ISOLATION OF GENES FROM COLD ACCLIMATED *Poncirus trifoliata* AND C*itrus unshiu*

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ISOLATION OF GENES FROM COLD ACCLIMATED *Poncirus trifoliata* AND C*itrus unshiu*

Cankui Zhang

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ISOLATION OF GENES FROM COLD ACCLIMATED
Poncirus trifoliata AND C*itrus unshiu*

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VITA

Cankui Zhang, son of Tianqi Zhang and Yueping Song, was born on November 18, 1971 in Luoyang city, Henan Province, the Peoples' Republic of China. He graduated from Mengjin No. 1 High School in 1990. He entered Henan University in September 1990 and earned a Bachelor of Science degree in Biology in July 1994. He entered Wuhan Institute of Botany, the Chinese Academy of Sciences in September 1994, and earned a Master of Science degree in Botany in July 1997. From 1997 to 2000, he worked as a research assistant professor in Wuhan Institute of Botany, the Chinese Academy of Sciences. In August 2000, he entered the University of Arkansas at Fayetteville to pursue a Doctor of Philosophy degree in Entomology. In August 2001, he transferred to Auburn University to pursue a Doctor of Philosophy degree in Horticulture. He married Ping Lang in November 1998. They have a son, Jingzhe Zhang, and a daughter, Jingran (zoe) Zhang.

DISSERTATION ABSTRACT

ISOLATION OF GENES FROM COLD ACCLIMATED *Poncirus trifoliata* AND *Citrus unshiu*

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Many adverse environmental conditions can affect the productivity and distribution of plant species. Low temperature is one of the most common stresses faced by many plants. Many plants are able to increase their freezing tolerance ability in response to a period of low, non-freezing temperature, a process called cold acclimation. During this process, various physiological and biochemical changes occurred in order to impart cold tolerance capacity to plants. Cell metabolom and proteome changes due to the adjustment at transcription level have been postulated and investigated in the process of cold acclimation in many plants, especially in *Arabidopsis*. Many genes have been isolated in response to low temperature stress in many herbaceous plants, while very limited information is available for woody plants, including economically important fruit crops, such as citrus.

Citrus species are cold-tender evergreen plants with a tropical and subtropical origin. *Citrus unshiu* (one kind of Satsuma) is one of the most cold hardy commercial

Citrus species which can tolerate temperature as low as –9 to –10 degree C. *Poncirus trifoliata*, with a maximum freeze tolerance ability of –30 degree C, is a deciduous relative of Citrus that is often used as rootstock to enhance freeze tolerance of the other Citrus species. In order to gain an understanding of the molecular mechanisms of these two species under cold temperature and compare their responses to low temperature, mRNA differential display and cDNA amplified fragment length polymophism (cDNA-AFLP) were used to identify the cold responsive genes under a gradually declined temperature regime. With relative quantitative RT-PCR, genes as follows were identified as differentially expressed in *P. trifoliata* and *C. unshiu*: betaine/proline transporter, water channel protein, aldo-keto reductase, early light induced protein, nitrate transporter, tetratricopeptide-repeat protein, F-box protein, ribosomal protein L15, chlorophyll a/b binding protein, photosystem II OEC 23, carbonic anhydrase, tumor related protein, pyrrolidone-carboxylate peptidase, β-galactosidase, translation initiaton factor eIF1, cytochrome C, trigger factor type chaperone family protein, polyprotein, leucine-rich repeat transmembrane protein kinase/receptor-like protein kinase, PAZ/PIWI domain containing protein, 40S ribosomal protein S23, amino acid permease 6, miraculin-like protein 2, 14-3-3 d-2 protein, nucleoside diphosphate kinase III, regulator of chromosome condensation like protein and glutathione S-transferase C-terminal domain containing protein. Osmotic adjustment, photo-oxidative protection and photosynthesis adaptation were suggested to be the main mechanisms for these plants to acclimate to low temperatures. The full length sequences of carbonic anhydrase, proline transporter and nitrate transporter have been obtained. The detailed characterization of these genes are ongoing.

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I. LITERATURE REVIEW

1.1 Cold acclimation

Plants are sessile organisms. Adverse environmental conditions such as salinity, drought, nutritional deficiency and extreme temperature (high and low), can potentially cause significant losses of plant productivity and constrain the distribution of some major crops. Low temperature is one of the most common stresses faced by many plants. The ability of plants to respond to cold temperatures is an important factor in their ecological and evolutionary dynamics. Plants vary greatly in their ability to survive freezing temperatures. Many tropical and subtropical plant species cannot tolerate low temperatures and their biogeographical range is limited. In contrast, temperate herbaceous species or perennials can survive freezing temperatures ranging from –5°C to –30°C or even lower (Thomashow 1998). Temperate plants have evolved mechanisms by which they can increase their ability to withstand such low freezing temperatures after a period of pre-exposure under low but non-freezing temperatures, a process called cold acclimation (Levitt 1980). During the cold acclimation process, cold hardening develops, providing the plant with a tolerance to low temperatures that would be lethal to an unhardened plant (Howarth and Ougham 1993). For some perennial plants such as grape, the combination of declining day length and decreasing temperatures in autumn are important factors influencing acclimation and cold hardiness (Wample et al. 2000), which is thought to be a gradual process.

1.2 Freezing injuries and tolerance mechanisms

1.2.1. Membranes

When exposed to low temperatures, plant cells encounter three main metabolic constraints: changes in the spatial organization of biological membranes, a retardation of biochemical and chemical reactions, and alterations in the availability and status of water (Vézina et al. 1997). Many studies have indicated that membrane disruption is the most severe injury caused by freezing (Levitt 1980; Steponkus 1984). Ice generally forms in intercellular spaces, mainly due to lower solute concentration of extracellular fluid and a higher freezing point (Thomashow 1999). The lower chemical potential of ice formed extracellularly is a driving force for the movement of water molecules from inside the cell, thus causing symplastic dehydration. Freezing injury is regarded to be a consequence of membrane lesions caused by freeze-induced dehydration (Steponkus 1984), although other factors may also contribute to cellular damage (Thomashow 1999). During thawing, the water potential gradient is reversed, and the cell's cytosol can be rehydrated. This dehydration/rehydration cycle has significant effects on cellular ultrastructure (Hincha and Schmitt 1992). There are three types of damages based on the ways that low temperature caused to cell membrane. "Expansion-induced lysis", a process caused by the osmotic contraction and expansion cycle during freezing and thawing, occurs in non-acclimated plants. "Lamellar-to-hexagonal II" phase transitions is another main injury in non-acclimated plants, a process involving the fusion of many cellular membranes. "Fracture-jump lesions" occur due to the low water potential and severe dehydration due to extreme freezing (Steponkus et al. 1993). In addition membrane lesions, many loosely bound, peripheral membrane proteins are released

during freezing under injurious conditions (Volger et al. 1978; Hincha and Schmitt 1985), thus causing negative effects to cellular metabolism.

1.2.2. Oxidative stress

At low temperatures, the balance between photosynthetic energy conversion and consumption is disrupted. When temperature declined, the ability of the plant to utilize captured light energy is substantially reduced, while the energy absorption and electron flow are less retarded, thus resulting in extra energy which can react with O_2 to produce $^1O_2$, hydrogen peroxide, and superoxide radicals O_2^- (Asada 1994; Prasad et al. 1994; Fadzillah et al. 1996; O'Kane et al. 1996; Foyer 1997), which damage cellular components (Elstner 1991; McKersie 1991). Acclimation to low temperature may be partly related to an enhanced antioxidant system that would prevent the accumulation of these reactive oxygen species (ROS) (Prasad 1996). Different plant species have evolved different mechanisms to cope with low temperature related oxidative stress. Under natural conditions, low temperature induced accumulation of glutathione (GSH) has been observed in spruce (*Picea abies* L.) and white pine (*Pinus strobes* L.) during winter time (Esterbauer and Grill 1978; Anderson et al. 1992). Under experimental conditions, GSH was also found to be induced in response to low temperature in soybean, squash and wheat (Vierheller and Smith 1990; Wang 1995; Kocsy et al. 2000). It also has been found that chilling-tolerant plants increase endogenous polyamine (PA) levels in response to chilling to a much greater extent than chilling-sensitive ones (Guye et al. 1986; Lee 1997; Shen et al. 2000). In a chilling sensitive cucumber cultivar, pretreatment of leaves with PA alleviated chilling injuries, while in a chilling tolerant cucumber cultivar, pretreatment of leaves with PA synthesis inhibitor enhanced chilling injuries.

The primary function of PA is probably inhibition of chill-induced activation of microsomal NADPH oxidase and consequential ROS generation (Shen et al. 2000). Because of their polycationic nature at physiological pH, PA can bind strongly to negatively charged cellular components such as nucleic acids, proteins, and phospholipids. Interactions of PAs with membrane phospholipids may stabilize membranes under conditions of stress (Smith 1985; Roberts et al. 1986). In addition to the above responses to deal with the oxidative stress under low temperature, plant cells can synthesize lipid-soluble antioxidants (α-tocopherol and β-carotene), water-soluble reductants (ascorbate and glutathione) and enzymes such as superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11), and glutathione reductase (GR; EC 1.6.4.2) (Zhang et al. 1995). These enzymes have important roles in detoxification of ROS. For instance, SOD can catalyze dismutation of superoxide to hydrogen peroxide and molecular oxygen (Bowler et al. 1992). Transgenic plants overexpressing SOD have exhibited enhanced tolerance to oxidative stress (Bowler et al. 1991; Perl et al. 1993). Sen Gupta et al. (1993) transformed a Cu-Zn-SOD from pea to tobacco and found that the photosynthetic rates of transgenic tobacco plants were approximately 20% higher than nontransformed plants when subjected to chilling temperature with moderate light intensity. Mn-SOD cDNA was transformed into alfalfa, resulting in an increase in freezing tolerance. Mn-SOD functions to minimize the deleterious accumulation of active oxygen and the enhanced SOD enzymatic activity may enable the injury to be contained within a few cells (McKersie et al. 1993).

1.3. Expression changes of genes and proteins in response to low temperature

1.3.1. Lipid composition

The lipid composition of the plasma membrane has to be changed under low temperatures in order to maintain membrane integrity and membrane associated protein with proper function. In rye seedlings, the ratio of unsaturated phosphatidylcholine and phophatidylethanolamine increased upon cold acclimation (Lynch and Steponkus 1987). Increased unsaturation of the membrane lipids in response to low temperature was also observed in cyanobacteria (Sato and Murata 1980). The increased unsaturation of membrane lipids might be due to the up-regulation of desaturase genes under cold stress (Los et al. 1993; Sakamoto and Bryant 1997). In addition to the abundant information devoted to lipid membrane composition in response to low temperature, research also indicated that the membranes of individual cellular compartments could experience a similar modification upon temperature stress. In *Brassica napus*, the content of the unsaturated fatty acid, linolenic acid (18:3) in the endoplasmic reticulum (ER) membrane increased about two fold upon exposure to low temperature, but the oleate desaturase and linoleate desaturase genes were only transiently regulated in response to low temperature. This suggests that enhanced translation or enhanced enzymatic activities are involved in ER lipid composition changes in response to cold (Tasseva et al. 2004). In green alga, the percentage of membrane polyunsaturated fatty acids was higher in winter time than in summer time. This increase in the degree of unsaturation might be important for green alga in order to decrease their threshold of low temperature survival and acclimate net photosynthesis and dark respiration rates to winter temperatures (Terrados and Lopez-

Jimenez 1996). The most direct evidence on lipid composition changes in the cold acclimation process was reported by Steponkus et al. (1988) in rye.

1.3.2. Heat shock proteins (HSPs)

HSPs are induced not only by high temperature but also by other stresses such as cold, drought, or salinity (Anderson et al. 1994; Coca et al. 1994). HSPs are part of a group of proteins induced by environmental stress either to protect the plant from damage or to help repair damage caused by the stress. There is very likely some overlap in function among the different stress proteins since one stress can induce protection against another (Lurie et al. 1994; Leshem and Kuiper 1996). In tomato, the expression of two heat shock-induced proteins, tom66 and tom111, was found to be first decreased then increased in response to low temperature. A clear correlation between the induction of these two genes and low temperature tolerance of the tissue was found (Sabehat et al. 1998). A similar mechanism of heat-shock-induced tolerance to chilling injury may exist in all plant organs. Protection against chilling injury by high-temperature treatment has been found in mung bean hypocotyls (Collins et al. 1995) and cucumber cotyledons and seeds (Lafuente et al. 1991; Jennings and Saltveit 1994). In these studies loss of protection was correlated with the disappearance of HSPs from the tissue (Lafuente et al. 1991; Collins et al. 1995). Presently, 11 heat shock protein families are known in plants (Nover and Scharf 1997). The function of heat shock protein is related to their molecular chaperon characteristics. Heat shock proteins can stabilize native proteins (Anderson and Guy 1995), refold stress-denatured proteins (Gaitanaris et al. 1990) and prevent aggregation of denatured proteins (Ellis and van der Vies 1991). Many members of the hsp70 family were found to be up-regulated in response to both heat shock and low

temperature in tomato and spinach. The timing of the increased up-regulation of heat shock proteins is consistent with the hypothesis that protein biogenesis or protein conformation are adversely affected by low temperatures (Li et al. 1999), indicating a possible role associated with heat shock proteins.

1.3.3. Antifreeze proteins (AFPs)

Antifreeze proteins are found in a wide range of overwintering plants and located in the apoplast where they inhibit the growth and recrystallization of ice that forms in intercellular spaces. During cold acclimation, the accumulations of AFPs is correlated with increased freeze tolerance in rye, wheat, and barley (Marentes et al. 1993; Antikainen and Griffith 1997). When AFPs bind to the surface of ice, they adsorb irreversibly onto a specific plane of the crystal lattice (Knight et al. 1991; Knight et al. 1995). Specific activities of AFPs include changes in ice crystal shape, thermal hysteresis, or inhibition of ice recrystallization, which varies among overwintering organisms and could be related to their freezing strategies (Griffith and Yaish 2004). Ice crystals grown in water or in a solution of substances that do not interact with ice are disc-shaped. By contrast, most AFPs bind to the prism face of ice, creating hexagonally shaped crystals (Griffith and Yaish 2004). At high concentrations, AFPs depress the freezing temperature of a solution noncolligatively without affect the melting temperature, a process known as thermal hysteresis (DeVries 1986). In bittersweet nightshade, a thermal hysteresis protein gene (STHP-64) was isolated and found to contain 10 consecutive 13-mer repeats at its C-terminus, a common feature of animal antifreeze proteins. Northern blots demonstrated that the STHP-64 transcript was not present in leaves until November and December, suggesting that cold acclimation induces

STHP-64 production (Huang and Duman 2002). At low concentrations, AFPs can inhibit the recrystallization of ice (Knight et al. 1984), which is the growth of larger ice crystals at the expense of smaller ice crystals. Larger ice crystals increase the possibility of physical damage within frozen plant tissues (Griffith et al. 1997). Two AFPs (*TaIRI-1* and *TaIRI-2*) were identified to be up-regulated in wheat in response to low temperature. TaIRI-1 protein was found to be able to inhibit the growth of ice crystals (Tremblay et al. 2005). Amino-terminal sequence comparisons, immuno-cross-reactions, and enzymatic activity analysis of AFPs in winter rye showed similarity between AFPs and pathogenesis-related (PR) proteins (Hon et al. 1995). Because the AFPs retain their enzymatic activities, they may also have antifungal properties that are important in disease resistance, particularly against low-temperature pathogens such as snow molds (Ergon et al. 1998; Hiilovaara-Teijo et al. 1999).

1.3.4. Dehydrins

Dehydrin, also known as a group-2 late embryogenesis abundant (LEA) protein, is one of several ubiquitous water-stress-responsive proteins in plants (Ingram and Bartels 1996). Dehydrins are highly hydrophilic, glycine-rich and boiling-stable proteins. They possess repeated sequence structures containing lysine-rich motifs which are speculated to form putative amphiphilic α helices. Dyhydrins are thought to bind to membranes and cellular proteins by hydrophobic interactions, and thus protect the functions of intracellular molecules by preventing their coagulation during symplastic dehydration (Close 1997). These proteins may accumulate during drought, salinization, late stages of seed development, freezing and in response to exogenous ABA (Close et al. 1993; Welin et al. 1994; Campbell and Close 1997; Close 1997). Many dehydrins have been isolated and

characterized not only in herbaceous plants but also in several woody species including *Prunus persica* (Arora and Wisniewski 1994), birch (Rinne et al. 1999), blueberry (Muthalif and Rowland 1994) and citrus (Hara et al. 2001). CuCOR19, a dehydrin isolated from *Citrus unshiu*, showed cryoprotective activities for lactate dehydrogenase (LDH) and catalase. The protective role of the CuCOR19 protein is thought to be related to its random coil structure, which can form a layer cohesive to other structures. Low temperatures can denature the association of some enzymes irreversibly. The binding of the coil structure in the CuCOR19 could prevent the disassociation of the enzymes. In addition to its cryoprotective role, CuCOR19 can bind water molecules which might be necessary to maintain water in the cell during symplastic dehydration (Hara et al. 2001). PCA60 is a dehydrin isolated from winter bark tissue of peach (Wisniewski et al. 1999). The extracted protein has been shown to preserve the *in vitro* enzymatic activity of lactate dehydrogenase during repeated freeze-thaw cycles. Another surprising characteristic of this dehydrin is its antifreeze activity shown by its involvement with ice crystal morphology and thermal hysteresis, the first time that antifreeze activity was demonstrated for a dehydrin (Wisniewski et al. 1999). In addition to its water-binding, macromolecule-stabilizing, and cryoprotective activities of dehydrins, at least one report showed that dehydrins may be involved in free radical scavenging (Hara et al. 2003). The overexpressed citrus dehydrin *CuCOR19* in tobacco causes an increase in the cold tolerance ability of transformed plants. Further analysis correlated this cold tolerance enhancement to the antioxidative activity imposed by the expressed protein (Hara et al. 2003). Another notable work about dehydrin was done by Puhakainen et al. (2004) in *Arabidopsis*. Chimeric double constructs with dehydrins *RAB18* and *COR47* or *LTI29*

and *LTI30* were introduced into *Arabidopsis* and resulted in increased accumulation of related dehydrin proteins. Compared to control plants, transgenic plants have more tolerance to cold temperature, and this increased resistance to cold temperature was speculated to be partly due to their protective role of cell membranes (Puhakainen et al. 2004).

1.3.5. Compatible solutes

Under environmental stresses including salinity, drought and low temperature conditions, plants can accumulate certain organic metabolites of low molecular weight known collectively as compatible solutes (Bohnert et al. 1995). Compatible solutes mainly include polyhydroxylated sugar alcohols, amino acids and their derivatives, tertiary sulphonium compounds and quaternary ammonium compounds (Bohnert and Jensen 1996). The main functions of compatible solutes involve membrane protection (Rudolph and Crowe 1985), cryoprotection of proteins (Carpenter and Crowe 1988), maintenance of osmotic potential (Yancey et al. 1982), and scavenging of free radicals (Smirnoff and Cumbes 1989). The levels of proline and sucrose increase in *Arabidopsis* (McKown et al. 1996), and spinach (Guy et al. 1992) during cold acclimation. The increase in synthesis of proline and sucrose might be associated with freezing tolerance enhancement (Stitt and Hurry 2002). The stress tolerance ability of *Arabidopsis* was increased by the suppression of proline dehydrogenase, an enzyme to catalyze the proline degradation, with its antisense cDNA (Nanjo et al. 1999), further demonstrating the protective role of proline during cold acclimation. In comparison of three wheat cultivars with different cold tolerance ability, the accumulation of sugars, amino acids and glycine betaine were the highest in the most cold tolerant cultivar, the lowest in the most cold sensitive cultivar

(Kamata and Uemura 2004). In *Arabidopsis*, levels of endogenous glycine betaine in the leaves were found to be greatly induced in response to cold acclimation, water stress and exogenous ABA application, indicating the involvement of glycine betaine in cold acclimation and water stress (Xing and Rajashekar 2001). The relationship between sugar content and freezing tolerance was investigated in cabbage. Concentration of sucrose, glucose, and fructose gradually increased during cold acclimation. However, the induced freezing tolerance was lost after only 1 day of deacclimation at control temperatures, and this change was associated with a large reduction in sugar content (Sasaki et al. 1996). Genetic engineering to increase levels of some compatible solutes, such as mannitol and proline, has been proven to be a promising approach to increase the ability of plants to tolerate environmental stress (Hayashi and Murata 1998).

1.4. Regulation of the cold acclimation response

1.4.1. The ICE/CBF/DREB1 regulatory pathway

Several cold responsive genes have been identified and characterized in *Arabidopsis*. These genes were designated as COR (cold-responsive or regulated), LTI (low temperature induced), KIN (cold inducible), RD (responsive to desiccation), and ERD (early dehydration inducible) (Thomashow 1994). Studies have shown that the promoter of some of these genes has a core sequence “CCGAC” designated as CRT (C-repeat) or DRE (dehydration responsive element) (Baker et al. 1994; Yamaguchi-Shinozaki and Shinozaki 1994). A family of transcription factors in *Arabidopsis* known either as C-repeat-binding factor (CBF1, CBF2, and CBF3) (Stockinger et al. 1997; Gilmour et al. 1998) or dehydration-responsive element-binding factor (DREB1B, DREB1C, and DREB1A)(Liu et al. 1998) have been identified. These transcription factors can bind to

the *cis* element and activate the transcription of the down stream cold responsive or dehydration responsive genes. Transgenic overexpression of the *CBF/DREB* gene in other plants turns on the expression of down stream cold responsive genes without cold acclimation and can increase the low temperature resistance ability (Jaglo-Ottosen et al. 1998; Liu et al. 1998). Since *CBF* transcripts begin accumulating within 15 min of plants' exposure to cold, Gilmour et al. (1998) proposed that another transcription factor constitutively exists, which can bind to the *cis* element of *CBF* gene under cold stimulus. Gilmour et al. (1998) named the unknown activator(s) "ICE" (inducer of CBF expression) protein(s). This *ICE* gene has been identified by another group and was found to encode a MYC-like bHLH (helix loop helix) transcription factor. ICE is a positive regulator of *CBF3* and has a critical role in cold acclimation (Chinnusamy et al. 2003). *CBF3* promoter includes five putative MYC recognition sequences, while *CBF1* and *CBF2* only includes one (Shinwari et al. 1998). The *ice1* mutation abolishes *CBF3* expression, and reduces the expression of CBF-target genes in the cold (Chinnusamy et al. 2003). Although ICE has a strong effect on the regulation of *CBF3*, it only slightly affects the induction of *CBF2* and *CBF1*. Actually, the expression of *CBF2* is enhanced in the *ice1* mutant after 6 and 12 h of cold treatment (Chinnusamy et al. 2003). The potential negative regulation of the *CBF* transcription factor genes may be important for ensuring that their expression is transient and tightly controlled (Novillo et al. 2004). Novillo et al. (2004) found a *CBF2*/*DREB1C Arabidopsis* mutant with a higher capacity to tolerate freezing than wild type plants before and after cold acclimation and higher tolerance to dehydration and salt stress. The increased cold tolerance was due to the enhanced expression of the *CBF1*/*DREB1B* and *CBF3*/*DREB1A* and the downstream

regulated *COR* genes. These results indicate that *CBF2/DREB1C* negatively regulates *CBF1*/*DREB1B* and *CBF3*/*DREB1A*, ensuring that their expression is transient and tightly controlled (Novillo et al. 2004).

In addition to the CBF/DREB signaling pathway in plant cold acclimation, there is evidence that multiple pathways may be involved in the acclimation process. Analysis of the *eskimo1* (*esk1*) mutant of *Arabidopsis* revealed that considerable freezing tolerance can be achieved in the absence of *COR* gene expression (without CBF/DREB signaling activation) (Xin and Browse 1998). Studies from the analysis of *sensitive-to-freezing* (*sfr*) mutants that are not able to fully acclimate also support the theory that CBF/DREB is not the only signaling pathway for plant cold acclimation. *sfr* mutants do not fully cold acclimated, and only retain about 50% of the wild-type capacity for cold acclimation, which means that the *sfr* mutation blocks some signaling pathway. Therefore, each mutant is still able to partially cold acclimate through signaling pathways that are not disrupted (Warren et al. 1996).

1.4.2. ABA-dependent and ABA-independent pathway

Expression of genes in response to low temperature is thought to be regulated by both ABA-dependent and ABA-independent signalling pathways. In *Arabidopsis*, ABA synthesis mutants, *aba1* or *abi*, are less freezing tolerant than wild-type plants (Heino et al. 1990; Gilmour and Thomashow 1991). The expression of many genes has been reported to be associated with ABA (Shinozaki and Yamaguchi-Shinozaki 2000; Ramanulu and Bartels 2002). The analysis of the promoter region of these ABA responsive genes reveals that many of the genes contain a fragment of (C/T)ACGTGGC consensus sequence, generally known as “ABA-responsive element (ABRE)” (Busk and

Pages 1998; Rock 2000). bZIP transcription factors are likely to bind to these elements in cold regulated genes and activate gene expression (Thomashow 1999). Contrary to the involvement of ABA in the changes of expression of specific genes, some genes are not dependent on ABA regulation. In the *aba1* mutant, cold induced expression of *COR78*, *COR47*, and *COR6.6* is normal (Gilmour and Thomashow 1991). Thus, Gilmour and Thomashow proposed that cold regulated expression of these genes occurs through an ABA-independent pathway. The important CBF/DREB transcription factor and its regulon are also not changed in response to exogenous ABA, further pointing to the existence of the ABA-independent regulation pathway under cold acclimation (Yamaguchi-Shinozaki and Shinozaki 1994). In moss *Physcomitrella patens*, ABA treated cells had slender chloroplasts and reduced amount of starch grains. When frozen to -4 °C, freezing injury-associated ultrastructural changes such as formation of a particulate domains and fracture-jump lesions were frequently observed in the plasma membrane of non-treated protonema cells but not ABA-treated cells. The ABA treated cells also had higher accumulation of free soluble sugars (Nagao et al. 2005). In another work from the same group, surprisingly, determination of ABA content by GC–MS revealed that low-temperature treatment did not increase accumulation of ABA, but that levels of freezing tolerance increased. These results suggest that *P. patens* does not require increases in levels of ABA for cold acclimation and possesses an ABA-independent cold-signaling pathway leading to the development of freezing tolerance (Minami et al. 2005). Whether and how ABA relates to the activation of expression of cold responsive genes is still unsolved. A clear conclusion about ABA's function in environmental stresses beyond its traditional function in plant development and growth

requires a total understanding of the cold acclimation mechanisms and whether ABA plays a critical role in regulating the activity of the mechanisms (Thomashow 1999).

1.4.3. Participation of Calcium in cold acclimation

The cytosolic concentration of the intracellular second messenger calcium (Ca^{2+}) can be transiently increased in response to low temperature in many plant species, including *Arabidopsis* (Lewis et al. 1997), tobacco (Knight et al. 1991), and tomato (Sebastiani et al. 1999). Elevation of calcium levels is mainly due to an influx of Ca^{2+} from external sources (Monroy and Dhindsa 1995), but evidence has shown that some calcium can be released from vacuole (Knight et al. 1996). In *Arabidopsis*, the calcium chelater (EGTA) blocks plasma-membrane calcium channels (La Gd3+), thus inhibiting cold acclimation as well as *kin* gene expression. Ruthenium red, an inhibitor of calcium release from intracellular storages, partially inhibited *kin* gene expression and development of freezing tolerance (Tähtiharju et al. 1997). In *Populus tomentosa* cuttings, treatment with $CaCl_2$ at the time of freezing acclimation enhanced the effect of freezing acclimation, but this enhancement was abolished by Ca^{2+} chelator EGTA, Ca^{2+} channel inhibitor $LaCl_3$ or CaM (calmodulin) antagonist chlorpromazine, indicating that the calcium-calmodulin messenger system was involved in the course of freezing resistance development (Lin et al. 2004).

1.5. Biology of citrus

Commercial citrus species and related genera are primarily evergreen species of subtropical and tropical origins belonging to the family Rutaceae (Swingle and Reece 1967). *Citrus* and related genera within the true citrus group are diploid (2n=18). Some triploids, tetraploids and hexaploids exist but generally occur at low percentages in the

population (Raghuvanshi 1968). The progenitor of the Satsuma group of mandarins (*C. unshiu* Marc.) probably originated in China but was transported to Japan (Saunt 1990). There are over 100 cultivars of Satsuma, which differ from each other primarily in time of maturity, fruit shape and fruit quality (Saunt 1990). Satsuma foliage and wood are the most freeze hardy of all commercially grown citrus cultivars, withstanding minimum wood temperatures of –9°C when fully acclimated (Yelenosky 1985). *Poncirus trifoliata* is a small tree with trifoliate, deciduous leaves, and can tolerate temperature as low as –30°C when fully acclimated (Yelenosky 1985). It is native to northern China, where low temperatures are common during winter months. *P. trifoliata* trees are used as rootstocks in commercial citrus production (Davis and Albrigo 1994). Selection of new citrus cultivars has occurred for thousands of years in ancient China. Citrus breeding is difficult, slow and time consuming. Most citrus and related species are very heterozygous and few important traits show single gene inheritance patterns (Davis and Albrigo 1994). Most citrus breeding programs have relied on traditional methods for developing new cultivars or rootstocks based on controlled hand crosses and selection of superior types from literally thousands of seedlings in the field (Hearn 1985). The entire process may take over 20 years. Therefore, many citrus breeders are searching for new methods to shorten this costly and time-consuming process. Molecular biotechnology is one of the most promising potential methods to do this. Much effort has been made to transfer disease resistance genes into citrus plants, thus far without much success due to the complexity of the techniques, and the specificity of citrus species (Ghorbel et al. 2000; Febres et al. 2003), and lack of knowledge about the resistance or acclimation mechanisms of woody plants at the molecular level. Thus, a better elucidation of the

responses at the molecular level of citrus plants under environmental stress will be a prerequisite for further transgenic effort. So far, a few genes have been identified and characterized in citrus or related species. Most of the identified genes belong to the dehydrin family, and were found to serve as free radical scavengers to protect cell membranes (Hara et al. 2003; Hara et al. 2004) and as cryoprotectants (Sanchez-Ballesta et al. 2004). Yet, most of these experiments were conducted following cold shock treatment in which plants were moved from warm temperature (25°C) to low temperature (4°C) directly, a process that does not occur in nature. A more objective transcriptome profiling analysis under a gradually declined temperature regime that mimics the natural decline in air temperature is necessary to fully understand the mechanisms of citrus plant in response to low temperature.

1.6. Techniques used to isolate genes

Many gene profiling techniques can be used to study the changes of transcripts in response to environmental stresses, such as Differential Display Reverse Transcription PCR (DDRT-PCR), cDNA Amplified Fragment Length Polymorphism (cDNA-AFLP), Serial Analysis of Gene Expression (SAGE), subtractive hybridization, DNA-chip, and cDNA microarray. Differential display and cDNA-AFLP have been widely used to identify genes whose expression levels have been altered under different environmental conditions because both techniques are straight forward and especially, don't need previous genomic information of the species of interest (Liang and Pardee 1992; Vos et al. 1995). Due to the lack of genomic information for *P. trifoliata* and *C. unshiu*, both techniques were utilized for the current research.

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II. Cold acclimation induced genes of trifoliate orange (*Poncirus trifoliata*) [1]

ABSTRACT

Commercial citrus varieties are sensitive to low temperature. *Poncirus trifoliata* is a close relative of *Citrus* species, and has been widely used as a cold hardy rootstock for citrus production in low temperature environments. mRNA differential display-polymerase chain reaction (DDRT-PCR) and quantitative relative RT-PCR were used to study gene expression of *P. trifoliata* under a gradual cold-acclimation temperature regime. Eight up-regulated cDNA fragments were isolated and sequenced. These fragments showed high similarities at the amino acid level to the following genes with known functions: betaine/proline transporter, water channel protein, aldo-keto reductase, early light induced protein, nitrate transporter, tetratricopeptide-repeat protein, F-box protein and ribosomal protein L15. These cold acclimation up-regulated genes in *P. trifoliata* are also regulated by osmotic and photo-oxidative signals in other plants.

Keywords Cold acclimation · differential display · gene expression · *Poncirus trifoliata* · quantitative relative RT-PCR

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[1] The nucleotide sequences reported in this paper have been submitted to Genbank under accession numbers of CN779663 (P1), CN779664 (P2), CN779665 (P3), CN779666 (P4), CN779667 (P5), CN779668 (P6), CN779669 (P7) and CN779670 (P8).

Introduction

Environmental factors such as freezing, drought, and high salt affect the growth, productivity and distribution of plants (Kasuga et al. 1999). Plants have evolved a wide variety of mechanisms that allow them to thrive in hostile environments, albeit their ability to survive in adverse environments varies greatly (Garwe et al. 2003). Distribution of many temperate fruit crops is restricted by low temperature conditions (Owens et al. 2002). Low temperatures can cause extracellular ice formation and damage to cell membrane (Gilmour et al. 1988), transporters and receptor proteins (Hazel 1995).

Many plants can acclimate to freezing by pre-exposure to low but nonfreezing temperatures (Fowler and Thomashow 2002). The cold acclimation process requires synthesis of new proteins (Tseng and Li 1991), alterations in lipid and carbohydrate composition, the accumulation of compatible osmolytes such as proline, betaine, and soluble sugars (Bohnert et al. 1995; Lynch and Steponkus 1987; Thomashow 1994), and activation of ion channels (Knight et al. 1996). Several plant genes induced by low temperature have been identified in alfalfa (Wolfraim et al. 1993), *Arabidopsis thaliana* (Gilmour et al. 1992) and strawberry (Yubero-Serrano et al. 2003). A low-temperature responsive dehydrin-like protein, wcor410, is involved in cryoprotection of the plasma membrane against freezing or dehydration stress (Danyluk et al. 1994). Several cold regulated (COR) proteins from different plants are involved in membrane stability (Thomashow 1998). COR proteins are hydrophilic and have been suggested to protect cells from low temperature, water deficit, and high salt stress conditions (Artus et al. 1996; Steponkus et al. 1998). C-repeat binding factor 1 (CBF1) is the DNA binding protein isolated from *A. thaliana*. The binding of this transcription factor to the C-

repeat/dehydration response element (*CRT/DRE*-motif) can activate the co-ordinate expression of several *COR* genes (Jaglo-Ottosen et al. 1998). Expression of *CBF1* in transgenic plants resulted in enhanced freezing tolerance (Jaglo-Ottosen et al. 1998; Liu et al. 1998).

Citrus is an economically important crop throughout the world, and its productivity is seriously affected by low temperature (Porat et al. 2002). Traditional breeding methods of major crops for improving freezing tolerance have had limited success in commercial *Citrus* species (Yelenosky, 1985). An alternative approach is to identify and characterize important cold induced genes and introduce these genes into susceptible crops to enhance freezing tolerance (Owens et al. 2002). *P. trifoliata* is a popular rootstock used in the citrus industry to impart greater cold tolerance to the scion (Yelenosky 1985). Dehydrins have been identified from *Citrus* and related species under low temperature stress (Cai et al. 1995; Hara et al. 1999; Porat et al. 2002). Treatment approaches for low temperature studies in plants are usually "cold shock" in which plants are moved from control temperature to 4 ˚C directly, versus "cold acclimation" which more closely resembles the natural conditions in the Southeastern United States. In our experiment, one year old plants were cold acclimated using a gradually declining day/night temperature regime as outlined by Yelenosky (1979) and Nesbitt et al. (2002) for citrus. This study was undertaken to elucidate the genetic basis of cold acclimation in *P. trifoliata* in order to develop a transgenic approach toward enhancing cold hardiness in *Citrus* cultivars.

Materials and methods

Plant growth conditions

One year old *P. trifoliata* plants were grown for 6 weeks in a growth chamber with a 12 h light period at 400 µmol $m^{-2}s^{-1}$ intensity. The regimen for 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. Plants were uniformly watered every day.

RNA extraction and mRNA differential display

Expanded leaves at the end of the second and the fifth week 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 10X DNase buffer and 4µl DNase I (2U/µl) 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 14000g and transferred to a new tube. The concentration of isolated RNA was measured using an Eppendorf Biophotometer (Brinkmann Instruments, NY). The quality of RNA was checked using formaldehyde-agarose gel electrophoresis. RNA from the end of second week was used as unacclimated control and RNA from the end of fifth week as treatment. mRNA differential display was performed using RNAimage kits and 144 primer pairs according to the protocol supplied with this kit (GenHunter, TN). 0.2µg of RNA was reverse transcribed in a 20µl reaction mixture at 42˚C for 60 min with M-MuLV reverse transcriptase (GenHunter). Amplification of cDNA fragments was

performed in a 20µl reaction mixture containing 2µl of the reverse transcribed cDNA, 0.2µM arbitrary primer (GenHunter), 0.2µM anchored oligo (dT)-primers (H-T11M, where M=A, G, C), 2µM of each dNTP, 10mM Tris-Cl, pH8.4, 50mM KCl, 1.5mM $MgCl_2$, 0.001% gelatin, 1µl α-[S^{35}] dATP (1000Ci/mmole) and 1 U *Taq* DNA polymerase (Qiagen, CA). The PCR program consisted of 40 cycles: 30 s at 94˚C, 2 min at 40˚C, 1 min at 72˚C; and a final 5 min elongation step at 72˚C. Amplified PCR products were separated on a 6% denaturing PAGE gel. Gel was transferred to a filter paper (Whatman, England) and dried at 80˚C for 1 hour in a gel dryer (BioRad, CA). PCR products on filter paper were exposed to BioMax Kodak film covered with two intensifying screens for 24 to 72 hours in a –80˚C freezer. The film was developed and the differentially expressed bands between control and treatment were excised from the filter paper according to the pattern on the film. The PCR products were extracted according to the GenHunter protocol, and reamplified using the original primer pair.

Cloning and sequence analysis of DNA fragments

Selected amplified DNA fragments were ligated directly into a PCR-Trap Vector (GenHunter) and transformed into competent *Escherichia coli (*GenHunter). Ten colonies were selected for each transformation event. 20µl lysis PCR was carried out according to GenHunter protocol. 10µl of lysis PCR products were separated on 1.5% agarose gel. Remaining 10µl of PCR product containing correct size inserts were digested with 0.2µl of *Taq*I (10U/µl), 10mM Tris-HCl, pH8.4, 50mM KCl, 1.5mM $MgCl_2$, 0.001% gelatin, and incubated at 65˚C overnight and products analyzed in agarose gel. Differentially restricted DNA fragments were used for plasmid isolation. Only fragments larger than

250bp were selected and sequenced in both directions using *Rseq* and *Lseq* primers (GenHunter) with ABI 3100 DNA sequencer (AU Genomics Lab). Analysis of nucleotide sequence of selected fragments was carried out using National Center for Biotechnology Information BLASTx search tool.

Quantitative Relative Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Quantitative relative RT-PCR was used to confirm the differential expression of DNA fragments isolated from control and cold acclimated plants. 2.5μg total RNA was reverse transcribed with 0.5mM dNTP, 5mM oligo(dT)-primers, 10mM Tris-HCl, pH 8.3, 50 mM KCl, 15mM $MgCl_2$, 1μl RNase inhibitor, and 100U M-MuLV-RT (Ambion). The mixture (20μl total reaction volume) was incubated at 42˚C for 1 hour. 1μl RT reaction was amplified in a 25μl solution with 10mM Tris-HCl, pH8.4, 50mM KCl, 1.5mM $MgCl_2$, 2.5mM dNTPs, 0.3μM actin primer, 0.6μM actin competitor, 0.5mM of each gene specific primers (Ambion) and *Taq* polymerase (Qiagen). PCR cycle program was as follows: 30 s at 94˚C, 30 s at 55˚C to 60˚C and 30 s at 72˚C for 30 to 35 cycles. Specific oligonucleotide primers were designed for each DD product for RT-PCR. The optimal PCR annealing temperatures and the cycle numbers are shown in **Table 1**. 10μl of the amplification products were separated using 1.8 % agarose gel electrophoresis and stained with ethidium bromide. The stained gel was used for quantitation of each band using a BioRad photo documentation system. A constitutively expressed actin gene was used as an internal standard in each reaction.

Results and discussion

Differential display polymerase chain reaction (DDRT-PCR) was used to study the responses of *P. trifoliata* to low temperature acclimation. A total of 144 different primer combinations were used and about 15,000 cDNA fragments were analyzed after autoradiography. Two hundred fifty five putative differentially expressed DNA fragments were cloned, sequenced and analyzed. DDRT-PCR can be a powerful technique to study differentially expressed genes. However, this technique can produce some spurious results (Marty et al. 1997). For example, we found two or three unique DNA fragments in some single bands. Because of this comigration of several separate fragments, ten clones obtained from each band on the gel were subsequently examined. These colonies were randomly selected from each transformation, and the sequence of the cloned insert in each of the colonies was determined.

Of the 255 putative differentially expressed DNA fragments, 14 (8 up regulated and 6 down regulated) fragments were confirmed to be differentially expressed in treated plants as determined by quantitative relative RT-PCR and show significant homology to known genes in the GenBank database using the BLASTx search utility. The nucleotide sequence of selected clones has been deposited in GenBank and their accession numbers along with the results of the BLASTx search are shown in **Table 2**. The subsequent results presented in this paper summarize our characterization of the 8 up-regulated transcripts.

Quantitative RT-PCR has been used extensively to study gene expression because of its high sensitivity and reproducibility (Taylor and Harrier 2003). Three different sets of independently isolated RNA were used to confirm the differential display results with

RT-PCR. Only reproducible differences between control and treatment in all three sets were considered positive. Agarose gel electrophoresis pattern of eight up-regulated differential display (DD) genes by RT-PCR with actin as an internal standard and a histogram representing relative quantity of each of the amplified bands on the gel are presented in **Fig 1**.

The inferred amino acid sequence of P1 shows 71% similarity to a betaine/proline transporter from *Avicennia marina.* Plants synthesize compatible osmolytes such as proline, glycine betaine and sugar under stress (Ishitani et al. 1993). In bacteria and animals, transporters have been identified that mediate the transport of either proline and betaine or GABA and betaine (Csonka 1989; Yamauchi et al. 1992). Increased level of proline in phloem sap during water stress in alfalfa suggests a role of transporter (Guerrier 1996). *HvProT* in barley encodes for proline transporter under salinity (Ueda et al. 2001). It is not only important to synthesize solutes, but its transport within cell may be equally important in stress abatement. These reports indicate a role for transport of proline and/or betaine in adaptation of plant cells to low water potential.

The inferred amino acid sequence of P2 shows 94% similarity to an *A. thaliana* water channel protein (aquaporin). Aquaporins are 25-30 kDa polypeptides with six putative membrane-spanning segments (Verkman et al. 1996), which facilitate movement of water across membrane by forming water-specific pores (Schäffner 1998). Ice plant aquaporin is down-regulated under low water potential (Yamada et al. 1995) and the down regulation of aquaporin might allow for cellular water conservation (Johansson et al. 1998). In contrast, the *rd*28 gene in *A. thaliana* is up-regulated under water stress

(Yamaguchi-Shinozaki K et al. 1992). Because of their role in water transport, it is logical to assume an important role for aquaporins in abatement of stress in plants.

The inferred amino acid sequences of P3 and P4 share 85% similarity with an aldo-keto reductase protein and 85% similarity with an early light inducible protein (ELIP) in *A. thaliana*, respectively. Water deficiency and low temperature can impose oxidative stress and increase production of reactive oxygen species (*ROS*) including the free radical superoxide (Moran et al. 1994; Powles et al. 1983). Free radicals can further react with cellular constituents such as lipids. Both aldo-keto reductase and early light inducible protein can detoxify these toxic chemicals (Burczynski et al. 2001; Hutin et al. 2003).

The inferred amino acid sequence of P5 demonstrates 55% similarity with an *Oryza sativa* nitrate transporter, NRT1-5. Nitrate transporters have been studied in species such as *A. thaliana* (Tsay et al. 1993) and ice plant (Popova et al. 2003). AtNRT1.1 is a low-affinity nitrate transporter in *A. thaliana* (Tsay et al. 1993) and is associated with stomatal opening and drought susceptibility (Guo et al. 2003), while McNRT1 nitrate transporter from ice plant is dependent on salt (Popova et al. 2003). Recent research on nitrate assimilation in *Synechococcus* sp. PCC7002 indicates that low temperature can inhibit the uptake of nitrate (Sakamoto and Bryant, 1998). The strongly up regulated expression of this gene in *Poncirus* during low temperature might indicate an active transport of nitrate which could be helpful to acclimate plants under adverse environment.

The inferred amino acid sequence of P6 shows 54% similarity with a putative tetratricopeptide-repeat containing protein (TPR) from *O. sativa*. The repeat motif serves

as protein-protein interaction module found in a number of functionally different proteins which facilitates specific interactions with a partner protein (Das et al. 1998). Soybean *gmsti* with a TPR containing motif is highly expressed on exposure to elevated temperature and plays a role in mediating the heat shock response with HSP70 proteins (Torres et al. 1995). Low temperature exposures are equally relevant for protein denaturation and folding. Hence, the role of TPR containing proteins in cold tolerance may be important.

The inferred amino acid sequences of P7 and P8 show 78% similarity to the F-Box protein family in *A. thaliana* and 94% similarity to ribosomal protein L15 in *O. sativa*, respectively. There is very limited information about these two genes related to low temperature, salinity and drought stresses. F-box proteins include protein-protein interaction domains that confer substrate specificity for ubiquitination. Ubiquitin-dependent proteolysis has been reported to selectively degrade certain proteins (Callis and Vierstra, 2000). Ribosomal protein plays a major role in controlling cell growth, division and development (Barakat et al. 2001). A low temperature induction of F-box protein and ribosomal L15 suggests the adjustment of cell metabolism at translation level in which certain proteins are selectively degraded and synthesized according to the need of cell.

P. trifoliata is an extensively used rootstock in the citrus industry. *Citrus* species are cold-tender evergreen plants with a tropical and subtropical origin and their capacity to survive freezing temperature does not approach that of other woody plants (Webber et al. 1967). *P. trifoliata* is a deciduous relative of *Citrus* and is a cold hardy plant (Yelenosky 1985). In our experiment, we have used a treatment condition that mimics

natural declines of temperature in the Southeast U.S. Effects of gradually declining temperature (acclimation) and sudden decrease in temperature (shock) were compared in a preliminary experiment using differential display. RNA was isolated from acclimated leaves and cold shocked leaves. RT-PCR using similar set of primers and RNA from control, cold shocked and cold acclimated plants was performed, and products were analyzed using denaturing PAGE in adjacent lanes (data not shown). As anticipated, DNA fragment representing expression patterns of cold shock and cold acclimation were different. We observed differential pattern of expression under cold acclimation and cold shock.

Salinity, drought, and cold are known to cause osmotic/dehydration stress in plants. In this study, three of the induced genes encountered during cold acclimation, have also been reported as important for the maintenance of osmotic balance in plant cells under salt or drought stress. This reinforces the notion that low temperature acclimation of plants involves some mechanisms required for growth and survival under low water potential. Plants can synthesize osmotic chemicals during salinity and drought stresses. Many plant transgenic researches have been focusing on the synthesis of these osmoprotectants but the stress tolerance abilities of transgenic plants have not been increased very efficiently even with overexpressed synthesis enzyme. It's reasonable to infer that plant might not have enough transporters to transport these newly synthesized chemicals to the function place in plant. The comparison of the cold tolerance ability between a transgenic plant with a synthesis enzyme such as P5CS and a transgenic plant with both the synthesis enzyme and transporter genes may shed more light on this aspect.

Besides the dehydration damage due to stress, another very serious problem for the plant cell is stress induced oxidative damage. As discussed, low temperature and high light induce oxidative stress in plants. Stresses like drought, salt, ozone and UV irradiation similarly can induce oxidative stress, indicating a common damage factor in plants exposed to environmental stress (Desikan et al. 2001). Plants possess capabilities to cope with oxidative stress through the use of ROS-scavenging enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and low molecular weight antioxidants including ascorbic acid, glutathione and phenolic compounds (Asada 1999). Three of the eight up regulated genes isolated have functions related to ROS scavenging. The damages related to certain stresses were reduced in transgenic tobacco with alfalfa aldose–aldehyde reductase (*MsALR*) and transgenic *Arabidopsis* with early light inducible protein indicate the participation of these proteins to adapt plants under stress conditions (Bartels 2001; Hutin et al. 2003). The correlation of the accumulation of ELIP and the tolerance to chilling-induced photooxidation in barley shed more light on this aspect (Bei-Paraskevopoulou and Kloppstech 1999). Transformation of model plants with the *Poncirus* early light inducible protein gene and aldo-keto reductase and the correlated concentration of the reactive chemicals and their derivatives should provide more understanding about the role of these genes in plants.

Low temperature is a serious problem for many plants, especially horticulturally important woody plants such as *Citrus*, but our understanding of the mechanisms of low temperature abatement at the molecular level is limited. Cold acclimation resulted in the up-regulation of several genes involved in maintenance of osmotic balance, scavenging of reactive oxygen species and photo-oxidative protection of plants. Recognition of

specific genes and their relevance in the process of stress abatement will allow their use in improvement of stress tolerance ability in *Citrus*.

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Table 1. Oligonucleotide primer sequences, PCR conditions and cycle numbers for the confirmation of differential displayed products with quantitative RT-PCR

DD product	Forward Primer (5'-3')	Reverse Primer (5'-3')	PCR annealing temperature	Cycle numbers
P1	AAGCTTCCTGCAATCAGTCAT	GGCAATTGGAGAATACACTTGA	55°C	33
P2	GCCATCCCATTCAAGTCCAA	GAATCGGCCATGAACATGTA	57°C	32
P3	CGTATGGCACAGTAATGGGTG	ACTCCAAGCAAATCCTTCCC	58°C	30
P4	GGTTGCGTCCCTGGTTCCTT	AGATTTATTTACAGTAGGAT	55°C	30
P5	ATAAAGGGAGTGGTGACGGC	AACACAGTATCCGGATTCCG	57°C	35
P6	TGATGGAAGTCAAGCAACTGG	CCACAAACATTTGGGAAGCA	58°C	33
P7	ATGAATGTGGCAGACTTGAGTC	AGCTGAGCATGTGTCATTGC	55°C	35
P8	ACGACCTTCAAGAAGGGCA	CCGCACATTATTATCACCTTC	54°C	35

Table 2. Isolated DD products, accession number and percentage similarity to known proteins by BLASTx search in NCBI

DD product	Accession Number	Length (bp)	Plant protein	similarity	Function
P1	CN779663	529	betaine/proline transporter	71%	Osmotic, Oxidative
P2	CN779664	259	water channel protein	94%	Osmotic
P3	CN779665	508	aldo-keto reductase	85%	Oxidative
P4	CN779666	313	early light inducible protein	85%	Oxidative
P5	CN779667	389	nitrate transporter NRT1	55%	Osmotic
P6	CN779668	645	tetratricopeptide repeat containing protein (TPR)	54%	Multifunction
P7	CN779669	444	F-Box protein family	78%	Multifunction
P8	CN779670	464	ribosomal protein L15	94%	Protein synthesis

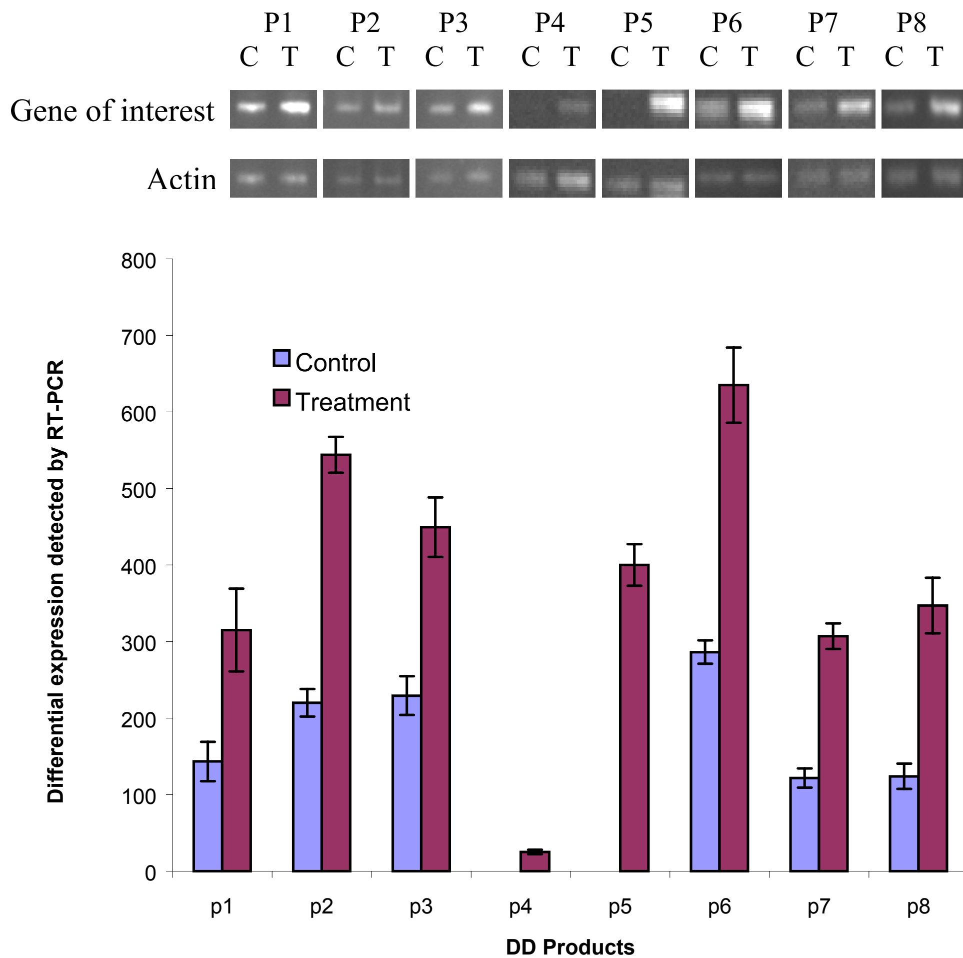

Fig. 1 (Top): Confirmation of differential expression of 8 DD products using relative quantitative RT-PCR with RNA from control and cold acclimated leaves. The cDNAs were synthesized from total RNAs isolated from control leaves (end of second week) and cold acclimation leaves (end of fifth week). Actin mRNA was used as an internal control. Specific RT-PCR primer pair information for all DD products is listed in table 1. (Bottom): A histogram showing the relative abundance of 8 DD products between control and cold acclimated plants. The intensities in each control and cold acclimation pair were normalized by setting the intensity of actin gene to 100. The values are the means of three independent experiments $\pm$ SE.

III. Down-regulated gene expression of cold acclimated *Poncirus trifoliata* [1]

ABSTRACT

Citrus sp. are important commercial fruit crops throughout the world that are occasionally devastated by subfreezing temperatures. *Poncirus trifoliata* (maximum freeze tolerance of -26˚C) is a close relative of commercial *Citrus* sp. (maximum freeze tolerance of -10˚C) that has been used in breeding programs to develop more cold-hardy genotypes and as a rootstock to enhance freeze tolerance of the scion. Species with greater freeze tolerance vary in gene expression during cold acclimation temperatures. mRNA differential display (DDRT-PCR) and quantitative relative RT-PCR were used to study the down regulation of gene expression in intact *P. trifoliata* exposed to a gradual cold acclimation regime to enhance our understanding of the mechanism that makes this species so freeze tolerant. Six down regulated genes were isolated and sequenced. These down regulated genes showed high homology to the following known function genes: chlorophyll a/b binding protein, photosystem II OEC 23, carbonic anhydrase, tumor related protein, pyrrolidone-carboxylate peptidase and β-galactosidase. Photoprotection and the global control of expression of genes related to photosynthesis appear to be important mechanisms for cold acclimation of *P. trifoliata*.

Key words: Differential display, down regulated genes, *Poncirus trifoliata*, cold acclimation and quantitative relative RT-PCR

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[1] The nucleotide sequences reported in this paper have been submitted in Genbank under accession numbers of CN807022 (PD1), CN807023 (PD2), CN807024 (PD3), CN807025 (PD4), CN807026 (PD5), CN807027 (PD6).

INTRODUCTION

The geographical distribution of plants is a function of their maximum freeze tolerance. Freezing temperatures can cause extracellular ice formation, which lowers apoplastic water potential, dehydrates the symplast, and destabilizes cellular membranes (Steponkus 1984). Plants have evolved a wide variety of mechanisms that allow them to thrive in hostile environments even though their ability to survive in adverse environments varies greatly among species (Garwe et al. 2003). Many plants acquire freeze tolerance when subjected to low, nonfreezing temperatures, a phenomenon known as cold acclimation (Levitt 1980; Guy 1990).

Acquisition of freezing tolerance requires physiological and biochemical changes including changes induced by altered gene expression (Guy et al. 1985; Thomashow 1998). A number of up-regulated genes have been identified in several plant species exposed to cold acclimating temperatures including alfalfa (Wolfraim et al. 1993), *Arabidopsis thaliana* (Gilmour et al. 1992) and barley (Sutton et al. 1992). Considerably less research has been devoted to down-regulation of genes during cold acclimation, even though studies have shown suppression of metabolic activity may be an important component of plant adaptation to low temperature. *Arabidopsis* transcriptome-profiling resulted in 306 genes identified as cold responsive, 88 of which were found to be down-regulated in response to low temperature (Fowler and Thomashow 2002).

Several *Citrus* sp. are important fruit crops throughout the world, but productivity is occasionally devastated by freezing temperatures in freeze-prone regions (Yelenosky 1985). *Poncirus trifoliata* (L.) Raf is a close relative of *Citrus* sp. in the Rutaceae family. *P. trifoliata* is used as a rootstock for commercial *Citrus* sp. to impart greater

cold tolerance to the scion, and has been crossed with commercial *Citrus* sp. in breeding programs to produce more cold-hardy germplasm, although these crosses have not led to genotypes with commercially acceptable fruit (Barrett 1978, 1982; Yelenosky 1985; Yelenosky et al. 1993). *P. trifoliata* is freeze tolerant to -26˚C (Spiegel-Roy and Goldschmidt 1996), whereas commercially grown Citrus sp. are freeze tolerant to at most -10˚C (Yelenosky 1985), a level that is very similar to that of many herbaceous species such as arabidopsis, which also has a maximum freeze tolerance of -10˚C (Gilmour et al. 1988). Gene expression during cold acclimation of woody plants that exhibit such low freeze tolerance is not well understood. Because *P. trifoliata* is used in breeding programs and as a rootstock for *Citrus* sp., there must be significant genetic overlap among these species, yet differences in freeze tolerance likely result from significant differences in gene expression during cold acclimation. We are studying gene expression of *P. trifoliata* during cold acclimation to identify genes that are involved in its greater freeze tolerance.

Transcriptome profiling of plants to environmental stresses can be studied via several different techniques, including differential display reverse transcription PCR (DDRT-PCR), serial analysis of gene expression (SAGE), subtractive hybridization, DNA-chip and cDNA microarray (Donson et al. 2002). DDRT-PCR, the technique we used in the present study, is a useful technique for gene expression studies in non-model organisms because detailed genomic information of the organism is not necessary and it is technically simple (Liang and Pardee 1992). In our study, we used a gradual cold acclimation regime that simulated natural declines in temperature during autumn in subtropical regions. Other research on *Citrus* sp. and other related species utilized a

much faster cold treatment that was equivalent to cold shock (Cai et al. 1995; Hara et al. 1999; Porat et al. 2002). Our research approach compliments theirs in helping identify differences in gene expression under different temperature acclimation regimes. We examined gene regulation by DDRT-PCR during a gradual process of cold acclimation in *P. trifoliata*. The genes identified by comparing DNA sequences provide insight into the process of cold acclimation and freeze tolerance of citrus.

MATERIALS AND METHODS

Plant culture

One-year-old *P. trifoliata* (L.) Raf. plants were grown for 5 weeks in a growth chamber with a 12 h light period at 400 μmol $m^{-2}s^{-1}$ intensity. The regimen for temperature decline was as follows: 32˚C day/21˚C night for 14 d; 27˚C day/16˚C night for 7 d; 24˚C day/13˚C night for 7 d and 18˚C day/7˚C night for 7 d. These temperature treatments have been shown to increase freezing tolerance of *P. trifoliate* and improve plant response to freezing temperatures (Young and Peynado 1962; Stathakopoulos and Erickson 1966). Plants were uniformly watered every day.

RNA extraction and mRNA differential display

Full expanded leaves were collected at the end of the 2nd and the 5th weeks at the same time of day (10th hour in day time cycle), 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 10X DNase buffer and 4μl DNase I (2U/μl) and incubated for 30 min at 37˚C to digest the

remaining genomic DNA. The resulting preparation treated with DNase inactivation reagent (20% volume) for 2 min, followed by centrifugation for 1 min at 14,000g. The resulting supernatant was transferred to a new tube, and the concentration of isolated RNA was measured using an Eppendorf Biophotometer (Brinkmann Instruments, NY). The quality of RNA was checked using formaldehyde-agarose gel electrophoresis. Total RNA prepared from leaves at the end of the second week of treatment was used as the unacclimated control and RNA from leaves harvested at the end of fifth week of cold acclimation was used as the treatment. mRNA differential display was performed using RNAimage kits and 144 primer pairs according to the protocol supplied with this kit (GenHunter, TN). RNA (0.2μg) was reverse transcribed in a 20μl reaction mixture at 42˚C for 60 min with M-MuLV reverse transcriptase (GenHunter). Amplification of cDNA fragments was performed in a 20μl reaction mixture containing 2μl of the reverse transcribed cDNA, 0.2μM arbitrary primer (GenHunter), 0.2μM anchored oligo (dT)-primers (H-T11M, where M=A, G, C), 2μM of each dNTP, 10mM Tris-Cl, pH8.4, 50mM KCl, 1.5mM $MgCl_2$, 0.001% gelatin, 1μl α-[S^{35}] dATP (1000Ci/mmole) and 1 U *Taq* DNA polymerase (Qiagen, CA). The PCR program consisted of 40 cycles: 30 s at 94˚C, 2 min at 40˚C, 1 min at 72˚C; and a final 5 min elongation step at 72˚C. Amplified PCR products were separated on a 6% denaturing PAGE gel. The gel was transferred to a filter paper (Whatman, England) and dried at 80˚C for 1 hour in a gel dryer (BioRad, CA). PCR products were detected by exposing the filter paper to BioMax Kodak film for 24 to 72 hours in a –80˚C freezer. The film was developed and the differentially expressed bands between control and treatment were excised from the filter paper

according to the pattern on the film. The PCR products were extracted according to the GenHunter protocol, and reamplified using the original primer pair.

Cloning and sequence analysis of DNA fragments

Selected, amplified DNA fragments were ligated directly into a PCR-Trap Vector (GenHunter) and transformed into competent *Escherichia coli* (GenHunter). Ten colonies were selected for each transformation event, and a 20µl PCR reaction was carried out on the lysed colonies according to the GenHunter protocol. Half of this PCR product was separated on a 1.5% agarose gel, and the remaining 10µl of PCR product (from colonies containing correct size inserts) were digested with 0.2µl of *Taq*I (10U/µl), 10mM Tris-HCl, pH8.4, 50mM KCl, 1.5mM $MgCl_2$, 0.001% gelatin, and incubated at 65˚C overnight and products analyzed on agarose gel. Differentially restricted DNA fragments were used for plasmid isolation. Only fragments larger than 250bp were selected and sequenced in both directions using *Rseq* and *Lseq* primers (GenHunter) with ABI 3100 DNA sequencer (AU Genomics Lab). Analysis of nucleotide sequence of selected fragments was carried out using National Center for Biotechnology Information BLASTx search tool.

Quantitative Relative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Quantitative relative RT-PCR was used to confirm the differential expression of DNA fragments isolated from control and cold acclimated plants. Total RNA (2.5µg) was reverse transcribed with 0.5mM dNTP, 5mM oligo(dT)-primers, 10mM Tris-HCl, pH 8.3, 50 mM KCl, 15mM $MgCl_2$, 1µl RNase inhibitor, and 100U M-MuLV-RT reverse

transcriptase (Ambion). The mixture (20µl total reaction volume) was incubated at 42˚C for 1 hour. 1 µl of the reverse transcriptase reaction was amplified in a 25µl reaction with 10mM Tris-HCl, pH8.4, 50mM KCl, 1.5mM $MgCl_2$, 2.5mM dNTPs, 0.3µM actin primer, 0.6µM actin competitor, 0.5mM of each gene specific primer and *Taq* polymerase (Qiagen). The PCR cycle program was as follows: 30 s at 94˚C, 30 s at 55˚C to 60˚C and 30 s at 72˚C for 30 to 35 cycles. Specific oligonucleotide primers were designed for each DD product for RT-PCR. The optimal PCR annealing temperatures and the cycle numbers are shown in **Table 1**. 10µl of the amplification products were separated using 1.8 % agarose gel electrophoresis and stained with ethidium bromide. The stained gel was used for quantitation of each band using a BioRad photo documentation system. A constitutively expressed actin gene was used as an internal standard in each reaction.

RESULTS AND DISCUSSION

Citrus sp. are some of the major fruit crops of the world, and are classified as cold-tender evergreens with tropical and subtropical origins (Webber et al. 1967). The distribution of citrus is mainly affected by temperature, especially subfreezing temperatures (Young 1961). Its capacity to survive freezing temperature does not approach that of northern woody plants, yet commercial citrus crops are grown in freeze-prone areas throughout the world. During the late 1970s to mid 1980s, freeze events were especially devastating and caused extensive damage to the citrus industry in the Southeastern US (Yelenosky 1985). *P. trifoliata*, a very cold-hardy relative of *Citrus* sp., has been extensively used in breeding programs to develop more cold-hardy genotypes and as a rootstock to impart a degree of cold tolerance to the citrus scion (Yelenosky 1985). Little research has been

conducted on this cold-hardy plant to gain an understanding of the molecular processes associated with cold acclimation.

mRNA Differential Display

In this study, DDRT-PCR was used to study low temperature acclimation in *P. trifoliata*. A total of 144 different primer combinations were used. Six down regulated genes were identified and pattern of expression confirmed by quantitative relative RT-PCR using comparisons between control (non-acclimated) and acclimated plants. As an example, the banding pattern of cDNA fragments amplified by the combination of one primer pair is shown in **Fig. 1**. To reduce the possibility of false positives associated with DDRT-PCR (Marty et al. 1997), each primer pair was used to amplify two different sets of RNA isolated from different *Poncirus* plants. RT-PCR products were applied in adjacent lanes on a 6% denatured PAGE gel. Differentially regulated cDNA fragments from both sets of RNA were extracted, cloned and sequenced. All sequenced fragments showed an 11A or 11 T tail (corresponding to the reverse transcription primer HT11M in the RNAimage kit from Genhunter, TN, USA) and the right arbitrary primer sequence, indicating successful cloning of the re-amplified cDNA isolated from filter paper. The results of sequence homology are shown in **Table 2**.

Quantitative RT-PCR has been used extensively to study gene expression because of its high sensitivity and reproducibility (Okamoto et al. 2003; Pires-Alves et al. 2003; Taylor and Harrier 2003). RNA isolated from three different groups of plants was used to confirm results of the differential display using RT-PCR. Only consistent differences between control and treatment in the three sets of RNA were considered significantly

different. **Fig. 2** (top) shows the agarose gel electrophoresis pattern of six down-regulated genes confirmed by RT-PCR using actin sequence as an internal standard. **Fig. 2** (bottom) shows histogram of RT-PCR results.

Chlorophyll a/b binding protein (LHC)

The inferred amino acid sequence of PD1 shows 96% similarity to *Chlorophyll a/b binding protein (LHC)* in cucumber seedlings. Light-harvesting chlorophyll *a/b* (LHC) proteins are major components of light-harvesting antennae of photo system II (PSII) in higher plants. LHC levels are known to change by the intensity of irradiance (Anderson et al. 1995). *LHC* RNA level in *Arabidopsis* was suppressed when plants were exposed to low temperature (Strand et al. 1997). In *Ammopiptanthus mongolicus*, a drought tolerant plant, expression of *LHC* and other photosynthetic genes were reduced under water stress suggesting reduction in energy requirement under water stress condition (Zhang et al. 2002). In maize (Hao et al. 1999) and salt meadow reed, *Phragmites communis* [*P. australis*] (Wang et al. 1998), *LHC II* gene expression was markedly reduced during osmotic stress suggesting that the down-regulation of photosynthesis related components might help with the dissipation of excess excitation energy. Plants respond to stress by slowing down the metabolic process, thus lowering the need for energy generated by photosynthesis. If photosynthetic energy levels are not changed accordingly, damaging active oxygen species could be produced.

Photo system II OEC 23

The inferred amino acid sequence of PD2 shows 91% similarity to *Photo system II OEC 23* in *Arabidopsis*. Photo system II (PSII) is a multi-subunit pigment-protein complex that catalyzes the splitting of water and releases molecular oxygen in the biochemical pathway of photosynthesis. The 23kDa protein is an extrinsic protein of PSII and part of the structure of oxygen-evolving complex in higher plants (Michael and Peter 1997). Murota et al. (1994) found almost complete dissociation of the 23kDa protein from isolated PSII, inhibition of photochemical reactions, and damage of the oxygen-evolving complex in tobacco grown under high concentration of NaCl.

To date most research on *OEC 23* has focused mainly on the dissociation and reassociation of the OEC. Our study provides the first evidence that changes in the transcriptional level of this component of the photosynthetic apparatus during cold acclimation. Because PSII drives a strong oxidizing reaction that is responsible for splitting water that generates oxygen, down regulation of *OEC23* may result in partial loss of function of PSII. This in turn might correlate with lower demand for energy minimizing the production of potentially damaging reactants under the stress situation.

Carbonic anhydrase

The inferred amino acid sequence of PD3 shows 82% similarity to carbonic anhydrase (CA) in mechanically wounded tobacco plants. Carbonic anhydrase is a zinc-containing enzyme which catalyses the reversible hydration of CO_2 to produce bicarbonate (Larsson et al. 1997). Known CAs can be grouped into α-CA, β-CA, and γ-CA types and plants appear to contain all three types (Hewett-Emmett and Tashian 1996). CA was proposed

to expedite diffusion of CO_2 into the chloroplast acting as a partner with Rubisco in CO_2 fixation (Graham et al. 1984), and to play a role in the buffering capacity of the chloroplast stroma by enhancing the rates of the dehydration/hydration reactions (Mayeau and Coleman 1991). In addition to reversibly converting CO_2 to bicarbonate, CA in tobacco also appears to have antioxidant activity as a plant defense response to biotic stress (Slaymaker et al. 2002). The authors further suggested that oxidative stress protection and the CA enzymatic activity are independent functions of plant CA. It has also been reported that wounding can cause changes in the expression of CA transcripts, indicating a new function related to maintenance of plant cellular homeostasis (Hara et al. 2000).

The *CA* found in this experiment was strongly expressed in control plants, but was undetectable in cold acclimated *Poncirus* plants. Pea *CA* transcripts were reported to be light-regulated (Mayeau and Coleman 1991). Our plant samples were collected at the same time during the day/night cycle, excluding the possibility of a confounding effect of light on CA expression. This is the first report about the potential role of CA in the adaptation of plants to low temperature. Whether this role is related to CA's participation in photosynthesis or the antioxidant process, or both, is not clear at this time.

Tumor related protein

The inferred amino acid sequence of PD4 shows 47% similarity with a tumor related protein expressed in tobacco callus and tumors. The function of this protein in callus and tumors is still not clear (Fujita et al. 1994). PD4 also shows 45% similarity to miraculin from grapefruit and trypsin inhibitor from *Theobroma obovatum,* respectively. Miraculin

is a taste modifier protein which can alters human taste perception (Theerasilp et al. 1989). Both miraculin and trypsin inhibitor are likely involved in biotic stresses. *LeMir,* a putative miraculin gene in tomato, was induced by a root-knot nematode. The encoded protein product might involve in the inhibition of the penetration of microorganisms around tomato root (Brenner et al. 1998). Transgenic tobacco plants expressing a cowpea trypsin inhibitor gene showed enhanced levels of insect resistance to a variety of insect pests (Boulter et al. 1989). Trypsin inhibitor activity was critical for the inhibition of growth and development of cyst nematodes in sugar beet hairy roots expressing the sporamin (trypsin inhibitor) gene (Cai et al. 2003).

Pyrrolidone-carboxylate peptidase

The inferred amino acid sequence of PD5 shows 72% similarity to pyrrolidone-carboxyl peptidase-like protein. The pyrrolidone carboxyl peptidases (Pcps) are a group of exopeptidases responsible for the removal of N-terminal pyroglutamate residues from a variety of peptides and proteins (Doolittle and Armentrout 1968). This enzyme was found to be widely distributed (Szewczuk and Kwiatkowska 1970), but its physiological role is still not clear. Pcp activity in archaea and eubacteria is thought to be involved in detoxification processes and nutrient metabolism (Awadé et al. 1994). In eukaryotic organisms, the enzyme is involved in the processing of biologically active peptides (Griffiths et al. 1980). Down-regulation of this gene in *Poncirus* under cold acclimation might indicate a new role for this gene in the plants.

β-galactosidase

The inferred amino acid sequence of PD6 shows 97% and 86% similarity with Beta-galactosidases from *Citrus sinensis* and *Lycopersicon esculentum*, respectively. ß-galactosidase modifies cell walls during development of many fruit crops such as apple (Yoshioka et al. 1994), kiwifruit (Ross et al. 1993), mango (*Mangifera indica*) (Ali et al. 1995) and Japanese pear (Tateishi et al. 2001). ß-galactosidase can cleave the ß-(1→4)-galactan bond directly and produce galactosyl residues from the cell wall during ripening (Smith et al. 1998), with free galactose (GAL) increasing simultaneously during this process (Gross and Sams 1984). Contrary to our observation of down regulation of ß-galactosidase in response to environmental stress, Kreps *et al*. (2002) also showed that in *Arabidopsis*, a putative β-galactosidase was induced 3.2 to 7.7 folds in response to cold, osmotic, and salt stress. It's unclear whether cell wall modification can help the plant increase its tolerance to stress or if other indirect functions exist for ß-galactosidase. Additionally, the down regulation of this gene may not correlate with changes in the level of translation of the gene, and thus the amount of functional protein may not directly relate to the transcriptional regulation observed in this study. The down-regulation of this gene in transgenic plants using a "reverse genetics" technique might help answer this question.

Research on cold stress in *Arabidopsis* showed induction of genes involved with protein synthesis, metabolism, transport facilitation, and protein targeting, while most of the down-regulated genes were related to photosyntheis (Jung et al. 2003). *Arabidopsis* transcriptome profiling identified 88 down-regulated genes in response to cold suggesting an important role for down regulated genes in cold tolerance. These down regulated

genes represent a wide range of functions, including transcription, signaling, cell wall biogenesis, defense, and photosynthesis (Fowler and Thomashow 2002).

In our study, three of the six down-regulated genes are associated with photosynthesis, one gene with cell wall biogenesis and two genes with biotic defense. Low temperatures have been found to slow the energy-consuming Calvin Cycle enzymes more than the energy-transducing light reactions (Wise 1995). Thus, low temperature stress may result in light stress. The excess of reducing power generated by light under low temperature stress conditions imposes oxidative stress and increases the production of reactive oxygen in photosynthetic organisms (Powles et al. 1983). Down regulation of the *LHC* and *OEC23* genes might reduce photosynthesis efficiency, thus avoiding oxidative damage to plant cells. However, production of reactive oxygen species cannot be totally avoided. Production of photo-oxidative protection chemicals can serve as an alternative mechanism for adaptation of plants to light stress resulting from low temperature stress. High light intensities are known to inhibit the transcription of *LHC* genes while activating the synthesis of the *ELIPs* (a sequence homolog of *LHC*) (Potter and Kloppstech 1993). It has been postulated that ELIPs function as substitutes for the inner LHC proteins, possibly in both PSI and PSII, when plants are grown under potentially harmful light conditions (Krol et al. 1995; Potter and Kloppstech 1993). A recent discovery of the photo-oxidative protection role of ELIPs in *Arabidopsis* sheds more light on this process (Hutin et al. 2003). The inverse relationship between *LHC* genes and *ELIPs* was also found in our study (Zhang *et al*., manuscript in preparation) and further demonstrates that the down regulation of some genes is an active adaptation mechanism of plants under adverse environments. The induction and repression of some

important genes related to photosynthesis are "balanced" in the plant cell and their global expression control can adapt the plant to stressful environments. Biotic stresses also induce physiological changes during plant growth and development. Carbonic anhydrase and tumor-related protein, detected in *Poncirus* after cold acclimation, has been associated with abiotic and biotic stresses in other plants, demonstrating similarity at the molecular level.

Subfreezing temperatures are a serious abiotic stress for perennial woody plants grown in most parts of the world. The mechanisms associated with low temperature adaptation at the molecular level, with respect to the down regulation of globally controlled genes are not very well understood. Several interacting pathways are activated during cold acclimation and ensure the winter survival of plants. A better understanding of these pathways should provide insight into strategies that impart higher levels of freezing tolerance to economically important fruit crops.

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Table 1. Oligonucleotide primer sequences, PCR conditions and cycle numbers for the confirmation of different displayed products with quantitative RT-PCR

DD product	Forward Primer (5'-3')	Reverse Primer (5'-3')	PCR annealing temperature	Cycle numbers
PD1	GCTTACCAGGTGGTCTTGATG	CCCTCGAACTGGTAGTAGCAGTA	55 °C	35
PD2	TGGAGATGAAGGTGGCAAGC	GGGAAAGATGGGAGTAGACAAT	52 °C	33
PD3	AAACTCTGGCACTCAAGGGT	GATTCCCCAACAGGACTCTG	52 °C	35
PD4	GCAACGAACCAACAGTATGG	GCTAGCCCGATCCATTATTT	50 °C	30
PD5	ACAGACATCTCTATCCACTGAAGC	CTCCAAGAGTGTGGCTACAAAT	55 °C	33
PD6	GCTTCTCAACGATGGTATCACG	GAAACCCAGCAAGCCTACAT	52 °C	31

Table 2. Isolated DD products, accession number and percentage similarity to known genes by BLASTx search in NCBI

DD product	Accession Number	Length (bp)	Plant gene	Similarity	Function
PD1	CN807022	468	*Chlorophyll a/b binding protein (LHC)*	96%	photosynthesis
PD2	CN807023	297	*Photosystem II OEC 23*	91%	photosynthesis
PD3	CN807024	331	*Carbonic anhydrase*	82%	Photosynthesis, antioxidant
PD4	CN807025	508	*Tumor related protein*	47%	biotic stress
PD5	CN807026	469	*Pyrrolidone-carboxylate peptidase*	72%	multiple
PD6	CN807027	482	*Beta-galactosidase*	97%	cell wall metabolism

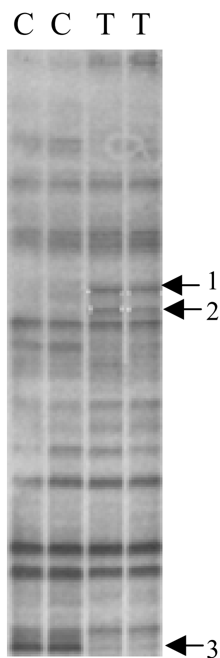

Fig. 1. Example of DDRT-PCR results. cDNAs were amplified from two separately isolated total RNAs by primer combination of $HT_{11}A$ and HAP_1. C_1 and C_2 are cDNAs amplified from the leaves collected at the end of second week (control); T_1 and T_2 are cDNAs amplified from the leaves collected at the end of fifth week (cold acclimated). Arrows 1 and 2 indicate two up regulated cDNA fragments; Arrows 3 indicates one down regulated cDNA fragment.

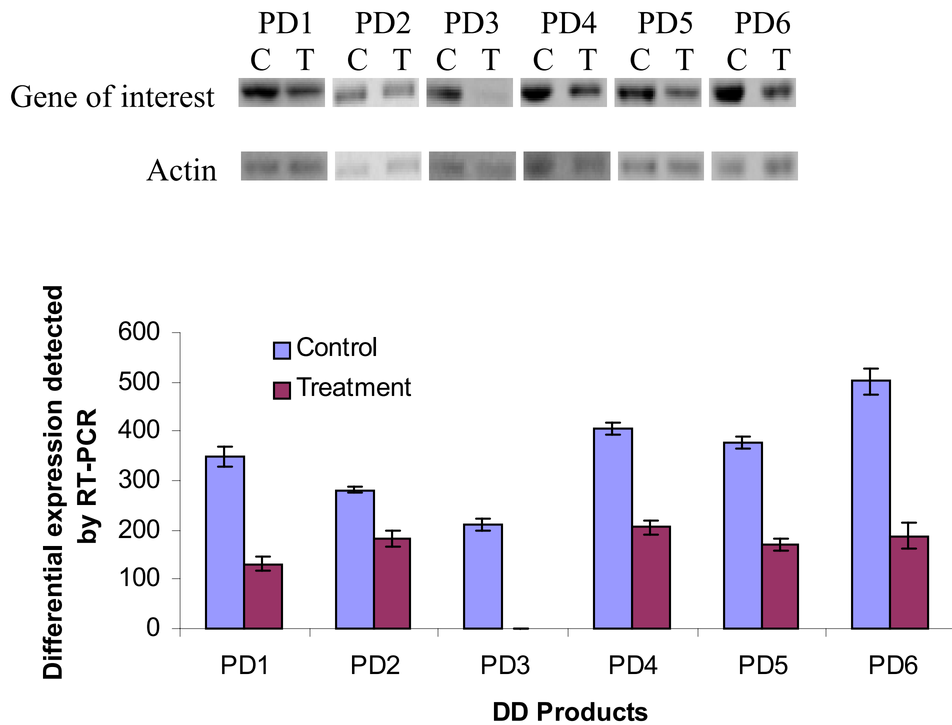

Fig. 2 (top): Confirmation of differential expression of 6 DD products using relative quantitative RT-PCR with RNA from control and cold acclimated leaves. The cDNAs were synthesized from total RNAs isolated from control leaves (end of first week) and cold acclimation leaves (end of fifth week). Actin gene was used as an internal control. Specific RT-PCR primer pair information for all DD products was listed in table 1. (Bottom): A histogram showing the relative abundance of 6 DD products between control and cold acclimated plants. The intensities in each control and cold acclimation pair were normalized by setting the intensity of actin gene to 100. The values are the means of three independent experiments $\pm$ SE.

IV. Identification of cold acclimated genes in leaves of C*itrus unshiu* by mRNA differential display

ABSTRACT

Citrus unshiu is freeze tolerant to –10ºC when fully acclimated after exposure to cold, nonfreezing temperatures. To gain an understanding of its cold tolerance mechanism, mRNA differential display reverse transcriptase polymerase chain reaction (DDRT-PCR) and quantitative relative RT-PCR were used to study gene expression of *C. unshiu* under a gradual cold-acclimation temperature regime. Six up-regulated and two down regulated genes were identified based on their amino acid sequences. The identified proteins encoded by the up-regulated genes were: 14-3-3 protein, 40S ribosomal protein S23, putative 60S ribosomal protein L15, nucleoside diphosphate kinase III protein, regulator of chromosome condensation-like protein, and amino acid permease 6. The proteins encoded by the two down-regulated genes were: miraculin-like protein and beta-galactosidase. Their individual function has been briefly reviewed based on published information. In addition to the findings in this study, we compared the function of cold responsive genes of *Poncirus trifoliata*, a very cold hardy relative of *Citrus* species that is freeze tolerant to –30ºC when fully acclimated, to the function of those genes in this study.

Keywords: Gene expression; Cold acclimation; 14-3-3 protein; nucleoside diphosphate kinase; amino acid permease

Abbreviations: CBF, C repeat-binding proteins; cDNA, DNA complementary to RNA; AFLP, amplified fragment length polymorphism; DDRT-PCR, differential display reverse transcriptase polymerase chain reaction; DNase, deoxyribonuclease; dNTP, deoxyribonucleoside triphosphate; M-MuLV, moloney murine leukemia virus; Oligo(dT), oligodeoxyribonucleotide thymidine; RNase, ribonuclease; RT, reverse transcriptase; SAGE, serial analysis of gene expression

1. Introduction

Crop quality and productivity are negatively affected by biotic and abiotic stresses. Low temperature is one of the most common environmental stresses and can potentially cause severe losses to major economically important plants. Disruption of cell membranes is the primary injury associated with freeze-induced dehydration (Thomashow 1998). Many plants increase their freezing tolerance in response to low, nonfreezing temperatures, a phenomenon known as cold acclimation. Changes at gene expression level were suggested to be associated with this process, and many genes related to cold acclimation have been cloned from several plants, including *Arabidopsis* (Gilmour et al. 1992), *Brassica napus* (Orr et al. 1992), and *Poncirus trifoliata* (Zhang et al. 2004). A number of the C repeat binding proteins (CBF) from *Arabidopsis* have been characterized. Overexpression of the gene encoding the protein was shown to increase tolerance of transformed plants to environmental stresses (Jaglo-Ottosen et al. 1998).

Although efforts have been taken to elucidate the cold adaptation mechanism of herbaceous plants, very limited information is available for woody plants under low temperatures. Citrus sp. are some of the most important fruit crops throughout the world. Yet, some of the most valued citrus crops are grown in relatively high-risk freeze areas (Yelenosky 1985). Research has been conducted in some *Citrus* varieties and relatives, and a few genes have been cloned (Cai et al. 1995; Hara et al. 1999; Sanchez-Ballesta et al. 2003; Zhang et al. 2004; Zhang et al. in press). *Citrus unshiu* is considered as one of the most cold hardy commercial *Citrus* species (Yelenosky 1985), but the changes in gene expression exposed to a gradually declined temperature regime, which mimics the natural temperature changes in the southeastern-U.S., have not been studied.

Transcriptome profiling of plants to environmental stresses can be studied using different techniques, which include differential display reverse transcription PCR (DDRT-PCR), serial analysis of gene expression (SAGE), subtractive hybridization, DNA-chip, and cDNA microarray. mRNA differential display has been widely used to identify genes whose expression levels have been altered under different environmental conditions because of its technical simplicity and lack of requirement for previous genomic information of the species of interest (Liang and Pardee 1992; Carginale et al. 2004). DDRT-PCR was used to clone genes in *C. unshiu* following a gradual cold acclimation regime. The identified genes will provide insights into the adaptation of important fruit crops to low temperature and may lead to strategies to develop transgenic citrus with enhanced levels of cold tolerance.

2. Materials and methods

2.1. Plant growth conditions

One year old *Citrus unshiu* plants were grown for 5 weeks in a growth chamber with a 12 h light period at 400 µmol $m^{-2}s^{-1}$ intensity. The regimen for 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. Plants were uniformly watered every day.

2.2. RNA extraction and mRNA differential display

Fully expanded leaves at the end of the second and fifth weeks 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 10X DNase buffer and 4μl DNase I (2U/μl) 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 14000g and transferred to a new tube. The concentration of isolated RNA was measured using an Eppendorf Biophotometer (Brinkmann Instruments, NY). The quality of RNA was checked using formaldehyde-agarose gel electrophoresis. RNA from the end of second week was used as an unacclimated control and RNA from the end of fifth week as treatment. mRNA differential display was performed using RNAimage kits and 64 primer pairs according to the protocol supplied with this kit (GenHunter, TN). 0.2μg of RNA was reverse transcribed in a 20μl reaction mixture at 42˚C for 60 min with M-MuLV reverse transcriptase (GenHunter). Amplification of cDNA fragments was performed in a 20μl reaction mixture containing 2μl of the reverse transcribed cDNA, 0.2μM arbitrary primer (GenHunter), 0.2μM anchored oligo (dT)-primers (H-T11M, where M=A, G, C), 2μM of each dNTP, 10mM Tris-Cl, pH8.4, 50mM KCl, 1.5mM $MgCl_2$, 0.001% gelatin, 1μl α-[S^{35}] dATP and 1 U *Taq* DNA polymerase (Qiagen, CA). The PCR program consisted of 40 cycles: 30 s at 94˚C, 2 min at 40˚C, 1 min at 72˚C; and a final 5 min elongation step at 72˚C. Amplified PCR products were separated in a 6% denaturing PAGE gel. The gel

was transferred to filter paper (Whatman, England) and dried at 80˚C for 1 hour in a gel dryer (BioRad, CA). PCR products on filter paper were exposed to BioMax Kodak film covered with two intensifying screens for 24 to 72 hours in a –80˚C freezer. The film was developed and the differentially expressed bands between control and treatment were excised from the filter paper according to the pattern on the film. The PCR products were extracted according to the GenHunter protocol, and reamplified using the original primer pair.

2.3. cDNA-AFLP analysis

cDNA was synthesized using RETROscript™ (Ambion, TX) according to the manufacturer's instructions, and digested using the *Mse*I/*EcoR*I enzyme combination. AFLP analysis was conducted according to the protocol of AFLP kit from Li-COR (Li-COR Biosciences, NE). Sequences of the adapters and primers used for cDNA AFLP analysis were: 5'-GACGATGAGTCCTGAG-3' (*Mse*I adapter 1); 5'TACTCAGGACT CAT-3' (*Mse*I adapter 2); 5'-CTCGTAGACTGCGTACC-3' (*EcoR*I adapter 1); 5'-AATTGGTACGCAGTCTAC-3' (*EcoR*I adapter 2); 5'-GATGAGTCCTGAGTAAC-3' (non-selective primer for *Mse*I); 5'-GACTGCGTACCAATTCA-3' (non-selective primer for *EcoR*I); 5'-GATGAGTCCTGAGTAACNN-3' (selective primer for *Mse*I, and NN represents 2bp extension); 5'-GACTGCGTACCAATTCANN-3' (selective primer for *EcoR*I, and NN represents 2bp extension). Pre-amplification was performed in a 25 µl reaction solution, containing 0.2 mM dNTPs, 0.2 µM non-selective primers, 1.2mM $MgCl_2$, 0.5U *Taq* polymerase (Invotrogen, CA) and 10 µl ligated cDNA fragments. The

PCR program consists 20cycles: 30 s at 94 ˚C, 60 s at 56 ˚C and 60 s at 72 ˚C. Selective amplification was performed in a 10µl reaction solution, containing 0.1mM dNTPs, 0.15 µM *EcoR*I primer and 0.3 µM *Mse*I primer, 1.2mM $MgCl_2$, 0.5 U *Taq* polymerase (Invitrogen) and 2.5 µl pre-amplification solution (diluted at 1:40). The PCR program was carried out with the following procedure: one cycle at 94 ˚C for 30 s, 65 ˚C for 30 s, and 72 ˚C for 60 s; 13 cycles with annealing temperature decreasing 0.7 ˚C per cycle followed by 23 cycles at 94 ˚C for 30 s, 56 ˚C for 30 s and 72 ˚C for 60 s. Selective reaction products were run on 6% polyacrylamide sequencing gel at 85 W for 3 hrs. Fragments were visualized by silver staining according to the Silver Sequence^{TM} DNA Sequencing System Technical Manual (Promega, WI).

2.4. Cloning and sequence analysis of DNA fragments

Selected amplified DNA fragments were ligated directly into a PCR-Trap Vector (GenHunter) and transformed into competent *Escherichia coli* (GenHunter). Ten colonies were selected for each transformation event. 20µl lysis PCR was carried out according to the GenHunter protocol. 10µl of lysis PCR products were separated in 1.5% agarose gel. The remaining 10µl of PCR product containing correct size inserts were digested with 0.2µl of *Taq*I (10U/µl), 10mM Tris-HCl, pH8.4, 50mM KCl, 1.5mM $MgCl_2$, 0.001% gelatin, and incubated at 65˚C overnight. Products were analyzed using agarose gel electrophoresis. Differentially restricted DNA fragments were used for plasmid isolation. Only fragments larger than 250 bp were selected and sequenced in both directions using

Rseq and *Lseq* primers (GenHunter) with ABI 3100 DNA sequencer (AU Genomics Lab). Analysis of nucleotide sequence of selected fragments was carried out using the National Center for Biotechnology Information BLASTx search tool.

2.5. Quantitative Relative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Quantitative relative RT-PCR was used to confirm the differential expression of DNA fragments isolated from control and cold acclimated plants. 2.5μg total RNA was reverse transcribed with 0.5mM dNTP, 5mM oligo(dT)-primers, 10mM Tris-HCl, pH 8.3, 50 mM KCl, 15mM $MgCl_2$, 1μl RNase inhibitor, and 100U M-MuLV-RT (Ambion). The mixture (20μl total reaction volume) was incubated at 42˚C for 1 hour. 1μl RT reaction was amplified in a 25μl solution with 10mM Tris-HCl, pH8.4, 50mM KCl, 1.5mM $MgCl_2$, 2.5mM dNTPs, 0.3μM actin primer, 0.6μM actin competitor, 0.5mM of each gene specific primers (Ambion) and *Taq* polymerase (Qiagen). The PCR cycle program was as followed: 30 s at 94˚C, 30 s at 55˚C to 60˚C and 30 s at 72˚C for 30 to 35 cycles. Specific oligonucleotide primers were designed for each DD product for RT-PCR. The optimal PCR annealing temperatures and the cycle numbers are shown in **Table 1**. 10μl of the amplification products were separated using 1.8 % agarose gel electrophoresis and stained with ethidium bromide. The stained gel was used for quantitation of each band using a BioRad photo documentation system. A constitutively expressed actin gene was used as an internal standard in each reaction.

3. Results and discussion

3.1. Identification and confirmation of differentially expressed transcripts in C. unshiu in response to low temperature

Understanding a plant's responses to a stress will require a comprehensive evaluation of stress-induced changes in gene expression. mRNA differential display, cDNA-AFLP and quantitative RT-PCR were used to study the responses of *C. unshiu* to low temperature stress. Sixty-four primer pair combinations were used and about 8,000 cDNA fragments were analyzed after autoradiography. The banding pattern of cDNA fragments amplified by the combination of one primer group is shown in **Fig. 1**. To decrease the "false positive" rate of mRNA differential display, each primer pair was used to amplify two different sets of RNA isolated from different *C. unshiu* plants. RT-PCR products were loaded in adjacent lanes in a 6% denatured PAGE gel. One hundred and sixty-five putative differentially expressed DNA fragments were cloned, sequenced, and analyzed. Because of the possibility of comigration associated with DDRT-PCR (Zhang et al. 2004), ten clones, obtained from each band on the gel, were examined. These colonies were randomly selected from each transformation, and the sequence of the cloned insert in each of the colonies was determined. Six genes were confirmed as up-regulated and two genes were confirmed as down-regulated in cold acclimated plants with quantitative RT-PCR. The genes show significant homology to known genes in the GenBank database using the $BLAST_X$ search utility. The nucleotide sequences of selected

clones have been deposited in GenBank, and their accession numbers along with the results of the $BLAST_X$ search are shown in **Table 2**.

Three different sets of independently isolated RNA were used to confirm the DD results with RT-PCR. Only reproducible differences in banding patterns between the control and treatment in all three sets were considered to be positive. The agarose gel electrophoresis pattern of six up-regulated and two down-regulated DD genes using actin mRNA as an internal standard and a histogram representing the relative quantity of each of the amplified bands on the gel are presented in **Fig 2**.

3.2. Up-regulated genes in C. unshiu in response to low temperature

The inferred amino acid sequence of O10 showed 96% similarity to a 14-3-3 protein from *Nicotiana tabacum*. The 14-3-3 proteins are small, highly conserved eukaryotic proteins involved in regulating multiple cellular enzymes, including plant calcium dependent protein kinases (Camoni et al. 1998) and plant plasma membrane H^+-ATPase (Camoni et al. 2000). In *Arabidopsis*, the binding of 14-3-3 protein to the calcium dependent protein kinase (CDPK) can activate the activity of the enzyme, and thus probably further activates CDPK signal transduction pathways in this plant (Camoni et al. 1998). In maize, 14-3-3 proteins are able to interact with the H^+-ATPase and the interaction depends on the phosphorylation status of the proton pump (Camoni et al. 2000). In *Arabidopsis*, two genes encoding 14-3-3 proteins were induced in response to low temperature. The 14-3-3 protein accumulation is correlated with an increase of cold tolerance ability and the genes were thought to have an adaptive role in this process

(Jarillo et al. 1994). The *14-3-3* in cold acclimated *C. unshiu* was induced about 2.3 fold in comparison to non-acclimated control. Considering its central role in signal transduction, the induced transcript might produce more 14-3-3 protein, and result in an increased down stream effect in the signal transduction cascade.

The inferred amino acid sequences of O26 and O151 showed 98% and 92% similarity to a 40S ribosomal protein S23 and a putative 60S ribosomal protein L15 from *Hyacinthus orientalis* and *Oryza sativa*, respectively. The *RPL15* transcript in *Syntrichia ruralis*, a desiccation tolerant moss, was stably maintained in desiccated and rehydrated gametophytes and may be involved in the maintenance of ribosomal integrity within the desiccated state and re-establishment of translational efficiency upon rehydration (Zeng and Wood 2000). *Ribosomal L15* gene was reported to be up-regulated in a citrus relative, *P. trifoliata*, under a similar low temperature acclimation process (Zhang et al. 2004). The participation of ribosomal proteins in cold acclimation process indicates that *de novo* protein synthesis is necessary for plants under adverse environments.

The inferred amino acid sequence of O53 showed 90% similarity to a nucleoside diphosphate kinase III protein (NDPK) from *Spinacia oleracea*. The NDPKs are enzymes whose major role is to maintain the balance between adenine and non-adenine triphosphates. In cultured sugarcane cells, the activity of NDPK was enhanced by heat shock (Moisyadi et al. 1994). It's postulated that under heat shock, NDPK can help plant cells maintain the nucleoside triphosphate pools which is an essential activity for plant survival. In peas, the expression of *mtNDPK* was not directly affected by different stresses including cold temperature, but the interaction of the protein encoded by this gene with another heat stress induced protein was detected, indicating a role of this

protein under stress condition (Escobar Galvis et al. 2001). The *NDPK* gene in the current study was induced almost 5 fold in cold acclimated *C. unshiu*. Under temperature stress conditions (low and high), plant cells might need to increase the synthesis of the NDPK protein so that cells can maintain the nucleoside triphosphate supply. The participation of NDPKs in the signal transduction pathway (Tanaka et al. 1998) in other organisms might indicate another reason for the up-regulated expression of this gene under stress conditions.

The inferred amino acid sequence of O58 showed 75% similarity to a regulator of chromosome condensation-like protein from *Arabidopsis*. The regulator of chromosome condensation protein is involved in the maintenance of chromatin conformation and can regulate chromosome condensation, and might be involved in monitoring and signaling of DNA replication (Enoch and Nurse 1990). The *Arabidopsis* gene expression profile under environmental stresses shows that regulator of chromosome condensation-like protein was highly induced by low temperature, indicating a role of this gene in plant low temperature adaptation (Seki et al. 2002). The role of this gene at low temperature stress is currently not clear, although the strongly up-regulated expression level in *C. unshiu* and *Arabidopsis* might indicate that adjustment of chromosome structure may be needed under stress conditions.

The inferred amino acid sequence of O37 showed 78% similarity to an amino acid permease 6 (AAP6) from *Arabidopsis*, 61% similarity to an AAP1 from *Brassica napus* and 52% similarity to AAP2 from *Arabidopsis*. In higher plants, inorganic nitrogen assimilated from roots and leaves was reduced to its organic form such as amino acids and these amino acids were transported to metabolically active organs for the plant to

utilize. Under desiccation and salinity conditions, mRNA levels of AAP6 were reduced (Rentsch et al. 1996). In *Mesembryanthemum crystallinum*, expression of AAP2 was induced in roots in response to salinity, while expression of AAP1 was induced in leaves in response to salinity (Popova et al. 2003). Although the exact roles of the proteins encoded by these genes under environmental stresses are still not clear, the induced level of the amino acid permease in *C. unshiu* indicates that active transport and metabolism of amino acids are necessary under low temperature conditions.

3.3. Down-regulated genes in C. unshiu in response to low temperature

The inferred amino acid sequence of O71 showed 95% similarity to a miraculin-like protein 2 from *Citrus* x *Paradisi*, 48% to tumor-related protein from tobacco, and 49% to aspartic protease inhibitor from potato. Miraculin is a taste modifier protein isolated from "miracle fruit". A putative miraculin gene in tomato, *LeMir,* was induced by a root-knot nematode, and this LeMir may have a role in defense against nematodes or other pathogens/pests (Brenner et al. 1998). In *P. trifoliata*, a similar gene was identified under cold acclimation conditions (Zhang et al. in press). Unlike the increased expression level of this protein under biotic stress or plant hormone treatment, the decreased expression level of this gene in *Citrus* and *P. trifoliata* might indicate a different function of the protein encoded by this gene under abiotic stress.

The inferred amino acid sequence of O8 showed 97% similarity to a beta-galactosidase from *Citrus sinensis*. Beta-galactosidase has been most studied for its role in fruit development. Down-regulation of this gene in tomato delays fruit softening

(Smith et al. 2002), but evidence indicates that this gene may be involved in plant stress adaptation. In *Arabidopsis*, a putative β-galactosidase was induced 3.2 to 7.7 fold in response to cold, osmotic, and salt stress (Kreps et al. 2002). Contrarily, the mRNA transcript was repressed under a gradually declined temperature regime in *P. trifoliata* (Zhang et al. in press). The decreased expression level of this gene in *Citrus* and *Poncirus* might indicate a unique role of this gene in woody plants under stress conditions.

3.4. Comparison of the function of differentially expressed genes between P. trifoliata and C. unshiu

Commercial *Citrus* species are cold-tender evergreen crops with tropical and subtropical origins. *C. unshiu* is one of the most cold hardy commercial *Citrus* species. *P. trifoliata* is a facultative deciduous relative of *Citrus* in the *Rutaceae* family that is often used in citrus breeding programs to develop cold hardy genotypes, and is used as a rootstock to enhance freeze tolerance of the scion in freeze-prone areas. *P. trifoliata* has a maximum freeze tolerance of about –30 ºC (Williams 1911). In order to gain an understanding of the molecular mechanisms of these two species under cold temperature and compare their different responses to low temperature, mRNA differential display was used to identify the cold responsive genes under a gradually declined temperature regime. Thus far in *P. trifoliata*, three groups of genes have been detected as related to cold temperature responses (Zhang et al. 2004; Zhang et al. in press). The first group include up-regulated proline/betaine transporter, nitrate transporter and water channel protein.

The proteins encoded by these genes are known to be associated with osmotic adjustment of plant cells under adverse environment such as drought, salinity and cold temperature. The second group include up-regulation of early light inducible protein and aldo-keto reductase. Proteins encoded by these genes were reported to alleviate the damages caused by photooxidative stress, which simultaneously exits in combination with other environmental stresses. The third group include down-regulation of chlorophyll a/b binding protein, photosystem II OEC23 and carbonic anhydrase. The proteins encoded by these genes are related to adjustment of plant photosynthesis under stress condition (Zhang et al. 2004; Zhang et al. in press). Down regulation of these genes can lower photosynthesis efficiency, and probably decreases generation of excess energy, which can produce reactive oxygen species that are toxic to plants. In *C. unshiu*, an amino acid transporter was isolated, which may indicate that active N-containing molecule translocation is necessary for *Citrus* and *Poncirus* to cope with stress. Genes encoding the regulator of chromosome condensation protein (only in *C. unshiu*) and ribosome proteins (in *C. unshiu* and *P. trifoliata*) were found to be up-regulated, indicating adjustments at the transcription and translation level are needed for plants to survive adverse conditions. Two genes (miraculin-like protein and beta-galactosidase) were down-regulated in both species. These genes were reported to be associated with biotic stresses and plant cell wall modification, but our work shows that these genes may have a potential role in plants under abiotic stress conditions and further demonstrates the similarity between biotic and abiotic stresses at the molecular level. Unlike *P. trifoliata*, genes involved in osmotic adjustment, photooxidative protection and photosynthesis repression were not detected in *C. unshiu*. This might be due to the limited number of genes that have been

isolated using DDRT-PCR or to differences in cold acclimation between these two species. Further work with northern blotting using low stringency conditions may provide more information.

3.5. Comparison of C. unshiu cold acclimated genes isolated with mRNA differential display and cDNA-AFLP

Due to the extensively reported high false positive rate characteristic of the differential display procedure, cDNA amplified fragment length polymorphism (cDNA-AFLP) was performed to further explore the alteration of gene expression in *C. unshiu* exposed to cold acclimation temperatures (Zhang, data unpublished). Two genes (translation initiation factor eIF1 and cytochrome C) were identified as up-regulated and four genes (trigger factor type chaperone family protein, polyprotein, leucine-rich repeat transmembrane protein kinase/receptor-like protein kinase and PAZ/PIWI domain containing protein) were identified to be down-regulated. Translation initiation factor eIF1 was reported to be related to low temperature (Berberich et al. 1995). Similar to ribosomal proteins detected with mRNA differential display, eIF1 is also an important component of the protein synthesis machinery in plant cells. It is likely that de novo synthesis of proteins and the maintenance of the protein synthesis machinery integrity is necessary for plant cells to tolerate environmental stress. The PAZ/PIWI domain containing protein was also reported to be associated with translational regulation in certain species (Morel et al. 2002), further demonstrating that the modulation at the translation level is important for plant adaptation to stress. A receptor-like protein kinase

with leucine-rich repeat transmembrane domain was induced by ABA, dehydration, high salt and cold treatment in *Arabidopsis*, suggesting that the protein encoded by this gene may function in the transmission of ABA and various environmental stress signals into intracellular reactions (Hong et al. 1997). Up-regulation of *14-3-3* and nucleoside diphosphate kinase in *C. unshiu* under low temperature further indicate that sensing and transduction of environmental signals are needed for plants to adapt to their environment. The low overlap of the identified genes by these two techniques demonstrates that more cold acclimated genes need to be identified to completely understand the adaptation mechanisms at the molecular level of *C. unshiu* exposed to cold acclimation temperatures. These results might also indicate that these two techniques can complement each other in order to get a complete gene expression profile of cold acclimated *C. unshiu*.

4. Conclusions

- Sixty-four primer pairs were used to generate about 8,000 cDNA fragments. One hundred and six-five putative differentially expressed fragments were cloned and sequenced. Eight genes (six up-regulated and two down-regulated) were confirmed as differentially expressed.
- The identified genes were mainly related to signal transduction (14-3-3), protein synthesis (S23 and L15), amino acid transport (AAP6), adjustment of chromosome structure (regulator of chromosome condensation-like protein), plant defense (miraculin) and cell wall metabolism (beta-galactosidase).

- The predicted functions of the genes identified from *C. unshiu* are different from the functions of the genes identified from its close relative, *P. trifoliata*. Osmotic modulation, photo-oxidative protection and photosynthesis adjustment were predicted as three main mechanisms for *P. trifoliata* in response to low temperature.
- The genes identified with differential display are different from the genes identified with cDNA-AFLP, indicating the two techniques can complement each other.
- More genes should be isolated with cDNA-AFLP, a more reliable transcriptome profiling technique, in order to get a more complete understanding of the molecular mechanisms of citrus and related species.
- More detailed characterization needs to be performed for some genes identified in both species. For example, the proline transporter gene identified in *P. trifoliata* might indicate that transport of proline is also important for plant adaptation to stress in addition to proline synthesis. The detailed characterization of the signal transduction important 14-3-3 gene identified in *C. unshiu* will also shed light on the understanding of the responsive mechanisms under cold acclimation.

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Table 1. Oligonucleotide primer sequences, PCR conditions and cycle numbers for the confirmation of different displayed products with quantitative RT-PCR

DD product	Forward Primer (5'-3')	Reverse Primer (5'-3')	PCR annealing temperature	Cycle numbers
O10	TTGCACCATGGCCAAACAG	GGTTGCCAGAATTGGTATGTAG	55 °C	32
O26	AGCTTTTGATCCAAATGATG	TTGAGACAAGTGAGCAATT	55 °C	32
O151	AAGCTTCTGCTGGGAAGA	GCAGAAATATCACTCCCC	55 °C	30
O53	CTTGCCATGGTATGGAAAG	TGGAGTCCATAAAGAGGTG	55 °C	30
O58	TTCTGCTGGGGATGGAAC	TTTTGGACAAAATCATGTTG	57 °C	32
O37	ATTCCGGCCATTAACAGTT	GGAAAAGTTGTAGCTGACAA	55 °C	32
O71	AAGCTTTCATATGGTGGT	CACAGGCGGTGTTATTTT	55 °C	32
O8	GCTTCTCAACGATGGTATCACG	GCCTACATGAATGTTTGAGG	55 °C	32

Table 2. Isolated DD products, accession number and percentage similarity to known genes by BLASTx search in NCBI

DD product	Accession Number	Length (bp)	Plant gene	Similarity	Function
O10	CV736155	452 bp	14-3-3 d-2 protein	96 %	Signal transduction
O26	CV736156	420 bp	40S ribosomal protein S23	98 %	Protein synthesis
O151	CV736157	429 bp	putative 60s ribosomal protein L15	92 %	Protein synthesis
O53	CV736158	442 bp	nucleoside diphosphate kinase III	90 %	Abiotic stress, signal transduction
O58	CV736159	375 bp	regulator of chromosome condensation-like protein	75 %	DNA replication, abiotic stress
O37	CV736160	360 bp	amino acid permease 6	78 %	Amino acid translocation
O71	CV736161	403 bp	miraculin-like protein 2	95 %	Biotic stress
O8	CV736162	503 bp	beta-galactosidase	97 %	Cell wall metabolism

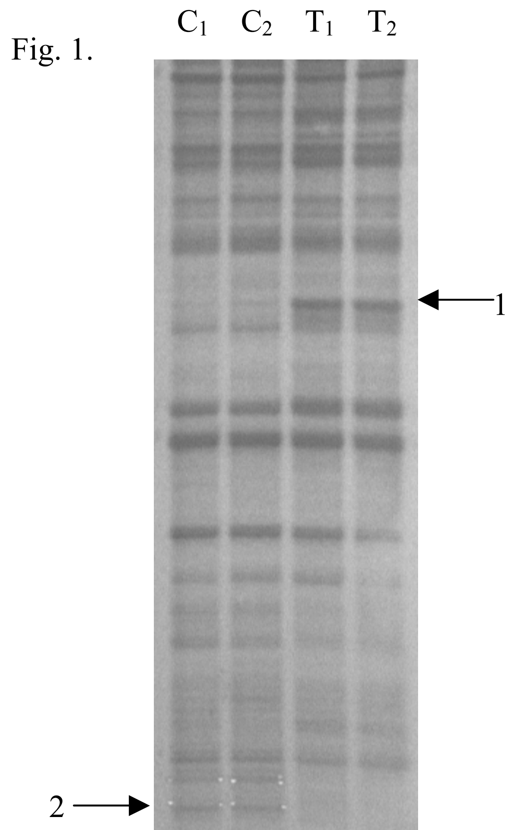

Fig. 1. Example of DDRT-PCR results. cDNAs were amplified from two separately isolated total RNAs by primer combination of $HT_{11}C$ and HAP_1. C_1 and C_2 are cDNAs amplified from the leaves collected at the end of second week (control); T_1 and T_2 are cDNAs amplified from the leaves collected at the end of fifth week (cold acclimated). Arrow 1 indicates one up regulated cDNA fragment; Arrow 2 indicates one down regulated cDNA fragment.

Fig. 2a.

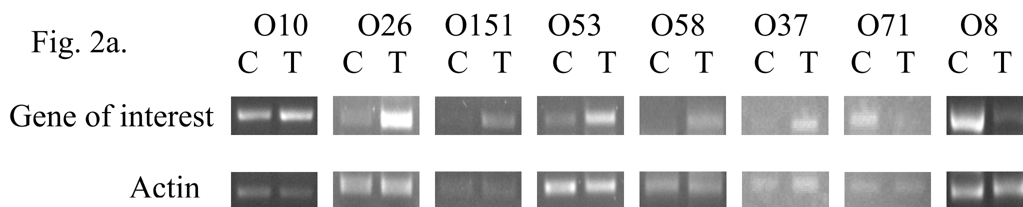

Fig. 2b.

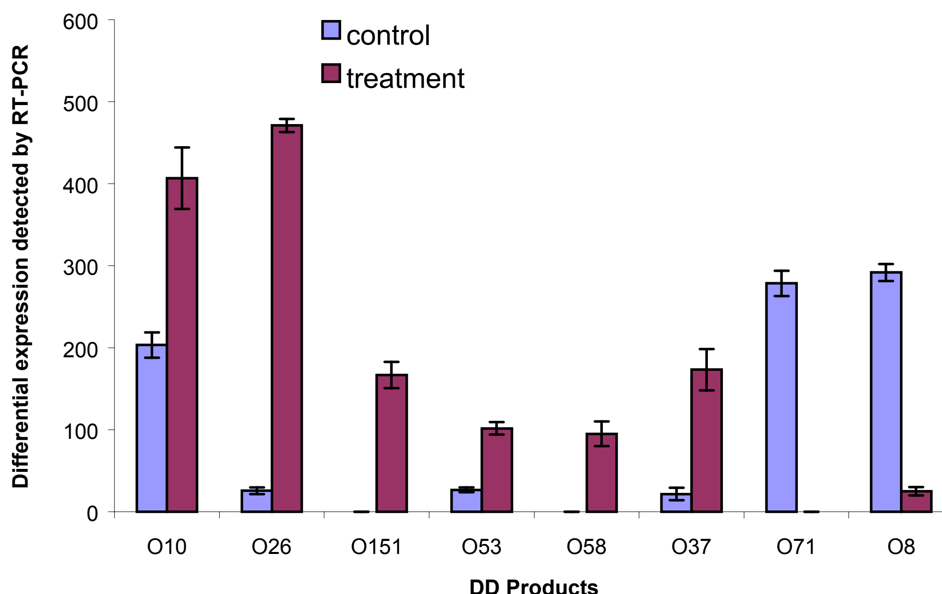

Fig. 2a: Confirmation of differential expression of 8 differential displayed (DD) products using relative quantitative RT-PCR with RNA from control and cold acclimated leaves. The cDNAs were synthesized from total RNAs isolated from control leaves (end of second week) and cold acclimation leaves (end of fifth week). Actin mRNA was used as an internal control. Specific RT-PCR primer pair information for all DD products is listed in table 1.

Fig. 2b: A histogram showing the relative abundance of 8 DD products between control and cold acclimated plants. The intensities in each control and cold acclimation pair were normalized by setting the intensity of actin mRNA to 100. The values are the means of three independent experiments $\pm$ SE.

V. Cold acclimation genes in C*itrus unshiu* "Owari"[1]

ABSTRACT

Citrus unshiu is a moderately cold hardy species, but low temperature affects its productivity. Very little is known about the mechanism of adjustment to low temperature stress in this species. cDNA-AFLP and RT-PCR were used to study changes in gene expression of this species during gradual cold-acclimation. Two up regulated and four down regulated genes were identified. The up-regulated genes show high similarities to translation initiation factor eIF1 and cytochrome C. The down-regulated genes show high similarities to trigger factor type chaperone family protein, polyprotein, leucine-rich repeat transmembrane protein kinase/receptor-like protein kinase and PAZ/PIWI domain containing protein. Expression of some of these cold responsive genes is also regulated by other stresses. The differences of gene expression between *Owari* and *Poncirus* during low temperature were also briefly discussed.

Key words: *Citrus unshiu,* cDNA-AFLP, cold acclimation and gene expression

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[1] The nucleotide sequences reported in this paper have been submitted in Genbank under accession numbers of CO578908 (OA123), CO578909 (OA36), CO578910 (OA120), CO578911 (OA68), CO578912 (OA69), CO578913 (OA87).

Introduction

Since plants cannot escape from exposure to a number of environmental extremes, plants have evolved mechanisms for adaptation to their surroundings. Subfreezing temperature is a stress to plants which results in serious inhibition of growth and development. One of the most serious injuries induced by cold temperature is cell dehydration associated with ice formation under freezing temperature (Levitt 1980). Ice formation usually initiates in extracellular space. The lower water potential of ice will be the driving force for dragging water from cytoplasm to the extracellular space, thus causing cell dehydration. Most plants from temperate regions can increase their cold tolerance ability upon exposure to a period of low, nonfreezing temperature, a process called "cold acclimation" (Guy 1990). During this process, many physiological and biochemical changes occur such as synthesis of new proteins (Tseng and Li 1991) and accumulation of compatible osmolytes (Bohnert et al. 1995). Several cold responsive genes have been identified in different plant species such as the *CBF* family in *Arabidopsis thaliana* (Gilmour et al. 1998), *hsp90* in *Brassica napus* (Krishna et al. 1995) and *cas15* in alfalfa (Monroy et al. 1993).

Considerable efforts have been taken to clarify the molecular mechanisms of herbaceous plants under cold acclimation, but our understanding of the molecular mechanisms of cold acclimation in woody plants is limited. Citrus is an economically important crop throughout the world, and its productivity is seriously affected by low temperature (Porat et al. 2002). Some preliminary cold acclimation molecular studies have been conducted on *Citrus* and closely related species, and genes such as dehydrin have been isolated (Cai et al. 1995; Hara et al. 1999; Porat et al. 2002). The treatment

approach used by these researchers was a "cold shock" (plants were moved from warmer temperature to low temperature directly) versus a "cold acclimation" temperature regime, which more closely approximates natural conditions. *Citrus unshiu* is one of the most cold hardy commercial *Citrus* species (Yelenosky 1985). We show both up and down-regulated genes during gradual process of cold acclimation in *C. unshiu* identified by cDNA-AFLP. The identified cold acclimated genes in *C. unshiu* shed more light to the understanding of the regulatory mechanisms of woody plant cold acclimation, and might also have the potential to enhance the cold tolerance ability of cold sensitive species via transgenic approaches.

Materials and methods

Plants: One year old *C. unshiu* plants were grown for 5 weeks in a growth chamber with a 12 h light period at 400 µmol $m^{-2}s^{-1}$ intensity. The regimen for 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. Plants were uniformly watered every day.

RNA extraction: Expanded leaves at the end of the second and the fifth week 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 10X DNase buffer and 4µl DNase I (2U/µl) 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 14000g and transferred to a new tube. The concentration of isolated RNA was measured using an Eppendorf Biophotometer (Brinkmann Instruments, NY). The quality of RNA was checked using formaldehyde-agarose gel electrophoresis. RNA from the end of second week was used as unacclimated control and RNA from the end of fifth week as treatment.

cDNA AFLP analysis: cDNA was synthesized using RETROscript^{TM} (Ambion, TX) according to the manufacturer's instructions, and digested using the *Mse*I/*EcoR*I enzyme combination. AFLP analysis was conducted according to the methods of Bachem et al. (1996). Sequences of the adapters and primers used for cDNA AFLP analysis were: 5'-GACGATGAGTCCTGAG-3' (*Mse*I adapter 1); 5'-TACTCAGGACTCAT-3' (*Mse*I adapter 2); 5'-CTCGTAGACTGCGTACC-3' (*EcoR*I adapter 1); 5'-AATTGGTACGCAGTCTAC-3' (*EcoR*I adapter 2); 5'-GATGAGTCCTGAGTAAC-3' (non-selective primer for *Mse*I); 5'-GACTGCGTACCAATTCA-3' (non-selective primer for *EcoR*I); 5'-GATGAGTCCTGAGTAACNN-3' (selective primer for *Mse*I, and NN represents 2bp extension); 5'-GACTGCGTACCAATTCANN-3' (selective primer for *EcoR*I, and NN represents 2bp extension). Pre-amplification was performed in a 25 µl reaction solution, containing 0.2 mM dNTPs, 0.2 µM non-selective primers, 1.2mM $MgCl_2$, 0.5U *Tag* polymerase and 10 µl ligated cDNA fragments. The PCR program consists 20cycles: 30 s at 94 ˚C, 60 s at 56 ˚C and 60 s at 72 ˚C. Selective amplification was performed in a 10µl reaction solution, containing 0.1mM dNTPs, 0.15 µM *EcoR*I primer and 0.3 µM *Mse*I primer, 1.2mM $MgCl_2$, 0.5 U Tag polymerase and 2.5 µl pre-

amplification solution (diluted at 1:40). The PCR program was carried out with the following procedure: one cycle at 94 ˚C for 30 s, 65 ˚C for 30 s, and 72 ˚C for 60 s; 13 cycles with annealing temperature decreasing 0.7 ˚C per cycle followed by 23 cycles at 94 ˚C for 30 s, 56 ˚C for 30 s and 72 ˚C for 60 s. Selective reaction products were run on 6% polyacrylamide sequencing gel at 85 W for 3 hrs. Fragments were visualized by silver staining according to the Silver Sequence™ DNA Sequencing System Technical Manual (Promega, WI).

Cloning and sequence analysis of DNA fragments: DNA fragments extracted from the gel were ligated with pGEM-T Easy vector system (Promega, WI) and introduced into *Escherichia coli* DH5α cells. cDNA fragments were sequenced using the ABI3100 sequencer at AU genomics lab. Analysis of nucleotide sequence of selected fragments was carried out using National Center for Biotechnology Information BLASTx search tool.

Quantitative RT-PCR: Quantitative relative RT-PCR was used to confirm the differential expression of DNA fragments isolated from control and cold acclimated plants. 2.5µg total RNA was reverse transcribed with 0.5mM dNTP, 5mM oligo(dT)-primers, 10mM Tris-HCl, pH 8.3, 50 mM KCl, 15mM $MgCl_2$, 1µl RNase inhibitor, and 100U M-MuLV-RT (Ambion). The mixture (20µl total reaction volume) was incubated at 42˚C for 1 hour. 1µl RT reaction was amplified in a 25µl solution with 10mM Tris-HCl, pH8.4, 50mM KCl, 1.5mM $MgCl_2$, 2.5mM dNTPs, 0.3µM actin primer, 0.6µM actin competitor, 0.5mM of each gene specific primers and *Taq* polymerase (Qiagen).

PCR cycle program was as follows: 30 s at 94˚C, 30 s at 55˚C to 60˚C and 30 s at 72˚C for 30 to 35 cycles. Specific oligonucleotide primers were designed for each DD product for RT-PCR. The optimal PCR annealing temperatures and the cycle numbers are shown in **Table 1**. 10μl of the amplification products were separated using 1.8 % agarose gel electrophoresis and stained with ethidium bromide. The stained gel was used for quantitation of each band using a BioRad photo documentation system. A constitutively expressed actin gene was used as an internal standard in each reaction. The differential expression of genes in response to cold acclimation is shown in **Table 2**. **Figure 1** (top) shows the agarose gel electrophoresis pattern of 6 low temperature responsive genes. **Figure 1** (bottom) shows histogram of RT-PCR results.

Results and discussion

Transcriptome profiling of plants to environmental stresses can be studied via DDRT-PCR, cDNA-AFLP, SAGE, subtractive hybridization, DNA-chip and cDNA microarray (Donson et al. 2002). cDNA AFLP analysis was used in this study due to its high reliability and non-requirement of prior genetic information of studied organism. A total of 32 primer pairs were used in this study. An average of 20 to 50 bands were obtained.

Up-regulated genes in cold acclimated "Owari": Cold acclimation is a complex and global process involving many physiological and biochemical changes. To acquire freezing tolerance, de novo protein synthesis is necessary for cold response (Guy 1990). The inferred amino acid sequence of OA 123 shows 96% similarity with the homolog of

translation initiation factor in *Coffea arabica* and rice, and 95% similarity with the translational initiation factor eIF1 from *Porteresia coarctata.* eIF1 in resurrection grass *Sporobolus stapfianus* was found to be induced during drought stress (Neale et al. 2000). A correlation between eIF1A and low temperature stress was shown in barley and maize (Berberich et al. 1995; Dunn et al. 1993). It can be postulated that the extensively de novo synthesis of proteins will help plant cells tolerate adverse environments.

The inferred amino acid sequence of OA36 shows 95% similarity with cytochrome c in *Arabisopsis thaliana.* Cytochrome c is a main component of the respiratory chain. In sunflower, it has been demonstrated that the gene encoding cytochrome c is regulated by both tissue type and environmental factors (Felitti and Gonzalez, 1998). In the green alga *Chlamydomonas reinhardtii, cytochrome c* responds to metabolic regulation (Felitti et al. 2000). The up-regulated expression of cytochrome c might indicate cell need to maintain energy production in order to meet the requirements of cellular metabolism during stress.

Down-regulated genes in "*Owari*" in response to cold acclimation: Although scientists have focused on up-regulated genes, down-regulation of gene expression also contributes to the adaptation of plants to stress. The inferred amino acid sequence of OA 120 shows 55% similarity with polyprotein in *A. thaliana*. In sunflower, a putative gene encoding polyprotein was down regulated in shoots and up regulated in roots by salinity stress, but under drought, the expression was down regulated in roots and up regulated in shoots and leaves (Liu and Baird 2003). Differences in expression of retrotransposons in response to

different biotic and abiotic stresses can result in genetic changes and alteration of gene expression and thus help the host adapt to stresses (Grandbastien 1998; Wessler 1996).

The inferred amino acid sequence of OA 68 shows 51% similarity with trigger factor type chaperone family protein in *A. thaliana*. Protein denaturation is a common consequence under stress condition. Chaperone family protein could help protein refolding. In *Bacillus subtilis,* trigger factor functions as a peptidyl-prolyl cis-trans isomerase, which catalyzes the in vitro refolding of ribonuclease T1 (Göthel et al. 1998). In wheat, the FKBP-type peptidyl-prolyl cis-trans isomerase (trigger factor) is heat induced and developmentally regulated (Kurek et al. 1999). Compared to the protective role of chaperone proteins in other species, the down regulation of this protein in *Owari* during low temperature might indicate that the denaturation of some proteins which are not suited to the acclimation of plant cells is needed.

The inferred amino acid sequence of OA 69 shows 52% similarity with leucine-rich repeat transmembrane protein kinase and a receptor-like protein kinase in *A. thaliana*. The mechanism of perception of extracellular signals in plants is largely unknown. A receptor-like protein kinase with a leucine-rich repeat transmembrane domain was induced by ABA, dehydration, high salt and cold treatment in *Arabidopsis*, suggesting that this gene may function in the transmission of ABA and various environmental stress signals into intracellular reactions (Hong et al. 1997). However, our finding, that expression of the receptor protein kinase was specifically down regulated, signifies that its role in low temperature stress in "*Owari*" is likely to be complicated.

The inferred amino acid sequence of OA 87 shows 76% similarity with the PIWI domain containing protein in *Oryza sativa* and 70% similarity with the PAZ/PIWI domain containing protein in *Arabidopsis*. The *PIWI/PAZ* family of genes, which contain conserved *PIWI/PAZ* domains, play important roles in stem cell self-renewal, RNA silencing, and translational regulation in various organisms (Cerutti et al. 2000; Kuramochi-Miyagawa et al. 2004; Morel et al. 2002). The function of PIWI/PAZ family proteins in plant cell exposed to environmental stresses is unknown. These recent results, together with our finding, show that it may be of importance to down regulate this gene in order to adapt plant cell to stress condition at the post transcription and translation level.

Poncirus trifoliata is a close relative of *Citrus* and is mainly used as the rootstock in citrus industry due to the cold tolerance ability it imparts to the scion. Our previous study of cold acclimation regulated genes in *P. trifoliata* by differential display RT-PCR have shown that genes with substantial homologies to betaine/proline transporter, water channel protein, aldo-keto reductase, early light inducible protein, nitrate transporter, tetratricopeptide-repeat protein, F-box protein and ribosomal protein L15 are induced during cold acclimation (Zhang et al. 2005a). In addition, we have shown that genes with homology to chlorophyll a/b binding protein, photosystem II OEC 23, carbonic anhydrase, tumor related protein, pyrrolidone-carboxylate peptidase and β-galactosidase are down-regulated during cold acclimation (Zhang et al. 2005b). Salinity, drought, and cold are known to cause osmotic/dehydration stress in plants. Three of the induced genes encountered during cold acclimation in *P. trifoliata* were reported as important to deal with cell dehydration caused by extracellular ice formation (Zhang et al.

2005a). In addition to dehydration, the accumulation of reactive oxygen species (ROS) is another common consequence of environmental stresses (Smirnoff 1993). Three of the eight up regulated genes isolated from cold acclimated *P. trifoliata* have functions related to ROS scavenging (Zhang et al. 2005a). Studies have shown that photosynthesis-related proteins are down regulated and it's postulated that these proteins might not be suitable to the new physiological condition caused by dehydration stress (Chandler and Robertson 1994). One up and two down regulated photosynthesis related genes were detected in cold acclimated *P. trifoliata*. Compared to those genes detected in cold acclimated *P. trifoliata*, genes isolated from cold acclimated *C. unshiu* were not involved in dehydration, photooxidative protection and photosynthesis, but involved in protein translation, respiration, cell metabolism, signal transduction and protein refolding.

In summary, the different types of genes detected in response to low temperature between *P. trifoliata* and *C. unshiu* might be an indication of their different stress tolerance ability, although the numbers of low temperature responsive genes detected in both species are a bit limited. More low temperature responsive genes should be isolated and the full-length cloning of the cDNA fragments and the detailed characterization of the obtained genes will be necessary to understand the underlined response mechanisms of plant cells to environmental stresses for both species.

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Table 1. Oligonucleotide primer sequences, PCR conditions and cycle numbers for the confirmation of differentially expressed products with quantitative RT-PCR

Differentially expressed product	Forward Primer (5'-3')	Reverse Primer (5'-3')	PCR annealing temperature	Cycle numbers
OA123	GCTACAACAAGATTCTCAAGGA	CCAATAGTTGGAGATGGATAAA	57°C	27
OA36	CCGCCATGTTCTTGTTAGC	GCGTCTTCTTTCTCTCTTTGG	56°C	28
OA120	TCATGGTTGGTACACAAAGG	GCTAACAATGCCTTGAACAA	58°C	33
OA68	ATCTCCATCAACCTCAATTTCA	GAATCTTCCCAGGACGAAAT	55°C	29
OA69	ACGGCGAATTGCAGAGAG	CTCTCATGGGTTCTGCAAA	55°C	33
OA87	GGAAATGATGGAAGAAGGATC	GCACCAGAAAGCATAGAAGAA	58°C	33

Table 2. Isolated cDNA AFLP products, accession number in Genbank and percentage similarity to known genes by BLASTx search in NCBI

Differentially expressed product	Accession Number	Length (bp)	Plant gene	Similarity	Function
OA123	CO578908	263	translation initiation factor	96%	protein synthesis
OA36	CO578909	292	cytochrome c	95%	cell respiration
OA120	CO578910	313	polyprotein	55%	stress-related
OA68	CO578911	302	trigger factor type chaperone family protein	51%	protein refolding
OA69	CO578912	306	leucine-rich repeat transmembrane protein kinase	52%	signal transduction
OA87	CO578913	287	PIWI domain containing protein	76%	translation regulation

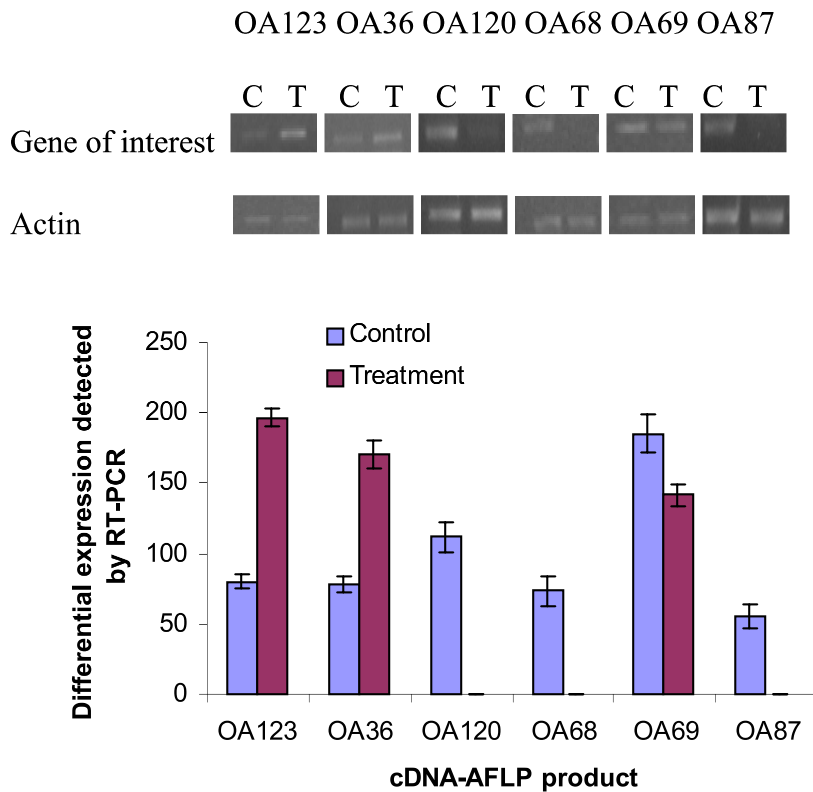

Fig. 1 (Top): Confirmation of differential expression of 6 cDNA AFLP products using relative quantitative RT-PCR with RNA from control and cold acclimated leaves. The cDNAs were synthesized from total RNAs isolated from control leaves (end of second week) and cold acclimation leaves (end of fifth week). Actin gene was used as an internal control. Specific RT-PCR primer pair information for all cDNA AFLP products was listed in table 1. (Bottom): A histogram showing the relative abundance of 6 cDNA AFLP products between control and cold acclimated plants. The intensities in each control and cold acclimation pair were normalized by setting the intensity of actin gene to 100. The values are the means of three independent experiments + SE.

VI. Conclusions, ongoing research and future perspectives

1. Conclusions

mRNA differential display was used to study the molecular mechanisms of *Citrus unshiu* and its relative, *Poncirus trifoliata*, under a gradually declined temperature regime. 144 primer pairs were used and about 15,000 cDNA fragments were analyzed after autoradiography in *P. trifoliata*, while 64 primer pairs were used and about 8,000 cDNA fragments were analyzed after autoradiograph in *C. unshiu*. In *P. trifoliata*, 14 cDNA fragments were confirmed to be positive with relative quantitative RT-PCR. Osmotic modulation, photooxidative protection and photosynthesis adjustment were postulated to be three of the main mechanisms in *P. trifoliata* under cold acclimation. In *C. unshiu*, 8 cDNA fragments were confirmed to be positive using relative RT-PCR, and these genes were reported to be associated with signal transduction, protein synthesis, amino acid transport, and cell wall metabolism.

In addition to differential display, another technique, cDNA-AFLP was also used to detect differentially expressed transcripts in *C. unshiu*. A total of 32 primer pairs were used in the study and about 10,000 bands were visualized upon silver staining. Six genes were confirmed to be positive using quantitative RT-PCR. The identified genes were related to protein translation, energy production, protein refolding and signal transduction.

2. Ongoing research

The isolation of genes with a new cDNA-AFLP protocol is still ongoing. A higher number of isolated genes will shed more light on the mechanisms of cold acclimation of *C. unshiu* in response to low temperature. Using RACE, full length sequences of carbonic anhydrase, proline transporter and nitrate transporter have been obtained. Carbonic anhydrase (*PtCA*) cDNA contains an open reading frame of 269 amino acids. The yeast complementation test for this gene is ongoing. Nitrate transporter (*PtNRT*) cDNA contains an open reading frame of 588 amino acids. 12 transmembrane domains have been predicted for this protein. Chloroplast membrane was predicted to be the functional site. A gene family including four members has been found for *Poncirus* proline transporter (*PtProT*). Their cDNAs contain an open reading frame around 391-400 amino acids. 10 transmembrane domains have been predicted for this protein.

3. Future research plan

- Due to the limited number of genes isolated by differential display, cDNA-AFLP should be utilized to isolate more genes in order to obtain a more comprehensive picture of the cold acclimation mechanisms in both *P. trifoliata* and *C. unshiu*. The comparison of the functions of genes from these two related species might help explain the differences in cold tolerance ability between these two species.
- Since no reports have been published to correlate the function of nitrate transporter to low temperature stress, functional analysis of this gene in oocytes, a plant transporter study model, will be indispensable to confirm the involvement of this gene under low temperature stress. Proline and proline synthesis have been

recognized for many years as important for plants to adapt to adverse environment, but very limited information is available to assign a role of the transport of this chemical in the process of plant cold acclimation. Comparison of the electrolyte leakage rate of transgenic *Arabidopsis* with P5CS (key enzyme related to proline synthesis), proline transporter gene and P5CS+ proline tranporter gene will be necessary to assign an exact role of the product encoded by proline transporter at the physiological level. Functional analysis of other interesting genes such as 14-3-3 will be helpful to understand the underlined mechanisms of these plant species in response to low temperature.

- Gene profiling works have been conducted in many species in response to low temperature, and many genes have been isolated and some of the functions of the isolated genes were also characterized. By contrast, less is known about stress responses of plants at the metabolite and metabolome level, thus a further profiling analysis of metabolites in these two species will enrich our understanding of cold acclimation mechanisms at a different level and may provide new insight into mechanisms utilized by plant species to cope with low temperatures stress.