Plant Stress ©2012 Global Science Books

Identification of Molecular Mechanism for Freeze Tolerance in Flower Buds of *Helleborus orientalis*

Zong Liu • Roger Sauve • Suping Zhou*

Department of Agricultural Sciences, School of Agriculture and Consumer Sciences, Tennessee State University, 3500 John A Merritt Blvd, Nashville, TN 37209, USA

Corresponding author: * **zsuping@tnstate.edu**

ABSTRACT

Recent changes in climate have led to very dramatic temperature fluctuations in winter and spring. Warmer winters and early springs and sudden temperature dips to subzero in mid to late spring are of common occurrence in the United States of America. Horticultural plants, including spring blooming ornamental species, fruit trees, and vegetables are very vulnerable to such weather conditions. This study was performed to identify genes conferring tolerance to the freeze-thaw stress in flower buds of *Helleborus orientalis*, using genome wide mRNA profiling (cDNA differential display) and quantitative real-time polymerase chain reaction analysis. Flowers were collected from field plants at frozen-defrosted-recovery stages and an incubator experiment was conducted under a corresponding temperature regime (0-4-10 ± 1°C). Based on expression patterns and putative functions of the isolated genes, programmed cell death, water transportation (aquaporin proteins), and oxidative phosphorylation are proposed to play key roles in the tolerance mechanism for freezing temperature; genes in the biosynthesis of steroids and terpenes were activated as soon as the temperature warmed up to above freezing point. This study provides novel understanding and gene sequences to be used for improving tolerance to subzero freezing temperature in floral plants.

Keywords: aquaporin, fluorescent differential display, oxidative phosphorylation, quantitative-PCR, programmed cell death, secondary metabolites

INTRODUCTION

In the temperate zone where low temperature occurs seasonally, perennial plants are acclimated and enter dormancy when temperature dips into continuous subfreezing regime in winter (Arora *et al*. 1997). Plants are wakened from dormancy and begin to put out new growth during the warmer days in late winter and spring (Kalberer *et al*. 2006; Gorsuchi *et al*. 2010). The actively growing tissues including leaf and flower buds are de-acclimated and they become vulnerable to injuries induced by the freeze-thaw stress during frosts in mid to late spring season (Kalberer *et al*. 2006). This incidence can cause great economic loss to horticultural crops, especially, fruit trees, perennial floral ornamentals and vegetables (Longstroth 2007). For instance, the Easter freeze in 2007 resulted in over two billion U.S. dollars damage in the United States of America (USA) (NOAA/USDA 2008). In this case a record-breaking warm March (27-30 °C) induced many plant species to start new growth, which were fatally killed by the advent of a cold wave of subfreezing temperatures (-12 to -6°C) on the 4th-10th of April (Longstroth 2007). Temperature of the freezing of water (0 to -2°C) can cause fatal injury to mesophilic plants. Climate change is predicted to bring earlier bud break and perhaps a greater risk of frost damage to developing leaves and flowers (Augsurger 2009). It is therefore an urgent task to develop strategies on how to protect plants from injuries caused by freezing temperature fluctuation during the growth period.

Most previous studies have been performed on understanding the mechanism for plants to develop cold hardiness during the process of acclimation to freezing temperature. Plants exposed to freezing temperature go through a two-step cycle: dehydration during freezing and rehydration when thawing. Freezing temperature induces water in the tonoplast and symplastic spaces to crystalize which causes severe dehydration of cells, mechanically rupturing cell membranes leading to leakage of water and cellular components (Zhang and Willison 1992). As temperature reaches above freezing point, cells enter the thawing stage when they need to be rehydrated immediately. However, in the water transportation system (tonoplast), the gas dissolved in the sap of the xylem comes out of solution because the solubility of gas is greatly reduced in ice compared to liquid sap when it is frozen, and the formation of emboli can occur in xylem cells upon thawing if the bubbles do not go back into solution but instead expand in a process called cavitation (Yang and Tyree 1992; Pratt 2005). Delayed rehydration resulting from blockage of the water transportation channel induces physiological dehydration stress to the cells, leading to cell death.

Tolerance to freeze-thaw stress is achieved by the products and activity of many genes (Guy 1990; Kurkela *et al.* 1988) and post-translational modification of proteins (Yeh *et al.* 2000). Accumulation of antifreeze proteins (Antikainen and Griffith 1997; Bravo and Griffith 2005), modification of structural components of membranes (Wu and Fletcher 2000; Tomczak *et al.* 2002), and production of osmolytes to improve osmotic responsiveness at thawing (Rajashekar *et al.* 2006) are all associated with enhancing tolerance to freeze and thaw stress.

Helleborus orientalis Lam. is an evergreen perennial flowering plant in the buttercup (*Ranunculaceae*) family. It is a cold-hardy and drought-tolerant species. In the middle Tennessee area, USA, plants bloom from March through April and early May. In the field, flower buds exposed to freezing cold temperature would continue to form normal seed pods. The objective of this study was to isolate genes that confer tolerance to the freeze-thaw cycle in flower buds of *H. orientalis*. Since yield and quality as well as profit of many crops depend on fruit set during the early season, information generated from this study would be very useful in improving plant productivity as it targets to improve freeze tolerance of flowers.

Received: 16 May, 2011. Accepted: 25 July, 2011.

Original Research Paper

MATERIALS AND METHODS

Collection of flower buds from field and incubator treatment plants

One-year old plants of *H. orientalis* 'London Fog' were purchased from Northwest Garden Nursery (Eugene, OR, USA) and planted in a shaded garden in Nashville, Tennessee in 2005. Plants bloomed during the early spring of 2007, and again in early March, 2008. On March 8th, 2008, there was a snow event after one week of warm temperatures. On the 9th, the air temperatures dropped to -5°C and remained below 0°C with intermittent snow. On March 10th, temperature warmed up to 0-5°C, and snow started to melt. By March 14th, all remaining snow had melted and the temperature warmed up to 10-15°C.

Flower buds (unopened) were collected at three different times representing the three sample groups tested. The first group comprised of frozen flower buds that were collected on March 9th. The second group represented the tissue samples that were defrosted and which were harvested on March 10th. The third group represented the tissue samples that were recovered and which were collected on March 14th. All samples were collected at 11:00 am.

Two flower buds were picked from each plant, and flowers from five plants were pooled to form one biological sample. Five sets (biological replicates) of samples were collected on each of the three days.

Incubator temperature treatments were performed in programmable incubators (ThermoFisher, Waltham, MA, USA). Potted plants were overwintered in a regular non-heated greenhouse. On March 12th, 2010, plants bearing flower buds were placed in two incubators which were programmed at 4 ± 1°C and a 12-h photoperiod provided by fluorescent lights (100 $\mu mol.m^{-2}.s^{-1}$ photosynthetically active radiation) for two days. The incubators were reprogrammed to 0 ± 1°C, and flower buds were slightly frozen after 24 h incubation, and they were collected as frozen flower tissues. At the time when the flower buds were collected from field plants, the air temperature was 4°C for the defrosted tissues and 10°C for the recovered tissues. Thus for the two temperature treatments, the incubator temperature was re-programmed to 4 ± 1°C, frozen flowers were all defrosted, and defrosted tissues were collected after 24 h. After this, incubator temperature was increased to 10 ± 1°C, and samples (recovery stage) were collected after an additional 24 h incubation. At each collection, two biological replicates were collected from each of the two incubators. Flowers were detached from plants while they were kept inside the incubators, and collected tissues were immediately frozen in liquid nitrogen. All samples were stored at -70 °C until analyzed.

Fluorescent cDNA differential display (FDD)

1. Optimization of total RNA extraction protocol

Frozen tissue was ground into a fine powder under liquid nitrogen, and total RNA was extracted using the RNA pure reagents kit provided by GenHunter (Nashville, TN, USA). Flowers of *H. orientalis* 'Longdon Fog' are dark purple in color, it was very difficult to obtain clean RNA as the extract was brownish. To obtain high quality total RNA, the crude RNA sample was mixed with 9 M LiCl (3:1; v/v), followed by incubation at 4 °C overnight. After centrifugation at 4°C at 13,000 × *g* for 20 min, the clean pellets were dissolved in DEPC treated water. Quality of total RNA was examined on a Nanodrop ND-1000 spectrometer (NanoDrop Technologies, Inc., Wilmington, DE, USA), and pure RNA was treated with DNaseI to remove genomic DNA.

2. FDD-polymerase chain reaction (FDD-PCR)

The DNA-free RNA was converted into single strand cDNA via reverse transcription using three one-base-anchored oligo dT primers (H-T11A, G, C). Then the three cDNA subpopulations were PCR-amplified with 240 primer combinations each consisting of one anchored primer labeled with 5′ fluorescein and one of the 80 arbitrary 13-mer primers in the RNA SpectraRed Kit (Genhunter). The 240 primer pair combinations could cover the majority of mRNA population in eukaryotic cells (Liang *et al.* 1994).

PCR products were separated on 6% polyacrylamide gels, and FDD gel image was obtained by scanning the gel on a FMBIO III scanner (MiraiBio, Hitachi Solutions America, Ltd, South San Francisco, CA, USA). Bands that were present only in one or two of the three samples, and bands of different intensity in the three samples were considered as candidate differentially expressed genes. They were labeled and DNAs were isolated from gels. Several bands that appeared uniformly in all the treatments were also collected to select internal reference genes in quantitative PCR (qPCR) assay.

3. Cloning of FDD-cDNA fragments

cDNA fragments were extracted from gel pieces and re-amplified using the same primers as in DD-PCR. After confirmation of the molecular size on 1.5% agarose gels, PCR products were cloned onto PCR-Trap cloning vectors (GenHunter), and positive clones were selected on Luria Bertani (LB) plate supplemented with 20 $\mu g.\ ml^{-1}$ tetracycline. Plasmid from the selected clone was isolated using QIAprep spin Miniprep Kit (Qiagen, CA, USA), and sequenced in both directions (Zhou *et al.* 2007).

4. Determination of putative gene identity

Putative identities of cloned genes were identified by blast search in the National Center for Biotechnology Information (NCBI) nucleotide database (other organisms). DNA sequences were also translated into peptide using the online tool on the ExPASy Proteomics Server (Gasteiger *et al.* 2003). The deduced peptide was blasted against the non-redundant protein databases (NCBI) to identify putative gene function.

Quantitative reverse-transcriptase-PCR (qRT-PCR) assay

Primers for qRT-PCR analysis were designed using the Primer Express Software (Applied Biosystems 1997). Primer sequences are in **Table 1**, and they were synthesized by the Invitrogen Corporation (Carlsbad, CA, USA). Total RNA was extracted and processed following the same procedure as in DD-PCR described above. Single strand cDNA was synthesized by reverse transcription of DNA-free RNA using Taqman® reverse transcription reagent kit (Applied Biosystems, Carlsbad, CA, USA).

The qPCR reaction mixture (in 40 µL) contained cDNA amount equal to 100 ng total RNA, primer pairs (100 µM each), and the Power SYBR Green PCR Master Mix (Applied Biosystems). PCR amplification was performed using a program of 40 cycles of 94°C for 30 s, and 60°C for 1 min on a 7000 Real Time PCR System (Applied Biosystems). For each treatment, four biological replicates were submitted to analysis. An endogenous reference gene (JK037034) was selected for having a stable expression level [threshold cycle (Ct) value] in the three groups of tissues. The frozen samples were used as the calibrator (control) to estimate the relative transcript abundance in flower bud tissues. Fold change compared to control was estimated using $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta C_t$ (treated)-ΔC_t (control). Ranges of fold given for each treatment were determined as $2^{-\Delta\Delta Ct + s}$ for upper limit, and $2^{-\Delta\Delta Ct - s}$ for lower limit, where s =the standard deviation of the ΔΔCt values among the four biological replicates (Applied Biosystems 1997).

RESULTS

Optimization of RNA extraction protocol for *H. orientalis* flowers

FDD-PCR requires high purity RNA for optimum performances. When assayed on a spectrometer, the ratio of the absorbance at 260 and 280 nm ($A_{260/280}$) of a pure RNA sample should be around 2 and $A_{260/230}$ at above 1.80. The initial RNA sample had a ratio of $A_{260/230}$ at 0.80, and $A_{260/280}$ at 1.88 (**Fig. 1A**), which indicates that the RNA solution contained a very large amount of phenol and other organic compounds. After further purification in LiCl, the ratio was 2.11 for $A_{260/230}$, and 2.12 for $A_{260/280}$ with a single

Table 1 Quantitative PCR primers and amplicon size.

NCBI Accession No.*	Primers (Forward/Reverse)	Amplicon size (bp)
HQ853479	5′-CAAGTTGGGCTTGAGCATTGA-3′/ 5′-CCAACACGGGCATATCTGTGT-3′	70
JK037065	5′-TTGGAAAGTAGCGATCCCTCTT-3′/ 5′-ACCGCCATCAGATCCTTCTCT-3′	78
JK037037	5′-CATACGGGAAAACCAGATTGTAGA-3′/ 5′-CCGCCATTTACAAGTATCAGCTTA-3′	110
JK037038	5′-ACCGGTGAAGCGAAAGCA-3′/ 5′-TTTGCATCAATCAAGCCTTCTTAG-3′	77
JK037039	5′-GAAAGCCAGAATGACTGAAGAAATG-3′/ 5′-AACATCATAATAGAAGACGAGACAATGG-3′	104
JK037033	5′-TTACCGCAAGCGTTTATTAAGCT-3′/ 5′-ACCAAAGATAAGACCCGATCCTT-3′	122
JK037045	5′-GGGAAGACAACAAATTGGTGATG-3′/ 5′-CACCTATTTGACAATAAACCAACAAGA-3′	92
JK037036	5′-CCTTGCAAACCCAGATTATGC-3′/ 5′-CATGACTCACGCACTCTTTTATATGA-3′	85
HQ853475	5′-TCGGGCTAGTGTACACCGTTT-3′/ 5′-ACCCACATCGCCTTTCTTAGG-3′	59
JK037056	5′-ATGTCATCCGTGATGCAGTTACTT-3′/ 5′-ACATCCATGGCGGTAACAGTCT-3′	67
JK037035	5′-GGAAGCTTTTGATCCGGAAAT-3′/ 5′-CCCTGCTTCAGCCTGAGAGTA-3′	84
JK037044	5′-GGATGCAAGGGTGAATACTATCTAGAT-3′/ 5′-GACAACACATTTTCCTATTACATCACAA-3′	121
JK037029	5′-TCCGGAGAGCTGTGGTTTTAA-3′/ 5′-GCCACGGTGGTGAAAGACTT-3′	84
HQ853478	5′-GGTGCTGGTTGCCTCATCA-3′/ 5′-TGTGGCAGGGAAGATTGCA-3′	60
JK037046	5′-GCAGAAGTAGTTGAGCCAGTTCAA-3′/ 5′-AGACGGGCATGACATTTCTTTT-3′	147
JK037048	5′-GCATTTTGCTGTGAGTGATTAAAAC-3′/ 5′-CACCACCTGGAAAAGCCTAAAAC-3′	147
JK037049	5′-AGGAAAGGTACAGCATCATCAACTAG-3′/ 5′-TGGGTTAAATAGTGAGAGAAGAAAGGT-3′	106
JK037053	5′-GAGCATGCATGATGACAGTACTCTAG-3′/ 5′-TCCTTCAAACCATAGGCAAAGTATAA-3′	93
JK037047	5′-GCTTACTTGGTGGGTCTATTCGA-3′/ 5′-GGCATGATGGTGACCCTCTT-3′	71
HQ853467	5′-ATTCATAGGCCATGGAGTCTTTG-3′/ 5′- CTTGGGTGAGGTTGTCGAAGAG-3′	62
JK037040	5′-CGGGCACACAATCCAAGTG-3′/ 5′-CCAGCATTATCGAATGTCAATAGAA-3′	66
JK037041	5′-CTTGATTGATGAGGCAGGTTCTC-3′/ 5′-TTTCAAGTTCCCTGGCTTCCT-3′	74
HQ853472	5′-GCGTTCTCGGATTGTTGAAGTC-3′/ 5′-CCTGCATAAGCATCCATTTCCT-3′	62
JK037055	5′-ACTAGGAGATTGAGAAGATGAATGAGAA-3′/ 5′-GGAAATATGCAAGAGTTGATCATGTAA-3′	88
JK037050	5′-TTTTGTGGCTGTGACATTTATGTG-3′/ 5′-CGGAGTGCAGGAAAATCAAGTAA-3′	76
JK037063	5′-CGAGGTGAAGCAGAGAACACTTC-3′/ 5′-TTCAGAACAAGGGATACTCCACATT-3′	87
JK037058	5′-TCCTCAACTTCCAGTTTCAAAACC-3′/ 5′-TTCTTGTGAAGAGGCATGTTGAAT-3′	78
JK037052	5′-TGCGCTATTCATGCCAAGAG-3′/ 5′-TCGCTCGCCCCTGATG-3′	71
HQ853474	5′-AAGCTGGATGATCGAGAACCA-3′/ 5′-GCTCATCCAACTCCGAAACTG-3′	62
HQ853470	5′-TCATTTATATGGCACTGGTGGATCT-3′/ 5′-GTTATGCTACCTTGAACCTTTGCA-3′	70
HQ853466	5′-CCAAGAACCGCGAACTTGA-3′/ 5′-GCCAAGGGCTCCAAGCAT-3′	59
JK037059	5′-GGCGGCTCTACTATTGCCTATCT-3′/ 5′-GCAATTGTGGACCCTTCTTGA-3′	76
JK037061	5′-AGACTGCTGAACCTGTTGCTGTT-3′/ 5′-TCTGCAGTCTGATAAAAGCCTTTCT-3′	85
HQ853465	5′-ATGCCCCGCCAACAGA-3′/ 5′- TTCTCCTCGGCCATGCA-3′	56
JK037057	5′-CTTGTTTTTTTAGGAAACGTTGCA-3′/ 5′-TGCCGGGCCTCATATCA-3′	67
JK037054	5′-CCGCCAGAAATACACTATCTACAACTA-3′/ 5′-CTATCTCTATGCATCTCTGTCCCTCTT-3′	97
HQ853476	5′-CAACCGCCTTGTTCATCACA-3′/ 5′-TCAACTCTTCTGGCGCTTCA-3′	60
JK037030	5′-CCAATGTAGAAACCGGCTCTGT-3′/ 5′-CAGGCTTGAGAGATAAATCGACAA-3′	87
HQ853481	5′-ATGACCCTGTTGTTGGTGTTACAA-3′/ 5′-GTGGTGCGTCCAATATTATGATGT-3′	67
HQ853484	5′-TAGGGCTCTGACGCATGAAGA-3′/ 5′-TCATTGATTGCAACAGCAAGTTT-3′	60
JK037027	5′-TTGCTACTCGGTTTTGATTTTATCAT-3′/ 5′-TCGAGGTAAAAGAACCCCTTTAGA-3′	128

*National Center for Biotechnology Information (NCBI) Genbank accession number of gene clones

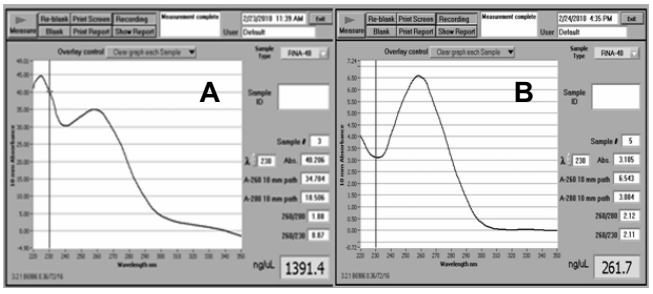

Fig. 1 Effect of extraction methods on purity of RNA extracts from flower buds of *H. orientalis*. RNA samples before (**A**) and after (**B**) purification in LiCl solution were measured on a Nanodrop-100 spectrometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). Total RNA was initially extracted from *H. orientalis* flower buds using the RNA pure reagents kit (GenHunter, Nashville, TN, USA). The low A_{260}/A_{230} ratio (**A**) indicates contamination of phenolic compounds in the RNA solution. After purification in 9 M LiCl (3: 1; v/v), pure RNA was obtained with higher ratio of A_{260}/A_{230} and A_{260}/A_{280} (**B**).

peak of absorbance at 260 nm (**Fig. 1B**). These results confirmed the high purity of RNA extracts after purification with LiCl. The same protocol was used to extract RNA from all the samples in this study.

Identification of genes differentially expressed at freeze-thaw-recovery stages

After removal of the genomic DNA, total RNA from each sample was transcribed into three cDNA sub-populations. Each of the cDNA sub-populations was then amplified using 240 primer pairs. On the FDD gels, products from the same primer pair were run side by side as frozen tissues (lane A), defrosted tissues (lane B) and recovered tissues (lane C). DNA banding patterns on FDD gel images were compared visually to identify cDNA fragments that were differentially expressed in the three types of tissues. **Fig. 2A** is the gel image of cDNA banding patterns using one pair of primers. Bands that were only present (or absent) or showing different signal intensity in one of the three lanes (A, B, C) should contain fragments of genes that were differentially expressed in the three types of tissues. For instance, band 1 was found only in the frozen tissue; band 2 increased in the defrosted tissue; band 3 was present in frozen and defrosted tissues, but absent in the recovered tissue; and band 4 appeared only in the recovered tissue. These bands were isolated from the FDD gel, reamplified using the same pair of unlabeled primers, and the PCR products were separated on 1.5% agarose gels (**Fig. 2B**) to determine the approximate length of the DNA fragments and if the reaction contained single or multiple DNA fragments. PCR products containing one band (>200 bp) were directly cloned into the PCR-TRAP vector, and the cloned

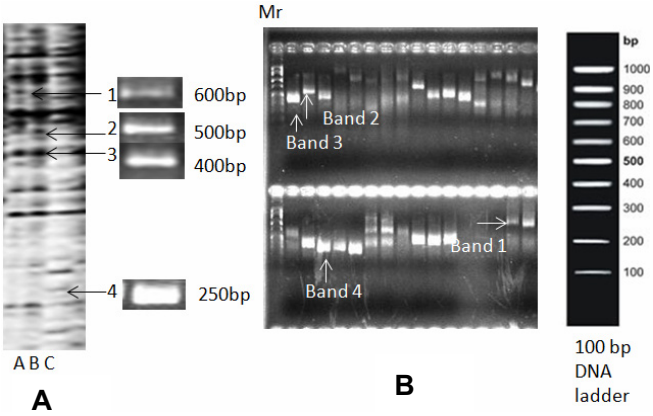

Fig. 2 Identification of temperature responsive genes in flowers of *H. orientalis* using fluorescent cDNA differential display. (**A**) A fluorescent differential display (FDD) gel image of cDNA populations from frozen flower buds (lane A), defrosted flower buds (lane B) and flower buds at recovery stage (lane C). Arrows point to cDNA bands (gene fragments) that were differentially expressed in frozen, defrosted, or recovered tissues: band 1 present only in the frozen tissue; band 2 increased in the defrosted tissue; band 3 present in frozen and defrosted tissues, but absent in the recovered tissue; band 4 appeared only in the recovered tissue. (**B**) An agarose gel image of re-amplified cDNA bands identified from FDD gels. DNA bands isolated from FDD gels were re-amplified using PCR and run on 1.5% agarose gel. The molecular size marker (Mr) lane was loaded with 100 bp DNA marker, the molecular size of each marker band is shown in the 100-bp DNA ladder (Fisher Scientific, Waltham, MA, USA).

gene was sequenced. The identity of the gene was determined by searching the sequence in the databases.

From this study, 26 gene clones (partial or full length) with putative functions were identified (**Table 2**), and the other clones had no significant similarity to gene sequences in the NCBI databases.

Changes in the relative transcript level of the cloned genes during freeze-thaw-recovery stages

In the qRT-PCR assay, one gene (JK037034) had a stable transcript level under the three temperature regimes (26.87 ± 0.06, 26.87 ± 0.12, 27 ± 0.11); it was selected as the internal reference gene to normalize the Ct value of each gene. Then the transcript abundance in the frozen tissue was used as control to calculate the relative transcript level during defrost and recovery stages. Based on the relative expression level (fold change), the cloned genes were divided into six groups (**Table 3**).

Group 1 has the genes that maintained constant transcript level. These genes (clones) are HQ853480 (protein phosphatase), HQ853473 (thaumatin-like protein), JK037026, JK037064 and JK037066. In the incubator experiment, HQ853480 and JK037066 were not stably expressed; instead, the transcript level of these two clones increased significantly at 10°C.

Group 2 contains genes that maintained stable transcript levels in frozen and defrosted flower buds, the transcript level had a significant increase (3-20-fold) at the recovery stage (10°C). These genes include HQ853479 (heat shock protein), JK037039 (gamma-tocopherol methyltransferase), JK037033 (plant terpene cyclase), JK037037 (protein binding protein putative), HQ853475 (putative aquaporin), HQ853478 (26S proteasome), and several unknown genes. In the incubator experiment, the following genes were induced continuously as temperature increased to 4 or 10°C, including HQ853475, JK037039, JK037033 and several unknown genes.

Group 3 contains genes that were induced continuously as temperature warmed up. Genes (clones) in this group are JK037047 (histone H3), HQ853467 (membrane protein), JK037040 (multicopper oxidase), JK037041 (Clp protease ATP), HQ853472 (CHCH superfamily), JK037063 (glutathione *S*-transferase GST), and two genes with unknown identity. All the genes except JK037055 followed the same expression pattern in field plants as well as in the incubator experiment.

Group 4 contains genes of which the transcript level was much higher in the defrosted samples compared to the frozen tissues, but had no further change in the recovery stage. This group has HQ853474 (cell elongation), HQ853470 (metal transporter protein), and two unknown genes. However, expression of these genes continued to increase as temperature warmed up in the incubator experiment.

Group 5 has six genes, including HQ853466 (chlorophyll *a*/*b*-binding), JK037059 (photosystem I reaction cen-

Table 2 Gene identity isolated from flower buds of *Hellebore orientalis* 'London Fog'.

NCBI accession No.*	Putative gene identity	Functions
HQ853472	NADH-ubiquinone oxidoreductase	Oxidative phosphorylation
HQ853480	serine/threonine phosphatases	Signal transduction
HQ853473	thaumatin-like proteins	Stress response
HQ853478	26S proteasome regulatory complex component	Posttranslational modification, protein turnover, chaperones
HQ853485	phosphoenolpyruvate carboxykinase (ATP)	C4 carbon fixation
HQ853474	Delta(24)-sterol reductase	Biosynthesis of steroids
HQ853470	ZIP zinc/iron transport family	Ion transport
HQ853465	cold and light inducible protein	Stress response
HQ853467	predicted membrane protein	Unknown function
HQ853484:	gamma carbonic anhydrase like 1	Photosynthesis
HQ853476	2-oxoglutarate-dependent dioxygenase	Alkaloid biosynthesis
HQ853479	heat-shock cognate protein	Stress tolerance
HQ853475	putative aquaporin	Membrane protein for regulation of water flow
HQ853466	chlorophyll a/b binding protein	Photosynthesis
HQ853481	Hypothetical protein	Unknown
JK037039	gamma-tocopherol methyltransferase	Biosynthesis of steroids
JK037033	plant terpene cyclases C1	Isoprenoid biosynthesis
JK037047	histone H3	Chromatin structural protein
JK037040	multicopper oxidase	Iron and copper homeostasis
JK037041	ATP-dependent caseinolytic protease (Clp)	An essential housekeeping enzyme in plant chloroplasts
JK037063	glutathione *S*-transferase GST	Detoxification enzyme
JK037059	photosystem I reaction centre subunit	PSI system
JK037061	sedoheptulose-1,7-bisphosphatase	Carbon fixation (Calvin cycle) enzyme
JK037057	ATP synthase	Energy regeneration
JK037030	programmed cell death	Stress response

*National Center for Biotechnology Information (NCBI) Genbank accession number of gene clones

Table 3 Gene expression in *Hellebore orientalis* flower buds at frozen, defrost and recovery stages.

NCBI Accession No.[z]	Field samples			Incubator experiment		
	Frozen	**Defrost**	**Recover**	**0 ± 1°C**	**4 ± 1°C**	**10 ± 1°C**
Group 1 Stably expressed at frozen, defrost and recover stages						
JK037026	1.00[y]	1.27	1.16	1.00	1.49	1.07
	0.8-1.3	0.9-1.3	0.8-1.7	0.9-1.1	0.8-1.7	0.7-1.4
HQ853480	1.00	0.75	1.34	1.00	1.35	3.04
	0.8-1.3	0.5-1.0	1.0-1.9	0.9-1.2	1.2-1.5	2.7-3.4
JK037064	1.00	0.73	0.92	1.00	2.01	1.26
	0.8-1.3	0.4-1.3	0.8-1.1	0.7-1.5	1.3-3.2	0.6-2.5
JK037066	1.00	0.93	1.24	1.00	1.04	2.10
	0.8-1.2	0.9-1.0	0.9-1.8	0.9-1.1	0.7-1.5	1.7-2.6
HQ853473	1.00	0.90	0.84	1.00	0.89	1.08
	0.8-1.3	0.8-1.1	0.6-1.1	0.7-1.4	0.7-1.2	1.0-1.2
Group 2 Stably expressed at frozen, defrost stages and increased in recover stage						
HQ853479	1.00	1.04	8.82	1.00	1.78	3.21
	1.0-1.1	0.8-1.4	7.7-10.0	0.7-1.5	1.6-2.0	2.1-4.8
JK037065	1.00	0.49	5.12	1.00	0.36	3.70
	0.7-1.4	0.3-0.7	4.5-5.9	0.9-1.2	0.2-0.5	3.2-4.3
JK037037	1.00	1.66	20.04	1	1.03	12.29
	0.7-1.5	1.4-2.0	16.9-23.8	0.9-1.1	0.8-1.3	10.6-14.2
JK037038	1.00	1.08	3.06	1.00	0.97	7.63
	0.8-1.3	0.8-1.4	2.3-4.1	0.8-1.3	0.8-1.2	6.2-9.4
JK037039	1.00	0.98	10.70	1.00	3.08	45.22
	0.6-1.6	0.4-2.5	10.0-11.5	0.7-1.5	2.4-3.9	36.1-56.7
JK037033	1.00	0.81	3.52	1.00	2.23	18.83
	0.9-1.1	0.7-0.9	3.0-4.2	0.7-1.4	1.9-2.9	14.5-24.4
JK037045	1.81	1.05	12.36	1.00	1.98	20.53
	0.9-1.3	0.6-1.9	10.7-14.3	0.8-1.2	1.7-2.3	14.5-29.1
JK037036	1.00	1.67	28.50	1.00	2.04	26.59
	0.8-1.3	1.3-2.1	25.2-32.2	0.8-1.2	1.9-2.2	29.9-44.8
HQ853475	1.00	0.66	4.32	1.00	2.91	14.51
	0.8-1.2	0.5-0.9	4.0-4.7	0.9-1.1	2.8-3.0	11.3-18.6
JK037056	1.00	1.41	2.85	1.00	1.13	2.90
	0.9-1.1	1.2-1.6	2.3-3.5	0.7-1.4	1.0-1.3	2.6-3.3
JK037043	1.00	1.81	5.37	1.00	0.91	4.43
	0.7-1.5	1.3-2.6	4.8-6.0	0.9-1.1	0.5-1.8	2.6-7.5
JK037035	1.00	1.02	6.25	1.00	0.91	9.93
	0.8-1.3	0.8-1.2	5.3-7.7	0.8-1.2	0.6-1.3	8.2-12.1
JK037044	1.00	1.22	7.58	1.00	0.73	1.73
	0.6-1.6	1.0-1.9	6.9-8.3	0.9-1.3	0.6-0.8	1.6-1.9
JK037029	1.00	0.43	2.59	1.00	0.82	1.88
	0.9-1.1	0.2-0.9	1.8-3.7	0.7-1.4	0.6-1.2	1.2-2.9
HQ853478	1.00	0.60	17.89	1.00	1.51	8.94
	0.7-1.4	0.5-0.8	12.9-24.8	0.6-1.8	1.2-1.8	5.7-14.1
JK037046	1.00	1.63	8.53	1.00	1.33	10.89
	0.6-1.6	1.1-2.4	5.3-13.7	0.9-1.1	1.1-1.6	9.0-13.1
JK037048	1.00	1.33	10.89	1.00	2.95	15.78
	0.9-1.1	1.1-1.7	9.0-13.1	0.9-1.2	1.6-5.5	13.6-18.4
JK037049	1.00	1.55	7.13	1.00	1.55	7.13
	0.9-1.2	1.1-2.1	5.8-8.7	0.9-1.5	1.1-2.1	5.8-8.7
Group 3 Continuously increased in defrost and recover stages						
JK037053	1.00	8.57	13.14	1.00	2.33	19.36
	0.6-1.6	7.4-9.9	10.5-16.4	0.9-1.2	1.8-2.9	14.0-26.7
JK037047	1.00	2.46	7.60	1.00	5.99	72.20
	0.8-1.3	2.0-3.0	5.8-9.9	0.901.1	4.0-8.9	56.1-93.0
HQ853467	1.00	4.26	22.87	1.00	6.81	35.08
	0.7-1.4	4.1-4.4	19.7-26.5	0.9-1.1	5.5-8.4	29.8-41.2
JK037040	1.00	3.95	30.59	1.00	3.09	10.88
	0.6-1.6	3.3-4.8	22.8-41.1	0.7-1.3	2.3-4.1	10.6-11.2
JK037041	1.00	5.63	6.27	1.00	3.75	8.74
	0.9-1.1	5.3-6.0	5.9-6.6	0.9-1.1	2.9-4.8	7.3-10.4
HQ853472	1.00	2.16	5.30	1.00	1.96	10.16
	0.6-1.6	1.6-2.9	4.0-6.9	0.7-1.4	1.6-2.3	9.0-11.5
JK037055	1.00	4.61	7.13	1.00	0.76	1.10
	0.6-1.8	3.1-6.8	6.9-7.4	0.8-1.2	0.5-1.2	0.9-1.3
JK037050	1.00	4.56	8.55	1.00	5.48	6.59
	0.8-1.2	4.3-4.8	6.8-10.6	0.7-1.5	2.6-11.3	4.6-9.4
JK037063	1.00	9.86	12.44	1	9.86	12.44
	0.7-1.5	7.1-13.8	10.2-15.2	0.7-1.5	7.1-13.8	10.2-15.2
Group 4 Increased in defrost stage and then remained stable in recovery stage						
JK037058	1.00	3.73	4.42	1.00	2.05	5.45
	0.7-1.5	2.9-4.7	3.7-5.3	0.9-1.1	1.3-3.2	5.0-5.9
JK037052	1.00	3.15	3.39	1.00	2.12	9.81
	0.8-1.3	2.9-3.4	2.5-4.5	0.9-1.1	1.9-2.5	7.5-12.7

Table 3 (Cont.)

NCBI Accession No.[z]	Field samples			Incubator experiment		
	Frozen	Defrost	Recover	0 ± 1°C	4 ± 1°C	10 ± 1°C
Group 4 Increased in defrost stage and then remained stable in recovery stage (Cont.)						
HQ853474	1.00	3.23	5.48	1.00	3.41	6.11
	0.7- 1.4	2.0-5.2	4.9-6.1	0.9-1.2	2.3-5.0	5.1-7.3
HQ853474	1.00	3.23	5.48	1.00	3.41	6.11
	0.7- 1.4	2.0-5.2	4.9-6.1	0.9-1.2	2.3-5.0	5.1-7.3
HQ853470	1.00	2.14	3.14	1.00	2.38	14.82
	0.4-1.6	1.4-3.3	2.7-3.6	0.9-1.1	1.5-3.7	13.3-16.5
Group 5 Increased during defrost stage but dropped at recovery stage						
HQ853466	1.00	14.78	11.98	1.00	18.53	32.20
	0.5-2.0	12.3-17.7	9.4-15.3	0.9-1.1	13.8-24.9	24.9-41.1
JK037059	1.00	8.00	5.37	1.00	66.94	166.57
	0.9-1.2	7.6-8.5	4.4-6.5	0.9-1.2	52.3-85.6	154.5-179.6
JK037061	1.00	9.12	5.07	1.00	4.08	14.10
	0.7-1.4	7.9-10.6	4.3-6.0	0.8-1.3	3.1-5.4	13.3-14.9
HQ853465	1.00	43.65	2.26	1.00	3.53	0.98
	0.9-1.1	41.8-45.6	2.0-2.5	0.8-1.3	2.8-4.5	0.8-1.2
JK037057	1.00	97.59	2.59	1.00	7.55	72.20
	0.9-1.1	80.9-117.6	2.4-2.8	0.9-1.1	6.0-9.4	56.1-93.0
JK037054	1.00	4.67	2.97	1.00	1.61	11.82
	0.8-1.3	3.9-5.6	2.8-3.1	0.7-1.5	1.1-2.3	10.7-13.1
Group 6 Decreased at defrost stage and then increased at recovery stage						
HQ853476	1.00	0.41	1.09	1.00	0.35	0.89
	0.8-1.2	0.3-0.5	0.9-1.3	0.9-1.1	0.3-0.5	0.7-1.2
JK037030	1.00	0.49	1.31	1.00	0.89	1.08
	0.7-1.3	0.4-0.6	1.2-1.4	0.7-1.4	0.7-1.2	1.0-1.2
HQ853481	1.00	0.60	5.60	1.00	0.31	6.49
	0.8-1.2	0.5-0.7	4.5-7.0	0.9-1.2	0.2-0.4	5.8-7.3
HQ853484	1.00	0.47	4.85	1.00	0.45	3.22
	0.7-1.5	0.4-0.6	4.1-5.7	0.8-1.3	0.3-0.6	2.1-5.0
JK037027	1.00	0.60	1.31	1.00	0.60	1.31
	0.9-1.1	0.5-0.7	1.1-1.5	0.7-1.4	0.5-0.7	1.1-1.5

[z]: National Center for Biotechnology Information (NCBI) Genbank accession number of gene clones;
[y]: Fold of gene transcript relative to frozen tissue. Values above 1.0 indicates increases, and below 0 decreases in transcript level. The fold and the range given for each temperature treatment were determined by evaluating the expression: $2^{-\Delta\Delta Ct}$ with $\Delta\Delta Ct + s$ and $\Delta\Delta Ct - s$, where s =the standard deviation of the $\Delta\Delta Ct$ values among the four biological replicates for each treatment.

ter subunit), JK037061 (sedoheptulose-1, 7-bisphosphatase), HQ853465 (cold induced, light), JK037057 (ATP synthase), and JK037054 of unknown identity. However, only expression of HQ853465 was validated in the incubator test, all the other genes (clones) increased continuously as incubator temperature warmed up from 0 to 10°C.

Group 6 contains five genes, HQ853476 (oxidoreductase), JK037030 (programmed cell death), HQ853484 (carbonic anhydrase-like), HQ853481 (hypothetical protein) and JK037027. Expression of genes in this group was suppressed in defrosted tissues. In the recovery stage, the transcript abundance in HQ853484 and HQ853481 exceeded (4 to 5-fold) the frozen flowers, while the other two genes (clones) had a similar transcript level in frozen and recovered tissues. Except for JK037030, the other four genes followed the same expression patterns in the flowers collected from field and incubator treatments.

DISCUSSION

Low temperature as well as the induced physiological and mechanical stresses affect the expression of multiple genes and proteins. As temperature warms up and plants enter recovery stage and prepare for new growth, a very different set of genes and proteins should be activated. Due to the complexity of the process, it is very challenging to identify those genes that are directly responsible for tolerance to the freeze-thaw stress. From *H. orientalis* flowers, over 50 plus gene fragments were identified in FDD-PCR analysis. Based on the putative function and expression under different temperature conditions, genes encoding for proteins that participate in apoptosis, aquaporin activity and oxidative phosphorylation could be good candidates for tolerance to the freeze and thaw stress.

Apoptosis, also known as programmed cell death, plays important roles in the elimination of sub-lethally damaged cells under various stresses. Experimental evidences show that suppressed activity of apoptosis is correlated to higher tolerance to stress injury. A study on *Sarcophaga crassipalpis* identified that cold hardening improves tolerance to lethal injury through blockage of cold-induced apoptosis (Yi and Lee 2011). In *H. orientalis* flowers, JK037030 (programmed cell death) was suppressed at the thawing stage followed by a slight increase at 10°C in the field samples; the transcript level remained stable in the incubator experiment. During the thawing of flower buds, field plants were exposed to a more complex environmental condition compared to the incubator experiment, apoptosis could be strongly suppressed for the maintenance of homeostasis and structural integrity of reproductive organs such as flower buds.

Aquaporins are members of the major intrinsic protein superfamily of integral membrane proteins which enable the transport of water, glycerol, and other solutes across membranes in various organisms (Van Dijck *et al.* 2005). In *Arabidopsis*, 30 genes have been found that code for aquaporin homologues. Some of these genes code for highly abundant constitutively expressed proteins and some are known to be temporally and spatially regulated during development and in response to stress (Kjellbom *et al.* 1999). Overexpression of aquaporin proteins in *Arabidopsis* plants increased the transgenic plant's tolerance to salt, drought and cold acclimation ability (Peng *et al.* 2007). The activity of these water channel proteins could affect the speed of dehydration at freezing stage and rehydration at thawing.

In *H. orientalis* flowers, expression of a putative aquaporin (HQ853475) remained stable in frozen and defrosted flower buds, it increased significantly at recovery stage (10°C) in field samples. We propose that the aquaporin gene

was activated to sustain water homeostasis in the flower buds as temperature warmed up when water loss from transpiration intensified. In the incubator experiment, constant air circulation could induce additional water-deficient stress, thus the aquaporin gene expression was induced more strongly and continuously to help maintain sufficient water supply to flower buds during defrost and recovery to normal growth.

As temperature warms up, cellular metabolisms become more active. In *H. orientalis* flowers, the activated genes participate in carbon fixation, synthesis of photosynthetic machinery components (PSI and PSII), cell proliferation (chromatin structural protein, ribosomal protein, and others) and secondary metabolism. For these reactions to proceed smoothly, adenosine triphosphate (ATP) is required to provide energy. Oxidative phosphorylation is a highly efficient way of releasing energy. The HQ853472 (NADH-ubiquinone oxidoreductase) gene was induced continuously as flower buds entered defrost and recovery stages under warmer temperature conditions, which could be an indication of active oxidative phosphorylation pathway to generate more ATP. Studies on other species also found that the temperature sensitivity of NADH-ubiquinone oxidoreductase was affected by the physiological property of plants. In the warm-season potato, expression of NADH-ubiquinone oxidoreductase gene in leaves was suppressed upon exposure to cold stress (Svensson *et al.* 2002).

H. orientalis is known to produce many species of alkaloids. Three genes were identified in the pathways of steroids and terpenoid biosynthesis, they are JK037039 (gamma-tocopherol methyltransferase), HQ853474 (Delta (24)-sterol reductase) and JK037033 (plant terpene cyclase). These genes were activated as soon as flowers were defrosted. The early activation of these genes suggest that secondary metabolites may play some role in tolerance to freeze and thaw stress in this species.

It is very unlikely to replicate the field condition in a laboratory setting. In this study, there are some discrepancies in the data between field samples and the incubator experiment. However, most of the genes followed similar expression patterns under both conditions, which confirmed that the measurement of gene expression is reliable.

According to this study, gene expression becomes active as temperature warms up, even from below freezing point to above zero but still very chilled (3-5°C) condition. Many genes are activated, as identified in this study; some are involved in producing molecules which protect essential metabolic activities from being interrupted. It is more important to know which genes are activated first, or the sequential order of the gene activation, to define the function of these genes. This should be the focus in future study.

ACKNOWLEDGEMENTS

This project was supported by Capacity Building Research Project and Evan Allen Funds from the United States Department of Agriculture, National Institute of Food and Agriculture.

REFERENCES

Arora R, Rowland LJ, Panta GR (1997) Chill-responsive dehydrins in blueberry: Are they associated with cold hardiness or dormancy transitions? *Physiologia Plantarum* **101**, 8-16

Antikainen M, Griffith M (1997) Antifreeze protein accumulation in freezing-tolerant cereals. *Physiologia Plantarum* **99**, 423-432

Applied Biosystems (1997) ABI PRISM 7700 Sequence Detection System, *User Bulletin* # **2**, 11-15

Augspurger CK (2009) Spring 2007 warmth and frost: Phenology, damage and refoliation in a temperate deciduous forest. *Functional Ecology* **23**, 1031-1039

Bravo LA, Griffith M (2005) Characterization of antifreeze activity in Antarctic plants. *Journal of Experimental Botany* **56**, 1189-1196

Gasteiger E, Gattiker A, Hoagland C, Ivanyi I, Appel RD, Bairoch A (2003) ExPASy: The proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Research* **31**, 3784-3788

Gorsuchi PA, Pandey S, Atkin OK (2010) Thermal de-acclimation: how permanent are leaf phenotypes when cold-acclimated plants experience warming? *Plant, Cell and Environment* **33**, 1124-1137

Guy CL (1990) Cold acclimation and freezing stress tolerance: role of protein metabolism. *Annual Review of Plant Physiology and Plant Molecular Biology* **41**, 187-223

Kalberer SR, Wisniewski M, Arora R (2006) Deacclimation and reacclimation of cold-hardy plants: Current understanding and emerging concepts. *Plant Science* **171**, 3-16

Kjellbom P, Larsson C, Johansson I, Karlsson M, Johanson U (1999) Aquaporins and water homeostasis in plants. *Trends in Plant Science* **4**, 308-314

Kurkela S, Francke M, Heino P, Lang V, Palva ET (1988) Cold induced gene expression in *Arabidopsis* L. *Plant Cell Reports* **7**, 495-498

Liang P, Averboukh L, Pardee AB (1994) Method of differential display. *Methods in Molecular Genetics* **5**, 3-16

Longstroth M (2007) The 2007 Easter Freeze in SW Michigan. *Horticulture* http://web3.canr.msu.edu/vanburen/feaster07.htm

NOAA/USDA (2008) The Easter freezes of April 2007: A climatological perspective and assessment of impacts and services. *NOAA/USDA Technical Report* 2008-01

Peng Y, Lin W, Cai W, Arora R (2007) Overexpression of a *Panax ginseng* tonoplast aquaporin alters salt tolerance, drought tolerance and cold acclimation ability in transgenic *Arabidopsis* plants. *Planta* **226**, 729-740

Pratt RB, Ewers FW, Lawson MC, Jacobsen AL, Brediger MM, Davis SD (2005) Mechanisms for tolerating freeze-thaw stress of two evergreen chaparral species: *Rhus ovata* and *Malosma laurina* (Anacardiaceae). *American Journal of Botany* **92**, 1102-1113

Rajashekar CB, Zhou HE, Zhang Y, Li W, Wang X (2006) Suppression of phospholipase *Dalpha1* induces freezing tolerance in *Arabidopsis*: Response of cold-responsive genes and osmolyte accumulation. *Journal of Plant Physiology* **163**, 916-926

Svensson AS, Johansson FI, Møller IM, Rasmusson AG (2002) Cold stress decreases the capacity for respiratory NADH oxidation in potato leaves. *FEBS Letters* **517**, 79-82

Tomczak MM, Hincha DK, Estrada SD, Wolkers WF, Crowe LM, Feeney RE, Tablin F, Crowe JH (2002) A mechanism for stabilization of membranes at low temperatures by an antifreeze protein. *Biophysical Journal* **82**, 874-881

Van Dijck P, Thevelein JM, Agre P, Carbrey JM, Tanghe A (2005) Gene expression regulation, fungal freezing *Candida albicans*/physiology aquaporins/metabolism. *Applied and Environmental Microbiology* **71**, 6434-6437

Wu Y, Fletcher GL (2000) Efficacy of antifreeze protein types in protecting liposome membrane integrity depends on phospholipid class. *Biochimica et Biophysica Acta (BBA) - General Subjects* **1524**, 11-16

Yang S, Tyree MT (1992) A theoretical model of hydraulic conductivity recovery from embolism with comparison to experimental data on *Acer saccharum*. *Plant, Cell and Environment* **15**, 633-643

Yeh S, Moffatt B, Griffith M, Xiong F, Yang DSC, Wiseman SB, Sarhan F, Danyluk J, Xue YQ, Hew CL, Doherty-Kirby A, Lajoie G (2000) Chitinase genes responsive to cold encode antifreeze proteins in winter cereals. *Plant Physiology* **124**, 1251-1264

Yi SX, Lee RE Jr. (2011) Rapid cold-hardening blocks cold-induced apoptosis by inhibiting the activation of pro-caspases in the flesh fly *Sarcophaga crassipalpis*. *Apoptosis* **16**, 249-255

Zhang M, Willison JHM (1992) Electrical impedance analysis in plant tissues: *In vivo* detection of freezing injury. *Canadian Journal of Botany* **70**, 2254-2258

Zhou S, Chen F, Sauve R (2007) Structure and temperature regulated expression of a cysteine protease gene in *Pachysandra terminalis* Sieb. & Zucc. *Journal of the American Society for Horticultural Sciences* **132**, 1-5