RESEARCH ARTICLE

The Peanut (Arachis hypogaea L.) Gene AhLPAT2 Increases the Lipid Content of Transgenic Arabidopsis Seeds

Silong Chen1, Yong Lei2, Xian Xu1, Jiaquan Huang2, Huifang Jiang2, Jin Wang1, Zengshu Cheng1, Jianan Zhang1, Yahui Song1, Boshou Liao2*, Yurong Li1*

1 Hebei Provincial Laboratory of Crop Genetics and Breeding, Cereal and Oil Crop Institute, Hebei Academy of Agricultural and Forestry Science, Shijiazhuang, China, 2 Key Laboratory of Biology and the Genetic Improvement of Oil Crops, Ministry of Agriculture, Oil Crops Research Institute of the Chinese Academy of Agricultural Sciences, Wuhan, China

* lboshou@hotmail.com (BSL); apeanutlab@gmail.com (YRL)

Abstract

Lysophosphatidic acid acyltransferase (LPAT), which converts lysophosphatidic acid (LPA) to phosphatidic acid (PA), catalyzes the addition of fatty acyl moieties to the sn-2 position of the LPA glycerol backbone in triacylglycerol (TAG) biosynthesis. We recently reported the cloning and temporal-spatial expression of a peanut (Arachis hypogaea) AhLPAT2 gene, showing that an increase in AhLPAT2 transcript levels was closely correlated with an increase in seed oil levels. However, the function of the enzyme encoded by the AhLPAT2 gene remains unclear. Here, we report that AhLPAT2 transcript levels were consistently higher in the seeds of a high-oil cultivar than in those of a low-oil cultivar across different seed developmental stages. Seed-specific overexpression of AhLPAT2 in Arabidopsis results in a higher percentage of oil in the seeds and greater-than-average seed weight in the transgenic plants compared with the wild-type plants, leading to a significant increase in total oil yield per plant. The total fatty acid (FA) content and the proportion of unsaturated FAs also increased. In the developing siliques of AhLPAT2-overexpressing plants, the expression levels of genes encoding crucial enzymes involved in de novo FA synthesis, acetyl-CoA subunit (AtBCCP2) and acyl carrier protein 1 (AtACP1) were elevated. AhLPAT2 overexpression also promoted the expression of several key genes related to TAG assembly, sucrose metabolism, and glycolysis. These results demonstrate that the expression of AhLPAT2 plays an important role in glycerolipid production in peanuts.

Introduction

Peanuts are one of the largest and most important edible oil-producing crops in the world, surpassed only by soybean and rapeseed in planting area during the last five years[1]; they are used to produce a large percentage of the vegetable oil of many countries. Peanut oil is of high nutritional value; it has high concentrations of unsaturated C18 fatty acids (FAs, approximately...
80%) [2], much of which (approximately 45%, or 18:1) is oleic acid, a desirable and healthy type of FA that has been implicated in cardiovascular and cerebrovascular health [3]. With rapid increases in its consumption by humans and its industrial use in renewable biomaterial and fuel production, the demand for vegetable oil has increased significantly [4]. Therefore, high oil content is desirable and has been a major goal of breeding and genetic engineering of oil crops including peanut. The oil content in peanut seeds generally ranges from 42% to 52% [5], which is relatively high compared with most other oilseed crops. However, the highest seed oil content in the peanut germplasm is approximately 63% [6]. It is a challenge to substantially increase the oil content of peanut seeds using conventional breeding and genetic engineering.

In oilseed plants, approximately 95% of seed storage oils are composed of triacylglycerols (TAGs), which are primarily synthesized during the seed’s maturation phase. In plants, TAGs play critical roles in diverse physiological processes and in multiple metabolic reactions [7]; in particular, TAGs that are stored in the endosperm support successful seedling establishment after germination [8]. Furthermore, TAGs supply approximately 30% to 35% of dietary calories to people in the developed world [9].

In higher plants, TAGs are synthesized from glycerol-3-phosphate (G3P) and FAs. Through many previous studies, the FA and TAG biosynthetic pathways have been well characterized [10]. TAG is synthesized de novo in several sequential acylation reactions at the sn-1-, sn-2- and sn-3-positions of the G3P backbone with acyl chains from acyl-CoAs through the conventional Kennedy pathway in the endoplasmic reticulum (ER). These reactions are catalyzed by three acyl-CoA-dependent acyltransferases [9]. Besides, in some plants, the synthesis of TAG can also be assembled by transacylation of the sn-2 FA from phosphatidylcholine (PC) onto sn-3-position of diacylglycerols (DAG), catalyzed by the phospholipid: diacylglycerol acyltransferase (PDAT) enzyme during seed maturation [11, 12]. Lyso phosphatidic acid acyltransferase (LPAT, EC 2.3.1.51) is one of the major TAG synthesis enzymes and catalyzes the second step of TAG formation, controlling the metabolic flow of lyso phosphatidic acid (LPA) to phosphatidic acid (PA) in Kennedy pathway [13].

Considerable knowledge has been gained regarding LPAT in eukaryotes. Genes that encode LPAT enzymes have been identified in various organisms, such as mammals, yeast and plants [14–20]. There are five isolated and annotated LPAT genes designated AtLPAT1–5 in the Arabidopsis genome [21]. They include the plastidial isoenzyme gene (AtLPAT1), the ER-located isoenzyme gene (AtLPAT2), the male gametophyte isoenzyme gene (AtLPAT3), and two less closely related members, AtLPAT4 and AtLPAT5, which encode proteins without in vitro detectable LPAT activity. Increasing evidence shows that different LPATs have distinct expression patterns and functions [22]. Three Arabidopsis LPATs (AtLPAT1, AtLPAT2, and AtLPAT3) are essential to normal plant development [21]. Both Arabidopsis AtLPAT4 and AtLPAT5 give rise to alternative transcripts and may have higher orders of complexity in PA synthesis [9]. It is worth stressing that studies have focused on orthologous LPAT genes from various plants, such as AtLPAT2 [21], rapeseed LPATs [23], TmLPAT2 [24], and EpLPAT2 [25], because of their important roles in TAG bioassembly.

Recent results suggest that LPAT also plays important roles in lipid production. An expanding line of research shows that the overexpression of LPAT in transgenic seeds is associated with increased TAG and total FA content. When the wild-type and mutant forms of the yeast genes SLC1 (sphingolipid compensation mutant) and SLC1-1, which are homologs of ER-localized LPATs in Arabidopsis, were overexpressed in Arabidopsis, rapeseed, and soybean, the TAG content of the seeds increased [26, 27]. Overexpression of rapeseed LPAT genes (BAT1.13 and BAT1.15) with homology to Arabidopsis AtLPAT2 in Arabidopsis seeds resulted in transgenic seeds with increased mass and TAG content [23]. Collectively, these data suggest that increasing the expression of LPAT in seeds may regulate the flow of carbon intermediates.
into TAG through the Kennedy pathway, resulting in enhanced TAG accumulation [23]. Therefore, LPAT could be a potential target gene for increasing seed oil content, serving as a valuable biotechnological tool. Nevertheless, more functional work remains to be done on the LPATs to understand their metabolic roles in TAG accumulation.

In oilseed plants, LPAT activity is a potential bottleneck for the incorporation of acyl-CoA into PA [21, 28]. Previous experimental studies performed on diverse species have revealed that LPAT enzymes have preferences in the utilization of certain FAs as substrates and are commonly considered some of the most stringent acyltransferases regarding acyl-specificity [24]. Biochemical studies on the LPATs from a number of oilseeds suggested that microsomal LPATs have a generalized preference for 16- and 18-carbon monounsaturated FAs over saturated FAs [29, 30].

The specific functions of LPATs in TAG biosynthesis in oil crops vary among different organisms [25, 31]. Despite studies on model organisms that show the potential of LPATs to change FA composition and increase the oil content of seeds, the precise functions of the majority of LPATs remain largely unknown. Therefore, the identification and functional characterization of new LPAT genes will provide useful information on the potential roles of LPATs in oil crops such as peanut, potentially contributing to genetic improvement.

In our previous studies, using cDNA library screening, two full-length transcripts encoding the LPAT-family genes AhLPAT2 and AhLPAT4 were isolated from developing peanut seeds, and their temporal and spatial expression patterns were analyzed [32, 33]. Gene expression analysis during seed development can be used to identify putative candidates involved in the synthesis of seed lipids such as TAG [31, 34–36]. Our results demonstrated that AhLPAT2 is highly similar to typical microsomal LPAT isoforms. AtLPAT2 was expressed ubiquitously in diverse organs but most highly in the seeds; it has a pattern of expression similar to that of RclLPAT2 [31], AtLPAT2 [21], BAT1.13 [23] and EpLPAT2 [25] but distinct from that of TmLPAT2 [24]. Moreover, we found that the AhLPAT2 expression level increases rapidly during the active phase of oil accumulation and then decreases markedly as the seed lipid content plateaus. In addition, AhLPAT2 transcript abundance positively correlated with seed oil content (p < 0.05). Unlike AhLPAT2, the AhLPAT4 gene is highly expressed, but only in the vegetative organs; its transcript level is considerably lower in seeds. The above lines of evidence indicate a correlation between AhLPAT2 and lipid biosynthesis in peanut seeds. However, this gene’s exact biological function in regulating seed oil accumulation is still unknown. Therefore, further work will be required to investigate the biological function of AhLPAT2.

Our objectives in this study were to identify and validate the potential contribution of AhLPAT2 to lipid accumulation in peanut. We further examined the expression differences of AhLPAT2 in developing seeds of high- and low-oil peanut cultivars. Then, the subcellular localization of AhLPAT2 was examined. We used Arabidopsis to survey the molecular function of the AhLPAT2 gene due to the difficulty of peanut plant transformation. In transgenic Arabidopsis, the effects of overexpression on seed oil content, FA composition and seed yield were analyzed. Our study provides a better understanding of the functional role of AhLPAT2 in seed oil accumulation. The results demonstrate that seed-specific overexpression of AhLPAT2 can be employed to increase TAG content and may facilitate improvements in oil production in peanut and other oil crops through genetic manipulation of the AhLPAT2 gene.

**Materials and Methods**

**Plant materials**

Seeds of the peanut cultivars Zhonghua12 and Te21 were planted at the Dishang Experimental Station of Food and Oil Crops Research Institute of the Hebei Academy of Agriculture and
Forestry Sciences in Shijiazhuang, China (37.56°N, 114.43°E) between May and September. Zhonghua12 is a high-oil cultivar whose seed oil content is approximately 59.4%; Te21 is a low-oil cultivar whose seed oil content is approximately 49.3% [37]. The treatment and collection of developing peanut seeds were performed as described by Yin et al. [38], with minor modifications. Seeds at various developmental stages (10, 20, 30, 40, 50, 60, 70 and 80 days after flowering, or DAF) were removed from the immature pods of the two genotypes, frozen in liquid nitrogen, and stored at −80°C for RNA isolation and analysis.

*Arabidopsis thaliana* (ecotype Col-0) was used for transformation in this study. The wild-type Col-0 and the transgenic plants were grown under standard conditions as described previously [39]. The Arabidopsis seeds were surface-sterilized in 3% sodium hypochlorite for 20 min and rinsed 10 times in distilled water. The sterilized seeds were then sown on plates containing Murashige & Skoog (MS) germination medium and kept for 3 days at 4°C in the dark to break dormancy. After 1 week, the seedlings were transferred to a pot filled with a mixture of peat/forest soil and vermiculite (1:1, v/v) and kept in the same controlled-environment growth chamber under the following conditions: 22°C with a diurnal photoperiod of 16 h light (50 μmol/m²/s) and 8 h dark per day and 70% relative humidity. For consistency in the reproducibility of the oil content measurements, the transgenic lines were always grown with Col-0 plants in the same chamber at the same time [40].

**The subcellular localization of AhLPAT2**

The full-length *AhLPAT2* ORF was fused without a stop codon downstream of the constitutive CaMV 35S promoter and upstream of *GFP* (NCBI accession number U87973) in a pEGFP expression vector, creating an *AhLPAT2*-GFP fusion protein. The coding sequence, including the *Xma*I (5') and *Bam*HI (3') restriction sites, was amplified using the primers 5'–TCCCCGGGATGGGTATTGCAGCTGCTGCT–3' and 5'–CGCGGATCCGGCTGTTGTTTGACATTCTT–3'. The PCR product and the pEGFP plasmid were cut with *Xma*I and *Bam*HI and ligated together using T4 ligase. The expression of *AhLPAT2* and *GFP* was under control of the CaMV 35S promoter. The sequences of the fusion constructs were verified by sequencing. The recombinant pEGFP *AhLPAT2*-GFP plasmid and the control pEGFP plasmid were bombarded into 20 to 25 onion epidermal segments using a Helios gene gun (Bio-Rad, Hercules, CA) according to the procedures described in [41, 42]. After incubation on MS medium at 25°C for 36 h in the dark, the transformed cells were observed by confocal microscopy (Nikon A1, Japan). DAPI (4', 6-diamidine-2'-phenylindole dihydrochloride; Roche, Germany) in methanol solution (1 μg/ml) was used to visualize nuclei.

**Construction of an AhLPAT2 vector for seed-specific expression and plant transformation**

The coding region of the *AhLPAT2* gene was amplified using the *AhLPAT2* forward primer 5'–GGCGAGCTCATGGGTATTGCAGCTGCTGCT–3', which contained a *Sac*I restriction site, and the *AhLPAT2* reverse primer 5'–TCCCCGGGATGGGTATTGCAGCTGCTGCT–3', which contained an *Xma*I restriction site. The amplified *AhLPAT2* gene with the flanking restriction sites was subcloned into the corresponding sites of the plant transformation vector pBarN in the sense orientation. pBarN was derived from the plant binary vector pCAMBIA-1301 (AF234297) [43] by introducing a *Hind*III/SacI fragment containing the rapeseed napin promoter [44] and an *Eco*RI/HindIII fragment containing the NOS terminator [45]. The hygromycin phosphotransferase coding region and its promoter were replaced with a fragment containing the 35S promoter and the *Bar* gene, which confers resistance to glufosinate-ammonium and was used as a selectable marker to screen for transgenic plants. The resulting
construct was designated Napin:AhLPAT2. The construct’s integrity was confirmed by sequencing. The plasmid was transformed into Agrobacterium tumefaciens strain GV3101 by electroporation (Gene Pulser II, Bio-Rad, Denmark) and then introduced into wild-type Arabidopsis using the floral dip method [46].

Selection of transgenic plants

To confirm transgene integration, T0 transgenic seeds were selected on plates containing MS medium supplemented with 100 mg/ml glufosinate. After 7 to 10 d, herbicide-resistant seedlings that had green leaves were identified as T1 transformants, and they were transferred to soil. The presence of the AhLPAT2 transgene was confirmed by PCR on genomic DNA isolated from the leaves of the T1 seedlings using the primers NapinHinF (5′–CCCAAGCTTAGCTTTCTTCATCGGTGATTGAT–3′) and AhLPAT2XmaR (5′–TCCCCCGGGCTACTGTTTGGACATTCTT–3′). Independent transgenic Arabidopsis lines exhibiting a 3:1 segregation of glufosinate (30 μg/ml) resistance in the T2 generation were selected, and homozygous T3 transgenic lines were established. T2 lines exhibiting greater oil deposition and AhLPAT2 expression than Col-0 were propagated to yield T3 seed lines. The quantitation of AhLPAT2 expression in transgenic Arabidopsis siliques was performed as described below. Seed oil content and FA analyses were conducted using seeds from the T1, T2 and T3 generations of transgenic Arabidopsis. Additional assays were performed, and data on plant height, the number of siliques per plant, seeds per silique, and silique length were collected from homozygous transgenic lines in the T3 generation.

Measurement of seed size and 100-seed weight

Arabidopsis seed size was determined as described previously [47]. The seeds from each line were dried in open tubes in a desiccator for 48 h prior to measuring and weighing. Arabidopsis seeds were photographed on white paper using a high-resolution scanner (Canon, Japan). After thresholding and converting the photographs to binary images, seed size was measured using the “Analyze particles” function of ImageJ (National Institutes of Health). The sizes of 40 to 50 seeds per silique per genotype were determined. To determine seed weight, batches of 100 seeds were weighed using a microbalance (Sartorius, Germany).

Determination of seed oil content and quantitative FA analysis

The oil content of air-dried peanut seeds at different developmental stages was determined using nuclear magnetic resonance (NMR) analysis as described previously [32]. Seed oil content was expressed as a percentage of dry seed weight. To determine the total oil content and FA composition of the Arabidopsis lines, the mature seeds were measured using gas chromatography (GC) coupled with a flame ionization detector (FID) as described previously [23]. Twenty milligrams of Arabidopsis seeds from each line were subjected to direct transmethylation by the addition of 1 ml of 5% (v/v) H2SO4 in methanol (CH3OH) containing 0.2% butylated hydroxytoluene (BHT) in glass tubes with Teflon-lined screw caps. For oil extraction and transmethylation, the samples were heated for 1.5 h to between 90°C and 95°C. FA methyl esters (FAMEs) were extracted with hexane. FAMEs were quantified using GC (Agilent 7890A, CA) on a 0.25 mm × 30 m column with Carbowax (Alttech) using helium as a carrier gas and 20 ml/min flow, and flame ionization was used for detection. The injector temperature was 250°C, and the detector temperature was 260°C. The injection volume was 1 μl. The oven temperature was maintained at 170°C for 1 min and then ramped to 210°C at 3°C per minute; it was then maintained at this temperature for an additional 8 min. FAMEs were identified by comparing their retention times with those of a standard mixture of FAMEs. One hundred
microliters of 2.5 mg/ml heptadecanoic acid (17:0) was used as the internal standard to quantify the individual FAs. Measurements of the total FA content and FA composition are given as micrograms of FA per milligram of dry seeds (μg/mg).

Seed protein analysis

The concentration of protein was determined using the Bradford method[48] and a Bradford protein assay kit (Tiangen, Beijing) according to the manufacturer’s instructions. Twenty milligrams of seeds were ground in 200 μl of extraction solution buffer. Finally, 75 μl of the supernatant was subjected to ultraviolet spectrophotometer analysis on a SPECORD 205 (Analytik, Jena), and the absorbance values at 595 nm were measured.

RNA isolation and SYBRGreen-mediated quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from developing peanut seeds at the indicated stages using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Total RNA was isolated from 100 mg of young siliques from wild-type Col-0 and the transgenic Arabidopsis lines 14 DAF using Trizol Reagent (Invitrogen, San Diego, CA). DNA contamination in the RNA samples was removed with RNase-free DNase.

For qRT-PCR, 1 μg of DNase-treated RNA from each sample was used for reverse transcription. The first-strand cDNA (1 μl) was used as the template for qRT-PCR amplification using SYBR Green Master Mix (TOYOBO, Japan). Triplicate quantitative assays were performed on an iQ 5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA) as follows: pre-denaturation at 95°C for 5 min; 40 cycles of 95°C for 15 s, 60°C for 20 s, and 72°C for 20 s; and a final extension at 72°C for 5 min. The gene-specific primers for AhLPAT2 in the peanut seeds were 5’–GGCTTATTGATTGGTGCTGGGT–3’ and 5’–ACCAACCAATGACCGGCA–3’. The gene-specific primers for qRT-PCR on the Arabidopsis samples are listed in Table S1 Table. The primers were properly optimized, and their efficiency was close to one. AtActin7 (NM_121018)[49] and AhUbiquitin[50] were used as reference genes in Arabidopsis and peanut, respectively, to quantify the relative transcription levels of each target gene. The relative transcript abundance of the target genes in the samples was calculated using the 2−ΔΔCT method[51]. The final values obtained were a measure of the fold changes in expression for the genes of interest.

Statistical analysis

All experiments were performed using at least three replicates. Significant differences were determined using paired Student’s t-tests in the SAS statistical package for Windows v. 9.0 (SAS Institute, NC, USA). Values of p<0.05 were considered significant.

Results

Identification of candidate gene AhLPAT2 relevant to lipid accumulation

To investigate the expression of genes that possibly participate in oil synthesis in developing peanut seeds, based on experimental results in the field over three years, we selected the high-oil-content peanut cultivar Zhonghua12 and the low-oil-content cultivar Te21. Our results suggest that oil content was stable in both genetic backgrounds over three years. The seed oil content of Zhonghua12 and Te21 differed significantly by more than 10% (p<0.01) (Fig 1A). Using experimental materials with such large differences in seed oil content, we identified many differentially expressed genes associated with seed oil content in peanut. The AhLPAT2
gene, isolated from developing peanut seeds by sequencing a full-length-enriched cDNA library and homology-based cloning, was originally identified from gene differential expression analysis using RNAs from different peanut organs and developing seeds at different stages [33]. Among the differentially expressed genes chosen and characterized, AhLPAT2, a gene encoding a putative LPAT protein, was identified as seed-preferred gene. The expression of AhLPAT2 had a similar trend to that of oil accumulation in seeds [33]. We focused on AhLPAT2 gene and further dissected its function in lipid accumulation in detail. In the present study, an attempt was made to test and analyze the AhLPAT2 transcript level at different developmental stages of Zhonghua12 and Te21. The trend in the AhLPAT2 expression pattern in developing seeds across the eight stages, from 10 DAF to mature seeds, was similar in the high-oil cultivar Zhonghua12 and the low-oil cultivar Te21. However, the AhLPAT2 transcript levels were significantly higher in Zhonghua12 seeds than in Te21 seeds at 10, 40, 50, 60, and 80 DAF.
and especially between 40 and 60 DAF (Fig 1B), when the storage oil accumulated at its maximum rate [38, 52]. Taken together with our previous data [33], AhLPAT2 is associated with seed oil accumulation.

The subcellular localization of AhLPAT2

To determine the subcellular localization of AhLPAT2, an AhLPAT2-GFP fusion gene driven by the CaMV 35S promoter was constructed (Fig 2), and GFP-tagged AhLPAT2 was transiently expressed in onion epidermal cells. GFP alone generated a strong fluorescent signal that was observed in the cytoplasm and the nucleus (Fig 2A–2D), whereas the signal from AhLPAT2-GFP was mostly detected in the cytoplasm of transformed cells (Fig 2E–2H). These data indicate that AhLPAT2 is a cytoplasmic protein.

The phenotypes of transgenic AhLPAT2-overexpressing Arabidopsis plants

To evaluate the function of AhLPAT2, we generated a binary plant transformation vector harboring the AhLPAT2 coding sequence controlled by the Napin promoter and introduced this construct into Agrobacterium tumefaciens, which was subsequently used to transform Arabidopsis. The presence of the AhLPAT2 transgene was confirmed in the T1 seedlings via PCR. Through segregation analysis of the herbicide-resistant transgenic plants, 45 single-copy AhLPAT2 sense plants were identified. To analyze the effect of the AhLPAT2 transgene on the lipid phenotypes of the seeds, 11 independent T2 homozygous single-copy AhLPAT2 plants were randomly selected. After using qRT-PCR with gene-specific primers to determine the AhLPAT2 expression levels in the siliques of the transformed Arabidopsis plants, we selected two independent homozygous lines with relatively high AhLPAT2 levels (FNH1-21 and FNH2-2) for further analysis. In general, the AhLPAT2 transcript levels in the developing siliques of line FNH2-2 were higher than in those of line FNH1-21 (Fig 3).

We examined whether AhLPAT2 overexpression changed any of the plants’ phenotypes. We did not observe any significant difference in plant height or seed development, including...
mature silique length and seed size, between the transgenic AhPAT2 lines and wild-type plants (S1 Fig). These results indicated that seed-specific overexpression of AhLPAT2 does not influence the overall growth and development of Arabidopsis plants.

Seed weight and yield per transgenic Arabidopsis plant

Because the amount of seed oil produced per plant is dependent on the oil content and the seed yield, and because the seed yield per plant is dependent on seed weight, the number of seeds per silique, and the number of siliques per plant, we examined the effect of AhLPAT2 overexpression on the seed yield per plant. The number of seeds per silique and the number of siliques per plant were not significantly changed in the two T3 homozygous transgenic lines (Fig 4A and 4B). By contrast, the 100-seed weight was significantly increased in the transgenic FNH2-2 line compared with Col-0 (Fig 4C). As a result, when the increase in the average seed weight of FNH2-2 was taken into account, the average seed yield per plant was 24.4% greater than that of wildtype (Fig 4D). However, the seed weight and yield of FNH1-21 were not severely affected by seed-specific overexpression of AhLPAT2 than FNH2-2, suggesting that the phenotypes of the transgenic lines derived from different independent transformation event are different.

The seed oil content of transgenic Arabidopsis

Analysis of 42 transgenic AhLPAT2 Arabidopsis overexpression plants showed that the seed oil content ranged from 25.5% to 36.0%, with a mean value of 32.2%; this mean is 7.4% higher than that of non-transgenic plants. Thirty-four of the plants had higher seed oil content than the non-transformed seeds (S2A Fig).

We also measured the total lipid content of Col-0 seeds and of seeds from the AhLPAT2 overexpressing lines, including the 11 independent T2 lines and the two T3 homozygous lines. The T2 plants were derived from representative T1 transgenic seeds. The homozygous Napin: AhLPAT2FNNH1 T2 lines exhibited oil content increases ranging from 1.7 to 3.8 percentage points on a mature seed weight basis, representing a net overall increase of 5.5% to 12.2%; the oil content of the FNH2 lines exhibited oil content increases ranging from 1.7 to 6.9 percentage points, representing net overall increases of 5.4% to 22.1% compared with that of the wild-type plants grown in the same growth environment at the same time (S2B Fig).
The average oil contents of the five transgenic FNH1 lines and the six FNH2 lines were 33.7% and 35.5%, respectively, which were significantly different from the average 31.2% oil content of the non-transformed plants (p < 0.05). Combining the results from the five FNH1 and six FNH2 lines, the mean seed oil content was 34.6%, which was 11.1% greater than the mean of the non-transformed plants (p < 0.05; S2B Fig). The homozygous FNH1-21 and FNH2-2 plants were analyzed in the next generation.

In the T3 homozygous seeds, AhLPAT2 overexpression caused a significant increase in oil content compared with Col-0 seeds. The oil content in the seeds of the two transgenic Arabidopsis lines (FNH1-21 and FNH2-2) was approximately 28.4%, which was 12.2% higher than that of Col-0 (25.3%; Fig 5A). The T3 homozygous transgenic seeds had an oil content similar to that of the T2 generations, with a 15.6% increase in the strongest line (FNH2-2). The high oil trait of the pooled segregating T2 generation was stable and heritable, as it was similar to the average of the corresponding homozygous T3 progeny (compare Fig 5A with S2B Fig). Combining the results from the T2 and T3 lines, these data indicate that AhLPAT2 promotes the accumulation of seed oil. Taken together with the increase in seed yield per plant in the transgenic lines, the overall seed oil production per plant in the Napin: AhLPAT2 lines increased by approximately 9.5% in the FNH1-21 line and by 43.5% in the FNH2-2 line compared with the wild-type plants (p < 0.05; Fig 5B); this increase was due to the higher seed yield per plant and

Fig 4. Effect of AhLPAT2 Overexpression on Seed Storage Reserve Content of Independent Homozygous T3 Lines. A. Seeds per silique. B. Numbers of siliques per plant. C. 100-seed weight. D. Seed yield per plant. Values are means ± SE (n = 10). Asterisks indicate significant differences between the wild-type and transgenic lines at p < 0.01 (**). doi:10.1371/journal.pone.0136170.g004
the increased seed oil content. However, the opposite result was obtained for the protein levels in the AhLPAT2 seeds (Fig 5C); presumably, the increased oil biosynthesis in the transgenic seeds may decrease the carbon flux to the protein biosynthetic pathways.

FA levels and composition in mature AhLPAT2 transgenic Arabidopsis seeds

We measured the FA content of whole mature seeds in the AhLPAT2 transgenic Arabidopsis lines. Compared with the corresponding wildtype, the total FA content of the seeds increased by 9.7% to 20.5% in T2 overexpressing seeds and by 11.8% to 24.6% in seeds of the corresponding T3 homozygous Napin:AhLPAT2 lines (S3 Fig and Fig 6A).

To determine if the increase in the total FA content in the seeds of the overexpression lines was derived from an increase in one or many specific FAs, we analyzed the changes in abundance of the major FAs in the transgenic seeds. We found that most of the FAs examined (except 22:1) accumulated to higher levels in the transgenic T2 and T3 seeds than in the wildtype seeds. The changes in FA composition in the T2 seeds were similar to those in the T3 homozygous seeds. The FAs with the greatest increases in the transgenic seeds were 18:0, 18:1, 18:2 and 18:3. The levels of the longer chain FA20:1 increased to a lesser degree (S4A Fig and Fig 6B). However, the changes of 18:3 showed characteristic differences between the transgenic T2 and T3 seeds in the overexpression lines. The 18:3 content in FNH2-2 line T3 seeds was not altered as compared with the wild type, while the amount of 18:3 was significantly increased by approximately 70% in transgenic T2 seeds compared with the wild type (Fig 6B and S4A Fig). This result needs to be confirmed by an exhaustive analysis of TAG molecular species. These results suggested that the seed-specific overexpression of AhLPAT2 can change the FA composition of the seeds.

Seed oil quality depends primarily on the chain length and saturation of the FAs. The FA saturation was significantly altered in the transgenic seeds. The saturated FA level increased slightly in the AhLPAT2 transgenic seeds. By contrast, the unsaturated FA level in the seeds of the two lines was significantly greater than that of the wild-type seeds. Therefore, the ratio of unsaturated to saturated FAs increased by more than 10% in the transgenic T3 seeds compared with the wild-type seeds (p < 0.05; Fig 6C). The most common fatty acyl carbon chain lengths in seed TAGs were 18C and 20C. We analyzed the changes in the FA carbon chain lengths in the transgenic seeds and found that the ratio of 18C to 20CFAs either did not change significantly or decreased slightly (Fig 6D). Similar results were obtained in both the T2 and T3 generations.
of single T-DNA insertion lines (S4B and S4C Fig). Taken together, these results indicate that AhLPAT2 is associated with altered proportions of unsaturated FAs in storage TAGs but not with changes in the lengths of the carbon chains.

Differential expression of representative FA and TAG biosynthetic genes in transgenic AhLPAT2 Arabidopsis siliques

To gain insight into the molecular mechanism by which AhLPAT2 facilitates FA biosynthesis and TAG assembly, we measured the expression levels of several representative FA and TAG biosynthetic genes in developing transgenic Arabidopsis siliques. These genes included key genes in the fatty acid synthetic pathway (FAD, ACP, and BCCP) and the Kennedy pathway (GPAT, LPAT, and DGAT), as well as the gene encoding the major oil body protein (Oleosin). During FA synthesis, delta-12 fatty acid desaturase (FAD2) is the main enzyme responsible for polyunsaturated lipid synthesis in developing seeds; it catalyzes the conversion of 18:1 to 18:2. The expression of AtFAD2 was the same in transgenic and wild-type siliques (Fig 7), indicating that the increased 18:2 content associated with AhLPAT2 was not caused by elevated expression of the gene encoding the enzyme that directly participates in FA desaturation. Acyl carrier protein (ACP) carries acyl intermediates and plays a central role in FA biosynthesis [53, 54]. Biotin carboxyl carrier protein (BCCP) is a subunit of ACCase; in the first committed step of FA biosynthesis, which is also the rate-limiting step [55, 56], it serves as a carrier protein for biotin and carboxybiotin for the carboxylation of acetyl-CoA to form malonyl-CoA [57, 58].
Fig 7. Altered Expression of Genes Involved in the FAs and TAG Biosynthesis Pathway. Values are means ± SE (n = 3). Total RNA was prepared from developing siliques of transgenic lines. Gene expression levels are shown relative to the expression of AtActin7 in each sample. The transcription level of each gene in the wildtype was set as 1.0. Asterisks indicate significant differences between the wild-type and transgenic lines at p < 0.01 (***) and p < 0.05 (*). doi:10.1371/journal.pone.0136170.g007
AtACP1 and AtBCCP2 were analyzed in young T3 siliques at 14 DAF. The transcript levels of the AtACP1 and AtBCCP2 genes were higher in the transgenic siliques (Fig 7), which can likely be ascribed to the increased total FA level in the transgenic seeds.

In the Kennedy pathway, G3P is sequentially acylated by G3P acyltransferase (GPAT), LPAT and DAG acyltransferase (DGAT). We noticed that the transcript levels of four related TAG biosynthesis genes in the Kennedy pathway, including AtGPAT9, AtLPAT2, and AtDGAT1, were up-regulated to varying degrees in the transgenic lines (Fig 7), which may be consistent with the increased TAG levels in the AhLPAT2-overexpressing seeds. There are four AtOleosin isoforms in Arabidopsis. Compared with the wildtype, the mRNA level of AtOleosin increased nearly 3.9-fold in the FNH2-2 siliques (Fig 7). In FNH1-21 transgenic seeds, despite the low expression of AtOleosin, the TAG level was elevated, possibly due to the action of the other AtOleosin isoforms.

Increased expression of key genes involved in sucrose metabolism and glycolysis in transgenic AhLPAT2 Arabidopsis siliques

Many previous studies have pointed out that biosynthesis of FA and TAG is tightly linked to photosynthesis, carbohydrate (sucrose) metabolism, and glycolysis which provide carbon source for FA synthesis [34, 59, 60]. It has been found that intermediates of sugar metabolism such as glucose-6-phosphate (Glc6P) and pyruvate are imported into plastid from the cytoplasm [61]. Pyruvate dehydrogenase complex in plastids can transform pyruvate into acetyl-CoA, which is the substrate for de novo synthesis of FA. Besides acetyl-CoA, sugar metabolism such as the pentose phosphate pathway also provides reducing power for FA biosynthesis, thus indicating that sugar metabolism is highly bound to FA biosynthesis. We attempted to detect whether the expression levels of genes involved in sucrose photoassimilation and glycolysis were altered. Sucrose synthase (SUS) plays a central role in photosynthetic carbon assimilation and partitioning. In the glycolytic pathway, fructose-bisphosphate aldolase (FPA) is an essential enzyme that catalyzes a reversible aldol reaction, and phosphoglycerate kinase (PGK) is a major enzyme used in the first ATP-generating step of the glycolytic pathway. ADP-Glc pyrophosphorylase (AGP) catalyzes the conversion of Glc1P to ADP-Glc, which is then incorporated into starch granules in developing seeds and acts as a regulatory enzyme for plant starch synthesis [62, 63]. In the developing siliques of the AhLPAT2 transgenic plants, the expression of AtSUS3 was highly or slightly increased compared with the wildtype (Fig 8). Compared with the wildtype, the mRNA abundance of AtFPA1 and AtPGK was at least 1.5-fold higher in the transgenic siliques (Fig 8). The mRNA level of AtAGP was also significantly higher in the transgenic lines (Fig 8). These data demonstrate that the mRNA levels of genes involved in sucrose metabolism, glycolysis, and starch synthesis are increased in the transgenic AhLPAT2-overexpressing Arabidopsis plants.

Discussion

Our results indicate that peanut AhLPAT2 plays important roles in the seed oil accumulation pathway and provide clues to understanding of the molecular mechanisms controlling oil biosynthesis and storage in peanut seeds. In agreement with previous results, our current data indicate that the transcript abundance of AhLPAT2 was higher in the seeds of a high-oil peanut cultivar than in a low-oil cultivar between 40 and 60 DAF. These patterns are consistent with a proposed general role for TAG metabolism in seeds. Similar to how quantitative trait loci (QTLs) and transcriptomics can be used to identify genes in the seed oil accumulation pathway [38, 64], our experimental materials with large differences in their seed oil content make it easier to identify differentially expressed genes associated with seed oil content. It has been
reported that in developing castor seeds, the *RcLPATB* gene[31] grouped with members of the so-called B-class LPAT enzymes and the *RcLPAT2* gene[65]; these genes are highly induced during endosperm expansion and maturation, when oil biosynthesis and accumulation are maximal.

We analyzed the subcellular localization of *AhLPAT2* in peanut. Inspection of the subcellular localization of peanut *AhLPAT2* in onion epidermal cells revealed that this protein is distributed rather randomly in the cytosol and is not concentrated on any membranes structures such as ER(Fig 2E and 2H). Analysis of amino acid sequences of *AhLPAT2* by PSORT tool revealed that the *AhLPAT2* protein lacked a nuclear localization signal in its sequence[33], which is consistent with the localization of *AhLPAT2* in the cytosol. In contrast, five AtLPAT1 to AtLPAT5 identified from Arabidopsis so far are membrane bound through transmembrane domains, especially AtLPAT2, which is ubiquitous and ER located[21]. At4g24160, the first soluble LPAT in plants, was found to be localized in the mitochondria and the chloroplast[66]. However, our results do not clarify, whether *AhLPAT2* is actually present in the same cells in developing Peanut seeds, flowers, or leaves or whether *AhLPAT2* expression is restricted to different tissue types within these organs. These questions will need to be addressed using monospecific *AhLPAT2* antibodies and immunomicroscopic analysis of native peanut tissues[67]. Regardless, it is tempting that *AhLPAT2* has been shown to play a role in the production of TAGs during seed development in transgenic plants.

*AhLPAT2* overexpression using a seed-specific promoter enhanced oil content, FA accumulation, and seed weight in Arabidopsis transgenic plants. Nevertheless, in our present study, the two transgenic lines selected show markedly different effects on expression levels of *AhLPAT2* and endogenous genes, seed yield, seed oil content, oil yield, and seed FAs content. It is possible that the *AhLPAT2* fragment may be integrated into different locus of the chromosomal
DNA of the two transgenic Arabidopsis lines. Furthermore, recent studies have revealed that the integration sites of the exogenous genes were unpredictable[68]. The site effect and the structure of the exogenous genes have significant effects on the gene expression and phenotype differences of the transgenic plants. In this study and under our experimental conditions, transgenic overexpression of AhLPAT2 resulted in a 12.2% increase in oil content and an 11.8% to 24.6% increase in total FA content in T3 Arabidopsis seeds without changing other agronomic traits. The change in the protein content of the transgenic AhLPAT2 seeds had the opposite effect. In addition to the seed quality traits, the seed yield-related traits were examined in the AhLAPT2 lines. An analysis of seed weight suggested that the overexpression of AhLPAT2 leads to a significant or slight increase in the average seed mass. The total seed yield in milligrams per plant increased by 0.3% to 24.4%. We hypothesize that the increase in seed weight could be the result of increased FA and oil accumulation in transgenic seeds[39]. These current results corroborated several previous predictions derived from our retrospective data [33]. Recent studies have also shown that the LPAT-catalyzed conversion of LPA to PA is a metabolic pivotal point in the provision of intermediates required for the synthesis of diverse glycerolipids including membrane lipids and neutral TAG[23]. Apparently, an increase in LPAT activity can result in the enlargement of the storage lipid sink through a feed-forward effect.

In addition, the above findings from our study are also consistent with the results from other studies that show that LPAT mainly catalyzes TAG synthesis for seed storage, except for participation in the synthesis of membrane lipids in eukaryotes[21, 69]. Many studies have demonstrated that LPAT overexpression in the model plant Arabidopsis leads to a significant increase in seed oil content. When SLC1and SLC1-1[27] were expressed in Arabidopsis, the seed oil content rose from 8% to 48%, showing that the TAG content of the seeds had increased [26]. The overexpression of rapeseed LPAT isoforms (BAT1.13 and BAT1.5) with homology to Arabidopsis AtLPAT2 in Arabidopsis seeds also resulted in 6% and 13% increases in average seed weight and total seed FA content, respectively, compared with non-transformed plants [23]. By contrast, reducing the expression of the homologous Arabidopsis LPAT gene resulted in a 30% lower seed mass and a 16% lower TAG content, frequently resulting in a wrinkled seed phenotype [70]. This study shows that the individual line with the largest change in transcript abundance also had the highest percentage of seed oil content and total oil yield per plant. Our results are consistent with the findings of previous studies [71, 72], which show a correlation between AhLPAT2 transcript abundance and oil content but do not provide a mechanism for the relationship between the increase in expression of AhLAPT2 and the increase in LPAT activity. However, several mechanisms have been proposed in which Arabidopsis DGAT catalyzes the last step of TAG formation[39], lending support to our present study.

An increase in seed oil content in transgenic Arabidopsis plants can lead to an increase in plant oil yield under the conditions used in this study. Most genetic studies and breeding programs for oilseed crops focus on increasing the amount of oil produced by a plant[73]. Presently, we found that the total oil yield (milligrams per plant) was significantly increased (p<0.05) in the AhLPAT2 lines, with a maximum increase of 43.5% in the best transgenic line compared with the wild-type control. The increase in seed oil production is mainly due to an increase in seed oil content and seed weight without a significant change (p>0.05) in seed number per plant, similar to the case of rapeseed microsomal LPATs expressed intragenic Arabidopsis[23]. Similar mechanisms of lipid accumulation may have been affected by rapeseed LPATs and peanut AhLPAT2, although further studies are needed to confirm this.

As expected, the results derived from Arabidopsis are also consistent with the results from other studies on oilseed crops. For instance, SLC-1 was introduced into a rapeseed cultivar with high erucic acid (22:1ω9) levels [26]. The resulting transgenic line showed a substantial increase
in seed oil content and an increase in the proportion of erucic acid. The transgenic line was later tested in the field and exhibited a 53% to 121% increase in total erucic acid yield (weight/plot) [74], and the six best T5 field-grown lines showed a 2.8% to 5.6% increase in oil content, representing overall net increases in oil content of 6.7% to 13.5% on a dry-weight basis [75]. In soybean, the SLC1 gene was expressed in somatic embryos under the control of the seed-specific phaseolin promoter. Some transgenic somatic embryos in both T2 and T3 transgenic seeds showed an average increase in oil content of 1.5% over the controls, with a maximum increase of 3.2% in one T3 line [27]. Taken together, these results suggest that increasing the expression of LPAT in seeds may promote the flow of carbon intermediates into TAG through the Kennedy pathway, resulting in increased TAG accumulation; these observations may be of value for increasing lipid yield via transgenesis [70]. It is possible that AhLPAT2 would promote lipid accumulation in peanut seeds as it did in transgenic Arabidopsis seeds, although more studies are needed to determine this result. Above all, these data indicated that improvement in seed quality traits in peanut may be achieved by the manipulation of the AhLPAT2 expression level. This manipulation may improve genetics not only for peanut but also for other oil crops.

We found that the AhLPAT2 gene could preferentially incorporate unsaturated FAs into lipids from Arabidopsis seeds. However, AhLPAT2 seems to lack a preference for the length of the FA carbon chain. This finding is consistent with those of other studies on diverse traditional oilseed crops, which suggest that LPAT, and especially microsomal LPAT, has a general preference for 16- and 18-carbon monounsaturated FAs over saturated FAs[65]. In Arabidopsis, AtLPAT2 was expected to preferentially utilize 18:1-CoA over other fatty acyl-CoAs [21]. In addition, a similar result was found for LAT1 in Limnanthes, whereas LAT2 showed a broader specificity, with 16:0-CoA being incorporated at a rate that was even higher than that of 18:1-CoA [76]. The EpLPAT2 enzyme of Echium pitardii displays high activity for 18:3n3-CoA and 18:2n6-CoA [25]. It has been shown that the acyl-CoA specificity of castor bean RcLPAT2 is strongly influenced by the acyl group at the sn-1 position of the acceptor LPA substrate [31]. However, many other studies pointed to the opposite conclusion. For example, the FA composition of the TAG fraction in BAT1.13 and BAT1.5 transgenic plants did not vary significantly in the proportions of the major classes of FAs or in the very-long-chain-FAs (20:1-CoA and 22:1-CoA) compared with the wild-type control plants [23]. Additionally, Brown, Slabas [29] also found that in the majority of species examined, there is no correlation between the final sn-2 composition of the oil and the observed selectivity of the LPAT enzyme. Similarly, Roscoe [70] reported that the FA composition of transgenic lines was minimally affected by overexpression of the yeast SLC1 gene or the homologous Arabidopsis LPAT gene. We speculate that this discrepancy could be due to differences between species types. Although our data strongly favor a role for AhLPAT2 in regulating FA composition, the acyl-CoA pool composition of the cell may also play a role in particular plants because the FA composition of TAG depends on both the substrate specificity of the acyltransferases and the acyl-CoA composition of the cell [77]. There is a need for further detailed studies and verification of the specificity of the AhLPAT2 enzyme using LPA and oleoyl-CoA as substrates.

In this work, the transcript levels for multiple selected ACCase and acyltransferase genes were increased in developing Arabidopsis siliques of AhLPAT2 seed-specific overexpression lines, thereby influencing FA and TAG accumulation. For example, the mRNA abundances of AtBCCP2 and AtACP1 were increased in overexpression lines compared with the wild type, which are involved in de novo FA biosynthesis. It seems likely that an increase in lipid accumulation in seeds requires upregulation of multiple related genes involved in carbon metabolism, FA synthesis, and end-product synthesis of TAGs from FAs. It has been proposed
that expression of transgenes encoding plant enzymatic functions can interfere with the normal expression patterns of corresponding endogenous genes as well as genes involved in the same or related processes. Although it is possible that the phenotype observed in overexpressing AhLPAT2 transgenic plants is due to solely to increased activity of this one enzymatic step, it is conceivable also that altering AhLPAT2 activity results in upstream metabolic pathways contributing to the biosynthesis of FA, to be incorporated into TAG. Moreover, we speculated that increases in AhLPAT2 activity may lower the size of the acyl-CoA pools, thereby signaling a need for enhanced FA synthesis. Together, our data suggest that increasing AhLPAT2 activity affects a series of genes involved in lipid metabolism, possibly through feedback or feed-forward effects[55]. However, qRT-PCR analysis showed that peanut AhLPAT2 did not strongly induce the expression of endogenous Arabidopsis AtFAD2. This finding suggests that AhLPAT2 may not have a strong effect on the AtFAD2 gene. Alternatively, another mechanism may also be involved in the AhLPAT2-dependent TAG assembly processes. Consistent with these results, the content of almost every major FA, not just 18:2, was higher in the transgenic seeds. Surprisingly, our results indicated that several important genes that directly participate in TAG assembly via the Kennedy pathway, such as AtGPAT9AtLPAT2 and AtDGAT1, were also expressed at higher levels in the AhLPAT2 overexpressing lines compared with wild-type plants. Similar results were observed in transgenic rapeseed [78] and potato [79] overexpressing CaLPAT from Crambe abyssinica. However, the mechanism by which this phenomenon works in peanut remains elusive; additional studies are needed to determine if the observed increase in storage lipids is caused by the exogenous AhLPAT2. We hypothesize that the interaction between exogenous AhLPAT2 and endogenous acyltransferase-encoding genes may have an additive effect on the percent seed oil content.

Previous studies have shown that when individual SUS isoforms are eliminated in pea [80] and maize [81], the seed weight and composition in these species are markedly changed. In this study, the expression of AtSUS3 was considerably induced in the transgenic lines. These observations, combined with our results and the molecular mechanism of the source-sink network in plants[82, 83], suggest that there is positive feedback between sucrose and lipids. In contrast to AtSUS3, the transgenic lines exhibited only moderate changes in the relative expression of key glycolytic genes such as AtFPA1, AtPGK and AtAGP. It is not surprising that increases in LPAT transcript abundance or activity may reduce the size of the acyl-CoA pool, thereby signaling a need for enhanced FA synthesis through, for instance, the promotion of ACCase activity[39].

In summary, in peanut, although several QTLs that influence seed oil content have been mapped, the specific genes associated with these QTLs have not yet been identified[6, 84]. In the present study, a novel AhLAPT2 gene from peanut was cloned, and its functions were analyzed. Our results show that seed-specific overexpression of AhLAPT2 has the potential to increase Arabidopsis seed oil content and weight. Analysis of seed yield per plant in transgenic Arabidopsis indicated that the total oil yield is also significantly increased. The plants had more unsaturated FAs and higher total FA content. In the siliques of homozygous transgenic lines, the relative expression levels of several genes involved in FA biosynthesis, TAG assembly, sucrose metabolism and glycolysis were also significantly increased. Therefore, AhLAPT2 is of interest for the genetic engineering of seed oil and FA composition and especially for decreasing the saturated FA content of edible vegetable oils.

**Supporting Information**

**S1 Fig. Effect of AhLAPT2 Overexpression on Arabidopsis Plant Development.** A. Plant height of mature Arabidopsis. B. Mature silique length. C. Mature seed size. Values are
means ± SE of measurements on individual plants (n = 10).

S2 Fig. Oil Content Analysis of Transgenic T1 and T2 Generation Transgenic Arabidopsis Seeds. A. Oil content distribution of transgenic T1 seeds. Col-0, wild-type control including 11 plants; FNH T1, Transgenic T1 Arabidopsis including 42 plants. The box contains 50% of the data points. The bars across boxes represent the medians. The top and bottom ends of the 'whiskers' represent the highest and lowest values observed. Black dots represent outliers. B. Seed oil content of homozygous T2 AhLPAT2 transgenic Arabidopsis plants. Seed oil content was determined by the NMR method. Mean FNH1 T2 indicates the mean of five FNH1 transformants; Mean FNH2 T2 indicates the mean of six FNH2 transformants; Mean FNH T2 indicates the mean of eleven FNH1 and FNH2 transformants. Values are average seed oil percentage ± SE (n = 5 and 6 for FNH1 and FNH2, respectively). Asterisks indicate significant differences between the wild-type and transgenic lines at p < 0.01 (**) and p < 0.05 (*).

S3 Fig. The Effect of AhLPAT2 Overexpression on Total FA Content in Independent T2 Homozygous Seeds. The values are means ± SE (n = 20). Asterisks indicate significant differences between the wild-type and transgenic lines at p < 0.01.

S4 Fig. The Effect of AhLPAT2 Overexpression on FA Profiles in Independent T2 Homozygous Seeds. A. The main FA composition. B. The ratio of unsaturated to saturated FAs. C. The ratio of 18- and 20-carbon FAs. The values are means ± SE (n = 20). Asterisks indicate significant differences between the wild-type and transgenic lines at p < 0.01 (**) and p < 0.05 (*).

S1 Table. Arabidopsis Gene-specific qRT-PCR Primers Used in this Study.

Acknowledgments

We thank Drs. Yuning Chen and Yueting Zhang (OCRI, CAAS) for provision technical guidance on gene expression analysis and plant transformation. We also thank Professor Shengyi Liu (OCRI, CAAS) and his laboratory staff for provision of plasmid pEGFP and technical assistance.

Author Contributions

Conceived and designed the experiments: BSL YRL JQH. Performed the experiments: SLC YL. Analyzed the data: SLC XX JNZ. Contributed reagents/materials/analysis tools: HFJ JW YHS ZSC. Wrote the paper: SLC XX.

References

1. FAO. Agricultural data, FAOSTAT 2014. Available: http://faostat3.fao.org/download/Q/QC/E.
2. Chu Y, Wu CL, Holbrook CC, Tillman BL, Person G, Ozias-Akins P. Marker-assisted selection to pyramidal nematode resistance and the high oleic trait in peanut. Plant Genome. 2011; 4:110.
3. Vassiliou EK, Gonzalez A, Garcia C, Tadros JH, Chakraborty G, Toney JH. Oleic acid and peanut oil high in oleic acid reverse the inhibitory effect of insulin production of the inflammatory cytokine TNF-α both in vitro and in vivo systems. Lipids Health Dis. 2009; 8:25. doi: 10.1186/1476-511x-8-25 PMID: 19558671
4. Lu C, Napier JA, Clemente TE, Cahoon EB. New frontiers in oilseed biotechnology: meeting the global demand for vegetable oils for food, feed, biofuel, and industrial applications. Curr Opin Biotechnol. 2011; 22:252–259. doi: 10.1016/j.copbio.2010.11.006 PMID: 21144729

5. Ozcan MM. Some nutritional characteristics of kernel and oil of peanut (Arachis hypogaea L.). J Oleo Sci. 2010; 59:1–5. PMID: 20032593

6. Huang L, Jiang H, Ren X, Chen Y, Xiao Y, Zhao X, et al. Abundant microsatellite diversity and oil content in wild Arachis species. PLoS ONE. 2012; 7:e50002. doi: 10.1371/journal.pone.0050002 PMID: 23185514

7. Cullinane M, Baysse C, Morrissey JP, O’Gara F. Identification of two lysophosphatidic acid acyltransferase isozymes enhances seed oil content in Arabidopsis. Plant Physiol. 1999; 120:913–919. PMID: 10289075

8. Penfield S, Rylott EL, Gilday AD, Graham S, Larson TR, Graham IA. Reserve mobilization in the Arabidopsis endosperm fuels hypocotyl elongation in the dark, is independent of abscisic acid, and requires PHOSPHENOXYPYRUVATE CARBOXYKINASE1. Plant Cell. 2004; 16:2705–2718. PMID: 15367715

9. Chapman KD, Ohlrogge JB. Compartmentation of triacylglycerol accumulation in plants. J Biol Chem. 2012; 287:2288–2294. doi: 10.1074/jbc.R111.290072 PMID: 22090025

10. Ohlrogge J, Browse J. Lipid biosynthesis. Plant Cell. 1995; 7:957–971. PMID: 7640528

11. Banas W, Sanchez Garcia A, Banas A, Styme S. Activities of acyl-CoA:diacylglycerol acyltransferase (DGAT) and phospholipid:diacylglycerol acyltransferase (PDAT) in microsomal preparations of developing sunflower and safflower seeds. Planta. 2013; 237:1627–1636. doi: 10.1007/s00425-013-1870-8 PMID: 23539042

12. Dahlyvist A, Stahl U, Lenman M, Banas A, Lee M, Sandager L, et al. Phospholipid:diacylglycerol acyltransferase: an enzyme that catalyzes the acyl-CoA-independent formation of triacylglycerol in yeast and plants. Proc Natl Acad Sci U S A. 2000; 97:6487–6492. PMID: 10829075

13. Misra N, Panda P, Parida B. Genome-wide identification and evolutionary analysis of algal LPAT genes involved in TAG biosynthesis using bioinformatic approaches. Mol Biol Rep. 2014; 1–14.

14. Pagac M, de la Mora HV, Duperrex C, Roubaty C, Vionnet C, Conzelmann A. Topology of 1-acyl-sn-glycerol-3-phosphate acyltransferases SLC1 and ALE1 and related membrane-bound O-acyltransferases (MBOATs) of Saccharomyces cerevisiae. J Biol Chem. 2011; 286:36438–36447. doi: 10.1074/jbc.M111.256511 PMID: 21849510

15. Yamashita A, Nakaniishi H, Suzuki H, Kamata R, Tanaka K, Waku K, et al. Topology of acyltransferase motifs and substrate specificity and accessibility in 1-acyl-sn-glycero-3-phosphate acyltransferase 1. Biochim Biophys Acta. 2007; 1771:1202–1215. PMID: 17707131

16. Eberhardt C, Gray PW, Tjoelker LW. Human lysophosphatidic acid acyltransferase: cDNA cloning, expression, and localization to chromosome 9q34.3. J Biol Chem. 1997; 272:20299–20305. PMID: 9242711

17. Cullinane M, Baysse C, Morrissey JP, O’Gara F. Identification of two lysophosphatidic acid acyltransferase genes with overlapping function in Pseudomonas fluorescens. Microbiology. 2005; 151:3071–3080. PMID: 16151217

18. Bourgis F, Kader JC, Barret P, Renard M, Robinson D, Robinson C, et al. A plastidial lysophosphatidic acid acyltransferase from oilseed rape. Plant Physiol. 1999; 120:913–922. PMID: 10398728

19. Oo KC, Huang AH. Lysophosphatidate acyltransferase activities in the microsomes from palm endosperm, maize scutellum, and rapeseed cotyledon of maturing seeds. Plant Physiol. 1989; 91:1288–1295. PMID: 16667178

20. Brown A, Coleman J, Tommey A, Watson M, Slabas A. Isolation and characterisation of a maize cDNA that complements a 1-acyl sn-glycerol-3-phosphate acyltransferase mutant of Escherichia coli and encodes a protein which has similarities to other acyltransferases. Plant Mol Biol. 1994; 26:211–223. PMID: 7948871

21. Kim HU, Li Y, Huang AH. Ubiquitous and endoplasmic reticulum-located lysophosphatidic acyltransferase, LPAT2, is essential for female but not male gametophyte development in Arabidopsis. Plant Cell. 2005; 17:1073–1089. PMID: 15772283

22. Frentzen M. Acyltransferases from basic science to modified seed oils. Lipid / Fett. 1998; 100:161–166.

23. Maisonneuve S, Bessoule JJ, Lessire R, Delseny M, Roscoe TJ. Expression of rapeseed microsomal lysophosphatidic acid acyltransferase isozymes enhances seed oil content in Arabidopsis. Plant Physiol. 2010; 152:670–684. doi: 10.1104/pp.109.148247 PMID: 19965969

24. Taylor DC, Francis T, Lozinsky S, Hoffman T, Glibin M, Mariil-lia EF. Cloning and characterization of a constitutive lysophos-phatic acid acyltransferase 2 (LPAT2) gene from Tropaeolum majus L. Open Plant Sci J. 2010; 4:7–17.
25. Mañas-Fernández A, Arroyo-Caro JM, Alonso DL, García-Matro F. Cloning and molecular characterization of a class A lysophosphatidate acyltransferase gene (EplPAT2) from *Echium* (Boraginaceae). Eur J Lipid Sci Technol. 2013; 115:1334–1346.

26. Zou J, Katavic V, Giblin EM, Barton DL, MacKenzie SL, Keller WA, et al. Modification of seed oil content and acyl composition in the brassicaceae by expression of a yeast sn-2 acyltransferase gene. Plant Cell. 1997; 9:909–923. PMID: 9212466

27. Rao SS, Hildebrand D. Changes in oil content of transgenic soybeans expressing the yeast SLC1 gene. Lipids. 2009; 44:945–951. doi: 10.1007/s11745-009-3337-z PMID: 19768478

28. Yu XH, Prakash RR, Sweet M, Shanklin J. Coexpressing *Escherichia coli* cyclopropane synthase with *Sterculia foetida* lysophosphatidic acid acyltransferase enhances cyclopropane fatty acid accumulation. Plant Physiol. 2014; 164:455–465. doi: 10.1104/pp.113.230953 PMID: 24204024

29. Brown AP, Slabas AR, Denton H. Substrate selectivity of plant and microbial lysophosphatidic acid acyltransferases. Phytochemistry. 2002; 61:493–501. PMID: 12409015

30. Knutzon DS, Lardizabal KD, Nielsen JS, Bleibaum JL, Davies HM, Metz JG. Cloning of a coconut endo-sperm cDNA encoding a 1-acyl-sn-glycerol-3-phosphate acyltransferase that accepts medium-chain-length substrates. Plant Physiol. 1995; 109:999–1006. PMID: 8552723

31. Arroyo-Caro JM, Chiléh T, Kazachkov M, Zou J, Alonso DL, García-Matro F. The multigene family of lysophosphatidate acyltransferase (LPAT)-related enzymes in *Ricinus communis*. Cloning and molecular characterization of two LPAT genes that are expressed in castor seeds. Plant sci. 2013; 199–200:29–40. doi: 10.1016/j.plantsci.2012.09.015 PMID: 23265316

32. Chen SL, Huang JQ, Lei Y, Zhang YT, Ren XP, Chen YN, et al. Identification and characterization of a gene encoding a putative lysophosphatidyl acyltransferase from *Arachis hypogaea*. J Biosci. 2012; 37:1029–1039. PMID: 23151793

33. Chen SL, Huang JQ, Lei Y, Ren XP, Wen QG, Chen YN, et al. Cloning and expression analysis of lysophosphatic acid acyltransferase (LPAT) encoding gene in peanut. Acta Agron Sin. 2012; 38:245–255.

34. Song QX, Li QT, Liu YF, Zhang FX, Ma B, Zhang WK, et al. Soybean GmbZIP123 gene enhances lipid content in the seeds of transgenic *Arabidopsis* plants. J Exp Bot. 2013; 64:4329–4341. doi: 10.1093/jxb/ert238 PMID: 23963672

35. Li RJ, Wang HZ, Mao H, Lu YT, Hua W. Identification of differentially expressed genes in seeds of two near-isogenic *Brassica napus* lines with different oil content. Planta. 2006; 224:952–962. PMID: 16575955

36. Shilman F, Brand Y, Brand A, Hedvat I, Hovav R. Identification and molecular characterization of homeologous 99-stearyol acyl carrier protein desaturase 3 genes from the allotetraploid peanut (*Arachis hypogaea*). Plant Mol Biol Rep. 2011; 29:232–241.

37. Zhang J, Liang S, Duan J, Wang J, Chen S, Cheng Z, et al. De novo assembly and characterisation of the transcriptome during seed development, and generation of genic-SSR markers in *Arachis hypogaea* L.) BMC Genomics. 2012; 13:90. doi:10.1186/1471-2164-13-90 PMID: 23963672

38. Yin D, Wang Y, Zhang X, Li H, Lu X, Zhang J, et al. De novo assembly of the peanut (*Arachis hypogaea* L.) seed transcriptome revealed candidate unigenes for oil accumulation pathways. PLoS ONE. 2013; 8:e73767. doi:10.1371/journal.pone.0073767 PMID: 24040062

39. Jako C, Kumar A, Wei Y, Zou J, Barton DL, Giblin EM, et al. Seed-specific over-expression of an Arabidopsis cDNA encoding a diacylglycerol acyltransferase enhances seed oil content and seed weight. Plant Physiol. 2001; 126:861–874. PMID: 11402213

40. Li Y, Beisson F, Pollard M, Ohrogge J. Oil content of Arabidopsis seeds: the influence of seed anatomy, light and plant-to-plant variation. Phytochemistry. 2006; 67:904–915. PMID: 16600316

41. Singlet C, Adang MJ, Lynch RE, Anderson WF, Wang A, Cardineau G, et al. Expression of a *Bacillus thuringiensis* cryIA(c) gene in transgenic peanut plants and its efficacy against lesser cornstalk borer. Transgenic Res. 1997; 6:169–176. PMID: 9090064

42. Scott A, Wyatt S, Tsou PL, Robertson D, Allen NS. Model system for plant cell biology: GFP imaging in living onion epidermal cells. Biotechniques. 1999; 26:1125, 1128–1132. PMID: 10376152

43. Hajdukiewicz P, Slabas AR, Denton H. Substrate selectivity of plant and microbial lysophosphatic acid acyltransferases. Phytochemistry. 2002; 61:493–501. PMID: 12409015

44. Singsit C, Adang MJ, Lynch RE, Anderson WF, Wang A, Cardineau G, et al. Expression of a *Bacillus thuringiensis* cryIA(c) gene in transgenic peanut plants and its efficacy against lesser cornstalk borer. Transgenic Res. 1997; 6:169–176. PMID: 9090064

45. Scott A, Wyatt S, Tsou PL, Robertson D, Allen NS. Model system for plant cell biology: GFP imaging in living onion epidermal cells. Biotechniques. 1999; 26:1125, 1128–1132. PMID: 10376152

46. Jako C, Kumar A, Wei Y, Zou J, Barton DL, Giblin EM, et al. Seed-specific over-expression of an Arabidopsis cDNA encoding a diacylglycerol acyltransferase enhances seed oil content and seed weight. Plant Physiol. 2001; 126:861–874. PMID: 11402213

47. Li Y, Beisson F, Pollard M, Ohrogge J. Oil content of Arabidopsis seeds: the influence of seed anatomy, light and plant-to-plant variation. Phytochemistry. 2006; 67:904–915. PMID: 16600316

48. Singlet C, Adang MJ, Lynch RE, Anderson WF, Wang A, Cardineau G, et al. Expression of a *Bacillus thuringiensis* cryIA(c) gene in transgenic peanut plants and its efficacy against lesser cornstalk borer. Transgenic Res. 1997; 6:169–176. PMID: 9090064

49. Scott A, Wyatt S, Tsou PL, Robertson D, Allen NS. Model system for plant cell biology: GFP imaging in living onion epidermal cells. Biotechniques. 1999; 26:1125, 1128–1132. PMID: 10376152

50. Hajdukiewicz P, Slabas AR, Denton H. Substrate selectivity of plant and microbial lysophosphatic acid acyltransferases. Phytochemistry. 2002; 61:493–501. PMID: 12409015

46. Clough SJ, Bent AF. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. 1998; 16:735–743. PMID: 10069079
47. Adamski NM, Anastasiou E, Eriksson S, O'Neill CM, Lenhard M. Local maternal control of seed size by KLUH/CYP78A5-dependent growth signaling. Proc Natl Acad Sci U S A. 2009; 106:20115–20120. doi: 10.1073/pnas.0907024106 PMID: 19892740

48. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976; 72:248–254. PMID: 942051

49. Jiang S, Kumar S, Eu YJ, Jamil SK, Stasolla C, Hill RD. The Arabidopsis mutant, fy-1, has an ABA-insensitive germination phenotype. J Exp Bot. 2012; 63:2693–2703. doi: 10.1093/jxb/err452 PMID: 22282534

50. Luo M, Dang P, Bausher MG, Holbrook CC, Lee RD, Lynch RE, et al. Identification of transcripts involved in resistance responses to leaf spot disease caused by Cercosporidium personatum in Peanut (Arachis hypogaea). Phytopathology. 2005; 95:381–387. doi: 10.1094/PHYTO-95-0381 PMID: 18943040

51. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods. 2001; 25:402–408. PMID: 11846609

52. Chen SL, Li YR, Xu GZ, Cheng ZS. Simulation on oil accumulation characteristics in different high-oil peanut varieties. Acta Agron Sin. 2008; 34:142–149.

53. Byers DM, Gong H. Acyl carrier protein: structure-function relationships in a conserved multifunctional protein family. Biochem Cell Biol. 2007; 85:649–662. PMID: 18059524

54. Upadhyyay SK, Misra A, Srivastava R, Surolia N, Surolia A, Sundd M. Structural insights into the acyl intermediates of the Plasmodium falciparum fatty acid synthesis pathway: the mechanism of expansion of the acyl carrier protein core. J Biol Chem. 2009; 284:22390–22400. doi: 10.1074/jbc.M109.14829 PMID: 19520851

55. Ohlrogge JB, Jaworski JG. Regulation of fatty acid synthesis. Annu Rev Plant Physiol Plant Mol Biol. 1997; 48:109–136. PMID: 15012259

56. Herbert D, Walker KA, Price LJ, Cole DJ, Pallett KE, Ridley SM, et al. Acetyl-CoA carboxylase—a graminicline target site. Pestic Sci. 1997; 50:67–71.

57. Thelen JJ, Mekhedov S, Ohlrogge JB. Biotin carboxyl carrier protein isoforms in Brassicaceae oilseeds. Plant Physiol. 2011; 156:1577–1588. doi: 10.1104/pp.111.175000 PMID: 21563329

58. Sasaki Y, Nagano Y. Plant acetyl-CoA carboxylase: structure, biosynthesis, regulation, and gene manipulation for plant breeding. Biosci Biotechnol Biochem. 2004; 68:1175–1184. PMID: 15215578

59. Meyer K, Stecca KL, Ewell-Hicks K, Allen SM, Everard JD. Oil and protein accumulation in developing seeds is influenced by the expression of a cytosolic pyrophosphatase in Arabidopsis. Plant Physiol. 2012; 159:1221–1234. doi: 10.1104/pp.112.198309 PMID: 22966496

60. Tan H, Yang X, Zheng X, Qu C, Mu J, et al. Enhanced seed oil production in canola by conditioning the principle of protein-dye binding. Anal Biochem. 1976; 72:248–254. PMID: 942051

61. Ballicora MA, Iglesias AA, Preiss J. ADP-Glucose pyrophosphorylase: A regulatory enzyme for plant starch synthesis. Photosynth Res. 2004; 79:1–24. PMID: 16228397

62. Viegas H, Mohnlmann T, Martini N, Neuaus HE, Geigenberger P. Embryo-specific reduction of ADP-Glc pyrophosphorylase leads to an inhibition of starch synthesis and a delay in oil accumulation in developing seeds of oilseed rape. Plant Physiol. 2004; 136:2676–2686. PMID: 15333758

63. Sun M, Hua W, Liu J, Huang S, Wang X, Liu G, et al. Design of new genome- and gene-sourced primers and identification of QTL for seed oil content in a specially high-oil Brassica napus cultivar. PLoS ONE. 2012; 7:e47037. doi: 10.1371/journal.pone.0047037 PMID: 23077542

64. Cagliari A, Margis-Pinheiro M, Loss G, Mastroberti AA, de Araujo Mariath JE, Margis R. Identification and expression analysis of castor bean (Ricinus communis) genes encoding enzymes from the triacylglycerol biosynthesis pathway. Plant Sci. 2010; 179:499–509. doi: 10.1016/j.plantsci.2010.07.015 PMID: 21802608

65. Ghosh AK, Chauhan N, Rajakumari S, Daum G, Rajasekharan R. At4g24160, a soluble acyl-coenzyme A-dependent lysophosphatidic acid acyltransferase. Plant Physiol. 2009; 151:869–881. doi: 10.1104/pp.109.144261 PMID: 19700561

66. Shockey JM, Gidda SK, Chapital DC, Kuan JC, Dhanoa PK, Bland JM, et al. Tung tree DGAT1 and DGAT2 have nonredundant functions in triacylglycerol biosynthesis and are localized to different subdomains of the endoplasmic reticulum. Plant Cell. 2006; 18:2294–2313. PMID: 16920778
68. van Leeuwen W, Rutting T, Borst-Vrenssen AW, van der Plas LH, van der Krol AR. Characterization of position-induced spatial and temporal regulation of transgene promoter activity in plants. J Exp Bot. 2001; 52:949–959. PMID: 11432912

69. Kim HU, Chen GQ. Identification of hydroxy fatty acid and triacylglycerol metabolism-related genes in lesquerella through seed transcriptome analysis. BMC Genomics. 2015; 16:230. doi:10.1186/s12864-015-1413-8 PMID: 25881190

70. Roscoe TJ. Identification of acyltransferases controlling triacylglycerol biosynthesis in oilseeds using a genomics-based approach. Eur J Lipid Sci Technol. 2005; 107:256–262.

71. van Erp H, Kelly AA, Menard G, Eastmond PJ. Multigene engineering of triacylglycerol metabolism boosts seed oil content in Arabidopsis. Plant Physiol. 2014; 165:30–36. doi:10.1104/pp.114.236430 PMID: 24696520

72. Wang Z, Huang W, Chang J, Sebastian A, Li Y, Li H, et al. Overexpression of SiDGAT1, a gene encoding acyl-CoA:diacylglycerol acyltransferase from Sesamum indicum L. increases oil content in transgenic Arabidopsis and soybean. Plant Cell Tiss Org. 2014; 119:399–410.

73. Liu J, Hua W, Yang HL, Zhan GM, Li RJ, Deng LB, et al. The BnGRF2 gene (GRF2-like gene from Brassica napus) enhances seed oil production through regulating cell number and plant photosynthesis. J Exp Bot. 2012; 63:3727–3740. doi:10.1093/jxb/ers066 PMID: 22442419

74. Taylor D, Katavic V, Zou J, MacKenzie S, Keller W, An J, et al. Field testing of transgenic rapeseed cv. Hero transformed with a yeast sn-2 acyltransferase results in increased oil content, erucic acid content and seed yield. Mol Breeding. 2002; 8:317–322.

75. Katavic V, Friesen W, Barton DL, Gossen KK, Giblin EM, Luciw T, et al. Utility of the Arabidopsis FAE1 and yeast SLC1-1 genes for improvements in erucic acid and oil content in rapeseed. Biochem Soc Trans. 2000; 28:935–937. PMID: 11171262

76. Brown AP, Carnaby S, Brough C, Brazier M, Slabas AR. Limnanthes douglasii lysophosphatidic acid acyltransferases: immunological quantification, acyl selectivity and functional replacement of the Escherichia coli pIsC gene. Biochem J. 2002; 364:795–805. PMID: 12049644

77. Tomosugi M, Ichihara Ki, Saito K. Polyamines are essential for the synthesis of 2-ricinoleoyl phosphatidic acid in developing seeds of castor. Planta. 2006; 223:349–358. PMID: 16133210

78. Xu K, Yang Y, Li X. Ectopic expression of Crambe abyssinica lysophosphatidic acid acyltransferase in transgenic rapeseed increases its oil content. Afr J Biotechnol. 2010; 9:3904–3901.

79. Yu D, Xie Z, Chen C, Fan B, Chen Z. Expression of tobacco class II catalase gene activates the endogenous homologous gene and is associated with disease resistance in transgenic potato plants. Plant Mol Biol. 1999; 39:477–488. PMID: 10092176

80. Craig J, Barratt P, Tatge H, Déjardin A, Handley L, Gardner CD, et al. Mutations at the rug4 locus alter the carbon and nitrogen metabolism of pea plants through an effect on sucrose synthase. Plant J. 1999; 17:353–362.

81. Chourey PS, Taliercio EW, Carlson SJ, Ruan YL. Genetic evidence that the two isozymes of sucrose synthase present in developing maize endosperm are critical, one for cell wall integrity and the other for starch biosynthesis. Mol Gen Genet. 1998; 259:88–96. PMID: 973888

82. Schwender J. Metabolic flux analysis as a tool in metabolic engineering of plants. Curr Opin Biotechnol. 2008; 19:131–137. doi: 10.1016/j.copbio.2008.02.006 PMID: 18378441

83. Schwender J. Experimental flux measurements on a network scale. Front in plant sci. 2011; 2:63.

84. Sarvamangala C, Gowda MVC, Varshney RK. Identification of quantitative trait loci for protein content, oil content and oil quality for groundnut (Arachis hypogaea L.). Field Crops Res. 2011; 122:49–59.