ISOLATION OF GENES FROM COLD TREATED *Poncirus trifoliata*

Except where reference is made to the work of others, the work described in this thesis is my own or was done in collaboration with my advisory committee. This thesis does not include proprietary or classified information.

_____

Shasha Meng

Certificate of Approval:

_____	_____
Robert C. Ebel Associate Professor Horticulture	Fenny Dane, Chair Professor Horticulture
_____	_____
Narendra K. Singh Professor Biological Sciences	Joe F. Pittman Interim Dean Graduate School

ISOLATION OF GENES FROM COLD TREATED *Poncirus trifoliata*

Shasha Meng

A Thesis

Submitted to

the Graduate Faculty of

Auburn University

in Partial Fulfillment of the

Requirements for the

Degree of

Master of Science

Auburn, Alabama
December 15, 2006

ISOLATION OF GENES FROM COLD TREATED *Poncirus trifoliata*

Shasha Meng

_____

Signature of Author

_____

Date of Graduation

VITA

Shasha Meng, daughter of Zhaoyu Meng and Xiuying Zhang, was born on February 23, 1981 in Beijing city, the Peoples' Republic of China. She graduated from Railway No. 2 High School in 1999. She entered Xiamen University in September 1999 and earned a Bachelor of Science degree in Biology in July 2003. In August 2003, she entered Auburn University to pursue a Master of Science degree in Horticulture.

THESIS ABSTRACT

ISOLATION OF GENES FROM COLD TREATED *Poncirus trifoliata*

Shasha Meng

Master of Science, December 15, 2006
(B.S., Xiamen University, 2003)

86 Typed pages

Directed by Fenny Dane

Low temperature is one of the most critical environmental factors limiting plant growth and productivity. To survive, plants have developed a complex stress response system for defense against various environmental factors. The purpose of this study was to determine genetic changes in cold treated *Poncirus trifoliata.* The cDNA amplified fragment-length polymorphism (cDNA-AFLP) patterns of 4°C treated (cold shock), non-treated, and cold acclimated plants were compared based on differences in gene expression.

Thirteen genes, isolated in this study, were grouped into five classes based on their expression patterns in cold acclimation and in 4°C treatment. Class I contains six genes confirmed as up-regulated in cold acclimated *Poncirus* only. They are: S7, homologue of a UDP-glycosyltransferase (UGT) from *Arabidopsis thaliana*; S47, homologue of a trehalose-6-phosphate synthase (TPS) from *Arabidopsis thaliana*; S126, homologue of a chalcone-flavanone isomerase family protein from *Oryza sativa*

(japonica cultivar-group); S159, homologue of a cinnamate 4-hydroxylase (C4H) CYP73 from *Citrus sinensis*; S160B, homologue of a formin-like protein AHF1 from *Arabidopsis thaliana*; S176, homologue of a putative serine carboxypeptidase (Ser-CP) from *Medicago truncatula*. Class II contains one gene which was confirmed as down-regulated in cold acclimated plants: S8, homologue of a putative syntaxin of plants 52 (SYP52) from *Oryza sativa* and a syntaxin of plants 51 (SYP51) from *Arabidopsis thaliana*. Class III contains four genes confirmed as up-regulated upon 55 h of cold treatment at 4°C: S17, homologue to a MAPK 3 from *Chorispora bungeana*; S53, homologue to the calcium ion binding site of a mannosyl-oligosaccharide 1, 2-alpha-mannosidase from *Arabidopsis thaliana*; S145, homologue to a TGA-type basic leucine zipper protein TGA2.1 from *Phaseolus vulgaris*; S149, homologue to a putative senescence-associated protein from *Pyrus communis*. Class IV contains one gene confirmed as up-regulated in *Poncirus* under both cold acclimation and 55 h of cold treatment at 4°C, which is S3, homologue of an auxin and ethylene responsive GH3-like protein from *Capsicum chinense* and an Nt-gh3 deduced protein from *Nicotiana tabacum*. Class V containing one gene confirmed as up-regulated in 55 h cold treatment at 4°C but as down-regulated upon cold acclimation, S134, homologue of a phosphoenolpyruvate carboxykinase (PEPC) from *Arabidopsis thaliana*.

Genes isolated in this study and others were divided into five groups based on their function: group I, genes involved in signal transduction and regulation of gene expression; group II, genes involved in protein synthesis; group III, genes involved in osmotic stress response; group IV, genes involved in oxidative stress; group V, genes involved in cell skeleton reorganization, vesicle trafficking and cell senescence.

ACKNOWLEDGEMENTS

The author would like to thank the members of her research committee Dr. Fenny Dane, Dr. Robert C. Ebel and Dr. Narendra K. Singh for their constructive advice and complete support during her research. She would also like to thank Dr. William Dozier for his help in her work. Thanks are also given to her group member, Ms. Rasima Bakhtiyarova, Ying Si, and other faculty members and graduate students who were able to give their help during this research.

Her deepest gratefulness goes to her parents, Zhaoyu Meng and Xiuying Zhang, and husband, Yuyao Shan, who always supported and encouraged her during her research.

Style manual of journal used Plant Cell Reports

Computer software used Microsoft word 98, Vector NTI and National Center for Biotechnology Information BLASTx search tool

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

I

IV

I. LITERATURE REVIEW

1.1 Effect of environmental factors on plant growth and productivity

Temperature stress (high and low) together with salinity and drought are the main environmental factors that limit plant growth and productivity (Lang et al. 2005, Kasuga et al. 1999). These abiotic stresses frequently lead to a series of secondary stresses, such as osmotic and oxidative stress and cellular damages. The ability of plants to respond to these stresses greatly decides the normal function and geographical distribution of the plants.

1.1.1 Effect of low temperature on plants

Low temperature can greatly impact plant biosynthetic activity and the physiological processes. In more serious conditions, permanent injury and plant death can be detected. The degree of damage depends on the duration of stress, developmental stage and plant species (Garwe et al. 2003). Low temperature stress can be classified into two types: (a) temperatures below freezing, (b) low temperatures above freezing. In both conditions, the plant membrane is the primary site to be damaged (Thomashow 1999). Studies showed that the severe dehydration associated with freezing causes major damage to the membrane. With a lower solute concentration, the extracellular fluid has a higher freezing point than the intracellular fluid. Consequently, ice formation begins first in the intercellular space when the temperature drops below zero. The formation of the extracellular ice then results in a

drop in water potential outside the cell. Finally, there is movement of unfrozen water down the chemical potential gradient from the intracellular to the intercellular spaces (Thomashow 1999). The cellular morphological changes observed during water loss are expansion-induced-lysis, lamellar-to hexagonal-II phase transitions, and fracture jump lesions on the membrane (Uemura and Steponkus 1995).

Except for injury on the membrane, other changes inside the cell can also cause cellular damages, such as freeze-induced production of reactive oxygen species (McKersie et al. 2000), the adhesions caused by the intercellular ice and protein denaturation (Thomashow 1999).

1.1.2 Effect of other environmental factors on the plant

Environmental factors such as drought and salinization can both cause osmotic stress. As a result, the signaling pathways and the cellular responses are very similar in these stresses. The damages observed under drought and salinization are disruption of homeostasis and iron distribution inside the cell (Zhu 2001).

1.2 Mechanisms plants use to survive adverse environmental conditions

To survive, higher plants have developed various defense mechanisms against environmental stresses. The response reaction is a polygenic trait involving a large number of genes (Guy 1990, Thomashow 1999). The changes of the expression levels of these genes lead to numerous molecular and physiological alterations. And the combined effect of the corresponding gene products will ensure the plant to maintain normal cellular function.

Environmental stresses (e.g. drought, salinity, cold, and heat) generate secondary stresses such as osmotic stress and oxidative stress inside the cell. The disruption of osmotic and ionic homeostasis together with the damage of functional and structural proteins and membranes can be recognized by different signal sensing molecules. Then, the modified receptor molecules activate different transcriptional factors. Accordingly, genes involved in detoxification, chaperones, osmoprotection and water and ion movement are expressed. As one aspect of the whole process, gene expression changes may lead to or accompany other defense mechanisms in plant cells such as plasma membrane changes, cytoskeleton changes and calcium concentration changes (Wang et al. 2003). Within hours, re-establishment of cellular homeostasis, functional and structural protection of proteins and membranes can be concluded and the plant gains stress tolerance or resistance. The general plant stress defense pathways are shown in Figure 1.

1.2.1 Altered gene expression

The complex stress signaling system in plants starts with the stress sensing receptors on the surface of the cell. There are different kinds of receptors sensing different stresses. The signals are then passed by the signal transduction cascades to the nucleus. In the nucleus, specific stress-related genes are transcribed in response to the signal. Some of these gene products are involved in signaling cascades and in transcriptional control. They can magnify the signals inside the cell, in such a manner that the cell can completely enter the “defense state”. Proteins like MyC (a family of transcription factors (TFs) with basic-helix loop-helix (bHLH) and Leu-zipper motif), Mitogen-Activated Protein Kinases (MAPKs) and Salt-Overly-Sensitive kinases

(SOSK) are examples of these kinds of proteins (Munnik et al. 1999; Shinozaki and Yamaguchi-Shinozaki 1997). Another group of gene products is directly involved in the protection of the membranes, such as heat shock proteins (Hsps), chaperones and late embryogenesis abundant proteins (LEA) (Larkindale et al. 2005). The third group of gene products is involved in water and ion uptake and transport, such as ion transporters (Zimmermann and Sentenac 1999). The interactions and cross-talk of all these molecules maintain plant growth and productivity under environmental stresses.

1.2.2 Plasma membrane changes

Recent studies showed that under stress, the total lipid content in plants decreased while the expression of genes involved in lipid metabolism increased. At the same time, the total fatty acid content still remains the same while the unsaturation level of the fatty acids increases. In addition, membrane repair processes are also found to be extremely efficient under stress conditions (Gigon et al. 2004). These plasma membrane changes allow the tolerant plants to maintain cellular morphology as well as homeostasis.

1.2.3 Cytoskeleton changes

The lipid decreases during stress will cause increasing fragility of the cell. To adapt to these changes, a dynamic cytoskeleton, and vesicle-based membrane traffic are essential for intra and intercellular maintenance (Komis et al. 2002). Beside its mechanical function, the cytoskeleton also functions as a sensor during cell division or differentiation. Under hyperosmotic stress, the shrinkage of the cell will lead to plasmolysis; certain plasmalemma areas attach to the cell wall, while others are

retracted. An actin filament (AF) polymerization is commonly observed at the sites where the most intense shearing forces are produced. The AF network is also involved in regulating cellular shape and protoplast volume (Komis et al. 2002).

1.2.4 Calcium concentration changes

Calcium is one of the most important signaling molecules in the cell. The pathways calcium participates in are extremely complex. Figure 2 shows genes and processes induced by Ca^{2+} (Reddy 2001). It is shown that stress can cause an elevation of cytosolic Ca^{2+} concentration in plant cells by recruiting Ca^{2+} from external sources. Some functions of intracellular Ca^{2+} storage under stress conditions are: (a) inducing AF formation directly by increasing the affinity for polymerization of G-actin subunits, (b) triggering of a series of Ca^{2+} dependent signal transduction pathways, and (c) directly inducing genes that are involved in conferring tolerance to stresses in plants, such as glyoxylase I from *Brassica juncea* (Deswal and Sopory 1999). Due to the complicated roles of Ca^{2+} in plant physiology in both normal and stress condition, more studies need to be done to discover the importance of this molecule.

1.2.5 Antifreeze proteins (AFPs)

A group of proteins are found to inhibit the growth and recrystallization of ice in the apoplast of the plant cell. The interaction changes the ice crystal shape and inhibits ice recrystallization. When there is a large amount of AFPs in the cell, the freezing temperature will be depressed without affecting the melting temperature. Two AFPs (*TaIRI-1* and *TaIRI-2*) were identified to be up-regulated under low

temperature in cereals (Tremblay et al. 2005).

1.3 Pathways and regulations

There are multiple stress signaling pathways, some of which are specific while others may be common in different cellular events.

1.3.1 Salt-Overly-Sensitive (SOS) pathway

SOS pathway is induced during salt stress, in which Ca^{2+} performs the secondary signals. Ca^{2+} will be sensed and bound by the calcium-binding protein SOS3. The SOS3-SOS2 kinase complex triggers the expression of an ion transporter, SOS1. By transporting the ion out of the cell, the cellular ionic homeostasis will be reestablished again (Zhu 2002; Chinnusamy et al. 2004). Figure 3 shows the SOS pathway in the plant.

1.3.2 Inducer of CBF Expression 1 (ICE1)- C-Repeat Binding Protein (CBF) pathway

Low temperature can induce the ICE1-CBF pathway. Although the signaling cascade is not completely clear, the cold-responsive transcriptome is activated during this process. Figure 4 shows the ICE1-CBF pathway in the plant (Chinnusamy et al. 2004).

1.3.3 Abscisic acid (ABA)-dependent and –independent pathways

Both low temperature and osmotic stresses can induce the ABA-dependent and –independent pathway. It is known that the ABA-dependent pathways activate gene

expression through ABA-responsive elements (ABRE), while the ABA-independent pathways activate gene expression through dehydration-responsive elements (DRE). Different kinds of transcriptional factors trans-activate these two types of elements of the stress-responsive genes. Figure 5 shows the ABA-dependent and –independent pathways (Chinnusamy et al. 2004; 2006).

1.3.4 Comparison of cold shock versus cold acclimation

As mentioned, the most common stress that plants constantly confront in nature is likely due to temperature changes. There are two forms of cold stresses frequently studied: cold shock and cold acclimation. Cold shock refers to the physiological response triggered by a sudden decrease in temperature while cold acclimation refers to the phenomenon of freezing tolerance that plant from temperate regions gain through exposure to low nonfreezing temperatures (Guy 1990), although many economically important crops are incapable of cold acclimation. Cold stress signals received by unidentified sensors in the plant (Chinnusamy et al. 2006) induce the expression and regulation of transcription factors and effector genes. A more complete understanding of the cold response pathway will be gained when plants are exposed to rapid and slow temperature decreases.

1.4 *Poncirus* and *Poncirus* stress response studies

Poncirus trifoliata is a citrus relative. This hardy orange usually grows as a small tree about 2-5 meters tall. It produces white flowers which typically appear in spring. As compared to citrus, *Poncirus* is tolerant to cold and many diseases. It can survive temperatures as low as -30ºC. These features make it good rootstock providing resistance to some diseases and increased cold-hardiness for citrus varieties.

The fruits of *Poncirus* which are extremely hardy are called trifoliate orange. Although the trifoliate oranges are edible, they are not often eaten (Yelenosky 1985).

Several research groups are presently conducting studies on citrus cold acclimation. Sahin-Cevik and Moore (2006) constructed a cDNA library from 2-day cold-acclimated *Poncirus* seedlings using subtractive hybridization method. Several cold acclimation response genes were detected. In their previous research, a group 2 LEA gene family in citrus was found in response to low temperature (Cai et al 1995). The Kingsville Citrus Center of Texas A&M University detected the CLTa (*Citrus* low temperature), a low temperature responsive transcript expressed in temperatures below 10°C, expression pattern in trifoliate orange during acclimation by real-time PCR and RT-PCR (Robbins and Louzada 2005). In another dehydrin study, Porat et al. (2002) found that dehydrins of *P. trifoliata*, *C. unshiu* and the various grapefruit form a closely related and unique dehydrin gene family that differs from most other plant dehydrins in having an unusual K-segment similar to that of gymnosperms and in having a serine cluster (S-segment) at an unusual position at the carboxy-terminus. *Citrus unshiu* dehydrin in response to chilling stress overexpressed in tobacco (*Nicotiana tabacum* L.) showed that dehydrin facilitates plant cold acclimation by acting as a radical-scavenging protein to protect membrane systems under cold stress (Hara et al. 2003). Recent researches on *Poncirus* stress defense mechanisms also showed that oxidative, drought, cold, and virus response pathways are greatly interacting with one another. Study on the effect of *Arbuscular mycorrhizal* (AM) fungus, *Glomus versiforme*, on *Poncirus* seedlings suggested that increased concentrations of antioxidant enzymes and non-enzymatic antioxidants found in AM plants may serve to protect the organism against oxidative damage, and enhance drought tolerance (Wu et al. 2006). The effect of water content on cytoplasmic

viscosity and the rate of intracellular ice formation showed that at low water contents, the high intracellular viscosity slowed ice crystallization making survival independent of cooling rate. While at higher water contents, the reduced viscosity required faster cooling to prevent ice crystal damage (Wesley-Smith et al 2004).

1.5 Techniques used to study differentially expressed genes

Nowadays, several techniques are available for the study of gene expression at different levels. Differential display reverse transcription PCR (DDRT-PCR), cDNA-amplified fragment length polymorphism (AFLP), subtractive hybridization and cDNA microarray are some of the more frequently used methods. Both DDRT-PCR and cDNA-AFLP require the isolation of RNA, reverse transcription of the total mRNA to cDNA, and restriction digestion of cDNA into smaller fragments. These fragments are then amplified in a PCR reaction using specially designed primers so that most of the genome can be amplified. Finally, a polyacrylamide gel is used to detect the differentially expressed genes between control and treated plants. These methods can give us a straightforward comparison between samples. But due to the mechanism of DDRT-PCR primer design, the false positive rate may be higher than that of the cDNA-AFLP (Lang et al. 2005). cDNA microarray at the other hand, can provide large amounts of information about gene expression differences between control and treatment in one single trial. However, the false positive rate and the high cost limit its usage. In our work, due to the lack of genomic sequence information for *P. trifoliata,* cDNA-AFLP was chosen to identify differentially expressed genes.

II. Introduction

In order to survive, plants do encounter different environmental factors, such as temperature stress, salinity and drought stresses. Different strategies are employed to protect plants against these stresses. These strategies include changes in stress related genes, reduction of the lipid content, and the modification of the cytoskeleton structure. Ca^{2+} concentration is known to be increased due to the import of Ca^{2+} from external sources and some specified proteins such as AFPs are expressed. All of these changes are controlled by different signaling pathways. Some of these pathways do respond to only one kind of stress, while others may interact with each other in different stresses. The main pathways are SOS pathway, ICE1-CBF pathway, ABA-dependent and –independent pathways (Chinnusamy et al 2004). Cold acclimation, the phenomenon of freezing tolerance that plants from temperate regions gain by pre-exposure to low nonfreezing temperatures, (Guy 1990) has been critical for plants to gain freezing tolerance. Cold acclimation involves precise regulation of expression of transcription factors and effector genes (Thomashow 1999). Significant progress has been made in several species (Fowler 2002; Guy 1990; Uemura and Steponkus 1995), but precise knowledge of cold induced gene expression in woody species is lacking.

To study the complicated cellular and molecular changes during plant cold stress, several methods have been developed such as DDRT-PCR, cDNA-AFLP, subtractive hybridization and cDNA microarray. In our study, cDNA-AFLP was chosen due to its low cost and high positive rate. The goal of this techniques is to

provide additional transcripts from a pool of genes differently expressed between control and cold treated plants. In cDNA-AFLP, different cDNA fragments are generated by restriction enzyme digestion. Two double stranded adaptors with different sequences are then ligated onto the cut ends of cDNA fragments. Because the sequences of the adaptors are known, special primers binding to the adaptors are designed. By this means, cDNA-AFLP is supposed to have a lower false positive rate (Vos et al. 1995; Breyne 2003).

The plant material used in this study to understand the cold response mechanism is *P. trifoliata*, a citrus relative which can survive temperatures as low as -30ºC. Due to its cold tolerance and several disease resistances, *Poncirus* is widely used as root stock in citrus industry.

III. **MATERIALS AND METHODS**

3.1. Plant material and growth conditions

For cold acclimation, one-year-old *Poncirus trifoliata* plants were grown for 6 weeks in a growth chamber under a 12/12-h (light/dark) photoperiod with light supplied at an intensity of 400 mmol m^{-2} s^{-1}. For cold acclimation treatment, the regimen for the temperature decline was as follows: 32°C (day)/21°C (night) for 14 days; 27°C (day)/16°C (night) for 7 days; 24°C (day)/13°C (night) for 7 days and 18°C (day)/7°C (night) for 7 days. For cold shock treatment, one year old *P. trifoliata* plants were exposed to 4°C for 10, 24 and 55 hours immediately following a control period of 32°C (day)/21°C (night) for 14 days. Plants were uniformly watered every day.

3.2. Total RNA extraction and cDNA AFLP

Fully expanded leaves of cold treated plants at the end of the second and the fifth week, of plant treated at 4°C for 10, 24 and 55 hours were collected, immediately immersed in liquid nitrogen and stored at –80˚C for later use. RNA was extracted from leaves according to the RiboPure kit protocol (Ambion, Austin, TX). Extracted RNA was mixed with 1/9 volume of 10×DNase buffer and 4 ml DNase I (2 U/ml) and incubated for 30 min at 37°C to digest the remaining genomic DNA. Digested RNA was treated with DNase inactivation reagent (20% volume) for 2 min, followed by centrifugation for 1 min at 14,000g and transferred to a new tube. The concentration of isolated RNA was measured using an Eppendorf Biophotometer

(Brinkmann Instruments, Westbury, N.Y.). The quality of RNA was checked using formaldehyde/agarose gel electrophoresis. RNA from the end of second week was used as control, RNA from the end of fifth week of the temperature regimen as cold acclimation, RNA extracted from leaves treated for 10, 24 and 55 hours at 4°C as cold shock. cDNA AFLP was performed using AFLP® kits (LI-COR Biosciences, Lincoln, NE). 0.2-10µg of RNA was reverse transcribed in a 40µl reaction mixture at 42˚C for 60 min with superscript III reverse transcriptase (Invitrogen, Carlsbad, CA), 0.2µM anchored oligo (dT)-primers, 8µl 5×first strand buffer, 2µM of each dNTP, 0.2 µM DTT for the first strand synthesis. 32 µl 5× second strand buffer, 10mM dNTP, 0.6 µM DTT, 15 units of *Escherichia coli* ligase, 50 units of *E.coli* polymerase I and 1.6 units of RNase-H were added to the previous mixture to generate the second strand of DNA. The mixture (total reaction volume: 160 µl) was then placed at 12˚C for one hour, followed by 22˚C for one hour. The template for cDNA-AFLP was then prepared according to Vos et al. (1995) and Bachem et al. (1996), using *EcoRI* and *MseI* as restriction enzymes. Sequences of primers and adapters used for AFLP reactions were provided by LI-COR.

3.3. Cloning and sequence analysis of DNA fragments

Selected amplified DNA fragments were ligated directly into a pGEM -T Easy Vector (Promega, Madison, WI) and transformed into competent *E. coli* (Promega). Three colonies were selected for each transformation event. Colony PCR was carried out by taking a small colony and suspending it in 60µl of H_2O in a PCR tube. The PCR reaction was set up as normal with primers provided by Promega to detect the correct insertion sizes. 10 µl of PCR product was separated on a 1.5% agarose gel. Only fragments larger than 200 bp were selected and sequenced in one direction using

either the T7 or SP6 primers (Promega) with an ABI 3100 DNA sequencer (AU Genomics Lab). The analysis of the nucleotide sequences of the selected fragments was carried out using the National Center for Biotechnology Information BLASTx search tool.

3.4. Quantitative Real-Time PCR

ABI 7500 Quantitative Real-Time PCR system was used to confirm the differential expression of DNA fragments isolated from control, cold shock and cold-acclimated plants. A 2.5-µg aliquot of total RNA was reverse transcribed with 0.5 mM dNTP, 5 mM oligo(dT)-primers, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 15 mM $MgCl_2$, 1 µl RNase inhibitor, and 100 U M-MuLV-RT (Ambion, Austin, TX). The mixture (total reaction volume: 20 ml) was incubated at 42°C for 1 h. Quantitative Real-Time PCR reaction was amplified in a 25 µl solution with 0.3 mM actin primer (Ambion, Austin, TX) for actin endogenous control, 0.5 mM of each gene specific primer and 12.5µl Power SYBR Green PCR Master Mix (Applied BioSystems, Foster City, CA). The PCR cycle program consisted of 1 cycle of 2 min at 50°C, one cycle of 10 min at 95°C, 40 cycles of: 15 s at 95°C and 60 s at 60°C. Specific oligonucleotide primers designed for selected cDNA-AFLP fragments and actin endogenous control were designed for each AFLP product for Real-Time PCR.

IV. RESULTS

4.1. Identification and confirmation of differentially expressed transcripts in *Poncirus trifoliata* in response to low temperature

One hundred and seventy-six putative differentially expressed DNA fragments were cloned, sequenced, and analyzed. Because of the possibility of comigration associated with cDNA-AFLP PCR (Lang et al., 2005), five clones, obtained from each band on the gel, were examined. These colonies were randomly selected from each transformation, and the sequence of the cloned insertion in each of the colonies was determined. The fragments show significant homology to known genes in the GenBank database using the BLASTx search utility.

To ensure optimal PCR efficiencies of different cold treatments for Relative Quantification (RQ) PCR, validation tests and melting curves were conducted for each primer pair in every reaction before and after confirmation of differentially expressed genes. Threshold cycle (Ct) values were recorded for each gene and actin in triplicate at all 4 time points (10 hours at 4 ºC, 24 hours at 4 ºC, 55 hours at 4 ºC and cold acclimation). Actin was used as housekeeping gene. *Poncirus* specific actin primers were designed to ensure a single melting peak during the dissociation step. Real time result of the evaluation test showed that the expression levels of actin were hardly influenced by the cold treatments (data not shown). The use of actin ensures an internal control in terms of cDNA quantity and integrity. Results for all selected gene primers were normalized with respect to results obtained with actin. According to Applied BioSystems (Foster City, CA), comparison of the expression of each gene

between control and cold treatments was determined with the delta-delta (ΔΔ) Ct, according to the following formula:

$$\Delta\Delta Ct = \Delta C_{actin} - \Delta C_{target}$$

From this formula, a positive result would indicate a decrease in the transcript level of the gene of interest in cold treatments, whereas a negative result would indicate an increase in expression level. Because primers in both control and treatments showed similar amplification levels, we can define the efficiency as 1. To transform the results into multiple rate (a more intuitive unit), the following formula was used:

$$Ratio = 2^{-\Delta\Delta Ct}$$

Five different classes of genes were identified according to their differential expression pattern: class I contains six genes which were confirmed as up-regulated in cold acclimated *Poncirus*; class II contains one gene which was confirmed as down-regulated in cold acclimated plants; class III contains four genes which were confirmed as up-regulated in 55h cold treatment at 4°C; class IV contains one gene which was confirmed as up-regulated in *Poncirus* under both cold acclimation and 55h cold treatment at 4°C; and class V contains one gene which was confirmed as up-regulated in 55h cold treatment at 4°C but as down-regulated upon cold acclimation.

4.2. Differentially expressed banding pattern of cDNA-AFLP fragments of *Poncirus trifoliata*

cDNA-AFLP and realtime PCR were used to identify and confirm differential expressed genes in *P. trifoliata* leaves under two different cold treatments: 55 hours at 4°C treatment and 6 weeks of cold acclimation. Reverse transcribed PCR products were loaded in adjacent lanes in a 6% denatured PAGE gel.

The banding pattern of cDNA fragments amplified by the combination of one primer group is shown in Figure 6. In most of the primer combinations, the numbers of bands detected per primer combination ranged from 50 to 70. There were certain combinations of primers that did not give good amplification results such as primer combination M-CAA×E-ACC (lane 14-16 in fig. 6) where a total of 20-40 bands were observed. In most cases, 20% of the bands were identical for each primer combination of all control, cold shock and cold acclimation treatments, while only. 10% of the bands occurred in lanes of 4 ºC treated plants. Five percent of the bands overlapped between control and 4 ºC treatments but were not detected in cold acclimation treatments.

4.3. Class I: genes up-regulated in cold acclimated *Poncirus*

Class I contains 6 genes isolated from *P. trifoliata*, which showed an increase in mRNA transcript levels following a cold acclimation treatment while no significant change was observed following 4°C treatments. The increase in transcript levels are indicated in Figure 7, while oligonucleotide primer sequences are shown in Table 1.

S7

The inferred amino acid sequence of S7 shows 77% similarity to UDP-glycosyltransferase (UGT) from *Arabidopsis thaliana*. UGTs contain a carboxy-terminal consensus sequence that is thought to be involved in binding of the protein to the UDP moiety of the sugar nucleotide. In mammalian cells, UGTs are membrane-bound enzymes localized in the endoplasmic reticulum (ER) with their catalytic sites facing the lumen (Ross et al. 2001). But no signal sequence or any clear membrane-spanning or targeting signals have been identified in plants to date (Li et al. 2001). This suggests that the UGTs function in the cytosol, within which, they may

associate as peripheral components of the endomembrane system (Burbulis and Winkel-Shirley 1999).

In plants, UGTs transfer a sugar from an activated sugar donor usually UDP-glucose, but also UDP-galactose and UDP-rhamnose (Martin et al. 1999; Miller et al. 1999; Jones et al 2003), to a broad array of substrates including hormones, secondary metabolites and xenobiotics such as pesticides and herbicides. The addition of sugar residues onto these molecules can increase both stability (through the protection of reactive nucleophilic groups) and water solubility (Jones and Vogt 2001). For example, if the glycosylation of a compound can alter the hydrophobic property of the molecule to hydrophilic, the compound then can not diffuse across lipid bilayers. So glycosylation of the aglycone in principle can be used to store the glycoside in a specific hydrophilic environment like endomembrane system and vacuole intracellularly or cell wall matrix extracellularly. The changes of local concentrations of these compounds through out the plant can therefore affect: first, the pathways of secondary metabolism by altering the concentration of substrates and products; second, the cold tolerance of the plant by decreasing the water potential within the cell.

Besides the changing of the physical properties of cellular compounds, glycosylation can also change the bioactivity of them. It has been reviewed that glycosylation can inactivate plant hormones like auxins, cytokinins, gibberellins and abscisic acid (Kleczkowski and Schell 1995). The reason is not clear, but changes in recognition site by hormone receptor or indirect events enabled by the glycosylation status might be the answer (Lim and Bowles 2004).

In some studies, scientists also found that over expression of a UGT, UGT73C5, in transgenic *A. thaliana* led to mycotoxin resistance (Poppenberger et al.

2003). This suggested the detoxification role of UGT in plants.

In this study, real-time PCR result confirmed a 5.5 fold increase of UGT transcript in cold acclimation as compared to control. On the other hand, no increase in expression levels was detected after 10, 24 and 55 hours at 4°C (Fig. 7). This suggests that UGTs, affecting secondary metabolism, cellular homeostasis and hormone active levels, might not be an instant adjustment during the fast and extreme low temperature changes. However, its transcription is necessary for plants to adapt to a slow cold acclimation regime.

S47

The inferred amino acid sequence of S47 shows 89% similarity to a trehalose-6-phosphate synthase (TPS) from *Arabidopsis thaliana*. TPS catalyzes the formation of trehalose 6-phosphate (T6P) from UDP-glucose and glucose-6-phosphate. This step is followed by the dephosphorylation of T6P to free trehalose which is found widespread in bacteria, fungi and insects (Elbein et al. 2003). In *E. coli*, trehalose helps the cells against osmotic stress. While in yeast, it functions as both storage carbohydrate and stress protection metabolites, especially under starvation (Eastmond and Graham 2003). Under normal conditions, most plant species do not easily accumulate detectable amounts of trehalose. The reason might be because of the presence of trehalase, an enzyme that breaks down trehalose (Goddijn and Smeekens 1998). Although it is unlikely that trehalose has a critical role in stress protection in higher plants, accumulating evidence suggests that its precursor, T6P, has an essential signaling function in plants. *Arabidopsis* over expressing AtTPS was found to acquire desiccation tolerance and insensitivity to increasing concentration of glucose and abscisic acid (ABA) (Avonce et al. 2004). *Arabidopsis* mutant with an insertion in the TPS1 gene led to an embryo-lethal phenotype. The supply of a low

concentration of sucrose partially overcame the block in development, which suggested that *Arabidopsis* T6P may be involved in some way in sugar metabolism (Eastmond et al. 2002). The study of downstream regulatory effects of T6P indicated that T6P might function as a cytosolic sugar "reporter". It enters the chloroplast and activates ADP-glucose pyrophosphorylase (AGPase) to promote starch biosynthesis (Kolbe et al. 2005). Also, in a recent study, TPS5, TPS6 and TPS7, three isoforms of TPS in *Arabidopsis*, were found binding to 14-3-3 protein which is up-regulated in cold acclimated *Citrus unshiu* (Lang et al. 2005).

In our study, S47 shows a 2.6 folds increase in cold acclimated *P. trifoliata*. The induction may indicate a change in carbon metabolism to increasing sugar concentration in the cytosol during cold. Indirectly, the dehydration of cells will be reduced due to lower intracellular water potential. More over, TPS may directly regulate downstream signaling pathway. The potential of some TPS isoforms to bind with 14-3-3, which is found up-regulated in cold acclimated *C. unshiu* strongly supports this possibility.

S126

The inferred amino acid sequence of S126 shows 85% similarity to a chalcone-flavanone isomerase family protein from *Oryza sativa (japonica cultivar-group)*. Chalcone isomerase (CHI) is an enzyme in the isoflavonoid pathway in plants. The reaction CHI catalyzes is the cyclization of chalcone and 6'-deoxychalcone into the key precursor of anthocyanins, (*2S*)-naringenin, and (*2S*)-5-deoxyflavanone, respectively (Gensheimer and Mushegian 2004). Some anthocyanins also exhibit antioxidant, antiasthmatic, antimalarial, antimicrobial, and other medically relevant properties (Dixon and Steele 1999).

In our study, S126 showed a 16.8 fold increase in transcript levels in cold

acclimated *Poncirus* as compared to control. The role of this gene at low temperature stress is currently not clear, although the strongly up-regulated expression level might indicate that adjustment of isoflavonoid pathway may be needed under stress conditions.

S159

The inferred amino acid sequence of S159 shows 100% similarity to a cinnamate 4-hydroxylase (C4H) CYP73 from *Citrus sinensis* and C4H from *Citrus x paradise*. C4H belongs to the CYP73A group of plant P450s. It is one of the core enzymes involved in phenylpropanoid metabolism that produces important, sometimes unique, secondary metabolites participating in plant development and defense response (Lu et al. 2006). The first reaction is the deamination of phenylalanine to generate *trans*-cinnamic acid by phenylalanine ammonia-lyase (PAL). C4H then convert the *trans*-cinnamic acid to *p*-coumaric acid. A thioesterification is then activated by 4-coumarate coenzyme A to produce p-coumaroyl-CoA which will be directed into different pathways (Ro et al. 2001).

C4H in most eukaryotic cells is found on membranes of organelles, especially on ER. The regulation of C4H seems to be transcriptional. Since C4H, PAL and 4CL have similar promoter regions, and tissue and cell type specific distribution patterns, together with similarities in the apparent mRNA and protein turnover rates, it is assumed that they might be regulated through a large structural and functional multienzyme complex where C4H serve as a structural scaffold, anchoring the enzyme-complex on the ER (Koopmann et al. 1999). In citrus, two C4H cDNA, C4H1 and C4H2, were cloned and their expression patterns are different. C4H1 was found continually expressed as a "house keeping gene" in the phenylpropanoid pathway while C4H1 transcripts were only found in wound induced leaves where the

mRNA level of C4H1 was still lower than C4H2 (Betz et al. 2001).

In our study, mRNA transcript level of S159 was found to be 2.5 fold higher than the control in cold acclimated *Poncirus*. The increasing expression of C4H during longer cold treatment could indicate a higher generation rate for secondary metabolites. If these secondary metabolites such as fatty acids (membrane components), lignins (cell wall constituents), flavonoids (pigments), isoflavonoids, furanocoumarins and norlignans (plant defense compounds) (Whitbred and Schuler 2000; Suzuki et al. 2002; Suzuki et al. 2004) keep on accumulating inside the cell, further morphological and molecular changes will be induced to help the plants defense to cold temperature. The increasing expression of C4H may also indicate an increased expression of PAL and 4CL. This prediction needs to be further investigated.

S160B

The inferred amino acid sequence of 160B shows 75% similarity to a formin-like protein AHF1 from *Arabidopsis thaliana*. Formin is defined as a multidomain protein containing a highly conserved formin homology domain 2 (FH2) that interacts with actin and a proline rich domain FH1. There are 21 formin isoforms in *Arabidopsis* (Cvrckova 2000; Cvrckova et al. 2004; Deeks et al. 2002). They can be separated into two subfamilies: class I and class II according to the presence or absence of an N-terminal transmembrane domain which can partially lead the formin to or near the plasma membrane (Cheung and Wu 2004; Favery et al. 2004; VanDamme et al. 2004; Ingouff et al. 2005). The general function of formins is to mediate AFs nucleation. During the process, formins interact with the barbed end of AFs, and allow actin elongation at the barbed end while remaining attached to the same end (Pruyne et al 2002; Sagot et al 2002; Li and Higgs 2003; Kovar and Pollard

2004). The actions help the actin to form bundles instead of branches in the cell.

AHF1 is a member of the class I formin. It has been shown that over expression of AHF1 in pollen tubes causes the formation of prominent actin cables, pollen tube swelling and deformation of the cell membrane at the tip of the pollen tube (Cheung and Wu 2004). A recent study found that the FH1 domain of AHF1 changes the activity of FH2 from a strong capper that blocks elongation at the barbed ends to a leaky capper that allows filament barbed-end growth. Indeed, the presence of the FH1 domain switches the actin chain's growing from only pointed-end direction to barbed-end elongation. AFH1 is also able to bind to the side of bundle AFs (Michelot et al. 2005).

In our study, S160B was found to induce a 2.3 fold increase in transcript levels in cold acclimated *Poncirus* as compared to control. Since AFH1 has roles of mediating the growth of AFs, it is a crucial factor in cytoskeleton reorganization, cell expansion and morphogenesis. One of the most serious problems in cold treated cells is the damage of the membrane due to its fragility. Many morphological damages can be observed such as expansion-induced-lysis, lamellar-to hexagonal-II phase transitions, and fracture jump lesions to occur on the membrane (Uemura and Steponkus 1995). Although the induction of these damages is due to dehydration, loss of cytoplasm modification is the direct cause. The reorganization of actin cytoskeleton in plant cells is known to be temporally and spatially regulated in response to external stimuli. According to our results, the need of reorganization of the cytoplasm is sensed somehow later in the cold treatment, since no mRNA increase was found after 10h, 24h and 55h at 4°C treatment. This latter increase in expression of AFH1 indicates that the reorganization of AF network is a necessity for *Poncirus* in its defense to low temperature.

S176

The inferred amino acid sequence of S176 shows 86% similarity to a putative serine carboxypeptidase (Ser-CP) from *Medicago truncatula*. Ser-CPs are proteases widely distributed in higher plants. They contain a conserved Ser-Asp-His triad which is essential for enzyme activity (Remington 1993). During catalysis, the active-site serine at the carbonyl C terminal of the scissile bond nucleophilic attacks either peptide or ester bonds to form a covalent acyl-enzyme intermediate which will be hydrolyzed (Schaller 2004). The cleavage of the peptides is important in secondary metabolite biosynthesis and enzyme activation.

In plants, Ser-CP has been extensively studied. The effect of the Ser-CP is mainly on turnover and mobilization of storage proteins as nitrogen and carbon resources during seed germination and C-terminal processing to wound response (Moura et al. 2001; Granat et al. 2003). Studies also suggested that plant Ser-CPs may also be involved in programmed cell death as they are implicated in the autolysis of cellular constituents (Domınguez et al. 2002). Various signaling events important for plant growth and development such as brassinosteroid signaling and seed development are also found impacted by Ser-CP (Li et al. 2001; Cercos et al. 2003).

In our study, S176 was found to increase 3.8 folds in cold acclimated *Poncirus* as compared to the control. In combination with the functions of the Ser-CP, this phenomenon may indicate that the secondary metabolites processing in cold acclimated plants is more active than it is under normal conditions.

4.4 Class II: gene down-regulated in cold acclimated *Poncirus*

The mRNA transcript levels of one gene was comfirmed as decreased in cold acclimated *Poncirus*. The decrease in transcript level is indicated in Figure 8, while oligonucleotide primer sequences are shown in Table 1.

S8

The inferred amino acid sequence of S8 shows 94% similarity to a putative syntaxin of plants 52 (SYP52) from *Oryza sativa* and 86% similarity to a syntaxin of plants 51 (SYP51) from *Arabidopsis thaliana*. In *Arabidopsis*, both *SYP51* and *SYP52*, which belong to the *SYP5* syntaxin gene family, share 82% sequence identity. Their identical expression pattern and biochemical behavior suggests that SPY51 and SYP52 serve redundant functions (Sanderfood et al. 2001).

Syntaxins, are part of a group of membrane proteins, soluble *N*-ethylmalemide sensitive factor adaptor protein receptor (SNAREs). A series of articles explained the function of the SNAREs in membrane fusion and vesicle trafficking (Fukuda et al. 2000; McNew et al. 2000; Parlati et al. 2000). Syntaxins are involved in the selection and fusion of the target membrane. In plants, SYPs on the cytoplasmic face of the target membrane form a three-helix bundle t-SNARE complex. This t-SNARE interacts with v-SNARE on the vesicle membrane to form a complex, which brings the two lipid bilayers close enough for fusion to proceed. Different SYPs have been found to reside on various endomembrane organelles. The specific binding between SNAREs indicates that although SYPs share high similarities, their functions are distinct from one another.

As the member of three syntaxin gene families in *Arabidopsis*, SYP51 was expressed on multiple organelles, including the *trans*-Golgi network (TGN) and prevacuolar compartment (PVC). Most proteins are thought to enter the ER and then

are transported to the TGN where the pathways for secretion and transport diverge. According to their targeting signals, vesicles that leave the TGN are then trafficked to different target membranes such as plasma membrane, the endosome, the vacuole or the cell (Sanderfoot and Raikhel 1999). Those recognized by clathrin-coated vesicles then enter PVC and ultimately the vacuole (Sanderfoot et al. 1998). In our study, the expression of SYP51 in cold acclimated *P. trifoliata* decreased to half of that in the control plant. Considering that the SYP51 resides on TGN and PVC, the reduction of the SYP5 may decrease the chance for cargos to be delivered to the TGN and the PVC. Consequentially, more components will be stocked in the cytosol without further processing. Indirectly, the metabolism of the plant is slowed down, and the water potential is decreased to defend from dehydration due to low temperature.

4.5. Class III: genes up-regulated in 55h cold treatment at 4°C

Class III contains 4 genes, all of which showed increased mRNA transcript level at 4°C treatment. Gene S17 shows an increased expression as early as 10 h, the first time point taken, and the induction increases until 55 h, the last time point for 4°C treatment, while no significant change was observed in cold acclimated *Poncirus* (Figure 9a). The mRNA for other three genes also increased at 4°C treatment as is shown in Figure 9b**.** Their oligonucleotide primer sequences are shown in Table 1.

S17

The inferred amino acid sequence of S17 shows 98% similarity to a MAPK 3 from *Chorispora bungeana*, a representative alpine survival perennial herb. The annual average temperature where *C. bungeana* grows is 5°C day/-4°C night and the plant can even survive embedded in snow (Zhang et al. 2006).

Chorispora bungeana MAPK3 (*Cb*MAPK3) shows high amino acid similarity

to *Arabidopsis thaliana* MAPK3 (*At*MAPK3). Both belong to the MAPKs gene family A, which is mostly involved in environmental and hormonal responses. In *Arabidopsis thaliana*, *At*MAPK3 was found to be induced by cold (4°C), oxidative stress (Kovtun et al. 2000), ABA signal, touch, salinity stress and bacterial-derived elicitor (flg22) (Mishra et al. 2006). The level of MPK3 transcript was also found to be increased 30 min after UV exposure, reaching a peak after ~1 h (~10-fold induction) followed by a return to the basal level 3 h after irradiation. Moreover, the results from *Arabidopsis* protein microarray showed that there are 48 potential substrates of AtMPK3. The candidates include TFs, transcription regulators, splicing factors, receptors, histones and others (Feilner et al 2005). Although the detailed picture of AtMAPK3 is not clear, as one of the terminal components of the "three-kinase" modules of MAPK cascades, AtMPK3 seems to mediate a great variety of intra- and extra-cellular stimuli transmission and amplification including cold stress, and result in the induction of downstream gene expression.

*Cb*MAPK3, the homolog of AtMPK3 in *Chorispora bungeana*, was found to have no tissue specificity. The expression pattern for *Cb*MAPK3 at 4°C showed that transcript levels of *Cb*MAPK3 increased rapidly within 1-2 h followed by a decline to the basal level at 24 h. When exposed to -4°C, the transcript levels also increased rapidly but maintained a high level up to 24 h. The study also showed that salt stress and ABA treatment, can also induce *Cb*MAPK3 expression (Zhang et al. 2006). In our study, the transcript level of S17 in *Poncirus trifoliata* increased greatly at 4°C. At 10 h exposure, the mRNA level showed a 5.6 fold increase as compared to control, followed by 10.1 fold increase at 24 h and 16.9 fold increase at 55h. Further increase may happen since 55 h was our last time point for plants exposed at 4°C. A decline of S17 expression is suggested to occur later during cold stress because cold acclimated

P. trifoliata S17 didn't show much of a increase as compared to control (Figure 9a).

The expression pattern of S17 indicates that MAP kinase3 contributes to cold tolerance in a relatively fast way during 4 °C shock as compared to weeks of cold acclimation in *P. trifoliata*. And this consequently may lead to the expression of other genes or to cross talking between other signal pathways to defend the plant against low temperature.

S53

The inferred amino acid sequence of S53 shows 100% similarity to the calcium ion binding site of a mannosyl-oligosaccharide 1, 2-alpha-mannosidase from *Arabidopsis thaliana*. In *Arabidopsis*, Golgi α-mannosidase II is considered to be involved in N-glycosylation of protein modification (Strasser et al. 2006). In our results, the S53 shows a 2.13 fold increase in mRNA transcript level in *Poncirus* treated for 55 h at 4°C (Figure 9b). Although the exact roles of the proteins under environmental stresses are still not clear, the induced level of the mannosyl-oligosaccharide 1, 2-alpha-mannosidase in *P. trifoliata* indicates that active protein glycosylation is necessary under low temperature conditions.

S145

The inferred amino acid sequence of S145 shows 96% similarity to a TGA-type basic leucine zipper protein TGA2.1 from *Phaseolus vulgaris*.

Plant bZIP TF found in all eukaryotes to date have a basic region that binds DNA and a leucine zipper dimerization motif. In *Arabidopsis*, 75 putative genes encoding proteins with the bZIP signature, the leucine zipper dimerization motif, were identified. They were further defined as ten groups according to the similar basic region and additional conserved motifs. Functions of each group varies from abscisic acid or stress signaling, pathogens defense to developmental regulation. Because of

the similarity among basic DNA-binding regions, many groups have redundant functions (Jakoby et al. 2002). The common properties of bZIPs are known to mainly interact with a 5' flanking DNA sequence element called G-box containing a 5'-ACGT-3' core. When plants receive the stimulus of environmental or physiological change, the specific binding between bZIP to G-box will lead to the expression of downstream genes. The activation of bZIP is usually considered to be post-transcriptional. In a review article published in 2001, Siberil et al. suggested that the G-box (5'-CACGTG-3') binding factors (GBF) were first synthesized in the cytoplasm as inactive monomers, then, phosphorylated by kinases to a conformation change according to a physical or physiological stimulus. The modified GBFs then bind with different protein carriers that allow them to travel through the pore complex on nuclear membranes. In the nucleus, dimerization occurred between bZIPs. They could interact then with their target G-box to initiate gene transcription (Siberil et al. 2001).

In *Phaseolus vulgaris*, the TGA-Type bZIP TF more specifically binds with the gene promoter for a bean abscission cellulose (BAC). Abscission in higher plants is important for reproduction and defense to environmental stress. Expression of the abscission-specific genes is considered tissue and hormonal specific. And the regulation of the specific binding of bZIP to BAC is considered to be induced by ethylene and inhibited by auxin (Tucker et al. 2002).

In our study, the mRNA transcript level of S145 is found to have a 2 fold increase after 55 h of 4ºC treatment in *Poncirus* while in cold acclimated plants no significant change has been observed. Although it is been mentioned that the activation of bZIP is usually post-transcripional, the transcriptional induction of this TP also might increase the inactive pool of different monomers in the cytoplasm. One

assumption is that, as cold stress stimuli are received by *Poncirus* cells, the signal is first transferred into the nucleus by different pathways, and then, defense response genes are expressed. Among them, bZIP TF may be one of the first genes that are increasingly transcribed to further induce other cold resistant genes. Once the stable monomer pool of inactive bZIP is established, bZIP dimmer successfully can induce the downstream genes, and the bZIP mRNA level can return to the normal level in cold acclimated leaves.

S149

The inferred amino acid sequence of S149 shows 97% similarity to a putative senescence-associated protein from *Pyrus communis*.

Leaf senescence is the last stage of development and leads to death. It can be caused by endogenous developmental factors like hormones or various environmental factors, such as stress and nutrient supply. During development or certain environmental stress, plant metabolic rate may be decreased. The decreasing metabolic rate then, may lead to less oxidative stress which is considered to be the most crucial factor in senescence (Munne-Bosch and Alegre 2002). Once these processes are sensed by the plant, senescence begins. The senescence associated genes (SAGs) are defined as genes found transcriptionally upregulated in senescing leaves. Senescence associated proteins are the products of these genes (Lim et al. 2003). Under the control of a complex and tightly regulated network of SAGs, the chloroplasts are the first organelles to breakdown while the nucleus which is needed for the genetic control for the cellular event and mitochondria which are the power station in the cell will remain intact until the last stage of the senescence. During this process, nutrients are reallocated to other growing parts of the plant or are stored for the next growing season (Quirino et al. 2000).

Because of the complexity of the network, no individual SAGs have been observed to have particular effects on the leaf senescence when inactivated or overexpressed. According to Lim et al. (2003), SAGs can be classified into six conceptual categories: class I, genes that control the developmental aging process; class II, genes that control other endogenous biological processes in plants in addition to leaf senescence; class III, genes that affect senescence in response to environmental factors; class IV, regulatory genes that upregulate senescence-associated activities or downregulate cellular-maintenance activities; class V, genes that are suggested to be involved in the degradation process of senescence regulatory factors; class VI, downstream genes that are involved in executing the senescence process, such as genes for cellular disintegration and nutrient recovery. The co-regulation of all these genes will finally lead to the death of the leaf (Lim et al. 2003).

In our study, S149 was found to increase 2.2 fold after 55 hours cold treatment at 4 ºC when no increase was found in cold acclimated *Poncirus*. This suggests that during sudden cold treatment, the plant may undergo some degree of senescence to accompany the decrease in metabolism rate. However, because *Poncirus* is a cold hardy species, its defense mechanism can adjust in various ways to components in the plant to cope with the low temperature. Senescence may not occur when acclimation occurs slowly.

4.6. Class IV: gene up-regulated in *Poncirus* under both cold acclimation and 55h cold treatment at 4°C

S3 is the only gene in this group (Figure 10). The inferred amino acid sequence of S3 shows 93% similarity to an auxin and ethylene responsive GH3-like protein from *Capsicum chinense* and an Nt-gh3 deduced protein from *Nicotiana*

tabacum. Auxin is a plant growth hormone involved in many aspects of plant physiological processes including cell elongation, division and root formation and some tropisms (Davies 1995). Since the molecular mechanisms of auxin response are still unclear, investigation of auxin induced genes became one strategy to understand its mechanism. The genes specifically induced by exogenous auxin in elongating tissues or dividing cells within 30 minutes are referred to as early or primary auxin-response genes. These genes can be divided to three classes: *Aux/IAA*s, *SAUR*s and *GH3*s (Hagen and Guilfoyle 2002). Soybean *GH3* was considered to be one of the most specifically auxin-regulated genes. In steady-state plant, the GH3 transcript is too low to be detected by Northern blot analysis while in exogenous auxin treatment, GH3 mRNA is induced within 5 minutes (Hagen and Guilfoyle 1985). In *Arabidopsis*, at least 20 GH3-like genes have been identified based on annotation of genomic sequence (Liu et al. 2005). Some of them were shown as important in photomorphogenesis, strengthening the potential link between phytochrome signaling and auxin responses (Hsieh et al. 2000; Nakazawa et al. 2001). Wiesler et al. (2002) suggested that the sensitivity of microtubules to assembly blockers can be modulated through altering their dynamics through auxin response (Wiesler et al., 2002)

The GH3-like protein (*Cc*GH3) found from a suppressive subtraction hybridization (SSH) library of *Capsicum chinense* contains several auxin- and ethylene-inducible elements in the putative promoter region. It was shown that auxin induced the gene expression in shoots, flower buds, sepals, petals and most notably ripening and mature pericarp and placenta. However, this CcGH3 protein was also expressed in fruit when auxin levels were decreasing while endogenous ethylene was applied. Ethylene-treated tomato fruits over-expressing CcGH3 showed hastened

ripening. These results suggest that CcGH3 may be a point of intersection in the signaling between auxin and ethylene (Liu et al 2005).

Nt-gh3 deduced protein was isolated from *Nicotiana tabacum* seedling in response to auxin treatment by differential display. The sequence of *Nt-gh3* showed 70% identity with the *GH3* sequence in soybean, and the *Nt-gh3* mRNA accumulated within a short time after auxin treatment (Roux and Perrot-Rechenmann 1997).

In our study, the mRNA level of S3 shows a dramatically increase during the whole cold treatment process in *Poncirus* as shown in Figure 10. Starting from the 10h treatment at 4°C the S3 mRNA transcript level increased 3.6 fold as compared to control. The induction of S3 kept on increasing to 771.6 fold in 55 h 4°C followed by a decrease to 21.6 fold in cold acclimated leaves. Although the exact function of GH3 protein in cold stress is not clear, it seems to have a critical role in the process.

4.7 Class V: gene up-regulated in 55h cold treatment at 4°C and down-regulated during cold acclimation.

Class V has only one gene, S134. The inferred amino acid sequence of S134 shows 96% similarity to a PEPC from *Arabidopsis thaliana*. PEPC is a ubiquitous enzyme that is present in higher plants, bacteria, cyanobacteria and green algae (Lepiniec et al. 1994; Toh et al. 1994). PEPC appears to be cytosolic enzyme catalyzing the following reversible reaction:

Oxaolacetate (OAA) + Mg^{2+}-nucleoside triphosphate → phosphoenolpyruvate (PEP) + Mg^{2+}-nucleoside diphosphate + CO_2

Thus, it is involved in C_4-dicarboxylic acid metabolism and crassulacean acid metabolism (CAM) in plants to fix atmospheric CO_2 and provides CO_2 for the Calvin cycle by decarboxylating OAA derived from malate and/or aspartate (Edwards et al.

1971; Dittrich et al. 1973). These reactions catalyzed by PEPC are at central positions that link the metabolism of amino acids, organic acids, sugars, lipids, and some secondary metabolites together. PEPC is also found functioning in a variety of non photosynthetic systems such as seed formation and germination, fruit ripening and C/N partitioning in C3 leaves (Chollet et al 1996; Lepineic et al. 1994).

In our study, S134 showed interesting dynamic changes during different cold treatment period in *Poncirus*. As is shown in Figure 11, the mRNA transcript level of S134 didn't change obviously in the early treatment at 4°C. But after 55 h at 4°C, the transcript of S134 increased 3.7 fold as compared to the control. This higher level of S134 didn't last long. In cold acclimated *Poncirus* after the plant has slowly acclimated to cold temperature to adjust to stress for weeks, the PEPC level decreased to 0.32 fold as compared to the control level. This finding suggests that in sudden cold stress without an adjustment period *Poncirus* cells may increase PEPC expression level to increase secondary metabolites. This suggests that the S149 senescence-associated protein's up-regulation might indicate a lower metabolism status inside the cell.

V. DISCUSSION

5.1 Isolation of differentially expressed genes using cDNA-AFLP

In order to study differentially expressed genes during cold treatments, two simple, low-cost methods (DDRT-PCR and cDNA-AFLP) were compared using cold hardy *Poncirus trifoliata* (Zhang et al. 2005; Lang 2005). The goal of these two techniques is to provide additional transcripts for selection from a pool of genes differently expressed between control and cold treated plants.

DDRT-PCR and cDNA-AFLP are gel-based transcript profiling systems based on electrophoretic fingerprinting of amplified cDNA fragments. Although DDRT-PCR has been widely used in differential gene expression studies (Zhang et al. 2005; Lang 2005; Roux and Perrot-Rechenmann 1997), several drawbacks are known including the high frequency of false positives and low sensitivity to transcripts that are expressed at low copy numbers. Because of mismatch priming during amplification, different-length excisions of the same transcript will occur. This can produce multiple clones with only some being differential, thus representing a source of false positives. Same-length excisions of different transcripts may also occur due to the mismatch priming. As compared to DDRT-PCR, cDNA-AFLP was reported to have better reproducibility (Breyne et al. 2003). In cDNA-AFLP, different cDNA fragments are generated by restriction enzyme digestion. Two double stranded adaptors with different sequences are then ligated onto the cut ends of cDNA fragments. Because the sequences of the adaptors are known, special primers binding to the adaptors are designed. By this means, cDNA-AFLP is supposed to have a lower number of false

positives.

However, the two techniques can complement each other, since different genes have been identified in *Poncirus* using cDNA-AFLP or DDRT-PCR (Lang et al. 2005)

5.2 *Poncirus trifoliata* cold response

In the citrus industry, temperature is a key factor limiting the growth and productivity of the plant. Most Citrus species are cold-tender evergreen crops, which originated in tropical and subtropical regions. These cold-tender species lack the capacity to tolerate freezing temperature (Webber et al. 1967). *Citrus unshiu*, one of the most cold hardy commercial citrus, can tolerant temperature as low as -10°C while *P. trifoliata*, a deciduous relative of *Citrus*, can tolerate temperatures as low as -30°C. Due to its ability to survive in low temperature, *P. trifoliata* is extensively used as rootstock to enhance freezing tolerance of the scion in freeze-prone areas (Yelenosky 1985). To understand the plant's cold response mechanism, we used cDNA-AFLP and RQ real time PCR to study *Poncirus* exposed to various cold treatments. The cold acclimation treatment mimics natural declines of temperature in the Southeastern U.S by gradually lowering day and night temperatures. The mRNA expression level at 55 hours 4°C treatment was used to investigate *Poncirus* response to cold shock. Usually, the cold shock response is induced as early as 4 hour at 4°C in herbaceous plant such as *Arabidopsis thaliana*. We decided to use 55 hours at 4°C because as woody plant, *Poncirus* may need more time to respond to environmental changes. This view is strengthened by the observation of S17 mRNA transcript expression levels during cold. S17 shares 98% similarity with a MAPK 3 from *Chorispora bungeana*, an extremely cold hardy herb. While the expression pattern for *Cb*MAPK3 at both 4°C and -4°C reached a peak within 1-2 h, the homologue S17 in

Poncirus didn't reach the expression peak until 55 hours at 4°C treatment (Fig. 9). Although no detailed information is available on the role of this gene in a known pathway, its delayed expression in *Poncirus* suggests that the stress response in complex woody plants is not as fast as in herbaceous annuals.

Thirteen cold-induced genes identified in this study and eight genes in previous *Poncirus* research (Zhang et al. 2005) show homology to genes involved in a variety of cellular functions in other species. These genes can be divided into five groups according to their function: group I: genes involved in signal transduction and regulation of gene expression; group II: genes involved in protein synthesis; group III: genes involved in osmotic stress response; group IV: genes involved in oxidative stress; group V: genes involved in cell skeleton reorganization, vesicle trafficking and senescence.

Group I genes are involved in signal transduction and regulation of gene expression. This group of genes is critical for plants in their defense to low temperature. In *Arabidopsis* for example, a DNA binding protein, CBF1, can bind to the C-repeat/dehydration response element. The binding will activate several cold-regulated proteins (COR). Plants genetically modified to over express CBF1 gained enhanced freezing tolerance (Jaglo-Ottosen et al. 1998). In *Poncirus*, several TFs such as zinc finger proteins and glycine rich RNA binding proteins were identified in response to cold (Sahin-Cevik and Moore 2006). In our study, 3 genes with homology to MAPK3, bZIP TF and auxin and ethylene responsive GH3-like protein, respectively, were found up regulated during *Poncirus* cold treatments (Fig. 9 and Fig 10). The first two genes were found up regulated in 4ºC treatments but not upon cold acclimation, while the auxin and ethylene responsive GH3-like protein was found up-regulated in all cold treatments (Fig. 10). MAPK 3 belongs to the MAPK

gene families. As one of the most well known signaling molecule families, the identification of MAPK in cold treated *Poncirus* indicates that additional signaling molecules might participate in the *Poncirus* cold response. bZIP TF is known to induce a large number of genes via *cis*-element binding. Various downstream genes from ABA pathways or stress signaling, from pathogen defense to developmental regulation suggest that these pathways are involved in *Poncirus* cold response. Ethylene is known to increase during *Poncirus* cold defense (Sahin-Cevik et al. 2006) and auxin known to modulate sensitivity of microtubules to assembly blockers (Wiesler et al. 2002).

In our study, an auxin and ethylene responsive GH3-like protein was up regulated. This increase was detected from 10 hours at 4 ºC treatment indicating an early and prolonged auxin and ethylene interaction with cold response (Fig 10). Although the number of group I genes identified in this study is limited as compared to those in *Arabidopsis*, the timing of the gene expression provides important information for genes involved in cellular communication, signal transduction and regulation of gene expression. Their induction may occur early during the cold response. Since similar genes such as MAPK3 and bZIP TF are involved in ABA and ethylene signaling pathways and in cold response pathways in both *Poncirus* and *Arabidopsis*, the timing of gene expression might be similar in herbaceous and woody perennial plants.

Group II genes involved in protein synthesis, F-box protein homologue and ribosomal protein in response to cold acclimation were detected by Zhang et al. (2005) in *Poncirus*. Limited information is available about the function of these genes with respect to their cold response function. F-box proteins with protein-protein interaction domains have been reported to selectively degrade certain proteins due to its substrate

specificity of ubiquitin-dependent proteolysis (Callis and Vierstra 2000). Ribosomal protein was found to have functions in controlling cell growth, division, and development (Barakat et al. 2001). Changes in protein synthesis and degradation in response to cold treatment was also found in *Arabidopsis* where HSPs act as chaperones to refold the denatured proteins to ensure a stable amount of certain proteins at low temperature (Sung et al. 2001). The findings in *Arabidopsis* and *Poncirus*, indicate that adjustment of protein metabolism at the translation and post-translation level during which proteins are selectively degraded, stabilized and synthesized, is needed in both herbaceous and woody plants to survive cold stress.

Group III genes are involved in osmotic balancing in cold treated *Poncirus*. In plants, many stress responses are associated with a coordinated and integrated set of metabolic alterations that are instrumental in impeding further alleviation of stress and induce a variety of novel proteins (genes) and secondary metabolites. These products have an important role in plant cold defense by acting as cryoprotectants to protect cell membranes and increase intracellular osmotic potential (Guy 1990; Crowe et al. 1990). In plant cells, changes in osmotic response have two aspects: changes in cellular component locations by transporters and synthesis of secondary metabolites. Previous studies on *Poncirus* identified genes acting as cellular component transporters such as hexose carrier, glucose-6-phosphate/phosphate translocator (Sahin-Cevik and Moore 2006), proline/betaine transporter, water-channel protein and nitrate transporter (Zhang et al. 2006). The induction of these transporters enables the osmoprotectants to move to their functional sites. Induction of enzymes involved in sugar metabolism such as fructose biophosphate aldolase was also found in cold treated *Poncirus* (Sahin-Cevik and Moore 2006). In our study several genes involved in secondary metabolite synthesis, modulation and transport were identified.

Chalcone-flavanone isomerase family protein homologue involved in isoflavonoid pathway (Fig. 7), PEPC homologue involved in C_4-dicarboxylic acid metabolism (Fig. 11) and homologue of C4H CYP73 (Fig. 7), one of the core enzymes involved in phenylpropanoid metabolism, were found induced in cold treatments. All three enzymes are critical in producing important, sometimes unique, secondary metabolites participating in plant development and defense response. Their increased expression provides the precondition for further accumulation of secondary metabolites. Gene S47, homologue of TPS, catalyzes the formation of T6P that might be the possible cytosolic sugar "reporter". Studies showed T6P promotes starch biosynthesis by its signaling function. S7, S176 and S53, homologues of UGT, putative Ser-CP and mannosyl-oligosaccharide 2-alpha-mannosidase (Fig. 7 and Fig. 9) respectively, are also known to be involved in modification and activation of important cellular molecules by increasing their stability, water solubility, activation or inactivation and protein glycosylation. The isolation of these genes implicated the active role played by plant secondary metabolites during cold response especially with respect to osmotic balancing.

Cold stress can result in oxidative stress due to the production of reactive oxygen species (ROS) (Zhang et al. 2006). In group IV, a total of two *Poncirus* genes involved in the oxidative stress defense during cold treatments were identified: aldo-keto reductase and early-light-inducible proteins (ELIPs). When plants encounter oxidative stress, a variety of ROS-scavenging enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and low-molecular weight antioxidants including ABA, glutathione and phenolic compounds are induced (Asada 2000). In Sahin-Cevik and Moore's study (2006), a cDNA homologue to glutathione S-transferase (GST), a detoxification enzyme originally found in *Solanum*

commersonii (Seppanen et al. 2000), was found expressed at higher levels in cold acclimated *Poncirus* (Sahin-Cevik and Moore 2006). Studies showed that in transgenic tobacco expressing alfalfa aldose-aldehyde reductase, the damage caused by oxidative stress was also reduced (Bartels 2001). A similar reduction was observed in *Arabidopsis* expressing ELIPs (Hutin et al. 2003) which also accumulated during chilling-induced photooxidation in barley (Bei-Paraskevopoulou and Kloppstech 1999). In Zhang et al.'s work (2005) homologues of aldo-keto reductase and ELIPs were isolated, indicating similar mechanisms exist in cold acclimated *Poncirus* (Zhang et al. 2005). The homologue genes expressed in both herbs and woody plants further provides evidence that herbaceous and woody species have closely related cold defense mechanism.

Cold response in woody plants is a complex regulation of different cellular events. Genes involved in cell skeleton reorganization, vesicle trafficking and senescence which induce AFH1, putative SYPs and senescence associated proteins are also found in this study in cold treated *Poncirus*. It is known that the membrane is the primary site of freezing damages. During cold stress, a decrease of the lipid content will cause increased fragility of the cell. To adapt to the changes, a dynamic cytoskeleton and vesicle-based membrane traffic is essential for maintaining the cell's shape. In various crops, microtubules reorganization has been found to correlate with cold response. Disassembly of microtubules was found to stimulate the activity of cold-induced calcium fluxes (Mazars et al. 1997). The data suggested a model (Nick, 2000), in which cold induced microtubule disassembling capacitates calcium influx through cold-sensitive ion channels. However, in our study, AFH1 was found to be increasingly expressed in cold acclimated *Poncirus* (Fig. 7). This conflict can be explained using Nick's model. The microtubule acts as a signaling mechanism

facilitating the Ca^{2+} influx to trigger the cellular pathways at the early stage of cold response. Later following cold acclimation, when the plant enters a more stable stage, microtubule's signaling function is replaced by its cell structure stable function to maintain the adapted cellular structure. Later during cold acclimation, actin filaments are produced to keep the plant cell membrane strong in order to counteract the fragility due to dehydration. This hypothesis is supported by a study in *Chlorophyton comosum* when hyperosmotic stress was induced, resulting in intense reorganization of the AF cytoskeleton. The plasmolysed cells displayed a significant increase in the overall AF content (Komis et al. 2002).

As a member of SNAREs gene families, SYP51 was found expressed on TGN and PVC in *Arabidopsis* (Sanderfoot et al. 2001). Most proteins are thought to enter the ER and are transported to the TGN where the pathways of secretion and transport diverge. According to their targeting signals, vesicles that leave the TGN are trafficked to different target membranes such as plasma membrane, the endosome, the vacuole or the cell membrane (Sanderfoot and Raikhel 1999). Those recognized by clathrin-coated vesicles then enter PVC and ultimately the vacuole (Sanderfoot et al. 1998). Although reason for lower gene expression of SYP51 in cold acclimated *Poncirus* is not clear (Fig. 8), a decreased metabolism and cellular water potential due to the accumulation of vesicle in cytosol might be implicated.

Senescence associated proteins are large group of proteins increasingly expressed during plant senescence. In this study, we recovered only a section of senescence associated protein. This leads to difficulties in identification of the exact molecular function of this protein. However, the expression pattern of this protein indicates an up regulation in 55 hours at 4ºC followed by a decrease to basal level upon cold acclimation suggesting that senescence is necessary in early cold defense

but not needed in cold acclimated response.

For horticulturally important woody plants like *Citrus*, low temperature is a serious problem causing reduction in plant growth and reproductivity. The molecular response of woody plant defense cold temperature is complex and our understanding of the mechanisms is limited thus far. Cold treatments resulted in the alternative gene expression of a series of genes in *Poncirus trifoliata* which will allow their use in improvement of stress tolerance ability in *Citrus* and other plant species.

VI. CONCLUSIONS

In the citrus industry, temperature is a key factor limiting growth and productivity of the plant. Most Citrus species originated in tropical and subtropical regions and lack the capacity to tolerate freezing temperatures (Webber et al. 1967). *Citrus unshiu*, one of the most cold hardy commercial citrus, can tolerant temperatures as low as -10°C while *P. trifoliata*, a relative of *Citrus*, can tolerant temperatures as low as -30°C. Due to its ability to survive low temperature, *P. trifoliata* is extensively used as rootstock to enhance freezing tolerance of the scion in freeze-prone areas (Yelenosky 1985). To understand the molecular cold response mechanism of *Poncirus*, we used two simple, low-cost methods (DDRT-PCR and cDNA-AFLP) (Zhang et al. 2005; Lang 2005). The basic goal for these techniques is to provide additional transcripts selected from a pool of genes expressed differently between control and cold treated plants. In this study, cDNA-AFLP combined with relative real-time PCR was used to investigate the molecular mechanisms of *Poncirus trifoliata* under both gradually declined temperature regime and 4°C treatment.

cDNA fragments were analyzed following sequencing. Thirteen cDNA fragments were confirmed positive. Together with our previous study, five groups of genes were classified according to their functions: Class I contains six genes confirmed as up-regulated in cold acclimated *Poncirus* only. They are: S7, homologue of a UDP-glycosyltransferase (UGT) from *Arabidopsis thaliana*; S47, homologue of a trehalose-6-phosphate synthase (TPS) from *Arabidopsis thaliana*; S126, homologue of a chalcone-flavanone isomerase family protein from *Oryza sativa (japonica*

cultivar-group); S159, homologue of a cinnamate 4-hydroxylase (C4H) CYP73 from *Citrus sinensis*; S160B, homologue of a formin-like protein AHF1 from *Arabidopsis thaliana*; S176, homologue of a putative serine carboxypeptidase (Ser-CP) from *Medicago truncatula*. Class II contains one gene which was confirmed as down-regulated in cold acclimated plants: S8, homologue of a putative syntaxin of plants 52 (SYP52) from *Oryza sativa* and a syntaxin of plants 51 (SYP51) from *Arabidopsis thaliana*. Class III contains four genes confirmed as up-regulated upon 55 h of cold treatment at 4°C: S17, homologue to a MAPK 3 from *Chorispora bungeana*; S53, homologue to the calcium ion binding site of a mannosyl-oligosaccharide 1, 2-alpha-mannosidase from *Arabidopsis thaliana*; S145, homologue to a TGA-type basic leucine zipper protein TGA2.1 from *Phaseolus vulgaris*; S149, homologue to a putative senescence-associated protein from *Pyrus communis*. Class IV contains one gene confirmed as up-regulated in *Poncirus* under both cold acclimation and 55 h of cold treatment at 4°C, which is S3, homologue of an auxin and ethylene responsive GH3-like protein from *Capsicum chinense* and an Nt-gh3 deduced protein from *Nicotiana tabacum*. Class V containing one gene confirmed as up-regulated in 55 h cold treatment at 4°C but as down-regulated upon cold acclimation, S134, homologue of a phosphoenolpyruvate carboxykinase (PEPC) from *Arabidopsis thaliana*. This finding can help us gain a better understanding of woody plant's cold response mechanism. The overall picture of the gene network found in this research is indicated in Figure 12.

Figure 1. Plant stress defense pathway (adapted from Wang et al. 2003).

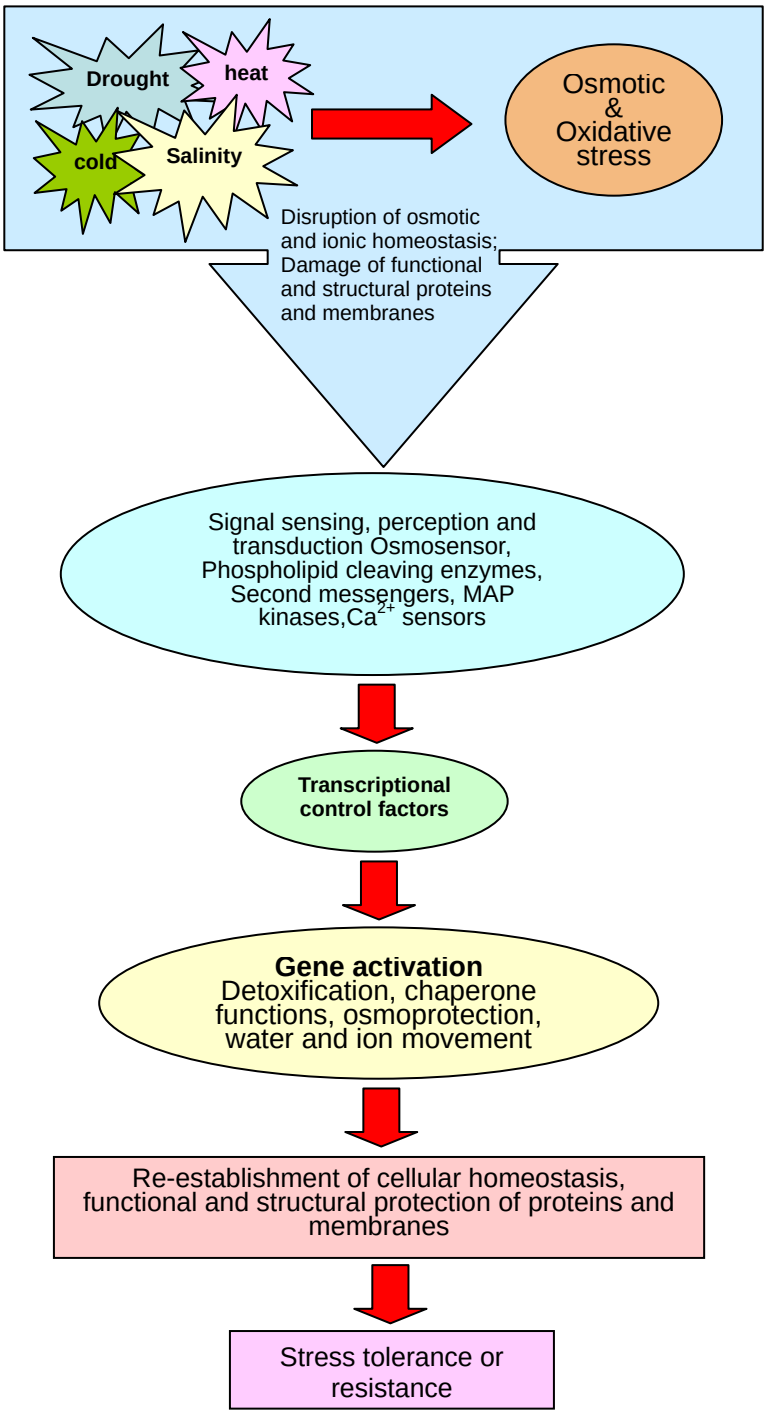


Figure 2. Ca^{2+} inducing cellular responses (adapted from Reddy 2001).

(CDPK=Calcium-dependent protein kinases CaM=Calmodulin EF=Elongation factor)

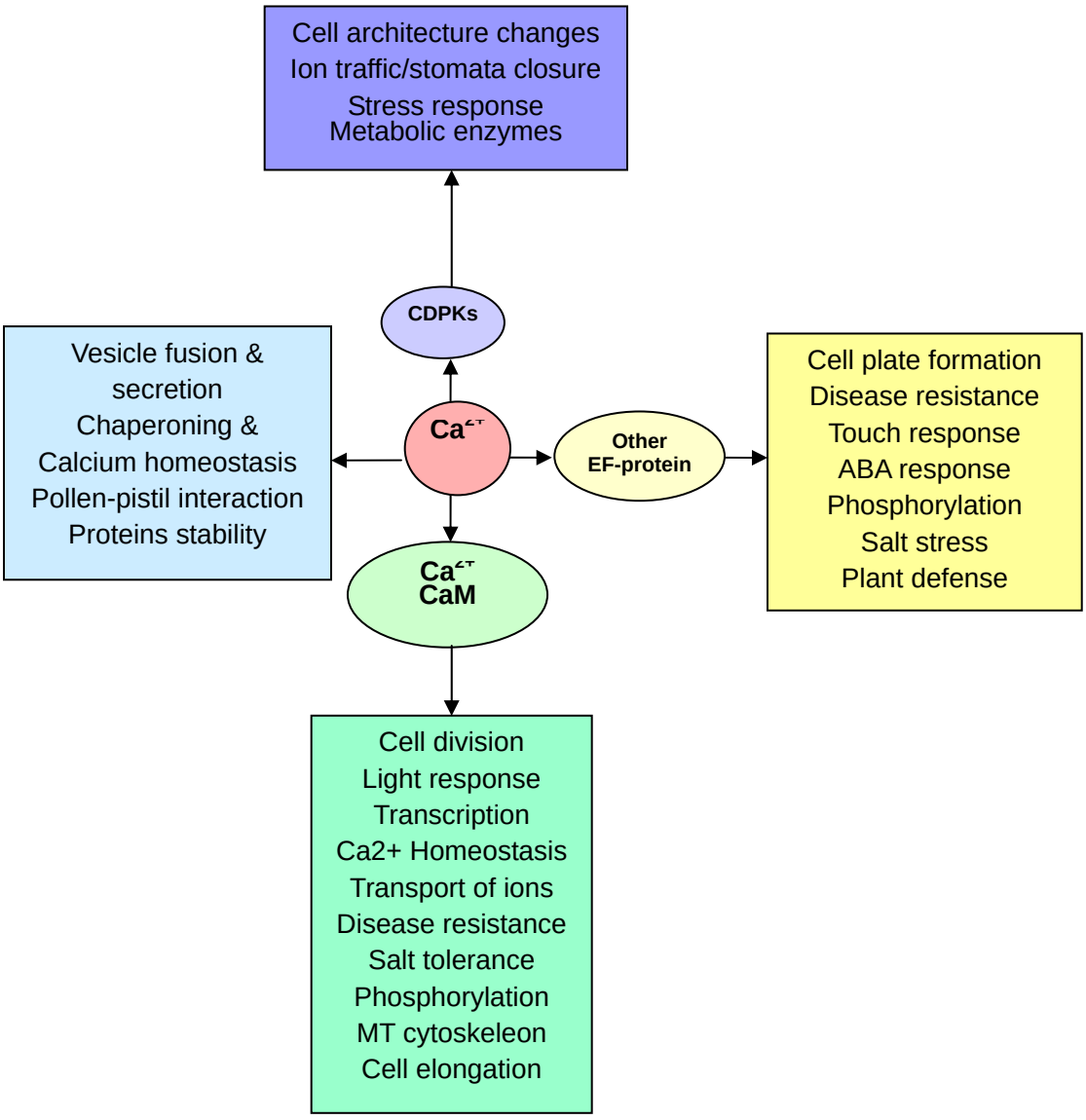


Figure 3. (Salt-Overly-Sensitive) SOS pathway in plants (adapted from Chinnusamy et al. 2004)

(NHX=Vacuolar Na^+/H^+ exchanger, HKT =High affinity K^+ transporter 1).

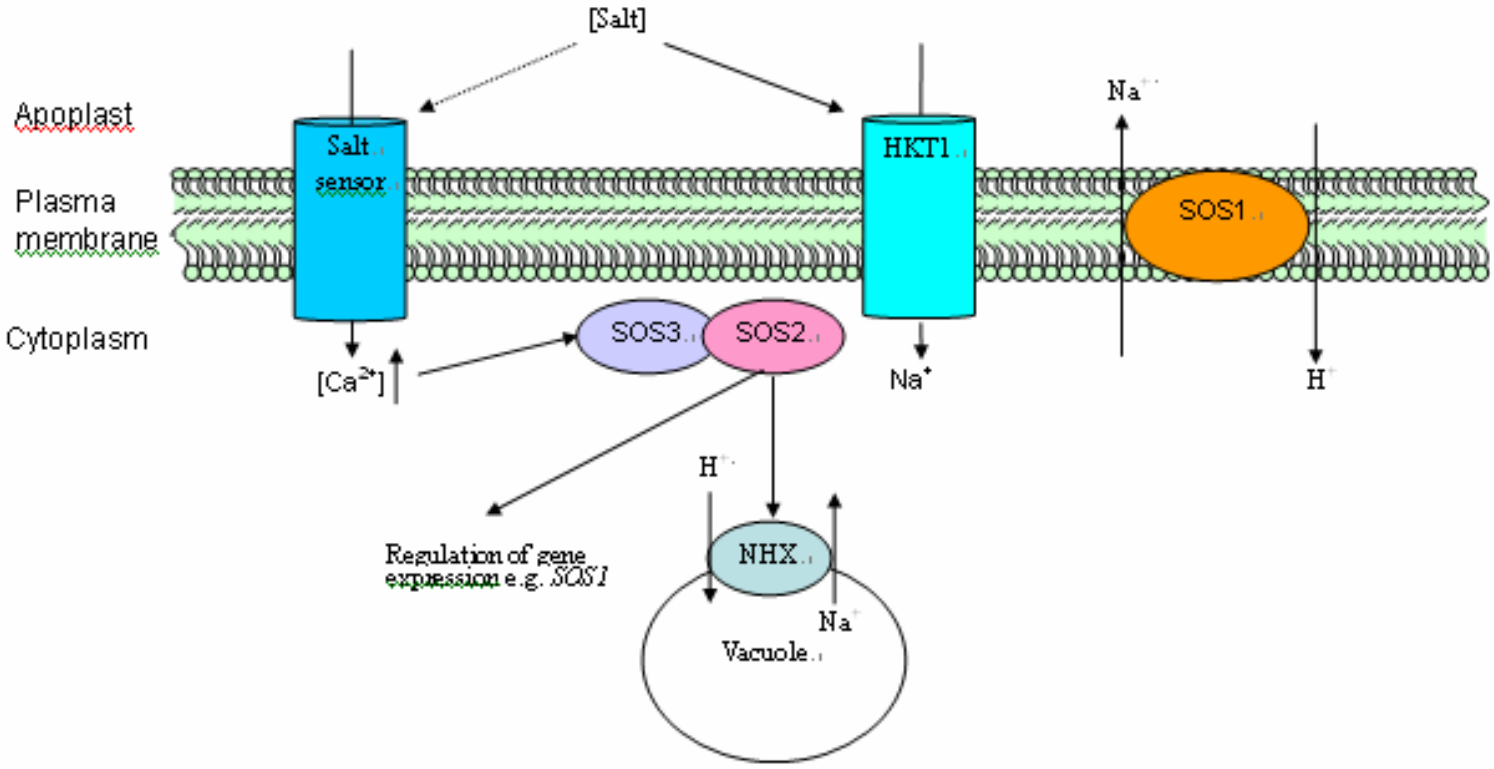


Figure 4. Inducer of C-repeat binding protein (CBF) Expression (ICE1)-CBF pathway in plants

(adapted from Chinnusamy et al. 2004)

DRE= dehydration responsive elements, CRT= C-Repeats,
COR=cold regulated.

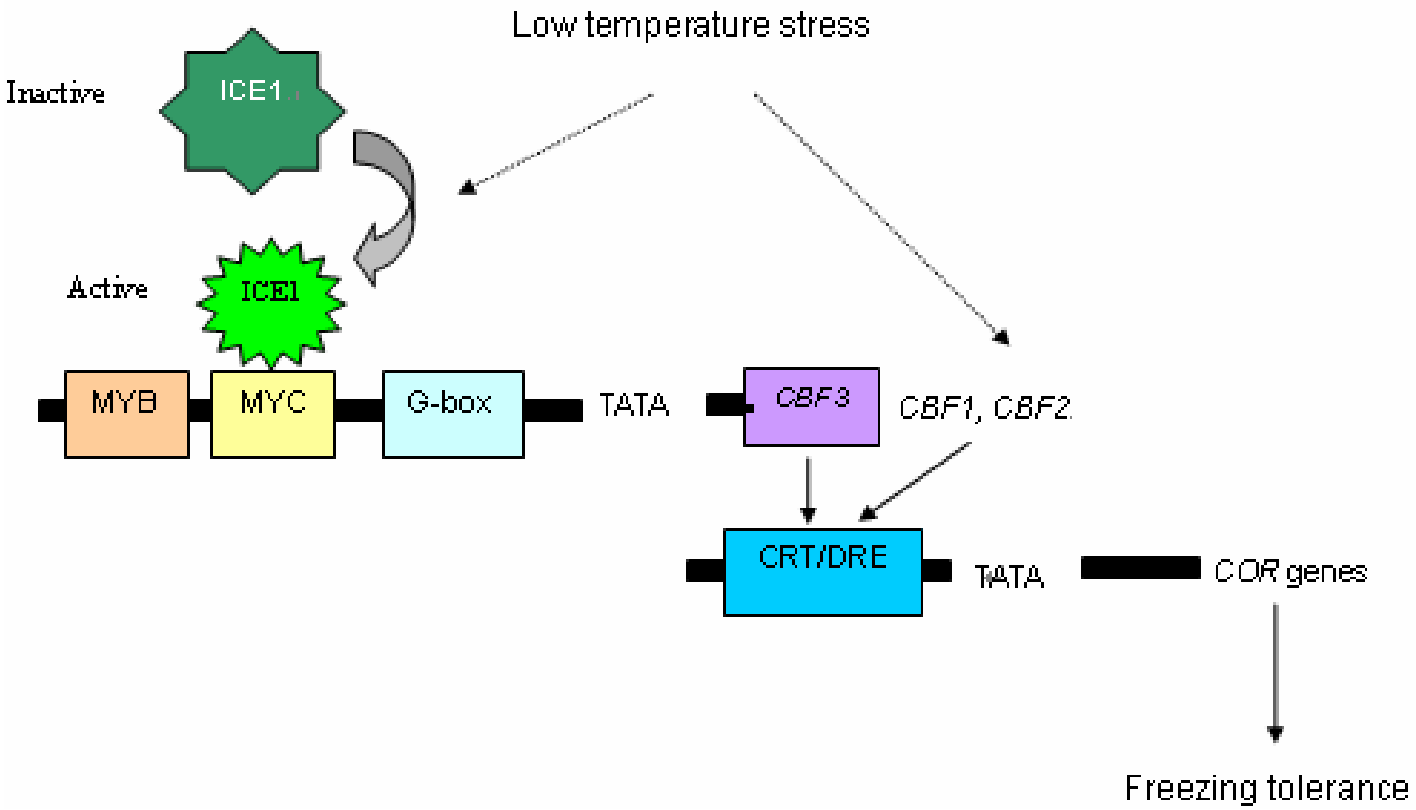


Figure 5. ABA-dependent and –independent pathways (adapted from Chinnusamy et al. 2004)

ABA= abscisic acid, ABI= ABA-insensitive, ABRE= ABA-responsive cis element, bZIP= basic leucine zippers, DREB= dehydration responsive element binding factors, IP_3= 1, 4, 5-trisphosphate, FRY= inositol polyphosphate 1-phosphatase, CBF=C-repeat binding protein, SCaBP5=SOS3-like Ca^{2+} sensor/binding proteins, PKS= protein kinase.

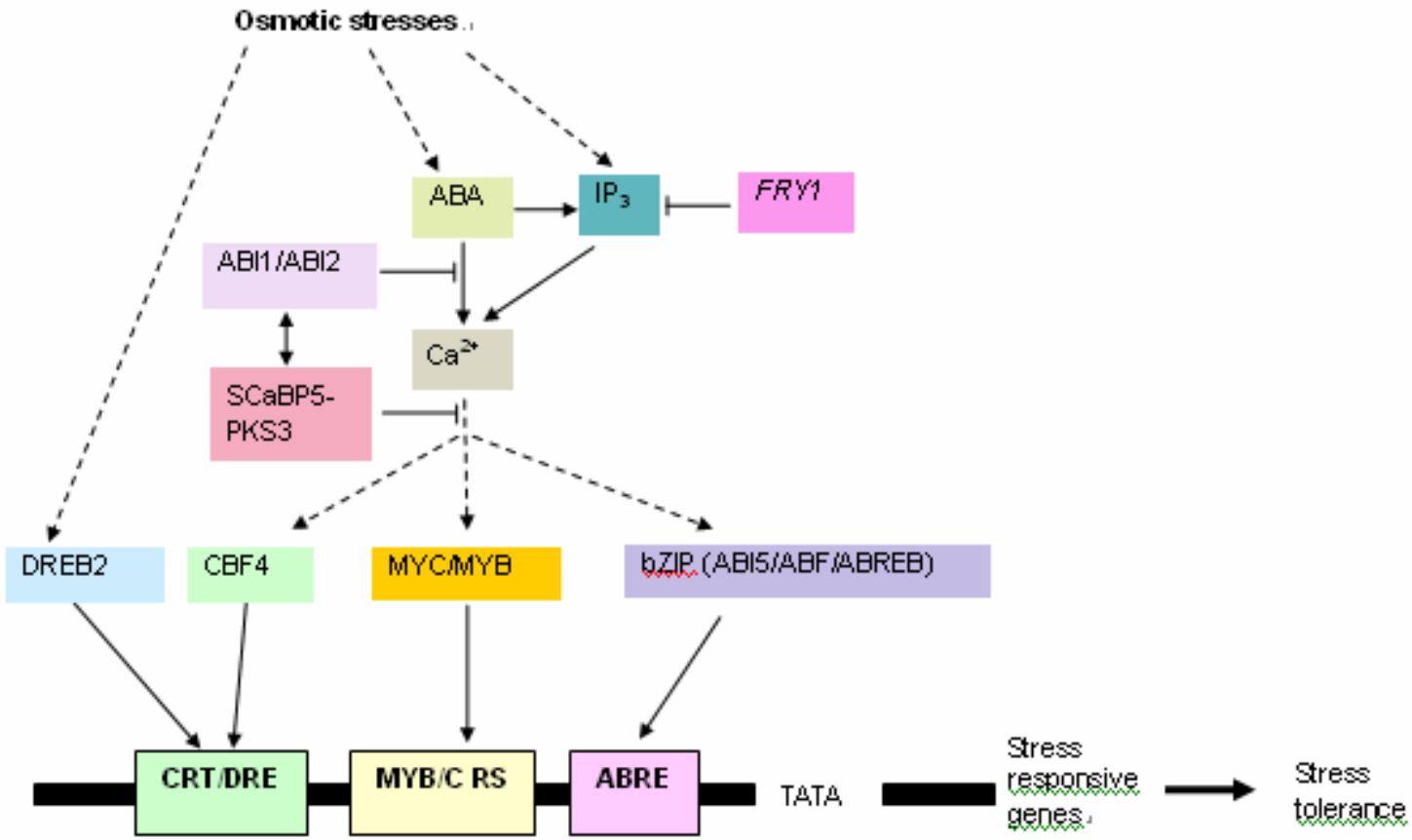
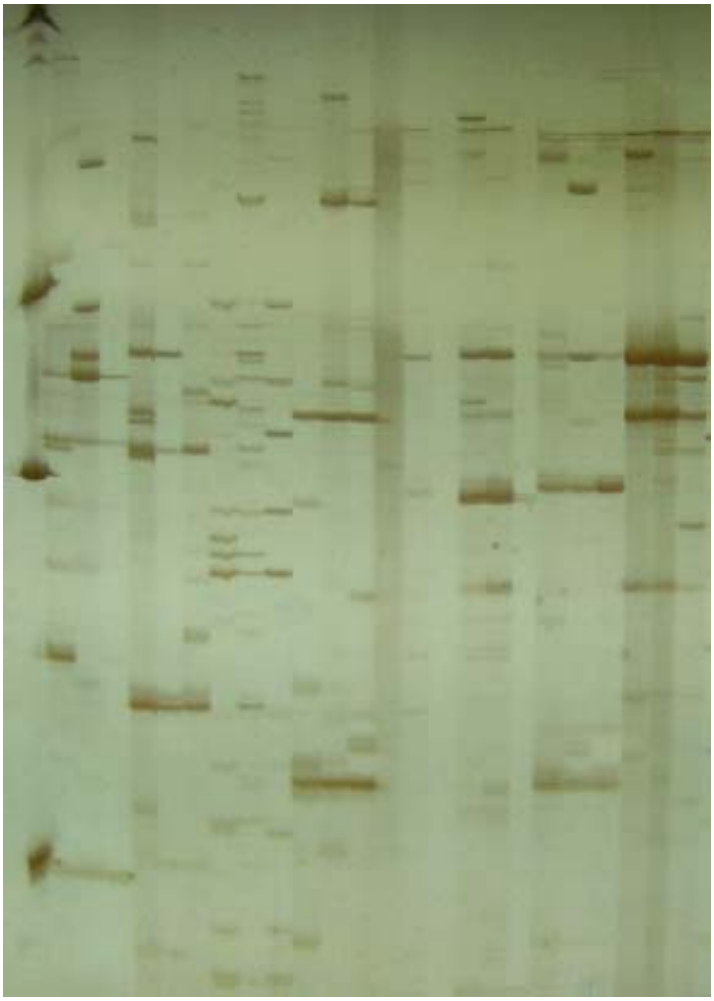


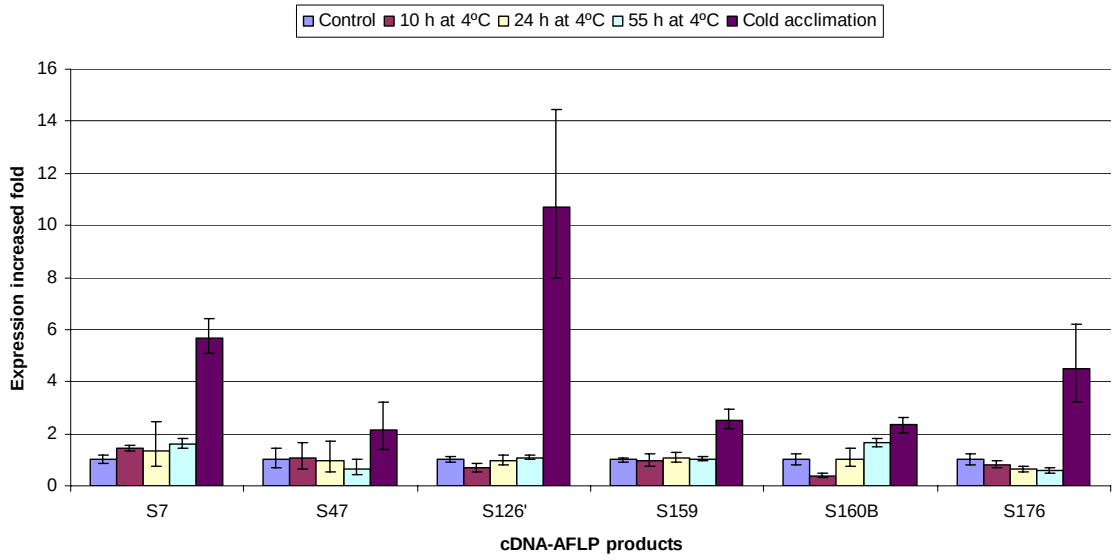
Figure 6. The banding pattern of cDNA fragments amplified by M-CAA primer group

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24



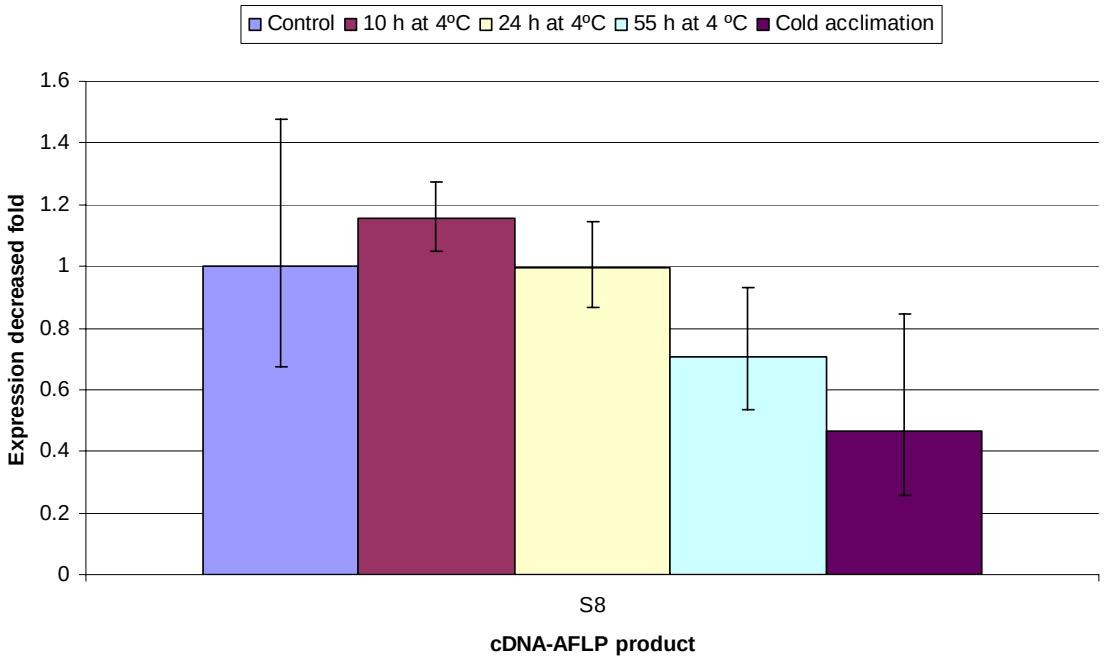
Lane 1: Ladder; lane 2-4: primer M-CAA×E-AAC control, 55 hr at 4ºC, cold acclimation; lane 5-7 primer M-CAA×E-AAG control, cold shock, cold acclimation; lane 8-10: primer M-CAA×E-ACA control, cold shock, cold acclimation; lane 11-13 primer M-CAA×E-ACT control, cold shock, cold acclimation; lane 14-16 primer M-CAA×E-ACC control, cold shock, cold acclimation; lane 17-19 primer M-CAA×E-ACG control, cold shock, cold acclimation; lane 20-22 primer M-CAA×E-AGC control, cold shock, cold acclimation; lane 23-25 primer M-CAA×E-AGG control, cold shock, cold acclimation.

Figure 7. Class I genes, mRNA expression level comparisons between cold treatments and control in *Poncirus trifoliata*



A histogram showing the relative gene expression levels of 6 class I products between control and cold treated plants. Control mRNA of each comparison is considered as 1 and each treatment pair were normalized by comparing the △△Ct to control. The values are the means of three independent experiments ± SE.

Figure 8. Class II gene, mRNA expression level comparisons between cold treatments and control in *Poncirus trifoliata*



A histogram showing the relative gene expression levels of class II product between control and plants treated under four different cold stress conditions. Control mRNA of each comparison is considered as 1 and the treatment *Poncirus* mRNA transcript levels were normalized by comparing the △△Ct to control. The values are the means of three independent experiments ± SE.

Figure 9 Class III genes, mRNA expression level comparisons between control and cold treatments of *Poncirus trifoliata*

9a

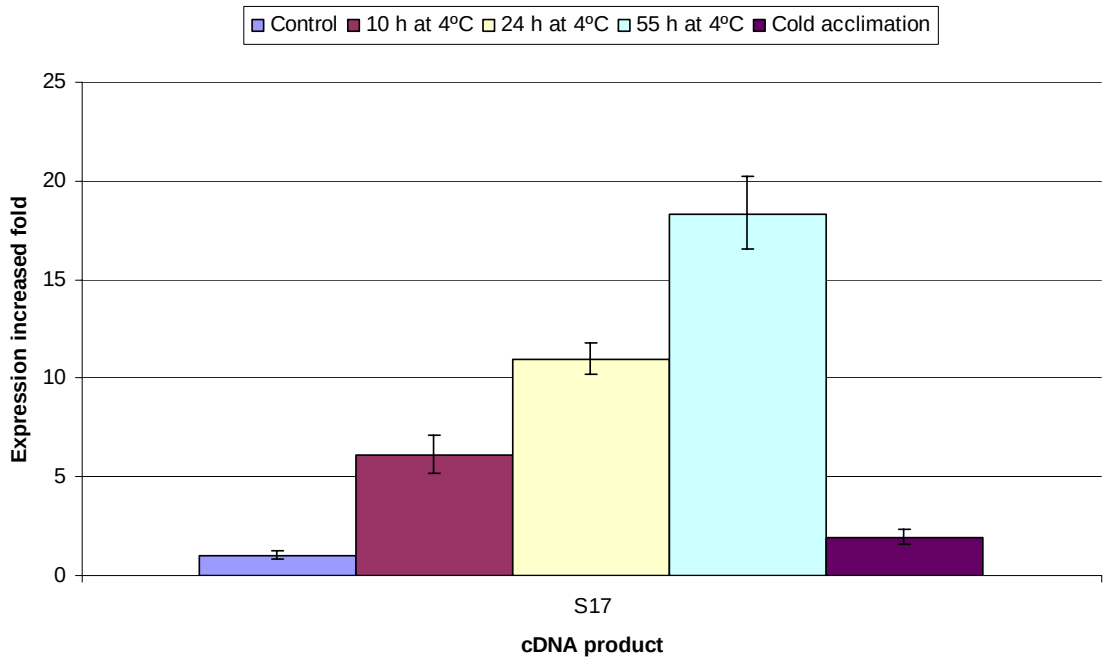


Figure 9a shows the relative gene expression levels of S17 at 4ºC for 10 hours, 24 hours, 55 hours and cold acclimation compared to control which is considered as 1. Cold treatments of S17 at different time periods were normalized by comparing the △△Ct to control.

9b

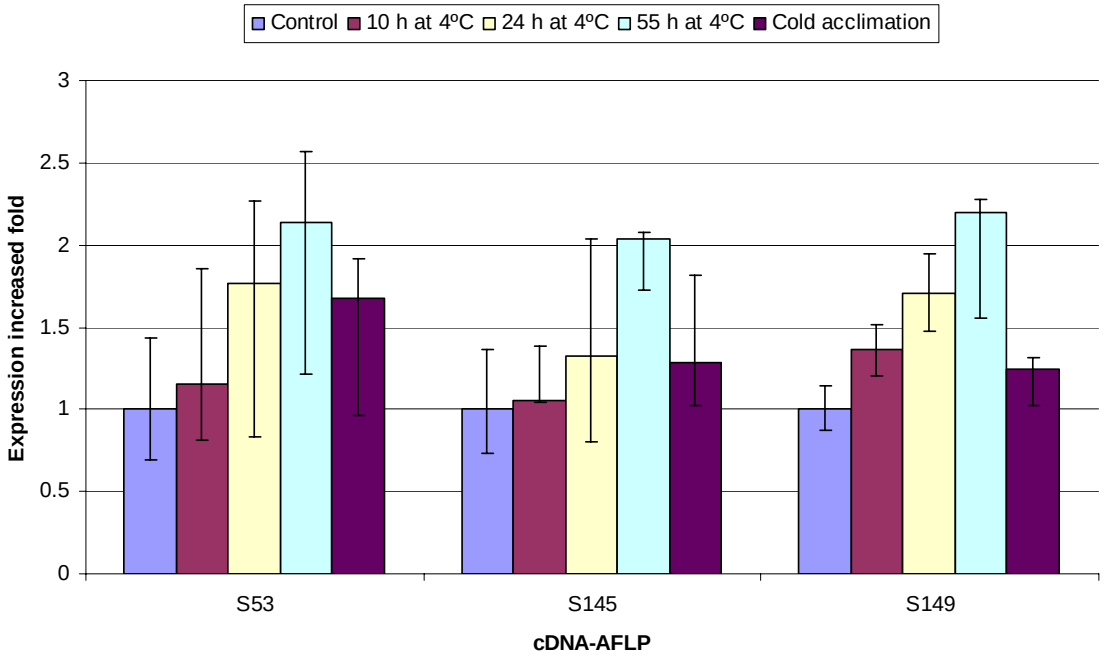


Figure 9b shows the relative gene expression levels of the other genes in class III under different cold treatments. Control mRNA of each comparison is considered as 1 and cold treatments of S53, S145 and S149 were normalized by comparing the △△Ct to control. The values are the means of three independent experiments ± SE.

Figure 10. S3 mRNA expression level comparisons between control and cold treatments of *Poncirus trifoliata*

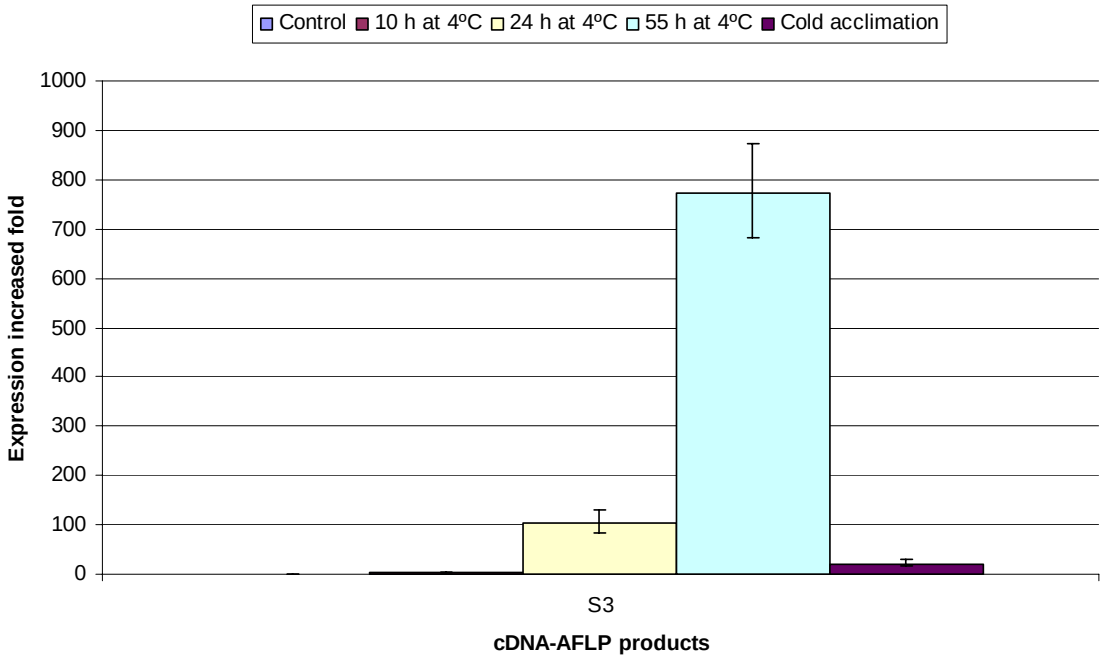
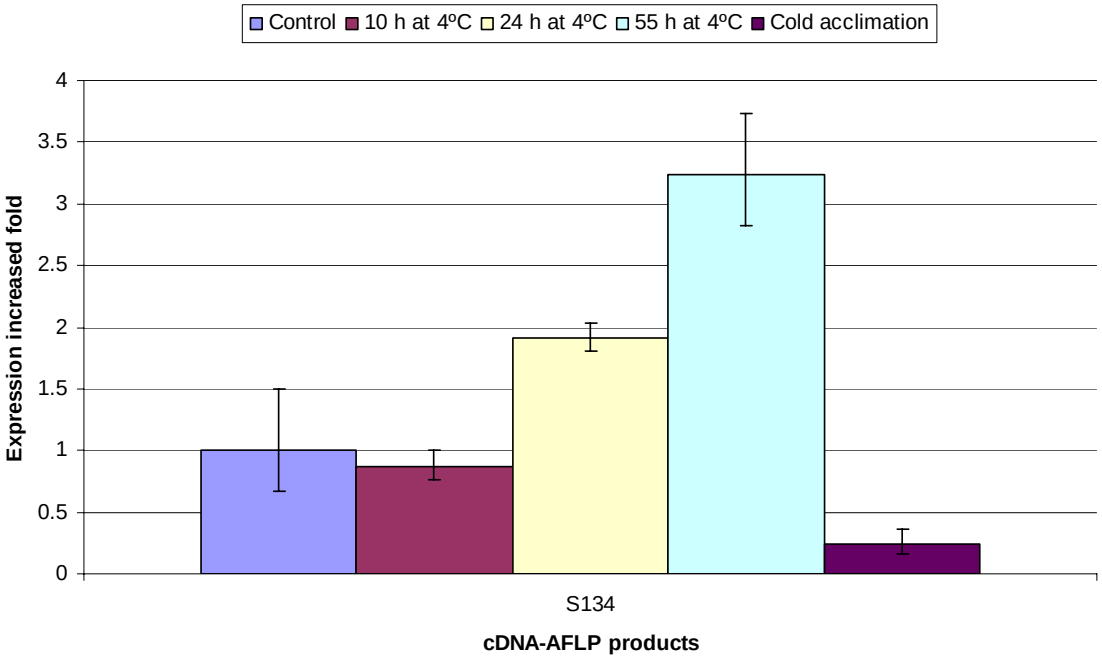


Figure 10 shows the relative gene expression levels of S3 at 4ºC for 10 hours, 24 hours, 55 hours and cold acclimation compared to control which is considered as 1. Gene expression of S3 at different cold treatments was normalized by comparing the △△Ct to control.

Figure11. S134 mRNA expression level comparisons between control and cold treatments of *Poncirus trifoliata*



Relative gene expression level of S134 at 4ºC for 10 hours, 24 hours, 55 hours and cold acclimation as compared to control, which is considered as 1. Different treatments of S134 mRNA were normalized by comparing the △△Ct to control.

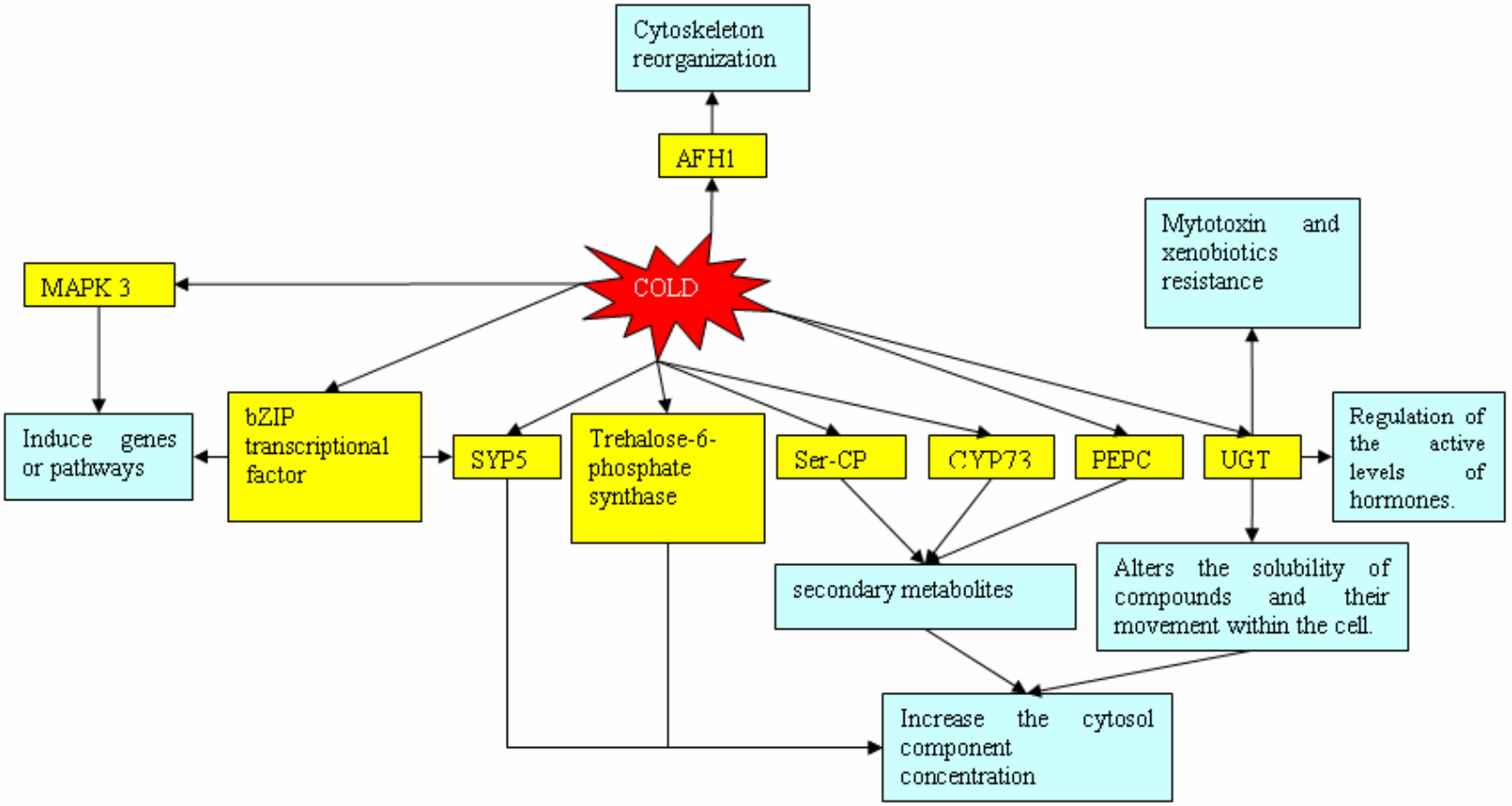
Table 1. Oligonucleotide primer sequences of 13 cDNA-AFLP products isolated from cold treated *Poncirus trifoliata*

	cDNA-AFLP products	Forward Primer (5'-3')	Reverse Primer (5'-3')
Class I	S7	CCCCATGATTTGCCAGCCTTA	GCTCTTTCAATCTCCATTCTCTCC
	S47	ATAGGCCAGCTCCAATCC	CCAAGCATGACAATCTGAC
	S126	TCTCCAAATCAAGTTCACGG	GTCATCATCTTGTGCAAGG
	S159	GAATGATCTAGGCTTGGCA	TGACTTCCGATACCTTCCTT
	S160B	CCCAAACGTTGGCTTATTGT	GGCATGGGAAGTATAGCAAT
	S176	TGAAAGGGCCCAGTTCTTCCA	GGCTGGAAGAGCATTGTTCTACTA
Class II	S8	ATGACTTGGACCAGCATG	CCCTTTGTGCGCTTATTC
Class III	S17	TTGCTATGGAACTCATGGAC	CAAATCCCGATGAATAACAT
	S53	CTGTAAGGCTTCACTGCTG	CTTTTATGAATATTTACTCGAAGTT
	S145	TCTGTTTGTTCTGCTCTTCAAGCC	GGGAATCTTCATATCAAGCTCAGG
	S149	ATAACGCAGGTGTCCTAAGA	TGCCACAAGCCAGTTATC
Class IV	S3	CAAGCAACTCCCAGAATATCAC	CAGCGTCATTGAGTACACAAGT
Class V	S134	TTTCCCCACCAAAGCTCATGCTC	CAAGCGGTGCATTGGCAA

Table 2. Isolated cDNA-AFLP products, percentage similarity to known proteins by BLASTx search in NCBI

Expression groups	cDNA-AFLP product	Plant protein	Function	Similarity
Class I	S7	UDP-glycosyltransferase	Increase stability of water, solubility and inactivation of some plant hormones	77%
	S47	trehalose-6-phosphate synthase	Change in carbon metabolism signaling molecule	89%
	S126	Chalcone-flavanone isomerase family protein	Adjustment of isoflavonoid pathway	85%
	S159	cinnamate 4-hydroxylase CYP73	Increase secondary metabolites	100%
	S160B	formin-like protein AHF1	Mediation actin filament nucleation	75%
	S176	putative serine carboxypeptidase	Secondary metabolite biosynthesis and enzyme activation	86%
Class II	S8	putative syntaxin of plants 52	Membrane fusion and vesicle trafficking	94%
Class III	S17	mitogen-activated protein kinase (MAPK) 3	Signal transduction	98%
	S53	mannosyl-oligosaccharide 1, 2-alpha-mannosidase	Protein glycosylation	100%
	S145	TGA-type basic leucine zipper protein	Transcriptional induction of downstream genes	96%
	S149	Putative senescence-associated protein	Cell senescence	97%
Class IV	S3	auxin and ethylene responsive GH3-like protein	Multifunction	93%
Class V	S134	phosphoenolpyruvate carboxykinase	Secondary metabolite metabolism	96%

Figure 12. The possible network of genes induced during cold stress in *Poncirus trifoliata* in this study



REFERENCES

Avonce N, Leyman B, Mascorro-Gallardo JO, Van Dijck P, Thevelein JM, Iturriaga G (2004) The *Arabidopsis* trehalose-6-P synthase AtTPS1 gene is a regulator of glucose, abscisic acid, and stress signaling. Plant Physiol 136:3649-3659

Bachem CWB, van der Hoeven RS, de Bruijn SM, Vreugdenhil D, Zabeau M, Visser RGF (1996) Visualization of differential gene expression using a novel method of RNA fingerprinting based on AFLP: Analysis of gene expression during potato tuber development. Plant J 9: 745–753

Barakat A, Szick-Miranda K, Chang I-F, Guyot R, Blanc G, Cooke R, Delseny M, Bailey-Serres J (2001) The organization of cytoplasmic ribosomal protein genes in the *Arabidopsis* genome. Plant Physiol 127:398–415

Bartels D (2001) Targeting detoxification pathways: an efficient approach to obtain plants with multiple stress tolerance? Trends Plant Sci 6:284–286

Bei-Paraskevopoulou T, Kloppstech K (1999) The expression of early light-inducible proteins (ELIPs) under high light stress as defense marker in Northern- and Southern European cultivars of barley (*Hordeum vulgare*). Physiol Plant 106:105–111

Betz C, McCollum TG, Mayer RT (2001) Differential expression of two cinnamate 4-hydroxylase genes in 'Valencia' orange (*Citrus sinensis Osbeck*). Plant Mol Biol 46:741-748

Breyne P, Dreesen R, Cannoot B, Rombaut D, Vandepoele K, Rombauts S, Vanderhaeghen R, Inze D, Zabeau M (2003) Quantitative cDNA-AFLP analysis for genome-wide expression studies. Mol Genet Genomics 269:173-179

Burbulis IE, Winkel-Shirley B (1999) Interactions among enzymes of the *Arabidopsis* flavonoid biosynthetic pathway. Proc Natl Acad Sci U S A 96:12929-12934

Cai Q, Moore GA, Guy CL (1995) An unusual group 2 LEA gene family in citrus responsive to low temperature. Plant Mol Biol 29:11-23

Callis J, Vierstra RD (2000) Protein degradation in signaling. Curr Opin Plant Biol 3:381–386

Cercos M, Urbez C, Carbonell J (2003) A serine carboxypeptidase gene (PsCP), expressed in early stages of reproductive and vegetative development in *Pisum sativum*, is induced by gibberellins. Plant Mol Biol 51:165–174

Cheung AY, Wu HM (2004) Overexpression of an *Arabidopsis* formin stimulates supernumerary actin cable formation from pollen tube cell membrane. Plant Cell 16: 257–269

Chinnusamy V, Zhu J, Zhu JK. (2006) Salt stress signaling and mechanisms of plant salt tolerance. Genet Eng (N Y) 27:141-177

Chinnusamy V, Schumaker K, Zhu JK (2004) Molecular genetic perspectives on cross-talk and specificity in abiotic stress signalling in plants. J Exp Bot 55:225-236

Chollet R, Vidal J, O'Leary MH (1996) Phosphoenolpyruvate carboxylase : A ubiquitous, highly regulated enzyme in plants. Annu Rev Plant Physiol Plant Mol Biol 47:273-298

Crowe JH, Carpenter JF, Crowe LM, Anchordoguy TJ (1990) Are freezing and dehydration similar stress vectors—a comparison of modes of interaction of stabilizing solutes with biomolecules. Cryobiology 27:219–231

Cvrckova F (2000) Are plant formins integral membrane proteins? Genome Biol 1: 1–7

Cvrckova F, Novotny M, Pickova D, Za´ rsky V (2004) Formin homology 2 domains occur in multiple contexts in angiosperms. BMC Genomics 5: 1–18

Davies P (1995) Plant hormone physiology, biochemistry and molecular biology, 2nd ed. Kluwer academic publishers, Dordecht, the Netherlands

Deeks MJ, Hussey PJ, Davies B (2002) Formins: Intermediates in signal-transduction cascades that affect cytoskeletal reorganization. Trends Plant Sci 7: 492–498

Deswal R, Sopory SK (1999) Glyoxalase I from *Brassica juncea* is a calmodulin stimulated protein. Biochim Biophys Acta 1450:460-467

Dittrich P, Campbell WH, Black CC (1973) Phosphoenolpyruvate carboxykinase in plants exhibiting crassulacean acid metabolism. Plant Physiol 52:357-361

Dixon RA, Steele CL (1999) Flavonoids and isoflavonoids - a gold mine for metabolic engineering. Trends Plant Sci 4:394-400

Domı´ nguez F, Gonza´ lez MC, Cejudo FJ (2002) A germinationrelated gene encoding a serine carboxypeptidase is expressed during the differentiation of the vascular tissue in wheat grains and seedlings. Planta 215:727–734

Eastmond PJ, Graham IA (2003) Trehalose metabolism: a regulatory role for trehalose-6-phosphate? Curr Opin Plant Biol. 6:231-235

Eastmond PJ, van Dijken AJ, Spielman M, Kerr A, Tissier AF, Dickinson HG, Jones JD, Smeekens SC, Graham IA (2002) Trehalose-6-phosphate synthase 1, which catalyses the first step in trehalose synthesis, is essential for *Arabidopsis* embryo maturation. Plant J 29:225-235

Edwards GE, Kanai R, Black CC (1971) Phosphoenolpyruvate carboxykinase in leaves of certain plants which fix CO 2 by the C 4 -dicarboxylic acid cycle of photosynthesis. Biochem Biophys Res Commun 45:278-285

Elbein AD, Pan YT, Pastuszak I, Carroll D (2003) New insights on trehalose: a multifunctional molecule. Glycobiology 13:17-27

Favery B, Chelysheva LA, Lebris M, Jammes F, Marmagne A, De Almeida-Engler J, Lecomte P, Vaury C, Arkowitz RA, Abad P (2004) *Arabidopsis* formin AtFH6 is a plasma membraneassociated protein upregulated in giant cells induced by parasitic nematodes. Plant Cell 16: 2529–2540

Feilner T, Hultschig C, Lee J, Meyer S, Immink RG, Koenig A, Possling A, Seitz H, Beveridge A, Scheel D, Cahill DJ, Lehrach H, Kreutzberger J, Kersten B (2005) High throughput identification of potential *Arabidopsis* mitogen-activated protein. kinases substrates. Mol Cell Proteomics 4:1558-1568

Fowler S, Thomashow MF (2002) *Arabidopsis* transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. Plant Cell 14:1675-1690

Fukuda R, McNew JA, Weber T, Parlati F, Engel T, Nickel W, Rothman JE, So¨ llner TH (2000) Functional architecture of an intracellular membrane t-SNARE. Nature 407: 198–202

Garwe D, Thomson JA, Mundree SG. (2003) Molecular characterization of XVSAP1, a stress-responsive gene from the resurrection plant Xerophyta viscosa Baker. J Exp Bot 54:191-201.

Gensheimer M, Mushegian A (2004) Chalcone isomerase family and fold: no longer unique to plants. Protein Sci 13:540-504

Gigon A, Matos AR, Laffray D, Zuily-Fodil Y, Pham-Thi AT (2004) Effect of drought stress on lipid metabolism in the leaves of *Arabidopsis thaliana* (ecotype Columbia). Ann Bot 94:345-351

Goddijn O, Smeekens S (1998) Sensing trehalose biosynthesis in plants. Plant J 1998 14:143-146

Granat SJ, Wilson KA, Tan-Wilson AL (2003) New serine carboxypeptidases in mung bean seedling cotyledons. J Exp Bot 160:1263–1266

Guy C (1990) Molecular responses of plants to cold shock and cold acclimation. J Mol Microbiol Biotechnol 1:231-242

Hagen G, Guilfoyle T (2002) Auxin-responsive gene expression: genes, promoters and regulatory factors. Plant Mol Biol 49:373-385

Hagen G, Guilfoyle TJ (1985) Rapid induction of selective transcription by auxins. Mol Cell Biol 5:1197-1203

Hara M, Terashima S, Fukaya T, Kuboi T (2003) Enhancement of cold tolerance and inhibition of lipid peroxidation by citrus dehydrin in transgenic tobacco. Planta 217:290-298

Hsieh HL, Okamoto H, Wang M, Ang LH, Matsui M, Goodman H, Deng XW (2000) FIN219, an auxin-regulated gene, defines a link between phytochrome A and the downstream regulator COP1 in light control of *Arabidopsis* development. Genes Dev 14: 1958–1970

Hutin C, Nussaume L, Moise N, Moya I, Kloppstech K, Havaux M (2003) Early light-induced proteins protect *Arabidopsis* from photooxidative stress. Proc Natl Acad Sci USA 100:4921–4926

Ingouff M, Fitz Gerald JN, Gue´ rin C, Robert H, Sørensen MB, Van Damme D, Geelen D, Blanchoin L, Berger F (2005) Plant formin AtFH5 is an evolutionarily conserved actin nucleator in cytokinesis. Nat Cell Biol 7: 374–380

Jakoby M, Weisshaar B, Droge-Laser W, Vicente-Carbajosa J, Tiedemann J, Kroj T, Parcy F (2002) bZIP transcription factors in *Arabidopsis*. Trends Plant Sci 7:106-111

Jaglo KR, Kleff S, Amundsen KL, Zhang X, Haake V, Zhang JZ, Deits T, Thomashow MF (2001) Components of the *Arabidopsis* C-repeat/dehydration-responsive element binding factor cold-response pathway are conserved in *Brassica napus* and other plant species. Plant Physiol 127:910-917

Jaglo-Ottosen K, Gilmour SJ, Zarka D, Schabenberger O, Thomashow MF (1998) *Arabidopsis* CBF1 overexpression induce COR genes and enhances freezing tolerance. Science 280:104–106

Jones P, Messner B, Nakajima J-I, Scha¨ffner A, Saito K (2003) UGT73C6 and UGT78D1—glycosyltransferases involved in flavonol glycoside biosynthesis in

Arabidopsis thaliana. J Biol Chem 278: 43910–43918

Jones P, Vogt T (2001) Glycosyltransferases in secondary plant metabolism: tranquilizers and stimulant controllers. Planta 213: 164–174

Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nat Biotechnol 17: 287-91

Kleczkowski K, Schell F (1995) Phytohormone conjugates: nature and function. Crit Rev Plant Sci 14: 283–298

Kolbe A, Tiessen A, Schluepmann H, Paul M, Ulrich S, Geigenberger P (2005) Trehalose 6-phosphate regulates starch synthesis via posttranslational redox activation of ADP-glucose pyrophosphorylase. Proc Natl Acad Sci U S A 102: 11118-11123

Komis G, Apostolakos P, Galatis B (2002) Hyperosmotic stress-induced actin filament reorganization in leaf cells of *Chlorophyton comosum*. J Exp Bot 53:1699-1710

Koopmann E, Logemann E, Hahlbrock K (1999) Regulation and functional expression of cinnamate 4-hydroxylase from parsley. Plant Physiol 119:49-56

Kovar DR, Pollard TD (2004) Insertional assembly of actin filament barbed ends in association with formins produces piconewton forces. Proc Natl Acad Sci USA 101: 14725–14730

Kovtun Y, Chiu WL, Tena G, Sheen J (2000) Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants Proc Natl Acad Sci U S A 97:2940-2945

Lang P, Zhang CK, Ebel RC, Dane F, Dozier WA (2005) Identification of cold acclimated genes in leaves of *Citrus unshiu* by mRNA differential display. Gene 359:111-118

Larkindale J, Hall JD, Knight MR, Vierling E (2005) Heat stress phenotypes of *Arabidopsis* mutants implicate multiple signaling pathways in the acquisition of thermotolerance. Plant Physiol 138:882-897

Lepiniec L, Vidal J, Chollet R, Gadal P, Crétin C (1994) Phosphoenolpyruvate carboxylase: structure, regulation and evolution. Plant Sci 99:111–124

Li F, Higgs HN (2003) The mouse Formin mDia1 is a potent actin nucleation factor regulated by autoinhibition. Curr Biol 13: 1335–1340

Li J, Lease KA, Tax FE, Walker JC (2001) BRS1, a serine carboxypeptidase, regulates BRI1 signaling in *Arabidopsis*. Proc Natl Acad Sci USA 98:5916–5921

Li Y, Baldauf S, Lim EK, Bowles DJ (2001) Phylogenetic analysis of the UDP-glycosyltransferase multigene family of *Arabidopsis thaliana*. J Biol Chem 276:4338-4343

Lim EK, Bowles DJ (2004) A class of plant glycosyltransferases involved in cellular homeostasis. EMBO J 23:2915-2922

Lim PO, Woo HR, Nam HG (2003) Molecular genetics of leaf senescence in *Arabidopsis*. Trends Plant Sci 8: 272-278

Liu K, Kang BC, Jiang H, Moore SL, Li H, Watkins CB, Setter TL, Jahn MM (2005) A GH3-like gene, CcGH3, isolated from *Capsicum chinense L.* fruit is regulated by auxin and ethylene. Plant Mol Biol 58:447-464

Lu S, Zhou Y, Li L, Chiang VL (2006) Distinct roles of cinnamate 4-hydroxylase genes in *Populus*. Plant Cell Physiol 47:905-914

Martin RC, Mok MC, Mok DWS (1999) A gene encoding the cytokinin enzyme zeatin O-xylosyltransferase of *Phaseolus vulgaris*. Plant Physiol 120: 553–557

Mazars C, Thion L, Thuleau P, Graziana A, Knight MR, Moreau M et al. (1997) Organization of cytoskeleton controls the changes in cytosolic calcium of cold-shocked *Nicotiana plumbaginifolia* protoplasts. Cell Calcium 22: 413–420

McKersie BD, Murnaghan J, Jones KS, Bowley SR (2000) Iron-superoxide dismutase expression in transgenic alfalfa increases winter survival without a detectable increase in photosynthetic oxidative stress tolerance. Plant Physiol 122:1427-1437

McNew JA, Parlati F, Fukuda R, Johnston J, Paz K, Paumet F, So llner TH, Rothman JH (2000) Compartmental specificity of cellular membrane fusion encoded in SNARE proteins. Nature 407: 153–159

Michelot A, Guerin C, Huang S, Ingouff M, Richard , Rodiuc N, Staiger CJ, Blanchoin L (2005) The formin homology 1 domain modulates the actin nucleation and bundling activity of *Arabidopsis* FORMIN1. Plant Cell 17:2296-2313

Miller KD, Guyon V, Evans JNS, Shuttleworth WA, Taylor LP (1999) Purification, cloning, and heterologous expression of a catalytically efficient flavonol 3-O-galactosyltransferase expressed in the male gametophyte of *Petunia hybrida*. J Biol Chem 274: 34011–34019

Mishra NS, Tuteja R, Tuteja N (2006) Signaling through MAP kinase networks in plants. Arch Biochem Biophys 452:55-68

Moura DS, Bergey DR, Ryan CA (2001) Characterization and localization of a wound-inducible type I serine-carboxypeptidase from leaves of tomato plants (*Lycopersicon esculentum Mill*). Planta 212:222–230

Munne-Bosch S, Alegre L (2002) Plant aging increases oxidative stress in chloroplasts. Planta 214: 608-615

Munnik T, Ligterink W, Meskiene I I, Calderini O, Beyerly J, Musgrave A, Hirt H (1999) Distinct osmo-sensing protein kinase pathways are involved in signalling moderate and severe hyper-osmotic stress. Plant J 20:381-388

Nakazawa M, Yabe N, Ichikawa T, Yamamoto YY, Yoshizumi T, Hasunuma K, Matsui M (2001) DFL1, an auxin-responsive GH3 gene homologue, negatively regulates shoot cell elongation and lateral root formation, and positively regulates the light response of hypocotyl length. Plant J 25: 213–221

Nick P (2000) Control of the response to low temperatures. In: Nick P (ed.) Plant Microtubules: Potential for Biotechnology (pp 121–135) Springer, Berlin, Heidelberg

Parlati F, McNew JA, Fukuda, R, Miller R, So¨ llner TH, Rothman JH (2000) Topological restriction of SNARE-dependent membrane fusion. Nature 407: 194–198

Poppenberger B, Berthiller F, Lucyshyn D, Sieberer T, Schuhmacher R, Krska R, Kuchler K, Glo¨ssl J, Luschnig C, Adam G (2003) Detoxification of the *Fusarium mycotoxin* deoxynivalenol by a UDP-glucosyltransferase from *Arabidopsis thaliana*. J Biol Chem 278: 47905–47914

Porat R, Pavoncello D, Lurie S, McCollum TG (2002) Identification of a grapefruit cDNA belonging to a unique class of citrus dehydrins and characterization of its expression patterns under temperature stress conditions. Physiol Plant 115:598-603

Pruyne D, Evangelista M, Yang C, Bi E, Zigmond S, Bretscher A, Boone C (2002) Role of formins in actin assembly: Nucleation and barbed-end association. Science 297: 612–615

Quirino BF, Noh YS, Himelblau E, Amasino RM (2000) Molecular aspects of leaf senescence. Trends Plant Sci 5:278-282

Reddy AS (2001) Calcium: silver bullet in signaling. Plant Sci 160:381-404

Remington SJ (1993) Serine carboxypeptidases: a new and versatile family of enzymes.

Curr Opin Biotechnol4:462-468

Ro DK, Mah N, Ellis BE, Douglas CJ (2001) Functional characterization and subcellular localization of poplar (*Populus trichocarpa x Populus deltoides*) cinnamate 4-hydroxylase. Plant Physiol 126:317-329

Robbins AL, Louzada ES (2005) Expression analysis of a cold responsive transcript from trifoliate orange by real-time PCR and RT-PCR. Plant Cell Rep 8:1-7

Ross J, Li Y, Lim E, Bowles DJ (2001) Higher plant glycosyltransferases. Genome Biol 2: 3004.1-3004.2

Roux C, Perrot-Rechenmann C (1997) Isolation by differential display and characterization of a tobacco auxin-responsive cDNA Nt-gh3, related to GH3. FEBS Lett 419:131-136

Sagot I, Rodal AA, Moseley J, Goode BL, Pellman D (2002) An actin nucleation mechanism mediated by Bni1 and profilin. Nat Cell Biol 4: 626–631

Sahin-Cevik M, Moore GA (2006) Identification and expression analysis of cold-regulated genes from the cold-hardy Citrus relative *Poncirus trifoliata* (L.) Raf. Plant Mol Biol 62:83-97

Sanderfoot AA, Ahmed SU, Marty-Mazars D, Rapoport I, Kirchhausen T, Marty F, Raikhel NV (1998) A putative vacuolar cargo receptor partially colocalizes with AtPEP12p on a prevacuolar compartment in *Arabidopsis* roots. Proc Natl Acad Sci U S A 95:9920-9925

Sanderfoot AA, Kovaleva V, Bassham DC, Raikhel NV (2001) Interactions between syntaxins identify at least five SNARE complexes within the Golgi/prevacuolar system of the *Arabidopsis* cell. Mol Biol Cell 12:3733-3743

Sanderfoot AA, Raikhel NV (1999) The specificity of vesicle trafficking: coat proteins and SNAREs. Plant Cell 11:629-642

Seppanen MM, Cardi T, Borg HM, Pehu E (2000) Characterization and expression of cold-induced glutathione S-transferase in freezing tolerant *Solanum commersonii*, sensitive *S. tuberosum* and their interspecific somatic hybrids. Plant Sci 153:125–133

Schaller A (2004) A cut above the rest: the regulatory function of plant proteases. Planta 220:183-197

Shinozaki K, Yamaguchi-Shinozaki K (1997) Gene expression and signal transduction in water-stress response. Plant Physiol 115:327-334

Siberil Y, Doireau P, Gantet P (2001) Plant bZIP G-box binding factors. Modular structure and activation mechanisms. Eur J Biochem 268:5655-5666

Strasser R, Schoberer J, Jin C, Glossl J, Mach L, Steinkellner H (2006) Molecular cloning and characterization of *Arabidopsis thaliana* Golgi alpha-mannosidase II, a key enzyme in the formation of complex N-glycans in plants. Plant J 45:789-803 Erratum in: Plant J 47:827

Sung DY, Vierling E, Guy CL (2001) Comprehensive expression profile analysis of the *Arabidopsis* hsp70 gene family. Plant Physiol 126:789–800

Suzuki S, Nakatsubo T, Umezawa T, Shimada M (2002) First in vitro norlignan formation with *Asparagus officinalis* enzyme preparation. Chem Commun 2002: 1088–1089

Suzuki S, Yamamura M, Shimada M, Umezawa T (2004) A heartwood norlignan, (E)-hinokiresinol, is formed from 4-coumaryl 4-coumarate by a *Cryptomeria japonica* enzyme preparation. Chem Commun 2004: 2838–2839

Thomashow MF (1999) Plant could acclimation: Freezing tolerance genes and regulatory mechanisms. Annu Rev Plant Physiol Plant Mol Biol 50:571–599

Tremblay K, Ouellet F, Fournier J, Danyluk J, Sarhan F (2005) Molecular characterization and origin of novel bipartite cold-regulated ice recrystallization inhibition proteins from cereals. Plant Cell Physiol 46:884-891

Toh H, Kawamura T, Izui K (1994) Molecular evolution of phosphoenolpyruvate carboxylase. Plant Cell Environ 17: 31–43

Tucker ML, Whitelaw CA, Lyssenko NN, Nath P (2002) Functional analysis of regulatory elements in the gene promoter for an abscission-specific cellulase from bean and isolation, expression, and binding affinity of three TGA-type basic leucine zipper transcription factors. Plant Physiol 130:1487-1496

Uemura M, Steponkus PL (1995) Cold acclimation of *Arabidopsis thaliana* (Effect on plasma membrane lipid composition and freeze-induced lesions). Plant Physiol 109:15-30

Van Damme, D, Bouget FY, Van Poucke K, Inze D, Geelen D (2004) Molecular dissection of plant cytokinesis and phragmoplast structure: A survey of GFP-tagged proteins. Plant J 40: 386–398

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: A new Technique for DNA fingerprinting. Nucleic Acids Res 23: 4407–4414

Wang W, Vinocur B, Altman A (2003) Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta 218:1-14

Webber HJ, Reuther W, Lawton HW (1967) History and development of the citrus industry In Reuther W, Webber HJ, Batchelor LD (ed), The citrus industry. University of California, Berkeley, USA Vol 1: pp 1-39

Wesley-Smith J, Walters C, Berjak P, Pammenter NW (2004) The influence of water content, cooling and warming rate upon survival of embryonic axes of *Poncirus trifoliata* (L.). Cryo Letters 25:129-138.

Whitbred JM, Schuler MA (2000) Molecular characterization of CYP73A9 and CYP82A1 P450 genes involved in plant defense in pea. Plant Physiol 124: 47–58

Wiesler B, Wang QY, Nick P (2002) The stability of cortical microtubules depends on their orientation. Plant J 32:1023-32.

Wu QS, Xia RX, Zou YN (2006) Reactive oxygen metabolism in mycorrhizal and non-mycorrhizal citrus (*Poncirus trifoliata*) seedlings subjected to water stress. J Plant Physiol 163:1101-1110

Yelenosky G (1985) Cold hardiness in *Citrus*. Hort Rev VII: 201-238

Zhang CK, Lang P, Dane F, Ebel RC, Singh NK, Locy RD, Dozier WA (2005) Cold acclimation induced genes of trifoliate orange (*Poncirus trifoliata*). Plant Cell Rep 23:764-749

Zhang T, Liu Y, Xue L, Xu S, Chen T, Yang T, Zhang L, An L (2006) Molecular cloning and characterization of a novel MAP kinase gene in *Chorispora bungeana*. Plant Physiol Biochem 44:78-84

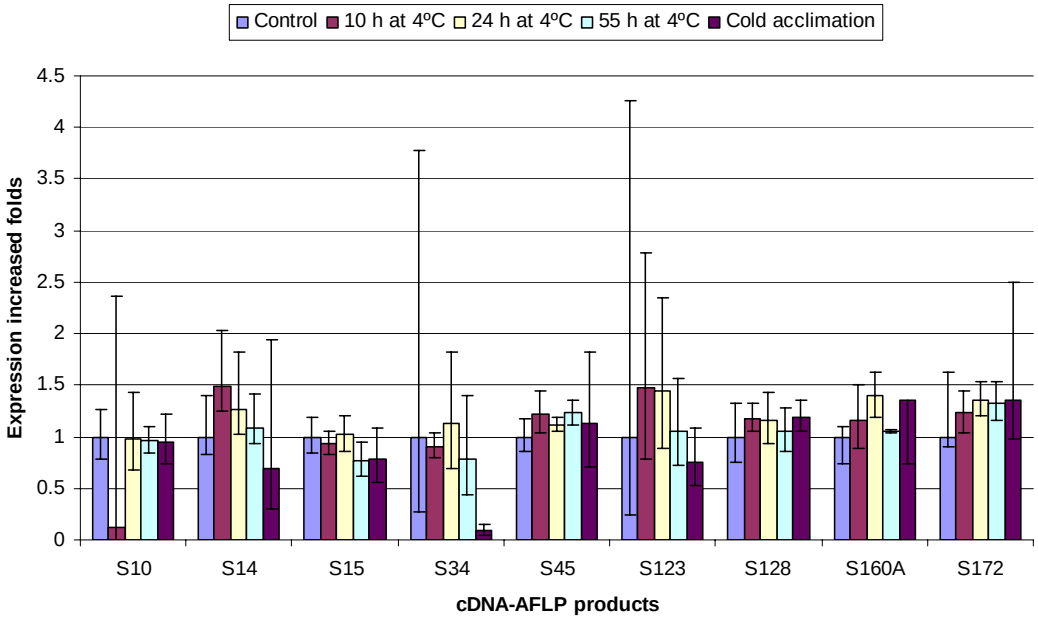
Zhu JK (2001) Plant salt tolerance. Trends Plant Sci 6:66-71

Zhu JK (2002) Salt and drought stress signal transduction in plants. Annu Rev Plant Biol 53:247-273

Zimmermann S, Sentenac H (1999) Plant ion channels: from molecular structures to physiological functions. Curr Opin Plant Biol 2:477-482

APPENDIX

Figure13. Genes that were not found to be up or down regulated in cold treated *Poncirus* in this study



During data analysis, any gene whose treatment expression is more than 2 fold or less than 0.5 fold of its expression in control were considered to be significant up or down regulated during cold treatments. The genes listed here are those that didn't show obvious change in gene expression. Control mRNA of each comparison is considered as 1 and each treatment pair were normalized by comparing the ΔΔCt to control. The values are the means of three independent experiments $\pm$ SE.

Table 3. Oligonucleotide primer sequences of 9 cDNA-AFLP products confirmed not to be changed during cold treatments from *Poncirus trifoliata*

cDNA-AFLP products	Forward Primer (5'-3')	Reverse Primer (5'-3')
S10	AAGAGGAAGATGAAGCAGG	GCACAGAGACTGAGTCTTGATT
S14	AACTTTGCGGACAGCCTT	GAATCAGCATTGCACAGCAT
S15	ACTCATGCATTCTGGAGCTG	TCTCAGCAACACAGCCTG
S34	GGAAGAGTAAGGGGCTTCAAGTGAG	GGGACGTTGAGCTTGCCA
S45	ATAAACCCCCAACAGTTCGT	GGTCAGTATAGCTGCAACATCA
S123	TACTGACAGTCGCCGAGT	CTCTTGAAGTCTGCGTGAA
S128	CATCACCTGCCAACTCAA	ATCAGGATGGATATGCTCC
S160A	TTTGTATCTGGATTGGCACC	GCTGATGGGAAAACCACT
S172	GTGGTTTAGAAATCTTAGATGGT	CTCAGGTAAAGCATATTCAATG

Table 4. 9 Isolated cDNA-AFLP products, percentage similarities to known proteins by BLASTx search in NCBI

cDNA-AFLP product	Plant protein	Function	Similarity
S10	Ubiquitin-protein ligase/ zinc ion binding	Protein digestion	86%
S14	RabGAP/TBC domain-containing protein-like	Signal transduction	98%
S15	Putative purple acid phosphatase	oxidative	86%
S34	Oxidoreductase	Multifunction	83%
S45	Ubiquitin-conjugating enzyme	Protein digestion	75%
S123	Glutathione dependent dehydroascorbate reductase precursor	oxidative	90%
S128	Beta-amylase	starch degradation	79%
S160A	Carbohydrate transporter/ organic anion transporter/ sugar porter	osmotic	89%
S172	DNA-3-methyladenine glycosylase (MAG)	DNA repair	90%