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Quantification of Major Plant Hormones and Expression of Genes Involved in ABA Biosynthesis and Activation/Deactivation during Strawberry Fruit Development

딸기 과실 발달 중 주요 호르몬 정량 및 ABA 생합성과 분해 관련 유전자 발현 연구

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UNDER THE DIRECTION OF DR. EUN JIN LEE SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

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ABSTRACT

Contents of eight plant hormones, expression levels of enzymes related with abscisic acid (ABA) biosynthesis, and expression levels of ripening related transcription factors (TFs) were studied in a non-climacteric fruit strawberry (*Fragaria* × *ananassa* Duch, cv. 'Seolhyang') during the six development stages. Eight plant hormones were ABA, indole-3-acetic acid (IAA), gibberellin 4 (GA_4), jasmonic acid (JA), methyl-jasmonate (MJ), jasmonoyl isoleucine (JA-Ile), salicylic acid (SA), and ethylene (ET). Developmental and ripening stages were;

small green (S1), green (S2), breaker (S3), pink (S4), red (S5) and fully red coloration (S6). Contents of IAA and GA_4 showed the highest levels at the S1 stage and gradually decreased afterwards. The ABA content was low at the S1 to S3 stages and then increased rapidly until the S6 stage, reaching the maximum level. In contrast, the MJ content did not show significant change, while the SA content was increased gradually. The contents of JA, JA-Ile, or ET were not sufficient to be detected or not detected. The expression levels of the ABA biosynthesis genes (*FaNCED1* and *FaABA2*), showed positive increases with the fruit ripening. On the other hand, the expression levels of the ABA degradation genes, *FaCYP707A1*and *FaUGT75C1*, were high when the ABA contents were low in the early stages of development. Among four ripening related TFs, *FaMYB1, FaMYB5, FaMYB10*, and *FaASR*, only the *FaMYB10* seemed to be closely related with ripening in strawberry fruit.

Key words: Plant hormones, *Fragaria* × *ananassa,* ABA biosynthesis pathway, ripening related TF, non-climacteric fruit

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INTRODUCTION

Fruits are grouped into climacteric or non-climacteric types by their respiration rise patterns and the climacteric group typically displayed the dramatic changes in respiration rate, color, texture, and aroma during the ripening stage (Giovannoni, 2001). It is well documented that the onset of ripening of climacteric fruits, such as tomato, apple, and avocado, is mainly triggered by ethylene (ET) and is closely linked with dramatic rise in ET production and respiration rates (Seymour et al., 1993).

In contrast to the climacteric fruits, the process and information on the non-climacteric fruits are less well known. The non-climacteric fruits, such as strawberry, citrus, and grape, do not show the dramatic rise in ET production and respiration rates during the ripening, nor ripening cannot be triggered by ET (Seymour et al., 1993; Adams-Phillips et al., 2004). In the non-climacteric strawberry fruit (cv. Chandler), it is reported that fruit can produce ET and two cDNAs encoding for major enzymes of the ET biosynthesis pathway (i.e., 1-aminocyclopropane-1-carboxylate oxidase [ACO], *FaACO1* and *FaACO2*) as well as three cDNAs encoding for ET receptors (i.e., ethylene response receptor [Etr], *FaEtr1* and *FaEtr2*, and ethylene response sensor [Ers], *FaErs1*) (Trainotti et al., 2005). In this study, good correlation was observed between ET production and expressions of those genes listed earlier during stages of fruit development

(Trainotti et al., 2005).

Indole-3-acetic acid (IAA) plays an important role in regulating fruit growth in strawberry. The expression profiles of auxin-responsive *Aux/IAA* genes (*FaAux/IAA1* and *FaAux/IAA2)* were very high at early stage of fruit development (Liu et al., 2011). The endogenous IAA levels has increased to the maximum at the small green color stage and then gradually decreased thereafter to the red color stage (Symons et al., 2012). It is well documented that IAA is mainly produced in achenes (seeds) of strawberry and, without seeds, the growth of fruit does not occur (Nitsch, 1950). Symons et al. (2012) have similarly indicated that IAA plays an important role for receptacle cell expansion, determining fruit size, and ripening of strawberry fruit. They reported that IAA levels were higher in the developing achenes than the receptacle tissues and were remarkably decreased when the achenes were removed from receptacles (Symons et al., 2012). When the achenes were removed from the fruit, the content of anthocyanins were decreased, indicating that IAA was also involved in color development in strawberry (Given et al., 1988).

Gibberellic acid (GA) has shown to be implicated in the regulation of non-climacteric fruit ripening in grape (Symons et al., 2006) and strawberry (Csukasi et al., 2011). The enlargement of receptacle cells is regulated by endogenous GA during strawberry fruit development (Csukasi et al., 2011). Three main bioactive GA_S (GA_1, GA_3, and GA_4) were identified in strawberry, the GA_4 being the most abundant species, and the total content was the highest at the white stage during

fruit development (Csukasi et al., 2011). In other study, only GA_1 was detected and the maximum content was observed at the green color stage and was decreased sharply thereafter (Symons et al., 2012). In general, both GA_1 and GA_4 levels were decreased as the strawberry fruit became more red-colored or ripened.

Abscisic acid (ABA) was also involved in ripening process of non-climacteric fruit such as bilberry (Karppinen et al., 2013), strawberry fruit (Symons et al., 2012), and sweet cherry (Kondo and Gemma, 1993). In strawberry growth, ABA contents was gradually increased from the green stage to the red stage (Jia et al., 2011; Symons et al., 2012) and the start of the increase in ABA levels coincides with the drop in both IAA and GA_1 levels (Symons et al., 2012). Between tissues, the ABA concentration was higher in the achenes than the receptacle tissues and as probably involved in seed maturation (Finkelstein, 2010; Symons et al., 2012). Exogenous application of ABA to strawberry fruit has disagreeable results. It has increased anthocyanin levels (Jiang and Joyce, 2003; Jia et al., 2011) and stimulated the accumulation of sucrose and other assimilates in the fruit tissue *in vitro* (Archbold, 1988; Ofosu-Anim et al., 1996). On the other hand, the application experiments did not show a promotion of ripening, which was indexed by color accumulation during the fruit development (Symons et al., 2012).

Molecular study on ABA biosynthesis and related enzymes (**Fig. 1**) were recently reported. Zhang et al. (2014) have studied enzymatic and functional analysis of β-glucosidase1 (BG1), catalyzing the hydrolysis of ABA glycosyl ester (ABA-GE) to release biologically active ABA, in strawberry. The onset of red

color development in strawberry fruit was concomitantly accompanied with sharply increased expression levels of the *FaBG1,* while down-regulation of *FaBG1*, resulting in a significant decline in endogenous ABA content, has inhibited fruit ripening as well (Zhang et al., 2014). Similarly, a decline of ABA concentration via down-regulation of 9-cis-epoxycarotenoid dioxygenase1 (*FaNCED1*) significantly inhibited fruit ripening in strawberry (Jia et al., 2011).

Jasmonic acid (JA) and salicylic acid (SA) are known to be involved in a defense mechanism in plants and antagonistically regulate each other as JA activates defense against necrotrophic pathogens, whereas SA induces defense against biotrophic pathogens that feed and reproduce on live host cells in an *Arabidopsis* model (Spoel et al., 2007). Both hormones are producing the signaling molecules and form a comprehensive network of interacting signal transduction pathways against pathogens and herbivorous insects (Becker and Spoel, 2006). However, it is not clear yet whether JA or SA is involved in fruit ripening process. Concentrations of endogenous JA and methyl-jasmonate (MJ) in cherry, a non-climacteric fruit, decreased as ripening progressed (Kondo et al., 2000) and endogenous MJ similarly decreased in strawberry (cv. 'Pajaro') (Mukkun and Singh, 2009). When exogenous MJ was applied, lycopene content was increased along with turning green color to red in never ripe tomato (Liu et al., 2012) or anthocyanin content was increased in strawberry fruit (Pérez et al., 1997). The roles of JA in fruit ripening seemed to be different depending on endogenous or exogenous sources of JA.

In climacteric fruit, exogenous SA has delayed the ripening of banana by reducing ET production (Srivastava and Dwivedi, 2000) and has reduced the production of free radicals and ET by suppressing lipoxygenase activity in kiwifruit (Zhang et al., 2003). Previous studies implied that SA could be involved in climacteric fruit ripening, however, whether SA was necessary or not for non-climacteric fruit ripening was remained unknown.

The MYB family is large, functionally diverse and represented in all eukaryotes and most MYB proteins function as transcription factors (TFs) with varying numbers of MYB domain repeats conferring their ability to bind DNA (Dubos et al., 2010). Thus, MYB proteins are key factors in regulatory networks controlling development, metabolism and responses to biotic and abiotic stresses (Dubos et al., 2010). In regard of ripening process, members of the MYB TFs responsible for anthocyanins accumulation and strawberry ripening have been previously reported by several researches (Kadomura-Ishikawa et al., 2015; Medina-Puche et al., 2013; Salvatierra et al., 2013). However, there are still many questions regarding the regulation of anthocyanins biosynthesis and fruit ripening by the TFs including *FaMYB1, FaMYB5*, and *FaMYB10*. In strawberry fruit, the *FaMYB1* expression was low during the small green and white stages and then was increased to the maximum level at the red stage (Kadomura-Ishikawa et al., 2015). Over- or down-expression of *FaMYB1* has resulted in a decreased or increased anthocyanins content, respectively, in strawberry fruit (Kadomura-Ishikawa et al., 2015). Similarly, down-regulated *FcMYB1* showed a decreased anthocyanin

content in strawberry *Fragaria chiloensis* (Salvatierra et al., 2013). These results imply that MYB1 expression functions to suppress the accumulation of anthocyanins in strawberry fruit. In contrary to MYB1, *FaMYB10* expression in developing strawberry receptacle was negligible from the small green to white stages and showed a very high level at the red stage. Increases of both ABA and anthocyanins content were accompanied by an increase in *FaMYB10* expression (Medina-Puche et al., 2013). Between these two, the expression of *FaMYB10* was independent from and not regulated by *FaMYB1* and vice versa (Medina-Puche et al., 2013). The molecular roles of ABA-, stress- and ripening-induced (ASR) proteins involved in strawberry fruit ripening were observed with an increased endogenous ABA content at transcriptional and translational levels (Chen et al., 2011).

In this study, we have quantified endogenous levels of the eight major plant hormones, such as IAA, GA, ABA, ET, JA, MJ, JA-Ile, and SA, in the strawberry fruit tissues and monitored the quantitative changes of hormones during the development and ripening stages of strawberry fruit. And expression levels of genes involved in biosynthesis and degradation pathways of ABA and TFs related with ripening process were investigated concomitantly in order to elucidate detailed ripening process of strawberry.

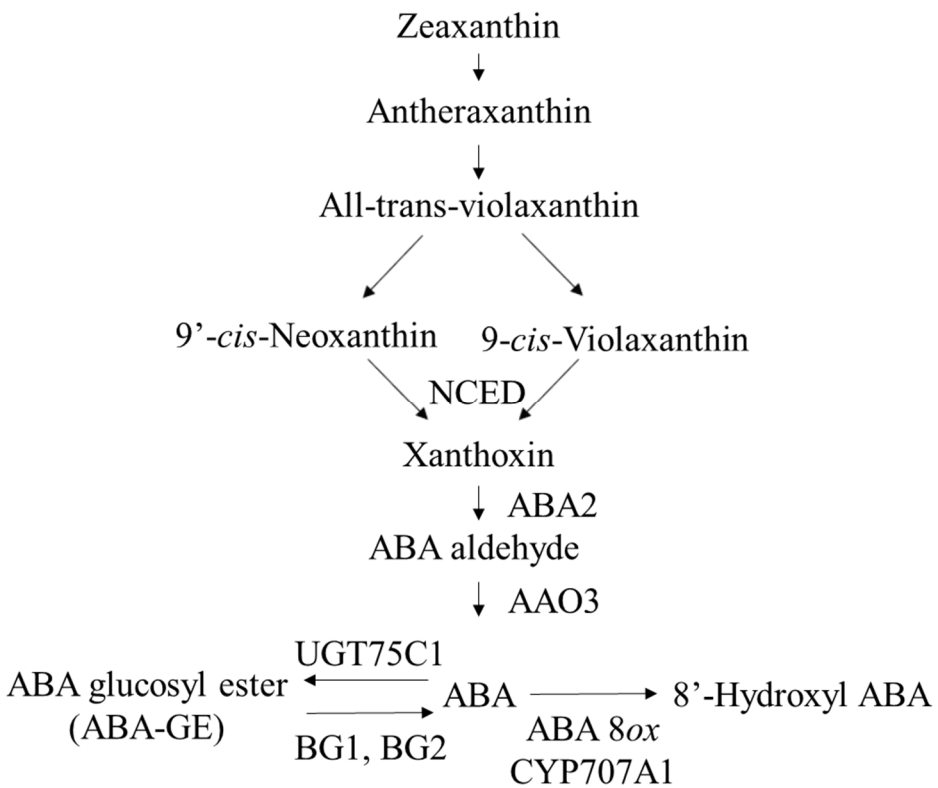

Fig. 1. ABA biosynthesis and degradation pathway in plant. Enzymes related with the gene expression are marked in red color.

MATERIALS AND METHODS

Plant material

Strawberry plants (*Fragaria* × *ananassa* Duch. cv. 'Seolhyang') were cultivated at a commercial greenhouse in Nonsan, Korea using standard practices. The fruits at different growth stages were harvested at the same time in the month of April, 2016. The growth and developmental stages were grouped as six developmental stages: S1, small green stage (11 days after post-anthesis, dpa); S2, green stage (20 dpa); S3, breaker (24 dpa); S4, pink (27 dpa); S5, red (31 dpa); S6, fully red (40 dpa) (**Fig. 2**). Strawberry fruit were harvested in the morning and immediately transported to the laboratory within 4 h and graded for uniform size and colors.

Calyx and peduncle were removed manually and about 50 g fruit was placed into a plastic bag and frozen at –90 ^{o}C until extraction and analyses. Only the ABA samples were freeze dried and stored in a dark covered container.

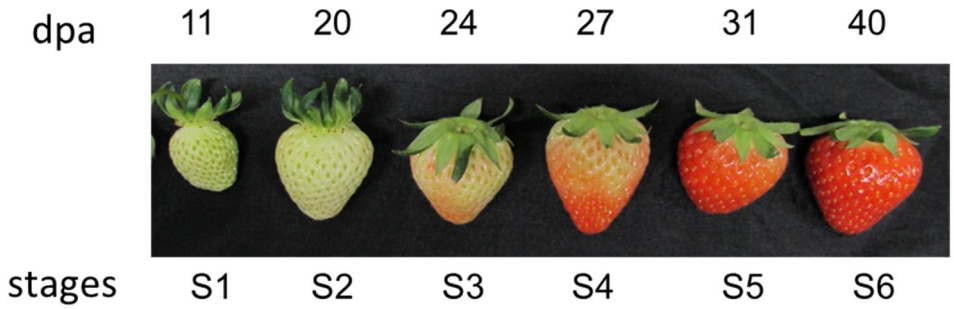

Fig. 2. Appearance of 'Seolhyang' strawberry fruit according to six development stages: S1, small green (11 days after post-anthesis (dpa)); S2, green (20 dpa); S3, breaker (24 dpa); S4, pink (27 dpa); S5 red (31 dpa); S6, full red (40 dpa).

IAA and GA_4 analysis

The IAA and GA extraction method by Csukasi et al. (2011) was followed. In brief, 10 g of frozen tissue were grounded into a fine powder using a motar and pestle in liquid nitrogen, and homogenized with 50 mL mixture of 1-propanol, water, and concentrated hydrogen chloride (2:1:0.002, v/v/v) and then incubated for 12 h at 4 ^{o}C. Extracts were then filtered through a Whatman No.1 filter paper, and then 50 mL dichloromethane was added and incubated for 30 min. This mixture was clarified by centrifugation for 10 min at 12,000 *g* and the lower phase containing hormones was collected and concentrated to dryness in a speed vacuum evaporator (CVE-2000, Eyela, Tokyo, Japan). The residue was re-dissolved in 200 μL methanol and filtered through a 0.45 μm PVDF membrane filter (Acrodisc, Pall life science, Ann Arbor, MI, USA) was injected and separated by a liquid chromatography-tandem mass spectrometry (LC-MS/MS) system below.

The LC-MS/MS analysis was performed on an Ultimate 3000 RS system interfaced with LTQ XL mass detector (Thermo Fisher Scientific, San Jose, CA, USA). The IAA and GA samples were separated using a U-VD Spher Pur C18-E column (1.8 μm, 50 mm × 2.0 mm) at 40 ^{o}C. The mobile phases were 0.1 % formic acid in water (v/v, solvent A) and 0.1 % formic acid in acetonitrile (v/v, solvent B). The solvent system was programmed as follows: 5 % of solvent B for 5 min, and then linear gradient of solvent B from 5 % to 100 % in next 15 min. After the main

separation, the column was flushed with 100 % solvent B for 5 min. The flow rate was 0.25 mL min^{-1} and 10 µL of sample was injected. The mass spectra data were collected covering a mass range from *mz* 100 to 1,000. Resolution was set to 100,000 and the transfer capillary temperature was set to 300 ^{o}C. Data were acquired and analyzed using Xcalibur 4.0 software. The concentrations of IAA and GA were calculated using external standards and expressed as a fresh weight basis.

ABA analysis

ABA was extracted described by Forcat *et al*. (2008) with modification. Two-hundred mg of frozen sample were ground. Samples were placed in a 2-mL microfuge tube with 400 µL of extraction solvent consisted of acetone:water:acetic acid (80:19:1, v/v/v) and homogenized for 3 min. After homogenizing, samples were placed on shaker and stirred for 30 min with additional 600 µL of extraction solvent and then centrifuged at 13,000 *g* for 2 min. The supernatant was removed to a 2-mL micro centrifuge tube and then re-suspended in a 500 µL of extraction solvent and centrifuged at 13,000 *g* for 2 min. The supernatants were combined together and lyophilized at room temperature using speed vacuum. Lyophilized sample was dissolved in 100 µL of solvent consisted of MeOH:acetic acid (99:1, v/v) and then combined with 1 mL solvent consisted of water:acetic acid (99:1, v/v) and centrifuged at 13,000 *g* for 1 min. The supernatant was extracted by using a 5

mL solid-phase extraction (SPE) cartridge. The cartridge was washed with 1 mL MeOH and 1 mL solvent consisted of water:MeOH:acetic acid (89:10:1, v/v/v). The sample was drawn through the SPE cartridge under vacuum and then elute the ABA was eluted from the SPE cartridge using 1mL of MeOH:water:acetic acid (80:19:1, v/v).

JA and JA-Ile analysis

The JA and JA-Ile were analyzed following the method as described by Böttcher et al. (2015). A hundred mg of frozen sample was mixed with 1 mL of isopropanol, water (6:4, v/v) containing with 2.5 mM diethyldithiocarbamic acid (DDC) was stirred for 2.5 h at 4 ^{o}C in a rotating mixer. Then the sample was centrifuged at 13,000 *g* for 10 min at 4 ^{o}C. The supernatant was collected and kept at 4 ^{o}C. The pellet was re-suspended in 1 mL of iso-propanol:water (6:4, v/v) containing 2.5 mM DDC for 1 h at 4 °C on the rotating mixer and centrifuged. The supernatant was collected and combined with the previous one. The organic solvent in the supernatant was removed *in vacuo* and the aqueous phase was acidified with 1 mL of water (pH 2). Acidified extract was passed through the Sep-Pak C18 Plus SPE cartridge. The cartridge was washed with 1 mL water (pH 2) before use. The captured sample was eluted with 2.5 mL of 80 % methanol containing 1 % acetic acid and dried *in vacuo*. The dried residue was re-suspended in 50 µL of 60 %

methanol containing 1 % acetic acid. Five μL sample was injected into and separated by the LC-MS/MS system under the same condition described above for IAA and GA_4 analyses.

ET analysis

Six or seven strawberry fruits were put into a 1-L of plastic container as one replicate. After 2 h at room temperature, 1 mL of gas samples were taken. The gas samples were injected into a gas chromatography (GC) (YL6400, Younglin Co., Korea) equipped with flame ionization detector and 6' × 1/8" SS Porapak N 80/100 column (Ohio valley specialty company, Mariettam, OH, USA). The injector temperature was 50 °C. The flow rate was 18 mL/min and the oven temperature was 50 °C. The detector temperature was maintained at 250 °C. Helium was used as the carrier gas. ET was identified using ET standard by comparing retention time.

MJ analysis

The MJ was analyzed using the method described by Mukkun and Singh (2009) with modification in sample amounts and extraction buffer volumes. About 50~60 g frozen fruit tissue was mixed with 100 mL of extraction buffer, consisted of 50 mL saturated sodium chloride (NaCl), 25 mL of 1 M citric acid, and 100 mL

of diethyl ether containing 10 mg L^{-1} butylated hydroxytoluene as an antioxidant and 50 μL of D_2 MJ as an internal standard. The mixture was homogenized for 30 sec and cleaned with 5 mL of saturated NaCl. The homogenate was centrifuged at 2,000 *g* for 10 min and the upper ether phase was filtered through a miracloth into a flask. The aqueous layer was mixed with 25 mL of diethyl ether and shaken for 30 min. The sample was centrifuged again for 10 min at 2,000 *g* and the diethyl ether phase was collected and combined into a new 50-mL tube and dried under nitrogen. The dried samples were dissolved with 3 mL of *n*-hexane:ether (2:1, v/v). The sample was loaded to a Sep-Pak C18 Plus cartridge. Before sample loading, the cartridge was washed with 3 mL *n*-hexane and eluted with 3 mL of *n*-hexane:ether (2:1, v/v). The MJ extract was filtered through the 0.45 μm PVDF membrane filter. The extract was dried under nitrogen and residues were re-dissolved in 50 μL of *n*-hexane:ether (2:1, v/v) and collected into a 1-mL micro tube. Two-μL extract was injected into a gas chromatography-mass spectrometry (GC-MS) below.

TRACE 1310 GC system coupled with a GC-MS detector (ISQ LT, Thermo Fisher Scientific, San Jose, CA, USA) was used to analyze the MJ. The ultra-performance capillary column (Agilent VF5MS, 0.25 μm, 60 mm × 0.25 mm) was coupled directly to the ion source (70 eV) of the GC-MS system. The injection port temperature was 300 ^{o}C. The oven temperature was held at 50 ^{o}C for 2 min, increased to 165 ^{o}C at a rate of 10 ^{o}C min^{-1}, increased to 185 ^{o}C at a rate of 2 ^{o}C

min^{-1}, and finally increased to 310 °C at a rate of 20 °C min^{-1} and maintained for 10 min. The MJ was identified by comparing its MS spectra with the MJ standard and National Institute of Standards and Technology Library.

SA analysis

The SA was analyzed using the method as described by Kim et al. (2006) with some modifications in sample amounts and extraction solvent volume. A hundred mg frozen sample was mixed in 2.5 mL acetonitrile and 0.5 mL 0.1 N HCl solution, shaken for 2 h at 25 °C, and centrifuged for 10 min at 3,000 *g*. The supernatant was evaporated under nitrogen at 30 °C and the residue was re-suspended in 1 mL 80 % methanol. The extract was filtered through the 0.45 μm PVDF membrane filter. The filtrate was collected and 15 μL was injected into an HPLC system.

An HPLC system (Model YL9100, Younglin Instrument, Seoul, Korea) equipped with a photo diode array detector (Model YL9160). A Symmetry C18 column (5 μm, 4.6 × 250 mm, Waters, USA) was used at 25 °C. The mobile phases were 0.3 % phosphoric acid in water (v/v, solvent A) and 100 % methanol (solvent B). The flow rate was 0.8 mL min^{-1} and the solvent system was programmed as follows: 0 % isocratic of solvent B for 5 min, subsequent gradient of solvent B from 0 % to 100 % in 40 min, and maintained 100 % B for 5 min. Data were acquired and analyzed using the YL-clarity 4.0 software.

RNA extraction and cDNA synthesis

Frozen fruit sample was completely grounded into a fine powder in liquid nitrogen. Total RNA was extracted using a HiGene total RNA Prep kit (Biofact, Korea) following the manufacturer's instructions. Five-hundred ng of total RNA was used for the cDNA synthesis for quantitative real-time (qRT)-PCR. cDNA synthesis was performed using amfiRivert Platinum cDNA Synthesis Master Mix Kit (GenDEPOT, Baker, TX, USA).

qPCR analysis

The qRT-PCR reaction was performed using 2X Real-Time PCR Master Mix including SYBER Green 1 (BioFACT, Daejeon, Korea) and the CFX Connect Real-Time System (BIO-RAD, Hercules, CA, USA) for 40 cycles under the following conditions: 94 °C for 15 min followed by 40 cycles of 95 °C for 20 sec, 60 °C for 20 sec, and 72 °C for 20 sec. Primer pairs were listed in **Table 1**. Specific primer sequences for *FaNCED1*, *FaNCED*2, and *FaCYP707A1* were obtained from previous reports (Concha et al., 2013) or other specific primers for *FaBG1*, *FaBG2*, *FaUGT75C1*, *FaABA2*, *FaMYB1*, *FaMYB5*, *FaMYB10*, and *FaASR* were directly designed from those full length cDNA sequences. Programs of Bioedit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and Primer 3 plus web server (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) were used for primer designs. The relative expression levels were calculated using

the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) and normalized by using the expression levels of the housekeeping gene 18S.

Table 1. Primer information used for qRT-PCR.

Gene		Primer sequence	Product size
FaNCED1	F	GTCGTGAAAAAGCCTTACCT	122
	R	AAGTTTTCTGTGATGGCGAA	
FaNCED2	F	GCTACTACTTCAGGCACATG	114
	R	TTTGGGGGTTGAAGAAACTG	
FaABA2	F	TAATCGAGGAGGAGAGAACA	235
	R	CTCAGAGCCTCAGATCACTC	
FaBG1	F	ACGCGGAGACATGCTTTCAG	137
	R	AAGGCTGCCTAGGAGAGAGC	
FaBG2	F	AAAGCACCGCATTGCCTTCT	121
	R	TTAGGAGGGAAGGCTCTGCG	
FaUGT75C1	F	CGTCACATACTCCACCGCAG	106
	R	TGAGACCGTCGTCGAATCCA	
FaCYP707A1	F	TATCCCAAAAGCAAAGAGGC	119
	R	TTCCGAATAGCATCAGGACT	
FaMYB1	F	AGTTGTCGACTGAGATGGAT	136
	R	GCAGTCTTCCAGCTATTAGC	
FaMYB5	F	GCGAACGGACAATGAGATAA	128
	R	CATGGTGATCAGCAGAAGAG	
FaMYB10	F	GACCACGAACCTTCATCAAA	119
	R	TTGTAGAGTCTGTGGTGGTT	
FaASR	F	AATCCGTTAGAAACCTCCTC	235
	R	GTGGTGCCATAAGTTTCAGT	
Fa18S	F	GTGCTCAAAGCAAGCCTACG	240
	R	ATCTGATCGTCTTCGAGCCC	

F, forward; R, reverse

Statistical analysis

The experiments were laid out in a completely randomized design with three replications. The statistical analysis was performed using the IBM SPSS Statistics, version 21 (Armonk, NY, USA) and treatment mean separation was calculated by one-way ANOVA and Duncan's multiple range test ($p < 0.05$) were presented.

RESULTS

Quantitative changes of hormones

Results on the IAA, GA_4, ABA, MJ, and SA analyses were presented in **Fig. 3.** Though eight hormones were measured, JA, JA-Ile, and ET were not detected or below the detection limits as listed below.

IAA contents. The IAA content was greatest at the S1 stage at a level of was 9.99 ng g^{-1} fresh weight (FW), and was gradually decreased thereafter. However, there were no significant difference between the S2 and S6 stages. The lowest IAA concentration was observed at the S5 and S6 stages at levels of 3.15 and 3.24 ng g^{-1} FW, respectively (**Fig. 3A**).

GA contents. In our strawberry samples, only GA_4 was detected and measured, while other major biologically active form GA_3 were not detected at any stages. GA_4 showed the highest content at the S1 stage with a level of 2.23 ng g^{-1} FW. Then GA_4 content was sharply decreased from the S2 stage and became the lowest level of 0.39 ng g^{-1} FW at the S6 stage (**Fig. 3B**).

ABA contents. The ABA content showed a rapidly increasing trend toward the end of growth and ripening stage (**Fig. 3C**). At the early stage, S1, the content was very low 16.61 ng g^{-1} and was the highest level at the matured S6 stage up to 445.39 ng g^{-1} FW.

JA, JA-Ile, and MJ contents. The JA and JA-Ile contents were detected but the concentrations were about the detection limits and, thus, could not be quantified. Only MJ was measured and the result was presented in (**Fig. 3D**). Though the MJ concentrations tended to be gradually decreased as the maturity progressed, there was no significant changes between the stages.

SA contents. The SA contents were relatively stable until the fruit color changed to pink (S4), then was rapidly increased from the red (S5) stages (**Fig. 3E**). This result indicated that the SA contents were coincidely increased the ripening of process in strawberry fruit.

ET contents. ET was not detected at any stages of strawberry fruit development.

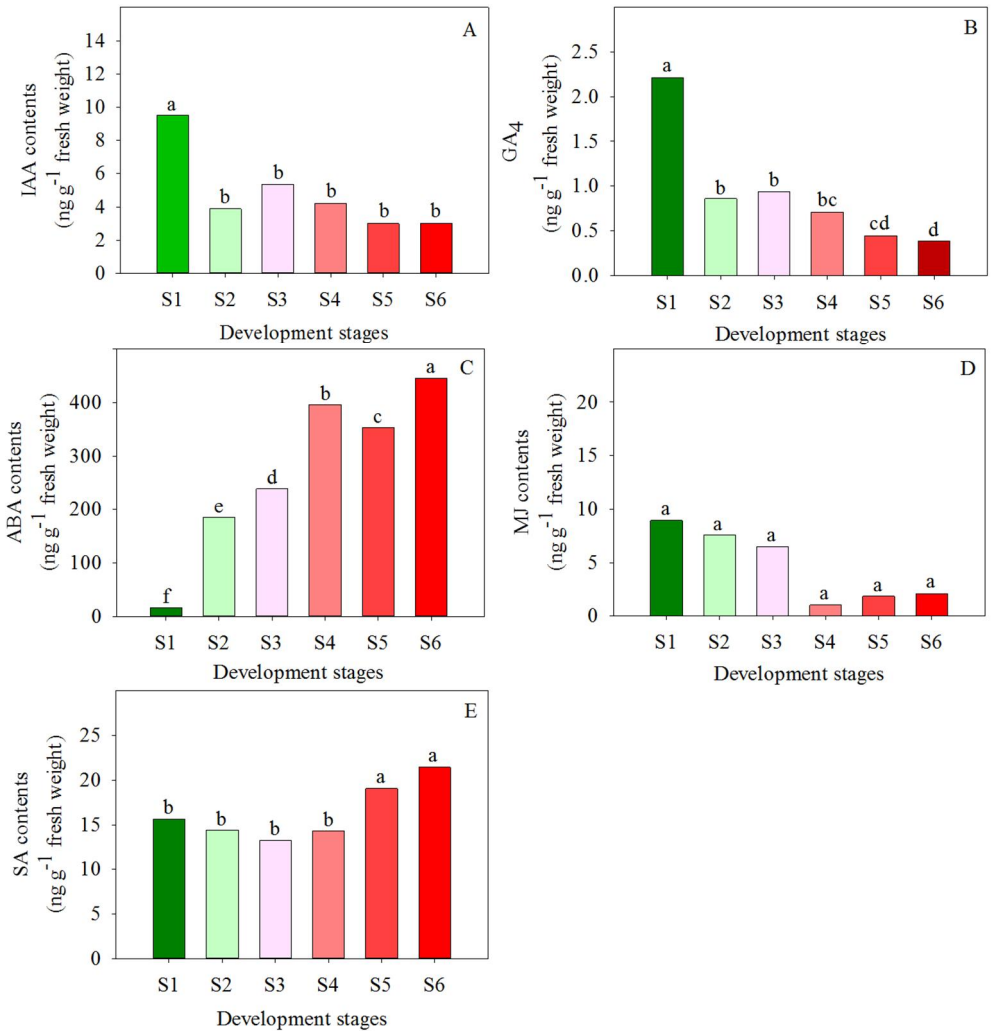

Fig. 3. Quantitative changes of eight hormones according to 'Seolhyang' strawberry fruit development stages. Different letters are significant at $p < 0.05$ by Duncan's test.

Expressions of ABA biosynthesis pathway gene

The mRNA levels of the three ABA biosynthesis pathway genes (*FaNCED1*, *FaNCED2*, *FaABA2*,) were presented in **Fig. 4**. The mRNA level of the *FaNCED1* were in an increasing trend toward maturity (**Fig. 4A**), while those of the *FaNCED2* showed a completely opposite trend (**Fig. 4B**). The expression level of *FaNCED2* was highest at the S1 stage and was gradually decreased with fruit ripening. There have been different views whether *FaNCED1* (Jia et al., 2011) or *FaNCED2* (Ji et al., 2012) was positively related to strawberry fruit ripening, but our result supported the main action of the *FaNCED1*. The expression level of the *FaABA2*, catalyzing xanthoxin to ABA aldehyde, showed somewhat increasing trend that the lowest level at the S1 and the highest one at the S6, however, there were no significant changes from the S2 to S5 stages (**Fig. 4C**). Therefore, we might summarize that the increase of ABA content was caused by the activation of enzymes of *FaNCED1 and FaABA2* within the ABA synthesis pathway.

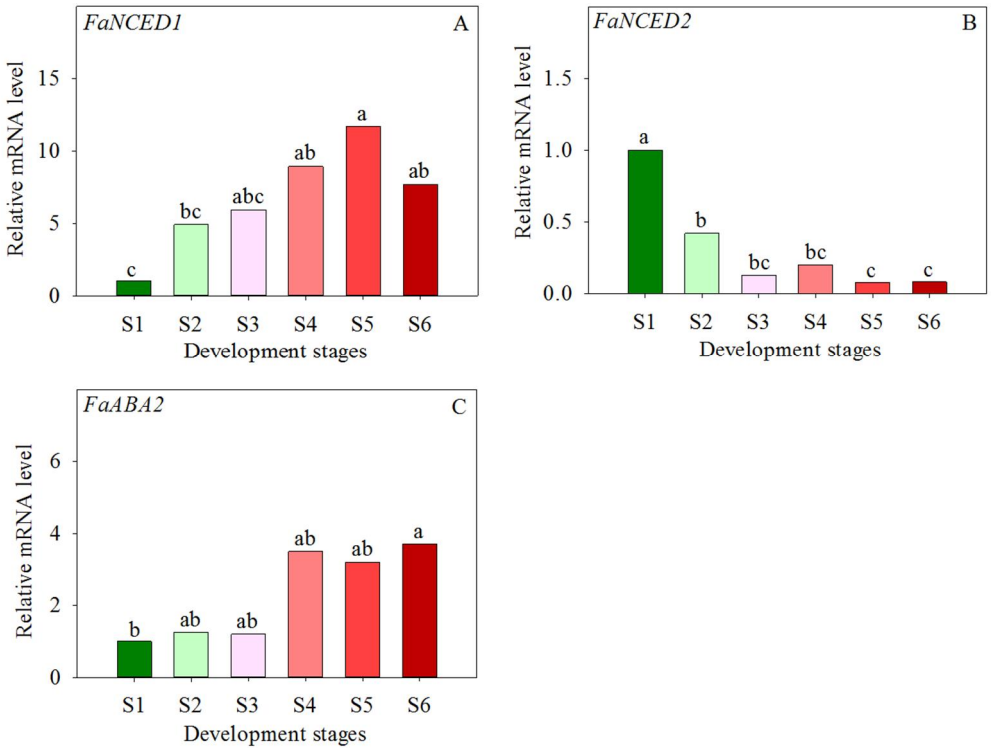

Fig. 4. Relative expression levels of genes involved in ABA biosynthesis pathway according to'Seolhyang' strawberry fruit development stages. Different letters are significant at $p < 0.05$ by Duncan's test.

Expressions of ABA activation/deactivation pathway genes

The mRNA levels of four enzymes related in activation/deactivation or degradation (*FaBG1, FaBG2, FaUGT75C1* and *FaCYP707A1)* were presented in **Fig. 5**. The expression of *FaBG1*, activating ABA from ABA-GE, showed the highest level at the S1 stage and then decreased sharply from the S2 stage and did not show further significant changes between the S2 and S6 stages (**Fig. 5A**). Similarly, the level of the *FaBG2* showed no expression changes in all stages (**Fig. 5B**). These suggested that the conversion of ABA-GE to ABA was not much involved during the ripening process or the ABA-GE was not major source in increasing the active ABA content in strawberry.

The expression levels of the *FaUGT75C1*, deactivating ABA to form ABA-GE, were low, sharply increased, and rapidly decreased at the S1, S2, and S3 stages, respectively (**Fig. 5C**). This indicated that the *FaUGT75C1,* which was supposed to lower the overall ABA content, was involved in early development stages from S1 to S3. The expression level of *FaCYP707A1*, deactivating ABA by forming 8'-hydroxyl ABA, showed a similar pattern as the *FaUGT75C1*. The levels of the *FaCYP707A1* showed the highest peak at the S2 stage and was decreased rapidly and remained low until the S6 stage (**Fig. 5D**). These results indicated that the low ABA content was partly caused by elevated activity of ABA deactivation enzymes, such as *FaUGT75C1* and *FaCYP707A1*.

When we combine the results above in synthesis and deactivation pathways, the increasing ABA content in the ripening strawberry fruits seemed to be resulted from both increased rates of synthesis by the *FaNCED1 and FaABA2* enzymes and reduced rates of deactivation by the *FaUGT75C1* and *FaCYP707A1* enzymes.

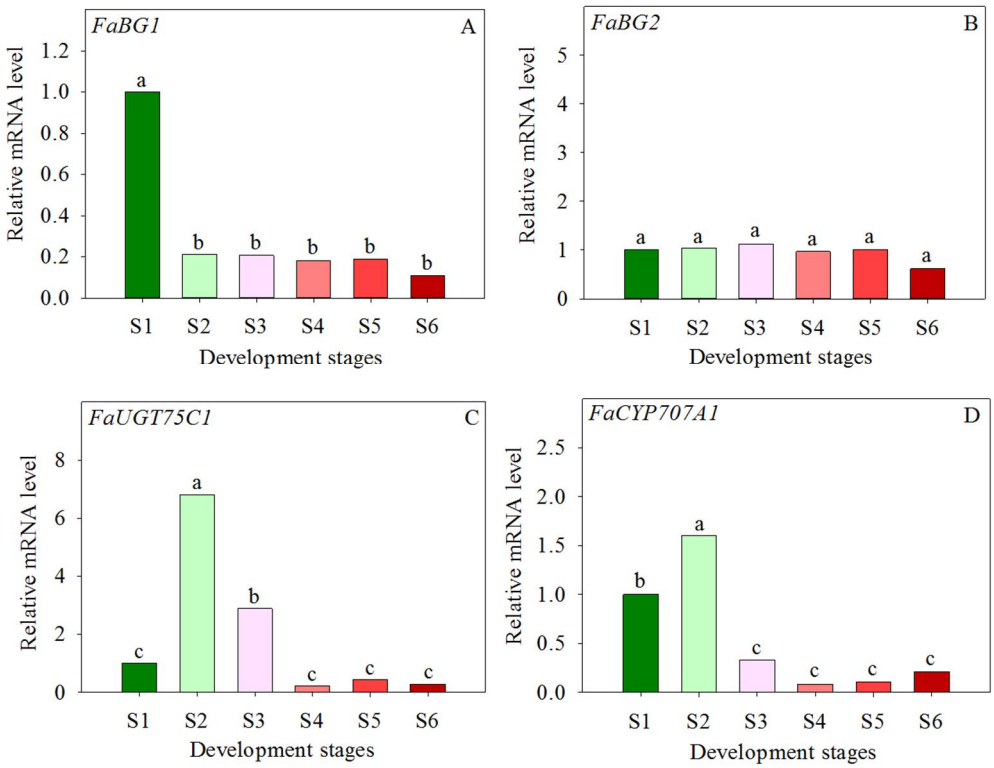

Fig. 5. Relative expression levels of genes involved in ABA degradation pathway according to 'Seolhyang' strawberry fruit development stages. Different letters are significant at $p < 0.05$ by Duncan's test.

Expressions of strawberry fruit ripening related TFs

Expression levels of MYB family TFs of *FaMYB1*, *FaMYB5*, *FaMYB10*, and *FaASR* which were known to be related to strawberry fruit ripening process were presented in **Fig. 6**. There were no significant changes in the *FaMYB1* levels during the ripening process (**Fig. 6A**). The levels of the *FaMYB5* did not showed significant changes (**Fig. 6B**). However, expression levels of *FaMYB*10 were extremely low between 0.6–2 at the S1 stage, steadily increased with ripening stages, and showed the highest level at the S5 stage when red color appeared (**Fig. 6C**). The expression levels of the *FaASR* was negatively highest at the S2 stage and decreased to be remained low level (**Fig. 6D**), showing an opposite pattern from that of the *FaMYB10.* These results indicated that only *FaMYB10* showed an increased mRNA expression levels during the ripening process and the other *FaMYB1, FaMYB5*, and *FaASR* were not.

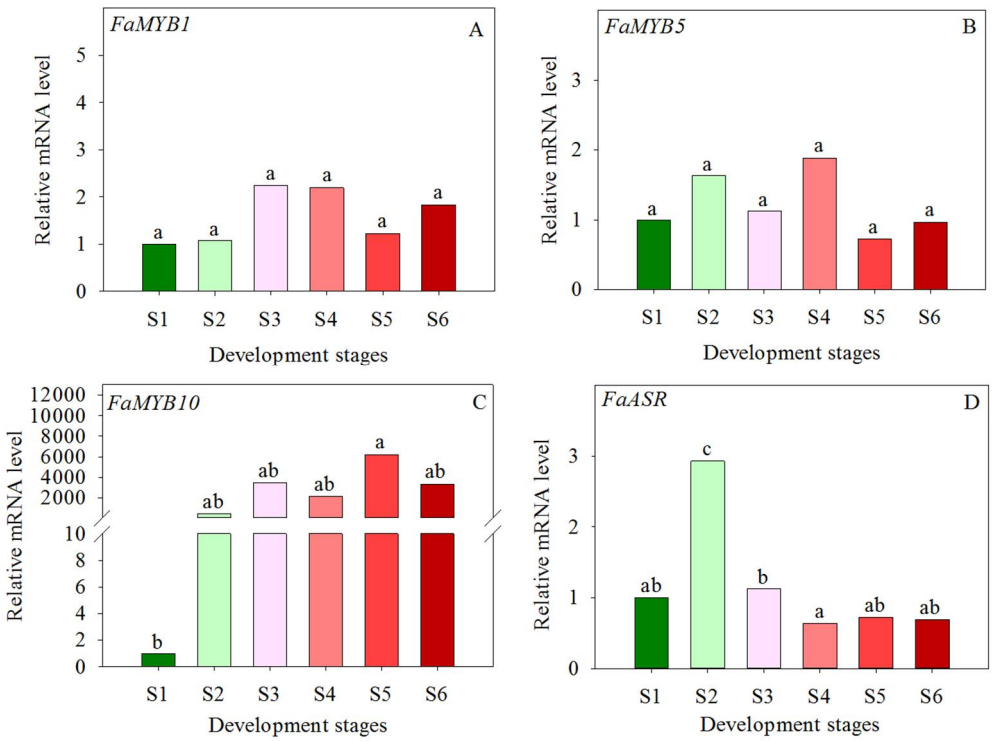

Fig. 6. Relative expression levels of ripening related TFs according to 'Seolhyang' strawberry fruit development stages. Different letters are significant at $p < 0.05$ by Duncan's test.

DISCUSSION

Plant hormone analysis

In this study, eight plant hormones were measured during the 6 stages of fruit growth and ripening (**Fig. 2**) and five hormones were quantified but remaining 3 were below the detection limits and could not be quantified.

IAA

In the previous studies, the IAA content was greatest at the 'small green' stage and declined afterwards (Nitsch, 1950; Symons et al., 2012) and our results showed the same trend (**Fig. 3A**). At the beginning of the measurement, however, we could not observe the increase, peak, and declining pattern as seen in those reports, probably because the S1 stage in our study was about four days later that the 'small green' stage in 11 dpa. While the IAA content was decreased, the ABA content were oppositely increasing as the fruit progressed to mature and ripening. It seemed that the IAA and ABA had an antagonistic regulation in fruit ripening in strawberry. In a recent study, the ABA has suppressed one strawberry MADS-box gene, *FaMADS1a,* expression but accelerated the ripening process and anthocyanin-related gene expressions, while IAA induced *FaMADS1a* expression with delayed ripening and reduced expressions of anthocyanin-related genes (Lu et al., 2018). Our results in the IAA and ABA concurred with those studies.

GA

The greatest level of the GA_4 content was found at the S1 stage and the level was gradually decreased as the maturity progressed (**Fig. 3B**), as similarly reported by Csukasi et al. (2011) and Symons et al. (2012). The GA_1 content also showed the similar pattern as the GA_4 (Symons et al., 2012). Though previous studies reported that the GA_1 and GA_4 (Csukasi et al., 2011; Symons et al., 2012) and GA_3 (Paroussi et al., 2002), only GA_4 was detected in our measurement. This was probably because the contents of GA_3 were extremely lower than that of the GA_4 and could not be much enough to be detected in our quantification method. The GA_4 content was peaked at the 'white' stage and was in the highest content in the receptacle tissues and expressions of enzymes involved in GA biosynthesis (*FaGA3ox*) and catabolism (*FaGA2ox*) in different tissues and developmental stages of strawberry fruit showed a stage-specific pattern during fruit development and was highest in the receptacle (Csukasi et al., 2011).

ABA

ABA contents. The ABA contents were low at the early stage and increasing as the ripening process was advanced, showing the opposite patterns of the IAA and GA_4 in our study (**Fig. 3C**). This result was concurred with the previous studies (Jia et al., 2011; Symons et al., 2012), who indicated that the ABA was

closely involved in the maturation of strawberry. From these findings, we initiated to determine the expression levels of enzymes involved in the ABA biosynthesis.

Expression of NCED enzymes. The NCED was reported as a key enzyme to control ABA biosynthesis in plants (Jia et al., 2011) and an increase in the expression level of it was coincided with the increasing content of ABA in a maturing avocado (Chernys and Zeevaart, 2000). There has been a disagreement on which NCED was positively related with ripening of strawberry, between NCED1 (Jia et al., 2011) and NCED2 (Ji et al., 2012). Our result showed that the *FaNCED1* steadily increased in the later stages of maturity (**Fig. 4A**), while the *FaNCED2* decreased oppositely (**Fig. 4B**). As a result, we support the *FaNCED1* was the key enzyme in the ABA biosynthesis and was positively related with fruit ripening in strawberry.

Expression of ABA2 enzyme. The expression of FaABA2 enzyme, converting xanthoxin to abscisic aldehyde, was the least at the S1 stage and steadily increased to be peaked at the S6 stage (**Fig. 4C**). Our results suggested that the increasing ABA contents during the ripening stages were caused by the increased expressions of both *FaNCED1* and *FaABA2*.

Expressions of enzymes controlling ABA activation/deactivation. After the biosynthesis, the content of active form of ABA could be negatively controlled by deactivation through conjugation and/or hydroxylation enzymes, or the inactive form of ABA-GE could become active by deconjugation of the glucose using BG1 and BG2 (**Fig.1**). The expression levels of *FaBG1* was increasing with maturity in

a previous report (Zhang et al., 2014), but result showed a reversed pattern, the greatest level at the early stage of the S1 and rapid subsequent reduction from the S2 stage **(Fig. 5A)**. The expression levels of the *FaBG2* did not show changes (**Fig. 5B**). Sucrose is known to have an important role in controlling of ripening process in a strawberry. Silencing the sucrose transporter gene has reduced sucrose and ABA contents and delayed the fruit ripening as well (Jia et al., 2013a).

In response to the down-controlled sucrose transporter gene, the expression level of the *FaBG1* was also decreased (Jia et al., 2013b). The trends of the *FaBG1* expression levels during ripening process were not consistent in previous studies. The *FaBG1* expression level was decreasing with ripening stage in strawberry (Jia et al., 2013a) and we observed the similar changes here (**Fig. 5A**).

The decrease of active ABA content was carried out by conjugation with glucose (*FaUGT75C1*) or hydroxylation (*FaCYP707A1*) (Sun et al., 2017). We confirmed that expressions of both *FaUGT75C1* and *FaCYP707A1* were highest at the S2 stage and rapidly reduced afterwards, rendering low levels of ABA at the early stage and high levels at the late stages (**Fig. 5C, D**). The results of the UGT75C1 in strawberry was similar to tomato, a climacteric fruit, the level was increased until the orange stage and then decreased subsequently as tomato turned to red (Sun et al., 2017).

In addition to the endogenous ABA accumulated in the maturing strawberry fruit, externally applied ABA could also promote the ripening process. The exogenously applied ABA promoted color accumulation, tissue softening, and

enzyme activity of the phenylalanine ammonia-lyase, the first and a key enzyme for the biosynthesis of the polyphenol compounds such as anthocyanins (Jiang and Joyce, 2003). Similarly, the external ABA treatment in grape has increased the gene expression level of biosynthesis and actual content of anthocyanin (Jeong et al., 2004). In support of these results, we have confirmed the increase of endogenous ABA contents during the growth and ripening stages and concomitant decreases of IAA and GA contents (**Fig. 3A, B, C**).

ET

The ET has been reported to be not involved in ripening of the non-climacteric fruits (McMurchie et al., 1972) and our result also confirmed the fact. However, a recent indicated that externally applied ethephon could promote natural strawberry fruit red–coloring and softening and, reversely, down-regulation of enzyme in the ET biosynthesis pathway, *S*-adenosyl-L-methionine synthetase 1, and constitutive triple response 1 has resulted in delayed coloration in a strawberry (Sun et al., 2013). Additionally, the down-regulations of these also caused a reduction in ethylene receptor 1, ethylene-insensitive 2, 3, chalcone synthase, and the ET signaling components such as polygalacturonase (Sun et al., 2013).

In addition, a treatment of an ET synthesis suppressor, 1-methylcyclopropene, has suppressed the activities of genes involved in ripening process, such as 1, 3-1, 4-b-D-glucanase and pectate lyase B (Balogh et al., 2005). Therefore, we

speculated that the traditional concept that ET was not involved in the ripening of the non-climacteric fruit might need to be reexamined.

JA, MJ, and JA-Ile

The JA (Davies, 2010) and MJ (Saavedra et al., 2017) have been known to be involved in protection of plants from external stresses. The endogenous levels of the JA and MJ were decreased in ripening cherry fruits (Kondo and Tomiyama, 2000) and similar result was confirmed in the strawberry (cv.' Pajaro') (Mukkun and Singh, 2009). However, exogenous application of MJ has shown to inhibit lycopene accumulation and stimulate β-carotene accumulation in the ripening of tomatoes (Saniewski and Czapski, 1983). Oppositely, exogenous MJ treatment during *in vitro* ripening of *Fragaria chiloensis* fruit has increased red coloration and the accumulation of anthocyanins and lignin (Concha et al., 2013). In this study, we could not find any supportive or negative changes in MJ content during the ripening process (**Fig. 3D**).

An isoleucine conjugate of JA, JA-Ile, inhibited root growth in the JA-insensitive *Arabidopsis* mutant jar1-1 (Staswick and Tiryaki, 2004). JA-Ile also regulated root growth by promoting interaction between coronatine insensitive 1 and jasmonate Zim domain 1 (Thines et al., 2007). In the other study, the contents of JA and JA-Ile were gradually decreased in grape (Böttcher et al., 2015). Against our expectation, too little levels of JA-Ile below the detection limit were measured and no conclusion could be drawn in our study.

SA

The SA is known as an endogenous hormone to be involved in plant growth, responses to environmental stresses, and defense system in plants (Davies, 2012). However, no function of SA during the ripening was elucidated so far. Since our data indicated that the endogenous SA contents were increased at the S4 and S5 stages (**Fig. 3E**), we might suggest that the SA could be related with the ripening of strawberry.

MYB and ASR TFs

In the non-climacteric fruit strawberry, the endogenous ABA and anthocyanin contents were positively related during the ripening, while and silencing of the genes in the biosynthesis pathway has resulted in reduced ABA and anthocyanin contents (Jia et al., 2011). Expression of MYB and ABA content were rapidly increased together in a stressed grape, which had fast coloration, so they were thought to be closely related (Jeong et al., 2004). In our study, only *FaMYB10* showed increases during the maturation and ripening periods (**Fig. 6C**). The suppression of *FaMYB1* did not alter the expression of the *FaMYB10* and anthocyanin contents, but silencing the *FaMYB10* has lowered the anthocyanin content (Medina-Puche et al., 2014). In our study, we could not find this inter-relationship between the *FaMYB1*and *FaMYB5* (**Fig. 6A, B**) and the expression

level of the *FaMYB10* were far greater than the others (**Fig. 6C**). Therefore, we believe that only *FaMYB10* was actively involved in the ripening process of strawberry in this study.

The *FaASR* has been reported to be involved in the maturation of strawberry and the expression level of *FaASR* was increased along with ABA content (Chen et al., 2011). And reversely, the exogenous application of ABA caused the increase the expression level of *FaASR* (Chen et al., 2011). Unlike this report, our result showed somewhat opposite trends, showing the peak at the S2 stage and decrease toward the later stages (**Fig. 6D**). We were not clear of this discrepancy but found a similar result in tomato that the expression of the ASR4 were continuously decreased as the maturity proceeded from the green to the red stage (Golan et al., 2014). We presume that different types of ASR genes could be involved in the fruit ripening of different species.

Conclusion

In this study, we have measured hormone contents, gene expression levels involved in the ABA biosynthesis, and TFs related with strawberry ripening. Among the hormones, ABA and SA showed a positive increase with the maturity progresses. Especially, the ABA showed the closest relationship with ripening with most abundant quantity, so that we considered it as the key hormone in strawberry ripening. The test on the expression of the enzymes in the ABA biosynthesis revealed that the *FaNCED1* and *FaABA2* have caused the increase of ABA. The low contents of ABA at the early stage of fruit growth seemed to be caused by the increased levels of ABA deactivation enzymes (*FaUGT75C1* and *FaCYP707A1*) through conjugation and hydroxylation. At the later stages, the decreased expression of these enzymes, in addition to the high levels of *FaNCED1* and *FaABA2*, was thought to contribute in the increases of ABA contents. Among the TFs that were related with ripening, only expression of the *FaMYB10* was confirmed to be closely related with ripening process.

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초 록

딸기(*Fragaria × ananassa*)는 호흡비급등형 과일로 간주되어왔다. 그러나 식물 호르몬의 역할과 딸기의 숙성 메커니즘 사이의 관계는 잘 연구되지 않았다. 따라서 본 실험에서는 abscisic acid (ABA), indole-3-acetic acid (IAA), gibberellin 4 (GA_4), jasmonic acid (JA), methyl-jasmonate (MJ), jasmonoyl isoleucine (JA-Ile), salicylic acid (SA) 및 ethylene (ET)의 성숙도에 따른 정량을 조사 하였다. 호르몬의 정량화는 small green (S1), green (S2), breaker (S3), pink (S4), red (S5) 그리고 fully red coloration (S6)의 여섯 발달 단계에서 측정하였다. IAA와 GA_4의 정량은 S1에서 각각 생체중 9.99 ng g^{-1}, 2.23 ng g^{-1}으로 가장 높았으며, 딸기의 숙성 과정에서 점차 감소 하였다. ABA의 측정량은 S1에서 S3까지 낮았고 S6에서 급격히 증가하여 생체중 445.39 ng g^{-1}의 최대 값을 보였다. 이와 대조적으로, MJ는 딸기의 숙성 단계에서 유의한 변화를 보이지 않았다. 딸기가 숙성할수록 SA의 정량은 점차적으로 증가했다. JA 및 JA-Ile은 검출되었지만 측정량은 미량으로 정량 하기에 충분하지 않았다. ET은 딸기의 모든 숙성 과정에서 측정되지 않았다. ABA 생합성 유전자인 *FaNCED1* 및 *FaABA2*는 딸기의 숙성과 양의 상관 관계를 보였다. 반면, *FaCYP707A1*

및 *FaUGT75C1*, ABA 분해 유전자는 딸기의 숙성 과정에서 음의 상관 관계를 보였다. *FaMYB1, FaMYB5* 및 *FaMYB10*의 전사 인자 발현 수준은 딸기 과일의 숙성에 따라 증가했다. 특히, *FaMYB10*은 딸기 과일의 주요 숙성에 관련된 전사 인자로 확인되었다. 따라서 '설향' 딸기 과일의 숙성은 ABA와 *FaMYB10* 과 같은 전사 인자에 의해 촉진 되는 것으로 확인되었다.

주요어: 식물 호르몬, 설향 딸기, ABA 생합성 유전자, 호흡비급등형 과일, 숙성관련 전사 인자

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