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Full Length Article

Differential Expression of β-*Ketoacyl ACP Synthase* Involved in Fatty Acid Synthesis in the Seed Development Stage of *Cleome viscosa*

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Abstract

The annual herbaceous weed plant *Cleome viscosa* L. is found globally in subtropical and tropical regions. Its seed oil has similar fatty acid composition and characteristics as *Jatropha curcas*, including viscosity, density, and saponification. However, the molecular mechanisms underlying fatty acid synthesis in *C. viscosa* are poorly understood. In plants, de novo fatty acid synthesis occurs in plastids. β-ketoacyl acyl carrier protein (ACP) synthase (KAS) is a key enzyme that activates the condensation reaction for fatty acid synthesis. There are three KAS-encoding genes, *KASI*, *KASII* and *KASIII*. This study identifies and investigates the expression patterns of these genes during seed development (1, 2, 3 and 4 weeks after fruit set [WAF]). Partial sequences of *KASI*, *KASII*, and *KASIII* were identified and found to share approximately 96% identity with homologous KAS genes from *Tarenaya hassleriana*. They showed similar expression patterns at 2 and 3 WAF and were expressed at all stages of seed development. *KASII* was most highly expressed at 3 WAF, while *KASI* and *KASIII* were expressed at higher levels at 2 WAF. Our findings supported the role that KAS enzymes play in producing fatty acids. KASIII is the first enzyme that is known to condense acetyl-CoA with malonyl-ACP to form 4:0-ACP. KASI uses C4:0-C14:0 ACP as a substrate to create C6:0-C16:0 ACP, while KASII catalyzes the final condensation of C16:O ACP to C18:O ACP.

Keywords: *Cleome viscosa*; β-*ketoacyl ACP synthase*; Seed development; Fatty acid synthesis

Introduction

Cleome viscosa L. is an annual weed of the Cleomaceae family that is commonly known as the Asian spiderflower and is found in tropical and subtropical regions of Asia, Africa, the Americas, and Oceania. *C. viscosa*'s leaves, seeds, and roots have anthelmintic, carminative, rubefacient, and antiseptic properties and are used to treat wounds, ulcers, and diarrhea (Upadhyay 2015). Its seeds are abundant in fatty acids (FAs), including palmitic oleic (10.2-13.4%), oleic acid (16.9–27.1%) and linoleic acids (47.0–61.1%), but are low in stearic acid (7.2–10.2%). In addition, 12-oxostearic acid is a minor component of seed oils (Kumari *et al*. 2012). The presence of unsaturated fatty acids in *C. viscosa* seeds may explain its medicinal properties, as their anti-inflammatory and antioxidant properties have been proven. Furthermore, the high percentage of linoleic acid in *C. viscosa* seeds makes it a potential source of gamma-linolenic acid (GLA), which supports skin health. According to Çamas *et al*. (2007), safflower (*Carthamus tinctorius* L.) seed oil is primarily comprised of linoleic and oleic acid which have been shown to have a positive effect on skin health, including the treatment of skin infections and the prevention of atherosclerosis.

In seed plants, de novo FA biosynthesis occurs in the plastids, catalyzed by the type II FA synthase that is found in bacteria, plants, and parasites. Each enzyme is encoded by one gene, producing a unique protein to catalyze a specific stage of the metabolic pathway. All plants contain three ketoacyl-acyl carrier protein (ACP) synthases (KASI, KASII, and KASIII) that act on different substrates. KASIII catalyzes the first FA chain elongation, using acetyl CoA and malonyl-ACP as substrates to produce acetoacetyl-ACP (4:0-ACP). Acetyl groups are transferred from acetyl CoA to the active cysteine residue of KASIII, producing malonyl CoA (Nofiani *et al*. 2019). The concentrations of medium-chain FAs (C8–C14) are elevated in the *Brassica napus* KASIII knockout. *KASIII* overexpression decreased C18:1 FAs and increased C18:2 and C18:3 FAs (Stoll *et al*. 2006). Unlike KASIII, KASI and KASII catalyze condensation reactions, using mainly acyl-ACPs for elongation via malonyl-ACP. KASI regulates chain elongation from 4:0-ACP to 16:0-ACP, while KASII is a key enzyme that catalyzes the final condensation reaction of palmitoyl-ACPs (16:0-ACP) to stearoyl-ACPs (18:0-ACP) (Li *et al*. 2009). These FAs are then transported to the endoplasmic reticulum for triacylglycerol (TAG) production.

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KAS gene expression in *Arabidopsis* seed development reportedly differs between wild-type and mutant strains. *KASI* exhibited a bell-shaped expression pattern, increasing in the early development stages, peaking between 8 and 11 days after flowering (DAF), and then decreasing. Conversely, *KASIII* expression did not change significantly between 5 and 13 DAF (Ruuska *et al.* 2002). KAS genes are highly expressed at 28 and 42 DAF but little expressed at 56 DAF in *Jatropha*, aligning with the development of oil bodies in endosperm cells between 28 and 56 DAF (Gu *et al.* 2012). In addition, there is a strong correlation between seed oil content and KAS gene expression. In *Jatropha*, mature seeds have the highest oil content (Jonas *et al*. 2020). Wu *et al*. (2021) also reported on the differential expression of KAS genes during seed development in *Camellia oleifera*. KAS genes were highly expressed in the developing seeds, with expression levels peaking at 25–30 DAF and then decreasing. These findings suggest that KAS genes play important roles in FA biosynthesis and seed development in *C. oleifera* and that their expression is regulated by miRNA–mRNA regulatory modules. Therefore, this study sought to isolate and evaluate the expression levels of *KASI*, *KASII*, and *KASIII* during FA synthesis in *C. viscosa* seeds at 1, 2, 3, and 4 weeks after fruit set (WAF) and to identify the key FA biosynthesis genes in *C. viscosa*.

Materials and Methods

Plant material

Cleome viscosa, reaching a height of around 1 meter, was tagged, and subjected to seed and leaves collection between July and August within its natural habitat. The study site was located in Amphor Mueang, Phitanulok, Thailand, with Latitude 16.73845 and Longitude 100.19604. The developing seeds (1, 2, 3 and 4 weeks after fruit set (WAF)) and young leaves at shoot tips (L) were harvested, immediately frozen in liquid nitrogen, and stored at -80°C until required for RNA extraction. All samples were collected in three biological replicates.

RNA extraction and complementary DNA (cDNA) synthesis

The total RNA was extracted from the leaves and seeds at different development stages with the Nucleospin RNA Plant Kit (Macherey-Nagel, Germany). The RNA was quantified with a NanoDrop spectrophotometer at 260 and 280 nm and examined on 1% agarose gels. One microgram of RNA was treated with DNase I and reverse-transcribed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) and oligo-dT primers according to the manufacturer's instructions.

Cloning and sequencing

Primers were designed for conserved gene regions based on the homologous *KASI*, *KASII* and *KASIII* gene sequences in the National Center for Biotechnology Information (NCBI) GenBank database and aligned with Clustal X software (v.2.0). Polymerase chain reactions (PCRs) were performed with a 50 µL reaction mixture of 1× PCR buffer, 2.5 mM magnesium chloride, 200 µM dNTPs, 1 unit of Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Fisher Scientific, USA), 10 µM of each primer, and 50 ng of cDNA template. The purified PCR products were then cloned into the pJET1.2/blunt cloning vector with the CloneJET PCR Cloning Kit (Thermo Fisher Scientific, USA) and sequenced. Next, cDNA sequences were analyzed with Gene Studio software and compared to their corresponding sequences with the NCBI BLAST tool. The nucleotide sequences were translated into protein sequences with the ExPASy translation tool (http://ca.expasy.org/tools/dna.html). Partial sequences were deposited in the NCBI GenBank database. A phylogenetic tree was constructed using the neighbor-joining method and 1,000 bootstraps (node cutoff: 50%) with the MEGA 11 software.

Gene expression analysis

The gene-specific primer pairs (*qKASI*, *qKASII* and *qKASIII*) were designed from their cDNA sequences (Table 1). Real-time quantitative PCR (RT-qPCR) was performed with a 20 µL reaction mixture containing 50 ng of cDNA, 1× SensiFAST SYBR No-ROX Mix (Bioline, USA), and 400 nM of each gene-specific primer pair. Thermocycling was performed with the CFX Connect Real-Time PCR Detection System (Bio-Rad, USA) and the following program: 95°C for 3 min, 35 cycles of 95°C for 15 s, 56°C for 15 s, and 72°C for 20 s. The actin gene was used as the internal reference. All RT-qPCR reactions were performed in three biological triplicates, including the internal and negative controls. The results were analyzed with the comparative delta-delta Ct ($2^{-\Delta\Delta Ct}$) method. All data are expressed as means ± the standard deviation after normalization. Statistical significance was determined using the Duncan test.

Results

Gene sequences and phylogenetic analyses

The *KASI*, *KASII* and *KASIII* primers were designed from *Tarenaya hassleriana*, *Arabidopsis thaliana*, *B. napus*, and *Ricinus communis* sequences, generating band sizes of approximately 220, 260 and 210 bp. The partial gene sequences were highly similar to the *KASI*, *KASII* and *KASIII* genes in other plants in the GenBank database and were used to design primers for RT-qPCR (Table 1). The cDNA sequences for *C. viscosa KASI*, *KASII* and *KASIII* genes were submitted to the NCBI GenBank database under the accession numbers OK493500, OK493501, and OK493502, respectively. The highest *KASI*, *KASII*, and

KASIII gene sequence similarity between *C. viscosa* and *T. hassleriana* (*ThKASI*, accession number XM_010540227; *ThKASII*, accession number XM_019202062; and *ThKASIII*, accession number XM_010555165) was 92, 91 and 92%, respectively. These findings suggest that the *KASI*, *KASII* and *KASIII* genes in *C. viscosa* likely perform similar functions as the *ThKAS* genes in *T. hassleriana*.

In addition, the deduced KASI, KASII and KASIII amino acid sequences from diverse plant species, including *T. hassleriana*, *Perilla frutescens*, *R. communis*, *A. thaliana*, and *Gossypium mustelinum*, were aligned with the ClustalX software to generate a neighbor-joining phylogenetic tree (Fig. 1). Phylogenetic analysis showed that the *C. viscosa* KASI, II, and III proteins were closely related to their *T. hassleriana* orthologs, forming a single branch, which suggests the functional conservation of each KAS gene class. KASI, II and III in *C. viscosa* and other plant species were subdivided into two groups. Clade 1 included KASI and II and Clade 2 included KASIII; both clades had well-supported bootstrap values.

Analysis of the differential expression of *KAS* genes

The expression patterns of *KASI*, *KASII* and *KASIII* in seeds (1, 2, 3 and 4 WAF) and leaves were analyzed with qRT-PCR, using the housekeeping actin gene as the internal comparison gene and leaves served as the control. We found that the three genes encoding KASI, II and III for FA biosynthesis were differentially expressed during seed development. *KASI* expression was at least twice as high in developing seeds as in leaves, showing the highest expression at 2 WAF, followed by 3, 1, and 4 WAF (Fig. 2). *KASI* expression dropped significantly between 3 WAF and 4 WAF. *KASII* expression increased gradually from 1 WAF to 2 WAF and peaked at 3 WAF, but was relatively low at 4 WAF (Fig. 3). KASIII is a condensing enzyme that catalyzes the first condensation reaction for FA synthesis using acetyl-CoA as a primer. *KASIII* expression occurred at all stages of seed maturation (Fig. 4). It was expressed the least at 1 WAF, and the most at 2 WAF, decreasing rapidly at 3 and 4 WAF. This contrasts with the *KASII* expression patterns; *KASII* was expressed strongly at 3 WAF. In addition, leave expression was 12–15% of the 2–3 WAF seed expression. However, *KASII* expression was approximately four times lower at 2 WAF than *KASIII* expression. These findings affirm that KAS genes are highly expressed during the intermediate stages of seed development.

Discussion

A cDNA clone encoding beta-ketoacyl-acyl carrier protein (ACP) synthases I–III (KASI–III) from *C. viscosa* was isolated and sequenced. The high sequence similarity with *T. hassleriana* and other plants suggested that these genes have been evolutionarily conserved (Yang *et al*. 2016). KAS genes contribute to plant growth and development, as well as de novo fatty acid production (Chi *et al.* 2010; Shi *et al.* 2022). The phylogenetic analysis of the KAS gene family divides into two distinct clades. *KASI* and *KASII* are found in Clade 1, while *KASIII* is in Clade 2. Judging by the KAS genes in *Linum usitatissimum*, *KASIII* diverged from *KASI* and *KASII* approximately 91.9–106.9 million years ago (MYA), significantly predating the divergence of *KASI* and *KASII*, which occurred 36.7–58.1 MYA (You *et al.* 2014). Additionally, the evolutionary development of *KASIII* suggests cyanobacterial ancestors incorporated via endosymbiosis (González-Mellado *et al.* 2010). The genes' functional divergence may have influenced the proteins' active site architecture. The thiolase superfamily includes elongation enzymes (KASI and KASII) with an invariant residue of Cys and two His (a CHH triad), while a Cys–His–Asn (CHN) triad is found in KASIII (Jiang *et al.* 2008).

The *KASI* expression pattern in *C. viscosa* is consistent with that in olive fruits (*Olea europaea* L.) according to investigations of the molecular mechanisms of fatty acid metabolism at 50, 80, 110, 140 and 170 DAF, called S1–S5,

Table 1: List of oligonucleotide primers

Primer name	Sequence (5′ –> 3′)
qKAS_I_R	GCTCTTGAGAATGCGGATCTT
qKAS_I_R	CCATGTTTGTAATAGCATAAGG
qKASII_F	ATGGGAGGCATGAAGGTCTTTA
qKASII_R	ACAAGCAGTTGAAATAGAATAGTTTG
qKAS_III_F	AATCTGTGGAGCAGTACCAAATA
qKAS_III_R	TAGCTTGATTGGCCTAGCAGTA

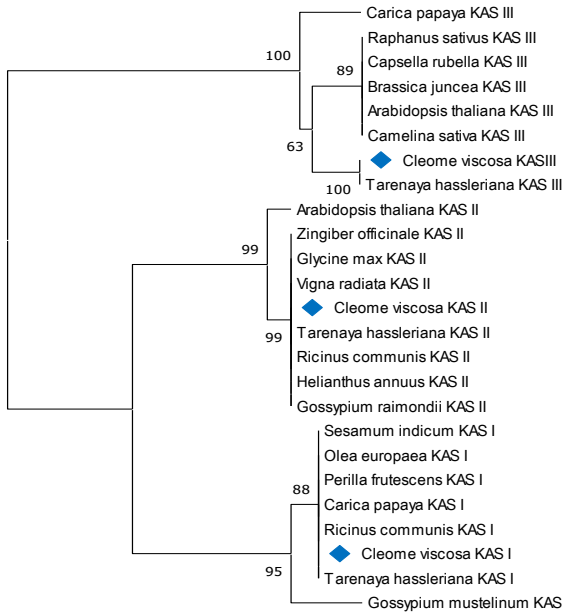

Fig. 1: Phylogenetic analysis of the deduced amino acid sequences of *C. viscosa* KAS I, KAS II and KAS III ortholog and other plants constructed by a neighbor-joining method with 1,000 bootstraps (node cutoff value was 50%) in the MEGA 11 software

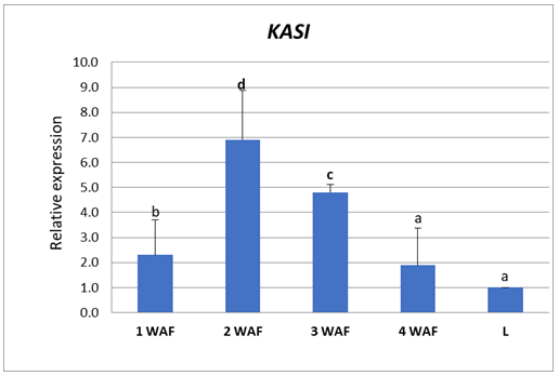

Fig. 2: Relative transcript levels of the *KASI* gene in seeds of *C. viscosa* were examined. The relative expression was detected during seed developmental stages [1-4 weeks after fruit set (WAF)]. Leaves (L) sample was set as the control group. Note: Error bars represent the standard deviation (SD) of three biological replicates, each with three technical replicates. Different letters indicate significant differences at a level of $p < 0.05$ by Duncan's test

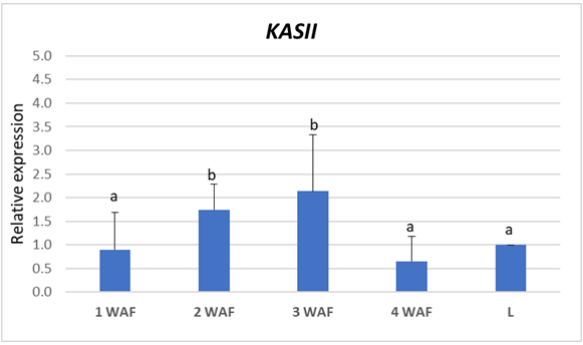

Fig. 3: Relative transcript levels of the *KASII* gene in seeds of *C. viscosa* were examined. The relative expression was detected during seed developmental stages [1-4 weeks after fruit set (WAF)]. Leaves (L) sample was set as the control group. Note: Error bars represent the standard deviation (SD) of three biological replicates, each with three technical replicates. Different letters indicate significant differences at a level of $p < 0.05$ by Duncan's test

conducted via transcriptome analysis. *KASI* was upregulated in S2 compared to S1, resulting in FA accumulation during S2 (Liu *et al.* 2021). Furthermore, high temperatures during soybean seed filling increased lipid content but decreased protein content, which was reflected in increased *KASI* expression in seeds at 21 and 28 days after treatment. High temperatures reduced the expression of the genes involved in seed storage protein synthesis while increasing the expression of the genes affecting seed lipid biosynthesis (Nakagawa *et al.* 2020). De novo fatty acid synthesis occurs mainly in the chloroplasts of higher plants, where KASI acts as a condensing enzyme that catalyzes the extension of the carbon chain in the chloroplasts. *KASI* mutation in *Arabidopsis* affected FA content in seeds and disrupted

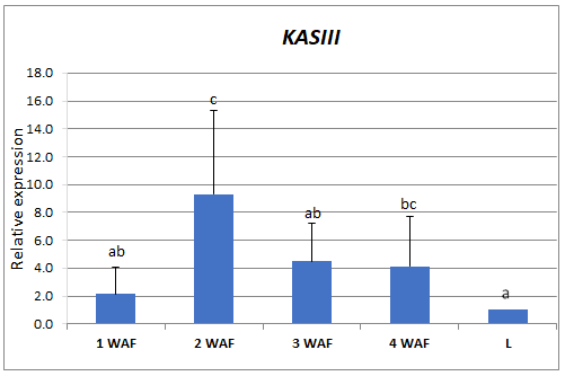

Fig. 4: Relative transcript levels of the *KASIII* gene in seeds of *C. viscosa* were examined. The relative expression was detected during seed developmental stages [1-4 weeks after fruit set (WAF)]. Leaves (L) sample was set as the control group. Note: Error bars represent the standard deviation (SD) of three biological replicates, each with three technical replicates. Different letters indicate significant differences at a level of $p < 0.05$ by Duncan's test

embryo development (Wu and Xue 2010). Moreover, *KASI* suppression in tobacco plants resulted in lower lipid and chlorophyll *a* and *b* content in the leaves and suppressed chloroplast division. Interestingly, overexpression in *KASI* transgenic lines led to an 8–25-fold increase in *KASI* expression compared to wild-type plants (Yang *et al.* 2016). Similarly, mutations in rice *KASI* led to an FA synthesis defect and, thus, impaired root cell elongation (Ding *et al.* 2015).

KASII showed similar expression patterns during seed development as in *Perilla frutescens* (Kim *et al.* 2016). Increased KASII and FAD3 proteins were associated with high alpha-linolenic acid content in perilla seeds that were grown at higher altitudes. The expression of genes involved in the synthesis of fatty acid in perilla seeds can be affected by geographic factors and abiotic stress conditions (Chumphukama *et al.* 2019). Concurrently, the high expression of the *KASII* and *FAD2-1* genes in mature seed *Jathapha cinerea* and *J. curcas* revealed that linoleic acid production was preferred over oleic acid synthesis (Lovio-Fragoso *et al.* 2018). In addition, the FA composition of *C. viscosa* seeds also showed that linoleic acid was the most prevalent, followed by oleic acid and palmitic oleic acid, respectively (Kumari *et al.* 2012).

High *KASII* expression during the latter stages of fruit development in oil palm could be explained by the increased need to synthesize lipids for storage in older mesocarp tissues (Ramli *et al.* 2012). The results of qRT-PCR revealed that the *KASII* expression in *Neocinnamomum caudatum* was higher during periods of rapid oil synthesis (81–96 DAF) and decreased dramatically as the fruit ripened (126 DAF) (Gan *et al.* 2018). Conversely, *KASII* expression levels in *Akebia trifoliate* were higher in the first stage than in the last stages (Zhong *et al.* 2022). In TAG biosynthesis, *KASII* expression

effectively converts palmitic acid (C16:0) to stearic acid (C18:0), indicating that KASII contributes to the balance between palmitate and stearate products (Ramli *et al.* 2012). A high-palmitic-acid sunflower line (CAS-5) contained 25–30% palmitic acid (of the total FAs) due to lower KASII activity (Pérez-Vich *et al.* 2016). Previous studies have identified a point mutation in the *KASII* gene of a high-palmitic-acid soybean, creating a stop codon that was predicted to result in nonfunctional KASII (Aghoram *et al.* 2006). Seed-specific RNA-interference-mediated downregulation of KASII increased the palmitic acid content of cottonseed oil to 65% of the total FAs (Liu *et al.* 2017). Hairpin-RNAi targeting of *KASII* in *Nicotiana benthamiana* leaf tissues makes more palmitic acid available for complex product metabolism, increasing the C16:C18 ratio in wax esters (Aslan *et al.* 2015).

KASIII is a condensing enzyme that catalyzes the first condensation reaction of FA synthesis by using acetyl-CoA as a primer. *KASIII* expression was present at all stages of seed maturation (Fig. 4). It was least expressed at 1 WAF, and most expressed at 2 WAF, decreasing rapidly at 3 and 4 WAF in contrast with *KASII*, which was more strongly expressed at 3 WAF. This pattern is consistent with *KASIII* expression at different seed development stages in *Jatropha curcas*, which peaked in the middle stages, with low expression during the early and late stages (Gu *et al.* 2012). Overexpressed *J. curcas KASIII* in *Arabidopsis* significantly increased the palmitic acid content and resulted in a higher C16:C18 FA ratio (Yu *et al.* 2015). In addition, the *KASIII* expression tendency was consistent with the FA accumulation rate in herbaceous peony seeds (Meng *et al.* 2021). Higher *KASI* and *KASII* expression in the early stages provides substantial precursor content for synthesizing long-chain FAs in oil tea (Wang *et al.* 2022). However, similar studies on sunflower *KASIII* expression found higher levels in developing seeds than in leaves, stems, roots, or seedling cotyledons (González-Mellado *et al.* 2010). The total oil content in developing sunflower seeds is very low during the early stages, slowly increasing until maturity, when the bracts become yellow and brown. The fully ripe seeds exhibited the lowest percentage of oleic acid (C18:1), whereas linoleic acid (C18:2) content was maximum. (Onemli 2012).

The addition of heterologous *KASIII* in *Arabidopsis* can increase the quantity of butyryl ACP substrate present for KASI, resulting in increased KASI activity (Yu *et al.* 2015). In this study, *KASII* was highly expressed at 3 WAF, while *KASI* and *III* were expressed at higher levels at 2 WAF. Previous studies of *J. curcas* investigated oil body development in developing seed endosperm, identified fatty acid and lipid biosynthetic genes from a normalized cDNA library, and determined their expression patterns in developing seeds. *J. curcas KASI*, *KASII* and *KASIII* are expressed in bell-shaped patterns, with peak expression in the middle stage (28 or 42 DAF) but low expression during the early and late stages (14 and 56 DAF) (Gu *et al.* 2012). The elevated levels of *KASIII* in overexpressing plants suggested a potential role of KASIII in the regulation of the fatty acid biosynthetic pathway (Dehesh *et al.* 2001). It is possible that the high expression of *KASI*, *KASII*, and *KASIII* in the early stage generated sufficient precursors for the synthesis of long-chain fatty acids, consistent with the rapid accumulation of fatty acids previously reported in other oil plants. The findings of this study will be valuable for the development of genetically modified crops with improved seed oil composition, content, and yield. Further investigations may be necessary to obtain accurate information on the timing of seed harvest and gene expression linked to fatty acid content.

Conclusion

The present study is the first one providing results about the expression of ketoacyl-acyl carrier protein (ACP) synthases (KAS) in *C. viscosa*, which is a weed with oil properties that are similar to *J. curcas*. The findings suggest that KASI, KASII and KASIII genes play crucial roles in fatty acid biosynthesis, particularly during the intermediate stages of seed development. The sequence similarity analysis revealed high homology with the equivalent genes in other plants, particularly *T. hassleriana*, and the phylogenetic analysis reaffirms the idea of functional conservation across different plant species, highlighting the importance of these genes for lipid metabolism. This study provides valuable information for the development of *C. viscosa* as a new industrial oilseed crop. Further studies may consider the specific functions and regulatory mechanisms affecting *KAS* genes in *C. viscosa*.

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Author Contributions

PS contributed to the analysis of the results and the writing of the manuscript. AP and AJ carried out the experiment and analyzed the data.

Conflicts of Interest

No potential conflict of interest was reported by the authors.

Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approval

Not applicable to this paper

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