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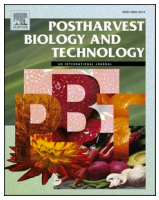

Transcriptomics analysis of mangosteen ripening revealed active regulation of ethylene, anthocyanin and xanthone biosynthetic genes

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ABSTRACT

Mangosteen (*Garcinia mangostana* Linn.) is known as the 'Queen of Fruits' due to its sweet and unique taste. The fruit also contains beneficial compounds such as anthocyanins and xanthones which have pharmacological properties including anticancer, and anti-inflammatory activities. Mangosteen has unique ripening behavior whereby the fruit undergoes full ripening (up to Stage 6 (S6), dark purple) only when harvested at the middle ripening stage (Stage 2 (S2), appearance of pink spots) onwards. This is unlike most other climacteric fruits which can ripen completely when harvested at the initial green stage (Stage 0, S0). However, information governing the ripening regulation of mangosteen is limited particularly at the molecular level. Hence, transcriptomics analyses on mangosteen pericarp was performed at different ripening stages; S0 (initial stage), S2 (middle stage), and S6 (final stage), revealing 250,680 unique transcripts, of which 6233 were differentially expressed transcripts (DETs). Furthermore, the benchmarking universal single-copy orthologs (BUSCO) analysis affirmed that the assembled transcriptome contains 93.8 % of gene representation in the Viridiplantae kingdom. Functional annotation indicated a high match with the species from the Malpighiales order, with most of the DETs participating in the metabolism process. Additionally, transcriptomics analysis indicated the fruit undergoes transitioning at the early ripening (S0 vs S2) by conserving carbon skeleton instead of energy production before ethylene surge later (S2 vs S6). Transcripts relating to the anthocyanin and xanthone biosynthesis were upregulated during ripening particularly at an early stage, consistent with ripening as an oxidative process with high requirements of antioxidants. This study contributes to the findings of novel transcripts important for the regulation of ripening which could lead to better commercial utilization and preservation of mangosteen in the future.

1. Introduction

Fruit ripening is a developmental process involving specific biochemical and physiological changes, including appearance, texture, flavor, and aroma which can enhance the desirable quality of fruit (Brumos, 2021; Li et al., 2021). Mangosteen (*Garcinia mangostana* L.), famously known as the "Queen of Fruits" ripens from a green to a dark purple color pericarp while developing an edible fleshy white pulp/aril. Mangosteen has economic importance for local industry as it has successfully generated USD 20.97 million and USD 75.21 million worth of agriculture in Malaysia (Department of Agriculture Malaysia, 2021) and Indonesia (Vikaliana et al., 2021), respectively in the year 2020. Moreover, the fruit possesses various bioactive compounds (e.g., anthocyanin and xanthone) evident in a wide range of applications including a potential cure for human-related diseases such as cancer and inflammation (Aizat et al., 2019a; Marx et al., 2021; Muniroh et al., 2021; Shibata et al., 2011), engineering (Aizat et al., 2019a; Anitha and Ramadevi, 2021; Ekabutr et al., 2019; Iradukunda et al., 2021), and food applications (Dunuweera et al., 2021; Mazlan et al., 2019; Owolabi et al., 2021).

There are seven stages of mangosteen ripening starting from yellowish green (Stage 0, S0) to a deep dark purple (Stage 6, S6) fruit based on the Malaysian maturity index (Osman and Milan, 2006). Interestingly, mangosteen has a unique ripening behavior unlike other fruit of its class (climacteric), whereby it will only ripen fully once harvested at the middle of the ripening process (Stage 2) onwards but not earlier during the mature green stage (Osman and Milan, 2006). This has led to a hypothesis that stage-specific molecules may play a

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significant role in the ripening regulation of mangosteen and therefore, the different ripening stages of mangosteen ripening need to be studied. A previous *de novo* transcriptomics study on mangosteen pericarp only use a single ripening stage (Stage 6), hence limiting the molecular insights of its ripening process (Matra et al., 2016). Meanwhile, other omics studies performed (proteomics and metabolomics) at the different time points of the fruit successfully unraveled proteins or metabolites that governs ripening (Jamil, 2021; Mamat et al., 2020; Matra et al., 2016), yet a global transcriptome study would unravel far more molecular information, especially with the lack of a complete genome sequence in this species.

Mangosteen can be mainly categorized into two different varieties, common mangosteen and Mesta. An evolutionary study between the varieties suggests a similar maternal lineage as mangosteen is known to undergo apomictic reproduction (Wee et al., 2022). Furthermore, comparative plastome analyses with other *Garcinia* species (e.g., *G. anomala, G. gummi-gutta, G. mangostana* var. Thailand, *G. oblongifolia, G. paucinervis,* and *G. pendunculata*) showcase a highly conserved gene content, gene order, as well as gene orientation (Wee et al., 2022). However, the phylogenomic analysis of *G. mangostana* var. common/-Mesta revealed different clustering from the *G. mangostana* var. Thailand (Wee et al., 2022), contradicting to the initial hypothesis of a close genetic relationship between the *G. mangostana* varieties with a single origin. Furthermore, the genome size of mangosteen was estimated at 5.92 Gb (Midin et al., 2018), considerably larger compared to the genome size of other plants such as tomato (~900 Mb) (Menda et al., 2013), apple (~600 Mb) (Peace et al., 2019), banana (~500 Mb) (D'Hont et al., 2012; Wu et al., 2016), and cassava (~700 Mb) (Mansfeld et al., 2021). This poses significant challenges, particularly when performing functional annotation, impeding the discovery of novel genes and biochemical pathways unique to mangosteen.

In transcriptomics studies, RNA sequencing (RNA-seq) is often preferred compared to other techniques such as microarray as it enables the global profiling and quantification of a complete set of transcripts without genome information at specific tissues, developmental stages, or responses to stresses (Mahmood et al., 2020; Rivera-Vicéns et al., 2022; Wang et al., 2009; Xiao et al., 2018). This approach allows the investigation of the gene expression as well as differential splicing of mRNA of the cells at a particular stage (Cui et al., 2019; Lowe et al., 2017; Ye et al., 2017). The *de novo* transcriptome assembly enables the reconstruction of transcript sequence with the absence of reference genome using pipelines such as Trinity and SOAPdenovo-Trans which have been reported to possess superior performance compared to ABySS and Inchworm for assembling transcriptomes in plants (Honaas et al., 2016). For instance, Goh et al. (2019) utilized Trinity *de novo* assembly to study the time-course transcriptome-wide gene expression changes during mangosteen seed germination.

Mangosteen mainly produces the bioactive xanthones during fruit ripening and the pericarp tissue contains the most abundant xanthones compared to other tissues (Aizat et al., 2019b; Mamat et al., 2020). However, xanthone biosynthetic genes and its related pathways in plants particularly in mangosteen, are still not fully elucidated (Ovalle-Magallanes et al., 2017; Remali et al., 2022; Resende et al., 2020). Therefore, this study employs RNA-seq leverage by *de novo* transcriptome assembly at different ripening stages (Stage 0, 2, and 6), which may assist in the discovery of genes involved in the xanthone biosynthetic pathway in mangosteen.

2. Methods

2.1. Sample preparation

Mangosteen fruit were obtained from the experimental plots at Universiti Kebangsaan Malaysia, Bangi, Malaysia (2°55′09.0″N, 101°47′04.8″E) between May to July 2014. These plants were of 20 years of age during harvesting. Three ripening stages which are Stage 0 (yellowish-green), Stage 2 (yellowish-green with uneven pink spots), and Stage 6 (dark purple) with two biological replicates (independent fruit) for each stage were selected for fruit sampling according to the Malaysian maturity index of mangosteen (Fig. S1) as described by Osman and Milan (2006). The fruit were processed by separating the pericarp tissue before grinding in liquid nitrogen except for the fruit at Stage 0 where the whole fruit were used as the tissues are inseparable (Osman and Milan, 2006). All tissues were kept in a −80 °C freezer until further examination.

2.2. RNA extraction, library preparation, and sequencing

In brief, total RNA from mangosteen pericarp was extracted using the modified CTAB method as detailed previously (Abdul-Rahman et al., 2017). Purified RNA samples at three ripening stages (Stage 0, 2, and 6) were sent for sequencing using Illumina HiSeq 4000 platform that generates 100 bp paired-end reads (Abdul-Rahman et al., 2017). Transcriptome sequences were deposited to NCBI under BioProject ID PRJNA339916 with three different BioSamples, which were SAMN05615258 (Stage 0), SAMN05615259 (Stage 2), and SAMN05615260 (Stage 6).

2.3. Transcriptome assembly and differentially expressed transcript (DET) analysis

Sequencing reads were pre-processed by removing adapter sequences from raw reads using Trimmomatic to yield high-quality clean reads (Phred score $\geq$ 25) for *de novo* assembly using Trinity v2.2.0 pipeline (Haas et al., 2013). DET analysis was performed between early (Stage 2/Stage 0) and late (Stage 6/Stage 2) ripening with edgeR (Robinson et al., 2009) based on the transcript abundance estimation using RSEM (Li & Dewey 2011) from the read alignment results of individual samples against the assembled transcriptome. These early and late ripening comparisons were chosen due to the progressive time series of mangosteen which proceeded from Stage 0 to Stage 2 and lastly Stage 6. Transcriptome abundance is expressed as Transcript Per Kilobase Million (TPM), which normalizes both gene length and read depth to allow for more accurate comparisons between samples. Statistically significant DETs were defined by FDR < 0.001 and $|\mathrm{Log_2FC}= > 2$.

2.4. Functional annotation and enrichment analysis

A Trinotate package (Bryant et al., 2017), which is a part of Trinotate pipeline, was used to annotate the assembled transcripts based on BLAST searches (E-value $\geq$ 1E–5) against the non-redundant (*nr*) and UniProt databases using BLASTx (nucleotide sequences) and BLASTp (predicted peptide sequences from Transdecoder). RNAMMER, PFam, SignalP, TmHMMM, eggnog, and gene ontology (GO) are among the tools employed during the annotation process. Further analyses including hierarchical clustering, profile plot, heatmap, and enrichment analysis (Fisher's exact test with Benjamin-Hochberg multiple test correction) were employed using the default settings in Perseus software v1.6.2.2 (Tyanova et al., 2016).

2.5. Reverse transcription-quantitative real-time PCR (RT-qPCR) analysis

Firstly, 1 μg of total RNA extract from an independent set of pericarp samples was used to produce the first-strand cDNA using SuperScript™ IV First-Strand Synthesis System (Thermo Fisher Scientific Inc., USA). The primers were designed using Primer-BLAST (Ye et al., 2012) as listed in Table S1. For the quantitative real-time PCR (RT-qPCR), the Biorad IQ5 multicolor real-time PCR detection instrument (Bio-Rad Laboratories, USA) was used along with Biorad SYBR Green Master Mix. The PCR reaction consists of cDNA template (1 ng), SYBR Green Master Mix (5 μL), 10 μM of each forward and reverse primers (0.25 μL), and

nuclease-free water to a 10 μL final volume. The amplification program used for this study was as follows: 95 °C for 15 s and 40 cycles of 95 °C for 15 s and 60 °C for 30 s. Validation of PCR specificity was performed using end-cycle melt curve analysis and no-template controls (NTCs). The gene validation using RT-qPCR was done using three random biological replicates with three technical replicates each. Internal reference actin (ACT) was used to normalize the expression of target genes using 2^{-ddCt} method (Livak and Schmittgen, 2001). The correlation between the relative expression data of RT-qPCR and RNA-seq was performed using Pearson product moment correlation coefficient and the significance at p-value < 0.001 was determined using R^2 coefficient determination and regression analysis in Excel (Microsoft, USA).

2.6. Pathway mapping of ethylene, anthocyanin, and xanthone

Pathway reconstruction for the biosynthesis of ethylene, anthocyanin, and xanthone was based on literature evidence as well as available databases, including Kyoto Encyclopedia of Genes and Genomes (KEGG) (www.genome.jp/kegg/) and Phytozome (https://phytozome-next.jgi.doe.gov/). Genes, enzymes, and metabolites involved in the ethylene and anthocyanin biosynthesis pathways were manually curated from the annotated datasets from previous studies (Jamil, 2021; Mamat et al., 2020). For xanthone-related genes including *BPS*, *benzophenone 3' hydroxylase* (*B3'H*), *1,3,7-trihydroxyxanthone synthase* (*THXS*), *xanthone-6-hydroxylase* (*X6H*), and *xanthone-specific prenyltransferase* (*PT*), were searched through various sources and databases including UniProt (www.uniprot.org), NCBI (www.ncbi.nlm.nih.gov), Phytozome, and available literature. Sequences of BPS, THXS, and PT were identified and used for a local BLAST search against mangosteen sequences found in this study which serves as the query database. Metabolism overview and plant hormone mapping were generated using the MapMan tool (Schwacke et al., 2019).

3. Results

3.1. Transcriptome assembly and functional annotation

A total of 656,913,570 raw reads were trimmed resulting in 650,887,650 clean reads for *de novo* assembly of a reference mangosteen pericarp transcriptome (Abdul-Rahman et al., 2017). In total, 250,682 transcripts and 181,646 unigenes were generated with an N50 length of 1158 bp and 68,352 peptide sequences were predicted from Transdecoder (Abdul-Rahman et al., 2017). The benchmarking universal single-copy orthologs (BUSCO) analysis showed that the transcriptome assembly has the highest completeness against Viridiplantae kingdom (93.8 %) followed by Embryophyta (91.7 %), Eudicotyledons (89.6 %), and Solanaceae (48.4 %) (Table 1).

Annotation searches using BLASTx against the *nr* protein database found hits to 65,090 transcripts as compared to 37,353 transcripts using the BLASTp searches of predicted peptide sequences (Table S2). Furthermore, 34,201 transcripts from mangosteen ripening found hits to Pfam (Table S3).

3.2. Differentially expressed transcript (DET) analysis

A total of 6233 transcripts were found to be differentially expressed (DET) during mangosteen ripening across the three stages (S0, S2, and S6). Noteworthy, a total of 535 DETs were expressed throughout ripening in both early (S0 vs S2) and late (S2 vs S6) ripening. The highest number of DETs were identified at early ripening with the majority of the DETs (3635) observed to decrease during the stage transition. However, most of the DETs (1019) at late ripening were upregulated, concurring with the onset of ethylene and hence ripening stimulation (Jamil et al., 2021; Palapol et al., 2009b). Fig. 1.

3.3. Transcriptome functional categories, expression profile, and enrichment analysis

Annotation analysis of the DETs (Fig. 2) revealed more than 40 % of the DETs were homologous with species from Malpighiales order with *Manihot esculenta* (~13 %), *Jatropha curcas* (~11 %), *Ricinus communis* (~10 %), *Populus trichocarpa* (~9 %), and *Populus euphratica* (~7 %) among the top BLAST matches (Ha et al., 2019; Munhoz et al., 2018; Srinivasa Rao et al., 2012). Mangosteen belongs to this Malphighiales order with the species distribution within this clusioid clade having the highest species diversity in the tropic region (Ruhfel et al., 2011). Other transcriptome studies such as in pepper also show high hit matches with species from similar clusioid clade indicating conserved genes within the same order (Rahmat et al., 2021; Trad et al., 2021; Zhang et al., 2020).

All 6233 DETs were then assigned with Gene Ontology (GO) classification (Fig. 3) which can be classified into three main groups: cellular component, molecular function, and biological processes. In the cellular component, the 'cell', 'cell part', and 'organelle' were among the highly represented by the DETs. In the molecular function, the majority of the DETs are associated with the terms 'binding' (e.g., compound/small molecule binding), 'hydrolase', and 'transporter' functions. For biological processes, DETs were dominantly represented by the terms 'cellular process', 'metabolic process', and 'biological regulation'. This GO analysis suggests that most DETs are located in cells and organelles, with functions related to binding, hydrolase and transporter activities, of which involved in cellular, metabolic and biological processes pertaining to mangosteen ripening.

Other than GO classification, the DETs were also aligned to the euKaryotic Ortholog Groups of proteins (KOG) database which predicts and classify possible functions into 25 KOG categories (Fig. 4A). The 'function unknown' category which consists of uncharacterized/predicted proteins, represented the largest group for both transcriptome and DETs (Goh et al., 2019). This is followed by 'replication, recombination and repair', 'signal transduction mechanism', and 'carbohydrate transport and metabolism' categories for DETs. Meanwhile, the transcriptome was represented by 'signal transduction mechanism', 'transcription', and 'posttranslational modification, protein turnover, chaperones' catagories.

Based on the pathway enrichment analysis (Fig. 4B) using KEGG pathways, the DETs were classified into four groups which were 'metabolism', 'environmental information processing', 'cellular processes', and 'organismal systems' during mangosteen ripening in pericarp. For metabolic pathways, most of the DETs were associated with carbohydrate metabolism and amino acid metabolism. This is followed by environmental information processing (signal transduction), cellular processes (transport and catabolism), and organismal systems (environmental adaptation).

Table 1
BUSCO comparison with several databases against transcriptomics data from mangosteen.

Database	Complete BUSCO		Complete-single-copy BUSCO		Complete-duplicate BUSCO		Fragmented BUSCO		Missing BUSCO		*N* BUSCO group search
	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	
Viridiplantae	93.8	403	59.1	254	34.7	149	4.4	19	1.8	8	430
Embryophyta	91.7	1260	54.2	745	37.5	515	5.7	78	2.6	37	1375
Eudicotyledons	89.6	1901	53.9	1144	35.7	757	6.2	131	4.2	89	2121
Solanaceae	48.4	1478	29.4	897	19.0	581	8.3	253	43.3	1321	3052

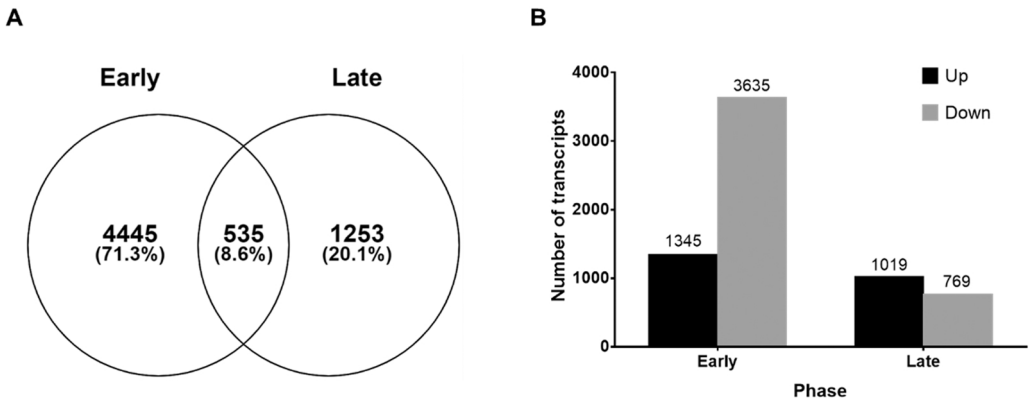

Fig. 1. Differentially expressed transcripts (DETs) (FDR < 0.001, $|Log_2FC| \geq 2$) represented by (A) Venn analysis at early (S0 vs S2) and late (S2 vs S6) ripening. (B) Number of upregulated (Up) and downregulated (Down) transcripts at each phase.

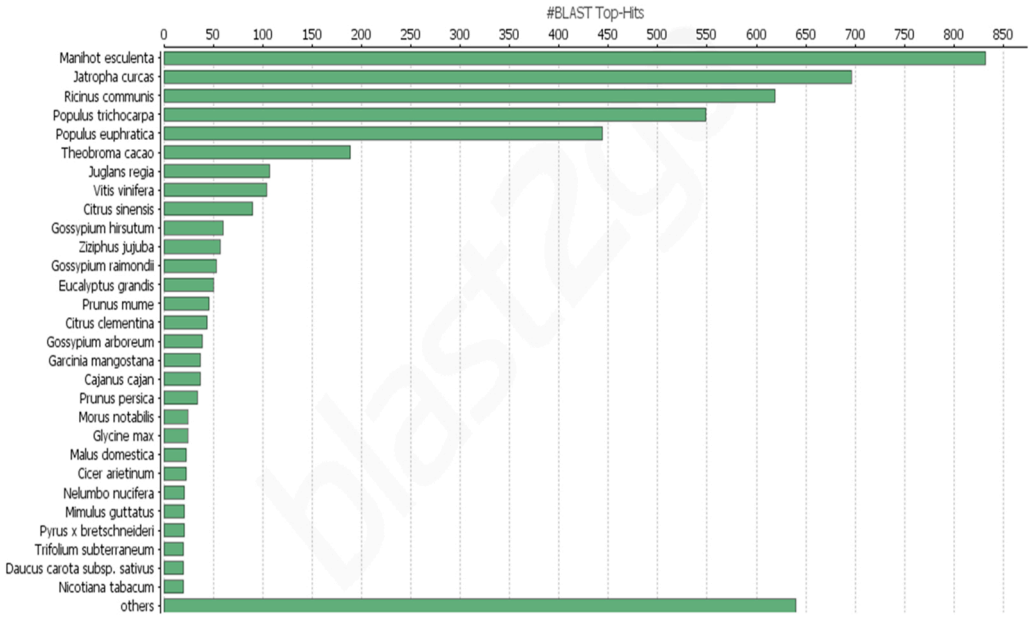

Fig. 2. Species distribution of identified differentially expressed transcripts.

The DETs involved in the metabolic pathway were also imported into the MapMan visualization tool to gain better insights from visualizing DET expression. Fig. 5 represents an overview of the DETs involved in different metabolic processes, including secondary metabolism, carbon metabolism, sugar metabolism, and cell wall metabolism. For example, DETs in carbohydrate metabolism such as glucose-1-phosphate adenylyltransferase (up to −8.49 Log_2FC), and granule-bound starch synthase 1 (up to −4.99 Log_2FC) involved in the starch synthesis were downregulated during early ripening. Similarly, DETs involved in cell wall modification including expansin and probable xyloglucan endotransglucosylase were declining down to −12.38 Log_2FC and −10.02 Log_2FC respectively during early ripening. Thus, Fig. 5 illustrates different transcript regulations during mangosteen ripening in the pericarp of which related to various biochemical pathways including hormonal and secondary metabolite regulation.

3.4. Regulation of ethylene during ripening

Ethylene is commonly associated with climacteric fruit and is synthesized from methionine to S-adenosylmethionine and finally to ethylene by ethylene-related enzymes (Pech et al., 2018). For example, *1-aminocyclopropane-1-carboxylate oxidase* (*ACO*) and 1-*aminocyclopropane-1-carboxylate synthase* (*ACS*) are exclusive enzymes dedicated to ethylene biosynthesis (Pattyn et al., 2021) and were found to be upregulated during ripening as observed in the mangosteen transcriptome. In early ripening (S0 vs S2), ten out of 12 *ACO* transcripts were significantly increased (up to 6.93 Log_2FC), coinciding with the upregulation at the protein level (Jamil et al., 2021), which may have initiated the first signal for ethylene production. This is followed by four upregulated *ACS* transcripts (up to 4.72 Log_2FC) at late ripening (S2 vs S6), resulting in a continuity of ethylene surge, concomitant with its postharvest changes (e.g., color development, soluble sugars, phenolic compounds, and loss of fruit firmness) (Jamil et al., 2021). Fig. 6.

3.5. Anthocyanin pathway

Ethylene accumulation induces color development in mangosteen pericarp during ripening by promoting genes relating to anthocyanin via phenylpropanoid and flavonoid pathways (Fig. 7). Four out of seven *phenylalanine ammonia-lyase* (*PAL*) transcripts, which is the entry gene for the phenylpropanoid pathway, were upregulated up to 3.53 Log_2FC during early ripening (S0 vs S2). The remaining three *PAL* transcripts were then upregulated up to 2.88 Log_2FC at late ripening (S2 vs S6) further promoting anthocyanin production during ripening (Jamil et al.,

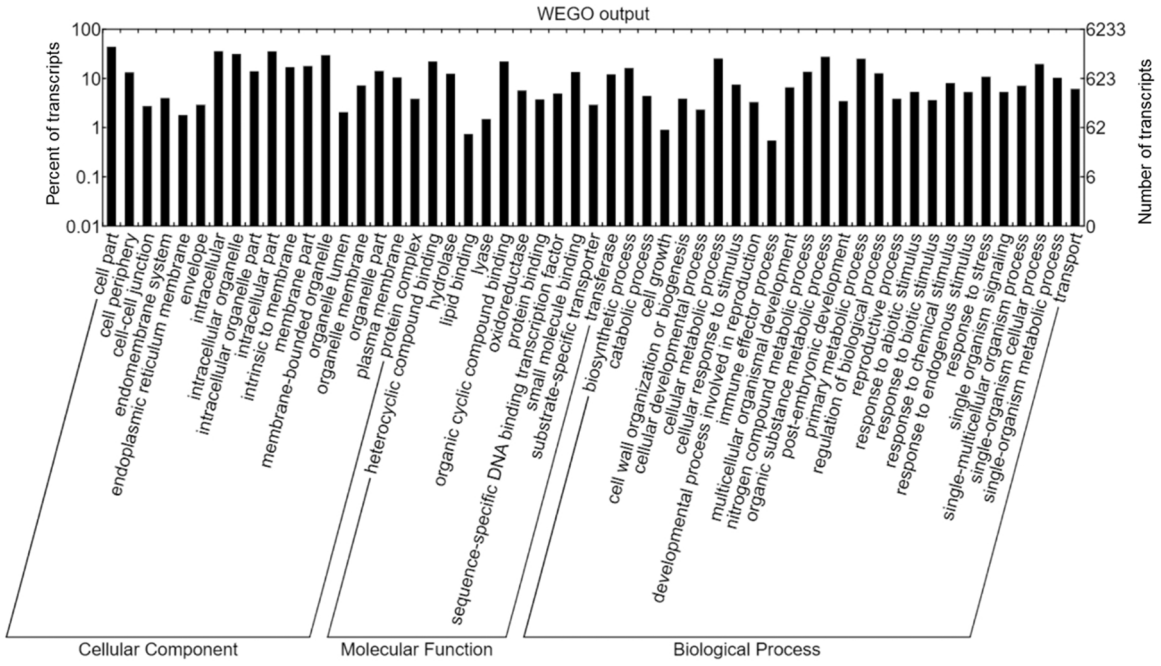

Fig. 3. GO classification of DETs using the Web Gene Ontology Annotation Plot (WEGO) web tool. The transcripts were classified into three main categories: biological processes, cellular component, and molecular function.

2021). Additionally, *chalcone synthase* (*CHS*) and *chalcone isomerase* (*CHI*) that controls the flavonoid biosynthesis were upregulated at early ripening up to 3.77 and 3.22 Log_2FC, respectively. Furthermore, upregulation of *flavanone 3-hydroxylase* (*F3H*, 3.22 Log2FC) during early ripening is vital to direct the carbon flow towards the biosynthesis of 3-hydroxylated flavonoids in mangosteen pericarp, which is responsible for the regulation of flavanols and anthocyanidins (Dai et al., 2022; Wang et al., 2020). Upregulation of anthocyanin-related genes such as *PAL*, *CHS*, and *CHI* at transcript level correlates with protein level during mangosteen ripening (Jamil et al., 2021). The *MYB* transcription factor (TF) (e.g., *MYB10* and *MYB4*) associated with anthocyanin production was also upregulated, similar to the trend observed in other fruit during ripening (Allan et al., 2008; Cheng et al., 2016; Jiang and Joyce, 2003; Palapol et al., 2009a).

3.6. Xanthone biosynthetic pathway in mangosteen

Xanthone derivatives (e.g., α-mangostin, mangiferin, patulone, and gentisin) may vary across the xanthone-producing species with more than 80 xanthones reported from mangosteen alone (Aizat et al., 2019b; Do and Cho, 2020; Ovalle-Magallanes et al., 2017). However, findings relating to xanthone gene expression in plants are limited (Remali et al., 2022). In this study, xanthone-related genes identified in the transcriptome data were mapped, which may be among the first to be performed in mangosteen. Gene mining was performed involving genes related to xanthone biosynthesis which include *benzophenone synthase* (*BPS*), *1,3,7-trihydroxyxanthone synthase* (*THXS*) and *xanthone-specific prenyltransferase* (*PT*). An increase in *BPS* throughout ripening (up to 4.22 Log_2FC) particularly during early ripening (S0 vs S2) is vital to ensure sufficient carbon skeleton supply for the formation of xanthones. Furthermore, an increase in xanthone accumulation via *BPS* may suggest their roles as a defense mechanism against pathogen attacks as well as a response toward the oxidative environment during ripening (Jamil et al., 2021; Remali et al., 2022; Tocci et al., 2018). Two of the *THXS* transcripts which belongs to the cytochrome P450 (CYP) family, have opposite regulation, with the first *THXS* (DN110382_c0_g1_i1) downregulated by −4.97 Log_2FC while the other *THXS* (DN76375_c0_g1_i2) was upregulated at early ripening by 7.61 Log_2FC. On the other hand, *PT* was significantly downregulated by −9.11 Log_2FC during early ripening before a significant increase (6.92 Log_2FC) at late ripening (S2 vs S6).

3.7. Regulation of transcription factors

Coordinating the fruit ripening process involves a combination between plant hormones, TFs, and epigenetic modification. For instance, 18 DETs relating to *bZIP* TFs were significantly reduced throughout mangosteen ripening (Fig. S2), implying a negative role for bZIP TFs in pericarp during fruit ripening (Liang et al., 2020). Similarly, the majority of the *bHLH* TFs were reduced during ripening as *bHLH* is involved in fruit-related growth and development (Brumos, 2021). On the contrary, *bHLH70* and *bHLH25* which were involved in the flavonoid biosynthetic pathway were significantly upregulated during early ripening. During early ripening, *NAC29*, *NAC10*, and *NAM-B1* which belong to the *NAC* TFs family increases up to 4.7 Log_2FC while *NAC8* and *NAC42* peaked at late ripening. On the other hand, two DETs from the *AP2/ERF* superfamily were upregulated while the remaining 20 DETs were significantly reduced during early ripening. Nevertheless, 11 out of 13 DETs belonging to the *AP2/ERF* family increased (up to 4.32 Log_2FC) from S2 onwards perhaps to induce the ethylene signaling pathway at late ripening, thus regulating the ethylene-responsive genes (Gao et al., 2020). The *WRKY* TFs (e.g., *WRKY40*, *WRKY41*, and *WRKY70*) were found to be decreasing (down to −11.22 Log_2FC) at early except for *WRKY72* (3.62 Log_2FC) and *WRKY75* (up to 3.21 Log_2FC). Interestingly, all 17 *WRKY* TFs were upregulated (up to 11.59 Log_2FC) at late ripening, concurrent with the *ACS* regulation. Additionally, 17 DETs belonging to *MYB* TFs were downregulated while 13 DETs were upregulated at early. At late ripening, *MYB4* and *MYB124* were found to significantly increase, in which is involved in the flavonoid pathway and stress tolerance respectively.

3.8. RT-qPCR analysis

The integrity of the DET data was validated by performing RT-qPCR for genes involved in ethylene (*SAM*, *ACO*, *ACS*), anthocyanin (*PAL, MYB10, UFGT, 4CL, CHS, CHI, F3'H, F3H, LDOX, DFR*), and xanthone (*BPS, PT, THXS*) pathways. As shown in Fig. 9, a good correlation during

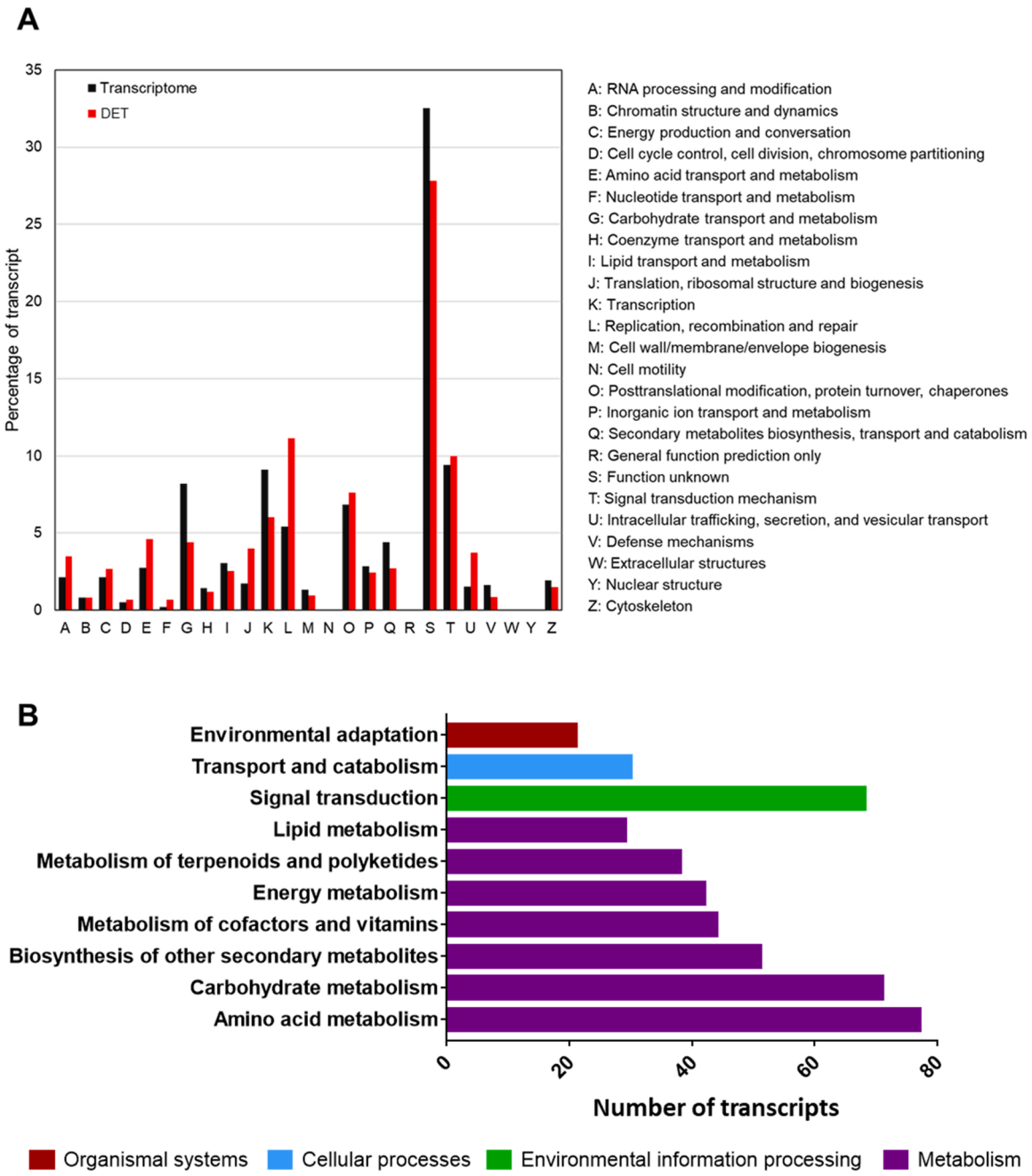

Fig. 4. Summary of functional annotations and enrichment analysis. (A) Transcriptome and DET were assigned the euKaryotic Orthologous Group (KOG) classification based on eggNOGv3 classification. (B) KEGG pathway assignment of the DETs. Enriched KEGG pathways are labeled on the vertical axis while the horizontal axis represents the number of DETs assigned to each pathway.

early ($R^2 > 0.85$) and late ($R^2 > 0.53$) ripening indicates a similar expression with the transcriptome data, thus reaffirming the expression of DETs that were detected in our transcriptomics analysis.

4. Discussion

The *de novo* transcriptome assembly approach is often employed as a sequence assembly method without the aid of a reference genome. This study employs three ripening stages from mangosteen pericarp tissue to represent the initial (S0), middle (S2), and final (S6) ripening process. The *de novo* transcriptome assembly was performed due to the absence of a mangosteen genome. This technique successfully assembled the transcriptome with more than 90 % conserved genes in the Viridiplantae kingdom, suggesting that the mangosteen transcriptome possesses most of the essential genes in the plant kingdom (Pech et al., 2018; Wang et al., 2018). In addition, our assembly has a higher N50 value than the previous *de novo* transcriptome assembly by Matra et al. (2016) which employs Ion Torrent instead of Illumina sequencing at a single ripening stage. Nevertheless, increasing the number of stages sequenced in this study may have led to a higher N50 value, evident in the doubling of N50 value upon sequencing four stages of mangosteen seed during germination (Goh et al., 2019). Furthermore, more than 60 % of the transcriptome, as well as the DETs, have unknown function, perhaps due to mangosteen having more genes that have yet to be characterized particularly given the genome size of mangosteen was up to 10-fold than other plants (Mansfeld et al., 2021; Menda et al., 2013; Midin et al., 2018; Peace et al., 2019; Wu et al., 2016). Even so, a total of 6233 DETs were identified, which provided valuable insights for future analysis

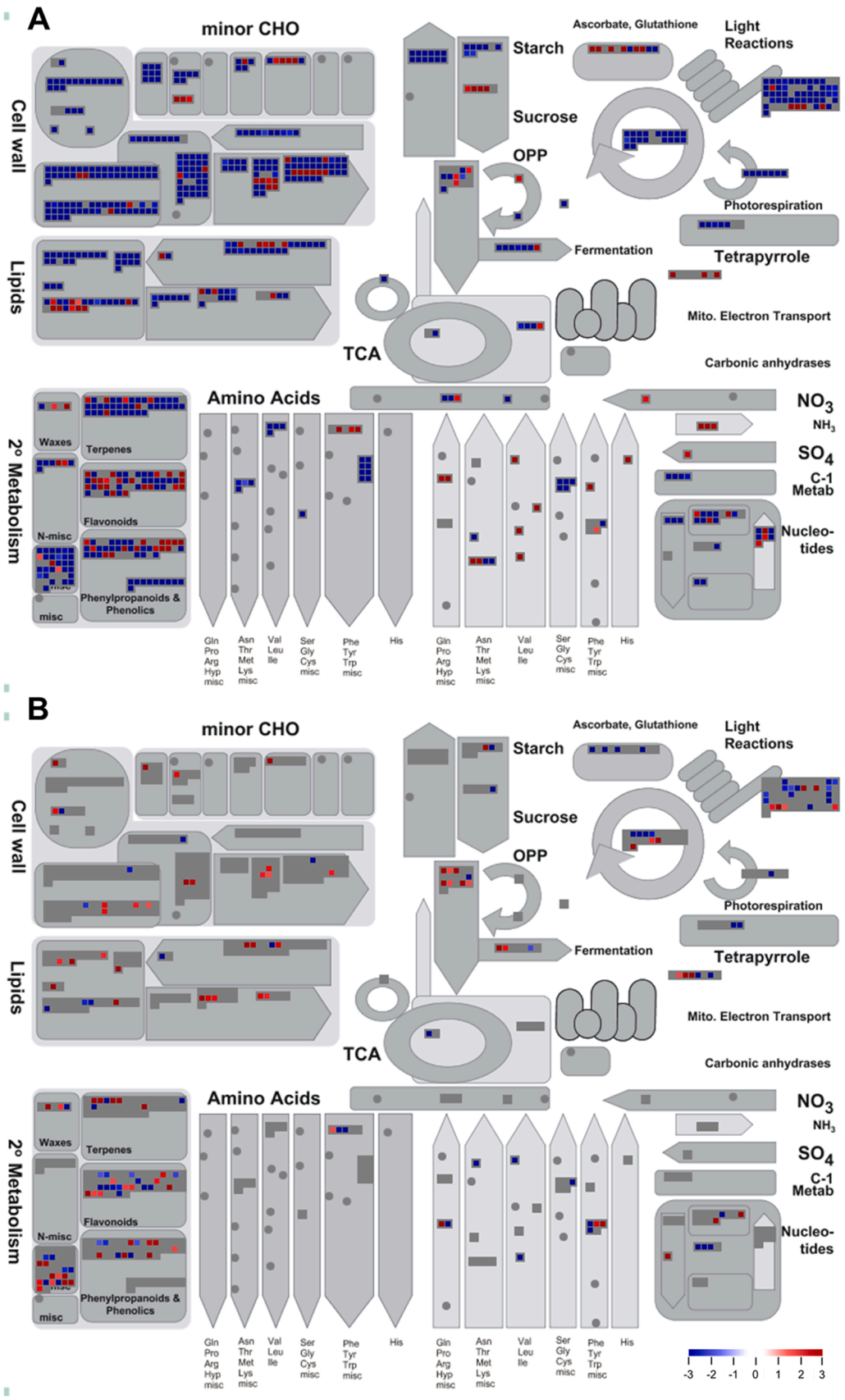

Fig. 5. Overview of DETs involved in metabolism pathway using MapMan visualization tool. The expression was represented in Log_2FC. Red or blue indicates up- or down-regulation, respectively. (A) early ripening (S0 vs S2); (B) late ripening (S2 vs S6).

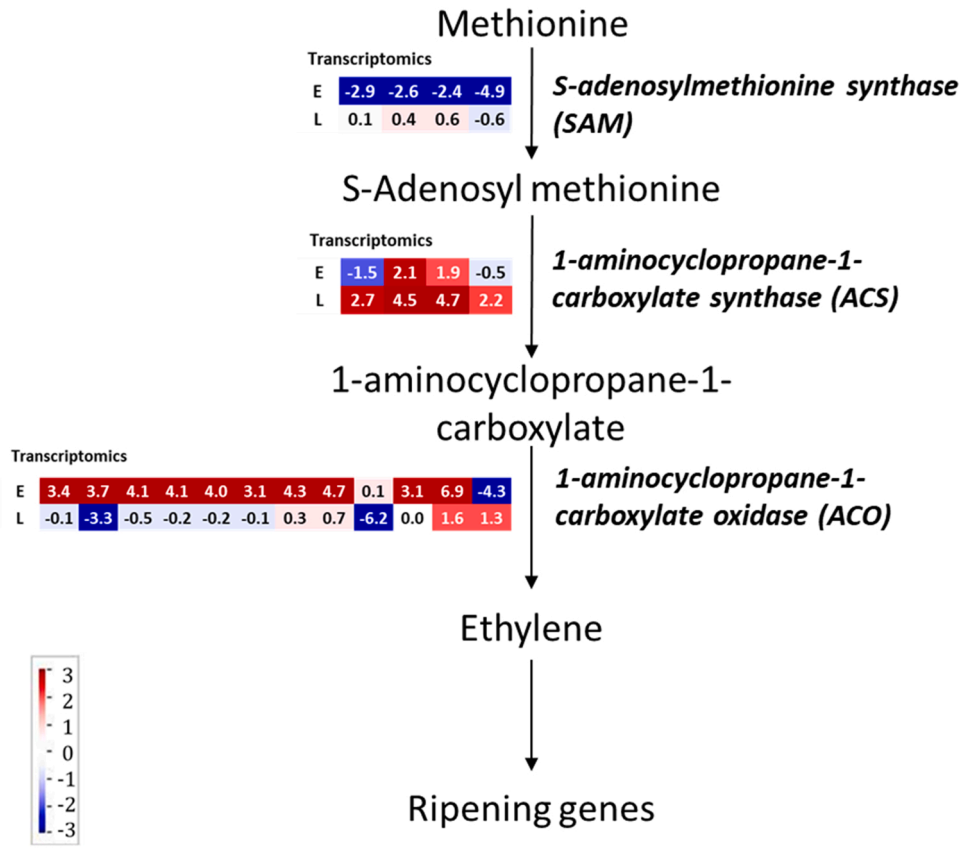

Fig. 6. Pathway mapping of ethylene biosynthesis in mangosteen. Bold gene indicates identified in transcriptomics datasets. Genes with expression indicate differentially expressed transcripts (DETs) at E = early ripening (S0 vs S2), or L = late ripening (S2 vs S6). The expression was represented in Log_2FC. Red or blue indicates up- or down-regulation, respectively.

relating to mangosteen with gene validation performed for the anthocyanin, ethylene, and xanthone pathways.

4.1. Hormonal regulation during ripening

Phytohormones play a vital role in signal transduction in response to stresses as well as improving fruit quality to be palatable for consumption. Ethylene is one of the phytohormones regulated by plants and is generally associated with climacteric fruit during the ripening process. Most of the climacteric fruit has reported the ACS, which has been well documented as the rate-limiting in ethylene biosynthesis during ripening concomitant with the burst of ethylene especially at late stages in mangosteen (Jamil et al., 2021; Li et al., 2019a; Liu et al., 2021b). Furthermore, prominent development of pink spots across the fruit at S2 onwards was in line with the increase in *ACS*, which may suggest that harvesting the fruit before this stage (S2) may compromise the quality and the flavor of the fruit, possibly due to reduced 1-aminocyclopropane-1-carboxylic acid (ACC) precursor for ethylene production but this requires further investigation. Additionally, the upregulation of *ACS* at late ripening indicates the transition from system I to system II of ethylene production at S2, signaling the regulation of ripening-related genes (e.g., color, texture, and aroma enrichment) (Aizat et al., 2013; El-Sharkawy et al., 2008; Li et al., 2013; Park et al., 2021). Surprisingly, ACS was not detected at the protein level (Jamil et al., 2021) suggesting the ACS activity in mangosteen was tightly regulated transcriptionally and post-translationally (Booker and DeLong, 2015; Sadka et al., 2019). Nevertheless, the regulation of ethylene also caused negative regulation for certain genes such as *calmodulin* (*CaM*), which is an essential calcium-binding protein, possibly due to complicated crosstalk between calcium and ethylene signaling to commence the ripening processes (Ding et al., 2018; Jamil et al., 2021; Yang et al., 2014). Even so, the decrease of *CaM* is vital in preventing possible translucent flesh disorder, which reduces the fruit quality due to the hardening of the flesh (Matra et al., 2019).

Other phytohormones including jasmonate, auxin (IAA), abscisic acid (ABA), gibberellic acid (GA), and salicylic acid (SA) are also regulated during fruit ripening. In this study, 164 DETs were involved in hormone signaling during mangosteen ripening. In jasmonate (JA) signaling, the regulation of *probable linoleate 9S-lipoxygenase 5 enzyme* (*9-LOX*, upregulated) and *linoleate 13S-lipoxygenase 2–1* (*13-LOX*, downregulated) from the major lipoxygenase (LOX) enzyme family were contradicting probably due to *9-LOX* and *13-LOX* having different pathway mechanisms (Viswanath et al., 2020). An increase in *9-LOX* expression enhances the plant defense mechanism against pathogen attack whereas *13-LOX* participated in the synthesis of jasmonates that are essential for response to tissue wounding (Aizat et al., 2018; Viswanath et al., 2020). Additionally, transcripts involved in salicylic acid (SA) biosynthesis such as *UDP-glycosyltransferase* and *salicylate carboxymethyltransferase* (*SAMT*) were upregulated particularly at late stage. The increase of *UDP-glycosyltransferase* at both transcript and protein (Jamil et al., 2021) levels regulate the production of SA in the pericarp. This is further affirmed by the identification of SA in a metabolomics study on mangosteen pericarp ripening by Mamat et al. (2019), perhaps to delay pericarp hardening and preserve the fruit by lowering metabolism during fruit ripening (Aizat et al., 2019a; Mustafa et al., 2018). Nonetheless, combination stimulus from both SA and JA pathways during ripening may increase defense responses against pathogenic attacks, benefiting fruit ripening under pathogen pressure (Darolt et al., 2020; Vos et al., 2013).

For abscisic acid (ABA) (Fig. S3), *ABA receptor* (*PYL4*) and *zeaxanthin epoxidase* (*ZEP*) genes that produces ABA precursor were downregulated throughout ripening. On the other hand, the *abscisic acid 8'-hydroxylase*

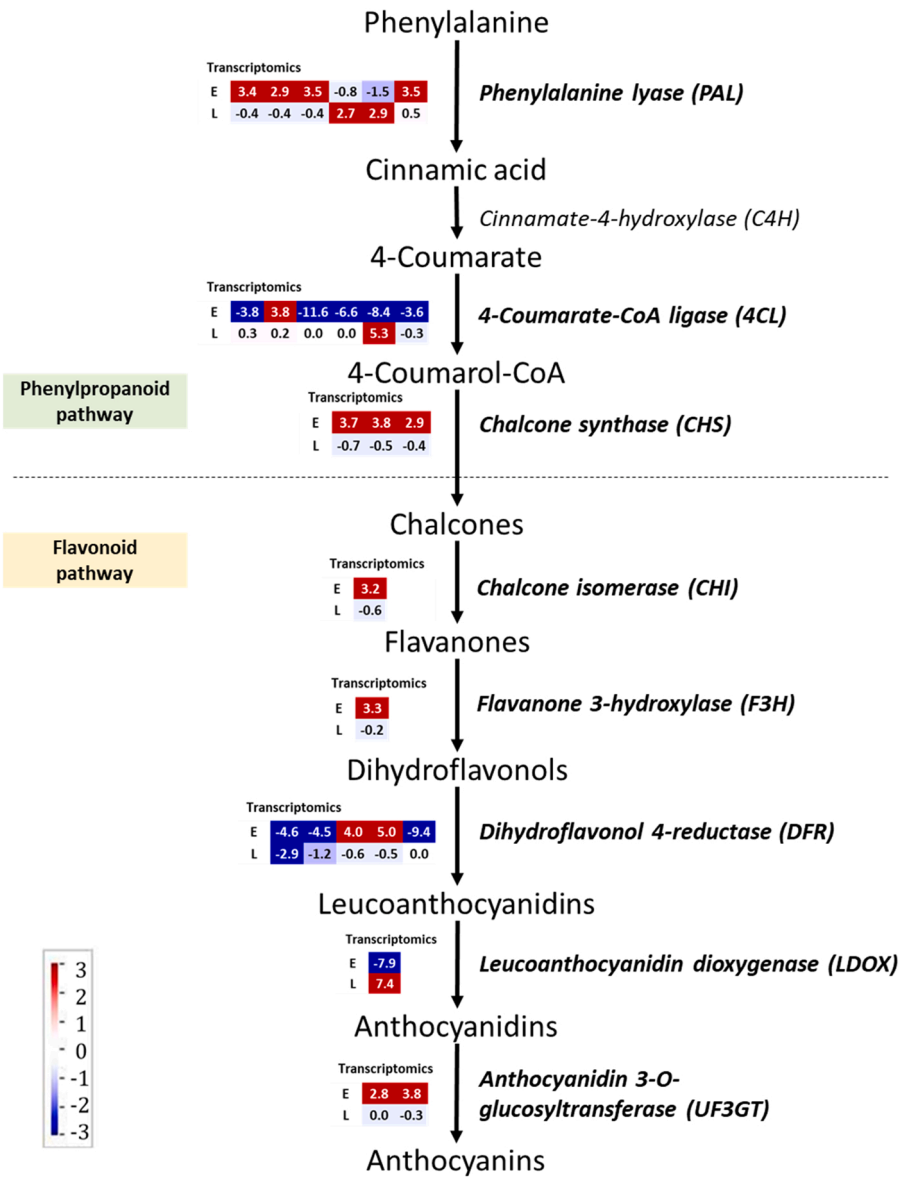

Fig. 7. Pathway mapping of anthocyanin biosynthesis via phenylpropanoid and flavonoid pathway in mangosteen. Bold gene names indicate identified in transcriptomics datasets. Genes with expression indicate differentially expressed transcripts at E = early ripening (S0vsS2), or L = late ripening (S2 vs S6). The expression was represented in Log_2FC. Red or blue indicates up- or down-regulation, respectively.

(*CYP707A*) and *protein-phosphatase 2C* (*PP2C*) that were involved in ABA signaling were upregulated during late ripening. Similarly, transcripts relating to gibberellin (GA) were mostly downregulated at early including *gibberellin 3-beta-dioxygenase* (*GA3ox*), *gibberellin 20 oxidase* (*GA20ox*), and *gibberellin-regulated protein* (*GRP*) while *gibberellin receptor* (*GIB1*) were upregulated indicating increased sensitivity towards GA signaling despite a reduction of transcripts relating to GA biosynthesis at early ripening. Interestingly, Mamat et al. (2020) reported an increase for abscisic acid (ABA) and gibberellin (GA) in pericarp with the highest reported at Stage 2 before decreasing towards the end of ripening, contrary to our transcriptomics analysis. This may be due to post-translational modification as well as possible crosstalk signaling between tissues in mangosteen, which requires further investigation. In short, the regulation of phytohormones play important roles in modulating physiological changes during mangosteen ripening, including anthocyanin biosynthesis as well as a diverse range of secondary metabolites (Kumar et al., 2014; Li et al., 2018; Liu et al., 2021a).

4.2. Energy metabolism during fruit ripening

Although fruit ripening and senescence are active processes, the expression of most genes related to energy-generating pathways such as glycolysis, TCA, and oxidative phosphorylation (OPP) decreased during early ripening (Fig. 5). Similarly, several fermentation-associated genes including *alcohol dehydrogenase* (*ADH*), *pyruvate decarboxylase* (*PDC*), and *lactate dehydrogenase* were also found to be downregulated (Table S3). However, as ripening progresses from S2 onwards, glycolysis-related genes such as *pyrophosphate-fructose 6-phosphate 1-phosphotransferase subunit alpha* (*PFP*) and *phosphofructokinase* (*PFK*) showed positive regulation coinciding with *ACS* upregulation at late ripening. PFK is involved in catalyzing fructose 6-phosphate to fructose 1,6-biophosphate which is one of the rate-limiting steps of glycolysis (Yao and Wu, 2016). This induces glycolysis by feeding glucose into the glycolysis and ultimately promoting the carbohydrate metabolism and accumulation of sugars as observed during the light treatment (Dong et al., 2019) and overexpression in tomato (Yu et al., 2022). Furthermore, metabolomics analysis further affirms active glycolytic activity at

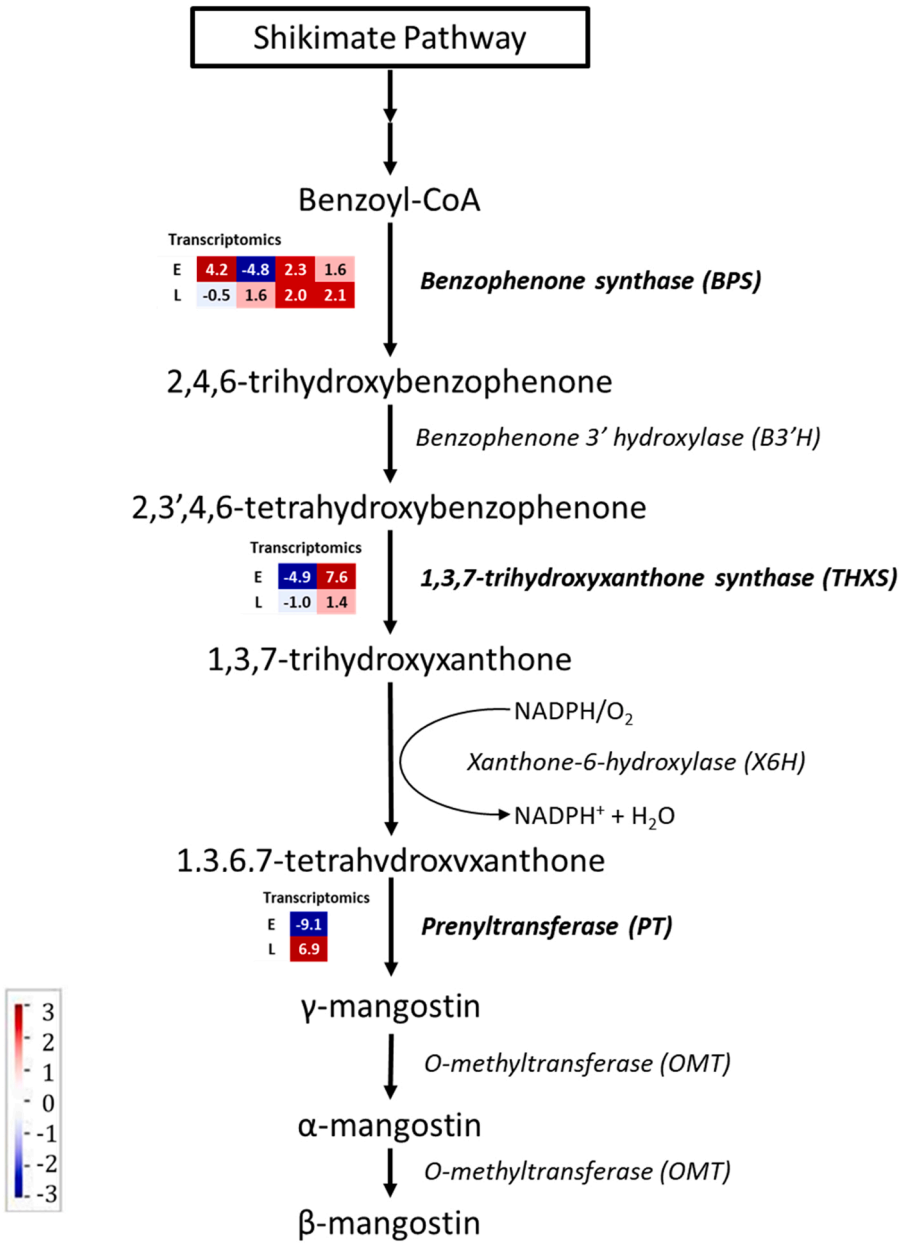

Fig. 8. Proposed pathway of xanthone biosynthesis in mangosteen modified from Remali et al. (2022). Bold gene names indicate transcripts identified in transcriptomics datasets. Genes with expression indicate differentially expressed transcripts at E = early ripening (S0vsS2) or L = late ripening (S2vsS6). The expression was represented in Log_2FC. Red or blue indicates up- or down-regulation, respectively.

late mangosteen ripening with the increase in D-glucose 6-phosphate, which is known to participate in the upstream part of glycolysis (Mamat et al., 2020). Moreover, PFP activity is tightly regulated in plants whereby fructose-2,6-biphosphate controls the regulation of PFP and even hampers PFP activity through overexpression (Basson et al., 2011). Therefore, the increase in *PFP* activity at late ripening may induce carbohydrate metabolism rather than affecting total sugar and organic acid content as observed in strawberries (Basson et al., 2011). Additionally, only a few transcripts related to OPP (e.g., *glucose-6--phosphate 1-dehydrogenase*, *G6PD*), glycolysis (e.g., *pyruvate kinase and glyceraldehyde-3-phosphate dehydrogenase*), and TCA (e.g., *citrate lyase*) were upregulated during early ripening, implying that the fruit was focusing on carbon conservation rather than energy generation at this point, as seen by the rise in *PFP* and *PFK* (Li et al., 2019b).

Other than regulating energy-producing pathways such as glycolysis, plants also produce energy and organic compounds via photosynthesis. During mangosteen ripening, the majority of genes involved in photosystems were downregulated, whereby a similar trend was observed at the protein level as well (Jamil et al., 2021). Transcripts of *chlorophyll a-b binding protein* (*CBP*) and *PsbP domain-containing protein 4* (*PPD4*) were considerably elevated early in the ripening process before declining later. As *CBP3* and *PPD4* are both apoproteins in photosystem II (PSII), their upregulation suggests pericarp sensitivity to light, promoting PSII excitation to oxidize water and transfer electrons to photosystem I (PSI) for NADPH synthesis, followed by the generation of ATP (Liu et al., 2013). The Calvin-Benson cycle uses these chemical energies to fix carbon dioxide and produce intermediary sugar phosphate and, finally, glucose (Bassham and Lambers, 2020).

4.3. Pericarp adaptaion to stress during ripening

The physiological changes occurred during fruit ripening involve a

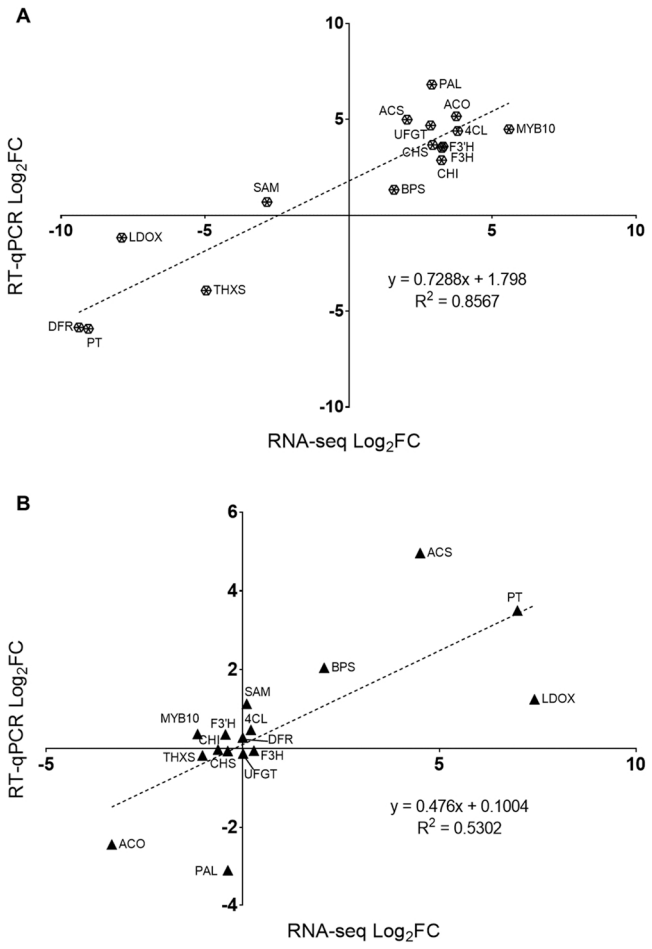

Fig. 9. Correlation analysis between RT-qPCR and RNA-seq during early (A) and late (B) ripening.

multitude of physiological as well as biochemical changes that may trigger stresses in plant. Pathogenic attacks and wounds towards the plant or fruit may also cause stress and responded by regulating phytohormones (e.g., methyl jasmonate) and gene/enzymes (e.g., pathogenesis-related protein) to protect the fruit during ripening. Stress due to the production of reactive oxygen species (ROS), which is a byproduct of biochemical reaction, is also common during fruit ripening whereby excessive build-up results in a damaging cellular oxidative environment. For example, an increased expression of *glutathione S-transferase* (*GST*), which is a detoxification enzyme, was observed in this study as well as at the protein level during early ripening, suggesting an early defense mechanism occurred concomitant with the increase of antioxidant activity in mangosteen pericarp (Jamil et al., 2021).

Aside from gene expression relating to stress response, mangosteen may regulate metabolites with antifungal/antibacterial properties (e.g., phenolics, flavonoids, and terpenoids) as part of the plant defense mechanism (Aizat et al., 2019b; Mamat et al., 2020). For instance, phenolic compounds such as anthocyanin are involved in color development during fruit ripening. Climacteric fruit such as tomato, banana as well as mangosteen acquired bright green at the beginning of ripening stages and progresses to orange/red, yellow, and purple respectively at the end of ripening due to chlorophyll degradation as well as build-up of color pigments (e.g., anthocyanin and carotenoids). The accumulation of cyanidin derivatives such as cyanidin-3-sophoroside and cyanidin-3-glucoside contributes to the red/purple pigments in mangosteen pericarp (Palapol et al., 2009b). The surge in ethylene during ripening signals the ethylene response factor (ERF) to induce ripening-related genes and TFs such as *MYB*, *bHLH*, and *WD40*, which have been associated with promoting anthocyanin production in various fruits (An et al., 2020; Kapoor et al., 2022). In mangosteen, both *ERF* and *MYB* were upregulated during mangosteen ripening whereby several studies associated the ERF-MYB complex with anthocyanin regulation in fruit (An et al., 2020; Yao et al., 2017). The *MYB10* upregulation found in this study may have promoted the expression of anthocyanin-related genes such as *PAL*, *CHS*, *CHI*, and *UF3GT*, likely to prepare the fruit and boost anthocyanin synthesis, resulting in a color shift from light green to dark purple. Similarly, *MYB113* was also reported to regulate anthocyanin synthesis in apple (Lin-Wang et al., 2011) and Arabidopsis (Gonzalez et al., 2008), suggesting similar roles in both *MYB113* and *MYB10*. The color shift in mangosteen pericarp also correlates with the accumulation of various secondary metabolites such as xanthones and their derivatives reported to be abundant in the fruit (Gonzalez et al., 2008; Xiao et al., 2018). In addition to promoting color change in mangosteen pericarp, anthocyanin also possesses potential health-benefiting properties including anti-inflammatory, anti-carcinogenic, protection against cardiovascular diseases, and diabetes (He and Giusti, 2010).

Another phenolic compound that is abundantly found in mangosteen is xanthone whereby more than 80 xanthone derivatives have been found in mangosteen alone and possess not only antioxidant attributes but other pharmaceutical activities including antifungal, antiviral, antitumor, and anticarcinogenic properties (Aizat et al., 2019b; Ovalle-Magallanes et al., 2017). Among the xanthone derivatives identified using the metabolomics approach include β-mangostin, gartanin, γ-mangostin, 9-hydroxycalabaxanthones, and α-mangostin whereby α-mangostin is the most prevalent of all (Do and Cho, 2020; Pham et al., 2019; Tjahjani et al., 2014). The proposed xanthone biosynthetic pathway in plants has been modelled from several xanthone-producing plants including *Garcinia mangostana* (mangosteen), *Gentiana lutea, Hypericum androsaemum,* and *Centaurium erythraea* (Remali et al., 2022). Some of the key genes in this pathway include *benzophenone synthase* (*BPS*), *benzophenone 3' hydroxylase* (*B3'H*), *1,3,7-trihydroxyxanthone* (*THXS*), *xanthone-6-hydroxylase* (*X6H*), *prenyltransferase* (*PT*), and *O-methyltransferase* (*OMT*) (Fig. 8) (Jamil et al., 2020; Khattab and Farag, 2020; Remali et al., 2022).

In this study, *BPS*, *THXS* and *PT* have been identified from the transcriptome data. The upregulation of these genes in mangosteen during ripening (Fig. 8) perhaps promoted the production of various xanthone derivatives as reported in a previous metabolomics study (Mamat et al., 2020). Interestingly, BPS was reported to decrease at the protein level (Jamil et al., 2021) suggesting post-translational modification during ripening to regulate the protein expression level (Spoel, 2018). Some xanthones such as α-mangostin and cratoxyarborenone C were indeed reduced in pericarp during similar ripening stages investigated in this study (Mamat et al., 2020).

Additionally, xanthone has been found abundantly in pericarp tissue as compared to aril or seed (Mamat et al., 2020). As xanthone biosynthesis differs between species, identification of these enzymes including their spatial and temporal expression as well as their regioselectivity and substrate preference are crucial to provide insights into the biochemical activity of any species. This is evident in the BPS preference to benzoyl-CoA in mangosteen for xanthone skeletons while 3-hydroxylbenzoyl-CoA is preferred for *Centaurium erythraea* (Belkheir et al., 2016; El-Seedi et al., 2010; Nualkaew et al., 2012; Remali et al., 2022). Nonetheless, the expression of transcripts relating to ROS scavenging system as well as the production of antioxidant metabolites/compounds (e.g., anthocyanin and xanthone) via the metabolic route, suggest that fruit ripening is indeed a molecular intensive process.

5. Conclusion

This study reported the assembly of mangosteen pericarp transcriptome from three different stages (covering early to late ripening). Most of the transcripts have unknown functions, as expected for a non-model organism. The regulation of DETs showed a shift in metabolic

activity including energy metabolism whereby the fruit may focus on carbon conservation at early ripening to produce secondary metabolites. Nevertheless, ripening is a molecular intensive process as evident in the increased expression of stress-response genes. Another stress-coping mechanism includes the accumulation of non-enzymatic antioxidants through the synthesis of various secondary metabolites. Additionally, genes and their expression relating to xanthone biosynthesis were successfully identified and mapped in mangosteen. Even so, understanding metabolic function and regulatory pathways is essential to highlight the biochemical basis of the ripening events and hence this should be thoroughly investigated in the future. In conclusion, this study provides a valuable molecular reference for future mangosteen studies, especially in the field of synthetic biology to bioengineer the production of valuable compounds such as xanthones for biomedical applications.

CRediT authorship contribution statement

Wan Mohd Aizat: Conceptualization, Visualization, Writing – Review & Editing, Supervision.: **Ili Nadhirah Jamil:** Writing- Original draft preparation, Validation, Formal analysis, Visualization.: **Azhani Abdul-Rahman:** Data-curation, Investigation, Methodology, Formal analysis, Visualization.: **Hoe-Han Goh:** Writing – Review & Editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Transcriptome sequences were deposited to NCBI under BioProject ID PRJNA339916.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.postharvbio.2023.112257.

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