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Cloning and Expression Analysis of Long-chain Fatty Acyl-CoA Synthetase Gene 9 from Cinnamomum camphora Postprint

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Abstract

The long-chain fatty acyl-CoA synthetase (LACS) subfamily plays an important role in plant fatty acid metabolism. Cinnamomum camphora (L.) Presl. is an oil-rich woody plant whose seed oil is rich in medium-chain fatty acids, but the biosynthetic mechanism remains unknown. Based on transcriptome data of C. camphora, this study identified the Arabidopsis AtLACS9 homologous candidate gene CcLACS9 through bioinformatics methods. The full-length cDNA sequence of CcLACS9 was cloned via PCR, and related software predicted that it encodes 697 amino acids and possesses three characteristic motifs of plant LACS subfamily members. In Δlacs-deficient yeast complementation tests, using oleic acid as the sole exogenous fatty acid, the mutant yeast transformed with CcLACS9 resumed normal growth, demonstrating that CcLACS9 possesses typical fatty acyl-CoA synthetase function. To investigate whether CcLACS9 participates in C. camphora seed oil biosynthesis, we further studied its tissue expression pattern and the relationship between its expression level and seed oil accumulation during seed development. Real-time quantitative PCR analysis revealed that the CcLACS9 gene was predominantly expressed in kernels and flowers, with the relative expression level in kernels being 17.74 times that in roots. Subsequently, we randomly measured and investigated 30 adult C. camphora trees for indicators such as thousand-seed weight, seed oil content, and proportion of medium-chain fatty acids in mature seeds. Based on kernel oil content, the test population was divided into three different grades (high, medium, and low), and three individual plants were selected from each grade for monthly correlation analysis of kernel oil content and relative expression level of CcLACS9. The results showed that during the early kernel development stage, both kernel oil content and CcLACS9 expression level continued to increase and showed a positive correlation; August was the peak period for CcLACS9 expression. After late September, kernel oil content tended to stabilize, but CcLACS9

expression level remained high while showing a downward trend, with no significant correlation between the two. The LACS subfamily is relatively conserved in plant evolution, and homologous genes have the same or similar functions in different plants. These research results suggest that CcLACS9 may have biological functions similar to AtLACS9, playing an important role in the synthesis and accumulation of kernel oil esters in C. camphora.

Full Text

Preamble

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Abstract

Long-chain fatty acyl-CoA synthetases (LACSs) play crucial roles in plant fatty acid metabolism. Camphor tree (*Cinnamomum camphora* (L.) Presl.) is an oil-rich woody plant whose seed oil is enriched in medium-chain fatty acids (MCFAs), yet the underlying biosynthetic mechanism remains unknown. In this study, we identified a candidate gene homologous to *Arabidopsis* AtLACS9 from camphor tree transcriptome data through bioinformatic analysis, designated as CcLACS9. The full-length cDNA sequence of CcLACS9 was cloned via PCR, and sequence analysis predicted that it encodes a polypeptide of 697 amino acids containing three characteristic motifs of the plant LACS subfamily. Using oleic acid as the sole exogenous fatty acid source, a Δlacs-deficient yeast complementation test demonstrated that CcLACS9 restored normal growth to the mutant yeast strain, confirming its typical fatty acyl-CoA synthetase activity. To investigate whether CcLACS9 participates in camphor tree seed oil biosynthesis, we examined its tissue expression patterns and the relationship between its expression levels and oil accumulation during seed development. Real-time quantitative PCR analysis revealed that CcLACS9 was predominantly expressed in kernels and flowers, with expression in kernels being 17.74-fold higher than in roots. We subsequently measured thousand-seed weight, seed oil content, and medium-chain fatty acid composition in mature seeds from 30 adult camphor trees. Based on kernel oil content, the population was divided into high, medium, and low grades, and three representative individuals from each grade were selected for monthly correlation analysis between kernel oil content and CcLACS9 expression. The results showed that during early kernel development, both oil content and CcLACS9 expression increased continuously, exhibiting a positive correlation. Expression peaked in August; after late September, oil con-

tent stabilized while CcLACS9 expression remained high but declined, showing no significant correlation. The LACS subfamily is evolutionarily conserved in plants, with homologous genes performing identical or similar functions across species. These findings suggest that CcLACS9 likely shares similar biological functions with AtLACS9, playing an important role in the synthesis and accumulation of kernel oil esters in camphor tree.

Keywords: camphor tree, long-chain fatty acyl-CoA synthetase 9, seed oil, expression analysis, yeast complementation assay

Introduction

Camphor tree represents a promising sustainable energy resource in China, with kernel oil comprising 55–65% of dry weight and consisting primarily of decanoic acid (C10) and lauric acid (C12) at proportions exceeding 90% (Zhao et al., 2012). These medium-chain fatty acids (MCFAs) exhibit low freezing points, excellent oxidative stability, rapid absorption, and favorable compatibility and spreadability, making them valuable for industrial, pharmaceutical, healthcare, cosmetic, and animal husbandry applications (Gong et al., 2012; Liu et al., 2016). Widely distributed across southern Chinese provinces, camphor tree demonstrates substantial development potential—annual seed oil production in Hunan Province alone exceeds 400 tons. However, large-scale utilization remains limited, primarily due to the lack of competitive elite varieties. As a highly heterozygous woody plant, camphor tree exhibits significant inter-individual variation in seed oil content and composition, making it crucial to establish constrained theoretical criteria for directed elite variety selection and breeding. Strengthening research on camphor tree lipid biosynthesis, particularly the identification of key genes, will help fill fundamental knowledge gaps and facilitate future elite variety development and industrial oil production.

Long-chain fatty acyl-CoA synthetases (LACS) catalyze the conversion of free fatty acids (C14–C20) into fatty acyl-CoA thioesters, participating in various lipid metabolic reactions essential for plant development, fatty acid elongation, triacylglycerol (TAG) formation in seeds, β-oxidation, biomembrane synthesis, and cell signal transduction (Watkins, 1997; Khurana et al., 2011). In higher plants, LACS proteins are evolutionarily conserved members of the adenylate-forming enzyme superfamily (AAE), characterized by a highly conserved AMP-binding domain comprising motif 1 (T[SG]-S[G]-G-[ST]-T[SE]-G[S]-X-P[M]) and motif 2 (Y[LWF]-G[SMW]-X-T[A]-E) (where X represents any amino acid), and a shared catalytic mechanism: (1) formation of an acyl-AMP intermediate with ATP consumption and pyrophosphate release, and (2) acyl transfer to the final receptor with AMP release (Babbitt et al., 1992; Stuible et al., 2000). The AMP-binding domain executes the first reaction and serves as a signature sequence for identifying AAE superfamily members (Stuible et al., 2000). LACS also contains a conserved domain of approximately 25 amino acid residues known as the acyl-CoA synthetase (ACS) signature sequence, which may constitute the fatty acid binding site and ACS activation

site (Mashek et al., 2007). Additionally, a specialized linker region of 45–70 residues distinguishes LACS from other ACS family members and is essential for normal biological function (Steinberg et al., 2000; Iijima et al., 1996).

With the widespread availability of plant genomic and transcriptomic data, increasing numbers of LACS subfamily members have been identified across species, laying the foundation for studying plant lipid metabolism. Functional characterization of LACS subfamily members has been most extensively conducted in the model plant *Arabidopsis thaliana*, where nine LACS-encoding genes have been identified. Defective yeast complementation tests confirmed that seven encoded proteins possess strong acyl-CoA synthetase activity, while all nine showed robust activity in vitro with distinct substrate preferences (Shockey et al., 2002). Expression analysis revealed that, except for AtLACS5 (specifically expressed in flowers), other members are ubiquitously transcribed across tissues but exhibit distinct organ or tissue specificity (Shockey et al., 2002). Studies demonstrate that AtLACS subfamily members have different subcellular localizations and play critical roles at various nodes of fatty acid-related lipid metabolism, being essential for normal organ and tissue development (Fulda et al., 2002; Lü et al., 2009; Schnurr et al., 2004; Jessen et al., 2011). Notably, AtLACS9 is predominantly expressed in developing seeds and rosette leaves, with its encoded protein (At1g77590) localized to the plastid membrane (Schnurr et al., 2002). Although T-DNA insertion in the *lacs9-1* mutant did not cause obvious developmental or phenotypic changes, long-chain fatty acyl-CoA synthetase activity in chloroplasts was reduced to approximately 10% of wild-type levels, indicating that AtLACS9 is the primary plastidial LACS but functions redundantly with another LACS for fatty acid export from plastids (Schnurr et al., 2002). Subsequent double mutant analysis confirmed functional redundancy between AtLACS9 and AtLACS1, demonstrating their important roles in seed oil biosynthesis (Zhao et al., 2010).

Materials and Methods

1.1 Plant Materials and Reagents

Experimental materials were collected from a small population of 10–20-year-old camphor trees at the Jiangxi Academy of Forestry. The CcLACS9 gene sequence was referenced from transcriptome data of five chemical types (linalool, eucalyptol, camphor, borneol, and isosafrole) of camphor tree leaf tissues constructed by the Camphor Tree Engineering Technology Center of the State Forestry Administration (Jiang et al., 2014).

RNA extraction kit (RNAiso for Polysaccharide-rich Plant Tissue), TaKaRa LA Taq®, reverse transcription kit (First Strand cDNA Synthesis Kit), qPCR kit (SYBR Green I qPCR Kit), agarose gel recovery kit, and PMD18-T cloning kit were purchased from TaKaRa. The yeast expression vector pYES2 and the defective yeast (*S. cerevisiae*) YB525 strain were kindly provided by Professor Tan Xiaoli of Jiangsu University. Various chain-length fatty acids were purchased

from Sigma-Aldrich.

1.2.1 Survey of Thousand-Seed Weight, Kernel Oil Content, and Composition in a Small Camphor Tree Population

Thirty camphor trees with similar growth vigor were randomly selected. In November 2015, 2 kg of seeds were collected, cleaned, and air-dried. Thousand-seed weight was measured using a 0.001 g precision electronic balance. Kernels were extracted, air-dried, and 100 g samples were used for oil extraction via Soxhlet method. Oil weight was measured to calculate individual seed oil yield, and fatty acid composition was determined by gas chromatography-mass spectrometry (GC-MS).

1.2.2 Screening of Candidate LACS9 Genes in Camphor Tree

The Pfam database (http://pfam.xfam.org/) was queried to download the hidden Markov model file for the AAE family characteristic domain (Pfam ID: PF00501) (Conti et al., 1996). HMMER3.0 (http://hmmer.janelia.org/) was used to annotate the transcriptome data from five chemical types of camphor tree leaf tissues. Based on annotation results, all contig sequences annotated as AAE superfamily members were retrieved from the camphor tree transcriptome. Using AtLACS9 as a reference sequence, local BLASTX was performed to identify homologous contigs, which were then assembled using CAP3 software to achieve electronic cloning of the camphor tree LACS9 gene.

1.2.3 Cloning of Camphor Tree LACS9 Gene

From April to July 2016, five tissues (stem, leaf, root, flower, and kernel) were collected from camphor trees, snap-frozen in liquid nitrogen, and total RNA was extracted using RNAiso for Polysaccharide-rich Plant Tissue according to the manufacturer' s instructions. Residual DNA was removed by DNase I digestion, and 50 ng of total RNA was used for first-strand cDNA synthesis with the PrimeScript 1st Strand cDNA Synthesis Kit. Based on the electronically cloned LACS9 sequence from the transcriptome data, specific primers were designed for PCR amplification: forward 5'-TTGCGAGAAATGGCTGAAT-3'and reverse 5' -AAGTTCCAACCAACGGATTPCR-3' . The 20 L reaction mixture contained 0.2 L LA Taq, 1 L cDNA, 2 L 10× LA Taq buffer, 0.5 L each of forward and reverse primers (10 mol/L), 1 L dNTPs (2.5 mmol/L), and 14.8 L ddH_2O. PCR conditions were: 95°C for 3 min; 35 cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 2 min; and final extension at 72°C for 10 min. PCR products were detected by 1% agarose gel electrophoresis, bands of expected size were excised, recovered, ligated into PMD18-T vector, and transformed into *E. coli* TOP10 competent cells by heat shock at 42°C. Positive clones were screened by colony PCR and sequenced by Shanghai Sangon Biotech.

1.2.4 Sequence Analysis and Identification of Camphor Tree LACS9

The ScanProsite Results viewer was used to search for conserved domain information in the annotated CcLACS9 protein. Multiple sequence alignment was performed using ClustalX to calculate sequence similarity between CcLACS9 and orthologous genes from other plants. Subcellular localization was predicted using SignalP 4.1 Server and PredictProtein. Phylogenetic analysis of plant LACS9 genes was conducted using MEGA6.0, incorporating LACS9 sequences from *Arabidopsis*, rapeseed, peanut, and other species.

1.2.5 Tissue-Specific Expression Analysis of Camphor Tree LACS9

From April to July 2016, flower, stem, leaf, kernel, and root tissues were collected for total RNA extraction. The *Actin* gene was used as an internal reference (forward: 5' -CCTCGACACACAGGCGTTAT-3' ; reverse: 5' -CCATGCTCGATGGGATATTTCA-3'). Real-time quantitative PCR (qRT-PCR) was performed to detect CcLACS9 expression in different tissues using primers forward 5' -ACCTGCCTTTGGCTCACA-3' and reverse 5' -AAGGCGATCCGTATCCAA-3' . Reactions were performed on a Bio-RAD C1000™ Thermal Cycler in 20 L volumes containing 50 ng cDNA, 10 L 2× SYBR Green qPCR mix, 1 L each of forward and reverse primers (10 mmol/L), and nuclease-free water. Cycling conditions were: 95°C for 2 min; 40 cycles of 95°C for 15 s, 58°C for 30 s, 72°C for 20 s; followed by a melting curve analysis. Each sample was analyzed in triplicate, and relative expression levels were calculated using the 2^(-ΔΔCT) method (Livak & Schmittgen, 2001).

1.2.6 Association Analysis Between CcLACS9 Expression and Kernel Development

Based on previous kernel oil measurements, the test population was divided into three grades: high (>60% oil yield), medium (50–60%), and low (<50%). Three representative plants were randomly selected from each grade. From May to November 2016, seeds were collected monthly and kernels were extracted. One portion of kernels was used for oil extraction and composition analysis, while another was used for total RNA extraction and qRT-PCR analysis of CcLACS9 expression during seed development. Oil extraction was performed by Soxhlet method and composition was analyzed by GC-MS. Correlation between LACS9 expression and oil content during seed development was analyzed using SPSS Statistics 19.0.

1.2.7 Defective Yeast Complementation Assay for Camphor Tree LACS9

Yeast strain YB525 (*faa1Δfaa4Δ*) lacks essential LACS activity for activating exogenous fatty acids and cannot grow normally in minimal medium, making it suitable for analyzing exogenous LACS activity (Zhu et al., 2009). The

recombinant plasmid pYES2-CcLACS9 and control vector pYES2 were transformed into YB525 competent cells, and positive transformants were selected on uracil-deficient solid medium. Random positive clones were cultured in uracil-deficient liquid medium to late logarithmic phase, collected by low-speed centrifugation, and washed twice with 2 mol/L sorbitol. Cells were transferred to uracil-deficient liquid medium containing 2% galactose (without glucose) and cultured with shaking for 4–5 h to induce target gene expression. Subsequently, 1% inoculum was transferred to uracil-deficient liquid medium containing 2% galactose and 0.1% Triton X-100 supplemented with 98 mol/L C18:1 oleic acid as the sole carbon source. Cultures were incubated at 30°C with shaking for approximately 84 h, and cell density was measured spectrophotometrically to assess growth.

Results

Survey of Seed Characteristics in the Test Population

Thousand-seed weight of the 30 camphor trees sampled in November ranged from 118.62 g to 184.23 g, with a mean of 149.92 g. Approximately two-thirds of individuals had thousand-seed weights between 140–180 g, showing a near-normal distribution with a variance of 16.92. Oil yield varied from 42.48% to 76.78%, averaging 57.37%, with a "polarized" distribution: 15 individuals exceeded 60% oil content, 12 were below 50%, and only 3 fell in the 50–60% range. Fatty acid composition analysis revealed that medium-chain fatty acids accounted for an average of 95.71% of total fatty acids in kernels, with most individuals ranging between 94–97%. Decanoic acid content (average 62.36%) was significantly higher than lauric acid (average 33.35%), with the two showing a strong negative correlation ($r = -0.86$, $P < 0.01$). Among the five surveyed traits, variance ranked as: thousand-seed weight > oil yield > decanoic acid content > lauric acid content > total medium-chain fatty acid content.

2.2 Screening and cDNA Cloning of Camphor Tree LACS9 Gene

Five unigene or contig sequences were identified in the leaf transcriptome data as potential orthologs of *Arabidopsis* LACS9 (Table 2). Assembly of these sequences using CAP3 software yielded a complete open reading frame for the electronically cloned camphor tree LACS9 gene. Based on this electronic clone, specific primers were designed for full-length cDNA amplification from cDNA templates of stem, leaf, root, flower, and kernel tissues. A clear single band was detected by 1% agarose gel electrophoresis

. Sequencing confirmed a 2,356 bp amplification product containing a 2,094 bp coding sequence encoding a 697-amino acid polypeptide, designated CcLACS9. EditSeq prediction indicated a molecular weight of 86.83 kDa and isoelectric point of 7.39 (slightly basic under physiological neutral conditions). Online ProtParam analysis revealed multiple strong hydrophilic regions, indicating CcLACS9 is a hydrophilic protein. The sequence was submitted to GenBank

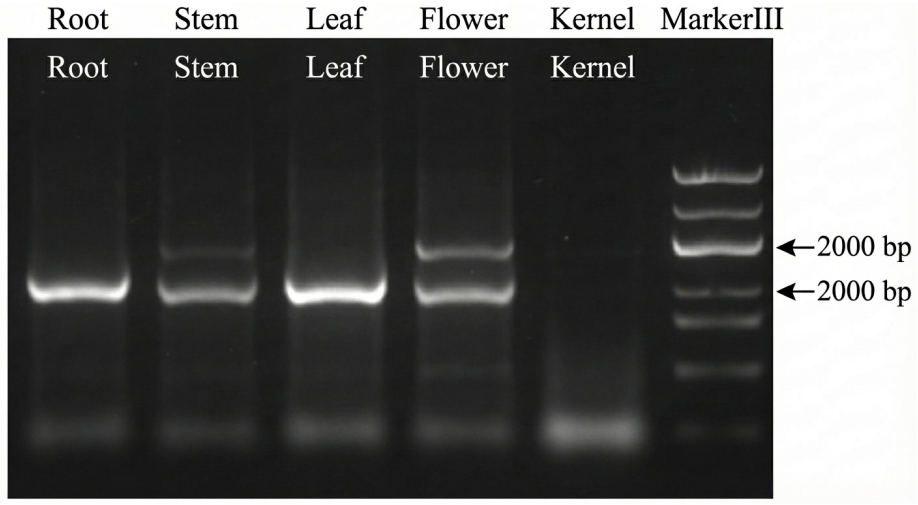

Figure 1: Figure 1

under accession number MF966481.

2.3 Sequence Identification and Phylogenetic Analysis of CcLACS9

The AMP-binding domain (PROSITE PS00455) [LIVMFY]-{E}-{VES}-[STG]-[STAG]-G-[ST]-[STEI]-[SG]-x-[PASLIVM]-[KR] is a hallmark of plant ACS family members. ScanProsite analysis revealed two highly conserved AMP-binding domains at positions 256–267 and 452–462 (marked with asterisks) in CcLACS9, along with a 30-residue ACS signature sequence at positions 531–560 (marked with stars), confirming CcLACS9 as a typical plant ACS family member. Additionally, a 71-residue conserved linker domain (positions 354–424, marked with diamonds) was identified between the AMP-binding domains and ACS signature sequence—a characteristic feature of eukaryotic LACS proteins with variable length across species and members. Thus, CcLACS9 possesses both ACS family common features and the LACS-specific linker domain, confirming its identity as a camphor tree LACS family member.

Multiple sequence alignment showed strong conservation of the AMP-binding domain, ACS signature sequence, and linker domain across plant LACS homologs [FIGURE:2]B. CcLACS9 shared sequence similarities of 80%, 81%, 80%, 78%, 78%, 75%, 78%, 75%, 75%, and 76% with oil palm (*Elaeis guineensis*), lotus (*Nelumbo nucifera*), *Amborella trichopoda*, diploid cotton (*Gossypium raimondii*), sesame (*Sesamum indicum*), *Arabidopsis thaliana*, physic nut (*Jatropha curcas*), rapeseed (*Brassica napus*), maize (*Zea mays*), and castor bean (*Ricinus communis*), respectively, indicating strong evolutionary conservation of LACS9 across plants. Phylogenetic analysis positioned CcLACS9 between monocot and dicot LACS9 genes, clustering closely with lotus LACS9—consistent with cam-

phor tree' s evolutionary status as a basal angiosperm. Subcellular localization prediction using SignalP 4.1 and PredictProtein indicated plastidial targeting, suggesting CcLACS9 likely performs functions analogous to AtLACS9 in exporting fatty acids from plastids and regulating kernel oil synthesis.

[FIGURE:2]

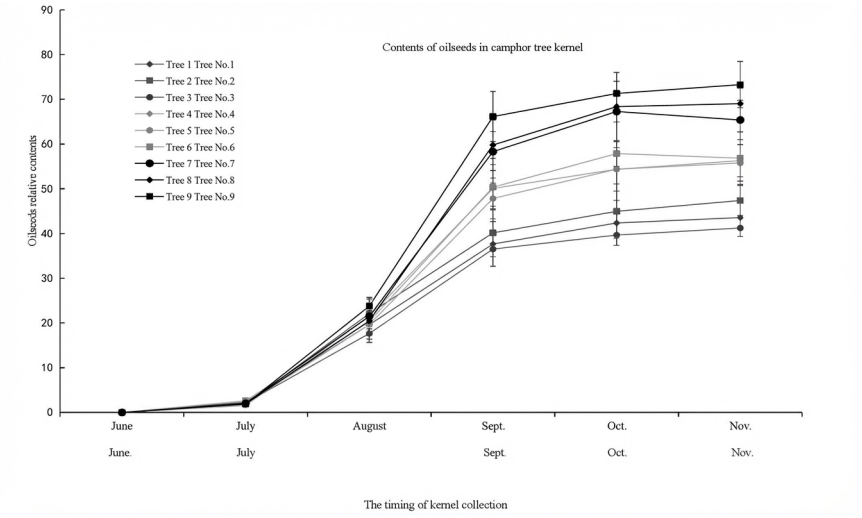

Figure 2: Figure 3

2.4 Tissue-Specific Expression of Camphor Tree LACS9

Total RNA was extracted from flower, leaf, stem, root, and kernel tissues, and qRT-PCR was performed using *Actin* as an internal reference. CcLACS9 was expressed in all tissues but with distinct abundance patterns

. Expression was most prominent in flowers and kernels, followed by leaves, with levels significantly higher than in other tissues. When root expression was set to 1, relative expression levels in stem, leaf, flower, and developing kernel were 4.75-, 9.31-, 15.82-, and 17.74-fold higher, respectively.

2.5 Positive Correlation Between Kernel Oil Content and CcLACS9 Expression During Early Development

Based on kernel oil content, the test population was divided into high (>60%), medium (50-60%), and low (<50%) grades. Three representative trees from each grade were sampled monthly from June to November 2016. During early seed development (June–August), kernel oil content increased rapidly as seeds expanded, then plateaued from September to November as seeds matured

. Oil content differences among grades became significant in August and per-

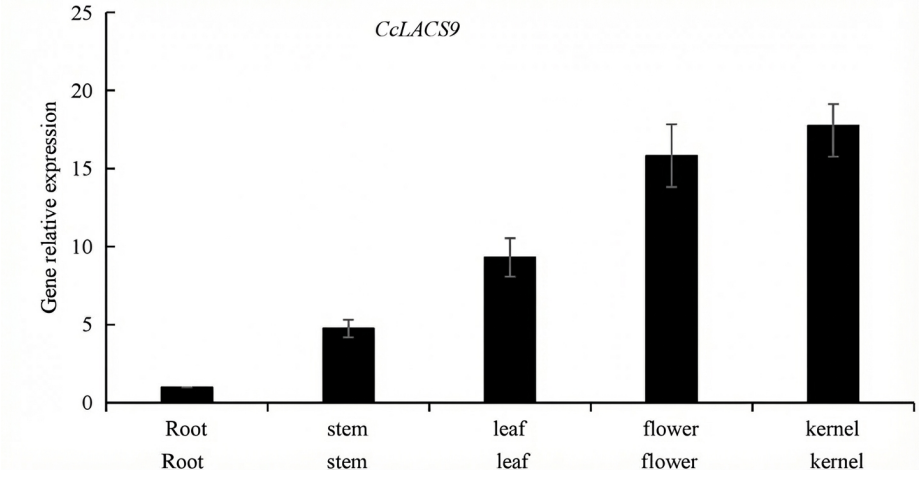

Figure 3: Figure 4

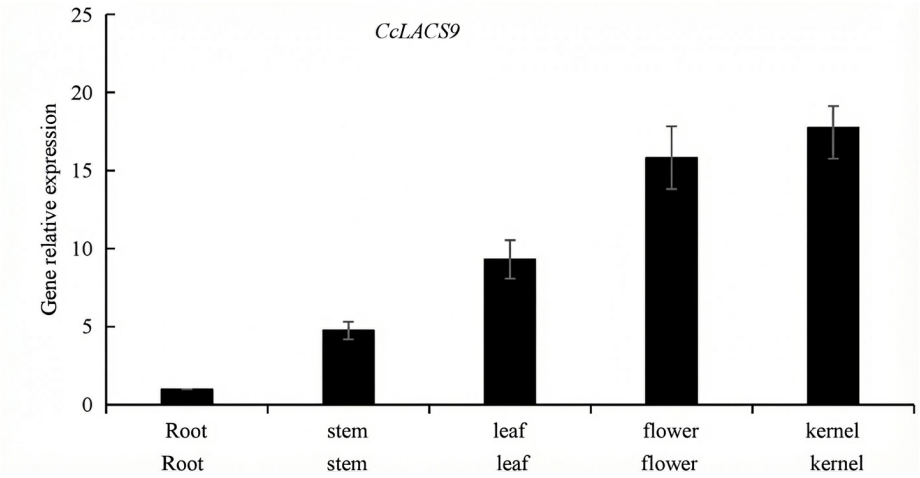

Figure 4: Figure 4

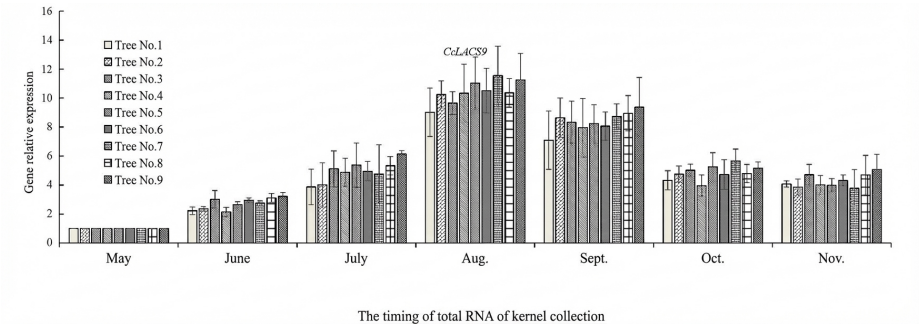

Figure 5: Figure 5

sisted through maturity (November), while fatty acid proportions remained relatively stable throughout development in all individuals.

Correspondingly, qRT-PCR analysis revealed that CcLACS9 expression was significantly upregulated from June to November compared to early May, peaking in August [FIGURE:6]. No significant differences in expression patterns were observed among individual trees or grades during the same period. SPSS analysis showed a strong positive correlation between kernel oil content and CcLACS9 expression during early development (June–August; r = 0.96), but no significant correlation during late development (September–November; r = 0.13), though both parameters remained at high levels.

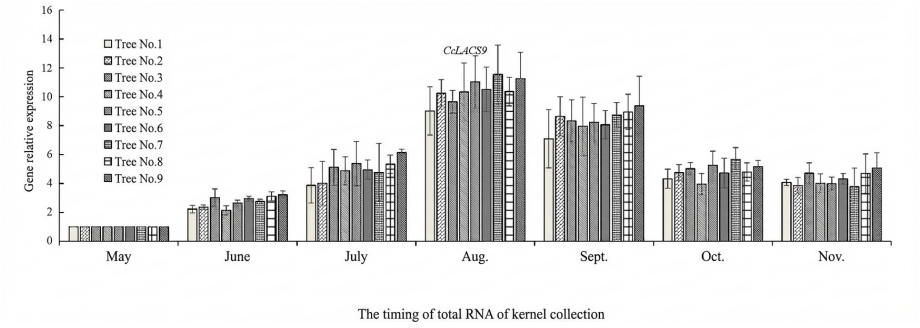

Figure 6: Figure 5

[FIGURE:6]

2.6 Functional Verification of Camphor Tree LACS9 by Defective Yeast Complementation

The CcLACS9 coding sequence was inserted into the pYES2 expression vector via restriction enzyme digestion and ligation. The recombinant plasmid was verified by restriction analysis and sequencing. Both pYES2-CcLACS9 and

empty pYES2 vector were transformed into YB525 yeast strain. Transformants were cultured in uracil-deficient liquid medium containing C18:1 oleic acid as the sole carbon source at 28°C with shaking for approximately 84 h to reach mid-logarithmic phase. OD600 measurements showed that YB525 cells transformed with pYES2-CcLACS9 grew normally, whereas those with empty pYES2 could not grow, demonstrating that CcLACS9 complements the LACS deficiency and possesses acyl-CoA synthetase activity [FIGURE:7].

[FIGURE:7]

Discussion

Multiple *Cinnamomum* species, including *C. camphora*, *C. micranthum*, *C. chartophyllum*, *Lindera megaphylla*, *L. communis*, *L. prattii*, and *L. robusta*, have kernel oil contents exceeding 50%, representing important potential biomass energy resources in China (Zhu et al., 2014). However, these resources remain underutilized, with camphor seeds often discarded as "negative byproducts"from urban landscaping. The lack of competitive elite varieties necessitates targeted breeding for high oil content. In our survey of 30 camphor trees, significant inter-individual variation was observed for thousand-seed weight (range: 65.61 g) and kernel oil yield (range: 34.3%), indicating substantial potential for elite variety selection in natural populations. No significant correlation was found between thousand-seed weight and oil yield, suggesting both traits should be considered comprehensively in breeding programs.

De novo fatty acid synthesis in plants initiates in plastids, where fatty acid synthase catalyzes sequential condensation reactions using malonyl-ACP and acetyl-CoA as substrates, extending acyl chains by two carbons per cycle to produce 16–18 carbon saturated fatty acyl-ACPs (Brown et al., 2006). Subsequent reactions by Δ9-stearoyl desaturase and acyl-ACP thioesterases generate free unsaturated fatty acids (Kachroo et al., 2007). For synthesis of triacylglycerols, waxes, cutin, and suberin, a large proportion of free fatty acids must be exported from plastids in the activated acyl-CoA form, a process mediated by LACS. AtLACS9 has been shown to play an important role in seed oil synthesis and accumulation in *Arabidopsis* (Schnurr et al., 2002). In this study, CcLACS9 shares 75% sequence similarity with AtLACS9, is predicted to localize to plastids, and shows kernel-predominant expression, suggesting it likely functions as an AtLACS9 ortholog in camphor tree seed oil synthesis and accumulation. Camphor fruits initiate in late April, with rapid expansion and peak oil accumulation occurring from May to August, transitioning to maturity after September. CcLACS9 showed continuous upregulation during early seed development (May–August), positively correlating with kernel oil content, further supporting its important role in camphor tree seed oil synthesis and accumulation. After seed maturation (post-September), the lack of correlation between CcLACS9 expression and oil content, despite maintained high expression levels, may reflect compensation for respiratory energy consumption and maintenance of dynamic oil content equilibrium. The absence of significant

correlation between inter-individual differences in CcLACS9 expression and oil content suggests additional factors participate in seed oil regulation.

Camphor tree seed oil, enriched in medium-chain fatty acids, serves as an excellent material for studying MCFA biosynthesis. Acyl-ACP thioesterase is a key gene regulating MCFA synthesis in camphor tree (Yuan et al., 1995), but whether CcLACS9 exhibits chain-length selectivity in activating free fatty acids remains unknown. We compared LACS9 sequences from unusual fatty acid (UFA)-accumulating species (camphor tree, oil palm, castor bean) with common species (*Arabidopsis*, soybean, sesame), revealing that LACS9 is a highly conserved gene family across plants, with inter-species similarities ranging from 75–81% and no distinctive characteristic motifs. Phylogenetic analysis using MEGA6.0 placed CcLACS9 between monocot (oil palm, maize) and dicot (sesame, tea oil camellia) LACS9 genes, consistent with camphor tree's basal angiosperm evolutionary position. LACS9 genes from UFA plants did not cluster in a specific clade. In microbial and animal studies, medium-chain acyl-CoA synthetases (MACS) specifically activate medium-chain fatty acids (Lindner et al., 2006; Kasuya et al., 2009; Meng et al., 2010). Poplar MACS also shows activity toward caproic, pelargonic, and capric acids in vitro (Cao et al., 2016). However, no plastid-localized MACS has been identified, casting doubt on their role in transporting medium-chain fatty acids across plastid membranes. Whether camphor tree possesses specific MACS for medium-chain fatty acid transport requires further investigation.

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