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Searching Cold Hardiness Related Genes through Transcriptome Profiling in Peach Tree (*Prunus persica*) Shoots during Cold Acclimation and Deacclimation

저온 순화 및 탈순화 동안의 전사체 분석을 통한

복숭아 나무 신초의 내한성 관련 유전자 조사

BY

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UNDER THE DIRECTION OF DR. HEE JAE LEE SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

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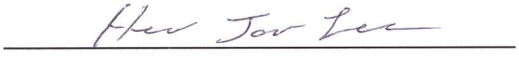
VICE-CHAIRMAN 

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Searching Cold Hardiness Related Genes through Transcriptome Profiling in Peach Tree (*Prunus persica*) Shoots during Cold Acclimation and Deacclimation

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ABSTRACT

Transcriptomes in the shoots of 5-year-old 'Soomee' peach trees were analyzed during cold acclimation (CA) and deacclimation (DA) for the identification of functional genes involved in cold hardiness. Shoots were collected from three trees at three physiological stages categorized based on seasonal change under field condition: early CA in the end of October, late CA in the middle of January, and late DA in the middle of March. Cold hardiness, expressed as temperature representing 50% injury occurred (LT_{50}), decreased during CA and increased during DA. Based on the transcriptome analysis, totally 17,208 assembled transcripts were identified in all samples examined. During CA and DA, 1,891 and 3,008 transcripts, respectively, were differentially expressed with |fold change| > 2 at $P < 0.05$. Of the differentially expressed transcripts or genes (DEGs), 1,522 and 2,830 DEGs during

CA and DA, respectively, were functionally annotated. A great proportion of the functionally annotated DEGs were enriched within molecular function gene ontology (GO) category both during CA and DA. The enriched DEGs mainly were associated with ‘metal ion binding’ and ‘transcription factor activity, sequence-specific DNA binding’ GO terms. Based on KEGG database, the most represented biochemical pathways during CA and DA were ‘metabolic pathway’ and ‘biosynthesis of secondary metabolites’. The results were validated by performing reverse transcription quantitative polymerase chain reaction on the selected 18 DEGs, showing significant fold changes during CA and DA. The ‘Soomee’ peach tree shoots exhibited higher relative expressions of the selected DEGs, when compared to less cold-hardy ‘Odoroki’ peach tree shoots. Irrespective of the cultivar, relative expressions of the up- and down-regulated DEGs during CA and DA, respectively, were more closely correlated with cold hardiness levels than those of the other DEGs. Therefore, the significant DEGs encoding functional proteins and enzymes including polygalacturonase inhibitor, squalene monooxygenase, and late embryogenesis abundant protein 2 might play crucial roles in determining cold hardiness level in peach tree shoots.

Keywords**:** cold acclimation, cold hardiness, deacclimation, differentially expressed genes, peach, transcriptome analysis

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INTRODUCTION

Abnormal cold stress during warm winter or early spring can damage fruit tree shoots and flower buds. Cold stress, including freezing, is one of the major environmental stresses on temperate fruit trees that limit their growth, productivity, and distribution. Some temperate fruit trees have a remarkable ability to adapt and withstand cold temperatures or freezing temperatures, which is called cold hardiness. In order to survive the harsh winter and grow again the following spring, temperate fruit trees change their cold hardiness through cold acclimation (CA) and deacclimation (DA) (Kalberer et al., 2006; Kozlowski and Pallardy, 2002; Thomashow, 1999). The modification of membrane lipid composition, the increases in compatible solutes, the enhancement of antioxidative mechanism, and the synthesis of protective proteins are the process of CA (Thomashow, 1999; Welling and Palva, 2008). Cold stress modifies the expression of various genes and their associated metabolites, thereby influencing many biological functions during the CA. Generally, the major changes during CA are reversed during DA (Kalberer et al., 2006).

The enzymes and protective proteins that function directly to protect cells from abiotic stresses, including cold stress, are important for the survival of plants by applying a given stress. However, due to the complexity of stress response regulated by multiple genes, it has been rarely successful, especially by single-function gene access to improve plant stress tolerance. Thus, high-throughput sequencing and functional genetics tools are useful to release the molecular mechanism in response to cold stress. In particular, a transcriptome sequencing analysis can provide a

framework data set related to determining metabolic pathways, clarifying gene expression patterns, and mining of new genes. Gene expression profiling has identified many genes regulated by cold stress on a variety of deciduous fruit trees including grape (Kim et al., 2016) and peach (Wang et al., 2019). In peach trees (*Prunus persica*), which are easily affected by cold rather than other temperate fruit trees, expressions of various genes including cold response genes have been investigated (Artlip et al., 2013; Bassett et al., 2015; Jiao et al., 2017; Wang et al., 2019) after peach genome was sequenced (Verde et al., 2013). However, most of the studies on cold stress in peach trees have focused on some recognized genes in specific tissues or on changes of their expression, as exposed to programmed low temperatures (Artlip et al., 2013; Bassett et al., 2015; Jiao et al., 2017).

In this study, transcriptomes were analyzed in the ‘Soomee’ peach tree shoots during CA and DA under filed condition. Transcripts, which were significantly up- and down-regulated or down- and up-regulated during CA and DA, respectively, were listed up as cold hardiness related genes and their expression patterns were compared in the ‘Soomee’ and ‘Odoroki’ peach tree shoots to confirm their involvement in cold hardiness. ‘Odoroki’ peach trees are known as more cold-sensitive cultivar than ‘Soomee’.

MATERIALS AND METHODS

Plant materials

Five-year-old ‘Soomee’ and ‘Odoroki’ peach trees grown under field conditions in the experimental orchard of the National Institute of Horticultural and Herbal Science (35° 82′ N, 127° 02′ E), Rural Development Administration, Wanju, Republic of Korea, were used in this study. The shoot internodes of the ‘Soomee’ and ‘Odoroki’ peach trees were separately collected at early CA (end of October), late CA (middle of January), and late DA (middle of March), immediately frozen in liquid nitrogen, and stored at –75°C until use. Bud-attached shoot internodes at the same physiological stages were also used for determining the levels of cold hardiness.

Cold hardiness determination

The cold hardiness of peach tree shoots was determined using an electrolyte leakage analysis following the methods of Pagter et al. (2008) and Lee et al. (2012) with slight modifications. Bud-attached shoot internodes were cut into 8 cm pieces and randomly divided into six groups. Four groups out of six were incubated in a programmable bath circulator (RW-2040G; Jeio Tech, Seoul, Republic of Korea), cooled at a rate of –2°C/h until they reached each target temperature, and maintained at the target temperature for 2 h. Four target temperatures ranging from –5°C to –35°C were selected. The incubated samples were thawed at 0°C, and all temperatures were recorded every second using a data logger (CR-1000 M; Campbell Scientific, Inc., Logan, UT, USA) with a copper-constantan

thermocouple. For comparison, the other two groups were separately incubated in a refrigerator at 4°C and in a freezer at –80°C.

Following the cold treatment, the shoot internodes were cut into 0.5 cm, and seven pieces of each sample were shaken in a 50 mL tube containing 10 mL distilled water at 125 rpm on an orbital shaker (Supertech Orbital Shaker; SeouLin Bioscience, Seoul, Republic of Korea) at room temperature for 24 h. The electrical conductivity (EC) of each aliquot was then measured using an EC meter (Orion Star A215; Thermo Fisher Scientific, Waltham, MA, USA). After autoclaving the samples at 120°C for 30 min, the EC was measured again. The percentage of injury was calculated as described by Arora et al. (1992), and adjusted using the equation reported by Yu et al. (2017). The median lethal temperature (LT_{50}) was calculated using the Gompertz function and used as a measure of cold hardiness. The adjusted injury values of each cultivar at each physiological stage were resampled 30 times as described by Arora et al. (2004) to obtain efficient LT_{50} estimates without repeating the entire experiment.

RNA extraction

Total RNA was extracted from shoots using cetyltrimethylammonium bromide buffer as described by Gambino et al. (2008) with slight modifications. A 900 μL aliquot of the extraction buffer was preheated to 70°C and added to a 2 mL microfuge tube containing 100 mg of shoot powder, mixed thoroughly, and incubated at 65°C for 10 min. An equal volume of chloroform:isoamyl alcohol (24:1, v/v) was added, vortexed for 5 s, and centrifuged at 11,000 × *g* at 4°C for 10 min. The 750 μL supernatant was recovered and again mixed with an equal volume

of the chloroform:isoamyl alcohol. The 600 μL supernatant was transferred to a new 2 mL tube and an equal volume of 6 M LiCl was added. The mixture was incubated on ice for 30 min and centrifuged at 21,000 × *g* at 4°C for 20 min to precipitate the RNA. The pellet was resuspended in 500 μL of preheated (70°C) SSTE buffer consisting of 0.5% sodium lauryl sulfate, 1 M NaCl, 1 M Tris-HCl (pH 8.0), and 10 mM EDTA while gentle shaking. An equal volume of the chloroform:isoamyl alcohol was added, and the mixture was centrifuged at 11,000 × *g* at 4°C for 10 min. The 400 μL supernatant was mixed with 280 μL cold isopropanol and centrifuged at 21,000 × *g* at 4°C for 15 min. The pellet was washed with 1 mL of 70% ethanol, dried, and resuspended in 20 μL of diethyl pyrocarbonate-treated water. Finally, the solution was heated at 70°C for 5 min to dissolve the RNA.

The quality and purity of the extracted RNA samples were assessed by determining their A_{260}/A_{280} ratios and RNA integrity numbers using a Nanodrop ND1000 (Thermo Fisher Scientific) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively.

cDNA library construction, sequencing, and assembling

A total of nine cDNA libraries were constructed from RNA extracted from the 'Soomee' peach tree shoots at the three physiological stages using a TruSeq RNA sample prep kit v2 (Illumina, San Diego, CA, USA). They were sequenced using a HiSeq 2500 system (Illumina) to generate 101-bp paired-end reads. The quality of the resulting data was confirmed using the FastQC v0.11.5 program (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and unwanted artifacts, including the adaptor sequence, low-quality reads, and short length reads

(< 36 bp), were removed from the raw data using the Trimmomatic v0.32 program (http://www.usadellab.org/cms/? page=trimmomatic). The statistic values, including the GC content and Phred quality score 30 (Q30), of the trimmed data were calculated. The trimmed reads were mapped to the reference peach genome (GCF_000346465.2) using the Bowtie2 aligner and the HISAT2 program (https://ccb.jhu.edu/software/hisat2/index.shtml), after which the aligned reads were assembled into transcripts or genes using the StringTie 1.3.3b program (https://ccb.jhu.edu/software/stringtie). The RNA sequencing (RNA-Seq) data were deposited in the National Center for Biotechnology Information (NCBI) database (accession No. PRJNA587386).

Identification and functional annotation of the differentially expressed genes (DEGs)

The abundances of the assembled transcripts in each sample were expressed by normalized fragments per kilobase of transcript per million mapped reads (FPKM) values. To effectively identify the DEGs, the assembled transcripts or genes showing zero FPKM in any sample were removed from the analysis. The filtered data were adjusted using the quantile normalization method after taking the $\log_2$ (FPKM + 1) value to reduce the range of the data and evenly distribute the data. Statistical analysis was performed on the fold change values using the Student's *t*-test at $P < 0.05$. The functional annotations and gene set enrichment analysis were also performed using the DAVID tool (http://david.abcc.ncifcrf.gov) based on their associated gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) database categories (http://www.genome.jp/kegg/).

Reverse transcription quantitative polymerase chain reaction (qPCR) analysis

To validate DEG identification results and analyze their expression patterns, qPCR analyses were performed on the transcripts showing significant fold change during CA and DA. The selected DEGs consisted of nine up- and down-regulated or nine down- and up-regulated genes and their specific primer sequences of the forward and reverse primers were used for qPCR (Table 1). First strand cDNAs were synthesized from the same RNA samples used for RNA-Seq, using an AccuPower RT premix (Bioneer, Daejeon, Korea). Primer sets were designed by NCBI Primer-BLAST. DNA duplication by primers were checked by electrophoresis on 1% agar in 0.02 M Tris-acetate buffer, including 0.5 mM EDTA. Relative expressions of the transcripts were determined with LightCycler 480 system (Roche Diagnostics, Basel, Switzerland) and AccuPower Greenstar qPCR master mix (Bioneer, Daejeon, Korea). The results were standardized to the expression level of the transcripts encoding glyceraldehyde-3-phosphate dehydrogenase as described by Zifkin et al. (2012). The relative expressions were plotted using SigmaPlot 12 version 8.0.2 (GraphPad Software Inc., San Diego, CA, USA).

Statistical analysis

Statistically significant differences among means were determined by Student's *t*-test at $P < 0.05$ using the R 3.2.2 software package (http://www.r-project.org).

Table 1. Primer sequence sets for qPCR of the selected nine up- and down-regulated DEGs during cold acclimation and deacclimation, respectively, and the selected nine down- and up-regulated DEGs during cold acclimation and deacclimation, respectively, in 'Soomee' peach tree shoots.

Gene (LOC No.)	Gene description	Primer sequence
Up- and down-regulated DEGs		
18766910	non-symbiotic hemoglobin	F: AGAGCAGGAAACATTGGTGG
		R: CTTCTGAGCTGATGGTGCAA
18769194	polygalacturonase inhibitor	F: ACCATCCTAAACCCAGCTCT
		R: AGTACCAGTCACAGCAGTCT
18780397	tonoplast dicarboxylate	F: ATGGATGATGTCATTGCGCT
	transporter	R: CTCCGCAGAATAGCATGGTT
18793247	extracellular ribonuclease	F: GAATGGGAAAAGCATGGCAC
	LE	R: TGGTTGTATGCCTGCACTTT
109947416	pEARLI1-like lipid	F: TGATGCTGCTGTGTGTCTTT
	transfer protein 3	R: GCATTGGAAGTCTGTTGGGA
18785901	14 kDa proline-rich protein	F: CACCAACACCACCAAAAACC
	DC2.15	R: TAGGGTCGAAGGAGGTTGTT
18768153	squalene monooxygenase	F: CCAAGCATGCTTCGACTACT
		R: TCAGTCTAGCTCCAACCCAA
18784088	late embryogenesis	F:AAACAGAGGGCAGAAGAAGC
	abundant protein 2	R: CTTTTCACAGCATCAGCAGC

F, forward; R, reverse.

Table 1. Continued.

Gene (LOC No.)	Gene description	Primer sequence
18768928	bidirectional sugar transporter SWEET1	F: CCTTTTGTTGCTGTGCCAAA R: TTCATCAGCAGTAGCAGCAG
Down- and up-regulated DEGs		
18793783	heterogeneous nuclear ribonucleoprotein A2 homolog 1	F: GCCGATGTAGTGACTTTTCCT R: GGTTCTTGTTCCCGTTCAGT
18789909	non-specific lipid-transfer protein 2	F: TTCTTGTCTTTGGGGGAAGC R: AAATGGGGAGCCACAAGTG
18770886	O-acyltransferase WSD1	F: TCTCAGGTTTCTGCAACACC R: AATGTATTTCCCGCATGCCT
18780769	protein E6	F: CACCACCAACAACAACAACC R: TGGCACGAACTCTTCTTCAC
109948145	vinorine synthase-like	F: ACTGCTTGCTATCTGACGTG R: CCATCCTGGTCCGTAAGTTG
18790018	aspartyl protease AED3	F: GCATCAATTCCATCTCGGGT R: AAGCTGTTCTCCAATGGCAA
18775166	phosphoenolpyruvate carboxykinase (ATP)	F: ATTCTGGCTGCAATATGGGG R: CAGCATCTTATCAGCCAGCA
18780494	protein SRG1	F: AGACGTGGAAGGTTTTGGAC R: ACTGGACCAATTTCACCACC

F, forward; R, reverse.

Table 1. Continued.

Gene (LOC No.)	Gene description	Primer sequence
18793298	cyclin-D1-1	F: ATGTCATCGGTTTGGAGCTC
		R: CCTCCAATCCAAAACACCCA

F, forward; R, reverse.

RESULTS AND DISCUSSION

Changes of cold hardiness

The LT_{50} values of ‘Soomee’ peach tree shoots significantly decreased during CA and increased during DA, indicating that cold hardiess increased and decreased during CA and DA (Fig. 1). The ‘Soomee’ peach tree shoots showed lower LT_{50} than the ‘Odoroki’ peach tree shoots during CA. Following the LT_{50} values, the ‘Soomee’ peach tree shoots was cold-hardier than the ‘Odoroki’ peach tree shoots during CA (Fig. 2).

Transcriptome sequencing data statistics

The transcriptomes of the ‘Soomee’ peach tree shoots during CA and DA were sequenced, generating reads 50,845,192 to 66,451,463 with GC content of 45.6-46.3% and a Q30 of 93.9-96.3% (Table 2), indicating that the trimmed data were significant. The Q30 means the probability of an incorrect base call 1 in 1,000 times. Virtually, all of the reads were almost perfect, having zero errors and ambiguities. The total bases of average of 5.12–6.68 × 10^9 were obtained for each sample, and almost all of the total reads were mapped to the reference peach genome (GCF_00346464.2), with a mapping ratio of 96.7-97.2% (Table 2).

DEGs analysis during CA and DA

Of the 18,344 assembled transcripts, 17,208 (93.8%) transcripts were identified in all the samples examined. In total, 1,891 and 3,008 transcripts were differentially expressed during CA and DA, respectively, with a |fold change| > 2 ($P < 0.05$)

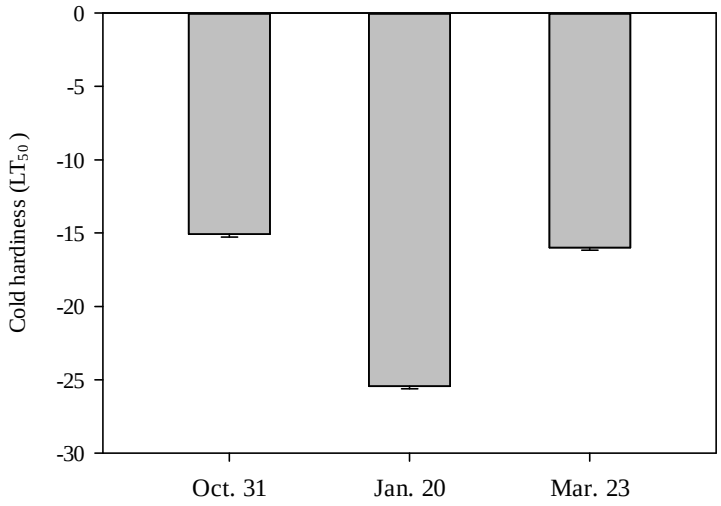

Fig. 1. Seasonal changes of cold hardiness in ‘Soomee’ peach tree shoots during cold acclimation and deacclimation. Vertical bars represent the standard errors of the means (n = 30).

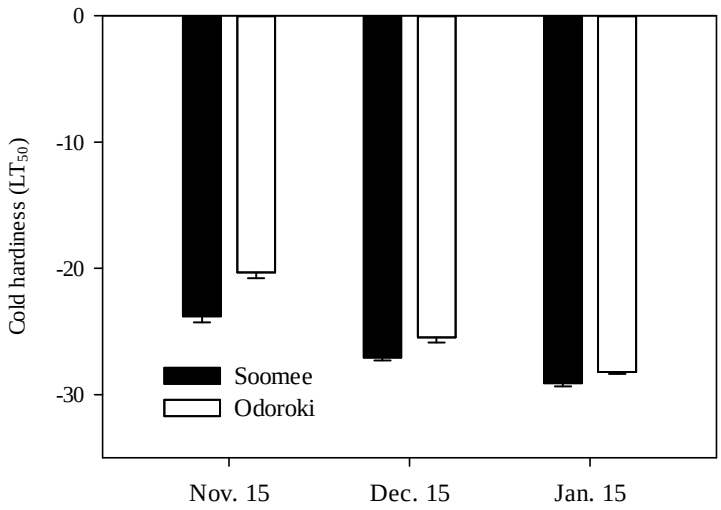

Fig. 2. Comparison of cold hardiness in ‘Soomee’ and ‘Odoroki’ peach tree shoots during cold acclimation. Vertical bars represent the standard errors of the means (n = 30).

Table 2. Transcriptome sequencing data statistics from ‘Soomee’ peach tree shoots during cold acclimation and deacclimation.

Physiological stage	Total read	Total base	GC content (%)	Q30 (%)	Mapping ratio (%)
Oct. 31	66,451,463	6,683,605,100	46.3	95.4	96.9
Jan. 20	54,420,355	5,463,673,888	45.8	93.9	96.7
Mar. 23	50,845,192	5,122,738,506	45.6	96.3	97.2

Read length is 101 bp, and each value is the mean of three biological replicates.

(Fig. 3). The number of significant DEGs was higher during DA than during CA, which indicates that DA is not merely a reverse of the CA process, but a physiological stage preparing trees for the resumption of growth and development. During CA and DA, 926 and 1,624 transcripts were up-regulated, respectively, while 965 and 1,384 transcripts were down-regulated, respectively (Fig. 3). The number of DEGs with a |fold change| > 10 ($P < 0.05$) was generally below 100 both during CA and DA.

Functional annotation and GO term enrichment of the DEGs during CA

GO terms have widely been applied to understanding the biological significance of DEGs. Among the 1,891 DEGs during CA, 1,522 could be functionally annotated and were classified into 38 functional terms within the three main GO categories: 10 biological process terms, 15 molecular function terms, and five cellular component terms were significantly enriched at $P < 0.05$ (Fig. 4). A great proportion of functionally annotated DEGs were associated with various molecular function terms (402 DEGs; 26.4%). Fewer DEGs were associated with the biological process (207 DEGs; 13.6%) and cellular component terms (194 DEGs; 12.7%), with a similar proportion of DEGs enriched in these two categories. In the biological process category, the GO term ‘metabolic process’ represented the most highly enriched group within the DEGs, followed by ‘cell wall organization’ (Fig. 4).

The exploration of DEGs associated with the ‘metabolic process’ term may allow the identification of genes involved in altering the secondary metabolic pathways during CA. Sanghera et al. (2011) reported that changes in the expression of genes in response to cold stress were followed by increases in the levels of

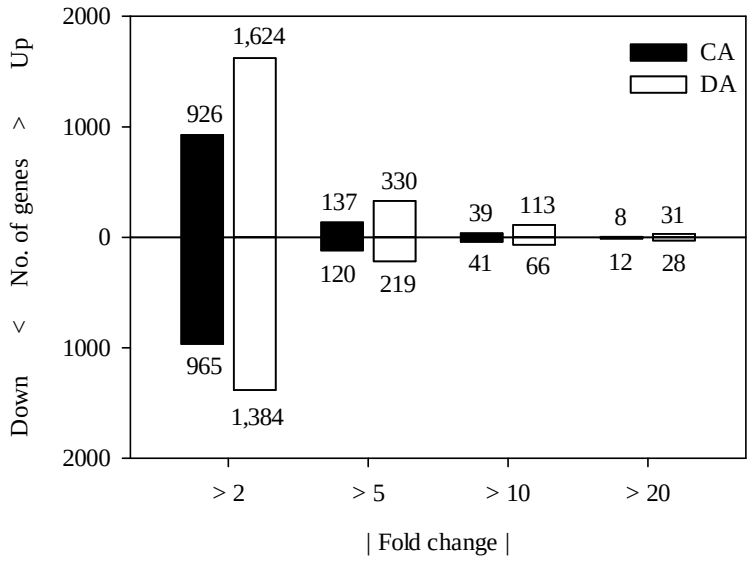

Fig. 3. Number of up- or down-regulated genes with a |fold change| > 2 ($P < 0.05$) in 'Soomee' peach tree shoots during cold acclimation (CA) or deacclimation (DA).

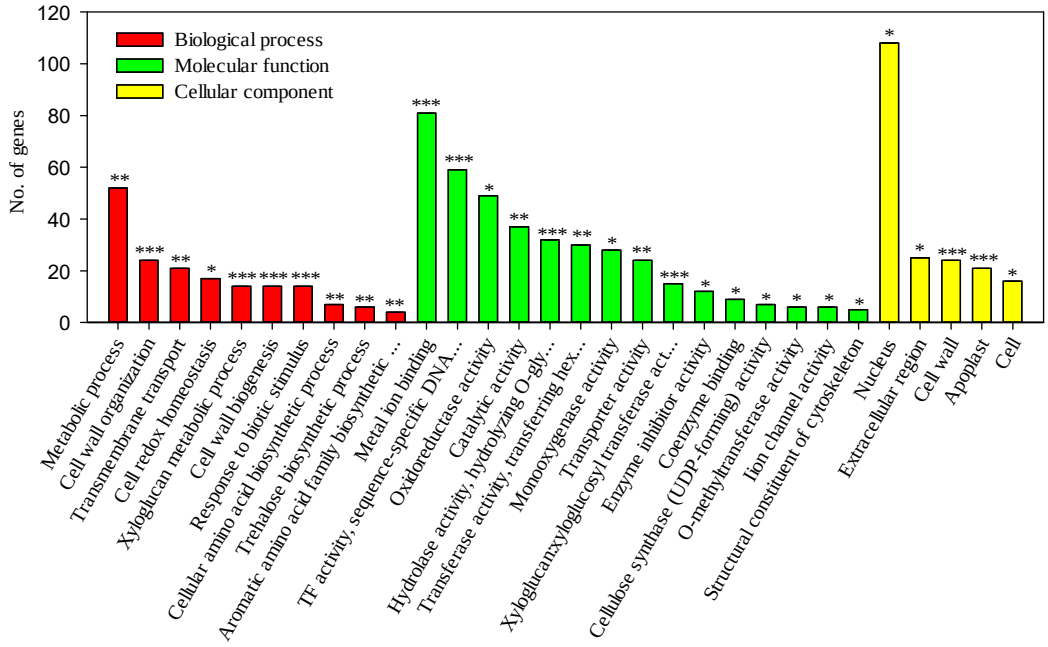

Fig. 4. Gene ontology terms enriched DEGs with a |fold change| > 2 ($P < 0.05$) in 'Soomee' peach tree shoots during cold acclimation. Asterisks (*, **, ***) on bars indicate significance at $P < 0.05$, 0.01, or 0.001, respectively.

hundreds of metabolites, some of which are known to have protective effects against cold stress. ‘Cell wall organization’ is a process that results in the assembly or disassembly of the cell wall or regulates the arrangement of its constituent parts, maintaining the shape of the cell and protecting it from osmotic lysis. CA was previously shown to induce changes in the cell wall polysaccharide composition and in the activities of the cell wall-modifying enzymes (Gall et al., 2015; Liu et al., 2018). In contrast to cell wall-modifying enzymes, Kalunke et al. (2015) reported that polygalacturonase inhibiting proteins are cell wall proteins that inhibit the pectin-depolymerizing activity of polygalacturonases secreted by microbial pathogens and insects. Li et al. (2003) also reported that polygalacturonase inhibitor proteins may play crucial role for general resistance to biotic and some abiotic stresses including cold stresses. In the present study, polygalacturonase inhibitor (LOC18769194) was identified and significantly differentially regulated during CA and DA, which showed significantly up- and down-regulated during CA and DA, respectively.

In the molecular function category, the enriched DEGs were mainly associated with the ‘metal ion binding’ and ‘transcription factor activity, sequence-specific DNA binding’ GO terms (Fig. 4), presumably enabling the identification of genes involved in signal transduction pathways during CA. Many proteins require bound metals to achieve their functions, and the metal-binding proteins are associated with a variety of cellular functions, including cell signaling (Dutta and Bahar, 2010). Transcription factors (or sequence-specific DNA binding factors) are proteins that control the transcription of genetic information from DNA into mRNA and play vital regulatory roles in abiotic stress responses in plants (Agarwal et al., 2017;

Wang et al., 2016). Proline-rich protein plays important role in abiotic stress tolerance of *Arabidopsis* (Zhan et al., 2012). Proline is a key determinant of cell wall protein that play important roles in plant development. The role of extensions, arabinogalactan proteins, and proline-rich proteins as important components of cell wall proteins that play pivotal roles in cell wall signal transduction cascades, plant development, and stress tolerance (Kishor et al., 2015). In the present study, proline-rich protein DC2.15 (LOC18785901) was identified and significantly up- and down-regulated during CA and DA, respectively.

In the cellular component category, the GO term ‘nucleus’ was dominant among the DEGs (Fig. 4), although other enriched terms included ‘extracellular region’, ‘cell wall’, and ‘apoplast’. The cold hardiness of plants often depends on the mechanical properties of the cell wall (Gall et al., 2015). During drastic cold exposure, freezing can induce the formation of extracellular ice, which leads to cell dehydration and subsequent collapse. Cell wall rigidity might determine the resistance of cells to this freezing-induced dehydration. CA can induce changes in lignin content and composition, thus preventing freezing damage and cell collapse (Gall et al., 2015; Liu et al., 2018), and may also induce changes in the composition of the cell wall polysaccharides and in the activities of cell wall-modifying enzymes (Gall et al., 2015). In the present study, extracellular ribonuclease LE (LOC18793247) was identified and significantly up- and down-regulated during CA and DA, respectively.

Functional annotation and GO term enrichment of the DEGs during DA

The GO term enrichment of the DEGs identified in the ‘Soomee’ peach tree

shoots were markedly different between the CA and DA stage (Figs. 4, 5). More functionally annotated DEGs were identified during DA than CA, and the DEGs during DA were classified into more terms within each GO category. During DA, 2,830 DEGs were functionally annotated and classified into 34, 40, and 26 functional terms within the GO categories of biological process, molecular function, and cellular component, respectively (Fig. 5). Of these, 22, 26, and 13 GO terms were significantly enriched in each GO category, respectively, at $P < 0.05$. The proportion of DEGs associated with the biological process terms (402 DEGs; 14.2%) was lower than those associated with the molecular function (1,161 DEGs; 41.0%) and cellular component terms (916 DEGs; 32.3%).

In the biological process category, the enriched DEGs were mainly associated with the 'carbohydrate metabolic process' and 'transmembrane transport' GO terms during DA (Fig. 5), unlike during CA (Fig. 4). The 'carbohydrate metabolic process' term is associated with the chemical reactions and pathways involving carbohydrate metabolism, including the formation of carbohydrate derivatives. Transmembrane transport is a process by which a solute is transported across a lipid bilayer. In contrast to CA, the GO terms 'ATP binding' and 'integral component of membrane' were dominant in the molecular function and cellular component categories, respectively, during DA. These GO terms suggest that the expression of the genes involved in the metabolic processes preparing for the resumption of growth and development is induced with decreasing cold hardiness during DA.

KEGG category enrichment of the DEGs

According to the results of the biochemical pathways analysis based on the

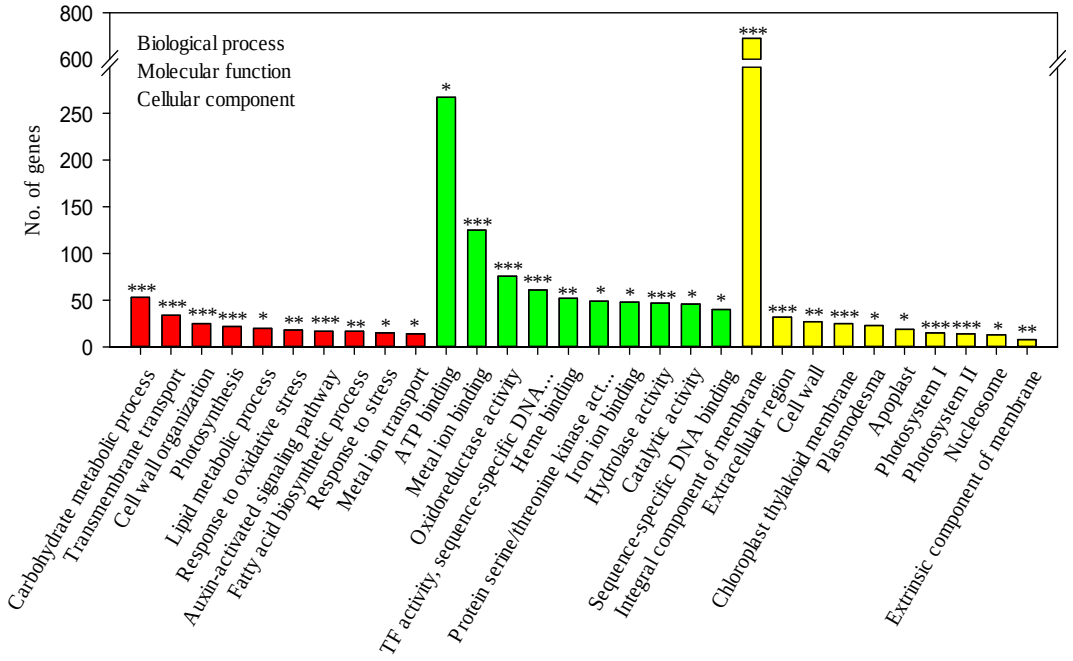

Fig. 5. Gene ontology terms enriched DEGs with a |fold change| > 2 ($P < 0.05$) in 'Soomee' peach tree shoots during deacclimation. Asterisks (*, **, ***) on bars indicate significance at $P < 0.05$, 0.01, or 0.001, respectively.

KEGG categories, 930 DEGs were assigned to 110 KEGG pathways during CA, while 1,467 DEGs were assigned to 116 KEGG pathways during DA (Fig. 6). The most represented pathways were ‘metabolic pathways (MapID 01100)’ (174 and 286 members during CA and DA, respectively) and ‘biosynthesis of secondary metabolites (MapID 01110)’ (120 and 185 members during CA and DA, respectively). The ‘metabolic pathways’-associated genes encoding squalene monooxygenase (LOC18768153) was greatly up- and down-regulated during CA and DA, respectively, while the gene encoding phosphoenolpyruvate carboxykinase (ATP) (LOC18775166) was significantly down- and up-regulated during CA and DA, respectively (Tables 3, 4). Squalene monooxygenase and phosphoenolpyruvate carboxykinase are involved in sterol biosynthesis and the decarboxylation of oxaloacetate to phosophoenolpyruvate in the gluconeogenesis pathway, respectively (Rylott et al., 2003; Wentzinger et al., 2002). These KEGG database annotations associated with the biochemical pathways provide a valuable resource for understanding the complex functions and utilities of the biological system involved in the response to cold stress in peach tree shoots.

Validation of in silico DEG data by qPCR

Although the number of DEGs in response to cold stress differed between CA and DA, most of the DEGs were identified during both phases. Considering the changes in cold hardiness, these common DEGs might play more important roles in determining the cold hardiness level in the ‘Soomee’ peach tree shoots rather than the other identified DEGs. Among the common DEGs with false discovery rate (FDR) < 0.05, 322 DEGs were significantly up- and down-regulated during

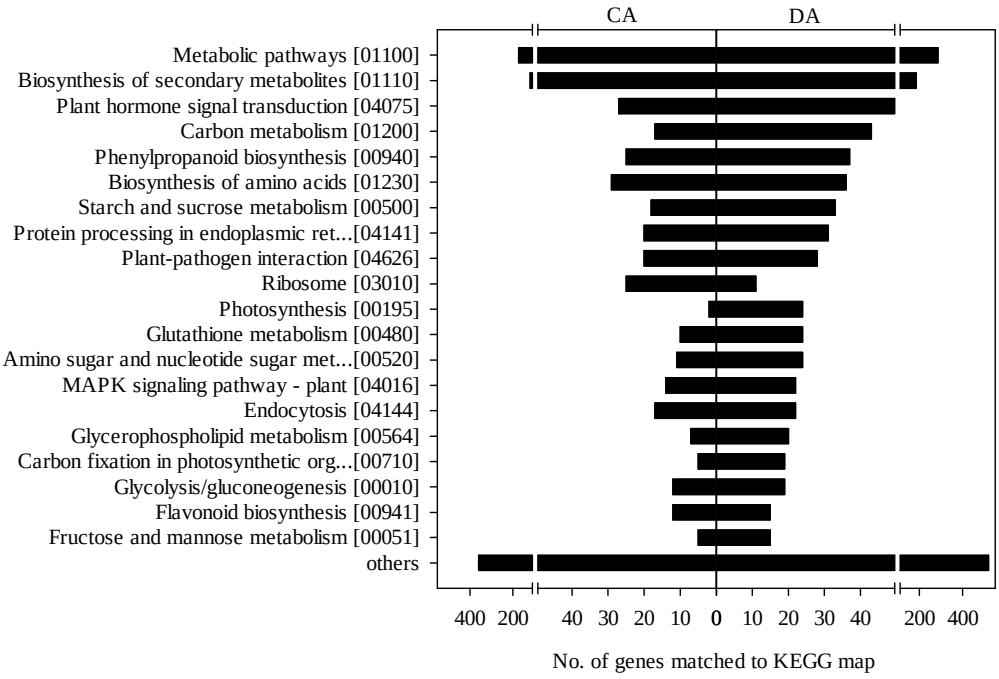

Fig. 6. Biochemical pathways, based on the KEGG database categories, enriched in DEGs with a |fold change| > 2 ($P < 0.05$) in 'Soomee' peach tree shoots during cold acclimation (CA) and deacclimation (DA).

Table 3. Correlation between cold hardiness (LT_{50}) and log_2 (FPKM + 1) of up- and down-regulated or down- and up-regulated DEGs during cold acclimation (CA) and deacclimation (DA) in the 'Soomee' peach tree shoots for the validation of the data by qPCR.

Gene (LOC No.)	Gene description	Fold change CA	Fold change DA	Correlation coefficient (r) LT_{50} and log_2 (FPKM + 1)
Up- and down-regulated DEGs				
18766910	non-symbiotic hemoglobin	61.9^{*}	-18.7^{*}	-0.81^{ns}
18769194	polygalacturonase inhibitor	47.3^{**}	-2.4^{ns}	-0.98^{ns}
18780397	tonoplast dicarboxylate transporter	20.9^{*}	-6.7^{**}	-0.99^{*}
18793247	extracellular ribonuclease LE	20.6^{*}	-14.8^{**}	-0.96^{ns}
109947416	pEARLI1-like lipid transfer protein 3	16.4^{**}	-7.5^{ns}	-0.94^{ns}
18785901	14 kDa proline-rich protein DC2.15	13.5^{*}	-58.7^{*}	-0.96^{ns}
18768153	squalene monooxygenase	12.4^{*}	-7.1^{**}	-0.62^{ns}
18784088	late embryogenesis abundant protein 2	6.0^{**}	-53.4^{**}	-0.90^{ns}

^{ns,*,**} Not significant or significant at $P < 0.05$ or 0.01, respectively.

Table 3. Continued.

Gene (LOC No.)	Gene description	Fold change CA	Fold change DA	Correlation coefficient (r) LT_{50} and log_2 (FPKM + 1)
18768928	bidirectional sugar transporter SWEET1	3.1*	−93.7**	−0.69ns
Down- and up-regulated DEGs				
18793783	heterogeneous nuclear ribonucleoprotein A2 homolog 1	−52.2**	16.3**	0.98ns
18789909	non-specific lipid-transfer protein 2	−16.7*	63.4**	0.91ns
18770886	O-acyltransferase WSD1	−10.3*	5.7**	0.99ns
18780769	protein E6	−9.5*	32.5**	0.91ns
109948145	vinorine synthase-like	−7.5**	9.7**	0.97ns
18790018	aspartyl protease AED3	−6.8*	12.7**	0.94ns
18775166	phosphoenolpyruvate carboxykinase (ATP)	−6.2**	15.6**	0.90ns
18780494	protein SRG1	−6.2**	15.2**	0.89ns
18793298	cyclin-D1-1	−5.2*	27.0**	0.80ns

ns,*,** Not significant or significant at $P < 0.05$ or 0.01, respectively.

Table 4. Correlation of cold hardiness (LT_{50}) with relative expression levels and log_2 (FPKM + 1) values of the selected DEGs during cold acclimation and deacclimation in the 'Soomee' peach tree shoots for the validation of the data by qPCR.

Gene (LOC No.)	Gene description	Correlation coefficient (r): log_2 (FPKM + 1) and qPCR	Correlation coefficient (r): qPCR and LT_{50}
Up- and down-regulated DEGs			
18766910	non-symbiotic hemoglobin	0.84^{ns}	-0.99^{*}
18769194	polygalacturonase inhibitor	0.97^{ns}	-0.99^{*}
18780397	tonoplast dicarboxylate transporter	0.99^{ns}	-0.99^{*}
18793247	extracellular ribonuclease LE	0.94^{ns}	-0.99^{*}
109947416	pEARLI1-like lipid transfer protein 3	0.95^{ns}	-0.99^{*}
18785901	14 kDa proline-rich protein DC2.15	0.96^{ns}	-0.99^{*}
18768153	squalene monooxygenase	0.86^{ns}	-0.92^{ns}
18784088	late embryogenesis abundant protein 2	0.93^{ns}	-0.99^{ns}
18768928	bidirectional sugar transporter SWEET1	0.90^{ns}	-0.93^{ns}
Down- and up-regulated DEGs			
18793783	heterogeneous nuclear ribonucleoprotein A2 homolog 1	0.97^{ns}	0.91^{ns}
18789909	non-specific lipid-transfer protein 2	0.99^{**}	0.91^{ns}
18770886	O-acyltransferase WSD1	0.87^{ns}	0.79^{ns}
18780769	protein E6	0.83^{ns}	0.53^{ns}

^{ns,*,**} Not significant or significant at $P < 0.05$ or 0.01, respectively.

Table 4. Continued.

Gene (LOC No.)	Gene description	Correlation coefficient (r) $\log_2$ (FPKM + 1) and qPCR	Correlation coefficient (r) qPCR and LT_{50}
109948145	vinorine synthase-like	0.87^{ns}	0.75^{ns}
18790018	aspartyl protease AED3	0.87^{ns}	0.67^{ns}
18775166	phosphoenolpyruvate carboxykinase (ATP)	0.96^{ns}	0.77^{ns}
18780494	protein SRG1	0.88^{ns}	0.58^{ns}
18793298	cyclin-D1-1	0.91^{ns}	0.50^{ns}

^{ns,*,**} Not significant or significant at $P < 0.05$ or 0.01, respectively.

CA and DA, respectively, while 327 DEGs were significantly down- and up-regulated. The FDR method has previously been used when the objective is to reduce the number of false positives and to increase the chances of identifying all the DEGs (Li et al., 2012).

To validate the DEGs and transcriptome data from the ‘Soomee’ peach tree shoots, 18 common DEGs showing the significant fold change during both CA and DA were selected and their expression patterns were analyzed using qPCR. The selected DEGs consisted of nine up- and down-regulated, and nine down- and up-regulated genes during CA and DA, respectively. Most of the selected genes were differently expressed during CA and DA (Figs. 7, 8), and their expressions were closely correlated with the in silico FPKM values of the selected DEGs or seasonal changes in the cold hardiness (LT_{50}) of the ‘Soomee’ peach tree shoots (Tables 3, 4). According to the correlation coefficients (r) between the relative gene expressions of each DEG and LT_{50} value, the relative expressions of the DEGs up-regulated during CA and down-regulated during DA were more closely correlated with cold hardiness than those down-regulated during CA and up-regulated during DA (Tables 3, 4). These results suggest that the DEGs up-regulated during CA and down-regulated during DA are more influential determinants of the cold hardiness level than those displaying the opposite pattern in the ‘Soomee’ peach tree shoots.

Relative expressions of the 18 common DEGs, in the ‘Soomee’ peach tree shoots were also compared with those in the less cold-hardy ‘Odoroki’ peach tree shoots (Figs. 9, 10). The ‘Soomee’ shoots had higher relative expression levels of these DEGs than the ‘Odoroki’ shoots, with the exception of the DEG encoding bidirectional sugar transporter SWEET1 (LOC18768928) (Fig. 9). These results

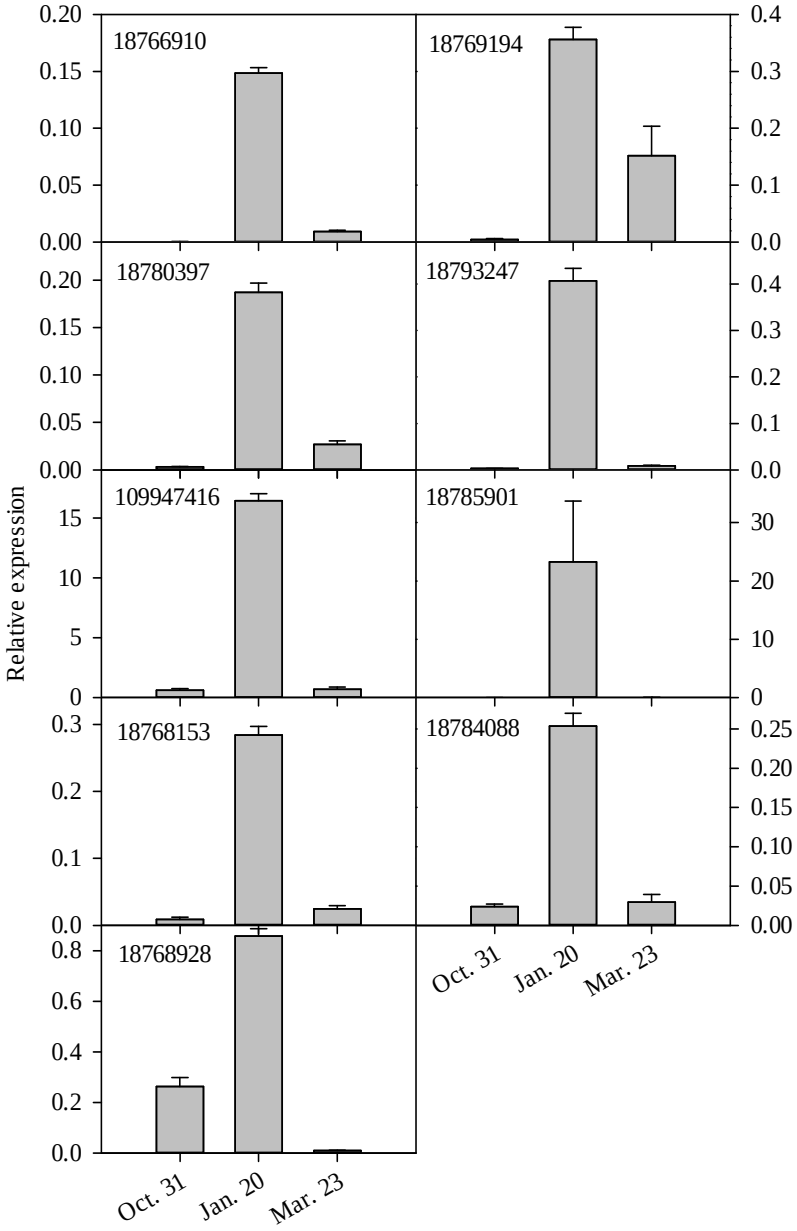

Fig. 7. Relative gene expressions for validation of the nine up- and down-regulated DEGs in ‘Soomee’ peach tree shoots during cold acclimation and deacclimation. Vertical bars represent the standard errors of the means (n = 3).

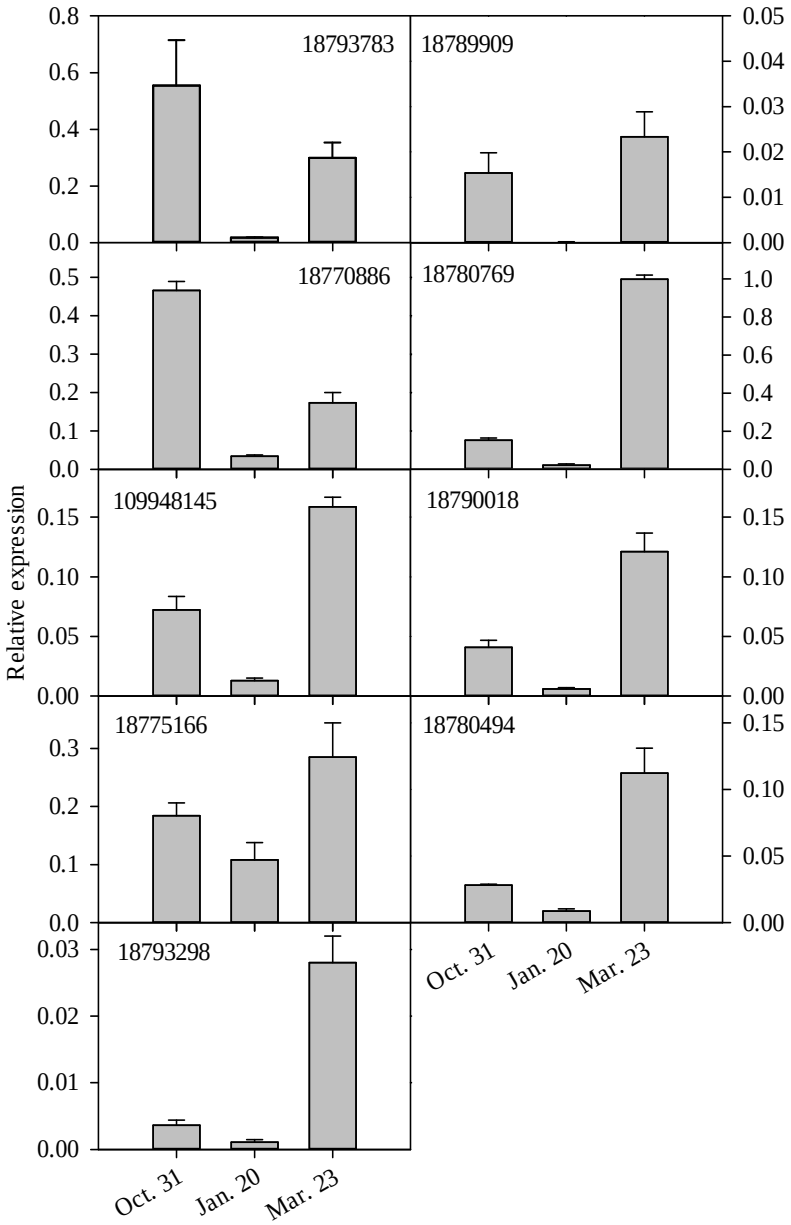

Fig. 8. Relative gene expressions for validation of the nine down- and up-regulated DEGs in ‘Soomee’ peach tree shoots during cold acclimation and deacclimation. Vertical bars represent the standard errors of the means (n = 3).

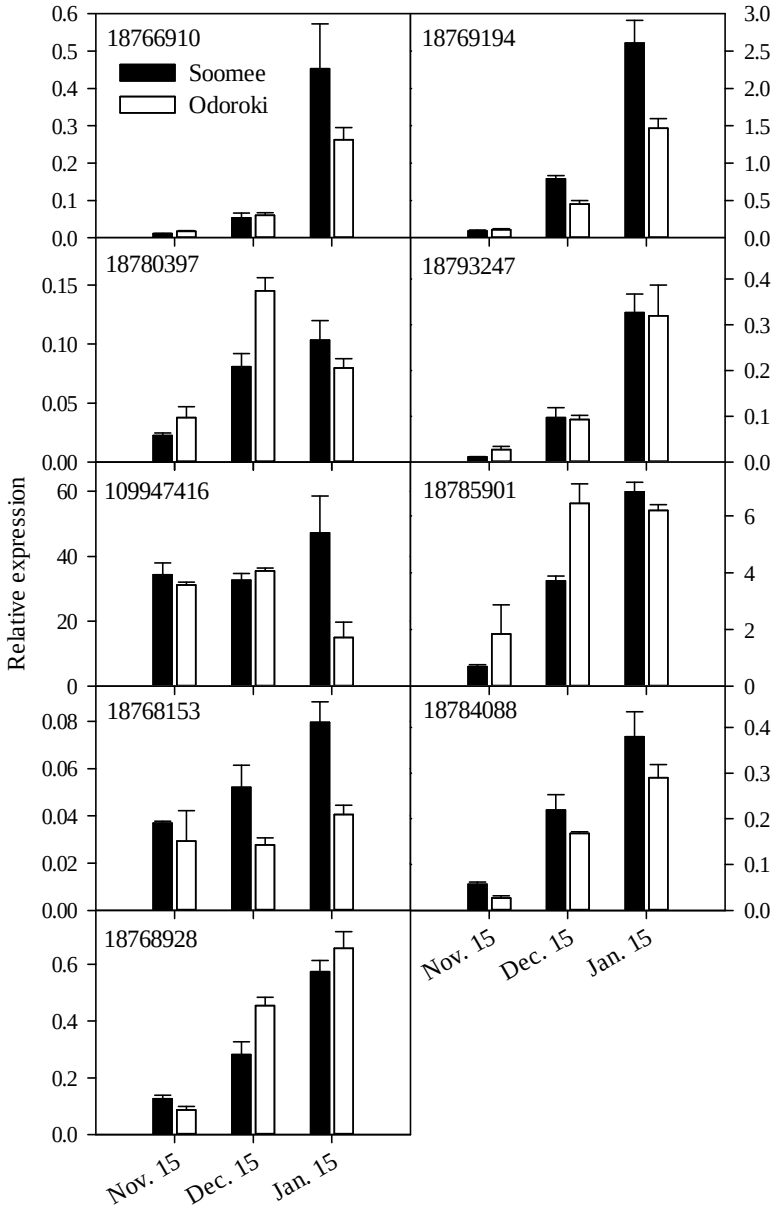

Fig. 9. Comparative gene expressions of the nine up- and down-regulated DEGs between 'Soomee' and 'Odoroki' peach tree shoots during cold acclimation. Vertical bars represent the standard errors of the means (n = 3).

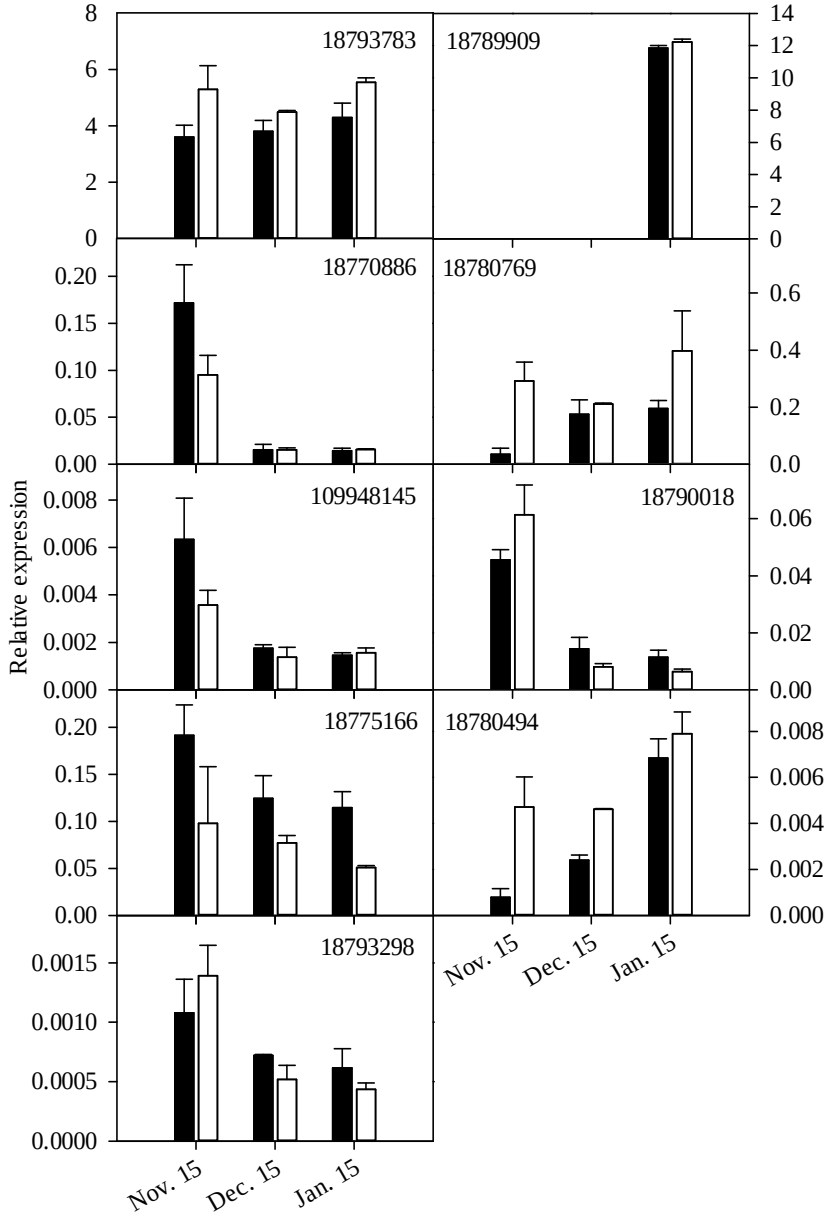

Fig. 10. Comparative gene expressions of the nine down- and up-regulated DEGs between 'Soomee' and 'Odoroki' peach tree shoots during cold acclimation. Vertical bars represent the standard errors of the means (n = 3).

demonstrate that the in silico data especially regarding the DEGs up-regulated during CA and down-regulated during DA, were useful for elucidating the molecular mechanisms involved in the cold hardiness of peach tree shoots.

Correlation of DEGs involved in cold hardiness of peach tree shoots

The correlation of DEG expression levels with the seasonal changes in cold hardiness that occur during CA and DA suggests that the DEGs common to these two processes with higher fold changes might be closely involved in determining cold hardiness in the peach tree shoots. However, higher fold changes do not necessarily imply that a DEG has a major impact on the determination of cold hardiness, since DEGs with low expression volumes (FPKM) may not play a crucial role in the response to cold stress despite their high fold change.

Most of the common DEGs were functional genes encoding enzymes and metabolic proteins, which can directly function to protect cells against stresses. The common DEGs exhibited higher correlation with cold hardiness level (Table 4), suggesting that they influentially function to determine cold hardiness in peach tree shoots. The DEGs included *polygalacturonase inhibitor*, *squalene monooxygenase*, and *late embryogenesis abundant protein 2*. However, their roles in enhancing cold hardiness are not known but have to be elucidated through further studies.

Numerous studies on functional genes have mainly focused on those encoding enzymes and protective proteins during exposure a given stress (Bassett et al., 2015; Chung et al., 2019; Grün et al., 2006; Lee et al., 2012; Nordin et al., 1993; Olvera-Carrillo et al., 2011). The stress-responsive functional genes include those that regulate accumulation of compatible solutes, passive and active transport systems

across membranes, protection and stabilization of cell structures from damage by reactive oxygen species, fatty acid metabolism enzymes, proteinase inhibitors, ferritin and lipid-transfer proteins, and and others functioning in the protection of macromolecules such as proteins and DNA from degradation (e.g., late embryogenesis abundant proteins, osmotins, and chaperones) (Ouellet and Charron, 2013; Thomashow, 1998; Visconti et al., 2019; Welling and Palva, 2008). A variety of common DEGs identified using transcriptome analysis during CA and DA in the present study will be useful in unraveling the molecular mechanisms in responses to cold stress, although their roles are required to be elucidated. Especially, the significantly up- and down-regulated DEGs encoding functional proteins and enzymes, including polygalacturonase inhibitor, squalene monooxygenase, and late embryogenesis abundant protein 2, might play crucial roles in determining cold hardiness level in peach tree shoots.

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ABSTRACT IN KOREAN

겨울철 저온은 온대 과수의 수량과 품질은 물론 재배지를 제한하는 주요 환경 스트레스 중의 하나이다. 온대 과수는 월동 중 순화와 탈순화 과정을 통해 내한성을 변화시키는데 막 지질과 단백질의 조성, 호환성 용질의 농도 등 다양한 생리·생화학적 변화를 수반한다. 이와 관련하여 다양한 유전자 발현의 변화를 아는 것은 내한성 기작을 밝히는 데 있어서 매우 중요하다. 본 연구에서는 5년생 복숭아 '수미' 품종의 가지를 대상으로 저온 순화와 탈순화 동안 전사체를 분석하고 내한성과의 상관 분석을 통해 내한성에 관련된 유전자를 조사하고자 하였다. 월동 중 '수미'의 가지에서 총 17,208개의 전사체가 발현되었다. 기능이 밝혀진 전사체 가운데 발현량에 있어서 2배 이상의 유의한 차이를 보이는 전사체는 저온 순화와 탈순화 동안 각각 1,522개와 2,830개였다. 이들 차별 발현 유전자(DEG)들은 생물학적 과정과 세포 성분 유전자 온톨로지(GO) 범주보다는 분자 기능 GO 범주에 더 많은 DEG들이 분포하는 것으로 나타났다. 분자 기능 GO 범주와 관련된 DEG들은 주로 '금속 이온 결합'과 '전사 인자 활동'과 관련이 있었다. KEGG 생화학적 반응 경로 분석에 있어서는 많은 DEG들이 '대사 반응 경로'와 '2차 대사 산물의 생합성'과 관련되어 있었다. 전사체 자료의 타당성은 발현량에 큰 차이를 보이는 18개의 DEG들을 대상으로 RT-qPCR을 통해 검증하였다. 저온 순화 처리에 따른 이들 DEG들의 발현 양상에 따르면 '오도로끼' 품종보다 내한성이 강한 '수미' 품종에서 발현량이 유의하게 높았고, 순화와 탈순화 동안 각각 상향과 하향된 DEG들이 다른 DEG보다 내한성에 더 밀접한 상관을 나타내었다. 이는

저온 순화와 탈순화 동안 *polygalacturonase inhibitor, squalene monooxygenase, late embryogenesis abundant protein2*와 같이 각각 상향과 하향된 DEG들이 복숭아의 내한성을 결정하는 데 중요한 역할을 한다는 것을 의미한다.