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Original Article

Gene expression of ethylene biosynthesis and ethylene signaling involved in chilling injury of Hom Thong Banana♣

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Abstract

This research studied the expression of ethylene-related genes involved with chilling injury (CI) of Hom Thong banana. Bananas fruit were treated with 1-methylcyclopropene (M) or ethylene (E), or left untreated (control; C) before being stored at 7±1 °C for four and eight days, with subsequent transfer to 25±1 °C for three days. Bananas treated with E were resistant to CI and had normal ripening. In contrast, bananas fumigated with M were susceptible to CI and did not ripen. The ripening index (peel color, firmness, and TSS) was related to a high expression of *MaACS1* and *MaACO1* genes after storage, whereas the expression of these genes in bananas treated with M was not apparent. The *MaERS3* gene in bananas treated with E was up-regulated after transfer to 25±1 °C while the expression levels of *MaCTR1* and *MaEIL2* were significantly down-regulated at 7±1 °C after four days. These results indicated that ethylene induces CI tolerance in Hom Thong banana to by suppression *MaCTR1* gene and inducing *MaACS* and *MaACO* genes.

Keywords: low temperature, ripening, ethylene, Hom Thong, 1-MCP

1. Introduction

Hom Thong banana is a crop that has high export potential from Thailand. Around 3,000 tons of fresh Hom Thong banana is currently exported per year. Banana fruit is harvested at the mature green stage and must be ripened with the consumer. Low-temperature storage induces chilling injury (CI) and leads to abnormal ripening. Bananas with CI show peel darkening and pitting. In these fruit, the peel does not change from green to yellow, and fruit softening does not occur. The CI symptoms in William banana are related to a decrease in ethylene binding (Jiang, Joyce, Jiang & Lu, 2004). Ethylene is a plant hormone that plays a role in fruit ripening of climacteric fruit, including banana. Therefore, CI symptoms in Hom Thong banana after low-temperature storage may involve ethylene biosynthesis and ethylene signaling.

Ethylene biosynthesis begins with S-adenosyl-L-methionine (SAM) which is converted to aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS). ACC is subsequently converted to ethylene by ACC oxidase (ACO). However, a high level of ethylene production is controlled by ACS turnover where ACC is converted to 1-(malonylamino) cyclopropane-1-carboxylic acid (MACC), a process that is called autoinhibition (Chang, 2016). When banana fruit begins ripening, ethylene production coincides with the onset of respiration (climacteric rise) followed by a rapid decrease (post climacteric) (Jones, Hulme & Wooltorton, 1965; Oeller, Wong, Taylor, Pike & Theologis, 1991). Ethylene biosynthesis in climacteric fruit is controlled via two systems. In system I, ethylene is produced at a low level. The ethylene

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from system I then induces ethylene biosynthesis in system II (autocatalysis) which is followed by the start of ripening (Alexander & Grierson, 2002).

Ethylene is perceived via a negative regulator. Ethylene is detected by ethylene receptors that are located in the ER membrane. The receptors are divided into many types, including Ethylene Response 1 (ETR1), Ethylene Response Sensor 3 (ERS3), Ethylene Insensitive 4 (EIN4) and then Constitutive Triple Response 1 (CTR1) which are inactive, resulting in cleavage of the ETHYLENE-INSENSITIVE2 (EIN2) C terminus, after that transcription factor (TF) such as Ethylene Insensitive-Like Protein1 (EIL1) or Ethylene Response Sensor (ERFs) interact with promoter region and transcriptional gene (Müller and Bosch, 2015; Chang, 2016).

Previous studies have found that low-temperature storage is associated with ethylene biosynthesis and ethylene signaling. For example, avocado fruit that are harvested from an orchard with chilling stress show ethylene production, and greater expression of *PaACS1-2*, *PaACO*, *PaETR*, and *PaERS1* genes when compared with fruit that was harvested from a non-chilled orchard (Hershkovitz, Friedman, Goldschmidt, Feygenberg & Pesis, 2009). Furthermore, 1-methycyclopropane (1-MCP), which inhibits ethylene perception, reduces CI and expression of the *EjETR1* gene in both okra and loquat cv. Luoyangqing (Huang *et al.*, 2012; Wang *et. al.*, 2010). In contrast, nectarine treated with ethylene had reduced CI and increased ACO enzyme activity and ethylene production (Zhou, Dong, Arie & Lurie, 2001). William banana treated with ethylene before storage at 7 ℃ for three days, showed fewer CI symptoms and normal ripening, whereas untreated fruit stored at 7 ℃ for eight days showed severe CI symptoms and abnormal ripening. This result suggests that *MaACO1*, *MaERS3*, and *MaEIL2* genes may be related to ripening and the degree of CI (Hong *et al.*, 2015). Furthermore, Saiyawan (2012) found that *MaACS* and *MaACO* genes were up-regulated and that fruit had normal ripening in both Namwa and Hom Thong cultivars after they were treated with ethylene before storage at 4 ℃. However, gene expression in relation to ethylene signaling does not appear to have been studied in Hom Thong banana. Moreover, the inhibition of ethylene perception in banana may prove the role of ethylene in CI and fruit ripening after low-temperature storage. The aim of this research was to study those ethylene-related genes that are associated with chilling injury in Hom Thong banana.

2. Materials and Methods

2.1 Plant material and treatments

Hom Thong bananas were harvested at the 70-80% maturity stage from an orchard at Banglane, Nakhon-Pathom province. Banana fruit were washed with 200 mg/L sodium hypochlorite and dipped in 200 mg/L of fungicide (prochloraz). Fruit were divided into three groups consisting of group 1 - the control, where fruit were kept in air at 25±1 ℃ for 18 hrs. In group 2, fruit were fumigated with 500 nl/L of 1-MCP (Floralifee/Rohm and Hass (EthyBloc®)) in a chamber for 18 h at 25±1 ℃. With group 3, fruit were dipped in 500 mg/L of ethephon (exogenous ethylene) for 2 min, dried at room temperature, and ripened at 25±1 ℃ for 18 hrs. After each of the treatments, fruit were stored at 7 ℃ for either four or eight days and then transferred to 25±1 ℃ for either one or three days. Samples of peel and pulp tissue were stored at -80 ℃.

2.2 Chilling injury and ripening evaluation

Chilling injury evaluation was modified from Jiang *et al.* (2004). Score was assessed by the extension of skin blackened areas; 1 = either green (mature) or yellow (ripe); 2 = greyish; 3 = small blackened areas (< 0.3 cm^2 of total area); 4 = intermediate blackened areas (0.3-0.8 cm^2 of total area); 5 = severe blackened areas (> 0.8 cm^2 of total area). Banana fruit ripening stage was modified from the banana ripening index of United States Department of Agriculture (USDA); 1 = peel dark green color; 2 = light green; 3 = yellowish green (more green than yellow); 4 = greenish-yellow (more yellow than green); 5 = yellow with green tip; 6 = yellow; 7 = yellow, flecked with brown.

2.3 Fruit qualities

Peel color was determined for each banana fruit using the brightness (L* value) and yellow color (b* value) index using a color meter (Konica Minolta, CR-400, Japan). Pulp firmness was measured using a firmness tester (chatillon, United States) and expressed as N/cm^2. Banana pulp was homogenized with DI water and centrifuged for 20 min at 12,000 rpm. The supernatant was collected and total soluble solids concentration (TSS) was measured using a hand refractometer (ATAGO, Japan).

2.3.1 RNA extraction and Real-time PCR analysis

1) RNA extraction

Total RNA extraction in banana peel was modified from the method of Chang, Puryear & Cairney (1993). Frozen tissue was ground to a fine powder using a mortar and pestle in liquid nitrogen. A 3 g sample of peel was added to 15 ml extraction buffer (2% cetyltrimethylammonium bromide (CTAB), 2M NaCl, 100 mM Tris-HCl pH 8.0, 25 mM EDTA, 2% polyvinyl pyrrolidone (PVP), and 5.0g/L spermidine) in a water bath at 65 ℃ and add 2% β-mercaptoethanol 300 µl. The sample was strongly shaken for 10 min and centrifuged at 7,000 rpm for 15 min at 4 ℃. The supernatant was added to 15 ml SEVAG (chloroform: isoamyl alcohol, 24:1), shaken for 10 min, then centrifuged at 7,000 rpm for 15 min at 4 ℃. Added 8M LiCl to the supernatant for the final concentration 3M LiCl and incubated overnight at 4 ℃. The sample was then centrifuged at 10,000 rpm for 20 min at 4 ℃. The precipitate was dissolved in 500 µl SSTE buffer at 65℃ (1M NaCl, 0.5% SDS, 10mM Tris-HCl pH 8.0 and 1mM EDTA), and 500 µl SEVAG was added. The RNA was centrifuged at 10,000 rpm for 5 min at room temperature. A 1 ml aliquot of absolute ethanol was added to the supernatant and the mixture was then incubated at -80℃ for 1 h, then centrifuged at 12,000 rpm for 20 min at 4 ℃. The precipitated RNA was washed with 1 ml 70% ethanol, drained well, and the RNA pellet dissolved in 20 µl of DMDC water (1 ml dimethyl dicarbonate in 100 ml of absolute ethanol and adjusted with DI water to 1 L). The quality of the total RNA was examined by spectrophotometer at 260 and 280 nm (NANODROP

2000C Spectrophotometer, Thermo Scientific, U.S.A.).

2) Elimination of DNA

DNA was eliminated from each sample using RNase free kit (Thermo Scientific, USA) consisting of 10X reaction buffer with $MgCl_2$, Dnase I, RNase-free water. Total RNA was kept at -80 °C for cDNA synthesis.

3) cDNA synthesis

cDNA was synthesized using a RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, USA) consisting of Oligo (dT) and nuclease-free water, 5X reaction buffer, RiboLock RNase Inhibitor (20U/µl), 10mM dNTP Mix and RevertAid M-MuLV RT (200U/ µl). The cDNA was kept at -80 °C.

4) Real-time PCR analysis

Real-time PCR analysis was measured in three replications using a Maxima SYBR Green qPCR Master Mix (2X), ROX solution kit (Thermo Scientific, USA) The mean value was analyzed using RT-qPCR (Bio-Rad, CFX 96, USA). The relative gene expression was calculated by the method of $2^{-\Delta\Delta Ct}$. The specific primers for ACS1, ACO1, ERS3, and EIL2 for real-time PCR were adopted from Hong *et al.* (2015). Primers for CTR1 and the reference gene (18S) were designed with primer3 version 4.0. The sequence of all primers is shown in Table 1. The mean ±SE of three replicates is presented. The expression levels were expressed as a ratio relative to the values at harvest (before storage) which were set at 1.

5) Statistical analysis

All data were analyzed statistically using IBM SPSS statistic 22 programs. The statistical difference was evaluated at the 5% level among treatments. The mean difference was determined according to Tukey HSD.

3. Results and Discussion

3.1 Chilling injury (CI) symptom and fruit ripening

Banana fruit treated with exogenous ethylene had delayed CI. In contrast, fruit fumigated with 1-MCP showed susceptibility to CI. With both fruit treated with ethylene and the control, there was slight CI after storage at 7 °C for four days. There was an increase in the greyish (2.5 points) color of the peel in these fruit after they were transferred to 25 °C. In contrast, bananas treated with 1-MCP showed a clearly greyish color during storage at 7 °C and showed increasingly severe CI symptoms after they were transferred to 25 °C. With fruit stored at 7 °C for eight days, it was found that those in the 1-MCP and control treatments showed severe CI symptoms (4-5 points) when compared with those treated with ethylene. However, all treatments storage for eight days showed severe CI symptoms after being transferred to 25 °C (Figures 1 and 2A).

Fruit treated with exogenous ethylene had normal ripening (Figure 2B). This was confirmed by the increases in the brightness (L* value) and yellow (b* value) color (Figure 3). Fruit firmness also decreased and TSS increased (Figure 4) markedly in the ethylene treated fruit at 25 °C following storage. The peel of the control fruit slightly developed from green to yellow whereas fruit firmness and TSS remained largely. Fruit treated with 1-MCP did not ripen (Figure 3 and 4). These results indicate that Hom Thong bananas that were treated with exogenous ethylene had a delay in the development of CI and had normal ripening. This finding is similar to that of Mohammed and Brecht (2002) with mango. In case of Mango stored in the immature stage showed greater CI symptoms than fruit stored at the mature stage. In nectarine, William banana, and Namwa banana, previous studies have shown that fruit treated with exogenous ethylene before storage at low temperature had reduced CI (Saiyawan, 2012; Wang *et al.*, 2006; Zhou, Dong, Arie & Lurie, 2001). In contrast, banana fumigated with 1-MCP before storage at low temperature showed severe CI symptoms and had abnormal ripening similar to that found with tomato (Biswas, Bast, Hewett & Heyes, 2014).

Figure 1. Chilling injury symptoms and ripening characteristics of Hom Thong banana stored at 7±1°C for either 4 or 8 days (4+0 and 8+0) and following transferred to 25±1°C for either 1 or 3 days (4+1, 4+3, 8+1 and 8+3).

Table 1. Sequences of specific primers used for real-time PCR analysis

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
MaACS1	CTGCTGGATGGACTTGAG	TACACGAAACTCTCGATCCT
MaACO1	GAAGGGGTACCTGAAGAAAG	GTCCTGGAAGAGCAAGATG
MaERS3	CTTACGCTGTCACCTGATCT	CACAACCAGTATCCTTGACC
MaEIL2	GACTCCAACTTCACTTGGTC	GGAAGCACTTCCTAGAGTCA
MaCTR1	CCTTTGGAACTGTCCATCGT	TGGTGGCTCAGTAACAGCAC
Ma18S	GGTGGAAGATCACCAGGCTA	TCCCCAAGTAACATCGAAGC

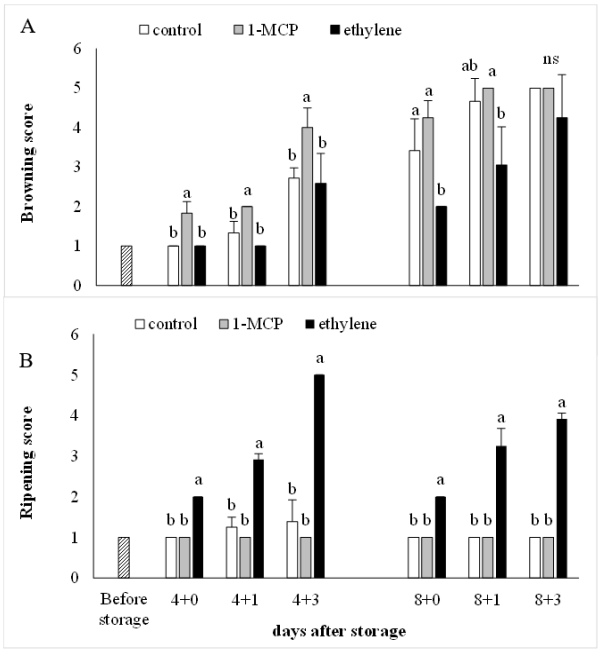

Figure 2. Browning score (A) and ripening score (B) of the peel of Hom Thong banana stored at 7±1°C for either 4 or 8 days (4+0 and 8+0) and following transferred to 25±1°C for either 1 or 3 days (4+1, 4+3, 8+1 and 8+3). Statistical difference at 5% level are shown among treatments. Means with different letters are significantly different and ns is non-significantly difference according to Tukey HSD.

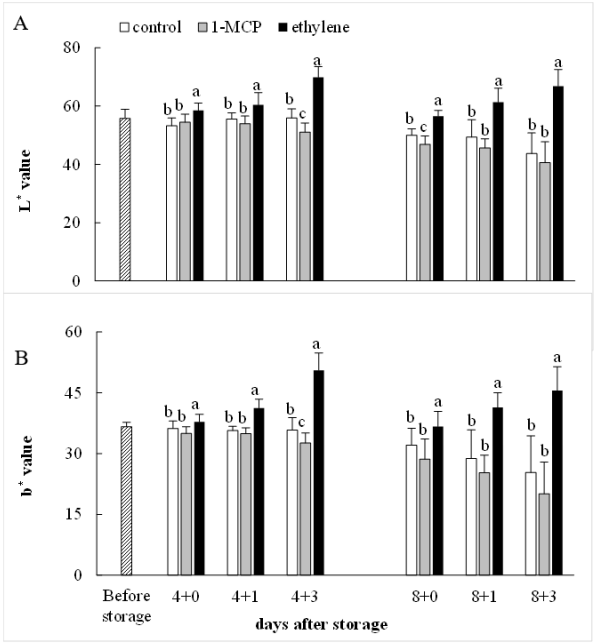

Figure 3. L* value (A) and b* value (B) of the peel Hom Thong banana stored at 7±1°C for either 4 or 8 days (4+0 and 8+0) and following transferred to 25±1°C for either 1 or 3 days (4+1, 4+3, 8+1 and 8+3). Statistical difference at 5% level among treatments. Means with different letters are significantly different according to Tukey HSD.

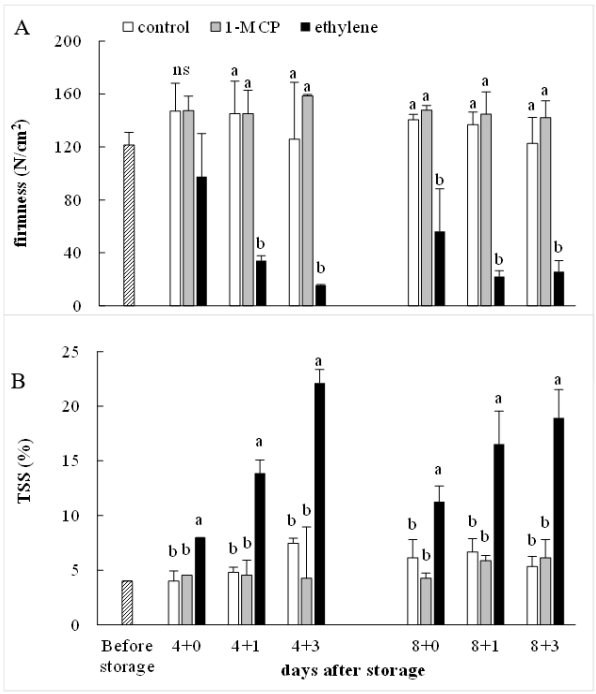

Figure 4. Firmness (A) and TSS (B) of the pulp of Hom Thong banana stored at 7±1°C for either 4 or 8 days (4+0 and 8+0) and following transfer to 25±1°C for either 1 or 3 days (4+1, 4+3, 8+1 and 8+3). Statistical difference at 5% level among treatments. Means with different letters are significantly different and ns is non-significant difference according to Tukey HSD.

3.2 Expression level of ethylene biosynthesis genes in peel of Hom Thong banana after low temperature storage

The expression of *MaACS1 and MaACO*1 genes of bananas treated with exogenous ethylene was up-regulated. The highest *MaACS*1 gene expression level was found in ethylene treated fruit that had been stored at 7°C for 8 days and then transferred to 25 °C for three days (Figure 5A). The *MaACO1* gene expression was also high in ethylene treated fruit after storage at 7 °C for either four or eight days that was then transferred to 25 °C (Figure 5B). These results suggest that *MaACS1* and *MaACO1* genes may induce ACS and ACO enzymes. *MaACS1* and *MaACO*1 gene expression of banana treated with 1-MCP was not detected during storage at low temperature or during ripening (Figure 5). Banana fruit is a climacteric fruit and responds to ethylene in two systems (Alexander and Grierson, 2002). 1-MCP, an ethylene inhibitor, blocks system I of ethylene and could not send the signal to ethylene in system II, resulting in the banana not ripening. The *MaACS1* and *MaACO1* gene expression level of the control banana increased slightly in the fruit that were stored at 7 °C for four days and then transferred to 25 °C for three days (Figure 5).

The development of CI symptoms depended on the combination of storage temperature and storage time. Therefore, at a critical point (in this study up to four days of storage), the banana cells could recover to being normal cells

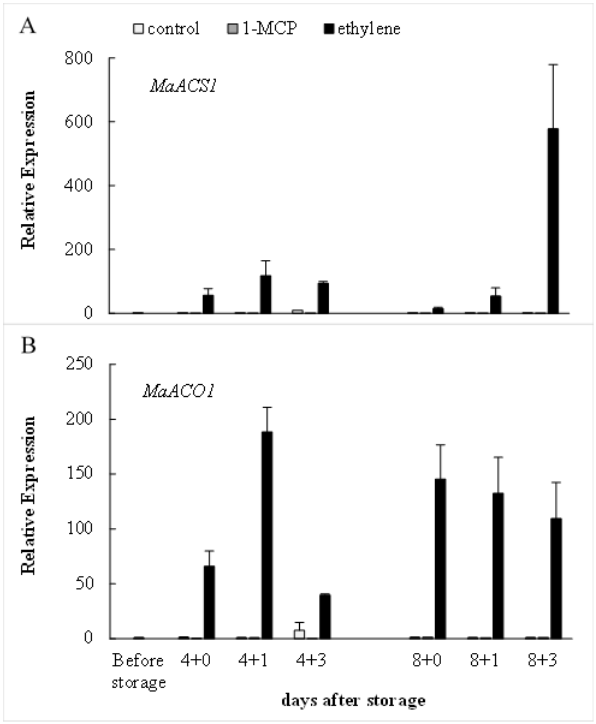

Figure 5. The expression level of *MaACS1* (A) and *MaACO1* (B) in the peel of Hom Thong banana stored at 7±1°C for either 4 or 8 days (4+0 and 8+0) and following transferred to 25±1°C for either 1 or 3 days (4+1, 4+3, 8+1 and 8+3). Each sample represents the mean ±SE of three replicates. Expression levels were expressed as a ratio relative to the values at harvest (before storage) which was set at 1.

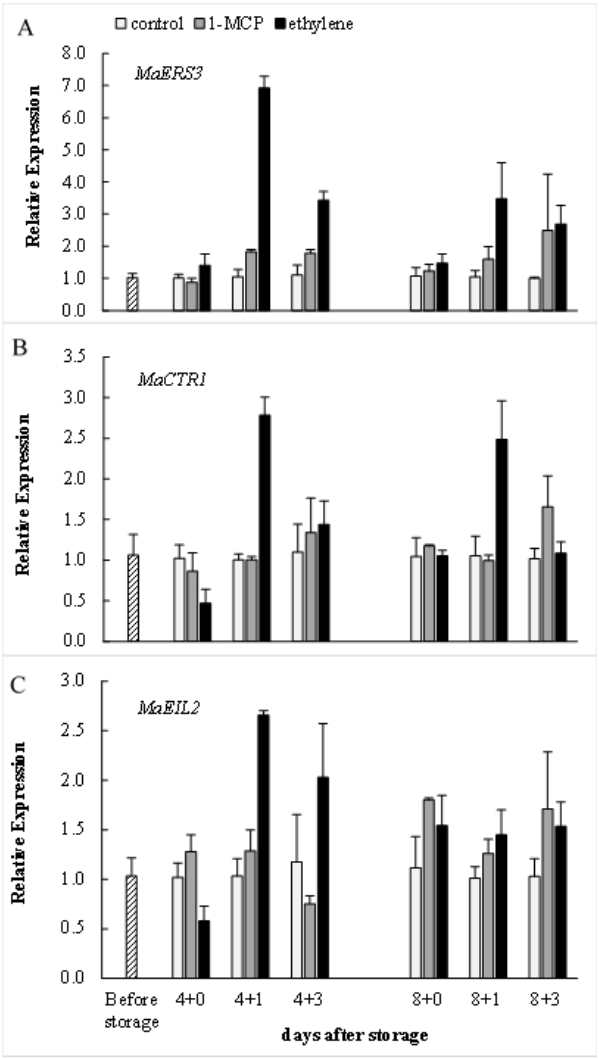

Figure 6. The expression level of *MaERS3* (A), *MaCTR1* (B) and *MaEIL2* (C) in the peel of Hom Thong banana stored at 7±1°C for either 4 or 8 days (4+0 and 8+0) and following transfer to 25±1°C for either 1 or 3 days (4+1, 4+3, 8+1 and 8+3). Each sample represents the mean ±SE of three replicates. Expression levels were expressed as a ratio relative to the values at harvest (before storage) which was set at 1.

after the temperature increased (Paull, 1990; Rasion and Orr, 1990) and had normal ripening after being transferred at 25 °C. However, in Hom Thong bananas stored at 7 °C for eight days, the cells were permanently damaged resulting in abnormal ripening. The expression levels of *MaACS1* and *MaACO1* genes in this study were the same as those reported by Saiyawan (2012) who found that Hom Thong bananas that were treated with exogenous ethylene before storage at 4 °C had higher ethylene production and faster than that of the untreated control. However, the ethylene production of banana fumigated with 1-MCP was low and consistent during storage (Saiyawan, 2012).

3.3 Expression level of ethylene signaling genes in peel of Hom Thong banana after low temperature storage

The *MaERS3* gene in banana peel was up-regulated by exogenous ethylene. This gene had the highest expression after storage for four days followed by transfer to 25 °C for one day. The expression level of *MaERS3* in fruit fumigated with 1-MCP slightly increased after being transferred to 25 °C, particularly for three days (Figure 6A). This result is similar to that of Hong *et al.* (2015) who found that the *MaERS3* gene was up-regulated in William banana ripened after low-temperature storage. The ERS3 protein is located on the endoplasmic reticulum membrane. Low temperature conditions can physically disrupt the physical membrane which can then no longer function (Lyons & Raison, 1970; Wang and Gemma, 1994). In fruit that had been fumigated with 1-MCP, the *MaERS3* gene was up-regulated following eight days of storage and then three days of ripening at 25 °C. However, this up-regulation did not occur following four days of storage and then three days of ripening. It may be bananas produce ERS3 for ethylene receiving but 1-MCP did not enough to bind ethylene receptor. The banana ripening was delayed (Blankenship & Dole, 2003).

The *MaCTR1* gene was down-regulated in bananas treated with exogenous ethylene after storage for four days then up-regulated after transfer to 25 °C for one day and then subsequently down-regulated to an expression level similar to that of the control. Similarly, the expression level of *MaCTR1* in fruit stored for eight days and ripened at 25 °C for one day

was up-regulated. For 1-MCP treated fruit there were no consistent effects of either storage or ripening conditions. However, the expression level of *MaCTR1* was higher following eight days of storage at 7 ℃ followed by ripening for three days at 25 ℃ (Figure 6B). In papaya, the expression level of *CTR2* and CI symptom development during storage at 12 ℃ were both lower than occurred under storage at 7 ℃ and the fruit had normal ripening (Zou *et al.*, 2014). This result suggested that Hom Thong banana treated with ethylene had normal ripening resulting from *MaCTR1* down-regulated. For 1-MCP treatment confirmed that the negative regulator of CTR1. *MaCTR1* gene in banana peel had stable during storage at 7 ℃ because 1-MCP inhibited ethylene perception. The *MaCTR1* gene expression of bananas treated with exogenous ethylene and 1-MCP for long time storage had up-regulated.

The expression levels of *MaEIL2* showed a similar pattern to that of *MaCTR1* on day four after storage and transferred to 25 ℃ for three days. Banana treated with exogenous ethylene and 1-MCP on day eight after storage showed slightly up-regulation *MaEIL2* gene. EIL2 is TF acts as a positive regulator of ethylene signaling, therefore, in the presence of ethylene, EIL2 should be highly accumulated. Our result revealed that the expression of MaEIL2 was down-regulated in banana treated with exogenous ethylene after storage for four days while 1-MCP treatment had up-regulated *MaEIL2* gene expression since day 4+0. This finding was similar to loquat cv. Luoyangqing that was susceptible to CI and had higher the expression of *EIL1* than that of cv. Baisha which was CI shows tolerance (Wang *et al.*, 2010). This report was an evidence that TF involved in CI and abnormal ripening of banana cv. Guangfen No.1. Guangfen No.1 banana stored at 7 ℃ for 12 days where MaNAC67-like protein was suppressed resulting in CI and unable to degrade of starch in pulp (Wang *et al.*, 2010).

The *MaERS3* and *CTR1* genes of untreated banana (control treatment) had a trend to down-regulate during storage at low temperatures. The expression level of all genes increased after transfer to 25 ℃. It was indicated that these genes signal to *MaEIL2*. However, bananas stored at 7 ℃ for eight days, the *MaCTR1* gene down-regulated and then up-regulate after transfer to 25℃ (Figure 7). This result suggested that the *MaEIL2* gene is inconsistent with the *MaCTR1* gene or banana has a gene family of EILs. The other EILs gene is expressed instead of EIL2. The previous study of Yan *et al.* (2011) suggested that the expression of *MaERS3* and *MaEIL1/2* genes was not detected in the pulp of Cavendish banana undergone natural fruit ripening. The present study suggested that exogenous ethylene induced CI tolerance in Hom Thong banana and promoted normal ripening by suppression of the *MaCTR1* gene.

The investigation of ethylene-related gene expressions and CI symptoms of Hom Thong banana focused on banana stored at 7 ℃ for four days (critical point). Hom Thong banana treated with ethylene before storage were CI tolerance by down-regulating *MaCTR1* and *MaEIL2* gene and up-regulating *MaERS3*, *MaACS1*, and *MaACO1* genes during low temperature storage. In contrast, banana fumigated with 1-MCP (inhibit ethylene perception) had severe CI symptoms and abnormal ripening. The expression of*MaERS3* and *MaCTR1* genes was at stable levels during low-temperature storage and then it was slightly up-regulated after being

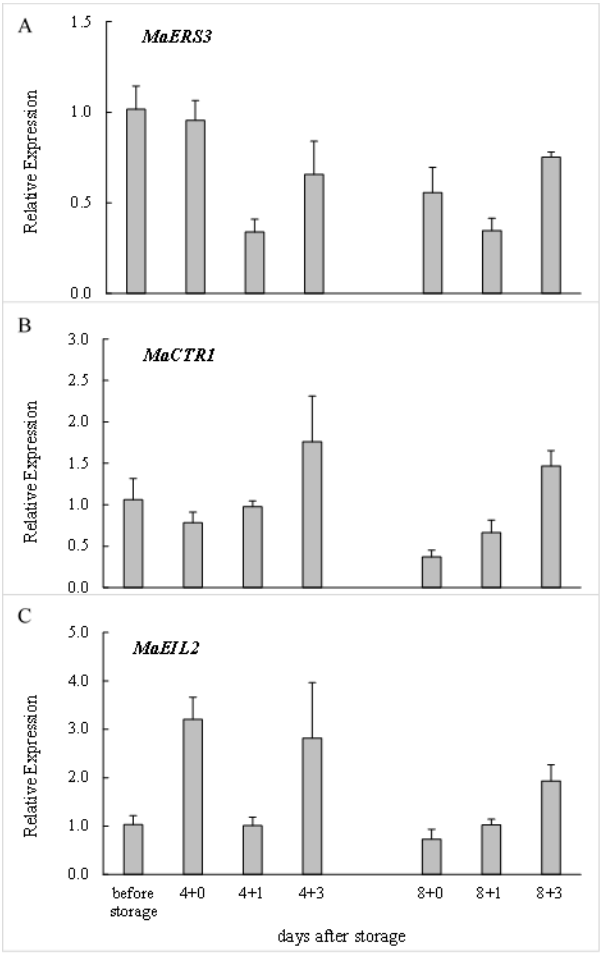

Figure 7. The expression level of *MaERS3* (A), *MaCTR1* (B) and *MaEIL2* (C) of Hom Thong banana in control treatment stored at 7±1℃ for 4 and 8 days (4+0 and 8+0) and transferred to 25±1℃ for 1 and 3 days (4+1, 4+3, 8+1 and 8+3). Each sample represents the mean ±SE of three replicates. Expression levels were expressed as a ratio relative to the harvest (before storage) which was set at 1.

transferred to 25 ℃. The expression level of *MaEIL2* gene was slightly up-regulated. However, the expression of *MaACS1* and *MaACO1* gene was not detected (Figure 8). The present study suggested that exogenous ethylene induces Hom Thong banana tolerance to CI and had normal ripening by repressing the *MaCTR1* gene. Thus, CI of banana fruit stored at low temperature is associated with ethylene action and ethylene signaling.

The peel color of banana fruits indicates fruit ripening and CI symptoms occur on the peel, therefore the gene expression of ethylene biosynthesis and ethylene signaling in the peel of Hom thong banana were studied in this research. However, the study on fruit ripening of Cavendish banana suggested that ethylene biosynthesis may be under a negative feedback regulation mechanism in the pulp and a positive feedback system may operate in the peel (Inaba *et al.*, 2007). In the future works, gene expression of ethylene biosynthesis and ethylene signaling of banana susceptible and tolerant to CI will be studied in both pulp and peel tissues.

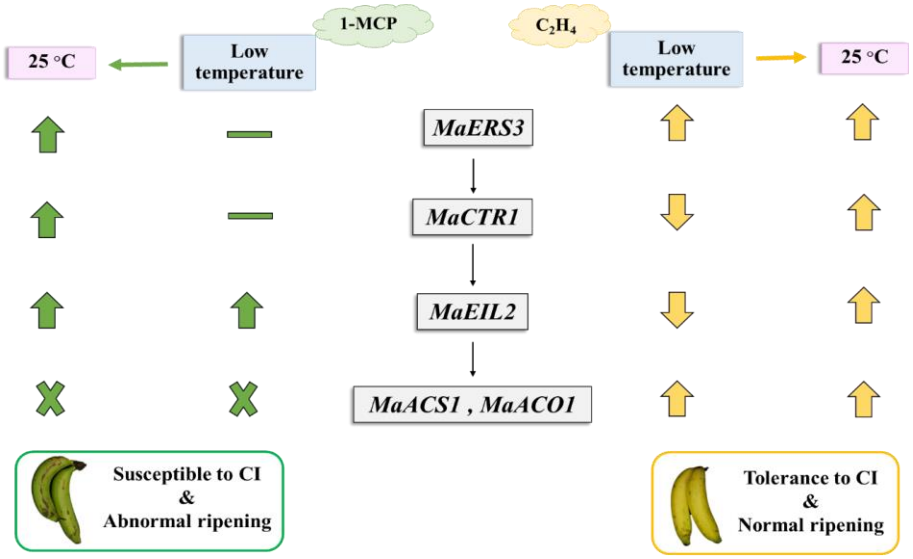

Figure 8. Model of ethylene biosynthesis and ethylene signaling in relation to chilling injury and fruit ripening in Hom thong banana focusing on storage at 7°C for 4 days (low temperature) and transfer to 25°C; green color sign represents banana fumigated with 1-MCP before storage which were susceptible to CI, yellow color sign represents banana treated with exogenous ethylene before storage were CI tolerance, up arrow represents gene up-regulate, down arrow represents gene down-regulate, dash sign represents stability of gene expression and cross sign represents gene were not detected the expression.

4. Conclusions

Exogenous ethylene induced Hom Thong banana tolerance to CI. This was associated with different expression levels of ethylene signaling genes and ethylene biosynthesis genes. For ethylene signaling genes, *MaCTR1* and *MaEIL2* were down-regulated whereas *MaERS3* was up-regulated. For ethylene biosynthesis genes, *MaACS1* and *MaACO1* were up-regulated.

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