Selection of Differentially Expressed Genes Using the Transcriptome Analysis of Ripening Grape Berries in Response to High Temperature

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ABSTRACT

Purpose : The transcriptome of ‘Campbell Early’ grape berries exposed to high temperature was analyzed to select useful genes by evaluating their differential expression through sequencing short reads on Solexa platform.

Research Method : We assembled 118 million high-quality trimmed reads using Velvet followed by Oases.

Findings : Functional categorization of expressed transcripts revealed the conservation of genes involved in various biological processes like responses to chemical (12.7%), responses to abiotic stimulus (11.8%), biosynthesis processes (11.8%), and cellular metabolic processes (10.4%) in grape berries exposed to high temperature. The major up-regulated genes included isocitratelase, cysteine proteinases superfamily protein, cupin family protein, and glycosyl hydrolase genes, and the major down-regulated genes included flavanone 3-hydroxylase, phenylalanine ammonia lyase, chlorophyll A-B binding family protein, and polygalacturonase inhibiting protein genes in grape berries exposed to high temperature. Among genes related to grape coloration, expressions of chalcone and stilbene synthase, flavanone 3-hydroxylase, leucoanthocyanidin dioxygenase, phenylalanine ammonia lyase genes were more strongly inhibited in berries kept at 35°C than 25°C.

Originality/Value : Transcript sets of grape berries generated in this study will provide a resource for gene discovery and development of functional molecular markers to be used in elucidating the mechanism of tolerance to high temperature.

Keywords: transcriptome, differentially expressed genes, high temperature, coloration

INTRODUCTION

Temperature, broadly considered as a major determining factor in crop production and quality, is expected to increase significantly because of climate change. Climate changes (elevated CO₂, high temperature and moderate drought) have affected grapevine phenology and caused adverse effects in berry development and composition of grapes (*Vitis* spp.), which is one

of the economically most important fruit crops and used as source for jam, juice, seed oil, dried fruits, jelly and wine making, and desert foods in the world (Salazar-Parra *et al.*, 2012).

Heat stress has been found to cause heavy yield losses, accelerate senescence, and diminish seed weight in legumes, which is particularly sensitive at the bloom stage (Siddique *et al.*, 1999). It was reported that temperatures above 35°C generally reduce photosynthesis efficiency in grapevine leaves (Kriedemann, 1986) and it stimulates sugar accumulation and significantly inhibits berry setting by exposure to high

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temperature (Greer and Weston, 2010).

Recently, next-generation sequencing technologies have led to a large amount of sequence data generated in a relatively short period of time resulting in dramatic development in genome and transcriptome research (Huang *et al.*, 2012; Xu, 2014). There are several studies on transcriptome analysis to show the effects of low temperature exposure on leaves and dormant buds (Kim *et al.*, 2016; 2017), the water and salinity stress on vines (Cramer *et al.*, 2007), and to elucidate the transcriptional characteristics in development stages (Zenoniet *et al.*, 2010) of *Vitis* spp. using RNA sequencing.

Transcriptome analysis of heat stress effects on *Arabidopsis* (Lim *et al.*, 2006), rice (Yamakawa *et al.*, 2007), and tomato (Frank *et al.*, 2009) have been reported. Although various genes with different expression patterns were studied from analysis of transcripts related to heat stress (Kim *et al.*, 2015; Liu *et al.*, 2012), transcriptome analysis from grapes exposed to high temperature has not been reported.

High temperatures by global warming have been known to have negative effects of berries in viticulture, accelerating the breakdown of malic acid (Lakso and Kliever, 1978) and transmute of mesophyll cells (Zhang *et al.*, 2005a). Recently, inhibition of normal development of berries and grapevines by high temperature during growing period has caused serious damages including bad coloration, low content of total soluble solids, and high acidity in fruits in grape production (Cramer *et al.*, 2011; Liu *et al.*, 2012; Wahid *et al.*, 2007). For grape production, fruit quality is determined mostly by skin coloration and total soluble solids of the flesh that are influenced by various environmental conditions such as temperature and light (Oanh *et al.*, 2010; Mori *et al.*, 2005; 2007; Son *et al.*, 2014; Bergqvist *et al.*, 2001; Azuma *et al.*, 2012). In Korea, it is reported that grape berries showed discoloration and reduced content of total soluble solids in high temperature conditions during day and night times of ripening stages (Kim *et al.*, 2010; 2012; Park, 2011).

One way to solve the problem is to select new grape cultivars tolerant of high temperature resulted from global climatic changes. Elucidation of molecular mechanisms of berries in response to high temperature is one of the important steps in developing novel cultivars of grapes tolerant of temperature changes, as well as in regulating the expression of genes related to stable ripening development regardless of climatic changes in grape production. However, the molecular mechanisms involved in grapevine tolerance to high temperature, especially at the ripening berry level, have been poorly understood. Therefore, this study was conducted to understand the mechanism of responses to high temperature in grapevines, to detect differentially expressed genes in grapevines following high temperature treatment, and to select useful genetic resources for breeding programs of heat-tolerant grapes.

MATERIALS AND METHODS

Plant materials and heat treatment

Healthy ‘Campbell Early’ (*Vitis labruscana* Bailey) grape clusters were harvested at ripening stages after veraison stage (the onset of berry ripening) of the modified E-L system (Coombe, 1995) at the experimental vineyard of Yeungnam University, Gyeongsan, Korea, after which they were placed in separate growth chambers (Ilshin Tech., Daejeon, Korea) equipped with 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, and 35°C. Berries were collected at 0, 6, 12, 24, 36 and 48h after treatment for RNA extraction.

RNA extraction and cDNA synthesis

Total RNA from grapevine berries was extracted using a protocol by Chang *et al.*, (1993) with some modifications. The concentration and quality of the total RNA was determined by a Nano Drop spectrophotometer (ND-1000, Technologies Inc., USA) and checked by

electrophoresis in 1% (w/v) agarose gels. First-strand cDNA was synthesized from 500 ng total RNA using a GoScript™ Reverse Transcription System (GoScript™ Reverse Transcription System, Promega, Madison, WI, USA) and was subsequently used as a template for PCR.

Transcriptome analysis

Libraries were subsequently prepared using the TruSeq SBS v5 protocol, and more than 118 mega sequencing paired-end reads were generated using an Illumina HiSeq 2000 (Illumina, San Diego, CA). After performing quality check and trimming (SolexaQA package) of the raw data, reference genes were mapped using Bowtie (version; 0.12.7). Gene expression was measured using the number of short reads, selection of differentially expressed genes (DEGs), clustering, and annotation. Differentially expressed genes were identified by the DESeq Bioconductor package (version 1.6.0) (Anders and Huber, 2010) with an FDR (false discovery rate) and were determined using the Benjamini-Hochberg multiple tests of 1% ($P < 0.05$) based on read counts and a minimal 1.5-fold change in at least one pair-wise comparison. Pearson's correlation hierarchical clustering was used to analyze gene expression patterns. Genes expressed by grapevines treated with high temperature were annotated using BLASTN and BLASTX. Annotation information was connected to gene ontology (GO) and KEGG pathway. All samples were analyzed in triplicate to ensure consistency of the results.

Real-time PCR analysis

Real-time PCR with first-strand cDNA synthesis from RNA was performed using C1000™ Thermal Cycler (CFX96™ Real-Time System, BioRad, Hercules, California, USA) system and SYBR Premix Ex (SYBR Premix Ex Taq, TaKaRa Bio Inc., Osaka, Japan) as the fluorescent dye in 20 µL reactions. The PCRs

were performed in thermocycler conditions starting with 30 s at 95°C, 40 cycles of 5 s at 95°C and 30 s at 60°C, followed by 5 s at 95°C. The relative transcripts levels were calculated for treated samples relative to the non-treated samples according to the standard-curve method. All samples were analyzed in triplicate to ensure consistency of the results.

RESULTS

Transcripts obtained from ripening berries of 'Campbell Early' grapevines treated with high temperature (25, 30 and 35°C) for 24 hours were analyzed to identify genes showing significant differential expression. The transcripts trimmed from the grapevine cDNA library are summarized in Table 01. The 118 million reads were trimmed to extract high quality sequences using SolexaQA. There were more than 6 million transcripts from high temperature treatment, and average read-length ranged from 87.63 to 90.63bp. Overall, the analyzed reads were found to cover 78.86-84.44% of the raw data.

Genes with differential expression patterns at high temperature compared to 25°C treatment were selected according to their transcriptional levels (Table 02). Among the selected differentially expressed genes (DEGs), 270 genes were found to be up-regulated, whereas 448 genes were down-regulated at high temperature. Among them, 77 genes showed commonly up- and down-regulation under high temperature treatment. UDP-Glycosyltransferase protein, RmlC-like cupins protein, RING/U-box superfamily protein, phosphotyrosine protein phosphatase superfamily proteins, and pectin lyase-like protein were redundant in both up- and down-regulations (data not shown).

Table 01: Statistical data from the transcriptome analysis of grape treated athigh temperatures.

Sample description	No. of reads	Total length (bp)	Avg. length (bp)	Trimmed/ Raw (%)
Campbell Early-25°C	6,767,335	613,346,344	90.63	84.44
Campbell Early-30°C	6,231,642	546,110,989	87.63	78.86
Campbell Early-35°C	6,672,262	588,673,605	88.23	79.79

Table 02: Genes with differential expression patterns athigh temperatures in ‘Campbell Early’ grape.

	Regulation pattern	No. of DEGs	No. of annotated DEGs
25°C vs. 30°C	Up	149	146
	Down	265	250
25°C vs. 35°C	Up	270	255
	Down	448	434

Annotated functions of DEGs from berries of grapes treated with high temperature were assigned by mapping unigenes onto the gene ontology (GO) structure using the TGICL (TIGR Gene Indices Clustering Tools). On the basis of GO analysis, 4,509 unigenes were identified

under high temperature treatments. Induced unigenes with assigned putative functions were classified into three ontologies; biological processes (531 genes), cellular components (973 genes) and molecular functions (224 genes) (Figure 01).

Figure 01: Gene ontology (GO) classification of induced transcripts in ‘Campbell Early’ grape berries treated with high temperature at the level 3.

Figure 02: Gene ontology (GO) classification of inhibited transcripts in ‘Campbell Early’ grape berries treated with high temperature at the level 3.

Genes encoding cellular macromolecule process (75), biosynthetic process (73), and protein metabolic process (65) were the most enriched among the biological processes GO terms. Within the cellular component GO terms, genes related to intracellular (128), intracellular part (125), and intracellular organelle (111) were enriched. Under the molecular functions GO terms, purine nucleotide binding (52), ribonucleotide binding (51), and purine nucleoside binding (46) were the most represented. Inhibited transcripts were also classified into three ontologies; biological processes (816 genes), cellular components (1,807 genes), and molecular functions (164 genes) (Figure 01). The categories of the majority of DEGs include those associated with cellular ketone metabolic process (42), organic acid metabolic process (41) and carbohydrate metabolic process (38) under the biological processes GO terms; intracellular (200), intracellular part (198), and intracellular organelle (176) under the cellular component GO terms; and active transmembrane transporter (22), coenzyme binding (21), and oxidoreductase activity (16) under the molecular functions GO terms.

Ten highly represented enzymes with putative functions from nucleotide sequences of the DEGs are shown in Table 03. Several frequently annotated enzymes such as pyruvate kinase, beta-aspartyl-peptidase, and H^+ -exporting were induced, while nitrilase, alcohol dehydrogenase, and tropinone reductase 1 were suppressed in response to stresses from high temperature in ripening ‘Campbell Early’ grapes. After exposure to high temperature for 24 hours, 10 genes highly induced or inhibited in grapes compared to the control were selected for further analysis (Table 04). Genes encoding isocitratelase, cystein proteinases protein, and cupin family protein were induced genes in response to high temperature. On the other hand, genes of flavanone 3-hydroxylase, PHE ammonia lyase 1, and chlorophyll A-B binding protein were shown to be inhibited in response to high temperature. The selected genes were also evaluated for their differential expression levels by reverse transcription followed with real-time PCR using the primer pairs listed in Table 05, which were designed based on the transcript sequences. The results of real-time PCR analysis using gene-specific primers showed

that the 10 up- and down-regulated genes were levels were similar to those determined from differentially expressed, and their expression the transcriptome analysis (Figure 03).

Table 03: Up- and down-regulated differentially expressed genes (DEGs) related to Enzyme Commission (EC) numbers identified by UniprotKB in high temperature-treated grapevines.

	EC	Enzyme	No. of DEGs
Up-regulated	2.7.1.40	Pyruvate kinase	2
	3.4.19.5	Beta-aspartyl-peptidase	2
	3.6.3.6	H ⁺ -exporting ATPase	2
	3.6.3.8	Ca ²⁺ -transporting ATPase	2
	6.1.1.3	Threonine---tRNA ligase	2
	6.1.1.6	Lysine---tRNA ligase	2
	1.1.1.1	Alcohol dehydrogenase	1
	1.1.1.40	Malate dehydrogenase (oxaloacetate-decarboxylating) (NADP ⁺)	1
	1.1.1.86	Ketol-acid reductoisomerase	1
	1.2.1.3	Aldehyde dehydrogenase (NAD ⁺)	1
Down-regulated	3.5.5.1	Nitrilase, cyanoalaninenitrilase	8
	1.1.1.1	Alcohol dehydrogenase	4
	1.1.1.206	Tropinone reductase I	4
	1.1.1.37	Malate dehydrogenase	4
	1.1.3.15	(S)-2-hydroxy-acid oxidase	3
	2.1.1.104	Caffeoyl-CoA O-methyltransferase	3
	3.6.3.14	H ⁺ -transporting two-sector ATPase	3
	1.2.1.3	Aldehyde dehydrogenase (NAD ⁺)	3
	1.1.1.14	L-iditol 2-dehydrogenase	2
	1.1.1.40	Malate dehydrogenase (oxaloacetate-decarboxylating) (NADP ⁺)	2

Table 04: The major 10 genes highly induced and inhibited in ‘Campbell Early’ grape 24 hours after exposure to high temperature.

Gene			Transcripts at		fold Change
			25°C	35°C	
Induced	U1	Isocitratelase	1	868	784.70
	U2	Cysteine proteinases superfamily protein	6	642	109.34
	U3	Cupin family protein	19	718	37.36
	U4	Glycosyl hydrolase 9B15	30	1,016	33.57
	U5	Beta-xylosidase 2	320	3,596	11.23
	U6	Aspartic proteinase A1	141	1,538	10.91
	U7	UDP-Glycosyltransferase superfamily protein	87	715	8.22
	U8	Dicer-like 2	181	1,410	7.79
	U9	Tubulin beta 8	395	2,571	6.51
	U10	BCL-2-associated athanogene 6	2,065	11,615	5.63
Inhibited	D1	Flavanone 3-hydroxylase	2,899	177	0.061
	D2	PHE ammonia lyase 1	2,110	161	0.076
	D3	Chlorophyll A-B binding family protein	1,307	134	0.102
	D4	Polygalacturonase inhibiting protein 1	577	65	0.113
	D5	Phenylalanine ammonia-lyase 2	1,974	274	0.139
	D6	Chalcone and stilbene synthase family protein	2,050	287	0.140
	D7	Myo-inositol-1-phosphate synthase 2	2,514	356	0.142
	D8	Cytochrome B5 isoform D	2,803	415	0.148
	D9	Nodulin MtN21 /EamA-like transporter family protein	934	151	0.161
	D10	Caffeoyl-CoA 3-O-methyltransferase	886	145	0.164

Table 05: Sequence of primers from transcripts used for real-time PCR analysis.

Name	Primer sequences	Name	Primer sequences
Up-regulated		Down-regulated	
U1	GGGAGGAGAGGAACAATGGAGT CCTCCCTGGACAGTCTTGAGAA	D1	GAGAGAGCAGGCAGAGTCCATC GGGATCTGATGCCAAGAATTTG
U2	GAAAATGAAGCGGTTTTTCGTG ATCCCAACAACCAAAACCAATG	D2	GGGAGCTTCACCCGTCTAGATT CGTGCTCGCGGTCTACTACTTT
U3	GGGTACATGGAGATGGCTTGTC AAGAGCATCCCACGTTTCAGAG	D3	ACACAGTAGCATCGCCATAGCA ATTCTTAGCGAGTCGTGCAAGG
U4	GGGCTCATACTTCATCCACCAC GTCGTTGTTTCCATCTCCAACC	D4	CCAACATCGTTGACTTCTCACG GGTCCAACGACGTCAAACCTCTT
U5	GGCGTACGTAAGAGGTCTCCAA ACAACATGCGGCTACCTTCAAT	D5	CTGTGACCAACCATGTCCAGAG CACGGCTTCAGCAGTTTTTCTT
U6	CCAAGTCCAATGGGAGAATCAG TACTCTTTTGGGGTCAGGGTGA	D6	TCCTGGACGAAATGAGGAAAAA ACCTGGTCCAAATCCAAACAGA
U7	ATTGACATGGAACATCGGTCT GAAAGAAGTTCGGCGAGTTTGA	D7	GTTGCCACCATCCTCAGTTACC CCAATCCAACACAAGCCCTTAG
U8	CAGCAAGCAGAAGCTGTGAAAA TCCCATATCTCCCAATACGTG	D8	CGGTTTCTTCTTCGGCTACAGA AAAGAGGGATGGGGATGAAAAA
U9	CGGAGTTGATTGACTCGGTTCT CAATTCTCAGCCTCCTTCTCA	D9	CAATGGTTTTGACAGCAGCAAG GGTGGCACTGAAGAAGGACAGT
U10	GTAGAGCCTCATGCAGCAGACA CCCTCTAGGCCAGAACCAGAAT	D10	GAGAGCAGGCAGAGTCCATCTC GGGATCTGATGCCAAGAATTTG

Primer set used for validation are listed in Table 4.

Figure 03: Verification of the expression levels of up-regulated (left) and down-regulated (right) genes in ‘Campbell Early’ grape berries in response to high temperature by quantitative real-time PCR analysis. Genes amplified by each primer set are listed in Table 3. Each vertical bar indicates the SEs (n = 3). Up-regulated (U1-U10) and down-regulated (D1-D10) genes are listed in Table, 04.

The DEGs were subdivided into four major groups in cluster analysis based on their expression profiles (Table 06). Among 883 DEGs, 338 DEGs were subdivided into cluster 2 which showed a highly inhibited expression pattern, and 137 DEGs were subdivided into cluster 4 which showed highly induced expression patterns by high temperature. The

cluster 2 with major significantly induced genes by high temperature in ‘Campbell Early’ grapes included those encoding terpene synthase 21, nitrilase 4, and flavanone 3-hydroxylase, while the cluster 4 with inhibited genes included those encoding nodulin MtN3 family protein, 3-ketoacyl-CoA synthase 2, and beta-xylosidase 2.

Table 06: Clustering and major DEGs identified in response to high temperature of ‘Campbell Early’ grapevine berries.

Expression pattern	DEGs
<p>Cluster 1 (198 genes)</p>	<p>O-fucosyltransferase family protein Disease resistance protein (TIR-NBS-LRR class) ARM repeat superfamily protein NAP1-related protein 2 Disease resistance protein (TIR-NBS-LRR class), putative Cytochrome P450, family 81, subfamily D, polypeptide 3 Pyridoxal phosphate (PLP)-dependent transferases protein Heavy metal transport/detoxification superfamily protein Heat shock protein 70 (Hsp 70) family protein Tubulin beta 8</p>
<p>Cluster 2 (338 genes)</p>	<p>Terpene synthase 21 Nitrilase 4 Flavanone 3-hydroxylase UDP-glucosyl transferase 78D2 Myo-inositol-1-phosphate synthase 2 Beta-galactosidase 3 Cytochrome B5 isoform D Chlorophyll A-B binding family protein Caffeoyl-CoA 3-O-methyltransferase Chalcone and stilbene synthase family protein</p>
<p>Cluster 3 (210 genes)</p>	<p>Cupredoxin superfamily protein Terpene synthase 04 Amino acid permease 2 Cysteine-rich RLK (RECEPTOR-like protein kinase) 8 SKU5 similar 5 GroES-like zinc-binding alcohol dehydrogenase family protein Ubiquitinating-conjugating enzyme 2 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase protein Sucrose synthase 4 Chitinase family protein</p>
<p>Cluster 4 (137 genes)</p>	<p>Nodulin MtN3 family protein 3-ketoacyl-CoA synthase 2 Beta-xylosidase 2 UDP-Glycosyltransferase superfamily protein Glycosyl hydrolase 9B15 Aspartic proteinase A1 Glutamine-dependent asparagine synthase 1 Cupin family protein Cysteine proteinases superfamily protein Isocitratelase</p>

Table 07: Coloration related genes and primer sequences of genes used for real time pcr analysis.

Gene	Primer sequences
Caffeoyl-CoA 3-O-methyltransferase (CCOAMP)	GAGAGAGCAGGCAGAGTCCATC GGGATCTGATGCCAAGAATTTG
Chalcone-flavanone isomerase (CFI)	ACAGTACGGGGTGCAGCTAGAG CCAAAGCTTCCTCCTCCTCTTC
Chalcone and stilbene synthase (CSS)	TCCTGGACGAAATGAGGAAAAA ACCTGGTCCAAATCCAAACAGA
Class-II DAHP synthetase (DAHP)	GCAGAGAGTCACCCAGTGGAAT CTGTGAGTCCAGCAGCAGACAT
Dihydroflavonol 4-reductase (DFR)	AATTATACGGCAGGGCCAATTT ACGTCCCTCTGCCTTAGGATTC
Flavanone 3-hydroxylase (F3H)	TTCAGCAACGAAATTCCTGTCA TTTCCTGCAAATCTCATCACGA
Leucoanthocyanidin dioxygenase (LDOX)	CCCATGGAGGAAAAGGAGAAGT CCACTCAAGCTGTCCACTAGCA
PHE ammonia lyase 1 (PAL1)	GGGAGCTTCACCCGTCTAGATT CGTGCTCGCGGTCTACTACTTT
Phenylalanine ammonia-lyase 2 (PAL2)	CTGTGACCAACCATGTCCAGAG CACGGCTTCAGCAGTTTTTCTT
Shikimate kinase 1 (SK1)	TCTTGTTGGGATGATGGGTCT GCTCCACCAATGTGTCACTGTC
UDP-glucosyl transferase 78D2 (UGT78D2)	GGCAGTTGGTCCTAAAGGGAGT CATTGCATCCAAACAGGTGGTA

Among high temperature induced DEGs, genes related to skin coloration of grape berries were selected from the results of transcriptome analysis. Gene-specific primer pairs were designed by nucleotide sequence alignment and employed for real-time PCR (Table 07). The expression of genes such as caffeoyl-CoA 3-O-methyltransferase (CCOAMP), chalcone-flavanone isomerase (CFI), chalcone and stilbene synthase (CSS), Class-II DAHP synthetase (DAHP), dihydroflavonol 4-reductase (DFR), flavanone 3-hydroxylase (F3H), leucoanthocyanidin dioxygenase (LDOX), phenylalanine ammonia lyase 1 (PAL1), PAL2, shikimate kinase 1 (SK1), UDP-glucosyl transferase 78D2 (UGT78D2) was investigated with each specific primer set in ‘Campbell Early’ grape berries during the

initial ripening stage where the grapes were kept at high temperatures under 16/8 h light/dark photoperiod (Figure 04) and under continuous dark conditions (Figure 05).

The expressions of all tested genes were inhibited when grapes were kept at 35°C during the initial ripening stages compared to berries kept at 25°C. The expressions of CSS, F3H, LDOX, and PAL genes were more highly inhibited in berries kept at 35°C than 25°C. On the other hand, the SK1 gene was more highly expressed in berries kept at 35°C than at 25°C. However, there was no significant difference between grape berries kept under alternate photoperiod condition and ones kept under dark condition at high temperatures in expression of genes related with coloration.

Figure 04: Comparison of expression of genes related to skin coloration by quantitative real-time PCR analysis in 'Campbell Early' grapes of initial ripening stages kept at 25, 30, and 35°C under light at 0-6, 24, and 48 h, and under dark condition at 12 h and 36 h.

Transcript levels were calculated using the standard curve method from triplicate data with the actin gene as the internal control and untreated berries (at time zero) as the reference sample. Results represent the mean fold increase in mRNA level over that of the untreated berries, which was defined as the 1× expression level. Results are the means of triplicate data from three experiments. Bars indicate the standard deviation. Caffeoyl-CoA 3-O-methyltransferase (A), chalcone-flavanone isomerase (B), chalcone and stilbene synthase (C), Class-II DAHP synthetase (D), dihydroflavonol 4-reductase (E), flavanone 3-hydroxylase (F), leucoanthocyanidin dioxygenase (G), PHE ammonia lyase 1 (H), phenylalanine ammonia-lyase 2 (I), shikimate kinase 1 (J), and UDP-glucosyl transferase 78D2 (K) genes.

Figure 05: Comparison of expression of genes related to skin coloration by quantitative real-time PCR analysis in 'Campbell Early' of initial ripening stages kept at 25, 30, and 35°C under continuous dark conditions.

Transcript levels were calculated using the standard curve method from triplicate data with the actin gene as the internal control and untreated berries (at time zero) as the reference sample. Results represent the mean fold increase in mRNA level over

that of the untreated berries, which was defined as the 1× expression level. Results are the means of triplicate data from three experiments. Bars indicate the standard deviation. Caffeoyl-CoA 3-O-methyltransferase (A), chalcone-flavanone isomerase (B), chalcone and stilbene synthase (C), Class-II DAHP synthetase (D), dihydroflavonol 4-reductase (E), flavanone 3-hydroxylase (F), leucoanthocyanidin dioxygenase (G), PHE ammonia lyase 1 (H), phenylalanine ammonia-lyase 2 (I), shikimate kinase 1 (J), and UDP-glucosyl transferase 78D2 (K) genes.

DISCUSSION

Biotic or abiotic stresses result in significantly reduced productivity and cause adverse growth effects in crop plants. Among various environmental factors, high temperatures caused by global warming is expected to become a major issue in yield reduction and poor quality of fruits in coming years (Wahid *et al.*, 2007; White *et al.*, 2006; Zhang *et al.*, 2005b). High temperatures may cause various types of harm to the crops, including smaller internodes, early senescence, increased tillering, and reduced total biomass in sugarcane plants (Ebrahimet *al.*, 1998). Zhang *et al.*, (2005a) reported that heat stress led to the damage of the mesophyll cells and increased permeability of plasma membrane in grape.

In the present study, we analyzed the transcriptome of ripening grape berries in response to high temperature conditions. Among DEGs responding to high temperature, genes encoding isocitratelase, cystein proteinases protein, and cupin family protein were induced; and lavanone 3-hydroxylase, PHE ammonia lyase 1, and chlorophyll A-B binding protein were inhibited genes in response to high temperature.

Isocitratelase (ICL), a key enzyme in the glyoxylate cycle, was reported to be induced under conditions of high temperature in *Mycogacterium avium* and *Mycobacterium tuberculosis* (HonerzuBentrupet *al.*, 1999). In Chinese cabbage, antioxidative enzyme 2-cysteine peroxiredoxin (2-Cys Prx) was reported to switch to a molecular chaperone upon heat shock stress (Kim *et al.*, 2009).

Using both cDNA microarray and RNA gel-blot analyses in rice, Rabbani *et al.*, (2003) found that 32, 62, and 57 genes were regulated by cold, drought, and high-salinity, respectively. Glycoside hydrolases included in carbohydrate

metabolism category are up-regulated commonly by cold, drought, and high-salinity stresses (Rabbani *et al.*, 2003).

Mori *et al.*, (2007) reported that anthocyanin biosynthetic genes such as chalcone synthase (CHS), F3H, and glutathione S-transferase (GST), with the exception of UDP-glucose flavonoid 3-O-glucosyltransferase (UGT), were inhibited under high temperature when compared with the control. PAL is a key enzyme in phenylpropanoid metabolism of grape berry. PAL and CHS activities have been shown to be accumulated in leaves of Arabidopsis at low temperature (Leyva *et al.*, 1995). Induction of flavonoids and phenylpropanoids was considered as the main acclamatory response of cells against heat stress in watermelon (Riveroet *al.*, 2001).

The expression of the chlorophyll a and b has been repressed more profoundly in developed leaves compared to developing leaves at high temperature in maize (Karim *et al.*, 1997; 1999). The heat shock proteins (HSPs), ranging in molecular weight from about 10 to 200 kDa, have chaperone-like functions and are involved in signal transduction during heat stress (Schofflet *al.*, 1999). In *Arabidopsis thaliana*, expression levels of 165 genes were changed by heat stresses, and especially those of HSPs were highly induced (Lim *et al.*, 2006). The transcriptome analysis of grapevine has showed that HSP20, monothiol glutaredoxin, and ripening-related protein were differentially expressed in response to heat stress at 45°C for 5 hr 30 min (Liu *et al.*, 2012), while genes encoding isocitratelase, cysteine proteinases superfamily protein, and cupin family proteins were differentially induced in this study.

In this study, we used grapevines exposed to high temperature at 35°C for 24 hr for transcriptome analysis. Among annotated DEGs responding to

high temperature, 270 genes were up-regulated and 448 genes were down-regulated specifically in ‘Campbell Early’ grapevines exposed to high temperature. We also investigated the expression pattern of several coloration related genes during the ripening in high temperature condition. During grape production, skin coloration of the flesh are one of the most important factors influencing quality. The expression of genes related to the skin coloration, such as DFR, F3H, and PAL genes were induced at 25°C in initial ripening stage. Azuma *et al.*, (2012) reported that high temperature or dark treatments inhibited the expression of flavonoid biosynthesis-related genes (CHS, CHI, F3H, DFR, LDOX, GST, and FLS4) in grape skin. Previous studies showed that expression of genes (PAL, CHS, STS, and F3H) related to skin coloration decreased in ‘Campbell Early’ and ‘Kyoho’ grape skin at 35°C (Kim *et al.*, 2015). In this study, genes involved in flavonoid and stilbene synthesis pathways were also inhibited in both transcriptome analysis and real-time PCR analysis of grapevines exposed to high temperature. Therefore, it suggests that discoloration of berry skin and poor quality of fruits resulted, from the inhibition of flavonoid and chalcone synthesis genes that are critical to grape production.

The expression levels of genes involved in flavonoid biosynthesis pathway were regulated by temperature and light in grape skin (Azuma *et al.*, 2012). Azuma *et al.*, (2012) found that the flavonol content increased under light irradiation when compared to dark condition in grapes. Czemmelet *et al.*, (2009) reported that expressions of both VvFLS1 and VvMYBF1 were highly induced in cell culture of grapevines after exposure to light. In this regard, flavonoid biosynthesis can be induced by sunlight and UV-B light in ‘Shiraz’ and ‘Cabernet Sauvignon’ grape berries even during developmental stages (Downey *et al.*, 2004; Matuset *et al.*, 2009).

Leyva *et al.*, (1995) reported that PAL and CHS mRNAs were accumulated in response to low temperature in a light-dependent manner in *Arabidopsis thaliana*. It suggested that flavonoid biosynthesis can be regulated by light and temperature in grape berries in developmental stages consistently with other plants including *Arabidopsis*.

CONCLUSIONS

In this study, transcriptomes of ‘Campbell Early’ grapevine berries exposed to high temperatures were analyzed by NGS. DEGs responsive to high temperature were selected from transcripts and their differential expressions under high temperature were confirmed by real time-PCR in grapes. The results suggested possible relationships between coloration of berry skins and high temperature growth conditions for grapevines. The results of this study will be helpful in elucidating the roles of genes with different expression patterns in tolerance to heat during grape ripening and in understanding the transcriptional network regulated by high temperatures in grapevines. Additionally, the investigation of transcriptional changes by high temperature at different ripening stages will provide further valuable information in breeding of grapes tolerant to high temperature in ripening stage. A number of valuable genes selected for specific expression in heat tolerant grapevines can be used as candidates of selection of biomarkers for breeding new grapevines tolerant to high temperature.

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REFERENCES

- Anders, S. and Huber, W. (2010). Differential expression analysis for sequence count data. *Journal of Genome Biology*.11: R106. <https://doi.org/10.1186/gb-2010-11-10-r106>.
- Azuma, A., Yakushiji, H., Koshita, Y. and Kobayashi, S. (2012). Flavonoid biosynthesis-related genes in grape skin are differentially regulated by temperature and light conditions. *Journal of Planta*.236: 1067-1080. <https://doi.org/10.1007/s00425-012-1650-x>.02.07.2015.
- Bergqvist, J., Dokoozlian, N. and Ebisuda, N. (2001). Sunlight exposure and temperature effects on berry growth and composition of Cabernet Sauvignon and Grenache in the central San Joaquin valley of California. *Journal of American Journal of Enology and Viticulture*.52:1-7.
- Chang, S., Puryear, J. and Cairney, J. (1993) A simple and efficient method for isolating RNA from pine trees. *Journal of Plant Molecular Biology*.11,113-116.
- Coombe, B.G. (1995) Adoption of a system for identifying grapevine growth stages. *Australian Journal of Grape and Wine Research*.1,104-110. <https://doi.org/10.1111/j.1755-0238.1995.tb00086.x>.
- Cramer, G.R., Ergül, A., Grimplet, J., Tillett, R.L., Tattersall, E.A.R., Bohlman, M.C., Vincent, D., Sonderegger, J., Evans, J., Osborne, C., Quilici, D., Schlauch, K.A., Schooley, D.A. and Cushman, J.C. (2007) Water and salinity stress in grapevines: early and late changes in transcript and metabolite profiles. *Journal of Functional Integrated Genomics*.7,111-134. <https://doi.org/10.1007/s10142-006-0039-y>.
- Cramer, G.R., Urano, K., Delrot, S., Pezzotti, M. and Shinozaki, K. (2011) Effects of abiotic stress on plants: a systems biology perspective. *Journal of BMC Plant Biology*.11, 163. <https://doi.org/10.1186/1471-2229-11-163>.
- Czemmel, S., Stracke, R., Weisshaar, B., Cordon, N., Harris, N.N., Walker, A.R., Robinson, S.P. and Bogs, J. (2009) The grapevine R2R3-MYB transcription factor *VvMYB1* regulates flavonol synthesis in developing grape berries. *Journal of Plant Physiology*.151, 1513-1530. <https://doi.org/10.1186/1471-2229-11-163>.07.02.2017.
- Downey, M.O., Harvey, J.S. and Robinson, S.P. (2004) The effect of bunch shading on berry development and flavonoid accumulation in Shiraz grapes. *Australian Journal of Grape and Wine Research*.10,55-73. <https://doi.org/10.1111/j.1755-0238.2004.tb00008.x>.
- Ebrahim, M.K., Zingsheim, O., El-Shourbagy, M.N., Moore, P.H. and Komor, E. (1998) Growth and sugar storage in sugarcane grown at temperatures below and above optimum. *Journal of Plant Physiology*.153,593-602. [https://doi.org/10.1016/S0176-1617\(98\) 80209-5](https://doi.org/10.1016/S0176-1617(98) 80209-5).
- Frank, G., Pressman, E., Ophir, R., Althan, L., Shaked, R., Freedman, M., Shen, S. and Firon, N. (2009) Transcriptional profiling of maturing tomato (*Solanum lycopersicum* L.) microspores reveals the involvement of heat shock proteins, ROS scavengers, hormones, and sugars in the heat stress response. *Journal of Experimental Botany*.60,3891-3908. <https://doi.org/10.1093/jxb/erp234>.
- Greer, D.H. and Weston, C. (2010) Heat stress affects flowering, berry growth, sugar accumulation and photosynthesis of *Vitis vinifera* cv. Semillon grapevines grown in a controlled environment. *Journal of Functional Plant Biology*.37,206–214. <https://doi.org/10.1071/FP09209>.

- HonerzuBentrup, K., Miczak, A., Swenson, D.L. and Russell, D.G. (1999) Characterization of activity and expression of isocitratelase in *Mycobacterium avium* and *Mycobacterium tuberculosis*. *Journal of Bacteriology*.181, 7161–7167.
- Huang, W., Li, L., Myers, J.R. and Marth, G.T. (2012) ART: A next-generation sequencing read simulator. *Journal of Bioinformatics*.28, 593-594. <https://doi.org/10.1093/bioinformatics/btr708>.
- Karim, M.A., Fracheboud, Y. and Stamp, P. (1997) Heat tolerance of maize with reference of some physiological characteristics. *Journal of Annals of Bangladesh Agriculture*.7, 27–33.
- Karim, M.A., Fracheboud, Y. and Stamp, P. (1999) Photosynthetic activity of developing leaves of *Zea mays* less affected by heat stress than that of developed leaves. *Journal of Physiologia Plantarum*.105,685–693. <https://doi.org/10.1034/j.1399-3054.1999.105413.x>.
- Kim, S.A., Ahn, S.Y. and Yun, H.K. (2016) Transcriptome analysis of grapevine shoots exposed to chilling temperature for four weeks. *Journal of Horticulture, Environment and Biotechnology*.57, 161-172. <https://doi.org/10.1007/s13580-015-0118-5>.
- Kim, S.A., Ahn, S.Y. and Yun, H.K. (2017) Transcriptomic changes in dormant buds of two grapevine cultivars following exposure to freezing temperature. *Journal of Horticulture, Environment and Biotechnology*.58, 152-161. <https://doi.org/10.1007/s13580-017-0147-8>.
- Kim, S.A., Ahn, S.Y., Han, H.H., Son, I.C. and Yun, H.K. (2015) Expression of genes affecting skin coloration and sugar accumulation in grape berries at ripening stages under high temperatures. *Journal of Advances in Environmental Research*.87,25-31. <http://dx.doi.org/10.4236/wjet.2015.33B002>.
- Kim, S.H., Choi, I.M., Cho, J.G., Han, J.H., Hwang, J.H., Seo, H.H. and Yun, H.K. (2012) Correlation analysis between fruit quality of ‘Campbell Early’ grapes and climatic factors. *Korean Journal of Agriculture and Forest Meteorology*.13,93-100. <https://doi.org/10.5532/KJAFM.2011.13.2.093>.
- Kim, S.H., Choi, I.M., Han, J.H., Cho, J.G., Son, I.C. and Do, K.R. (2010) Effect of climatic condition of 2010 on fruit yield and quality. *Korean Journal of Horticultural Science and Technology*.29(S1),127-128.
- Kim, S.Y., Jang, H.H., Lee, J.R., Sung, N.R., Lee, H.B., Lee, D.H., Park, D.J., Kang, C.H., Chung, W.S., Lim, C.O., Yun, D.J., Kim, W.Y., Lee, K.O. and Lee, S.Y. (2009). Oligomerization and chaperone activity of a plant 2-Cys peroxiredoxin in response to oxidative stress. *Journal of Plant Science*.177,227-232. <https://doi.org/10.1016/j.plantsci.2009.05.010>.
- Kriedemann, P.E. (1986) Photosynthesis in vine leaves as a function of light intensity, temperature, and leaf age. *Journal of Vitis*.7,213-220.
- Lakso, A.N. and Kliwer, W.M. (1978) The influence of temperature on malic acid metabolism in grape berries. II. Temperature responses of net dark CO₂ fixation and malic acid pools. *American Journal of Enology and Viticulture*.29, 145-149.

- Leyva, A., Jarillo, J.A., Salinas, J. and Martinez-Zapater, J.M. (1995) Low temperature induces the accumulation of *Phenylalanine ammonia-lyase* and *chalcone synthase* mRNA of *Arabidopsis thaliana* in a light-dependent manner. *Journal of Plant Physiology*. 108, 39-46. <http://dx.doi.org/10.1104/pp.108.1.39>.
- Lim, C.J., Yang, K.A., Hong, J.K., Choi, A.S., Yun, D.J., Hong, J.C., Chung, W.S., Lee, S.Y., Cho, M.J. and Lim, C.O. (2006) Gene expression profiles during heat acclimation in *Arabidopsis thaliana* suspension-culture cells. *Journal of Plant Research*. 119, 373-383. <https://doi.org/10.1007/s10265-006-0285-z>.
- Liu, G.T., Wang, J.F., Cramer, G., Dai, Z.W., Duan, W., Xu, H.G., Wu, B.H., Fan, F.G., Wang, L.J. and Li, S.H. (2012) Transcriptomic analysis of grape (*Vitis vinifera* L.) leaves during and after recovery from heat stress. *Journal of Plant Biology*. 12, 174. <https://doi.org/10.1186/1471-2229-12-174>. 03.07.2014.
- Matus, J.T., Loyola, R., Vega, A., Pena-Neira, A., Bordeu, E., Arce-Johnson, P. and Alcalde, J.A. (2009) Post-veraison sunlight exposure induces MYB-mediated transcriptional regulation of anthocyanin and flavonol synthesis in berry skins of *Vitis vinifera*. *Journal of Experimental Botany*. 60, 853-867. <https://doi.org/10.1093/jxb/ern336>.
- Mori, K., Goto-Yamamoto, N., Kitayama, M. and Hashizume, K. (2007) Loss of anthocyanins in red-wine grape under high temperature. *Journal of Experimental Botany*. 58, 1935-1945. <https://doi.org/10.1093/jxb/erm055>. 23.12.2014.
- Mori, K., Sugaya, S. and Gemma, H. (2005) Decreased anthocyanin biosynthesis in grape berries grown under elevated night temperature condition. *Journal of Scientia Horticulturae*. 105, 319-330. <https://doi.org/10.1016/j.scienta.2005.01.032>. 06.01.2017
- Oanh, V.T.K., Matsumoto, K., Hwang, Y.S. and Chun, J.P. (2010) Changes of cell wall polysaccharides during berry ripening in grapes (*Vitis* spp.). *Journal of Horticulture, Environment and Biotechnology*. 51, 513-519.
- Park, S.J. (2011) Study on environmental factors on sugar contents and coloration of grapes. Annual Reports of Fruits Research, National Institute Horticultural and Herbal Science. 896-901.
- Rabbani, M.A., Maruyama, K., Abe, H., Khan, A., Katsura, K., Ito, Y., Yoshiwara, K., Seki, M., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2003) Monitoring expression profiles of rice genes under cold, drought, and high-salinity stresses and abscisic acid application using cDNA microarray and RNA gel-blot analyses. *Journal of Plant Physiology*. 133, 1755-1767. <http://dx.doi.org/10.1104/pp.103.025742>. 08.12.2016.
- Rivero, R.M., Ruiz, J.M., Garcia, P.C., Lopez-Lefebvre, L.R., Sanchez, E. and Romero, L. (2001) Resistance to cold and heat stress: accumulation of phenolic compounds in tomato and watermelon plants. *Journal of Plant Science*. 160, 315-321. [https://doi.org/10.1016/S0168-9452\(00\)00395-2](https://doi.org/10.1016/S0168-9452(00)00395-2). 03.07.2014.
- Salazar-Parra, C., Aguirreolea, J., Sa'nchez-Dí'az, M., José' Irigoyen, J. and Morales, F. (2012) Climate change (elevated CO₂, elevated temperature and moderate drought) triggers the antioxidant enzymes response of grapevine cv. Tempranillo, avoiding oxidative damage. *Journal of Physiologia Plantarum*. 144, 99-110. <https://doi.org/10.1111/j.1399-3054.2011.01524.x>. 03.10.2016.

- Schoffl, F., Prandl, R. and Reindl, A. (1999) Molecular responses to heat stress. In: Shinozaki, K., Yamaguchi-Shinozaki, K. (Eds.), *Molecular Responses to Cold, Drought, Heat and Salt Stress in Higher Plants*. R.G. Landes Co, Austin, Texas, 81–98 pp.
- Siddique, K.H.M., Loss, S.P., Regan, K.L. and Jettner, R.L. (1999) Adaptation and seed yield of cool season grain legumes in Mediterranean environments of south-western Australia. *Australian Journal of Agricultural Research*.50, 375–387. <https://doi.org/10.1071/A98096>.
- Son, I.C., Han, J.H., Kim, S.H., Oh, S.I., Moon, K.H. and Choi, I.M. (2014) Effects of the elevated temperature and carbon dioxide on vine growth and fruit quality of ‘Campbell Early’ grapevines (*Vitislabruscana*). *Korean Journal of Horticultural Science and Technology*.32, 781-787. <https://doi.org/10.7235/hort.2014.13059>.
- Wahid, A., Gelani, S., Ashraf, M. and Foolad, M.R. (2007) Heat tolerance in plants: An overview. *Journal of Environmental and Experimental Botany*.161,199-223. <https://doi.org/10.1016/j.envexpbot.2007.05.011>.
- White, M.A., Diffenbaugh, N.S., Jones, G.V., Pal, J.S. and Giorgi, F. (2006) Extreme heat reduces and shifts United States premium wine production in the 21st century. *Proceedings of National Academy of Science of the United States America*.103, 11217-11222. <https://doi.org/10.1073/pnas.0603230103.07.03.2016>.
- Xu, J. (2014) *Next-generation sequencing: Current Technologies and Applications*.Caister Academic Press, Ontario, Canada.
- Yamakawa, H., Hirose, T., Kuroda, M. and Yamaguchi, T. (2007) Comprehensive expression profiling of rice grain filling-related genes under high temperature using DNA microarray. *Journal of Plant Physiology*.144,258-277. <http://dx.doi.org/10.1104/pp.107.098665>. 26.09.2016.
- Zhang, J.H., Huang, W.D., Liu, Y.P. and Pan, Q.H. (2005a) Effects of temperature acclimation pretreatment on the ultrastructure of mesophyll cells in young grape plants (*Vitisvinifera*L. cv. Jingxiu) under cross-temperature stresses. *Journal of Integrated Plant Biology*.47, 959-970. <https://doi.org/10.1111/j.1744-7909.2005.00109.x>.
- Zhang, Y., Mian, M.R., Chekhovskiy, K., So, S., Kupfer, D., Lai, H. and Roe, B.A. (2005b) Differential gene expression in *Festuca* under heat stress conditions. *Journal of Experimental Botany*.56,897–907. <https://doi.org/10.1093/jxb/eri082>.
- Zononi, S., Ferrarini, A., Giacomelli, E., Xumerle, L., Fasoli, M., Malerba, G., Bellin, D., Pezzotti, M. and Delledonne, M. (2010) Characterization of transcriptional complexity during berry development in *Vitisvinifera* using RNA-Seq. *Journal of Plant Physiology*.152,1787-1795. <http://dx.doi.org/10.1104/pp.109.149716>.