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New insight into ovary abortion during ovary development of hazelnut through a combined proteomic and transcriptomic analysis

Jianfeng Liu[a], Jiyang Xing[a], Jun Fang[b], Pengfei Ai[c], Yunqing Cheng[a,*]

[a] Jilin Provincial Key Laboratory of Plant Resource Science and Green Production, Jilin Normal University, Siping, Jilin Province, 136000 China
[b] The Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences, Harbin, Helongjiang Province, 150081 China
[c] College of Bioscience & Bioengineering Hebei University of Science and Technology, Shijiazhuang, Hebei Province, 050080 China

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ABSTRACT

Hazel (*Corylus* spp.) is the most economically important species in the Betulaceae family. Frequent ovary abortion during the prefertilization stage causes the high drop ratio of pistillate flowers and smaller fruit numbers in a fruit cluster, resulting in yield loss. To better understand the mechanism of ovary abortion formation in hazel, we conducted transcriptomic and proteomic analyses and identified genes and proteins that were altered in response to ovary abortion. A total of 89,846 unigenes and 4343 proteins were identified using RNA-seq and iTRAQ technology, respectively. Among them, 1895 unigenes and 710 proteins were differentially expressed and accumulated in the developing and abortive ovary respectively. Furthermore, we employed the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis, revealing pathways that are significantly enriched in both transcriptomic and proteomic assays. These include the phenylalanine metabolic pathway, biosynthesis of phenylpropanoid, biosynthesis of secondary metabolites, and ascorbate and aldarate metabolism. Transcriptome-proteome integrative analysis revealed 42 correlations. Among these, 32 correlations with same changing trend (that are involved in metabolism stress response, vascular strand development, water transport, and seed development) are likely associated with abortive ovary formation. Importantly, ethylene (ETH), jasmonic acid (JA), and salicylic acid (SA) signal transduction pathways may contribute to the regulation of the abortive ovary formation via the up-regulation of ethylene-responsive transcription factor 1 (ERF1/2), jasmonate ZIM domain-containing protein (JAZ), transcription factor MYC2 (MYC2), transcription factor (TGA), and pathogenesis-related protein 1(PR-1). Our work will facilitate the identification of ovary abortion related genes and proteins and provide insights into the molecular mechanism of fruit development.

1. Background

Hazel (*Corylus* spp.) is the most economically important species in the Betulaceae family. Its edible kernel in fruit is an important raw material in food processing industry. The European hazel, *Corylus avellana* L. (2n = 2x = 22), is the most important and widely distributed species in most hazelnut production countries, including Turkey, Italy, Azerbaijan and Spain (Guo et al., 2009). European hazel was introduced to Northeast China in the twentieth century. However, European hazel, which originates from temperate areas, is not suitable for culture in Northeast China due to the area's extremely low temperatures in winter. *C. heterophylla* Fisch. ex Besser (2n = 2x = 22) and its hybrids with European hazel (hybrid hazel; *C. heterophylla* × *C. avellana*) (2n = 2x = 22) are the most important *Corylus* germplasm in China (Guo et al., 2009). In the past several years, the culture area of hybrid hazel (*C. heterophylla* × *C. avellana*) has increased dramatically in China and is currently more than 50,000 hm^2 with a concomitant increase in fruit yield, setting the stage for China to become a major global producer of hazelnut. Thus, hazelnut industry plays a much more important role in increasing farmers' income in China, especially in mountainous areas.

The development and fertilization of the ovaries are necessary to produce edible hazelnut, and their characteristics are unique compared to most angiosperm. As pistillate inflorescences of hazel bloom, their ovaries have barely initiated differentiation. Generally, the development of many ovaries will stop soon after pollination and the abortive ovary begins to form 30 days after blooming, and the fertilization process of the abortive ovary has not been completed yet (Liu et al., 2014a). It was reported that the abortive ovaries ratio ranged from 29% to 72% (Liu et al., 2014a, b). If the drop ratio of pistillate inflorescences were concluded, less than 15% of pollinated pistils of hazel would be capable of developing into normal edible fruit (Liu et al., 2014a;

* Corresponding author.
E-mail address: yqcheng@jlnu.edu.cn (Y. Cheng).

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Beyhan and Marangoz, 2007). Thus, the ovary abortion phenomenon is of vital importance because it incurs yield losses in hybrid hazel (*C. heterophylla* × *C. avellana*) (Liu et al., 2014a). It is known that the abortive ovary is incapable of finishing the fertilization process due to arrested pollen tubes and the absence of a mature embryo sac showing atypical small size, growth arrest, lignification, dehydration, and a short life span (Liu et al., 2014a). However, the molecular mechanism of hazel ovary abortion still remains unclear.

With the development of large-scale transcriptional and proteomic profiling technologies over the past decade, many important genes and proteins associated with fruit development and senescence have been identified, which is beneficial for the better understanding of their complicated regulation network (Song et al., 2016; Vashisth et al., 2015; Ma et al., 2014). Auxin-like 2, 4-dichlorophenoxyacetic acid (2, 4-D) works as a high-efficiency anti-stalling agent in the post-harvest fresh fruit industry. In order to illuminate the molecular mechanism underlying the effects of 2,4-D on fruit quality preservation, mature fruits of Olinda Valencia orange [*Citrus sinensis*(L.) Osbeck] were dipped either in water or in 2, 4-D aqueous solution, and subsequent proteomic and transcriptomic analysis was carried out. In total, 3413 differentially expressed genes (DEGs) and 99 differentially accumulated proteins (DAPs) were identified between the control and the treated samples, and seven proteins were found with similar expression patterns at both the transcriptional and post-transcriptional levels. It was found that 2, 4-D retarded fruit senescence by altering the levels of many endogenous hormones and by improving stress defense capabilities by up-regulating defense-related genes and proteins (Ma et al., 2014). Accumulated evidence indicates that regulation at the translation and protein degradation levels play crucial roles in controlling protein abundances (Ritchie et al., 2015). Thus, the next-generation DNA sequencing and proteomics technologies provide an unprecedented opportunity for researchers to survey total mRNA and protein abundances changes in different samples. The transcriptome-proteome integrative analysis of developing and abortive ovaries will provide new insight into the molecular mechanism of hazel ovary abortion. Given that there was a negative linear correlation between yield and ovary abortion ration (Liu et al., 2014a), our work will also provide fundamental information for further identification of ovary abortion related genes and proteins as well as breeding for high yields in hazelnut.

2. Materials and methods

2.1. Sample collection

The trial was conducted at a hazel orchard near Siping Jilin province China from 2015 to 2016. Eight-year-old hybrid hazel trees of (*C. heterophylla* × *C. avellana*) cultivar “Dawei” were used as materials. According to our previous study results, abortive ovaries began to form approximately 30 days after blooming. At this period, the diameters of abortive ovaries varied from 1.5 to 2.0 mm before lignification, dehydration and withering. The developing ovaries were twice as big in diameter (Liu et al., 2014a). The morphological characteristics of the fresh developing and abortive ovaries are shown in Fig. 1A and Fig. 1B respectively, while Fig. 1C shows a fruit with both developing and abortive ovaries. During the experiment, about 1500 fruit clusters from 60 trees were randomly collected. Each pistillate inflorescences or fruit cluster contained about 3 developing ovaries and 5 abortive ovaries. In the lab, the developing and abortive ovaries were manually separated, collected with three biological replicates, and frozen in liquid nitrogen before RNA, protein extraction for sequencing and qRT-PCR.

2.2. RNA purification and sequencing

RNA of six ovary samples (including developing and abortive ovaries, each with three biological replicates) were extracted using the RNA Easyspin Isolation System (Aidlab Biotech Beijing China), and RNase-free DNase I was used to eliminate the residual genomic DNA in raw RNA extract according to the manufacturer’s protocol (Promega Beijing, China). The pretreatment of mRNA before sequencing was performed as described previously (Cheng et al., 2015). Agilent 2100 Bioanaylzer and ABI StepOnePlus Real-Time PCR System were used to quantify and qualify of the samples’ libraries. Then, the six libraries were sequenced using Illumina HiSeq 4000, and raw reads were produced. Trinity (version: v2.0.6) (Grabherr et al., 2011) was used to perform *de novo* assembly with clean reads with min_contig_length set to 150 and min_kmer_cov set to 3 and all other parameters set default. Tgicl (version: v2.0.6) (Pertea et al., 2003) was used to cluster transcripts to Unigenes with repeat_stringency, minmatch and minscore set to 0.95, 35 and 35 respectively and all other parameters set default. We used Blast (Altschul et al., 1990) to align Unigenes to NT, NR, COG, KEGG and SwissProt to get the annotation, and used Blast2GO (Version: v2.5.0; parameters: default) (Conesa et al., 2005) with NR annotation to get the GO annotation and use InterProScan5 (Quevillon et al., 2005) to get the InterPro annotation. Clean reads were mapped to Unigenes using Bowtie2 (version: v2.1.0) (Langmead and Salzberg, 2012) with options “q; phred64; sensitive; dpad 0; gbar, 99,999,999; mp 1, 1; np, 1; score-min L, 0, -0.1; I, 1; X,1000; no-mixed; no-discordant; p,1; k, 200”, and then, gene expression levels were calculated with RSEM (Li and Dewey, 2011a, b) and all other parameters were set to default. DEGs were detected using NOIseq method (Tarazona et al., 2011), and threshold of Fold Change ≥ 2.00 and Probability ≥0.8 was used to judge DEGs.

2.3. Protein preparation and iTRAQ labeling

iTRAQ analysis was implemented by BGI (Shenzhen, China). A total of six protein samples of ovary (including developing and abortive ovaries, each has three biological replicates) were extracted based on method of phenol extraction coupled with ammonium acetate precipitation as described previously (Isaacson et al., 2006). Protein concentrations were determined using the Bradford method (Hammond and Kruger, 1994).

Protein digestion was performed using Trypsin Gold with the ratio of protein: trypsin = 20:1 as previously described (Wang et al., 2016). After that, the peptides were vacuum centrifuged to dryness, and iTRAQ labeling of peptide samples were carried out using iTRAQ Reagent 8-plex Kit (AB SCIEX, Framing-ham, USA) according to the manufacturer's protocol.

2.4. SCX chromatography and LC-ESI-MSMS analysis based on TripleTOF™ 5600

Each fraction was re-suspended in buffer A (5% ACN, 0.1% FA) and centrifuged at 20,000 g for 10 min, and the final concentration of peptide was about 0.5 mg/mL on average. 10 μL supernatant was loaded on a LC-20AD nanoHPLC (Shimadzu, Kyoto, Japan) by the autosampler onto a 2 cm C18 trap column. Then, the peptides were eluted onto a 10 cm analytical C18 column (inner diameter 75 mm). The samples were loaded at 8 μL /min in 4 min, then the 41 min gradient was run at 300 nL/min starting from 5 to 35% B (95%ACN, 0.1%FA), followed by 5 min linear gradient to 80%, and maintenance at 80% B for 5 min, and finally return to 5% in 1 min.

Data acquisition was performed with a TripleTOF 5600 System (AB SCIEX, Concord, ON) fitted with a Nanospray III source (AB SCIEX, Concord, ON) and a pulled quartz tip as the emitter (New Objectives, Woburn, MA). Data was acquired using an ion spray voltage of 2.5 kV, curtain gas of 30 PSI, nebulizer gas of 15 PSI, and an interface heater temperature of 150 °C. The MS was operated with a RP of greater than or equal to 30,000 FWHM for TOF MS scans. For IDA, survey scans were acquired in 250 ms and as many as 30 product ion scans were collected if exceeding a threshold of 120 counts per second (counts/s) and with a 2+ to 5+ charge-state. Total cycle time was fixed to 3.3 s. Q2