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ORIGINAL ARTICLE

Expression of Genes Controlling Unsaturated Fatty Acids Biosynthesis and Oil Deposition in Developing Seeds of Sacha Inchi (*Plukenetia volubilis* L.)

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Abstract Sacha inchi (*Plukenetia volubilis* L., Euphorbiaceae) seed oil is rich in α-linolenic acid, a kind of n-3 fatty acids with many health benefits. To discover the mechanism underlying α-linolenic acid accumulation in sacha inchi seeds, preliminary research on sacha inchi seed development was carried out from one week after fertilization until maturity, focusing on phenology, oil content, and lipid profiles. The results suggested that the development of sacha inchi seeds from pollination to mature seed could be divided into three periods. In addition, investigations on the effect of temperature on sacha inchi seeds showed that total oil content decreased in the cool season, while unsaturated fatty acid and linolenic acid concentrations increased. In parallel, expression profiles of 17 unsaturated fatty acid related genes were characterized during seed development and the relationships between gene expression and lipid/unsaturated fatty acid accumulation were discussed.

Keywords Sacha inchi · Seed development · Lipid accumulation · Unsaturated fatty acids · Temporal expression pattern · Temperature

Abbreviations

ALA	Alpha-linolenic acid (18:3n-3)
DAP	Days after pollination
DGAT	Acyl-CoA: diacylglycerol acyltransferase
DW	Dry weight
FA	Fatty acid desaturase
FATA	Acyl-acyl-carrier protein thioesterase A
FFA	Free fatty acids
FW	Fresh weight
G3PDH	Glycerol-3-phosphate dehydrogenase
LNA	Linoleic acid (18:2n-6)
MBOAT	Membrane-bound *O*-acyltransferase
NL	Neutral lipids
OLA	Oleic acid (18:1n-9)
PAM	Palmitic acid (16:0)
PAP	Phosphatidate phosphatase
PL	Phospholipids
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PUFA	Polyunsaturated fatty acid(s)
SAD	Stearoyl-acyl-carrier protein desaturase
SFA	Saturated fatty acid(s)
STA	Stearic acid (18:0)
TAG	Triacylglycerol(s)
UI	Unsaturation index
USFA	Unsaturated fatty acid(s)

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Introduction

Sacha inchi (*Plukenetia volubilis* L.), also known as "inca inchi" or "inca peanut", is a perennial, oleaginous, woody twining vine of the Euphorbiaceae family, native to the tropical Peruvian jungles at altitudes between 200 and 1,500 m. This plant yields four to seven lobed capsules with one dark lenticular seed per lobe, which is rich in protein (27–30 %) and oil (40–60 %) [1–5]. Flour and oil obtained from sacha inchi seeds have been consumed by Peruvian Indian natives for hundreds of years [6]. The fatty acid (FA) composition of sacha inchi seed oil makes it special, because it contains 45.2–51.39 % of α-linolenic acid (18:3 *cis*-Δ9,12,15; ALA), a kind of n-3 fatty acid, which differs markedly from major vegetable oils [7].

Because polyunsaturated fatty acids (PUFA) such as ALA cannot be synthesized in mammals, they are essential FA (i.e., required in the diet) and the recommended n-6:n-3 FA ratio in the human diet is approximately 2:1 to 6:1 [8, 9]. Several studies have strongly suggested that ALA are important in relation to the pathogenesis (and prevention) of coronary heart disease and hypertension during pregnancy and breastfeeding, besides showing a hypocholesterolemic effect when used as food supplements [10, 11]. However, traditional oilseed crops (e.g., soybean, peanut, maize, sunflower, and rapeseed) have a low ALA content (0–10 %) with a high n-6:n-3 ratio. The high n-6:n-3 ratio in our modern diet (15:1 to 20:1) has been thought to be a major contributor to lipid metabolism disorder, cardiovascular disease and autoimmune diseases [12, 13]. There is an immediate need for developing ALA-enriched oilseed crops to ameliorate the global dietary n-3 fatty acid deficiency. Sacha inchi seed oil is an excellent source of α-linolenic acid. Developing sacha inchi as a domesticated oilseed crop would be a promising endeavor.

In oilseeds, lipids are stored primarily as triacylglycerols (TAG), with the major compositions 16- and 18-carbon saturated and unsaturated fatty acids. In general, the main processes leading to the accumulation of ALA begin with the de novo formation of acyl chains (up to 18 carbons) in the plastid, followed by desaturation reaction at the Δ9 position and FA release, further produce two carbon double bonds at Δ12 and Δ15 positions by desaturation in the endoplasmic reticulum (ER) with acyl editing [14]. Studies have shown that the low temperatures could induce the accumulation of PUFA in developing seeds. When exposed to high daily temperatures during seed filling, PUFA content of soybean seed oil was significantly reduced [15, 16]. High temperatures also considerably decreased the PUFA content of seed oil in flax [17, 18]. It is often thought that oilseed plants (such as oil palm) grown in warm climates are inclined to accumulate higher saturated FA than those grown in temperate zone. However, sacha inchi seems to be a typical tropical plant. Why sacha inchi seeds accumulate such high quantities of ALA is unknown. Investigating the profiles of lipid and ALA accumulation in sacha inchi seeds might provide insights into understanding the physiological and molecular mechanisms underlying ALA biosynthesis to facilitate the exploration and utilization of oilseed crop resources in agriculture.

In this study, we characterized a time-course for seed development and storage reserve (in particular lipids and ALA) accumulation during sacha inchi seed development, and investigated the temperature effect on lipid and ALA accumulation of sacha inchi seeds. Further, based on our previous gene (expression sequence tags) EST library [19] we inspected temporal expression profiles of 17 lipid genes involved in different steps of the pathway leading to ALA-based oil biosynthesis within sacha inchi developing seeds using quantitative real-time PCR technology. Results obtained here are helpful to reveal the physiological and molecular mechanisms underlying ALA biosynthesis in sacha inchi seeds.

Materials and Methods

Plant Material and Sample Collection

Two-year-old sacha inchi trees introduced from Peru by seeds were grown in Xishuangbanna Tropical Botanical Garden (21°56′N, 101°15′E, 600 m above sea level) at the Chinese Academy of Sciences, Yunnan, China under natural climate conditions. The average daily temperature annually is in the range 8–35 °C at the study site. There is a distinct cool season from November to February with an average daily temperature range of 8–20.7 °C (the average temperature is 18.2 °C through the cool season) and a distinct hot season from June to September with an average daily temperature range of 22.4–35 °C (the average temperature is 25.1 °C through the hot season) [20]. The development of sacha inchi fruits and seeds from pollinated female flowers to mature seeds was observed and recorded from March of 2010 to October of 2011. Mature female flowers were hand-pollinated when the stigma was fully expanded, and recorded as 0 days after pollination (DAP). Capsules at different developmental stages were harvested and the dissected seeds were collected for testing the content of storage materials or immediately frozen in liquid nitrogen and stored at −80 °C for further analysis (such as lipid and fatty acid composition determination, or RNA extraction). Leaf tissue was collected from fully expanded young leaves and root tips were collected, dissected and washed from the same individuals for RNA extraction. To

investigate the effect of temperature on lipid accumulation and the biosynthesis of different fatty acids in developing seeds, seven sacha inchi individuals were randomly selected and tagged for experiments and sample collection. Six mature female flowers were hand-pollinated in late October of 2010 and in late May of 2011, respectively. The mature seeds were sampled from each individual tagged in February and September of 2011, respectively, for lipid and fatty acid composition analysis. Since this allows the filling process of sacha inchi seeds sampled through the cool season and hot season, respectively. And the effect of temperature on lipid accumulation and the biosynthesis of different fatty acids in developing seeds can be tested.

Measure of Seed Development and Storage Materials

Seeds dissected were immediately weighed as fresh weight (FW), then reweighed to determine dry weight (DW) after being dried at 103 °C for 17 h. Seed size was measured using 1 mm^2 counting paper. The protein content of the seeds was measured as described by Bradford [21], carbohydrate content was measured using the modified anthrone reagent [22]. Oil content was determined by a minispec mq-one Seed Analyzer (Bruker Optik GmbH, Germany).

Determination of Lipid and Fatty Acid Composition from Developing Seeds

Total lipid was extracted from sacha inchi seeds as described previously by Xu et al. [23], and separation of individual lipid fractions was achieved by using NH2 solid-phase extraction (SPE) columns (Dima, Beijing) as described by Gutiérrez et al. [4]. Fatty acid composition of the separated lipid fractions was determined by gas chromatography. Fatty acid methyl esters (FAME) were prepared by heating the dry fraction materials at 85 °C for 60 min in 2 % (v:v) sulfuric acid in dry methanol, as described by Pomeroy et al. [24]. The resulting FAME were dissolved in 1 mL of dichloromethane (0.01 % BHT) for GC (GC-2014, Shimadzu) equipped with a flame ionizing detector (FID) and DB-23 capillary column (J&W Scientific, Folsom CA; 30 m × 0.25 mm i.d.; film thickness = 0.2 μm). The process was operated at an oven temperature of 170 °C, which was then raised to 220 °C at a rate of 5 °C/min and then kept at 220 °C for 5 min. Injector and detector temperatures were 250 and 280 °C, respectively. Helium was used as the carrier gas and kept at a constant flow of 1.2 mL/min and a split ratio of 20:1. Peak identification was performed by comparing the relative retention times with those of commercial standards purchased from Sigma Aldrich (St. Louis, MO, USA) under the same conditions. The fatty acid content was determined using a computing integrator and presented as the percentage of the oil. Molar percentage (mol%) of each fatty acid was calculated as (100 × mol FA)/(sum of mol FA for all FA). Unsaturation index (UI) was calculated as in Pan et al. [25].

Total RNA Extraction and CDNA Preparation

Total RNA was extracted from leaf, root, and developing seeds at 12 developmental stages using RNAiso Reagent (Takara, Dalian, China). RNA pellets were dissolved in RNase free water, quantified by absorbance at 260 nm in a spectrophotometer (NanoDrop ND-2000) and checked for quality by Goldview™ (Bioteke, China) agarose gel electrophoresis. PrimeScript RT reagent Kit with gDNA Eraser (Perfect Real Time, TaKaRa Code. DRR047) was used for reverse transcription and to remove genomic DNA according to the manufacturer's instructions.

Selection of Genes and Primer Design

To obtain the promising target genes associated with unsaturated fatty acid biosynthesis and accumulation, we searched the EST database of sacha inchi constructed in our previous study [19] and focused on lipid genes which were associated with unsaturated fatty acid biosynthesis. In total, 17 USFA-related genes including nine fatty acid desaturase genes were obtained (Table 1). The gene-specific primers were designed using the Primer Express software for Real-Time PCR version 3.0 (Applied Biosystems, Foster City, CA, USA) using the default parameters. Length of PCR products varied from 80 to 150 bp. Information on optimized primer pairs is listed in Table 1.

Quantitative Real-time PCR (qRT-PCR) Based Gene Expression Analysis

All real-time reactions were performed on an Applied Biosystems 7500 Real-Time PCR System. qRT-PCR was conducted with an initial step of 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. PCR product specificity was confirmed by melting-curve analysis and by electrophoresis on 4 % agarose gel. The amplification efficiency of each primer pair was determined empirically by ten-fold serial dilutions of cDNA and only those with an efficiency of over 0.8 were used. Triplicate experimental sets of qPCR reaction samples, including the reference control genes and quadrupled negative controls (reaction samples without cDNA templates), were prepared in a final volume of 20 μL that contained 1 μL of cDNA template, 2× FastStart Universal SYBR Green Master mix with ROX (Roche, Germany), and 300 nM of forward and reverse primers. The sacha

Table 1 Selection of unsaturated fatty acid related genes, primer sequences and size of amplification products

USFA-related gene cellular location and activity[a]	Abbreviation	Forward and reverse primers	Amplicon size (bp)
Plastid location			
Acyl-acyl-carrier protein thioesterase A	FATA[b]	F:5′-TGATTACCCGTTCCCATAACAGA-3′	100
		R:5′-TGAGAAGAAGAGGAGGAAATCGA-3′	
Stearoyl-acyl-carrier protein desaturase	SAD1[b]	F:5′-CGAGTGGACATGCGACAAAT-3′	150
		R:5′-TCTGGCTGTGTTCCCATGAG-3′	
	SAD2[b]	F:5′-CACGATGCCAGCTCACTTGA-3′	145
		R:5′-CTCTAACCCCCATCGTCCAA-3′	
Oleate desaturase	FAD6-1	F:5′-TGATGCCATGGTTGGGCTAT-3′	100
		R:5′-GCATTCCACTCGTCCGAAGA-3′	
	FAD6-2	F:5′-CAGGCCAATTCGGAGTCAAA-3′	105
		R:5′-GGAGTGGCCACAGCTTGAAC-3′	
Linoleate desaturase	FAD7-1[b]	F:5′-GGTCATTACCAGTTTCAAGGAGTCA-3′	137
		R:5′-ATCATTTTCTCCACTAACAACGTCC-3′	
	FAD7-2[b]	F:5′-GGTGTTATCAGAATGTAGTATGAGGC-3′	116
		R:5′-TTTTTGAAACCGGTAACTGTTTA-3′	
Cytoplasm location			
Glycerol-3-phosphate dehydrogenase	G3PDH[b]	F:5′-GGCGTCGAGGCTATTTCACT-3′	115
		R:5′-CCCCCATTAGAACACAACAGTTAA-3′	
Soluble acyl-CoA: diacylglycerol acyltransferase	DGAT3[b]	F:5′-CTGCATTGGTGTGGGTTTGA-3′	120
		R:5′-TTAAACGGCATCAGCAGCAA-3′	
Endoplasmic reticulum location			
Oleate desaturase	FAD2-1[b]	F:5′-TGCATTCCTTGTTCTCATCACAT-3′	100
		R:5′-GGTTGCCAGAGCTCCTTTCA-3′	
	FAD2-2	F:5′-GGTTCTCCATTCCGCACTTCTA-3′	117
		R:5′-GACTTCGGTTTCGGGACAAA -3′	
Linoleate desaturase	FAD3[b]	F:5′-GACTTGGTAAAAAGCGTTAGCCAGG-3′	97
		R:5′-TTGCCAATTTCTTATACAGATCAGG-3′	
Membrane-bound *O*-acyltransferase	MBOAT[b]	F:5′-TGCAGCATATGGCAGCGTAT-3′	95
		R:5′-ACTTGGCAGGTCTTGCTGGTT-3′	
Phospholipase A_2	PLA_2	F:5′-CATGTGATGGGCTTGATGCT-3′	90
		R:5′-TTTGGCTGCATTCTTGGCTTA-3′	
Phospholipase C	PLC	F:5′-CCACGTGGCAACACCTGTAA-3′	95
		R:5′-CCCTAACCCCTAATCGGTCAA-3′	
Phosphatidate phosphatase	PAP[b]	F:5′-GGGCAGGTCAAGGGCTACA-3′	100
		R:5′-CCCCACAAATCCCTGAGGTA-3′	
Acyl-CoA: diacylglycerol Acyltransferase 2	DGAT2[b]	F:5′-GGTCTTTCACCCGCAACAAG-3′	80
		R:5′-ACACCACCCGGAATCACAAT-3′	
Constitutive control gene			
Actin	Act	F:5′-GCCAGATGGGCAGGTTATCA-3′	100
		R:5′-GTGTATGCCAGCAGCTTCCA-3′	

[a] Tentative assignments of the gene name and the cellular activity

[b] These genes were first cloned in our laboratory

Table 2 Summary of transcript profiles of candidate USFA-related genes

Patterns	Gene[a]	Maximum ratio of expression			
		Seed vs seed[b]	Seed vs leaf[c]	Seed vs root[d]	Leaf vs root[e]
Cluster 1	PLC	4.5	4.2	8.4	1.99
	DGAT2	73.4	44.2	28.7	0.65
	DGAT3	9.1	0.79	8.5	10.8
Cluster 2	FAD2-1	1,000	0.67	1.5	2.2
Cluster 3	SAD-1	38.2	13.99	21.9	1.6
	SAD-2	4,006	26,200	348.3	0.01
	FATA	23.9	1.99	4.5	2.3
	FAD2-2	11,121	1,759	6,004	3.4
	FAD3	2,479	6,033	226.1	0.04
	MBOAT	105.8	1.6	16.3	10.3
	G3PDH	91.2	26.0	25.1	0.96
	PAP	376.1	19.5	66.2	3.4
Cluster 4	FAD6-1	19.8	0.16	5.8	35.3
	FAD6-2	10.6	0.07	2.6	34.3
Cluster 5	FAD7-1	12.4	8.2	2.8	0.35
	FAD7-2	5.4	0.92	1.4	1.6
	PLA_2	27.1	1.4	2.1	1.5

[a] The gene abbreviated names are described in Table 1

[b] Ratio of the maximum to the minimum expression in seed

[c] Ratio of the maximum expression in seed to the expression in leaf

[d] Ratio of the maximum expression in seed to the expression in root

[e] Ratio of expression in leaf to root

inchi actin gene was used as the internal control to normalize the relative amount of mRNAs for all samples. All reactions were run in triplicate on MicroAmp 96-well plates (Applied Biosystems), using a unique sample (leaf) as an endogenous calibrator control in each, and were analysed using the Applied Biosystems SDS 7500 system software (Applied Biosystems). The RQ values were calculated using the Livak equation, $RQ = 2^{-\Delta\Delta Ct}$, and the results expressed as a ratio depending on the calibrator sample used in all experiments.

Gene Clustering and Visualization

Quantitative expression data of each gene were subjected to gene expression clustering analysis using the *k*-mean clustering method [26, 27] provided within the software expression analyzer and display (EXPANDER) [28]. Input data were first standardized using mean = 0 and variance = 1, and fixed norm and then pursued clustering with *k*-mean method. Clustering into five groups gave the best clustering quality scores. To graphically view the expression patterns of clusters the option of mean patterns with error bars operated by the EXPANDER was chosen which allows each cluster to be displayed in a separate panel with error bars representing standard deviations.

Statistical analysis

Data were analyzed using the statistical software package SPSS (version 17.0) by one-way ANOVA for each fatty acid composition variable with season as main fixed factors. Prior to analysis, data were checked for normality and homogeneity of variance, and were log10-transformed when necessary to satisfy the assumption of ANOVA. Differences among means were determined by one-way ANOVA using the least significant difference (LSD0.05) test or Tamhane's *post hoc* tests. Graphics were generated using Origin software (version 8.0).

Results

Seed Development and Storage Material Accumulation

Based on our field observation, sacha inchi seeds took approximately 112 days from fertilization to maturation under nature conditions. The developing seeds grew gradually at the initial stage before ca. 14 DAP (see Fig. 1a). The fresh weight of seeds and seed size increased rapidly throughout the middle stage; the fresh weight reached a peak (1,732.35 mg per seed) and the full size of seed (338.33 mm^2 in area) appeared at ca. 35 DAP. As young seeds consisted mostly of water, there was only a small portion of dry weight during this period. The fresh weight reached another peak (1,908.66 mg per seed) at ca. 92 DAP. Correspondingly, the dry weight accumulated gradually throughout the middle and late stage of seed development and reached its maximum (1,300 mg per seed) at ca. 92 DAP. The seed filling ended up and seed started desiccation with a color change from light green to a deep brown after 92 DAP (see Fig. 1b).

Accumulations of main storage materials including carbohydrate, protein and lipid were examined at different development stages (Fig. 1c). Similar to the increase in dry weight, lipid accumulation was slow at the early stage of seed development and the lipid content was only 2.655 % (on dry weight basis) by 35DAP (Fig. 1c). After that, it increased rapidly and reached its maximum at 105DAP, accounting for 49.61 % (Fig. 1c). However, the lipid content dropped slightly in dry mature seeds at 112DAP (Fig. 1c). Protein content began to rise after 21DAP, about two weeks before fast lipid accumulation, i.e., prior to 35DAP only minimal amounts of lipid could be detected (Fig. 1c). Protein content in seed samples ranged from 3.91 to 27.91 % (on dry weight basis), the lowest at 7, and the

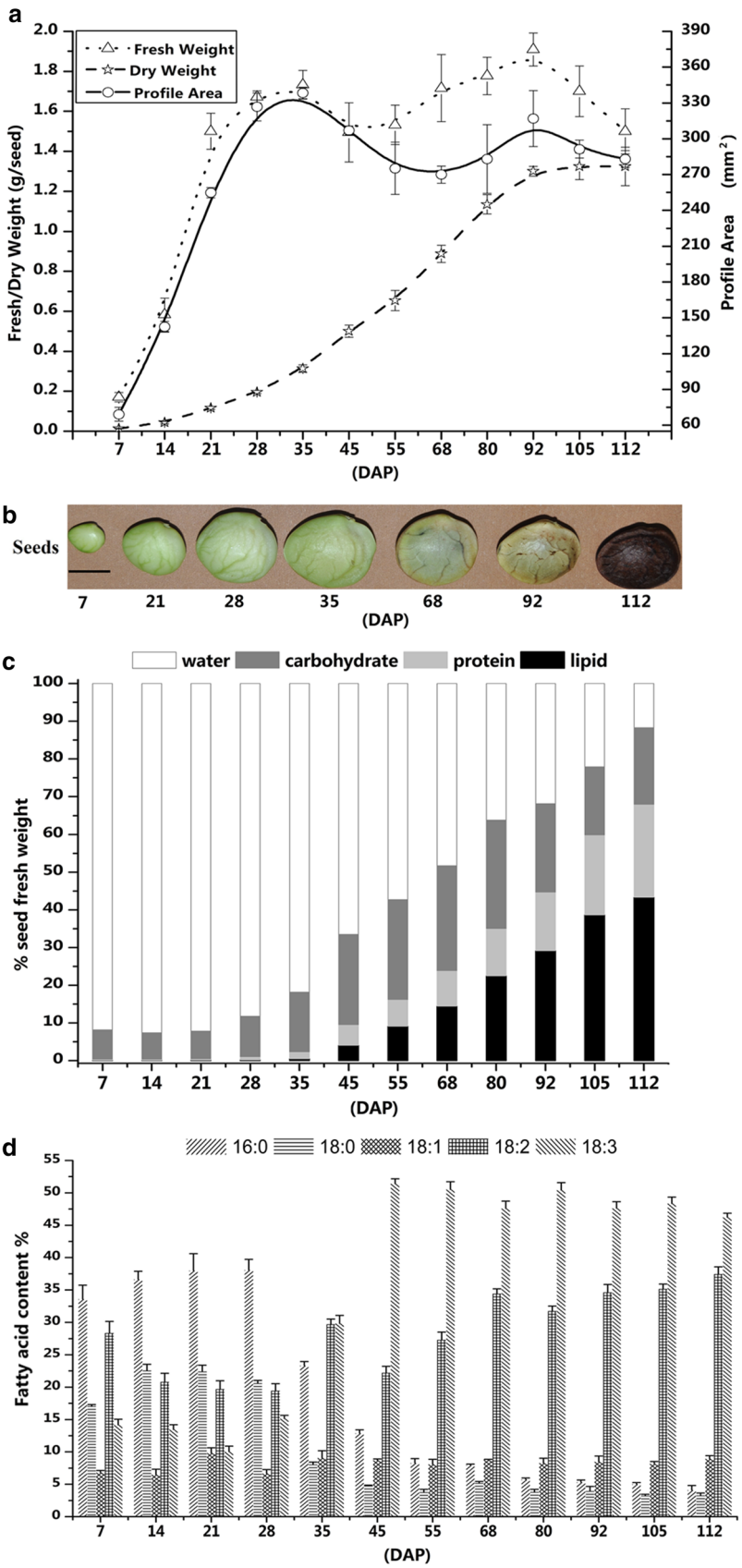


Fig. 1 Characterization of sacha inchi seed development. **a** Changes in fresh weight, dry weight and profile area during seed development. **b** Appearance of sacha inchi seeds at different developmental stages, *scale bar* 1.0 cm. **c** Changes in water, carbohydrate, protein and oil content during seed development. **d** Changes in fatty acid composition during seed development. *DAP* days after pollination, 16:0 palmitic acid, 18:0 stearic acid, 18:1 oleic acid, 18:2 linoleic acid, 18:3 linolenic acid. Each data point represents the mean ± SD of at least three biological replicates and *error bars* indicate the standard deviation

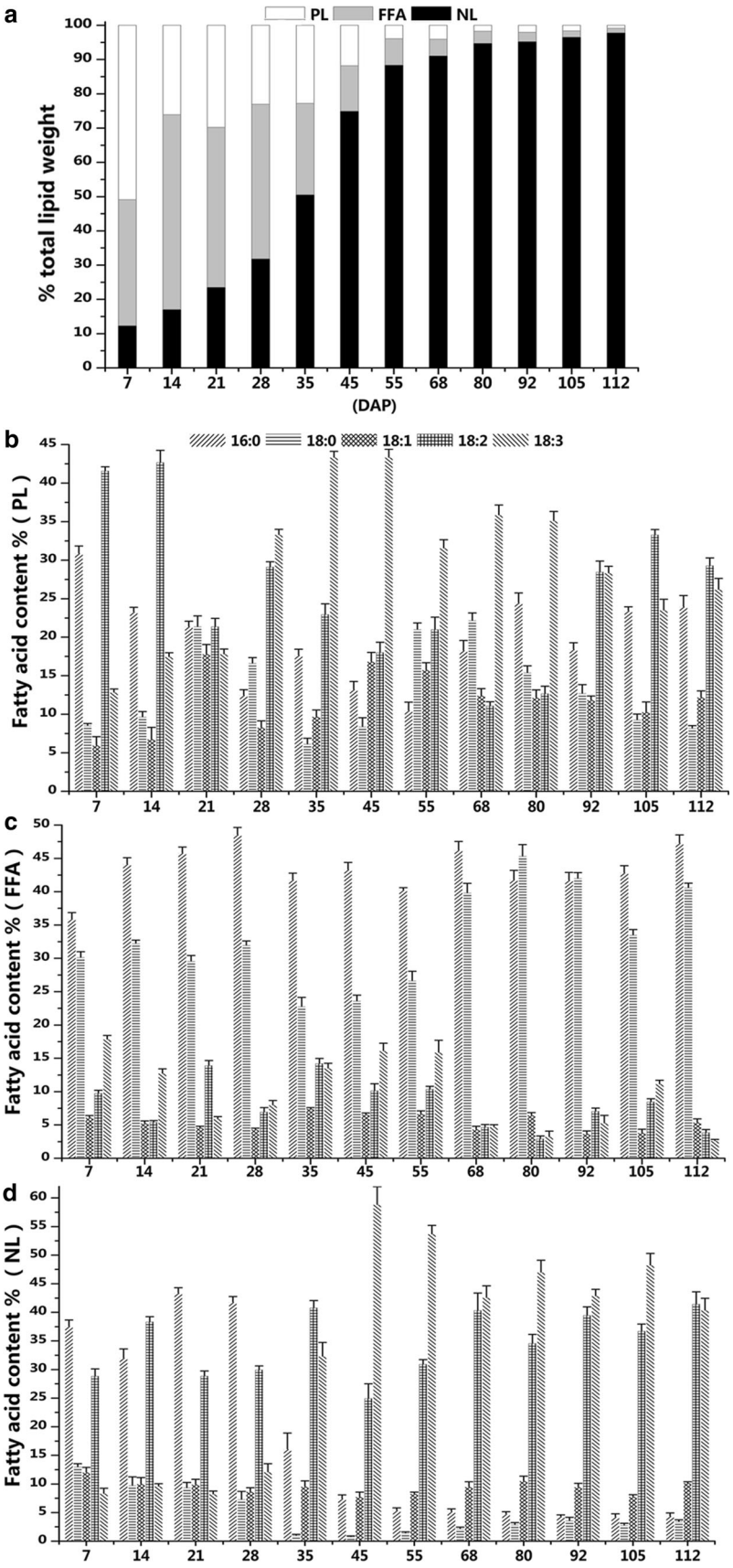


Fig. 2 Content and fatty acid composition changes of lipid fractions of sacha inchi seed oil. **a** PL, FFA and NL content changes in total lipids during sacha inchi seed development. **b** Fatty acid composition changes (mol%) in PL, FFA and NL fractions of sacha inchi seed oil at different developmental stages. **c** DAP days after pollination, PL phospholipids, FFA free fatty acids, NL neutral lipids, 16:0 palmitic acid, 18:0 stearic acid, 18:1 oleic acid, 18:2 linoleic acid, 18:3 linolenic acid. **d** Each data point represents the mean ± SD of at least three biological replicates and *error bars* indicate the standard deviation

highest at 112 DAP (Fig. 1c). In contrast, carbohydrate content decreased gradually prior to 35 DAP (from 95.85 to 87.43 %), and rapidly after fast storage material accumulation (from 87.43 to 23.02 %).

Lipid and Fatty Acid Accumulation in Developing Sacha Inchi Seeds

The main fatty acids identified in sacha inchi seed oils included palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2n-6) and linolenic (18:3n-3) acids. The accumulation of each fatty acid at different developing stages of seeds is illustrated in Fig. 1d. The contents of saturated palmitic and stearic acids were 38.1 and 20.6 % of total fatty acids at 28 DAP, respectively, and sharply decreased thereafter to 4.1 and 3.3 % as the seeds matured (at 112 DAP), respectively. Reversely, the content of total unsaturated fatty acids (USFA), including oleic, linoleic and linolenic acids, varied from 41.3 % (at 28 DAP) to 92.6 % (at 112 DAP). In particular, the proportion of linoleic to linolenic acids increased from 19.5 and 15.2 % (at 28 DAP) to 37.5 and 46.2 % at 112 DAP, respectively. These results mean that the desaturation of fatty acids is associated with oil accumulation in developing seeds.

The proportion of lipid fractions, including neutral lipids (NL) comprised primarily of TAG, free fatty acids (FFA) and phospholipids (PL) is an important qualitative factor for determining edible production of seed oils. The contents of lipid fractions at different stages of seed development are given in Fig. 2a. The accumulation of NL increased dramatically from the early stage (12.4 % at the 7 DAP) to the mature stage (97.9 % at the 112 DAP). The content of both FFA and PL decreased dramatically from the early stage (36.8 %, 50.9 % at the 7 DAP, respectively) to the mature stage (1.3 %, 0.9 % at the 112 DAP, respectively). The fatty acid profile of the NL fraction was similar to the corresponding total lipid after 35 DAP (Figs. 1d, 2d), because of the quantitative primacy of this fraction in the total oil (Fig. 2a). In the FFA fraction, palmitic and stearic acids were predominant at any stage of seed development, and the saturated fatty acids (SFA) ranged from 64.7 to 88.0 % of all the fatty acids (Fig. 2c). Contrary to this, higher unsaturated fatty acids (USFA) content was observed in the PL fraction (Fig. 2b). Changes of fatty acid composition (nmol/mg dry weight) in lipid fractions at different seed developmental stages were shown in the supplementary material Table S1.

Expression Profiles of Unsaturated Fatty Acid (USFA) Related Genes

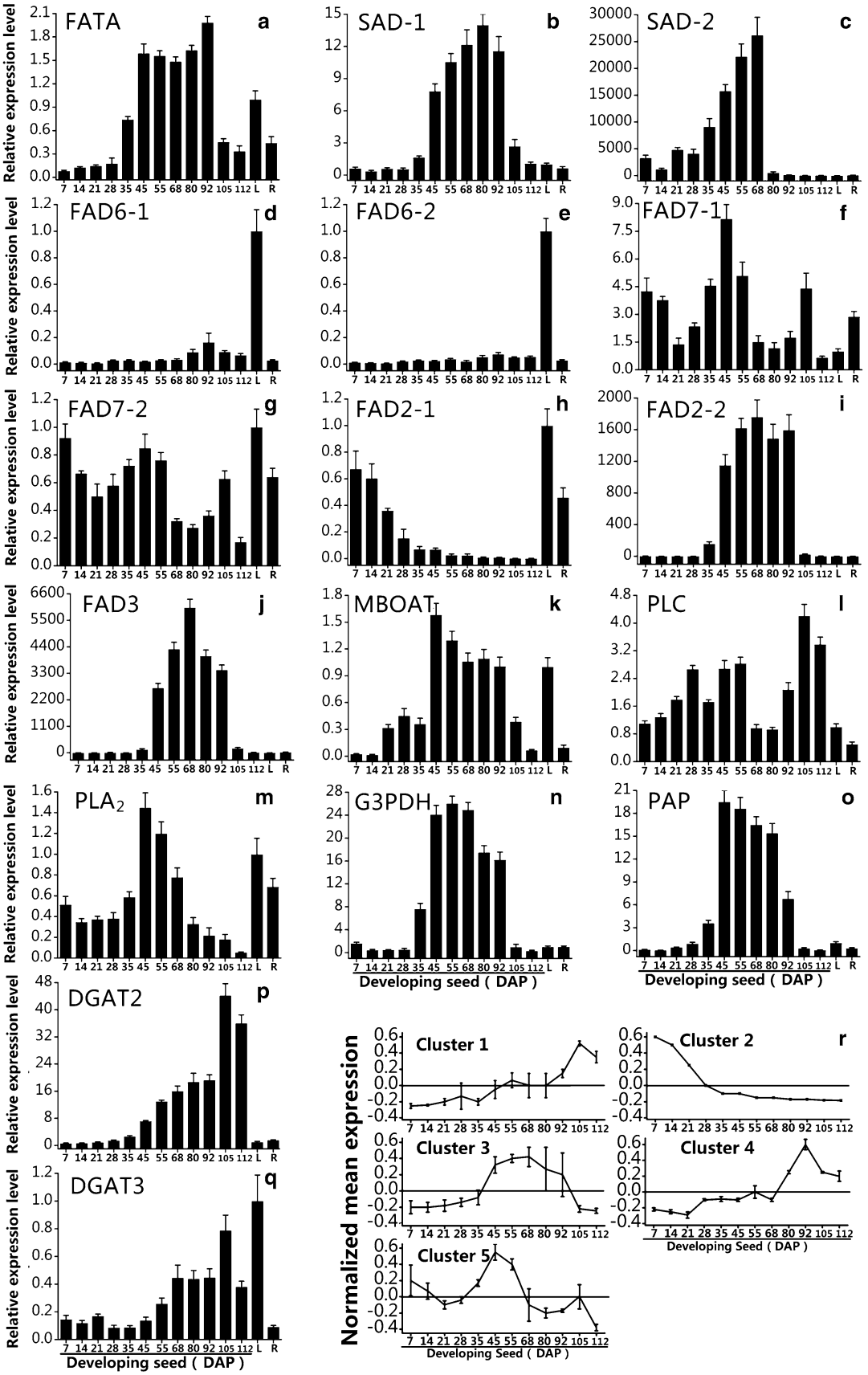
By using quantitative real-time PCR, we examined the temporal expression profiles of 17 USFA-related genes at the different stages of seed development. The results were illustrated in Fig. 3a–q. All 17 USFA-related genes showed higher expression levels in developing seeds than in roots with the relative maximum expression ratio (seed vs root) ranging from 1.4 to 6,004 fold (Table 2); 12 genes (SAD-1, SAD-2, FAD7-1, FATA, FAD2-2, FAD3, MBOAT, PLC, PLA_2, G3PDH, PAP and DGAT2) were more highly expressed in developing seeds than in leaves with the relative maximum expression ratio (seed vs leaf) ranging from 1.4 to 26,200 fold (Table 2). In particular, the expression levels of SAD-2, FAD2-2 and FAD3 were extremely higher within developing seeds than their expression in leaves and roots.

To examine relationships among the temporal expression patterns of the genes tested, we performed clustering analysis and identified five groups with their own expression patterns by using *k*-mean clustering method. The visualization results were described in Fig. 3r. Genes within the same cluster meant that they had similar expression patterns during the development of seeds. On the whole, cluster 3 covering eight genes and cluster 5 covering three genes displayed higher expression in the middle stages of seed development; while clusters 1 and 4 represented higher expression in the late stages of seed development, including 3 and 2 genes, respectively. In addition, clusters 2 covering only one gene showed higher expression in the early stages of seed development.

Fig. 3 Expression profiles of key enzymes controlling deposition of sacha inchi oil. **a–q** Expression of unsaturated fatty acid (USFA) related genes in developing seeds, leaf and root of sacha inchi. Abbreviated names for the genes are described in Table 1. Each data point represents the mean ± SD of three replicates. DAP days after pollination, *L* leaf, *R* root. **r** Expression patterns of unsaturated fatty acid (USFA) related gene clusters during sacha inchi seed development. Abbreviated names for the genes are described in Table 1. Each cluster is represented by the mean expression pattern over all the genes assigned to it. *Error bars* ± SD. *DAP* days after pollination

Effect of Temperature on Lipid Accumulation in Sacha Inchi Seeds

To examine whether environmental temperature influences lipid accumulation and shapes fatty acid composition of seed oils during sacha inchi seed filling, we compared the lipid content and fatty acid composition of seeds set through the cool and hot seasons, respectively. The oil content of seeds set through the cool season (48.2 %) is statistically significant lower ($P < 0.05$) compared with the hot season (49.8 %). Further, inspecting the proportions of different fatty acids in mature seeds set in the two seasons, we found that the proportions of fatty acids (except for linoleic acid) displayed statistically significant differences (Table 3). The SFA (including palmitic and stearic acids)


FATA
a
SAD-1
b
SAD-2
c
FAD6-1
d
FAD6-2
e
FAD7-1
f
FAD7-2
g
FAD2-1
h
FAD2-2
i
FAD3
j
MBOAT
k
PLC
l
PLA2
m
G3PDH
n
PAP
o
DGAT2
p
DGAT3
q
Relative expression level
7 14 21 28 35 45 55 68 80 92 105 112 L R
Developing seed (DAP)
Cluster 1
Cluster 2
r
Cluster 3
Cluster 4
Cluster 5
Normalized mean expression
7 14 21 28 35 45 55 68 80 92 105 112
Developing Seed (DAP)

were significantly higher ($P < 0.05$) in the hot season than the cool season (Table 3). However, the USFA (including oleic and linolenic acids) and unsaturation index (UI) were significantly higher ($P < 0.05$) in the cool season than the hot season (Table 3).

For mature sacha inchi seeds containing mainly neutral lipids, the NL fraction revealed similar fatty acid composition changes to those observed in the total lipids (Table 3). In the FFA fraction, there was a statistically significant increase in stearic acid and SFA in the cool season, while USFA concentrations decreased (Table 3). However, an obvious increase in linolenic acid was observed in the PL fraction in the cool season (Table 3). Significantly increased unsaturation index (UI) ($P < 0.05$) could be detected in each lipid fraction of sacha inchi oil in the cool season.

Discussion

In angiosperms, TAG are most commonly accumulated within oleaginous seeds (specifically in endosperms or cotyledons). We characterized a time-course for seed development and lipid accumulation during sacha inchi seed development. Similar to most seed plants [29], three major physiological periods of seed development were observed in sacha inchi. Firstly, it was a period of rapid gain of fresh weight and seed size (Fig. 1a). Increase in fresh weight from 7 to 35 DAP was more than ten times, and by 35 DAP the seed has reached its full size. Between 35 and 92 DAP, the rapid dimensional and fresh weight increase ceased, and it was a rapid dry weight gain period (Fig. 1a). Finally, from 92 to 112 DAP, it was a natural desiccation period (Fig. 1a). As the seed matured and approached quiescence, the fresh weight and seed size decreased again, while the dry weight remained relatively stable. Our observation also showed that the biosynthesis of carbohydrate mainly occurred at the early stage and the accumulation of lipids and proteins mainly occurred at the middle and late stages during seed development of sacha inchi (Fig. 1c), similar to previous observations in the developing seeds of arabidopsis and castor bean [30, 31]. The proportions of lipid, protein and carbohydrate in mature sacha inchi seeds are in consistent with the previous research [32]. Before 35 DAP, FFA and PL comprised the main total lipid proportion, and SFA was prominent in TL and NL. After 35 DAP, there was a period of rapid gain of NL and USFA began to enrich in TL and NL (Figs. 1d, 2a, d). These observations mean that FFA and PL contributed predominantly to the total lipids in young seeds, but were replaced by NL with seed development. The content of total lipids at the mature seeds resulting from this study is comparable to previous reports [1–5]. Also, the contents of FFA and PL at the mature seeds are in agreement with those of common edible oils such as canola, soybean and sunflower [33], and can be easily removed by refining for edible oil production.

Although the molecular mechanism underlying FA desaturation is complex in plants, in general, the pathway of FA desaturation mainly involves in plastids and endoplasmic reticulum. Desaturation of 18:0 to 18:1-ACP catalyzed by a stromal stearoyl-ACP desaturase (SAD) is one

Table 3 Fatty acid profiles of total lipids and lipid fractions of sacha inchi seeds maturing in two distinct seasons

Total lipids								
Growing season	16:0 mol%	18:0 mol%	18:1 mol%	18:2 mol%	18:3 mol%	SFA mol%	USFA mol%	UI
Cool season	4.0 ± 0.6[a]	3.1 ± 0.5[a]	8.8 ± 1.1[a]	37.5 ± 1.2[a]	46.7 ± 0.7[a]	7.1 ± 1.0[a]	92.9 ± 1.0[a]	223.3 ± 3.6[a]
Hot season	4.5 ± 0.5[b]	3.9 ± 0.3[b]	11.2 ± 1.0[b]	37.4 ± 0.9[a]	43.0 ± 0.7[b]	8.4 ± 0.7[b]	91.6 ± 0.7[b]	214.6 ± 2.9[b]
NL fraction								
Growing season	16:0 mol %	18:0 mol %	18:1 mol %	18:2 mol %	18:3 mol %	SFA mol %	USFA mol %	UI
Cool season	3.9 ± 0.2[a]	2.5 ± 0.4[a]	8.2 ± 0.3[a]	38.1 ± 1.1[a]	45.4 ± 1.1[a]	6.4 ± 0.3[a]	93.6 ± 0.8[a]	224.4 ± 3.9[a]
Hot season	4.6 ± 0.3[b]	3.4 ± 0.4[b]	10.8 ± 0.9[b]	38.3 ± 0.9[a]	42.9 ± 0.8[b]	8.0 ± 0.6[b]	92.0 ± 0.6[b]	215.6 ± 4.1[b]
FFA fraction								
Growing season	16:0 mol %	18:0 mol %	18:1 mol %	18:2 mol %	18:3 mol %	SFA mol %	USFA mol %	UI
Cool season	48.0 ± 0.9[a]	43.0 ± 1.5[a]	4.0 ± 0.1[a]	5.2 ± 0.6[a]	3.1 ± 0.5[a]	91.0 ± 1.2[a]	9.0 ± 1.2[a]	22.1 ± 2.6[a]
Hot season	46.6 ± 2.0[a]	38.3 ± 1.7[b]	5.2 ± 0.6[b]	6.8 ± 1.5[a]	3.5 ± 0.3[a]	84.9 ± 1.4[b]	15.1 ± 1.4[b]	28.2 ± 3.0[b]
PL fraction								
Growing season	16:0 mol %	18:0 mol %	18:1 mol %	18:2 mol %	18:3 mol %	SFA mol %	USFA mol %	UI
Cool season	20.8 ± 1.4[a]	11.9 ± 0.3[a]	13.6 ± 0.8[a]	29.8 ± 0.9[a]	23.9 ± 1.4[a]	32.7 ± 0.9[a]	67.3 ± 0.9[a]	142.7 ± 3.1[a]
Hot season	20.6 ± 1.2[a]	13.4 ± 0.9[a]	14.2 ± 1.0[a]	30.7 ± 0.6[a]	21.1 ± 0.5[b]	34.0 ± 0.7[a]	66.0 ± 0.7[a]	136.2 ± 2.6[b]

Results are given as the average for at least three independent experiments ± standard deviation. Within a column, means followed by the same letter are not statistically different at the 0.05 probability level

of the key steps regulating unsaturated FA levels in plastids, and the generated 18:1$^{\Delta 9}$ FA are exported from plastids to the endoplasmic reticulum, where two sequential double bonds ($^{\Delta 12,15}$) are catalyzed by microsomal desaturase enzymes FAD2 and FAD3, respectively. To identify the critical lipid genes related to the formation of USFA in sacha inchi seeds, we characterized the expression profiles of 17 unsaturated fatty acid related genes during the time-course of seed development and USFA/TAG accumulation by qRT-PCR. Among the 17 lipid genes, there were 8 genes located in clusters 3, including SAD-1, SAD-2, FAD2-2, FAD3, FATA, MBOAT, PAP and G3PDH, displaying a major flat-rise pattern associated with oil accumulation in developing seeds. In particular, the genes SAD-2 (which catalyzes desaturation of 18:0 FA into 18:1$^{\Delta 9}$ FA), FAD2-2 (which converts 18:1$^{\Delta 9}$ into 18:2$^{\Delta 9,12}$ by inserting a double bond at the n-6 position) and FAD3 (which converts 18:2$^{\Delta 9,12}$ into 18:3$^{\Delta 9,12,15}$ by inserting a double bond at the n-3 position) were highly expressed in developing seeds (see Table 2), and their expression pattern were closely associated with the USFA accumulation in NL (storage lipids) fraction in the late stage of seed development. These observations suggested that the SAD-2, FAD2-2 and FAD3 played a major role in the desaturation of USFA in sacha inchi seeds. The high transcripts of these genes might be the mechanism underlying the biosynthesis of USFA in TAG within developing sacha inchi seeds. Besides, the expression patterns of another four genes FATA (involved in transport of 18:1 FA from plastids and endoplasmic reticulum), MBOAT (involved in acyl-editing for incorporating USFA into TAG), PAP and G3PDH (involved in TAG assembly in the Kennedy pathway) were associated with oil accumulation. In addition, the three genes DGAT2, DGAT3 (involved in TAG assembly in the Kennedy pathway) and PLC (involved in formation of diacylglycerol) located in cluster 1 were highly expressed after 35 DAP, and closely associated with oil accumulation in the late stage of seed development. These results indicated that their involvement in biosynthesis of lipids in developing seeds of sacha inchi. In addition, another ER located oleate desaturase gene FAD2-1 was mainly expressed in leaf, root and young seeds, and its expression showed a declining pattern with seed development, meaning that it might not be responsible for the formation of 18:2 FA in storage lipids within sacha inchi seeds. Unsurprisingly, the two plastid located oleate desaturase genes FAD6-1 and FAD6-2 (involved in desaturation of oleic FA in membrane lipids) and another two plastid located desaturase genes FAD7-1 and FAD7-2 (involved in desaturation of dienoic FA in membrane lipids) were weakly expressed in developing seeds, meaning that they might not be responsible for desaturation of main fatty acids in storage lipids in sacha inchi seeds. In cluster 5, the PLA_2 gene, which hydrolyses the acyl group from the sn-2 position of phospholipids, yielding FFA and lysophospholipids with an acyl chain at the sn-1 position as the enzymatic products, was highly expressed in developing seeds and displayed a expression pattern similar to FAD7-1 and FAD7-2, suggesting its function in the unsaturated fatty acid biosynthesis of membrane lipids.

Studies have revealed that environmental temperature could be a critical factor which influences lipid accumulation and shapes fatty acid composition of seed oils during seed filling [34]. Sacha inchi blooms and sets fruit throughout a year in Xishuangbanna area. The effects of temperature on total oil content and on fatty acid composition of lipid fractions of sacha inchi seeds were determined in two distinct seasons. The results showed that total oil content decreased in the cool season, suggesting that the low environmental temperature is not beneficial to lipid accumulation in sacha inchi seeds; while USFA and linolenic acid concentrations increased, indicating that the low environmental temperature is favorable to the accumulation of USFA in sacha inchi seeds. In soybean and flax, the low environmental temperature, similarly, enhanced the proportion of USFA in seed oils [35–41]. Though studies has revealed that some desaturase enzymes (such as the FAD2 and FAD3 genes) could be regulated at the transcriptional level or at the post-translation level in response to low temperature induction [42–44], the molecular mechanisms underlying the effect of temperature on lipid and fatty acid accumulation is unknown in sacha inchi seeds. Further studies on elucidating the function of critical desaturase enzymes such as SAD, FAD2 and FAD3 at the both biochemistry and molecular levels could be interesting to dissect the molecular and physiological mechanisms underlying the effect of temperature on lipid and fatty acid accumulation in sacha inchi seeds. It was also observed that low temperature increased SFA concentration in the FFA fraction and linolenic acid concentration in the PL fraction, for the fact that more highly unsaturated fatty acid was incorporated into phospholipids at lower temperature in plants [45]. Cellular membranes are composed of a complex mixture of phospholipid molecular species, and accumulation of polyunsaturated fatty acids in plant membranes is an important physiological response to low temperature exposure in plants. The importance of the polyunsaturated fatty acids at low temperature has been shown in mutant lines that are deficient in the desaturation of fatty acids [46, 47] and in transgenic plants [48–51].

For the first time, the relationship between fatty acid accumulation and the expression of 17 unsaturated fatty acid (USFA) related genes was investigated in developing sacha inchi seeds during seed development and USFA/TAG accumulation. In addition, the effect of temperature on oil content and FA composition of sacha inchi seeds was

estimated. The results provide integrative information for understanding ALA biosyntheses in sacha inchi seeds and identifying rate-limiting enzyme genes which are critical to metabolic engineering of transgenic oilseeds for high ALA production.

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Conflict of interest We declare that we have no competing interests.

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