Comparison of transcriptomic and proteomic analyses to construct a model for the promotion of starch synthesis by ABA in *Euryale ferox* Salisb. seeds

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Abstract

Background: *Euryale ferox* Salisb. is an annual aquatic herb and the only species belonging to the genus *Euryale* in the Nymphaeaceae family. *E. ferox* seeds are used in medicine and diets. Starch is the main factor affecting *E. ferox* seed quality, but its regulatory mechanism has not been elucidated.

Results: Herein, four time points of seed development, including after flowering T1 (10 days), T2 (20 days), T3 (30 days) and T4 (40 days), were investigated by using RNA-Seq and iTRAQ technology. Using weighted gene co-expression network analyses (WGCNAs), co-expressed genes and hub genes were identified for each module. Of particular importance are the discoveries of specific modules for seed starch during the seed developmental stages. The candidate regulators of seed starch are involved in the hormonal signaling pathways. Three ABA signaling receptor kinases, *EfPYR1*, *EfSnRK2.1* and *EfSnRK2.2*, were identified as hub genes functioning in starch synthesis during the seed maturation process. The changes in expression pattern, ABA and starch content also indicated that ABA is positively correlated with starch.

Conclusions: Together, these results indicate that *E. ferox* seed accumulation of starch is promoted by ABA, providing new insights into the regulatory mechanism of starch synthesis in *E. ferox* seeds.

Background

Euryale ferox Salisb. (Nymphaeaceae), an ancient species and an annual aquatic herb with large, prickly, floating leaves, is a basal member of the phylum Magnoliophyta that originated in China and Southeast Asia (Fig. 1). The genus *Euryale* consists of only a single species, *E. ferox*, which is characterized by both sexual reproduction and cleistogamy [1]. *E. ferox* can be grown in lakes, bottoms and raceways, signifying a very important and unique type of hydrophytic plant. In China, *E. ferox* is a popular aquatic vegetable, whose seeds are called gorgon fruit and are considered a tonic [2]. *E. ferox* is also a class of plant with medicinal value, and a variety of medicinal ingredients have been isolated and identified in its seeds [3, 4]. In addition, *E. ferox* has been proven to treat many diseases, such as chronic diarrhea, hypofunction of the spleen, excessive leukorrhea and kidney problems, for several thousand years in traditional Chinese medicine [4, 5]. With the development of technology, we have a deeper understanding of the effects of *E. ferox*.

Starch is the main component of *E. ferox* seeds. The photo-contracted compound produced by the source organ is provided to the storage organ (seed) in the form of sucrose and then formed by the catalytic action of a series of enzymes in the grain. The biosynthesis and accumulation of grain starch is directly related to the production and quality of *E. ferox*. The biosynthesis of starch from sucrose occurs in the developing endosperm, and several important enzymes are involved in the pathway of starch synthesis [6, 7], such as sucrose synthase (SUS), UDP-glucose pyrophosphorylase (UGPase), AGPase, and starch synthase. Starch synthase includes two forms: granule-bound amylose synthesis is controlled by starch synthase (GBSS), and amylopectin synthesis is controlled by soluble starch synthase (SSS) and starch

branching enzyme (SBE) [8–10]. In previous reports, the activities of SUS, AGPase, SSS, and SBE were associated with grain weight [11–14]. During grain filling, some enzymes express different levels of these enzymes between the inferior and superior caryopses [15]. In addition, starch synthesis is affected by hormones and other factors.

Plant hormones play important roles in regulating grain filling, especially abscisic acid (ABA) [13, 16, 17]. In previous reports, ABA-related mutants were used to establish a relationship between ABA signaling and sugar responses in several sugar response screens [18]. ABA biosynthesis and subsequent ABA signaling through ABI4 regulates glucose responses [19].

ADP-glucose pyrophosphate (AGPase) subunits are involved in starch biosynthesis induced by sugars [20]. In angiosperms, AGPase consists of two small and two large subunits, which are used as glycosyl donors by starch synthases [21, 22]. The allosteric regulation of AGPase activity plays an important role in starch biosynthesis in plants [23]. AGPase transcript levels also control starch production [24]. The expression of the genes encoding AGPase subunits is differentially regulated by tissue specificity and physiological signals. Sugar-induced expression has been described for the small (ApS) and large (ApL1, ApL2 and ApL3) subunits [25–28].

A combination of both sucrose and ABA results in much higher expression levels, suggesting a synergistic interaction [29]. Genetic analysis also confirmed that most glucose-responsive mutants are associated with beta or ethylene. It was previously reported that high concentrations of glucose inhibited the growth of *Arabidopsis thaliana* seedlings, but ethylene clouds lead to the redevelopment of seeds. Through analysis of endogenous ABA in sugar-responsive mutants (*gin1*, *gin5*, *isi4*, and *sis4*), the ABA content was found to be lower than in wild type plants [30]. Sugar sensitivity can be restored by adding a particular concentration of ABA to these sugar-insensitive mutants. However, these experiments did not directly prove the relationship between sugar and signaling; Chen et al. subsequently found that glucose directly controls expression of ABA biosynthesis genes [31]. After glucose treatment, the transcription levels of ABA1, ABA2, ABA3, NCED3 and AAO3 were enhanced. These studies suggest that there may be a unique pathway in which glucose directly regulates the expression and accumulation of ABA synthetic genes in higher plants. The interaction between ABA and sugar signaling continuously promotes the growth and development of plants.

In this study, *E. ferox* seeds, as the research material, were sampled at four different stages of its development (10, 20, 30, and 40 days after flowering). By using RNA sequencing (RNA-seq) and iTRAQ technology (Fig. 2), we analyzed the mechanism of interaction between ABA and starch biosynthesis. The regulatory mechanisms lay a foundation for improving starch content in plants.

Results

The differences in starch content and amyloid morphology during the development of *E. ferox* seeds

Ten to 40 days after flowering, fruit weights of *E. ferox* showed an increasing trend. The 10-20 day weight gain was the largest (141.3%), and the increase was reduced after 20-40 days. The total starch content of the seeds increased during seed maturation. The total starch content increased the fastest after 20-30 days of flowering. When the kernels reached maturity at 40 days, the total starch content was as high (62.94%). Amylopectin and amylose content also increased. However, the amylopectin/amylose ratio was decreased (Fig. 3, Table S1).

Ten days after flowering, the seed cells began to enter initial proliferation. At this time, cell volume was small, amyloid in the cells was low, and the amyloid was spherical and arranged neatly within the cells. The starch granules had irregular polyhedral shapes with sharp edges and corners. With seed development, cell volume expanded, and amyloid volume in the seed increased. When the seed was mature, the amyloid was arranged closely, and the degree of cell filling was increased (Fig. 4).

The above observations are consistent with the results of the determination of the seed starch content. Ten days after flowering, the starch content in the seeds was reduced. During the 10-20 days after flowering, starch accumulated in large amounts, and the content increased rapidly. At 20-25 days after flowering, the seeds gradually matured, and the starch increase was more gradual.

Overview of the quantitative proteomics analysis

iTRAQ was applied to analyze the proteins extracted from the seeds of *E. ferox* collected at 10, 20, 30 and 40 days after flowering. Three biological replicates were used to the proteomic analysis (Fig. S1, Table S2). We used liquid-mass coupling (LMS) to analyze the samples labeled by enzymatic hydrolysis. In order to obtain credible protein, the peptide matching error was confined to less than 1 ppm. The screening results were as follows: the credible protein 1 screens out 3175 credible proteins; protein 2 screens out 3083 credible proteins ; and credible protein 3 screens out 3118 credible proteins .

The v-look up function was used to differentially screen the three results. The t-test was performed on data with three repeats to compare the values in each group, and the difference multiples FC and the p-values of each comparison group were calculated to determine significant differences. Then, the double standards with 2 times difference multiples and p-value less than 0.05 were used to screen differentially expressed proteins. One hundred fifty-two (20 vs. 10 days), 736 (30 vs. 10 days), 774 (40 vs. 10 day), 460 (30 vs. 20 days), 530 (40 vs. 20 days) and 157 (40 vs. 30 days) DEPs were identified (Fig. S2). The results indicated that with the maturity of *E. ferox* seeds, increasing proteins were involved in the development process, with more proteins involved in the later stages of development than in the early stages (Fig. S3).

Overview of the transcriptomic analysis

RNA-seq was performed for four groups at 10, 20, 30 and 40 days, and each group included three biological replicates. A total of 4897 DEGs were confirmed among the nine samples at the four developmental stages in *E. ferox* seeds [32]. KEGG pathway analysis was used to identify the biological roles of DEGs with functional annotation and enrichment modules. According to the KEGG pathway

enrichment analysis, the DEGs in related modules were enriched in environment information processing, especially those related to plant hormone signal transduction in *E. ferox* seeds. (Fig. S4).

Comprehensive analysis of the WGCNA network of *E. ferox* seed RNA-Seq data

The WGCNA package was used to obtain a comprehensive understanding of DEGs in *E. ferox* seed at the different developmental stages and to identify important genes that are highly associated with seed starch [33]. All 4897 DEGs were retained for WGCNA. Modules were defined as clusters of highly interconnected genes, and genes within the same cluster had high correlation coefficients among them. This analysis identified 23 distinct modules shown in the dendrogram in Fig. 5a. Different colors represent specific modules containing a cluster of highly correlated genes. Twenty-three modules that correlated with distinct samples due to specific expression profiles were identified (Table S3).

In addition, correlation among different modules was also considered, and 7 broad clades were identified in 14 modules (Fig. 5b). A heat map showed that there was a high degree of correlation among the three black dotted boxes, such as the light green module, dark green module, turquoise module and the royal blue module. GO analysis in these modules identified series terms that were involved in response to signal transduction and protein phosphorylation, indicating that these clusters may participate in the process of seed development.

Genes in the module were related to starch data. Notably, ME light yellow, ME tan, ME light green and ME brown were highly related and selected for analysis. ABA-signaling regulatory genes were identified as a candidate hub for these modules (Fig. 5c). These ABA regulating genes had the highest connectivity in the dataset, indicating that the ABA signaling regulatory network may play a major role in seed starch. These genes included SnRK2, which functions primarily as a positive regulator of ABA signaling. A member of the PYR (pyrabactin resistance)/PYL(PYL1-like)/RCAR (regulatory components of ABA receptor) family of proteins functions as an abscisic acid sensor (Table S4). These results suggest that ABA signaling has an important relationship with starch biosynthesis in *E. ferox* seeds.

Comparative analysis of transcriptome and proteome data

To further explore the development of *E. ferox* seeds, we conducted a correlation analysis between the transcriptomic and quantitative proteomic data (Fig. 6). A total of 4897 DEGs and 3494 DEPs were identified in transcriptome and proteome, respectively, of which 433 members were determined to correlate with transcript-to-protein based on identification. Among the 443 cor-DEGs-DEPs genes, 17 (T2 vs T1), 31 (T3 vs T1), 168 (T4 vs T1) and 51 (T4 vs T2) genes showed the same trend, while 107 (T2 vs T1), 3 (T3 vs T1), 55 (T4 vs T1) and 5 (T4 vs T2 day) genes showed the opposite trend at the two levels (Table S5). We suggest that some of these genes might play important roles in *E. ferox* seed starch biosynthesis.

To study the 443 cor-DEGs-DEPs gene, we used GO annotations and KEGG to predict their functions. The results covered a wide range of cellular components, molecular functions and biological processes (Fig. S4). Consistent with the DEG function enrichment and WGCNA, some cor-DEGs-DEPs genes were also related to plant hormone signal transduction. All differentially expressed genes and proteins associated with plant hormones were counted (Fig. 7). We found that these genes, c54854.graph_c0 (*EfPYR1*), c51120.graph_c0 (*EfSnRK2.1*) and c47171.graph_c1 (*EfSnRK2.2*), were differentially expressed during the development of *E. ferox* seeds and the related ABA signaling. These results suggest that these genes regulate ABA signaling and affect starch synthesis in *E. ferox* seeds.

Building a co-expression network

WGCNA can also be employed to construct gene networks, in which each node represents a gene, and the connecting lines (edges) between genes represent co-expression correlations. *EfPYR1*, *EfSnRK2.1* and *EfSnRK2.2* were hub genes that exhibited the most connections in the network as indicated by their high KME (eigengene connectivity) values. We used *EfPYR1*, *EfSnRK2.1* and *EfSnRK2.2*, representing three nodes, and the thirty gene connecting lines (edges), representing between co-expression correlations, to build a co-expression network (Fig. 8a). The thirty genes were highly expressed in *E. ferox* seeds development (Fig. 8b). We found that the weight of *EfPYR1* was higher than that of *EfSnRK2.1* or *EfSnRK2.2*.

To evaluate the correlation of thirty genes from transcript to protein in *E. ferox* seed development, the PCC (Pearson correlation coefficient) was calculated (Fig. 8c, Table S6). Among the thirty genes, some genes (c55828.graph_c0, c52934.graph_c0, c5498 1.graph_c2, etc.) showed strong positive correlations, and some genes (c52200.graph_c2, c54936.graph_c0, c53379.graph_c2, etc.) showed negative correlations. The results indicated that these genes play important roles in starch synthesis of seeds during *E. ferox* seed development.

Identification of the expression patterns of key regulators and changes in hormone content in *E. ferox* seeds

To analyze key gene expression patterns, three DEGs related to abscisic acid synthesis during the growth and development of *E. ferox* seeds were selected for qRT-PCR (Fig. 9, Table S7). As shown in Fig. 9, the expression levels of the three cor-DEG-DEP genes, *EfPYR1*, *EfSnRK2.1* and *EfSnRK2.2*, increases with the growth of *E. ferox* seeds. At the same time, these genes had similar expression patterns to the transcriptome and protein data, indicating that results of the transcriptomic analysis are reliable.

These DEGs were hormone-related and are involved in abscisic acid pathways. The phytohormone abscisic acid (ABA) functions as a crucial signal to promote seed maturation and dormancy. As such, we measured the content of ABA in different *E. ferox* seed development stages. Increased ABA levels were observed with the growth of the *E. ferox* seeds and maturation (Fig. 9, Table S8). In this study, we found that ABA was related to starch synthesis. With *E. ferox* seed maturation, ABA and starch levels increased. These studies further confirmed that reliable prediction of functions of *EfPYR1*, *EfSnRK2.1* and

EfSnRK2.2 genes regulate starch by ABA during *E. ferox* seed maturation (Fig. 9). The contents of ABA and starch in *E. ferox* seeds increase in parallel during *E. ferox* seed development.

Discussion

Sucrose is not only a carbon source for starch synthesis but also a signaling molecule. Alone or in coordination with ABA, sucrose can regulate the expression of genes involved in starch synthesis. In sweet cherries (*Prunus avium*), ABA and starch content increases in the buds during their endodormancy period [34]. The ABA to single-node cuttings of grapevines increased the content of starch and the expression of starch synthesis genes [35]. ABA reportedly plays important roles in starch synthesis during the development of wheat grain [36, 37], barley [38] and rice [39]. In this study, we constructed a dynamic transcriptome and proteome landscape of early *E. ferox* seed development by sampling 4 time points from 0 days, 10 days, 20 days, 30 days, and 40 days. The dynamic transcriptome and proteome data provided herein clearly demonstrated four key developmental stages within the early seed. We found, by WGCNA analysis, that the increase in starch content was closely related to the ABA hormone in *E. ferox* seeds. This phenomenon was consistent with previously reported observations. These analyses all proved that ABA promotes starch synthesis to regulate the sink activity and dormancy status of seeds.

The purpose of this study was to investigate the potential regulatory mechanism of starch synthesis in *E. ferox* seeds. To further identify the key genes involved in these regulatory mechanisms, we performed WGCNA analysis on transcriptome data. We identified three hub genes, *EfPYR1*, *EfSnRK2.1* and *EfSnRK2.2*, associated with ABA hormone regulation. Further, co-expression analysis was performed in conjunction with proteomic data. Three hub genes were differentially expressed at most different stages during seed formation. In previous reports, ABA was shown to play a crucial role in promoting seed maturation [40–42]. The PYL receptors can bind to ABA, which interacts with the type 2C protein phosphatase (PP2Cs) to form a stable complex, leading to the release of SNF1-related kinase 2 (SnRK2s) from PP2C-SnRK2 complexes [43–48]. Then, SnRK2s are activated to modulate ABA responses [49–53].

These hub genes are generally thought of as not just a simple information flow from the transcriptome to the proteome. There is a complex regulatory network in seed starch synthesis. We identified the abovementioned genes in the seed forming stage, indicating that a network for complex hormonal synthesis and signaling functions to regulate seed starch synthesis (Fig. 10). In a previous report, ABA was demonstrated to play a major role in the sugar signaling network by ABI4 in that it enhanced ApL3 responses to subsequent sugar signals [39], indicating a novel and central role for ABA mediated glucose responses in plants mediated by GIN5 and GIN6/ABI4. Therefore, analogous with the effect of ABA in cereal seeds, our results in starch synthesis suggest that ABA improves *E. ferox* seed starch content by favoring the synthesis of starch.

Conclusions

In summary, we constructed a complete picture of the molecular dynamics of *E. ferox* seed maturation by RNA-Seq transcriptome profiling with proteome analysis. We provided comprehensive insight into the regulatory mechanism of ABA, which promotes seed starch synthesis during *E. ferox* development. Furthermore, the contents of ABA in the *E. ferox* seeds increased by enhanced expression of three hub genes, EfPYR1, EfSnRK2.1 and EfSnRK2.2, leading to increases in starch content in *E. ferox* seeds. Analysis of the comprehensive data set in this study provides a useful genomic resource for *E. ferox* seed maturation research and molecular insights into the ABA and starch regulatory networks in *E. ferox* seeds.

Methods

Plant material and sample collection

The formal identification of the *Euryale ferox* used by Floar Reipublicae Popularis Sinicae(http://www.efloras.org/florataxon.aspx?flora_id=2&taxon_id=200007078): Submerged leaves not prickly; leaf blade sagittate or elliptic, 4–10 cm, base deeply cordate. Floating leaves prickly on petioles and along veins; leaf blade abaxially dark purple and adaxially green, to 1.3(–2.7) m in diam., subleathery, abaxially sparsely pubescent, adaxially glabrous, base emarginate or sinuate; veins abaxially strongly ribbed; primary veins prickly on both surfaces. Flower to 5 cm in diam. Peduncle stout, densely prickly. Sepals triangular-ovate, 1–1.5(–3) cm, abaxially dense-ly prickly; prickles retrorse. Petals outer purple-violet fading to inner white, oblong-lanceolate, 1(–2.5) cm. Ovary 7–16-loculed, each locule with 6–8 or more ovules. Fruit dark purple, globose, 5–10 cm in diam., spongy, densely prickly. Seeds black, 8 to many, globose, 6–10 mm in diam.; testa thick, rigid. Fl. Jun–Aug. Lakes, ponds. Anhui, Fujian, Guangdong, Guangxi, Guizhou, Hainan, Hebei, Heilongjiang, Henan, Hubei, Hunan, Jiangsu, Jiangxi, Jilin, Liaoning, Nei Mongol, Shaanxi, Shandong, Shanxi, Sichuan, Taiwan, Yunnan, Zhejiang [Bangladesh, India, Japan, Kashmir, Korea, Russia (Far East)].

The *Euryale ferox* plants was collected from suzhou, China. The collection of this plant materials comply seriously with the Chinese and international guidelines. *E. ferox* (*Euryale ferox* Salisb.) has not been listed in the appendices I, II and III of the Convention on the Trade in Endangered Species of Wild Fauna and Flora which was validated from Jan 2th, 2017 (<https://cites.org/eng/app/appendices.php>). The collector had deposited in 2015 with barcode ID: [QFNU0018115](http://www.cvh.ac.cn/en/spm/QFNU/QFNU0018115). (<http://www.cvh.ac.cn/en/spm/QFNU/QFNU0018115>). Guo CY undertook the formal identification. *Euryale ferox* plants were grown in Yangzhou University Aquatic Vegetable Test Base, normal cultivation management. Seeds of *E. ferox* were collected at 10, 20, 30 and 40 days (stages T1, T2, T3, and T4, respectively) after anthesis. Seed samples with three biological replicates per developmental stage were utilized. All samples were frozen in liquid nitrogen and immediately stored at -80 °C.

Observation of Amyloid in *E. ferox* seeds

Euryale ferox seeds were cut into approximately 5 mm x 5 mm x 5 mm small blocks, immediately placed in 0.1 mol/L glutaraldehyde fixation fluid, refrigerated at 0 to 4 °C, and used to observe the starch grain morphology. Cut kernels were removed from glutaraldehyde fixative before observation and washed 3-4 times with double distilled water. Then, 30%, 50%, 70%, 80%, 90%, 95%, 100% and then 100% (addition of anhydrous Na₂SO₄) ethanol was added for dehydration and drying after the completion of dehydration (CPD-300 type critical point dryer). After drying, the observed surface of the *Euryale ferox* seed was placed onto the stage, face up, and the surface was sprayed with gold in an SCD 500 ion sputter. Finally, the amyloplast and starch granule structure were subjected to S-4800II field emission scanning electron microscopy (FESEM) for morphological observation and imaging.

Starch content determination

The total starch content, amylose content, amylopectin content, and amylose and amylopectin content ratios of *Euryale ferox* seeds were determined using the Megazyme Total Starch Kit (K-TSTA 09/14, Megazyme International Ireland Ltd.) and the Megazyme Straight Ratio Kit (K-AMYL 09/14, Megazyme International Ireland Ltd.).

Total starch content

Mill cereal, plant or food products were passed through a 0.5 mm screen. An accurate weight of ~ 100 mg of test sample, in duplicate (one as a sample blank), was placed into Corning culture tubes (16 x 120 mm) [C(q)], and the exact weights were recorded. The tubes were tapped so that the samples dropped to the bottom of the tubes. Then, 10 mL of 100 mM sodium acetate buffer, pH 5.0, containing 5 mM CaCl₂ [B(a)] was added to each tube using a Brand Bottle-top dispensette [C(m)]. The tubes were vigorously stirred on a vortex mixer for 5 s. Next, 0.1 mL of undiluted thermostable α-amylase [A(1)] (Megazyme cat. no. E-BSTAA) was added to one of the tubes using a HandyStep® dispenser [C(l)] with a 5 mL tip. The second tube (sample blank) received 0.1 mL of 100 mM sodium acetate buffer (pH 5.0) [B(a)]. The tubes [C(j)] were vortexed for 3 s, the tubes were loosely capped and immediately transferred to a boiling water bath. After approx. 2 min, tube caps were tightened, and the contents were vigorously mixed on a vortex mixer. After an additional 5 and 10 min, tube contents were vortexed again for 5 s and returned to the boiling water bath. After 15 min (from addition of α-amylase), the tubes were removed from the boiling water bath, and the contents were vigorously mixed for 5 s on a vortex mixer. The tubes were then placed in a water bath at 50°C and allowed to equilibrate to temperature for 5 min. Then, 0.1 mL of undiluted AMG [A(2)] (Megazyme cat. no. E-AMGDF; 3,300 U/mL) was added to the sample tube using a HandyStep® dispenser with a 5 mL tip and vortexed for 3 s. To the second tube (the sample blank), 0.1 mL of 100 mM sodium acetate buffer (pH 5.0) [B(a)] was added. The tubes were incubated at 50°C for 30 min with no further mixing. Then, the tubes were removed from the water bath and allowed to cool to room temperature for 10 min. The tubes were inverted a few times to ensure that the condensed water on the inside of the lid was mixed with liquid in the tube. Then, 2.0 mL of each solution (sample and sample blank) was transferred to microfuge tubes [C(o)] that were centrifuged at 13,000 rpm for 5 min (the remaining 8.2 mL of incubation solution was retained; refer to the NOTE below). Using a Gilson Pipetman

dispenser, a 1.0 mL aliquot of the supernatants was accurately transferred to 12 x 120 mm tubes containing 4 mL of 100 mM sodium acetate buffer (pH 5.0), and the contents were mixed. Then, 0.1 mL aliquots of each sample were transferred in duplicate to the bottoms of 16 x 120 mm glass test tubes. A single 0.1 mL aliquot of the blank sample was also transferred to a 16 x 120 mm glass test tube, and 3.0 mL of GOPOD reagent was added with subsequent incubation of the solutions at 50°C for 20 min. The absorbance was then measured at 510 nm and compared with the reagent blank.

Amylopectin content and amylose/amylopectin content ratios

Starch Pretreatment

The starch or flour samples were accurately weighed (20-25 mg to the nearest 0.1 mg) into 10 mL screw capped Kimax® sample tubes. The sample weight was recorded to the nearest 0.1 mg. To the tube, 1 mL DMSO was added while gently stirring at low speed on a vortex mixer. The tube was capped, and the tube contents were heated in a boiling water bath until the sample was completely dispersed (approx. 1 min) until no gelatinous lumps of starch remained. The contents of the sealed tubes were vigorously mixed at high speed on a vortex mixer, and the tubes were placed in a boiling water bath and heated it for 15 min with intermittent high-speed stirring on a vortex mixer. The tubes were stored at room temperature for approx. 5 min, and 2 mL of 95% (v/v) ethanol was added with continuous stirring on a vortex mixer. An additional 4 mL of ethanol was added, and the tubes were capped and inverted to mix. A starch precipitate formed. The tubes were allowed to stand for 15 min (or overnight if desired). The tubes were centrifuged at 2,000 *g* for 5 min, the supernatants were discarded and the tubes were drained on tissue paper for 10 min until all of the ethanol was removed. The pellet was used in subsequent amylose and starch measurements. A volume of 2 mL DMSO was added to the starch pellet with gentle vortex mixing. The tubes were placed in a boiling water bath for 15 min and mixed occasionally to ensure that there were no gelatinous lumps. 7. Upon removing tubes from the boiling water bath, 4 mL Con A solvent (Buffer 3; page 4) immediately added and mixed thoroughly, and then the tube contents were completely transferred (by repeated washing with Con A solvent) to a 25 mL volumetric flask. The volume was diluted with Con A solvent (**this is Solution A**). When necessary, this solution was filtered through Whatman® No. 1 filter paper (this step was necessary for the whole flour samples).

B. Con A Precipitation of Amylopectin and Determination of Amylose

A total of 1.0 mL **Solution A** was transferred to a 2.0 mL Eppendorf® microfuge tube. A volume of 0.50 mL Con A solution (bottle 1) was added, and the tube was capped and gently mixed by repeated inversion. The tube was allowed to stand for 1 h at room temperature. The tube was centrifuge at 14,000 *g* for 10 min in a microfuge at room temperature. A volume of 1 mL of the supernatant was transferred to a 15 mL centrifuge tube. A total of 3 mL of 100 mM sodium acetate buffer, pH 4.5, was added. This buffer reduced the pH to ~ 5. The contents were mixed, lightly stoppered (with a marble) and heated in a boiling water bath for 5 min to denature the Con A. The tube was placed in a water bath at 40°C and allowed to equilibrate for 5 min. A volume of 0.1 mL of a amyloglucosidase/ α -amylase enzyme mixture (page 3; solution 2) was added and incubated at 40°C for 30 min. The tube was centrifuged at 2,000 *g* for 5 min. To 1.0 mL aliquots of the supernatant, 4 mL of GOPOD Reagent (Reagent B) was added. The

solution was incubated at 40°C for 20 min, and the **Reagent Blank** and the **D-Glucose Controls** were incubated concurrently. The absorbance of each sample and the D-glucose controls were read at 510 nm and compared with the reagent blank.

Protein extraction, iTRAQ labeling and LC-ESI mass spectrometry (MS)/MS analysis

All analyses were performed by a Triple TOF 5600 mass spectrometer (SCIEX, USA) equipped with a Nanospray III source (SCIEX, USA). The samples were loaded by a capillary C18 trap column (3 cm × 100 µm) and then separated by a C18 column (15 cm × 75 µm) on an Eksigent nanoLC-1D plus system (SCIEX, USA). The flow rate was 300 nL/min, and the linear gradient was 90 min (from 5 – 85% B over 67 min; mobile phase A = 2% ACN/0.1% FA and B = 95% ACN/0.1% FA).

The data were acquired with a 2.4 kV ion spray voltage, 35 psi curtain gas, 5 psi nebulizer gas, and an interface heater temperature of 150°C. The MS scanned between 400 and 1500 with an accumulation time of 250 ms. For IDA, 30 MS/MS spectra (80 ms each, mass range 100 – 1500) of MS peaks above intensity 260 and with a charge state of between 2 and 5 were acquired. A rolling collision energy voltage was used for the CID fragmentation for the acquired MS/MS spectra. The mass was dynamically excluded for 22 s.

WGCNA Network Analysis

We utilized the *Euryale ferox* seed Illumina RNA-seq data that were previously generated and analyzed by Liu et al. The gene co-expression network using differentially expressed genes was constructed using the WGCNA package in R [41]. A total of 23 different modules were derived as described. Module hub genes were used as seed nodes to extract the gene co-expression network, and the resulting gene-gene interactions were used to visualize the subnetworks using Cytoscape. In standard WGCNA networks, the power was set to 6, minModuleSize was set to 30, and initial clusters were merged on eigengenes. The merge CutHeight value was set to 0.25 across all networks. The total connectivity was calculated for all genes in each network.

Function annotations

The DEGs or DEPs were mapped to Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (<http://www.genome.jp/kegg/>).

Measurements of hormone content

The endogenous hormone contents in *Euryale ferox* seeds were measured. Each sample was prepared by three replicate samples. High-performance liquid chromatography-mass spectrometry was used to measure hormone. (AB 5500, Beijing, China) following the protocol described previously [54].

Quantitative real-time PCR analysis

Total RNA was extracted from triplicate samples from the four developmental stages of *E. ferox* seeds (total of 12 samples) using an RNA kit (RNA simply Total RNA Kit, Tiangen, Beijing, China) according to the manufacturer's instructions. Five micrograms of each sample was reverse transcribed into cDNA using the Prime Script RT reagent Kit (TaKaRa). Gene-specific primers for the three genes sequences were designed using Primer Premier 5 software. The β -Actin gene was amplified with forward primer 5'-GACTCTGGTGATGGTGT-3' and reverse primer 5'-CACTTCATGATG GAGTTGT-3'. The amplifications were performed in 20 μ l reactions consisting of 10 μ l 2 $\times$ SYBR® Premix EX TaqII (Tli RNaseHPlus) (TaKaRa, Dalian, China), 2.0 μ l of a mixture of the forward and reverse primers, 2.0 μ l cDNA template, and 6.0 μ l sterile ddH₂O. A detection System (Bio-Rad, Hercules, CA, USA) was used with the following cycling parameters: 95 °C for 3 min, followed by 39 cycles of 95 °C for 30 s and T_m for 30 s.

Abbreviations

WGCNA

Weighted Gene Co-expression Network Analysis

DEG

Differentially expressed gene

DEP

Differentially expressed protein

FPKM

Fragments per kilobase of exon per million fragments mapped

Declarations

Availability of data and materials

Not applicable.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

W. conceived and designed the experimental design. X.L1(Xiang Li)., X.X., X.L2(Xian Liu)., and X.Z. contributed to the experimental work. L. L.contributed to the interpretation of the results and coordinated the study. All the authors read and approved the final manuscript.

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Figures

Figure 1

Growth and development of *Euryale ferox*.

Figure 2

Euryale ferox fruit, seed and kernel during four developmental stages (T1, T2, T3 and T4).

Figure 3

Changes in *Euryale ferox* seeds during four development stages. (a) Seed weight; (b) Amylopectin content; (c) Amylose content; (d) Ratio of amylopectin to amylose content.

Figure 4

Changes in amyloids and starch grains in seed development in *Euryale ferox* seeds.

Figure 6

Correlations between protein and messenger ribonucleic acid (mRNA) expression. The x-axis represents protein expression levels, and the y-axis represents gene expression levels. Scatterplots and correlation coefficients between differentially expressed proteins (DEPs) and differentially expressed genes (DEGs).

Figure 7

DEPs and DEGs associated with hormones during development of *Euryale ferox* seeds.

Figure 8

Network hubs regulating genes in different seed developmental stages. (a) Network hubs (EfPYR1, EfSnRK2.1 and EfSnRK2.2) regulating genes in differentiation stage; (b) Expression patterns of regulating genes; (c) Correlations of regulating genes; Each correlation is shown by circle shapes of blue and red. Blue indicates a positive correlation, and red indicates a negative correlation.

Figure 9

Expression patterns of hub genes and the content of ABA in *Euryale ferox* seeds.

Figure 10

Schematic diagram showing the putative role of ABA in promoting starch synthesis in *Euryale ferox* seeds.

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