Comparative transcriptome analysis of unripe and ripe banana (cv. Nendran) unraveling genes involved in ripening and other related processes

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Abstract

Banana is one of the most important fruit crops consumed globally owing to its high nutritional value. Previously, we demonstrated that the ripe pulp of the banana cultivar (cv.) Nendran (AAB) contained a high amount of pro-vitamin A carotenoids. However, the molecular factors involved in the ripening process in Nendran fruit are unexplored. Hence, we commenced a transcriptome study by using the Illumina HiSeq 2500 at two stages i.e. unripe and ripe fruit-pulp of Nendran. Overall, 3474 up and 4727 down-regulated genes were obtained. A large number of identified transcripts were related to genes involved in ripening, cell wall degradation and aroma formation. Gene ontology analysis highlighted differentially expressed genes that play a key role in various pathways. These pathways were mainly linked to cellular, molecular and biological processes. The present transcriptome study also reveals a crucial role of up-regulated carotenoid biosynthesis pathway genes namely, lycopene beta cyclase and geranylgeranyl pyrophosphate synthase at the ripening stage.

Introduction

Banana is amongst one of the most essential staple food cultivated in both tropical and subtropical countries and consumed worldwide [1]. The banana plant is a flowering monocot belonging to the family Musaceae and mainly originated from intra- and inter-cross among Musa acuminata (A genome) and Musa balbisiana (B genome) [2]. This resulted in several genome groups viz. AA, AB, AAA, AAB, AABB, AAAB and ABBB [3]. Ripening process in banana leads to various changes in gene expression that results in changes in flavor, texture and color of these fruits [4, 5]. These irrevocable biological and physiological changes due to ripening often result in shortening of
the shelf life of a banana, causing losses at its postharvest level [6]. Previously, some chemical treatments were extensively employed to minimize postharvest losses but due to economical and health concerns, these were not favored [7]. Previous studies have reported that ripening in banana involves various gene families that are associated mainly with cell wall degradation and few genes have also been identified which are associated with transcription factors (TFs), signal transduction and ethylene biosynthesis [8–10]. However, the role of molecular factors related to carotenoid accumulation during the ripening process is not explored much in banana. Carotenoids play a significant role during fruit ripening and banana represent a low to moderate amount of their content. Screening of banana germplasm is found to have a significant variation in carotenoid content in their pulp tissue [11]. Banana cultivar (cv.) Nendran (AAB) is identified with high pro-vitamin A content in ripe fruit-pulp [12]. Hence, it will be alluring to study the molecular mechanism associated with ripening and pro-vitamin A accumulation in Nendran.

DNA sequencing has grown as an inescapable medium for studies related to molecular biology. The availability of the draft sequence of the banana genome (523 megabase) from Musa acuminata a double haploid provided imperative information for genetic improvement of the banana plant [13]. Being a quick and economical method, next-generation sequencing (NGS) tools provide high throughput transcriptome analysis with techniques like RNA-Seq [14]. In comparison to whole-genome sequencing, transcriptome profiling is favored as it is confined to study only a subset of the genome (transcribed portions of the genome) [15]. This analysis helps to understand the expression of genes in varying biological environments like in cells and tissues [15]. Further, factors related to stresses and different metabolic pathways can be well studied using genome-scale NGS based technologies. In banana (Dwarf Cavendish), transcriptome analysis has been done to understand the molecular mechanisms involved during the ripening process [16]. Transcriptome analysis of different varieties of Musa spp. has been performed on leaf, root, pulp, rhizome in response to fungal infection, to analyze the role of TF in ripening and to study metabolic processes under low potassium stress [17]. These analyses could further enhance our understanding of the molecular mechanism underlying biosynthesis and defense, and can also contribute to elucidate evolutionary aspects of its genes, and genomes. Databases such as Arabidopsis Next-gen sequences DBs and prediction algorithms have been used to provide information on genes associated with fruit ripening [18]. Further, transcriptome analysis of fruits like kiwi [19] blueberry [20], Cucumis melo [21], orange [22, 23] watermelon [24] and tomato [25] have led to identifying pathways and genes associated with fruit ripening and development. Recent developments in genomics comprising molecular markers (simple sequence repeats) have also enhanced our current knowledge in understanding various functional characteristics of the plant genome, which can help to improve banana by breeding approaches [16]. In-silico databases also use to harbor information of novel molecular markers that can be utilized for genetic improvement programs in banana.

The present study is commenced to get the global expression profile about the key molecular factors involved in ripening, carotenoid accumulation and other related processes in the economically and nutritionally important banana cv. Nendran. To elucidate the role of various up- and down-regulated genes in Nendran, we have generated and analysed transcriptomic data at two fruit developmental stages i.e. unripe and ripe using NGS technology hosted on the Illumina platform.

Materials and methods

Plant material

Banana cv. Nendran was used for experimental purposes. The fruit samples from unripe (6 weeks/w) and ripe (15 weeks/w) stages of Nendran were collected from the banana germplasm...
plot at National Agri-Food Biotechnology Institute (NABI), Mohali, Punjab. Sampling was performed during the summer and winter seasons. The tissues were then kept in liquid nitrogen and stored at 80°C till further use.

RNA extraction, cDNA library preparation and illumina sequencing
Total RNA was isolated from fruit pulp using the RNA extraction kit (Sigma-Aldrich, USA). In total three biological replicates were taken for each sample and used further for the isolation of RNA. Isolated RNA was treated with DNase I kit (Ambion Thermo Scientific, USA) to eliminate DNA contamination. Total RNA was analyzed by agarose gel electrophoresis for size and integrity. The quantification of total RNA was done with a NanoQuant (Infinite 200 PRO NanoQuant, Austria). The sample for RNA sequencing was derived from the pooling of the RNA samples in two groups i.e. replicates isolated from the fruit-pulp of 6w (unripe) and 15w (ripe) stages. DNA-free RNA was used for cDNA first-strand synthesis by using revert aid first-strand cDNA synthesis kit (Thermo Scientific, USA) as per manufacturer’s protocol. Oligo dT primer was used for cDNA preparation. Consequently, the integrity of RNA used for library preparations was checked with a value of ≥ 8.5 using Bioanalyzer (Agilent, USA). The quality control (QC) passed RNA samples were then processed for library preparation. The paired-end libraries were prepared from the total RNA using Illumina TruSeq stranded mRNA library prep kit as per the instructions (Illumina Inc., USA). The generated libraries were sequenced on Illumina HiSeq 2500 platform.

Transcriptome assembly and RNA seq analysis
Raw reads obtained from sequencing were processed to obtain high-quality reads. Moreover, all reads were trimmed by using the Trimmomatic 0.35 tool [26] to remove low-quality reads and any adapter sequences if present. The resultant high-quality reads of each sample were used for mapping on Musa acuminata DH-Pahang v2 on banana genome hub [27] (https://banana-genome-hub.southgreen.fr/download). The reads were mapped using STAR 2.6 [28]. Cufflinks v2.2.1 [29] program was used to assemble the STAR aligned transcripts to quantify their expression. Cufflinks, Cuffmerge and Cuffdiff were then used for further mapping and expression analysis of differentially expressed genes (between unripe and ripe samples). Cuffdiff software was also used to quantify the abundance of transcripts in the form of Fragment Per Kilobase of transcript per Million mapped reads (FPKMs). The genes were additionally categorized as differentially expressed by considering statistical significance (p<0.05, p<0.01) and false discovery rate for significant expression.

Functional annotation of Differentially Expressed Genes (DEG) and pathways
For functional annotation of DEG and to identify putative pathways associated with them, we annotated identified DEG’s with banana genome hub, NCBI protein database and GO databases. Significant GO IDs were extracted from the banana genome hub ontology browser. The g:Profiler web server was employed for functional enrichment analysis [30]. Further, the WEGO tool [31] was used to calculate the statistical enrichment of DEGs in various pathways using FDR values of < 0.05 (threshold).

Quantitative real-time PCR (qRT-PCR) validation
Total RNA was isolated from ripe and unripe fruit pulp samples and cDNA was prepared as discussed above. The qRT-PCR study was implemented with ABI 7500 Sequence Detector
Housekeeping gene Actin1 (GenBank Accession No. AF246288) was used to normalize the variant expression of chosen genes [32, 33]. The primers were firstly tested for single-band amplification using conventional end-point PCR. The expression of each gene was tested in unripe and ripe conditions of Nendran fruit samples. A melting curve study was carried out using qRT-PCR. The total volume of each reaction was adjusted to 10 μl and contained 1X SYBR Green Master Mix (Applied Biosystems, USA); 5 pmol of each primer (forward and reverse); 0.5 μl cDNA template and sterile distilled H2O. PCR conditions followed during real-time PCR experiment were: step (1) 50˚C 2 min, step (2) 95˚C 10 min, step (3) (95˚C 0.15 min, 60˚C 1 min) x 40 cycles, followed by the thermal dissociation curve. The relative expression level was analyzed using the 2−ΔΔCt method [34]. Primer details along with corresponding gene IDs are mentioned in the S1 Table. All the primers used in the qRT-PCR analysis were unique to each gene and were designed using Primer3 software [35]. All experiments were executed in biological triplicates and each experiment entailed three technical replicates. Statistical significance was determined by using the Student’s paired t-test.

Results

Transcriptome sequencing, alignment and analysis of banana fruit samples

The whole transcriptome sequencing i.e. RNA-seq (paired-end) of fruit (ripe and unripe) samples of cv. Nendran was performed using Illumina HiSeq 2500 platform. On average for each sample, 96,013,558 reads in NEN-Ripe and 107,849,342 in NEN-Unripe samples were recovered from two cDNA libraries. Approximately 89.30% of total reads have a Phred quality score > 30 (a measure of the quality of nucleobases). After exclusion of low-quality reads, 95,320,622 and 107,351,986 reads were obtained from NEN-Ripe and NEN-Unripe samples, respectively. Clean reads were then selected for aligning to the banana genome. By mapping the selected transcripts, 94% (Ripe) and 92% (Unripe) reads matched with the banana genome (Table 1).

Further, the expression is evaluated in FPKMs using the Cufflink software package [29]. Based on log2 fold change parameter and p-value ≤ 0.01, we obtained 3206 up-regulated (≥ 2 fold) and 4352 (≤ -2 fold) down-regulated genes in unripe vs. ripe samples (Fig 1A). Similarly, with p-value ≤ 0.05, a total of 3474 up- and 4727 down-regulated genes were obtained (Fig 1A). The scatter plot of the expressed genes at unripe and ripe stages of fruit-pulp is presented in Fig 1B. Different colors were used in scatter plot to specify up-regulated genes, down-regulated genes and genes in which expression was not affected. We evaluated unripe and ripe samples and created scatter plot of expressed genes where specific colors were used to exemplify down-regulated, up-regulated and non-regulated genes. The DEGs were examined between control (Unripe) and test (Ripe) samples using the FPKM method log2 fold change ≥ 2 as a threshold. The signifying log2 values of gene expression and screening conditions are represented in Fig 1B.

Functional enrichment of differentially expressed genes

The significantly differentially expressed genes were then mapped to the banana genome hub database (https://banana-genome-hub.southgreen.fr/). Further, the study on gene ontology
(GO) and classification of DEGs was performed to get the information of genes involved in cellular, molecular and biological processes in ripened fruit pulp of Nendran. All the DEGs with annotated GO terms were visualized using the WEGO tool (Figs 2 and 3). In the cellular component, most of the genes were classified into the extracellular region, cell part and membrane part, while in the molecular function, most of the genes were involved in catalytic activity, binding and molecular function regulators. In biological processes, genes were mainly involved in response to stimuli, biogenesis or cellular component organization, biological regulation, etc. Overall, cellular component organization, localization, developmental process and response to stimuli were the most considerably enriched processes in DEGs (S1 Fig).

**Identification of differentially expressed genes**

To identify transcripts that are expressed differentially in response to ripening, the top 50 up-regulated and down-regulated genes were selected for further analysis. Gene expression of the most up-regulated transcripts varied from 14 to 6.6 folds (Table 2). The acyl carrier protein and cytochrome P450 encoding genes are shown to be up-regulated significantly. Categorically, other genes encoding for stress and pathogenesis-related proteins were also up-regulated in ripen fruit-pulp samples. Similarly, the genes that were down-regulated are mostly linked with TF, hydrolase, and cellulose related genes. A detailed list of top up- and down-regulated genes is depicted by a heat map (Fig 4). The top 50 up- and down-regulated genes are listed in Tables 2 and 3, respectively.

**Differential expression pattern of genes involved in the carotenoid biosynthesis pathway**

The fruit ripening response and the expression of carotenoid biosynthesis pathway genes in cv. Nendran was correlated and presented in S2 Fig. It was observed that expression of *isopentenyl-diphosphate delta isomerase 1-like* (IPP) (1 fold), *lycopene beta cyclase* (LCYβ) (1.29 fold), *geranylgeranyl pyrophosphate synthase* (GGPS) (6 fold), *prolycopene isomerase* (CRTISO) (1.5 fold) and *isopentenyl-diphosphate delta isomerase 1-like* (IPP) (1 fold), *lycopene beta cyclase* (LCYβ) (1.29 fold), *geranylgeranyl pyrophosphate synthase* (GGPS) (6 fold), *prolycopene isomerase* (CRTISO) (1.5 fold) were up-regulated in ripened fruit pulp of Nendran.
fold), cytokinin dehydrogenase (5 fold), 4-hydroxy-3-methylbut-2-enyl diphosphate synthase (HDS) (4 fold) and phytoene desaturase (PDS) (1.44 fold) was significantly enhanced at the ripe stage of fruit compared to the unripe stage. While the gene expression of 9-cis-epoxycarotenoid dehydrogenase (NCED), carotenoid 9%,10(9%,10')-cleavage dioxygenase 1-like (CCD1), lycopene epsilon cyclase (LCYe), β-carotene 3-hydroxylase 2 (BCH2) and phytoene synthase 2 (PSY2) was highly down-regulated at ripened stage (ranging from -1 to -9 folds) (S2 Fig).

Genes involved in ripening, aroma and flavor

The banana ripening process is known to be involved in softening of fruit tissues that lead to the formation of aromatic compounds. The softening is mainly governed by the degradation of cell wall components [36]. This process is associated with a repertoire of genes, which are differentially expressed to regulate these events. Therefore, we have also analyzed the expression of genes that are linked with the ripening, flavor and aroma formation.

The expression pattern of methyltransferase, expansins, pectin lyase (PL), xyloglucan endotransglucosylase/hydrolase protein 32 (XTH), polygalacturonase (PG) which are ripening associated genes has been evaluated in the transcriptome data generated at the ripe stage of Nendran. The expression of the XTH gene family was highest (13 fold) followed by PL (12 fold) and trans-resveratrol di-O-methyltransferase-like (11 fold) in ripe fruit-pulp tissue in comparison to the unripe stage of fruit-pulp.
From the putative \textit{XTH} gene family, 15 genes were up-regulated with the highest fold change (13 fold) and 17 genes were down-regulated (-7 fold). Similarly, six genes from the \textit{PL} family were highly expressed during ripening stage of the banana. Nine \textit{expansin} genes were identified and their expression was increased up to 9 fold. Few members of the \textit{PG} gene family were highest in expression (up to 6 fold), while other gene families like glucosidases (GSMUA\_Achr11G06230\_001) (7 fold) are also expressed in the fruit-pulp of cv. Nendran. However, the expression of some of the members of these gene families like \textit{pectinesterase} (5 fold) was considerably enhanced but still on the lower side when compared to the expression of other ripening associated families such as \textit{XTH}. The expression details of ripening associated genes are provided in the \textit{S2 Table}. Genes involved in softening of the cell wall were amongst the highly up-regulated genes (\textit{PL, PE, XTH}) indicating that softening is the main event during the ripening of banana.

The presence of various volatiles viz. butyl acetate, isoamyl alcohol, and isoamyl acetate attributes to the aroma of banana fruit. Fatty acid biosynthesis and other pathways like the phenylpropanoid pathway mainly produce these volatile compounds. In this study, the expression pattern of genes involved in the biosynthesis pathways of fatty acid, unsaturated fatty acid and amino acid formation was analyzed. We have checked the expression of \textit{alcohol dehydrogenase} (\textit{ADH}) that mediates the conversion of alcohol from sugars. \textit{ADH} genes are usually expressed during the fruit ripening process and reported to play a major role in the development of flavor. In total 5 transcripts annotated for \textit{ADH} (\textit{ADH1—ADH5}) were identified and among them \textit{ADH1} (GSMUA\_Achr2G08040\_001) has shown one-fold increased expression, while others have not indicated any significant change in expression levels. Likewise,
Table 2. List of top 50 up-regulated genes in Nendran ripe fruit samples.

| Musa acuminata IDs | Unripe FPKM | Ripe FPKM | Log2 (fold change) | P-value |
|-------------------|-------------|-----------|--------------------|---------|
| NA                | 0.084       | 1631.93   | 14.2455            | 0.00435 |
| Ma05_g16870       | 0.2272      | 2196.7    | 13.2392            | 0.22265 |
| Ma03_g13710       | 0.3616      | 2292.69   | 12.6306            | 0.22785 |
| Ma01_g17320, Ma01_g17330 | 0.0881 | 368.309   | 12.0301            | 0.17305 |
| Ma03_g11500       | 0.1433      | 473.92    | 11.881             | 0.00435 |
| Ma04_g17140       | 0.3154      | 663.35    | 11.0384            | 0.0315  |
| Ma02_g15050, Ma02_g15080, Ma02_g15140 | 0.2177 | 387.42    | 10.7974            | 0.04925 |
| Ma02_g01380       | 0.691       | 1051.4    | 10.5714            | 0.03525 |
| Ma11_g02090       | 0.0557      | 45.48     | 9.674              | 0.01545 |
| Ma06_g36490       | 0             | 676.19    | 9.4034             | 5.00E-05 |
| Ma06_g30000       | 3.1304      | 1572.4    | 8.9724             | 0.0368  |
| Ma11_g03030       | 0.0297      | 13.42     | 8.8177             | 0.0439  |
| Ma08_g30710, Ma08_g30720 | 2.3397 | 1055.65   | 8.8176             | 0.0006  |
| Ma01_g08810, Ma01_g08820 | 5.11   | 2165.23   | 8.727              | 5.00E-05 |
| NA                | 0.0754      | 30.64     | 8.6664             | 0.00435 |
| Ma10_g24000       | 0.0244      | 9.9       | 8.666              | 0.04935 |
| Ma04_g33410, Ma04_g33420, Ma04_g33440 | 5.7266 | 2293.89   | 8.6459             | 5.00E-05 |
| Ma06_g36070       | 0.0738      | 27.39     | 8.5367             | 0.0104  |
| Ma04_g29630, Ma04_g29640 | 6.4843 | 2380.04   | 8.5198             | 0.00375 |
| Ma04_g20350       | 3.3082      | 1206.63   | 8.5107             | 0.00055 |
| Ma04_g04750       | 21.9975     | 6325.5    | 8.1677             | 0.03775 |
| Ma09_g31190       | 2.5786      | 741.18    | 8.1671             | 0.00955 |
| Ma09_g25760       | 0.5324      | 135.71    | 7.9938             | 0.00875 |
| Ma08_g11500       | 2.4939      | 631.55    | 7.9844             | 0.00035 |
| Ma11_g03350       | 0.2043      | 50.78     | 7.9537             | 0.037   |
| Ma06_g29240, Ma06_g29250 | 0.1632 | 40.08     | 7.9398             | 0.01065 |
| Ma05_g17850       | 1.8005      | 431.87    | 7.9061             | 0.0191  |
| Ma08_g08190       | 0.3605      | 78.63     | 7.7691             | 0.01995 |
| Ma04_g18390       | 4.12        | 756.6     | 7.5207             | 0.00025 |
| Ma02_g05950       | 3.5512      | 617.78    | 7.4427             | 0.0025  |
| Ma08_g20650       | 0.0493      | 8.37      | 7.4071             | 0.01545 |
| Ma08_g11790       | 0.072       | 11.7      | 7.3433             | 0.00455 |
| Ma03_g10270       | 0           | 159.22    | 7.3239             | 5.00E-05 |
| Ma07_g15550, Ma07_g15600 | 2.0088 | 300.7     | 7.2259             | 0.0034  |
| Ma06_g16120, Ma06_g16130 | 2.204  | 318.29    | 7.1741             | 0.00385 |
| Ma06_g07540       | 0.282       | 39.53     | 7.1312             | 0.00865 |
| Ma09_g30030       | 0.4778      | 62.16     | 7.0235             | 0.02725 |
| Ma06_g33980       | 4.2437      | 547.44    | 7.0112             | 0.00185 |
| Ma04_g35390       | 1.1122      | 141.47    | 6.9909             | 0.00045 |
| Ma08_g17680       | 0.2461      | 30.13     | 6.9358             | 0.03105 |
| NA                | 0           | 120.5     | 6.9248             | 0.00535 |
| Ma10_g31360       | 0.2208      | 24.87     | 6.8157             | 0.0103  |
| Ma04_g17460       | 0.1326      | 14.71     | 6.7938             | 0.00435 |
| Ma04_g02270       | 7.4929      | 825.12    | 6.7829             | 0.0047  |
| Ma11_g01760       | 0.1312      | 14.42     | 6.7803             | 0.01715 |
| Ma04_g10050       | 0.2748      | 30.15     | 6.7779             | 0.0362  |
| Ma07_g24340       | 7.0938      | 740.83    | 6.7064             | 0.005   |

(Continued)
lipoxigenases (LOX) genes are also involved in aroma development and the expression of one gene belonging to LOX (GSMUA_Achr9G12470_001) was up-regulated (5 fold) in ripened banana. Further analysis of transcriptome data revealed that various transferases like benzoyltransferases, methyltransferases and acyltransferases were significantly up-regulated in ripe banana indicating their potential role in the aroma. The highest expression was observed in putative 3-N-debenzoyl-2-deoxytaxol N-benzoyltransferase that was increased maximum by 12 fold in a ripe banana. Similarly, 3-oxoacyl-[acyl-carrier-protein] reductase and acyltransferase genes also exhibited 11 and 2 fold increased expression, respectively (S3 Table).

Ethylene exposure also accelerates ripening in banana. ACC synthase (ACS) and ACC oxidase (ACO) are the main regulators that govern ethylene biosynthesis in fruits [37]. ACO is also considered to be the rate-limiting step in ethylene production [38]. In the current study, expression change in ACS and ACO genes to understand their role in fruit ripening of cv. Nendran is explored. It has been observed that the expression of ACS (3 fold) and ACO (1 fold) was up-regulated in a ripe fruit-pulp than that of unripe fruit-pulp (S4 Table).

Identification of Transcription Factors (TFs)

TFs regulate the expression of genes. Hence, we have downloaded TFs from the PlantTFDB database [39]. This database harbors 2896 TFs from Musa acuminata which are classified into 57 families. It was examined that most of the TFs belong to the multigene family and mainly the TFs were related to MYB, bHLH, ERF, NAC and C2H2 gene family. These gene families showed varied expression at the ripening stage (up and down). A list of the top 12 TF family members is given in Fig 5 and a detailed list of all transcription factors family members along with fold change expression is provided in the S5 Table.

Validation of differential gene expression by qRT-PCR

The validation of differential expression of selected genes belonging to different pathways was checked by qRT-PCR assay. Total ten genes were selected based on their significant differential expression pattern and potential role in acting as TFs stress response and carotenoid pathway genes. All the genes exhibited a comparable trend of expression in unripe and ripe stages as attained by transcriptomic data. It was observed that expression of putative 3-oxoacyl-[acyl-carrier-protein] reductase, chloroplastic (ACP), trans-resveratrol di-O-methyltransferase (TRM) and expansin-A2 (EXP) was up-regulated which is in accordance with the transcriptome data. Similarly, the expression of genes involved in the carotenoid pathway LCYβ and GGPS was highly up-regulated in ripe fruit-pulp of cv. Nendran as compared to unripe sample (Fig 6). Further, the expression of Actin 1 (LOC103992183) was also analysed and no change in expression pattern was observed in both ripe and unripe conditions.

Discussion

Banana is a staple fruit crop worldwide. Therefore, the understanding of molecular mechanisms that are associated with various traits is crucial. Reports showed that ripening in various
Fig 4. Clustered heatmap depicting top 50 up-regulated (left panel) and down-regulated genes (right panel) in ripe fruit pulp.
Red color indicates up-regulated genes and green color indicates down-regulated genes in Nendran fruit samples.

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Table 3. List of top 50 down-regulated genes in Nendran ripe fruit samples.

| Musa acuminata IDs | Unripe FPKM | Ripe FPKM | Log2 (fold change) | P-value |
|--------------------|-------------|-----------|--------------------|---------|
| Ma08_g14870        | 57.9970     | 12.3140   | -2.2357            | 0.0422  |
| Ma08_g29710        | 18.6179     | 3.6973    | -2.3231            | 0.0459  |
| Ma05_g15560        | 198.1540    | 38.7050   | -2.3560            | 0.04935 |
| Ma05_g29200        | 82.2009     | 15.9326   | -2.3672            | 0.0323  |
| Ma02_g15190        | 6.4337      | 1.2186    | -2.4005            | 0.04805 |
| Ma08_g23830, Ma08_g23840 | 53.1387 | 9.9826    | -2.4263            | 0.04685 |
| Ma08_g14790        | 90.0315     | 16.7500   | -2.4508            | 0.0317  |
| Ma01_g09160        | 2.6453      | 0.4839    | -2.5402            | 0.04245 |
| Ma11_g16990        | 12.0605     | 2.0735    | -2.5468            | 0.03935 |
| Ma03_g24790        | 11.7536     | 2.0114    | -2.5559            | 0.0428  |
| Ma07_g06110        | 15.2059     | 2.5859    | -2.5646            | 0.038   |
| Ma01_g15000        | 16.7579     | 2.8328    | -2.5649            | 0.04005 |
| Ma08_g13700        | 55.7171     | 9.4159    | -2.5649            | 0.04005 |
| Ma11_g22760        | 24.0255     | 4.0311    | -2.5753            | 0.0439  |
| Ma07_g05150        | 13.5948     | 2.2438    | -2.5990            | 0.04545 |
| Ma05_g03730        | 18.2312     | 2.9992    | -2.6038            | 0.046   |
| Ma07_g21650        | 31.7084     | 5.1810    | -2.6136            | 0.02875 |
| Ma08_g27050        | 23.9941     | 3.9084    | -2.6180            | 0.037   |
| Ma05_g16930        | 23.3441     | 3.7838    | -2.6252            | 0.0362  |
| Ma00_g01530        | 20.2872     | 3.2693    | -2.6335            | 0.0239  |
| Ma06_g01110        | 30.6854     | 4.9257    | -2.6379            | 0.04155 |
| Ma01_g14940        | 136.9260    | 21.7356   | -2.6553            | 0.03425 |
| Ma07_g15700        | 179.5230    | 28.0934   | -2.6759            | 0.0445  |
| Ma08_g05740        | 29.6195     | 4.6249    | -2.6791            | 0.04335 |
| Ma05_g05010        | 105.5100    | 16.4617   | -2.6802            | 0.0486  |
| Ma11_g02290        | 12.5888     | 1.9597    | -2.6835            | 0.0328  |
| Ma06_g19920        | 10.2797     | 1.5929    | -2.6901            | 0.02795 |
| mito04_g00800      | 10.7582     | 1.6646    | -2.6922            | 0.0327  |
| Ma02_g00470        | 175.1800    | 27.0540   | -2.6949            | 0.02425 |
| Ma06_g28590        | 32.6655     | 5.0367    | -2.6972            | 0.0341  |
| Ma07_g27270        | 54.7385     | 8.3345    | -2.7154            | 0.02555 |
| Ma08_g20470        | 54.1184     | 8.2262    | -2.7178            | 0.04435 |
| Ma02_g18970        | 29.7441     | 4.4848    | -2.7295            | 0.04965 |
| Ma03_g02940        | 23.3713     | 3.5238    | -2.7296            | 0.0315  |
| Ma09_g22220        | 45.9947     | 6.9188    | -2.7329            | 0.03515 |
| Ma06_g04700        | 16.4684     | 2.4648    | -2.7402            | 0.0328  |
| Ma05_g11240        | 186.9100    | 27.9561   | -2.7411            | 0.02375 |
| Ma05_g06160        | 84.3011     | 12.5721   | -2.7453            | 0.0389  |
| Ma10_g20280        | 15.5856     | 2.3120    | -2.7530            | 0.04475 |
| Ma09_g05470        | 20.7883     | 3.0835    | -2.7531            | 0.04635 |
| Ma07_g08320        | 193.6760    | 28.4678   | -2.7662            | 0.04545 |
| Ma04_g18080, Ma04_g18100 | 18.5819 | 2.7309    | -2.7665            | 0.01615 |
| Ma02_g01240        | 460.2760    | 67.5250   | -2.7690            | 0.02775 |
| Ma10_g07330        | 34.0326     | 4.9770    | -2.7736            | 0.0273  |
| Ma09_g22050        | 38.0633     | 5.5625    | -2.7746            | 0.0358  |
| Ma11_g22400        | 20.6719     | 2.9902    | -2.7894            | 0.03615 |
| Ma04_g12450        | 95.9664     | 13.8414   | -2.7935            | 0.04895 |

(Continued)
fruit crops including banana is initiated by a set of genes that brings physico-chemical changes in the quality of fruit [40]. These changes mainly alter cell wall degradation, synthesis of volatile compounds and alteration in phenolic constituents [40]. Sequencing technologies now provide ways to identify ripening and associated process-related genes and subsequently those can be used for genetic manipulation for the improvement of banana. Henceforth, insights into the genes responsible for ripening are necessary to understand the molecular basis of these genes that can also be implemented to improve post-harvest losses of this highly perishable crop.

In the present study, cv. Nendran of banana has been selected as it is having a high content of carotenoid at the ripening stage of fruit-pulp [12]. Further, early ripening (15w of bunch emergence) of fruits in this nutritional rich cultivar has also influenced us to understand the molecular basis of this observation. Using transcriptome analysis hosted on Illumina (HiSeq

![Fig 5. Transcription factors expressively correlated with cv. Nendran ripe fruit-pulp. Orange bars represent down-regulated genes while green bars represent up-regulated genes.](https://doi.org/10.1371/journal.pone.0254709.g005)
2500) 3474 up- and 4727 down-regulated genes were obtained in the Nendran ripe fruit-pulp samples. Functional enrichment of the genes revealed their probable role in various metabolic processes.

Nendran has been reported as an excellent source of \( \beta \)-carotene and its activity was correlated with enhanced antioxidant activity [12]. Ripening plays a crucial role in the biosynthesis of carotenoids in the fruit-pulp tissue of banana [41]. In the current study, the expression pattern of carotenoid genes indicated that \( \text{LCY} \beta \), \( \text{GGPS} \), and \( \text{PDS} \) were up-regulated in ripe fruit pulp. Moreover, the role of \( \text{LCY} \beta \) in \( \beta \)-carotene biosynthesis has already been demonstrated by

**Fig 6. Quantitative real-time PCR of selected genes.** Expression profile of selected genes in ripe Nendran banana. Gene expression was normalized using \( \text{Actin1} \) as an internal control. Orange bars represent the expression of unripe while green bars depict the expression of ripe genes. Statistical analysis was executed using Student’s paired t-test and statistical significance was checked at \( ** P \leq 0.001; *** P \leq 0.0001 \). \( \text{TRM} \) (Trans-resveratrol di-O-methyltransferase); \( \text{EXP} \) (Expansin-A2); \( \text{ACP} \) (Putative 3-oxoacyl-[acyl-carrier-protein] reductase, chloroplastic); \( \text{PSY2} \) (phytoene synthase 2); \( \text{LCY} \beta \) (lycopene beta cyclase); \( \text{GGPS} \) (geranylgeranyl pyrophosphate synthase); \( \text{TIP2} \) (Probable aquaporin \( \text{TIP2} \)); \( \text{HSP} \) (Heat shock 70 kDa protein 8); \( \text{PHD} \) (PHD-type domain-containing protein); \( \text{bHLH} \) (transcription factor bHLH62). (Corresponding gene IDs given in S1 Table).

https://doi.org/10.1371/journal.pone.0254709.g006
the regulation of lycopene flux [42]. Similarly, GGPS serves as a precursor for important compounds like tocopherols, carotenoids, and chlorophyll [43]. Expression of GGPS was up-regulated in ripe pulp samples. PDS is one of the first enzymes in the carotenoid biosynthesis pathway. It has also been reported as a positive regulator for ripening in tomato fruit [44]. Mutation in PDS gene by CRISPR/Cas technology resulted in decreased chlorophyll and carotenoid content in banana cv. Rasthal [32]. The transcriptome data have shown a ~2 fold increase in expression of the PDS gene (GSMUA_Achr3G21400_001) in the ripe fruit. It indicates the possible role of PDS in early fruit ripening and high carotenoid deposition in cv. Nendran. BCH gene is reported to involve in the biosynthesis of zeaxanthin which is a precursor of abscisic acid [45]. In our analysis expression of gene annotated as β-carotene 3-hydroxylase 2 (BCH2) (GSMUA_Achr11G02930_001) in Musa was down-regulated by ~ -6 fold. Other carotenoid pathway genes like CCD, NCED are considered carotenoid degrading enzymes [46]. In this study expression of these genes was down-regulated in ripened banana, signifying their less role in the ripening and carotenoid degradation process in Nendran.

Softening is mainly initiated with the inception of ripening [47], which leads to cell wall degradation. Cell wall hydrolysis plays a crucial role in plant growth and development, stress response and ripening process. Utmost of the genes involved in this process are mainly members of multigene families and are associated with specified functions like cell wall metabolism [48]. The significant cell wall degrading proteins are pectin methyl esterase, polygalacturonase (PG), XTH, expansins, PL, galactosidases and endoglucanases [49]. XTH gene family members have been reported to play important role in the ripening of fruits like tomato and apple [50, 51]. Studies in fruit crops such as mango and banana have reported the role of these genes in cell wall loosening [52, 53]. The expression pattern of most of the genes belonging to XTH and expansins gene families was reported to be highly up-regulated during ripening conditions. Amongst them, some members of these gene families were also down-regulated indicating that they might not be playing any significant function in the ripening process.

Ethylene is considered one of the major plant hormones that control many aspects of fruit ripening [54]. The initial step in ethylene biosynthesis is the conversion of S-adenosyl methionine to 1-aminocyclopropane-1-carboxylic-acid catalyzed by ACS [55]. The ACS and ACO genes are reported to regulate ethylene biosynthesis in the tomato and apple during the fruit ripening stage [56, 57]. Transcriptome data in this study revealed up-regulation of both genes in ripe fruit-pulp tissue specifying their potential role in ethylene synthesis and ripening.

We also analysed the expression of transcription gene families in fruit-pulp of cv. Nendran and found that most of the members of these multigene families of TFs were down-regulated at the ripening stage. These TFs may not be required at this stage hence, their expression is declined during the ripening process.

**Conclusion**

The comparative analysis of transcriptome at the unripe and ripen stages of fruit-pulp of cv. Nendran provides a comprehensive landscape of differentially expressed genes that are mostly associated with ripening, carotenoid biosynthesis, aroma and other related processes. The expression data acquired by RNA-seq were validated by qRT-PCR analysis. The results of this study suggested that many differentially expressed genes in the unripe and ripe banana are associated with aroma and ripening processes. Gene families in ripening like PL, expansins, XTH etc. were showed differential expression patterns. Genes like acyltransferases known to be responsible for cell wall hydrolysis and production of aromatic volatiles and flavor have shown higher expression at the ripening stage. The expression pattern of the carotenoid synthesis pathway genes indicated that LCYβ and GGPS were highly up-regulated during the ripening
stage while CCDs and NCEDs were downregulated. Overall, the present study has provided information about the promising role of genes such as acyltransferases, LCYβ and GGPS to develop a better understanding of the ripening process and their link with carotenoid synthesis, aroma and flavor formation in banana fruit-pulp.

**Supporting information**

S1 Fig. List of enriched GO terms in differentially expressed genes in fruit-pulp of Nendran. X-axis represents significance of gene ontology term enrichment and y-axis represents the log P-values.

(PDF)

S2 Fig. Heatmap of differentially expressed genes involved in carotenoid biosynthesis pathway. Red color represents up-regulated genes and green color represents down-regulated genes.

(PDF)

S1 Table. Primers used in quantitative real-time PCR study.

(XLSX)

S2 Table. Expression pattern (log2 fold change) of the genes involved in ripening.

(XLSX)

S3 Table. Expression pattern (log2 fold change) of the genes involved in aroma and flavor.

(XLSX)

S4 Table. Expression pattern (log2 fold change) of the genes involved in ethylene synthesis.

(XLSX)

S5 Table. List of Transcription Factors (TFs) family members with the expression in log2 fold change.

(XLSX)

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