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LCYB GENE EXPRESSION AND MORPHOPHYSIOLOGICAL TRAITS OF *MUSA ACUMINATA* CULTIVARS

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SUMMARY

Banana (*Musa acuminata*) is a tropical fruit plant vigorously growing in Southeast Asia, particularly Indonesia. Despite its prevalence in different *Musa acuminata* cultivars, the *LCYB* gene expression and morphological and physiological traits remain unexplored. Therefore, the presented study sought to examine the manifestation of the *LCYB* gene and analyze various morphophysiological features. The promising research focused on probing the five cultivars of banana, specifically *M. acuminata* var. breviformis, *M. acuminata* var. tomentosa, *M. acuminata* var. malaccensis (Ridl.), *M. acuminata* var. microcarpa (Becc.), and *M. acuminata* var. rutilifers. The morphological observations revealed these cultivars have no prominent distinctions in stem size, fruit characteristics, banana blossom, and leaf traits. Remarkably, cultivar Rutilifers showed the highest carotenoid and total chlorophyll content levels. Inversely, the banana cultivars Breviformis and Microcarpa exhibited comparatively lower contents of physiological parameters than the other cultivars. Likewise, by analyzing the *LCYB* gene expression, it was evident that the cultivar Tomentosa displayed the superior level, followed by the cultivar Malaccensis. Conversely, the cultivar Microcarpa exhibited the lowest *LCYB* gene expression. To summarize the results, the applicable study enunciated a significant relationship between the *LCYB* gene expression and the chlorophyll and carotenoid contents across the various banana cultivars.

Keywords: Banana (*Musa acuminata*) cultivars, chlorophyll and carotenoid content, *LCYB* gene expression, *Musa acuminata*, Tomentosa

Key findings: *LCYB* gene expression and morphophysiological traits significantly differed among *Musa acuminata* Indonesian local cultivars.

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INTRODUCTION

In Southeast Asia, particularly Indonesia, the banana (*Musa acuminata*) grows abundantly and primarily comprises two species, *M. acuminata* and *M. balbisiana*. Genome A exists in *M. acuminata,* while genome B confirms in *M. balbisiana* species. Worldwide, rapid progress can be visible in the genetic research of bananas. However, there is still a lack of studies focusing on local Indonesian banana cultivars that connect important physiological traits like chlorophyll and carotenoid contents with lycopene β-Cyclase (*LCYB*) gene expression.

Nevertheless, these two aspects surfaced as closely interrelated in the endogenous β-carotene biosynthesis of bananas (Ashraf *et al.*, 2015). Therefore, it is imperative to investigate the relationship between these two traits in several Indonesian local banana cultivars, such as *M. acuminata* var. breviformis, *M. acuminata* var. tomentosa, *M. acuminata* var. malaccensis (Ridl.), *M. acuminata* var. microcarpa (Becc.), and *M. acuminata* var. Rutilifers. There exists an association between *LCYB* gene expression and chlorophyll and carotenoid contents in crop plants (Welsch *et al.,* 2000).

In tomato (*Solanum lycopersicum*), the transgenic plants with varied levels of *DcLCYB1* displayed superior or inferior levels of chlorophyll, carotenoids, and β-carotene in the leaves and storage roots, respectively (Pecker *et al.,* 1996). As anticipated, the chlorophyll content also rose in the transgenic lines (Joyard *et al.,* 2009), coordinating regulation in the carotenoid synthesis (Toledo-Ortiz *et al.,* 2010; Stange and Flores, 2011; Moreno *et al.,* 2013). Additionally, in bananas, the genetic studies of the *LCYB* gene have, so far, been central to in silico characterization in the DH-Pahang accession (*M. acuminata*, A genome) and the DH-PKW accession (*M. balbisiana*, B genome) (Wiprayoga *et al.,* 2023). In several local *M. acuminata* cultivars, the research on *LCYB* gene expression using real-time PCR (qRT-PCR) has no previous reports.

Through gene expression analysis at the transcription stage, determining and comparing the mRNA's varied levels can occur. The mRNA level, specifically *LCYB*, in various banana genotypes under maximum favorable growth conditions is fundamental for scrutiny, as gene regulation at the expression level affects the end outcome of a molecule. A high level of *LCYB* expression can influence the translation level of the LCYB enzyme and elevate β-carotenoid content (Giorio *et al.,* 2022). Carotenoid compounds, apart from being involved in β-carotene biosynthesis, are also vital in photosynthesis and light harvesting (Diretto *et al.,* 2007). Previously, the relationship between chlorophyll content and *LCYB* gene expression in several *Musa acuminata* cultivars has no existing exploration. Therefore, this study aimed to examine *LCYB* gene expression in the five banana (*M. acuminata*) cultivars and analyze morphophysiological traits, such as chlorophyll-carotenoid content.

MATERIALS AND METHODS

The plant material used in this research consisted of five banana (*Musa acuminata*) cultivars procured from the collection of the Indonesia Research Center for Genetic Engineering (IRCGE), National Research and Innovation Agency (NRIA), Indonesia (6°33'S 106°43'E), i.e., *M. acuminata* var. breviformis, *M. acuminata* var. tomentosa, *M. acuminata* var. malaccensis (Ridl.), *M. acuminata* var. microcarpa (Becc.), and *M. acuminata* var. rutilifers. Fresh banana leaves came from the experimental garden of NRIA.

Chlorophyll-carotenoid measurements

Chlorophyll content measurement used 100% (v/v) acetone (Sigma, USA), according to Fendiyanto *et al.* (2019a, b). Five banana cultivars' leaves (0.75 g) sustained grinding using the liquid N_2 and then extracted using 10 ml of acetone (100%, v/v, Sigma, USA).

Additionally, the extract mixture incurred complete centrifugation at 4,200 rpm/min. The supernatant's subsequent absorbance measurement utilized the Spectrophotometer at 470, 646, and 662 nm. Chlorophyll a and b, carotenoids, and total chlorophyll quantifying employed the following equations:

$$Chl\text{-}a = 11.75\ A662 - 2.350\ A646$$

$$Chl\text{-}b = 18.61\ A646 - 3.960\ A662$$

$$Car = 1000\ A470 - 2.270\ Chl\text{-}a - 81.4\ Chl\text{-}b/227$$

$$Tot\text{-}Chl = 20.2\ (A645) + 8.02\ (A663) \times (V/[1000 \times W])$$

Legends
Chl-a: Chlorophyll-a (mg/g FW)
Chl-b: Chlorophyll-b (mg/g FW)
Car: Carotenoid (mg/g FW)
Tot-Chl: Total Chlorophyll Content (mg/g FW)
V: Volume (10 mL)
W: Weight (100 mg)

Morphological analysis

In the presented study for morphological analysis, data recording ensued on plant height, stem diameter and length, average leaf area, leaf (midrib distance, width, stretch, and margin length), banana blossom (extent, margin length, and scope), inflorescence length, and fruit length after 10 days of banana blossom emergence. All five banana cultivars attained direct measuring using bioimaging with the Image-J software (Fendiyanto *et al.,* 2019a, b).

RNA Isolation

The process of total RNA extraction involved Genezol reagents (ATP Biotech, Taiwan) as outlined in the procedure by Fendiyanto *et al.* (2021). Leaf samples of five *M. acuminata* cultivars underwent the RNA isolation process. The leaves, weighing from 0.5 to 1 g, bore pulverization with liquid nitrogen, with the resulting homogenate transferred to 2 ml tubes. Subsequently, adding 1 mL of Genezol to the homogenate continued to mix vigorously using a vortex mixer. The mixture, allowed to stand for 5 min at room temperature, attained the addition of 200 µL of chloroform (Sigma, USA), inverting the solution gently 15 times. The solution proceeded to incubation for 3 min and, subsequently, centrifuged at 11,000 rpm for 15 min at 4 °C (Satrio *et al.,* 2021, 2023). Transferring the RNA in the aqueous phase to a new tube incurred an additional 500 µL of isopropanol (Merck, USA) to precipitate the RNA. The RNA precipitation step comprised incubating the solution for 10 min, followed by centrifugation at 11,000 rpm for 10 min at 4°C, forming the RNA pellet. Washing the RNA pellet used 1 mL of 75% (v/v) ethanol (Merck, USA) before continuing to dissolve and resuspend in 30 µL of ddH_2O (containing DEPC).

During the RNA isolation process, DNase treatment ensued following the procedure outlined in the RevertAid Reverse Transcription KIT (Thermoscientific, USA). The isolated total RNA's quantification continued by dissolving 2 µL of total RNA in 398 µL of ddH_2O-DEPC 0.01%, determining the concentration with a spectrophotometer (UV-Vis, GeneQuant 1300, USA) at wavelengths of 260 and 280 nm. The assessed purity of the isolated total RNA employed the 260/280 nm ratio. Additionally, RNA integrity's evaluation by electrophoresis transpired, running RNA samples on a 1.5% agarose gel for 60 min using 1x TAE buffer.

cDNA synthesis and *LCYB* primer design

The process of generating cDNA progressed based on the RevertAid First Strand cDNA Synthesis kit protocol from Thermo Scientific, USA. Engaging PCR used *Actin* and *LCYB* primers, according to the procedure by Miftahudin *et al.* (2021), to amplify specific genes.

For primers designed to assess *LCYB* gene expression, conservative regions identified depended on the sequence similarity of *LCYB* between *M. acuminata* (Accession number KP406755.1) and *M. troglodytarum* (Accession number KP406754.1). These primers were crafted using the Primer 3 tool within the Ugene software platform (Okonechnikov *et al.,* 2012; Fendiyanto *et al.,* 2021). The *LCYB* primers employed in this study were: *LCYB-forward*: 5'-AACTCCTCGAGCTTGTTCCA-3' and *LCYB-reverse*: 5'- CCCATCGCTGCAAATCAAGA-3', resulting in an amplification product spanning 460 bp.

Analysis of *LCYB* gene expression

The relative expression analysis of *LCYB* in the five banana cultivars applied real-time PCR (Quant Studio, Applied Biosystems, USA). The Ct values' further quantification to determine the *LCYB* gene expression levels employed the $2^{-\Delta\Delta Ct}$ using the following equations (Satrio *et al.,* 2019; Miftahudin *et al.*, 2021):

$$\Delta Ct = Ct\ LCYB - Ct\ Actin$$

$$\Delta\Delta Ct = \Delta Ct\ LCYB.Var - \Delta Ct\ LCYB.Mic$$

$$LCYB\text{-}exp = 2^{-\Delta\Delta Ct}$$

LCYB-exp: Relative gene expression of LCYB in x cultivar
ΔCt *LCYB.Var*: The intensity of *LCYB* gene results in x cultivar
ΔCt *LCYB.Mic*: The intensity of *LCYB* gene results in Microcarpa cultivar as a control
ΔCt: Relative *LCYB* gene expression based on the housekeeping gene (*Actin*)
x: Cultivar of *M. acuminata*, respectively

Data analysis

The data underwent analysis of variance (ANOVA), and, in cases where significant differences manifested among the data means, a subsequent Welch's T-Test proceeded at a significance level of $\alpha = 0.05$ using the R program (Fendiyanto *et al.,* 2019b). Statistical analysis generation used the R version 3.5.1 program (https://cran.r-project.org; Satrio *et al.,* 2023).

RESULTS AND DISCUSSION

Morphological characters of banana

Based on the morphological traits, it is evident that the morphological appearance of the five banana cultivars showed nonsignificant differences in stem size, fruits, banana heart, and leaves. For morphological traits, such as plant height, stem diameter and length, average leaf area, leaf vein distance, leaf width, and lengths of the leaf, leaf edge, banana blossom, and fruit after 10 days of banana blossom emergence, the banana cultivars revealed nonsignificant variations (Figure 1). Nevertheless, the cultivar Breviformis showed lower values for leaf characteristics than the other four cultivars.

Chlorophyll and carotenoids content

For the chlorophyll test, it is apparent that the banana cultivars exhibited significant differences for chlorophyll a and b, carotenoids, and total chlorophyll content. The chlorophyll test also indicated that the banana cultivar Tomentosa appeared with the highest chlorophyll a content, while the lowest in the cultivar Microcarpa (Figure 2a). Based on the chlorophyll b content, the highest values were notable in the cultivars Rutilifers and Tomentosa, whereas the lowest was in the cultivar Microcarpa (Figure 2b). The banana cultivar Rutilifers showed the highest and significantly different values for total chlorophyll and carotenoid content, and the two cultivars, Breviformis and Microcarpa, exhibited lower and statistically different amounts compared with the other three banana cultivars (Figure 2c-d).

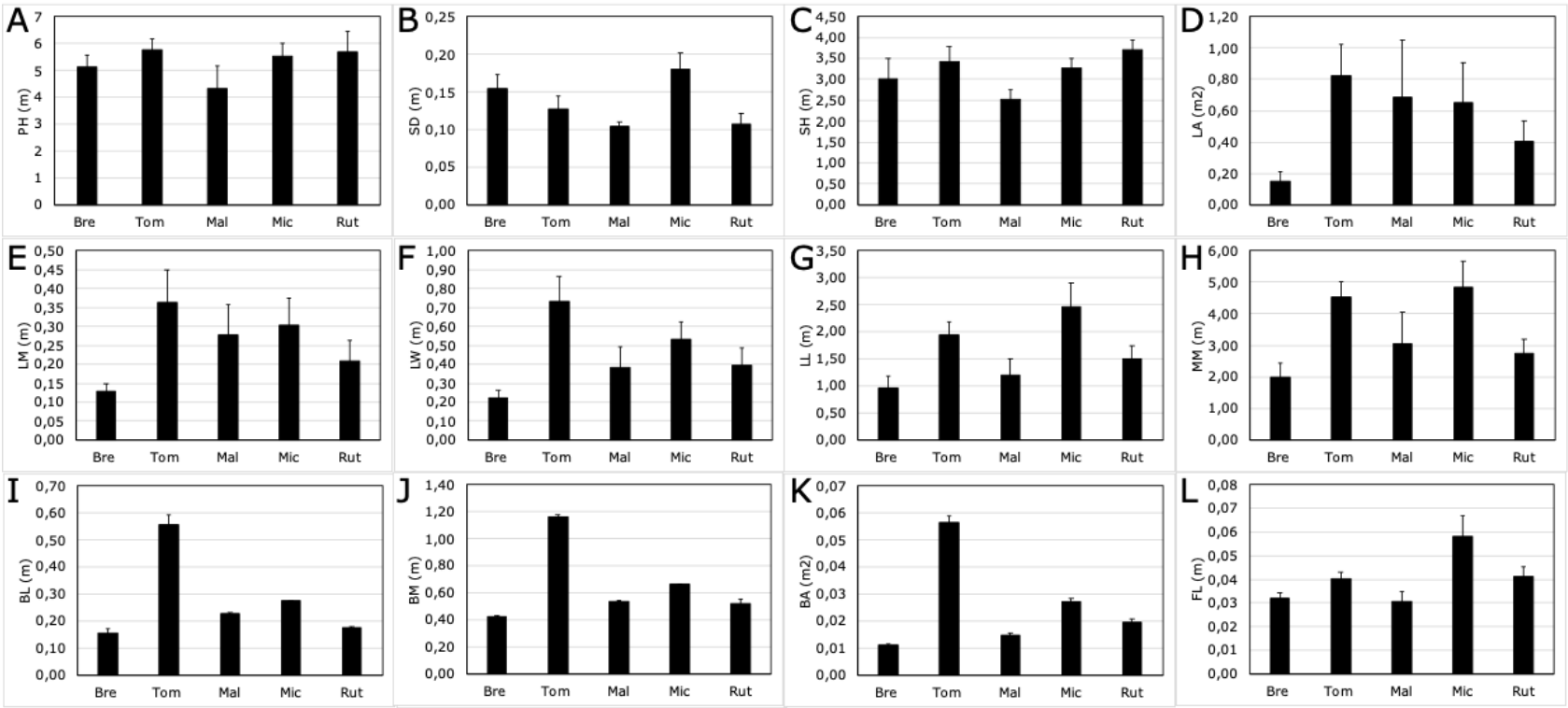

Figure 1. Morphological characters of banana (*Musa acuminata*) cultivars.
Plant Height (PH, A), Stem Diameter (SD, B), Stem Height (SH, C), Leaf Area (LA, D), Leaf Margins (LM, E), Leaf Width (LW, F), Leaf Length (LL, G), Margin Length (MM, H), Banana Blossom Length (BL, I), Banana Blossom Margin (BM, J), Banana Blossom Area (BA, K), and Flower Length (FL, L) are measured in this research. Columns and bars represent the means and SE (n = 5, Three biological and technical replications, respectively). Asterisks indicate significant differences among *M. acuminata* cultivars. Welch's t-tests (p, 0.05) were performed for all *M. acuminata* subspecies. Bre: *Musa acuminata* var. breviformis, Tom: *Musa acuminata* var. tomentosa, Mal: *Musa acuminata* var. malaccensis (Ridl.), Mic: *Musa acuminata* var. microcarpa (Becc.), Rut: *Musa acuminata* var. Rutilifers.

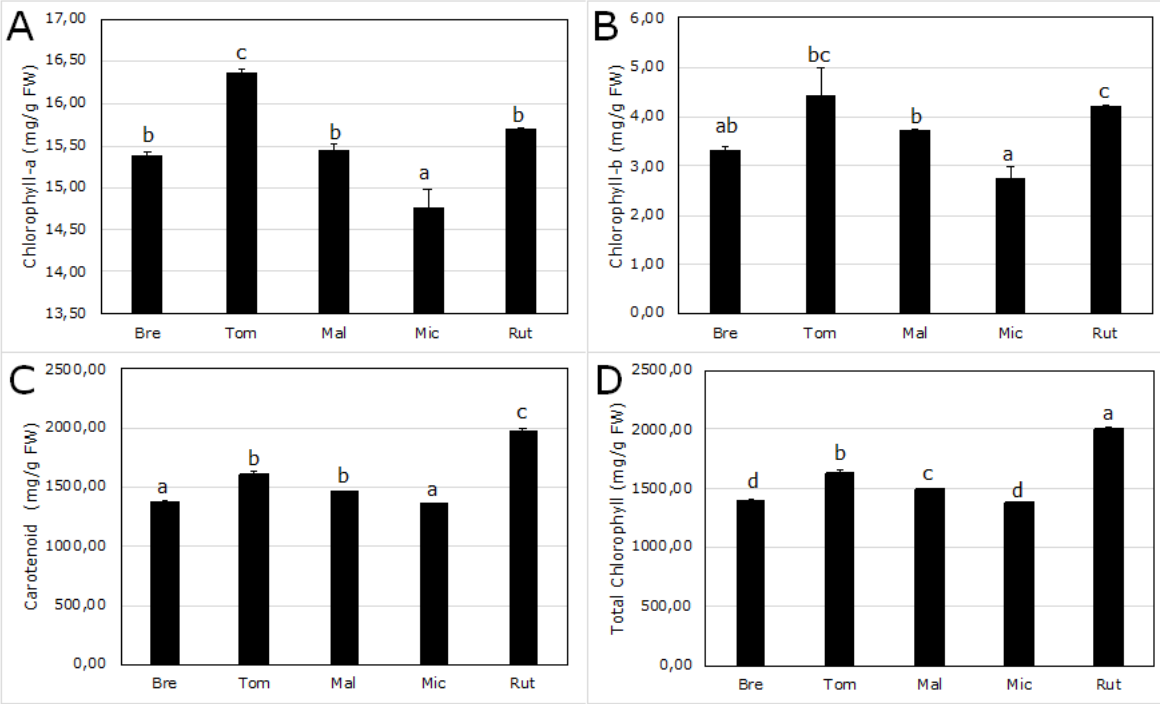

Figure 2. Chlorophyll content among banana (*Musa acuminata*) cultivars.
Chlorophyll-a (A), Chlorophyll-b (B), Carotenoids (C), Total Chlorophyll (D). Columns and bars represent the means and SE (n = 5, Three biological and technical replications, respectively). Different letters indicate significant differences among *M. acuminata* cultivars. Duncan Multiple Rate Test (DMRT, $p < 0.05$) was performed for all *M. acuminata* cultivars. Bre: *Musa acuminata* var. breviformis, Tom: *Musa acuminata* var. tomentosa, Mal: *Musa acuminata* var. malaccensis (Ridl.), Mic: *Musa acuminata* var. microcarpa (Becc.), Rut: *Musa acuminata* var. Rutilifers.

Gene expression of *LCYB*

The *LCYB* gene encodes the enzyme Lycopene beta cyclase, which controls the pathway for beta-carotene formation in banana plants. The highest expression levels of the *LCYB* gene surfaced in the banana cultivar Tomentosa, followed by the cultivar Malaccensis. However, the lowest *LCYB* gene expression came from the cultivar Microcarpa. The expression levels of the *LCYB* gene showed significant differences among the banana cultivars, viz., Rutilifers, Breviformis, Tomentosa, and Malaccensis, compared with the cultivar Microcarpa (Figure 3). The values of *LCYB* gene expression demonstrated distinct amplification plots and delta RN values by comparing with the gene actin as a housekeeping gene and the negative control (non-template mix) (Figure 4).

DISCUSSION

The *LCYB* gene encodes an enzyme involved in the synthesis of carotenoids in crop plants, including bananas (Diretto *et al.,* 2007). Specifically, *the Lycopene β-cyclase* (*LCYB*) genes are responsible for producing the enzyme lycopene β-cyclase, which is vital in the biosynthesis pathway of the carotenoids, creating various carotenoid pigments in crop plants (Diretto *et al.,* 2006; Poerba *et al*., 2019). Carotenoids are natural pigments that contribute to the vibrant colors in fruits and vegetables, such as red, orange, and yellow. The carotenoids are also essential in crop plants, including protection against excessive light, antioxidant activities, and participation in photosynthesis (Ruiz-Sola *et al.,* 2016; Giorio *et al.,* 2022). In the presented findings, it was well-defined that banana cultivars Tomentosa

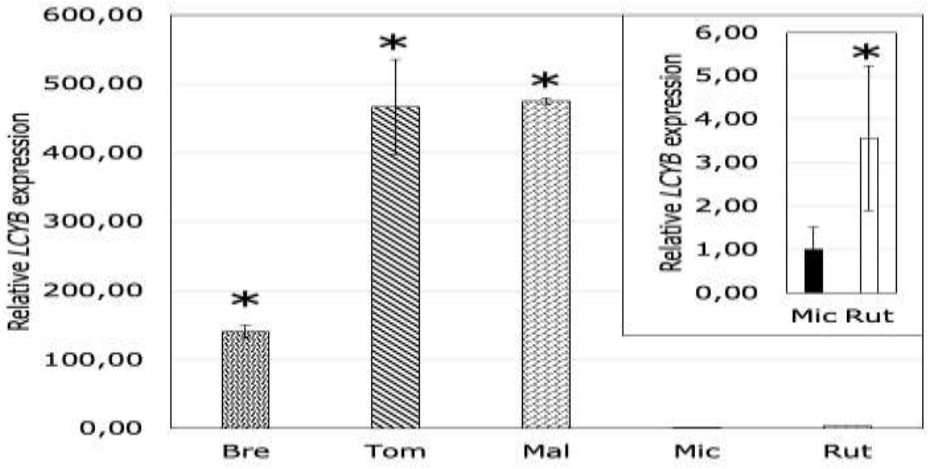

Figure 3. Relative gene expression of *Lycopene β-cyclase* (*LCYB*) in various banana (*Musa acuminata*) cultivars using qRT-PCR.
For gene expression, leaves of *M. acuminata* were used. Columns and bars represent the means and SE (n = 3, Three biological and technical replications, respectively). Asterisks indicate significant differences between *M. acuminata* cultivars and the Microcarpa (Becc.) cultivar. Welch's t-tests (p, 0.05) were performed for all *M. acuminata* subspecies. Bre: *Musa acuminata* var. breviformis, Tom: *Musa acuminata* var. tomentosa, Mal: *Musa acuminata* var. malaccensis (Ridl.), Mic: *Musa acuminata* var. microcarpa (Becc.), Rut: *Musa acuminata* var. Rutilifers.

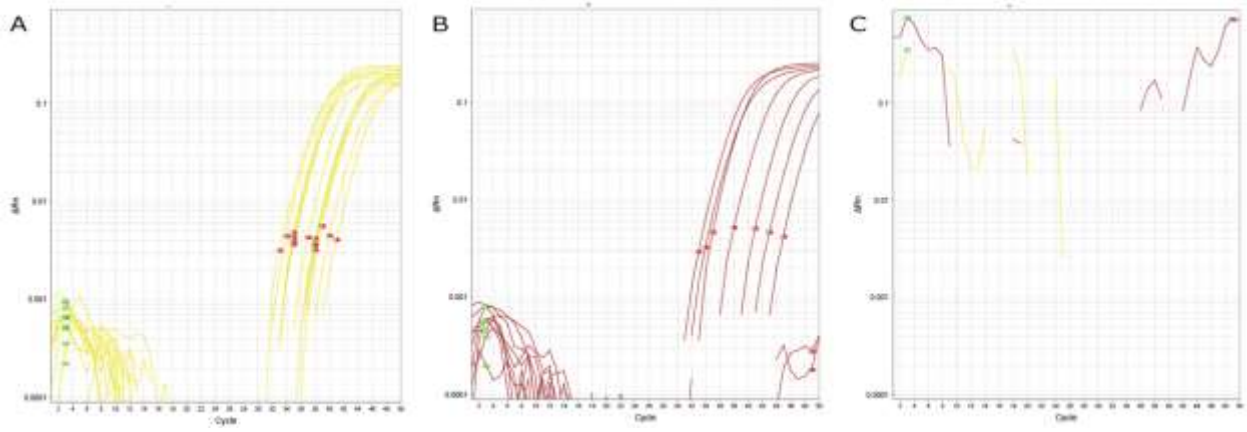

Figure 4. Amplification plot of Ct value in *Actin* (A) and *LCYB* (B) genes of *Musa acuminata* cultivars. ΔRn value is the Rn value of an experimental reaction minus the Rn value of the baseline signal generated by the qRT-PCR instrument. The cycle number is 50 and ΔRn is measured among 0.0001 to 0.1 values. Non-template reaction and ddH_2O (Negative controls) are used in this experiment (C).

and Malaccensis revealed the maximum content of carotenoids and a high level of *LCYB* gene expression (Figures 2, 3).

LCYB is a chief player in converting lycopene, a linear carotenoid, into beta-carotene, a cyclic carotenoid (Giorio *et al.,* 2022). This conversion is a pivotal step in the biosynthesis of beta-carotene, which serves as a precursor for other carotenoids like lutein and zeaxanthin. The regulation of *LCYB* genes can vary among the different crop plant species, and understanding their expression

and activity offers insights into the color development and nutritional properties of fruits and vegetables. However, banana cultivars Microcarpa and Rutilifers revealed a similar mode of action of the *LCYB* gene expression; however, both cultivars have lower *LCYB* expression but also have high carotenoid content in their leaves (Figures 2, 3). It is normal because the observed carotenoids were not only carotene or lycopene but also had lutein (Giorio *et al.,* 2022). However, it also needs verification in future studies. Past research on *LCYB* genes enunciated that it has a significant role in plant biology and crop enhancement as it aids in comprehending mechanisms governing carotenoid content. This knowledge can contribute to developing crops with improved nutritional profiles and color characteristics (Ru *et al.,* 2020). The mode of action in *LCYB* gene expressions among the *M. acuminata* local cultivars could be varied (Figure 3).

Carotenoids contribute to the red, orange, and yellow hues in fruits and vegetables (Demurtas *et al.,* 2015; Nisar *et al.,* 2015). Lycopene, a type of carotenoid, is responsible for the red color in tomatoes, watermelons, and specific banana cultivars (Hughes *et al.,* 2016; Park *et al.,* 2016). Lycopene B-cyclase is an enzyme aiding lycopene conversion into beta-carotene, another type of carotenoid (Cunningham and Gantt, 1998; Li *et al.,* 2014). In this context of bananas, the expression of the *LCYB* gene influences the magnitude of lycopene and other carotenoid content found in the fruits. Lycopene contributes to the red appearance of bananas, which can be desirable for particular cultivars from a marketing perspective (Ronen *et al.,* 1999, 2000). It is relevant that the genetic characteristics and carotenoid substance of bananas can vary among the species and cultivars (Figure 3).

LCYB gene expression indicates the process of encoding genetic information in the *LCYB* gene, then used to produce the LCYB enzyme in the *Musa acuminata* genotypes (Sun *et al.,* 2007; Asif *et al.,* 2013). This process involves transcription and translation, synthesizing the LCYB protein (Liu *et al.,* 2014). During transcription, the *LCYB* gene's DNA sequence becomes a template to generate messenger RNA (mRNA) in the cell nucleus. The newly formed mRNA undergoes modifications, including removing non-coding regions (introns) and adding a 5' cap and a 3' poly-A tail, crucial for mRNA stability and functionality (Woitsch and Romer, 2003). The processed mRNA gets transported from the nucleus to the cytoplasm for protein synthesis. The level of *LCYB* gene expression can also incur influences from various factors, including environmental cues and the developmental stages of the banana plant fruits (Sun *et al.,* 2007). Gene expression affects the LCYB enzyme production and, thus, impacts the conversion of lycopene to beta-carotene in bananas.

The *LCYB* gene is crucial in carotenoid biosynthesis, facilitating the conversion of lycopene into beta-carotene and affecting carotenoid content (Li *et al.,* 2008; Apel and Bock, 2009). The interplay between chlorophyll and carotenoids is elemental for photosynthesis, as chlorophylls capture light energy and convert it into chemical energy. However, the higher chlorophyll content signifies better photosynthetic activity, leading to increased energy production for plant growth and development (Fraser *et al.,* 2002). It is worth mentioning that the relationship between chlorophyll content and *LCYB* gene expression can vary based on factors like plant species, developmental stage, environmental conditions, and other regulatory aspects (Römer *et al.,* 2000; Martin *et al.,* 2017; Li *et al.* 2018; Venkatachalam *et al.,* 2019).

CONCLUSIONS

In conclusion, a relationship between *LCYB* gene expressions and chlorophyll characters among all cultivars existed. The banana cultivar Rutilifers demonstrated the highest and distinct levels of carotenoid and total chlorophyll content. In contrast, Breviformis and Microcarpa cultivars exhibited comparatively lower and significantly different contents than the other cultivars. It was also noticeable that the cultivar Tomentosa showed the most elevated levels, followed by the

banana cultivar Malaccensis. Conversely, the cultivar Microcarpa exhibited the minimum levels of *LCYB* gene expression. The practical study revealed a significant relationship between the *LCYB* gene expression and the chlorophyll content across the various banana cultivars examined.

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