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Article

UID Transcriptomic Discovering Hub Genes Involving in Seed Development and Lipid Biosynthesis in Sea Buckthorn

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Abstract

Sea buckthorn is a vital woody oil species valued for soil conservation and bioactive seed oil rich in unsaturated fatty acids and other compounds. However, low oil content in the seeds and small seeds are the main bottleneck restricting the development and utilization of sea buckthorn. In this study, we tested seed traits of 12 sea buckthorn cultivars and identified the key genes and transcription factors involving in seed development and lipid biosynthesis by integration of UID RNA-seq (Unique Identifiers, UID), WGCNA and qRT-PCR analysis. Results identified five cultivars (CY02, CY11, CY201309, CY18, CY21) with significantly higher oil contents and five cultivars (CY10, CY201309, CY18, CY21, CY27) with significantly heavier seeds. A total of 10873 genes were significantly differentially expressed between S1 and S2 seed developmental stages of 12 cultivars. Response regulator (*RRS-6*), auxin response factor (*ARF-7* and *ARF-20*) in the KEGG pathway of “Circadian rhythm – plant” and “Plant hormone signal transduction” were related to seed development. GPAT family, DGAT family and PDAT family in the KEGG pathway of “Glycerolipid metabolism” were related to lipid biosynthesis. WGCNA identified five modules related to seed oil content and seed weight/size, and 417 candidate genes were screened from these modules. In which, multiple hub genes and transcription factors were identified, such as ATP synthase, ATP synthase subunit D and Acyl carrier protein 1 were related to seed development; plastid-lipid-associated protein, acyltransferase-like protein, and glycerol-3-phosphate 2-O-acyltransferase 6 were involved in lipid biosynthesis, and transcription factors of *Dof1.2*, *bHLH137* and *ERF4* were associated with seed enlargement and development. These findings provide crucial insights into the genetic regulation of seed traits in sea buckthorn, offering targets for future breeding efforts aimed at improving oil yield and quality.

Keywords: sea buckthorn; seed development; lipid biosynthesis; UID RNA-seq; WGCNA

1. Introduction

Sea buckthorn (*Hippophae rhamnoides* L.), a perennial shrub or small tree belonging to the Elaeagnaceae family, mainly distributes in the temperate regions of the Eurasian continent [1]. As an important woody plant for soil and water conservation and desertification control, it could grow on marginal land such as sandy land, barren mountains and saline-alkali land, which can avoid land competition with field oil crops [2]. Sea buckthorn seed oil contains more than 140 bioactive components including unsaturated fatty acids, flavonoids, organic acids, alkaloids, sterols, triterpenes and various vitamins, which has high medicinal and edible value [3]. With the increase of global consumption of sea buckthorn seed oil, the demand gap is widening. The main reason of the low yield of seed oil of sea buckthorn is from its small seed and low seed oil content [4]. There are

significant differences in seed size, grain weight, and seed oil content among different cultivars of sea buckthorn [5]. The mining and comparative analysis of key genes during the seed development of different cultivars of sea buckthorn may be helpful to understand the regulatory mechanism of seed development and lipid biosynthesis, which is of great significance to improve the yield and quality of its seed oils.

Seed size and oil content are crucial indexes in the evaluation of yield and quality in plants. To date, the genes regulating seed size and oil content have been identified in various plants, including *Arabidopsis*, soybean, maize and peanut. For example, through genome-wide association studies (GWAS) of over 1,800 soybean materials, *GmST05* was identified; its overexpression and knockout in soybean significantly increased and decreased seed size, respectively [6]. Overexpression of *GmAP2-1* and *GmAP2-4* in soybeans could increase seed size and weight [7]. Overexpression of the *AtSHB1* gene in Canadian rapeseed produced larger seeds [8]. Overexpression *OsFBN1* increased the tiller number but decreased the panicle length, grain-filling percent and JA levels compared to the wild type and RNAi silencing lines under heat stress [9].

Transcription factors play a crucial role in regulating seed size and oil content. A zinc-finger transcriptional regulator overlapping with several seed weight quantitative trait loci (QTLs), known as *GmPLATZ*, was discovered to be essential in promoting seed weight/size in soybeans, and its overexpression obviously enhanced seed weight/size [10]. bHLH proteins were specifically expressed in seeds 5-20 days after flowering, leading to a maximum increase of 13.81% in grain weight in wheat and 18.55% in rice [11]. The expression patterns of the rice 16 *Dof* gene during seed development were different, indicating that the *Dof* gene might be involved in the ethylene and JA signaling pathways, thereby affecting grain filling and quality [12]. Overexpression of *LuWRI1a* increased seed weight/size, oil content in *Arabidopsis* and also increased seed oil reserves in transgenic flax [13]. The transcription factor inhibited the expression of downstream auxin genes, regulating silique cell wall development and seed weight in rapeseed [14].

Weighted gene co-expression network analysis (WGCNA) is an efficient method to analyze and mine high-throughput sequencing data, which can accurately identify hub genes, co-expression modules and regulatory networks that are essential for seed development and lipid metabolism [15]. In the WGCNA analysis, the genes between the samples were divided into modules according to a certain group of co-expressed genes, and then these modules and phenotype data were used to cluster and find the hub genes in the module. In soybean, 946 protein-related and 4,815 fatty acid-associated hub genes were identified by the WGCNA analysis, including known regulators like *VPS35*, *ABI3b*, and *LEC1d* homologs, as well as novel candidates potentially bridging *WRI1* and *LEAF* networks for oil/protein accumulation [16]. Similarly, in peanut and maize, WGCNA linked seed weight/size traits to modules containing auxin-responsive *ZmARF12* (a cell division repressor) and ribosomal/transcription factor (TF) hubs, demonstrating its power to uncover conserved and species-specific regulatory mechanisms governing seed composition and morphology [17,18].

High-throughput sequencing technology has been applied to identify key genes involved in seed development and lipid metabolism in sea buckthorn [19]. High-throughput RNA sequencing revealed dynamic gene expression patterns during sea buckthorn seed development, identifying 1,737 lipid metabolism-related unigenes and key regulators, such as delta9-desaturases (*ACP-Δ9D*), *DGAT1*, and *WRI1* that orchestrate fatty acid biosynthesis and triacylglycerol accumulation [5,20]. Integrated small RNA and transcriptome sequencing further uncovered miRNA-transcription factor co-regulatory networks, including 27 miRNAs and 31 transcription factors linked to lipid synthesis and seed development, with critical hubs like miR164d-*ARF2* and novelmiRNA-58-*GPD1* modulating lipid precursors and seed oil content [21]. However, the genes associated with seed development and oil accumulation in sea buckthorn have yet to be systematically explored, and there is a paucity of research employing WGCNA to investigate genes associated with seeds development and oil accumulation in sea buckthorn.

In this study, seeds from 12 sea buckthorn cultivars at two developmental stages were collected, then UID RNA-seq and WGCNA analysis were performed to identify the key genes and transcription

factors regulating seed development and lipid biosynthesis. Furthermore, candidate hub genes were identified through quantitative real-time PCR analysis. The detailed functional investigation of the networks and hub genes and transcription factors could further improve the understanding of the key genes that control seed development and lipid biosynthesis, which were significant for quality and yield improvement of sea buckthorn.

2. Results

2.1. Seed Traits

At the S1 developmental stage of seeds, the oil contents of ‘CY02’, ‘CY32’, ‘CY10’, ‘CY11’, ‘CY201309’, ‘CY18’ and ‘CY27’ were relatively higher than those of the other five cultivars (0~S1 vs. S1~S2), while the seed oil contents of ‘CY02’, ‘CY11’, ‘CY201309’, ‘CY18’ and ‘CY21’ were relatively higher than those of the other seven cultivars at the S2 stage (0~S1 vs. S1~S2) (Figure 1A). Compared to the S1 stage, the seed oil contents increased 1.05 to 1.51-fold for all the cultivars at S2 stage.

At the S1 stage, the values of TSW of ‘CY10’, ‘CY201309’, ‘CY18’, ‘CY21’ and ‘CY27’ were significantly higher than those of the other seven cultivars, while the values of TSW of ‘CY04’, ‘CY10’, ‘CY201309’, ‘CY18’, ‘CY21’ and ‘CY27’ were relatively higher than those of the other six cultivars at the S2 stage. From S1 to S2 stage, the values of TSW of each cultivars increased 1.02- to 1.81-fold (Figure 1B). The TD and LD values of ‘CY10’, ‘CY201309’, ‘CY18’, ‘CY21’ and ‘CY27’ seeds were larger than those of the other seven cultivars (Figure. 1C-D). The TD and LD values had a similar expression trend with thousand grain weight at developing seeds, namely higher TSW showed larger TD and LD.

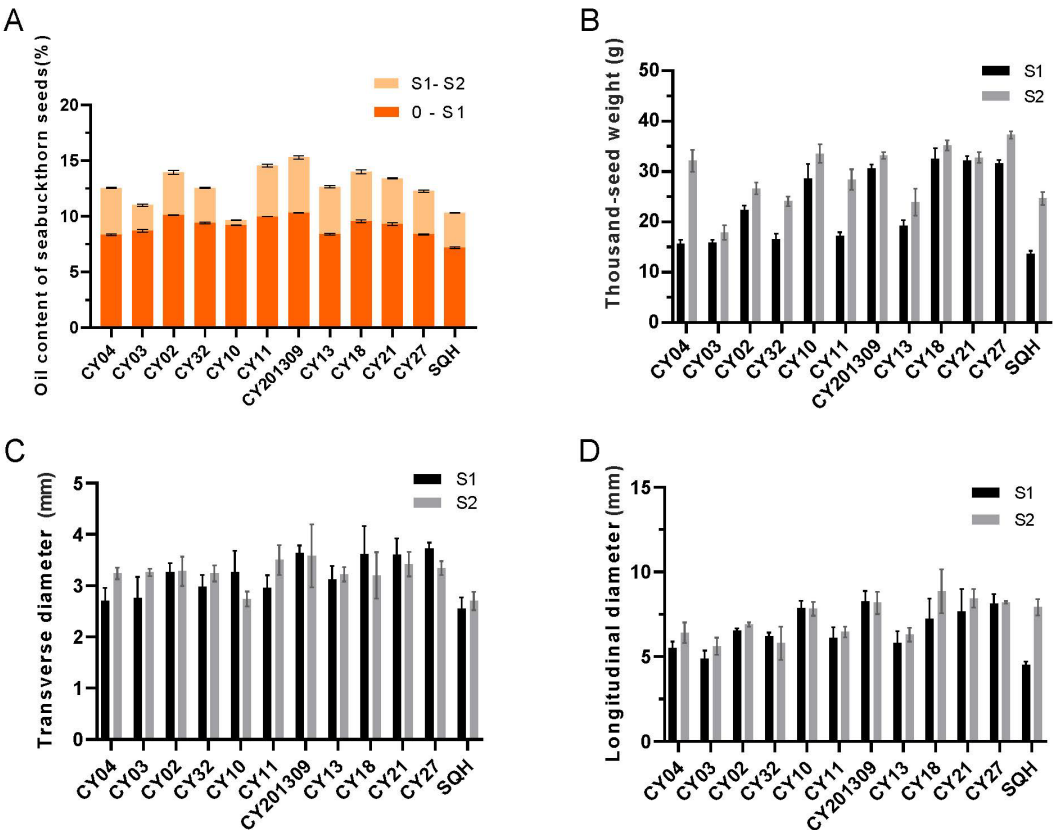


Figure 1. The oil content, thousand-seed weight (TSW) and size of developing sea buckthorn seeds. (A) Oil contents in seeds from twelve cultivars ‘CY04’, ‘CY03’, ‘CY02’, ‘CY32’, ‘CY10’, ‘CY11’, ‘CY201309’, ‘CY13’, ‘CY18’, ‘CY21’, ‘CY27’, and ‘SQH’ at two development stages. (B) TSW in seeds from twelve cultivars at two development stages. (C) The transverse diameters from twelve cultivars at two development stages. (D) The

longitudinal diameters from twelve cultivars at two development stages. Error bars indicate standard deviations of three biological replicates. *Indicate significant differences of data between the two cultivars at the same developmental stage at the level of 0.05.

2.2. Analysis of UID Transcriptome Data

In total, about 4.2 billion raw reads were generated, with an average of 59 million reads per sample. After removing adapter sequences, duplicated sequences, ambiguous reads and low-quality reads, a total of 492.01 Gb clean data were generated; 98.34-99.11 % of bases had a Q of ≥ 20 , 94.08-96.40 % of bases had a Q of ≥ 30 , and the reads had a 43.58–46.92% GC content. Genes with normalized reads lower than 0.5 RPKM were removed from the analysis. There were 10,662 unigenes (98.06%) annotated in the uniprotAc database, 6,749 unigenes (62.08%) annotated in the Refseq database, 6,759 unigenes (62.16%) annotated in the Pfam database, 9,649 unigenes (88.74%) annotated in the interpro database, 1325 unigenes (6.84%) annotated in the eggNOG database, 7,992 unigenes (73.50%) annotated in the Gene Ontology (GO) database and 1035 unigenes (9.52%) annotated in the KEGG database.

2.3. Identification of Differentially Expressed Genes and Functional Annotation

Differential expression analysis showed that 10873 DEGs were screened between S1 and S2 stages for 12 cultivars, which included 6,830 up- and 4,043 down regulated genes. The GO terms of DEGs related pathways belong to three categories: biological process (BP), cellular component (CC), and molecular function (MF) (Figure 2). For the BP ontology, the representational enriched terms were photosynthesis, monocarboxylic acid biosynthetic process and monocarboxylic acid metabolic process. For the MF ontology, the representational enriched terms were cofactor binding, oxidoreductase activity and lyase activity.

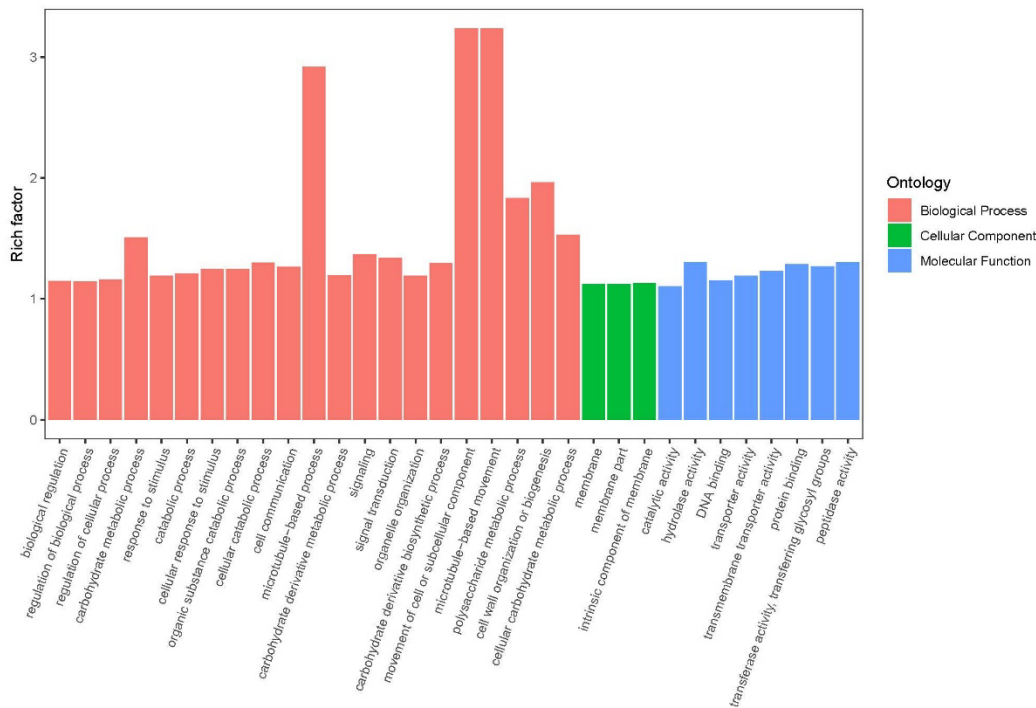


Figure 2. Gene Ontology analysis of DEGs.

DEGs were mapped to the KEGG database and the enriched KEGG data were filtered ($p < 0.05$). According to the KEGG annotations, 26 KEGG pathways were significantly enriched in upregulated and downregulated, the DEGs were mapped to the reference canonical pathways in the KEGG database (Figure 3). As a result, a total of 1990 upregulated DEGs were enriched into 15 pathways.

The upregulated DEGs significantly enriched in the pathway of “Stilbenoid, diarylheptanoid and gingerol biosynthesis”, “Photosynthesis - antenna proteins”, “Other types of O-glycan biosynthesis”, “Thiamine metabolism” and “Phenylalanine, tyrosine and tryptophan biosynthesis” (Figure 3A). A total of 354 downregulated DEGs were enriched into 11 pathways. The downregulated DEGs significantly enriched in the pathways of Spliceosome, Galactose metabolism, Carotenoid biosynthesis and Biosynthesis of unsaturated fatty acids (Figure 3B).

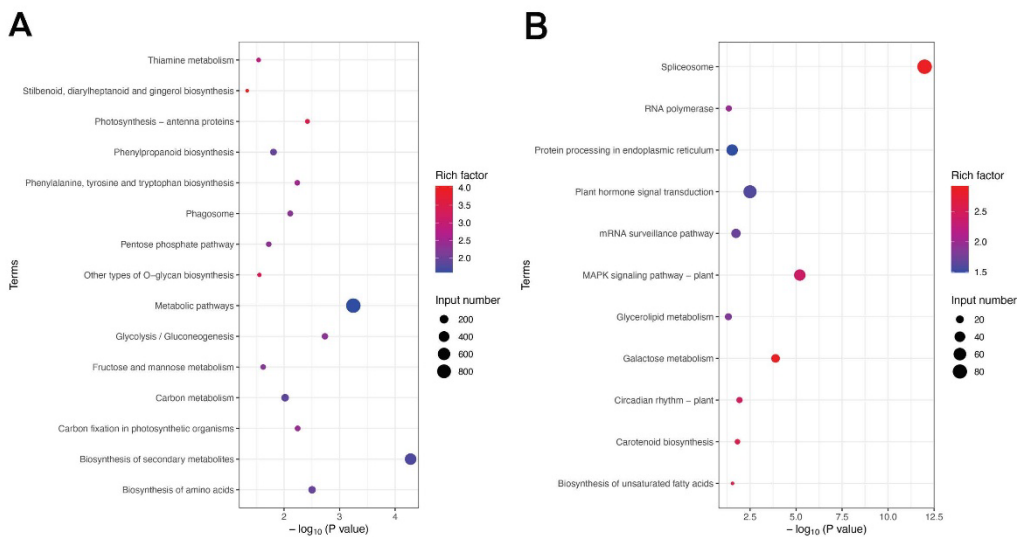


Figure 3. KEGG enrichment analysis of DEGs. Top 20 KEGG up (A) and down (B) pathways of S1 vs S2. The abscissa is the P value, and the ordinate is the KEGG path name. The greater the rich factor (P value), the more significant the enrichment level of DEGs in this pathway. The color of the circle represents the degree of enrichment of the rich factor. The size of the circle represents the number of DEGs contained in that KEGG pathway.

2.4. Differentially Expressed Genes (DEGs) Involved in Seed Development

A total of 99 DEGs associated with seed development were identified, including two abscisic acid (ABA) genes, two brassinosteroid insensitive 1 (BRI1) genes, five choline ethanolamine kinase (CEK) genes, two cellulose synthase A (CESA) genes, three cytokinin oxidase (CKX) genes, three DA1-related protein (DA1) genes, two fertilization independent endosperm (FIE) genes, three GRAS genes, four histone deacetylase (HDA) genes, five haiku (IKU) genes, four histidine phosphotransfer (HPT) genes, seven methyltransferase (MET) genes, 16 response regulators (RRS) genes, 10 scarecrow-like (SCL) genes, one shatterpppoo (SHP) genes, two target of rapamycin (TOR) genes and 28 ubiquitin specific protease (UBP) genes, and the total RPKM of each transcript for these genes was greater than 20 (Supplementary Table S1). The heatmap of candidate genes related to seed development showed that most of these genes were highly expressed in both S1 and S2 stages of 12 cultivars (Figure 4). For five higher TSW cultivars (‘CY10’, ‘CY201309’, ‘CY18’, ‘CY21’ and ‘CY27’), the genes of RRS-6, MET-3 and HPT-2 highly expressed at the S1 stage, the SCL-5, HPT-4 and HAIKU-5 genes highly expressed at the S2 stage, and the genes of GRAS-2, GRAS-3, ABA-1, UBP-19, FIE-2, UBP-22, SCL-10, UBP-8 and UBP-24 highly at the S1 and S2 stages. The expression levels of above candidate genes appeared to have a similar trend for TD and LD in most cultivars.

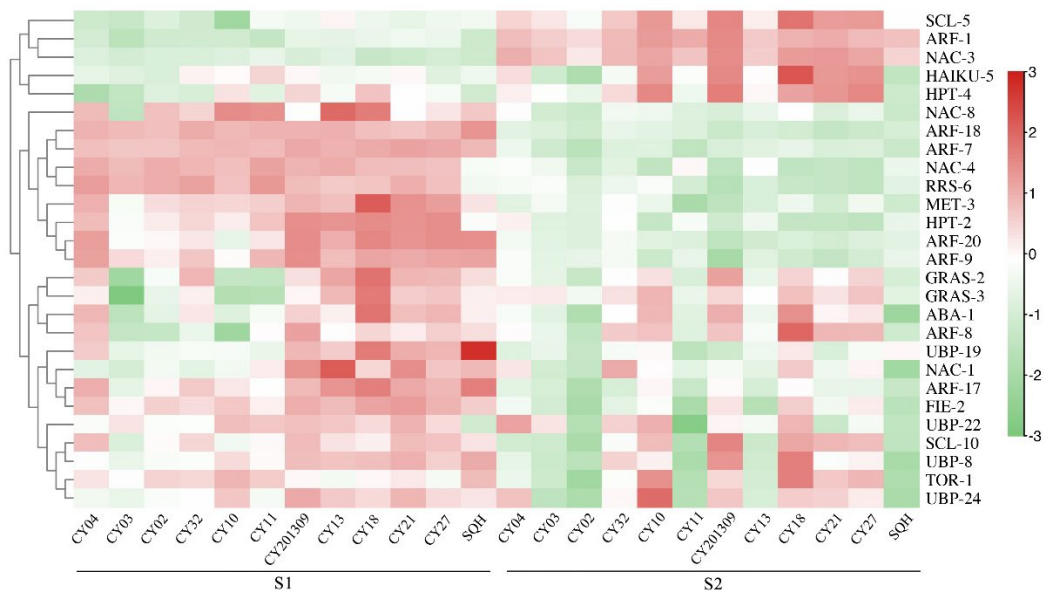


Figure 4. Heatmap analysis of significant DEGs associated with seed development from two developmental stages in twelve cultivars. The expression value (in RPKM) for the DEGs during S1 and S2 development in both cultivars was log2 transformed, and the total RPKM value was greater than 20. lipid biosynthesis.

A total of 31 transcription factors associated with seed development were identified, including 20 auxin response factor (ARF), one leafy cotyledon (LEC), eight nac transcription factor (NAC) and two transparent testa 1 (TT1). The heatmap of transcription factors related to seed development showed that most of these genes were expressed in both developmental stage S1 and S2 of 12 cultivars (Figure 4). For five higher TSW cultivars (‘CY10’, ‘CY201309’, ‘CY18’, ‘CY21’ and ‘CY27’), the ARF-7, ARF-18, NAC-4, ARF-9, ARF-20 and LEC highly at the S1 stage, the NAC-3 and ARF-1 highly expressed at the S2 stage, and the ARF-8, NAC-1 and ARF-17 highly expressed at the S1 and S2 stages. The above DEGs expression appeared to have a similar trend for TD and LD in most cultivars.

2.5. Differentially Expressed Genes (DEGs) Involved in Lipid Biosynthesis

A total of 78 DEGs associated with seed lipid biosynthesis were identified, including seven glycerol-3-phosphate dehydrogenase (GPD1) genes, 17 glycerol-3-phosphate acyltransferase (GPAT) genes, one lysophosphatidylcholine acyltransferase (LPCAT) gene, one lysocardiolipin and lysophospholipid acyltransferase (LCLAT) gene, three diacylglycerol acyltransferase 1 (DGAT1) genes, three phospholipid diacylglycerol acyltransferase (PDAT) genes, rive lecithin-cholesterol acyltransferase (LCAT) genes, two threonine-protein kinase BAM (BAM) genes, one phosphoglycerate mutase (PGAM) gene, seven oleosin (OLE) genes, 11 long chain acyl-CoA synthetase (ACSL) genes, six acyl carrier protein (ACP) genes and 14 ketoacyl-CoA synthase (KCS) genes (Figure 5). The total RPKM of each transcript for these genes was greater than 20 (Supplementary Table S2). The expression levels of OLE family genes exhibited significant differences between the S1 and S2, with a marked surge in expression levels at the S2 stage. The increase was particularly pronounced in some cultivars, for example, for the higher OC cultivars (‘CY11’ and ‘CY201309’), OLE-6 exhibited significant stage-specific expression differences, particularly in the CY11 and CY201309, in which its expression level at the S2 stage was 201-fold and 104-fold higher than that at the S1 stage.

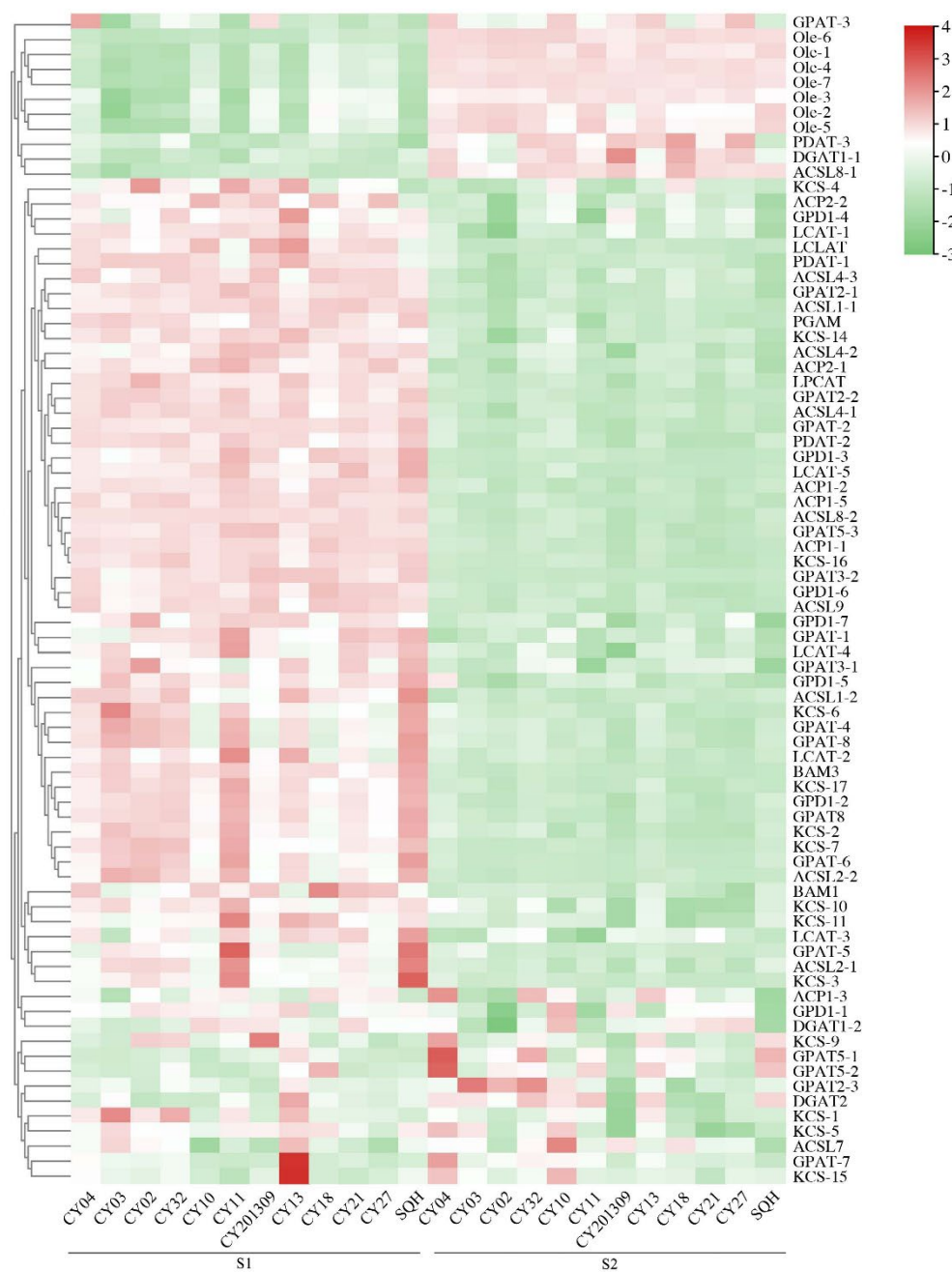


Figure 5. Heatmap analysis of significant DEGs associated with lipid biosynthesis from two developmental stages in twelve cultivars. The expression value (in RPKM) for the DEGs during S1 and S2 development in both cultivars was log2 transformed, and the total RPKM value was greater than 20.

The expression levels of *GPD1-6* were the highest among *GPD1* family genes, showing significantly higher values in most cultivars at the S1 stage, compared to S2. *GPD1-2* exhibited significant stage-specific expression differences, particularly in the high oil content cultivar CY11, in which its expression level at the S1 stage was 40-fold higher than that at the S2 stage. Both *GPAT-4* and *GPAT-8* demonstrated elevated expression at the S1 stage, with their expression levels in CY11 being 46- and 47-times higher in S1 than in S2, respectively. In contrast, the *DGAT* gene family showed significantly higher expression at S2 compared to S1. *ACSL2-2*, *ACSL4-1*, and *ACSL4-3* displayed relatively high expression levels at the S1 stage, and markedly decreased at the S2 stage. *KCS-17* exhibited exceptionally high expression at the S1 stage, reaching levels 26-fold greater than those observed at the S2 stage. Conversely, *KCS-10* and *KCS-14* showed increased expression at the

S2 stage in some cultivars. Notably, for the CY11 cultivar, the expression levels of *ACP1-1* and *ACP1-2* were dramatically reduced by 20-fold at the S2, compared to S1.

A total of 102 differentially expressed TFs were identified between S1 and S2 seed developmental stages of 12 cultivars. 12 transcription factors of *LEC1-1*, *Dof-11*, *Dof-14*, *WRI1-3*, *FOXO-7*, *SPT6-1*, *AP2/ERF-3*, *Dof-3*, *AP2/ERF-1*, *WRI1-5*, *Dof-7* and *FUS3* were upregulated in high oil content cultivars relative to other cultivars at the S1 stage. A total of eight transcription factors of *SPT5-1*, *SPT5-2*, *SPT16-1*, *Dof-19*, *FOXO-8*, *FOXO-11*, *ABI4-1* and *ABI4-2* were upregulated in high oil content cultivars relative to other cultivars at the S2 stage (Figure 6, Supplementary Table S3).

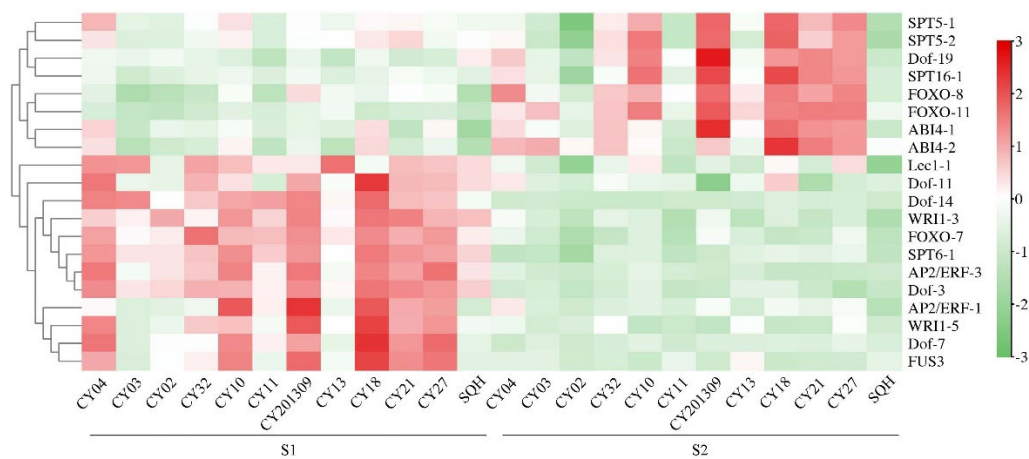


Figure 6. Heatmap analysis of significant TFs associated with lipid biosynthesis from two developmental stages in twelve cultivars. The expression value (in RPKM) for the DEGs during S1 and S2 development in both cultivars was log2 transformed, and the total RPKM value was greater than 20.

2.6. Co-Expression Network Analysis of DEGs by WGCNA

WGCNA analysis showed that the gene numbers of different modules varied greatly, ranging from 48 to 2389. A total of 17 different modules were identified (marked with different colors) (Figure 7), five of which was significantly associated with OC, TSW, TD and LD. The thistle1 module was significantly positively correlated with TSW (correlation coefficient $r = 0.92$), TD ($r = 0.88$) and LD ($r = 0.73$), the yellow4 module was significantly positively correlated with TD ($r = 0.83$), and the darkmagenta module was significantly positively correlated with TD ($r = 0.80$) (Figure 8).

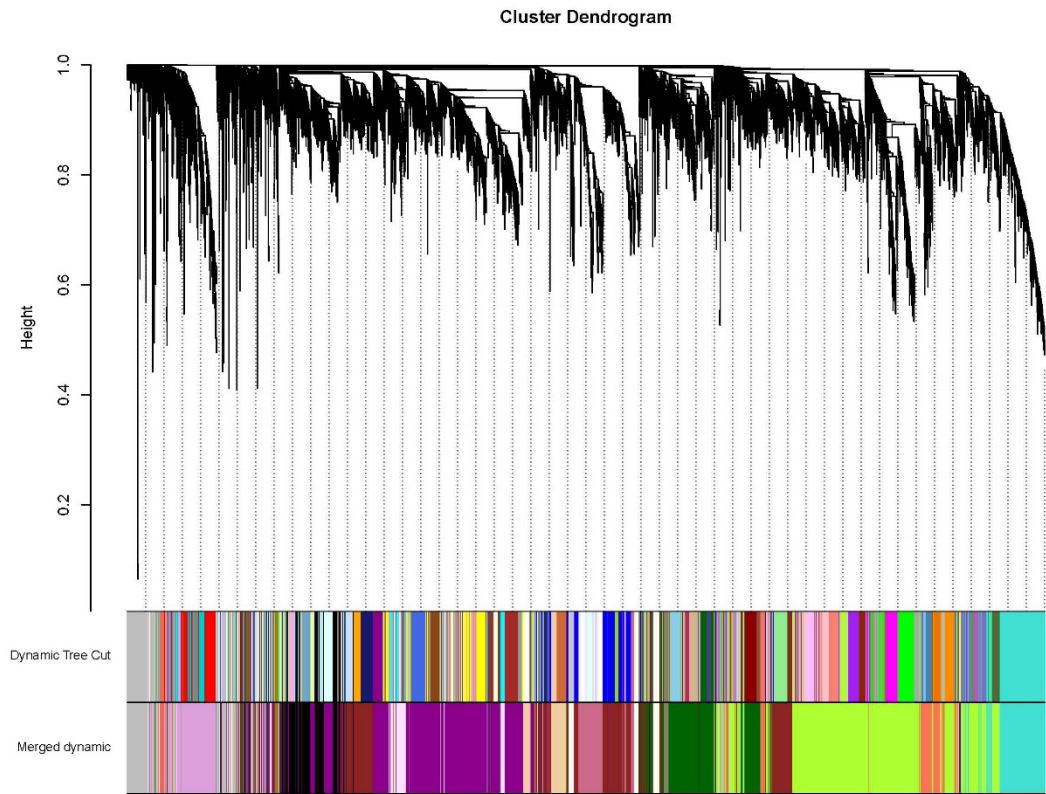


Figure 7. Weighted gene co-expression network analysis and visualization. (A) Hierarchical cluster tree showing co-expression modules identified by WGCNA. Each leaf (short vertical line) in the tree represented one gene. The major tree branches constitute 17 modules labeled by different colors.

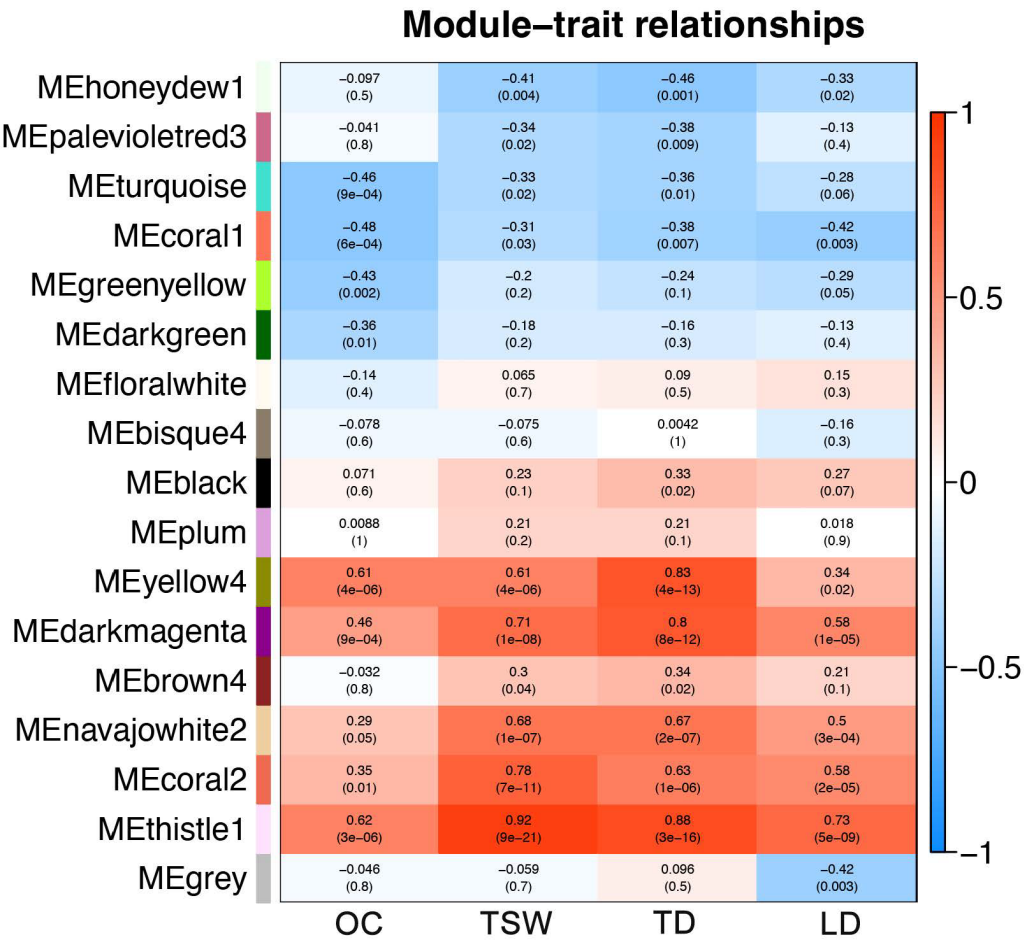


Figure 8. Module-trait relationships. Each row corresponded to a module, and each column corresponded to a trait. OC: oil content; TSW: thousand-seed weight; TD: transverse diameters; LD: longitudinal diameters.

In the co-expression network of DEGs, 177 hub genes with the highest kme values (kME > 0.95) were screened in five modules (Figure 9). In which, some hub genes were involved in lipid metabolism, such as *ACP1* (Hic_asm_3.979), *PAP* (Hic_asm_12.1324), *PAT* (Hic_asm_0.74), *GPAT6* (Hic_asm_3.866) and transcription factors of *DOF1.2* (Hic_asm_12.3128), *bHLH* (Hic_asm_0.1283), *bHLH137* (Hic_asm_22.1742) and *ERF4* (Hic_asm_3.2216) were associated with seed development.

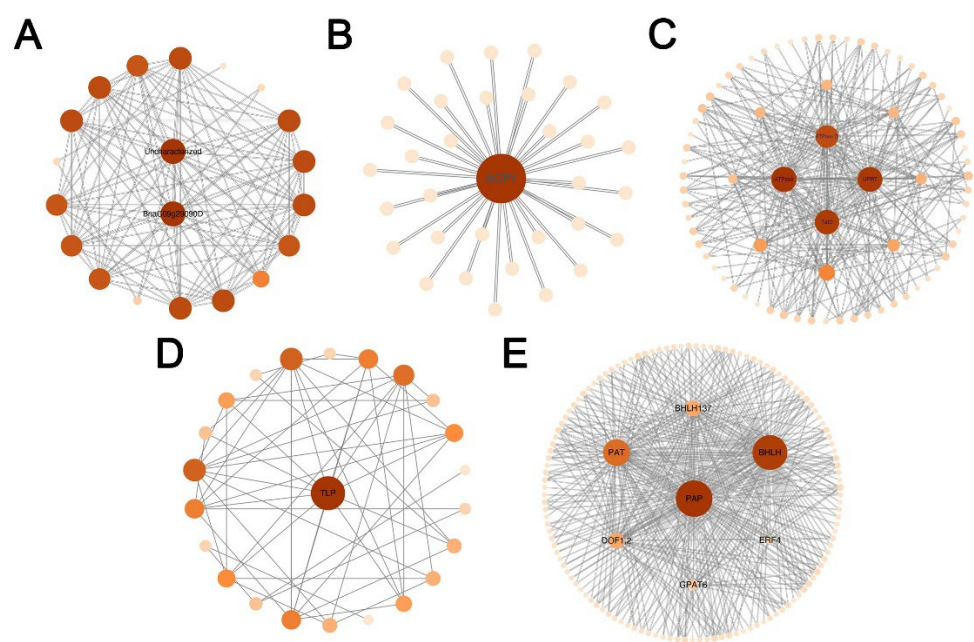


Figure 9. Gene co-expression network analysis in five modules of yellow4 (A), darkmagenta (B), navajowhite2 (C), coral2 (D) and thistle1 (E).

2.7. qRT-PCR of key DEGs

According to the results of qRT-PCR analysis, the changes in the expression of selected 12 genes related to lipid biosynthesis and seed development showed a similar tendency, compared to the RNA-seq data (Figure.S1, S2), indicating that UID transcriptomic profiling data were highly reliable.

3. Discussion

3.1. Key Genes Associated with Seed Development

ATP enzymes play an important role in promoting plant growth and development, it is a key membrane protein responsible for maintaining the proton gradient and ion balance within cells, thus affecting cell expansion and growth [22]. ATPase boosts plant growth by improving photosynthesis via stomatal control [23], enhancing stress resilience (salt/drought) [24], modulating hormone signals [25], and increasing adaptation in transgenic plants [26]. In this study, *ATPase* (Hic_asm_12.2935) and *ATPase D* (hic_asm_22.1085) were identified as both DEGs and hub genes in “MEnavajowhite” module. The correlation coefficient with TSW, TD and LD were 0.68, 0.67 and 0.5, respectively. Our findings revealed up-regulation of *ATPase* (Hic_asm_12.2935) and *ATPase D* (hic_asm_22.1085) genes during S2 development, with heightened expression levels observed at CY10, indicating that ATP enzymes contribute to cell enlargement and seed development of sea buckthorn.

ACP protein plays an important role in seed oil and fatty acid metabolism, and affects seed development and maturation by regulating fatty acid synthesis and desaturation process. Overexpression of the *ACP* gene in *Arabidopsis* significantly alters the fatty acid profile in seeds, increasing the proportion of unsaturated fatty acids, thereby enhancing the quality of seed oils [27]. Studies have shown that *ACP* in *Arabidopsis* can regulate the metabolism of long-chain fatty acids, thereby affecting the plant’s response to low-oxygen conditions and seed development and maturation [28]. During the S1 stage of sea buckthorn seed development, expression level of *ACP1* (Hic_asm_3.979) showed gradual upward trends, decreasing rapidly after the S2 stage. In the S1 stage of seed development, the expression of the *ACP1* gene in CY02, CY201309 and CY18 were rapidly upregulated, which was consistent with the trends of TSW, TD, and LD increasing gradually at this stage. Our findings indicated that *ACP* contributes to seed development of sea buckthorn.

3.2. Key Genes Associated with Lipid Metabolism

The *GPAT* gene family plays an important role in lipid biosynthesis, in which *GPAT6* primarily facilitates triacylglycerol (TAG) synthesis, a process critical for energy storage and membrane integrity [29,30]. The knockout mutant, *gpat6*, caused a massive reduction in seed production. The knockout mutant, *gpat6*, caused a massive reduction in seed production [31]. Secondly, *GPAT6* acts in concert with other acyltransferases to regulate lipid components and affect the fatty acid profile of the plant (Chen et al., 2016). In this study, *GPAT6* (Hic_asm_3.866) associated with lipid metabolism were identified as both DEGs and hub genes in module “MEthistle1”, its rapid increase was particularly pronounced in the higher OC cultivars (CY11, CY201309, CY18 and CY21). These indicated *GPAT6* promote lipid synthesis and accumulation during sea buckthorn seed development.

The *PDAT* gene is involved in TAG synthesis and shows different substrate specificities in different plants, which further affects the quality and composition of oil [32]. In *Brassica napus* [33] and *Gossypium* [34], it was found that the content of oils and the proportion of specific fatty acids were significantly increased by overexpressing *PDAT1* genes from other plants. Through functional studies of different acyltransferases, the scientists found that their interaction and substrate selectivity in vegetable oil synthesis have a significant impact on the final oil composition [35]. Certain acyltransferases exhibit a diversity of substrates during catalysis, which allows plants to synthesize a variety of different fatty acid derivatives, thus enriching the chemical composition of vegetable oils [36]. During the S1 stage of sea buckthorn seed development, expression level of *PAT* (Hic_asm_0.74) showed gradual upward trends, decreasing rapidly after the S2 stage. In the S1 stage of seed development, the expression of the *PAT* gene in CY10, CY201309, CY18 and CY21 were rapidly upregulated, which was consistent with the trends of TSW, TD, and LD increasing gradually at this stage. These suggests that *PAT* gene contributes the lipid biosynthesis in sea buckthorn seeds.

3.3. Key Genes Associated with Lipid Metabolism

Transcription factors (TFs) play pivotal roles in orchestrating seed development and grain weight determination. Among these, members of the bHLH family emerge as central regulators, governing seed size, shape, and developmental timing through stage-specific gene expression modulation and protein complex formation [37–40]. In our study, *bHLH* and *bHLH137* showed significantly higher expression in CY201309. *bHLH137* were upregulated in high TSW cultivars (CY201309 and CY27) relative to other cultivars at the S1 stage. Notably, *bHLH137* exemplified this functional significance in this study. Previous reports have found that its heterologous expression in *Pyrus bretschneideri* promoted cell expansion [41], while interactions with partners like PbGIF1 amplified its transcriptional activation capacity. Furthermore, *bHLHs* integrated hormonal signaling particularly auxin and gibberellin pathways to fine-tune seed development [42,43]. These multifaceted roles make bHLH proteins a major target for genetic improvement of seed traits.

ERF family transcription factors regulate seed size by interacting with stress-response networks and growth signals [44]. *ERF4* embodied these two functions: It regulated fruit ripening by inhibiting ethylene biosynthesis genes (*ACS1/ACO1*) through JAZ-MYC2 interaction [45], while its overexpression induced cell expansion, a mechanism directly implicated in its role in seed development [46]. Thus, *ERF* proteins link environmental adaptation and developmental programs and may have enhanced seed weight under complex conditions. In summary, *ERF4* may play multiple roles in regulating seed size and weight, involving transcriptional regulation and interactions with other growth factors.

The Dof zinc finger protein family plays an important role in plant growth and development [47,48]. They exhibit tissue-specific expression patterns linked to growth and stress resilience [49,50]. Studies on rice, soybean, and wheat have revealed their effects on root structure and abiotic stress tolerance [51,52], while transgene overexpression enhanced biomass and environmental adaptability [53–55]. The elevated expression of Dof1.2 in specific genotypes such as CY02 and CY201309 in this study highlights its potential role in pathways related to seed size and oil synthesis. Dof protein might

be the hub for integrating cell expansion and oil accumulation. The results provide a new strategy for improving the seed traits of sea buckthorn.

4. Materials and Methods

4.1. Plant Materials

Twelve sea buckthorn cultivars (*Hippophae rhamnoides* L.) with different phenotypes in seed weight/size and oil content were selected in 2022 in an experimental field of Dryland Agriculture and Forestry Research Institute (N41°29', E120°22') in Liaoning province of China, including 'CY04', 'CY03', 'CY02', 'CY32', 'CY10', 'CY11', 'CY201309', 'CY13', 'CY18', 'CY21', 'CY27', and 'SQH'. We collected the fruits of sea buckthorn at 30 and 60 days after flowering, considered as S1 and S2 development stage.

4.2. Determination of Seed Traits

The chloroform–methanol method was used to determine the oil content of sea buckthorn seed at two stages [56]. Five g freeze-dried seed powder (M1) was extracted with methanol and chloroform (v/v 1:2), then followed with vortexing or ultrasonication for 20 min. After three times of extraction, 1/5 volume of KCl solution (m/m: 0.9%) was added to the pooled extract. The mixture was vortexed for three minutes, then the solution was allowed to delaminate for 5 minutes. The bottom layer solution was collected into new glass sample bottles (M2) and evaporated to a constant weight (M3). Oil content (OC) was calculated by using the formula: oil content (%)=(M3-M2)/M1×100%. Three biological replicates were performed for each sample to determine oil content.

A total of 1000 intact seeds were randomly selected to measure thousand-seed weight (TSW), with visual inspection to exclude damaged or underdeveloped individuals. To ensure operational accuracy, seeds were divided into 10 subgroups of 100 seeds each. Each subgroup was weighed individually, and the total weight was calculated as the sum of all subgroups. Seed weight was determined using an analytical balance (Sartorius CPA225D, accuracy ±0.0001 g), calibrated daily with standard weights. TSW was calculated using the formula: TSW (g) = (Total weight of subgroups / Number of subgroups) × 10. Data were expressed as mean ± standard deviation (SD) from three independent replicates subgroup. The coefficient of variation (CV) was calculated to assess seed weight heterogeneity.

longitudinal diameters (LD) and transverse diameters (TD) were measured using a digital caliper (Mitutoyo 500-196-30, accuracy ±0.01 mm). LD was defined as the maximum longitudinal axis and TD as the maximum transverse axis perpendicular to length. Each seed was measured three times by the same operator, and the mean value was calculated to minimize operational error.

4.3. Total RNA Extraction, Library Preparation and UID Transcriptome Sequencing

Total RNA was extracted from developing seeds harvested at S1 and S2 stages using the Spin Column Plant Total RNA Purification Kit (Sangon Biotech, Shanghai, China). The extracted RNA was subsequently purified with the Dynabeads™ Oligo (dT) 25 mRNA kit (Thermo Fisher Scientific Baltics, UAB). The integrity of the RNA was verified through 1.5% agarose gel electrophoresis. Quantification of the qualified RNA samples was conducted using the Qubit 3.0 fluorometer with the Quant-iT RNA Assay Kit, Broad Range (Thermo Fisher, Q10213). For the preparation of stranded RNA sequencing libraries, 2 µg of total RNA was utilized, employing the KC-Digital Stranded mRNA Library Prep Kit for Illumina® (Catalog No. DR08502, Wuhan Seqhealth Co.). The kit mitigates duplication bias during PCR and sequencing processes by employing unique identifiers consisting of 8 random bases to label pre-amplified cDNA molecules. Library products ranging from 200 to 500 base pairs were subsequently enriched, quantified, and sequenced using the DNBSEQ-T7 sequencer (MGI Tech Co., Ltd., China) with the PE150 model.

The initial raw sequencing data underwent quality control using Trimmomatic (version 0.36), whereby low-quality reads were removed, and adaptor-contaminated reads were trimmed.

Subsequently, the resulting clean reads were processed with proprietary scripts to mitigate duplication bias introduced during library preparation and sequencing. Specifically, the clean reads were initially clustered based on UID sequences, grouping reads with identical UID sequences into the same cluster. Within each cluster, reads were subjected to pairwise alignment, and those exhibiting sequence identity exceeding 95% were extracted into a new sub-cluster. Following the generation of all sub-clusters, multiple sequence alignment was conducted to derive a consensus sequence for each sub-cluster. Subsequently, any errors and biases introduced by PCR amplification or sequencing were mitigated. Functional annotation of unigene sequences was performed through comprehensive BLASTX (version 2.6.0+) alignment analyses against four major biological databases: the NCBI non-redundant protein database (Nr, release 2023-08) using an E-value threshold of $1e-5$, the manually curated Swiss-Prot protein database (release 2023_07), the Kyoto Encyclopedia of Genes and Genomes pathway database (KEGG, version 102.0), and the evolutionary classification system of the Cluster of Orthologous Groups database (COG, updated 2023).

4.4. RNA-seq Data Analysis

Transcript or gene expression levels were quantified using RPKM (Reads per Kilobase per Million Reads) values, which normalize the counts of short sequences by accounting for read depth and transcript length. A pairwise transcriptome comparison was then performed between the control and experimental groups. DEGs (Differentially Expressed Genes) were conducted utilizing the Edge software. Genes exhibiting a false discovery rate (FDR) of less than 0.01 and a fold change of 2.0 or greater were deemed significantly differentially expressed between the control and experimental groups.

4.5. Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Enrichment Analysis

The identified DEGs were subsequently annotated using the KEGG database [57], a prominent public resource for metabolic pathways and functional information on gene products (<http://www.genome.jp/kegg>). Pathway analysis was performed by comparing pathways significantly associated with DEGs to the genomic background. This comparison was achieved by calculating a P-value via the hypergeometric distribution, with adjustments for multiple testing accomplished through FDR correction. Concurrently, MapMan software was employed to enhance the intuitive visualization of DEGs within the metabolic pathway.

4.6. Weighted Gene Co-expression Network Analysis (WGCNA)

Utilizing input files containing RPKM values for all genes and phenotypic data from 24 samples, the WGCNA package in R software (Version 3.4.4) was applied to identify modules of highly correlated genes [58]. The resulting data were visualized using Cytoscape software (Version 3.6.1). The Pick Soft Threshold function was utilized to determine the optimal soft power, culminating in the construction of the co-expression network at a soft power of $\beta=10$. We designated a merge CutHeight parameter of 0.25 to consolidate distinct modules exhibiting a similarity exceeding 75%. Combining the transcriptomic and phenotypic data of 24 samples, WGCNA analysis of the modules was performed using R software. A cluster tree was plotted against the expression of genes, with high correlation among genes within the same cluster. Gene division modules were performed using a dynamic programming algorithm. Subsequently, genes demonstrating analogous expression patterns were amalgamated into the same module. We computed the kME (Module Eigengene) value to assess the effective connectivity among hub genes. In this investigation, modules with a kME value greater than 0.7 were selected, as these genes more accurately represent the overall expression trend of the module. Ultimately, hub genes and transcription factors within the module were identified based on the kME value and the degree of connectivity of the genes within the network.

4.7. Quantitative Real-Time PCR (qRT-PCR) Analysis

Total RNA was extracted from frozen developing seeds at S1 and S2 from 12 cultivars. Complementary DNA (cDNA) synthesis was conducted using 1 µg of total RNA with the HiScript II QRT SuperMix Kit (Vazyme, China), following the manufacturer's protocol. qRT-PCR was performed in triplicate for three biological replicates. The expression levels of the target genes were normalized using the sea buckthorn genes *UBQ5*, as reference genes. Relative expression levels were determined using the $2^{-\Delta\Delta Ct}$ comparative threshold cycle (Ct) method. All gene-specific primers utilized in this study are listed in Supplementary Table S4.

5. Conclusions

The seed oil yield of seabuckthorn depends on seed development and lipid biosynthesis. A total of 10873 genes were significantly differentially expressed between S1 and S2 seed developmental stages of 12 cultivars. *RRS-6*, *ARF-7* and *ARF-20* in the KEGG pathway of "Circadian rhythm – plant" and "Plant hormone signal transduction" were related to seed development. GPAT family, DGAT family and PDAT family in the KEGG pathway of "Glycerolipid metabolism" were related to lipid biosynthesis. WGCNA identified five modules related to seed oil content and seed weight/size, and 417 candidate genes were screened from these modules. In which, multiple hub genes and transcription factors were identified, such as *ATPase*, *ATPase D* and *ACP1* of hub genes related to seed development, *PAP*, *PAT*, and *GPAT6* of hub genes involved in lipid biosynthesis, as well as *DOF1.2*, *bHLH137* and *ERF4* of transcription factors associated with seed growth and development.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Figure S1: The quantitative real time polymerase chain reaction verification of twelve genes related to seed development and lipid biosynthesis. Figure S2: The quantitative real time polymerase chain reaction verification of twelve genes related to seed development and lipid biosynthesis. Table S1: The candidate genes involved in seed development. Table S2: The candidate genes involved in lipid biosynthesis. Table S3: The candidate genes involved in transcription factors. Table S4: Information of all primer sequence in qRT-PCR.

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