The 6th International Conference on Biochemistry and Molecular Biology

Dynamic expression of sulfate transporter genes in durian flesh during fruit ripening

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Abstract:

Durian (*Durio zibethinus*) is a famous fruit for its very strong odor which is composed of numerous sulfur-containing compounds. Since sulfur is an essential macronutrient, plants take sulfate from soil via root and translocate to other parts. This process is facilitated by several members of the sulfate transporter (SULTR). However, the genes involved in sulfate transport system in fruits have not been revealed. Here, we investigated the dynamic expression of *SULTR*s in durian fleshes at on-tree developmental stages (immature and mature) and postharvest ripening (unripe, mid-ripe, and ripe) of Chanee cultivar. We found seven putative *SULTR*s in our in-house transcriptome, matched with the sequences in the durian genome database derived from the Musang King cultivar. A phylogenetic analysis showed that all isoforms are clustered into four groups which exhibit different subcellular localization. The gene expression analysis, determined by both RNA-seq and real-time reverse transcription-quantitative PCR, suggests that the plasma membrane-localized *DzSULTR1;2* might facilitate sulfate transport into flesh cells since its transcript level increased continuously during the early ripening process. Subsequently, sulfate is reduced to sulfur-containing metabolites, corresponding with our observation on an increased cysteine level in the ripe fruit. Our results provide additional information on sulfate transport in the fruit.

Introduction:

Sulfur is considered as an essential element required for plant growth and development.[1] It is used for the biosyntheses of sulfur-containing amino acids (cysteine and methionine), vitamins, coenzymes, and various secondary metabolites such as glucosinolates and sulfoflavonoids.[2,3] Plants acquire the essential sulfur as sulfate from soil and distribute to other organs. During growth and development, sulfate is reserved in the vacuoles of source organs and can be remobilized through the phloem to the expanding organs.[4]

Various sulfate transporters (SULTRs) are encoded by a large family of genes. These SULTRs, exhibiting different sulfate affinities and localizations, are responsible for sulfate assimilation and accumulation in cells.[5,6] They can be divided into four main groups which are high-affinity transporters group 1, low-affinity transporters group 2, diverse transporter function group 3, and vacuolar sulfate exporters group 4.[7] Previous studies indicated that SULTRs consist of 12, 12, 16, 28, and 8 members in Arabidopsis, rice, poplar, soybean, and maize, respectively.[8,9,10,11,l2]

The SULTR family is well characterized in Arabidopsis. Three plasma membrane-localized AtSULTR1;1, AtSULTR1;2 and AtSULTR1;3 were reported to encode high-affinity sulfate transporters. AtSULTR1;1 and AtSULTR1;2 are co-localized in roots and play a role

in the sulfate uptake from soil.[13] *AtSULTR1;1* mRNA is abundantly expressed under sulfur deficiency, while *AtSULTR1;2* mRNA is more prominent in a wide range of sulfur conditions.[13] AtSULTR1;3 is phloem-specific sulfate transporter related to source-to-sink translocation of sulfur nutrient.[4] Low-affinity sulfate transporters, AtSULTR2;1 and AtSULTR2;2 are involved in sulfate transport regulation in vascular tissue of roots and leaves.[14,15] Lastly, tonoplasts-localized sulfate transporter AtSULTR4;1 and AtSULTR4;2 facilitate the efflux of sulfate from the vacuoles into the cytoplasm.[16]

Durian (*Durio zibethinus* Murr.) is well-known as a fruit that releases very strong odor during ripening. The strong odor consisting of high intensity of sulfur-containing volatiles was reported.[17,18,19] It has been reported that sulfur-containing volatiles were derived from methionine degradation by the activity of methionine gamma-lyase into methanethiol, an important precursor for sulfur volatile biosynthesis.[20,21] These observations suggest that sulfate must be translocated and accumulated in the fruit. To date, sulfate transport encoded by the family of the genes has been characterized in various plants leading to understand sulfate assimilation. However, this process in fruit which is an important sink organ is rarely investigated. Hence, durian fruit could be considered as a fruit model to comprehend sulfate assimilation and also sulfur metabolism. Here, we identified sulfate transporters found in durian fleshes and analysed their expressions during fruit ripening.

Methodology:

Plant materials and sample preparation

Durian fruit used in this study were collected from an orchard in Trat province located in the eastern part of Thailand in April, 2017. Five stages of fruit were considered, including two on-tree developmental (immature and mature) and three postharvest ripening (unripe, mid-ripe and ripe) stages. Four fruit at each stage was obtained representing four replicates. The immature and mature stages were collected at 70 and 90 days after anthesis (DAA), respectively. For postharvest ripening, all fruit were harvested at commercial maturity stage (90 DAA). The unripe stage, fruit were left at 30°C for one day. At the mid-ripe and ripe stages, they were left until reached firmness of 3.4 ± 0.81 N and 1.55 ± 0.45 N, respectively. When the fruit reached the considered stages, the fleshes were collected, immediately frozen in liquid nitrogen, freeze-dried, ground into powder, and used for gene expression and metabolite analysis.

Chemicals

All organic solvents and chemicals for High-Performance Liquid Chromatography (HPLC) were obtained from Sigma (St. Louis, MO).

HPLC

To determine cysteine, metabolite extraction and the combination of mono-bromobimane fluorescent labeling and HPLC were performed.[22,23] Then, the concentrations were calculated from the standard curve for absolute quantification.

Phylogenetic analysis

The phylogenetic tree was inferred using the neighbor-joining method and conducted using MEGA7 with 1,000 bootstrap replicates.[24,25] Amino acid sequences of durian sulfate transporter (DzSULTR) were obtained from blasting nucleotide sequences of in-house transcriptome against NCBI database (http://www.ncbi.nlm.nih.gov) using durian genome derived from Musang King cultivar. All sequences in this study were retrieved from the NCBI database under the following GenBank accession numbers for *Durio zibethinus* 'Musang King': XP_022744196.1 (DzSULTR1;2), XP_022743635.1 (DzSULTR1;3), XP_022740513.1 (DzSULTR2;1a), XP_022752025.1 (DzSULTR2;1b), XP_022753085.1

(DzSULTR2;2), XP_022771139.1 (DzSULTR3;1a), XP_022722611.1 (DzSULTR3;1b), XP_022725854.1 (DzSULTR3;3), XP_022724887.1 (DzSULTR3;4a), XP_022714932.1 (DzSULTR3;4b), XP_022720785.1 (DzSULTR3;4c), XP_022731117.1 (DzSULTR3;5a), XP_022751704.1 (DzSULTR3;5b), XP_022742422.1 (DzSULTR4;1), and XP_022765231.1 (DzSULTR4;2), *Arabidopsis thaliana*: NP_192602 (AtSULTR1;1), NP_565166 (AtSULTR1;2), NP_564159 (AtSULTR1;3), NP_196580 (AtSULTR2;1), NP_565165 (AtSULTR2;2), NP_190758 (AtSULTR3;1), NP_192179 (AtSULTR3;2), NP_173722 (AtSULTR3;3), NP_188220 (AtSULTR3;4), NP_568377 (AtSULTR3;5), NP_196859 (AtSULTR4;1), and NP_187858 (AtSULTR4;2), *Glycine max*: XP_003526594 (GmSULTR1;1a), XP_003526594 (GmSULTR1;1b), XP_003532966 (GmSULTR1;2a), XP_003547605.1 (GmSULTR1;2b), XP_003543770.2 (GmSULTR1;3a), XP_006596866.1 (GmSULTR1;3b), XP_003531364 (GmSULTR2;1a), XP_003538517 (GmSULTR2;1b), XP_003552820 (GmSULTR2;1c), XP_003526596 (GmSULTR2;2a), XP_003544185 (GmSULTR2;2b), XP_003543772 (GmSULTR2;2c), XP_006601860 (GmSULTR2;3), XP_003521258 (GmSULTR3;1a), XP_003554265 (GmSULTR3;1b), XP_003518908 (GmSULTR3;2a), XP_003536673 (GmSULTR3;2b), XP_003529415 (GmSULTR3;3a), XP_003556073 (GmSULTR3;3b), XP_003528541 (GmSULTR3;3c), XP_003529722 (GmSULTR3;4a), XP_003531685 (GmSULTR3;4b), XP_003543650 (GmSULTR3;4c), XP_003546346 (GmSULTR3;4d), XR_136691 (GmSULTR3;5a), XR_416059 (GmSULTR3;5b), XP_003520027 (GmSULTR4;1), and XP_003552670 (GmSULTR4;2), *Oryza sativa*: NP_001049259 (OsSULTR1;1), NP_001049261 (OsSULTR1;2), BAC98594 (EMBL accession number) (OsSULTR1;3), NP_001049258 (OsSULTR2;1), NP_001049257 (OsSULTR2;2), NP_001064623 (OsSULTR3;1), NP_001049042 (OsSULTR3;2), NP_001054098 (OsSULTR3;3), NP_001056778 (OsSULTR3;4), NP_001172445 (OsSULTR3;5), NP_001044083 (OsSULTR3;6), and NP_001062644 (OsSULTR4;1), *Populus tremula* × *P. alba*: ABK35751.2 (PtaSULTR1;1), ABB59580.1 (PtaSULTR1;2), ABK35753.1 (PtaSULTR2;1a), ABK35755.1 (PtaSULTR2;1b), ABB59581.1 (PtaSULTR2;2), ABB59578.1 (PtaSULTR3;1a), ABK35750.1 (PtaSULTR3;1b), ABB59577.2 (PtaSULTR3;2a), ABK35756.1 (PtaSULTR3;2b), ABK35746.2 (PtaSULTR3;3a), ABK35748.1 (PtaSULTR3;3b), ABB59575.1 (PtaSULTR3;4a), ABB59574.1 (PtaSULTR3;4b), ABK35749.1 (PtaSULTR3;5), ABK35752.1 (PtaSULTR4;1), ABK35757.1 (PtaSULTR4;2), *Zea mays*: XP_008648264 (ZmSULTR1;1), EU974789 (ZmSULTR1;2), NP_001296802 (ZmSULTR2;1), NP_001141114.1 (ZmSULTR3;1), NP_001169671 (ZmSULTR3;3), NP_001148179 (ZmSULTR3;4), ONM38590 (ZmSULTR3;5), and NP_001306629.1 (ZmSULTR4;1).

RNA isolation

Total RNA of durian flesh tissue was extracted and purified using Purelink Plant RNA Reagent (Invitrogen™) according to the manufacturer's instruction. Genomic DNA was eliminated by treatment with DNase I (Thermo Scientific™). The quality and quantity of RNA samples were assessed with the ratios of A260/280 and A260/230 between 1.8 to 2.0 and 2.0 to 2.2, respectively.

Illumina sequencing

Equal amounts of total RNA from four fruit at each stage (unripe and ripe) were pooled. Messenger RNA isolated by oligo (dT) was used as templates for cDNA synthesis. Then the cDNA libraries were sequenced using Illumina HiSeq 4000 platform. After sequencing, low-quality, adaptor-polluted and high content of unknown base reads were filtered. Next, the de novo assembly with filtered reads were performed.

De novo assembly and annotations

All the assemblies were performed on a Trinity (version: v2.0.6), consisting of three software modules including Inchworm, Chrysalis, and Butterfly.[26] The result sequences of Trinity are called transcripts. Then, Tgicl was used to perform gene family clustering called Unigenes.[27] The Unigenes were aligned and annotated using Blast (version: v2.2.23 with default parameters) against NT, NR, COG, KEGG and SwissProt databases.[28]

Differentially Expressed Gene (DEG) analysis

To investigate significant differential expression of genes in RNA-sequencing between two stages of durian, DEG analysis was performed using PossionDis software.[29] The parameters, Fold Change ≥ 2.00 and FDR ≤ 0.001, were used to select the genes.

Expression analysis of sulfate transporter genes

To facilitate the real-time reverse transcription-quantitative PCR (real-time RT-qPCR) analysis, all genes were investigated under the same reaction condition. All primer sequences in this study (forward primers (5′→3′): *DzSULTR1;2*: ACTCATCACTCCTCTGTTC, *DzSULTR1;3:* CACCTGTAACTGACATTGATAC, *DzSULTR3;1a*: TGACGAAGA GGAAGACAAG and *DzSULTR3;1b*: CAGATAGATGCGCCTGTTTAC and reverse primers (5′→3′): *DzSULTR1;2*: AAGAAGGCTCCCATACAG, *DzSULTR1;3:* CCAGGATTTGCCAGAATAAG, *DzSULTR3;1a*: GGTGTCAATGTTACCAACAG and *DzSULTR3;1b*: CCACGTTAGGTAGCTTATGAAAG) were designed according to nucleotide sequences of our in-house transcriptome data. One microgram of total RNA was transcribed as cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific™) according to the manufacturer's instructions. The PCR reaction contained 1 µl of diluted cDNA corresponding to 1 ng of cDNA, 5 µl of 2x SsoFast™ EvaGreen® Supermixes (Bio-Rad Laboratories Inc., USA), and 200 nM of each gene-specific primer in a final volume of 10 µl. Amplification of real-time RT-qPCR products was carried out with a CFX95 Real-time System (Bio-Rad Laboratories Inc., USA) under the following conditions: enzyme activation at 98°C for 30 sec, followed by 40 cycles of denaturation at 98°C for 5 sec and annealing/extension at 58°C for 5 sec. A melting curve analysis for testing specific products was obtained by heating the products from 55°C to 95°C in increment of 0.5°C. Four biological replicates for each sample were used for real-time RT-qPCR analysis. *Elongation factor 1 alpha* (*EF-1α*) gene was used as a reference gene. The relative expression of each gene compared with the reference gene was calculated using $2^{-\Delta Ct}$ method.[30]

Results and Discussion

To our knowledge, this is the first report on the expression analysis of the *SULTR* genes in any fruits. To investigate the evolutionary relationship of SULTRs, the comparison of the phylogenetic distribution of SULTR in *D. zibethinus* and the other species was performed. A phylogenetic tree was constructed using MEGA7 with Neighbor-Joining method and 1,000 replicates bootstrap (Figure 1). Fifteen putative SULTR proteins were obtained from durian genome database derived from Musang King cultivar.[20] Among these SULTRs, seven putative isoforms, labeled as (●) in Figure 1, were matched in our in-house transcriptome derived from Chanee cultivar. Therefore, these seven isoforms might be considered as fruit specific isoforms. The phylogenetic analysis clearly demonstrated that DzSULTRs can be classified into four groups based on their basic structural features which are clustered with SULTR proteins from Arabidopsis, soybean, rice, poplar and maize (Figure 1).[8,9,10,12,31] Thus, orthologs of the SULTRs could be identified and their functions might be the same under different sulfur nutritional conditions. Group 1 included AtSULTR1;1, AtSULTR1;2, AtSULTR1;3, GmSULTR1;2b and ZmSULTR1;2 which have been characterized and involved in sulfate uptake.[4,12,13] Two durian sulfate transporters, namely DzSULTR1;2, and DzSULTR1;3, also

belong to this group (Figure 1). DzSULTR2;1a, DzSULTR2;1b and DzSULTR2;2 were clustered in group 2 (Figure 1). Group 2 SULTRs encode low-affinity transporters.[15] *AtSULTR2;1* was expressed in the xylem parenchyma and pericycle cells of root and in the phloem of leave suggested to mediate sulfate uptake from the apoplast within the vascular bundle.[15] AtSULTR2;2 was also localized in root phloem and leave vascular bundle. Both of them involved in the distribution of sulfate from vascular bundle to the palisade cells.[14] In contrary, *ZmSULTR2;1* transcript was highly expressed in tassels of maize.[12] The large family group 3 SULTR is composed of DzSULTR3;1a, DzSULTR3;1b, DzSULTR3;3, DzSULTR3;4a, DzSULTR3;4b, DzSULTR3;4c, DzSULTR3;5a, and DzSULTR3;5b (Figure 1). The reduction of sulfate uptake in chloroplast was found when loss of *AtSULTR3;1, AtSULTR3;2, AtSULTR3;3, and AtSULTR3;4* in Arabidopsis.[32] Furthermore, co-expression of *AtSULTR3;5* and *AtSULTR2;1* can enhance the activity of root-to-shoot sulfate transporter.[33] The last group consisting DzSULTR4;1 and DzSULTR4;2 were clustered in group 4 (Figure 1). This group was reported as vacuolar SULTR which promote sulfate efflux from vacuole and maintain sulfate concentration in cells.[16]

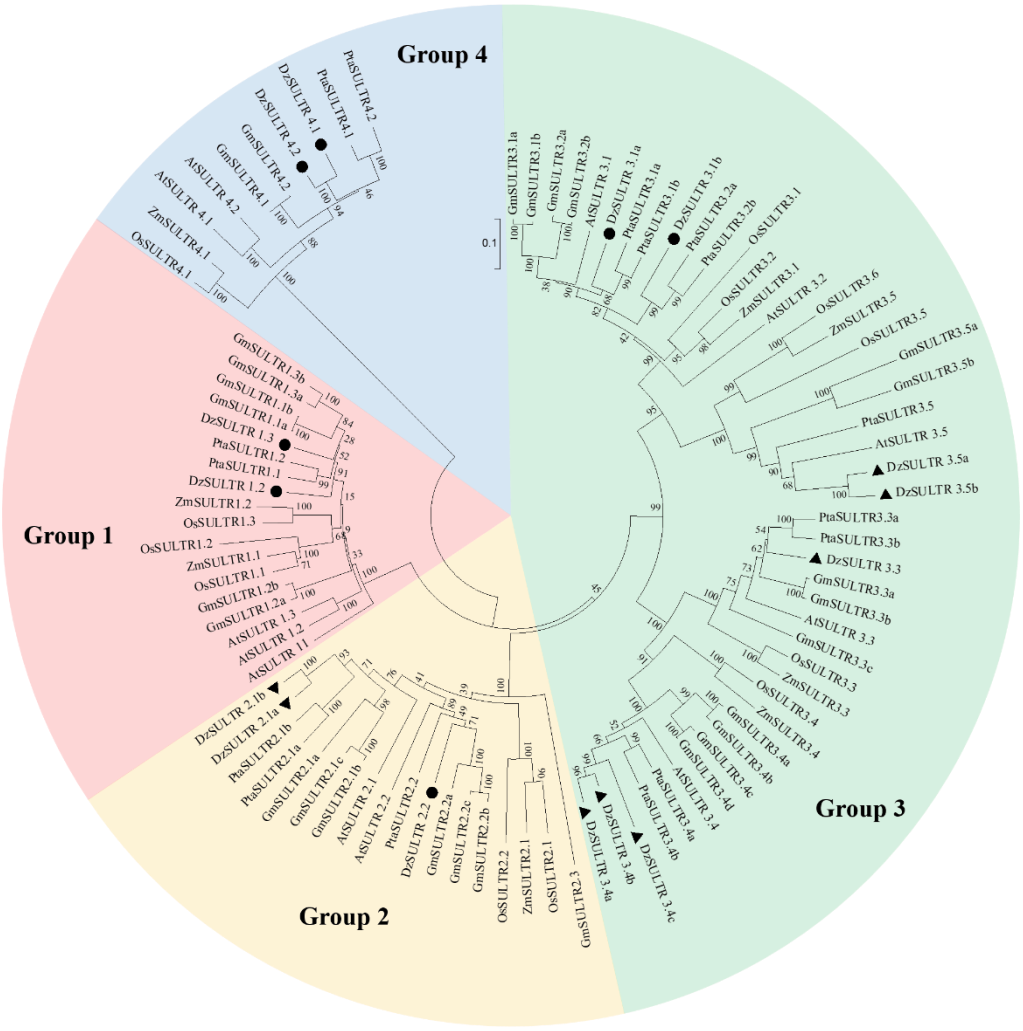

Figure 1. Phylogenetic tree of putative sulfate transporters (SULTRs) from *D. zibethinus* 'Musang king'. The DzSULTR family includes fifteen putative members and were clustering into four main groups. The isoforms matched with our in-house transcriptome data were labeled (●) including DzSULTR1;2, DzSULTR1;3, DzSULTR2;2, DzSULTR3;1a, DzSULTR3;1b, DzSULTR4;1, and DzSULTR4;2. The other isoforms of DzSULTRs found in the durian genome were also labeled (▲) in this figure. The tree was constructed by the neighbor-joining method. The bootstrap values were expressed as the percentage of 1,000 replicates.

To confirm the expression of *DzSULTR* genes, four genes (*DzSULTR1;2*, *DzSULTR1;3*, *DzSULTR3;1a* and *DzSULTR3;1b*), showing significant changes between unripe and ripe stages in gene expression according to our in-house transcriptome data, were selected and further confirmed their expressions by real-time RT-qPCR analysis. Dynamic of gene expression was observed during five ripening stages of durian fleshes including immature, mature, unripe, mid-ripe and ripe. The expression levels of *DzSULTR1;2*, *DzSULTR1;3*, *DzSULTR3;1a* and *DzSULTR3;1b* were changed during ripening (Figure 2). The expressions of plasma membrane-localized SULTRs group 1, *DzSULTR1;2*, *DzSULTR1;3*, were increased during postharvest ripening, reaching the maximum level at the mid-ripe stage and dramatically decreased at the ripe stage (Figure 2). According to previous reports, *AtSULTR1;2*, and *GmSULTR1;2b* were found to be localized in root cells and are related to sulfate uptake from the soil.[4,13] However, *ZmSULTR1;2* was predominantly expressed in the tassel of maize and might play the important role in sulfate uptake under sulfur deficiency during flower development.[12] Interestingly, *DzSULTR1;2* expression level was the highest among all transcripts studied. Hence, *DzSULTR1;2* might be the major functional isoform and possibly responsible for sulfate uptake across plasma membrane into flesh cells during postharvest ripening. The group 3 SULTRs, *DzSULTR3;1a* and *DzSULTR3;1b*, exhibited different expression patterns. The transcript level of *DzSULTR3;1a* was highly increased at the ripe stage while *DzSULTR3;1b* was not significantly different among three stages after harvesting (Figure 2). *DzSULTR3;1a*, *DzSULTR3;1b* and *AtSULTR3;1* belong to the same clade (Figure 1). Therefore, they might possess similar function as *AtSULTR3;1* which is localized at plastid membrane and responsible for sulfate transport into plastid, especially chloroplasts.[32]

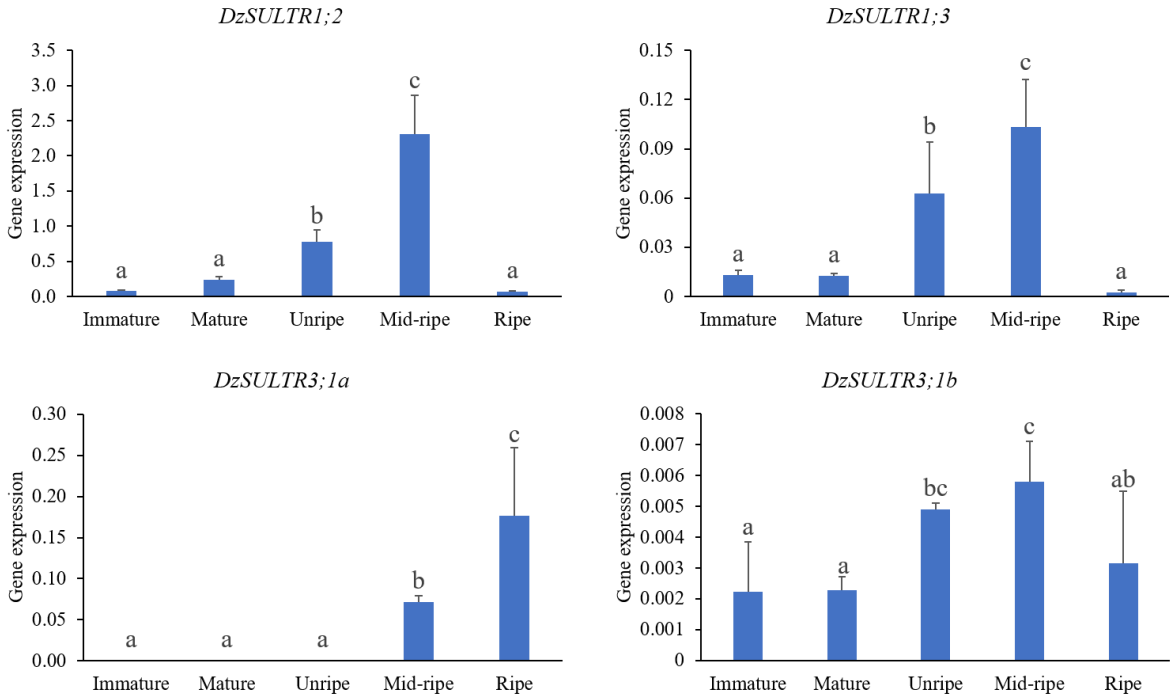

Figure 2. Relative expression of *DzSULTR* genes in durian fleshes during on-tree developmental stages (immature and mature) and postharvest ripening (unripe, mid-ripe, and ripe) was accomplished by real-time RT-qPCR analysis. ANOVA test (Duncan's test, $\alpha = 0.05$) was used for determining the statistical significant.

It has been reported that sulfate was absorbed and transported by various SULTRs into chloroplasts, where it is reduced and assimilated into cysteine.[32] Therefore, we further measured the amount of cysteine which is the key metabolite in sulfur metabolism at the mature, unripe and ripe stages using HPLC. As expected, cysteine level in the fleshes was

significantly increased at the ripe stage (Figure 3) corresponding with increased the *DzSULTR3;1a* transcript during postharvest ripening (Figure 2). Mutations of *AtSULTR3;1*, *AtSULTR3;2* and *AtSULTR3;3* caused the reduction of cysteine content in the seeds suggesting that these SULTRs might facilitate sulfate translocation between seed compartments.[34] Thus, the up-regulation of *DzSULTR3;1a* and *DzSULTR3;1b* correlated with increasing in cysteine content can imply that these genes might facilitate sulfate transport into plastids.

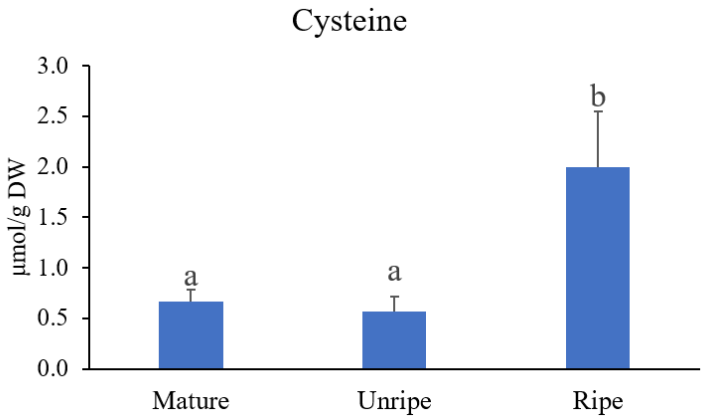

Figure 3. The amount of cysteine in durian fleshes during three ripening stages. Four biological replicates of each stage were used for cysteine measurement. ANOVA test (Duncan's test, $\alpha = 0.05$) was used for determining the statistical significant.

Conclusion

In summary, durian SULTRs were classified into four main groups. Dynamic expression of *SULTR* genes were investigated and found that four genes (*DzSULTR1;2*, *DzSULTR1;3*, *DzSULTR3;1a* and *DzSULTR3;1b*) were up-regulated after postharvest ripening concurring with the increased cysteine content at the ripe stage. Therefore, DzSULTR1;2 might be a major form involving in sulfate translocation across plasma membrane in the fruit. Moreover, DzSULTR3;1a and DzSULTR3;1b might play a crucial role in sulfate transport into plastids relating to cysteine biosynthesis during postharvest ripening of durian fruit. Our results provide the information of sulfate transport in durian fruit that is involved in the initial step for sulfur metabolism in the sulfur-rich fleshes of durian.

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Acknowledgements

This work was supported by the FY2018 Thesis Grant for Master Degree Student, National Research Council of Thailand.