibitor) and shaken vigorously for 2 h at room temperature, and the insoluble tissue was removed by centrifugation at $30,000 \times g$ and 4 °C for 30 min. The subsequent supernatant was collected. The protein concentration was determined using a 2D Quant kit (GE Healthcare). The samples were frozen in liquid nitrogen and stored at -80 °C until further use.

Two-dimensional electrophoresis (2-DE)

Two-dimensional electrophoresis (2-DE) was carried out according to the method of Granier [30]. Each of the inflorescence analyses was performed in triplicate. Each 1200 µg protein sample was added to 450 µl of a solution [7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 40 mM DTT, and 0.5% (v/v) pH 4-7 IPG buffer] and loaded onto Immobiline Dry Strips (pH 4-7 nonlinear, 24 cm) (GE Healthcare, Waukesha, WI, USA) by rehydration. Separation was performed on an IPGphor II unit (GE Healthcare, Waukesha, WI, USA) with the following parameters: 30 V for 12 h, 50 V for 1 h, 100 V for 1 h, 300 V for 1 h, 600 V for 1 h, 1000 V for 2 h, and 8000 V for 10 h. After isoelectric focusing, the strips were equilibrated in reducing buffer [6 M urea, 2% SDS, 2.5 M Tris-HCl (pH 8.8), 30% glycerol, and 0.002% (w/v) bromophenol blue and 65 mM DTT] for 15 min. The strips were subsequently equilibrated in alkylation buffer [6 M urea, 2% SDS, 2.5 M Tris-HCl (pH 8.8), 30% glycerol, 0.002% (w/v) bromophenol blue and 4% iodoacetamide (IAA)] for 15 min. The second dimension separation of proteins was performed on SDS-PAGE gels (12.5% polyacrylamide) using an Ettan™ Dalt six apparatus (GE Healthcare, Waukesha, WI, USA). The electrophoresis was carried out at 25 °C and 2 W/gel for 45 min and then 13 W/gel for 4.5 h until the bromophenol blue dye front arrived at the bottom of the gels. The gels were stained with Coomassie brilliant blue R-250. The proteins were visualized by Coomassie brilliant blue R250 staining, and gel images were acquired using an image scanner (GE Healthcare, Waukesha, WI, USA). Image analysis was performed with Image Master 2D Platinum software version 7.0 (GE Healthcare, Waukesha, WI, USA) to detect and match the protein spots. Spots were considered reproducible when they were well resolved in the three biological replicates. Protein spots were selected based on an abundance ratio ≥ 2 and a threshold of $p \leq 0.05$.

In-gel digestion and MALDI-TOF/TOF analysis

The protein spots were manually excised from the gels. The gel spots were washed twice. CBB destaining solution at room temperature for 30 min was used to remove the water and destain the gel spot. The destaining solution was removed, and dehydration solution 1 was added for 30 min. Dehydration solution 1 was removed, and dehydration solution 2 was added for 30 min. Dehydration solution 2 was removed, and 10 µl of working solution was added for 30 min. Cover solution was added for digestion overnight (approximately 16 h) at 37 °C. The supernatants were transferred into another tube, extracting the solution to the gel at 37 °C for 30 min, and then centrifuged for 5 min. The peptide extracts and the supernatant of the gel spot were combined and then completely dried. The samples were resuspended in 5 µl of 0.1% TFA followed by mixing in a 1:1 ratio with a matrix consisting of a saturated solution of α-cyano-4-hydroxy-trans-cinnamic acid in 50% ACN and 0.1% TFA. A 1 µl mixture was spotted on a stainless steel sample target plate. Peptide MS and MS/MS were performed on an ABI 5800 MALDI-TOF/TOF Plus Mass Spectrometer (Applied Biosystems, Foster City, USA). Data were acquired in a positive MS reflector using a CalMix5 standard to calibrate the instrument (ABI5800 Calibration Mixture). Both the MS and MS/MS data were integrated and processed using GPS Explorer V3.6 software (Applied Biosystems, USA) with default parameters. Based on combined MS and MS/MS spectra, proteins were successfully identified based on 95% or higher confidence intervals of their scores in the MASCOT V2.3 search engine (Matrix Science Ltd., London, U.K.) using the following search parameters: NCBI nr-Viridiplantae database; trypsin as the digestion enzyme; one missed cleavage site; fixed modifications of carbamidomethyl (C); partial modifications of acetyl (Protein N-term), deamidated (NQ), dioxidation (W), oxidation (M); 100 ppm for precursor ion tolerance and 0.3 Da for fragment ion tolerance.

Metabolite extraction and LC-MS/MS analysis

First, 50 mg of each sample was placed in an EP tube. Then, 1000 µl of extraction liquid ($V_{\text{methanol}}: V_{\text{acetonitrile}}: V_{\text{water}} = 2:2:1$, which was stored at -20

°C before extraction) and 20 µl of internal standard were added. The sample was homogenized in a ball mill for 4 min at 45 Hz and then treated with ultrasound for 5 min (incubated in ice water). After homogenization 3 times, the sample was incubated for 1 h at -20 °C to precipitate the proteins. Then, the sample was centrifuged at 13000 rpm for 15 min at 4 °C. Next, 200 µl of the supernatant was transferred to LC-MS vials, 20 µl from each sample was taken and pooled as the QC samples, and 200 µl of the QC sample was used for the UHPLC-QE Orbitrap/MS analysis. LC-MS/MS analyses were performed using an UHPLC system (1290, Agilent Technologies) with a UPLC HSS T3 column (1.7 µm 2.1 × 100 mm, Waters) coupled to a Q Exactive system (Orbitrap MS, Thermo). The mobile phase was as follows: positive: 0.1% formic acid in water and negative: 5 mM ammonium acetate in water (A) and acetonitrile (B); the procedure was carried with the elution gradient as follows: 0 min, 1% B; 1 min, 1% B; 8 min, 99% B; 10 min, 99% B; 10.1 min, 1% B; 12 min, 1% B, which was delivered at 0.5 ml min⁻¹. The injection volume was 1 µl. The QE mass spectrometer was to acquire MS/MS spectra on an information-dependent basis (IDA) during an LC/MS experiment. In this mode, the acquisition software (Xcalibur 4.0.27, Thermo) continuously evaluates the full scan survey MS data as it collects and triggers the acquisition of MS/MS spectra depending on preselected criteria. ESI source conditions were set as following: sheath gas flow

rate as 45 Arb, aux gas flow rate as 15 Arb, capillary temperature 320 °C, full ms resolution as 70000, MS/MS resolution as 17500, collision energy as 20/40/60 eV in NCE model, ion spray voltage floating (ISVF) as 3.8 kV or -3.1 kV in positive or negative modes, respectively.

Quantitative real-time PCR (qRT-PCR)

To investigate the relationship between the transcriptional and translational expression of related genes after treatment, we used qRT-PCR to analyze DAPS and DAMs (Supplemental Table S1). Total RNA was isolated using the same method described by Schultz [31]. cDNA was synthesized from 1 µg of total RNA with PrimeScript reverse transcriptase (TaKaRa, Japan) according to the manufacturer’s instructions. qRT-PCR was performed using SYBR Green real-time PCR master mix (Toyobo, Japan) with an ABI 7500 system (Roche, USA). The relative expression of the target genes was calculated using the comparative Ct method.

Abbreviations

Full name	abbreviation
Diferentially abundant protein species	DAPS
Diferentially abundant metabolites	DAMs
Quantitative real-time PCR	qRT-PCR
primary stem heading stage haplometrotic inflorescences axis	ZD
primary stem heading stage female with marker inflorescences axi	ZB
primary stem heading stage monoecious inflorescences axis	ZL
Pyrroline-5-carboxylic acid	P5C
Pyrroline-5-carboxylic acid synthetase	P5CS
Pyrroline-5-carboxylic acid reductase	P5CR

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

All authors agree to publish the paper

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Competing interests

All authors declare that they have no conflict of interest.

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Authors' contributions

MFJ and HFL designed the experiments. ZY performed the experiments. PM, CRH, ZGL, HZB, LGR, DJJ, LR, LLL, ZHB, WQ and LXT assisted with the experiments. ZY wrote the manuscript. All authors have read and approved the manuscript.

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Tables

Table 1 Significantly enriched pathway annotation of DAPS identified by 2-DE

Description	ZB/ZD Ratio	ZB/ZD qPCR Ratio	ZL/ZD Ratio	ZL/ZD qPCR Ratio
Heat shock protein (DAPS)	0.167 ± 0.117	0.804 ± 0.267	0.002 ± 0.1133	0.585 ± 0.356o
14-3-3 protein (DAPS)	0.472 ± 0.118	0.891 ± 0.346	3.146 ± 0.2249	1.094 ± 0.256
Enolase (DAPS)	0.523 ± 0.313	0.933 ± 0.346	0.940 ± 0.2013	0.958 ± 0.242
Inositol-1-monophosphatase (DAPS)	2.052± 0.113	1.257 ± 0.279	0.218 ± 0.0134	0.734 ± 0.156
phospholipase D alpha (DAPS)	0.612 ± 0.213	0.624 ± 0.452	0.509 ± 0.1209	0.740 ± 0.291
Phosphoglycerate kinas (DAPS)	0.005 ± 0.125	0.665 ± 0.273	0.399 ± 0.1001	0.604 ± 0.372
Triosephosphate isomerase (DAPS)	0.031 ± 0.134	0.699 ± 0.273	1.344 ± 0.2133	1.028 ± 0.262
Isocitrate dehydrogenase (DAPS)	1.053 ± 0.122	1.729 ± 0.163	0.009 ± 0.3514	0.482 ± 0.153
Fructose-bisphosphate aldolase (DAPS)	1.272 ± 0.167	1.527 ± 0.245	0.622 ± 0.2132	0.797 ± 0.513
cysteine synthase (DAPS)	3.217 ± 0.101	2.085 ± 0.145	2.413 ± 0.2213	1.832 ± 0.214
L-Malic acid (DAMs)	1.071 ± 0.021	1.654 ± 0.237	0.925 ± 0.050	0.796 ± 0.367
Pyruvic acid (DAMs)	1.087 ± 0.032	0.875 ± 0.212	1.252 ± 0.089	0.934 ± 0.154
Quinic acid (DAMs)	0.608 ± 0.184	0.754 ± 0.243	0.871 ± 0.140	0.871 ± 0.273

Table 2 Comparison of expression pattern at the mRNA, protein, and metabolites

No.	Pathway	Number of DAPS
1	carbon metabolism	10
2	carbon fixation in photosynthetic organisms	5
3	biosynthesis of amino acids	8
4	proteasome	5
5	glycolysis / Gluconeogenesis	6
6	gysteine and methionine metabolism	6

Figures

Figure 1

Changes of morphological (A), 2-DE master gel of inflorescence(B)

Figure 2

Hierarchical clustering of the 73 differentially expressed proteins. A, 4 euphylla stage changes in the expression of different proteins in different inflorescences. B, 5 euphylla stage changes in the expression of different proteins in different inflorescences. C, main stem heading stage changes in the expression of different proteins in different inflorescences. D, second-level branching stage changes in the expression of different proteins in different inflorescences.

Figure 3

Biological process cellular component and functional category respectively distribution of the 73 identified proteins.

Figure 4

The functional category of DAPS identified by the 2-DE

Figure 5

KEGG pathway distribution of the 73 identified proteins.

Figure 6

Heat map and cluster tree of identified metabolite concentrations in extracts from inflorescence of main stem heading stage castor. Each column represents a biological repeat with eight biological replicates per inflorescence and each metabolite is represented by a single row: ZD, main stem heading stage haplometrotic inflorescences; ZB, main stem heading stage female with marker inflorescences; ZL, main stem heading stage monoecious inflorescences

Figure 7

The category of DAM identified by the metabonomics

Figure 8

KEGG pathway distribution of the DAM.

Figure 9

Differential metabolite KEGG pathway bubble Plot. Each bubble in the bubble diagram represents a metabolic pathway. The abscissa and bubble size of the bubble indicate the size of the impact of the pathway in the topological analysis. The larger the size, the larger the impact. The ordinate of the bubble and the color of the bubble indicate the P value of the enrichment analysis (take the negative natural logarithm, $-\ln P$ -value), the deeper the color, the smaller the P value, and the more significant the enrichment.

Supplementary Files

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