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RESEARCH REPORT

Comparative transcriptomic analysis provides novel insights on the hormonal regulation of postharvest apples under cold storage

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Abstract
Postharvest storage is a crucial phase during which fruit undergoes ripening and senescence. This study is based on the hypothesis that ethylene-auxin interactions play a significant role in determining apple storage life and quality during the postharvest period. To test this hypothesis, fruit flesh firmness and hormone levels were analyzed alongside transcriptomic changes observed through RNA sequencing. A comprehensive investigation of hormone metabolism-related gene expression across the whole transcriptome was conducted in 'Golden Delicious' apples over a six-month storage period to elucidate the molecular mechanisms underlying postharvest ripening and senescence. Understanding changes in the ripening process was facilitated by pretreatment with ethylene, the ethylene inhibitor 1-methylcyclopropene (1-MCP), auxin, and the auxin inhibitor 1-N-naphthylphthalamic acid (NPA) prior to cold storage. The results demonstrated that the auxin inhibitor suppressed ethylene production along with auxin levels, while the ethylene inhibitor suppressed auxin levels together with ethylene production throughout all storage periods. Findings also revealed that ethylene and auxin hormones regulate key metabolic processes associated with apple ripening during storage. Additionally, many transcriptional regulatory genes involved in hormone signaling and metabolism, such as *AIL5*, *ABI3*, *AP2*, *ERF-011*, *NAC*, *RAP210*, and *REVEILLE*, exhibited high activity during storage. Furthermore, this study revealed the high expression levels of *ABC transporter* and *LRR receptor protein kinase* for the first time in apples under storage conditions. These findings provide deeper insights into the physiological and molecular changes occurring in climacteric fruit during postharvest storage.

Keywords 1-MCP · Auxin · Ethylene · NPA · Transcriptomics

1 Introduction

Fruit are largely classified into two groups, climacteric and non-climacteric, based on differences in ripening metabolism, primarily influenced by ethylene biosynthesis. In climacteric fruit, ethylene biosynthesis increases during ripening, reaching its peak alongside a rise in respiration rate. In contrast, non-climacteric fruit exhibit a decline in ethylene production during ripening and senescence (Iqbal et al. 2017). Apples are among the most well-known examples of climacteric fruit, and the effects of ethylene synthesis or signaling inhibitors, such as 1-methylcyclopropene (1-MCP), in delaying ripening and senescence have been prominently observed both before and after harvest.

Fruit ripening and senescence are highly complex processes requiring precise coordination and involving significant changes in peel color, flesh firmness, taste, flavor, and aromatic compound levels (Li et al. 2016). Recent research

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highlights the crucial role of ethylene-auxin interactions in regulating ripening and senescence (Trainotti et al. 2007; Bottcher et al. 2013; Su et al. 2015; Li et al. 2016; Busatto et al. 2021). In certain fruits, such as strawberry, grape, and tomato, exogenous auxin application has been observed to delay ripening during the pre-ripening stage (Bottcher et al. 2010; Symons et al. 2012; Su et al. 2015). However, in pear and apple trees, auxin application has been shown to increase ethylene production, leading to early softening and accelerated ripening (Kondo and Takano 2000; Yuan and Carbaugh 2007; Li et al., 2008). Additionally, when 1-MCP is applied to apples during development and ripening, an increase in the expression of genes related to auxin signaling has been observed (Tadiello et al. 2016).

In 'Granny Smith' and 'Golden Delicious' apple varieties, five developmental stages—spanning fruit development, harvest, and one week post-harvest—were examined using two different microarray platforms (Tadiello et al. 2016). This study demonstrated that ripening and senescence are regulated by hormones and transcription factors. Among the upregulated genes were *MADS box*, *NAC (NAM/ATAF/CUC)*, zinc finger-type transcription factors, *AUX/IAA* (auxin/indole-3-acetic acid transcription inhibitor), and *ERF* (ethylene response factor), along with photosynthesis-related genes encoding light-harvesting and chlorophyll a/b-binding proteins. In another study, three apple varieties—'Golden Delicious,' 'Granny Smith,' and 'Fuji'—treated with 1-MCP were compared to untreated controls at six different time points after harvest (Busatto et al. 2021). This research focused on genes associated with hormone signaling and metabolism, revealing expression changes in ethylene-related genes, including *ACS* (1-aminocyclopropane-1-carboxylic acid synthase), *ACO* (1-aminocyclopropane-1-carboxylic acid oxidase), *ERS* (ethylene response sensor), and *ERF*, following 1-MCP treatment during storage. Genes related to auxin signaling and homeostasis that exhibited expression changes included *AUX/IAA*, *ARF* (auxin response factor), *GH3* (Gretchen Hagen3), and *ILL* (IAA-leucine resistant (ILR)-like).

A transcriptomic study comparing control and 1-MCP treatments in 'Fuji' apples stored under cold conditions (2, 4, and 8 months) further demonstrated that 1-MCP suppresses the expression of genes involved in ethylene synthesis and signaling, such as *ACS*, *ETR* (ethylene receptor), *EIN (*ethylene insensitive), and *ERF* (Zhang et al. 2022). Additionally, it inhibited the expression of enzymes responsible for cell wall degradation and revealed effects on glycolysis and the tricarboxylic acid cycle.

This study is based on the hypothesis that ethylene-auxin interactions play a key role in the postharvest period of apples, influencing storage life and quality. To test this hypothesis, the effects of ethylene, the ethylene inhibitor 1-MCP, auxin (*IAA*), the auxin inhibitor NPA, and combined 1-MCP + NPA treatments on fruit flesh firmness and hormone levels were investigated over a six-month storage period, with a focus on transcriptomic changes related to hormone metabolism. These findings contribute to a deeper understanding of the physiological and molecular changes occurring in climacteric fruit during storage.

2 Materials and methods

2.1 Hormone and inhibitor applications and storage

Apple fruit cv. 'Golden Delicious' harvested at the optimum time for the region were divided into six groups and subjected to treatments with the hormones ethylene and auxin, the ethylene inhibitor 1-MCP, the auxin inhibitor NPA, and 1-MCP + NPA. The fruit (600 kg) was obtained from the Egirdir Fruit Research Institute in Isparta, Southwest Turkey. The optimum harvest time for the cultivar in the region was determined by previous studies and corresponds to a soluble dry matter content of 10–12%, titratable acidity of ≥ 0.55%, firmness of ≥ 85 N, and 145–150 days after full bloom. Each analysis was performed in three replicates, with each replicate containing 20 fruits. All treatments were applied by immersing the fruit in a solution containing 0.1% Tween 20 and the respective substance for 3 min at room temperature (21°C and 50–60% relative humidity). The auxin treatment was performed using 20 mg L^{-1} indole acetic acid (IAA, Sigma, USA), the auxin inhibitor treatment used 1 µg L^{-1} 1-N-naphthylphthalamic acid (NPA, Naptalam, Sigma, USA), and the ethylene treatment used 0.5 g L^{-1} ethylene solution (ethephon, Bayer, Australia). After treatments, the fruit were allowed to dry and then stored in plastic boxes (53 mm × 36.5 mm × 31.5 mm) at 0°C and 90 ± 5% relative humidity for 6 months. The 1-MCP treatment was applied for 12 h in a gas-tight chamber at a concentration of 625 ppb. Before the 1-MCP treatment and the control treatment, the fruit were immersed in a solution containing 0.1% Tween 20 for 3 min at room temperature and dried to ensure exposure to the same conditions as the other treatments. Fruit flesh firmness, auxin, and ethylene production were determined in samples taken on day 1, month 3, and month 6 of storage, and transcriptomic analyses were performed using RNAseq.

2.2 Determination of fruit flesh firmness

Measurements were taken at two different points along the equatorial circumference of the fruit at the beginning and

during storage. A Lloyd LF Plus texture analyzer (Ametek, U.K.) with Nexygen software (version 4.1) was used for the measurements. A cylindrical probe with a diameter of 11 mm and a 1 KN load cell were used to penetrate the fruit to a depth of 10 mm at a constant speed of 100 mm min^{-1}. The maximum force obtained in Newtons (N) was considered as the firmness of the fruit flesh.

2.3 Determination of the auxin level

Auxin determination was performed using LC-MS-MS (Shimadzu, LCMS-8030 Plus) with modifications according to Han et al. (2012). Samples were dried to a powder under nitrogen gas, and then 2 g of the sample was weighed and homogenized with 20 mL of methanol containing 1% formic acid for 2 min using a vortex. This homogeneous mixture was then incubated in the dark in an ultrasonic bath at 42 kHz for 30 min. After this process, the samples were centrifuged at 3000 x g (15 °C) for 10 min. The supernatant (20 mL) was diluted with 180 mL of pure water. This solution was passed through SPE C18 cartridges (500 mg 6 mL^{-1}, Finisterre by Teknokrama TM) at a rate of approximately 1–2 drops s^{-1} using a vacuum manifold, and then approximately 1–2 drops s^{-1} of 5 mL of water were passed through the cartridges. The cartridges were washed with 6 mL of methanol containing 1% formic acid at a rate of approximately 1–2 drops s^{-1}, and the resulting solution was evaporated under a nitrogen gas stream at 40 °C until dry. The residue was dissolved in 1 mL of methanol, filtered through a 0.45 µm filter, and ready for injection into the instrument.

In the analyses, various concentrations of the main representatives of auxins, namely indole-3-acetic acid (IAA, Sigma, USA), indole-3-butyric acid (IBA, Sigma, USA), and naphthalene acetic acid (NAA, Sigma, USA), were used as standards for calibration. An Inertsil ODS-4 (2.1 × 50 mm), 3 µm HPLC column was used at 40 °C for separating IAA, IBA, and NAA.

2.4 Determination of ethylene production

The fruit were weighed to approximately 1 kg and placed in gas-tight glass jars with a volume of 5 L, tightly sealed. After waiting for 2 h at room conditions (20 ± 1 °C), 15–20 mL of gas sample was taken from the jars with a gas-tight plastic syringe and directly injected into the gas chromatograph. Measurements were conducted using a fused silica capillary column (GC-GASPRO, 30 m, × 0.32 mm I.D.) with a flame ionization detector (FID) on an Agilent GC-6890 N gas chromatograph and Chemstation A.09.03 [1417] software. The carrier gas flow was maintained at a constant flow rate of 1.7 mL min^{-1}. The temperature of the oven FID detector was set at 250 °C. The flow rates for high-purity hydrogen (H_2) and dry air used as carrier gasses in the FID were 30 and 300 mL min^{-1}, respectively. The amount of ethylene produced by the fruit was evaluated using the following formula.

$$\text{Ethylene production}\left(\mu L\ C_2H_4\ kg^{-1}h^{-1}\right) = \left[\left(C_2H_{4\ \text{measurement}} \times \left(V_{\text{jar}} - V_{\text{fruit}}\right)\right) / \left(h \times M \times 1000\right)\right]$$

h: time elapsed inside the jar (hours); M: weight of the fruit inside the jar (kg).

2.5 Transcriptomic analysis by RNA sequencing

RNA isolation from apple seeds was performed using the RNeasy Plant Mini Kit (Qiagen, USA), and DNA cleanup was performed according to the instructions of the RNase-Free DNase Kit (Qiagen, USA). The quantity and quality of total RNA were determined using Nanodrop (USA) and visualized by 2% agarose gel electrophoresis. mRNA libraries were prepared using The Ribo-Zero rRNA Removal Kit (Plant) (EpiCentre, Biotechnologies, America) and The NEBNext® Ultra™ RNA Library Prep Kit for Illumina (New England Biolabs, Beijing, China) compatible with the Illumina HiSeq4000 system.

Quality control of the sequences was performed using FastQC and CLC Genomics Workbench version 11 programs. Subsequently, sequences with low-quality reads and adapter regions were removed using the CLC Genomics Workbench version 11 tool. The reference genome for the 'Golden Delicious' variety of apple was obtained from the Phytozome database and was used as the reference genome for read assembly. Clean reads were mapped to the reference genome using the CLC Genomics Workbench version 11 program to assemble contigs, and unigenes was created by assembling contigs. In addition, the expression level differences in mRNA sequences were determined by calculating reads per kilobase per million mapped reads (RPKM) values. The datasets analyzed during the current study have been uploaded to the NCBI Sequence Read Archive (SRA) under the Bioproject accession number PRJNA1145589.

After determining the differences in the expression levels of the libraries, the expression differences were examined in groups by comparing the data of: (a) control sample-ethylene-treated product-1-MCP treated with an ethylene inhibitor; (b) control sample-auxin-treated product-product treated with an auxin inhibitor; and (c) control sample-ethylene-treated product-auxin-treated product-product treated with both ethylene and auxin inhibitors. Furthermore, hierarchical clustering heatmaps were created using the log2

RPKM values of the genes in these comparison groups using PermutMatrix software (Caraux and Pinloche 2005).

Using the Blast2GO program within OmicsBox (Conesa et al., 2008), gene ontology and function analyses were conducted for the identified genes. For this purpose, gene transcripts were loaded into the Blast2GO program and a BlastX search was performed. Subsequently, a file with a.dat extension containing gene ontologies was obtained and examined for the blast search. The top 25 genes with the highest and lowest log2 fold change values were selected from the samples collected on day 1, month 3, and month 6. These genes were analyzed for Gene Ontology (GO) classification in order to examine both upregulated and downregulated genes. The analysis covered the three main GO categories: biological processes, cellular components, and molecular functions. In addition, PCA was performed separately for the samples taken on day 1, month 3, and month 6 using Workbench 11.0 software to determine if there was a cumulative difference between the groups. A Venn diagram was created to determine the common and unique genes among the groups. Using KAAS (KEGG Automatic Annotation Server) with a *p*-value of 0.01 and selecting the top 25 genes with the highest and lowest log2 fold change values, KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis was performed to determine the pathways associated with the genes on day 1, month 3, and month 6. The results obtained for metabolic pathways were mapped using iPath 3.0, whereas the other results were visualized using KEGG tools.

2.6 RT–PCR verification

The First Strand cDNA Synthesis Kit (Thermo Fisher Sci., USA) was used to synthesize cDNA from RNA molecules. For gene expression measurements, iTaq Universal SYBR Green Supermix (Bio-Rad, USA) containing SYBR Green I was used. The primers were designed using NCBI Prime-Blast and are shown in Supplementary Table 3. The amplification reactions consisted of the following steps: initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 10 s and annealing and extension at 60 °C for 30 s. For each RT-PCR analysis, negative control samples containing all reaction components except cDNA and an internal control primer were included. Specific primers for the apple actin gene with the GDR number MDP0000752428 (The Genome Database for Rosaceae) were used as the internal control. Amplifications were performed using three biological and two technical replicates. The relative expression of the examined genes was determined using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak 2008).

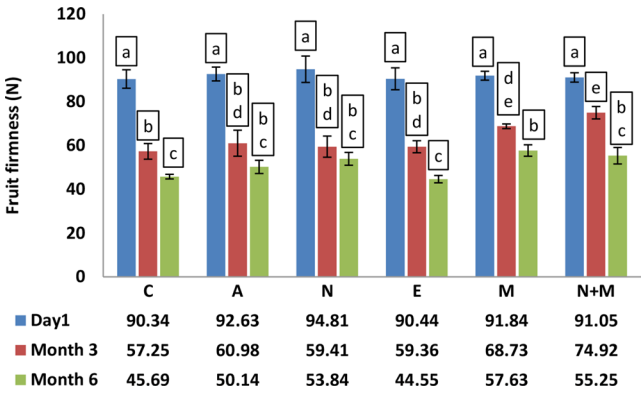

Fig. 1 Changes in fruit flesh firmness in apples under different storage periods (1 day, 3 months and 6 months) and treatments (C: Control treatment, A: Auxin treatment, N: Auxin inhibitor treatment, E: Ethylene treatment, M: Ethylene inhibitor treatment, N+M: Auxin inhibitor+ethylene inhibitor treatment). Different letters on the columns indicate statistical differences on 5% significance level

2.7 Statistical analysis

The trial was conducted using a completely factorial randomized design. Analyses were conducted with three replications, each consisting of 20 fruit, throughout the storage period. The data obtained from the experiment were subjected to analysis of variance using JMP7 software. The significance of the treatments was determined at a 5% probability level using Tukey Test of Oneway ANOVA by use of SPSS 11.0 (Statistical Package for Social Sciences, SPSS Inc., IL, USA).

3 Results

3.1 Determination of fruit flesh firmness

The effects of treatments and storage duration on fruit flesh firmness were significant ($p<0.05$), whereas the interaction between storage duration and treatment was not significant. Fruit firmness decreased across all treatments as storage progressed. On day 1, firmness values ranged from 90.34 N (C treatment) to 94.81 N (N treatment). By month 3, firmness declined to a range of 57.25 N (C treatment) to 74.92 N (N+M treatment). At the end of storage (month 6), firmness values further decreased, ranging from 44.55 N (E treatment) to 57.63 N (M treatment) (Fig. 1).

Regardless of treatment, fruit firmness declined by an average of 29% at month 3 and 44% at month 6 compared to initial values. The M treatment best preserved fruit firmness, maintaining values of 91.84 N on day 1 and 57.63 N at month 6, followed closely by the N+M treatment (91.05 N on day 1, 55.25 N at month 6). In contrast, the greatest firmness loss was observed in the E treatment (90.44 N on day 1,

44.55 N at month 6), followed by the C treatment (90.34 N on day 1, 45.69 N at month 6).

3.2 Determination of the auxin level

In this study, tissue levels of IAA, IBA, and NAA were analyzed separately under different treatments (Fig. 2 and Supplementary Fig. 1). Among the auxins, IBA exhibited the most pronounced response to the treatments. Auxin application (A) on day 1 significantly increased IBA levels across all time points (Fig. 2). Conversely, treatment with the auxin inhibitor (NPA) on day 1 led to a significant reduction in IBA levels compared to other time periods. In samples treated with ethylene (E), IBA levels were higher than in the control (C) group, though the increase was not statistically significant. However, in samples treated with an ethylene inhibitor (M) or a combination of ethylene and auxin inhibitors (N+M), IBA levels were significantly lower than in the control across all time points.

Over time, IBA levels declined in all treatment groups except for those receiving NPA (N), where levels increased during storage (Fig. 2). This pattern was unique to IBA and was not observed for NAA or IAA. The sustained increase in IBA levels under NPA treatment suggests that, unlike NAA, IBA biosynthesis may partially escape the inhibitory effects of NPA over prolonged storage. Similar to NAA, IBA levels were also affected by 1-MCP (ethylene inhibitor) treatment, showing particularly lower values than those observed under NPA treatment, especially during extended storage periods. The effects of treatments, storage duration, and their interaction on auxin content were statistically significant ($p<0.05$).

3.3 Determination of ethylene production

The effects of treatments, storage period, and the interaction between storage period and treatment on the ethylene content of apples were significant ($p<0.05$). During storage, the ethylene production of apples increased parallel to the progression of ripening. The ethylene production values ranged from 0.17 g $kg^{-1}s^{-1}$ (N treatment) to 0.86 g $kg^{-1}s^{-1}$ (E treatment) on day 1, and 0.48 g $kg^{-1}s^{-1}$ (M treatment) to 78.69 g $kg^{-1}s^{-1}$ (E treatment) on month 3. At the end of storage, the values ranged from 3.33 g $kg^{-1}s^{-1}$ (N+M treatment) to 115.13 g $kg^{-1}s^{-1}$ (E treatment) (Fig. 3). The N+M and M treatments were the most effective in suppressing ethylene production throughout storage, followed by the N treatment. On average, the highest ethylene production was found in the E treatments.

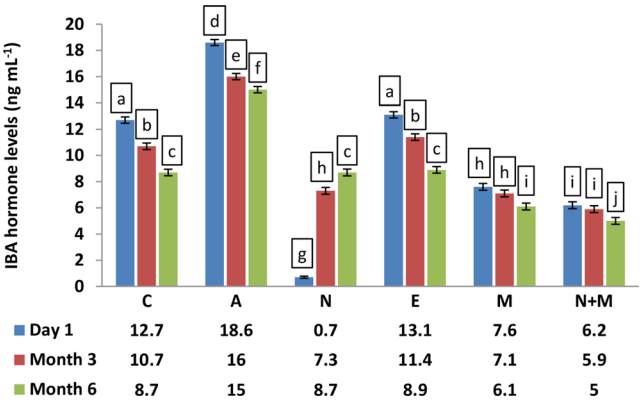

Fig. 2 Changes in IBA hormone levels in apples under different storage periods (1 day, 3 months and 6 months) and treatments (C: Control treatment, A: Auxin treatment, N: Auxin inhibitor treatment, E: Ethylene treatment, M: Ethylene inhibitor treatment, N+M: Auxin inhibitor+ethylene inhibitor treatment). Different letters on the columns indicate statistical differences on 5% significance level

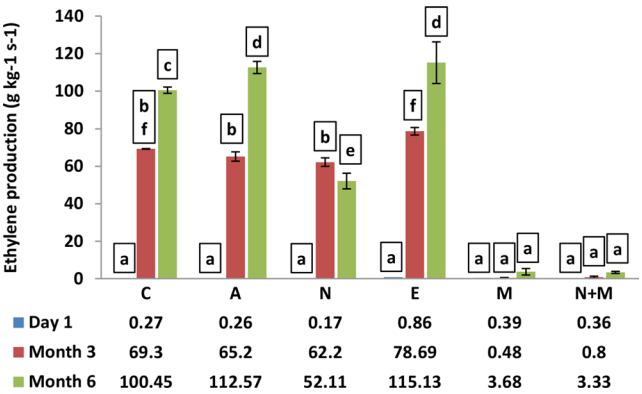

Fig. 3 Changes in ethylene production in apples under different storage periods (1 day, 3 months and 6 months) and treatments (C: Control treatment, A: Auxin treatment, N: Auxin inhibitor treatment, E: Ethylene treatment, M: Ethylene inhibitor treatment, N+M: Auxin inhibitor+ethylene inhibitor treatment). Different letters on the columns indicate statistical differences on 5% significance level

3.4 Transcriptomic analysis by RNA sequencing

The quality assessment resulted in high-quality (≥Q30) and clean reads. Raw reads ranged from 17,156,336 to 65,981,015, with an average GC base ratio of 56%. The high-quality data's forward and reverse reads were merged. RNA-seq analysis, including biological replicates, was performed using the CLC Genomics Workbench 11.0 program. The total length of the obtained transcripts ranged from 91,105,548 to 126,404,002, with over 91% matching.

3.4.1 Venn diagram

After one day of cold storage, a total of 240 genes exhibited increased expression, while 449 genes showed decreased expression when the five treatment groups (A, N, E, M,

N+M) were compared with the untreated control (C) (Supplementary Fig. 2). At three months, the number of differentially expressed genes (DEGs) was lower, with 154 genes upregulated and 56 genes downregulated compared with the control (Supplementary Fig. 3). By six months, gene expression changes were more pronounced, with 449 genes showing increased expression and 353 genes showing decreased expression (Supplementary Fig. 4). In total, 802 genes were identified as differentially expressed over the storage period, with 689 genes at 24 h, 210 genes at 3 months, and 802 genes at 6 months.

At day 1, when individual treatments were compared, ethylene (E) upregulated the expression of 81 genes and downregulated 249 genes. The ethylene inhibitor (M) upregulated 72 genes and downregulated 32 genes. Auxin (A) upregulated 7 genes and downregulated 1 gene, while the auxin inhibitor (N) upregulated 6 genes and downregulated 5 genes. The combination of inhibitors (N+M) resulted in the upregulation of 2 genes and the downregulation of 1 gene. In the Venn diagram analysis (1 C-1E-1 M-1 A-1 N-1 N+M), the genes MD05G1135300, MD15G1286600, and MD01G1080400 were identified as commonly upregulated, while MD00G1039200, MD08G1037800, and MD16G1263300 were commonly downregulated.

At the three-month sampling period, ethylene (E) upregulated 6 genes and downregulated 16 genes. The ethylene inhibitor (M) upregulated 15 genes and downregulated 4 genes. Auxin (A) upregulated 6 genes and downregulated 4 genes, while the auxin inhibitor (N) upregulated 3 genes and downregulated 12 genes. The combination of inhibitors (N+M) upregulated 15 genes and downregulated 6 genes. In the Venn diagram analysis (3 C-3E-3 M-3 A-3 N-3 N+M), 74 upregulated genes were found to be common across treatments, while MD11G1072500 was the only gene commonly downregulated (Supplementary Fig. 3).

At the six-month sampling period, ethylene (E) upregulated 32 genes and downregulated 111 genes. The ethylene inhibitor (M) upregulated 63 genes and downregulated 49 genes. Auxin (A) upregulated 18 genes and downregulated 14 genes, while the auxin inhibitor (N) upregulated 80 genes and downregulated 46 genes. The combination of inhibitors (N+M) upregulated 182 genes and downregulated 29 genes. In the Venn diagram analysis (6 C-6E-6 M-6 A-6 N-6 N+M), the commonly upregulated genes were MD10G1069200 and MD12G1187000, while the commonly downregulated genes were MD02G1099300, MD07G1029400, MD17G1057900, and MD15G1040500 (Supplementary Fig. 4).

3.4.2 Heat maps

A heat map was generated by selecting the 25 genes with the highest and lowest $\log_2$ fold change values, with p-values less than 0.01 (Supplementary Figs. 5–7). Among the genes exhibiting significant expression changes were *RAP2.10* (ethylene-responsive transcription factor), *ERF-011-like* (ethylene-responsive transcription factor), *AP2-like* (ethylene-responsive transcription factor), *ABI3* (B3 domain transcription factor), and *REVEILLE 3* (protein involved in circadian regulation), all of which are associated with hormone signal transmission and metabolism. In addition to transcription and RNA splicing, a wide range of genes related to ribosome structure and translation were highly active. Other responsive genes included those involved in photosynthesis, respiration, carbohydrate and fat metabolism, as well as genes regulating oxidative stress defense responses.

By the end of three months of cold storage, the gene expression profiles remained largely similar to those observed on day 1. A wide range of kinase and phosphatase genes responsible for signal transduction were activated (Supplementary Figs. 8–10). Several key genes related to hormone metabolism showed significant expression changes compared with the control. Notably, *ethylene-responsive transcription factor 4* exhibited reduced expression under ethylene (E), auxin (A), ethylene inhibitor (M), and the combination of auxin and ethylene inhibitors (N+M). Similarly, *SAUR36* (an auxin-responsive protein) showed decreased expression under E, A, and N+M treatments, while *ARG7-like* (an indole-3-acetic acid-responsive gene) displayed reduced expression under A and N treatments.

At the end of six months of storage, despite similarities with the genes showing expression changes from day 1, ethylene-responsive transcription factors were largely absent from the list of genes exhibiting the most severe expression changes, except for *RAP2.10*, which showed decreased expression under ethylene treatment and when an ethylene inhibitor was applied (Supplementary Figs. 11–13). Other hormone metabolism-related genes with significant expression changes included *ARG7-like*, which exhibited increased expression under all treatments compared with the control, and *SAUR36*, which showed increased expression in response to auxin and auxin inhibitor applications.

3.4.3 Primary component analysis (PCA)

Cumulative differences between the groups were determined by performing principal component analysis (PCA) separately for day 1, month 3, and month 6. It was observed that control (C) and auxin inhibitor (N) treated samples clustered close to each other at the end of day 1. All treatment

groups showed different characteristics in months 3 and 6. The treatment groups clustered together and differed greatly from the control group (Supplementary Fig. 14).

3.4.4 Gene ontology analysis (GO)

Gene ontology analysis performed for functional characterization on day 1 revealed that the predominant biological processes involving active genes were cellular metabolic processes. The primary molecular functions of these genes included binding to organic cyclic and heterocyclic compounds, while their cellular localizations were mainly within intracellular anatomical structures (Supplementary Fig. 15). Similarly, gene ontology analysis conducted after three months of storage indicated that the active genes were primarily involved in the metabolic processes of organic substances. Their molecular functions remained largely focused on binding to organic cyclic and heterocyclic compounds, and their cellular localizations were predominantly within intracellular anatomical structures (Supplementary Fig. 16). By the end of six months of storage, the functional characterization results remained consistent with those observed on day 1 and month 3. The key biological processes continued to be centered on the metabolic processes of organic substances, with molecular functions related to binding organic cyclic and heterocyclic compounds, and the cellular localization of active genes remained largely within intracellular anatomical structures (Supplementary Fig. 17).

3.4.5 KEGG pathway analysis

KEGG pathway analysis was performed using the KAAS (KEGG Automatic Annotation Server) tool, selecting the top 25 genes with a p-value of 0.01 and the highest and lowest log2 fold change values (Fig. 4, Supplementary Tables 1, and Supplementary Fig. 18). On day 1, 14 genes were associated with metabolic pathways, and 5 genes were linked to ribosomes. Other key pathways included five genes involved in protein processing in the endoplasmic reticulum, one gene related to ribosome biogenesis in eukaryotes, and one gene associated with ABC transporters. By month 3, the number of genes involved in metabolic pathways decreased to 9, while the number of genes related to ribosomes increased to 8. During this period, two genes associated with glycosyltransferases, two genes related to peptidases and their inhibitors, and three genes involved in translation factor activity were identified. Additionally, the number of genes associated with plant hormone signal transduction increased by two. At month 6, the number of genes related to metabolic pathways increased significantly to 24, and the number of ribosome-associated genes also rose to 11. During this period, five genes involved in antigen processing and presentation, four genes linked to plant hormone signal transduction, and three genes related to neutrophil extracellular trap formation were identified.

The analysis results indicated that pathway diversity was higher at months 3 and 6 compared to day 1 (Supplementary Tables 1 and Supplementary Fig. 18). Genes involved in genetic information processing were the most active across all three time points. Pathways related to energy metabolism, amino acid metabolism, and environmental information processing remained consistently active throughout storage. However, pathways involved in cellular signaling and the biosynthesis of secondary metabolites were uniquely active during the 6-month storage period.

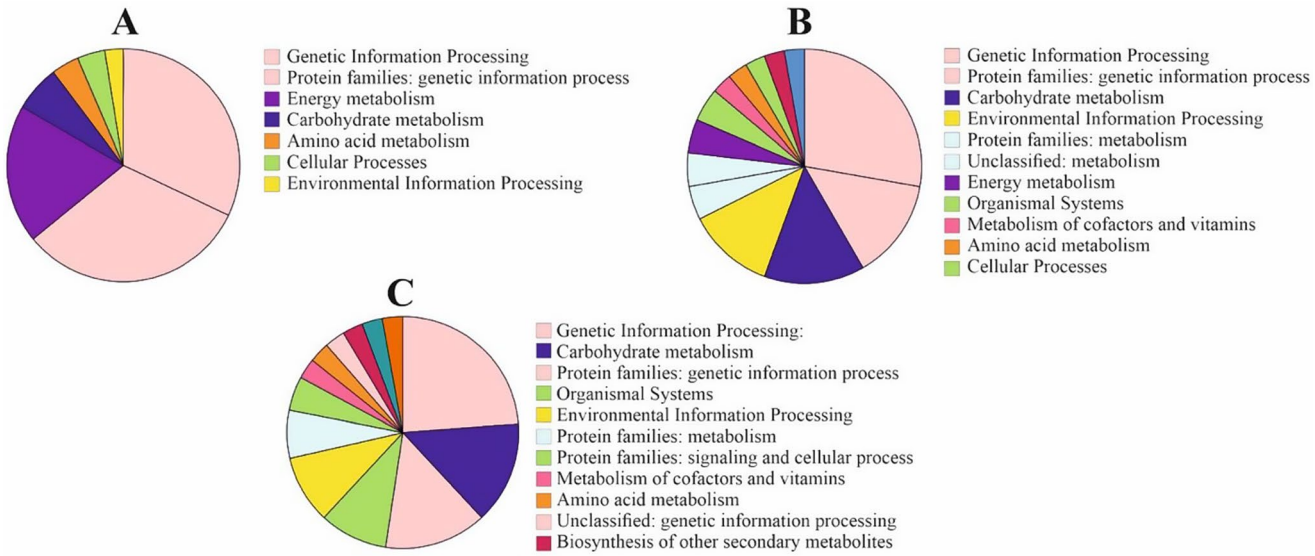

Fig. 4 Summary of KEGG analysis. **A**; after 1 day, **B**; 3 months and **C**; 6 months storage

Genes exhibiting significant expression changes in the oxidative phosphorylation pathway on day 1 included *COX1*, which encodes subunit 1 of the cytochrome c oxidase complex, a key enzyme in cellular energy production (Supplementary Fig. 20). Other highly expressed genes included *ATPF0A* and *ATPF0B*, which encode subunits of the F-type H+-transporting ATPase complex responsible for ATP synthesis, and *ATPF1D*, which encodes the delta subunit, a regulatory component of this ATPase complex. Additionally, *ND5*, which encodes chain 5 of the NADH-ubiquinone oxidoreductase complex involved in the electron transport chain and energy production, showed substantial expression changes.

At month 3, apple genes displaying significant expression changes were primarily associated with energy metabolism and photosynthesis (Supplementary Fig. 21). Several of these genes, including *ATPF0A*, *ATPF0B*, and *ATPF1D*, were part of the *ko01100* metabolic pathway. Notably, genes related to Photosystem II—*psbJ*, *psbK*, and *psbZ*—were identified. These genes encode components of the Photosystem II complex, which converts light energy into chemical energy during photosynthesis. The *petB* gene, which encodes the cytochrome *b6* protein, also showed high expression changes. This protein is involved in the photosynthetic electron transport chain and plays a crucial role in energy production.

After 6 months of storage, genes involved in carbon fixation (*ko01100* metabolic pathway) exhibited notable expression changes (Supplementary Fig. 22). The *ALDO* gene encodes the fructose-bisphosphate aldolase enzyme, which catalyzes the cleavage of fructose-1,6-bisphosphate into two three-carbon molecules during glycolysis and gluconeogenesis. The *TPI* gene encodes triosephosphate isomerase, which regulates the interconversion of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate in glycolysis. Additionally, the *E1.1.1.40* gene, encoding malate dehydrogenase, catalyzes the oxidation of malate to oxaloacetate while reducing NADP+ to NADPH, linking this reaction to both the Krebs cycle and photosynthesis. The *rbcS* gene, encoding the small subunit of the ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) complex, was also highly expressed. This enzyme facilitates carbon fixation by binding CO_2 to ribulose-1,5-bisphosphate during photosynthesis.

3.5 RT–PCR verification

The validation of bioinformatics results was conducted on 20 genes related to plant hormone metabolism using RT-PCR to confirm the expression levels identified through RNA-Seq analysis, as shown in Fig. 5. The expression patterns observed in RT-PCR were consistent with those obtained from RNA-Seq, indicating strong agreement between the two methods.

4 Discussion

In apples, which belong to the climacteric fruit group, various studies have demonstrated that as ripening and senescence progress, ethylene production increases, leading to fruit softening (Li et al. 2016; Iqbal et al. 2017). Additionally, during ripening, structural components such as pectin in the cell wall, which contribute to resilience, undergo enzymatic degradation, resulting in fruit softening (Varanasi et al. 2013; Dheilly et al. 2016; Zhang et al., 2017). To the best of our knowledge, no evidence in the literature suggests that fruit flesh firmness decreases due to auxin or auxin inhibitor applications. In the present study, fruit flesh firmness decreased over time across all treatments. However, after six months of storage, the greatest firmness loss occurred in the ethylene-treated samples, followed by the control (Fig. 1). Thewes et al. (2021, 2022) also reported softening in stored apples due to ethylene application. The observation that the auxin inhibitor treatment maintained the highest firmness both on day 1 and after six months, second only to ethylene inhibitor applications, suggests that auxin, in conjunction with ethylene, may play a role in fruit softening.

Ethylene is a natural ripening hormone that influences fruit ripening even at very low concentrations. Inhibiting or slowing ethylene biosynthesis in fruits and vegetables is one of the most effective methods to delay ripening and extend postharvest life (Blankenship and Dole 2003; Erbas and Koyuncu 2016). As expected, ethylene production in apples increased over time during storage compared with initial levels (Fig. 3). NPA+1-MCP and 1-MCP applications were the most effective treatments in suppressing ethylene production throughout all storage periods, followed by NPA alone. Conversely, the highest ethylene levels were observed in the ethylene and auxin treatments. This observation aligns with findings from Jung et al. (2011) and Thewes et al. (2021, 2022), who applied exogenous ethylene to stored apples. Furthermore, our results support the hypothesis that the auxin inhibitor suppressed ethylene production, as NPA-treated samples consistently showed lower ethylene levels across all three storage periods, closely resembling the effects of 1-MCP and NPA+1-MCP treatments. Although exogenous auxin application has not been widely studied in stored apples, it has been shown to increase ethylene production during fruit development in pears and apples, leading to early softening and accelerated ripening (Li et al., 2008; Kondo and Takano 2000; Yuan and Carbaugh 2007). Similarly, high ethylene and auxin levels commonly

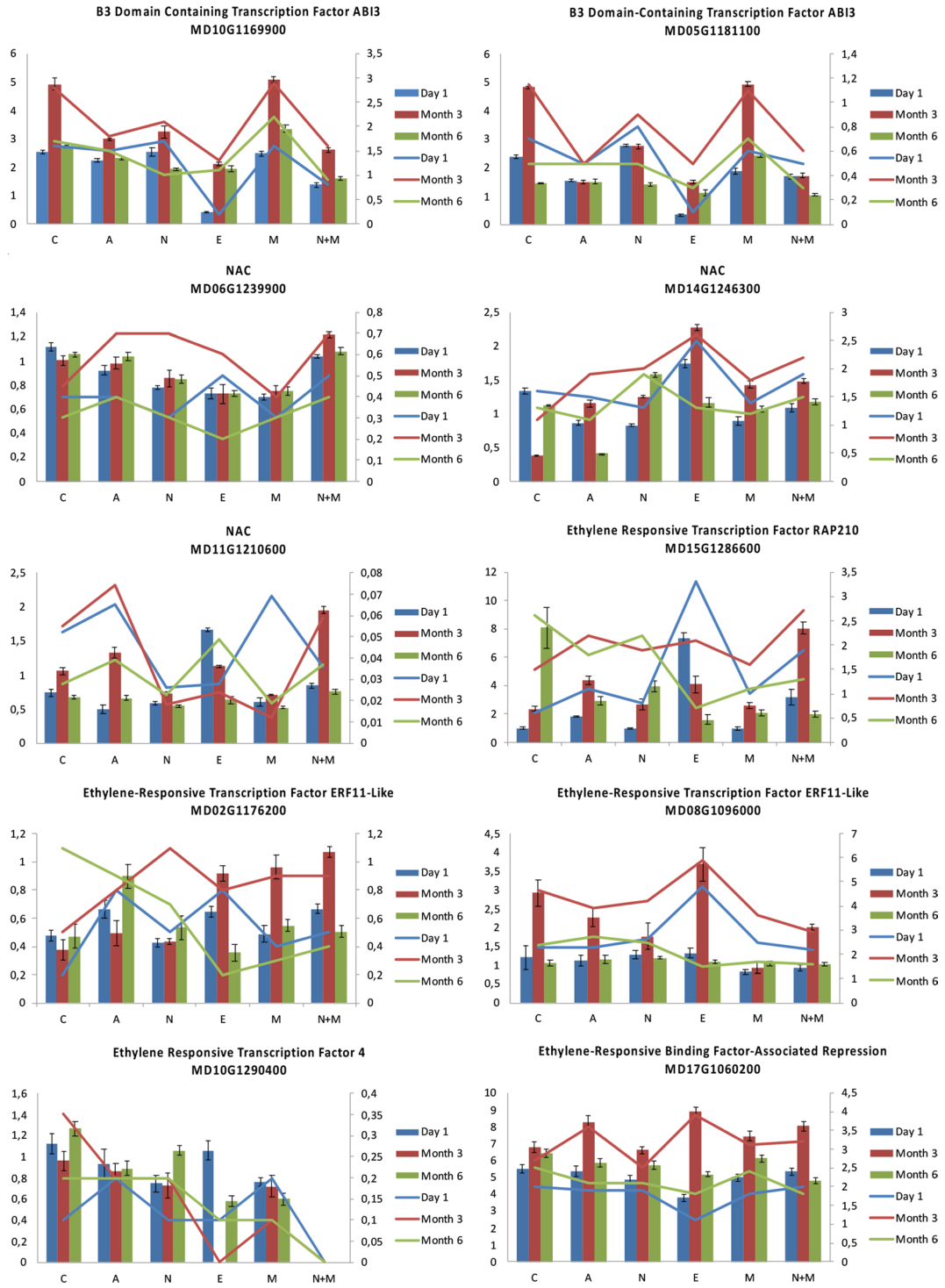

Fig. 5 Verification of RNA-Seq results by RT-PCR for selected hormone metabolism-related genes. The x-axis represents different treatments (C: Control, A: Auxin, N: Auxin inhibitor, E: Ethylene, M: Ethylene inhibitor, N+M: Auxin inhibitor+Ethylene inhibitor). The left y-axis (column graph) shows relative expression levels obtained by RT-PCR, while the right y-axis (line graph) represents transcript counts from RNA-Seq analysis

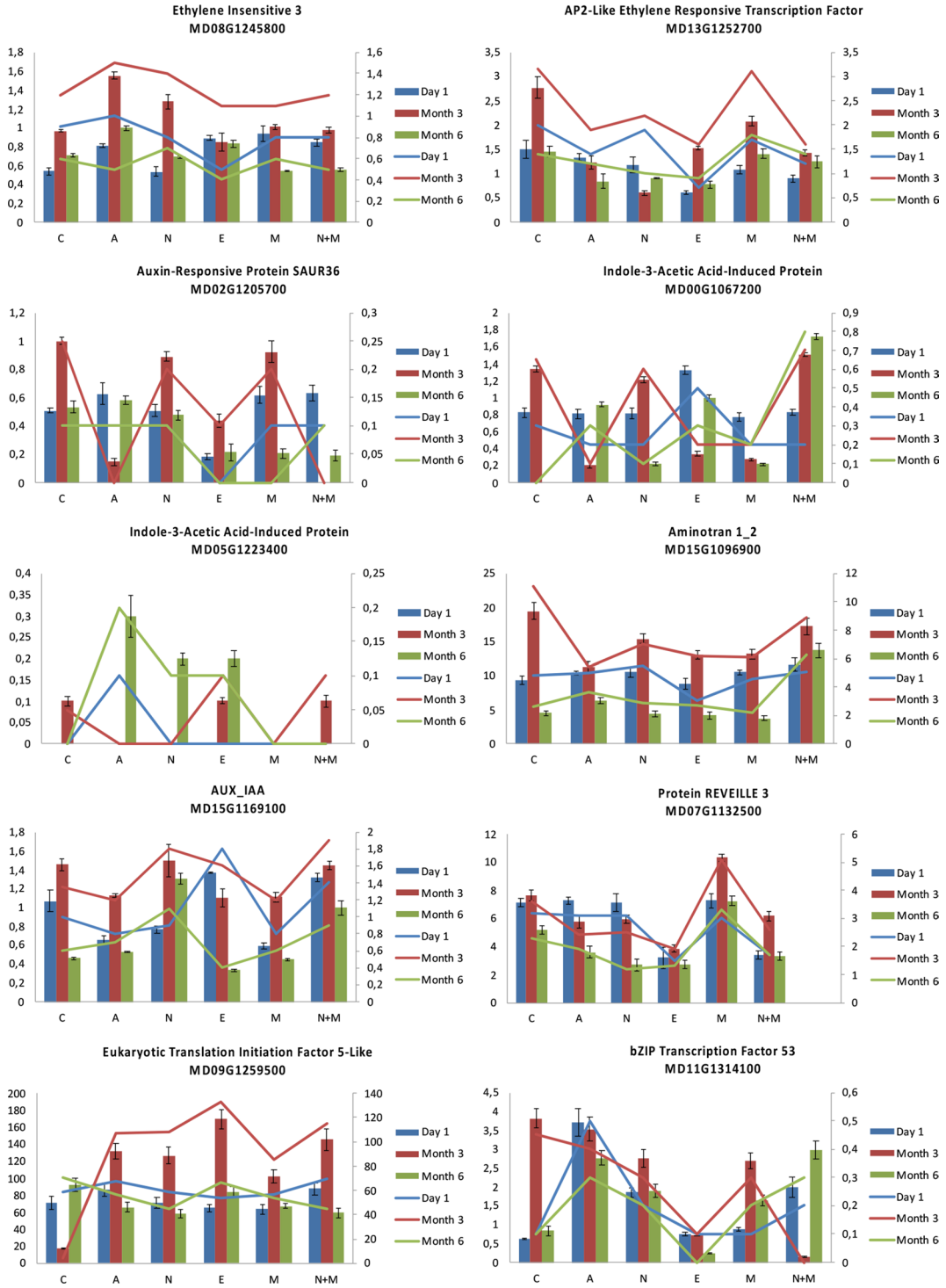

Fig. 5 (continued)

co-occur during ripening in tomatoes and grapes (Bottcher et al. 2010, 2013). Suppressing the SEPALLATA1/2 gene in transgenic apple trees increased auxin concentrations, which in turn activated auxin biosynthetic genes from the AUX/IAA and ARF families, delaying ripening by inhibiting ethylene synthesis (Schaffer et al. 2012). In another study comparing the ripening of 'Minneiska' and 'Scifresh' apple varieties, auxin levels in fruit cells decreased concurrently with the activation of ethylene biosynthesis (Shin et al. 2016). These pre-harvest observations align with our findings on ethylene production under auxin application after six months of storage. Additionally, consistent with

Fernández-Cancelo et al. (2022), auxin concentration was highest immediately after harvest while ethylene was at its lowest. Over time, auxin levels decreased as ethylene concentrations increased, except in NPA-treated samples, where auxin levels initially declined but later exhibited a gradual increase during storage.

The effects of NPA application diminished after three months of storage and disappeared by six months, as indicated by IBA and IAA levels (Fig. 2 and Supplementary Fig. 1). NPA was more effective at suppressing NAA levels. In contrast, 1-MCP effectively controlled ethylene production even over the longest storage period. One of the most striking findings in this study was that after six months of storage, ethylene production remained low in auxin inhibitor-treated samples, whereas auxin levels in the tissue were reduced in 1-MCP and NPA + 1-MCP treatments compared with the control, except for IAA levels at six months, which were similar to the control. This suggests that the auxin inhibitor suppressed both auxin and ethylene production, whereas the ethylene inhibitor reduced NAA and IBA levels, as well as IAA levels, throughout all storage periods except at day 1 and three months. These findings provide strong evidence for the interaction between ethylene and auxin in apple ripening and postharvest storage.

KEGG pathway analysis revealed that genes active during storage are associated with various biological and metabolic processes, including energy metabolism, photosynthesis, amino acid synthesis, carbohydrate metabolism, lipid metabolism, and antioxidant defense systems (Supplementary Tables 2 and Supplementary Fig. 19). Specifically, genes such as *COX1* (cytochrome c oxidase complex subunit 1), *ND5* (NADH-ubiquinone oxidoreductase chain 5), and *ATPF0A/B* (F-type H + transporting ATPase subunit a/b) were linked to energy metabolism, while *psbJ*, *psbK*, and *psbZ* were associated with photosystem II. Additionally, antioxidant defense genes such as *katE* (catalase), *gpx* (glutathione peroxidase), and *por* (protochlorophyllide reductase) were identified. Many genes related to carbohydrate and amino acid metabolism were also detected. Similar to the findings of Tadiello et al. (2016) and Busatto et al. (2021), fundamental metabolic processes remained highly active during storage.

Another key finding from the transcriptome analysis is that nearly all genes related to hormone metabolism in apples stored under different storage periods and treatments were involved in ethylene and auxin signal transduction and downstream transmission processes. These findings indicate that ethylene and auxin play a crucial role in governing the metabolic processes of ripening in stored apples. Furthermore, numerous transcriptional regulatory genes associated with hormone metabolism remained active throughout storage. While ethylene biosynthetic genes were not detected at the three sampling points—possibly due to the selection of seed tissue instead of fruit cortex—genes responsible for ethylene signaling and transduction were highly active throughout the study. The seed is the tissue that initiates hormonal changes in fruits, with these changes being transmitted from the center to the cortex and peel tissues (Kumar et al. 2014). Seed tissue was used in the transcriptomics part of the study to detect early gene expression changes. However, Eccher et al. (2015) proposed that ethylene is produced within the cortex, with the signal being transmitted to the seed tissue, suggesting that the cortex serves as the primary sensor of nutritional stress upon detachment from the tree. This supports our observation of various ethylene-responsive transcription factors, including Ethylene Responsive Transcription Factor RAP2.10 (MD15G1286600), AP2-like Ethylene Responsive Transcription Factor (MD13G1252700), Ethylene-Responsive Transcription Factor ERF11-Like (MD02G1176200, MD08G1096000), Ethylene Responsive Transcription Factor 4 (MD10G1290400), NAC Transcription Factors (MD06G1239900, MD14G1246300, MD11G1210600), Ethylene-Responsive Binding Factor-Associated Repression (MD17G1060200), REVEILLE 3 (MD07G1132500), and Ethylene Insensitive 3 (MD08G1245800), all of which coordinate ethylene responses in seed tissue. Conversely, auxin-related genes identified included Auxin-Responsive Protein SAUR36 (MD02G1205700), Indole-3-Acetic Acid-Induced Proteins (MD00G1067200, MD05G1223400), and AUX-IAA (MD15G1169100). Other hormone-regulation-related genes included ABA Insensitive 3 (MD10G1169900), B3 Domain-Containing Transcription Factor ABI3 (MD05G1181100), Probable LRR Receptor-Like Serine/Threonine-Protein Kinase (MD16G1091400), ABC Transporter C Family Member 10-Like (MD06G1099100), and bZIP Transcription Factor 53 (MD11G1314100). In addition to hormone metabolism, which was the main focus of this study, genes related to stress responses and metabolic processes, such as oleosin (MD07G1248300), dehydrin (MD15G1253900), heat shock protein (MD08G1068500), mannan mannosidase (MD08G1028100), pectinesterase (MD17G1154500), aminotransferase (MD15G1096900), programmed cell death protein (MD06G1160300), LEA (MD17G1204200), and catalase (MD06G1009000), also exhibited significant expression changes throughout cold storage.

Several auxin-responsive genes demonstrated strong interactions with ethylene signaling components. *AUX/IAA* (MD15G1169100) and *SAUR36* (MD02G1205700) were upregulated under ethylene treatment, indicating that ethylene may induce auxin signaling components to regulate ripening. *SAUR36*, a member of the auxin-sensitive SAUR gene family, is a well-established aging regulator with

known effects on leaf abscission in *Arabidopsis* (Hou et al., 2013). This study clearly demonstrated that its expression is also regulated by ethylene, reinforcing the notion that auxin plays a critical role in fruit softening. Additionally, Indole-3-Acetic Acid-Induced ARG7-like (MD00G1067200, MD05G1223400) showed increased expression under all treatments, further suggesting that auxin-related pathways are dynamically regulated in response to ethylene and auxin balance.

AP2/ERF transcription factors form one of the largest transcription factor families in plants and are widely expressed in various tissues, playing significant roles throughout the plant lifecycle (Klee and Giovannoni 2011). This family is classified into four subgroups: AP2 (APETALA2), DREB (Dehydration-Responsive Element Binding Protein), ERF (Ethylene Responsive Factor), and RAV (ABI/VP). AILs (AINTEGUMENTA-LIKE), a subfamily of AP2/ERF transcription factors, have been shown to be crucial for embryogenesis, flowering, and fruit development (Horstman et al. 2014; Krizek 2015). AP2/ERF transcription factors regulate cell wall modification, anthocyanin synthesis, and aroma compound formation during fruit development. Among the genes with which they interact is pectin esterase (Zhai et al. 2022), which exhibited significant expression changes in this study. *AIL5* has been identified as a gene with increased expression during postharvest ripening in bananas (Chen et al. 2022) and has also been highly expressed in somatic embryos of lychee (Litchi chinensis Sonn.) (Men et al. 2021). *AIL5* has been shown to be highly expressed during flowering in apples, continuing throughout fruit development (Dash and Malladi 2012). In this study, *AIL5* responded to ethylene, auxin, and auxin inhibitor applications, with its expression consistently decreasing under these treatments compared with the control across all storage periods.

RAP2.10, a transcription factor belonging to the AP2 family, is classified as a DREB-type transcription factor that responds to ethylene. Various studies have demonstrated that RAP2 transcription factors are active under abiotic stress conditions and regulate programmed cell death (Yang et al. 2020; Coego et al. 2014). In this study, *RAP2.10* (MD15G1286600) expression showed a steady increase over time under control and auxin inhibitor applications, whereas it exhibited a consistent decrease under ethylene treatment, suggesting a role in auxin-mediated ripening pathways. ERF11 transcription factors act as negative regulators of fruit ripening by deactivating the *ACS1* and *ACO1* genes through the activation of histone deacetylase enzymes (Han et al. 2016). The expression of both *ERF11* homologs (MD02G1176200, MD08G1096000) analyzed in this study displayed distinct patterns under different treatments. *ERF11* expression increased under ethylene treatment at month 3, supporting its function as a negative regulator of ethylene biosynthesis via histone deacetylation. Furthermore, *ERF11* expression also increased under auxin treatment, suggesting that auxin may indirectly regulate ethylene biosynthesis through epigenetic modifications. These results indicate that ERFs not only mediate ethylene responses but also function at the intersection of ethylene and auxin pathways.

Ethylene Insensitive 3 (*EIN3*, MD08G1245800), activated by *CTR* and *EIN2*, is a key transcription factor in ethylene signaling that regulates ripening-related genes by activating downstream ethylene response factors (ERFs) (Dolgikh et al. 2019). In our study, *EIN3* expression increased under auxin treatment at all time points and under ethylene treatment at day 1 and month 6. This suggests that auxin modulates ethylene responsiveness by upregulating *EIN3*. Similar interactions have been observed in *Arabidopsis*, where auxin enhances ethylene sensitivity via stabilization of *EIN3* (Zhu et al. 2011). The suppression of *EIN3* expression under ethylene inhibitor (1-MCP) treatment further supports its role as a convergence point for ethylene and auxin pathways in postharvest apple ripening.

In the upper steps of ethylene signal transduction are the CTR (Constitutive Triple Response) and EIN2 (Ethylene Insensitive 2) receptors. MD10G1169900, a B3 domain-containing transcription factor ABI3 (ABA Insensitive 3), has been shown to be suppressed in the presence of ABA in previous studies (Zhao et al. 2019) and in response to ethylene in our study. This suggests that ABA and ethylene act antagonistically during ripening. Although the precise mechanism of ABI3 remains unclear, it has been associated with seed development and storage compound accumulation in *Arabidopsis* (Grimberg et al. 2018). Fernández-Cancelo et al. (2022) reported a strong correlation between ethylene, ABA, apple fruit color, and firmness, suggesting that both hormones are required to trigger fruit ripening. Consistent with this observation, in the present study, *ABI3* expression significantly decreased in ethylene (E)-treated samples after one day of storage. However, under ethylene inhibitor treatment, *ABI3* expression remained high at all time points, suggesting that ethylene suppresses ABA signaling. These findings highlight *ABI3* as a key regulator of ABA-ethylene interactions during postharvest ripening in climacteric fruit.

Receptor-like protein kinases are involved in receptor-mediated signaling pathways that localize to the cell membrane and transmit extracellular signals into the cell. The LRR receptor-like serine/threonine-protein kinase, a key player in brassinosteroid and ABA signal transduction, has been shown to be affected by both ABA and ethylene during strawberry ripening (Hou et al. 2018). In our study, this kinase exhibited decreased transcript levels under ethylene and 1-MCP treatments, as well as under ethylene and auxin inhibitor (NPA) treatments. The suppression of this gene

under both hormone inhibitors suggests that it may act as an upstream regulator integrating multiple hormone signals. This finding aligns with Fernández-Cancelo et al. (2022), further supporting the role of ABA-ethylene crosstalk during apple ripening and the LRR protein kinase may function as an early regulator of this interaction.

REVEILLE (RVE) proteins, a subgroup of MYB transcription factors, are primarily known for their roles in circadian rhythm regulation and flowering time control in Rosaceae (Liu et al. 2023). They have also been implicated in fruit development in melon and grapes (Xiang et al. 2022). In this study, RVE proteins were involved in fruit ripening in apple, with their expression decreasing under ethylene, auxin, and auxin inhibitor treatments while increasing under ethylene inhibitor treatment. These expression patterns suggest that RVE proteins are involved in auxin-ethylene interplay, further reinforcing the significance of hormonal crosstalk in fruit development and ripening.

ABC (ATP-binding cassette) transporter C family members are membrane transporters responsible for the active movement of various molecules, including plant hormones. Alongside PIN, PILS, and AUXIN/LAX proteins, ABC transporters are among the most important transporters for auxin in plant tissues (Balzan et al. 2014). While no studies have directly linked ABC transporters to ethylene transport, numerous reports have demonstrated their role in jasmonic acid transport (Theodoulou et al. 2005; Zhang et al. 2013, 2020; Nguyen et al. 2017). In this study, the expression of a previously uncharacterized ABC transporter (MD06G1099100) increased significantly under auxin and ethylene treatments from day 1 to month 3, marking it as a potential transporter involved in hormone movement within apple tissues. Fernández-Cancelo et al. (2022) reported cold-induced jasmonic acid accumulation, which appears to contribute to the climacteric burst in 'Golden Reinders' apples under cold storage. The continuous expression of ABC transporters throughout cold storage in our study supports a possible synergistic interaction between jasmonic acid and ethylene in ripening. Given the established role of ABC transporters in jasmonic acid movement, it is plausible that ethylene modulates auxin and jasmonic acid transport, forming a multi-hormonal regulatory network during storage.

Nonspecific lipid transfer proteins (nsLTPs) are a highly complex and heterogeneous group of proteins associated with various biological processes, including plant-pathogen interactions, hormone metabolism, cutin and wax formation in fruit, cell wall expansion, seed development, germination, and nodulation (Missaoui et al. 2022). Safi et al. (2015) demonstrated that the application of ABA, salicylic acid, jasmonic acid, and ethylene increased *nsLTP* expression in wheat. In tomatoes, high *nsLTP* expression has been linked to increased cutin content and cuticle thickness, which delays water loss and fruit softening during storage (Wang et al. 2022). In the present study, *nsLTP* expression was significantly elevated following the combined application of two inhibitors after six months of storage.

Two recent transcriptome analyses have investigated gene expression changes in stored apples. The first study examined three apple cultivars—'Golden Delicious', 'Granny Smith', and 'Fuji'—treated with the ethylene inhibitor 1-MCP, comparing them to controls across six time points (at harvest, 24 hours, 1 week, 2 weeks, 3 weeks, and 2 months of storage) (Busatto et al. 2021). Notably, the two-month storage period was conducted under cold conditions, whereas the earlier time points were at 20°C. This study focused on hormone signaling and metabolism, observing expression changes in ethylene-related *ACS*, *ACO*, *ERS*, and *ERF* genes with 1-MCP application during storage. Genes related to auxin signaling and homeostasis, including *AUX/IAA*, *ARF*, *GH3*, and *ILL*, also exhibited expression changes. While *ERF* and *AUX/IAA* gene expression in our study aligned with Busatto et al. (2021), differences in other genes likely resulted from variations in storage conditions and sampling times. Moreover, our study focused on seed tissue rather than fruit pulp, potentially contributing to observed differences in gene expression patterns.

Another transcriptome study on 'Fuji' apples stored under cold conditions (2, 4, and 8 months) compared control and 1-MCP treatments, revealing that 1-MCP affected glycolysis and the tricarboxylic acid cycle. This study also reported changes in ethylene synthesis and signaling genes (*ACS*, *ETR*, *EIN*, and *ERF*) and demonstrated that enzymes associated with pectin degradation were suppressed under 1-MCP treatment (Zhang et al. 2022). Unlike these previous studies, the present study analyzed the combined effects of ethylene, auxin, auxin inhibitor, and two hormone inhibitors, providing novel insights into hormonal crosstalk and signal transduction.

Ethylene and auxin production patterns vary by cultivar and developmental stage, both pre- and postharvest (Busatto et al. 2022; Fernández-Cancelo et al. 2022; 2023). In this study, key genes involved in ethylene biosynthesis, such as *ACC* and *ACO synthase*, as well as auxin metabolism regulators like *GH3* and *ARFs* (Yue et al. 2020; Wang et al. 2021), were not detected at specific sampling periods (1 day, 3 months, and 6 months). One possible explanation for this absence is the focus on seed tissue, which serves as a genetic regulatory hub in fruit (Kumar et al. 2014). This may account for the identification of unique transcription factors and signaling molecules that regulate hormone biosynthesis in the cortex tissue. Despite the complexity of hormonal interactions, genes associated with basal metabolism, as well as ethylene and auxin metabolism, were consistently detected across different storage durations and treatments.

In addition to auxin- and ethylene-related regulation, key transcription factors and signaling components were identified as potential regulators of hormonal crosstalk, including ABA Insensitive 3 (ABI3), B3 Domain-Containing Transcription Factor ABI3, Probable LRR Receptor-Like Serine/Threonine-Protein Kinase, ABC Transporter C Family Member 10-Like, and bZIP Transcription Factor 53. Further studies with more frequent sampling and broader cultivar selection would enhance our understanding of hormonal regulation in postharvest apples.

Our findings also indicate that genes associated with photosynthesis, aerobic respiration, transcription, and translation maintained high expression levels regardless of treatment or storage duration, ensuring cellular functionality throughout storage. A key transcriptome finding corresponding with respiration rate measurements was the suppression of the acyl-coenzyme A gene, which is involved in the citric acid cycle and exhibited a substantial increase following ethylene application under 1-MCP treatment at six months. Furthermore, the inhibition of ethylene signaling by 1-MCP at the transcriptome level correlated with physiological changes observed in fruit, reinforcing the observed suppression of hormone-related genes.

The interplay between auxin and ethylene was evident in multiple aspects of postharvest ripening and senescence. Several transcription factors, hormone-responsive genes, and signal transduction components demonstrated regulatory crosstalk between these hormones. Ethylene-responsive EIN3, which integrates auxin signaling, emerged as a key regulatory intersection between ethylene and auxin, influencing plant growth, development, and ripening. Similarly, ERF transcription factors exhibited dual regulation by ethylene and auxin, emphasizing their role in mediating hormonal interactions. Auxin-responsive genes such as *AUX/IAA* and *ARF* were modulated by ethylene, further illustrating the dynamic interaction between these pathways.

Additionally, LRR receptor kinase was implicated as a key regulatory component in ethylene-auxin crosstalk, possibly acting as an upstream integrator of multiple hormone signals. The identification of an ABC transporter as a candidate for ethylene-auxin interaction suggests a transport-based mechanism underlying this hormonal interplay. Furthermore, the involvement of *ABI3* and *REVEILLE 3* highlights their roles in coordinating ethylene, auxin, and ABA signaling, underscoring the complexity of plant hormone regulatory networks.

5 Conclusion

This study's fruit firmness measurements revealed similar softening patterns under ethylene and auxin treatments, while the auxin inhibitor (NPA) maintained firmness at levels comparable to 1-MCP after six months of storage. A key finding supporting ethylene-auxin crosstalk was the hormonal analysis, which showed that the auxin inhibitor suppressed ethylene production along with auxin levels, while the ethylene inhibitor reduced auxin levels alongside ethylene production. Transcriptomic analysis revealed that most hormone-related genes exhibiting expression changes under various storage conditions were involved in ethylene and auxin signaling pathways and their downstream processes. These results indicate that postharvest ripening in apples is primarily regulated by ethylene and auxin. Key regulatory nodes integrating ethylene and auxin signaling included *EIN3*, *ERF11*, *RAP2.10*, *AUX/IAA*, *SAUR36*, *LRR receptor kinase*, *ABC transporter*, and *ABI3*. Notably, the high expression levels of ABC transporters and LRR receptor-like protein kinases—previously associated with plant hormone metabolism—were influenced by ethylene and auxin treatments, providing novel insights into their role in coordinating postharvest ripening and senescence. Other significantly differentially expressed genes included oleosin, dehydrin, heat shock protein, mannan mannosidase, pectinesterase, aminotransferase, programmed cell death protein, LEA, and catalase. Fundamental cellular processes such as photosynthesis, respiration, transcription, and translation remained active in fruit tissues throughout storage. While the auxin inhibitor, either alone or combined with the ethylene inhibitor, did not significantly impact overall fruit quality and storage life, this study uncovered new molecular processes occurring in apples under cold storage. Further functional validation (e.g., gene knockouts or overexpression studies) will be essential to elucidate the precise roles of these genes in ethylene-auxin interactions.

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Author contributions UCA conceptualized the experiment, conducted the analyses, coordinated the project, acquired funding, and prepared the original draft manuscript. MCB contributed to conception and design, conducted the analyses, reviewed the draft critically for intellectual content, and approved the final version to be published. YCA contributed to conception and design, conducted the analyses, reviewed the draft critically for intellectual content, and approved the final version to be published. PB contributed to conception and design, conducted the analyses, reviewed the draft critically for intellectual content, and approved the final version to be published. MAK contributed to conception and design, conducted the analyses, reviewed the draft critically for intellectual content, and approved the final version to be published. DE contributed to conception and design, conducted the analyses, reviewed the draft critically for intellectual content, and approved the final version to be published. EH conducted the analyses, reviewed the draft critically for intellectual content, and approved the final version to be published. BA conducted the analyses, reviewed the draft critically for intellectual content, and approved the final version to be published. ST conducted the analyses, reviewed the draft critically for intellectual content, and approved the final version to be published. All the authors were in agreement in accountability, accuracy and integrity of the work. All the authors have approved the final version of the manuscript.

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Data availability The data that support the findings of this study are openly available in the NCBI Sequence Read Archive (SRA) at https://www.ncbi.nlm.nih.gov/sra/PRJNA1145589, reference number PRJNA1145589.

Declarations

Ethical approval This declaration is not applicable.

Conflict of interest The authors declare that they have no competing interests.

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