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Integrative metabolomics and transcriptomics analyses reveal pivotal regulatory mechanisms of 1-methylcyclopropene in maintaining postharvest storage quality of 'Fuji' apples

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Abstract

In this study, integrative metabolomics and transcriptomics analyses were conducted to investigate the effects of 1-methylcyclopropene (1-MCP) on apple fruit quality during long-term cold storage. The results showed that 1-MCP (1 μL/L) treatment could maintain fruits apparent quality (i.e. external color and firmness), inhibit the increase of rot rate and soluble solids content/titratable acidity ratio, decrease ethylene release, and respiratory intensity during cold storage, and extend shelf life. Moreover, 1-MCP had long-term effects on the accumulation of many qualities related to metabolite and gene expression in fruits. 1-MCP affected genes related to metabolism at the early stage of storage, specifically those of the glycolysis and tricarboxylic acid cycle pathways. Genes related to the degradation of sucrose, starch, and cellulose were inhibited, and some starch and cellulose synthesis genes were up-regulated by 1-MCP. Apart from ethylene synthesis and signal transduction being inhibited by 1-MCP, several enzymes (pectinesterase, pectate lyase, polygalacturonase) were involved in pectin degradation, and degradation products of the cell wall (i.e. D-galacturonic acid and D-glucuronic acid) were also strongly inhibited, further maintaining fruit firmness. Cysteine, as precursor glutathione (GSH) related to plant resistance, up-regulated the synthase gene. However, the expression of genes related to cyanoalanine synthase and amino acid utilization pathways was suppressed by 1-MCP. Collectively, 1-MCP could maintain the postharvest quality of apple fruits.

Keywords: 1-Methylcyclopropene; apple; fruit quality; metabolomics; transcriptomics.

Introduction

Apples (*Malus* × *domestica* Borkh.) are one of the most important commercial fruit crops grown in temperate regions because of their palatable flavor and rich nutrients (Giovannoni, 2004). The 'Fuji' apple, a dominant cultivar in China, is famous for its late ripening and storable characteristic (Chen *et al.*, 2020). In actual production and trade, fruit quality is the most important index determining commercial value. Postharvest storage is a critical stage from growing to market, during which fruit quality must be maintained as much as possible. Thus, to enable long-term availability and marketability, flesh apples are always subjected to cold storage (McGlasson *et al.*, 1979; Sevillano *et al.*, 2009). However, during long-term cold storage, the fruit will face many physiological disorders, such as loss of nutrients and juiciness, color browning, and loss of flavor and taste, which inevitably lessens its economic benefits (Jan *et al.*, 2012; Ignat *et al.*, 2014; Storch *et al.*, 2017). Specifically, researchers have shown that complicated multilayered factors including cell structures (Brummell *et al.*, 2004), sugars (Cao *et al.*, 2013; Wang *et al.*, 2013), lipids (Zhang and Tian, 2010; Brizzolara *et al.*, 2018), hormones (Zhao *et al.*, 2009; Park *et al.*, 2015; Shi *et al.*, 2015), and transcription factors (Bolt *et al.*, 2017; Zhao *et al.*, 2020) are involved in fruit deterioration during long-term cold storage.

To solve the above-mentioned problems, a series of preservation technologies have been developed. For example, one study applied aqueous hexanal to improve 'Royal Delicious' apple quality, which retained higher firmness, reduced decay, and improved overall quality during cold storage (Sulaimankhil *et al.*, 2021). In another study, high storage humidity and a perforated polyethylene liner were shown to contribute to delaying fresh fruit weight loss, reducing internal ethylene concentration, and preventing fruit shriveling (Lee *et al.*, 2019). Finally, *Listeria innocua* bacterial counts on Fuji apple surfaces were shown to decrease under a controlled atmosphere (CA, 33 °F, 2% O_2, 1% CO_2)

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with continuous gaseous ozone to ensure fresh apple safety (Sheng *et al.*, 2018).

The ripening and senescence progress of climacteric fruit is strongly regulated by ethylene (Lv *et al.*, 2020). For apples, as a typically climacteric fruit, the key point for quality maintenance is to inhibit internal ethylene accumulation, which comprises reducing the binding probability of ethylene and its receptors and the removal of exogenous ethylene (Martinez-Romero *et al.*, 2007). At present, it is widely accepted that 1-methycyclopropene (1-MCP) is a competitive inhibitor of ethylene that can suppress ethylene signal transmission downstream and deactivate maturation-related processes by irreversibly binding with ethylene receptors (Zhao *et al.*, 2021). Compared with other preservatives, 1-MCP is characterized by easy synthesis, high efficiency, and non-toxicity (Watkins, 2006). Extensive research has shown that 1-MCP treatment can delay the quality decline of climacteric fruit during storage and play positive roles in all aspects of preservation (Lv *et al.*, 2020; Al Shoffe *et al.*, 2021; Zhao *et al.*, 2021). For example, 1-MCP can maintain fruit firmness by creating a more compact cell structure, preventing the glue layer from degrading in the cell wall and increasing the intercellular space, preventing fruit swelling and maintaining the integrity of organelles and membranes (Baritelle *et al.*, 2001). 1-MCP also delays fruit degreening and inhibits carotenoid accumulation (Sabir and Agar, 2011). Additionally, 1-MCP may improve the disease resistance of fruit by promoting disease course protein expression, and enhancing antioxidant enzyme activity and phenolic metabolism (Lum *et al.*, 2016). There is no doubt that 1-MCP treatment is positively associated with delaying ripening and maintaining quality, for example, rosaceous fruits respond to 1-MCP (Zhang *et al.*, 2020).

Advanced omics technology can be used to understand comprehensive biological information. At present, there are many omics reports on apples. Zhao *et al.* (2020) identified 12 clusters of differentially expressed genes (DEGs) in apple fruits involved in multiple biological processes, including sugar, malic acid, fatty acid, liquid, complex phytohormones, and the stress–response pathway, as well as a total of 151 metabolites created during long-term storage. Moreover, Onik *et al.* (2018) used comparative transcriptomic profiling to compare pre- and post-ripened apple samples. A total of 9187 differentially expressed genes were identified in post-ripened fruits, which are mainly involved in hormonal signaling pathways including ethylene, abscisic acid, auxin, gibberellin, brassinosteroid, and anthocyanin biosynthesis. Two bud sport apple cultivars, 'Jonathan' and 'Sweet Jonathan,' were reported to have 4313 differentially expressed genes between the two cultivars during fruit development, and functional analysis revealed that stress–response genes and signal-transduction-related genes were enriched (Zhao *et al.*, 2019). Furthermore, one review suggested that in relatively chilling-tolerant pomegranate fruits, where 573 common chilling-tolerance-associated genes were identified, the up-regulation of transcripts was involved in the activation of jasmonic acid and ethylene hormone biosynthesis and signaling, stress-related transcription factors, and other biological processes (Kashash *et al.*, 2019).

Although many works have focused on the preservation function of 1-MCP in various horticultural products, integrated information revealing the regulatory mechanisms of 1-MCP in apple fruit, especially under long-term cold storage and in simulated business models, is limited. The purpose of this study is to combine metabolomics and transcriptomics analyses to reveal pivotal regulatory mechanisms of 1-MCP in maintaining the postharvest storage quality of Fuji apples under long-term cold storage, which is expected to provide a further comprehensive understanding of 1-MCP treatment in maintaining postharvest fruit quality.

Materials and Methods

Plant materials and treatments

Fuji apples at commercial maturity (approximately 200 d after flowering, without any mechanical damage or physiological disorders, for example, apple watercore) were harvested from a local farm located in Luochuan County, Shaanxi, China. One half of the collected apples was pretreated with 1 μL/L 1-MCP for 24 h at 0 °C and the other half was used as the control. The pretreated and the control apples were then immediately delivered to cold storage and stored at (0±0.5) °C and 85%–90% relative humidity for 8 months.

Six boxes of the 1-MCP-treated and control fruit were removed from storage after 2, 4, and 8 months of storage, and immediately transported to our laboratory. For each treatment, three boxes of fruit were used for transcriptomic and metabolomic assays, and the other three boxes were used for shelf-life quality determination. For the metabolomic and transcriptomic assays, fruit flesh at the equator was collected and immediately frozen in liquid nitrogen and stored at −80 °C for further analysis. The other three boxes of fruit were subjected to further storage at room temperature for 35 d to simulate shelf life, during which certain physiological indicators were measured. Ten fruits free from pests and diseases and without mechanical injuries were picked out from one box randomly as one biological replicate, and all analyses were performed using three replicates.

Measurement of physiological indicators

Fruit physiological indicators including respiration rate, ethylene production, weight loss rate, fruit firmness, soluble solids content (SSC), and titratable acidity (TA) were measured every 7 d according to a previous study (Zhang *et al.*, 2019).

Metabolomic analysis

Metabolomic analysis was performed using an ultra performance liquid chromatography (UPLC; Shim-pack UFLC SHIMADZU CBM30A, Shimadzu, Kyoto, Japan) and tandem mass spectrometry (MS/MS; 6500 QTRAP, Applied Biosystems, Foster City, CA, USA) system as described by Chen *et al.* (2013). The differential metabolites were selected based on the combination of variable importance in project (VIP) values obtained from the orthogonal partial least squares-discriminant analysis (OPLS-DA) model and the fold change of each metabolite, where metabolites with $|\text{Log}_2\text{Fold change}| \geq 1$ and VIP≥1 were considered as differential metabolites. The principal components analysis, Pearson correlation coefficient, OPLS-DA and VIP value analysis, and hierarchical clustering were conducted by R (3.5.0). The peak area was used to represent the relative content of each metabolite. Heat maps were drawn by TBtools (Chen *et al.*, 2018).

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Transcriptomic analysis

Total RNA was extracted using an E.Z.N.A.® Plant RNA Kit (Omega Bio-Tek, Doraville, GA, USA) according to the protocol. Then the cDNA libraries were prepared using a TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (TransGen Biotech, Beijing, China). RNA-seq was conducted at Beijing Novogene Bioinformatics Technology Co., Ltd. (Beijing, China) on an Illumina HiSeq-PE150 platform. The clean reads were aligned to the reference genome database using HISAT2 (http://ccb.jhu.edu/software/hisat/index.shtml). Transcript assembly was performed using StringTie software. The fragments per kilobase of exon per million mapped reads (FRKM) method was used to estimate the transcript abundance. DESeq2 was used for the differential expression analysis. Benjamini and Hochberg's approach was employed to adjust *P*-values. Genes with an adjusted P-value<0.05 and $|\log_2 \text{foldchange}|>1$ were recognized as DEGs. All the DEGs were subjected to Gene Ontology (GO) enrichment analysis and mapped to the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database using clusterProfiler.

Quantitative real-time polymerase chain reaction analysis

Five genes from the DEGs were selected to perform the quantitative real-time polymerase chain reaction (qRT-PCR) analysis to verify the reliability of RNA-seq results. The reaction was carried out by using a TransStart Top Green qPCR SuperMix (TransGen Biotech, Beijing, China) kit and the CFX96 touch real-time PCR detection system (Bio-Rad, Hercules, CA, USA) according to the instructions. The gene *MdActin* was used as the reference gene and the relative expression levels of the selected genes were calculated using the $2^{-\Delta\Delta CT}$ method. Three replicates for each treatment were conducted and three measurements were performed on each replicate. Primers used for the qRT-PCR analysis are presented in Table S8.

Physiological data analysis

For the physiological data analysis, Student's *t*-test was used to analyze the significant difference between the control and treatment groups in the Statistical Product Service Solutions (SPSS, Version 23.0) software. The heat maps were drawn by TBtools.

Results

Fruit appearance and physiological variations during storage in response to 1-MCP treatment

Fruit physiological characteristics (i.e. weight loss rate, rot rate, ethylene production, respiration rate, firmness, SSC, TA, and SSC/TA ratio) were measured during the shelf life stage. We observed that the characteristic red color of the apple skin faded and turned yellow over storage, which was suppressed by 1-MCP treatment (Figure 1A). The variation in the color index was determined by photographs. The L^* value reflects the brightness of the skin color, which reached a peak value after 4 months of storage and then decreased. A greater value of a^* indicates a more red skin color. The results showed that the value of a^* decreased gradually as the storage time increased. The value of b^* reflects the degree of yellowness of the fruit skin, which increased during the storage time. Although the color index values of the control and treatment groups showed a similar variation trend, the treatment group changed more slowly than the control group, indicating that 1-MCP could maintain the fruit skin color. The results also indicated that 1-MCP treatment can inhibit the increase of fruit rot rate and solid acid ratio; delay the decline of fruit firmness; and reduce ethylene release, respiratory intensity, and the appearance of ethylene and respiratory peaks during low-temperature storage and shelf storage over time, respectively (Figure 1B–1D), thus delaying the decline of fruit texture and taste, and prolonging the storage and preservation period. However, 1-MCP treatment did not slow down the weight loss of the fruit during storage. In summary, 1-MCP treatment retained the commercial value and retarded the quality deterioration rate of the apple fruit compared to the control groups after 4 months of storage.

Metabolomic analysis

Overview of metabolites

Based on the broad target metabolomics technology, 461 metabolites were detected in all eighteen groups (2 treatments×3 periods×3 replicates), which belonged to eighteen substance categories. As Figure S1A shows, the squared Pearson correlation coefficients (R^2) of the three biological replicates in all the groups were great. Then, principal component analysis (PCA) showed that PC1 (21.77%) parted groups due to storage time and revealed that storage time may have a great influence on the accumulation pattern of metabolites, whereas PC2 (15.80%) separated the control groups from the 1-MCP treatment groups, which indicated that 1-MCP treatment was responsible for the effect on apple storage (Figure S1B).

We found that amino acids accounted for the highest number of substances (seventy-six metabolites were detected), followed by organic acids (sixty-seven metabolites were detected), flavonoids, phenylpropanoids, lipids, alkaloids, saccharides, and so on (Figure S1C). Compared to the control group, the proportion of the total content of amino acids and derivatives, vitamins and derivatives, lipids, phenylpropanoids, and flavonoids in the 1-MCP-treated group accounted for more than 50% (Figure S1D), which indicated that 1-MCP treatment might affect the maintenance or accumulation of these substances. Among them, the difference in accumulation of amino acids and derivatives was the most obvious (from 40% to 60%), which particularly increased after 4 months and 8 months of storage compared to the control group.

Tracking metabolites profiles

The different metabolites of the 1-MCP treatment group and control group during storage are shown in Figure 2. After 2 months of apple fruit storage (1-MCP_2), there was little variation in the amount and categories of metabolites. The levels of four nucleotides and derivatives (guanosine 5′-monophosphate, cytosine, cytidine-5-monophosphate, and cytidine), two phenylpropanoids (terpineol and caffeoyl tartaric acid), anthocyanins (geranium glycosides), flavonoids (*c*-hexosyl-apigenin-*o*-dihexoside), and other benzamides in the 1-MCP-treated groups were lower than those in the control groups by a factor of 2–5. However, the levels of amino acids and their derivatives (2-aminoisobutyric acid) and flavonoids (diosmin) were up-regulated in the 1-MCP_2 group (by a factor of 2–4). After continued cold storage for 4 months

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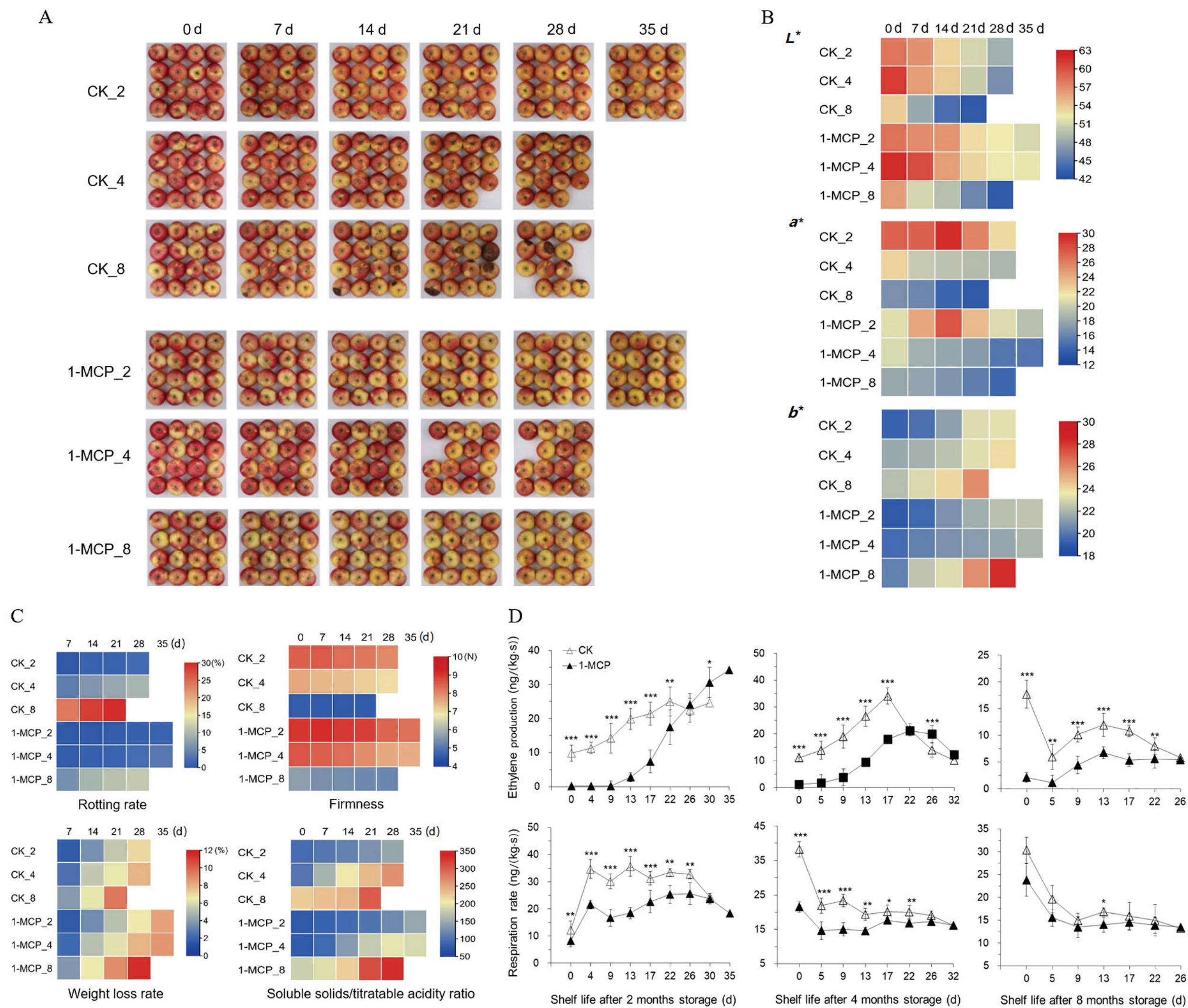

Figure 1. The variation of fruit appearance and physiological traits at different storage and shelf life stage after 1-MCP treatment. (A) Digital photos of fruit appearance at different storage times. (B) Variation of fruit peel color index (also see Tables S1–S3). (C) Variation of fruit rotting rate, firmness, weight loss rate, and soluble solids/titratable acidity ratio (also see Tables S4–S7). In panels B and C, longitudinal direction shows the different low-temperature storage period, the horizontal direction shows an additional 35 days of stimulated shelf life stage. (D) Variation of fruit respiration rate and ethylene production. CK indicates the control group; 1-MCP the 1-MCP treatment group. Statistical significance between the control and 1-MCP treatment is indicated by asterisk according to Student's *t*-test (**P*<0.05, ***P*<0.01, ****P*<0.001). CK_2, CK_4, and CK_8 represent the control group storage for 2, 4, and 8 months. 1-MCP_2, 1-MCP_4, and 1-MCP_8 represent the 1-MCP-treated group storage for 2, 4, and 8 months; 0, 7, 14, 21, 28, and 35 d represent different shelf-life stages.

(1-MCP_4), a total of sixty-two differential metabolites were screened out, including forty-one up-regulated metabolites and twenty-one down-regulated metabolites mainly belonging to thirteen metabolites types, which were the most accumulated metabolites in terms of amount and type. The up-regulated metabolites included 5-aminovaleric acid, histamine, nicotinic adenine dinucleotide, 6-methylmercaptopurine, indole and its derivatives, and others, whereas amino acids, nucleotides and their derivatives, maltotriose, carbohydrates, glucuronic acid, and organic acids and their derivatives were down-regulated. The three most up-regulated metabolites were cysteine, glutathione reductant, and tryptamine (12.2-, 10.6-, and 4.1-fold increases, respectively). The substances in which the content level decreased most obviously in the down-regulation metabolites were glucuronic acid, D-galacturonic acid, and 5-*O*-*p*-vanillic acid *O*-hexoside (141-, 1.4-, and 7.3-fold decreases, respectively). When the apple fruit was stored for 8 months (1-MCP_8), there were thirty-four differential metabolites, and many were still amino acids and their derivatives. The most up-regulated metabolites were 7-*O*-hexoside, DL-methionine, and L-methionine (5.1-, 4-, and 3.8-fold increases, respectively), whereas caffeoyl tartaric acid, 5-aminovaleric acid, and cytidine were the most down-regulated metabolites (3.1-, 2.9-, and 2.8-fold decreases, respectively). Overall, there were decreases in the number of metabolites and differences in substance content after 8 months of storage. In particular, amino acids, as the top metabolite, showed the most obvious difference; the content first increased, then decreased, and finally increased again in the 1-MCP treatment group. Moreover, two differential metabolites (caffeoyl tartaric

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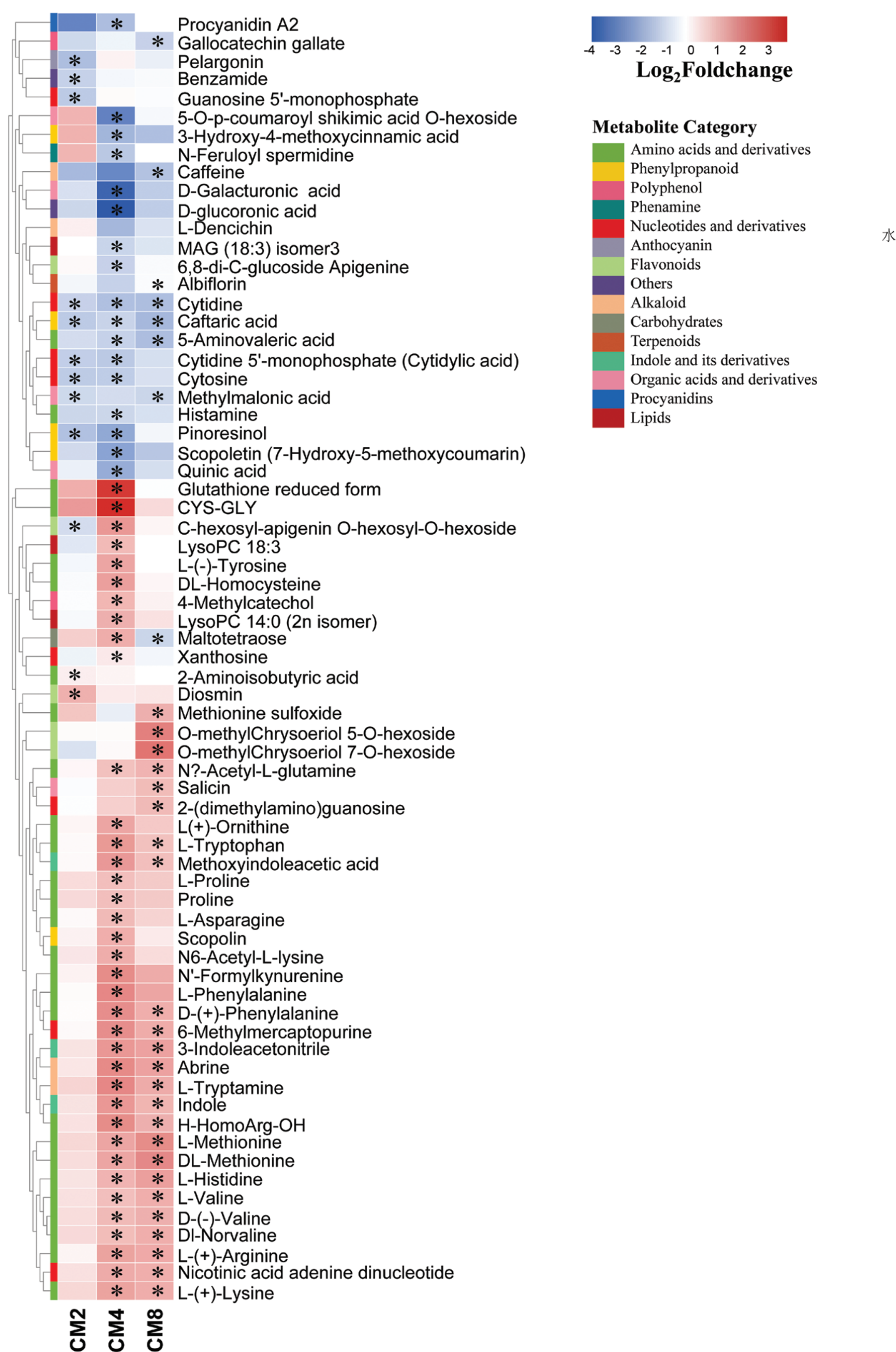

Figure 2. Effects of 1-MCP treatment on metabolites during 2-/4-/8-month storage of apple fruit. The heat map shows the fold change (log2 fold change) of metabolites in each comparison group. CM2, CM4, CM8 represent MCP2 *vs.* CK2, MCP4 *vs.* CK4, MCP8 *vs.* CK8, respectively; the former is the 1-MCP treatment group, the latter the compared group; red and blue indicate up-regulated and down-regulated in the 1-MCP treatment group, respectively; * indicates the screened differential metabolites in each combination of comparison. Different color represents different metabolite category in the left of figure, and given clustering analysis according fold change of every metabolite.

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acid and cytidine) were continuously down-regulated in the 1-MCP treatment group, whereas consistently up-regulated metabolites were not detected (Figure S2).

Transcriptome analysis

Summary data of RNA-seq

To investigate possible transcriptome changes in the apples during postharvest storage after 1-MCP treatment, after 2, 4, and 8 months of storage, the treated and control groups were subjected to transcriptome sequencing analysis. The raw RNA-seq data have been submitted to the NCBI Sequence Read Archive (SRA) database under accession number SRP319226. After quality assessment and data filtering, between 36 968 992 and 60 639 782 clean reads were obtained from all eighteen samples, and the average clean data were approximately 6.69 GB. The Q30 percentage of the clean reads for each sample was no less than 93.00%, and the average GC content was 47.17%. More than 90% of the clean reads were uniquely mapped to the reference genome, whereas the percentage of multiple mapped reads was less than 4% (Table S9). In addition, all of the squared Pearson correlation coefficients (R^2) of the three biological replicates in the six groups were greater than 0.9 (Figure S3), and the RT-PCR results of five selected genes were highly correlated with the RNA-seq data (Figure S4), indicating that the RNA-seq results were reliable for further analysis.

DEGs analysis

Based on the RNA-seq data, six groups—the 1-MCP-treated groups after 2, 4, and 8 months of storage (i.e. MCP2, MCP4, and MCP8) and the control group stored for the same amount of time (i.e. CK2, CK4, and CK8)—were compared, as shown in Figure 3. More DEGs were recognized with increasing storage time, but the 1-MCP-treated group had relatively fewer DEGs than the control group. On the other hand, more down-regulated DEGs were detected in the 1-MCP-treated groups, especially after 8 months of storage (Figure 3A). The results indicated that 1-MCP was more likely to inhibit the increasing trend of selected gene expression, which might be related to the delay of fruit senescence metabolism. The DEGs that existed in the nine comparison groups were selected and subjected to clustering analysis, and a heatmap (Figure 3B) showed that the gene expression pattern of group CK8 was quite different from the others. We also compared the differences and similarities of DEGs between the 1-MCP-treated and control groups at the three storage stages; the results showed that ninety-two genes were up-regulated, and 191 genes were suppressed under 1-MCP treatment throughout storage (Figure 3C).

GO and KEGG enrichment

To gain insight into the effects of 1-MCP treatment on the quality of Fuji apples, the DEGs of the treated and control groups at different storage stages were selected and then classified with GO terms (Figure 3D; Figures S5A and S6A). A maximum number of DEGs was observed in the 'Cellular Component' (CC) term, and it is worth noting that the terms related to photosynthesis and photosystems were only enriched in up-regulated genes in the three comparison groups (Figure 3D). The results of the GO analysis suggested that 1-MCP treatment could promote the expression of genes related to photosynthesis, whereas the expression of genes related to the cell wall, enzyme activity, cofactor binding, fatty acids, and proteasomes was inhibited. In addition, 1-MCP treatment showed broad effects on transcription factors. To obtain a better understanding of the above-mentioned gene interactions and their impact on biological functions, the DEGs were assigned to different KEGG pathways (Figure 3E; Figures S5B and S6B). The most enriched pathways included 'photosynthesis-antenna proteins,' 'photosynthesis,' 'zeatin biosynthesis,' and 'cyanoamino acid metabolism' (Figure 3E).

Taken together, 1-MCP treatment mainly affected metabolic pathways during postharvest storage, which included photosynthesis-related pathways, porphyrin, chlorophyll, and carbohydrate metabolism, plant hormone signal transduction, amino acid metabolism, fatty acid metabolism, and phenylpropanoid biosynthesis. At the early stage, 1-MCP treatment mainly affected starch and sucrose metabolism, glycolysis and pyruvate pathways, and fatty acid synthesis. The effects of 1-MCP treatment on the metabolism of some amino acids and secondary metabolic pathways were observed in the middle and later stages of storage. In addition, photosynthesis-related pathways, porphyrin and chlorophyll metabolism, phytohormone signal transduction, cyanoalanine metabolism, and lactose metabolism were regulated by 1-MCP during the entire storage period.

The responses of sugar and acid metabolites and related genes transcription to 1-MCP treatment

The sugar metabolism of fruit is not only an important process of bioenergy production but also a precursor source of many other important metabolites. The sugars accumulate from photosynthesis and are stored as starch and sucrose in fruit through starch and sucrose metabolic pathways (Li *et al*., 2012), which are then converted into glucose and fructose and further participate in other metabolic pathways. As Figure 1D shows, during the storage stage, the ratio of soluble solids to acid ultimately increased. Furthermore, to analyze the related genes (Figure 4A), the results showed that in the 1-MCP treatment group, *sorbitol dehydrogenase* (DVH24_ 022604) and *sucrose phosphate synthase* (DVH24_ 009535, DVH24_ 000768) were down-regulated, whereas the expression of *sucrose synthase* (DVH24 _ 029571, DVH24_ 018269), *starch synthase* (DVH24_011725, DVH24_016976), and *granule binding starch enzyme* (DVH24_ 008353, DVH24_011984) were up-regulated after 2 and 4 months of storage. In the starch degradation pathway, 1-MCP treatment mainly lowered the expression of *β-amylase* (DVH24_001971, DVH24_025558, DVH24_035791), and maltotetraose content was first higher and then lower than that in the control group with prolonged storage time. In cellulose metabolism, *cellulose synthase* (VH24_016467, DVH24_016470, DVH24_001798) and *endoglucanase* (DVH24_020524, DVH24_012987, DVH24_040086) were down-regulated in the 1-MCP treatment group. In the glycolysis pathway, 1-MCP treatment negatively affected *glyceraldehyde-3-phosphate dehydrogenase* (DVH24_004955), *phosphoglycerate mutase* (DVH24_016858, DVH24_042494), *pyruvate kinase* (DVH24_001736, DVH24_007752), and *enolase* (DVH24_ 008996). *Phosphofructokinase* (PFK5), as the limiting enzyme in the glycolysis process (Rider *et al*., 2004), *PFK5* (DVH24_014427, DVH24_021233) was up-regulated in the 1-MCP_2 group.

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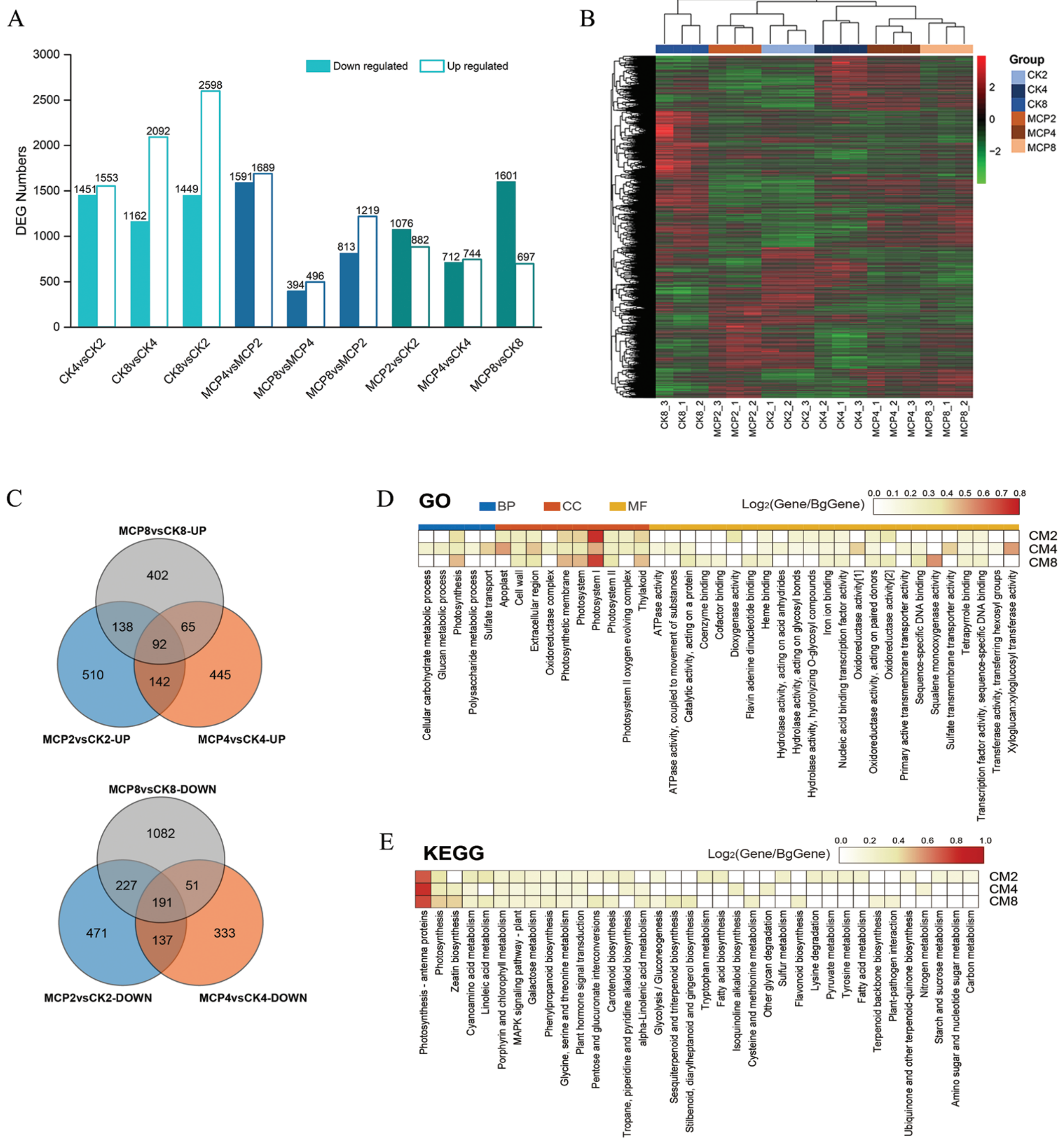

Figure 3. Transcriptome responses of apple fruit to different cold storage stages after 1-MCP treatment. (A) Number of DEGs in each comparison. (B) Hierarchical clustering of DEGs in different treatment group. The color of the bar represents the expression level of the gene, which is the normalized value of the gene FPKM: from red to green means higher to lower expression of genes. (C) Venn diagram showing the overlap/non-overlap of DEGs in the three different storage stages. UP and DOWN means the up-/down-regulated genes. (D) GO analysis of all DEGs in three combinations of 1-MCP compared with control group of fruit; 'Gene/BgGene' indicates the proportion of genes enriched in this function of all genes enriched in this function. The greater the proportion, the redder the color. BP, Biological Process; CC, means Cell Component; MF, means Molecular Function. (E) KEGG enrichment analysis of all DEGs in three combinations of 1-MCP compared with control group of fruit; 'Gene/BgGene' represents the proportion of DEG enriched in this pathway among all genes enriched in this pathway. The more greater the proportion, the redder the color. CK indicates control group of fruit, MCP indicates 1-MCP treatment group of fruit; 2, 4, 8 indicates storage time (month). CM2 represents MCP2 *vs.* CK2, CM4 represents MCP4 *vs.* CK4, and CM8 represents MCP8 *vs.* CK8. (F) Comparison of DEGs comparing 1-MCP-treated fruit to control fruit during three storage stages. CK represents the control group, MCP represents the 1-MCP treatment group, and 2, 4, 8 represent the storage time (month), respectively.

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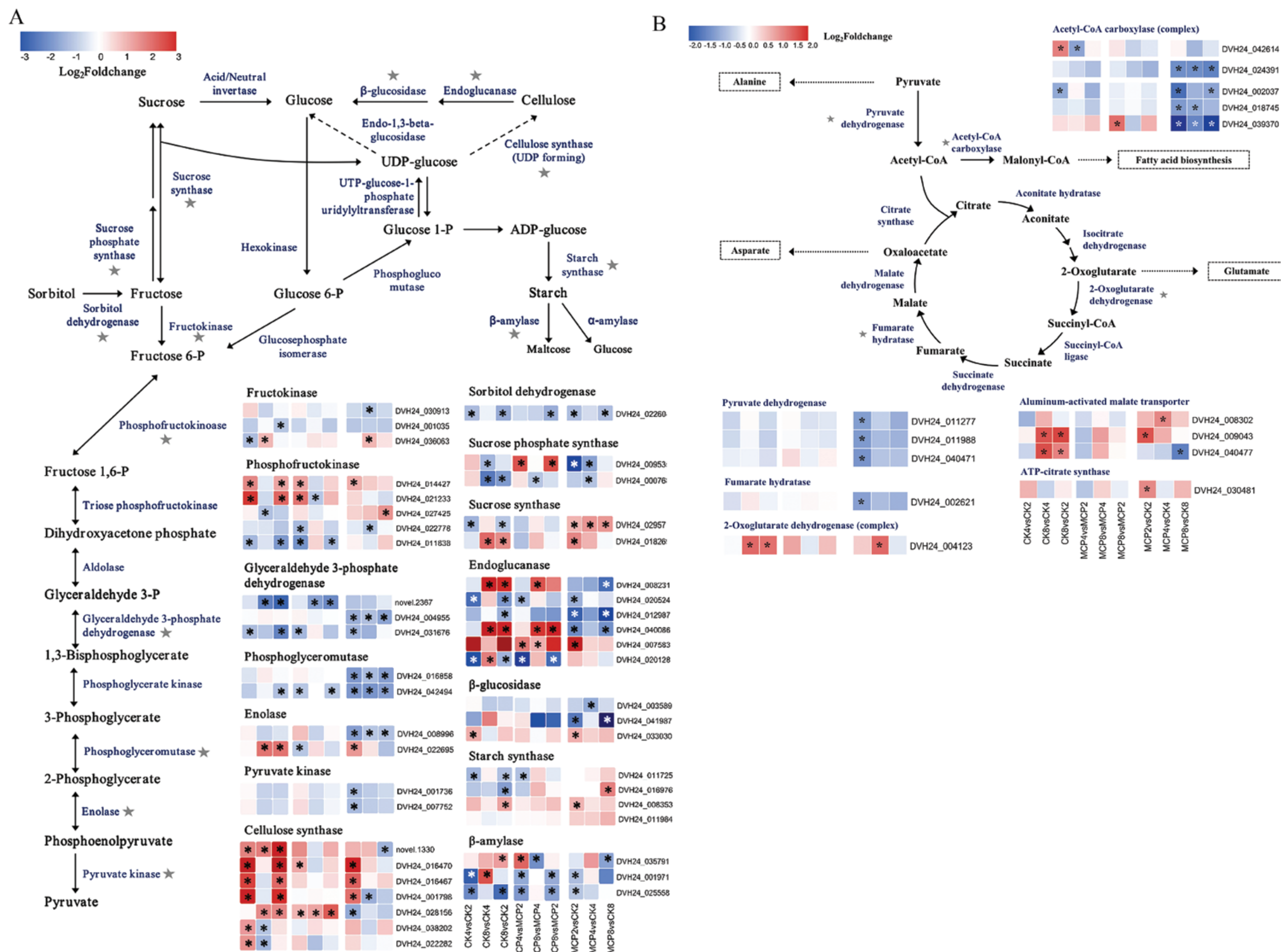

Figure 4. (A) Responses of sugar and acid metabolism and related gene transcription to 1-MCP treatment. (B) Responses of the TCA cycle pathway to 1-MCP treatment during long-term storage. The heat map shows the fold change of genes in different comparison combinations and converts by logarithmic processing, base 2. Red or blue indicate the gene was up- or down-regulated in the comparison group, respectively; * indicates DEGs with *P*<0.05 significance. The circle indicates the scaled data of metabolites content, the higher content level the redder the color.

As shown in Figure 2, during the initial stage of storage, some organic acids changed very little until the middle storage stage, where four organic acids and their derivatives—D-galacturonic acid, 3-hydroxybutyric acid, 5-*O*-*p*-coumaroyl mangrove acid *O*-hexoside, and quininic acid—were down-regulated in the treatment group; as storage time increased, this difference became less obvious. Moreover, as shown in Figure 4B, 1-MCP treatment and storage had little influence on the gene expression of tricarboxylic acid cycle (TCA)-cycle-related enzymes. 1-MCP treatment inhibited the expression of *pyruvate dehydrogenase* (DVH24_011277, DVH24_011988, DVH24_040471) and *fumarate hydratase* (DVH24_002621), which stimulated the accumulation of fumaric acid. 1-MCP promoted the expression of *α-ketoglutarate dehydrogenase complex* (DVH24_004123) associated with glutamate metabolism after 4 months of storage. Acetyl-CoA enters the fatty acid metabolism pathway through *acetyl-CoA carboxylase*; the key enzyme includes multiple subunits (Choi-Rhee and Cronan, 2003). Biological *carboxylase* (BC), biotin carboxyl carrier protein (BCCP), and their related genes (DVH24_024391, DVH24_002037, DVH24_018475, DVH24_039370) were inhibited by 1-MCP treatment during storage, indicating that the related metabolic pathways were blocked. Aluminum-activated malic acid transporter (ALMT) genes related to malic acid transport were differentially regulated by 1-MCP, where genes DVH24_008302 and DVH24_009043 accumulated in the early and middle stage of fruit storage, but the gene (DVH24_040477) was down-regulated in the later stage of storage.

In summary, 1-MCP mainly lowered the expression of some genes in the glycolysis pathway, promoted the expression of *sucrose synthase*, *starch synthase*, and other genes, and blocked the degradation of sucrose, starch, and other substances in the metabolic pathway of glucose and fructose, but it had little influence on the gene expression of TCA-cycle-related enzymes during fruit storage. The results showed that 1-MCP could maintain the content of malic acid, citric acid, and fumaric acid, and reduce the content of succinic acid during storage.

Alterations in pentose and glucuronate interconversion pathway-related genes and metabolites

As products of cell wall degradation, D-galacturonic acid and D-glucuronic acid showed lower levels in the 1-MCP-treated apples after 4 months of storage compared to the control group; the expression of genes in related pathways is shown in Figure 5. *Pectinesterase* (DVH24_013755, DVH24_033564,

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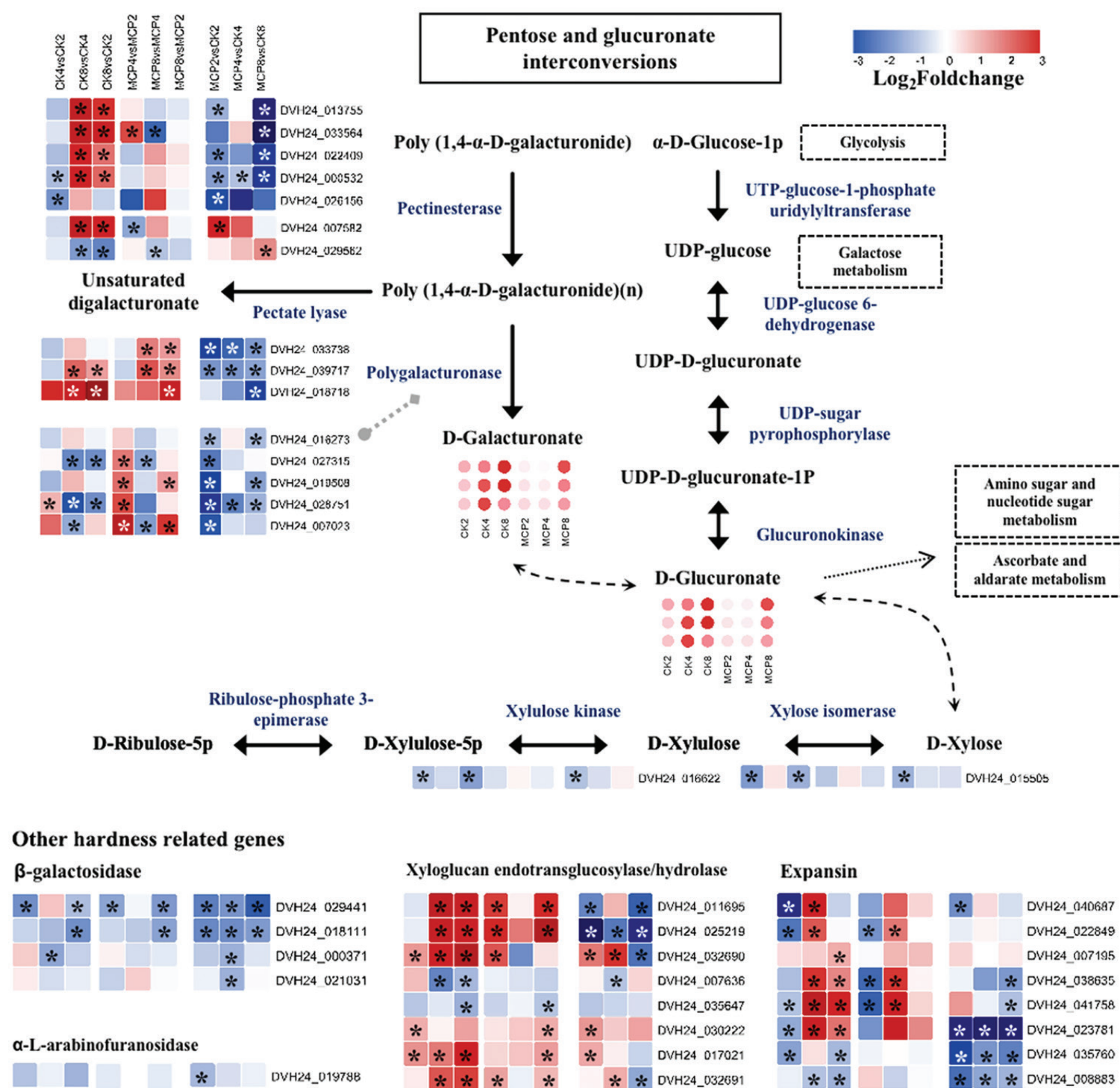

Figure 5. The transcriptomic mapping and key metabolites associated with pentose and glucuronate interconversions pathways during long-term storage after 1-MCP treatment. The heat map shows the fold change of genes in different comparison combinations and converts by logarithmic processing, base 2. Red or blue indicate the gene was up- or down-regulated in the comparison group, respectively; * indicates DEGs with P<0.05 significance. The circle indicates the scaled data of metabolites content, the higher content level the redder.

DVH24_022409, DVH24_000532, DVH24_026156) was down-regulated by 1-MCP, whereas *pectinesterase inhibitor* (DVH24_007582, DVH24_029562) was up-regulated in the treated samples. The expression of *pectate lyase* (DVH24_033738, DVH24_039717, DVH24_018718) was remarkably inhibited by postharvest 1-MCP treatment. Moreover, *Polygalacturonase* (DVH24_019508, DVH24_028751) and *β-galactosidase* (DVH24_029441, DVH24_018111, DVH24_000355) were also down-regulated in the 1-MCP-treated fruit. In addition, *Xylulose kinase* (DVH24_016622) and *xylose isomerase* (DVH24_015505) were down-regulated during the metabolism of ribose phosphate to xylose in the 1-MCP_2 group. According to previous studies, *α-*l*-arabinofuranosidase*, *xyloglucan endotransglucosylase/hydrolase* (XTH), and expansin (EXP) are involved in the process of cell wall expansion and fruit softening (Cosgrove, 2000; Asif *et al*., 2014). The transcriptional level of *α-*l*-arabinofuranosidase* (DVH24_019788) was lower in the 1-MCP_2 group. Both *xyloglucan endotransglucosylase/hydrolase* and *expansin* are gene families containing numerous members, and different members are involved in many kinds of physiological processes. According to our data, the level of *XTHB* (DVH24_025219) was increased with fruit senescence, whereas it was suppressed by 1-MCP treatment during storage. *XTH2* (DVH24_032690) and *XTH8* (DVH24_017021) were up-regulated in the early stage but down-regulated in the 1-MCP_8 group. Furthermore, most XTHs (DVH24_011695, DVH24_025219, DVH24_032690,

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DVH24_017021, DVH24_032691) were inhibited during long-term storage after 1-MCP treatment. Apart from this, 1-MCP suppressed the expression of *expansin* (DVH24_023781, DVH24_008883). The transcription level of another *EXPA1* member (DVH24_040687) decreased after 2 months of storage, and a remarkable down-regulation of *EXPA4* (DVH24_038635) and *EXPA8* (DVH24_041758) was observed after only 8 months of storage. Overall, the increasing trend of *expansin* (DVH24_040687, DVH24_022849, DVH24_023781, DVH24_038635, DVH24_041758) was suppressed in the 1-MCP treatment storage group.

Analysis of DEGs related to cysteine and methionine metabolic pathways

According to the metabolome analysis, the accumulation of compounds belonging to amino acids and their derivatives was obviously different at the later stage of storage time in the treated fruit compared to the control. Furthermore, the number of differential metabolites increased with increased storage time from one metabolite (2-aminoisobutyric acid) to 16–25 metabolites, including 5-aminovaleric acid, *N*-acetyl-L-glutamine, L-phenylalanine, L-tryptophan, L-valine, L-histidine, L-methionine, *N′*-formylkynurenine, homoarginine, glutathione, and others. The treated group had higher levels of amino acids related to protein synthesis and related metabolites. Among these highly varied compounds, glutathione, homocysteine, asparagine, and methionine sulfoxide are all related to the metabolism of cysteine and methionine; therefore, we further explored the effect of 1-MCP treatment on the expression of related pathway genes (Figure 6).

Cysteine synthesis in plants mainly involves two steps: *O*-acetylserine is first synthesized under the catalysis of serine acetyltransferase (SAT), and then cysteine synthase (CSase) catalyzes the synthesis of cysteine from *O*-acetylserine and H_2S. Cyanoalanine synthase (CAS) and CSase are a pair of functionally redundant enzymes. 1-MCP treatment inhibited the expression of *CAS* (DVH24_014675, DVH24_014237), whereas *CSase* (DVH24_015300, DVH24_036740) was up-regulated in the 1-MCP_2 and 1-MCP_8 groups, compared to the control. These results indicated that 1-MCP treatment was beneficial for the synthesis of cysteine.

Cysteine can be used to synthesize glutathione (GSH), which is important for maintaining plant resistance, especially resistance to active oxygen damage. γ-Glutamate-cysteine ligase and glutathione synthase are key enzymes in the GSH synthesis pathway. The level of *γ-glutamate-cysteine ligase* (DVH24_015960) increased first but then decreased after 4 months of storage with fruit senescence, whereas *novel.885* increased after 8 months of storage. GSH may have been oxidized by glutathione peroxidase to form oxidized glutathione (G–S–S–G), and *glutathione peroxidase* (DVH24_008031)

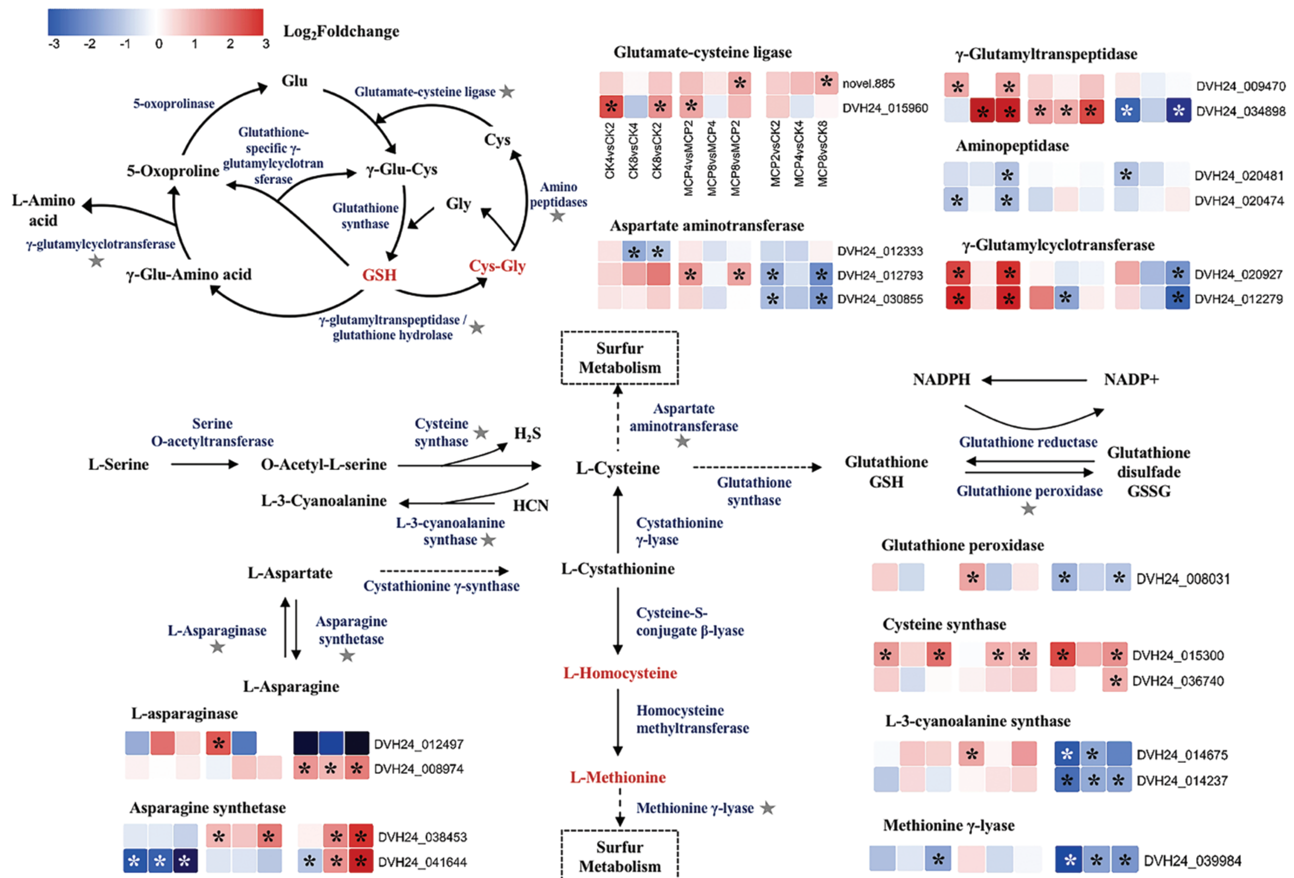

Figure 6. DEGs involved in cysteine and methionine metabolism pathway during long-term storage after 1-MCP treatment. The heat map shows the fold change of genes in different comparison combinations and converts by logarithmic processing, base 2. Red or blue indicate the gene was up- or down-regulated in the comparison group, respectively; * indicates DEGs with $P<0.05$ significance.

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was obviously suppressed by the 1-MCP treatment during postharvest storage.

Glutathione is also involved in the transmembrane transport of amino acids in organisms. After 4 months of storage, GSH and cysteine glycine (CYS-GLY) showed higher levels in the treated fruit, and 1-MCP treatment had no effects on the levels of serine, glutamic acid, cysteine, glycine, glutamyl cysteine, or 5-oxoproline. The results suggested that the accumulation of glutathione and CYS-GLY in the treated group after 4 months of storage may be related to the process of amino acid transmembrane transport. Two important enzymes, γ-glutamyltranspeptidase and γ-glutamylcyclotransferase, are involved in this transport process. The expression levels of *γ-glutamyltranspeptidase* (DVH24_034898) and *γ-glutamylcyclotransferase* (DVH24_020927, DVH24_012279) were inhibited by 1-MCP treatment, especially at the later stage of storage.

Moreover, apples after 4 months of storage had higher levels of L-methionine and L-homocysteine. The expression levels of *methionine-γ-lyase* (DVH24_039984) was down-regulated by 1-MCP treatment during the entire storage time, which might account for the accumulation of methionine. Homocysteine is the intermediate product of the methionine synthesis pathway, which is obtained by multistep catalysis from aspartic acid. However, in this study, we did not find a change in the expression level of genes in the related pathway. There was no difference in the accumulation of aspartic acid between the treated and control groups, but the L-asparagine content was higher in the 1-MCP_4 group. Asparagine synthetase could transform L-asparagine into aspartic acid, and L-asparagine could generate aspartic acid by L-asparaginase. The results showed that *asparagine synthetase* (DVH24_038453, DVH24_041644) was up-regulated after 1-MCP treatment, whereas l-*asparaginase* (DVH24_012497) was remarkably inhibited. These results indicated that 1-MCP affected the conversion between L-asparagine and aspartic acid.

Responses of ethylene synthesis and signal transmission pathway to 1-MCP treatment

1-MCP competitively binds to ethylene receptors, resulting in the inhibition of fruit ripening. Figures 1C and 1D show that 1-MCP treatment inhibited both ethylene production and the increase of the respiration rate. Combining the metabolomics and transcriptomics analyses, methionine (the precursor of ethylene) accumulation of the 1-MCP_2 group was higher than after 4 and 8 months of storage, but *S*-adenosine L-methionine (SAM) content showed no evident changes at all stages. For the genes of the ethylene biosynthesis metabolic pathway, two *SAMS* (DVH24_041206, DVH24_025274) and *ACS* (DVH24_030213), *ACO1* (DVH24_016918) and its homologous gene (DVH24_003003) were inhibited in the 1-MCP-treated group. However, a few *ACO* homologous genes (DVH24_030551, DVH24_038224, DVH24_029428) were up-regulated after 8 months of storage, but the expression levels were still relatively low. Moreover, a series of ethylene receptors and transcription factors responded to ethylene signals. For example, the expression levels of *ETR* (DVH24_001804, DVH24_013455), *EBF* (*EIN3 binding F-box*) (DVH24_017778, DVH24_029157), and *ERF* (DVH 24_012791, DVH24_040461) were lower in the 1-MCP-treated group at the beginning of fruit storage. *CTR* (DVH24_016680, DVH24_033515), a negative regulatory element of the ethylene response factor, was up-regulated in the 1-MCP-treated group during storage, whereas 1-MCP treatment genes had little effect on the expression of *EIN2* and *EIN3* genes (Figure S7).

Discussion

Fuji apple is a variety with great economic value, so we used this material in this study to evaluate fruit quality variation after 1-MCP treatment during long-term cold storage. The overall effects of postharvest treatment with 1-MCP were revealed by integrative metabolomics and transcriptomics analyses.

1-MCP retarded quality deterioration

Fruit softening, as a key determinant of postharvest quality, is a process closely related to the remodeling of the cell wall structure (Wang *et al.*, 2018). Cellulose, hemicellulose, and pectin are the dominant components of plant cell wall tissue, and mainly consist of polysaccharides. Many key enzymes involved in sugar metabolism show a strong correlation with fruit hardness, and their relative gene expression will be affected by different treatments (Tucker *et al.*, 2017). For example, D-galacturonic acid and D-glucuronic acid are important products of the pentose and glucuronate metabolic pathways. Apples contain higher levels of D-galacturonic acid in water-soluble pectin extracts, are easier to soften, and show a higher correlation with fruit texture variations (Billy *et al.*, 2008). D-Galacturonic acid, which is the product of pectin metabolism, could also be regarded as an indicator of fruit softening levels during storage. In this study, it was found that D-galacturonic acid and D-glucuronic acid accumulated gradually with increased storage time, whereas 1-MCP-treated fruit samples had obviously lower levels of these two compounds after 4 months of storage. Additionally, the gene expression of pectin degradation-related enzymes *PE*, *PL*, and *PG* was strongly inhibited, and the levels of pectin degradation products remained low during fruit storage. These results may partially explain the mechanism of how 1-MCP delays the decrease of fruit firmness during postharvest storage, resulting in a longer shelf life.

1-MCP regulated sugar and acid metabolism to delay apple fruit ripening

1-MCP inhibited the utilization pathway of glucose and fructose and decreased the expression of some genes in the glycolysis pathway, whereas it mainly increased sucrose and starch content by lowering the expression of related genes. In addition, 1-MCP contributed to halting the degradation of sucrose, starch, and other substances in the fruit, whereas it had little effect on the TCA cycle. Lee *et al.* (2012) found that a 24-h 1-MCP treatment of 'Empire' apples had little effect on most carbohydrates and organic acids, whereas it had a significant effect on levels of amino acids and volatile metabolites; moreover, it up-regulated the content of the metabolites of flesh browning, which was different from our test results, because the duration of processing samples was different. In our results, long-term treatment had a cumulative effect on flesh quality control and ultimately postponed fruit decay. Previous study also reported that 1-MCP treatment maintained the levels of malic acid and citric acid during storage at

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2 °C, maintained the activities of phosphoenolpyruvate carboxylase (PEPC) and malic dehydrogenase (MDH) related to organic acid metabolism, and led to higher V-ATPase and V-PPase activities and lower PEPCK activities; moreover, the gene expression of related enzymes was also regulated at the transcriptional level (Liu *et al.*, 2016). In summary, 1-MCP mainly regulated glucose and acid metabolism to delay fruit ripening and maintain fruit flavor.

1-MCP inhibited cysteine and methionine metabolism pathways further to delay fruit senescence

The metabolism of cysteine and methionine in the fruit can be connected to various other metabolic pathways. As an important sulfur-containing compound in plants, cysteine plays a vital role in the process of sulfur metabolism by affecting compounds such as methionine, glutathione, sulfur-containing vitamins, coenzymes, and sulfur-containing signaling molecules (Gotor *et al.*, 2015). CSase is a key enzyme involved in the synthesis of cysteine. Cyanide, which can be produced during the later metabolic stage of ethylene and aromatic amino acids, inhibits the respiratory chain of fruit. CAS can catalyze cysteine and cyanide to form cyanoalanine, which is important for eliminating harmful cyanide in plants (Siegien and Bogatek, 2006; Ebbs *et al.*, 2010). According to our results, 1-MCP promoted the expression of CSase-related genes but suppressed that of CAS-related genes. However, we did not find any change in cysteine content in the fruit, suggesting that the GSH synthesis pathway might be promoted by 1-MCP because GSH could be formed from cysteine over multiple steps. Apart from the significantly reduced GSH, a small amount of G–S–S–G could also be detected. A higher ratio of GSH/G–S–S–G is beneficial to reductive environmental maintenance and protein synthesis. In our study, the content of GSH was higher than that in the untreated fruit at the middle stage of storage, indicating a lower internal oxidation state of the apples, which might be related to a better fruit quality during the early and middle storage stages. The data showed that 1-MCP treatment induced the accumulation of methionine, the precursor of ethylene synthesis, which indicated that the synthesis of ethylene was inhibited, and the senescence of fruit was delayed. In addition, methionine could be catalyzed by methionine-γ-lyase for further sulfur metabolism. We found that methionine-γ-lyase-related genes were strongly suppressed by 1-MCP treatment during storage. The blockage of multiple methionine utilization pathways may have been the reason for the accumulation of methionine in fruit.

1-MCP inhibited ethylene release in apples

Ethylene is the key determinant of fruit ripening. Research has found that 1-MCP could slow the release of ethylene and inhibit ethylene synthesis genes and ethylene receptors and transcription factors in the ethylene signaling pathway, further delaying apple ripening (Martinez-Romero *et al.*, 2007). Other researchers also reported that ethylene release continuously increased in all samples, but 1-MCP had an inhibitory effect on it in sweet cherry fruit compared to the control groups (Ren *et al.*, 2011). Moreover, the literature showed that 1-MCP restrained the expression of ethylene-related genes *PcACS1*, *PcACS4*, *PcACS5*, and *PcACO1* in pear fruit but up-regulated the expression of *PcACO2*; thus, 1-MCP regulated genes related to ethylene synthesis differently (Zhao *et al.*, 2018). As reported previously, Yang *et al.* (2013) showed that 1-MCP inhibited the expression of ethylene receptor genes such as *ETR1*, *ERSs*, *CTR1*, and *EIN2* and some key transcription factors of the signal transmission pathway. Thus, 1-MCP maintained apple fruit quality by inhibiting ethylene release.

Conclusions

Our research indicated that 1-MCP treatment could prolong the shelf life of apples, especially after long-term cold storage. After integrative analyses of metabolomics and transcriptomics, it was determined that 1-MCP mainly reduced the levels of metabolites and inhibited the changes of selected genes in fruit ripening and senescence-related pathways, including the metabolic pathways of ethylene, sugars, organic acids, and amino acids, to maintain the postharvest quality of apples. These findings provide a solid theoretical basis for precision technology of controlling fruit postharvest storage and further meeting consumer expectations.

Supplementary Material

Supplementary material is available at *Food Quality and Safety* online.

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Conflict of Interest

The authors declare no conflict of interest.

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Author Contributions

Hongliang Zhu and Chunjiao Zhang: Conceptualization, Methodology and Supervision. Lingling Zhang and Peiyu Zhang: Writing Original draft preparation and Writing, Reviewing and Editing, Software, Validation. Modi Gao and Yi Zhao: Software, Data curation, Visualization and Investigation.

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