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Expression of Genes Related to Skin Coloration and Sugar Accumulation in Grape Berries at Ripening Stages under High Temperatures

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Abstract. The expression of genes related to ripening of berries was investigated in 'Campbell Early' and 'Kyoho' grapes under different temperature conditions. There was low expression of genes associated with flavonoid compound accumulation and coloration in the berries at the initial ripening stages under high temperatures. Expression of the chalcone synthase, flavone 3-hydrogenase, and polygalacturonase genes was dependent on treated temperatures, and all genes were highly inhibited in berries kept at 35 ℃. While the expression of genes related to stilbene compound accumulation and coloration increased greatly at 30 ℃, it was reduced at 35 ℃ in berries in the active coloration stage. Interestingly, the stilbene synthase gene was highly expressed at 35 ℃ in 'Kyoho' grapes in the ripening stages. Expression of the beta-amylase and polygalacturonase genes increased gradually in the ripening stages, while that of the polygalacturonase gene tended to decrease at 35 ℃ in both grape berries. Future studies investigating the expression levels of various genes can be conducted based on transcriptome analysis of berries at the early veraison stages to obtain meaningful information regarding the grape ripening process under elevated temperatures.

Keywords: Gene, grape, skin coloration, sugar accumulation, high temperature, veraison

1. Introduction

Grape (*Vitis vinifera*) is one of the most economically important fruit crops in the world [1]. Grapes are used to make wine, jam, juice, jelly, grape seed extract, raisins, vinegar, and grape seed oil, as well as consumed raw [2]. During grape production, skin coloration and total soluble solids of the flesh are the most important factors influencing quality. It is well known that coloration of grape berry skin is influenced by various environmental conditions; therefore, coloration is used as an index for ripening [3], [4]. Several studies have reported that normal coloration of grape berry skin resulted from the accumulation of anthocyanins under cool conditions during the ripening stages of grape fruits. While cool conditions are favorable to berry skin coloration, a continuous high temperature generally inhibits accumulation of pigments such as anthocyanins in the skin of grape berries [5]-[7].

Sugar content in grapes is often used as an indicator of maturity and a basis for pricing of grapes during harvest. As a part of the ripening process, changes in the molecular mass and solubility of individual cell-wall constituents of grape berries may also be accelerated from certain stages [8]. While higher anthocyanin contents are found at cooler temperatures, there is little difference in concentrations of sugar under varying temperature regimes, except for a small increase in total soluble solids in the juice at higher temperature [9], [10].

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Table I: NCBI gene accession numbers and sequences of gene primers used for quantitative real-time PCR analysis

Gene	Accession No.	Primer sequences
β-actin	AB372563	5'-ACGAGAAATCGTGAGGGATG-3' 5'-ATTCTGCCTTTGCAATCCAC-3'
β-amylase (*BMY*)	XM_002274576.2	5'-TCCACTCCCAGAATGGGTTAGA-3' 5'-TTTTAAAACCCGCTCCTTGTCA-3'
Polygalacturonase (*PG*)	EU078975.1	5'-AGGTGTGACTCTGACCCCCATA-3' 5'-CGATTGGGAGACTTCTTGTGCT-3
Phenylalanine ammonia-lyase (*PAL*)	X75967.1	5'-TGAACAATGGCGAAAGTGAGAA-3' 5'-TCTCTTGCGCTCTCAACCTCTT-3'
Chalcone synthesis (*CHS*)	EF192464.1	5'-AGTTCAAGCGCATGTGTGAAAA-3' 5'-CTTCAACCACCACCATGTCTTG-3'
Flavanone 3-hydroxylase (*F3H*)	EF192467.1	5'-TGGTCAATTTCTACCCCCAATG-3' 5'-AAGTCTTTCCGCCATCCCTAGT-3'
Stilbene synthase (*STS*)	X76892.1	5'-GGTGCCATTGCAGGAAACTTAC-3' 5'-CAAGTGGGTCAAAAGCCTGAGT-3'

A great deal of study of anthocyanin biosynthesis in grape berries has shown that anthocyanins are synthesized from phenylalanine through activation of various enzymes and expression of genes in the anthocyanin biosynthetic pathway [11], [12]. However, few investigations of the effects of temperature on expression of the anthocyanin biosynthetic pathway genes in grape berries have been conducted to date. Poor coloration occurred in temperate zones with climates favorable to grape growth and ripening. Coloration of 'Kyoho' grape berry skin was also reportedly inhibited or delayed at high temperatures in Korea [10], [13] and Japan [14]. Owing to the potential for climate shift in response to global warming to lead to exposure of grapes to continuous high temperature, the effects of high temperatures on yield and grape berry quality should be considered. Therefore, this study was conducted to examine the expression of genes related to anthocyanin synthesis and sugar accumulation, and to determine at what point from to collect samples for transcriptome analysis of grape berries to determine the effects of high temperatures.

2. Materials and Methods

2.1. Plant Materials and Temperature Treatment

Healthy 'Campbell Early' and 'Kyoho' (*Vitis labruscana* Bailey) grape clusters were harvested at 4 different stages after 35 (veraison) using the modified E-L system [15] at the experimental vineyard of Yeungnam University, Gyeongsan, Korea, after which they were placed in separate growth chambers (Ilshin Tech., Daejeon, Korea) equipped with a temperature controller. The berries were detached from the cluster by cutting the peduncle to avoid berry dehydration and decay, and then maintained at 25, 30, or 35℃. Berries were collected at 0, 12, 24, 48, and 72 h after treatment for RNA extraction.

2.2. RNA Isolation and Real-Time PCR

Total RNA was extracted from grapevine berries using a slightly modified pine tree method [16]. The first-strand cDNA was then synthesized from the total RNA (500 ng) using a GoScriptTM Reverse Transcription System (Promega, Madison, WI, USA) and subsequently used as a template for PCR. Real-time PCR was conducted using a C1000TM Thermal Cycler (BioRad, Hercules, CA, USA) using SYBR Premix Ex (TaKaRa Bio Inc., Osaka, Japan) as the fluorescent dye. Amplification was conducted by subjecting the samples to one cycle of 95℃ for 30 s, followed by 40 cycles of 95℃ for 5 s and 60℃ for 30 s. Transcript levels were calculated using the standard-curve method and normalized against the grapevine beta-actin gene (AB372563) as an internal control, after which melting curves of the amplified products were recorded. Untreated berries (at time zero) were tested as the reference sample. For each gene, the reference sample was defined as the 1x expression level, and the results were expressed as the fold increase in mRNA

over the reference sample. All reactions were performed in triplicate to ensure consistency. Gene specific primer pairs were designed and employed for real-time PCR (Table I).

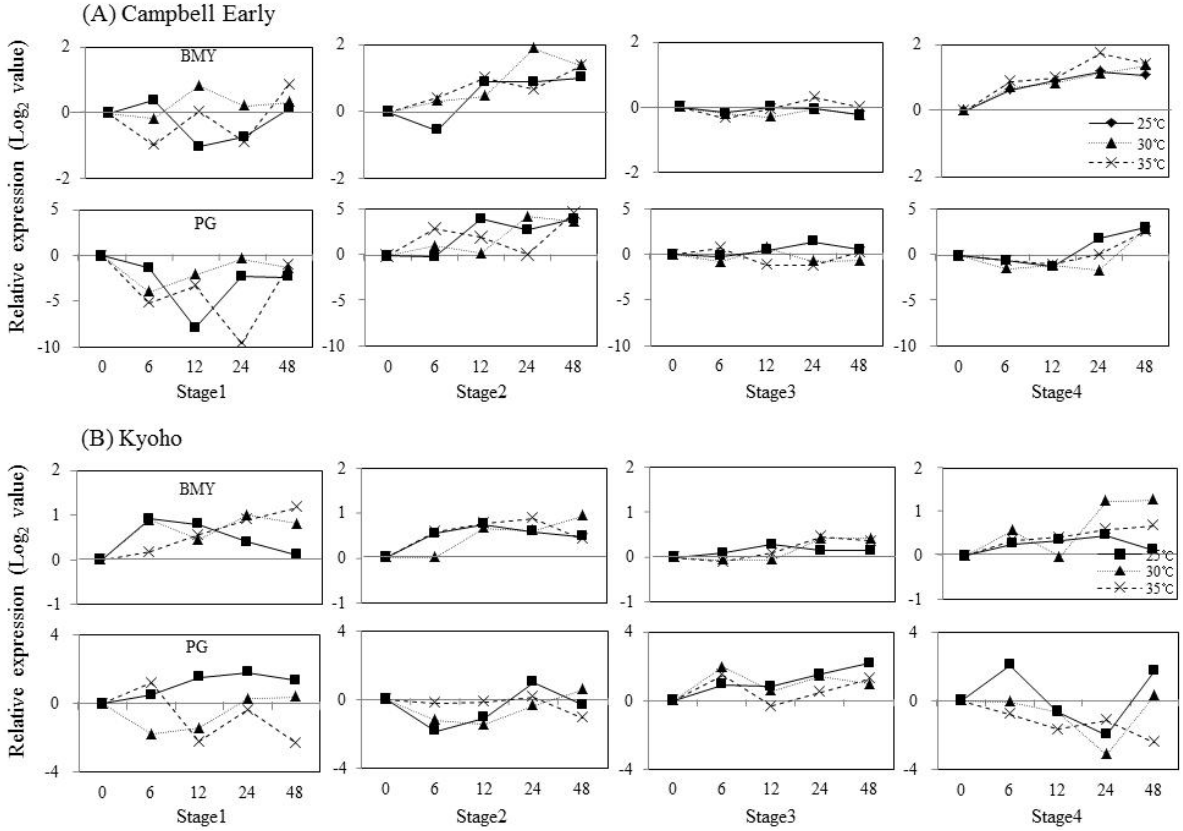


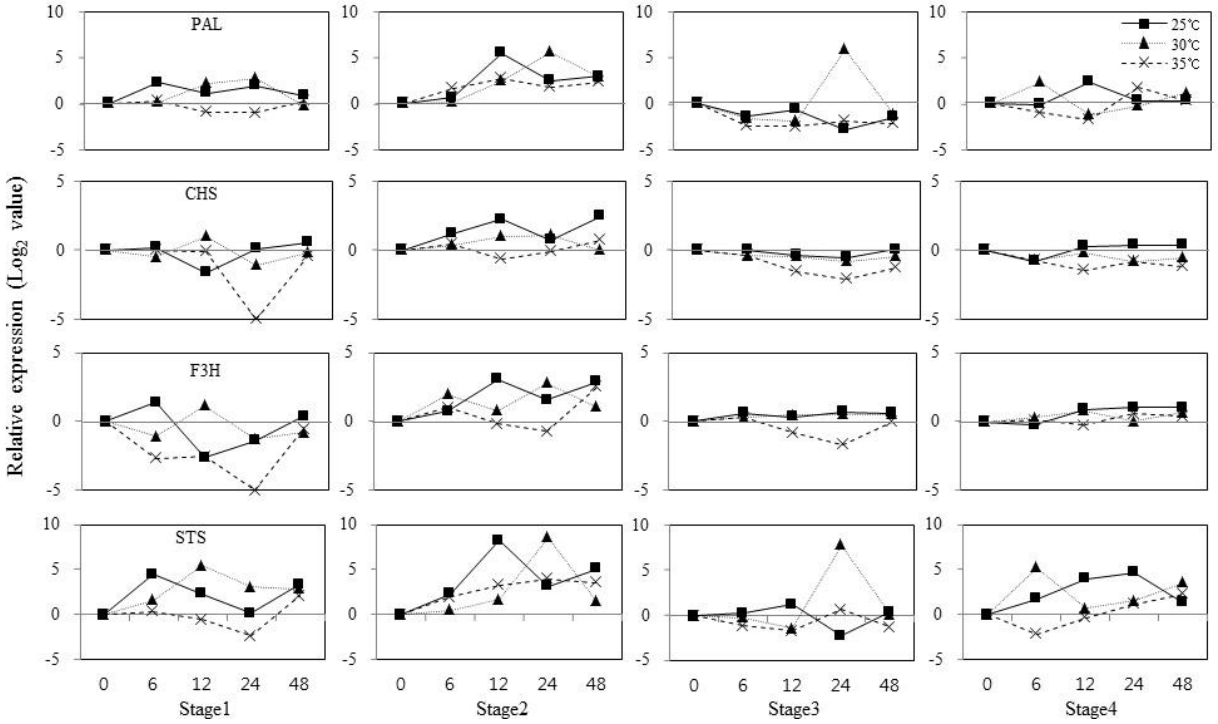
Fig. 1: Expression of beta-amylase (*BMY*) and polygalacturonase (*PG*) genes at different temperatures in the berries of (A) 'Campbell Early' and (B) 'Kyoho' grapes

3. Results and Discussion

To analyze the changes in expression of genes related to the ripening of grapes kept at different high temperatures, the expression of genes such as phenylalanine ammonia-lyase (*PAL*), chalcone synthase (*CHS*), stilbene synthase (*STS*), flavanone 3-hydroxylase (*F3H*), β-amylase (*BMY*), and polygalacturonase (*PG*) was investigated with each specific primer set in 'Campbell Early' and 'Kyoho' grape berries during four ripening stages from veraison. As grape berries ripened, *BMY* gene expression increased gradually in all tested stages in 'Campbell Early' and 'Kyoho' grapes, and there was no significant difference in levels observed at 35 ℃ and 25 ℃ (Fig. 1). However, expression of the *PG* gene tended to be inhibited at high temperature relative to 25 ℃–30 ℃ in both types of grapes. These findings suggest that it would be better to use berry samples collected during the early stage of veraison to obtain meaningful transcript data for elucidation of the changes induced by high temperatures.

In 'Campbell Early' grapes, expression of the *STS* gene showed greater upregulation in the early stages of ripening than in later stages (Fig. 2). Additionally, the *STS* gene was more highly expressed in berries kept at 25 ℃ than at 35 ℃ in the early ripening stages, whereas there was a slight increase in all treated samples during the late ripening stages. Expression of the *F3H* gene was inhibited during ripening at 30 ℃. The *CHS* gene was inhibited at 35 ℃ in all grapes, regardless of ripening stage. Even though the *PAL* gene was highly expressed in all tested stages, its level was lower at 35 ℃ than at 25 ℃–30 ℃. In 'Kyoho' grapes, expression of the *PAL* gene decreased slightly with ripening, and there was little reduction at high temperature (Fig. 2). In agreement with the results observed for 'Campbell Early' grapes, the expression of the *CHS* gene was not high compared to other genes, and it was lower at 35 ℃ than at 25 ℃–30 ℃. The *F3H* gene was slightly expressed in all tested stages, and greatly inhibited at high temperature during the ripening stages, except for the late ripening stage. The *STS* gene was up-regulated at 6 h after temperature treatment, while it gradually decreased at 35 ℃ relative to 25 ℃–30 ℃.

A)



B)

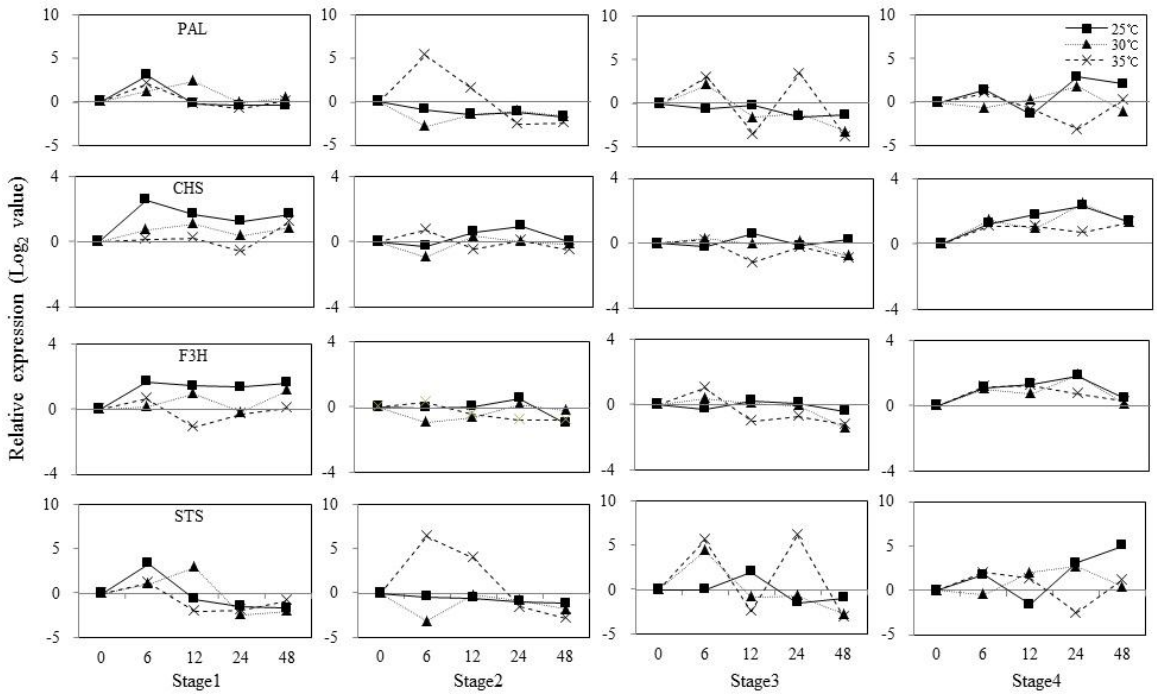


Fig. 2: Expression of phenylalanine ammonia lyase (*PAL*), chalcone synthase (*CHS*), stilbene synthase (*STS*), and flavone 3-hydrogenase (*F3H*) genes at different temperatures in the berries of 'Campbell Early' (A) and 'Kyoho' (B) grapes

As berries ripen, they undergo various changes including softening and coloring of the berry, accumulation of total soluble solids, and reduction of acid contents. The changes in berries are mainly affected by climate and the potential nutrition balance of vines. Many studies have investigated the effects of various temperatures on the metabolism of sugars, acids, and anthocyanins in controlled environments [7], [9], [17].

The increase of total soluble solids in the grapes originates from the storage of carbohydrates, which are broken down into sucrose, glucose and fructose molecules [8]. Moreover, it has been reported that there were no significant changes in cell-wall polysaccharide content, but that modification of polysaccharide components occurred during ripening of grape berries [18]. It has also been suggested that the rapid accumulation of glucose resulted in an increased glucose/fructose ratio and significant increase of soluble solid contents during ripening [19]. Previous studies showed that total sugar contents increased in 'Delaware' grape skin and decreased in 'Muscat Bailey A' grape skin at 30 ℃ relative to 20 ℃, but that no further differences were observed at high temperatures [20]. It has also been reported that there was an increase in total soluble solids in the juice at higher temperature [9], [21]. In this study, *BMY* gene expression increased gradually with no significant reduction at 35 ℃, whereas expression of the *PG* gene was inhibited at 35 ℃ relative to 25 °–30 ℃ in both grape cultivars. Because many enzymes show different expression patterns with respect to the accumulation of sugars in grapes during ripening, the changes in expression levels of various genes should be investigated to analyze the effects of temperature on fruit ripening in grapes.

The skin colors of grape berries vary with varieties and are determined by the anthocyanin contents of skin and pulp of berries. The levels of these compounds are affected by harvesting season, temperature during ripening period, and hormonal changes in grapes [22], [23]. Anthocyanins are synthesized from phenylalanine through enzyme activities and gene expression in grape skins [11], [12], [24], [25]. However, few studies have investigated the effects of temperature on the regulation of anthocyanin biosynthesis in grapes. In this study, expression of genes related to berry skin coloration (*PAL*, *CHS*, *STS*, and *F3H*) increased greatly at 30 ℃, and decreased at 35 ℃ during the active coloration stage. Interestingly, *STS* was highly expressed at 35 ℃ in 'Kyoho' grapes during the ripening stages. The expression levels of other *MYB* related genes and many flavonoid biosynthesis pathway genes were regulated independently by temperature and light [26]. However, it has been reported that the composition of proanthocyanidin was not significantly influenced by temperature regimes because proanthocyanidins were accumulated before veraison [27].

Since temperatures are expected to increase in most parts of the world [28], the effects of climatic changes on yield and fruit quality attributes is of great importance to cultivation of grapes and other fruit crops [10], [29]. Although many grape varieties can reach coloring in unfavorable weather, coloring of some, such as 'Kyoho', is sensitive to temperature [30]. It has been predicted that elevated temperature could change the geographical distribution of grape cultivation in subsequent decades [31]. Among various environmental factors involved in the mechanism of skin coloration and sugar accumulation, temperature has been the primary focus, as well as its combinations with light [21], [26], and water regime [27]. It has also been reported that soluble solid content was highest in the elevated CO_2 group, and lowest in the elevated temperature group [10].

Because many factors influence the ripening of grapes, systematic approaches including the transcriptome analysis from ripening berries can confer useful information for investigation of biochemical changes. For the most effective approach, it is important to properly select a critical point to show various responses. In this study, we determined the most effective ripening stage of grape berry to show changes in responses to high temperature. The results presented herein will be valuable to future studies conducted to elucidate the responses of ripening grape berries to high temperature at the molecular level.

4. Acknowledgements

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