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Screening Differential Expressions of Defense-related Responses in Cold-treated 'Kyoho' and 'Campbell Early' Grapevines

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Abstract. Low temperature is one of the major environmental factors that affect productivity including reduced growth and budding of vines, and changes of metabolic processes in grape (*Vitis* spp.). To screen the specific expression of abiotic stress-related genes against cold treatment in 'Kyoho' and 'Campbell Early' grapevines, expression of various defense-related genes was investigated by RT-PCR and real-time PCR. Among the 67 genes analyzed by RT-PCR and real-time PCR, 17 and 16 types of cDNA were up-regulated, while 5 and 6 types were down-regulated in cold-treated 'Kyoho' and 'Campbell Early' grapevines, respectively. Genes encoding carotene (*Cart3564* and *Cart4472*), chalcone isomerase (*CHI*), cytochrome P450 (*CYP*), flavonol synthase (*FLS*), endo-*β*-glucanase precursor (*Glu*), glutathione peroxidase (*GPX*), glutathione-*S*-transferase (*GST*), leucine-rich repeats (*LRR*), manganese superoxide dismutase (*Mn-SOD*), phenylalanine ammonia lyase (*PAL*), polygalacturonase-inhibiting protein (*PGIP*), proline rich protein 2 (*PRP2*), small heat shock protein (*sHSP*), temperature induced lipocalin (*TIL*), and thaumatin-like protein (*TLP*) were up-regulated, while those encoding CBF like transcription factor (*CBF1*), chitinase-like protein (*CLP*), cold induced protein (*CIP*), glycerol-3-phosphate acyltransferase (*GPAT*), and mitogen-activated protein kinase (*MAPK*) were down-regulated by low temperature treatment in both in 'Kyoho' and 'Campbell Early'.

***Additional key words*:** abiotic stress, low temperature, real-time PCR, *Vitis*

Introduction

Grapes (*Vitis* spp.) are the most widely cultivated and economically important fruit crop worldwide (Vivier and Pretorius, 2002). *Vitis vinifera* cultivars grow well in temperate, semi-arid climates that can sometimes experience freezing or subfreezing temperatures during winter (Tillett et al., 2012). One of the most variable environmental factors to which plants are exposed is temperature. Changes in temperature affect many metabolic processes in plant cells, and may also modify structural components in plants (Hughes and Dunn, 1990).

Low temperature is one of the major environmental factors affecting the growth and development of plants and significantly constraining the spatial distribution of plants and agricultural productivity. When a plant is exposed to suboptimal low temperatures, many cellular functions are disturbed (Feng et al., 2009), leading to various changes in the hormone levels (Chandler and Robertson, 1994), membrane lipid composition (Lynch and Steponkus, 1987), and antioxidants levels (Pennycooke et al., 2005). The accumulation of compatible osmolytes, such as soluble sugars, betaine, and proline, also occurs in cold-stressed plants (Dorffling et al., 1997; Koster and Lynch, 1992; Nomura et al., 1995).

Screening expression of specific genes that respond to cold can provide helpful resources in breeding programs of cold-tolerant grapes, and can aid in our understanding of molecular mechanisms of responses to cold in plants. Therefore, in this study, we screened differentially expressed genes in grapevines following cold treatment to provide useful information and increase the efficiency at developing new grape cultivars with improved cold tolerance through molecular breeding programs.

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Materials and Methods

Plant Materials and Cold Treatment

Grapevines of 'Kyoho' (sensitive to cold stress) and 'Campbell Early' (moderately tolerant to cold stress) (Kang et al., 2002) with 8-10 true leaves were grown in a greenhouse maintained at 4°C under 16:8 light:dark illumination for 0, 1, 2, 3, and 4 weeks, respectively. Leaves were harvested from the vines at 1, 2, 3, and 4 weeks, immediately frozen in liquid nitrogen, and then stored at -80°C for future use. Leaves harvested from untreated vines were used as an untreated control.

RNA Isolation and Semi-quantitative RT-PCR and Real-time PCR Analysis

Total RNA was extracted from grapevine leaves using a modified version of the method described by Chang et al. (1993). The differential expression of genes was screened by semi-quantitative RT-PCR using gene specific primer pairs. First-strand cDNA was synthesized from the total RNA (500 ng) using a PrimeScript^{TM} 1st strand cDNA synthesis kit (Takara Bio Inc., Japan) and subsequently used as a template for PCR. The actin gene primers were used as an internal control. PCR was conducted by subjecting the samples to the following conditions: initial denaturation for 5 min at 94°C followed by 30 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min and then final extension for 7 min at 72°C. The PCR products were subsequently identified by 1% (W/V) agarose gel electrophoresis with 0.5X TBE running buffer. Quantitative real-time PCR was conducted on a C1000^{TM} Thermal Cycler (BioRad, USA) using SYBR Premix Ex (TaKaRa Bio Inc., Japan) as the fluorescent dye. All reactions were performed in triplicate to ensure consistency of the results. Amplification was carried out as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 60°C for 30 s. Transcript levels were calculated using the standard-curve method and normalized using grapevine actin gene (AB372563.1) as internal control and melting curves of the amplified products were recorded.

Results and Discussion

Overall, the expression of 67 genes related to defense to biotic or abiotic stress in plants were evaluated following cold treatment of 'Kyoho' and 'Campbell Early' grapevines using semi-quantitative reverse transcription PCR (RT-PCR).

Among the genes screened, those encoding carotene (*Cart3564* and *Cart4472*), chalcone isomerase (*CHI*), cytochrome P450 (*CYP*), flavonol synthase (*FLS*), endo-β-glucanase precursor (*Glu*), glutathione peroxidase (*GPX*), glutathione-*S*-transferase (*GST*), leucine-rich repeats (*LRR*), manganese superoxide

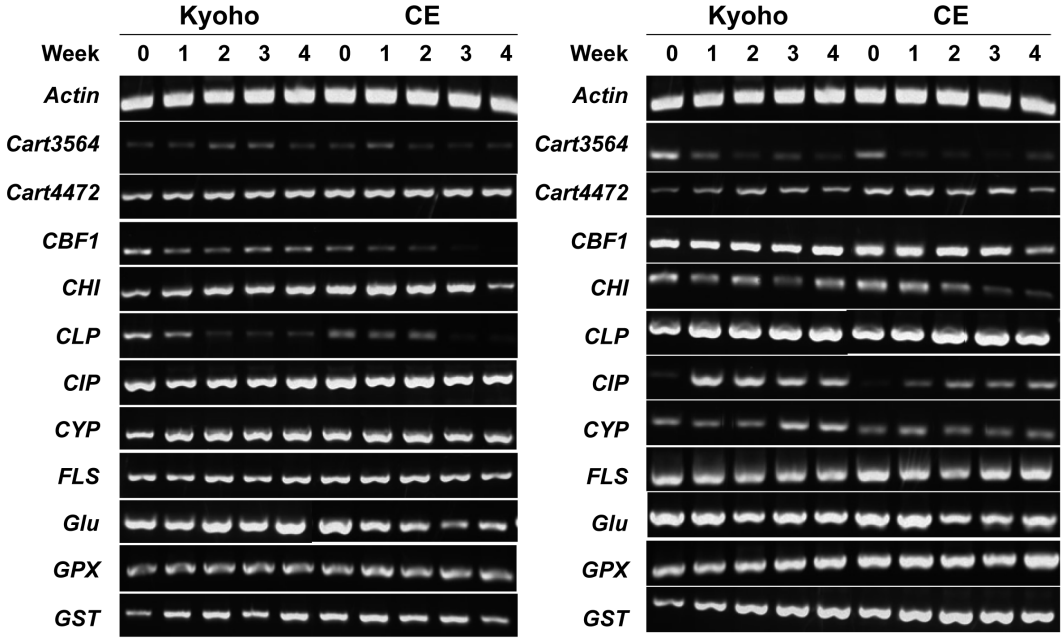

Fig. 1. Semi-quantitative RT-PCR analysis to screen genes induced in shoots of 'Kyoho' and 'Campbell Early' grapevines in response to cold. *Cart3564* and *Cart4472*, carotene; *CBF1*, CBF like transcription factor; *CHI*, chalcone isomerase; *CLP*, chitinase-like protein; *CIP*, cold induced protein; *CYP*, cytochrome P450; *FLS*, flavonol synthase; *Glu*, endo-β-glucanase precursor; *GPX*, glutathione peroxidase; *GST*, glutathione-*S*-transferase; *GPAT*, glycerol-3-phosphate acyltransferase; *LRR*, leucine-rich repeats; *Mn-SOD*, manganese superoxide dismutase; *MAPK*, mitogen-activated protein kinase; *PAL*, phenylalanine ammonia lyase; *PGIP*, polygalacturonase-inhibiting protein; *PRP*2, proline rich protein 2; *P5CS*, pyrroline-5-carboxylate synthase; *sHSP*, small heat shock protein; *TIL*, temperature induced lipocalin; *TLP*, thaumatine-like protein.

dismutase (*Mn-SOD*), phenylalanine ammonia lyase (*PAL*), polygalacturonase-inhibiting protein (*PGIP*), proline rich protein 2 (*PRP2*), small heat shock protein (*sHSP*), temperature induced lipocalin (*TIL*), and thaumatin-like protein (*TLP*) were up-regulated, while genes encoding CBF like transcription factor (*CBF1*), chitinase-like protein (*CLP*), cold induced protein (*CIP*), glycerol-3-phosphate acyltransferase (*GPAT*), and mitogen-activated protein kinase (*MAPK*) were down-regulated by low temperature treatment in both 'Kyoho' and 'Campbell Early' (Fig. 1 and Table 1).

Table 1. Sequences of gene specific primers used for RT-PCR analysis in this study.

Name (accession no.)	Primer sequences
Carotene 3564 (XM002265752.2)	5'-TGCTACTGAAGTTATGATTGGAAA-3' 5'-CTCTAAACATGCAACTAATCAGC-3'
Carotene 4472 (XM002274326.2)	5'-TATTGACCGCAACTTTCGTATTTA-3' 5'-TCATGAAGTTGAAAGTACCAGAGA-3'
CBF like transcription factor (*CBF1*) (AY390372.1)	5'-CTTAAGAAATCCAGGATATGGCTA-3' 5'-TTAAACCTTGCATATTGAACATTG-3'
Chalcone isomerase (*CHI*) (XM002282072.2)	5'-TCCCATCTCTCCTTCAACCA-3' 5'-TATCCCCGAAGATGTCTCCA-3'
Chitinase-like protein (*CLP*) (XM002269123.1)	5'-GAAGCCATTGGTGAAGGTGT-3' 5'- GGGTGGCGTTCTGTTCTATG-3'
Cold induced protein (*CIP*) (XM002283501.1)	5'-CCAAGTGTTTGGGAGTCCAT-3' 5'-GCAGAACCCCTCTTCTTTGA-3'
Cytochrome p450 (*CYP*) (CAB85635.1)	5'-AAGCAACGGTTACAGCTAAG-3' 5'-GCCATATCTGTCTTCCATGT-3'
Endo-β-glucanase precursor (*Glu*) (AB601116.1)	5'-TTGGTAACCCTGAAAGCTGA -3' 5'-ACACCATATCCATGGTAGCC-3'
Flavonol synthase (*FLS*) (XM002285805.1)	5'-AGCGGTACTCAGCAAAGGTT-3' 5'-TGAGAAGGTTGAGTGGGTTG-3'
Glutathione peroxidase (*GPX*) (XM003631370.1)	5'-CAGGGGTTTCCAGTTATTCG-3' 5'-CACCCTCATCATGGTGCTTA-3'
Glutathion-*S*-transferase (*GST*) (AY156048)	5'-GGCGATCAAAGTCCATGGTAG-3' 5'-GCTTCTCCAATCCCTTAACCC-3'
Glycerol-3-phosphate acyltransferase (*GPAT*) (XM002276065.1)	5'-TCTTCCTCTTCATTGTCTTCTTCT-3' 5'-ACATGTAATAGTCAAAGGGCTCTC-3'
Leucine-rich repeats (*LRR*) (XM002285517.2)	5'-TCGTGGAGTGGCTATGACTG-3' 5'-GTGTTGAGAGAACCGCCATT-3'
Manganese superoxide dismutase (*Mn-SOD*) (EU280161.1)	5'-ATAACCCCCTACAACAAAGCCCTA-3' 5'-CTTTCCATACGTTCTTCAGGTAAT-3'
Mitogen-activated protein kinase (*MAPK*) (AY395740.1)	5'-CATAGACACGATTAAAGTGATTGG-3' 5'-GATCTTCACTTCTCCTCTGTGATT-3'
Phenylalanine ammonia lyase (*PAL*) (X75967.1)	5'-CCAGTTCTCAGAGCTTGTTAATGA-3' 5'-ATACATGTTCCCTATCCACCACTT-3'
Polygalacturonase-inhibiting protein (*PGIP*) (AF305093.1)	5'-CTCTCCTCCTCTCTCCTCCT-3' 5'-CGGTGAGGTTAGAGAGCTTG-3'
Proline rich protein2 (*PRP2*) (XR078193.2)	5'-TTGGTAACCCTGAAAGCTGA-3' 5'-ACACCATATCCATGGTAGCC-3'
Pyrroline-5-carboxylate synthase (*P5CS*) (AJ005686.1)	5'-ACTTCAAGAGAGGAGATCCCTAAT-3' 5'-GAATATGATCAATGGCAGAATGTA-3'
Temperature induced lipocalin (*TIL*) (DQ222993.1)	5'-AGATAGCTTCATTTCCCTCATTTT-3' 5'-AAATAGATTTAATCCACCAAATGC-3'
Thaumatin-like protein (*TLP*) (XM002282928.2)	5'- GTCAACCAATGCACCTAC-3' 5'- GGTGGATCATCCTGTGGA-3'
Small heat shock protein (*sHSP*) (XM003634002.1)	5'-AGTCTCTTCGCCCCAATTTC-3' 5'-ACTGAAAGCGCACAAGCACT-3'
β-actin (AB372563.1)	5'-ACGAGAAATCGTGAGGGATG-3' 5'-ATTCTGCCTTTGCAATCCAC-3'

To confirm the RT-PCR results, the relative expression levels of 22 genes were also estimated by real-time PCR in two grapevine cultivars. The mRNA accumulation patterns of 22 genes were consistent with the results of RT-PCR (Figs. 2 and 3). Overall, 17 and 16 types of mRNA in 'Kyoho' and 'Campbell Early', respectively, were up-regulated, while five types of mRNA in 'Kyoho' and six in 'Campbell Early' were down-regulated by low temperature treatment. Among the tested genes, the transcript levels of *Glu*, *GST*, *PGIP*, *PRP2*, and *TLP* increased greatly in 'Kyoho' grapevines treated with low temperature for 2 or 4 weeks, while the transcript levels of *Glu*, *GST*, *PGIP*, *PRP2*, and *TLP* increased greatly in 'Campbell Early' treated with low temperature for 2 or 3 weeks (Fig. 2). However, the total levels of *CBF1*, *CLP*, *CI*, *GPAT*, *MAPK*, and *P5CS* gene expression decreased in response to low temperature treatment when compared

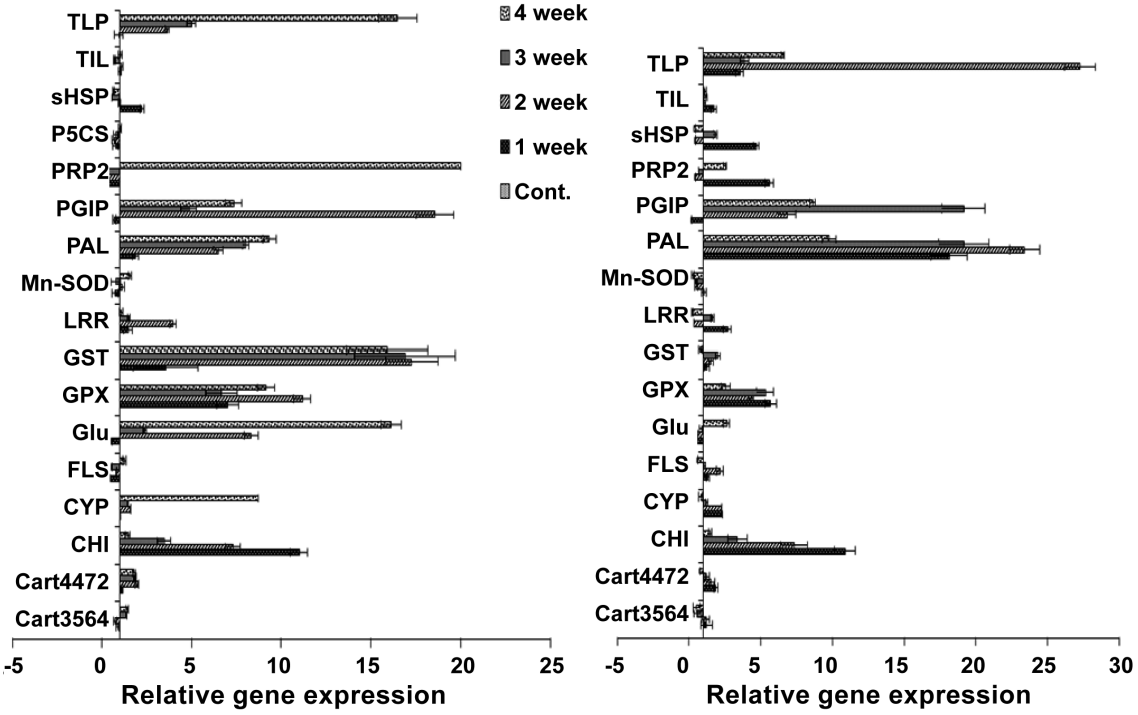

Fig. 2. Quantitative real-time PCR analysis of up-regulated gene expression in 'Kyoho' (left) and 'Campbell Early' (right) in response to cold. *Cart3564* and *Cart4472*, carotene; *CHI*, chalcone isomerase; *CYP*, cytochrome P450; *FLS*, flavonol synthase; *Glu*, endo-β-glucanase precursor; *GPX*, glutathione peroxidase; *GST*, glutathione-*S*-transferase; *LRR*, leucine-rich repeats; *Mn-SOD*, manganese superoxide dismutase; *PAL*, phenylalanine ammonia lyase; *PGIP*, polygalacturonase-inhibiting protein; *PRP*2, proline rich protein 2; *P5CS*, pyrroline-5-carboxylate synthase; *sHSP*, small heat shock protein; *TIL*, temperature induced lipocalin; *TLP*, thaumatine-like protein. Vertical bars indicate the SEs (n = 3).

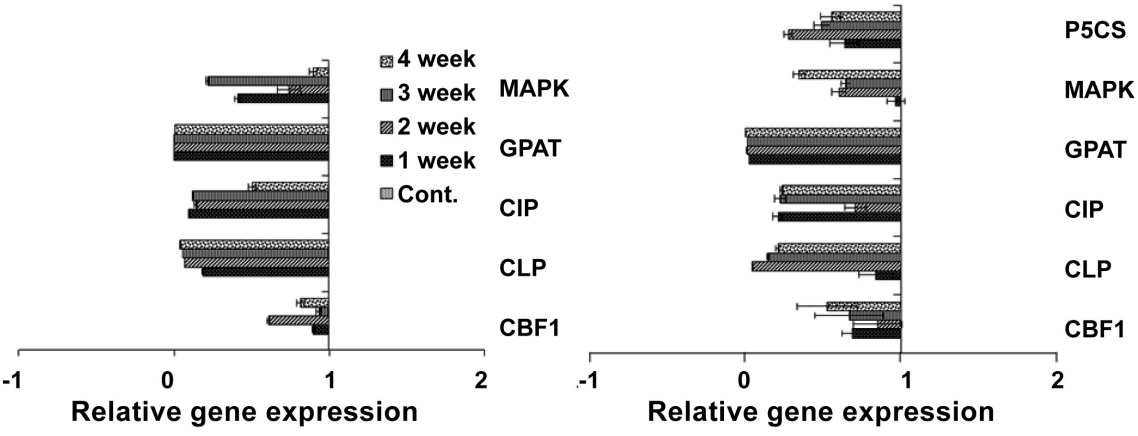

Fig. 3. Quantitative real-time PCR analysis of down-regulated gene expression in 'Kyoho' (Left) and 'Campbell Early' (Right) in response to cold. *CBF1*, CBF like transcription factor; *CLP*, chitinase-like protein; *CIP*, cold induced protein; *GPAT*, glycerol-3-phosphate acyltransferase; *MAPK*, mitogen-activated protein kinase; *P5CS*, pyrroline-5-carboxylate synthase. Vertical bars indicate the SEs (n = 3).

with untreated controls in both 'Kyoho' and 'Campbell Early' grapevines (Fig. 3).

The Venn diagram (Fig. 4) shows the distribution of significantly induced and repressed gene expression features during the low temperature treatment period for both grapevines. Among the genes tested in this study, some genes showed up-regulated expression patterns, while others showed down regulated expression patterns by low temperature in grapevines. Among them, *P5CS* gene showed up-regulation in 'Campbell Early' and down-regulation in 'Kyoho' grapevine. It is worth noting that the expression of the *P5CS* gene following cold treatment differed between 'Kyoho' (sensitive) and 'Campbell Early' (moderately tolerant) grapevines. The different expressions of this gene likely resulted from the difference in responses to cold and cold-acclimation capabilities. Accordingly, these candidate sequences with different expression to cold could be a valuable tool for development of functional molecular markers to assist in selection during breeding programs.

Low temperatures activated a number of cold-inducible genes such as those that encode active oxygen species (AOS) accumulation or scavenging, cell membrane proteins, molecular chaperones such as HSP, transcription factors (Mahajan and Tuteja, 2005), and PR proteins (Hon et al., 1995). All cold, drought, and salt stresses also stimulate the accumulation of compatible osmolytes and antioxidants (Hasegawa et al., 2000). Huang et al. (2011) reported that accumulation of anthocyanin was enhanced by cold stress, and that transcript levels of *CHI* were dependent on interspecific backgrounds being elevated to much higher levels in *Poncirus* rootstock during cold acclimation. *PAL* and *CHS* mRNAs of Arabidopsis were accumulated coordinately in response to low temperatures in a light-dependent manner (Leyva et al., 1995).

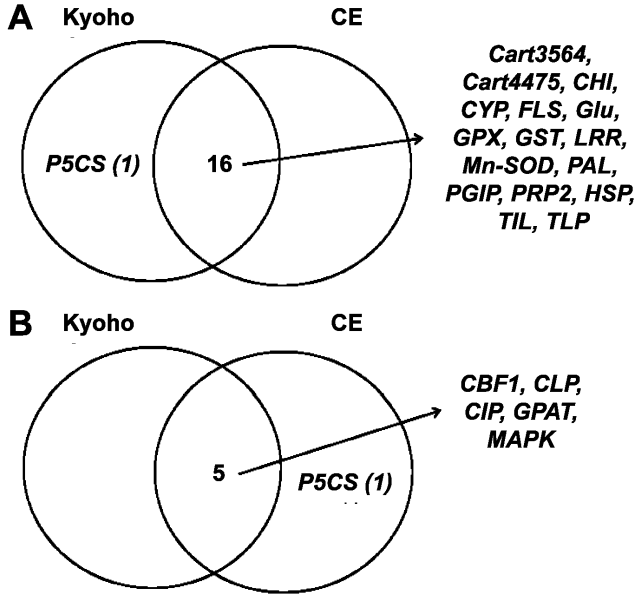

Fig. 4. Venn diagrams of differentially expressed genes in shoots of 'Kyoho' and 'Campbell Early' grapevines in response to cold. A, up-regulated genes; B, down-regulated genes. *Cart3564* and *Cart4472*, carotene; *CBF1*, CBF like transcription factor; *CHI*, chalcone isomerase; *CLP*, chitinase-like protein; *CIP*, cold induced protein; *CYP*, cytochrome P450; *FLS*, flavonol synthase; *Glu*, endo-β-glucanase precursor; *GPX*, glutathione peroxidase; *GST*, glutathione-*S*-transferase; *GPAT*, glycerol-3-phosphate acyltransferase; *LRR*, leucine-rich repeats; *Mn-SOD*, manganese superoxide dismutase; *MAPK*, mitogen-activated protein kinase; *PAL*, phenylalanine ammonia lyase; *PGIP*, polygalacturonase-inhibiting protein; *PRP*2, proline rich protein 2, *P5CS*, pyrroline-5-carboxylate synthase; *sHSP*, small heat shock protein; *TIL*, temperature induced lipocalin; *TLP*, thaumatine-like protein.

AOS are known to be generated in response to chemical and several environmental stresses, including chilling and freezing (Senaratna et al., 1988; Tsang et al., 1991; Wise and Naylor, 1987), drought (Perl-Treves and Galun, 1991; Price and Hendry, 1991), and pathogen attack (Koch and Slusarenko, 1990; Montalbini and Buonaurio, 1986). *Mn-SOD* transcripts gradually increased in wheat seedlings and remained high for 49 d of low-temperature stress (Wu et al., 1999). AOS and salicylic acid are known to induce *GST* levels (Marrs, 1996). Moreover, Jain et al. (2010) found that the transcript levels of Arabidopsis *GST* genes were induced by desiccation, salt, and cold stress, and that *OsGSTU10*, which was down-regulated by desiccation stress, was up-regulated by cold stress in Arabidopsis.

It has been reported that *PGIP* transcripts accumulated in apple during cold storage (Yao et al., 1999), as well as in cold-treated Arabidopsis (Ferrari et al., 2003). It was also suggested that low temperatures can increase susceptibility to diseases and that cold induction of defensive proteins might provide protection against pathogen infections. Antimicrobial proteins such as β-glucanase and chitinase, which are highly homologous with pathogenesis-related (PR) proteins, were found to be accumulated in the leaf apoplast of winter rye after cold exposure (Hon et al., 1995).

Chi et al. (2009) suggested that although plant temperature-induced lipocalin and *HSP* were thought to participate in plant responses to heat and cold stress, they are considered to function in different protection systems. The expression of *TIL* was up-regulated in wheat and Arabidopsis treated with heat shock and cold acclimation (Charron et al., 2002). Additionally, the expression of Arabidopsis *TIL1* was significantly increased upon cold acclimation in Arabidopsis leaf (Kawamura and Uemura, 2003).

In this study, 67 plant defense related genes responding to biotic or abiotic stress in the pathway of PR protein, cell modification, AOS accumulation or scavenging, anthocyanin synthesis, and molecular chaperones were selected for screening for differential expression in two grapevines during cold treatment. The expression of various genes associated with

cold stress was induced or repressed by cold treatment. Differential expression was also screened between 'Kyoho' and 'Campbell Early' grapevines responding to cold stress.

Through comparative analysis of genes induced by cold stress between 'Kyoho' (sensitive) and 'Campbell Early' (moderately tolerant) grapevines, important information useful to understanding the cold response mechanism was obtained. Although this information can be used for the breeding of new grapevine cultivars tolerant to low temperature, a number of valuable genes, which specifically expressed in cold tolerant grapevines, should be selected through further studies such as transcriptome analysis in the future. Sequences of genes with different expression in response to cold could be a valuable tool for development of molecular markers to increase selection efficiency in breeding programs.

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