



Effects of Ethephon and Abscisic Acid Application on Ripening-Related Genes in 'Kohi' Kiwifruit (*Actinidia chinensis*) on the Vine

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The effects of ethephon and abscisic acid (ABA) application on ripening-related genes of pre-harvest 'Kohi' kiwifruit (*Actinidia chinensis*) were studied to clarify the possibility of the fruit ripening on the vine. The fruits were treated on the vine at mature stage with 250 $\mu\text{L}\cdot\text{L}^{-1}$ ethephon or 100 $\mu\text{mol}\cdot\text{L}^{-1}$ ABA, and the expression levels of chlorophyll synthase (AcCLS), chlorophyllase1 (AcCLH1), polygalacturonase (AcPG), expansin (AcEXP), β -amylase (Ac β -AM), sucrose synthase (AcSUSY), MADS-box SEPALLATA4/RIPENING INHIBITOR-like (AcSEP4/RIN) and FRUITFUL-like (AcTDR4/FUL) genes were analyzed. The expression levels of AcPG, AcEXP, Ac β -AM, and AcSUSY increased in the ethephon-treated fruit, but those of AcCLH1 at 6 and 9 days after treatment and AcCLS decreased. Moreover, the expression levels of AcSEP4/RIN and AcTDR4/FUL, the latter of which is associated with ethylene biosynthesis, were higher in the ethephon-treated fruit. The expression level of each gene in ABA-treated fruit was not significantly different from that of the untreated control. The results suggest that ethephon application increases the expression levels of AcPG, AcEXP, Ac β -AM, AcSUSY, AcSEP4/RIN, and AcTDR4/FUL in 'Kohi' kiwifruit on the vine.

Keywords: kiwifruit; *Actinidia chinensis*; ethephon; ABA; fruit ripening; ethylene

1. Introduction

Fruit ripening involves physiological, biochemical, and structural changes such as cell-wall degradation, pigment synthesis, and increases in both sugar and flavor (Seymour et al., 1933; Mworio et al., 2012). Molecular investigations of fruit development have concentrated mainly on fruit ripening (Gray et al., 1992). It is considered that fruit ripening commences with the synchronized expression of numerous genes, including various MADS-box transcription factors, and functional genes related to the degradation of cell walls, starch, chlorophyll and sucrose accumulation. For example, EXP and PG genes are involved with cell-wall degradation. Expansin influences cell wall degradation by disrupting noncovalent linkages in cellulose–hemicellulose (McQueen and Cosgrove, 1994). The AcPG gene is related to the depolymeriza-

tion and solubilization of the pectic backbones of cell-wall polysaccharides in kiwifruit (*Actinidia chinensis*) in the ripening stage (Wang et al., 2000).

Furthermore, β -AM and SUSY genes are soluble sugar-related genes. β -amylase breaks starch into maltose during fruit ripening, resulting in a sweet flavor (Robyt and Whelan, 1968). Ma β -AM expression was strongly induced during the ripening of banana (*Musa acuminata*), and the increase in Ma β -AM expression levels was significantly correlated to the degradation of starch (Nascimento et al., 2006). Sucrose synthase (SUSY gene) catalyzes the reversible conversion of sucrose and a nucleoside diphosphate into the corresponding nucleoside diphosphate glucose and fructose (Edurne et al., 2012). Sucrose synthase plays an important role in sucrose accumulation in pear (*Pyrus pyrifolia*) (Zhang et al., 2014). The expression levels of the CuSUSY gene

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increased during fruit ripening in satsuma mandarin (*Citrus unshiu*) (Komatsu et al., 2002). In addition, *CLS* and *CHL* genes are chlorophyll related gene. Chlorophyll synthase (*CLS* gene) is a key enzyme in chlorophyll biosynthesis, and chlorophyllase (*CHL* gene) is a key enzyme in chlorophyll degradation. Finally, MADS-box transcription factors play important roles in the regulation of vegetative growth, flowering, floral organ development, seed development, senescence, fruit ripening, and organ abscission in plants (Smaczniak et al., 2012). MADS-box in kiwifruit includes *SEP/RIN*, *FRUITFUL TDR4/FUL*, *AGAMOUS (AG)-like TAGL1*, and *APETALA3 (AP3)*, all of which are associated with ethylene biosynthesis and ripening (McAtee et al., 2015).

Kiwifruit has two important market species: *Actinidia deliciosa* and *Actinidia chinensis*. In general, ethylene production in *A. chinensis* is higher than that in *A. deliciosa* in the ripening stage (Asiche et al., 2016). Ethylene and ABA are important plant hormones in the processes of fruit ripening. Ethephon is a widely used chemical replacing ethylene treatment when ethylene is not available and ethephon application accelerated ripening in kiwifruit after harvest (Mworio et al., 2010). In addition, ABA may also be associated with kiwifruit ripening, because it stimulated banana fruit ripening (*Musa sapientum*) (Jiang et al., 2000).

However, the effects of ethylene and ABA application on kiwifruit on the vine are not clear. It is considered that the ripening of kiwifruit on the vine may be more marketable. In this study, the effects of ethephon and ABA application on the ripening-related genes such as *AcPG*, *AcEXP*, *Acβ-AM*, *AcSUSY*, *AcCLS*, *AcCLH1*, *AcSEP4/RIN*, and *AcTDR4/FUL* in 'Kohi' kiwifruit on the vine were studied.

2. Materials and methods

2.1. Plant material

Three-year-old 'Kohi' kiwifruit (*A. chinensis*) vines top-grafted on 'Hayward' kiwifruit (*A. deliciosa*) vines were used in the experiment in 2014. The vines were grown in an open field at Chiba University, located at 35°N latitude, 140°E longitude, and at an elevation of 37 m. Three hundred fruits from the vines (100 fruits per vine) were randomly divided into 3 groups (100 fruits per group) at mature stage (155 days after full bloom during the commercial harvest period for general ripening in the room following harvest, and soluble solids content was 10%–11% on the vine). In the first group, the fruits were dipped into 250 μL·L⁻¹ ethephon solution with ethanol with a 5% concentration for 1 min on the vine. The fruits in the second group were dipped into 100 μmol·L⁻¹ ABA solution with 5% ethanol similarly with ethephon treatment. In the third group, the fruits were dipped into 5% ethanol as an untreated control. The concentrations of ethephon and ABA that were used in our study were effective to promote the ripening in some fruits (Abeles et al., 1992). Twenty fruits from each group were sampled at 0, 3, 6, 9, and 12 days after treatment (DAT). The pulp was sampled and frozen by liquid N₂ at -80 °C for the analysis of gene expression.

2.2. RNA extraction, cDNA synthesis, and quantitative real time RT-PCR (qRT-PCR) analysis

Total RNA was extracted from a sample (500 mg FW; 3 replications) with a modification of the MagExtractor PCR & Gel

Table 1 Primers used for quantitative real-time RT-PCR

Gene	Forward/Reverse primer (5'–3')	Reference
<i>AcSEP4/RIN</i>	F: GAGGCTCAAGACAAGGGTTG R: AAGCTGCTCAAGCTCCTTTG	HQ113364.1 (Varkonyi-Gasic et al., 2011)
<i>AcTDR4/FUL</i>	F: GGAGAGTGCAGCTGAAGAGG R: AGATCTCGCGAGCTTTCTTG	Achn247791 (Huang et al., 2013)
<i>AcCLH1</i>	F: TGTAAGCCACCGTGTGGTA R: GTCAGCGTCTCCTCCATA	Achn035481 (Huang et al., 2013)
<i>AcCLS</i>	F: GGCCCGTGTCTTACTGGATA R: CCTGACGGAATAGGACGGTA	Achn001951 (Huang et al., 2013)
<i>Acβ-AM</i>	F: GAAACCCTCTCTGGGGACTC R: CACGATCCTCCATGTTCTT	Achn387071 (Huang et al., 2013)
<i>AcSUSY</i>	F: GTGTACGGGACCATCGATT R: TCTGGTTCTCGTTCGGTTTC	Achn064451 (Huang et al., 2013)
<i>AcPG</i>	F: GCAGAATGCCTGACTTCCTC R: CAAAGCTCGGAATGAAGGAG	Achn144321 (Huang et al., 2013)
<i>AcEXP</i>	F: TCCTGCTTTGAGCTGATGTG R: GCACCAGATGTCTTGGGTCT	Achn194511 (Huang et al., 2013)
<i>Elongation factor-1α</i>	F: GCACTGTCATTGATGCTCCT R: CCAGCTTCAAACCACCAGT	Nieuwenhuizen et al., 2009

Clean Up (Toyobo, Osaka, Japan) method reported by Vogelstein and Gillespie (1979). cDNA synthesis for *AcPG*, *AcEXP*, *Acβ-AM*, *AcSUSY*, *AcCLS*, *AcCLH1*, *AcSEP4/RIN*, and *AcTDR4/FUL* genes was performed according to the instruction manual for ReverTra Ace[®] qPCR RT Master Mix (FSQ-201, Toyobo). Quantitative RT-PCR (Step One Plus, Life Technologies, Tokyo, Japan) was performed using a KAPA SYBR FAST Master Mix (Kapa Biosystems, Boston, MA, USA) according to the instruction manual. Gene-specific primers for each gene (Table 1) were used for qRT-PCR analysis. The expression level of each gene (3 replications) was determined by a comparative 2^{-ΔΔCT} method. The expression level was normalized to that of the *Elongation factor-1α* gene (Nieuwenhuizen et al., 2009).

2.3. Statistical analysis

Data (presented as means ± SE of 3 replications) were subjected to analysis of variance procedures and separated by the Tukey–Kramer test at $P \leq 0.05$ using the SAS statistical analysis package (SAS Institute, Cary, NC, USA).

3. Gene expression analysis

3.1. *AcPG* and *AcEXP*

In the ethephon-treated fruit, the expression of *AcPG* was significantly high at 9 and 12 DAT (Fig. 1, A), while that of *AcEXP* was significantly high at 12 DAT (Fig. 1, B). The ABA-treated fruit showed no significant difference with the control.

3.2. *Acβ-AM* and *AcSUSY*

The expression levels of *Acβ-AM* and *AcSUSY* were significantly high in the ethephon-treated fruit after treatment (Fig. 2). In the ABA-treated fruit, the expression levels of *Acβ-AM* showed no significant difference except for 12 DAT (Fig. 2, A). The expression levels of *AcSUSY* in ABA-treated fruit were lower than the control at 12 DAT, but were higher at 6 DAT (Fig. 2, B).

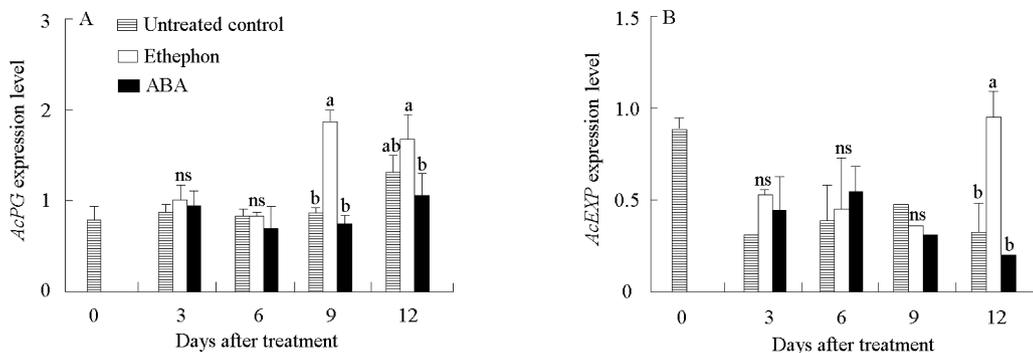


Fig. 1 The expression levels of *AcPG* and *AcEXP* after ethephon and ABA applications in 'Kohi' kiwifruit

Different letters show significant differences ($P \leq 0.05$) by the Tukey–Kramer test on each date after treatment and 'ns' indicates an insignificant difference. Data are the means \pm SE of three replications.

3.3. *AcCLS* and *AcCLH1*

The expression levels of *AcCLS* and *AcCLH1* were shown in Fig. 3. *AcCLS* expression decreased significantly from 6 to 12 DAT in the ethephon-treated fruit compared with the control (Fig. 3, A). The expression levels of *AcCLH1* increased at 12 DAT, although they decreased at 6 and 9 DAT (Fig. 3, B). The ABA-treated fruit showed no significant difference with the control on the expression levels of *AcCLS* (Fig. 3, A). *AcCLH1* expres-

sion levels in ABA-treated fruit decreased at 3 and 9 DAT, but increased at 12 DAT (Fig. 3, B).

3.4. *AcSEP4/RIN* and *AcTDR4/FUL*

The expression levels of MADS-box genes *AcSEP4/RIN* and *AcTDR4/FUL* fluctuated after treatment (Fig. 4). The expression levels of *AcSEP4/RIN* and *AcTDR4/FUL* at 3 and 9 DAT were higher in the ethephon-treated fruit than in other treatments.

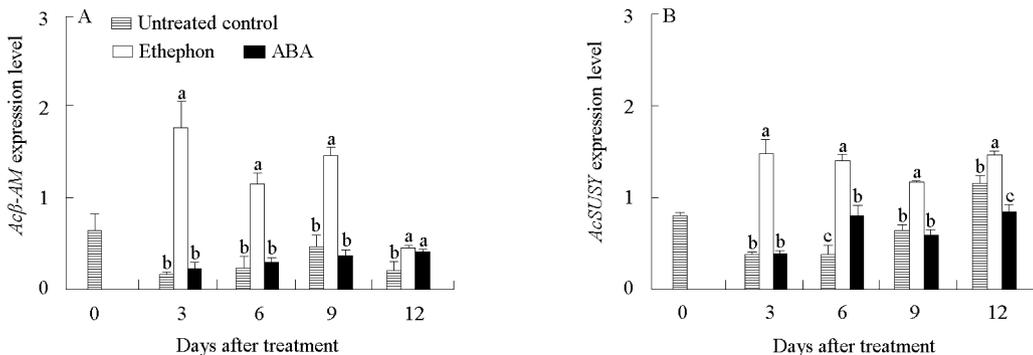


Fig. 2 The expression levels of *Acβ-AM* and *AcSUSY* after ethephon and ABA applications in 'Kohi' kiwifruit

Different letters show significant differences ($P \leq 0.05$) by the Tukey–Kramer test on each date after treatment. Data are the means \pm SE of three replications.

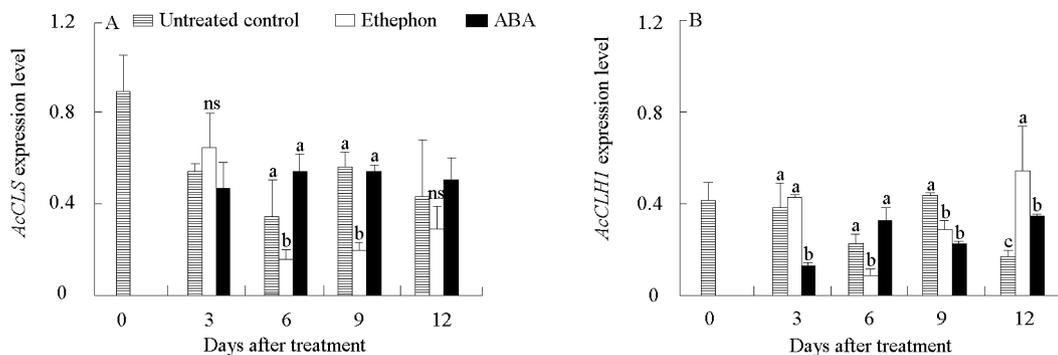


Fig. 3 The expression levels of *AcCLS* and *AcCLH1* after ethephon and ABA applications in 'Kohi' kiwifruit

Different letters show significant differences ($P \leq 0.05$) by the Tukey–Kramer test on each date after treatment and 'ns' indicates an insignificant difference. Data are the means \pm SE of three replications.

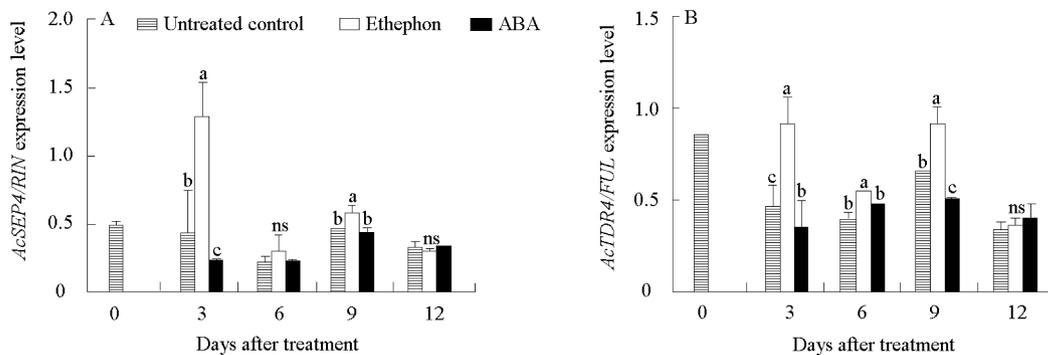


Fig. 4 The expression levels of *AcSEP4/RIN* and *AcTDR4/FUL* after ethephon and ABA applications in 'Kohi' kiwifruit. Different letters show significant differences ($P \leq 0.05$) by the Tukey–Kramer test on each date after treatment and 'ns' indicates an insignificant difference. Data are the means \pm SE of three replications.

4. Discussion

The ethylene signal is known as the trigger that induces fruit softening, color change, and flavor in climacteric fruit (Hua and Meyerowitz, 1998). Our previous report showed that ethephon application significantly increased the expression levels of *AcACS1* and *AcACO1*, the activities of ACS and ACO, and the production of ACC and ethylene in 'Kohi' kiwifruit on the vine (Kongsuwan et al., 2016). In our study, ethephon application significantly increased the expression levels of *AcEXP* and *AcPG* in kiwifruit on the vine. Kongsuwan et al. (2016) observed that ethephon application significantly encouraged fruit softening in 'Kohi' kiwifruit on the vine. Therefore, these results suggest that ethephon application may induce fruit softening through *AcEXP* and *AcPG*. A previous study also showed that *AcEXP* and *AcPG* respond quickly to propylene application in 'Sanuki Gold' kiwifruit (*A. chinensis*) (Mworira et al., 2012). Brummell et al. (1999) suggested that the expression of *SLEXP1* is involved in the softening of tomato (*Solanum lycopersicum*) fruit. Wang et al. (2000) reported that a high expression level of *AcPG* positively correlated with softening in kiwifruit (*A. chinensis*).

Chlorophyllase activity is tightly associated with chlorophyll breakdown during fruit ripening, and overexpression of *ClCLH* enhances chlorophyll breakdown in lemon (*Citrus limon*) (Shemer et al., 2008). In our study, the expression levels of *AcCLS* were significantly lower in the ethephon-treated fruit than in the other treatments from 6 to 12 DAT. In contrast, the expression levels of *AcCLH1* at 12 DAT in the ethephon-treated fruit were significantly higher than those in the other treatments. This result suggests that ethephon application induced the expression of a chlorophyll degradation gene in 'Kohi' kiwifruit on the vine. Zhang et al. (2012) showed that ethephon application increased chlorophyllase activity in kiwifruit (*A. deliciosa*).

Ethephon treatment increased soluble solids content in 'Kohi' kiwifruit on the vine (Kongsuwan et al., 2016). The expression levels of *Acβ-AM* and *AcSUSY* were significantly high in the ethephon-treated fruit. Our study supports the result of McAtee et al. (2015) that propylene applications increased the expression of *Acβ-AM* and *AcSUSY* in kiwifruit (*A. chinensis*). Peroni et al. (2008) reported that the expression of *Miβ-AM*, which is involved in starch degradation during ripening in mango (*Mangifera indica*), was low during fruit development but increased gradually throughout ripening. Komatsu et al. (2002) suggested that sucrose synthase had important roles on sugar accumulation in satsuma mandarin fruit.

The expression levels of *AcSEP4/RIN* and *AcTDR4/FUL*, which are associated with ethylene biosynthesis in kiwifruit (McAtee et al., 2015), were significantly high in the ethephon-treated fruit in our study, while the expression levels of *AcTDR4/FUL* and *AcPG* increased at 9 DAT in the ethephon-treated fruit. The increase in *AcTDR4/FUL* coincided with the upregulation of *AcPE* and *AcEXP*. This finding may reveal a close relationship between MADS-box genes and genes involved in cell-wall degradation (McAtee et al., 2015). Fujisawa et al. (2011) suggested that *SISEP4/RIN* regulates fruit softening and ethylene production by the direct transcriptional regulation of cell-wall-modifying genes and ethylene biosynthesis genes during ripening in tomato (*S. lycopersicum*).

ABA is an important factor in the fruit ripening process and promoting ethylene biosynthesis in fruits such as apple (Kongsuwan et al., 2012), banana (Jiang et al., 2000), mango (Zaharah et al., 2013) and tomato (Zhang et al., 2009). In our study, ABA application did not significantly influence the ripening-related genes in 'Kohi' kiwifruit on vine. However, the expression levels of *Acβ-AM*, *AcSUSY*, and *AcCLH1* differed temporarily. Therefore, the ripening-related genes in 'Kohi' kiwifruit may be regulated primarily by ethylene rather than ABA. Seta (2012) suggested that the effects of ABA on fruit ripening may vary depending on fruit species, stage of fruit, time for application and the concentration of ABA.

In conclusion, ethephon application to kiwifruit on the vine could increase the expression levels of *AcPG*, *AcEXP*, *Acβ-AM*, *AcSUSY*, *AcSEP4/RIN*, and *AcTDR4/FUL*, which may be related to the ripening of 'Kohi' kiwifruit.

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