Transcriptome analysis and identification of genes associated with ω-3 fatty acid biosynthesis in *Perilla frutescens* (L.) var. *frutescens*

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Abstract

Background: Perilla (Perilla frutescens (L.) var. frutescens) produces high levels of α-linolenic acid (ALA), a ω-3 fatty acid important to health and development. To uncover key genes involved in fatty acid (FA) and triacylglycerol (TAG) synthesis in perilla, we conducted deep sequencing of cDNAs from developing seeds and leaves for understanding the mechanism underlying ALA and seed TAG biosynthesis.

Results: Perilla cultivar Dayudeulkkae contains 66.0 and 56.2 % ALA in seeds and leaves, respectively. Using Illumina HiSeq 2000, we have generated a total of 392 megabases of raw sequences from four mRNA samples of seeds at different developmental stages and one mature leaf sample of Dayudeulkkae. De novo assembly of these sequences revealed 54,079 unique transcripts, of which 32,237 belong to previously annotated genes. Among the annotated genes, 66.5 % (21,429 out of 32,237) showed highest sequences homology with the genes from Mimulus guttatus, a species placed under the same Lamiales order as perilla. Using Arabidopsis acyl-lipid genes as queries, we searched the transcriptome and identified 540 unique perilla genes involved in all known pathways of acyl-lipid metabolism. We characterized the expression profiles of 43 genes involved in FA and TAG synthesis using quantitative PCR. Key genes were identified through sequence and gene expression analyses.

Conclusions: This work is the first report on building transcriptomes from perilla seeds. The work also provides the first comprehensive expression profiles for genes involved in seed oil biosynthesis. Bioinformatic analysis indicated that our sequence collection represented a major transcriptomic resource for perilla that added valuable genetic information in order Lamiales. Our results provide critical information not only for studies of the mechanisms involved in ALA synthesis, but also for biotechnological production of ALA in other oilseeds.

Keywords: Perilla frutescens, Seed, ω-3 fatty acid, α-linolenic acid, Triacylglycerol, Transcriptome

Background

Perilla frutescens, commonly called perilla, is a cultivated crop of the mint family Lamiaceae. Two distinct varieties, P. frutescens var. frutescens, the oilseed crop for source of perilla oil, and P. frutescens var. crisp for the aromatic leafy herb, are cultivated in East Asia countries mainly in Korea, Japan and China [1]. P. frutescens var. frutescens, hereafter called perilla, contains 35–45 % triacylglycerol (TAG) in seeds. It is a rich source of polyunsaturated fatty acids (FA) showing 54–64 % of ω-3 FA (α-linolenic acid, ALA or 18:3) and 14 % ω-6 FA (linoleic acid, LA or 18:2) [2]. Major oil seed crops (e.g., soybean, rapeseed, maize, peanut and sunflower) have relatively low ω-3 FA content (below 10 % in total FAs) in seed TAGs. The ω-3 and ω-6 FAs confer various health benefits for human [3]. The recommended ω-6/ω-3 FA ratio in human diet is 2:1 or lower [4, 5]. However, a typical human diet has high ω-6/ω-3 FA ratio (approximately 15:1) which is considered as a major contributor to
cardiovascular diseases [5]. Perilla seed oils have an approximately 0.2:1 ratio of ω-6/ω-3 FAs. This extremely low ratio of ω-6/ω-3 FAs makes perilla a desirable dietary source of vegetable oils [2]. Perilla oil also has many industrial uses, such as for drying oil in paint, varnish and ink manufacturing or as a substitute for linseed oil [6]. Perilla seed cakes are used as animals and birds feed.

Most research for perilla has been focused on identification of metabolites and their biological activities for human health [7, 8]. Some of the genes involved in the biosynthesis of anthocyanins, flavones and monoterpenoids have been studied [9, 10]. Recent reports on the generation of transcriptome using high-throughput sequencing were primarily for identification of genes for anthocyanin pathways associated with red or green leaf varieties of perilla [11, 12]. In contrast, studies on the molecular basis of seed FA and TAG synthesis in perilla have been limited. A seed-specific omega-3 fatty acid desaturase cDNA has been cloned [13] and characterized in perilla [14]. An oleosin promoter from perilla was found to have a seed-specific activity in transgenic Arabidopsis [15]. Besides perilla, flax (Linum usitatissimum), sacha inchi (Plukenetia volubilis L.), and chai (Salvia hispanica L., a member of mint family Lamiaceae) also contain high percentage of ALA in seed oil [14]. Seed transcriptome data of Chai [16] and sacha inchi (Plukenetia volubilis L.) [17] have been published, but a few genes contributing to the accumulation of ω-3 FA have been characterized for their expression profiles during seed development.

In this study, we adopted Illumina HiSeq 2000 platform aiming at analyzing the seed transcriptome of perilla. A leaf transcriptome was also included which allows comparison and detection of differentially expressed gene (DEG) in developing seeds of perilla. We have identified 54,079 unique transcripts from a total of 392 mega-base raw sequences, including transcripts for the majority of enzymes involved in lipid biosynthesis and metabolism. We further characterize the expression profiles of 43 key genes involved in FA and TAG in developing seeds and leaf using quantitative PCR (qPCR) assays. To our knowledge this work describes the first seed transcriptome of perilla. We harvested developing seeds 1–4 weeks after flowering (WAF) during seed development and found that seeds matured at 5 WAF (Fig. 1a). These harvested seed samples were analyzed for their FA content and composition by gas chromatograph (GC). During seed development, total FAs were measured at a very low level of 2.8 μg.mg⁻¹ in seeds at 1 WAF and increased steadily to 43 μg.mg⁻¹ at 2 WAF, 205 μg.mg⁻¹ at 3 WAF and 353 μg.mg⁻¹ at 4 WAF (Fig. 1b). We measured an average of total FA at 415 μg.mg⁻¹ in mature seeds (5 WAF and older), which is about 40 % of seed dry weight. Mature seeds at 5 WAF contained 66 % of ALA, 13.8 % of LA, 11.2 % of oleic acid (18:1 A9), 1.7 % of stearic acid (18:0), and 7.3 % of palmitic acid (16:0) (Fig. 1c). ALA was also found to be a predominant FA in young seeds, showing 30.9 and 50.5 % at 1 and 2 WAF, respectively (Fig. 1c). After 2 WAF, ALA level gradually reached a plateau level of 66 %. Oleic acid was 7.7 % in seeds at 1

Results and discussion
Fatty acid profile in developing seeds and leaf
To investigate the relationships between FA profile and gene expression, we studied seed development in perilla. We harvested developing seeds 1–4 weeks after flowering (WAF) during seed development and found that seeds matured at 5 WAF (Fig. 1a). These harvested seed
WAF and increased to 15.1 % at 2 WAF and maintained similar levels thereafter to the maturation stage. The remaining three FAs, palmitic acid, stearic acid and LA, had slight declining accumulation patterns during seed development. These FAs had levels at 25.7, 6.8 and 29 %, respectively at 1 WAF, then declined to about half of their beginning levels at 2 WAF, and maintained the levels throughout the remaining stages of seed development. In a leaf sample, we also detected a high level of ALA at 56.2 %, similar to that of mature seeds (Additional file 1: Table S1). Besides ALA, there are six FAs in leaves, represented at 14.9 % for LA, 3.3 % for oleic acid, 2 % for stearic acid, 4.3 % for hexadecatrienoic acid (16:3), 2.2 % for palmitoleic acid (16:1), and 17.2 % for palmitic acid (Additional file 1: Table S1). Our observed spatial and temporal patterns of FAs in developing seeds and leaf tissues were similar to described [14].

**Transcriptome sequencing of perilla and de novo assembly**

RNA samples from four different stages of developing seeds (1–4 WAF) and leaf were sequenced using Illumina Hiseq2000 system, which generated total 392,479,798 reads. After trimming the adapter and low quality reads and removing those shorter than 25 bp, a total 372,171,322 high quality reads were obtained from combined four different stages of developing seeds and one leaf libraries (Table 1). These reads were assembled into 191,545 contigs (or transcripts) (N50 = 1900 bp) and 80.7 % of them (154,621 transcripts) were annotated (Additional file 1: Table S2). Using a sequence similarity cutoff of 95 %, the assembled sequences were clustered into 54,079 unique transcripts, with an average length of 871 bp and total size of 47.1 Mb (Additional file 1: Table S2). Transcripts and unique transcripts were searched against the Phytozome database (http://phytozome.jgi-doe.gov) using BLASTx with E-value cut-off of 1E-10. The search resulted 154,621 transcripts and 32,237 unique transcripts (66.5 %) are highly matched with proteins from Mimulus guttatus (Monkey flower), followed by 1709 (5.3 %), 1431 (4.4 %), and 977 (3.0 %) transcripts matched with proteins from Solanum tuberosum, Solanum lycopersicum and Vitis vinifera, respectively. The remaining 6691 (21 %) transcripts matched protein sequences from 37 plant species (Additional file 2: Figure S2). It is not a surprise that most perilla transcripts have high sequence homology to M. guttatus [18], as both species are under the same Lamiales order. The results allow the translation of genomics and genetics research findings between M. guttatus and perilla.

**Functional annotation of perilla transcriptome**

We validated and annotated the unique transcripts with BLASTx homology search in Phytozome database. Among total 32,237 annotated unique transcripts, 21,429 transcripts (66.5 %) are highly matched with proteins from Mimulus guttatus (Monkey flower), followed by 1709 (5.3 %), 1431 (4.4 %), and 977 (3.0 %) transcripts matched with proteins from Solanum tuberosum, Solanum lycopersicum and Vitis vinifera, respectively. Among total 32,237 annotated unique transcripts, it is not a surprise that most perilla transcripts have high sequence homology to M. guttatus [18], as both species are under the same Lamiales order. The results allow the translation of genomics and genetics research findings between M. guttatus and perilla.

**Analysis of differentially expressed genes (DEG) in perilla developing seeds**

To examined the difference in gene expression between seeds and leaves, we performed a DEG analysis using bow-tie2 (v2.1.0) [19]. The up- or down-regulated genes were determined by comparison with the level of corresponding genes in leaf. The number of transcripts with > 2-fold change with a false discovery rate (FDR) < 0.01 was presented in Additional file 2: Figure S3. In developing seeds at 1, 2 and 3 WAF, the numbers of up-regulated genes were about 28–48 % less than that of down-regulated genes, showing 1184, 1052 and 1032, respectively; whereas the number of down-regulated genes presented at 1640, 2027 and 2151, respectively (Additional file 2: Figure S3). When seeds reached to maturation at 4 WAF, the number of up- and down-regulated genes had almost identical numbers, 2059 or 2058 (Additional file 2: Figure S3). As we can see, the numbers (1032–1184 counts) of up-

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**Table 1 Summary of sequencing data of P. frutescens seeds and leaf transcriptomes**

|                | Seed       | Leaf       | Total      |
|----------------|------------|------------|------------|
|                | 1 week     | 2 weeks    | 3 weeks    | 4 weeks    |
| Total number of raw reads | 59,619,730 | 64,434,520 | 98,130,006 | 67,528,198 |
| Total number of clean reads | 57,081,328 | 61,445,940 | 92,851,684 | 63,305,772 |
| Trimmed/raw (%) | 95.7       | 95.4       | 94.6       | 93.7       |

*Trimmed/raw: Total trimmed read/total raw read*
regulated genes were similar in seeds at the first three stages (1–3 WAF), and increased to 2-fold (2,059 counts) in 4 WAF. Whereas the numbers of down-regulated genes (2027–2059 counts) were similar in seeds at late three stages (2–4 WAF). The DEG detected in this study provides a global view of seed transcriptome which is important for further investigation of the molecular basis of seed development not only in perilla, but also in other oilseeds.

**Clustering of DEGs**

Hierarchical clustering was performed with the 6012 DEGs using Another Multidimensional Analysis Package (AMAP) library in R [20] to examine the similarity and diversity of expression profiles. Similarity of expression pattern of genes was estimated with pearson’s correlation. The results are displayed by Java Treeview (Additional file 2: Figure S4A). The normalized values are represented by different colors, with red representing positive values and green representing negative values. The analysis resulted in twelve clusters (Additional file 2: Figure S4B). Cluster 1 (374 DEGs) and 6 (602) had a similar declining pattern showing a higher level in seeds at 1 WAF, and decreased levels during the rest stages of the development (Additional file 2: Figure S4B). These DEGs may be important for early seed development. DEGs in Cluster 2 (1851) were down-regulated in seeds at all stages indicating that these genes were involved in cell metabolism in leaf. In contrast, DEGs in Cluster 3, 4 and 5 were all up-regulated with slightly different trends showing concave/flat, concave/rise and convex/flat, respectively. These DEGs were likely seed specific genes. Genes in Cluster 7–12 were less differentially expressed between leaf and seeds (Additional file 2: Figure S4B). Cluster 7 (51) had a convex/flat pattern with slightly higher expression levels in seeds at early (1 WAF) and late (4 WAF) stages. Cluster 8 (106) and 10 (118) had similar concave/flat expression patterns and both peaked in 2 WAF seeds. Cluster 9 (478) and 11 (131) were both flat/rise and peaked at 4 WAF. Cluster 12 (37) showed concaved/rise with a peaked expression at 3 WAF. The above variable temporal patterns indicate that multiple mechanisms were involved in regulating gene expression during perilla seed development. Similar temporal patterns of DEGs were also observed in other oilseeds [21–23].

**Analysis of seed abundant DEGs in Cluster 3, 4, 5 and 10**

Gene Ontology (GO) analysis was further used to classify functions of transcripts in cluster 3, 4, 5, 10 DEG. Using DAVID (http://david.abcc.ncifcrf.gov/tools.jsp) based on the Arabidopsis Information Resource Gene Ontology classification [24], a total of 2870 DEGs were categorized into 43 functional groups under main GO terms: cellular component, molecular function and biological process. DEGs in all four Cluster 3, 4, 5 and 10 showed similar functions. In the biological process, most transcripts were assigned to “nitrogen compound metabolic process (264 counts),” followed by “cellular metabolic process (235),” “biosynthetic process (221) and “primary metabolic process (141).” In the cellular components category, the majority of transcripts were associated with the terms “cell periphery (264),” followed by “protein complex (57),” and “organelle membrane (31).” In the molecular function group, the majority of transcripts were related to the terms “ion binding (299),” “transferase activity (281)” “hydrolase activity (249),” “oxidoreductase activity (180)” and “transmembrane transporter activity (81)” (Additional file 1: Table S5). Additional file 1: Table S6 lists the top 50 DEGs from Cluster 3, 4, 5 and 10. Among them, the most abundant genes are seed storage proteins (cruciferin, cupsin and late embryogenesis abundant (LEA) proteins) and lipid biosynthesis genes, including oleosins, hydroxysteroid dehydrogenase 1 for TAG biogenesis, acyl carrier protein and FAB2, FAD7/8 and FAD2 for FA synthesis.

**Analysis of acyl-lipid genes in developing seeds**

The most comprehensive database of plant acyl-lipid genes and pathways have been constructed for Arabidopsis (http://arabidopsisacyllipids.plantbiology.msu.edu/pathways/pathways) [25]. To identify acyl-lipid genes involved in seed oil biosynthesis in perilla, we searched perilla assembled genes using Arabidopsis acyl-lipid genes as queries. Among 975 queries, a total of 540 unique transcripts were identified from perilla transcriptome (Additional file 1: Table S7), which is about 55 % match up. A similar result (57 % match up) was obtained when searching lesquerella (Physaria fendleri) transcriptome using Arabidopsis acyl-lipid genes as queries [26]. Considering lesquerella and Arabidopsis both belong to the same Brassicaceae, whereas perilla and Arabidopsis are from different order, our results indicate that acyl-lipid genes are conserved among different plant species. Furthermore, we have focused on 43 major genes whose functions are likely responsible for FA and TAG biosynthesis based on our knowledge from model Arabidopsis (Additional file 1: Table S8). Deduced amino acid sequences of perilla genes had varied sequence identities with those of Arabidopsis genes, showing a relatively higher range of 74–92 % for FA biosynthesis than 41–87 % for TAG assembly. Perilla oleosins involved in oil-body formation showed 51–69 % identity compared with those of Arabidopsis. Our data indicate that between perilla and Arabidopsis, genes for FA biosynthesis in plastid are more conserved than those for TAG assembly in ER. The high content of ALA in perilla seed TAG (Fig. 1b and c) is probably resulted from some the genes in ER modified through evolution and become favorable for generating ALA in seed oils.
**Genes for FA biosynthesis in plastids**

Knowledge of genes and pathways involved in seed oil biosynthesis has been extensively studied. FAs are synthesized in plastid and then exported to cytosol to be activated to acyl-Coenzyme A (CoA) for TAG assembly in ER [25, 27, 28]. Key genes and pathways involved in de novo FA biosynthesis in perilla plastids are proposed (Fig. 2). Acetyl-CoA generated from pyruvate by the plastidial pyruvate dehydrogenase complex (PDHC) is used as a starting substrate for FA synthesis (Figs. 2 and 3b). The PDHC is a large multienzyme containing three components: E1 (pyruvate dehydrogenase or PDH, composed of E1α and E1β subunits), E2 (dihydrolipoyl acyltransferase or DHLAT), and E3 (dihydrolipoamide dehydrogenase or LPD) [29]. In perilla transcriptomes, we have identified five subunit genes for PDHC: PDH (E1α), PDH (E1β), EMB3003 (E2), LTA2(E2) and LPD1 (E3) (Additional file 1: Table S8). Spatial and temporal analysis of gene expression of these five subunits indicated that they all expressed in leaf and seeds; during seed development, they all had similar bell-shaped patterns with peaks at 2 WAF (Fig. 3c). The results suggest that genes encoding subunit of PDHC are coordinately regulated for synthesizing acetyl-CoA in seed and leaf (Fig. 3).

Once acetyl-CoA is synthesized, a heteromeric complex enzyme, acetyl-CoA carboxylase (ACC) catalyzes acetyl-CoA to form malonyl-CoA (Figs. 2 and 4a) [30]. A malonyl-CoA ACP transferase (MCMT) then further converts malonyl-CoA to malonyl-ACP. Perilla MCMT ortholog (Locus_14579) was identified showing 86 %
homology with Arabidopsis MCMT (At2g30200) and it is expressed high in 2 WAF developing seeds (Fig. 8a). ACC is composed of 4 subunits, 3 of them, biotin carboxyl-carrier protein (BCCP), biotic carboxylase (BC) and alpha-carboxyltransferase (α-CT) are encoded from nuclear genome; and beta-carboxyltransferase (β-CT) is encoded in plastid genome [31]. Six subunit genes were identified from perilla seeds and leaf transcriptomes (Additional file 1: Table S8). They are isoforms of BCCP1 and BCCP2, BC, isoforms of α-CTa and α-CTb, and β-CT. Spatial and temporal analysis of gene expression revealed that all of them were expressed significantly in leaf. During seed development, BCCP1 (Locus_29162) had flat-rise from 2 to 4 WAF, and BCCP2 (Locus_17340) had a bell-shaped pattern with high levels at 2 and 3 WAF. The BC also had a bell-shaped pattern, but with a high level only at 2 WAF. Isoforms α-CTa (Locus_8492) had a bell-shaped pattern similar to that of BCCP2. However α-CTb (Locus_2178) did not expressed in developing seeds. The β-CT (Locus_53041) was expressed moderately from 2 to 4 WAF (Fig. 4b). Thus, with exception of α-CTb, the other 5 genes α-CTa, β-CT, BC and BCCP1 or BCCP2 may coordinately work together in seeds.

FA elongation is conducted by an acyl-chain specific condensing enzyme subunit, 3-keto-acyl-ACP synthase (KAS), and 3 common components, 3-ketoacyl-ACP reductase (KAR), 3-hydroxylacyl-ACP dehydratase (HAD) and 2-enoyl-ACP reductase (EAR) (Fig. 5a) [32]. KAS III, I and II specifically catalyze the reaction of acyl-chain elongation for 2:0-ACP to 4:0-ACP, 4:0-ACP to 16:0-ACP and 16:0-ACP to 18:0-ACP, respectively. Based on the sequence homology with Arabidopsis genes, we have identified perilla orthologs for all these subunits.
Three KAS isoforms and three component of fatty acid synthase (FAS) showed similar temporal expression patterns during seed development (Fig. 5b). WRINKELD1 (WRI1) is a transcription factor for regulating some of the genes associated to FA biosynthesis in plastids. Arabidopsis WRI1 regulates α-PDH, BCCP2, MCMT, KASIII and EAR genes in FA biosynthesis in seed development (Fig. 2) [33]. Perilla WRI1 ortholog (Locus_23825) showed 82% identity with that of Arabidopsis and it had a seed-specific expression pattern with a peak at 2 WAF (Fig. 3a). Overexpression of Brassica and maize WRI increased in 10–40% of seed oils in transgenic plants [34, 35]. Perilla WRI1 could be used for engineering oilseeds for increased FA production in plastids which would provide increased FA supply for TAG assembly.

Stearoyl-ACP desaturase (SAD) catalyzes 18:0-ACP to 18:1-ACP in plastid (Fig. 2). Arabidopsis has seven SAD family genes included FAB2 (At2g43710), and FAB2 plays a major role in producing 18:1 [36]. Perilla ortholog (Locus_13564) of Arabidopsis FAB2 was detected in the seed transcriptome, and a homologue of At1g43710, DES6 (Locus_9486), was detected in the leaf transcriptome. Detailed analysis of gene expression confirmed that indeed perilla FAB2 and DES6 were differentially expressed in seeds and leaf, respectively (Fig. 5b). 16:0-ACP, 18:0-ACP and 18:1-ACP are hydrolyzed to the acyl moiety from ACP by two fatty acid thioesterases. FATA and FATB are specific to 18:1-ACP and 16:0 or 18:0-ACPs, respectively. Two fatty acid thioesterases FATA (orthologous Locus_29919 of At3g25110) and FATB (orthologous Locus_6603 of At1g08510) were both detected in perilla seeds and leaf.
Fig. 5 Synthesis of FAs and acyl-CoAs. a Pathway of synthesis of FA and acyl-CoA b Expression of genes for FA and acyl-CoA synthesis. Abbreviations are described as in Fig. 2.
However, the temporal expression of FATA and FATB were complementary to each other, showing a bell-shaped pattern with high levels at 2 and 3 WAF for FATA and inverted bell curve with high levels at 1 and 4 WAF for FATB (Fig. 5b). The higher expression of FATA at 2–3 WAF would suggest more 18:1 were terminated and released to ER, coinciding with the stages when seeds underwent rapid TAG synthesis. The highest transcript level of FATB detected in seeds at 1 WAF would suggest a swift demand of 16:0 and 18:0 for membrane biosynthesis at the onset of seed development, consisting with the higher levels of 16:0 and 18:0 detected in seeds at 1 WAF (Fig. 1c) [37]. Long chain acyl-CoA synthase (LACS) is located membrane of plastid outer envelope and/or ER and catalyzes free fatty acid to add Coenzyme A (CoA) for producing fatty acyl-CoA. Two perilla LACSs, LACS8 (Locus_3838 ortholog of At2g04350) and LACS9 (Locus_23636 ortholog of At1g77590), were identified. Expression of the LACS9 exhibited a bell-shaped pattern with a maximum level at 2 WAF (Fig. 5b), which may associate with the increased demand of FA-CoA formation in cytosol [38] when developing seeds entering rapid growth phase. LACS9 was localized in plastid outer envelope [38]. For the LACS8, more transcripts were detected in seeds at 3–4 WAF than 1–2 WAF (Fig. 5b), therefore, the ER-localized LACS8 might be involved in TAG synthesis [39].

Desaturases associated with ER

ER contains two desaturases, FAD2 and FAD3. These enzymes catalyze desaturation of FAs attached to PC from PC-18:1 to PC-18:2 (FAD2) and from PC-18:2 to PC-18:3 (FAD3) [40–42] (Fig. 6a). During the search of perilla transcriptomes, we uncovered three loci, Locus_773, Locus_22029 and Locus_5107 encoding desaturases. Results of sequence alignment showed Locus_773 having 79 % of identity with the first plant FAD2 from Arabidopsis (AtFAD2, Additional file 2: Figure S5A) [41]. Further phylogenetic analysis of 32 plant FAD2s revealed that the perilla sequence fell in a clade belong to constitutive type FAD2s (Additional file 2: Figure S5B). We designated this sequence perilla FAD2 (PfrFAD2, Fig. 6c, Genbank ID: KP070823). During seed development, PfrFAD2 expressed at a low level at 1 WFA, but it elevated to 33.8-, 50.0- and 21.2-fold at 2, 3 and 4 WFA, respectively (Fig. 6d). In leaf, PfrFAD2 expressed at a significantly level showing 6.3-fold higher than that of FA-CoA (FAD3) development (2–4 WAF), only a low level of the transcript was detected in young seeds at 1 WAF (Fig. 6d) where no seed TAG was measured. However, we found that PfrFAD7/8 was highly expressed in leaf, indicating the importance of its function in leaf. Perilla contains high level of 18:3 FAs not only in seeds (Fig. 1c) but also in leaf (Additional file 1: Table S1). In leaves, glycerolipid (GL) moiety as PA or diacylglycerol (DAG) can flux between chloroplast and ER [31]. There are two linoleate desaturases, FAD7 and FAD8, structurally related with FAD3 in chloroplasts of Arabidopsis [47, 48]. Perilla leaf contains a high level of 18:3 up to 56.2 %, indicating that the majority of GL are transported from ER to chloroplast where GL-18:3 are then converted to GL-18:3 by FAD7/8 (Fig. 6a). Compared with PfrFAD3, PfrFAD7/8 N-terminus contains extra amino acid sequences encoding chloroplast transit peptide which supports the role of desaturation for membrane lipid in chloroplasts (Fig. 6b). Overall, our results of sequence and gene expression analyses provide essential information that PfrFAD3 and PfrFAD7/8 may be major enzymes for synthesizing 18:3 in seed TAGs and leaf membrane glycerolipids, respectively.

TAG biosynthesis in ER

Multiple mechanisms are involved in TAG biosynthesis in ER [25, 27, 28]. Acyl-CoAs in the cytosol can be incorporated into TAG through the glycerol-3-phosphate (G3P) pathway or the Kennedy pathway [49, 50], which involves three sequential acylation of acyl-CoAs into...
Fig. 6 Polyunsaturated FA synthesis. a Polyunsaturated fatty acid synthesis pathway in ER and chloroplast. GL, glycerolipids. b Amino acid alignment of perilla (Pfr) FAD3 and FAD7/8. Red rectangles indicate His box, green line indicates a chloroplast transit peptide. c Phylogenetic analysis of plant FAD2, FAD3 and FAD8 desaturases. d Expression of FAD2, FAD3 and FAD7/8. Abbreviations are described as in Fig. 2.
G3P. Firstly, G3P is acylated by glycerol-3-phosphate acyltransferase (GPAT), followed by a second acylation by the acyl-CoA:acylglycerol-3-phosphate acyltransferase (LPAT), yielding phosphatidic acid (PA). PA is then hydrolyzed to form diacylglycerol (DAG), which is finally used as a substrate for the diacylglycerol acyltransferase (DGAT) to produce TAG. The acyl-CoAs can also be incorporated directly into phosphatidylcholine (PC) by the acyl editing reactions [25, 51, 52]. These acyl editing reactions can be catalyzed either by forward and reverse reactions of lyso-PC acyltransferase (LPCAT) to yield acyl-CoA, or by a phospholipase A-type activity to yield a free FA that then is activated to acyl-CoA. Since PC is the site for modified FA synthesis including 18:2, ALA, rapid de-acylation and re-acylation of PC results in an acyl-CoA pool enriched with unsaturated FAs which are then utilized for TAG synthesis [53, 54]. Besides, many plants utilize PC-mediated pathways to synthesize TAG. The enzyme Phospholipid:DAG acyltransferase (PDAT) synthesizes TAG by transacylation of the sn-2 FA from PC onto sn-3 position of DAG [55]. FAs at the sn-1 and sn-2 position of PC in perilla can be converted to TAG through DAG by phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT) which exchanges phosphocholine between PC and DAG [56, 57]. CDP-choline:DAG cholinephosphotransferase (CPT) catalyzes the reaction of CDP-choline with DAG to generate PC. This reaction can be reversible [58–60]. PDCT and the reverse reaction of CPT would facilitate the FA on PC to be incorporated to TAG. A schematic drawing of TAG biosynthesis in perilla seeds is presented in Fig. 7a.

Genes involved in Kennedy pathway and acyl editing reactions

Based on the putative Arabidopsis GPAT9 (At5g60620) sequence [61], a perilla GPAT9 (PfrGPAT9) transcript (Locus_10180) was found from the transcriptomes showing 81 % sequence identity to At5g60620 (Additional file 1: Table S8). PfrGPAT9 transcript levels were comparable among leaf and developing seeds at different stages, although a bell-shaped pattern peaked at 2 WAF, the overall changes were about 2-fold or less (Fig. 7b). Perilla LPAT2 (PfrLPAT2, Locus_6587), was identified using Arabidopsis LPAT2 (At3g57650) known to be involved in seed TAG biosynthesis [62]. Perilla and Arabidopsis LPAT2s share 81 % sequence identity. (Additional file 1: Table S8). PfrLPAT2 expression showed a continuous increase from 1 to 4 WAF during seed development, and its expression is higher in leaves than seeds (Fig. 7b). The spatial and temporal expression patterns of perilla GPAT9 and LPAT2 suggest their constitutive functions with house-keeping roles in both membrane lipid and TAG synthesis. DGAT is the last enzyme in Kennedy pathway and often thought to be the rate limiting step in determining synthesis of TAG [63]. Perilla Locus_14696, Locus_12629 and Locus_1560 were revealed to encode PfrDGAT1, PfrDGAT2, and PfrDGAT3 and showed 79, 67 and 42 % sequence identity with Arabidopsis DGAT1 (At2g19450), DGAT2 (At3g51520) and DGAT3 (At1g48300), respectively, (Additional file 1: Table S8). PfrDGAT1 and PfrDGAT2 were expressed predominantly in seed, whereas DGAT3 expressed both in seeds and leaf at similar levels (Fig. 7b). PfrDGAT1 and PfrDGAT2 are probably involved in TAG biosynthesis in seeds, whereas PfrDGAT3 is a house-keeping enzyme.

As polyunsaturated FAs (PUFA) are major components in TAG of perilla seeds, the acyl editing mechanism [28, 64] would enrich acyl-CoA pool with PUFA-CoAs, facilitating the incorporation of PUFAs into TAGs. Although there are two Arabidopsis LPCATs, (LPCAT1, At1g12640 and LPCAT2, At1g63050) were reported [64, 65], we found only one perilla LPCAT (Locus_43749) in transcriptomes and it expressed both in leaf and developing seeds (Fig. 7b). The finding of PfrLPCAT would suggest acyl-editing through PfrLPCAT likely utilized in perilla.

Genes involved in PC-mediated pathways for TAG biosynthesis

TAG can be synthesized directly between DAG and PC by Phospholipid:diacylglycerol acyltransferase (PDAT) through acyl-CoA independent pathway [28, 64]. PDAT transfers FA of sn-2 position in PC to sn-3 position of DAG and synthesize TAG (Fig. 7a) [55, 66]. This mechanism has been demonstrated well with a castor PDAT. Castor seed oil contains 90 % ricinoleic acid (18:1OH) which is synthesized on the sn-2 of PC [58, 67]. When a castor PDAT (RcPDAT) was introduced into Arabidopsis expressing a castor fatty acid hydroxylase gene (RcFAH12, [59, 60]), the transgenic Arabidopsis with dual RcFAH12 and RcPDAT enhanced 18:1OH level in TAG [68, 69]. Two perilla PDAT orthologs, PfrPDAT1 (Locus_7255) and PfrPDAT2 (Locus_29208), corresponding to Arabidopsis PDAT1 and PDAT2, respectively, were detected. PfrPDAT1 expressed in seeds and leaves, whereas PfrPDAT2 shows seed-specific expression (Fig. 7b). The spatial and temporal expression profiles of PfrPDATs are similar to that of Arabidopsis PDATs [66]. Our data of PfrPDATs provide molecular basis for further investigation of the role of PfrPDATs in ALA-containing TAG synthesis.

Perilla seed oil contains 60 % ALA (18:3) distributed at all three sn-1, 2, 3 positions of TAG, with somewhat higher at the sn-2 position [68]. However, perilla LPAT2 showed no activity in acylating ALA to the sn-2 position of TAG [68]. This indicates that the majority of ALA in the sn-2 TAG could be formed through PC-mediated DAG pathways, such as PDCT and reverse reaction of CPT, rather than Kennedy pathway (Fig. 7). We identified a full-length perilla PDCT (PfrPDCT, Locus_15867)
Fig. 7 TAG synthesis and oil-body formation. 

**a** TAG biosynthesis through Kennedy pathway and PC-mediated pathway in seed. 18X indicate unsaturated FA (18:1, 18:2 and 18:3).

**b** Expression of genes involved in TAG biosynthesis. Abbreviations are described as in Fig. 2.
cDNA showing 62 and 64 % identity with PDCTs from Arabidopsis and castor (Fig. 8a). *PfrPDCT* had a bell-shaped temporal expression pattern during seed development and also expressed significantly in leaf (Fig. 8b). The role of Arabidopsis PDCT (At3g15820) in contributing of unsaturated FA in TAGs has been demonstrated [57]. The castor PDCT has also shown to evolve to effectively convert 18:1OH-PC to 18:1OH-DAG for 18:1OH-containing TAG synthesis in transgenic Arabidopsis [56]. Besides, we have also identified two perilla CPT orthologs, CPT1 (*PfrCPT1*, Locus_7821) and CTP2 (*PfrCPT2*, Locus_25627) based on Arabidopsis AAPT1 (At1g13560) and AAPT2 (At3g25585). Both *PfrCPT1* and *PfrCPT2* expressed in seeds and leaves, but *PfrCPT2* had a relative higher level in leaves than seeds (Fig. 8b). The identification of *PfrPDCT* and *PfrCPTs* in this study helps to explain the ALAs at the sn-2 of perilla TAGs that are likely acquired by PC-mediated DAG formation through *PfrPDCT* and *PfrCPTs*.

**Oil body protein, oleosins**

TAGs is covered by single layer of phospholipids of ER and amphipathic oleosin (OLN) proteins. Arabidopsis encodes five seed-specific oleosin genes in the genome [70]. We have identified four different isoforms of oleosin from perilla seed transcriptome with molecular weight of 15, 16, 18, 19 KD (*PfrOLN*-15, −16, −18 and −19). Full-length of *PfrOLN*-15 (AF210697.1) and *PfrOLN*-19 (AF237625.1) isoforms are closer to Arabidopsis OLN1 (AT3G01570) and they share a conserved harpin domain (~72 hydrophobic or neutral residues) with other oleosins (Fig. 9a and b). All four *PfrOLNs* showed a seed-specific expression (Fig. 9c). However, the expression of *PfrOLN*-15 had a linear-rise pattern showing a maximum 673.9-fold induction at 4 WFA compared with that of 1 WAF. The other *PfrOLNs* had much less dynamic changes showing either linear-rise (*PfrOLN*-16, −18) or bell-shaped (*PfrOLN*-19) patterns with a maximum induction between 103.4- and 188.5-fold

![Fig. 8 Characterization of perilla PDCT (phosphatidylcholine:diacylglycerol cholinephosphotransferase).](image-url)
Fig. 9 Characterization of perilla oleosins. a Phylogenetic tree of perilla and Arabidopsis oleosins (OLN1 to OLN5 is AT3G01570, AT3G27660, AT4G25140, AT5G40420, AT5G51210 respectively) b Amino acid sequence alignment of perilla and Arabidopsis oleosins c Expression of genes encoding four perilla oleosins
Pfroln-15 may be the major oleosin isoform in oil-body of perilla seeds (Fig. 9c).

In general, the expression profiles of genes involved in fatty acid and TAG biosynthesis detected by RNAseq analysis (Additional file 1: Table S8) and qPCR (Figs. 3, 4, 5, 6, 7, 8 and 9) are comparable, except for oleosin genes. Pfroln-15 showed a highest expression in developing seeds using qPCR whereas Pfroln-19 and Pfroln-19 were highest using RNAseq. The discrepancy of expression level between RNAseq data and qPCR data was likely caused by chimeric transcripts generated by assembly program, which is inevitable in a assemble process purely based on de novo transcriptome data.

**Conclusions**

Perilla frutescens (L.) var. frutescens, a valuable oilseed crop, contains high amount of ALA in seeds and leaves. Deep sequencing of cDNAs from developing perilla seeds and leaves was carried out to identify genes involved in the synthesis of seed TAG enriched with ALA. A total of 54,079 unique genes from 392 mega-base raw sequences were assembled. The majority (66 %, 21,429 out of 32,237) of the matched genes showed highest homology to Mimulus guttatus genes, confirming the close relationship between the two species. Genes involved in the synthesis of FA and TAG were identified and annotated by detailed sequence alignments. We have identified nearly all of the known genes for de novo FA biosynthesis in plastid, export from the plastid and TAG assembly in ER. In addition, we characterized the expression profiles of 43 key genes in TAG metabolism using quantitative PCR (qPCR). Two α-3 fatty acid desaturase genes, PfFAD3 and PfFAD7/8 were identified as key genes for ALA synthesis in seeds and leaves, respectively. The identification of PfDGATs, PfPDATs, PfPDCT and PfCPTs provides additional key genes not only for future studies on the mechanisms of ALA-containing TAG synthesis in perilla, but also for use as targets in genetic engineering of other oilseeds to produce a high level of ALA.

**Methods**

**Plant materials and RNA extraction**

Seeds of Perilla frutescens (L.) var. frutescens cultivar ‘Dayudeulkkae’ were obtained from the National Institute of Crop Science, Miryang Republic of Korea. Perilla plants were grown in the greenhouse at temperatures between 18 and 28 °C. After fertilization, developing seeds from 1, 2, 3, 4 weeks and mature leaves were collected, immediately frozen in liquid nitrogen and stored at −80 °C prior to RNA extraction. Total RNAs from developing seeds and leaves of three replicates were extracted using the Plant RNA Reagent (Invitrogen) and treated with DNase I (Takara) according to manufacturer’s instructions. RNA quality was examined using 1 % agarose gel and the concentration was determined using a Nanodrop spectrophotometer (Thermo). The RNA integrity number determined by Agilent 2100 Bioanalyzer was greater than 7.0 for all RNA samples to construct cDNA libraries.

**Fatty acid content analysis**

The fatty acid content of seeds and leaves were analyzed by gas chromatographic analysis with a known amount of 15:0 fatty acid as an internal standard. Samples were transmethylated at 90 °C for 90 min in 0.3 mL of toluene and 1 mL of 5 % H2SO4 (v/v methanol). After transmethylation, 1.5 mL of 0.9 % NaCl solution was added, and the fatty acid methyl esters (FAMEs) were transferred to a new tube for three sequential extraction with 1.5 mL of n-hexane. FAMEs were analyzed by gas chromatography using a GC-2010 plus instrument (Shimadzu, Japan) with 1 30 m × 0.25 um (inner diameter) HP-FFAP column (Agilent, USA), during which the oven temperature was increased from 170 to 180 °C at 1 °C/min.

**cDNA library construction and massive parallel sequencing**

RNA-Seq paired end libraries were prepared using the Illumina TruSeq RNA Sample Preparation Kit v2 (catalog #RS-122-2001, Illumina, San Diego, CA). Based on the instruction provided by the kit, mRNAs were purified from total RNA using poly (A) selection, and then chemically fragmented and converted into single-stranded cDNA. Using random hexamer priming, a second strand is generated to create double-stranded (ds) cDNAs. Library construction begins with generation of blunt-end cDNA fragments from ds-cDNAs. Then Adenine nucleotide (A)-base added to the blunt-end in order to make them ready for ligation of sequencing adapters. After the size selection of ligates, the ligated cDNA fragments which contain adapter sequences are aligned via PCR using adapter specific primers. The library was quantified with KAPA library quantification kit (Kapa biosystems KK4854) following the manufacturer’s instructions. Each library was sequenced using Illumina HiSeq2000 platform, which created 100 bp paired-end sequencing reads.

**De novo assembly and unique transcripts annotation**

Raw sequencing data composed of 100 bp paired-end reads filtered by Phred quality score (Q ≥ 20) and read length ( ≥25 bp) with SolexaQA [71]. We used all the sequence reads from different tissue samples to optimize the de novo assembly using the software tools Velvet (v1.2.07) [72] to assess k-mer sizes and assembled contigs. The contigs were joined into transcript isoforms using Oases (v0.2.08) [73]. Velvet and Oases are based on the de Bruiijn graph algorithm. We took several hash length into consideration to select the best de novo
assembly. The unique transcripts of perilla were defined by merging the best de novo assembly and validated by direct comparison with gene sequences in the Phytozone (http://www.phytozone.net/) using BLASTx (e-value ≤ 1E-10). The proteins with the highest sequence similarity were retrieved for analysis.

Short read mapping and expression profiles in experimental samples

Reads for each sequence tag were mapped to the assembled unique transcripts using Bowtie software (v2.10) [19]. The number of mapped clean reads for each unique transcript was counted and then normalized with DESeq package in [74]. Only those representative transcripts with mapped reads counts of 1000 or above in at least one experimental sample were retained for further analysis. Fold change and binomial-Test were used to identify differentially expressed genes between each sample. FDR (false discovery rate) was applied to identify the threshold of the p-value in multiple tests and analysis and this value was calculated via DESeq. All correlation analysis, hierarchical clustering was performed using AMAP library in R [20].

GO analysis

Gene Ontology (GO) analysis was carried out via DAVID (http://david.abcc.ncifcrf.gov/tools.jsp) [24]. The gene lists by annotated TAIR ID of transcripts of up- and down-regulated DEG were analyzed with counts ≥ 5 and FDR ≤ 0.01 of each GO terms.

Quantitative PCR

Total RNA were reverse transcribed with the PrimeScrip™ 1st strand cDNA synthesis kit (Takara, Japan) according to manufacturer’s protocol. Real-time PCR was performed using the SYBR® Premix Ex Taq™ II (Takara, Japan) on the CFX96 Real-Time PCR system (Bio-Rad) with gene-specific primer pairs (Additional file 1: Table S5). Perilla ACTIN (AB002819.1) was used as the internal reference gene. The relative expression value was calculated via the ΔΔCt method.

Full-length cDNA cloning and sequence analysis

A cDNA containing full-length open reading frame (ORF) for FAD2, FAD3 and PDCT were amplified using KOD polymerase from total RNA of developing seeds or leaves samples using primers (Additional file 1: Table S9). PCR products were cloned into pCR-Blunt vector (Invitrogen) for Sanger sequencing. The amino acid sequence alignment of proteins was performed with CLUSTALW program of DNASTAR software with default parameters. Phylogenetic tree was built with the CLUSTALW method with DNASTAR MegAlign program.

Additional files

Additional file 1: Table S1. Fatty acid composition of perilla developing seeds and leaves. All data are averages of three measurements ± SE.
Table S2. Statistical summary of de novo transcriptome assembly and annotation. Table S3. Annotation of 54079 perilla representative transcripts and their expression in developing seeds and leaf. Transcripts represents as Locus. Raw read count and normalized read count were represented as expression level for each developing seeds and leaves samples. Table S4. Nucleotide sequences of 54079 perilla representative transcripts in FASTA format. Table S5. Gene ontology analysis of DEG cluster 3, 4, 5 and 10 transcripts of perilla. Table S6. List of gene products for the top 50 DEGs in Cluster 3, 4, 5 and 10. Table S7. Number of identified genes involved in acyl-lipid metabolism of perilla transcriptome. Table S8. List of expressed genes involved in fatty acid and TAG biosynthesis in perilla. Table S9. Information of perilla gene primers (5′ → 3′) used in the qPCR analysis and cDNA cloning. (ZIP 27440 kb)

Additional file 2: Figure S1. Fatty acid composition of perilla developing seeds and leaves. All data are averages of three measurements ± SE.
Figure S2. Statistical summary of 6012 DEGs based on log ratio RPKM data. (A) Heatmap. (B) Line plot for 12 clusters. Fold changes of DEGs in developing seeds (1–4 weeks after flowering, WAF) are calculated based on leaf value. Figure S3. Characterization of perilla FAD2. (A) Amino acid sequence alignment of perilla and Arabidopsis FAD2. Red boxes indicate His conserved motifs. (B) Phylogenetic tree of plant FAD2s. Abbreviations: Ah, Arachis hypogaea; At, Arabidopsis thaliana; Bc, Brassica carinata; Bj, Brassica juncea; Br, Brassica rapa; Cs, Camelina sativa; Ct, Carthamus tinctorius; El, Euphorbia lagascae; Gm, Glycine max; Gh, Gossypium hirsutum; Ha, Helianthus annuus; Jc, Jacaranda curcas; Lj, Linum usitatissimum; Os, Oryza sativa; Pfr, Perilla frutescens; Rc, Ricinus communis; Sl, Sesamum indicum; Sa, Spinacia oleracea; Vi, Vigna mungo; Vf, Vitis labruscra. Figure S4. Perilla FAD3 and FAD7/8 amino acid sequence alignment with Arabidopsis FAD3, FAD7 and FAD8. Red box indicate His conserved motifs. Abbreviations are described in Additional file 2: Figure S5. ATfAD3 (AT5G29980), ATfAD7 (AT3G11170), ATfFAD8 (AT5G05580). (PPTX 1366 kb)

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Availability of supporting data

The sequence raw data from this study have been submitted to the NCBI Sequence Read Archive (SRA) (http://www.ncbi.nlm.nih.gov/sra) under the BioProject ID PRJNA287080.

Authors’ contributions

HUK conceived and designed research. HUK and GQC wrote the article. DS and JHL analyzed de novo assembly and differentially expressed genes using Bioinformatics tools. HUK, KYL and SH conducted the experiments and contributed to the study design. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.
Ethics approval and consent to participate
Not applicable.

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