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Genome-wide identification and characterization of 1-amino-cyclopropane-1- carboxylate synthase (ACS) gene family in *Carica papaya* and expression insights in response to hormone stress

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Abstract

ACC-synthase (1-aminocyclopropane-1-carboxylate synthase), also known as the ACS gene, plays a pivotal role in ethylene production, which is of great importance in the fruit ripening process for producing saleable yield (marketable fruit). The ACS gene family presumably controls stress responses, plant growth and development, and particularly fruit ripening. Computational biology was used as an essential tool to identify seven ACS genes in *Carica papaya* (red hermaphrodite) using an RNA-seq database (NCBI GEO). Further, the phylogenetic relationships of ACS genes determined gene family resemblance in the genomes of *Hordeum vulgare*, *Musa acuminata*, *C. papaya*, and *Arabidopsis thaliana*; therefore, the identified gene families were further classified into four distinct clades (Type-I, Type-II, Type-III, and Type-IV) in alignment with the well-established Arabidopsis classification. Moreover, encompassing gene structure, domain motifs, cis-element phylogenetic profiling, synteny, and transcriptomic profiling unveiled latent structural and functional attributes within *CpACS* genes. Through segmental duplication of *CpACS*, insights into evolutionary duplication events were predicted. The paralogous behavior of ACS genes in *C. papaya* and a comprehensive transcriptomic analysis demonstrated both up- and down-regulation patterns in response to ethylene treatment at different time points during the fruit ripening process, using the papaya manual handbook V2 (2021). Gene expression showed upregulation of two essential *CpACS* genes, *CpACS5* and *CpACS6*. RT-qPCR validates the expression of these important genes during fruit ripening. However, one gene, *CpACS7*, is expressed in the later stages of fruit development. Our results demonstrated novel avenues for

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understanding the expression pathways of the ACS gene family in red hermaphrodite papaya, and most of these genes were linked to regulating various abiotic stresses, plant growth, and fruit development.

Keywords *Carica papaya*, red-hermaphrodite, ACC-synthase, Ethylene, GWAS, ACS gene, fruit ripening

Introduction

Carica papaya (Papaya) has significant economic value as a tropical fruit, especially in developing countries such as South and Central America, Southeast Asia, and Africa, where it is a cash crop [1] with growing demand in the oceanic region [2–4]. There is a tremendous increase in red hermaphrodite papaya production worldwide, including major producing regions such as Brazil, Mexico, India, Indonesia, Hawaii, and Australia (FAO, 2021). Since papaya is a highly perishable fruit, the knowledge of ethylene production and ripening regulation on the molecular level can effectively reduce significant post-harvest losses that can be as much as 30% of harvested fruit [5]. Ethylene plays a central role in papaya ripening and its quality, shelf-life, and fruit marketability [6]. Due to the climacteric nature of papaya, the fruit ripens suddenly as ethylene is produced, leading to changes in color, texture, and taste [7].

Ethylene is a gaseous plant hormone involved in controlling several processes in plant development, such as seed germination, flower formation, leaf ageing, fruit maturation, and responses to environmental factors, including pathogen attack and physical injury [8, 9]. Ethylene is responsible for changes in fruit ripening, such as increases in sugar concentration and changes in color, hardness, and texture. Climacteric fruits, such as papaya, showed an increase in ethylene levels as they progressed from the growth to the ripening phase. The autocatalytic production of ethylene also checks the ripening process. It makes it an influential factor in ripening, with the capability to control ethylene exposure to delay or hasten ripening as per commercial needs [10]. This key hormone has been widely studied for its ability to improve fruit quality and shelf life, a significant challenge in tropical fruit production and marketing [11]. It is known to be an active participant in the control of fruit ripening and preservation after harvest [12].

Ripening fruits have been categorically divided into climacteric or non-climacteric according to their ability to synthesize and respond to ethylene. Tomatoes, bananas, apples, peaches, and kiwifruit are fruits that show a sharp increase in ethylene synthesis, and the rate of respiration as ripening starts is called the climacteric [13]. On the other hand, fruits like citrus, grapes, strawberries, etc., depending on ripening, are non-climacteric, as there is no sudden increase in ethylene levels or respiration during ripening. The biosynthesis of ethylene is a well-established biological process that undergoes several steps. The first and the most stringent step within this pathway is the formation of 1-aminocyclopropane-1-carboxylate (ACC) from S-adenosylmethionine (SAM) catalyzed by the enzyme ACC synthase (ACS). This is then oxidized to ethylene by ACC oxidase (ACO), completing the biosynthesis of ethylene [14]. Many plants harbor a multigenic ACS gene family that encodes the ACC synthase enzyme. While members of this gene family share similar domains, their expression varies in response to developmental signals and environmental factors, indicating the complexity of regulating ethylene biosynthesis [10]. ACS belongs to the pyridoxal-5′-phosphate (PLP) dependent aminotransferase family and needs vitamin B6 as its cofactor [15].

ACS has been most reported in climacteric fruits like tomatoes, where four ACS genes were observed to control the shift from system 1 (auto-inhibited) to system 2 (auto-catalyzed) ethylene, which is vital for the ripening of the commodity [16]. As in other fruits, the level of ACS genes is differentially expressed to control ethylene production. In apples, *MdACS1* contributes to most of the ethylene produced during ripening [17, 18]. Other members, namely *MdACS3a* and *MdACS6*, are differentially expressed across developmental stages, thereby ensuring low ethylene release in young fruits and a higher rate as it nears the harvesting stage [19]. Such regulation has been reported in peach and pear fruits, where *PpACS1* and *PpACS1b* were shown to be involved in fruit ripening and softening [20, 21]. The differences in *MdACS1* alleles have been known to cause large variations in ethylene production rates, which influence apple ripening and fruit quality after harvest [22, 23]. These observations confirm that members of the ACS gene family are responsible for regulating ethylene biosynthesis during climacteric fruit ripening, with prospects for designing strategies for genetic engineering to increase the desirable shelf life and improve fruit quality.

The study of the ACS gene family in *C. papaya* is of great significance for improving fruit ripening, the ultimate goal of fruit research, and for extending our knowledge to other productivity-related commercial issues [24]. Thus, regulation of ethylene biosynthesis and, therefore, the ACS genes is crucial for improving post-harvest handling and reducing spoilage, a major issue for these tropical fruits in supply chains [25]. Various ripening fruits contain distinct PEFs [26]. A well-coordinated biosynthesis of ethylene can also be useful for implementing other handling practices, including Controlled Atmosphere (CA) storage, which can enable the elongation of the papaya storage period to a considerable extent [27].

In addition, controlling ethylene synthesis and genetic or chemical modification may accelerate ripening and ensure proper synchronization of the supply chain, thereby reducing the actual economic cost for farmers and middlemen [28].

The current study aims to understand ethylene regulation by identifying and characterizing the ACS gene family in papaya. It provides preliminary information for developing better strategies to improve fruit quality. Thus, the cultivation of papaya cultivars with ACS gene suppression in response to changing environmental conditions and the growing global demand for tropical fruits in the context of climate change is becoming increasingly important [29, 30]. For instance, drought, extreme temperatures, and pests increase ethylene production in fruits, resulting in early ripening or shedding, thus reducing yields and fruit quality [31]. This suggests that modification of ACS gene expression using genetic engineering/biotechnology tools could lead to the generation of papaya with longer shelf-life or greater stress tolerance to temperature changes during transport and storage [32].

This study presents a comprehensive genome-wide analysis and characterization of the ACS gene family in *C. papaya* L. (red hermaphrodite) regarding ethylene biosynthesis and the ripening process. The identified ACS genes were obtained from the papaya genome and further compared for their evolutionary relationships, gene organization, protein domains, synteny maps, and expression analysis. This study enhances our understanding of the molecular mechanisms that regulate ACS gene expression, including ethylene production. The results of this study present functional implications that will help advance expertise in papaya breeding programs to improve fruit quality, delay ripening, and address post-harvesting technology.

Materials and methods

Gene sequence retrieval and characterizing physiochemical properties

The query sequence of the ACC-synthase (ACS) gene from *Arabidopsis thaliana* was retrieved from the TAIR database (https://www.arabidopsis.org/). This sequence was used as a query for a BLAST search against the *C. papaya* genome available on the NCBI database (https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_000150535.2/) [33]. The aminotransferase query protein sequence was used to identify putative ACS genes in *Carica papaya*. A BLASTP search (E-value threshold: 1e – 5) was performed against identified *Arabidopsis thaliana* ACS (*AtACS*) protein sequences. Subsequently, Maximum Likelihood phylogenetic analysis was conducted using the Q.pfam+R5 model with 1,000 bootstrap in IQ-Tree, together with conserved domain analysis, to validate candidate genes and remove redundant sequences. The non-redundant ACS genes were identified and characterized. The data in terms of location and features of the identified ACS genes in *C. papaya* were obtained from NCBI, including mRNA length, direction, and chromosomal location. Additional physiochemical parameters such as the molecular weight, theoretical pI value, grand average of hydropathicity (GRAVY), and instability index were examined using the Protparam tool available at (https://web.expasy.org/protparam/) [34].

Classification of ACS genes using phylogenetic analysis

To classify the identified genes across species such as *Arabidopsis thaliana*, *Hordeum vulgare*, *Musa acuminata*, *and Carica papaya*, a comparative phylogenetic tree was constructed from peptide sequences of ACS genes. All the obtained sequences were analyzed using IQ-Tree followed by Maximum Likelihood (ML), with bootstrap values based on 1000 replications, and a Q.pfam+R5 model was used to determine the genes associated with ancestral relationships within the tree clades [35]. The genes were renamed using the model organism *A. thaliana*, and identified ACS genes. The phylogenetic tree visualized using iTOL (https://itol.embl.de/) provide s a clear graphical representation [36].

Gene structure and domain analysis

The intron-exon organization of the ACS genes was analyzed using the Gene Structure Display Server (GSDS) (https://gsds.gao-lab.org/) to display the gene organizati on [37]. The conserved motifs of the identified ACS genes and associated domains in *C. papaya* were studied by Multiple Expectation Maximization for Motif Elicitation (MEME) (https://meme-suite.org/meme/tools/) [38]. The analysis was carried out with a maximum of 20 motifs, and motif lengths ranged from 6 to 50 amino acids. The identified motifs were finally drawn using TB-tools (https://bio.tools/tbtools).

Analysis of MiRNA of *CpACS* genes

To predict miRNA binding sites of ACS genes, the online tool psRNATarget (https://www.zhaolab.org/psRNATarget/) was used with the genes' coding sequences (CDS) to identify binding sites using default settings [39]. These identified miRNAs were expected to regulate the ACS genes on the post-transcriptional level.

Cis-elements and subcellular localization

To identify cis-acting regulatory elements involved in gene regulation, the 1000-bp upstream sequences of the ACS genes were extracted from the NCBI database and subjected to the PlantCARE (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/) database [40]. The identified cis-elements ranged in size from 5 to 20 base

pairs, and the heatmap was generated using TB-tools to show the presence and location of the regulatory elements. The subcellular localization of the *CpACS* genes was analyzed using WoLF PSORT (https://wolfpsort.hgc.jp/), a web-based tool for protein location prediction at the cellular level.

Gene duplication and synteny analysis

Examining the evolution of ACS genes in *C. papaya* involves extensive analysis of gene duplication and synteny. To estimate the time of gene divergence, Ka/Ks ratios were used. Applying default parameters, the Ka/Ks substitution rates were estimated in Tb-tools [41]. To estimate the time of divergence between paralogous genes, the Ks value was used in the T = Ks/2λ formula, where $\lambda = 1.3 \times 10^{-8}$. All gene duplication events were detected by using MCScanX v1. 0, employing default settings. To analyze the synteny relation between genes of *C. papaya* (Cp) and genes of *A. thaliana* (At), and *H. vulgare* (Hv). The TB-tools were employed to demonstrate syntenic relationships across different plant species.

Gene expression and validation using transcriptomic analysis and RT-qPCR

Transcriptomic analysis data were used to investigate the role of 1-aminocyclopropane-1-carboxylate synthase in *C. papaya*. The goal was to identify genes associated with ethylene production during fruit ripening. The NCBI Gene Expression Omnibus (GSE128577) was used to retrieve RNA-sequencing data. The normalized expression matrix data was downloaded and used for downstream expression analysis. The papaya fruits (*Carica papaya* L. cv. "Golden") were collected at the pre-climacteric stage (approximately 150 days after anthesis, with up to one-quarter yellow peel) and sampled across defined ripening conditions (0 h, 12 h control, 24 h control, 12 h ethylene, and 24 h ethylene). Papaya fruits were collected at three different times of year, representing biological triplicates, and subjected to ethylene treatment at 100 ppm for 12 h, followed by an additional 12 h of ripening. Five conditions were established and tested, including a control group at 0, 12, and 24 h, and ethylene-treated (ET) fruits at 12 and 24 h each with biological replicates (three independent harvests), and samples were pooled from at least five fruits per replicate. RNA-seq libraries were produced from pulp tissues and sequenced using the Illumina HiSeq 2500 platform. The original study processed raw reads by quality filtering, alignment to the papaya reference genome, gene-level count analysis, and normalization using DESeq2. Differential expression analyses were used to focus on samples collected at 12 and 24 h after ET to identify ethylene-responsive genes, with particular emphasis on members of the *CpACS* family involved in ethylene biosynthesis. This analysis provided insight into the transcriptional regulation of ethylene production and its role in papaya fruit ripening, including pathways related to the disassembly of the cell wall and the softening of the fruit pulp. RNA was extracted from *C. papaya* fruit pulp tissues using TRIzol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Fruit samples were collected from the control and experimental groups at 0-, 12-, and 24-hours during ripening. The quality and concentration of extracted RNA were assessed before downstream analyses. For transcript-level validation, cDNA was synthesized from 1 µg of total RNA using M-MLV reverse transcriptase (Takara, Osaka, Japan). RT-qPCR (quantitative real-time PCR) was performed using TB Premix Ex Taq II with ROX Reference Dye II (#RR820A, Takara) on an Applied Biosystems 7500 Fast Real-Time PCR System following standard protocols. Gene expression levels were normalized against an internal reference gene, and relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Primer sequences used for RT-qPCR analysis are provided in Dataset S1. This experimental validation complemented the RNA-seq–based transcriptomic analysis and confirmed the identification of ethylene-responsive genes, particularly members of the *CpACS* family involved in ethylene biosynthesis during papaya fruit ripening (Table 1).

Table 1 Primer's sequence of candidate genes with reference gene primer UBQ

CpACS-5	
ACS-5-F:	ACCCGTTTCCTTGTCTGGTC
ACS-5-R:	TAGCATTCTCCGCAAGCCAA
CpACS-6	
ACS-6-F:	TCCCATGCATCAGTTGTCCC
ACS-6-R:	TCTTTTAACCCCACCGCAGT
CpACS-7	
ACS-7-F:	CTCGCTCAGCAATGTAACGC
ACS-7-R:	CAAATGCCTCGGTTTGCCC
UBQ	
UBQ-F:	ACT CAC CGG CAA GAC CAT
UBQ-R:	GTGGAGAGTCGATTCCTTTTG

Results

Identification and physiochemical properties of *CpACS* genes

Identification of ACS genes in *C. papaya* yielded 7 non-redundant genes. The physiochemical properties are described in detail. The gene *CpACS7* has the smallest peptide length of 400 amino acids and a molecular weight of 44,284.82 Da, while the gene *CpACS5* has the largest, which has a peptide length of 552 amino acids and a molecular weight of 60,484.2 Da. The molecular weights of the proteins varied from 44,284.82 (*CpACS7*) Da to 60,484.2 Da (*CpACS5*). The stability of the proteins

was predicted using the instability index (II); proteins with an II value < 40 were classified as stable, and those with an II value > 40 as unstable. Among the seven identified proteins, four (*CpACS1*, *CpACS2*, *CpACS4*, and *CpACS5*) were predicted to be unstable, with II values exceeding 40; *CpACS2* is the most unstable, with an II value of 51.12. The last 3 proteins (*CpACS3*, *CpACS6*, and *CpACS7*) were predicted as stable, as indicated by their II values, which were below 40, with *CpACS3* having the smallest II value of 34.81 shown in (Fig. 1). The GRAVY (Grand Average of Hydropathy) index was used in the study to assess the hydropathicity of the discovered proteins. Most of the proteins were found to be soluble, and most of the reported proteins were hydrophilic; a negative GRAVY score supports this. The most hydrophilic protein was *CpACS2*, with a GRAVY value of -0.332, while the protein *CpACS6*, and *CpACS7* with a GRAVY value of 0.022 and 0.009 considered as approximately neutral hydropathic nature with a slight tendency toward hydrophobicity. The isoelectric points (pI) of these proteins ranged from 5.88 (*CpACS7*) to 7.59 (*CpACS3*). Out of the seven genes, 42.86% (3 genes) were located on the negative strand, and 57.14% (4 genes) were located on the positive strand (Table 2).

Comparative genomics and phylogenetic analysis of identified ACS genes

The peptide sequence was obtained from different plant species, such as *H. vulgare*, *M. acuminata*, *C. papaya*, and *A. thaliana*, and was plotted in a comparative phylogenetic tree to identify ACS genes. The model-organism *A. thaliana* ACS-identified genes were used to identify and rename them according to their origin within the clade. A total of 49 ACS genes were identified in 4 plant species, including five from *H. vulgare*, 26 from *M. acuminata*, 11 from *A. thaliana*, and 7 from *C. papaya*. The renamed genes were categorized into 4 clades based on ACS gene type: Type-I, Type-II, and Type-III, as shown in (Fig. 2). The clade Type-IV contains the highest number of genes, which is 24 in number, and three of the particular genes of *CpACS*. Similarly, the Type-I and Type-III clades each have a single, while clade Type-II have two *CpACS* gene. The similar gene origins in the phylogenetic tree show the ancestral relationship and may have similar functions.

Genetic architecture and domain motif analysis

The genomic architecture and intron-exon distribution across various gene loci are depicted in (Fig. 3), as per the findings presented in this investigation. Notably, among the identified *CpACS* genes, five (*CpACS1*, *CpACS2*, *CpACS3*, *CpACS4*, and *CpACS5*) exhibit a four-exon structure devoid of introns, while two genes show a different structure. One *CpACS* gene, *CpACS6*, contains seven exons, and *CpACS7* contains the most, nine. The motif analysis reveals conserved motifs and their corresponding domains in each *CpACS* gene. As illustrated in (Fig. 4), motifs 2, 3, 5, and 7 exhibit conservations in all of *CpACS* genes, while other motifs, such as 1, 4, 6, and 8, are present in most of the *CpACS* genes (71%). The identified genes have conserved domain known as aminotransferase which present predominantly in all *CpACS* genes.

Cis-regulatory element and sub-cellular localization for regulatory transcription function

Analysis of the cis-regulatory elements of the *CpACS* gene revealed a complex regulatory pattern involving multiple signaling pathways and stress responses. A high number of AREs, DRE core, and TC-rich repeats in the promoters of most *CpACS* genes suggests that these genes may regulate abiotic stress responses, including drought, anaerobic conditions, and wounding. Ethylene-responsive elements (EREs) are found in *CpACS1* and *CpACS5*, suggesting these genes may play a strong role in ethylene signaling, which broadly governs critical processes such as fruit ripening and stress adaptation.

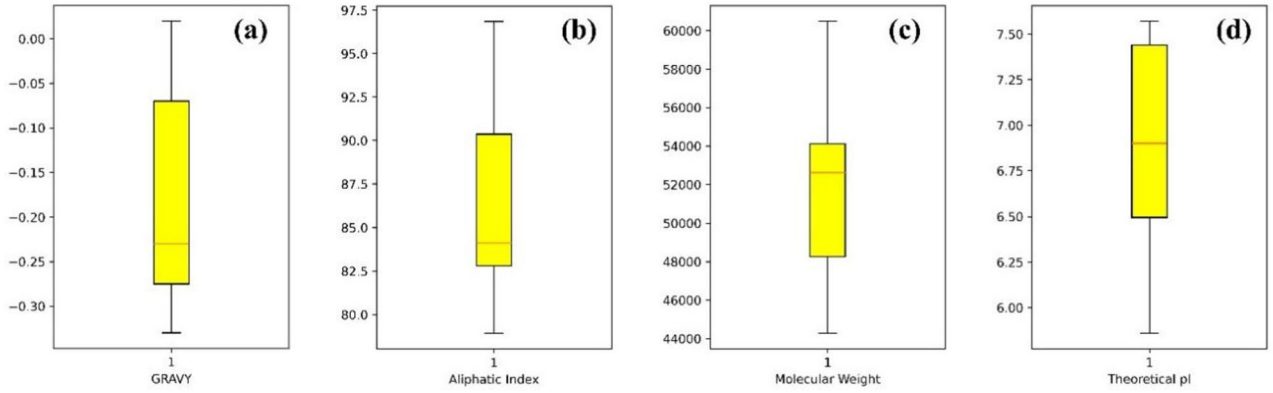


Fig. 1 The graphical overview and distribution of (**a**) GRAVY (Grand Average of Hydropathy), (**b**) Aliphatic index, (**c**) Molecular weight, and (**d**) Theoretical pI values across all ACS genes in *C. papaya*

Table 2 Physiochemical properties of identified *CpACS* genes

Accession No.	Gene IDs	Scaffold	Scaffold Location		Strand	Size		Mw	pI	GRAVY	II	Subcellular Location
			Start	End		CDS	Peptide					
LOC110811354	*CpACS1*	supercontig_1322	2117	6608	-	1467	488	55100.72	6.81	-0.287	49.1	Cytoplasm
LOC110815304	*CpACS2*	supercontig_922	179,343	181,008	-	1329	442	50000.57	6.14	-0.332	51.12	Nucleus
LOC110809089	*CpACS3*	supercontig_129	501,672	503,508	-	1401	466	52612.2	7.59	-0.261	34.81	Nucleus
LOC110810587	*CpACS4*	supercontig_1466	5177	6978	+	1419	472	53145.72	7.54	-0.235	44.12	Nucleus
LOC110817493	*CpACS5*	supercontig_29	453,971	457,522	+	1659	552	60484.2	7.5	-0.15	51.96	Chloroplast
LOC110808937	*CpACS6*	supercontig_131	700,730	709,977	+	1266	421	46524.84	6.83	0.022	37.69	Chloroplast
LOC110808111	*CpACS7*	supercontig_151	441,056	444,475	+	1203	400	44284.82	5.88	0.009	39.1	Cytoplasm

Abbreviations: II Instability index, *Mw* Molecular weight, *GRAVY* Grand Average of Hydropathy, *pI* Isoelectric point

Also, the presence of hormone-responsive motifs such as ABRE for abscisic acid, CGTCA- and TGACG-motifs for jasmonic acid, P-box and TATC-box for gibberellin indicates that these genes are regulated in harmony with multiple hormones involved in controlling growth, development, and defense responses. Among them, Box 4, G-box, and GT1-motif under *CpACS4* indicate the possibility that these elements were regulated by light and Circadian rhythm to control genes affecting photosynthesis, stress response, and other physiological processes. These genes often contain numerous TATA-box elements, indicating their transcription initiation potential; most notably, *CpACS6* contains 210, *CpACS2* contains 147, and *CpACS1* contains 113 TATA-box elements. The CAAT box elements, which bind general transcription factors, are most notable in *CpACS5*. The presence of tissue-specific regulatory motifs, such as the CAT-box associated with the meristem and the GCN4_motif associated with endosperm, also points towards tissue-specific regulation. Further analysis shows that CpACS3 and CpACS5 contain a greater diversity of transcription factor binding sites, including the MYB and MYC classes (Fig. 5). Other motifs associated with environmental responses or developmental control suggest that transcription factors regulate genes in a stimulus-dependent manner. Therefore, since the stress-responsive, hormonal, and light-regulatory motifs are present in promoter *CpACS* genes, their expression profile indicates that it is strongly regulated in response to stress-limiting environmental signals within *C. papaya*; ACS is paramount in ethylene biosynthesis and stress tolerance during the fruit ripening process.

The study on the subcellular localization of the *CpACS* genes indicates that the genes are distributed across multiple organelles, suggesting that their products participate in a variety of cellular processes. *CpACS5*, and *CpACS6* have high presence in chloroplasts, suggesting that some of them may play photosynthesis-related functions. The three genes *CpACS2*, *CpACS3*, and *CpACS4* exhibit strong nuclear signals, suggesting they are involved in gene regulation, possibly associated with ethylene biosynthesis. Since *CpACS1*, and *CpACS7* proteins were detected in the cytoplasm, they are assumed to be involved in cytosolic signaling.

Analysis of Ka/Ks and MiRNA

In the investigation of ka/ks values (non-synonymous and synonymous substitution rates) within the *CpACS* genes, a spectrum was observed ranging from 0.082047802 (*CpACS1_CpACS4*) to a peak of 0.505268428 (*CpACS5_CpACS6*), the latter being the highest among all comparisons. Remarkably, the paralogous-gene pair *CpACS5_CpACS6* manifested the lowest value at 71.25064078 million years ago (MYA), while the highest value of 163.2955104 million years ago was discerned for

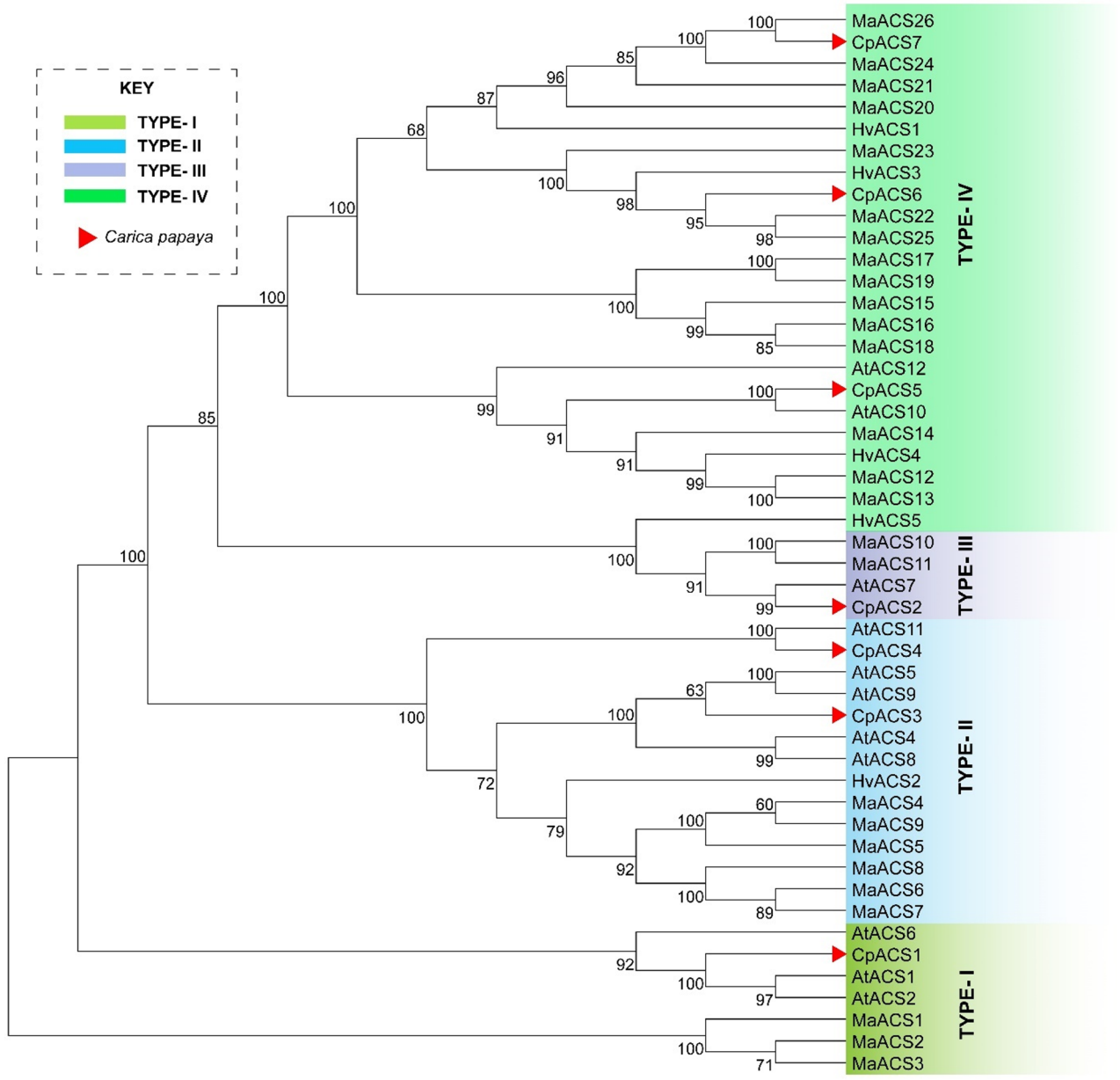


Fig. 2 Evolutionary analysis using MEGA-11 software investigated the phylogenetic relationships of ACS gene family in *H. vulgare* (Hv), *M. acuminata* (Ma), *C. papaya* (Cp), and *A. thaliana* (At). The identified ACS genes were categorized in three clades according to type of ACS gene as Type-I, Type-II, Type-III and Type-IV

the gene pair *CpACS1_CpACS4*, indicative of segmental duplication events (Fig. 6). Within the framework of evolutionary selection, a ka/ks value less than 1 indicates purifying selection, whereas a value greater than 1 indicates a trend towards positive or diversifying selection. The miRNA analysis predicted three potential microRNAs, such as cpa-miR172a, cpa-miR172b, and cpa-miR159a, that target two *CpACS* genes. In particular, the *CpACS2* gene targeted my two mature miRNAs, including miR172a and miR172b, while the *CpACS3* gene targeted my single miRNA, like miR159a.

Gene duplication and syntenic relationship

In the context of the paralogous behavior of *CpACS* genes, notable instances of paralogy are observed on the papaya scaffold. All identified *CpACS* genes show subsequent linkage with other genes on different chromosomes in the papaya. The similar interactions among these ACS genes in *C. papaya* are shown in (Fig. 7a). Plotting dual synteny analysis reveals orthologous behavior between papaya genes and those of other plant species. In papaya, scaffold-supercontig_29 and scaffold-supercontig_129 display orthologous relationships with chromosomes

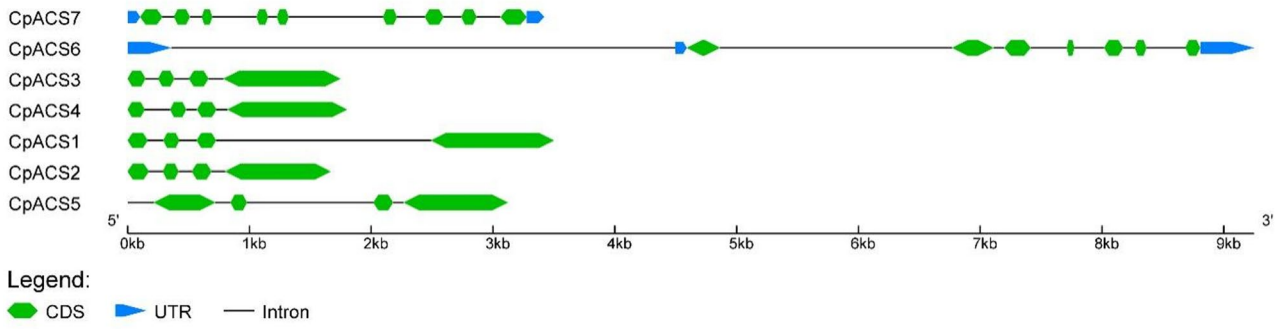


Fig. 3 The investigation of intron-exon structures and their distribution across distinct *CpACS* genes involved a detailed analysis of each gene's intron-exon composition, delineating the specific numbers and arrangement of introns and exons

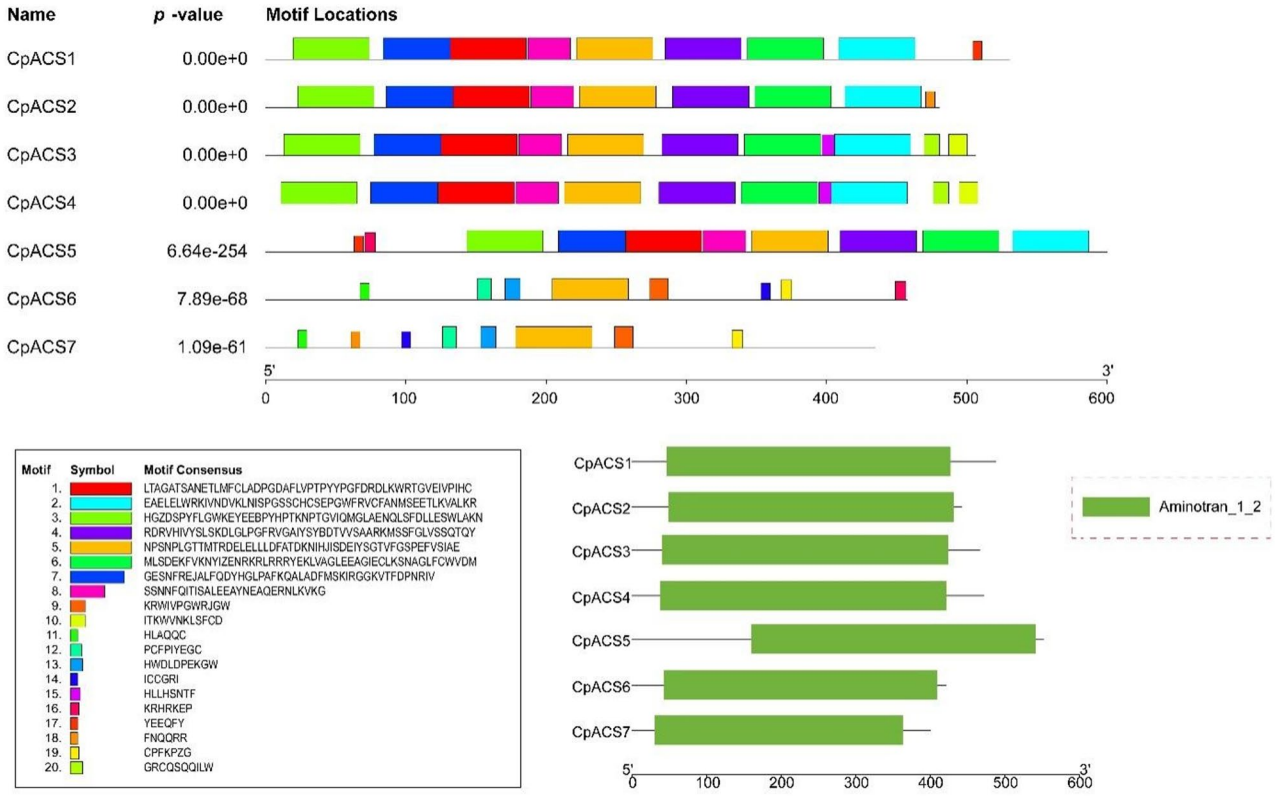


Fig. 4 The graphical representation illustrates the distribution of the complete set of 20 motifs within *CpACS* genes. The conserved domains, predominantly aminotransferase is featured in this distribution

1, 2, 3, 4, and 5 of Arabidopsis (Fig. 7b). In the case of barley, chromosome 4 H exhibits orthologous behavior with scaffold-super contig 129 of papaya (Fig. 7c). These linkages across different plant species reveal ancestral relationships among genes at different evolutionary locations.

Expression analysis of *CpACS* genes in the fruit ripening process

RNA-seq expression analysis reveals gene regulation in response to ethylene treatment. The treatments were administered at 12- and 24-hour intervals to determine the gene's role in fruit ripening. Treatment with 100-ppm ET resulted in upregulation of three candidate genes, including *CpACS5*, *CpACS6*, and *CpACS7*. At 12- and 24-hour intervals, the expression of *CpACS5* and *CpACS6* is significantly up-regulated, indicating that these two genes are important regulators of ethylene at the early stage of fruit ripening compared to the control group (Fig. 8). These genes play a role as positive regulators, while the *CpACS7* gene shows downregulation when treated with ET at both 12- and 24-hours. The gene indicates a slowdown in ripening when ET is applied at later stages of fruit development. These genes are responsible

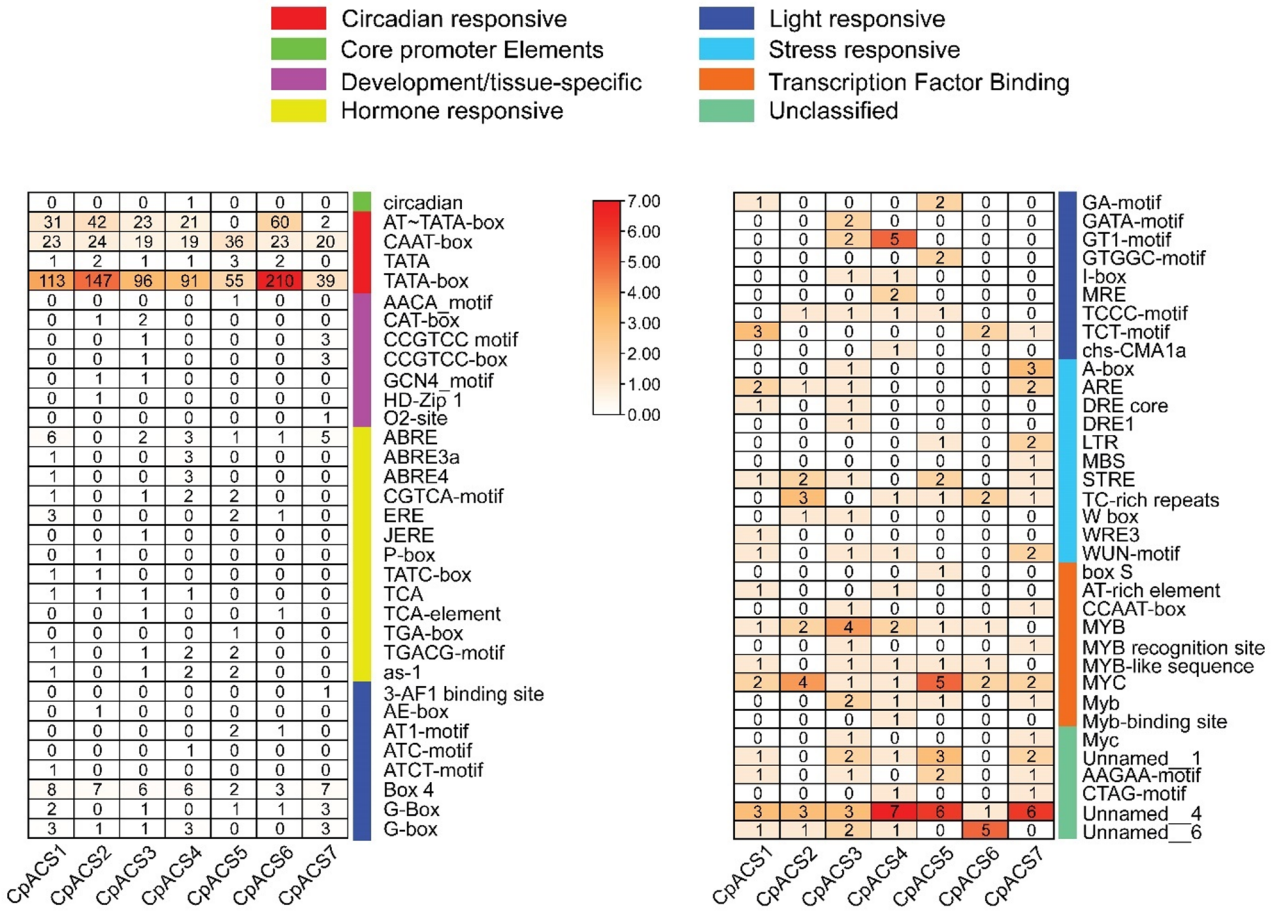


Fig. 5 The graphical depiction herein illustrates the cis-regulatory elements associated with *CpACS* genes, elucidating their functional implications across distinct tiers by examining individual gene promoter regions

for the responsiveness of the ethylene-mediated pathway and for the disassembly of the fruit cell wall.

The consistent expression of *CpACS5* suggests that it remains engaged in autocatalytic ethylene production, and the high expression of *CpACS6* indicates that it is involved in the control of downstream events related to fruit ripening. Conversely, *CpACS7* showed a significant reduction in transcript abundance upon ethylene exposure at both time points, suggesting a possible negative control role or involvement in a set of feedback controls that alter the ripening process at subsequent developmental phases. The suppressive action of *CpACS7* in response to prolonged ethylene exposure might represent a regulatory mechanism that reduces excessive ethylene levels and slows ripening once critical developmental sites are reached. Combined, these genes appear to play a role in ethylene responsiveness and would presumably contribute to the stimulation of ethylene-mediated pathways that mediate fruit softening and cell wall disassembly. To confirm the RNA-seq expression profiles, RT-qPCR was performed at 12 and 24 h of ethylene exposure, validating the quality of the transcriptomic data and the importance of these *CpACS* genes in papaya fruit ripening (Fig. 9).

Discussion

In recent years, the identification and characterization of ACS genes in several plant species have broadened our understanding of ethylene biosynthesis, stress responses, and developmental processes [42–46]. Nevertheless, compared with other plants, studies on the ACS gene family in *C. papaya* remain limited, and more information is needed. Therefore, we investigated the function of the *CpACS* genes in detail during plant development and fruit ripening and found their significant importance for developing essential knowledge for possible deployment in the papaya breeding program and other tropical fruit crops. We found that evolutionary processes, such as gene duplication, contributed to diversification and were instrumental in shaping gene families, including the ACS gene family in *C. papaya*. The current study provided us with limited knowledge of the ACS genomic structure, phylogeny, and the functional characterization of *CpACS* genes involved in ethylene production.

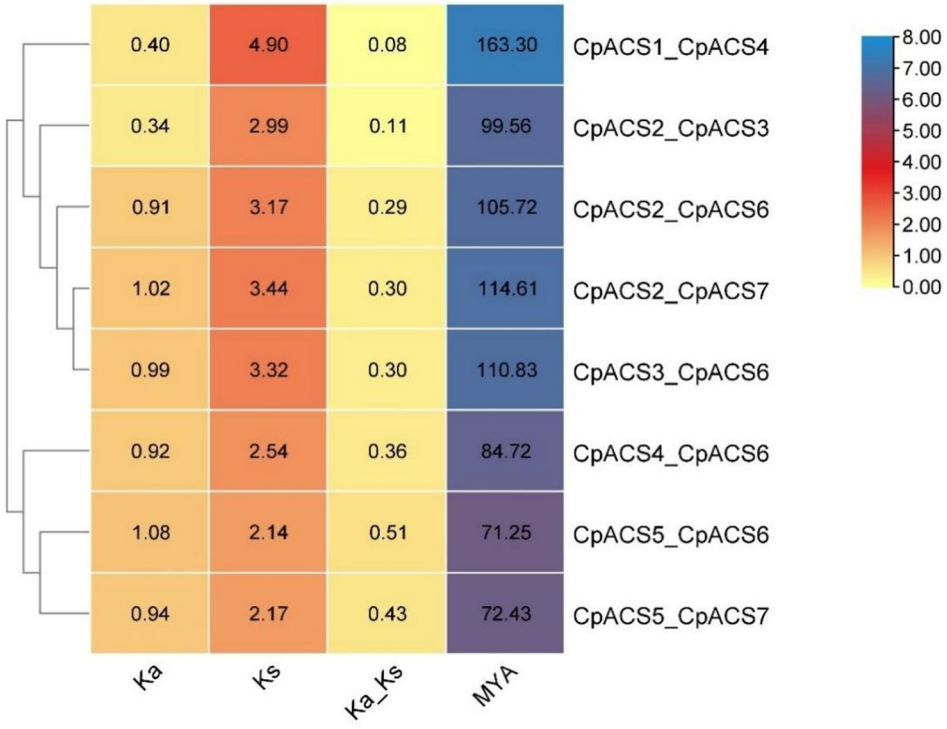


Fig. 6 The representation of Ka/Ks is the ratio of non-synonymous substitution (Ka) over synonymous substitution (Ks) mutations. The gene duplication over selection and evolutionary pressure to paralogous pairs of *CpACS* genes was determined based on Ks and Ka values

The phylogenetic analysis of ACS genes cloned from other plant species, including barley (*H. vulgare*), Cavendish banana (*M. acuminata*), and the model plant Arabidopsis (*A. thaliana*), was used to investigate evolutionary relationships and possible modes of action for these genes during ripening. This includes 49 ACS genes identified in four species: 26 from *M. acuminata*, 11 from *A. thaliana*, 5 from *H. vulgare*, and 7 from *C. papaya*. The clades comprised interspersed genes, indicating a common ancestry and possible functional similarities among species. These orthologs in each species suggest that ACS genes may have functional significance specific to ethylene biosynthesis, stress responses, and developmental processes that have been evolutionarily conserved throughout this lineage. Genomic structural analysis of *CpACS* genes indicated that five of them (*CpACS1* to *CpACS5*) consist of four exons, whereas *CpACS6* consists of seven exons and *CpACS7* comprises nine. The motif analysis of all *CpACS* revealed that motifs 2, 3, 5, and 7 are conserved in all *CpACS* genes, while others are present in most. Additionally, the *CpACS* genes harbor a predominant domain named as aminotransferase which is conserved in all *CpACS* genes [47].

The promoter elements suggest a convergence of multiple signaling pathways and stress responses, creating a complex regulatory system. An abundance of AREs, DRE cores, and TC-rich repeats suggests possible involvement in abiotic stress sensing and regulation, while ethylene-responsive elements (EREs) in the promoters of both *CpACS1* and *CPAC5* indicate a putative signal transduction pathway role in processes such as fruit ripening. Hormone-responsive elements such as ABRE (abscisic acid) and CGTCA/TGACG (jasmonic acid) are specifically associated with growth and defense regulation. The light-regulated motifs and multiple copies of TATA boxes indicate potential transcriptional activity, and consensus tissue-specific motifs suggest they are likely to play important or specialized regulatory roles in various plant tissues.

Subcellular localization of *CpACS* genes suggests their involvement in various cell processes., *CpACS5*, and *CpACS6* localize to chloroplasts and may be involved in photosynthesis. The nuclear signals of *CpACS2*, *CpACS3*, and *CpACS4* indicate a role in gene regulation, possibly in ethylene biosynthesis. *CpACS1*, and *CpACS7* localize to the cytoplasm-related proteins that may be involved in cytosolic signaling.

Gene duplication is a key force driving plant evolution, as it generates genetic diversity and shapes the genome to enable the acquisition of new functions. Most gene pairs showed ka/ks values ranging from 0.082 (*CpACS1_CpACS4*) to 0.505 (*CpACS5_CpACS6*), indicating purifying selection acting on these genes. At the same time, the gene pair *CpACS5_CpACS6* showed potential functional diversification, as indicated by an elevated ka/ks value; however, based on the divergence times of each gene,

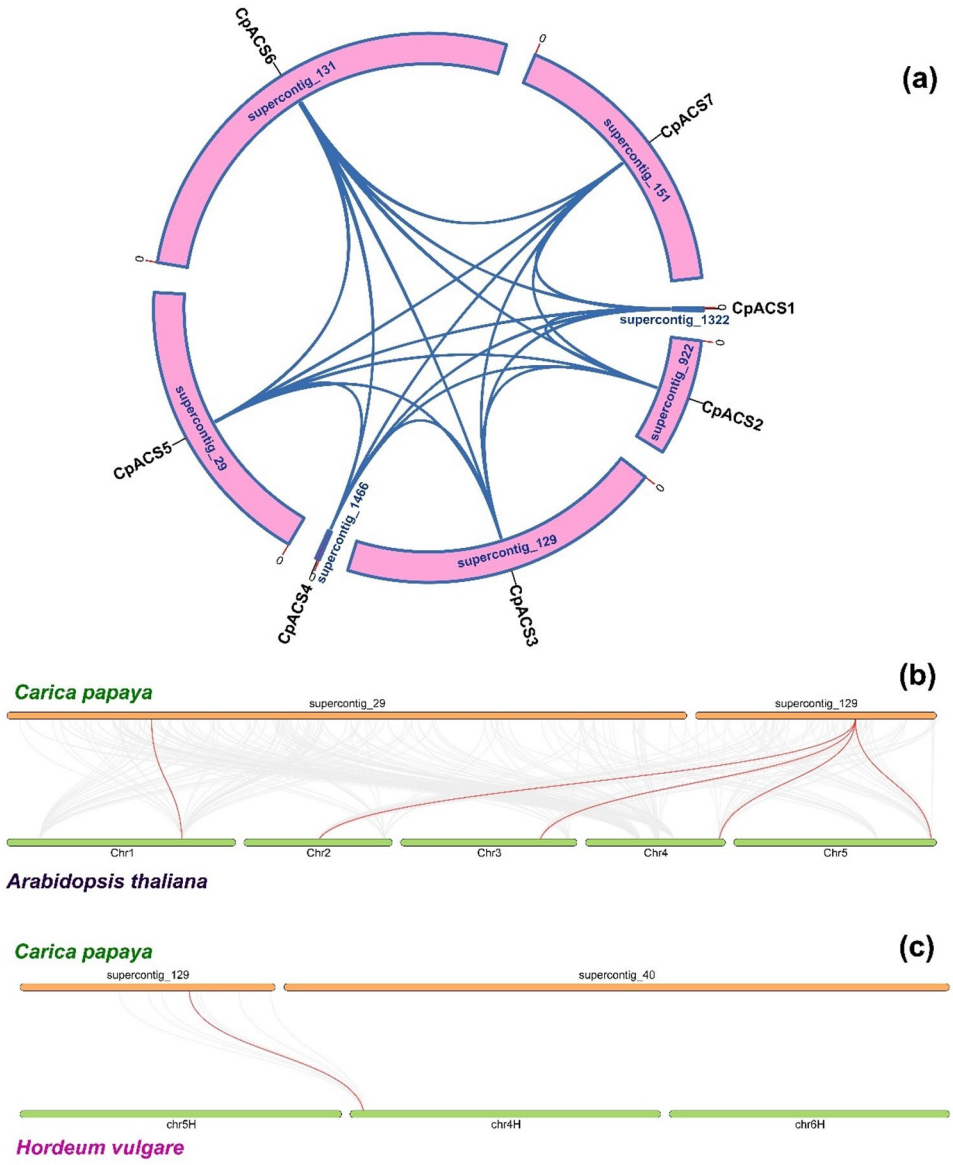


Fig. 7 The paralogous (**a**) and orthologous behavior exhibited by identified genes associated with diverse crops, such as *Arabidopsis* (**b**) and barley (**c**) manifests a discernible pattern in the distribution of gene duplications

which aligned with our intuition as segmental duplication events. This is a major mechanism underlying plants' ability to cope with environmental stresses, both abiotic and developmental. The CpACS2 and CpACS3 genes, which are target genes of miRNAs such as cpa-miR172a, cpa-miR172b, and cpa-miR159a, provide better insight into post-transcriptional regulation in plants. Hence, cpa-miR172a and cpa-miR172b target CpACS2, and both of them have been known to play a role in development, like controlling flowering time in Arabidopsis through regulation of AP2-like genes [48] in the juvenile to adult phase transition during development, and are hence involved in plant maturation and phenotypic plasticity [49]. On the other hand, cpa-miR159a was shown to target *CpACS3* and is reported to control MYB transcription factors, which are important in development and stress. Information on the role of cpa-miR159a in modulating MYB33 and MYB65, which impacts developmental defects and stress physiology in Arabidopsis, is also reported [50]. The transcriptomic data for the *CpACS* gene were analyzed in this study to examine the role of ethylene in the fruit ripening process. The results show that two key genes, *CpACS5* and *CpACS6*, are significantly up-regulated during fruit ripening, and that one gene, *CpACS7*, is also expressed in this pattern but in later stages. This might be linked to other gene expression or adapt other pathways of expression. The expression patterns of these genes were validated by RT-qPCR.

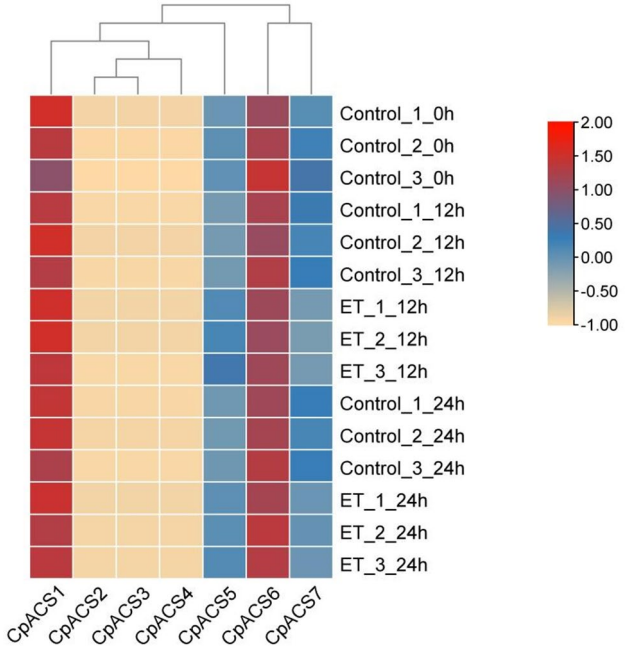


Fig. 8 The heatmap shows the regulation of genes at different time intervals (0 h, 12 h, and 24 h) when exposed to varying amounts of ethylene to identify the genes involved in the fruit ripening process. This comprehensive study shows that the three key genes, *CpACS5*, *CpACS6* and *CpACS7*, are involved in fruit ripening

This study primarily focuses on bioinformatics analysis and gene expression profiling. Further studies should focus on understanding the functional role of these *CpACS* through specific gene silencing, such as CRISPR and RNA interference (RNAi), or gene overexpression, to help us understand the regulatory roles of these genes in ethylene biosynthesis and ripening. Besides, integrative multi-omics studies that can integrate transcriptomic data with proteomic and metabolomic data could provide a deeper insight into downstream signaling, including cell-wall remodeling and softening. Mechanistic modeling of coordinated regulation of *CpACS* genes can be used to design interventions to regulate ethylene production, ultimately improving the quality of postharvest fruit and shelf life in papaya and other climacteric fruits.

Conclusion

A comprehensive analysis of the ACS gene in *C. papaya* is linked to ethylene production and fruit ripening. Seven ACS genes were identified in the *C. papaya* genome, and their phylogenetic relationships were characterized to classify them into distinct clades alongside other plant species. This investigation studied the gene structure and physicochemical properties. Analysis of conserved motifs, cis-regulatory elements, and gene duplication provided more profound insights into the functional diversity and regulatory intricacies governing ACS-gene expression. MiRNA analysis unveiled specific regulatory networks associated with key *CpACS* genes, offering critical insights into post-transcriptional regulation and their roles in modulating fruit ripening mechanisms. Additionally, differential gene expression and RT-qPCR patterns during fruit ripening, induced by ethylene application to fruit, revealed up-regulation of CpACS genes

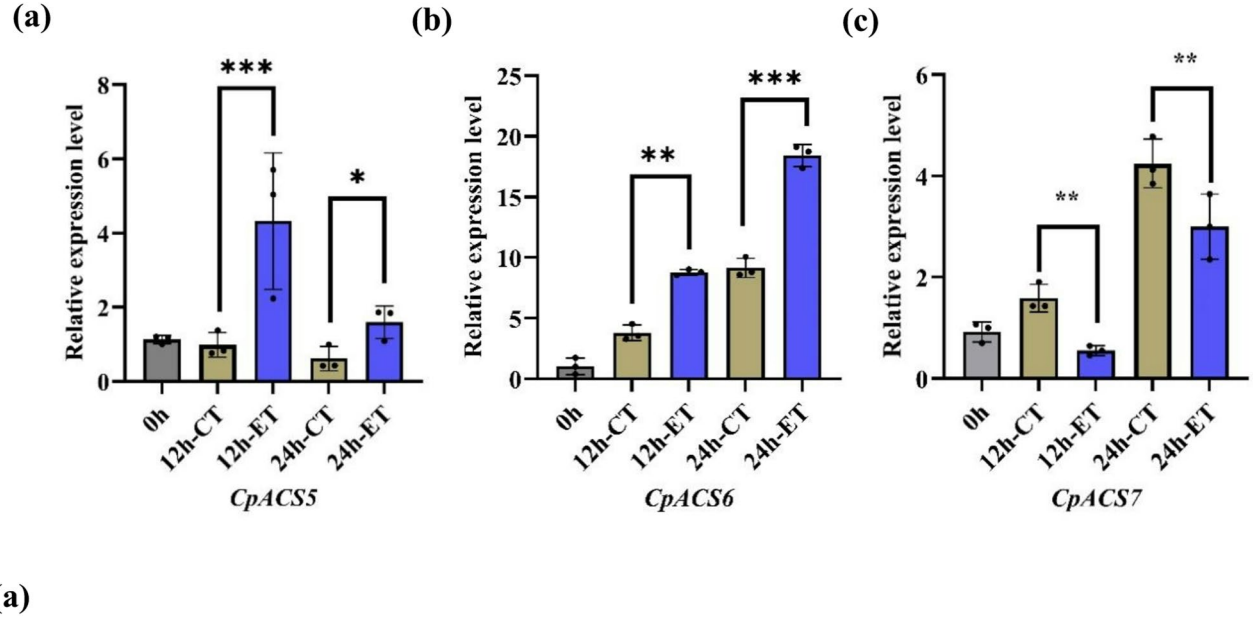


Fig. 9 Relative expression of **(a)***CpACS5*, **(b)***CpACS6*, and **(c)***CpACS7* exposed to control (CT) (0 h, 12 h, and 24 h) and Ethylene-Treatment (ET) at 12 h and 24 h. Transcript levels were measured by RT-qPCR, normalized and are presented as $2^{-\Delta\Delta Ct}$ values relative to the control. Bars show means ± SE of three biological replicates. (Significance level of p-value: $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ ***; Student's t-test)

and provided insights into fruit ripening and ethylene biosynthesis and expression.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-026-09368-y.

Supplementary Material 1.

Authors' contributions

Conceptualization: ** MM, HGN, AA, MTA. **Methodology: ** MA, NS, ZA, MA, IU, HA, SU **. Formal Analysis: ** MAH, WL, MTA. Data Curation **: MA, NS, ZA. **Writing—Original Draft Preparation: ** MA, NS, ZA, MA, IU, HA, SU, MM, HGN, AA, MAH, WL. **Writing—Review and Editing: ** FSB, MAK, MTA. Funding acquisition: MTA. **Supervision: ** MTA. All authors have read and agreed to the submitted version of the manuscript.

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Data availability

The datasets used and/or analyzed in the current study, available in the manuscript, and the genomic sequences of Carica papaya for the current study were retrieved from NCBI (https://www.ncbi.nlm.nih.gov/datasets/genome/GCF/_000150535.2/). The transcriptomic dataset retrieved and analyzed in the current study is available in the NCBI Gene Expression Omnibus (GEO) repository under accession GSE128577 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE128577). This dataset was aimed at identifying the key ACS genes involved in ethylene-mediated fruit ripening at different time points.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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