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# Effect of 1-MCP on storage quality and the mechanism involved in ethylene signal transduction in a new early-maturing apple variety 'Taihangzaohong' fruits during cold storage

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## Summary

1-Methylcyclopropene (1-MCP) can reduce the rate of fruit softening and prolong storage time. In this study, the fruit of a new early-maturing apple variety, 'Taihangzaohong', was treated with air (control), 2 µL/L 1-MCP, 100 µL/L ethylene ($C_2H_4$) or 2 µL/L 1-MCP + 100 µL/L $C_2H_4$ for 24 hours and then stored at 4 °C for 70 days. The postharvest physiological indices and the expression of 13 genes related to ethylene biosynthesis and signal transduction were monitored every 10 days. The results showed that 1-MCP can delay the softening rate and maintain the fruit quality of this early-maturing apple variety by reducing ethylene production by reducing the expression of *MdACO1, MdACO2,* and *MdACS1*, as well as by preventing ethylene signal transduction by decreasing the expression of *MdETR2* and *MdERS1* and increasing the expression of *MdCTR1*. Understanding the significant changes in these genes and their functions may help us explore the mechanisms controlling apple fruit softening and its response to exogenous 1-MCP and ethylene stimuli, as well as inhibition at the receptor level during ripening and senescence.

**Keywords:** 1-MCP; 'Taihangzaohong' early-maturing apple; storage quality; ethylene synthesis; signal transduction

## Introduction

A new early-maturing apple variety, 'Taihangzaohong' (*Malus domestica* Borkh. cv. Taihangzaohong) originated from a seedling apple tree accidentally discovered in the orchard of Tianhuyu Village, Jingxing Mining District, Shijiazhuang City, Hebei Province, China in the year 2000 by the Apple Rootstock Innovation Team of Hebei Agricultural University. By investigating the varieties planted around the orchard, combined with the results of the SSR molecular marker test, it is believed that 'Jonathan' and 'Morris' may be its parents. 'Taihangzaohong' is now cultivated in Baoding and Shijiazhuang city, Hebei Province, China. It has the following characteristics: the peel colour is bright red, the fruit shape is round, and the taste is delicate, mild sour and sweet, which makes this fruit popular with farmers. However, 'Taihangzaohong' apple fruits soften quickly after harvest, which seriously affects their edible and commodity value, as is common for early-maturing apple varieties worldwide. The shelf life of early-maturing apple varieties is short (Onik et al., 2018), resulting in an unreasonable variety structure in China and the reduced sale of early-maturing varieties. Therefore, prolonging the shelf life of early-maturing varieties is the key to adjusting the variety structure.

The softening of apple fruits is affected by many factors, and the physiological and biochemical changes of the softening process are regulated by genes. For climacteric fruits, the change in ethylene production is the main factor regulating fruit softening (Giovannoni, 2004; Onik et al., 2018). Researchers have studied fruit softening from various aspects, but the regulatory mechanism is not fully understood, especially for different fruits.

The process of ethylene biosynthesis is as follows: ACC (1-aminocyclopropene-1-carboxylic acid) is synthesized from SAM (S-adenosylmethionine) by the action of ACC synthetase (ACS); then, ethylene is synthesized from ACC through ACC oxidase (ACO) (Tsuchisaka and Theologis, 2004). ACS is the rate-limiting enzyme in this process (Zhao et al., 2020). When the *MdACS1* gene in apple was silenced, ethylene production was significantly reduced, thus slowed the softening rate and extended the storage period of apple fruit (Dandekar et al., 2004).

Ethylene signal transduction also plays an important role in regulating fruit softening. ETR and ERS are ethylene receptors, and when ethylene binds to these receptors, it will inhibit their negative regulatory activity. ETR1 (ethylene receptor1) activates the negative regulatory activity of CTR1 (constitutive triple response 1) and then inhibits EIN2 (ethylene in sensitive 2), which has positive regulatory activity. EIN2, which is located downstream and at the end of the ethylene signal transduction pathway, is a positive regulator of ethylene signal transduction (Yang et al., 2013). EIN2 can relay the ethylene signal to the transcription factors ethylene-insensitive 3 (EIN3) and ethylene-insensitive 3-like (EIL) (Yang et al., 2013). In the absence of ethylene, the ethylene receptor (ETR) binds to CTR1 to form an ETR-CTR1 complex, which activates the negative regulatory activity of CTR1 and inhibits the downstream ethylene response; when ethylene is present, ethylene binds to the receptor, CTR1 cannot be activated, and the production of ethylene is positively regulated by EIN2 and ERF1 (Lin et al., 2008).

1-MCP (1-methylcyclopropene) is an ethylene inhibitor that can block the binding of ethylene receptors to ethylene, causing the ethylene signal to be blocked, thereby slowing the fruit softening rate (Pre-Aymard, 2003; Lwin et al., 2021; Dong et al., 2015). 1-MCP is nontoxic, so it is widely used for maintaining fruit quality. The use of 1-MCP in apple (Watkins et al., 2000; Pre-Aymard, 2003), pear (Yudou et al., 2019; Lwin et al., 2021; Zhao et al., 2020), jujube (Ozturk et al., 2021), citrus (Estables-Ortiz et al., 2016), plum (Velardo-Micharet et al., 2017), nectarine (Ozkaya et al., 2016), kiwifruit (Xu et al., 2019), durian (Thongkum et al., 2018), banana (Song et al., 2020; Zhu et al., 2019) and others has been studied.

At present, there are many studies on extending the storage period of apples but relatively few studies on early-maturing varieties. In this study, the early-maturing apple variety 'Taihangzaohong' was used as the test material. The fruits were treated with 1-MCP, ethylene ($C_2H_4$) or 1-MCP+$C_2H_4$ to study the change in fruit hardness and ethylene content and the expression of ethylene synthesis and signal transduction genes. The objective of this study was to determine the effects of 1-MCP on the fruit quality of the early-maturing apple variety 'Taihangzaohong' and to find a way to extend the shelf life of early-ripening apples. The related gene expression related to ethylene synthesis and signal transduction was investigated to study the relationships between ethylene function and storage quality in early-maturing apple during storage to elucidate the softening mechanism of early-ripening apple. Our current study will provide information on the regulation of gene expression during ethylene biosynthesis, perception and signal transduction in early-maturing

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'Taihangzaohong' apple fruits during cold storage or under the influence of 1-MCP treatment, which may be helpful for understanding the softening mechanism of early-maturing apple varieties and the effects of 1-MCP on postharvest quality.

## Materials and methods

### Test material and treatment

The apples used in this experiment were harvested from the Apple Demonstration Garden of Hebei Agricultural University, Nanshennan Village, Shunping County, Baoding City, Hebei Province on July 23, 2018. The apple trees were 9-year-old trees of the early-maturing variety 'Taihangzaohong'; the dwarfing interstock was SH40, and the base stock was *Malus Robusta*. The tree shape was slender spindle, and the row spacing in and between rows was 2 m × 4 m. The trees grew vigorously and were managed regularly.

Fruits with uniform size, no mechanical damage and consistent maturity at the periphery of the canopy were selected and harvested and then returned to the laboratory. The apple fruits were treated with 2 μL/L 1-MCP, 100 μL/L ethylene ($C_2H_4$) or 2 μL/L 1-MCP + 100 μL/L $C_2H_4$ at 24 °C for 24 h under sealed conditions. Air-sealed treatment was used as the control. After treatment, the fruits were transported to a refrigeration house (4 °C) for storage, this time was set as 0 DAS (DAS – days after storage); samples were taken every 10 days until 70 DAS, and 15 fruits were sampled for each treatment every time. The 15 fruits were equally divided into three biological replicates.

### Test methods

#### *Determination of the ethylene release rate*

The apple fruits were placed in a hermetic glass container, each container contained five fruits. After being sealed for 6 hours, ethylene gas was collected, and 1 mL gas was extracted by a medical syringe and stored for later use. Each treatment was repeated 3 times. The ethylene release rate was measured by a FULI-9790II gas chromatograph. The chromatographic conditions were as follows: the chromatographic column was a stainless steel packed column; a hydrogen flame ionization detector was used; the column oven temperature was 90 °C, the vaporization chamber temperature was 140 °C, the carrier gas flow rate was N2-120 mL/min (0.12 Mpa), H2-100 mL/min (0.1 Mpa), and synthetic air-100 mL/min (0.1 Mpa), and the injection volume was 1 ml.

#### *Measurement of the hardness, the weight of a single fruit, and contents of soluble solids and malic acid in the fruits*

The fruit hardness was measured by a GY-1 fruit hardness tester. Four symmetrical sides on the surface of the fruit were chosen, the skin was peeled off. Pressed the indenter of the hardness tester into the pulp to the scale line, and the scale line pointed by the needle at this time was the hardness of the pulp. The average value of the four sides was taken as the result, with units of kg/cm$^2$.

The single fruit weight was measured using an electronic balance, and the data was accurate to two decimal places.

For the determination of the contents of soluble solids and malic acid, the pulp was collected and the juice was squeezed from both the sunny side and shaded sides of the fruit. The soluble solids content was measured by a PAL-1 handheld brix meter, and the malic acid content was measured by a GMK-855 malic acid meter.

#### *The relative expression level of related genes*

The total RNA of fruit samples was extracted using a Tiangen DP441 kit, the purity of the RNA was detected by super trace ultraviolet spectrophotometry, and 1% agarose gel electrophoresis was used to detect the integrity of RNA. The RNA was reverse transcribed into cDNA by a reverse transcription kit. The relative expression levels of four ethylene synthesis genes (*MdACO1, MdACO2*, *MdACS1,* and *MdACS3*), three ethylene receptor genes (*MdETR1, MdETR2,* and *MdERS2*) and six other ethylene signal transduction genes (*MdCTR1, MdEIN2, MdEIL1, MdEIL2, MdEIL3* and *MdEBF1)* were detected using a full-scale gold fluorescence quantitative kit. The 2-ΔΔCT method was used for the analysis of the data. The actin gene from apple (*MdACTIN*) was the reference gene. The nucleotide sequences of primers used for Real-time PCR were showed in Tab. 1.

#### *The statistical analysis of the data*

The statistical analysis of the data and the significant difference analysis were conducted by Excel 2007 and SPSS 26.0. To be specific, the average values and the standard deviations of the results of three biological repetitions were calculated by the 'AVERAGE' and 'STDEV' functions of Excel 2007. The significant difference analysis of different treatments was conducted by one-way ANOVA method in SPSS 26.0, with the significance level of 0.05. The charts were drew by the drawing function of Excel 2007.

**Tab. 1:** Nucleotide Sequences of Primers used for Real-time PCR

| Gene | Forward primer (5'-3') | Reverse primer (5'-3') |
|---|---|---|
| *MdACO1* | TCTACAACCCAGGCAACGACTCAT | AGCTTCAAATCTTGGCTCCTTGGC |
| *MdACO2* | AAGGTTCAAGGAAATGGTGGCAGC | AGAAGAAGGTGCTTTCCCAGTCCA |
| *MdACS1* | AGAACTACATAGCCGAGAACCA | CCAACAGAACAAGCCAGCATT |
| *MdACS3* | TTGATAGAGATTTGAGGTGGAGGA | GTGGGTTTGATGGATTTGTGATTAG |
| *MdETR1* | TATCGCATACACAGAACGAGGCGT | ATTGCACAAGGGTACACCGAGACT |
| *MdETR2* | AAATGGAAACGGTGTGGGAG | TGCTGGAATCGTGACCTCTG |
| *MdCTR1* | ACAAGATTTTCATGCCGAAC | TATGGACAAGTTTGGAGGCT |
| *MdERS2* | CAACTAGGGATATGCGAC | CACTGGCATCCAAAGACTTC |
| *MdEIN2* | AGCAGACTTACCAACCGAAA | GCACTACTTCCTCCATCGTC |
| *MdEIL1* | TCCTGATAGATGTCCACCTCCA | TACGTCCTGCCTCTCTCTTTGTC |
| *MdEIL2* | CCAGTTGAATTGTTCATTCGGGAG | GTTCACTGGTGGTTGACTTGC |
| *MdEIL3* | GGAGTTTGGGCTTCTGTGAT | TGCTGTCGCCTCTGGTTCAT |
| *MdEBF1* | GCCTTCGCAAATGTCTATTCG | CCACCAGTTGAAAGAACACCA |
| *MdACTIN* | CTGAACCCAAAGGCTAATCG | ACTGGCGTAGAGGGAAAGAA |

## Results and analysis

### The ethylene release rate

As shown in Fig. 1, with prolonged storage, the ethylene release rate of the control fruits gradually increased and it increased rapidly after 10 DAS and reached the highest value at 50 DAS. The ethylene release rate of the $C_2H_4$-treated fruits was significantly higher than that of the control. At 50 DAS, the $C_2H_4$-treated and control fruits almost completely rotted, so there were no fruits for testing.

The ethylene release rate of the 1-MCP-treated fruits was significantly lower than that of the control, and it was very low until the end of the storage period; that of the 1-MCP+ $C_2H_4$-treated fruits was higher than the 1-MCP-treated fruits but lower than the $C_2H_4$-treated and control fruits during most of the storage period.

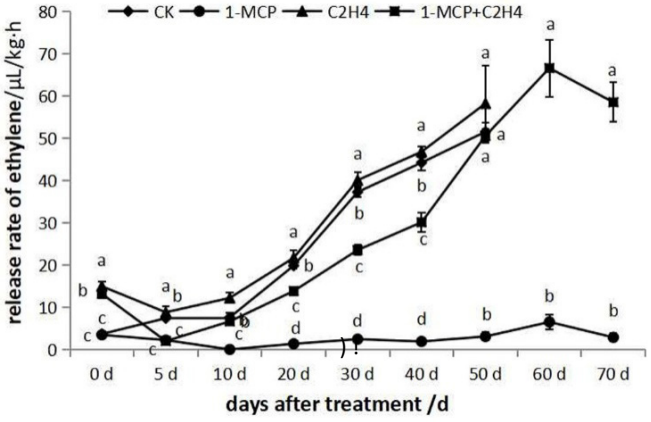


**Fig. 1:** Changes of the ethylene release rate of different treatments (2018). Note: Different lowercase letters represent significant differences.

### *Change of the physical indices of fruits*

As shown in Fig. 2A, with the prolongation of the storage, the hardness of all treated fruits showed a decreasing trend, but the decrease level was different. The hardness of the 1-MCP-treated fruits was significantly higher than other treatments during the whole storage time. The hardness of the control fruits decreased significantly before 30 DAS, and fell to 5.37 kg/cm$^2$ after 40 DAS and lost its edible value. The hardness of the $C_2H_4$-treated fruits was minimal. The hardness of the 1-MCP+ $C_2H_4$-treated fruits was larger than that of the $C_2H_4$-treated fruits.

As shown in Fig. 2B, in the later storage period, the weight of a single 1-MCP treated fruit was significantly higher than other group fruits. The content of soluble solids in 1-MCP-treated fruits was significantly higher than that of the control fruits after 40 DAS (Fig. 2C). The malic acid content of 1-MCP-treated fruits was significantly higher than that of other group fruits after 40 DAS (Fig. 2D).

### Changes of the expression of ethylene synthesis and signal transduction genes

#### *Changes of the expression of ethylene synthesis genes (MdACO1, MdACO2, MdACS1 and MdACS3)*

As shown in Fig. 3A, the relative expression of *MdACO1* in all treated fruits first increased and then decreased during storage. Its expression in 1-MCP-treated fruits was lower than that of CK except at 50 DAS. Its expression in $C_2H_4$- and 1-MCP+$C_2H_4$-treated fruits reached a maximum at 20 DAS and 10 DAS, respectively, and both were significantly higher than the control.

As shown in Fig. 3B, overall, the relative expression of *MdACO2* in all treated fruits first increased and then decreased, and the maximum level in the CK, 1-MCP-treated and $C_2H_4$-treated fruits was reached at 10 DAS. Its expression in the 1-MCP-treated fruits was significantly lower than that of CK at 10, 30, 40 and 50 DAS. Its ex-

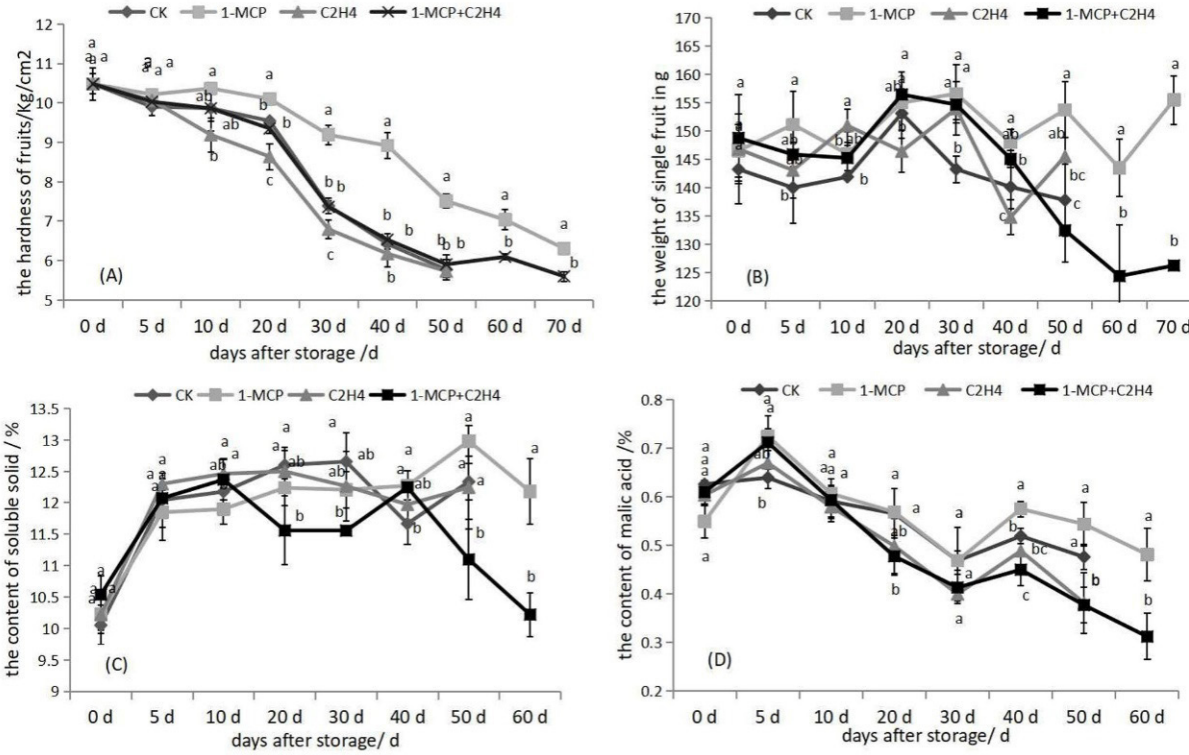


**Fig. 2:** Change of fruit hardness (A), weight of a single fruit (B), and the contents of soluble solids (C) and malic acid (D) (2018). Note: Different lowercase letters represent significant differences.

pression in the $C_2H_4$-treated fruits was significantly higher than that in control fruits at 0, 5, 20, and 30 DAS.

As shown in Fig. 3C, during storage, the relative expression of the *MdACS1* gene in the fruits of each treatment showed a trend of firstly increasing and then decreasing. After treatment with 1-MCP, its expression was significantly lower than that of the control and was at a very low level. After treatment with $C_2H_4$, its expression was significantly higher than that of the CK.

As shown in Fig. 3D, the relative expression of the *MdACS3* gene in all treated fruits during storage firstly increased and then decreased, and the maximum level was reached at 10 DAS. Its expression in the 1-MCP-treated fruits was higher before 20 DAS and lower after 20 DAS than the control fruits. Its expression in the $C_2H_4$-treated fruits was significantly lower than that in the control fruits after 30 DAS.

***Changes of the expression of ethylene signal transduction genes***

*(1) Changes of the expression of ethylene receptor genes (MdETR1, MdETR2 and MdERS2)*

As shown in Fig. 4A, the relative expression level of the *MdETR1* gene in 1-MCP-treated fruits was higher than that in the control fruits at 0, 5 and 50 DAS. Its expression in $C_2H_4$-treated fruits was lower than that of the control at 5 and 30 DAS.

As shown in Fig. 4B, after treatment with 1-MCP, the relative expression of *MdETR2* in the 1-MCP-treated fruits was significantly lower than that of the control during the whole storage period. Its expression in the $C_2H_4$ treatment group was significantly higher than that of the control at 0 and 20 DAS. Its expression in the 1-MCP+$C_2H_4$ treatment group was significantly lower than that of the control at 0, 5, 20 and 40 DAS.

As shown in Fig. 4C, 1-MCP treatment significantly decreased the relative expression of the *MdERS2* gene during storage. Its expression was higher in the $C_2H_4$-treated fruits than that of the control at 0 and 10 DAS. The 1-MCP+$C_2H_4$ treatment significantly decreased its expression at 0, 5, 20, and 40 DAS and increased it at 10 and 30 DAS.

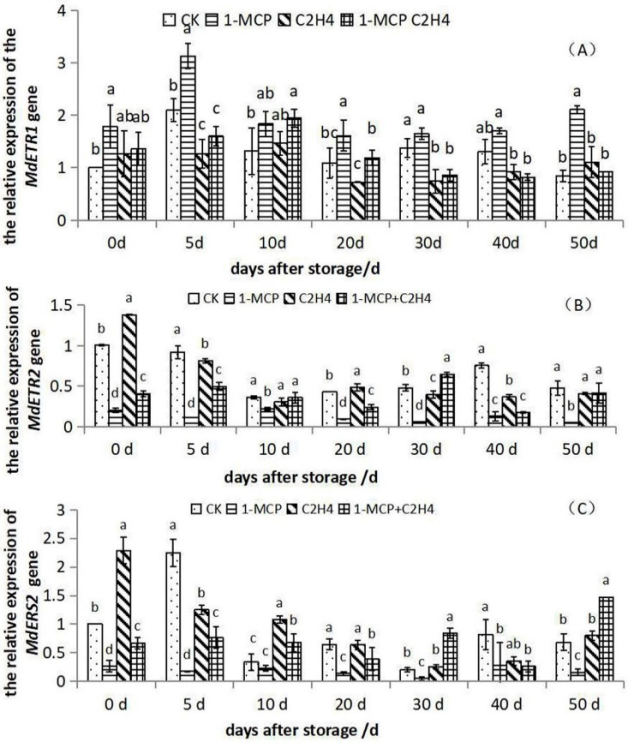


**Fig. 4:** Changes of the relative expression of ethylene receptor genes (A-*MdETR1*, B-*MdETR2* and C-*MdERS2*) (2018).
Note: Different lowercase letters represent significant differences.

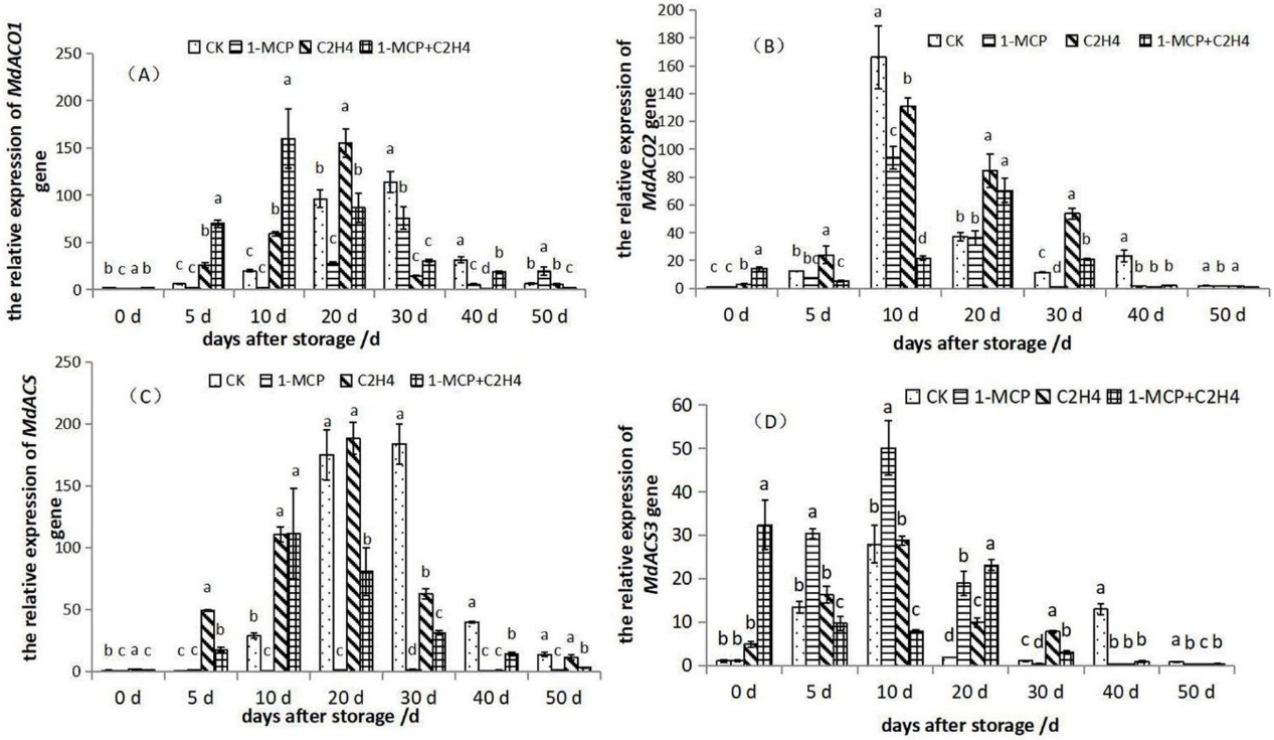


**Fig. 3:** Changes of the relative expression of ethylene synthesis genes (A-*MdACO1*, B-*MdACO2*, C-*MdACS1*and D-*MdACS3*) (2018).
Note: Different lowercase letters represent significant differences.

*(2) Changes in the relative expression of other ethylene signal transduction genes (MdCTR1, MdEIN2, MdEIL1, MdEIL2, MdEIL3 and MdEBF1)*

As shown in Fig. 5A, the relative expression of the *MdCTR1* gene in the control and $C_2H_4$-treated fruits showed a trend of firstly decreasing and then increasing, and there was no significant difference between them. Its expression was higher in the 1-MCP-treated fruits than in the control fruits at 5, 20, 30, and 50 DAS. Its expression was higher in the 1-MCP+$C_2H_4$-treated fruits than in the control fruits at 20, 40, and 50 DAS.

As shown in Fig. 5B, after 1-MCP treatment, the relative expression of the *MdEIN2* gene was significantly higher than that of the control at most periods. Its expression in fruits treated with $C_2H_4$ and 1-MCP+$C_2H_4$ was not significantly different from that of the control.

As shown in Fig. 5C, the relative expression of the *MdEIL1* gene was significantly increased by 1-MCP treatment during the whole storage period. Its expression in the $C_2H_4$-treated fruits was significantly higher at the earlier stages but lower at the later stages than that of the control. After 1-MCP+$C_2H_4$ treatment, its expression fluctuated frequently.

As shown in Fig. 5D, overall, the expression level of *MdEIL2* firstly increased and then decreased in all treatments. Its expression was significantly higher in 1-MCP-treated fruits than the control fruits at earlier stages but was lower at later stages. $C_2H_4$ treatment had a similar effect on its expression. The 1-MCP+$C_2H_4$ treatment had less impact on its expression.

As shown in Fig. 5E, the relative expression of the *MdEIL3* gene in the 1-MCP-treated fruits was higher than that of the control at 5, 10, 20, and 50 DAS but was lower at 30 and 40 DAS. After $C_2H_4$ treatment, its expression in the fruits firstly increased and then decreased. The 1-MCP+$C_2H_4$ treatment had less impact on its expression.

As shown in Fig. 5F, the relative expression of the *MdEBF1* gene in 1-MCP-treated fruits was significantly higher than that of the control group throughout the whole storage period. Its expression in $C_2H_4$-treated fruits was significantly higher at the earlier period (0-20 DAS) but was lower than that of the control at the later period (30 and 40 DAS). The 1-MCP+C2H4 treatment had less impact on its expression.

## Discussion

### The effects of 1-MCP on the storage quality of 'Taihangzaohong' early-maturing apple fruits during cold storage

The results of this study showed that after treatment with 1-MCP, the decrease rate of the hardness of 'Taihangzaohong' apple fruits was significantly lower than other fruits (Fig. 2A), and the ethylene release rate was inhibited by 1-MCP (Fig. 1). This result is consistent with the conclusions of previous studies (Pre-Aymard, 2003; Ozturk et al., 2021; Xie et al., 2017; Estables-Ortiz et al., 2016). The weight of a single fruit and the contents of soluble solids and malic acid in the 1-MCP-treated fruits were higher than those in the control. These results indicated that 1-MCP plays a certain

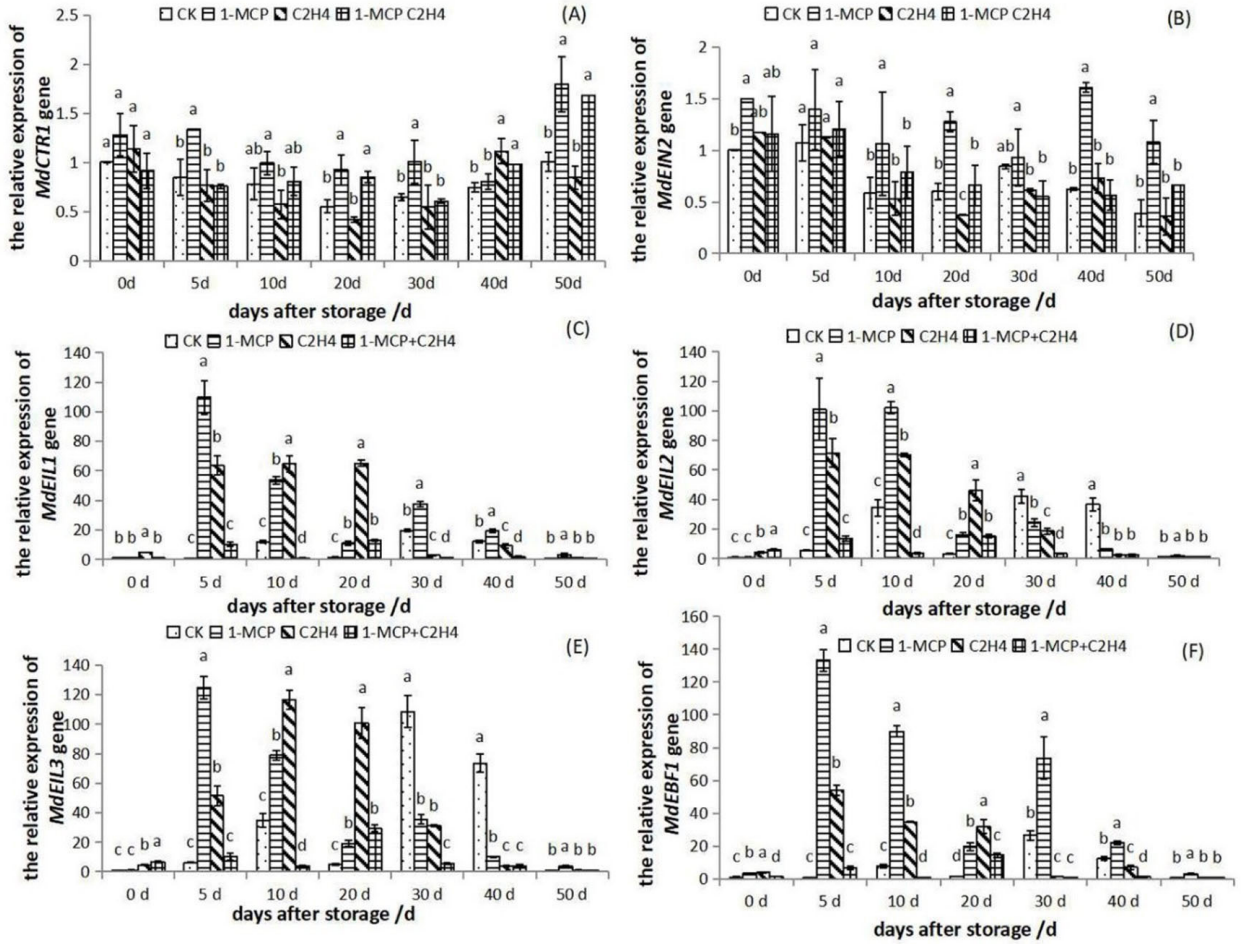


**Fig. 5:** Changes of the relative expression of other ethylene signal transduction genes (A-*MdCTR1*, B-*MdEIN2*, C-*MdEIL1*, D-*MdEIL2*, E-*MdEIL3* and F-*MdEBF1*) (2018).
Note: Different lowercase letters represent significant differences.

role in prolonging the shelf life of the early-maturing apple variety 'Taihangzaohong'. The hardness of $C_2H_4$-treated apple fruits was lower than that of untreated and 1-MCP+$C_2H_4$-treated fruits, indicating that $C_2H_4$ accelerates the softening of the fruits and that 1-MCP can offset the effect of $C_2H_4$ on fruits to a certain extent. The research also suggested that the softening of fruits from this apple variety is closely related to ethylene. According to the results of this study, the rate of fruit softening was positively related to the ethylene release rate (Fig. 1), and this result was consistent with those of previous studies (Zhao et al., 2020; Argenta et al., 2016; Chiriboga et al., 2013; Dong et al., 2018).

**The effects of 1-MCP on ethylene synthesis gene expression in 'Taihangzaohong' early-maturing apple fruits during cold storage**

Two key enzymes, 1-aminocyclopropane-carboxylase (ACC) synthase (ACS) and ACC oxidase (ACO), which catalyse the last steps of the ethylene biosynthetic pathway, were early targets of fruit ripening research (Giovannoni, 2004; Chen et al., 2020). In apple, both enzymes are encoded by multigene families. *ACS* and *ACO* genes play an important role in the process of ethylene synthesis, and *ACS* acts as a rate-limiting enzyme in this pathway (Yang et al., 2013). *ACS* and *ACO* genes have been isolated and extensively studied in apple fruit (Costa et al., 2010; Huang et al., 2010; Zheng et al., 2007). The expression of *MdACS1* and *MdACS3* in apple has attracted much attention. The significant increase of the expression of *ACS1* during fruit ripening and as a result of ethylene treatment was pronounced; *ACS1* and *ACO1* are correlated with the ethylene climacteric burst of apple fruit, and treatment with 1-MCP generally produced effects opposite those of ethylene (Yang et al., 2013; Costa et al., 2010), which is consistent with the results of our study.

In this study, with the extension of storage time, the relative expression of the *MdACO1, MdACO2* and *MdACS1* genes in the control fruits first increased and then decreased (Fig. 3A-3C), indicating that $C_2H_4$ synthesis was promoted, which is consistent with the ethylene release rate results (Fig. 1). Compared with the control fruits, the ethylene content of 1-MCP-treated fruits was much lower, and the relative expression of these three genes was also inhibited. The effect of exogenous $C_2H_4$ on endogenous $C_2H_4$ was opposite that of 1-MCP, and exogenous $C_2H_4$ increased the expression of these three genes in an earlier phase of storage, indicating that exogenous $C_2H_4$ increased the production of endogenous $C_2H_4$ by increasing the expression of the these genes. The effect induced by $C_2H_4$ was counteracted by 1-MCP to a certain extent because the effect induced by 1-MCP+$C_2H_4$ was smaller than that induced by $C_2H_4$ alone.

Previous studies indicated that the expression of *ACS3* did not change upon ripening and 1-MCP treatment (Shibuya et al., 2004; Tatsuki et al., 2007; Wiersma et al., 2007). Others have reported a negative feedback regulation where the expression of *ACS3* was stimulated by 1-MCP (Costa et al., 2010; Varanasi et al., 2011). The expression of *ACS3* can also be influenced by different fruit varieties and developmental stages (Tatsuki et al., 2007; Yang et al., 2013), which may be why different studies have produced different results regarding *ACS3*. In our study, 1-MCP treatment increased the expression of *MdACS3* at earlier phases of the storage period but decreased it at later phases. Excessive ethylene has a negative feedback effect on the expression of the *MdACS3* gene (Tatsuki et al., 2007), which may be why the expression of the *MdACS3* gene in control fruits decreased at 10 DAS in our study (Fig. 3D). Studies have reported that during fruit storage, the expression of the *MdACS3* gene showed a downward trend (Li and Yuan, 2008), possibly because excessive ethylene restrained its expression, which is consistent with the results of our study.

According to above analysis, 1-MCP reduced the production of endogenous $C_2H_4$ by repressing the expression of *MdACO1*, *MdACO2* and *MdACS1* genes but not *MdACS3*, and thus prolonged the shelf life of this apple variety.

**The effects of 1-MCP on the expression of ethylene signal transduction genes in 'Taihangzaohong' early-maturing apple fruits during cold storage**

*(1) The effects of 1-MCP on ethylene receptor gene expression (MdETR1, MdETR2 and MdERS2)*

Ethylene plays a major role in the process of fruit softening. Ethylene stimulates the expression of maturation-related genes through ethylene signal transduction (Xie et al., 2017; Li et al., 2016). The ethylene signal transduction also plays an important role in regulating the release of ethylene. The ethylene signal is perceived by ethylene receptors (ETRs, ERSs) (Kendrick and Chang, 2008). Ethylene receptors act as negative regulators of the ethylene response, and ethylene binding inactivates them (Hua and Meyerowitz, 1998). At least five receptor genes have been reported in apple (*MdETR1, MdETR2, MdETR5, MdERS1, MdERS2*) (Li and Yuan, 2008; Wiersma et al., 2007; Thongkum et al., 2018). The expression of these genes was induced by exogenous ethylene and decreased by 1-MCP during fruit ripening (Yang et al., 2013; Tatsuki et al., 2009), although some of these genes have been found to be constantly expressed in apple fruits and other fruit tissues (Kevany et al., 2007; Tatsuki et al., 2009a; Wiersma et al., 2007).

In our study, we detected the expression of three ethylene receptor genes (*MdETR1, MdETR2* and *MdERS2*), and 1-MCP negatively influenced the expression of *MdETR2* and *MdERS2* in 'Taihangzaohong' apple fruits (Fig. 4B-4C). The results indicating that 1-MCP decreased the speed of softening of 'Taihangzaohong' apple fruits by reducing the expression of *MdETR2* and *MdERS2* and preventing the binding of ethylene to these receptors, thus blocking ethylene signal transduction. The expression of these two genes also coincided well with ethylene production (Fig. 1). The delayed ethylene production may also be directly caused by the lack of an ethylene response due to the binding of 1-MCP to its receptors, and the binding of ethylene receptors with 1-MCP may actually have decreased the degradation of the receptor protein that was triggered by ethylene binding and therefore decreased the sensitivity of the fruit to the ethylene signal (Costa et al., 2010; Yang et al., 2013). In this study, the ethylene release rate increased sharply after 10 DAS, and the relative expression of the *MdETR1* gene showed little change in the control fruits. Its expression was higher in 1-MCP-treated fruits than in the control fruits in some phases, which was inconsistent with the results of previous studies and the results of *MdETR2* and *MdERS2*, indicating 1-MCP plays an important role by regulating the expression of *MdETR2* and *MdERS1* but not *MdETR1* in this variety.

*(2) The effects of 1-MCP on the relative expression of other ethylene signal transduction genes (MdCTR1, MdEIN2, MdEIL1, MdEIL2, MdEIL3 and MdEBF1)*

CTR1 (constitutive triple response 1) is a negative regulator of the ethylene response and acts downstream of ethylene receptors by repressing the positive regulator EIN2 (ethylene insensitive 2) (Alonso et al., 1999). Ethylene binds to the ethylene receptor to inhibit the expression of the *CTR1* gene and promotes the expression of the positive regulatory factor *EIN2*. Then, *EIN2* relays the ethylene signal to the transcription factor *EIN3* (ethylene-insensitive 3) and *EIL* (ethylene-insensitive 3 like), which in turn activates *ERF1* (ethylene response factor 1), thereby promoting fruit ripening. These important ethylene perception elements have been studied in a number of fruit species (Yang et al., 2013). 1-MCP can delay the change in fruit hardness during fruit ripening due to the irreversible binding of 1-MCP and $Cu^{2+}$; thus, the binding of ethylene to the receptor was blocked, thereby delaying fruit ripening (Yan et al., 2010). Receptors

that do not combine with ethylene remain active and bind to CTR1; therefore, CTR1 is active. The ability of ETR1 to bind to downstream CTR1 is high (Xie et al., 2017). Application of 1-MCP to plum fruit down-regulated *ETR1* and *CTR1* expression levels in an early-maturing cultivar but only inhibited the expression of *CTR1* in a late-maturing cultivar (El-Sharkawy et al., 2007).

In this study, the relative expression of the *MdCTR1* gene in fruits treated with 1-MCP was higher than that in the control fruits (Fig. 5A). This result was in line with expectations because the ethylene content was low in 1-MCP-treated fruits, and the active ethylene receptor can bind to *MdCTR1*, thus promoting the expression of *MdCTR1*.

Active CTR1 can phosphorylate the C-terminus of EIN2. EIN2 is a key factor in the degradation of EBF1; after EIN2 enters the nucleus, it can degrade EBF1 and promote the accumulation of EIN3, thus stimulating downstream signal transduction (Xie et al., 2017). In this study, the relative expression of the *MdEIN2* and *MdEBF1* genes in 1-MCP-treated fruits was higher than that of the control fruits at most storage times (Fig. 5B and Fig. 5F). These results were not consistent with expectations, but a report indicated that *EIN2* acts as a negative regulator of ethylene signalling (Yan et al., 2011).

The relative expression of the *MdEIL1* gene was increased by 1-MCP. The relative expression of the *MdEIL2* and *MdEIL3* was increased at an earlier phase but decreased at a later phase by 1-MCP (Fig. 5C-5E). It has been proposed that EILs are not regulated by ethylene in fruit but rather by ripening signals (Huang et al., 2010). EILs are believed to be a positive regulator within the ethylene signal (Tieman et al., 2001), however it was also suggested that EILs may function differently among different gene families or different tissues (Mbeguie-A-Mbeguie et al., 2008; Tacken et al., 2010), and a report indicated that EILs act as negative regulators (Yan et al., 2011). Since multiple EIL genes have been discovered in apple fruit, the significant regulation of ripening from other family genes cannot be ruled out, and this needs further study.

Prior to the herein described experiment, we conducted a pre-experiment in 2017. This included two treatments (CK and 2 µL/L 1-MCP treatment), and detected the release rate of ethylene, the hardness of fruits, the content of soluble solid and malic acid, and the expression level of *MdACO1*, *MdACO2*, *MdACS1*, *MdACS3*, *MdETR1*, *MdCTR1*, *MdEIN2*, *MdEIN3*, *MdEBF1* genes (Supplemental Figures 1-5). For the sake of completeness, the results have been added as Supplemental Material. The change trends of the same index or the same gene in the 2017 and 2018 data sets coincided with each other. Thus, a variation due to environmental factors between years is unlikely. However, the 2017 data has not been included in the same statistical analysis as the 2018 data presented in this paper due to some deviations in the experimental setup (less treatments, less parameters recorded).

## Conclusion

The expression of *MdACO1, MdACO2, MdACS1, MdETR2, MdERS1* and *MdCTR1* correlated well with the process of ripening and the response to ethylene and 1-MCP treatments. Based on this study, we can obtain the following conclusions: 1-MCP can delay the softening rate of the early-maturing apple variety 'Taihangzaohong' by reducing ethylene production and reducing the expression of *MdACO1, MdACO2*, and *MdACS1,* as well as by preventing ethylene signal transduction by decreasing the expression of *MdETR2* and *MdERS1* and increasing the expression of *MdCTR1*.

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**Conflict of interest**

The authors have no conflicting interests, and all authors have approved the manuscript and agree with its submission to Journal of Applied Botany and Food Quality.

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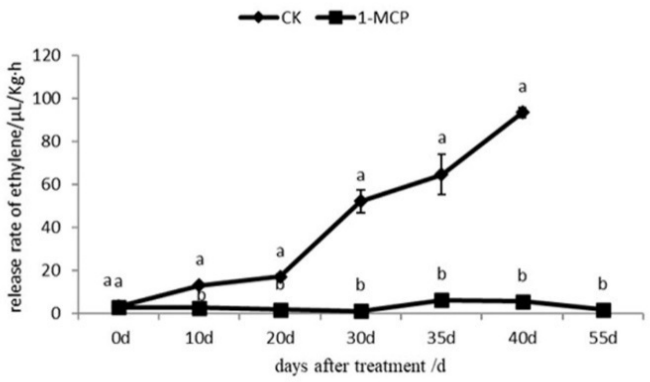
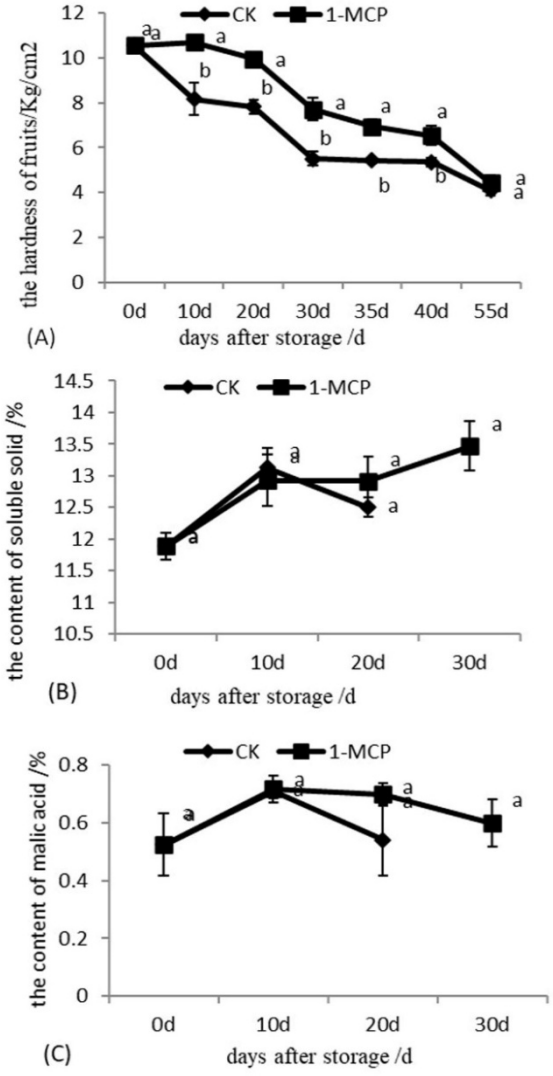
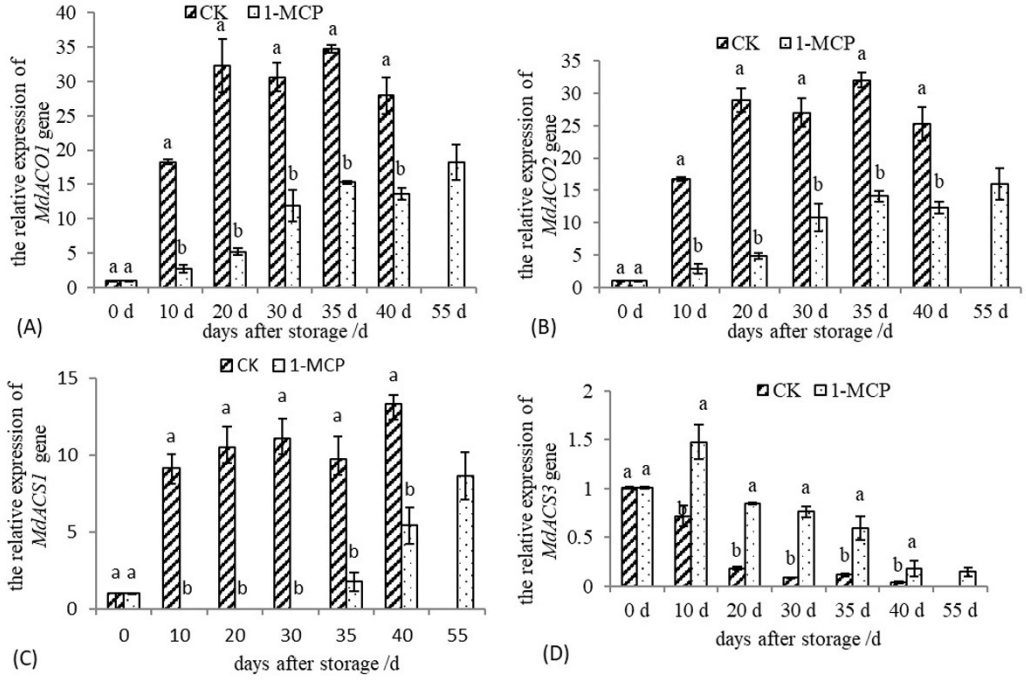
**Supplemental Figures (Data from pre-experiment in 2017)**



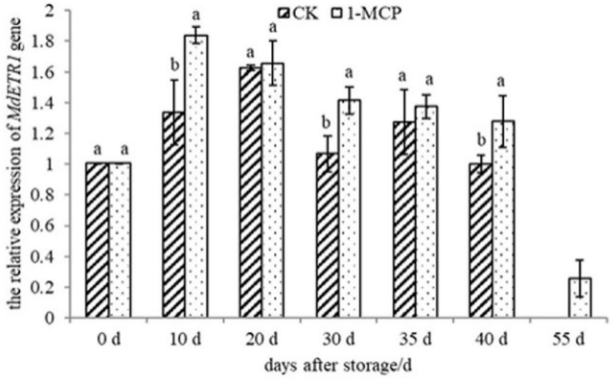

**Supplemental Fig. 1:** Changes of the ethylene release rate of different treatments. (2017)
Note: Different lowercase letters represent significant differences.



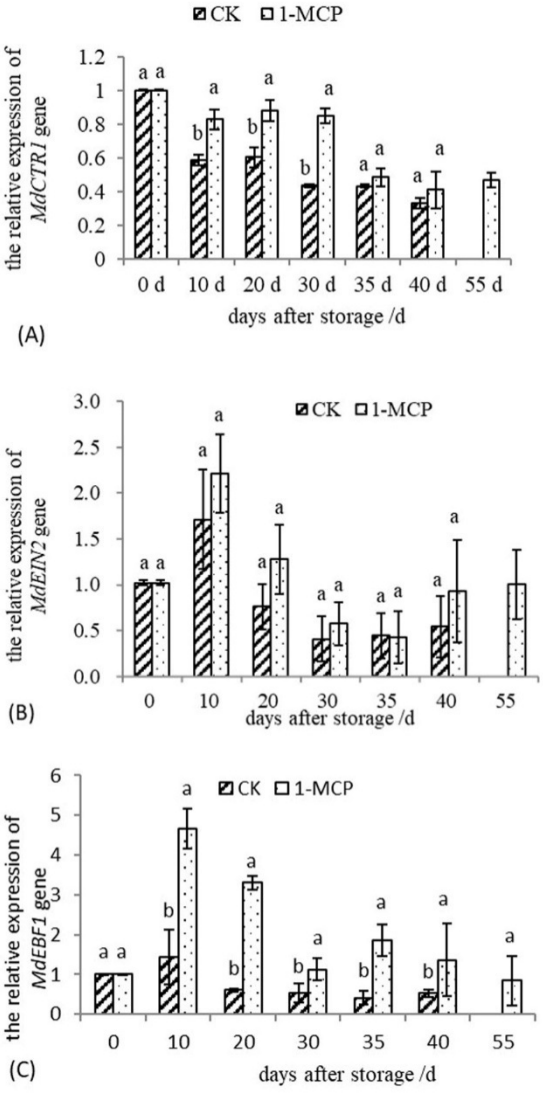

**Supplemental Fig. 2:** Change of the fruit hardness (A), the contents of soluble solids (B) and malic acid (C). (2017)
Note: Different lowercase letters represent significant differences.




**Supplemental Fig. 3:** Changes of the relative expression of ethylene synthesis genes (A-*MdACO1*, B-*MdACO2*, C-*MdACS1*and D-*MdACS3*). (2017)
Note: Different lowercase letters represent significant differences.




**Supplemental Fig. 4:** Changes of the relative expression of ethylene receptor genes *MdETR1*. (2017)
Note: Different lowercase letters represent significant differences.




**Supplemental Fig. 5:** Relative expression of other ethylene signal transduction genes (A-*MdCTR1*, B-*MdEIN2* and C-*MdEBF1*). (2017)
Note: Different lowercase letters represent significant differences.