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Reference gene validation and differential expression of *Psy* and *ACCase* in oil palm with contrasting oil and carotene profiles at different ripening stages

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Abstract

Reliable normalization and accurate quantification are essential for gene expression studies in oil palm mesocarp. This study assessed three candidate reference genes (*Actin, GAPDH*, and *EF1α*) for stability using four algorithms (ΔCt, BestKeeper, NormFinder, and GeNorm). *Actin* was consistently identified as the most stable single housekeeping gene (HKG) (stability value 0.021), while the combination of *GAPDH* and *EF1α* provided the most stable normalization pair (stability value 0.017), supporting their use as internal controls for normalization. Normalized expression analysis of two key biosynthetic genes, *acetyl-CoA carboxylase* (*ACCase*) and *phytoene synthase* (*Psy*), was conducted in contrasting phenotypes differing in oil yield and carotene content. The high-yielding, high-carotene genotype displayed lower ΔCt values for both *ACCase* (4.67 vs. 6.19) and *Psy* (4.75 vs. 5.26), corresponding to 2.86-fold and 1.42-fold higher expression, respectively. These expression differences aligned closely with phenotypic outcomes, as the superior traits achieved a 40.8% increase in oil yield and a 155.3% increase in carotene content. Expression patterns showed stage-specific specialization: *ACCase* was upregulated during the unripe stage, enhancing fatty acid biosynthesis, while *Psy* was most differentially expressed during ripening, coinciding with carotenoid accumulation. Together, these findings suggest that transcript abundance of oil and carotenoid pathways may be coordinated in elite oil palm and provide a framework for linking gene expression with metabolic output, which may inform future efforts to improve oil yield and quality.

[Keywords: carotenoid biosynthesis, *Elaeis guineensis*, housekeeping genes]

Introduction

Oil palm (*Elaeis guineensis* Jacq.) is the most productive oil-bearing crop globally, dominating the vegetable oil market due to its unparalleled yield per hectare (Apriyanto et al., 2022). The economic value of the fruit mesocarp is derived from two primary components: triacylglycerols (palm oil) and carotenoids (Morcillo et al., 2021). The biosynthesis of these compounds is a complex, developmentally regulated process, with ripening marked by a dramatic metabolic shift from oil accumulation to pigment production, visually signaled by a color change from black/purple to deep red/orange (Tranbarger et al., 2011; Lieb et al., 2017; Puteh et al., 2022; Antoniassi et al., 2024).

The genetic regulation of these traits is a major focus for breeding programs. The enzyme acetyl-CoA carboxylase (ACCase) catalyzes the first committed step in de novo fatty acid biosynthesis, serving as a critical flux-controlling point for oil production (Budiani, 2014; Afifi et al., 2025). Similarly, phytoene synthase (Psy) acts as the pivotal rate-limiting enzyme at the entry point of the carotenoid pathway, governing the synthesis of beta-carotene (Zhou et al., 2022; Zhang et al., 2025). Precise quantification of the expression of these genes via quantitative real-time PCR (qPCR) is therefore fundamental to understanding the molecular mechanisms underlying trait variation. However, the accuracy of qPCR is profoundly dependent on rigorous preliminary validation. This includes confirming RNA integrity and purity, establishing primer specificity through standard PCR and melt curve analysis, and determining amplification efficiency for each primer set (Grätz et al., 2022; Chen et al., 2023). Without this foundation, gene expression data can be misleading.

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A critical, yet often overlooked, step is the validation of stable reference genes for normalization. The stability of these genes is not universal; it can vary significantly across experimental conditions, tissues, developmental stages, and, crucially, genotypes. The use of an inappropriate reference gene is a major source of error in qPCR studies (Chan et al., 2014; Yeap et al., 2014; Afiq et al., 2019; Masura et al., 2022). While several studies have validated reference genes in oil palm under various abiotic stresses (Kwan et al., 2016; Zuhar et al., 2021), a significant gap still exists: a comprehensive validation for comparing genotypes with contrasting oil and carotene content across the critical transition of fruit ripening.

The present study aimed to bridge this gap by providing a complete molecular toolkit for reliable gene expression analysis in oil palm mesocarp. The study began with the confirmation of high-quality RNA templates. Subsequently, primer pairs for key genes were designed and validated, specificity was confirmed via endpoint PCR and melt curve analysis, and precise PCR efficiencies were calculated. The most stable reference genes under these specific experimental conditions were then systematically identified using a comprehensive suite of algorithms: the comparative ΔCt method, BestKeeper, geNorm, and NormFinder, to achieve a consensus on the optimal genes for normalization.

Finally, using these validated reference genes, the expression patterns of *ACCase* and *Psy* were characterized across two phenotypic groups with contrasting oil and carotene content in both unripe and ripe fruits. Gene expression data were integrated with oil yield and carotene content measurements. These findings provide an essential methodological foundation for accurate gene expression studies in oil palm and offer novel insights into the transcriptional regulation of key economic traits, revealing the molecular basis underlying superior yield and quality.

Material and Methods

Plant material and sample preparation

Oil palm (*Elaeis guineensis* Jacq.) Tenera clones, derived from a Dura × Pisifera (D×P) cross of the La Mé population, were established in 2010 at the Ciomas Research Farm, Indonesian Oil Palm Research Institute (IOPRI), Bogor, Indonesia. The trees were grouped according to two distinct phenotypes: high oil–high carotene and low oil–low carotene, based on documented consistent oil yield and carotene content over at least two harvest seasons. From each group, two representative trees were selected for mesocarp tissue collection. Individual fruitlets were randomly sampled from each tree at two developmental stages: unripe (black/purple exocarp, ~90 DAA) and ripe (deep red/orange exocarp), following Forero et al. (2018). Fresh mesocarp tissue was immediately frozen in liquid nitrogen and stored until RNA extraction.

RNA extraction, quantification, and quality assessment

Total RNA was isolated from ~100 mg of frozen mesocarp powder using the hot borate method (Chang et al., 1993). RNA concentration and purity were assessed using a NanoDrop™ spectrophotometer, with an acceptable purity ratio defined as A260/A280 between 1.8 and 2.1 and A260/A230 > 2.0 (Esteva-Socias et al., 2019). RNA integrity was evaluated by electrophoresis with 2X RNA Loading Dye (Thermo Scientific) on a 1.5% agarose gel stained with FloroSafe DNA Stain (1st BASE Biochemicals), confirming intact 28S and 18S rRNA bands.

Primer design and validation

Primers for three candidate reference genes (*Actin*, *Elongation Factor 1-alpha* [*EF1α*], and *Glyceraldehyde-3-phosphate dehydrogenase* [*GAPDH*]) were obtained from published studies. Primers for the target genes (*ACCase* and *Psy*) were designed using Primer3Plus based on *E. guineensis* sequences retrieved from NCBI and the GenomSawit database (Table 1). Primer specificity was confirmed by endpoint PCR with MyTaq™ HS Red Mix (Bioline) on a T100 Thermal Cycler (Bio-Rad), followed by electrophoresis on a 2% agarose gel (Promega). Single amplicons of the expected size were observed for all primer pairs.

cDNA synthesis and quantitative real-time PCR (qPCR)

First-strand cDNA was synthesized from ~1 µg of total RNA using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo Co., Ltd.), following the manufacturer's protocol. The resulting cDNA was diluted and used as a template for qPCR. qPCR reactions (10 µL) contained 5 µL SensiFAST™ SYBR® Hi-ROX Mix (Bioline), 3.2 µL nuclease-free water, 1 µL cDNA, and 0.4 µL of each primer. Amplification was performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems) using the following program: 95°C for 10 min; 40 cycles of 95°C for 15 s and 60°C for 60 s. Melting curve analysis was carried out immediately after amplification (95°C for 15 s, 60°C for 60 s, followed by a gradual increase to 95°C at 0.3°C/s) to confirm primer specificity. All qPCR reactions were performed in technical triplicates, and the mean Ct values were used for analysis. Results are presented as mean ± standard deviation

Table 1. Primer sequences used in this study

Gene ID	Forward sequence	Reverse sequence	Reference/Accession ID
Actin	CCCACCTGAACGGAAATACA	CGGATGGCACCTCAGTCTTA	Putranto et al., 2016
EF1α	GGTGTGAAGCAGATGATTTGC	CCTGGATCATGTCAAGAGCC	Aberlenc-Bertossi et al., 2008
GAPDH	ACTGCTACTCAGAAGACTGTT GATG	TGCTGCTAGGAATGATGTTA AAGCT	Rasid et al., 2008
ACCase	CCTGCTCGCCTCTTGGGTTAG	TGCCTCGGAAAGCTTATCTT GTG	EG_Chr7_p5.00_sc00077_p003 3; XR_833131.3; XM_010928874.3; XM_010928873.3; XM_010928872.3; NM_001303559.1
Psy	CGGATCTAGTGAGTTGTTCTTC GA	GGAATTATGCTGTATATACCA TATCCGCAAT	p5_sc00017_p0037_1; XM_010941957.4; Syuhada et al., 2021

from two biological replicates per phenotypic group. Due to the limited sample size, formal statistical testing was not performed; however, descriptive statistics (mean, SD, fold-change ranges) are provided to indicate variability within and between groups.

PCR efficiency and reference gene validation

PCR efficiency was determined using a four-point serial dilution of pooled cDNA (1:5, 1:10, 1:20, and 1:40). Standard curves were generated by plotting Ct values against the log10 of the dilution factors. Amplification efficiency (E) was calculated from the slope using the following formula (Plotka et al., 2017):

$$E = \left(10^{-\frac{1}{slope}} - 1\right) \times 100\% \quad (1)$$

Only primer pairs with efficiencies of 90–110% (slopes between –3.1 and –3.6) were included in subsequent analyses. Reference gene stability was evaluated across all samples using the ΔCt method (Silver et al., 2006), BestKeeper (Pfaffl et al., 2004), geNorm (Vandesompele et al., 2002), and NormFinder (Andersen et al., 2004). A comprehensive ranking was then generated to identify the most suitable reference gene(s) for normalization.

Gene expression analysis

The relative expression levels of the target genes (*ACCase* and *Psy*) were calculated using the comparative ΔΔCt method and fold change (FC). A separate analysis was conducted for the unripe and ripe fruit to assess the effect of phenotypic trait group (high vs. low oil and carotene content) within each ripening stage. The low-oil-yield, low-carotene fruit sample was used as the calibrator for each stage. This approach directly tests for expression differences between the high- and low-yield phenotypic groups at the same point in development. Data were analyzed using StepOne Software v2.3. For each ripening stage, data are presented as mean FC in the high-yield plants relative to the low-yield calibrator from two biological replicate trees per phenotypic group.

Oil and carotene quantification

Oil content was determined from mesocarp tissue following the extraction method of Sinta et al. (2025) and expressed as a percentage of dry weight. Total carotene content was quantified spectrophotometrically according to the Indonesian National Standard (SNI 7709:2019) and expressed as parts per million (ppm) of crude palm oil (CPO).

Results and Discussion

RNA quality and primer validation

High-quality RNA is a prerequisite for reliable qPCR analysis, particularly in oil palm mesocarp, which contains high polysaccharides, lipids, and polyphenolics that can interfere with amplification. The RNA extracted from both low-oil/low-carotene and high-oil/high-carotene fruits showed intact rRNA bands and A260/280 ratios within the acceptable range, indicating purity and suitability for downstream applications. Detailed RNA concentration, purity values, and representative gel images showing intact 28S and 18S rRNA are provided in Supplementary Figure S1 and Table S1. Standard endpoint PCR for all candidate reference genes (*Actin*, *EF1a*, *GAPDH*) and target genes (*ACCase*, *Psy*) yielded single, specific amplicons of the expected size, confirming primer specificity before qPCR analysis (Supplementary Figure S2). Melt curve analysis further confirmed single specific products for all amplifications (Supplementary Figure S3).

PCR efficiency and assay optimization

A dilution series (5×, 10×, 20×, and 40×) of pooled cDNA samples (unripe and ripe) was used to evaluate the optimal template concentration for qPCR. Standard curves across a 40-fold dilution series demonstrated excellent linearity, with amplification efficiencies within the acceptable 90–110% range (Shakeri, 2022): *Actin* (108.6%), *GAPDH* (104.7%), *EF1α* (90.2%), *ACCase* (109.4%), and *Psy* (97.6%) (Figure 1).

Notably, amplification of *Psy* failed at the 40x dilution. While high-abundance reference genes (*Actin*, *GAPDH*, *EF1α*) and the *ACCase* target gene amplified consistently across all dilutions, the inability to detect *Psy* at the highest dilution factor reflects its inherently lower expression level in the mesocarp tissue. At the 5× dilution, *Psy* exhibited a higher Ct value (28.4) compared to reference genes (*Actin*: 25.5; *GAPDH*: 27.7; *EF1α*: 24.7) and *ACCase* (25.7), confirming its lower abundance in the pooled sample.

However, it should be noted that qPCR amplification efficiency and sensitivity can also be influenced by primer-specific factors such as GC content, secondary structure, and amplicon characteristics (Bustin et al., 2009). While our *Psy* primer pair demonstrated excellent efficiency (97.6%) within the acceptable range and produced specific, single-peak melt curves (Supplementary Figure S3), these technical factors could theoretically contribute to reduced amplification at high dilutions.

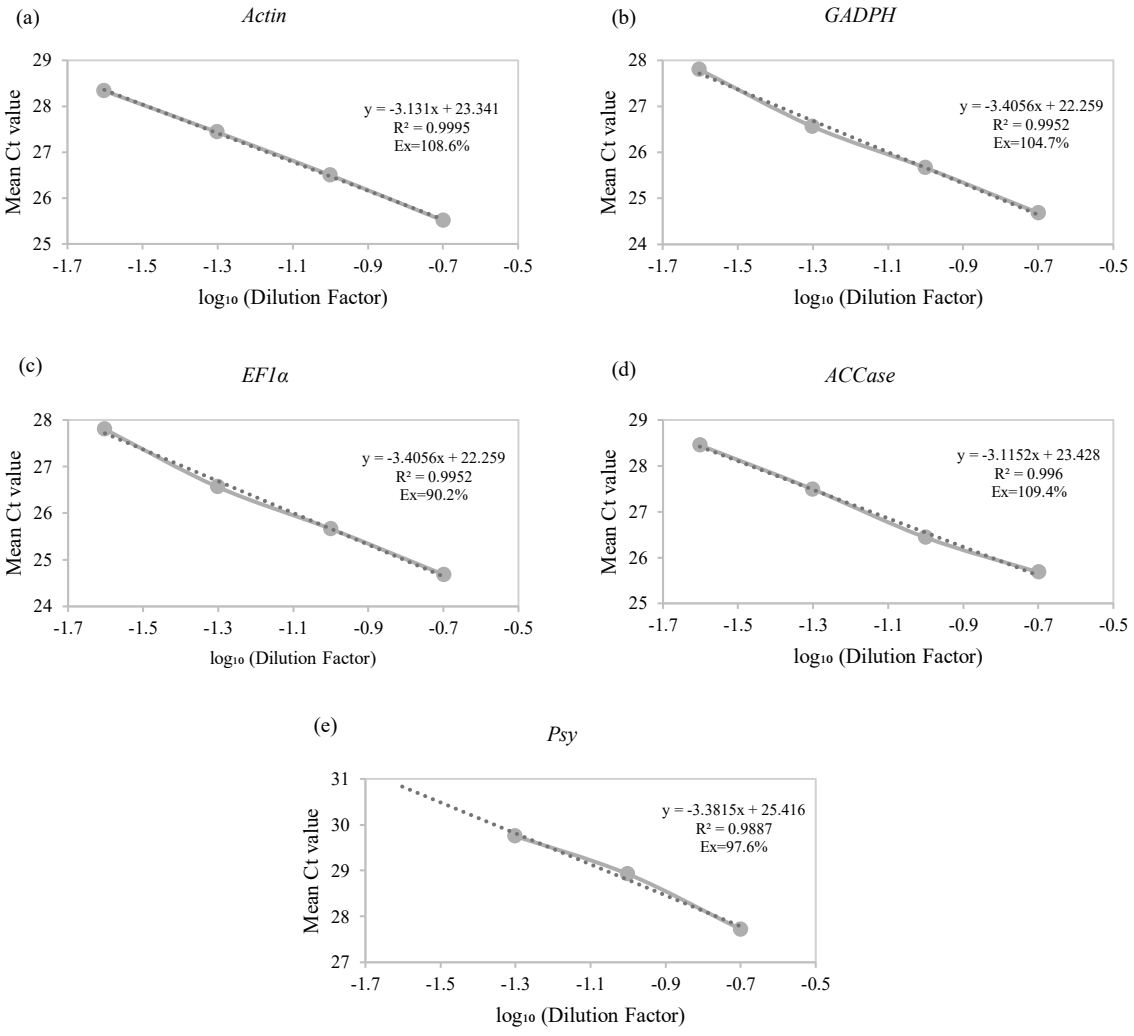

Figure 1. Standard curves for five primer pairs: (a) *Actin*, (b) *GAPDH*, (c) *EF1α*, (d) *ACCase*, and (e) *Psy*, generated from a 40-fold dilution series of cDNA (5×, 10×, 20×, 40×)

Nevertheless, this pattern aligns with the metabolic prioritization of oil biosynthesis over carotenoid accumulation in oil palm, particularly during early fruit development (Ting et al., 2020; Wong et al., 2014). Such failures at high dilutions have been noted in low-expression genes in other plant systems (Moebes et al., 2022). This observation is also consistent with the biological role of *Psy* as a regulatory gene with tissue- and stage-specific expression patterns (Tranbarger et al., 2011; Wan Nur Syuhada et al., 2021). The successful amplification of *Psy* at lower dilutions (5×–20×) and its high assay efficiency (97.6%) confirm that the data obtained are reliable for comparative expression analysis. Consequently, 5× dilutions were used for subsequent assays, ensuring optimal template input for both high- and low-expression genes.

Comprehensive stability analysis of reference genes

The stability of three candidate reference genes (*Actin, GAPDH, EF1α*) was assessed using ΔCt, BestKeeper, NormFinder, and GeNorm (Figure 2). While individual algorithms produced slightly different rankings, *Actin* and *GAPDH* consistently ranked as the most stable genes. GeNorm analysis further supported the combination use of *Actin* and *GAPDH*. Comprehensive ranking across all algorithms identified *Actin* as the most stable, followed by *GAPDH* and *EF1α*. These findings suggest that for experimental designs comparing samples across developmental stages and phenotypic groups, *Actin* alone is a reliable single reference gene (stability value = 0.021 in NormFinder with grouping). However, for studies prioritizing maximum normalization accuracy, the combination of *GAPDH* and *EF1α* (stability value = 0.017) represents a more robust strategy, consistent with MIQE guidelines recommending the use of multiple reference genes (Bustin et al., 2009). *EF1α* showed lower stability than *Actin* and *GAPDH* when biological groupings were considered, underscoring the importance of validating reference gene stability under specific experimental conditions rather than relying on published data from different tissues or treatments.

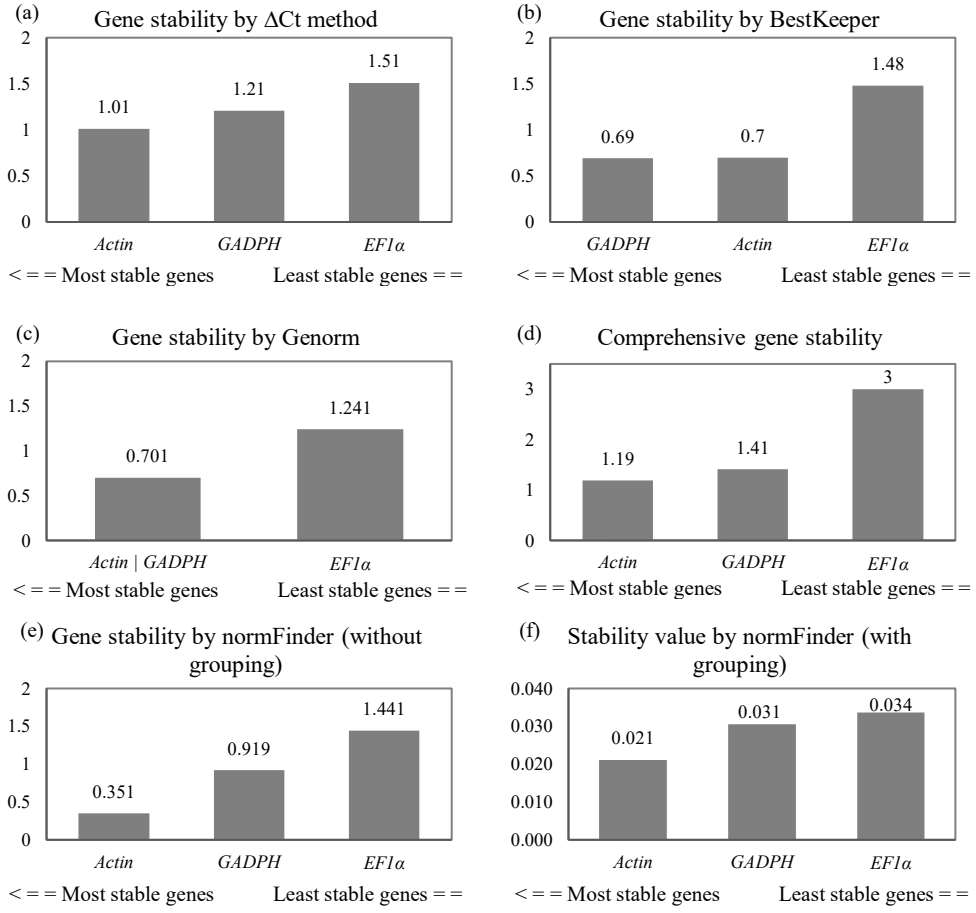

Figure 2. Stability ranking of three candidate reference genes (*Actin*, *GAPDH*, *EF1α*) in oil palm mesocarp evaluated using four algorithms: (a) ΔCt, (b) BestKeeper, (c) GeNorm, comprehensive analysis (d), NormFinder with (e) and without grouping (f). Lower stability values indicate higher expression stability.

Further examination using NormFinder revealed the impact of experimental design on stability rankings. Without grouping, *Actin* was ranked as the most stable gene, followed by *GAPDH*, with *EF1α* as the least stable. However, when grouping parameters (unripe vs. ripe; high vs. low oil–carotene) were introduced, *Actin* remained the most stable single reference (stability = 0.021), while *GAPDH–EF1α* was identified as the best two-gene combination (stability = 0.017). Since lower stability values indicate higher stability, both *Actin* alone and the *GAPDH–EF1α* pair were suitable for normalization in this study. These results further demonstrate that reference gene stability is context-dependent (Andersen et al., 2004; Expósito-Rodríguez et al., 2008). This multi-algorithm, multi-condition validation follows MIQE guidelines (Bustin et al., 2009) and minimizes bias that could arise from relying on any single statistical method (Aithal et al., 2015; Bocian et al., 2025; Robinson et al., 2025).

Expression profiles of ACCase and Psy in contrasting phenotypes

The normalized relative expression profiles of *ACCase* and *Psy* were compared between the two phenotypic groups at both unripe and ripe stages (Figure 3). Normalization with different housekeeping genes (HKGs) produced variation in fold-change (FC) values. When *Actin* was used as the reference gene, *ACCase* expression showed moderate FC (2.86 at the unripe stage and 2.59 at the ripe stage), while normalization with the combination of *GAPDH* and *EF1α* resulted in much higher FC values (35.30 at unripe and 3.72 at ripe). A similar pattern was observed for *Psy*, where *GAPDH+EF1α* normalization yielded FC values of 15.37 (unripe) and 2.05 (ripe), compared with 1.25 and 1.42, respectively, when normalized with *Actin*.

Expression analyses were performed for each phenotypic group and developmental stage, using two biological replicate trees, with multiple fruitlets pooled per tree. Data are presented as mean fold-change ± standard deviation (Supplementary Table S2). At the unripe stage, *ACCase* expression in the high-yield phenotype was 2.86 ± 0.31-fold (*Actin* normalization) or 35.30 ± 4.21-fold (*GAPDH+EF1α* normalization) relative to the low-yield calibrator. For *Psy* at the unripe stage, fold-change values were 1.25 ± 0.18 (*Actin*) and 15.37 ± 2.05 (*GAPDH+EF1α*). At the ripe stage, variability was lower across all measurements (standard deviations ranging from 0.09 to 0.42), reflecting more consistent expression patterns during later development.

The choice of normalization strategy substantially affected the magnitude of fold-change values, representing a 12-fold difference for *ACCase* at the unripe stage and a 12.3-fold difference for *Psy* at the unripe stage. Despite these dramatic quantitative differences, the biological conclusion remained consistent across all normalization approaches: both *ACCase* and *Psy* were more highly expressed in the high-oil/high-carotene phenotype at both developmental stages. This consistency demonstrates that while absolute FC values are highly sensitive to reference gene selection, the directional conclusion (higher/lower expression) is robust when stable reference genes are used. As reported in previous studies, normalization with less stable or variably expressed HKGs can lead to inflated or underestimated FC values (Vandesompele et al., 2002; Kozera & Rapacz, 2013; Batool et al., 2024), underlining the importance of validating HKG stability prior to expression analysis and of prioritizing consistent biological patterns across multiple normalization strategies.

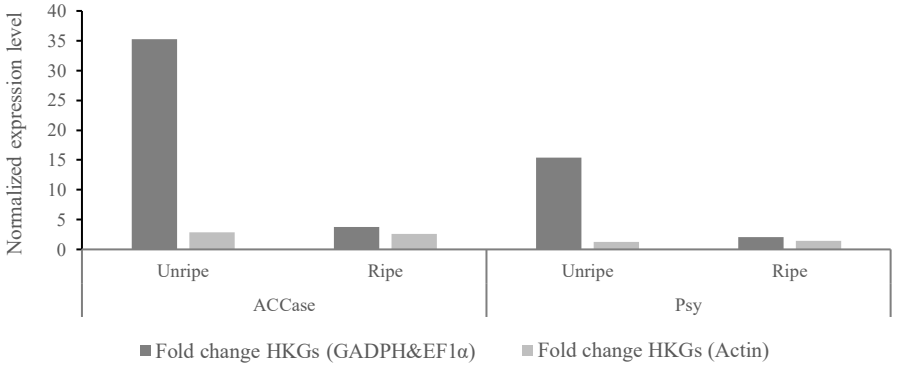

Figure 3. Normalized relative expression of *ACCase* and *Psy* in oil palm mesocarp of high- and low-oil/carotene phenotypes at unripe and ripe stages. Expression was normalized using (■) *GAPDH + EF1α* and (■) *Actin* as reference genes. Bars represent fold change (FC) values, confirming higher expression of both genes in the high-oil/high-carotene phenotype across stages, with *ACCase* showing stronger upregulation than *Psy*. All values are expressed relative to the low-yield, low-carotene calibrator at the corresponding stage.

Table 2. Comparison of ΔCt values of *ACCase* and *Psy* at their highest expression stage with corresponding oil yield (%) and carotene content (ppm) in contrasting oil palm phenotypes.

Sample ID	ΔCt *ACCase*	ΔCt *Psy*	Oil yield (%)	Carotene content (ppm)
Low yield–low carotene	6.19	5.26	21.85	491.15
High yield–high carotene	4.67	4.75	30.77	1254.21

Notably, *ACCase* exhibited stronger upregulation than Psy at both developmental stages. At the unripe stage, *ACCase* FC reached 35.30 (*GAPDH+EF1α* normalization) compared to 15.37 for *Psy*; with *Actin* normalization, the values were 2.86 and 1.25, respectively. A similar trend was observed at the ripe stage, where *ACCase* remained higher than *Psy* under both normalization strategies (3.72 vs. 2.05 with *GAPDH+EF1α*; 2.59 vs. 1.42 with *Actin*). This consistent trend suggests that fatty acid biosynthesis (via *ACCase*) is showing higher transcript abundance than carotenoid biosynthesis (*Psy*), particularly during early development (Tranbarger et al., 2011). These findings align with the metabolic prioritization of oil accumulation in oil palm mesocarp, where oil represents the major storage product (Ting et al., 2020).

To strengthen the biological interpretation, ΔCt values of *ACCase* and *Psy* at their most differentially expressed stage were compared with oil yield and carotene content (Table 2). As expected, the high-oil/high-carotene sample exhibited lower *ΔCt* values for both *ACCase* (4.67) and *Psy* (4.75), indicating higher transcript abundance compared with the low-oil/low-carotene sample (ΔCt 6.19 for *ACCase* and 5.26 for *Psy*). These differences in gene expression paralleled the phenotypic profiles, where oil yield increased from 21.85% in the low-yield sample to 30.77% in the high-yield sample, and carotene content rose dramatically from 491.15 ppm to 1,254.21 ppm. The consistent trend between lower ΔCt (higher expression) and improved oil/carotene traits is consistent with the proposed functional roles of *ACCase* in fatty acid biosynthesis and *Psy* in carotenoid biosynthesis, as also reported in other oil crops and carotenoid-rich fruits (Salas et al., 2006; Welsch et al., 2010).

However, several limitations of the current dataset should be acknowledged. Although the limited biological replication (n=2 per phenotype) prevented formal correlation testing, the direct comparison suggests that transcript abundance of these genes contributes to oil and carotene variation in oil palm. It is also acknowledged that, with only one genotype representing each phenotypic group, the observed expression differences could reflect genotype-specific effects rather than phenotype-associated regulation. Nonetheless, the consistency of these differences with the expected functional roles of *ACCase* and *Psy*, and their alignment with stage-specific metabolic priorities (oil accumulation during the unripe stage, carotenoid accumulation during ripening), supports the biological relevance of the findings.

These expression differences are correlative, not causal. While they are consistent with the proposed roles of *ACCase* in fatty acid biosynthesis and *Psy* in carotenogenesis, confirming a causal relationship would require additional evidence, such as enzyme activity measurements, metabolite flux analysis, or functional validation studies. The present findings should therefore be interpreted as correlative associations that provide a foundation for future mechanistic investigations.

Despite these limitations, this study addresses a critical research gap by providing validated reference genes and optimized qPCR workflows for oil palm mesocarp, alongside a dual-pathway analysis of oil and carotenoid biosynthesis in divergent oil palm phenotypes. The stronger upregulation of *ACCase* compared with *Psy* underscores the dominant role of fatty acid synthesis during mesocarp development, while the stage-specific expression of *Psy* highlights its contribution to ripening-associated carotenoid accumulation. These results suggest that transcript abundance of these genes may contribute to oil yield and carotenoid variation. Future studies with larger populations and diverse genetic backgrounds will be necessary to validate these associations, quantify statistical correlations, and if validated, potentially integrate *ACCase* and *Psy* into marker-assisted breeding pipelines. Beyond breeding, understanding the regulation of fatty acid and carotenoid biosynthesis may also support metabolic engineering strategies to enhance oil quality and nutritional value.

Conclusion

This study establishes a robust framework for gene expression analysis in oil palm by identifying *Actin*, *GAPDH*, and *EF1α* as stable reference genes. The higher expression of *ACCase* and *Psy* in high-performing plants—with distinct temporal expression peaks aligning with oil and carotenoid accumulation phases—is consistent with the enhanced oil yield and carotene content observed phenotypically. These findings provide a molecular foundation for understanding the genetic regulation of these economically important traits in oil palm and offer a basis for future mechanistic and breeding studies.

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