

Optimizing RNA Isolation from Durian Leaves and Evaluating the Effect of DNase Treatment on RNA-seq Transcriptomes

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Abstract—RNA extraction from durian (*Durio zibethinus* L.) leaves is hindered by latex and abundant polyphenols and polysaccharides. Here, we optimized a TRIzol-based workflow specifically for durian leaves and systematically assessed whether a post-extraction DNase workflow alters mRNA-seq profiles under RIN-matched conditions. Of four workflows tested, only a modified TRIzol method incorporating Polyvinylpyrrolidone (PVP), β -mercaptoethanol, and isopropanol precipitation consistently produced high-purity, intact RNA, whereas DNase treatment reduced RNA yield. RIN-matched RNA samples generated comparable sequencing output and quality. Despite similar sequencing quality, Principal Component Analysis (PCA) clearly separated DNase-treated and non-treated libraries. DNase-associated differences were enriched in signaling and regulatory functions as well as stress- and defense-related pathways. Overall, this study provides a practical RNA extraction strategy for durian leaves and shows that DNase treatment can measurably shift isoform-level expression in RNA-seq analyses.

Keywords—Trizol, PVP, CTAB, transcriptome, mRNA sequencing

I. INTRODUCTION

Durian (*Durio zibethinus* L.) is a high-value tropical fruit crop in Southeast Asia, but RNA-based and transcriptomic studies are constrained by persistent technical barriers to RNA isolation. Durian leaf tissues contain abundant latex and high levels of secondary metabolites that can co-precipitate with RNA or bind nucleic acids after oxidation, resulting in low yield, poor purity, and inhibition of downstream enzymatic reactions. Similar matrix effects have been reported in other metabolite-rich plant tissues, where standard phenol/guanidinium-based reagents and some

commercial kits may fail or require substantial modification to consistently recover functional RNA [1].

High-quality, intact RNA is essential for accurate RNA sequencing (RNA-seq) and differential expression analysis. Carryover contaminants such as polysaccharides, polyphenols, and residual proteins can reduce library yield and complexity, inhibit PCR, and introduce bias in transcript quantification. For recalcitrant plant tissues, successful extraction protocols typically combine strong denaturants and RNase inhibitors with reagents that mitigate metabolite interference, for example, reducing agents (e.g., β -mercaptoethanol) to limit oxidation, Polyvinylpyrrolidone (PVP) to sequester phenolics, and optimized salt and precipitation steps to minimize polysaccharide carryover [1, 2].

A recurring practical concern in plant RNA-seq workflows is residual genomic DNA (gDNA) contamination after extraction. DNase treatment is commonly used to remove gDNA; however, its performance can be inconsistent when RNA preparations contain inhibitory carryover, and additional handling may reduce RNA recovery or integrity [3]. In stranded mRNA library preparation, poly(A) selection enriches mRNA before cDNA synthesis and can reduce the contribution of gDNA to sequencing reads, suggesting that pre-library DNase treatment may not always be required [4].

In this study, the standard TRIzol extraction protocol was modified by incorporating PVP directly during the tissue homogenization step. The addition of PVP facilitated the binding of oxidized phenolic compounds and reduced the formation of RNA–phenolic complexes, a common challenge in durian leaves due to their high mucilage and polyphenol content compared to many other plant species. Introducing PVP at the initial stage significantly improved RNA purity, yielding RNA of sufficient quality for RNA-seq analysis without requiring additional purification steps [5, 6].

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Therefore, this study aims to (i) establish a simple and efficient RNA extraction strategy suitable for durian leaf tissues and (ii) systematically compare transcriptomic profiles of *D. zibethinus* cv. Monthong RNA samples extracted using a TRIzol-based method with and without DNase treatment. By evaluating RNA-seq outputs under controlled RNA quality conditions, this work provides practical guidance for durian transcriptomics and clarifies whether routine DNase treatment materially influences expression profiles in metabolite-rich plant tissues.

II. MATERIALS AND METHODS

A. Extraction and Quality Assessment

Total RNA was extracted from durian (*Durio zibethinus* L.) leaf tissues using an optimized, multi-step protocol designed for metabolite-rich tissues. Four RNA extraction/precipitation combinations were initially evaluated: (i) CTAB extraction with LiCl precipitation, (ii) TRIzol extraction with LiCl precipitation, (iii) CTAB extraction with isopropanol precipitation, and (iv) TRIzol extraction with isopropanol precipitation (Table I), following previously described protocols [7–10].

Based on yield and consistency, TRIzol extraction with isopropanol precipitation was selected for further optimization. During tissue grinding, 0.01 g of PVP was added to bind phenolic compounds. After homogenization, 1000 μ L of TRIzol reagent was added, followed by the addition of 10 μ L β -mercaptoethanol (final concentration 1% v/v) as a reducing agent (v). RNA was then precipitated with isopropanol according to the manufacturer's protocol.

TABLE I. RNA EXTRACTION PROTOCOLS USED IN THIS STUDY

Method	Sample preparation	RNA isolation	RNA precipitation	Ref.
(i)	LN	CTAB	LiCl	[7]
(ii)	LN	TRIzol	LiCl	[8]
(iii)	LN	CTAB	Isopropanol	[9]
(iv)	LN	TRIzol	Isopropanol	[10]
(v)	LN + PVP	TRIzol + β -mer	Isopropanol	[11]
(vi)*	LN + PVP	TRIzol + β -mer	Isopropanol	[11]

β -mer and LN indicate β -mercaptoethanol and Liquid Nitrogen, respectively. * indicates DNase-treated with column purification.

For DNase treatment in method (vi), RNA was purified and treated with DNase I using a FARB Mini Column (Favorgen Biotech) according to the manufacturer's instructions.

RNA integrity was assessed using an Agilent 2100 Bioanalyzer. To ensure comparable RNA quality between treatments, only samples with RNA Integrity Number (RIN) ≥ 7 from both DNase-treated and non-DNase-treated groups were used for RNA-seq library preparation [12].

B. Experimental Design for Transcriptome Analysis

D. zibethinus cv. Monthong plants were used. The experiment followed a completely randomized design with two groups and three biological replicates per group

($n = 3$): (i) DNase-treated RNA and (ii) non-DNase-treated RNA.

Leaves were surface sterilized with 0.01% (w/v) sodium hypochlorite, rinsed with sterile distilled water, harvested, and immediately frozen in liquid nitrogen until RNA extraction.

C. cDNA Library Preparation and RNA Sequencing

Qualified RNA samples were submitted to U2Bio Co., Ltd. (Bangkok, Thailand) and processed by Macrogen Inc. (Seoul, South Korea) for library preparation and paired-end 150 bp (PE150) sequencing on an Illumina platform. Libraries were constructed using the TruSeq Stranded mRNA Library Prep Kit following the TruSeq Stranded mRNA Reference Guide (#1000000040498 v00). Approximately 6 Gb of sequence data were generated per sample. The service provider evaluated standard sequencing quality metrics, including Q20/Q30 (%), GC content, total bases, and total read counts, prior to data release.

D. Transcriptome Analysis

1) Quality control and read preprocessing

Raw paired-end reads were assessed with FastQC. Trimming and filtering were performed using Trimmomatic v0.39 with identical parameters for all samples: end trimming (quality threshold 3), sliding-window trimming (4 bp window; minimum average quality 15), and removal of reads shorter than 50 bp.

2) De novo transcriptome assembly

Quality-filtered reads were assembled *de novo* using Trinity v2.15.1 in non-strand-specific mode (minimum contig length 200 bp). *In silico* normalization was enabled (maximum coverage 200 $\times$). To reduce falsely fused transcripts, a maximum pair distance of 500 bp was applied. Additional parameters included minimum k-mer coverage of 1 (Inchworm), maximum reads per graph 200,000, minimum glue value of 2, and maximum cluster size of 25 (Chrysalis). Butterfly was run with a path reinforcement distance of 25, minimum identity of 98%, maximum differences of 2, and maximum internal gap of 10.

Assembly completeness was evaluated using BUSCO v5.6.0 with the Embryophyta dataset (OrthoDB v10) and a BLAST e-value cutoff of 1.0×10^{-3} . BUSCO results were reported as percentages of complete, duplicated, fragmented, and missing orthologs.

3) Transcript clustering and coding region prediction

Redundant transcripts were clustered using CD-HIT v4.8.1 (95% identity; bandwidth 20; word length 10). Coding regions were predicted with TransDecoder. Open reading frames (ORFs) ≥ 60 amino acids were retained using the universal genetic code. Pfam searches were enabled, and the retain long ORFs option was applied using the 500 longest ORFs as the training set.

4) Functional annotation and expression analysis

Functional annotation was performed using BLASTx (E-value $\leq 1 \times 10^{-5}$). Gene Ontology (GO) terms were

assigned and classified, and KEGG pathway annotation was conducted. Transcript abundance was estimated, and differential expression analysis was performed to compare DNase-treated and non-DNase-treated samples using identical parameters across all samples.

III. RESULT AND DISCUSSION

A. Performance of RNA Extraction Methods in Durian Leaf Tissue

Multiple RNA extraction protocols (methods i–vi) were evaluated for durian leaf tissues. CTAB–LiCl, TRIzol–LiCl, and CTAB–isopropanol failed to yield detectable RNA, whereas TRIzol–isopropanol successfully yielded detectable RNA (Lane 1–4). A modified TRIzol protocol incorporating PVP, β -mercaptoethanol, and isopropanol precipitation consistently produced intact RNA ($A_{260}/_{280} > 1.9$; $A_{260}/_{230} > 2.0$; Fig. 1, Lane 5). DNase treatment reduced RNA yield (Fig. 1, Lane 6). Importantly, the PVP-supplemented TRIzol method was performed without silica-based column purification, thereby minimizing RNA loss due to retention on the silica membrane matrix, a common issue in metabolite-rich tissues such as durian. Eliminating the column step better preserved overall RNA composition and reduced the risk of selective transcript loss prior to RNA-seq analysis.

B. Sequencing Output and Read Quality

Paired-end RNA-seq data (PE150) were generated for DNase-treated (+DNase01–03) and non-DNase-treated (–DNase01–03) samples. Sequencing yielded 7.01–8.32 Gb per sample (46.4–55.1 million reads), with comparable depth between groups. Total bases, read counts, and GC content (44.0–47.8%) did not differ significantly between DNase-treated and non-treated libraries (Welch's t-test, $p > 0.05$). All libraries showed high base-call quality (Q20: 98.7–98.9%; Q30: 95.0–95.7%). Overall, sequencing depth, nucleotide composition, and read quality were highly consistent

across treatments, supporting robust downstream assembly and expression analyses.

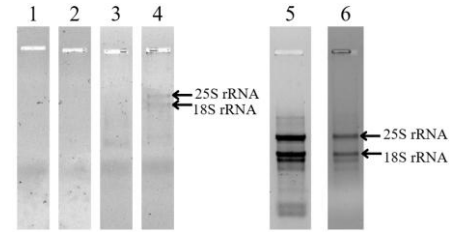


Fig 1. Agarose gel electrophoresis of total RNA extracted from durian leaf tissues using different extraction methods. Lane 1: CTAB + LiCl; Lane 2: TRIzol + LiCl; Lane 3: CTAB + isopropanol; Lane 4: TRIzol + isopropanol; Lane 5: modified TRIzol with PVP and β -mercaptoethanol without column purification; Lane 6: RNA from method (v) after DNase treatment with silica-based column purification. The positions of 25S and 18S rRNA are indicated.

C. Transcriptome Analysis

1) Quality control, preprocessing, and assembly

FastQC of raw paired-end reads showed consistently high per-base quality across all libraries. After adapter removal and quality filtering with Trimmomatic, per-base quality and quality profiles were highly comparable among samples.

De Novo Assembly of the six libraries produced 257,844 transcripts representing 164,925 putative genes, with a total assembled length of 223.5 Mb and GC content of 40.28% (Table II). Using the longest isoform per gene, the assembly had an N50 of 747 bp, mean transcript length of 613 bp, and median length of 385 bp. Read mapping back to the assembly was 52%–59% across samples, with similar alignment rates among libraries. Assembly completeness assessed by BUSCO (embryophyta_odb10) indicated 92.63% complete BUSCOs (19.64% single-copy; 72.99% duplicated), with 4.28% fragmented and 3.10% missing, supporting a high-quality and largely complete transcriptome.

TABLE II. SUMMARY OF SEQUENCING OUTPUT AND RAW READ QUALITY FOR DNASE-TREATED AND NON-DNASE-TREATED RNA SAMPLE

Sample ID	Total bases (bp)	Total reads	GC (%)	AT (%)	Q20 (%)	Q30 (%)
+DNase01	7,011,848,986	46,436,086	46.6	53.4	98.8	95.5
+DNase02	7,054,684,364	46,719,764	47.8	52.2	98.7	95.2
+DNase03	8,321,961,528	55,112,328	44	56	98.9	95.3
–DNase01	7,450,995,642	49,344,342	45.1	54.9	98.9	95.7
–DNase02	7,158,317,778	47,406,078	45.2	54.8	98.8	95.1
–DNase03	7,265,012,264	48,112,664	46.3	53.7	98.7	95

2) Global expression patterns assessed by PCA

Principal component analysis (PCA) of isoform-level expression data showed that PC1 and PC2 explained 34% and 23% of the total variance, respectively (Fig. 2). The non-DNase-treated libraries (–DNase01–03) clustered tightly and were separated from the DNase-treated libraries primarily along PC2. In contrast, DNase-treated samples (+DNase01–03) showed greater dispersion, indicating increased variability among libraries following DNase treatment. Overall, PCA suggests that DNase treatment is associated with a shift in global isoform-level

expression patterns. The increased data dispersion observed in the DNase-treated group is consistent with the possibility that the filter column step may introduce selective RNA loss. This effect may particularly impact structurally unstable transcripts, resulting in the preferential depletion of certain RNA populations. Consequently, the gene expression profiles of the column-purified (DNase-treated) samples exhibited greater variability compared to the non-column (non-DNase) samples. This interpretation is consistent with the observed reduction in RNA yield following

DNase-column treatment (Fig. 1), supporting the possibility of partial transcript loss during purification.

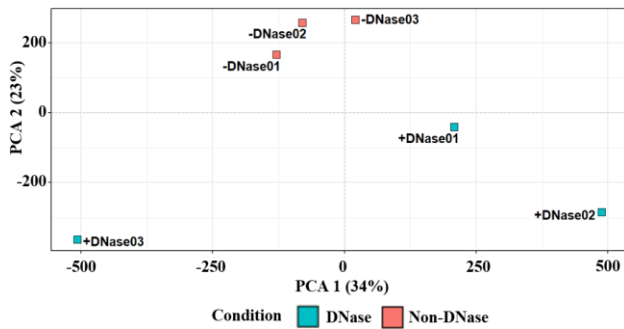


Fig 2. Principal Component Analysis (PCA) of isoform-level expression profiles from DNase-treated and non-DNase-treated samples. Each point represents one biological replicate. Principal components PC1 (34%) and PC2 (23%) explain the indicated proportions of total variance. Red squares represent non-DNase-treated samples (-DNase01-03), while blue squares represent DNase-treated samples (+DNase01-03). Separation between groups along PC2 indicates treatment-associated shifts in global isoform-level expression patterns.



Fig 3. Volcano plot of differential isoform expression between DNase-treated and non-DNase-treated samples. Each point represents an isoform. The x-axis shows log₂ fold change and the y-axis shows -log₁₀(FDR). Red points indicate significantly differentially expressed isoforms, while gray points indicate non-significant isoforms. Dashed lines mark significance and fold-change thresholds.

The volcano plot summarizes differential expression between DNase-treated and non-DNase-treated libraries (Fig. 3). Most isoforms were centered around log₂ fold change ≈ 0 and were not significant (grey points), indicating broadly similar expression for the majority of transcripts. However, a subset of isoforms exceeded the significance thresholds (red points) on both sides of the plot. Of 82,245 detected isoforms, 536 (0.65%) were significantly differentially expressed (FDR < 0.05, $|\log_2\text{FC}| \geq 1$), including 274 upregulated and 262 downregulated isoforms. These significant isoforms were concentrated at higher absolute fold changes, consistent with DNase treatment affecting the abundance of a discrete set of transcripts.

Hierarchical clustering of the differentially expressed isoforms revealed a clear separation between DNase-treated and non-DNase-treated libraries (Fig. 4). The three non-DNase samples clustered together and were distinct from the three DNase-treated samples, indicating a consistent treatment-associated expression signature. The heatmap also showed two major isoform clusters with opposing patterns: one cluster exhibited

higher normalized expression in non-DNase samples and lower expression in DNase-treated samples, while the other cluster showed the inverse trend. Overall, the clustering and reciprocal expression blocks support that DNase treatment is associated with systematic shifts in the expression of a defined subset of isoforms and that within-group profiles are reproducible across biological replicates.

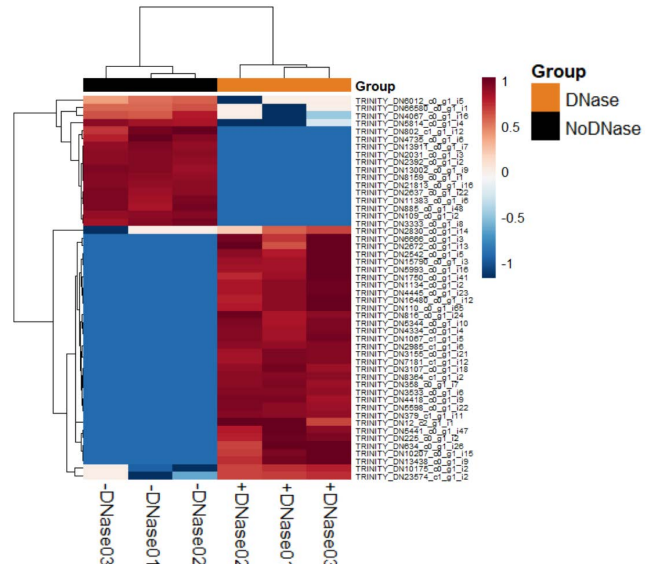


Fig 4. Heatmap of matched Differentially Expressed (DE) isoforms between DNase-treated and non-DNase-treated samples. Rows represent isoforms and columns represent biological replicates (+DNase01-03 and -DNase01-03). Expression values are shown as Z-scores scaled per isoform. Red indicates higher relative expression, blue indicates lower expression, and white represents values near the mean (Z-score ≈ 0). Hierarchical clustering (Euclidean distance) is shown as dendrograms for both isoforms and samples, with clear separation between DNase-treated and non-treated groups.

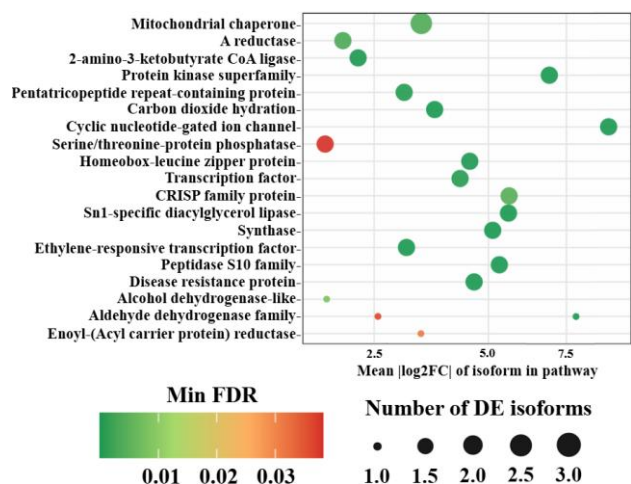


Fig 5. Pathway level summary of differentially expressed isoforms between DNase-treated and non-DNase-treated samples. Bubbles represent functional categories plotted by mean log₂ fold change, with size indicating the number of differentially expressed isoforms and color indicating the minimum False Discovery Rate (FDR).

Pathway-level summarization indicated that DNase treatment affected a number of isoforms (Fig. 5). The largest mean log₂ fold changes were observed for regulatory/ signaling-related categories, particularly

cyclic nucleotide-gated ion channels and the protein kinase superfamily, which also showed low minimum FDR values (green bubbles). Several additional regulatory groups (e.g., transcription factor-related categories and CDPK family proteins) showed intermediate-to-high mean log₂FC with similarly low FDR. In contrast, categories linked to core metabolic/housekeeping functions (e.g., serine/threonine-protein phosphatases, alcohol/aldehyde dehydrogenase family proteins, and enoyl- (acyl carrier protein) reductase) generally exhibited lower mean log₂FC values and, in some cases, higher minimum FDR (warmer colors). Overall, the figure supports isoform-specific quantitative modulation associated with DNase treatment, with the strongest effects concentrated in signaling and regulatory components.

High quality RNA is a prerequisite for reliable RNA-seq, and widely used guidelines emphasize RNA integrity assessment (e.g., Bioanalyzer RIN) and minimizing inhibitors and DNA carryover because these factors influence library yield and quantitative accuracy [13]. These requirements are particularly relevant for durian leaves, where latex and abundant polyphenols and polysaccharides complicate extraction and can inhibit downstream enzymatic steps. Accordingly, we optimized a TRIzol-based workflow for metabolite-rich tissues. The inclusion of PVP and β -mercaptoethanol is a well-established strategy for such matrices: PVP sequesters phenolic compounds, while β -mercaptoethanol maintains a reducing environment that limits phenolic oxidation and helps suppress RNase activity, thereby improving RNA purity and integrity [5, 6]. Based on our results, the incorporation of PVP into the TRIzol extraction step effectively addressed the intrinsic challenges associated with durian leaf tissues, which are characterized by exceptionally high levels of phenolic compounds and mucilage [5, 6]. Early removal of these interfering substances eliminated the need for additional column-based purification steps, which are often associated with substantial RNA loss in metabolite-rich plant species [7, 9]. Consequently, this modified approach yielded RNA of sufficient purity and quantity to support stable and reliable RNA-seq analysis [13].

We also evaluated whether post-extraction DNase treatment materially alters RNA-seq outcomes under controlled RNA quality (RIN-matched) conditions. Despite comparable sequencing output and base quality between treatments, PCA and hierarchical clustering consistently separate DNase-treated from non-treated libraries, indicating systematic treatment-associated shifts in global expression patterns. The observed differences in gene expression profiles between the DNase-treated and non-DNase-treated groups may, in part, be attributed to the silica-based column purification step applied in the DNase-treated samples. Column binding can result in uneven or selective RNA retention, potentially altering both the quantity and composition of RNA entering library preparation. As a consequence, the RNA-seq data derived from the DNase-treated group exhibited significantly greater dispersion compared to the non-

DNase-treated samples. Such global structure assessments are commonly used to diagnose sample-level differences and technical effects in RNA-seq datasets [14].

Importantly, only a small fraction of isoforms was differentially expressed and the affected isoforms remained detectable in both conditions, supporting quantitative modulation rather than selective transcript loss. A practical explanation is that DNase digestion and the associated cleanup/handling steps introduce proportional changes in recoverable RNA (e.g., partial loss during purification), which can propagate into isoform-level abundance estimates especially in difficult plant matrices [15].

From a workflow perspective, DNase treatment is often recommended to ensure RNA is free of genomic DNA [16]. However, in stranded mRNA library preparation, poly(A) selection is an explicit upstream step, which is expected to reduce genomic DNA carryover into the final library [4, 17]. Therefore, when RNA integrity and purity meet recommended thresholds and poly(A) selection is used, routine DNase treatment may provide limited additional benefit for expression profiling, while still posing a risk of reduced yield and subtle quantitative shifts. Collectively, our results suggest DNase treatment should be applied selectively and consistently based on study objectives (e.g., stringent DNA removal requirements) rather than automatically included in all durian leaves RNA-seq workflows.

IV. CONCLUSION

We established a simple and efficient RNA extraction strategy for durian (*Durio zibethinus* L.) leaf tissue by optimizing a TRIzol-based workflow for metabolite-rich samples, enabling consistent recovery of high-purity, intact RNA suitable for RNA-seq. Using RIN-matched RNA, we compared transcriptomic profiles generated with and without post-extraction DNase treatment. Although sequencing output and read quality were comparable between groups, DNase treatment reduced RNA yield and produced reproducible shifts in global isoform-level expression, affecting only a small subset of isoforms. Notably, the additional silica-based column purification step associated with DNase treatment may contribute to partial RNA retention or selective loss, potentially increasing variability in downstream RNA-seq data. Avoiding filter column purification throughout the workflow may therefore better preserve RNA integrity and transcript composition in metabolite-rich tissues such as durian leaves. Overall, these findings provide practical guidance for durian transcriptomics and show that routine DNase treatment can measurably influence quantitative expression profiles even when RNA quality is controlled; therefore, it should be applied selectively based on study objectives and downstream analyses.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

C.C., the first author, performed all experiments and drafted the manuscript. P.P. supervised the RNA extraction experiments. N.S. supervised the experimental work. C.C. and K.T. analyzed the transcriptome data. S.S. supervised the overall study, secured funding, reviewed and edited the manuscript, and served as the corresponding author. All authors have read and approved the final manuscript.

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