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Transcriptome analysis to elucidate hexanal's mode of action in preserving the post-harvest shelf life and quality of banana fruits (*Musa acuminata*)

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ABSTRACT

Banana is a climacteric fruit whose ripening once initiated is irreversible and proceeds very fast making the fruits highly perishable. Application of various post-harvest technologies has been shown to slow down the ripening process in climacteric fruits such as banana. One of these technologies is hexanal, a naturally occurring compound that delays the ripening process in banana fruits without compromising the quality. As the molecular mechanisms underlying the mode of action of hexanal in delaying ripening are yet to be elucidated, we undertook a comparative transcriptomic analysis using banana fruits treated with either hexanal, ethylene or untreated controls. Results of our study show that hexanal significantly delayed the rate of pulp softening throughout the storage period. Sequencing results showed that 776 genes were up-regulated and 2146 were down-regulated upon hexanal treatment while 4 genes were up-regulated and 76 were down-regulated upon ethylene treatment at day one of storage. Additionally, 2423 genes were up-regulated and 2862 were down-regulated upon hexanal treatment while a total of 4820 genes were up-regulated and 5395 were down-regulated upon ethylene treatment at day four. We found that hexanal treatment transiently suppressed the expression of genes involved in ethylene biosynthesis, cell membrane deterioration and cell wall degradation by day four of storage contrary to the observed induction of the same genes in ethylene-treated fruits. The particular genes are; Aminocyclopropane-1-Carboxylic Acid Oxidase, 1-Aminocyclopropane-1-Carboxylic Acid, Phospholipase D, Polygalacturonase, Expansin and Xyloglucan Endotransglucosylase. Later on at day 18 of storage, genes involved in ethylene biosynthesis, cell wall and cell membrane degradation and aroma synthesis were induced in the hexanal-treated fruits. These findings reveal that hexanal works by temporarily suppressing the expression of genes involved in ethylene biosynthesis, cell wall degradation and cell membrane deterioration.

1. Introduction

Ripening is a crucial phase in the maturation process of fruits. As the fruit ripens, expression of various genes involved in different metabolic pathways are triggered leading to several physiological and biochemical changes which transforms the fruit into an edible state [6]. Key among them include softening, color change, sugar metabolism, synthesis of aroma volatiles, and increased susceptibility to pathogens [29]. Banana is a climacteric fruit and once ripening is initiated, it is irreversible and proceeds very rapidly, making the fruit highly perishable [1]. This in turn causes post-harvest deterioration, which results in substantial economic losses for all the banana value chain actors.

Fruit softening during ripening is one of the critical changes which enhances banana acceptability by consumers. In banana, softening is high during the later stages of ripening due to extensive cell wall degradation associated with the disassembly of primary cell wall and the middle lamella [5]. Additionally, other mechanisms such as loss of turgor, degradation of starch into sugars and degradation of the cell membrane also contribute to softening in banana fruits [10]. However, significant softening in banana fruit is the result of degradation of the cell

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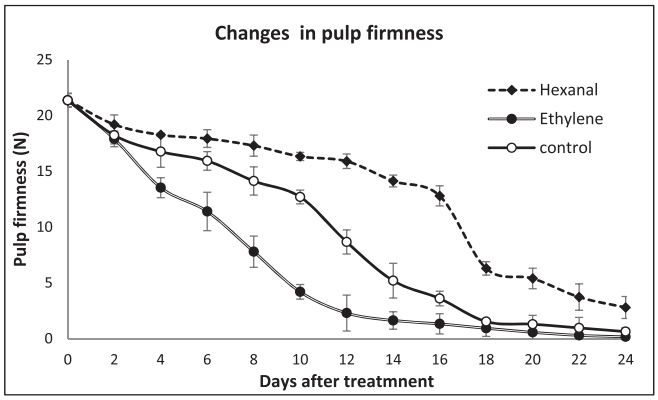

Fig. 1. Effects of hexanal and ethylene treatment on pulp firmness of 'Grand Nain' banana fruits.

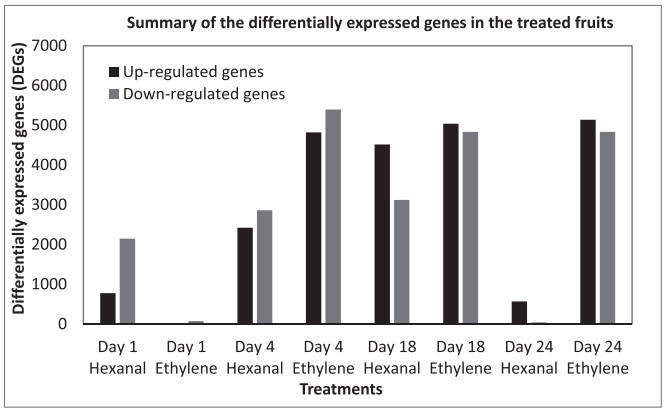

Fig. 3. Summary of the differentially expressed genes in the hexanal and ethylene treated fruits in each day of storage in comparison to the untreated control fruits.

wall and the cell membrane [5, 20]. Degradation of the cell wall is caused by additive action of pectolytic enzymes such as polygalacturonase (PG), pectin methylesterase (PME) and pectate lyase (PL) [11]. The PG and PME enzymes regulate the breakdown of pectin polysaccharides while PL operate by catalyzing the cleavage of glycosidic bonds of unsaturated regions of pectins by β-elimination reaction [3,23]. Therefore, the suppression of these enzymes in fruit tissues will considerably reduce the rate of softening, improve fruit quality and extend the post-harvest shelf life [9].

Studies have recently shown that hexanal can enhance banana fruit shelf life by up to 9 days without compromising its quality [30,31]. Being a relatively new technology, hexanal's mode of action is not well understood. Several studies have suggested that it works by inhibiting the enzyme phospholipase D action, which catalyzes hydrolysis of membrane phospholipids and initiates membrane deterioration and thus fruit softening [22]. Further, a study by Tiwari and Paliyath [27], showed that hexanal is a weak inhibitor of ethylene, a hormone which triggers ripening in climacteric fruits. In our previous study [31], fruits treated with hexanal showed a consistent trend of delayed softening, reduced rate of respiration and ethylene production throughout storage compared to the untreated controls. Hexanal likely works by suppressing cell wall and cell membrane degradation and the ethylene-dependent ripening of fruit. A study by Tiwari and Paliyath [27], showed that treatment of fruits with hexanal was more advantageous as compared to other existing post-harvest technologies such as 1-Methylcyclopropane (1-MCP). Tomato fruits treated with hexanal had higher levels of lycopene, β-carotene and major volatile components as compared to those treated with 1-MCP [27]. Further studies in banana fruits reported that 1-MCP treatment caused poor peel color and flavor development [13], which is not the case in hexanal treated fruits [30].

In this study, we characterized the action of hexanal in delaying fruit ripening. Genome-wide transcriptome analysis shows that hexanal treatment suppressed the expression of Aminocyclopropane-1-Carboxylic Acid Oxidase (ACO), 1-Aminocyclopropane-1-Carboxylic Acid Synthase (ACS), Phospholipase D (PLD), Polygalacturonase (PG), Xyloglucan Endotransglucosylase (XTH) and expansins, which are genes involved in suppressing ripening in fruits. On the other hand, ethylene treatment strongly induced these ripening genes such as ACS, ACO, PLD, PG and expansins.

2. Materials and methods

2.1. Plant material and post-harvest treatment

Banana fruits var. 'Grand nain' (*Musa acuminata*) were obtained from a small-scale farm in Meru County, located in Agro ecological zone (AEZ) II of Kenya. The fruits were harvested at the mature green stage based on the disappearance of angularity and the number of days after anthesis (approximated at 104 days). Upon harvesting, the fruits were quickly

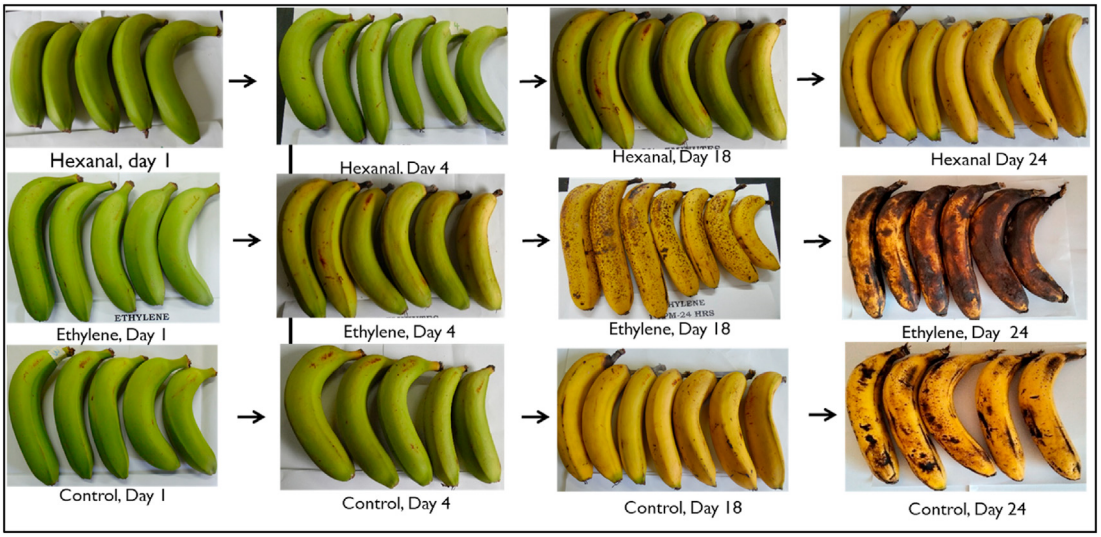

Fig. 2. Progression of ripening in 'Grand nain' banana fruits treated with hexanal, ethylene, or left untreated and allowed to ripen under ambient room conditions.

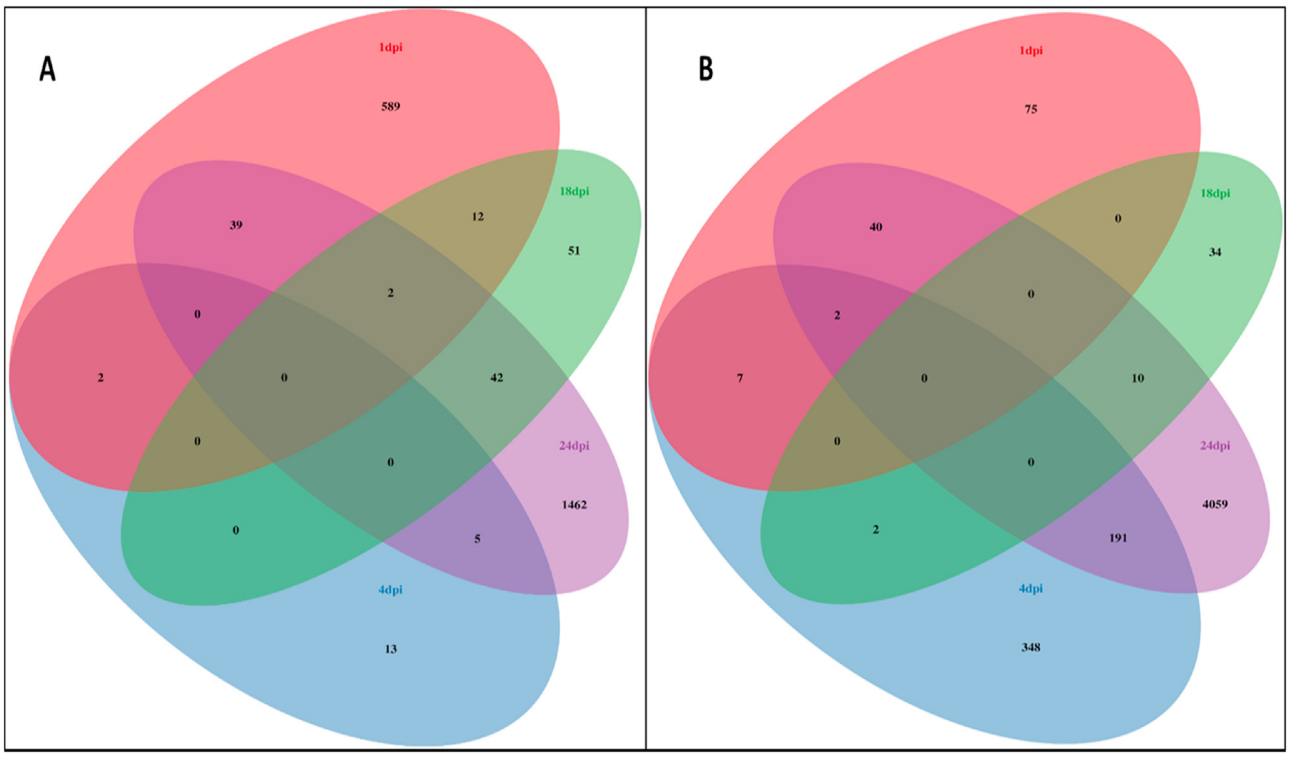

Fig. 4. Venn diagram illustrating the corresponding number of differentially expressed genes in day 1, 4, 18 and 24 in the hexanal treated fruits (A) and ethylene treated fruits (B).

transported to the post-harvest laboratory, washed, dried, selected for uniformity and randomly batched into three for various treatments.

The first batch was dipped in 2% hexanal for 5 minutes, the second batch was treated with 1 ppm ethylene in an airtight container for 24 hours while the final batch was left untreated to act as the control. After the treatments, all the fruits were left to undergo ripening under ambient room conditions. Three fruits from each treatment combination were randomly sampled at four different time points (day 1, day 4, day 18, day 24 of storage) and the pulp tissues were diced very fast, snap-frozen in liquid nitrogen and stored at −80 °C for subsequent RNA extraction. The sampling time points were informed by the physical data on pulp firmness. Day 1 is the starting point for the samples immediately after treatment, while day 4 and 18 are the time points the pulp firmness decreased sharply in the ethylene and hexanal treated fruits respectively. As from day 24, there was no significant differences in pulp firmness among the treatments. The molecular studies were conducted at Biosciences eastern and central Africa (BecA) laboratories based at the International Livestock Research Institute (ILRI) in Nairobi, Kenya.

2.2. Analysis of pulp firmness

The fruits were first peeled and the probe allowed to penetrate the flesh to a depth of 1 cm and the corresponding force required to penetrate this depth determined. The firmness was measured along the equatorial region of each fruit using a penetrometer (CR-100D, Sun Scientific Co. Ltd, Japan). Four locations along the equatorial zone of the fruit were used and average value of firmness calculated. Firmness was expressed as Newtons (N/mm).

2.3. RNA extraction and quality check

RNA was extracted from each pulp tissue using the cetyltrimethylammonium bromide (CTAB) method [5]. The extracted RNA was first treated with DNase I (Thermo Scientific, USA) to degrade any possible DNA contamination. The integrity of RNA was checked in the gel electrophoresis and NanoDrop™ 2000 spectrophotometer (Thermo Scientific, USA), where the 260:280 ratio was above 1.8.

2.4. Library preparation and sequencing

RNA libraries were prepared from 500 ng of Dnase 1 treated total RNA using the Truseq Stranded Total RNA Ribo-Zero Plant sample preparation protocol as per the manufacturer's instruction. Ambion® ERCC Spike-in mix (Thermo Fisher scientific, USA) was used as the internal control check necessary in gene expression studies [28]. The library concentration was checked using the Qubit® fluorometer (Thermo Scientific, USA) using the High Sensitivity reagents, while the integrity was checked using Agilent Bioanalyzer 2200 TapeStation system. The libraries were normalized to 10 nm and sent to Genohub Company (USA) for sequencing using Illumina NovaSeq 6000 system (Illumina, USA) for 150×2 pair-ended sequencing. The paired-end reads obtained were exported to the high-performance cluster (HPC) at the International Livestock Research Institute (ILRI) for subsequent bioinformatics analysis.

2.5. Data analysis

Physical data on pulp firmness was subjected to analysis of variance (ANOVA) using the Genstat statistical package (version 18). The means were separated by Least Significance Difference (LSD) at $p \leq 0.05$ using Fisher's protected test.

For differential expression analysis, raw reads were subjected to quality control using FastQC v0.11.7 [4] followed by preprocessing using cutadapt v1.18 [19]. For the trimming procedure using cutadapt, the first bases were removed (-u 14) and quality cutoff was set at 28(-q 28). The forward and reverse adapters were removed using the parameters -a and -A respectively. Reads with a minimum length of 50 base pairs (–minimum-length = 50) were retained for downstream analyses. Preprocessed reads were aligned to the reference genome after building the genome indices using star v2.5.3a [2]. The alignments were used in generating

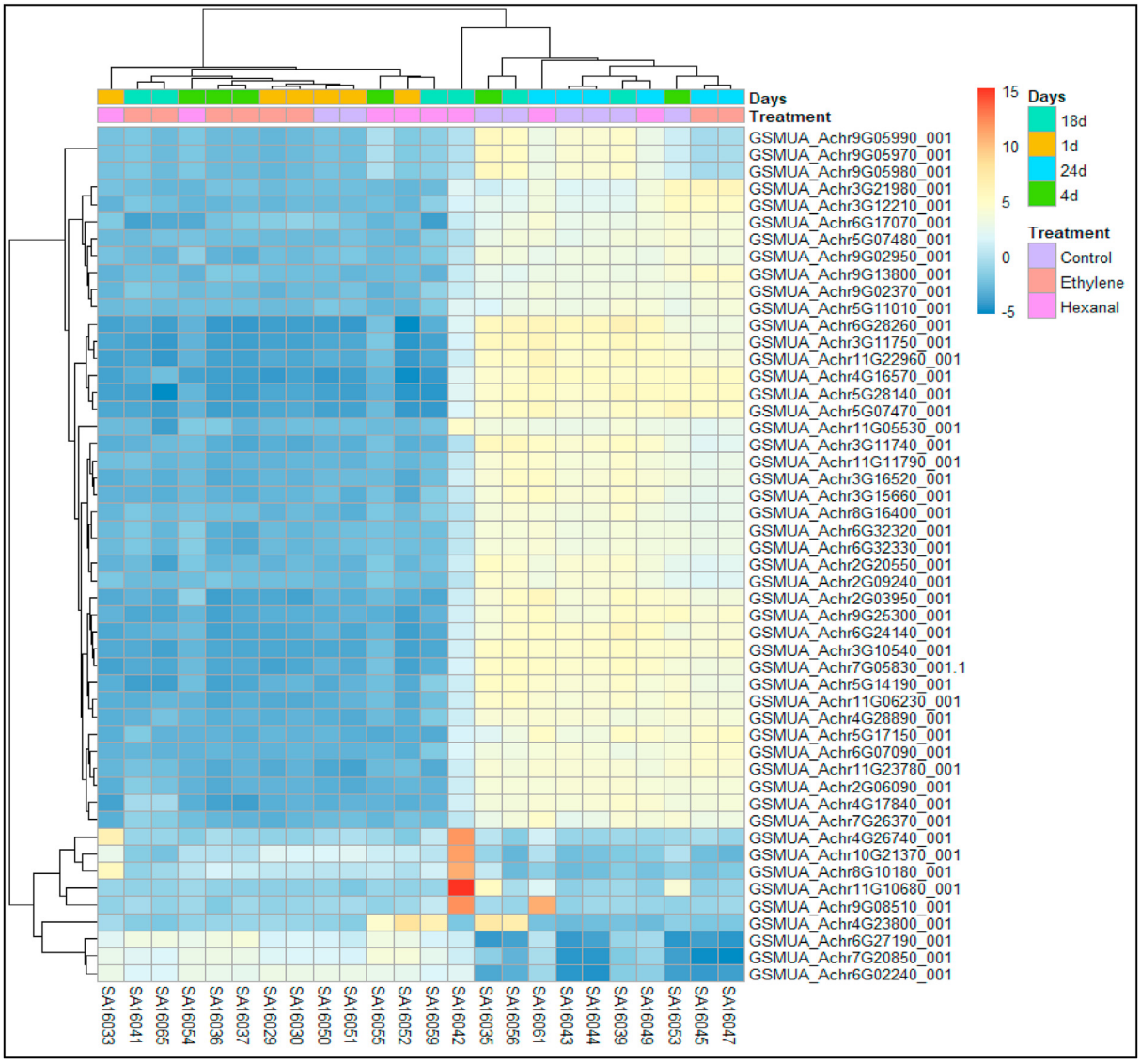

Fig. 5. Heatmap showing the topmost 50 genes differentially expressed across the treatments (hexanal, ethylene and controls) in all the days (days 1, 4, 18 and 24) of storage.

gene counts using featureCounts, which is part of the subread v1.6.2 suite [32]. Differential gene expression analysis was performed using the Bioconductor package DESeq2 in R v.3.6 [15,24] a statistical computing language. Data exploration using principal component analysis allowed for the detection of outlier samples which were subsequently removed from downstream analysis. Lowly expressed genes, those with low number of gene counts per sample were also removed at a threshold of 100 gene counts. The count data was transformed using variance stabilizing transformation function which normalizes the raw counts by using size factors. For each comparison or contrast, a list of significant and differentially expressed genes was obtained using the Benjamini-Hochberg (BH) multiple testing procedure on the *p*-values to obtain adjusted *p*-values (threshold 0.05). The log2 fold change was used to distinguish between downregulated and upregulated genes and the resulting genes visualized using the Bioconductor package Enhanced-Volcano [7]. To identify and visualize differentially expressed genes which had overlaps in the different conditions, we used VennDiagram [12]. Mapman program version 3.6.0RC1 (https://bit.ly/3gcmdHw) was employed to visualize changes in the various metabolism pathways following hexanal treatment.

3. Results

3.1. Pulp firmness

Pulp firmness was significantly ($p < 0.05$) affected by hexanal and ethylene treatments. Hexanal treatment significantly slowed the rate of pulp softening compared to the untreated control fruits. Overall, ethylene treated fruits lost their pulp firmness faster compared to the untreated controls. By the 12th day of storage, ethylene treated fruits had lost their pulp firmness to 2.3 N as compared to 8.6 N, in the untreated controls and 16 N in the hexanal treated fruits. On the other hand, fruits treated with hexanal remained firmer throughout the storage period. By the 18th day of storage, the pulp firmness of the hexanal treated fruits had decreased to 6.3 N compared to 0.9 N and 1.5 N in the ethylene treated fruits and controls respectively.

3.2. Effects of hexanal treatment on banana ripening

The ripening process was delayed in the fruits treated with hexanal compared to the untreated control and those treated with exogenous ethylene (Figs. 1 and 2). However, the hexanal treated fruits started to

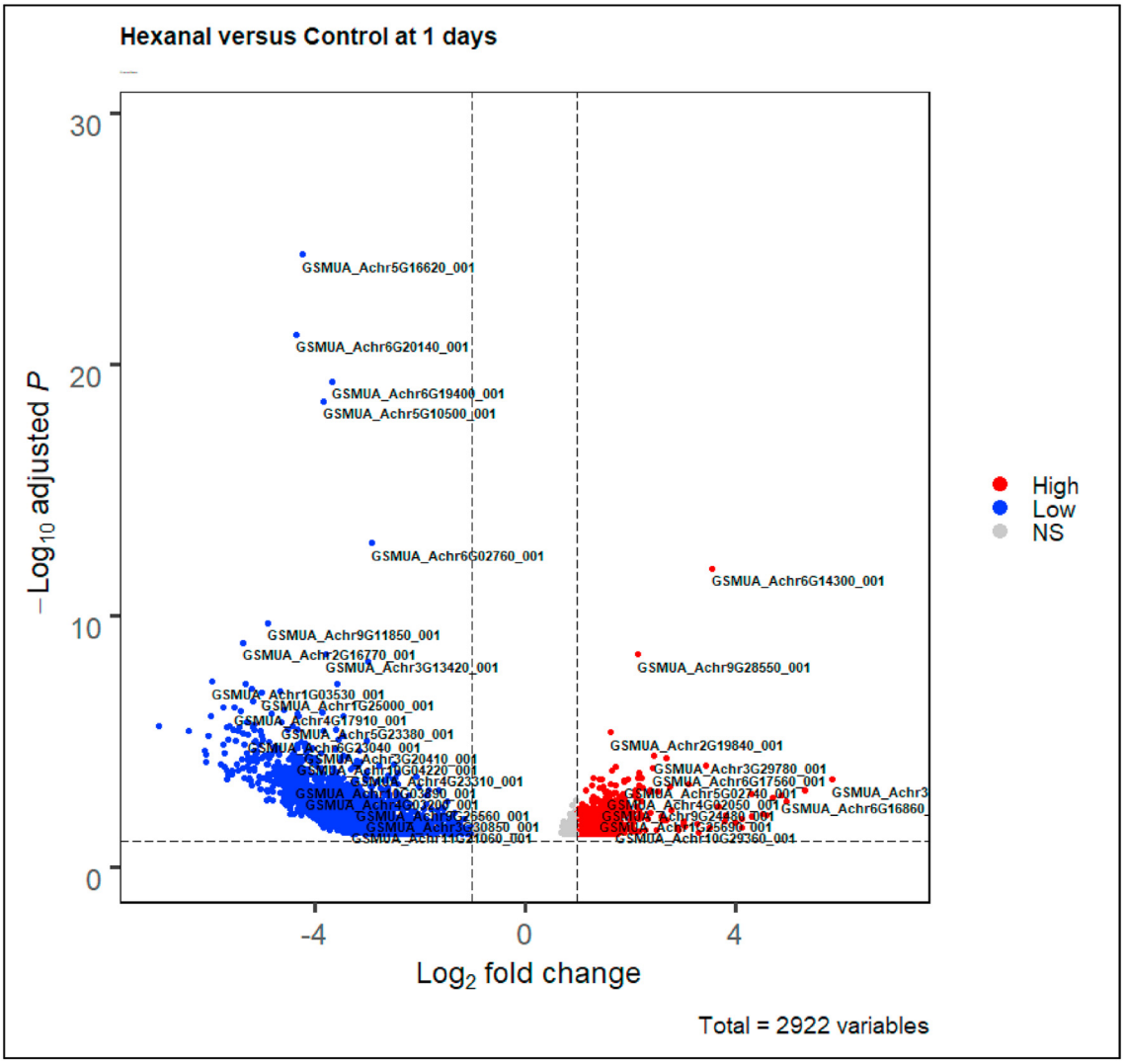

Fig. 6. Differential gene expression between hexanal treated and untreated fruits at day 1 of storage using Bioconductor Enhanced volcano package in R. The graph shows distribution of the upregulated genes between hexanal treated fruits and untreated fruits (red dots) and downregulated genes (blue dots) with the expection of the non-differentially expressed or unchanged genes (gray dots). Each dot in the Enhanced volcano diagram represents one differentially expressed genes. The X-axis represents the log2fold change and the y-axis shows the -log10 adjusted P-value. (For interprentation of the references to color in this figure legend, the reader is referred to the web version of this article.)

ripen later on in storage on day 18, as indicated by the peel color changing from green to yellow. On the other hand, fruits treated with exogenous ethylene ripened rapidly compared to the untreated control, and by day 18 of storage, they were completely ripe compared to the untreated control (Fig. 2).

3.3. *Comparative transcriptome analysis and differential gene expression*

To characterize the transcriptional profile of banana fruits treated with hexanal, data analysis was conducted at 1, 4, 18, and 24 days of storage (DOS) between the hexanal-treated, ethylene-treated, and untreated samples. Upon sequencing, a total of 8.5 billion read pairs was obtained. The analysis identified differentially regulated genes, 776 up-regulated and 2146 down-regulated DE genes at 1 day of storage (dos), 2423 up-regulated and 2862 down-regulated DE genes were detected at 4 dos, while 4516 up-regulated and 3121 down-regulated DE genes were detected at 18 dos, while 568 up-regulated and 41 down-regulated DE genes were detected at 24 dos in the hexanal-treated fruits compared with untreated ones (Fig. 3). Specifically, 589 genes were only found in day 1, while 13, 51 and 1462 genes were unique only in days 4, 18 and 24 respectively, in the hexanal treated fruits (Fig. 4). Similarly, in the ethylene-treated fruits; 4 up-regulated and 76 down-regulated DE genes were detected at 1 dos, 4820 up-regulated and 5395 down-regulated DEG at 4 dos while 5038 up-regulated and 4835 down-regulated DEGs at 18 dos while 5138 up-regulated and 4835 down-regulated DEGs were detected at 24 dos compared with the untreated fruits (Fig. 3). A total of 75 genes were unique in day 1 while 348, 34 and 4059 genes were only found in days 4, 18 and 24 of storage respectively, in the ethylene treated fruits (Fig. 4). Overall, most of the up-regulated/down-regulated genes are those involved in plant hormone signal transduction, nitrogen metabolism, photosynthesis, MAPK signaling pathway, arachidonic acid metabolism, alpha-linolenic acid metabolism, and secondary metabolites. This study focused majorly on genes involved in the banana ripening and senescence process and therefore, we discuss only the genes involved in ethylene biosynthesis, softening, respiration, flavor, and aroma volatiles.

3.3.1. *Commonly up-regulated/down-regulated genes across the treatments*

A summary of the most commonly up-regulated and down-regulated genes was generated through a heatmap (Fig. 5). Genes involved in cell

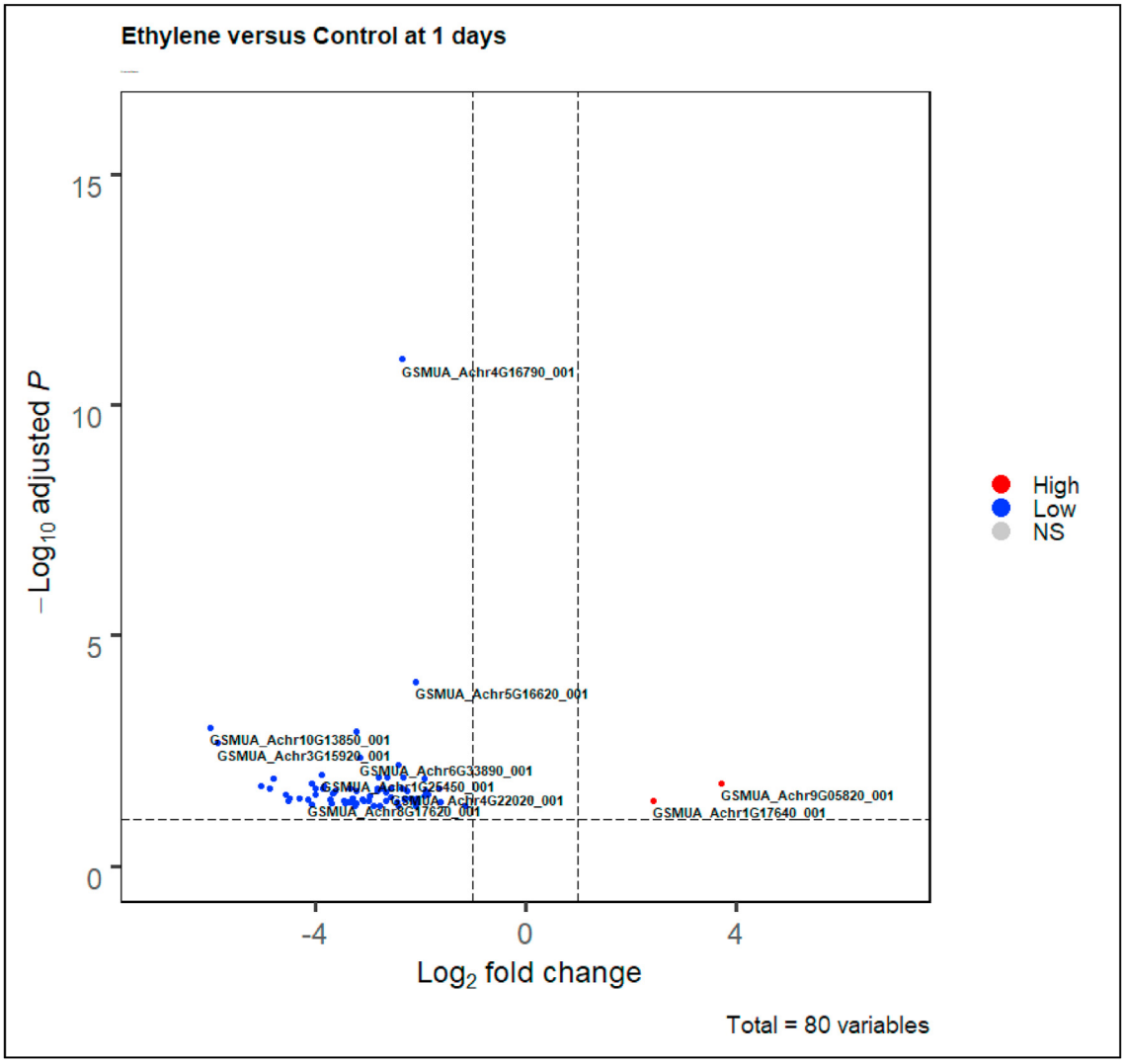

Fig. 7. Differential gene expression between ethylene treated and control fruits at day one of storage using Bioconductor EnhancedVolcano package in R. The graph shows the distribution of the upregulated genes between ethylene treated fruits and untreated fruits (red dots) and downregulated genes (blue dots) with the exception of the non-differentially expressed or unchanged genes (gray dots). Each dot in the EnhancedVolcano diagram represents one differentially expressed gene. The x-axis represents the Log2 fold change, and the y-axis shows the –Log10 adjusted P-value. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

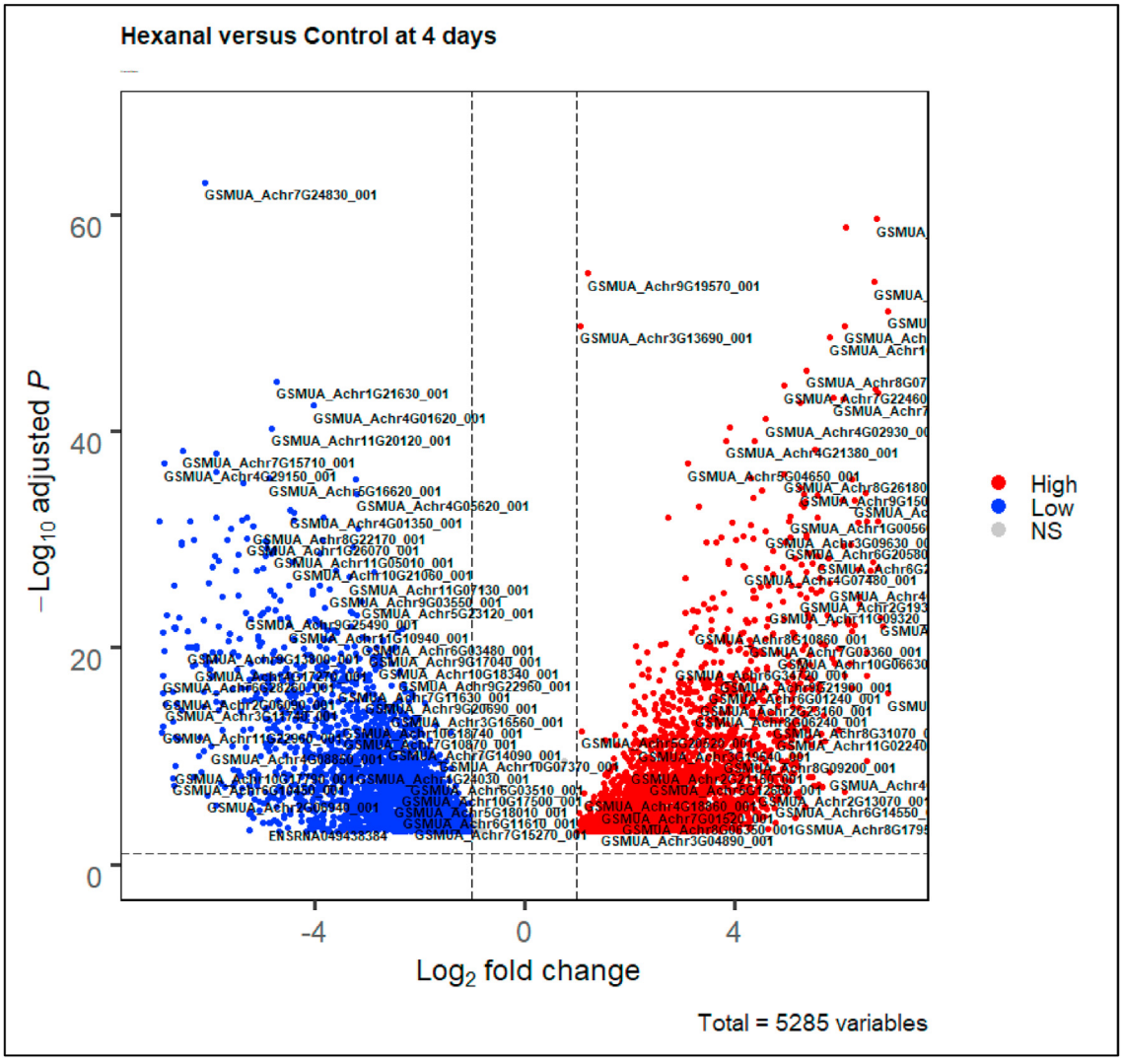

Fig. 8. Differential gene expression between hexanal treated and control fruits at day four of storage using Bioconductor EnhancedVolcano package in R. The graph shows the distribution of the upregulated genes between hexanal treated fruits and untreated fruits (red dots) and downregulated genes (blue dots) with the exception of the non-differentially expressed or unchanged genes (gray dots). Each dot in the EnhancedVolcano diagram represents one differentially expressed gene. The x-axis represents the Log2 fold change, and the y-axis shows the –Log10 adjusted P-value. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Down-regulated genes at day 4 of storage in the hexanal treated fruits.

Musa gene ID	Fold change	Description
GSMUA_Achr6G07090_001	−8.066411	Hypothetical protein
GSMUA_Achr3G12210_001	**−7.986645**	**Mannan endo-1,4-beta-mannosidase 9**
GSMUA_Achr5G08860_001	−7.924749	Phytosulfokines 3 [Source
GSMUA_Achr5G07480_001	**−7.909507**	**Expansin-A2**
GSMUA_Achr11G14670_001	**−7.852065**	**4-coumarate–CoA ligase-like 3**
GSMUA_Achr4G07870_001	**−7.816073**	**Mannan endo-1,4-beta-mannosidase 1**
GSMUA_Achr2G08720_001	−7.712897	Non-symbiotic hemoglobin 2
GSMUA_Achr11G06230_001	**−7.698712**	**4-coumarate–CoA ligase-like 6**
GSMUA_Achr3G26220_001	**−7.557151**	**Probable xyloglucan endotransglucosylase**
GSMUA_Achr9G25300_001	−7.548052	expressed protein
GSMUA_Achr4G28890_001	−7.385965	Pyruvate kinase isozyme G, chloroplastic
GSMUA_Achr3G29200_001	**−7.376818**	**calmodulin-binding protein**
GSMUA_Achr4G16570_001	**−7.370286**	**Putative O-methyltransferase ZRP4**
GSMUA_Achr5G08100_001	**−7.366191**	**Glucan endo-1,3-beta-glucosidase GVI**
GSMUA_Achr5G26460_001	−7.333241	Serine/threonine-protein kinase PBS1
GSMUA_Achr9G02390_001	−7.214481	Expressed protein
GSMUA_Achr9G02400_001	−7.214481	Cytochrome c subfamily, putative
GSMUA_Achr1G05010_001	−7.156602	WRKY transcription factor 22
GSMUA_Achr9G02370_001	−7.153570	Chitinase 6
GSMUA_Achr5G07470_001	**−7.148954**	**Expansin-A2**
GSMUA_Achr3G21980_001	−7.094123	4,5-DOPA dioxygenase extradiol-like protein
GSMUA_Achr5G28140_001	**−7.092154**	**Pyruvate decarboxylase isozyme 2**
GSMUA_Achr3G16520_001	−7.090656	Peroxidase 43
GSMUA_Achr8G30230_001	−7.090466	F-actin-capping protein subunit alpha
GSMUA_Achr5G14190_001	−7.089067	Omega-3 fatty acid desaturase, chloroplastic
GSMUA_Achr3G14210_001	−7.074001	Whole genome shotgun sequence of line PN40024
GSMUA_Achr3G11750_001	−7.068577	3-oxoacyl-[acyl-carrier-protein] reductase
GSMUA_Achr6G07190_001	−6.960259	3-hydroxybenzoate 6-hydroxylase 1
GSMUA_Achr3G10540_001	−6.907184	Cytochrome P450 71A9
GSMUA_Achr6G28260_001	**−6.901440**	**Probable pectate lyase 22**
GSMUA_Achr2G06090_001	−6.900857	Putative Transcription factor bHLH62
GSMUA_Achr11G22960_001	**−6.896571**	**Expansin-A8**
GSMUA_Achr3G11020_001	−6.886086	Pirin-like protein
GSMUA_Achr11G11790_001	**−6.885001**	**Putative Polyneuridine-aldehyde esterase**
GSMUA_Achr4G09840_001	−6.868048	3-hydroxy-3-methylglutaryl-coenzyme A reductase 3
GSMUA_Achr4G29150_001	**−6.865447**	**1-aminocyclopropane-1-carboxylate synthase**
GSMUA_Achr3G11740_001	−6.847148	Putative Predicted protein
GSMUA_Achr3G15660_001	−6.828465	Putative Pathogenesis-related protein 1
GSMUA_Achr5G29460_001	−6.711948	Ferritin-3, chloroplastic
GSMUA_Achr11G06790_001	−6.683474	Hydrolyzing O-glycosyl compounds
GSMUA_Achr5G11010_001	−6.665280	Probable gibberellin receptor GID1L2
GSMUA_Achr7G26370_001	**−6.658694**	**Indole-3-acetic acid-amido synthetase GH3.8**
GSMUA_Achr9G16670_001	**−6.580616**	**Polygalacturonase At1g48100**
GSMUA_Achr10G17790_001	−6.649837	Homeobox-leucine zipper protein ROC8
GSMUA_Achr5G03490_001	−6.647396	Putative Dihydroflavonol-4-reductase
GSMUA_Achr4G17840_001	**−6.589201**	**Beta-amylase 3**
GSMUA_Achr3G02040_001	**−5.956975**	**Xyloglucan endotransglucosylase**
GSMUA_Achr8G14160_001	−5.252622	**Probable xyloglucan endotransglucosylase**
GSMUA_Achr4G16280_001	−4.814683	**Phospholipase DDHD1**
GSMUA_Achr1G18250_001	−3.313718	**1-aminocyclopropane-1-carboxylate oxidase 3**

wall hydrolysis such as Expansins (GSMUA_Achr5G07480_001, GSMUA_Achr5G07470_001, GSMUA_Achr11G22960_001), Pectate Lyase 22 (GSMUA_Achr6G28260_001) and Mannan Endo-1,4-Beta-Mannosidase 9 (GSMUA_Achr3G12210_001) were identified. Additionally, genes involved in the synthesis of aroma volatiles such as 4-Coumarate–CoA Ligase-Like 6 (GSMUA_Achr11G06230_001), Anthocyanin 5-aromatic acyltransferase (GSMUA_Achr4G26740_001), O-Methyltransferase ZRP4 (GSMUA_Achr4G16570_001), and Polyneuridine-Aldehyde Esterase (GSMUA_Achr11G11790_001) were also identified among the commonly differentially expressed genes (Fig. 5).

3.3.2. Differentially expressed genes related to ethylene biosynthesis pathway following hexanal and ethylene treatments

In climacteric fruits such as banana, ripening is characterized by autocatalytic increase in ethylene production. In this study, GSMUA_Achr1G18250_001 and GSMUA_Achr4G29150_001 genes coding for 1-Aminocyclopropane-1-Carboxylate Oxidase (ACO) and 1-Aminocyclopropane-1-Carboxylate Synthase (ACS), key enzymes in the ethylene biosynthesis pathway were significantly ($P < 0.05$) down-regulated by −3.3 and −6.8 folds respectively, four days post hexanal treatment (Fig. 8, Table 1). On the other hand, ACO and ACS genes were up-regulated by 4.5 and 7.3 folds, respectively, in fruits treated with exogenous ethylene by day four post treatment (Fig. 9, Table 2).

After 18 days of storage, ACS and ACO genes involved in the ethylene biosynthesis pathway were highly up-regulated by 5.5 and 2.8 folds, respectively, in the hexanal treated fruits (Fig. 10, Supplemental table 3). This indicates that hexanal temporarily suppresses the ripening process in banana fruits.

3.3.3. Differentially expressed genes related to the cell wall and cell membrane degradation following hexanal and ethylene treatments

Hexanal treatment significantly ($P < 0.05$) down-regulated most of the genes coding for various cell wall hydrolases after one day of storage (Fig. 6, Supplemental table 1). Genes coding for PG, Endoglucanase and XTH were down-regulated by 3.3-, 5.2-, and 3.6-folds, respectively, following hexanal treatment after one day of storage (Fig. 6, Supplemental table 1). A similar trend was observed at day four of storage, with XTH, PG, PL and expansin genes being significantly down-regulated in the hexanal treated fruits (Fig. 8, Table 1). However, these genes (XTH, PL, PG, expansins) were up-regulated later in storage in day 18 (Fig. 10, Supplemental table 3).

After one day of storage, ethylene treatment caused GSMUA_Achr8G14160_001 coding for XTH and GSMUA_Achr2G05670_001 coding for Endoglucanase enzymes to be down-regulated by 3.2- and 2.3- folds respectively (Fig. 7, Supplemental table 2). At day four of storage, genes coding for various cell wall degradation enzymes such as PG, PL, XTH and expansin were significantly ($P < 0.05$) up-regulated in the ethylene treated fruits (Fig. 9, Table 2).

In the present study, GSMUA_Achr1G14590_001, which codes for Phospholipase D gene, a key enzyme in the cell membrane degradation pathway was significantly down-regulated by 3.3- and 4.8- folds in the hexanal treated fruits after one and four days of storage, respectively (Fig. 8, Table 1, Supplemental table 1). Notably, the transcripts of PLD increased by 6.2 fold after 18 days of storage in hexanal-treated fruits (Supplemental table 3). In fruits treated with exogenous ethylene, the up-regulation of PLD occurred earlier after four days of storage (Fig. 9, Table 2). Considering that PLD is a key enzyme in cell membrane degradation, our findings suggest that hexanal treatment inhibited PLD activity, delaying its action by 14 days.

3.3.4. Differentially expressed genes related to aroma and flavor compounds following hexanal and ethylene treatments

Hexanal treatment suppressed the expression of 4-Coumarate-CoA Ligase-Like 3, Flavonoid 3′, 5′-Hydroxylase, O-Methyltransferase Zrp4, Glycosyltransferase and Acetyltransferase by up to four days (Table 1).

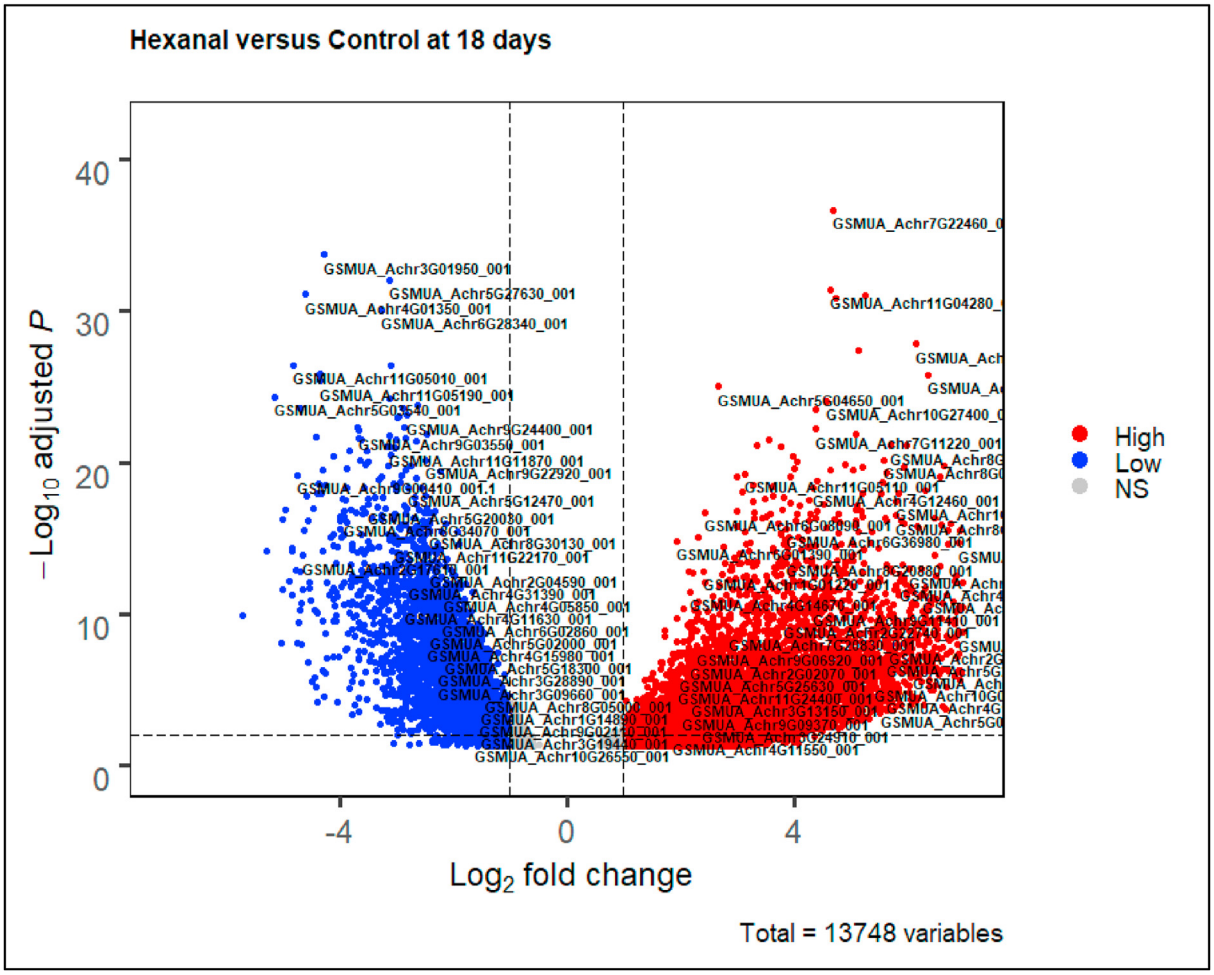

Fig. 9. Differential gene expression between hexanal treated and control fruits at day 18 of storage using Bioconductor EnhancedVolcano package in R. The graph shows the distribution of the upregulated genes between hexanal treated fruits and untreated fruits (red dots) and downregulated genes (blue dots) with the exception of the non-differentially expressed or unchanged genes (gray dots). Each dot in the EnhancedVolcano diagram represents one differentially expressed gene. The x-axis represents the Log2 fold change, and the y-axis shows the –Log10 adjusted P-value. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Transcripts of 4-Coumarate-CoA Ligase-Like 3 and 4-Coumarate-CoA Ligase-Like 6 increased by 8.9 and 9.1 folds in fruits treated with ethylene at day four of storage (Table 2). Later on, some gene families involved in synthesizing aroma volatiles such as Anthocyanin 5-Aromatic Acyltransferase, Glutathione S-Transferase, Zeatin O-Glucosyltransferase, 4-coumarate–CoA ligase-like 3, and 3-Hydroxybenzoate 6-Hydroxylase 1 were up-regulated in the hexanal treated fruits by day 18 of storage (Fig. 9, Supplemental table 3).

3.3.5. Summary of changes in overall plant metabolism pathways in plants in the hexanal treated fruits on day 1 and 18 post-treatment

Most of the genes related to various metabolic processes were down-regulated upon hexanal treatment at day one of storage (Fig. 11). However, later on, at day 18 of storage, most of these genes involved in the different metabolic processes were up-regulated (Fig. 12). Mapman analysis showed that most of these genes are involved in various ripening related processes such as cell wall hydrolysis, lipid metabolism, secondary metabolism processes such as flavonoid, terpenes and phenylpropanoid (Figs. 11 and 12).

4. Discussion

Hexanal has shown great potential to delay banana softening and ripening in general without compromising the post-harvest quality [30, 31]. In this study, we performed a genome-scale RNA-Seq analysis to characterize the transcript profile changes upon hexanal treatment. We found that hexanal treatment delays ripening by down-regulating Phospholipase D (PLD), Xyloglucan Endotransglucosylase (XTH), Expansin, Pectate Lyase (PL), and Polygalacturonase (PG) genes at one and four days post-treatment. This correlated with the observed reduced rate of pulp softening in the hexanal treated fruits compared to those treated with either ethylene or untreated controls. Additionally, hexanal application down-regulated the expression of 1-Aminocyclopropane-1-Carboxylate Oxidase (ACO) and 1-Aminocyclopropane-1-Carboxylate Synthase (ACS), key enzymes controlling the ethylene biosynthesis pathway up to day four post-treatment.

The current study's findings show that hexanal and ethylene treatment in banana fruits perturbed the ripening process. Indeed, genes involved in different ripening processes such as ethylene biosynthesis, respiration, softening, starch breakdown, cell membrane degradation, synthesis of secondary metabolites, and aroma volatiles were highly enriched in differentially regulated genes. At day one of post-treatment, most DEGs were suppressed, indicating that ripening was not initiated in both ethylene and hexanal treated fruits. Of interest was the suppression of PLD gene, the key enzyme in the cell membrane degradation pathway following hexanal treatment. The PLD gene was down-regulated in hexanal-treated fruits until 18 days of storage, an indication that hexanal acts by delaying cell membrane degradation in ripening fruit.

Table 2
Up-regulated genes at day 4 of storage in the ethylene treated fruits.

Musa gene ID	Fold change	Description
GSMUA_Achr11G06230_001	**9.124285**	**4-coumarate–CoA ligase-like 6**
GSMUA_Achr3G11740_001	9.087838	Putative Predicted protein
GSMUA_Achr3G11750_001	8.997658	Putative 3-oxoacyl-[acyl-carrier-protein] reductase
GSMUA_Achr4G07870_001	**8.962026**	**Mannan endo-1,4-beta-mannosidase 1**
GSMUA_Achr11G14670_001	**8.934630**	**4-coumarate–CoA ligase-like 3**
GSMUA_Achr5G14190_001	8.930586	Omega-3 fatty acid desaturase, chloroplastic
GSMUA_Achr6G28260_001	**8.848502**	**Probable pectatelyase 22**
GSMUA_Achr3G16520_001	8.670661	Peroxidase 43
GSMUA_Achr11G22960_001	**8.651980**	**Expansin-A8**
GSMUA_Achr3G15660_001	8.551304	Putative Pathogenesis-related protein 1
GSMUA_Achr5G07470_001	**8.503574**	**Expansin-A2**
GSMUA_Achr3G10540_001	8.418075	Putative Cytochrome P450 71A9
GSMUA_Achr11G11790_001	8.322081	Putative Polyneuridine-aldehyde esterase
GSMUA_Achr4G16570_001	**8.273729**	**Putative O-methyltransferase ZRP4**
GSMUA_Achr5G07480_001	**8.230896**	**Expansin-A2**
GSMUA_Achr4G09840_001	8.217694	3-hydroxy-3-methylglutaryl-coenzyme A reductase 3
GSMUA_Achr5G26460_001	8.195684	Serine/threonine-protein kinase PBS1
GSMUA_Achr4G28890_001	8.186530	Pyruvate kinase isozyme G, chloroplastic
GSMUA_Achr3G12210_001	**8.176467**	**Putative mannan endo-1,4-beta-mannosidase 9**
GSMUA_Achr5G28140_001	8.175771	Pyruvate decarboxylase isozyme 2
GSMUA_Achr8G12090_001	8.071015	bZIP family transcription factor, putative, expressed
GSMUA_Achr11G06790_001	8.013862	Hydrolase, hydrolyzing O-glycosyl compounds, putative
GSMUA_Achr6G35440_001	7.994881	Putative Vacuolar amino acid transporter 1
GSMUA_Achr3G29200_001	7.893561	Putative Vacuolar amino acid transporter 1
GSMUA_Achr5G17790_001	7.825083	carboxyl-terminal peptidase, putative, expressed
GSMUA_Achr5G21880_001	**7.812157**	**Probable xyloglucanendotransglucosylase**
GSMUA_Achr2G03950_001	7.795815	Putative peroxisomal-coenzyme A synthetase
GSMUA_Achr9G02400_001	7.771324	Putative expressed protein
GSMUA_Achr4G32050_001	7.767113	Putative Transmembrane protein 56-B
GSMUA_Achr1G12640_001	7.732491	Putative Zinc finger CCCH domain-containing protein 18
GSMUA_Achr9G27290_001	7.666850	integral membrane protein, putative, expressed
GSMUA_Achr6G24140_001	7.656789	Probable purple acid phosphatase 20
GSMUA_Achr6G07190_001	7.597139	Putative 3-hydroxybenzoate 6-hydroxylase 1
GSMUA_Achr10G26050_001	7.584836	Putative Probable WRKY transcription factor 42
GSMUA_Achr10G15290_001	7.567973	Putative WRKY transcription factor 6
GSMUA_Achr9G02950_001	7.561559	Pleiotropic drug resistance protein 3
GSMUA_Achr4G17840_001	7.546894	Beta-amylase 3, chloroplastic
GSMUA_Achr11G06900_001	7.484945	Putative expressed protein [Source
GSMUA_Achr5G03540_001	**7.450570**	**Probable xyloglucanendotransglucosylase**
GSMUA_Achr7G26370_001	7.348724	Probable indole-3-acetic acid-amido synthetase GH3.8
GSMUA_Achr4G29150_001	**7.331809**	**1-aminocyclopropane-1-carboxylate synthase**
GSMUA_Achr6G23280_001	**7.033801**	**Putative Probable glycosyltransferase At5g03795**
GSMUA_Achr4G16280_001	**6.415545**	**Putative Phospholipase DDHD1**
GSMUA_Achr3G26220_001	**6.284669**	**Probable xyloglucanendotransglucosylase**
GSMUA_Achr9G16670_001	**6.218343**	**Polygalacturonase At1g48100**
GSMUA_Achr8G14160_001	**5.823889**	**Probable xyloglucanendotransglucosylase**
GSMUA_Achr3G02040_001	**5.048936**	
GSMUA_Achr1G18250_001	**4.461206**	**Probable xyloglucanendotransglucosylase 1-aminocyclopropane-1-carboxylate oxidase 3**
GSMUA_Achr6G10730_001	**4.038721**	**Expansin-A15**
GSMUA_Achr6G12910_001	**3.494066**	**1-aminocyclopropane-1-carboxylate oxidase**

Expression of genes responsible for banana softening such as expansin, polygalacturonase (PG), pectin esterase (PE), pectate lyase (PL) and mannan endo-1,4-beta-mannosidase were down-regulated in the hexanal-treated fruits. The plant cell wall consists of polysaccharides such as cellulose, hemicelluloses, and pectins [11]. During the ripening process, the pectins polysaccharides are degraded by various enzymes including polygalacturonase, pectin esterase and pectate lyase [10]. The polygalacturonase hydrolyzes the α-1,4-glycosidic bonds between galacturonic acid residues, following de-esterification of pectin by pectin esterase [33]. On the other hand, expansins enzymes are engaged in loosening hydrogen bonds between cellulose microfibrils and matrix glycans [10]. Mannan endo-1,4-beta-mannosidase enzyme is involved in the cleavage of the mannan backbone in hemicellulose polysaccharides found in the cell wall [17,26]. The softening process is known to begin at the onset of ripening [6], possibly explaining the low expression of these genes in the hexanal treated fruits in days 1 and 4 of storage. These findings show that fruit softening was significantly delayed by hexanal treatment. However, the application of exogenous ethylene gas is known to trigger fruit softening, as evidenced in this study. Several genes, such as XTH, PL, expansins, PG and Endo-1, 4-Beta-Mannosidase have been previously reported to be responsible for banana softening [5,18]. This study showed that these genes involved in cell wall degradation were up-regulated following ethylene treatment as compared to the suppression of the same genes in the hexanal treated fruits. Additionally, genes involved in cell membrane degradation, such as Phospholipase D and calmodulin-binding protein genes were induced.

Ethylene initiates the ripening process in climacteric fruits such as banana [14,25], so it is expected that its application exogenously will fasten the ripening process by strongly up-regulating genes involved in ethylene biosynthesis. We discovered marked differences when we compared ethylene biosynthesis genes in the hexanal-treated fruits and those treated with ethylene. In untreated fruits, the expression of ACO and ACS genes involved in ethylene biosynthesis were not differentially expressed at day four of storage. However, upon ethylene treatment, these genes' expression were 4.5 and 7.3 folds higher respectively than untreated fruits. Notably, hexanal treatment resulted in a strong suppression in the expression of these key enzymes. The ACS and ACO are both rate-limiting enzyme in ethylene biosynthesis pathway. Since ethylene production is critical for initiation of ripening in climacteric fruits such as banana, the suppression of ACS and ACO genes in the hexanal treated fruits indicates that the ripening process had been suppressed.

Ripening is associated with the acquisition of certain aromas [6]. In banana fruits, aroma is attributed to various volatiles such as butyl acetate, isoamyl alcohol, isoamyl acetate and elemecine [8]. In our study, we discovered that hexanal treatment delayed the synthesis of volatile aroma genes up to 18 days of storage while exogenous ethylene treatment induced these genes by day four of storage. Banana volatiles are produced majorly by the fatty acid biosynthesis pathway, phenylpropanoid pathway, and isoleucine biosynthesis pathway [8]. In this study, genes involved in production of various aroma volatiles such as 4-coumarate-CoA ligase-like 3, Anthocyanin 5-aromatic acyltransferase, glutathione S-transferase, Zeatin O-glucosyltransferase, and Beta-fructofuranosidase 1, were significantly up-regulated by day four in fruits treated with exogenous ethylene as compared to the

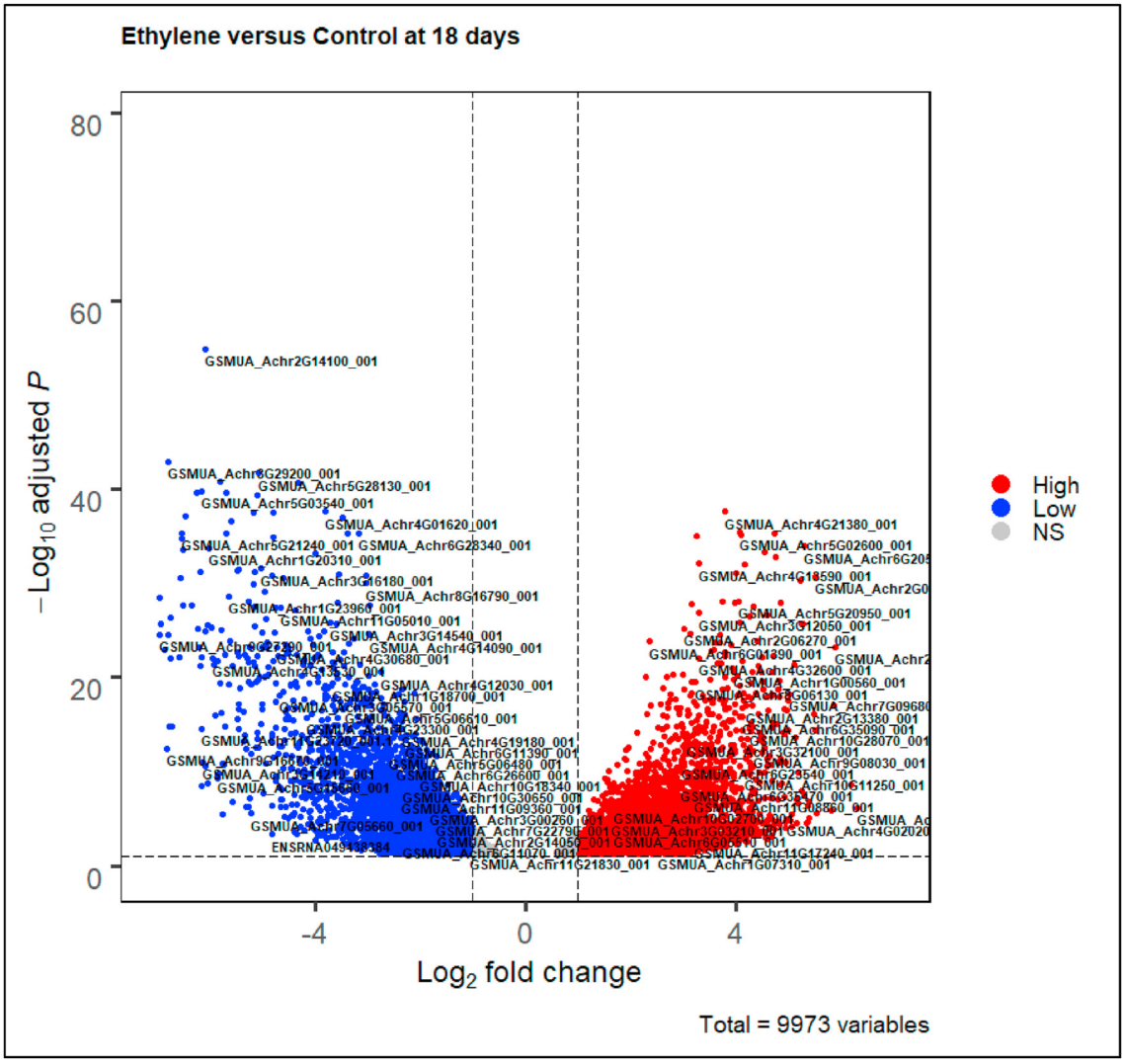

Fig. 10. Differential gene expression between ethylene treated and control fruits at day 18 of storage using Bioconductor EnhancedVolcano package in R. The graph shows the distribution of the upregulated genes between hexanal treated fruits and untreated fruits (red dots) and downregulated genes (blue dots) with the exception of the non-differentially expressed or unchanged genes (gray dots). Each dot in the EnhancedVolcano diagram represents one differentially expressed gene. The x-axis represents the Log2 fold change, and the y-axis shows the –Log10 adjusted P-value. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

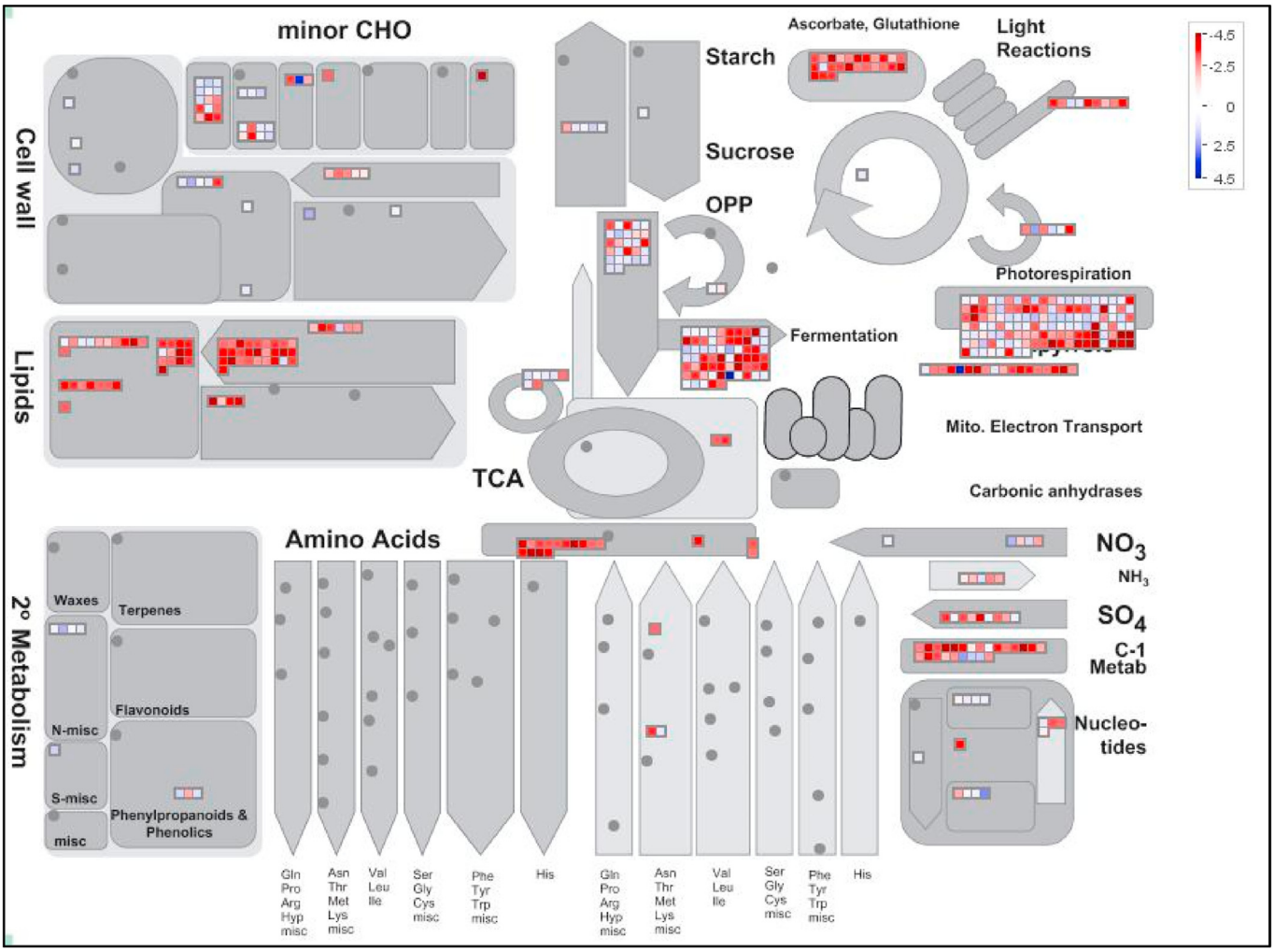

Fig. 11. Schematic of the metabolism pathway using the MapMan visualization platform at day 1 post storage in the hexanal treated fruits. MapMan analysis was performed using *Musa acuminata* gene ID. The logarithm of gene expression ratios (treatment/control) base 2 were used in MapMan analysis. The red or blue squares indicate the up or down-regulated genes involved in corresponding metabolism. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

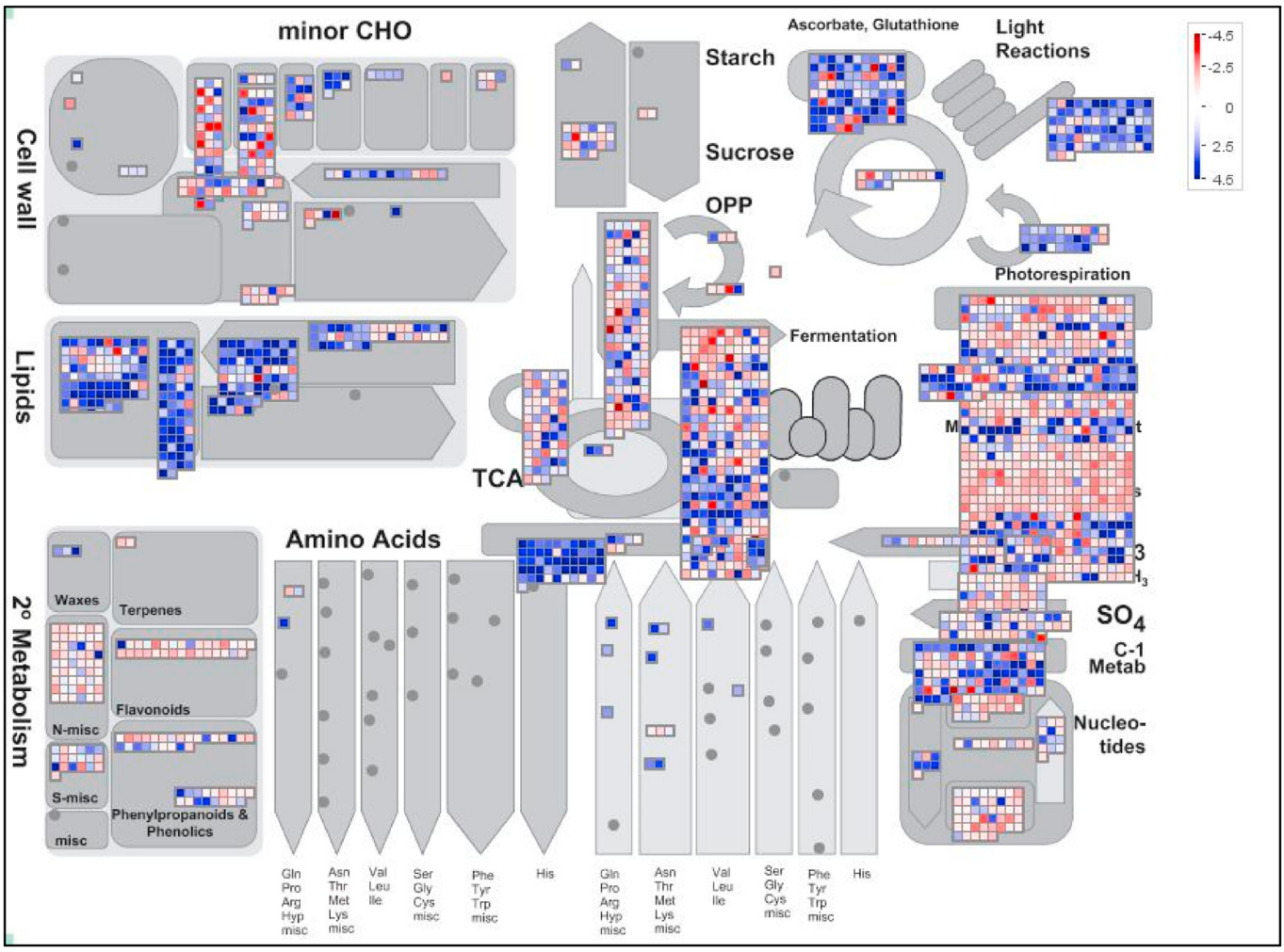

Fig. 12. Schematic of the metabolism pathway using the MapMan visualization platform at day 18 post storage in the hexanal treated fruits. MapMan analysis was performed using *Musa acuminata* gene ID. The logarithm of gene expression ratios (treatment/control) base 2 were used in MapMan analysis. The red or blue squares indicate the up or down-regulated genes involved in corresponding metabolism. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

hexanal-treated fruits. Interestingly, by the 18th day of storage, most of the gene families involved in aroma synthesis were up-regulated in the hexanal treated fruits.

By day 18 post treatment, fruits treated with ethylene were completely over-ripe while those treated with hexanal had started to ripen. Fruits treated with exogenous ethylene ripen within 4–5 days and become unsuitable for sale within 1–3 days after turning yellow [16]. On the other hand, in fruits treated with hexanal, most of the ripening related genes, including those involved in cell wall degradation and cell membrane deterioration, were induced later compared to the case with fruits treated with ethylene or the untreated controls. The observed temporal suppression of ripening genes shows that hexanal doesn't irreversibly inhibit the activities of phospholipase D enzyme, hence the fruit will finally undergo normal ripening and softening. Previous studies by Refs. [21,22,31] have shown that hexanal can enhance the treated fruits' quality attributes. The improved fruit quality may be due to hexanal treatment modulating the expression profile of genes involved in various pathways such as synthesis of aroma volatiles and starch degradation. In this study, the previously suppressed genes involved in synthesizing aroma volatiles following hexanal treatment, were later induced by the 18th day of storage. These findings show that hexanal treatment acts by antagonising ethylene action therefore, delaying the onset of ripening and its associated processes but does not affect the progression of ripening once it starts nor the quality of fruits.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jafr.2021.100114.

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