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Quality and expression of ethylene response genes of 'Daebong' persimmon fruit during ripening at different temperatures

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ABSTRACT

This study was conducted to investigate the changes in quality and ethylene related genes expression in astringent persimmon 'Daebong' during ripening at 15 °C and 25 °C after an application of 100 μg kg^{-1} ethylene. Ethylene production in treated fruit was higher than in untreated fruit throughout the ripening period. Total soluble solids content was reduced slightly during ripening in both treatment and control fruit. However, the reducing sugar content was increased as ripening proceeded. Firmness and water soluble tannin contents were reduced with similar patterns in treated fruit during ripening at both temperatures. Ethylene treated fruit showed more rapid expression of *DKEIL*, *DKERF2*, *DKERF5* and *DKERF8* genes at 25 °C than 15 °C. In conclusion, ethylene treatment improved the ripening quality of astringent persimmon regardless of temperature. In addition, the expression of ethylene related genes was higher at 25 °C than 15 °C, which may be associated with faster ripening.

1. Introduction

Persimmon fruit is mainly grown in Asian countries, with 95% of the world production being from Korea, China and Japan (Pang et al., 2007; Qinggang et al., 2013). Persimmon fruit contains glucose, fructose, beta-carotene, high levels of functional materials, such as vitamin C, gallic acid and catechin (Hiroshi and Akira, 2007). In Korea, persimmon ranks the fourth in fruit production following apple, pear and citrus. Persimmon fruit are either astringent or non-astringent; Asia originated cultivars are astringent persimmon. In non-astringent persimmon, soluble tannin is reduced naturally during maturation while in astringent persimmon, soluble tannin is maintained during maturation (Yamada et al., 2002; Akagi et al., 2009).

It is necessary to remove astringency to use persimmon as a fresh fruit. Many studies on astringency removal include ethylene (Kato, 1987; Nakano et al., 2003; Lim et al., 2015), CO_2 (Arnal and Delrio, 2003; Salvador et al., 2007), ethanol (Ortiz et al., 2005), and high temperature (Ruth and Lillian, 1993) treatment.

Although the effect of ethylene treatment on astringency removal and fruit ripening of persimmon have been studied, no research to select optimal temperature conditions and period for the most effective utilization of ethylene has been done. Thus, more studies to select the optimal conditions to ensure the removal of astringency and fruit ripening still needed. In addition, it has been well known that ethylene has high effect on the removal of astringency and fruit ripening, but its mechanism on the removal of astringency and fruit ripening is not sufficiently studied.

Yoo et al. (2009) reported that the biological response of ethylene is mediated through a signaling pathway composed of the ethylene receptors (*ETR*, *ERS*) and the *CTR1* gene, the ethylene insensitivity genes (*EIN2*, *EIN3*, *EIL*), and the ethylene response element (*ERF*).

The *ERF* gene family, which is found only in plants, accounts for about 6% of the entire Arabidopsis genes (Sakuma et al., 2002) and its expression is regulated by plant hormones such as ethylene (Brown et al., 2003; Neal and Reuber, 2004). *DKCTR1*, *DKEIL1*, and *DKERF*s were identified as the genes related to ethylene signal transduction in persimmon (Pang et al., 2007).

It has been recognized that the ethylene response involves a change in gene expression. In most cases, the ethylene regulatory process is induced by extrinsic and intrinsic factors, and in some cases by inhibition of ethylene biosynthesis. However, despite the important role of ethylene in the process, little research has been done in persimmon to characterize genes involved in ethylene perception and signal transduction.

The main production and distribution season of persimmon in Korea is from October to November, when the marketing temperature is generally in the range of 15 °C to 25 °C. This study was therefore designed to investigate the effect of ethylene at 15 °C and 25 °C on the quality and ethylene signal transduction pathway of astringent persimmon.

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2. Material and methods

2.1. Plant material and ethylene treatment

Astringent persimmon fruit (*Diospyros kaki* Thunb. 'Daebong'), an astringent persimmon cultivar (PCA), were harvested from Jeollanam-do, Yeongham, Korea on 21 Oct. 2015. The fruit were transported to Kangwon national University horticulture laboratory within 12 h of harvest. A total of 240 fruit were treated with ethylene on the same day after harvest. Ethylene was applied at 100 μg kg^{-1} (Akaura, 2010), in a sealed 62 L container for 6 d at 15 °C and 25 °C. Four containers were used for this study, and each container contained 60 fruit. Control fruit were treated similarly but without ethylene. Fruit were kept at either 15 °C or 25 °C, and data were collected at 2 d intervals. Firmness, color, total soluble solids content and ethylene production were measured immediately after treatment at each temperature. Samples of fruit pulp were frozen using liquid nitrogen and stored in deep freezer (−80 °C) until analysis. Quantitative analysis of water soluble tannin, glucose, fructose and gene expression was carried out using frozen samples of the pulp.

2.2. Measurement of ethylene production

Three fruit were placed in air tight 4 L volume containers for 3 h and ethylene content was analyzed using GC2010 Shimadzu (Shimadzu Corporation, Japan) equipped with BP 20 Wax column (30 m × 0.25 mm × 0.25 μm, SGE analytical science, Australia) and a flame ionization detector (FID). The rate of ethylene production was expressed as μg kg^{-1} h^{-1}.

2.3. Measurement of fruit quality

Fruit were measured for firmness, total soluble solids content, fruit skin color, soluble tannin content, fructose content and glucose content. The firmness of the fruit was measured in an equatorial end area using a Rheo meter (Sun Scientific Co. Ltd., Japan), fitted with a 3 mm diameter round stainless steel probe with a flat end (Agusti et al., 2004). Fifteen fruit were measured for each treatment and results were expressed in N. Total soluble solids content was measured from 5 g of homogenized persimmon pulp juice from 15 fresh fruit using a digital refractometer (Model-Atago, USA). Glucose and fructose content was measured according to the method used by Park et al. (2016); 5 g of the frozen pulp sample obtained from 15 fruit was placed in 50 mL of distilled water, ground with a homogenizer and the juice solution was centrifuged at 2000 rpm for 10 min and the supernatant was filtered with 0.45 μm membrane filter. Analysis was performed using HPLC with RI detector (Waters 410 Differential Refractometer, Waters, MA, USA) and a Sugar-Pak ™ column (6.5 × 300 mm, Waters, USA) with an injection volume of 10 μL. Fruit skin color was measured from 15 fruit and each treatment were taken using a Minolta Colorimeter (Model CR-400, Japan) and calibrated with a black and white standard tile. Necessary measured values were Hunter L^* and a^* values. Soluble tannin content was measured with the modified Folin-Dennis method (Taira, 1995). Samples of 5 g were added directly into a solution of 25 mL of 80% methanol. 6 mL of distilled water and 1 mL of the sample were mixed. Afterward, the reagent of 0.25 mL of 2 N Folin-Ciacalteau was added and vortexed. After 3 min, 1 mL of saturated Na_2CO_3 plus and 1.5 mL of distilled water was added. After 1 h incubation at 25 °C, the mixed sample was measured using a spectrometer at 725 nm absorbance. The results were expressed as g kg^{-1} on a fresh weight basis.

2.4. Gene expression analysis

Transcript accumulation of *DkCTR1, DkEIL1, DkERF1, DkERF2, DkERF3, DkERF5, DkERF7* and *DkERF8* was evaluated via quantitative real-time RCR (RT-PCR). Total RNA was isolated from frozen pulp samples with the Robospin Plant TM Kit (GeneAll, Korea) according to the manufacturer's instructions, and treated with RNA-free DNAase I to remove genomic DNA. The quality and content of the extracted RNA were measured using a Nano-drop and then cDNA was synthesized with oligo d $(T)_{18}$ primer and SuperScript® III Reverse Transcriptase (Life Technologies, USA) from 5 μg of total RNA. Subsequently, the cDNA was utilized to conduct real time PCR using gene-specific primers. Specific primers were as reported in Table 1 and adapted from an earlier study (Yin et al., 2012). 1 μL of cDNA template was amplified using the Platinum SYBR Green qPCR supermix-UDG (Invitrogen, the Netherlands) in a 20 μL qPCR reaction according to the manufacturer's protocol. The samples were amplified wit 1 μL of cDNA template was amplified using the Platinum SYBR Green qPCR supermix-UDG (Invitrogen, the Netherlands) in a 20 μL qPCR reaction according to the manufacturer's protocol. The samples were amplified with PCR as follows: 3 min 95 °C, 30 cycles of 10 s at 95 °C, 30 s at 60 °C, 1 min at 72 °C followed by 5 min at 72 °C. Melting curve analyses were performed on the PCR products. *DkActin* was used as the reference gene to calculate relative expression levels, using the ΔΔCt method (Livak and Schmittgen, 2001). Three RT-PCR runs were performed per each treatment.

Table 1
Real-time PCR primers of ethylene signaling related genes.

Gene	Primary PCR (5′-3′)	Secondary PCR (5′-3′)
DkCTR1	GGCTTGTAACCCCACCAATA	CCATTGAAGCCCAGAGAAC
DkEIL1	GCCTACCCTGGTCAAGTGAA	GAGACCAGCATGGGACAAGT
DkERF1	GCTGCTGTCGGAGAGTGAT	TCTCGGGCCTTACAAAGAAG
DkERF2	AAGAGGCGGTGACAAACAAG	AAGGTCACAATCCCTTTGGA
DkERF3	AAGAGGCGGTGACAAACAAG	TCACCACATTCCATCATCCA
DkERF5	GGCCGTAGACAGGTTCTTGA	AAAAAGGGAAACTCCTCAACG
DkERF7	GACGACGGAGATGGAGACAT	ATCAACATCAGAGGCGAAGG
DkERF8	ATCTGGAAGGGGGACAATTC	AGAGTAGCGCGGCAAAATTA

2.5. Statistical analysis

All results were presented as means ± standard errors and differences between treatment groups were tested for significance using *t*-test. Statistical analyses were performed with SPSS statistics program (Version 21, SPSS, USA).

3. Result and discussion

3.1. Ethylene production

The ethylene production was 0.35 μg kg^{-1} h^{-1} at harvest and it was increased to 0.46 μg kg^{-1} h^{-1} and 0.53 μg kg^{-1} h^{-1} in the control and treated fruit respectively after 2 d of ripening (Fig. 1). There was no significant difference within the ripening temperature conditions. After ripening for 4 d at 15 °C, ethylene production of the control and the treated fruit decreased to 0.42 μg kg^{-1} h^{-1} and to 0.48 μg kg^{-1} h^{-1} respectively. On the other hand, in the 25 °C condition, control group and the treatment group was increased to 0.47 μg kg^{-1} h^{-1} and 0.59 μg kg^{-1} h^{-1} respectively (Fig. 1). As the ripening proceeded, ethylene production increased and the ethylene production in the treated fruit was higher than that of control fruit. Persimmon is climacteric fruit, but ethylene production remains low during ripening (Nakano et al., 2002; Pang et al., 2007). In this study, the time point at which ethylene production increases and the point at which minimum in firmness where similar. Salvador et al. (2007) reported that the maximum point of ethylene production and the maximum point of Pectin-methylesterase (PME) activity were matched; the activity of PME, which is closely related to cell wall degradation is believed to be promoted by ethylene.

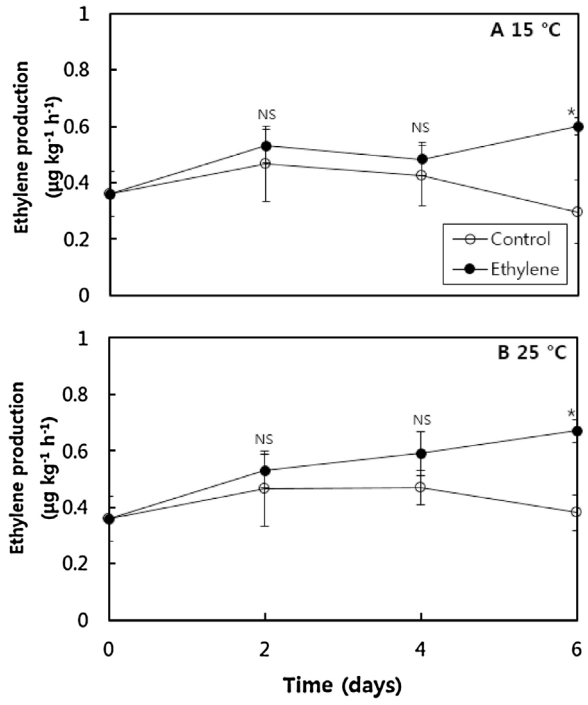

Fig. 1. Ethylene production of 'Daebong' persimmon fruit as affected by ethylene treatment during ripening at 15 °C and 25 °C. Vertical bars represent standard errors of the means (n = 15). A and B indicate 15 °C and 25 °C treatment group, respectively. NS,* and ** indicate non significant at P > 0.05, significant at P < 0.05 and P < 0.01, respectively.

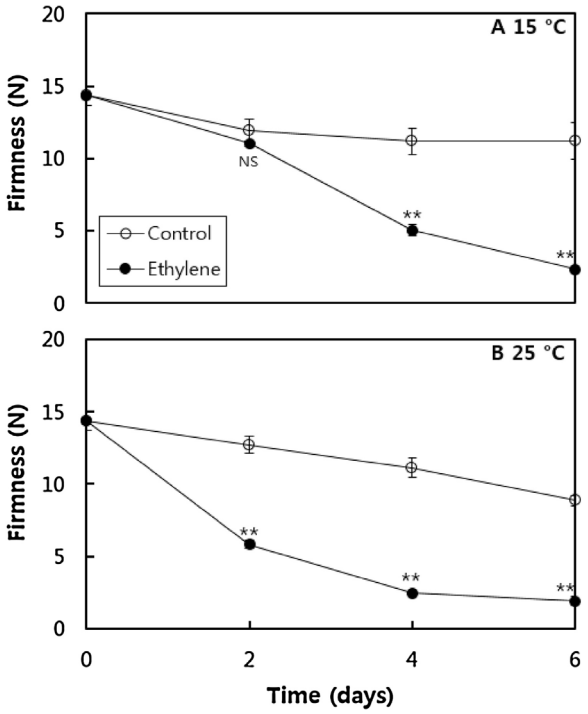

Fig. 2. Firmness of 'Daebong' persimmon fruit as affected by ethylene treatment during ripening at 15 °C and 25 °C. Vertical bars represent standard errors of the means (n = 15). A and B indicate 15 °C and 25 °C treatment group, respectively. NS,* and ** indicate non significant at P > 0.05, significant at P < 0.05 and P < 0.01, respectively.

3.2. Firmness and color

Fruit firmness is a typical fruit ripening indicator. Fig. 2 shows the changes in persimmon fruit firmness during ripening. Firmness was 14.35 N at harvest. At 15 °C, Firmness decreased to 11.21 N in the control fruit and to 2.35 N in the treated fruit after ripening for 6 d. At 25 °C, firmness decreased to 8.86 N and 1.91 N in the control and treated fruit, respectively (Fig. 2). Firmness of the control fruit decreased slightly during ripening. However, that of the treated fruit reduced dramatically at 15 °C after 4 d, and at 25 °C after 2 d of ripening. This result shows higher ripening temperatures were associated with higher exogenous ethylene activity. In agreement with the present study, Salvador et al. (2007) suggested that ethylene is involved in a decrease in firmness linked with a decrease in soluble tannins responsible for the astringency. According to Fisher and Bennett (1991) and Lieberman (1979), the decrease in firmness during storage was closely related to changes in cell wall components, the reason for the accelerated softening at higher temperature in the present study is that the changes in the cell wall components at higher temperature was higher during ripening.

Besada et al. (2010) and Itamura et al. (1991) reported that softening of the flesh in persimmon is directly related to the action of ethylene. It is reported that the polysaccharides constituting the cell wall of persimmon are converted to low molecular weight by the action of the enzyme induced by ethylene, but the exact mechanism has not been revealed (Jeong et al., 2001). From the current data, softening is the typical indicator of ethylene effect in persimmon fruit ripening more particularly at 25 °C; the point of rapid decrease in firmness is associated with maximum *DkERF8* gene expression.

At harvest, the Hunter L^* value was 54.27 and reduced continuously as ripening proceeded (Fig. 3). At 15 °C, control fruit decreased slightly to 49.93 within 6 d whereas, ethylene treated fruit decreased to 40.89 within 6 d. Similarly, at 25 °C the Hunter L^* value of control fruit and ethylene treated fruit decreased from 54.27 to 47.98 and 36.03 on 6 d, respectively (Fig. 3). This result indicated that in both ripening condition ethylene treatment fruit Hunter L^* value was highly reduced compared to control fruit during the entire ripening period. In case of ethylene treatment at 15 °C, there was significant reduction after 4 d, whereas; at 25 °C, the Hunter L^* value decreased after 2 d. Immediately after harvest, Hunter a^* value was 30.81 and changed slightly in control fruit in both temperatures (Fig. 3). However, there was slight increase of Hunter a^* value the last 2 d after ethylene treatment, than a rapid decrease after 4 d in both temperatures. Maturation and pigment development of persimmon can be promoted by ethylene treatment, resulting in increased Hunter a^* values (Lee and Chujo, 1991; Park and Kim, 2002a, 2002b). Also, in our study as the ripening period proceeded, there was a reduction in Hunter a^* value. This result is similar with No et al. (2014) who reported the low Hunter a^* value with a long period astringency removal treatment.

3.3. Total soluble solids, glucose and fructose content

The total soluble solids content was 15.1% immediately after harvest and reduced slightly to 13.6% after 6 d of ripening in both the control and treatment groups, and there was no significant difference in both temperature conditions (data not shown). Persimmon fruit contain simple sugars such as fructose, glucose and sucrose, and the result of present study is also related with Yoshihiro et al. (1985) who reported the breakdown of sucrose to fructose and glucose through enzyme activities during storage. In the present study, the decrease of soluble solids content was not different between the control and ethylene treated fruit. Therefore, it was concluded that the consumption by respiration during the ripening period and the decrease by the enzyme activity were more effective than the aging effect by the exogenous ethylene treatment. In addition, Arnal and Delrio (2003) reported that TSS reduction is related to the loss of astringency following CO_2 treatment due to the change of soluble tannins into insoluble form.

At both temperatures, the glucose content increased during ripening; from 3.1 g kg^{-1} immediately after harvest to 6.3 g kg^{-1} in the control fruit and 7.7 g kg^{-1} in the treated fruit at 15 °C, and to

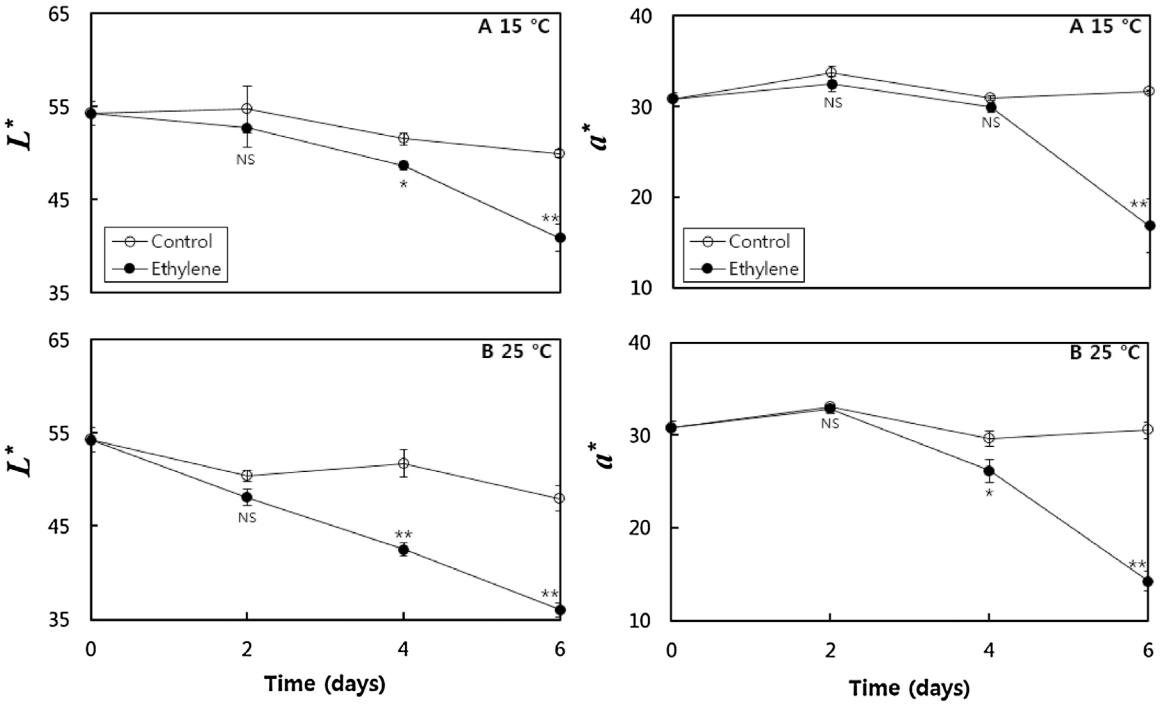

Fig. 3. Hunter L^* and a^* value of 'Daebong' persimmon fruit as affected by ethylene treatment during ripening at 15 °C and 25 °C. Vertical bars represent standard errors of the means (n = 15). A and B indicate 15 °C and 25 °C treatment group, respectively. NS,* and ** indicate non significant at P > 0.05, significant at P < 0.05 and P < 0.01, respectively.

6.7 g kg^{-1} and 8.0 g kg^{-1} in control and treated fruit, respectively, at 25 °C, after 6 d of ripening (Fig. 4). The changes of fructose content were similar to that of glucose content. Fructose content increased from 2.5 g kg^{-1} immediately after harvest to 5.0 g kg^{-1} and 6.0 g kg^{-1} after 6 d of ripening at 15 °C in the control and treated fruit, respectively (Fig. 4). At 25 °C, it was increased to 5.5 g kg^{-1} and 6.6 g kg^{-1} in the control and treated fruit respectively. The contents of glucose and fructose in treated fruit were higher than those of the control fruit during ripening. It increased after 4 d of ripening at 15 °C, and after 2 d of ripening at 25 °C. More than 90% of free sugars accounts for glucose and fructose in persimmon fruit. The increase in free sugars during the ripening period was interpreted as a result of sucrose being converted into glucose and fructose under the action of invertase as the fruit ripened.

3.4. Water soluble tannin

Soluble tannins are the main astringent compounds in persimmon

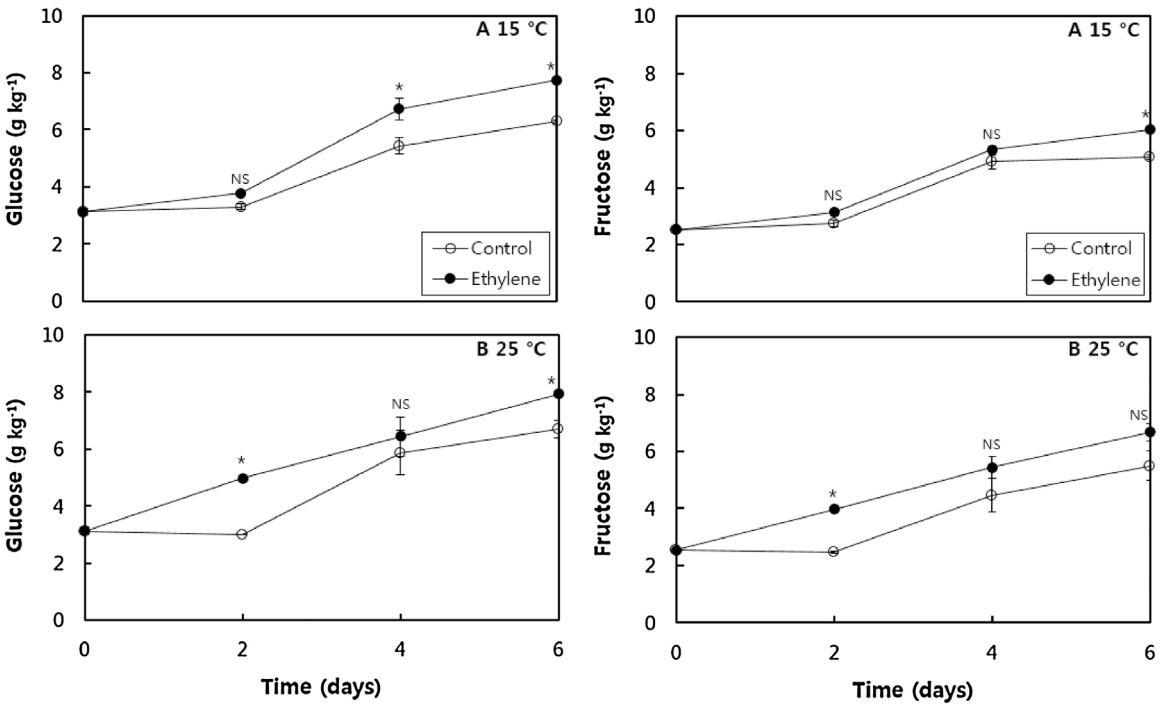

Fig. 4. Glucose and fructose content of 'Daebong' persimmon fruit as affected by ethylene treatment during ripening at 15 °C and 25 °C. Vertical bars represent standard errors of the means (n = 15). A and B indicate 15 °C and 25 °C treatment group, respectively. NS,* and ** indicate non significant at P > 0.05, significant at P < 0.05 and P < 0.01, respectively.

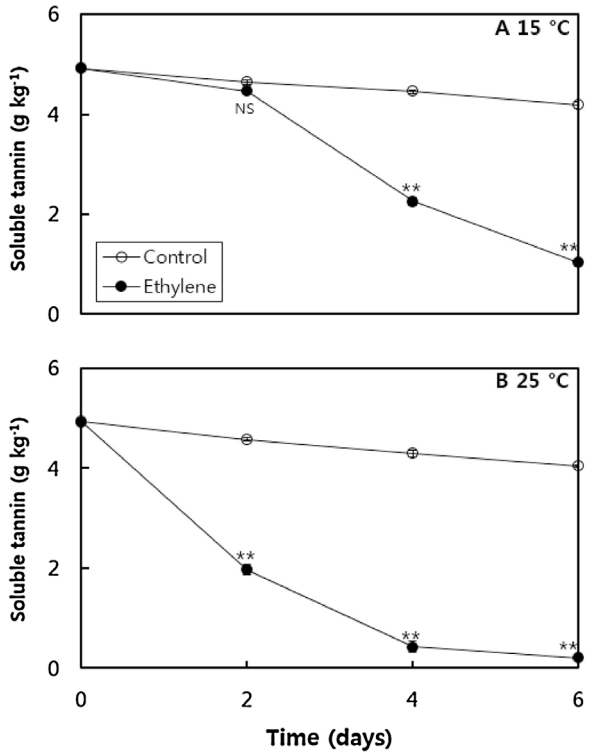

Fig. 5. Soluble tannin content of 'Daebong' persimmon fruit as affected by ethylene treatment during ripening at 15 °C and 25 °C. Vertical bars represent standard errors of the means (n = 15). A and B indicate 15 °C and 25 °C treatment group, respectively. NS,* and ** indicate non significant at $P > 0.05$, significant at $P < 0.05$ and $P < 0.01$, respectively.

fruit which have been used to evaluate the effectiveness of astringency removal treatments and the astringency level of fruit (Yamada et al., 2002; Salvador et al., 2007). In the present study, the water soluble tannin content at harvest was 4.8 g kg^{-1} (Fig. 5). At 15 °C, the soluble tannin was reduced slightly to 4.2 g kg^{-1} within 2 d of ripening and significant differences were not observed between the control and treated fruit. After 2 d the water soluble tannin content in the control fruit did not show a significant difference. However, water soluble content in the treated fruit declined to 1.06 g kg^{-1} on day 6. At 25 °C, soluble tannin contents decreased sharply after 2 d and reached 0.47 g kg^{-1} on the 4 d. On the other hand, 4 g kg^{-1} of soluble tannin was measured on the 6 d in untreated fruit (Fig. 5). The astringent taste of persimmon is associated with soluble tannin and the loss of astringency is due to reaction between the acetaldehyde produced in the fruit and the soluble tannins (Seo et al., 1999; Plaza et al., 2012). Kato (1990) reported that ethanol treatment combined with ethylene had a greater effect than ethanol alone on astringency removal. The finding of present study on ethylene treated fruit is similar to that of Yin et al. (2012) who reported the reduction in soluble tannin content from 14.5 g kg^{-1} to 3.9 g kg^{-1} with ethylene treatment after 3 d of ripening. Oshida et al. (1996) also reported rapid decrease of soluble tannin content during the on-tree treatment of astringency removal by enclosing fruit in polyethylene bags (1 fruit/bag) containing 5 mL of 5% (v/v) ethanol in water. The slight changes in soluble tannin content in the control group could be due to slow ripening of persimmon.

3.5. Ethylene-related genes expression

Ethylene action is achieved by regulating ethylene receptors and triggering of signal transduction reactions, and ultimately by controlling relevant gene expression in the fruit (Solano et al., 1988; Bleecker and Kende, 2000). Therefore, real-time PCR was used to investigate the

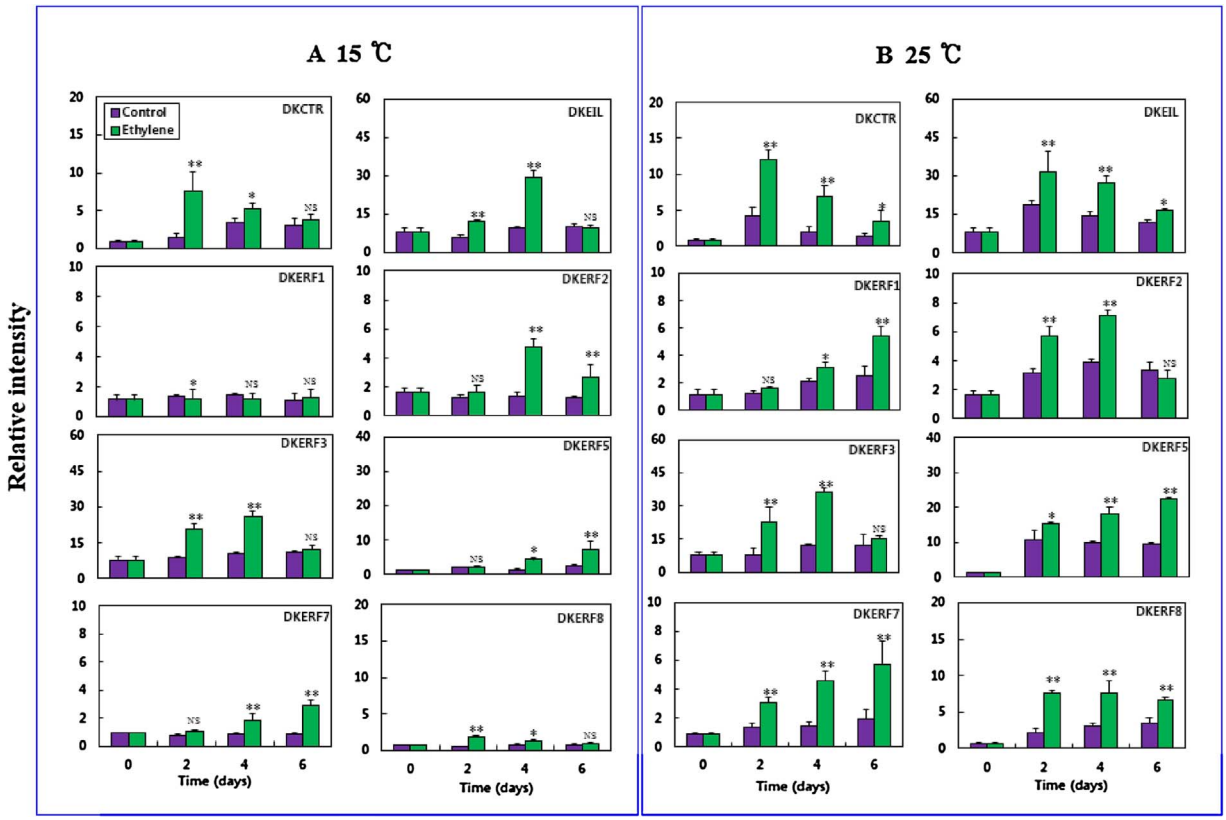

Fig. 6. Transcript accumulation of targeted genes of 'Daebong' persimmon fruit as affected by ethylene treatment during ripening at 15 °C and 25 °C. Vertical bars represent standard errors of the means (n = 3). A and B indicate 15 °C and 25 °C treatment group, respectively. NS,* and ** indicate non significant at $P > 0.05$, significant at $P < 0.05$ and $P < 0.01$, respectively.

effect of the expression of eight ethylene receptor genes by ethylene treatment and temperature on fruit quality. Expression of ethylene response genes during ripening was generally higher in the ethylene treated fruit than in the control fruit regardless of temperature (Fig. 6) Ethylene treated fruit showed stronger expression consistently in *DKCTR*, *DKERF2*, *DKERF3*, *DKERF5*, *DKERF7* and *DKERF8* transcript levels toward the end of the ripening period at both temperatures. In ethylene treated fruit, *DKERF 1, 2, 5, 7,* and *8* genes were expressed about twice more at 25 °C than at 15 °C. In addition, the expression patterns of *DKEIL*, *DKERF2*, *DKERF5* and *DKERF8* genes were observed to occur rapidly at 25 °C. Increased levels in expression of *DKCTR*, *DKEIL* and *DKERF* gene families have been shown to correlate with fruit ripening and softening in astringent persimmon (Yin et al., 2012). Similarly we also observed that ethylene treatment led to increases in expression of *DKCTR*, *DKEIL* and *DKERF* genes and encouraged fruit ripening and softening. Hence, these results strongly suggest that the regulation of *DKCTR*, *DKEIL* and *DKERF* genes is one of key ways to control fruit ripening and softening of persimmon. Ethylene treatment also leads to increase in the expression of *DKCTR* gene which is a negative regulator of the ethylene response pathway (Kieber, 1997). It is interesting that a negative regulator of ethylene response pathway is significantly up-regulated by ethylene treatment, but this result is not surprising. Ethylene has a central role in regulating and coordinating many physiological processes including color development, texture change and metabolism shift during ripening in climacteric fruit (Klee and Giovannoni, 2011). Since larger quantities of ethylene can be produced by exogenous ethylene treatment, they can promote modifications of physiological processes. It is possible for the negative expression regulator to be activated for suppression of the ripening process in response to the suddenly increased ethylene content. Because similar pattern of *CTR* gene expression in response to ethylene treatment is also observed in apple (Yang et al., 2013), these results indicate that *CTR* gene may also be involved in the molecular regulation of the ripening process.

4. Conclusions

Removal of astringency from astringent cultivars and ripening for fresh consumption is accompanied by fruit softening. Firmness and water soluble tannin content, which are typical indicators of ripening in astringent persimmon, were 2.35 N and 1.06 g kg^{-1} in 15 °C on 6 d; 2.45 N and 0.47 g kg^{-1} in 25 °C on 4 d, respectively. Using ethylene for astringency removal at 25 °C ripened fruit faster at 2 d than was that seen at 15 °C. Our results suggest that more rapid and higher expression of *DKEIL*, *DKERF2*, *DKERF5* and *DKERF8* genes at 25 °C than at 15 °C may be responsible for the difference. *DKERF5* and *DKERF8* genes in particular were associated with astringency removal and fruit softening, respectively (Yin et al., 2012). These results indicated that ripening quality was improved after 6 d of ripening at 15 °C and within 2–4 d of ripening at 25 °C for utilization of fresh fruit. The findings of this study contribute to understanding of the effect of temperature conditions on the changes in quality and expression of ethylene response genes during ripening and astringency removal by using ethylene.

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