Article

Alpha-Linolenic Acid Content and Expression of KASII and FAD3 in Perilla Seeds Correlated Cultivated Areas of Northern Thailand.

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Abstract: Perilla frutescens is commonly used as an n-3 fatty acid source for people living in Northern Thailand. However, cultivated areas are limited because it apparently requires specific growth conditions. Our results suggested that perilla seeds grown in higher altitude in Maehongson province contained more ALA contents than seeds collected from Chiangrai and Nan province. Furthermore, mass spectrometry (MS) proteomic and gene expression analysis revealed that the increase of KASII and FAD3 proteins associated with the mRNA expression and the ALA production. Predictive bioinformatics analysis demonstrated two important transcription factor binding sites, AP2 and B3, which responsible for KASII and FAD3 genes, respectively. The AP2 and B3 transcription factor families were known to be responsible for abiotic stress such as drought and temperature changes. In conclusion, high production of ALA by perilla apparently relies on the altitudes. Moreover, plant responses to abiotic stress environments contribute to the increase of KASII and FAD3 gene and protein expression. However, responsive transcription factors will be further studied for a proof of concept and additionally improving the growing methods in order to increase perilla productivity.

Keywords: Perilla frutescens; oil seed; n-3 fatty acids; functional foods; alpha-linolenic acid; proteomics

1. Introduction

Perilla frutescens, also called perilla, is one of the mint families, Lamiaceae. Perilla is commonly known as a rich source of unsaturated fatty acids, especially α-linolenic acid (ALA) which is comparable to other oilseeds such as flax (Linum usitatissimum) and inca peanut (Plukenetia volubilis) [1]. The ALA is a precursor for other n-3 fatty acids biosynthesis in human body. Once obtained from food sources, it can be further metabolized into eicosahexaenoic acid (EPA) and docosahexaenoic acid (DHA) by the action of Δ12 and Δ15 desaturases [2]. Perilla oil contains as high as 50-65% of ALA [1,3] and thus it can be promoted as part of diets. A regular consumption of food containing high n-3 fatty acids has beneficial in terms of health promotions and preventions, for example
reducing the risk from cardiovascular disease [4,5] and other noncommunicable diseases [6,7] as well as helping the brain function including learning and memories [8-10].

From a practical point of view, the n-3 fatty acids from plants are not only benefit for direct consumptions but also can potentially be promoted as food supplements. Increase consumption of n-3 fatty acids can drive the n6/n3 imbalance ratio in modern diet to a more proper proportion where ideally recommended ratio is 1/1 [5,6]. The n-3 fatty acids producing plants are also recognized as an alternative to fish oil and thus becoming a valuable choice for vegetarian [3]. Furthermore, the important of n-3 fatty acids is not only food-based ingredients but also widely used as a component, dominantly from virgin flaxseed oils, in cosmetics and personal care products as part of their lipid-based formulations [11]. On the other hand, perilla seed oil has not been reported to be used in industrial scale. This could be due to its limited availability. Important driving factors that limited the widespread may include restrictively cultivated lands, distinctive growing conditions as well as efficiency of plantation that can effect crop yield.

In Thailand, perilla cultivated areas are relatively limited. A perilla commonly grows well in mountain areas because annually average temperature is relatively low and the humidity is appropriate. Apparently, the ability of ALA production of perilla is varied in each cultivated area. In this study, we determined how ALA contents in perilla seeds from each location, i.e. Maehongson, Chiangrai and Nan, are different in correspond to the altitudes of each cultivated area, particularly in the aspect of gene expression. During oil-seed development, many genes encoding enzymes in lipid biosynthesis are temporally up-regulated [12-14]. The key genes involving in n-3 fatty acids biosynthesis, β-ketoacyl-acyl carrier protein synthase II (KASII) and fatty acid desaturase-3 (FAD3), were selected as candidates for surrogate markers in this study. KASII gene is not a tissue-specific expression. Its basal expression level can be found throughout all developmental stages of both perilla leaves and seeds [15]. While the expression of FAD3 has a seed-specific expression [16]. According to the study of Kim et al. [12] and Liao et al. [17], the expression of FAD3 was higher in developing seeds than in leaves. Indeed, at a certain time, gene expressions would not be a sole indicator or a marker representing the ALA production. On the other hand, proteins are effective molecules affecting enzymatic reaction in perilla seeds. Therefore, not only mRNA transcripts but also protein levels were determined so that it could be answered whether protein expressions correlated with mRNA abundances in corresponding to ALA contents. Quantitative PCR and quantitative mass-spectrometry-based proteomics were applied for determination of both mRNAs and proteins levels. Gene expression of KASII and FAD3 could be alternative biomarkers for crop selection and to determine the high n-3 fatty acid producing crops for new season cultivation. In addition, it could be partly indicated the quality of cultivated areas. Furthermore, this finding can be useful for crop modification in order to increase crop yield. Nevertheless, these surrogate markers are still needed to be validated in further study.

2. Results

2.1. Fatty Acid Compositions in Perilla Oil

Perilla is generally grown during May-July of each year and mature seeds were collected in December. The lipid content ranged from 28-37% (g/100g seeds). The ALA content of perilla seeds collected from Maehongson, Chiangrai and Nan were 79.3 ± 4.4%, 69.1 ± 5.5% and 63.3 ± 7.3%, respectively. The n-6 linoleic acid (LA) content was found to be 10-20% and was presented in a reverse order to the ALA content where the higher the ALA level, the lower the linoleic acid content available. The physical characteristics of seeds, oil contents and growing condition in local field such as geographical height and average annual growth temperature, information is derived from National Statistical Office of Thailand year 2016, were summarized in Table 1.
2.2. Messenger RNA Expression of KASII and FAD3 and the ALA Content Production

Mature seeds of perilla from three locations were selected for mRNA extraction. By using real-time PCR, the expression of gene encoding KASII and FAD3 were analyzed. The result indicated that seed sample from Maehongson showed significantly higher expression of KASII by 3.4 ± 0.3 fold (p<0.01) in relative to the expression of genes from Nan sample (control), while the sample from Chiangrai showed similar expression to the control (Figure 1a). However, the expression of FAD3 was showed no significant different among all areas (Figure 1b). Based on ALA rich plants [1,14] inca peanut and flax were selected for further analysis to compare the expression of KASII and FAD3 related to ALA production with perilla seed from Maehongson. As shown in Figure 2a and 2b, there were no significant different in the level of expression of gene encoding KASII and FAD3 among three seed oils.

![Figure 1](https://preprints.org/doi:10.20944/preprints201808.0439.v1)

_Figure 1._ The mRNA expression of (a) KASII gene and (b) FAD3 gene in mature perilla seed collected from different locations. Sample from Nan was set as a control group. The fold change represented the mean ± SE of duplicate experiment. *p<0.05, **p<0.01.

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Table 1. Perilla growing locations.

| Field locations | Latitude       | Longitude     | Altitude (m) | Mean annual temperature (°C) | ALA content (wt%)  
<table>
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<tr>
<td>Maehongson</td>
<td>19° 25’ N</td>
<td>97° 59’ E</td>
<td>1,164</td>
<td>26.9</td>
<td>79.3 ± 4.4</td>
</tr>
<tr>
<td>Chiangrai</td>
<td>20° 8’ N</td>
<td>99° 49’ E</td>
<td>431</td>
<td>25.5</td>
<td>69.1 ± 5.5</td>
</tr>
<tr>
<td>Nan</td>
<td>18° 34’ N</td>
<td>100° 52’ E</td>
<td>207</td>
<td>27.3</td>
<td>63.3 ± 7.3</td>
</tr>
</tbody>
</table>

1 mean ± SE for duplicate injection
Figure 2. The mRNA expression of (a) KASII gene and (b) FAD3 gene in different mature. Flax seed sample was set as a control group. The fold change represented the mean ± SE of duplicate experiment.

2.3. Protein Expression of KASII and FAD3 by MS-Based Proteomics

The high expression of gene encoding FAD3 may reflect the activity of n-3 desaturase as well as effect on the accumulation of ALA contents in various oil crops. The mRNA expression levels do not always correlate to protein expression levels because of transcriptional regulation, post-translational regulation and mRNA stability. Therefore, we further examined whether protein expression levels correlate to mRNA expression. Herein, KASII and FAD3 protein abundance in perilla seeds were analyzed with LC MS/MS and subsequently quantified by MS1 filtering method using Skyline software.

MS1 filtering relied on quantification of the identified peptides from LC MS/MS. Tryptic peptides peak area of KASII from Maehongson perilla seed sample, FMLYMLTAGA and GFVMGEGAVLLEELEHAK, were higher than Chiangrai and Nan samples (Figure 3). Correspondingly, the peak area of FAD3 tryptic peptide from Maehongson perilla seeds (Figure 4), SGADGEVFDGQQQYEGIGK, were also higher than in Chiangrai and Nan samples. Presumably, the geographic of perilla cultivation influenced on the expression of genes involving n-3 fatty acid production pathways. Altitudes, actually, are associated with abiotic stress, for instance, drought, change of humidity and low temperatures so that the expression of KASII and FAD3 are postulated that correlate with abiotic stress regulatory-transcription factors. Hence, the prediction for putative abiotic stress transcription factor binding sites of the promoter of KASII and FAD3 upstream region has been carried out to support our hypothesis.
Figure 3. The tryptic peptides represent the protein expression of KASII in mature perilla seeds collected from different locations. Peak area of tryptic peptides analyzed by Skyline, (a) FMLYMLTAGA and (b) GFVMGEAVLLEEHAK. The peptide mapping in protein KASII was demonstrated in (c).
Figure 4. The tryptic peptides represent the protein expression of FAD3 in mature perilla seeds collected from different locations. Peak area of tryptic peptides analyzed by Skyline, SGADGEVFDGQQQYEGIGK (a). The peptide mapping in protein FAD3 was demonstrated in (b).

2.4. Bioinformatics Analysis for Transcription Factor Binding Sites

For the prediction of the regulatory transcription factors, which responsible for the correlation of altitudes and their targeted gene expressions, we seek for transcription factor target within 1.0 kb upstream sequence regions of the targeted genes. However, P. frutescens genome sequencing is uncompleted so far. Therefore, we applied A. thaliana, completed genome sequencing plant model, to identify which transcription factor binding sites located on upstream region of KASII and FAD3. We then examined whether KASII and FAD3 are conserved genes among oil corps and completed genome sequencing plant model, A. thaliana. Firstly, we performed multiple alignment of two proteins among P. frutescens, A. thaliana and other oil crops. Multiple alignment of KASII protein sequences from various oil crops showed nearly 95% similarity (Supplementary Figure S1). Moreover, multiple sequence alignment of FAD3 protein sequences demonstrated approximately 95-98% similarity (Supplementary Figure S2) and particularly, both KASII and FAD3 demonstrated almost 100% similarity to A. thaliana. Therefore, it was postulated that upstream regions of genes are attributed to highly conservative degree. The upstream 1.0 kb sequences region of KASII and FAD3 were derived and predicted with prediction tool in Plant Transcription Database. The prediction criteria was set up at highly stringent parameters seeking for transcription binding site within 1 kb upstream of target genes. The binding site of transcription factor AP2 for KASII gene was identified with p-value of less than 1×10^{-6}. The conserved sequence of AP2 binding site was also found in (+) strand GTCTTTCTTTTCTTCTTTTT or CAGAAAGAAAAGAAGAAAAA in (-) strand. Similarly, the putative transcription factor binding site of B3 was identified with p-value cutoff of less than 1×10^{-6} on 1.0 kb upstream region of FAD3 and the binding site sequence is AAGGAAAAACCAAAGAAATA. The PWM logo was shown in Figure 5. This, consequently, presumably indicated that both KASII and FAD3 genes are regulated by AP2/B3 transcription factor families, which are responsible for abiotic stress environments such as drought and coldness.
3. Discussion

Perilla is remarkably known for its high ALA production. In Thailand, the cultivated areas are distributed in the Northern regions especially mountainous areas where they are located at higher altitudes than the other parts. On this regards, the correlation between ALA productivity and growing locations was determined through the expression of key genes, KASII and FAD3, encoding for fatty acid biosynthesis in mature seeds.

In term of genetic factors, several genes encoding enzymes involving lipid biosynthesis are expressed differently during seed developments of oil-producing plants [12-14,18]. In particular, a number of genes contributing to the production and accumulation of lipids are up regulated. The transcriptome analysis of inca peanut seeds revealed that KAS genes were highly expressed in middle-late stages during seed development where the oil accumulation was getting started [14,19]. According to the study of Venglat et al [13], KAS genes in flax seeds were highly expressed at the middle stage of embryo development. This expression pattern of KAS genes was in agreement with the occurrence in other oil crops such as Jatropha (Jatropha curcas) [20]. Recent transcriptome analysis of genes contributing lipid biosynthesis in perilla has been indicated 43 key genes getting involved [12].

To produce ALA in a high level, the desaturase enzymes are needed to be active. The mutation of FAD2 and FAD3 genes could affect the desaturation process in developing seeds [21]. In particular, the mutation of FAD3 gene significantly resulted in the reduction of ALA in flax [22]. In the embryos of flax seed at mature stage, the genes encoding fatty acid desaturase in many isoforms were highly expressed [13]. Particularly, it had been reported that the expression of gene encoding SAD, FAD2 and FAD3 were high, which was closely associated to the high production and accumulation of oil in storage cells of inca peanut [14]. These finding were also in well-agreement with the high expression of FAD3 gene in perilla. As reported by Rao et al [1] and Kim et al. [12], the increase of ALA to LA ratio during perilla seed maturation indicated that the n-3 desaturase was very active. They were also found that, throughout the late stage of seed development until the end of seed maturation, the content of ALA reached a steady state and remain constant.

According to the results, the expression of KASII and FAD3 genes was in agreement with the content of ALA and the altitudes, although it was not significantly different among the three areas. This could partly suggest that the altitudes may be one of many factors affecting the productivity of ALA in perilla. As perilla is short-day plants, it needs a long-night period to induce flowering [23] and cold stress for polyunsaturated fatty acids production [24,25]. Thus during growing season of short-day length, the terrain provides a proper low temperature and dry conditions [26]. Commonly, the annually average temperatures of Thailand in high mountain areas are low and yearly lowest during October to December. The exposure of plant to low temperature during seed maturation and lipid accumulation could then shape the profile of polyunsaturated fatty acids in many developing oil seeds [14,27,28]. On the other hands, the production of ALA possibly resulted from the interaction of many factors including climate, soil types and conditions, agricultural conditions, genetic diversity and the ability of plants to adapted to different environments [25-27,29].

Although the high expression of gene encoding FAD3 may reflect the activity of n-3 desaturase and the accumulation of n-3 fatty acids, the production of ALA may also depend on protein...
abundance. So that rather than solely measured mRNA expression level, we further used targeted proteomics to determine protein abundance related to target gene. It was found that both KASII and FAD3 in Maehongson perilla seeds expressed higher protein abundance than Chiangrai and Nan perilla seeds. Presumably, the high altitudes might play a role for increasing ALA contents due to lower temperature and drought. This could explain the increases of KASII and FAD3 expression are attributed to the increase of drought which is then contributed to the rising of n-3 fatty acid production eventually. During seed oil accumulation, a number of transcription factors play crucial roles in regulation of gene expressions of lipid synthetic genes. On this regards, the bioinformatics tool had been used to predict the transcription factor binding sites are responsible for the seed oil biosynthesis. The transcription factor binding sites at 1.0 kb upstream of KASII and FAD3 genes were found to be AP2 and B3 families, respectively. In particular, the AP2 and B3 transcription factors themselves play many roles in seed oil deposition especially in seed oil biosynthesis pathway [17]. Upon the abiotic stress environment, previous study also mentioned that the expression of FAD3 is responsible for the encounter of A. thaliana to drought and subsequently resulted in the increase of ALA production [30]. The explanation on this matter could be relevant to the increase of gene functions in cellular components in order to generate high level of polyunsaturated fatty acids to incorporate into membrane lipid components which are a tolerance respond to drought stress [31]. Not only major roles in seed oil biosynthesis, in fact, the AP2 and B3 families also dominantly respond to drought, high-salt content and temperature change in oil seeds [32]. As mentioned previously, the possibility in that the increasing of KASII and FAD3 expression corresponded to the increment of ALA production may associate with the altitude of growing sites and can be explained by the counteracts of perilla to environments such as drought and low temperature [25].

4. Materials and Methods

4.1. Chemicals and Sample Collection

All chemicals used were reagent grade except that chemicals used in RNA and DNA work were molecular grade. Mature perilla seeds (grown and collected in field conditions) were collected from three locations including Maehongson, Chiangrai and Nan province. Inca peanut was a gift from Chiangrai Agriculture Development Co., Ltd. Flax seeds were obtained from the Royal Project Doi Kam, Chiangrai.

4.2. Fatty Acid Composition and Lipid Analysis

Two kilograms of sun-dried seed samples from each location was subjected for oil extraction using cold-pressed method. Oil was then centrifuged at 4,500 rpm for 10 min to remove all other unwanted residues. Yellowish pure perilla oil was kept at 4 °C for further investigation. Fatty acid compositions were analyzed using GC/MS (Agilent Technologies, USA 6890) by the Institute of Product Quality and Standardization, Maejo University.

4.3. RNA Extraction and cDNA Preparation

Total RNA were extracted from seed samples as described previously [33]. In brief, 30 mg of seeds were mixed in equal amount with PVP-40. Seeds were then ground in liquid nitrogen before adding extraction buffer (8 M LiCl, 2% (w/v) PVP-40 and 5% (v/v) mercaptoethanol) and ethanol. Chloroform was used in order to remove high molecular weight impurities and lipids. The pellet was redissolved in solubilization buffer (1.4% (w/v) SDS, 0.75 M NaCl, 0.025 M EDTA, 2% (v/v) mercaptoethanol) and chloroform extraction was repeated followed by 5,000 rpm centrifugation for 3 min. The supernatant were transferred to a new microcentrifuge tube and gently mixed with TRI reagent (Molecular Research Center). Centrifugation at 12,000 rpm for 2 min was carried out and aqueous phase were transferred to a new tube for RNA precipitation using isopropanol. RNA pellets were then resuspended in RNase-free water. The cDNA was prepared using ReverTra Ace qPCR RT kit (TOYOBO) and kept at -20 °C until use.
4.4. Gene Selection and Primer Design

Two genes associated to fatty acid biosynthesis were selected in this study. A conserve region of selected genes were compared with other plant species on NCBI database using CLUSTAL omega multiple sequence alignment. The KASII gene was compared among plant species including *Perilla frutescens*, *Sesamum indicum*, *Jatropha curcas*, *Theobroma cacao* and *Glycine max*. The FAD3 gene was compared among plant species including *P. frutescens* (Accession numbers: U59477, AF213482, AF047039 and KC990786), *S. indicum*, *Nicotiana tabacum*, *Camelina sativa* and *Solanum tuberosum*. Elongation factor-1α gene (*EF-1α*) was used as a constitutive control gene. Primer pairs for sequence analysis were designed using Primer3 software [34]. The sequences of forward (F) and reverse (R) primers were as followed: *EF-1α* F: 5'-TACTACTGCACTGTATTGATGC-3' R: 5'-CAATCTTGTANACRTTCTGAAATG-3', KASII F: 5'-ATACCHATTGGGTITGGGAGG-3' R: 5'-CRA TCATDGAYTTTGTGACTCAC-3', FAD3 F: 5'-CTTCAACCCCTACAGCGATTTG-3' R: 5'-TAACCGTGGTGATGTAATG-3'. The underline bases were degenerative bases which were B: G/C/T, D: A/G/T, H: A/C/T, N: A/G/C/T, R: A/G and Y: C/T. Gene-specific primers for real-time PCR were design for the gene length between 100-150 base pairs. The sequences of forward and reverse primers were as followed: *EF-1α* F: 5'-CAAGGATGGTACAGACGTGA-3' R: 5'-TCATCGTACCCTGCCCTGAGT-3', KASII F: 5'-ATGGGAGAAGGTGCTGGA-3' R: 5'-CTTCAACCCCTACAGCGATTTG-3' R: 5'-CTTCAACCCTACAGCGATTTG-3'.

4.5. Gene Cloning and Sequence Analysis

Amplification was carried out at the optimum condition for each gene using the following temperature conditions: 1 cycle of pre-incubation at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. The degenerative primers were used at 1 μM. The PCR products were analyzed on 2% agarose gel electrophoresis in 1× TBE buffer and visualized by RedSafe gel staining (iNtRON Biotechnology). The purify PCR products were clone into pTZ57R vector (InsTAclone, Thermo Fisher Scientific). Five selected clones of each gene were sequences using the service from Macrogen Inc. DNA sequences data were analyzed using CLUSTAL omega multiple sequence alignment.

4.6. Gene Expression Analysis

To evaluate the expression of gene related to fatty acid biosynthesis, a real-time PCR was conducted with THUNDERBIRD SYBR qPCR mix (TOYOBO). Each reaction consisted of 1× SensiFAST SYBR Lo-ROX mix, 0.3 μM of each primer, 1× ROX dye and 5 μL of 10-time dilution of cDNA sample in a total volume of 20 μL. The reactions were run in triplicate on the Applied Biosystems 7500 real-time PCR system (Thermo Fisher Scientific). The PCR program was set as the following conditions: 1 cycle of polymerase activation at 95 °C for 2 min followed by 40 cycles of denaturation for 5 s at 95 °C, then annealing and extension at 60 °C for 35 s. The data were exported as threshold values (Ct) and further analyzed as fold change. The expression of *EF-1α* was used as a reference gene.

4.7. Proteomic Approach for Protein Expression Analysis

Proteins were extracted by grinding perilla seed in liquid nitrogen. Then, the debris was added with 1% SDS to dissolve insoluble proteins and shaken at 37 °C overnight. Next, the protein solution was centrifuged at 12,000 rpm for 15 min and the protein solution in supernatant was collected. After that, 10% Trichloroacetic acid (TCA) in acetone was added to the supernatant until the final concentration was reached 1%. The mixture was then kept at -20 °C for at least overnight. The protein pellet was collected by centrifugation at 12,000 rpm, 4 °C for 15 min. The protein pellet was dissolved in cold acetone, then vortex vigorously to wash the remaining TCA and kept in -20 °C for 30 min. The protein pellet was collected by centrifugation at 12,000 rpm at 4 °C for 15 min. The
supernatant was removed and the pellet was air-dried for at least 5 min before dissolving in 8 M urea, 10 mM AmBiC and 1% SDS. The protein concentration was determined using BCA assay.

4.8. Protein Electrophoresis and In-Gel Digestion

Thirty micrograms of perilla protein extract was run in SDS-PAGE at 30 mA for 120 min. The separating gel was sliced into 12 small pieces along protein lanes. SDS and Coomassie dye in protein slices was washed by soaking gel slices in 50% acetonitrile (ACN) in 25 mM AmBic buffer for 2 h. The solution was removed and 100% ACN was added several times until the gel pieces were clear. After that, gel slices were incubated in 100 μL of 4 mM dithiothreitol (DTT) in 50 mM AmBic at 60 °C for 15 min. Then 20 mM iodoacetamide (IAA) was added into the gel slices and further incubated at room temperature for 1 h. Gel washing process was then repeated by adding 50% ACN in 25 mM AmBic buffer followed by 100% ACN. After the gel slices became opaque, they were air-dried. These processes were repeated three times. Twenty micrograms of trypsin (sequencing grade, Sigma) were added into the gel slices and further incubated overnight. To extract tryptic-digested peptides, 100 μL of 75% ACN in 1% TFA was added to the gel slices and incubated for 5 min in a shaker. The solution was then transferred into a new clean tube. The extracted peptides were dried in Speed Vac. The sample was kept in ~80 °C for further analysis.

4.9. Mass Spectrometry Analysis

Peptide samples were dissolved in 0.1% formic acid. The solutions were analyzed using the HCTultra PTM Discovery System (Bruker Daltonics Ltd., U.K.) equipped with the UltiMate 3000 LC System (Dionex Ltd., U.K.). The digested peptides was separated by nanocolumn (PepSwift monolithic column 100 μm i.d. × 50 mm), in which mobile phase systems comprised of 0.1% formic acid (Eluent A) and 80% ACN in 0.1% formic acid (Eluent B). An elution was performed for 13 min using a linear gradient from 10% to 70% Eluent B at a flow rate of 300 nL/min, followed by a regeneration step (90% Eluent B) and an equilibration step (10% Eluent B). Peptide fragment mass spectra were acquired in data-dependent AutoMS (2) mode with a scan range of 300–1500 m/z, 3 averages, and up to 5 precursor ions selected from the MS scan 50–3000 m/z.

4.10. Protein Identification, Database Search and Protein Abundance Quantification

The raw LC-MS raw data sets from gel pieces were converted to Mascot generic file (mgf file) and subjected to search with In-house MASCOT search engine (Matrixscience, London, UK, licensed for Faculty of Tropical Medicine, Mahidol University) against Arabidopsis thaliana Swissprot database (most updated version). Search parameters were set as following, Enzyme: trypsin, Fix modification: Carbamidomethyl at cysteine residue (57.03 Da), variable modification: Oxidation at methionine (32 Da), Peptide tolerance: 20 ppm, MS/MS tolerance: 0.25 Da, peptide charge was set as 1+, 2+, 3+ and monoisotopic mode. The identified protein pass through score 100 lists containing y, b-ion peak lists and intensity of precursor mass were exported into DAT files and subjected to Skyline version 4.0 for use as library spectrum, then quantification of targeted proteins was performed with MS1 filtering method.

4.11. Protein Similarity Search and Transcription Factor Binding Site Search

Perilla KASII (O48943) and FAD3 (Q92PP7) protein sequences were retrieved from Uniprot (Swissprot) database and multiple aligned by tcoffee algorithm. To predict transcription factor binding region, upstream sequences of model A. thaliana of KAS II (AT1G74960 1.0kb) and FAD3 (AT2G29980 1.0kb) genes were retrieved from ThaleMine database [35] and analyzed for transcription factor binding site prediction against Plant transcription database with Transcription factor binding site algorithm [36,37]. Threshold p-value was set up at less than 1x10⁻⁶. The pair wise matrix score also derived from PlantRegMap database.

4.12. Statistics
Gene expression data were expressed as mean ± SE of duplicate experiment. Statistical significance was calculated using one-way ANOVA, *p<0.05 and **p<0.01.

5. Conclusions

The expression of KASII and FAD3 gene were highest in the seed sample from Maehongson and were correlated with the ALA content. In this case, the KASII and FAD3 gene expression in mature perilla seed can possibly be used as a molecular marker for fast determination of crops producing a desirable ALA and thus it is beneficial to crop selections for plant propagation in a new season. The MS-based proteomic analysis also confirmed the high expression KASII and FAD3 in seed samples from Maehongson. It was also found that the transcription factor binding sites AP2 and B3 were specific for KASII and FAD3, respectively. These connection might be responsible for sensitive respond to environmental changes in which the increasing of KASII and FAD3 expression and the ALA production were possibly associated with the altitudes of growing sites. This finding would also provide useful information for further genetic manipulations as well as plant treatments in ALA producing-perilla. Furthermore, the increase of ALA productivity in limited areas and restricted conditions could play a part in added the value of the product for further industrial applications.

Supplementary Materials: The following are available online at www.mdpi.com... Figure S1: Multiple alignment of KASII amino acid sequence of P. frutescens. KASII protein sequence similarity of P. frutescens (PfKASII) were compared with A. thaliana (AtKASII) and other oil seeds including Jatropha curcas (JacKASII), G. max (GmKASII), Thobroma cacao (ThbKASII). The results demonstrated that PfKASII has more than 97% similarity compared with AtKASII.

Figure S2: Multiple alignment of FAD3 amino acid sequence of P. frutescens. FAD3 protein sequence similarity of P. frutescens (PfFAD3) were compared with A. thaliana (AtFAD3) and other oil seeds including L. usitatissimum (LiuFAD3), G. max (GmFAD3), S. indicum (SiFAD3), T. cacao (ThcFAD3). The results demonstrated that PfFAD3 has 95% similarity compared with AtFAD3.


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Conflicts of Interest: The authors declare no conflict of interest.

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