Study on the kiwifruit (*Actinidia chinensis*)

ripening on the vine

January 2017

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千葉大学学位申請論文

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LIST OF ABBREVIATIONS

Ac	*Actinidia chinensis*
β-AM	β-amylase
ABA	abscisic acid
ACC	1-aminocyclopropane-1-carboxylate
ACO	1-aminocyclopropane-1-carboxylate oxidase
ACS	1-aminocyclopropane-1-carboxylate synthase
AP3	APETALA3
cDNA	complementary deoxyribonucleic acid
CLH	chlorophyllase
CLS	chlorophyll synthase
DAFB	days after full bloom
DAT	days after treatment
DNA	deoxyribonucleic acid
EXP	expansin
FW	fresh weight
FUL/TDR4	FRUITFUL-like
GC-MS-SIM	gas chromatography-mass spectrometry-selected ion monitoring
HPLC	high performance liquid chromatography
NDGA	nordihydroguaiaretic acid
NCED	9-*cis*-epoxycarotenoid dioxygenase
PCR	polymerase chain reaction

PG	polygalacturonase
RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
SE	standard error
SPME	solid-phase microextraction
SUSY	sucrose synthase
SSC	soluble solids concentrations
SEP4/RIN	SEPALLATA4/RIPENING INHIBITOR-like

CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

1.1.1 Introduction

Ripening characteristic of fruits is divided into two groups including climacteric and non-climacteric fruits. In the climacteric fruit, ripening is accompanied by respiration peak and concomitant burst of ethylene. The non-climacteric, respiration shows no dramatic change and ethylene production remains at very low level.

Kiwifruit is classified as a climacteric fruit because it is ripened in response to exogenous ethylene, and its ripening is characterized by a period of autocatalytic ethylene production (Park and Kim 1995). The ripening of kiwifruit appears to be different from that of other typical climacteric fruit because fruit produces little ethylene while on the vine (Patterson et al., 2003). However, kiwifruit ripens with ethylene treatment after harvesting (Mworia et al., 2010; Sfakiotakis et al., 1997).

If kiwifruit can be ripened on the vine, the fruit could be more marketable. Ethephon has been applied to both *A. deliciosa* and *A. chinensis* kiwifruit commercially to accelerate ripening after harvest (Mworia et al., 2010; Park et al., 2006; Pranamornkith et al. 2012; Zhang et al., 2012). ABA application induced *MdACS1* and *MdACO1* gene accumulation and increased ACS and ACO activities, ACC concentration in 'Tsugaru' apple (Kongsuwan et al., 2011). NDGA is an ideal inhibitor of NCED enzyme and blocks ABA biosynthesis (Zhang et al., 2009b). NDGA was treated to clarify the roles of ABA in kiwifruit ripening. Fruit ripening involves physiological, biochemical, and structural changes such as cell wall degradation, pigment synthesis and the increase of sugar and flavor (Mworia et al., 2012; Seymour et al., 1993). Gray et al. (1992) suggested that molecular investigations of fruit development have mainly concentrated on fruit ripening. However, the interaction

between ABA and ethylene biosynthesis and ripening-associated genes in 'Kohi' (*A. chinensis*) kiwifruit on the vine are not yet been clarified.

1.1.2 Objectives

1.1.2.1 To study of the possibility of the fruit ripening on the vine and the effects of ethylene or ABA on the ripening of 'Kohi' kiwifruit on the vine

1.1.2.2 To study of the effects of ethephon or ABA application on the ripening-related genes in 'Kohi' kiwifruit on the vine

1.1.3 Scope of studies

1.1.3.1 ABA treatment at the 100 µmol/L or ethephon treatment at 250 µL/L or NDGA at the 100 µmol/L effected on the activities of ACS and ACO, ACC concentration and ethylene production in 'Kohi' kiwifruit on the vine

1.1.3.2 Isolation of *AcNCED1* gene of 'Kohi' kiwifruit which is a key enzyme for abscisic acid biosynthesis pathway and study of the role of ABA and the expression of *AcNCED1* gene on ethylene biosynthesis in 'Kohi' kiwifruit on the vine

1.1.3.3 The effects of ethephon or ABA application on the ripening-related genes including *MADS* box transcription factor gene (*AcSEP4/RIN* and *AcTDR4/FUL*), cell wall related gene (*AcEXP* and *AcPG*), sugar related gene (*Acβ-AM* and *AcSUSY*) and chlorophyll related gene (*AcCLS* and *AcCLH1*) in 'Kohi' kiwifruit on the vine

1.1.4 Expected Outcomes

1.1.4.1 The ripening of 'Kohi' kiwifruit on the vine

1.1.4.2 To clarify the relationship between ABA and ethylene biosynthesis in 'Kohi' kiwifruit on the vine

1.1.4.3 To understand the effects of ethephon or ABA application on the ripening-related genes in 'Kohi' kiwifruit on the vine

1.2 LITERATURE REVIEW

1.2.1 Kiwifruit

1.2.1.1 Fruit production

Kiwifruit was produced more than 1.37 million tons in the top 10 producing countries in the world in 2014 (Table 1). Italy has the largest production of kiwifruit in the world, followed by New Zealand and Chile. Japan has the production of kiwifruit about 28,000 tons.

Table 1.1 Top 10 Countries of kiwifruit production in 2014.

Ranking	Countries	Production (Tons)	Percentage (%)
1	Italy	384,844	27.96
2	New Zealand	376,400	27.34
3	Chile	240,000	17.44
4	Greece	161,400	11.73
5	France	65,253	4.74
6	Turkey	36,781	2.67
7	Iran	32,000	2.32
8	Japan	28,000	2.03
9	United States	26,853	1.95
10	Portugal	25,000	1.82
Total		1,376,531	100

Source: FAOStat.org (Tons)

1.2.1.2. Cultivar of kiwifruit

Kiwifruit is native in Southeast Asia. There is more than 50 species in the genus *Actinidia,* and many have commercial potential. The two economically important species are *Actinidia deliciosa* and *Actinidia chinensis*. *A. deliciosa* is the species widely known around the world as kiwifruit and is commercially grown in many countries.

A. deliciosa, including cultivars such as 'Hayward', 'Allison', 'Bruno', and 'Skelton' are widely known as large fruit size, green flesh, and long storage life (Thompson et al., 2000*). A. chinensis*, is the second important species, including 'Hort 16A', 'Sanuki Gold', and 'Kohi', which has yellow flesh, high soluble solids, and low organic acid concentrations, but short storage life (Xu et al., 1998, 2000).

1.2.1.3 Structure of fruit

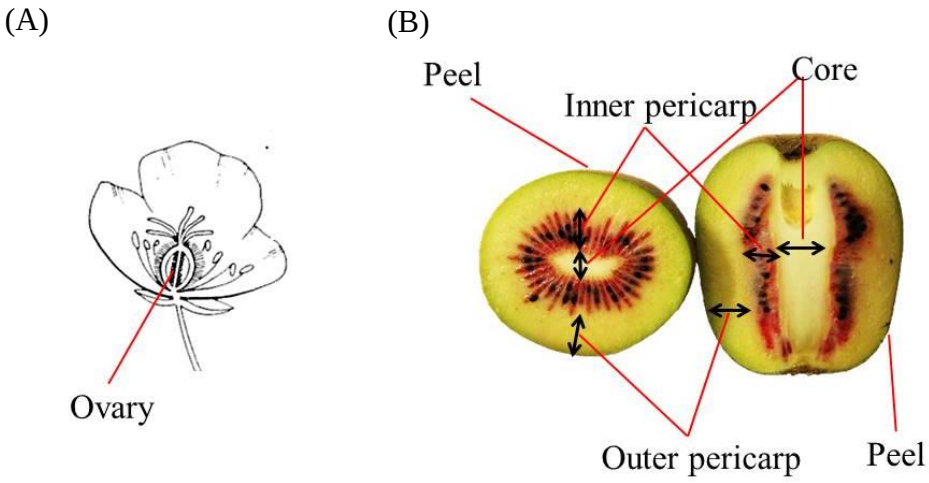

Figure 1.1 (A) Kiwifruit female flower and (B) structure of 'Kohi' (*A.chinensis*) fruit.

The kiwifruit female flower is shown in Figure 1.1(A). Fruit develops from ovary and becomes the three main structures including core, inner pericarp, and outer pericarp. The inner pericarp surrounds the seed and the outer pericarp accumulates starch. The structure of 'Kohi' (*A. chinensis*) fruit is shown in Figure 1.1(B).

1.2.1.4 Stage of fruit development

The growth stages for 'Hort16A' (*Actinidia chinensis)* are shown in Table 1.2 following by the Biologische Bundesantalt, Bundessortenamt und Chemische Industrie (BBCH) scale, which describes phenological changes in plant growth and fruit development (Richardson et al., 2011). The growth stages of kiwifruit from stage 65 to 90 described by BBCH. At stage 65; the fully opened flower, stage 70; fruit set, stage 80; fruit mature, stage 89; the fruit is 'eating ripe' and stage 90; fruit senescence.

Table 1.2 Growth stages for *Actinidia chinensis* 'Hort16A' (Richardson et al., 2011).

Stage	**BBCH description**	**BBCH *Actinidia chinensis* 'Hort16A'**	**Days after anthesis**
65	Fully open flowers	Fully open flower	0
70	Fruit set	Fruit set; petals have abscised, fruit start to growth	10
75	50% fruit growth	Fruit reached 50% final weight	60
80	Fruit mature	Mature fruit; seeds 95% black; outer pericarp starts to change color	155
87	Softening starts	Start of flesh softening	210
89	Eating ripe	Fruit softening; fruit firmness ≤10 N	237
90	Plant senescence	Production of autocatalytic ethylene	270-285

1.2.1.5 Postharvest fruit ripening

There are 4 phases in kiwifruit ripening after harvest (Fig. 1.2). Fruit in phase 1 does not produce endogenous ethylene but are highly sensitive to the application of exogenous ethylene after harvest. Fruit in phase 2 shows rapid softening. Fruit in phase 3 starts with the onset of endogenous 'autocatalytic' ethylene production, and fruit become to the eating-ripe window for consumers, fruit softening and production of aroma volatiles. Fruit in phase 4 is unacceptably soft and often exhibit 'off flavor'

notes. The duration of the softening phases depends on species, environmental conditions, and harvest time (early or late season). Application of exogenous ethylene in phase 1 accelerates and synchronizes fruit ripening in phases 1 and 2.

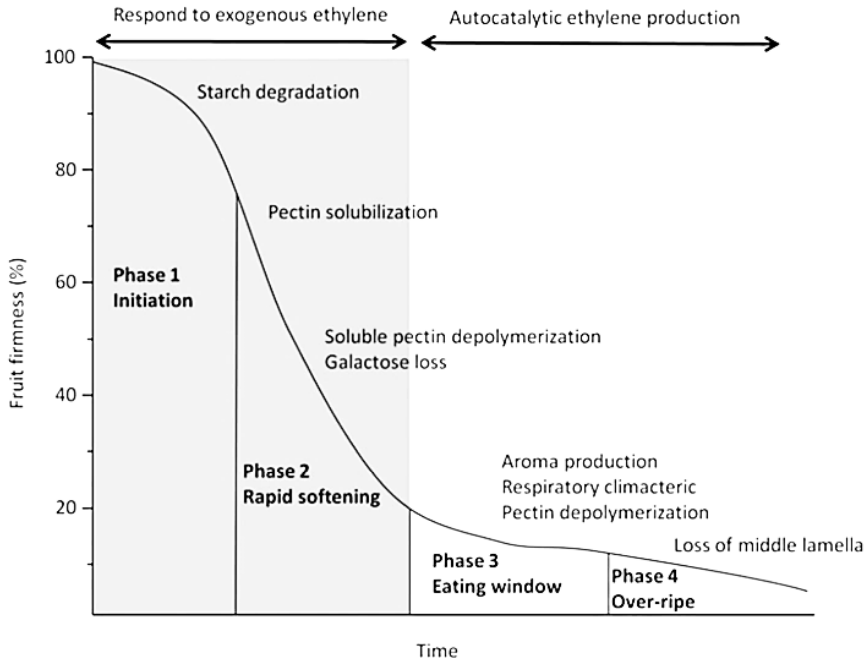

Figure 1.2 Schematic representation of postharvest kiwifruit ripening in relation to the timing of key physiological events (1-4 are softening phases) based on Schroder and Atkinson (2006).

1.2.2 Abscisic acid (ABA)

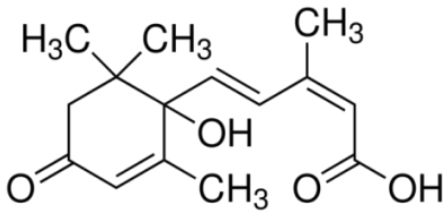

Figure 1.3 Chemical structure of ABA.

1.2.2.1 ABA biosynthesis

ABA is a plant hormone, which is involved in various physiological processes in plant including plant growth, seed development and dormancy, plant responses to various environmental stresses and fruit ripening (Kondo et al., 2002; Zeevaart and Creelman 1988). ABA is derived from zeaxanthin. The 9-*cis*-violaxanthin and 9'-cisneoxanthin changed to form xanthoxin by 9-*cis*-epoxycarotenoid dioxigenase (NCED), and then converted to ABA as shown in Figure 1.4 (modified from RIKEN Plant Hormone Research Network, 2010).

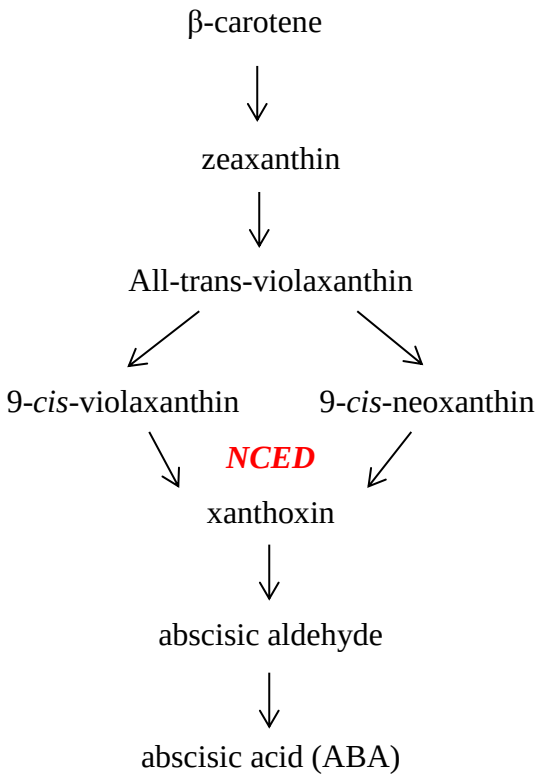

Figure 1.4 Abscisic acid biosynthesis (NCED: 9-*cis*-epoxycarotenoid dioxigenase).

1.2.2.2 ABA and fruit ripening

ABA is an important factor in the processes of fruit ripening and promotes ethylene biosynthesis in wide range of fruits (Table 1.3). Biochemical and genetic studies have suggested that *NCED* is the key enzyme in the ABA biosynthetic pathway in plants. Zhang et al. (2009b) reported that ABA triggers ethylene biosynthesis by regulating the expression of *ACS* and *ACO* genes during the fruit ripening process. ABA application increased ethylene biosynthesis in 'Tsuguru' apple fruit at climacteric stage after harvest (Kongsuwan et al., 2012). The expression *NCED1* in peaches and grapes are initiated by ABA biosynthesis at the onset of fruit ripening suggesting *NCED* genes were expressed at the beginning of maturation, and then ABA began to accumulate (Zhang et al., 2009a).

Table 1.3 The effects of ABA on fruit ripening.

Fruit	Effect of ABA application	Reference
Apple	Increase of jasmonates (JA) concentration	Kondo et al., 2001
	Increase of ACS and ACO activities and ACC concentration	Kongsuwan et al., 2012
	Induce of *MdACS1* and *MdACO1* accumulation	
Banana	Stimulation of activities of pectin methyl esterase (PME), pectate lyase (PL) and cellulase	Lohani et al., 2004
Sweet cherry	Enhance of the total sugar and anthocyanin	Kondo et al., 1993
Grape	Promotion of ABA concentration, ripening and softening	Zhang et al., 2009a
Kiwifruit	ABA content in hardy kiwifruits follows almost the same trend of change with ethylene, respiratory intensity, pectase and amylase	Shuqian et al.,2014
Litchi	Promotion of anthocyanin synthesis	Wang et al., 2007
Mango	Promotion of the activities of ACS and ACO	Zaharah et al., 2013
	Accumulation of ACC concentration	
	Enhance of fruit softening and activity of *endo*-polygalacturonase and reduce pectin esterase activity	
Peach	Promotion of ABA concentration, ripening and softening	Zhang et al., 2009a
Strawberry	Acceleration of fruit color and softening	Jiang and Joyce, 2003
	Stimulation of ethylene production	
	Acceleration of anthocyanin, phenolic content and phenylalanine ammonia-lyase (PAL) activity	
Tomato	Increase of the ABA content in both flesh and seed	Zhang et al., 2009b
	Increase of expression of *ACS* and *ACO* genes	
	Promotion of ethylene synthesis and fruit ripening	

1.2.2.3 Nordihydroguaiaretic acid (NDGA)

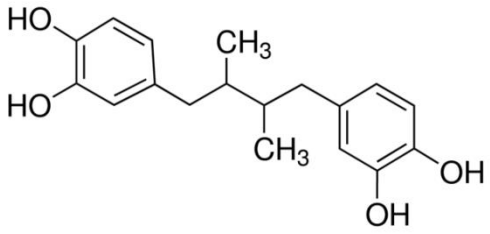

Figure 1.5 Chemical structure of NDGA.

Nordihydroguaiaretic acid (NDGA) is an ideal inhibitor of the NCED enzyme with regard to its permeating speed and can block ABA biosynthesis. Zhang et al., (2009b) reported that 100 µM NDGA inhibited ABA accumulation in mature green tomato and suggested that inhibition of ABA synthesis by NDGA suppressed ethylene production and delayed fruit ripening.

1.2.3 Ethylene

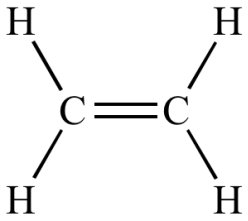

Figure 1.6 Chemical structure of ethylene.

1.2.3.1 Ethylene biosynthesis

Ethylene is involved in several physiological and developmental processes in higher plants, including ripening of fruit, abscission of organs and tissues, senescence, wound response as well as in other abiotic stress (Yang and Hoffman, 1984). Ethylene is synthesized from methionine in three steps. The first step, conversion of methionine to S-adenosyl-L-methionine (SAM) catalyzed by the enzyme SAM synthetase; the second step, formation of 1-aminocyclopropane-1-carboxylic acid (ACC) from SAM

via ACC synthase activity; and the last step, the conversion of ACC to ethylene, which is catalyzed by ACC oxidase shown in Figure 1.7 (modified from RIKEN Plant Hormone Research Network 2010).

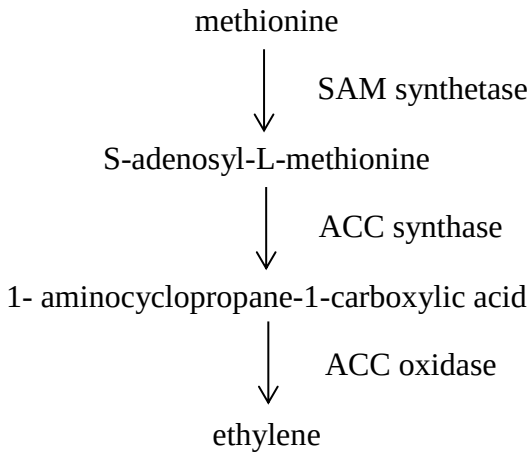

Figure 1.7 Ethylene biosynthesis.

1.2.3.2 Ethylene and fruit ripening

The ripening of fruit is divided into two groups including climacteric and non-climacteric fruits. In climacteric fruit, ripening is accompanied by a peak in respiration and a concomitant burst of ethylene. Non-climacteric, respiration shows no dramatic change and ethylene production remains at a very low level. There are two systems of ethylene production have been defined in plants including ethylene system I and II. Ethylene system I functions during normal growth, development and during stress responses. Ethylene system I is auto inhibitory such as exogenous ethylene which inhibits synthesis, and inhibitors of ethylene action can stimulate ethylene production. Ethylene system II operates during floral senescence, fruit ripening and fruit senescence. Ethylene system II is stimulated by ethylene and is therefore autocatalytic, and inhibitors of ethylene action inhibit ethylene production (Alexander et al., 2002).

1.2.3.3. Regulation by ethylene of *ACO* and *ACS* genes expression

In ripening climacteric fruits, the ethylene biosynthesis enzymes 1-amino-cyclopropane carboxylic acid oxidase (ACO) and 1-amino-cyclopropane carboxylate synthase (ACS) are induced and contribute to the regulation of ethylene biosynthesis (Yang and Hoffman, 1984). The level of *ACS* and *ACO* transcripts are increased at the onset of ripening and in response to exogenously applied ethylene. The *ACS1* and *ACO1* genes involve in fruit ripening such as apple (Kongsuwan et al., 2011), banana (Liu et.al., 1999), kiwifruit (Mworia et al., 2010), peach (Cin et al., 2006), and tomato (Nakatsuka et al., 1997).

1.2.3.4. Ethephon

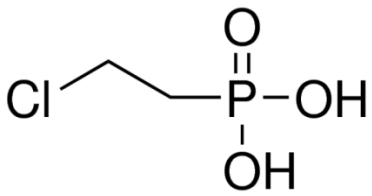

Figure 1.8 Chemical structure of ethephon.

Ethephon or 2-chloroethylphosphonic acid is the most widely used as plant growth regulator. Ethephon is commercially applied to accelerate of climacteric fruit and promote color development of fruit. The effects of ethephon on fruit are shown in Table 1.4.

Table 1.4 The effects of ethephon on fruit ripening.

Fruit	Effect of ethephon application	Reference
Kiwifruit	Acceleration of CO_2 production, SSC accumulation and total phenolic content, decrease firmness and TA	Zhang et al., 2012
Banana	Faster and uniform ripening with development of color and consumer acceptability	Mahajan et al 2010
Peach	Induce of ACO expression and abscisic acid stress ripening-like protein (ASR)	Zhang et al., 2012
Broccoli	Acceleration of rates of chlorophyll degradation	Costa et al., 2005
Mango	Acceleration of ripening	Sergent et al., 1993
Guava	Decrease of acid content during ripening and storage	Mahajan et al., 2008
Pear	Acceleration of fruit ripening and decrease acidity Improve of quality of fruit ripening	Dhillon et al., 2011
Blueberry	Stimulation of anthocyanin accumulation and fruit softening.	Ban et al., 2007
Tomato	Increase of SSC and red color	Logendra et al., 2004
Apple	Increase of ACC and ethylene Enhancement of red peel color and an increase in concentration of flavonoid compounds. Anthocyanin, proanthocyanidins and flavonols	Li et al., 2002

1.2.4 Physiology change during fruit ripening

Fruit ripening is the composite of the processes occurring from latter stages of growth and development through the early stages of senescence. Fruit ripening involves physiological, biochemical, and structural changes such as cell wall degradation, pigment synthesis, and the increase of sugar and flavor (Mworia et al., 2012; Seymour et al., 1993). The compositional changes accompanying fruit ripening are shown in Figure 1.9. Ethylene production, respiration, sugar, color changes to red or yellow, soluble pectin, and polymerized tannin increase with fruit ripening. In contrast, chlorophyll loses acidity, starches, flesh firmness, insoluble pectin, and unpolymerized tannins decrease with fruit ripening (Inoue, 2010). These changes occur simultaneously

and are caused to synchronized expression of numerous genes at the onset of ripening (Fujisawa et al., 2011).

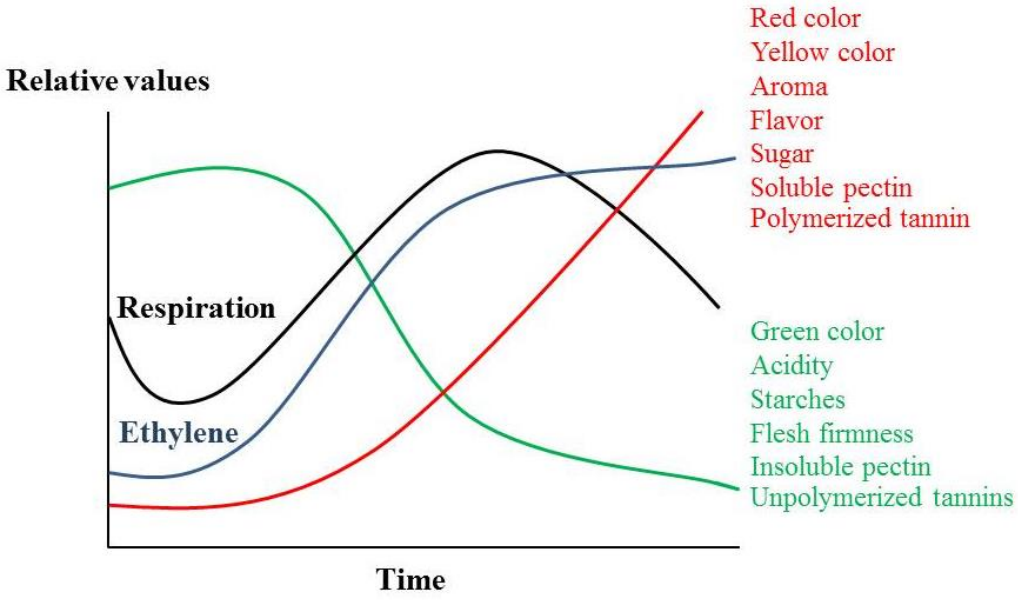

Figure 1.9 The compositional changes accompanying fruit ripening.

1.2.5 Ripening-associated gene

Fruit ripening involves physiological, biochemical, and structural changes such as cell-wall degradation, pigment synthesis, and increases in both sugar and flavor (Mworia et al., 2012; Seymour et al., 1993). These changes occur simultaneously and caused by the synchronized expression of numerous genes at the onset of ripening (Fujisawa et al., 2011). Molecular investigations of fruit development have concentrated mainly on fruit ripening (Gray et al., 1992). Ripening associate gene and function is shown in Table 1.5.

Table 1.5 Ripening-associated gene and function.

Gene related	Gene name	Function on plant	Reference
Abscisic acid	*NCED*	a key enzyme in ABA biosynthesis	Zhang et al.,2009b
Ethylene	*ACS*	a key enzyme in ethylene biosynthesis	Yang and Hoffman, 1984
	ACO		
Chlorophyll	*CLS*	Chlorophyll synthase is a key enzyme in chlorophyll biosynthesis	Costa et al., 2005
	CLH	Chlorophyllase is a key enzyme in chlorophyll degradation.	
Sugar	*β-AM*	β-amylase breaks starch into maltose during the ripening of fruit	Robyt et al., 1968
	SUSY	Sucrose synthase catalyzes the reversible conversion of sucrose and a nucleoside diphosphate into the corresponding nucleoside diphosphate-glucose and fructose	Edurne et al., 2012
Cell wall	*EXP*	Expansin influences cell wall degradation by disrupting noncovalent linkages in cellulose–hemicellulose	McQueen-Mason and Cosgrove, 1994
	PG	Depolymerization and solubilization of the pectic backbones of cell-wall polysaccharides	
MADS-box Transcription factors	*SEP/RIN* *TDR4/FUL* *AG* *AP3*	Associated with ethylene biosynthesis and ripening	McAtee et al., 2015

CHAPTER 2

EFFECT OF ETHEPHON OR ABSCISIC ACID APPLICATION ON 'KOHI' KIWIFRUIT (*ACTINIDIA CHINENSIS*) RIPENING ON THE VINE

2.1 INTRODUCTION

Kiwifruit is classified as a climacteric fruit because it ripens in response to exogenous ethylene, and its ripening is characterized by a period of autocatalytic ethylene production (Park and Kim 1995). The ripening of kiwifruit appears to be different from that of other typical climacteric fruit because fruit produces little ethylene while on the vine (Patterson et al., 2003). However, kiwifruit ripens with ethylene treatment after harvesting (Sfakiotakis et al., 1997; Mworia et al., 2010). There are two commercially important species of kiwifruit: *Actinidia deliciosa* and *Actinidia chinensis*. *A. deliciosa*, including cultivars such as 'Hayward', is widely known for its large fruit size, green flesh, and long storage life (Thompson et al., 2000). *A. chinensis*, including such cultivars as 'Hort 16A', 'Sanuki Gold', and 'Kohi', has yellow flesh, high soluble solids and low organic acid concentrations, but a short storage life (Xu et al., 1998, 2000). In general, *A. chinensis* produces more ethylene than *A. deliciosa* (Asiche et al., 2016). These results may hint that the fruit of *A. chinensis* could ripen more easily than that of *A. deliciosa* and also, the fruit has a shorter storage and shelf life than that of *A. deliciosa*. Ethylene is a kind of plant hormone that is involved in fruit ripening in many plants (Guo and Ecker, 2004). Ethephon has been applied to both *A. deliciosa* and *A. chinensis* commercially to accelerate ripening after harvest (Mworia et al., 2010, Park et al., 2006, Pranamornkith et al., 2012, Zhang et al., 2012). If kiwifruit can be ripened on the vine, the fruit may be more marketable.

ABA concentrations are very low in unripe climacteric fruit such as tomato (*Solanum lycopersicum*) (Zhang et al., 2009a), peach (*Pruns persica*) (Zhang et al., 2009b), and avocado (*Persea americana*) (Chernys and Zeevaar, 2000) but increase

during fruit ripening. Therefore, ABA may also play an important role in regulating fruit ripening. The previous research suggested that ABA stimulated the processes of fruit ripening and promoted ethylene biosynthesis in climacteric fruits such as banana (*Musa sapientum* L.) (Jiang et al., 2000) and apple (*Malus domestica*) (Kongsuwan et al., 2012). However, the interaction between ABA and ethylene biosynthesis in 'Kohi' kiwifruit on the vine is unclear. Nordihydroguaiaretic acid (NDGA) is an ideal inhibitor of NCED enzyme and blocks ABA biosynthesis (Zhang et al., 2009a). NDGA was treated to clarify the roles of ABA in kiwifruit ripening.

In this study, the possibility of the fruit ripening on the vine and the effects of ethylene or ABA on the ripening of 'Kohi' kiwifruit on the vine were investigated.

2.2 MATERIALS AND METHODS

2.2.1. Plant material

Three-year-old 'Kohi' kiwifruit (*A. chinensis*) vines top-grafted on 'Hayward' kiwifruit (*A. deliciosa*) vines at a Chiba University field, located at 35°N, Lat. 140°E, and elevation 37 m, were used in the experiment. Four hundred fruits were randomly divided into four groups at 155 DAFB (mature stage). The mature stage of kiwifruit has 95% black seeds and the color change of outer pericarp commences (Richardson et al., 2011). In the first group, the fruits were dipped into a 250 µL/L ethephon solution with 5% ethanol for 1 minute on the vine. In the second and third groups, the fruits were dipped into either 100 µmol/L ABA or NDGA solution with 5% ethanol, similar to the ethephon treatment in the first group. In the fourth group, the fruits were dipped into 5% ethanol as an untreated control. The fruit drop rate was measured after treatment. Twenty fruits per treatment were harvested at 0, 3, 6, 9, and 12 Days after treatment (DAT). Fruit firmness, soluble solids and malic acid concentrations, and ethylene production were measured immediately after harvest. The pulp was sampled and frozen by liquid N_2 at -80°C for analysis of ACS and ACO activities, ACC and ABA concentrations, volatile compounds, and RNA extraction.

2.2.2 Fruit firmness and concentrations of soluble solids and malic acid

The firmness of 20 fruits was measured on each side of the fruit after peeling the skin using a penetrometer (FT 327, EFFEGI, Italy). Soluble solid concentration was determined using a refractometer (PL-1, ATAGO, Japan). Titratable acidity was calculated as malic acid concentration.

2.2.3 ACS and ACO activities, ACC concentrations, and ethylene production

ACS and ACO activities, as well as ACC and ethylene concentrations, were analyzed using gas chromatography with a flame ionization detector (model GC-2014; Shimadzu, Kyoto, Japan) according to the method of Kondo et al. (1991). Ethylene production was determined by incubating 20 fruits per treatment (5 replications of 4 fruits) in a 2 L plastic box at 25°C for 2 h.

2.2.4 ABA concentrations

One-gram pulp samples (three replications) were homogenized in 20 mL of cold 80% methanol (v/v) with 0.3 µg ABA-d_6 as an internal standard. ABA was extracted and analyzed by the method of Setha and Kondo (2009). The methyl ester of ABA was analyzed by gas chromatography−mass spectrometry−selected ion monitoring (GC-MS-SIM; model QP5000; Shimadzu). The column temperature was a step gradient of 60°C for 2 min, and thereafter was raised to 270°C at 10°C per min; it was held at 270°C for 35 min. The ions were measured as ABA-d_0 methyl ester/ABA-d_6 methyl ester at m/z 190, 260, 194, and 264. The ABA concentration was determined from the ratio of peak areas for m/z 190(d_0)/194(d_6).

2.2.5 Isolation of *NCED1* gene from 'Kohi' kiwifruit

RNA was extracted using the method reported by Kondo et al. (2012). First-strand cDNA was synthesized from total oligo (dT) according to the manufacturer's instructions (ImProm-II™ Reverse Transcription System, Promega, Madison, WI, USA). The cDNA was used as a template for amplifying the *NCED1* gene with degenerate primers (forward, 5′- GAACCRTGGCCRAAAGTTTC-3′; reverse, 5′-CAATYTGMAGYTCYGAYTTCCA -3′) designed from the conserved sequences of plant *NCEDs* (accession numbers KC816734 and AJ439079). PCR was performed under the following conditions: 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 50°C for 2 min, and 72°C for 3 min, with the final reaction being terminated at 72°C after 10 min. The PCR product was transformed into *Escherichia coli* DH5α by TA cloning techniques (pGEM®-T Easy Vector Systems, Promega, USA). Positive colonies were selected, amplified, and then sequenced. The phylogenetic tree was constructed using the Clustal w (http://clustalw.ddbj.nig.ac.jp/). The number for each interior branch is the percent bootstrap values calculated from 1,000 replicates. The scale bar corresponds to 0.1 amino acid substitutions per residue.

2.2.6 RNA extraction, cDNA synthesis, and quantitative real-time RT-PCR analysis

RNA was extracted (300 mg FW; three replications) by the method reported by Henderson and Hammond (2013). cDNA synthesis was carried out according to the method of Kondo et al. (2014). Gene-specific primers for each gene (Table 2.1) were used for RT-PCR. The relative expression level of each gene was determined by a relative standard curve method. Quantitative real-time PCR (model: Step One Plus, Life Technology, Tokyo, Japan) was performed using a KAPA SYBR FAST Master Mix

(Kapa Biosystems, Boston, MA, USA) according to the instruction manual. The expression level was normalized to that of the elongation factor-1α gene (Nieuwenhuizen et al., 2009).

Table 2.1 Primers used for real-time RT-PCR

Gene		Forward/reverse primer (5'-3')	Reference
AcNCED1	(F)	5' GAACCATGGCCGAAAGTTTC-3'	Accession number
	(R)	5'CAATTTGCAGTTCTGATTTCCA-3'	(LC123596)
AcACS1	(F)	5′-GCTCACGTTCATCACCTCGA-3′	Huang et al. (2013)
	(R)	5′-GACTGTATACAATGTGAACT-3′	
AcACO1	(F)	5′-GCTATGAAGGAATTTGCCGA-3′	Huang et al. (2013)
	(R)	5′-CAGCTCTGGCTGAGGACACG-3′	
Elongation	(F)	5′-GCACTGTCATTGATGCTCCT-3′	Nieuwenhuizen et al.
factor-1α	(R)	5′-CCAGCTTCAAAACCACCAGT-3′	(2009)

2.2.7 Volatile compound analysis

Volatile compounds were analyzed according to a previous report (Wang et al., 2015). One-gram pulp samples (three replications) were put into a 5-mL vial to which was added 10 μL of 5 μg cyclohexanal as an internal standard. Volatile compounds were extracted by injecting a 10-μM polydimethylsiloxane solid-phase microextraction (SPME) fiber (Supelco, Bellefonte, PA, USA) into the vial and exposing it to the headspace for 30 min at 40°C. Volatile compounds were analyzed using a gas chromatographer with a flame ionization detector (model GC-4000; GL Sciences, Tokyo, Japan; DB-Wax; 60m×0.25mm i.d. capillary column; Agilent, Santa Clara, CA, USA). The oven, detector, and injection temperatures were 50, 250, and 230°C, respectively. The compounds were identified by commercial reference compounds with a GC-MS QP2010 standard gas chromatograph mass spectrometer (Shimadzu).

2.2.8 Statistical analysis

Data are presented as means ± SE of three replications, 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).

2.3 RESULTS

2.3.1 Fruit firmness and concentrations of soluble solids and malic acid

Fruit firmness in ethephon-treated fruit decreased significantly at 3 DAT, and the fruit became edible at 9 DAT on the vine (Fig. 2.1). The soluble solids concentration (SSC) in ethephon-treated fruit increased significantly at 3 DAT, and the malic acid concentration decreased at 6 DAT. The SSC was higher in ABA-treated fruit than in the untreated control at 12 DAT. The fruit drop rate in the ethephon-treated fruit was 4% when the fruit reached edible condition at 9 DAT and was significantly higher than other treatments. The fruit drop rate in the ABA-treated fruit did not differ significantly with that of the untreated control. The fruit drop rate in the NDGA-treated fruit at 12 DAT was the lowest in all groups.

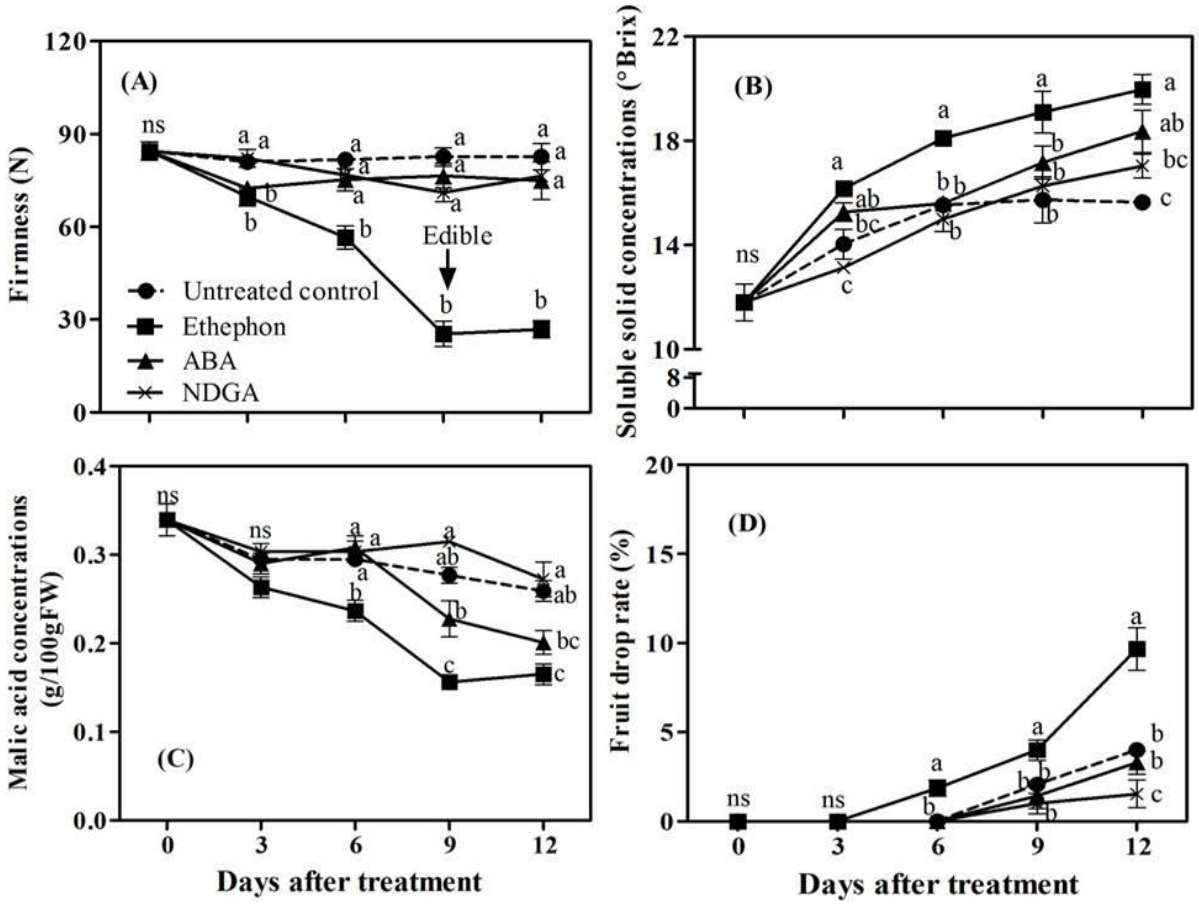

Figure 2.1 (A) Firmness, (B) soluble solid, (C) malic acid concentrations, and (D) fruit drop rate after ethephon, ABA, and NDGA applications in 'Kohi' kiwifruit (*Actinidia chinensis*). Different letters show significant differences ($P \leq 0.05$) by Tukey-Kramer test at each date. Data are the means ± SE of three replications

2.3.2 Volatile compounds

In 'Kohi' kiwifruit, the major volatile compounds were n-hexanal and (E)-2-hexenal (Fig. 2.2). n-hexanal decreased from 0 to 12 DAT in all treatments while (E)-2-hexenal slightly fluctuated from 0 to 6 DAT and then decreased in all treatments. Both n-hexanal and (E)-2-hexenal decreased significantly in ethephon-treated fruit at 9 DAT. ABA, NDGA, and the untreated control fruits did not differ significantly from each other in the concentrations of volatile compounds. (E)-2-hexenal in the ethephon-treated fruit was lowest at 9 and 12 DAT.

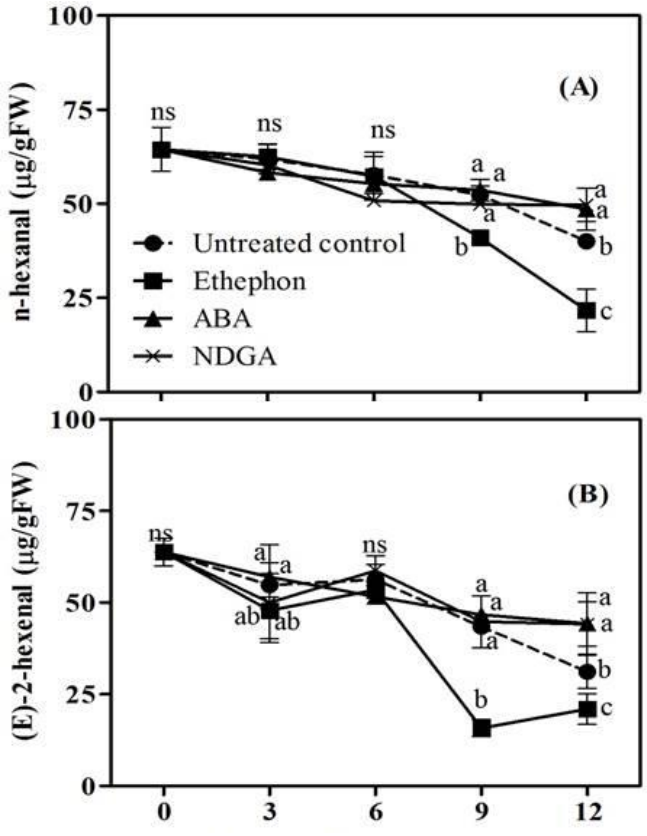

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Figure 2.2 (A) n-hexanal and (B) (E)-2-hexenal after ethephon, ABA, and NDGA applications in 'Kohi' kiwifruit (*Actinidia chinensis*). Different letters show significant differences ($P \leq 0.05$) by Tukey-Kramer test at each date. Data are the means ± SE of three replications.

2.3.3 Ethylene metabolism

The expression levels of *AcACS1* were significantly higher in the ethephon-treated fruit from 3 to 9 DAT than those of the other treatments. The expression levels of *AcACO1* gene in the ethephon-treated fruit increased significantly compared to the other treatments from 3 to 12 DAT. The expression levels of *AcACS1* and *AcACO1* in the ethephon-treated fruit were highest at 9 DAT (Fig. 2.3). The ABA- and NDGA-treated fruits did not show a clear tendency in *AcACS1* expression compared to the untreated control fruit. ACS activities were significantly increased in the ethephon-treated fruit at 9 and 12 DAT compared to the other treatments. ACO activities were highest in the ethylene-treated fruit at 3, 6, and 9 DAT. The ACC concentration in the ethephon-treated fruit was highest at 9 DAT. ACC concentrations in ABA, NDGA, and the untreated control did not differ significantly over time except at 6 DAT. Ethylene production in ethephon-treated fruit was higher from 3 to 9 DAT than that of the other treatments and was highest at 9 DAT.

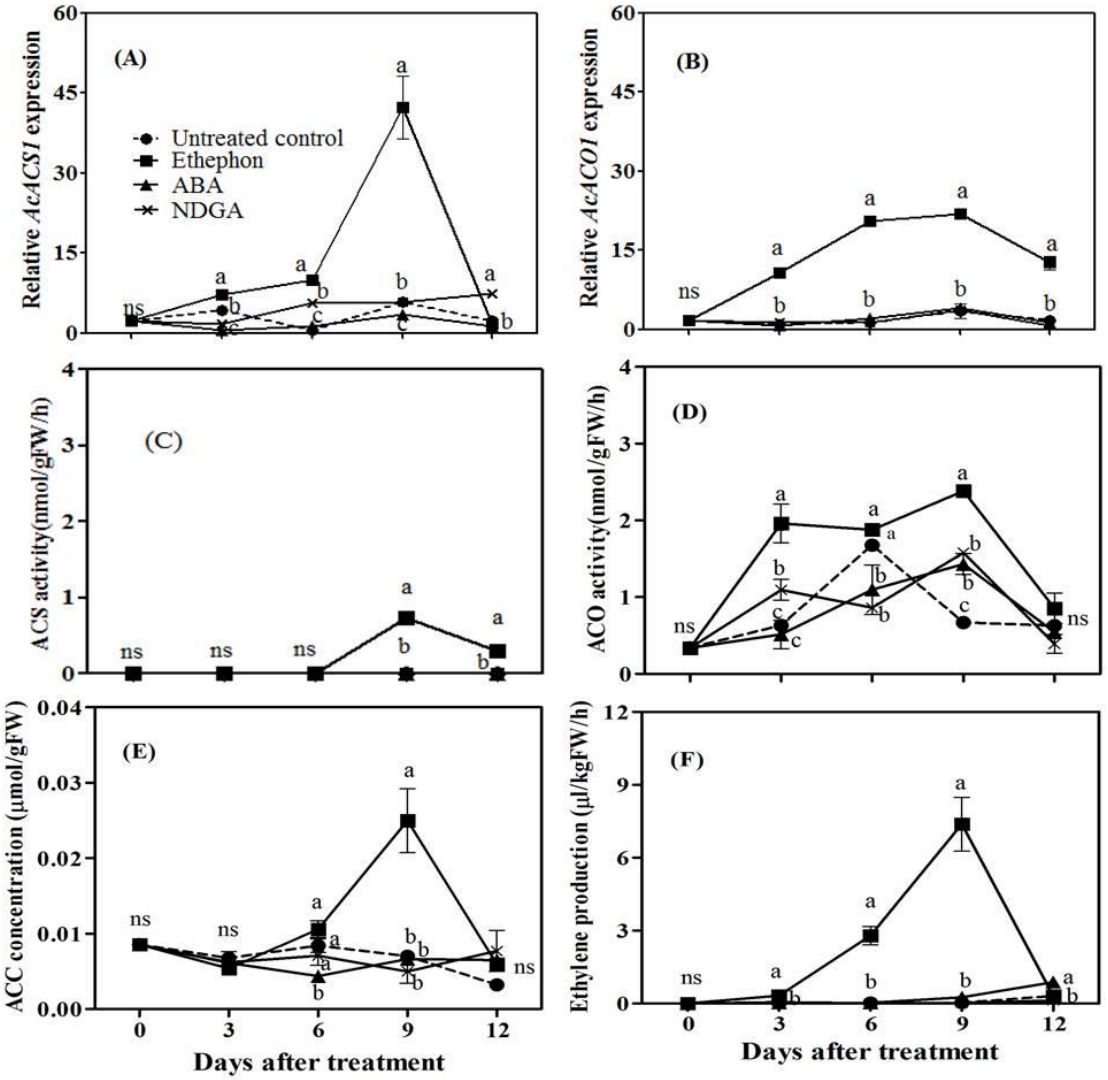

Figure 2.3 Ethylene metabolisms after ethephon, ABA, and NDGA applications in 'Kohi' kiwifruit (*Actinidia chinensis*). Different letters show significant differences ($P \leq 0.05$) by Tukey-Kramer test at each date. Data are the means ± SE of three replications.

2.3.4 ABA concentrations and the expression levels of *AcNCED1*

Sequences encoding plant *NCEDs* were determined by a homology search of the NCBI databases using the BLAST program. *AcNCED1*, *which* was isolated in our study (accession number LC123596) *showed* 99% homology at the amino acid sequence level with *NCED* isolated from almond (Prunus dulcis) tissues (accession number ACH58414). Phylogenetic analysis showed that the *AcNCED1* was closest *Pisum sativum* (accession number BAC10549) (Fig. 2.4).

ABA application significantly increased the expression levels of the *AcNCED1* gene at 3 and 6 DAT (Fig. 2.5). ABA concentrations were highest in ABA treated-fruit at 3, 6, and 9 DAT. Ethephon application did not increase endogenous ABA concentrations at any of the measurement dates.

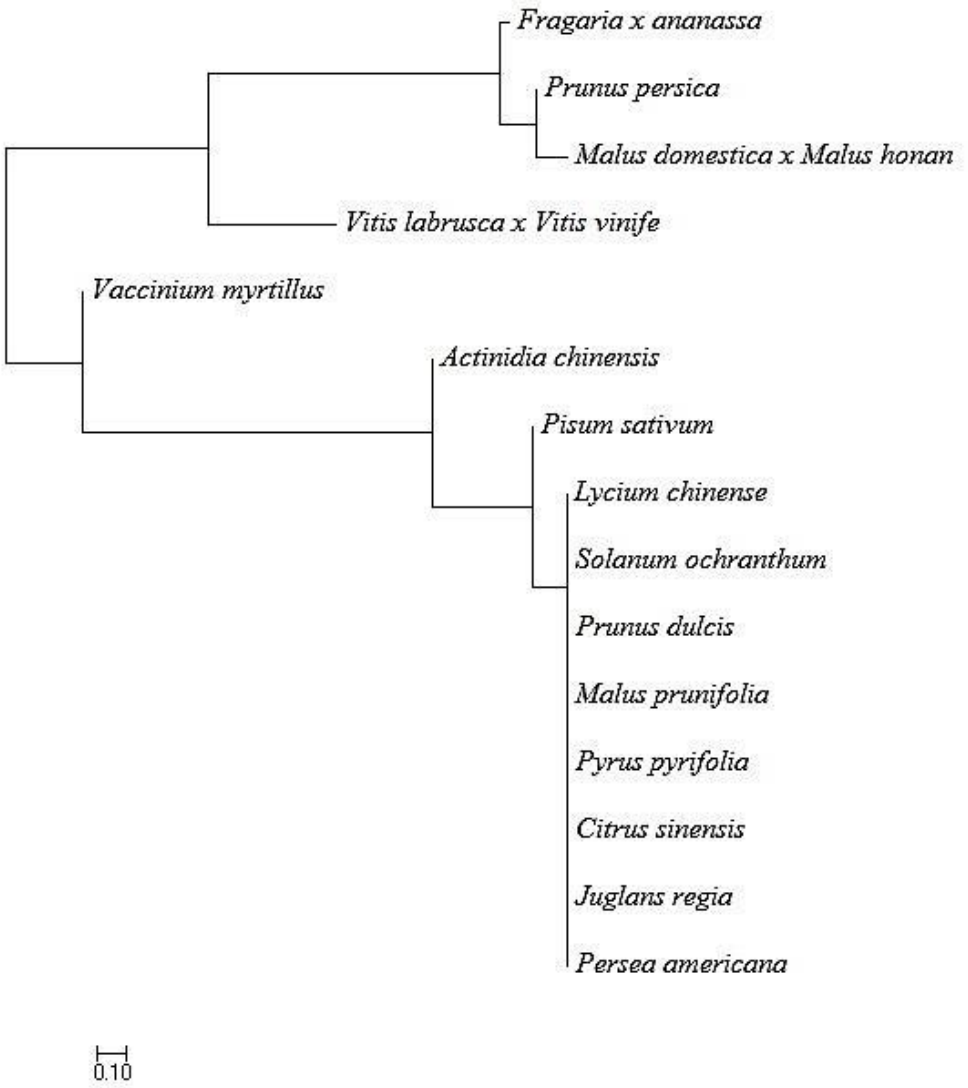

Figure 2.4 Phylogenetic analysis of *Actinidia chinensis NCED1* and other plant *NCED1*. The GeneBank accession numbers for the sequences are as follows: *Fragaria* x *ananassa*; (ADP06891), *Vitis labrusca* x *Vitis vinifera* (ABV01923), *Prunus dulcis* (ACH58414), *Pyrus pyrifolia* (AFM77567), *Malus prunifolia* (AIG92852), *Prunus persica* (ABV01922), *Persea americana* (AAK00632), *Malus domestica* x *Malus honanensis* (AGQ03804), *Pisum sativum* (BAC10549), *Vaccinium myrtillus* (AGT01913), *Lycium chinense* (AIY62759), *Solanum ochranthum* (ADQ74073), *Juglans regia* (AFO59594), *Citrus sinensis* (AAY89370), *Actinidia chinensis* (BAU71154).

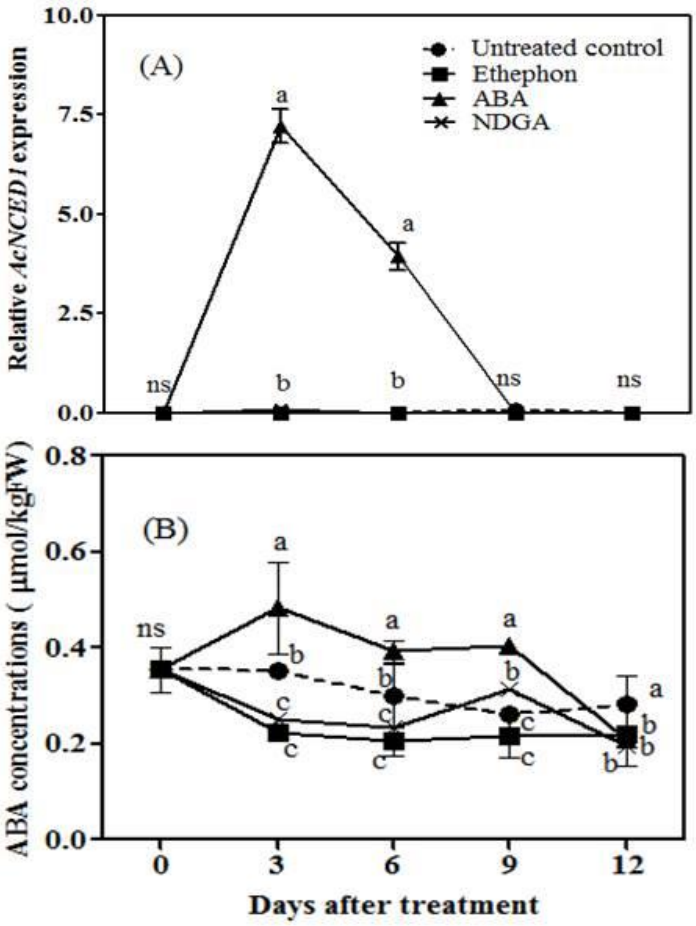

Figure 2.5 (A) Quantitative real time RT-PCR of *AcNCED1* and (B) ABA concentrations after ethephon, ABA, and NDGA applications in 'Kohi' kiwifruit (*Actinidia chinensis*). Different letters show significant differences ($P \leq 0.05$) by Tukey-Kramer test at each date. Data are the means ± SE of three replications.

2.4 DISCUSSION

Kiwifruit contains little endogenous ethylene at harvest but is highly sensitive to exogenous ethylene after harvest (Schroder and Atkison 2006). Ethylene application promoted fruit softening in kiwifruit after harvest (Ritenour et al., 1999). In addition, the results of our study indicated that ethephon treatment can increase SSC in 'Kohi' kiwifruit on the vine. Together, these results suggest that the ethylene signal can enhance both the conversion from starch to sugars and the reduction of organic acid on the vine.

In general, *ACS1* and *ACO1* genes are associated with the ripening of many fruits, such as apple (Dong et al., 1991) and banana (Liu et al., 1999). In our study, ethephon application significantly increased the expression levels of *AcACS1* and *AcACO1*, the activities of ACS and ACO, and the production of ACC and ethylene on the vine. This result suggests that ethephon application may effectively stimulate the expression of *ACS* and *ACO* genes. In this study, agrees with the result of Mworia et al. (2010) that propylene, which is a derivative of ethylene, upregulated the expression of *AcACS1* and *AcACO1* genes and increased ethylene production in kiwifruit.

The aroma volatiles of kiwifruit are among the crucial factors in consumer acceptance

of the fruit's sweetness and acidity (Garcia et al., 2012). (E)-2-hexenal and hexanal were the main aldehydes for 'Hort16A' (*A. chinensis*) and 'Hayward' (*A. deliciosa*) kiwifruit (Garica et al., 2012). In this study, both n-hexanal and (E)-2-hexenal were lowest in the ethephon-treated fruit. These results are consistent with previous findings that ethephon treatment decreased (E)-2-hexenal content in banana (Sonmezdag et al.,

2013). Garcia et al. (2013) suggested that aldehydes tended to decrease as kiwifruit ripened (*Actinidia* spp.). This fact explains the change in flavor from 'green' and 'grassy' to 'fruity'. Hatanaka (1993) reported that (E)-2-hexenal and hexanal were green note compounds generated by lipoxygenase action. In this study, aldehydes (n-hexanal and (E)-2-hexenal) decreased when kiwifruit became ripe. Bartley and Schwede (1989) suggested that the reduced levels of aldehydes are likely to be the result of diminished lipoxygenase activity in ripening fruit.

In this study, ABA application induced the expression of the *AcNCED1* gene and the increase of ABA. Therefore, exogenous ABA may stimulate *NCED*, an upstream enzyme in the ABA synthesis pathway. ABA application increased endogenous ABA, but fruit firmness and malic acid concentrations did not differ significantly with the untreated control. Therefore, the ripening in 'Kohi' kiwifruit may be regulated mainly by ethylene rather than ABA. In contrast, Zaharah et al. (2012) reported that the accumulation of ABA during the climacteric rise stage may have initiated ethylene production during the ripening of 'Kensington Pride' mango (*Mangifera indica*) fruit. ABA application accelerated ethylene biosynthesis by promoting the ACS and ACO activities as well as ACC accumulation in mango fruit (Zaharah et al., 2013). The application of ABA to mature green tomato induced ethylene synthesis and accelerated fruit coloring and softening (Zhang et al., 2009a). The effects of ABA on fruit ripening may vary between fruit species, such as apple (Kondo et al., 1991), banana (Jiang et al., 2000), grape (*Vitis rotundifolia*) (Sandhu et al., 2011), peach (Kobashi et al., 1999), strawberry (*Fragaria* × *ananassa*) (Jiang and Joyce, 2003), and tomato (Zhang et al., 2009a).

CHAPTER 3

THE EFFECTS OF ETHEPHON OR ABSCISIC ACID APPLICATION ON RIPENING-RELATED GENES IN 'KOHI' KIWIFRUIT (*ACTINIDIA CHINENSIS*) ON THE VINE

3.1 INTRODUCTION

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). Ethephon application accelerated ripening in kiwifruit (*A. chinensis*) after harvest (Mworia et al., 2010). ABA may also be associated with kiwifruit ripening, because it stimulated banana fruit ripening (*Musa sapientum L*.) (Jiang et al., 2000). However, the effects of ethylene or 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. 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., 1993; Mworia et al., 2012). These changes occur simultaneously and are caused by the synchronized expression of numerous genes at the onset of ripening (Fujisawa et al., 2011). Molecular investigations of fruit development have concentrated mainly on fruit ripening (Gray et al., 1992). *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). *EXP* and *PG* genes are involved with cell-wall degradation. Expansin influences cell wall degradation by disrupting noncovalent linkages in cellulose–hemicellulose (McQueen-Mason and Cosgrove, 1994). The *AcPG* gene is related to the depolymerization and solubilization of the pectic backbones of

cell-wall polysaccharides in kiwifruit (*A. chinensis*) in the ripening stage (Wang et al. 2000). *β-AM* and *SUSY* are soluble sugar-related genes. β-amylase breaks starch into maltose during fruit ripening, resulting in a sweet flavor (Robyt and Whelan, 1968). *Maβ-AM* expression was strongly induced during the ripening of banana (*Musa acuminate*), and the increase in *Maβ-AM* expression levels was significantly correlated to the degradation of starch (Nascimento et al., 2006). Sucrose synthase (*SUSA 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 increased during fruit ripening in satsuma mandarin (*Citrus unshiu*) (Komatsu et al., 2002). Chlorophyll synthase (*CLS* gene) is a key enzyme in chlorophyll biosynthesis, and chlorophyllase *(CLH)* gene) is a key enzyme in chlorophyll degradation.

In this study, the effects of ethephon or ABA application on the ripening-related genes in 'Kohi' kiwifruit (*A. chinensis*) on the vine were studied.

3.2 MATERIALS AND METHODS

3.2.1 Plant material

Three-year-old 'Kohi' kiwifruit (*A. chinensis*) vines top-grafted on 'Hayward' kiwifruit (*A. deliciosa*) vines were used in the experiment. The vines were grown in an open field at Chiba University, located at 35°N Lat., 140°E Long., and at an elevation of 37 m. Three hundred fruit were randomly divided into three groups at 155 days after full bloom on the vine. In the first group, the fruit were dipped into a 250 µL/L ethephon solution with 5% ethanol for 1 minute on the vine. The fruit in the second group were dipped into 100 µmol/L ABA solution with 5% ethanol similarly with ethephon treatment. In the third group, the fruit were dipped into 5% ethanol as an untreated control. The concentrations of ethphone or ABA that were used in our study were effective to promote the ripening in some fruits (Abeles et al., 1992). Twenty fruit from each group were sampled at 0, 3, 6, 9, and 12 days after treatment (DAT). The pulp was sampled and frozen by liquid N_2 at -80°C for the analysis of gene expression.

3.2.2 RNA extraction, cDNA synthesis, and quantitative real time RT-PCR analysis

Total RNA was extracted from a sample (500 mg FW; three replications) with a modification of the MagExtractor PCR & Gel Clean Up (Toyobo, Osaka, Japan) method reported by Vogelstein and Gillespie (1979). cDNA synthesis was performed according to the instruction manual for ReverTra Ace® qPCR RT Master Mix (Code No. FSQ-201) (Toyobo). Quantitative RT-PCR (model; 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 3.1) were used for RT-PCR analysis. The expression level of each gene was determined by a comparative C_T ($\Delta\Delta C_T$) method. The expression level was normalized to that of the *Elongation factor-1α* gene (Nieuwenhuizen et al., 2009).

Table 3.1 Primers used for real-time RT-PCR

Gene		Forward/reverse primer (5'-3')	Reference
AcSep4	(F)	5'-GAGGCTCAAGACAAGGGTTG3'	HQ113364.1
	(R)	5'-AAGCTGCTCAAGCTCCTTTG-3'	
AcTDR4	(F)	5'-GGAGAGTGCAGCTGAAGAGG-3'	Achn247791
	(R)	5'- AGATCTCGCGAGCTTTCTTG-3'	
AcCLH1	(F)	5'-TGTAAGCCACCGTGTTGGTA-3'	Achn035481
	(R)	5'- GTCAGCGTCTCCTTCCCATA-3'	
AcCLS	(F)	5'-GGCCCGTGTCTTACTGGATA-3'	Achn001951
	(R)	5'-CCTGACGGAATAGGACGGTA-3'	
Acβ-AM	(F)	5'-GAAACCCTCTCTGGGGACTC-3'	Achn387071
	(R)	5'-CACGATCCTCCATGTTCCTT-3'	
AcSUSY	(F)	5'-GTGTACGGGACCATCGATTT-3'	Achn064451
	(R)	5'- TCTGGTTCTCGTTCGGTTTC-3'	
AcPG	(F)	5'-GCAGAATGCCTGACTTCCTC-3'	Achn144321
	(R)	5'-CAAAGCTCGGAATGAAGGAG-3'	
AcEXP	(F)	5'-TCCTGCTTTGAGCTGATGTG-3'	Achn194511
	(R)	5'- GCACCAGATGTCTTGGGTCT-3'	
Elongation factor-1α	(F)	5′-GCACTGTCATTGATGCTCCT-3′	Nieuwenhuizen et al. (2009)
	(R)	5′-CCAGCTTCAAAACCACCAGT-3′	

3.2.3 Statistical analysis

Data (presented as means ± SE of three 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).

3.3 RESULTS

In the ethephon-treated fruit, expression of the *AcPG* gene was significantly high at 9 and 12 DAT, while that of the *AcEXP* gene was significantly high at 12 DAT (Fig. 3.1). The ABA-treated fruit showed no significant difference with the untreated control.

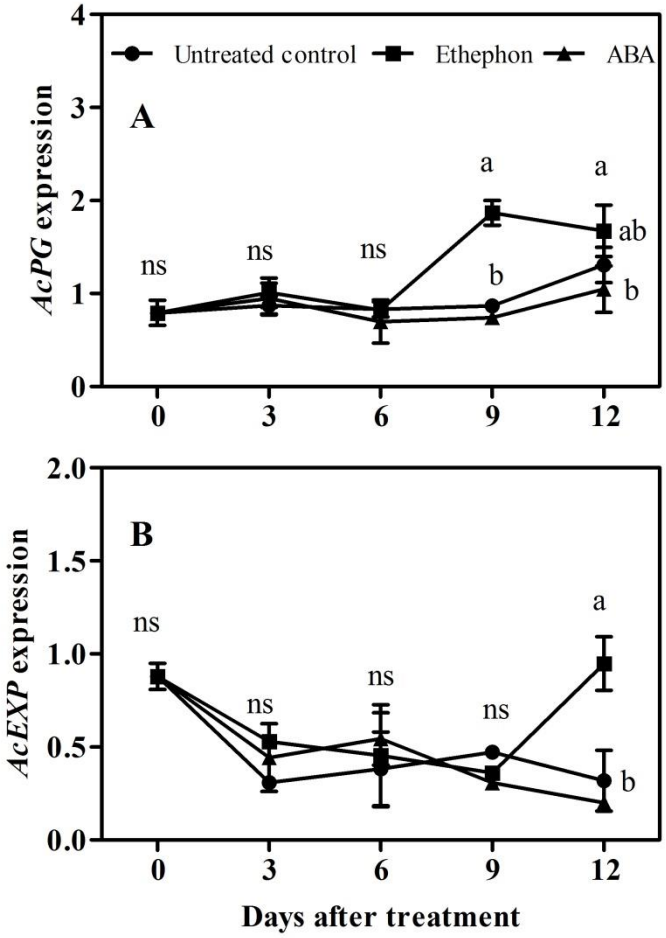

Figure 3.1 The expression levels of (A) *AcPG* and (B) *Ac EXP* genes after ethephon and ABA applications in 'Kohi' kiwifruit (*Actinidia chinensis*). Different letters show significant differences (*P≤0.05*) by Tukey-Kramer test at each date. Data are the means ± SE of three replications.

The expression levels of the *Acβ-AM* and *AcSUSY* genes were significantly high in the ethephon-treated fruit after treatment (Fig. 3.2). The ABA-treated fruit generally showed no significant difference with the untreated control.

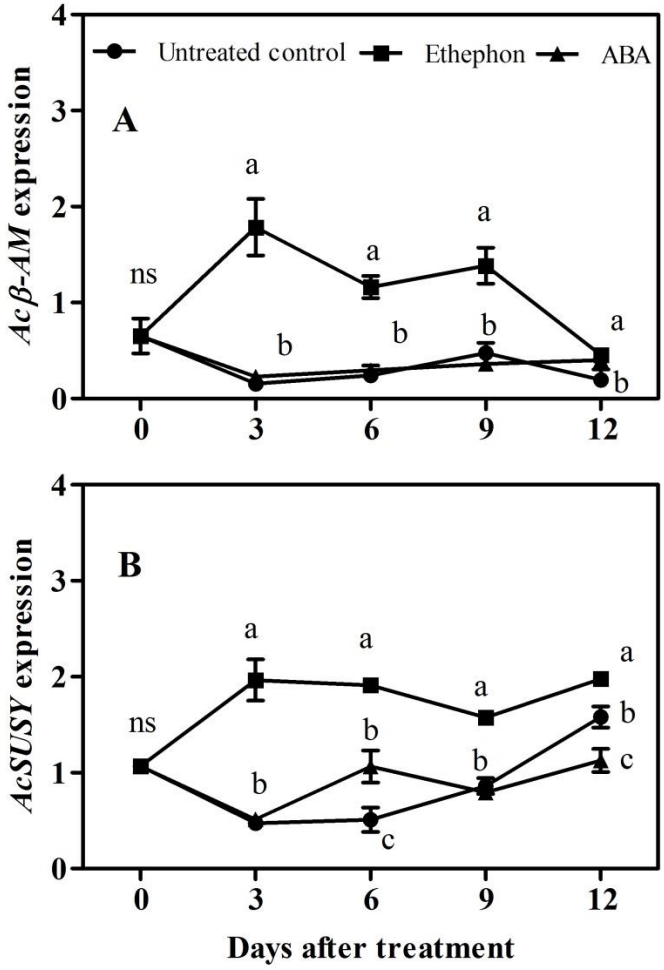

Figure 3.2 The expression levels of (A) *Acβ-AM* and (B) *AcSUSY* genes after ethephon and ABA applications in 'Kohi' kiwifruit (*Actinidia chinensis*). Different letters show significant differences (*P≤0.05*) by Tukey-Kramer test at each date. Data are the means ± SE of three replications.

The expression levels of *AcCLS* and *AcCLH1* genes are shown in Fig. 3.3. *AcCLS* expression decreased significantly from 6 to 12 DAT in the ethephon-treated fruit. The ABA-treated fruit showed no significant difference with the untreated control. *AcCLH1* expression levels did not show a clear tendency with either treatment.

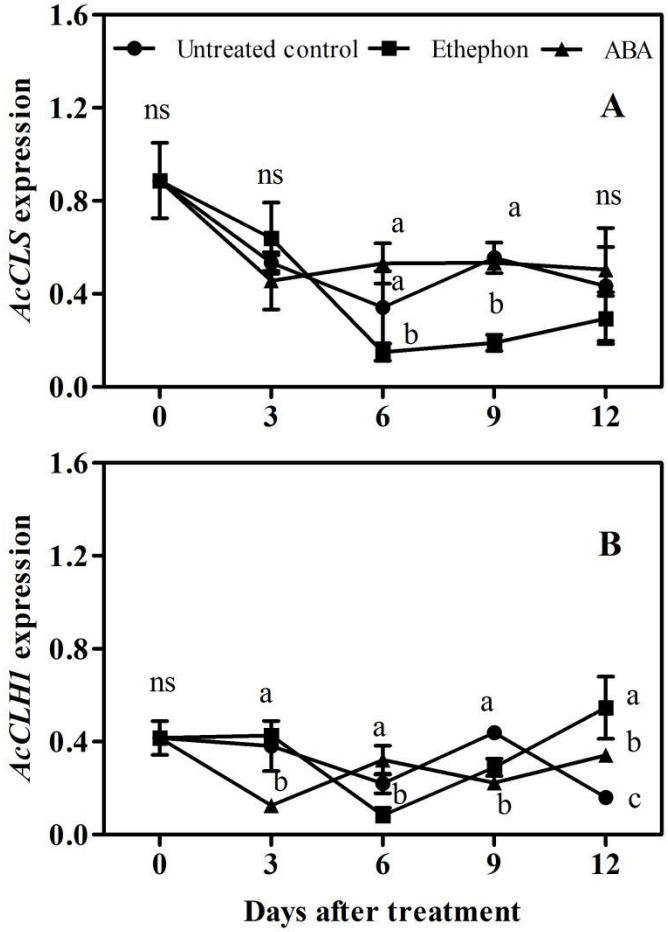

Figure 3.3 The expression levels of (A) *AcCLS* and (B) *AcCLH1* genes after ethephon and ABA applications in 'Kohi' kiwifruit (*Actinidia chinensis*). Different letters show significant differences (*P≤0.05*) by Tukey-Kramer test at each date. Data are the means ± SE of three replications.

The expression levels of *MADS*-box genes *AcSEP4/RIN* and *AcTDR4/FUL* fluctuated after treatment (Fig. 3.4). The expression levels of the *AcSEP4/RIN* and *AcTDR4/FUL* genes at 3 and 9 DAT were higher in the ethephon-treated fruit than in the fruit of the other treatments.

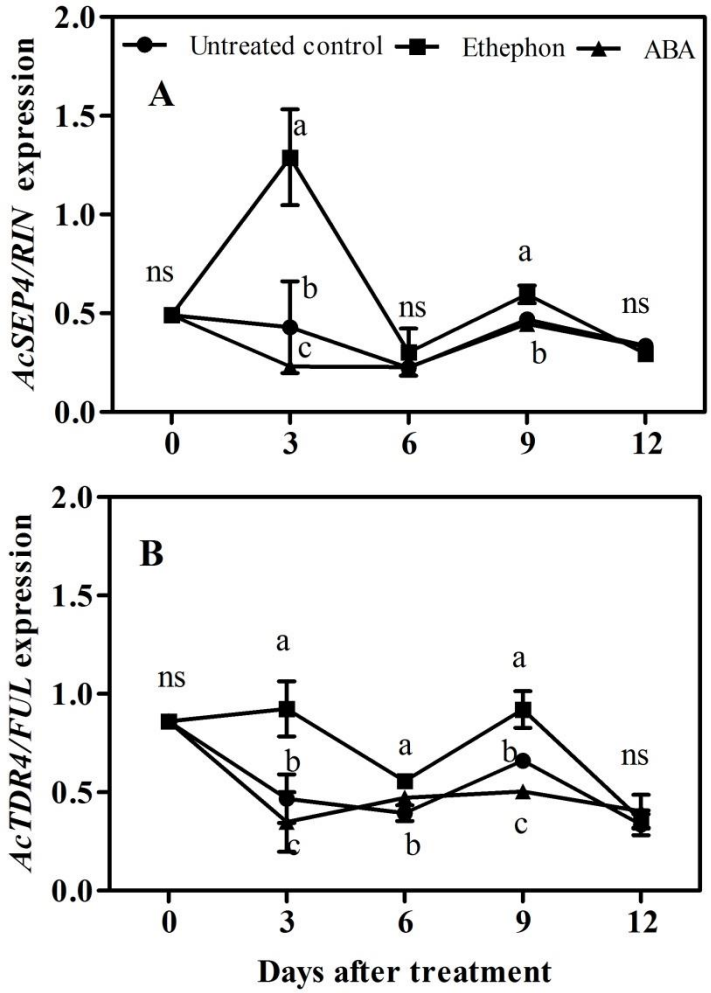

Figure 3.4 The expression levels of (A) *AcSEP4/RIN* (B) *AcTRAD4/FUL* genes after ethephon and ABA applications in 'Kohi' kiwifruit (*Actinidia chinensis*). Different letters show significant differences (*P≤0.05*) by Tukey-Kramer test at each date. Data are the means ± SE of three replications.

3.4 DISCUSSION

The ethylene signal is known as the trigger that induces fruit softening, color change, and flavor in climacteric fruit (Hua and Meyerowitz, 1998). In this study, ethephon application significantly increased the expression levels of *AcEXP* and *AcPG* genes in kiwifruit on the vine. This result suggests that ethephon application may induce fruit softening through the *AcEXP* and *AcPG* genes. A previous study also showed that the *AcEXP* and *AcPG* genes respond quickly to propylene application in 'Sanuki Gold' kiwifruit (*A. chinensis*) (Mworia 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 the *AcPG* gene positively correlated with softening in kiwifruit (*A. chinensis*).

Chlorophyllase activity is tightly associated with chlorophyll breakdown during fruit ripening, and overexpression of the *ClCLH* gene enhances cholorophyll breakdown in lemon (*Citrus limon*) (Shemer et al., 2008). In this study, the expression levels of the *AcCLS* gene were significantly lower in the ethephon-treated fruit than in the other treatments from 6 to 12 DAT. In contrast, the expression levels of the *AcCLH1* gene 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*).

The expression levels of the *Acβ-AM* and *AcSUSY* genes were significantly high in the ethephon-treated fruit. This study supports the result of McAtee et al. (2015) that propylene applications increased the expression of *Acβ-AM* and of *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 the *AcSEP4/RIN* and *AcTDR4/FUL* genes, which are associated with ethylene biosynthesis in kiwifruit (McAtee et al., 2015), were significantly high in the ethephon-treated fruit in this study, while the expression levels of *AcTDR4/FUL* and *AcPG* genes 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 *SlSEP4/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*). In conclusion, ethephon application to kiwifruit on the vine could increase the expression levels of the *AcPG*, *AcEXP*, *Acβ-AM*, *AcSUSY*, *AcCLH1*, *AcSEP4/RIN*, and *AcTDR4/FUL* genes, which may be related to the ripening of 'Kohi' kiwifruit.

GENERAL DISCUSSION

Ethylene regulates fruit ripening through expression of genes with the physiological changes such as rise in respiration, autocatalytic ethylene production, fruit softening, color, aroma and flavor (Oetiker et al., 1995). The ripening of kiwifruit appears to be different from other typical climacteric fruit because fruit produces little ethylene on the vine and the fruit did not ripen without the exogenous application of ethylene (Patterson et al., 2003). Exogenous ethylene application in kiwifruit after harvest can induce fruit softening, ethylene biosynthesis and fruit ripening (Sfakiotakis et al., 1997; Mworia et al., 2010). In this study, the possibility of the fruit ripening on the vine, the effects of ethylene or ABA application on the ripening, and the ripening-related genes of 'Kohi' kiwifruit on the vine were investigated.

This study indicated that ethephon application increased SSC in 'Kohi' kiwifruit on the vine, while decreased acidity and fruit firmness. These results suggested that the ethylene signal can enhance both the conversion from starch to sugars, reduction of organic acid and promote fruit softening in kiwifruit on the vine.

The expression of *AcACS1* and *AcACO1* genes are associated with the ripening in kiwifruit (Mworia et al., 2010). In this research, ethephon application significantly increased the expression levels of *AcACS1* and *AcACO1*, the activities of ACS and ACO, and the production of ACC and ethylene on the vine. From the result, ethephon application may effectively stimulate the expression of *AcACS* and *AcACO* genes and correlated with ripening mechanism on 'Kohi' kiwifruit.

(E)-2-hexenal and hexanal were the main volatile compound in aldehydes group for 'Hort16A' (*A. chinensis*) and 'Hayward' (*A. deliciosa*) kiwifruit (Garica et

al., 2012). In this study, both n-hexanal and (E)-2-hexenal were lowest in the ethephon-treated fruit. These results are consistent with previous findings that ethephon treatment decreased (E)-2-hexenal content in banana (Sonmezdag et al., 2013). Aldehydes that was decreased in ripening kiwifruit(*Actinidia* spp.) suggested that the change in flavor from 'green' and 'grassy' to 'fruity' (Garcia et al., 2013).

ABA application induced the expression of the *AcNCED1* gene and the increase of ABA concentration in 'Kohi' kiwifruit. Therefore, exogenous ABA may stimulate *NCED*, an upstream enzyme in the ABA synthesis pathway. ABA application increased endogenous ABA accumulation, fruit firmness and malic acid concentrations did not differ significantly with the untreated control. Therefore, the ripening in 'Kohi' kiwifruit may be regulated mainly by ethylene rather than ABA. In contrast, ABA application accelerated ethylene biosynthesis by promoting the ACS and ACO activities as well as ACC accumulation in mango fruit (Zaharah et al., 2013). The effects of ABA on fruit ripening may vary depending on fruit species, stage of fruit, method of application, time for application and the concentration of ABA (Setha, 2012).

Ethephon application significantly increased the expression levels of *AcEXP* and *AcPG* genes in kiwifruit on the vine. This result suggests that ethephon application may induce fruit softening through the *AcEXP* and *AcPG* genes. 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 the *AcPG* gene positively correlated with softening in kiwifruit (*A. chinensis*).

It has been reported that chlorophyllase activity is tightly associated with chlorophyll breakdown during fruit ripening, and overexpression of the *ClCLH* gene

enhances cholorophyll breakdown in lemon (*Citrus limon*) (Shemer et al., 2008). In this study, the expression levels of the *AcCLS* gene were significantly lower in the ethephon-treated fruit decreased the expression levels of the *AcCLS* gene but increased, the expression levels of the *AcCLH1* gene. This result suggests that ethephon application induced the expression of a chlorophyll degradation gene in 'Kohi' kiwifruit on the vine.

Ethephon application induced the expression levels of the *Acβ-AM* and *AcSUSA* in 'Kohi' kiwifruit. 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 the *AcSEP4/RIN* and *AcTDR4/FUL* genes, which are associated with ethylene biosynthesis in kiwifruit (McAtee et al., 2015), were significantly high in the ethephon-treated fruit in this study, while the expression levels of *AcTDR4/FUL* and *AcPG* genes 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). *SlSEP4/RIN* gene was regulated 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*) (Fujisawa et al., 2011).

In conclusion, ethephon application to kiwifruit on the vine could increase the expression levels of the ripening-related genes.

SUMMARY

The effects of ethephon and ABA application on the ripening and ripening-associated genes of pre-harvest ‘Kohi’ kiwifruit (*A. chinensis*) were studied. ABA concentrations and *AcNCED1* gene expression increased in ABA-treated fruit. Malic acid concentrations and fruit firmness decreased in ethephon-treated fruit, but SSC and ethylene biosynthesis increased. The accumulated fruit drop rate in ethephon-treated fruit was 4% at the edible stage at 9 DAT. The production of n-hexanal and (E)-2-hexexnal decreased in ethephon-treated fruit. The expression of *AcACS1*, *AcACO1*, *AcCLH1*, *AcPG*, *AcEXP*, *Acβ-AM* and *AcSUSY* genes increased in ethephon-treated fruit but the expression of *AcCLS* gene decreased. Moreover, the expression of *AcRIN/SEP4* and *AcTDR4/FUL* which is associated with ethylene biosynthesis had high level in ethephon-treated fruit. ABA application did not have significant difference from the untreated control. The results of this study suggest that ethephon application could induce ripening-associated genes in ‘Kohi’ kiwifruit and ‘Kohi’ kiwifruit may be ripened by on-vine ethephon application at 9 DAT, thus obviating ripening treatment after harvest.

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