THE ROLE OF PLANT WATER DEFICITS ON COLD TOLERANCE DURING

COLD ACCLIMATION OF A COLD TOLERANT (*PONCIRUS TRIFOLIATA*)

AND COLD SENSITIVE (*CITRUS UNSHIU*) SPECIES

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Kari Ann Dansereau


Certificate of Approval:


Donald J. Eakes<br>Professor<br>Horticulture

Robert C. Ebel, Chair<br>Associate Professor<br>Horticulture

Amy N. Wright<br>Associate Professor<br>Horticulture

George T. Flowers<br>Interim Dean<br>Graduate School

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Kari Ann Dansereau

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______________________________
Signature of Author

______________________________
Date of Graduation

VITA

Kari Ann Dansereau was born in Colorado Springs, Colorado on August 27, 1978. She is the mother of Farrah Joy Glover and the daughter of Mr. and Mrs. Richard W. Dansereau. She has two sisters, Kristin and Katy. In 1996 she graduated from Baker High School in Mobile, Alabama, and went on to Faulkner State Community College in Bay Minette, Alabama for two years. In the spring of 2001 she enrolled at Auburn University and graduated with a B.S. in Horticulture in the spring of 2005. She went on to pursue a Masters of Science degree in Horticulture at Auburn University. She received her Master's Monday, December 17, 2007.

## THESIS ABSTRACT

# THE ROLE OF PLANT WATER DEFICITS ON COLD TOLERANCE DURING COLD ACCLIMATION OF A COLD TOLERANT (*PONCIRUS TRIFOLIATA*) AND COLD SENSITIVE (*CITRUS UNSHIU)* SPECIES

Kari Ann Dansereau

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*Citrus unshiu* (Satsuma mandarin) is considered one of the most cold hardy commercial *Citrus* species; whereas, *Poncirus trifoliata* (Trifoliate orange), a close relative of *Citrus* sp., is capable of surviving freezes to -26˚C. Citrus develop plant water deficits during cold acclimation that may play a role in contrasting cold tolerance between these two species. The accumulation of 14-3-3 proteins has been associated with an increase in cold tolerance, and the genes expressed were thought to have an adaptive role in this process. 14-3-3 proteins have been identified and induced 2.3 fold when comparing cold-acclimated to non-acclimated Satsuma mandarin. This study was conducted to determine differences between these two species that may explain the

differences in gene expression of 14-3-3.

*C. unshiu* and *P. trifoliata* trees were kept unacclimated by holding day/night air temperatures at 25˚C/20˚C, or acclimated by dropping day/night air temperatures weekly from 25˚C/20˚C to 20˚C/15˚C to 15˚C/10˚C, and then to 10˚C/5˚C for 9 weeks. During acclimation, stomata closed similarly for both species. Stomata never completely closed, which allowed low levels of transpiration and $CO_2$ assimilation. Root resistance was higher for *C. unshiu* compared to *P. trifoliata*, and was higher for acclimated versus unacclimated plants by the end of the experiment. Plant water deficits, as measured by stem water potential, declined similarly for both species during cold acclimation, while remaining similar and constant when unacclimated. The similarity in plant water deficits between species during cold acclimation and the responses of stomates and root resistance cannot explain the contrasting cold hardiness of these two species. The difference of cold tolerance is likely due to a difference in gene expression during cold acclimation.

14-3-3 protein was observed using quantitative RT-PCR for both acclimated and unacclimated species. Both acclimated *C. unshiu* and *P. trifoliata* showed down-regulation of 14-3-3 protein when plants were fully acclimated to chambers at 5˚C and held for 21d, which differs from a study that showed up-regulation of 14-3-3 protein in *C. unshiu* when acclimated to 7˚C for 7d. A possible conclusion would be that 14-3-3 is an important factor during mid to late-acclimation, but the gene is no longer needed when plants are fully acclimated or cold-hardened. More detailed characterization of the signal transduction important 14-3-3 gene identified in *C. unshiu* and *P. trifoliata* will shed light on the understanding of the responsive mechanisms under cold acclimation.

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# CHAPTER I

# LITERATURE REVIEW

## Freeze Risk along the Gulf Coast for Satsuma Mandarin Production

Citrus is a cold-sensitive evergreen which under appropriate environmental conditions exhibits a limited degree of acclimation to low temperatures and resistance to freezing injury (Young, 1970). In many parts of the world such as the southeastern U.S., citrus are grown in areas that are highly vulnerable to freezes that cause injury or tree death and significant economic losses (Attaway, 1997; Ebel et al., 2005). A series of freezes and especially the freeze in 1940 devastated the citrus industry established on the northern coast of the Gulf of Mexico, and freezes following discouraged its resurgence (Winberg, 1948). During the 1940's the only method the growers used to protect the trees were banking soil around the trunk, growing trees under longleaf pine trees, and through the use of smudge pots. The process of banking soil allowed the trunks to survive, but did not protect the canopy and resulted in a recovery period of several years (Attaway, 1997). During the 1983 freeze many growers used irrigation as a cold protectant rather than more costly heating practices (using petroleum fuels) (Yelenosky, 1985). Irrigation, along with banking soil protected trees well during the radiation-type severe freezes of 1981 and 1982 (Buchanan et al., 1982; Parsons et al., 1983), but was less effective during the advective Christmas freeze of 1983 (Yelenosky, 1985). Advective freezes are the more sever freezes that occur in this region (Attaway, 1997).

New methods are underway that can mitigate freeze-risk and revitalize the Satsuma mandarin industry in this region. Evaporative cooling by overtree water misting, at the problem stage to delay bloom and thus avoid late frost damage has been successful (Lipe et al., 1977). Microsprinkler irrigation inside the canopy can protect scaffold limbs thus shortening the time it takes to get back into full production after severe freezes that kill whole, unprotected trees (Bourgeois and Adams, 1987; Bourgeois et al., 1990; Nesbitt *et al.*, 2002). Expense deters widespread commercial use, but plastic high tunnel houses can provide substantial freeze protection (Ebel et al., 2005).

Knowing the degree of cold hardiness in a species is critical for accurately predicting the extent of injury during a freeze (Yelenosky, 1985). The 1940 freeze reached a minimum of about -7.0˚C; however the canopies were unhardened and completely killed. We have preliminary evidence that Satsuma mandarin selections from China may be cold hardy to -12˚C (R.C. Ebel, unpublished data; Zhang et al., 2005). Genetic engineering may present an opportunity to dramatically alter maximum cold hardiness of citrus (Iba, 2002; Wisiniewski et al., 2003). If Satsuma mandarins were genetically modified to have maximum cold hardiness of -15˚C, severity of freeze injury would be reduced to 9%, 4%, and 7% for slight (some foliage die back), moderate (extensive foliage die back and some stem die back), and severe (extensive die back or tree death) freeze injury, respectively (Ebel et al., 2005). Microsprinkler irrigation has been used for freeze protection, but because of poor coverage due to high wind velocities of advective freezes; microsprinkler heads are placed inside the canopy to protect scaffold limbs. Although effective, the majority of the canopy bearing fruiting wood remains exposed (Bourgrois and Adams, 1987; Bourgeois et al., 1990; Nesbitt et al.,

2002). Severe freezes would kill the leaves and cause dieback of the fruiting wood thus eliminating productivity the year of the freeze, but with the survival of the scaffold limbs the trees would produce substantially the second year after the freeze. Thus, with the use of microsprinklers, severe freezes would cause damage similar to that of moderate freezes of unprotected trees. Microsprinklers adjust the freeze-risk by reducing the severe freeze injury to moderate freeze injury. Even with microsprinkler irrigation, growers can expect to lose their crop 1 out of 4 years (25%) with commercial cultivars (cold hardy to -10˚C). However, the ratio might improve to almost 1 out of 5 years (22%) with the more cold-hardy selections from China (cold hardy to -12˚C), (Ebel et al., 2005). To equip plants with freeze tolerance; genetic modification to -15˚C would improve freeze-risk to about one in 9 years (11%). Upon severe damage several years are required to regain significant productivity upon replanting and low yield until the trees fill their allotted space, thus microsprinkler irrigation is strongly encouraged in this region to enhance economic viability of this industry (Ebel et al., 2005). To understand the potential of genetic modification to reduce freeze-risk of Satsuma mandarin we need to study the freeze injury mechanism to identify methods that may increase cold hardiness. Gene expression during cold acclimation of woody plants that exhibit high freeze tolerance is not well understood. *Poncirus trifoliata* is one species that has been documented for having high freeze tolerance (Yelenosky, 1985); *P. trifoliata* is used in breeding programs and as a rootstock for *Citrus* sp. There must be a significant genetic overlap among these species, yet differences in freeze tolerance likely result from significant differences in gene expression during cold acclimation (Lang et al., 2004; Zhang et al.,

2005). Studies have been conducted on *P. trifoliata* during cold acclimation to identify genes that are involved in its greater freeze tolerance.

## The Effect of Low Temperature on Plant Growth

Temperature, along with light and water, is one of the most critical factors in the physical environment of plants. This is especially so because plants are unable to maintain their tissues at a constant temperature. Environmental temperature therefore exerts a profound influence on cellular metabolism and, as a result, plant growth. Every individual enzymatic reaction, every metabolic function, every physical process- has temperature limits above and below which it cannot function. Temperature affects the integrity of cell structure (especially the structure and properties of membranes) and influences the direction of specific developmental events, such as metabolism (Alscher, 1990). During temperature decline, plants endure structural modifications in cells or functions that increase the fitness of the organism in low temperature stressful environments.

Plants that are able to withstand some freezing in nature are thought to do so by the nucleation and growth of ice outside the cells (Pearce, 1988). The extracellular ice grows mainly by withdrawing water from within the cell, thus dehydrating them. The amount of water withdrawn, and the extent of intercellular dehydration, increases as the temperature falls (Mazur, 1969; Gusta et al., 1975). Cells are killed when their tolerance to dehydration is exceeded. This is apparently due to failure of the structure of membranes (Steponkus, 1984; Pearce and Wilson, 1985), also called plasmolysis. The osmotic potential of cells has been frequently reported to increase during the acclimation

process and lead to better cold tolerance (Levitt, 1980). Much attention has been placed on changes in lipid composition (Thomashow, 1999) and the structural response of the plasma membrane to low-temperature stress in plants that exhibit extracellular freezing (Gordon-Kamm and Steponkus, 1984a, 1984b; Pearce, 1982; Pearce and Wilson, 1985; Singh, 1979; Wolfe and Steponkus, 1981). Ultrastructural studies of cells and tissues that exhibit extracellular freezing are numerous. Bark tissues of black locust (*Robinia pseudoacacia*) and mulberry (*Morus bombycis*) showed that the plasmalemma undergoes significant changes in structure and conformation during periods of acclimation and deacclimation; and that the plasmalemma response to a low-temperature stress is distinctively different in acclimated and non-acclimated cells and tissues (Pomeroy and Siminovitch, 1971; Niki and Sakai, 1981, 1982).

Membrane systems of the cell are a primary site of freeze-induced injury and this injury results largely from the severe dehydration associated with freezing (Levitt, 1980; Steponkus, 1984). Cellular dehydration can directly cause the formation of deleterious nonbilayer membrane structures such as hexagonal II-phase lipids. In addition, it can cause the generation of reactive oxygen species that can damage membranes and other cellular components (Levitt, 1980). Membrane lipids consist primarily of diacyglycerides containing 2 fatty acids. Some fatty acids are unsaturated, while others are fully saturated with hydrogen. Because saturated fatty acids- and lipids that contain them solidify at higher temperatures than unsaturated fatty acids, the relative proportions of unsaturated and saturated fatty acids in membrane lipids have a strong influence on the fluidity of membranes (Nishida, 1996). The point, at which the membrane changes from the fluid state to a gel (or semicrystalline) state, is known as the transition temperature. Chilling-

sensitive plants, such as *Citrus unshiu* tend to have a higher portion of saturated fatty acids and correspondingly a higher transition temperature (Alscher, 1990); whereas Chilling-resistant species, such as *Poncirus trifoliata* tend to have lower proportions of saturated fatty acids and therefore a lower transition temperature (Steponkus, 1978; Kratsch, et al., 2000). During acclimation to low temperature, the proportion of unsaturated fatty acids increases and transition temperature decreases (Nishida, 1996). The integrity of membrane channels is disrupted during the transition from the liquid state to the semicrystalline state. This results in loss of compartmentation and solute leakage, and the operation of integral proteins that make up respiratory assemblies and photosystems. Membranes of chilling-resistant or acclimated plants are able to maintain membrane fluidity to much lower temperature and thereby protect these critical cellular functions against damage (Nishida, 1996). Exposure of cold-sensitive plants to low positive temperatures induces destructive changes in the cell ultrastructure: swelling of chloroplast stroma (Kratsch, 2000), rupture of chloroplast envelope and lamellae, a decrease in the number of ribosomes, and failure in the tonoploast intactness (Ishikawa, 1996).

## Mechanisms of Injury in *Citrus* Species during Cold Acclimation

Cold hardiness is the ability of a plant or plant organ to resist freezing or subfreezing conditions (Fuchigami, 1996; Soule, 1985). Cold acclimation is the process of adapting to the change from summer to winter, also called hardening (Westwood, 1993). Citrus trees acclimate slowly and deacclimate rapidly in response to temperature, which affects cold hardiness (Yelenosky, 1985; Young, 1970). 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).

For practical purposes, citrus remains uninjured if ice does not form in the tissues during cold acclimation. This does not include the fruit peel, which is chill sensitive or injured at temperatures above 0˚C (Eaks, 1960; Purvis and Yelenosky, 1982). Thus any mechanism that prevents or delays ice formation in citrus tissues essentially increases freeze tolerance (Yelenosky, 1985). Ice avoidance as a result of freezing point depression (due to solute accumulation and/or decreases in tissue water) is considered less of a factor in the cold hardiness of citrus than either passive frost-protection methods or active supercooling (Yelenosky, 1985). Ice avoidance because of freezing point depression probably does not account for more than a 1˚C added protection (Yelenosky, 1985).

It is suspected that once ice has formed in citrus tissues, a critical amount of time, inversely related to minimum temperature, is required before irreversible injury is to be induced. Lethal injury may be induced within 3-5 minutes after the initial surge of ice crystallization in unhardened citrus seedlings less than one year old (Yelenosky and Horanic, 1969). Ice propagates quickly along citrus stems, usually from a single site, and probably occurs as a result of ice formation in the xylem (Yelenosky, 1975). The starting point of ice formation depends on the degree of acclimation, and starts at the basal portion of the stem in acclimated plants, but at the distal portion of the stem in non-acclimated plants (Yelenosky, 1975; Nesbitt et al., 2002). Citrus are injured by subfreezing temperatures only after ice forms within the tree (Spiegal-Roy and

Goldschmidt, 1996; Yelenosky, 1985), which usually occurs between -2.2 to -6.7˚C (Young, 1966). Ice can be initiated anywhere in the canopy, and propagates rapidly to the rest of the canopy (Lucas, 1954; Yelenosky, 1975, 1991). In citrus, the relatively slow rate of freezing results in the first ice being formed exclusively in the apoplast where water is purest (Westwood, 1993). Citrus plants can survive extensive extracellular freezing (Young and Peynado, 1967), but if freezing is rapid, at -10˚C/min, ice crystals may form suddenly in the symplast and death of those tissues invariably occurs (Young and Mann, 1974). Actively growing plants are typically unacclimated and are relatively hydrated (Yelenosky et al., 1984). During freezes, stem water freezes exerting an outward pressure on bark until it cracks, this causes direct injury to symplasts (Yelenosky et al., 1984). Citrus trees dehydrate as they acclimate to low temperatures (Yelenosky, 1978, 1982; Young, 1970; Young and Peynado, 1965), and there is enough room in the apoplast for ice crystals to form without directly penetrating the symplast. As temperature decreases further, the matric potential of ice drops below that of the osmotic potential of the symplast causing water to move towards the ice, which dehydrates the symplast (Ebel et al., 2005). Since the rate of water exit from the cells is critical to whether freezing occurs inside or outside the cells, factors affecting the permeability of membranes to water are important. Membranes of hardened plants are more permeable to water than nonhardened plants (Ebel et al., 2004).

Weiser (1970) has summarized data relating to plant cell death caused by slow freezing: Supercooling → Freezing of extracellular water → Rapid propagation of ice through the stem→ A rise in tissue temperature (exotherm) from release of heat of fusion → Further cooling after readily available water is frozen → Movement of water from the

protoplast out of the cell in response to the external vapor pressure deficit → Continued extracellular freezing of water → Growth of ice crystals → Shrinkage of protoplasts, plasmolysis, and concentration of cell solutes → Slowed migration of water to external ice as most or all freely water is frozen → Continued temperature reduction until the critical temperature is reached → Granulation of protoplasm → Death.

## Plant Water Relations during Cold Acclimation

It has been recognized early on that citrus develop plant water deficits during cold acclimation, and the development of these plant water deficits may play a role in cold tolerance (Yelenosky, 1985). Plants are subjected to a variety of environmental stresses, however, in the long run; water deficits reduce plant growth and yield more than all other stresses combined, because it is ubiquitous within the plant (Bunce, 1981). Water movement can be described as an analogy to Ohm's law, which states that flow is proportional to the driving force, the water potential, and inversely proportional to the resistances in the flow path. Factors affecting water movement can therefore be discussed in terms of effects on water potential and on resistances (Blizzard and Boyer, 1980; Newman, 1976; Tinker, 1976). Water deficits are characterized by decreases in water content, turgor, and total plant water potential, resulting in wilting, partial or complete closure of stomata, and decrease in cell enlargement and plant growth. If severe, water deficits cause cessation of photosynthesis, disturbance of many metabolic processes, and finally death (Bunce, 1982). The cause of water deficits is clear, but the manner in which they develop is complex.

Several environmental parameters influence water loss rates, such as, temperature, stomatal behavior, transpiration, and root resistance (resistance of water to move from the roots into the xylem) (Jarvis and Mansfield, 1981; Ball et al., 1987; Aphalo and Jarvis, 1993). Stomata are the adjustable portals through which plants gain $CO_2$ due to the saturation vapor pressure in the intercellular spaces of the leaf (Farquhar and Sharkey, 1982), simultaneously losing water vapor (Kriedemann and Barrs, 1981). Stomata respond to a wide range of aerial environmental factors: light, $CO_2$, humidity, temperature, wind, and leaf water status; therefore constitute a critical interface between the inside of the leaf and the plant's aerial environment (Cowan, 1977). In citrus, temperature and humidity are particular factors that influence stomatal aperture, which alter rate of water loss (Kriedemann and Barrs, 1981). The water loss resulting from stomatal behavior arises from an imbalance between water supply and demand. Water demand depends on the leaf-to-air vapor pressure gradient and is a function of leaf temperature and atmospheric vapor pressure gradient. The supply of the plant depends on the magnitude of liquid phase supply resistances, which in turn depend at least partially on soil water status and soil temperature (Rawlins, 1963; Smart and Bingham, 1974). Young and Bell (1974) reported that cold-acclimated citrus begin to exhibit increased diffusive resistance and decreased stomatal aperture at or around 12˚C. Yelenosky (1985) similarly reported citrus trees stop growing when ambient temperatures are below 12˚C. Based solely on stomatal behavior, closing stomata seemingly should minimize plant water deficit, but it is well recorded that in nature plant water deficits still develop during cold acclimation.

It is well known that stomatal resistance at the leaf-air-interface primarily controls movement of water through and out of the plant. Transpiration is the process by which plants release water vapor. Drying soil, cold soil, and inadequate aeration of the root medium all drastically reduce transpiration because they decrease absorption of water and produce a leaf water deficit that causes stomatal closure. Slatyer (1967) justified this theory by assuming that if beginning at zero time, for a plant in darkness with roots in water culture ($\Psi_{root} = 0$, $\Psi_{leaf} = 0$), transpiration rises to a steady state in about 30 minutes. After 1 hr, transpiration and absorption are occurring at the same rates, and the $\Delta\Psi$ between leaf and root, substrate is stabilized. The temperature of the root substrate is then reduced enough to increase $R_{root}$ and decrease water absorption. As absorption lags behind transpiration, leaf $\Psi_w$ decreases and water absorption returns to its original rate because of the driving force, $\Delta\Psi$ is increased. It is assumed that the decrease in leaf $\Psi_w$ was not large enough to cause stomatal closure. However, if the root substrate is cooled more, further reducing water absorption, before $\Delta\Psi$ has fallen low enough to bring water absorption back to the original level, leaf $\Psi_w$ will have decreased so much that partial closure of stomata occurs. This reduces transpiration until it is in balance at a lower level with the new reduced rate of absorption and a much greater $\Delta\Psi$. In summary, according to this concept, water flow through plants is controlled primarily in the vapor phase between the evaporating surfaces and the bulk air. The stomata are the principal regulators of water movement through plants; and increase in resistance to water flow elsewhere, especially in the soil and the root reduces transpiration indirectly by reducing leaf turgor enough to cause stomatal closure. Thus, although transpiration is controlled

directly by stomatal aperture, plant turgor is often reduced by decreased absorption of water.

The success of plants depends on the existence of a vascular system capable of moving from roots to shoots the large quantities of water lost by transpiration and the presence of a control or feedback mechanism that keeps absorption and transpiration approximately balance (Aphalo and Jarvis, 1993). In simple terms, water deficits develop during periods when water loss in transpiration exceeds absorption. This occurs to some extent every sunny morning in plants that are transpiring at normal rates, but becomes more severe when water absorption is limited by drying, decreasing temperatures, or a combination of the two (Kramer, 1982; Slatyer, 1967).

## The Role of Root Hydraulic Conductivity

Roots impose about 2/3 of the total liquid water flow in the soil-plant – atmosphere, the water potential gradient from soil to atmosphere is known to be the driving force (Rieger and Litvin, 1999; Düring et al., 1996). Soil temperature has pronounced effects on many physiological processes in plants such as root growth, ion absorption, hydraulic conductivity and associations with symbiotic organisms (Kramer, 1969; Nielsen, 1974; Syvertsen et al., 1983). If root temperatures are above or below optimum temperatures, the hydraulic conductivity of roots may be reduced and thus may indirectly affect stomatal conductance, $CO_2$ assimilation, and subsequent translocation of carbohydrates (Kadoya et al., 1981). In the case of citrus, roots acclimate to cool soil (15˚C) through an increase in conductivity of individual roots (Syvertsen, 1983). Root

signaling has been regarded as an “early warning system” which starts to induce adaptation processes *before* water status declines (Düring, 1996).

## Gene Expression during Cold Acclimation

Cold acclimation is a physiological modification that is induced by gradual exposure to chilling temperatures which enable the plant to maintain homeostasis and ultimately, to survive and reproduce in a stressful environment (Levitt, 1980). The ability to cold-acclimate is a polygenic trait involving a large number of genes whose expression is controlled mainly by low temperature (Browse and Xin, 2001; Suzuki et al., 2000). Alterations in expression of these genes lead to numerous molecular and physiological changes characteristic of the cold acclimation process. The combined effect of the corresponding gene products is reflected in the amount of genes that are affected by low temperature, which according to a recent estimate is up to 25% of the transcriptome in *Arabidopsis thaliana* (Kreps et al., 2002). Therefore, cold acclimation must include the activation of mechanisms that protect cells against the potentially deleterious consequences of dehydration.

Cold acclimation induces changes in gene expression. Several plant genes induced by low temperatures have been identified in alfalfa (Wolfraim et al., 1993), *A. thaliana* (Gilmour et al., 1992), and strawberry (Yubero-Serrano et al., 2003). During cold acclimation, there are changes in mRNA transcription, increases in protein synthesis, and qualitative changes in the pattern of proteins synthesized (Gilmour et al., 1992). A major class of cold-induced genes encode homologs of late embryogenesis active proteins (LEA-proteins) that are synthesized late in embryogenesis and during

dehydration stress. A low-temperature-responsive dehydrin-like protein, wcor410, is involved in cryoprotection of the plasma membrane (Danyluk et al., 1994). Proteins encoded by cold-regulated genes (COR) share common physical properties: they are hydrophilic, remain soluble upon boiling in dilute aqueous buffer, and exhibit relatively simple amino acid sequences that form amphipathic $\alpha$- helices. The expression of these cold-induced genes results in increased freezing tolerance (Thomashow, 1999).

The promoter regions of certain COR proteins are activated in response to low temperature and dehydration stress, and are involved in membrane stability (Thomashow, 1998). Analysis of these promoter regions of *COR* genes of *Arabidopsis* led to the identification of a DNA regulatory element called the *d*ehydration *r*esponsive *e*lement (DRE). The DRE has a conserved core C-repeat sequence of CCGAC that communicates responsiveness to low temperature and dehydration. Specific proteins that bind to the DRE are called C-repeat *b*inding *f*actors (CBFs). *CBF1* is the DNA binding protein isolated from *A. thaliana*. The binding of this transcription factor to the C-repeated/dehydration response element (*CRT/DRE*-motif) can activate the co-ordinate expression of several *COR* genes. Expression of *CBF1* in transgenic plants resulted in enhanced freezing tolerance (Jaglo-Ottosen et al., 1998; Liu et al., 1998). It has been concluded that cold acclimation is regulated by DRE-containing genes that are induced by CBF transcriptional factors (Thomashow, 1999). Interest in understanding the molecular basis of cold acclimation is driven by both a desire to understand the mechanisms that plants have evolved to tolerate environmental stresses and the prospect that such knowledge might provide new strategies to improve the environmental stress

tolerance of agriculturally important plants that are cold sensitive such as commercial *Citrus* sp.

**Freeze Tolerance of Trifoliate Orange and Satsuma Mandarin**

One of the most important factors in the acclimation process and the subsequent tolerance of freezing in citrus is the cold-hardy rootstock (Gardner and Horanic, 1958; Yelenosky, 1978). *P. trifoliata* is a very cold tolerant citrus species that is capable of surviving freezes to -26˚C (Spiegel-Roy and Goldschmidt, 1996); whereas Satsuma mandarin, one of the most cold hardy of commercial citrus species, cannot survive below -10˚C (Yelenosky, 1985). Although *P. trifoliata* is in another genus, there is sufficient genetic capability with Satsuma mandarin and other species that allow cross grafting and also cross-fertilization (Ebel et al., 2004; Zhang, 2005). The evidence that *Poncirus trifoliata* is more cold hardy is based on field observations alone. It is well documented for both trifoliate orange and Satsuma mandarin that drought and cold temperature induce plant water deficits and decreased stomatal behavior. However, it is not known whether cold temperatures induce higher water deficits in more cold hardy species such as trifoliate orange, that in turn activate genes that increase cold tolerance; and if the difference in cold tolerance between trifoliate orange and Satsuma mandarin is due to differences in water relations. If this were to be proven true this would partially explain the greater freeze tolerance of trifoliate orange compared to Satsuma mandarin.

The purpose of this experiment is to compare plant water deficits of freeze tolerant (*P. trifoliata)* and freeze sensitive (Satsuma mandarin) species during cold acclimation; also to determine correlation's between plant water deficits and capabilities

of these species during cold acclimation. The results of this experiment will partially determine if there is a specific gene expression between the roots and the plant that initiate the plants ability to begin the cold acclimation process.

## Literature Cited

Alscher, R.G. 1990. Stress Responses in Plants: Adaptation and Acclimation Mechanisms. Wiley-Liss, New York.

Aphalo, P.J. and P.G. Jarvis. 1993. The boundary layer and the apparent responses of stomatal conductance to wind speed and to the mole fractions of CO2 and water vapour in the air. Plant. Cell and Environ. 16:771-783.

Attaway, J.A. 1997. A history Florida citrus freezes. Florida Science Source. Lake Alfred Fla.

Ball, J.T., I.E. Woodrow, and J.A. Berry. 1987. A model predicting stomatal conductance and it's contribution to the control of photosynthesis under different environmental conditions. Progress in Photo. Res. 4:221-228.

Bourgeois, W.J. and A.J. Adams, 1987. Low-volume scaffold branch irrigation for citrus freeze protection. HortScience 22:48-50.

Bourgeois, W.J., A.J. Adams, and D.R. Snipe. 1990. Effectiveness of scaffold branch irrigation for freeze protection of Louisiana citrus during 1989 freeze. Proc. Fla. State Hort. Soc. 103: 62-64.

Browse, J. and Z.G. Xin. 2001. Temperature and sensing and cold acclimation. Curr. Opin. Plant Biol. 4:241-246.

Bruce, J.A. 1981. Relationships between maximum photosynthetic rates and photosynthetic tolerance of low leaf water potentials. Can. J. Bot. 59:769-774.

Bruce, J.A. 1982. Effects of water stress on photosynthesis in relation to diurnal acclimation of carbohydrate in source leaves. Can. J. Bot. 60:195-200.

Blizzard, W.E. and J.S. Boyer. 1980. Comparative resistance of the soil and the plant to water transport. Plant Physiol. 66:809-814.

Buchanan, D.W., F.S. Davies, and D.S. Harrison. 1982. High and low volume under tree-irrigation for citrus cold protection. Proc. Fla. State Hort. Soc. 95:23-26.

Cowan, I.R. 1977. Stomatal behavior and environment. Adv. Bot. Res. 4:117-228.

Danyluk, J., M. Houde, and F. Sarhan. Differential expression of a gene encoding an acidic dehydrin in chilling-sensitive and freezing-tolerant *Gramineae* species. FEBS Lett. 344:20-24.

Düring, H., B.R. Loveys, and P.R. Dry. 1996. Root signals affect water use efficiency and shoot growth. Acta Hort. 427:1-13.

Eaks, I.L. 1960. Physiological studies of chilling injury in citrus fruits. Plant Physiol. 35:632-636.

Ebel, R.C., P.A. Carter, W.A. Dozier, D.A. Findley, M.L. Nesbitt, B.R. Hockema, and J.L. Sibley. 2004. Pattern of exotherm and electrolyte leakage measured at high frequency of Satsuma mandarin exposed to subfreezing temperatures. HortScience 39:1-3.

Ebel, R.C., B.L. Campbell, M.L. Nesbitt, W.A. Dozier, J.K. Lindsey, and B.S. Wilkins. 2005. A temperature index model to estimate long-term freeze-risk of Satsuma mandarins grown on the northern coast of the Gulf of Mexico. J. Amer. Soc. Hort. Sci. 130:500-507.

Farquhar, G.D. and T.D. Sharkey. 1982. Stomatal conductance and photosynthesis. Annu. Rev. Plant Physiol. 33:317-345.

Fuchigami, L.H. 1996. Cold hardiness, p. 146 In: F.B. Salisbury (ed.) Units, Symbols, and Terminology for Plant Physiology: A Reference for Presentation of Results in Plant Sciences, Oxford Univ. Press, New York.

Gardner, F.D. and G.E. Horanic. 1958. Influence of various rootstocks on the cold resistance of the scion variety. Proc. Fla. State Hort. Sci. 71:81-86.

Gilmour, S.J., N. Artus, and M.F. Thomashow. 1992. cDNA sequence analysis and expression of two cold-regulated genes of *Arabidopsis thaliana*. Plant Mol. Biol. 18:13-21.

Gordon-Kamm, W.J and P.L. Steponkus. 1984a. The behavior of the plasma membrane following osmotic contraction of isolated protoplasts: implications in freezing injury. Protoplasma 123:83-94.

Gordon-Kamm, W.J. and P.L. Steponkus. 1984b. The influence of cold acclimation on the behavior of the plasma membrane following osmotic contraction of isolated protoplasts. Protoplasma 123:161-173.

Gusta, L.V., M.J. Burke, and A. Kapoor. 1975. Determination of unfrozen water in winter cereals at subfreezing temperatures. Plant Physiol. 56:707-709.

Iba, K. 2002. Acclimative response to temperatures stress in higher plants: approaches of gene engineering for temperature tolerance. Annu. Rev. Plant Biol. 53:225-246.

Ishikawa, H.A. 1996. Ultrastructural features of chilling injury: Injured cells and the early events during chilling of suspension-cultured mung bean cells. Am. J. Bot 83:825-835.

Jaglo-Ottosen, K.R., S.J. Gilmour, D.G. Zarka, O. Schabenberger, and M.F. Thomashow. 1998. *Arabidopsis CBF1* overexpression induces *COR* genes and enhances freezing tolerance. Sci. 280:104-106.

Jarvis, P.G. and T.A. Mansfield. 1981. Stomatal Physiology. Cambridge Univ. Press, London and New York.

Kadoya, K., F. Mizutani, and A. Hino. 1981. Effects of root environmental conditions on stomatal behavior and translocation of photosynthates. Proc. Intl. Soc. Citriculture. 2:498-501.

Kramer, P.J. 1969. Plant and Soil Water Relationships, p. 482. McGraw-Hill, Inc.

Kramer, P.J. 1982. Water and plant productivity or yield, p. 41-47. In: M. Rechcigl, Jr. (ed.) Handbook of Agricultural Productivity, Vol. 1, CRC Press, Boca Raton, Florida.

Kratsch, H.A., and Wise R.R. 2000. The ultrastructure of chilling stress. Plant Cell Environ. 23:337-350.

Kreps, J.A., Y. Wu, H.S. Chang, T. Zhu, X. Wang, and J.F. Harper. 2002. Transcriptome changes for *Arabidopsis* in response to salt, osmotic, and cold stress. Plant Physiol. 130:2129-2141.

Kriedemann, P.E. and H.D. Barrs. 1981. Citrus orchards, p. 325-417. In: T.T. Kozlowski (ed.) Water Deficits and Plant Growth, Vol. 6, Academic Press, New York.

Lang, P., C.K. Zhang, R.C. Ebel, F.Dane, and W.A. Dozier. 2004. Identification of cold acclimated genes in leaves of *Citrus unshiu* by mRNA differential display. Gene 359:111-118.

Levitt, J. 1980. Responses of plants to environmental stresses. Second Edition, Vol.1. Chilling, freezing, and high temperature stresses. Academic Press, New York.

Lipe, W.N., O. Wilke, and O. Newton. 1977. Freeze protection of peaches by evaporative cooling in the post-rest, pre-bloom period. J. Amer. Soc. Hort. Sci. 102:370-372.

Liu, Q., M. Kasuga, Y. Sakuma, H. Abe, S. Miura, K. Yamaguchi-Shinozaki, and K. Shinozaki. 1998. Two transcription factors, *DREB1* and *DREB2*, with an *EREBP/AP2* DNA binding domain separate two cellular signal transduction pathways in drought-and-low-temperature-responsive gene expression, respectively, in *Arabidopsis*. Plant Cell 10:1391-1406.

Lucas, J.W. 1954. Subcooling and ice nucleation in lemons. Plant Physiol. 29:246-251.

Mazur, P. 1969. Freezing injury in plants. Annu. Rev. Plant Physiol. 20:419-448.

Nesbitt, M.L., R.C. Ebel, D. Findley, B.S. Wilkins, F. Woods, and D. Himelrick. 2002. Assays to asses freeze injury of Satsuma mandarin. HortScience. 37:871-877.

Newman, E.I. 1976. Water movement through root systems. Philos. Trans. R. Soc. London, Ser. 273:463-478.

Nielsen, K.F. 1974. Roots and root temperatures. In: E.W Carson (ed.) The Plant Root Environment, Univ. Press, Virginia.

Niki, T. and A. Sakai. 1981. Ultrastructural changes related to frost hardiness in the cortical parenchyma cells from mulberry twigs. Plant Cell Physiol. 22:171-183.

Niki, T. and A. Sakai. 1982. Effect of cycloheximide on the freezing tolerance and ultrastructure of cortical parenchyma cells from mulberry twigs. Can. J. Bot. 61:2205-2211.

Nishida, I. and N. Murata. 1996. Chilling sensitivity in plants and cyanobacteria: the crucial contribution of membrane lipids. Annu. Rev. Plant Physiol. Plant Mol. Bio. 47:541-568.

Parson, L.R., T.A. Wheaton, D.P.H. Tucker, and J.D. Whitney. 1983. Low volume microsprinkler irrigation for citrus cold protection. Proc. Fla. State Hort. Soc. 95:20-23.

Pearce, R.S. 1982. Ultrastructure of tall fescue (*Festuca arundinacea* Schreb. cv 5170) cells fixed while exposed to lethal or non-lethal extracellular freezing. New Phytol. 92:259-272.

Pearce, R.S. 1988. Extracellular ice and shape in frost-stressed cereal leaves: A low-temperature scanning-electron-microscopy study. Planta 175:313-324.

Pearce, R.S. and J.H.M. Wilson. 1985. A freeze-etch study of the effect of extracellular ice freezing on the cellular membranes of wheat. Planta 163:304-316.

Pomeroy, M.K. and D. Siminovitch. 1971. Seasonal cytological changes in secondary phloem parenchyma cells in *Robinia pseudoacacia* in relation to cold hardiness. Can. J. Bot. 49:787-795.

Purvis, A.C. and G. Yelenosky. 1982. Sugar and proline accumulation of grapefruit flavedo and leaves during cold hardening of young trees. J. Amer. Soc. Hort. Sci. 107:222-226.

Rawlins, S.L. 1963. Resistance to water flow in the transpiration stream. Bull.-Conn., Agric. Exp. Stn., New Haven 664:69-85.

Rieger, M. and P. Litvin. 1999. Root system hydraulic conductivity in species with contrasting root anatomy. J. Exp. Bot. 50(331):201-209.

Singh, J. 1979. Ultrastructural alterations in cells of hardened and non-hardened winter rye during hyperosmotic and extracellular freezing stresses. Protoplasma 98:329-341.

Slatyer, R.O. 1967. Plant-Water Relationships. Academic Press, New York.

Smart, R.E. and G.E. Bingham. 1974. Rapid estimates of relative water content. Plant Physiol. 53:258-260.

Soule, J. 1985. Glossary for Horticultural Crops. Wiley, New York.

Spiegal-Roy, P. and E.E. Goldschmidt. 1996. Biology of Citrus, p. 30. Cambridge University Press, Cambridge, UK.

Steponkus, P.L. 1978. Cold hardiness and freezing injury of agronomic crops. Adv. Agron. 30:51-98.

Steponkus, P.L. 1984. Role of the plasma membrane in freezing injury and cold acclimation. Annu. Rev. Plant Physiol. 35:543-584.

Suzuki, I., D.A. Los, Y. Kanesaki, K. Mikami, and N. Murata. 2000. The pathway for perception and transduction of low-temperature signals in *Synechocystis*. EMBO J. 19:1327-1334.

Syvertsen, J.P., R.M. Zablotowicz, and M.L. Smith, JR. 1983. Soil Temperature and flooding effects on two species of citrus. Plant and Soil 72(3): 3-12.

Thomashow, M.F. 1998. Role of cold-responsive genes in plant freezing tolerance. Plant Physiol. 118:1-7.

Thomashow, M.F. 1999. Plant cold acclimation: Freezing tolerance genes and regulatory mechanisms. Annu. Rev. Plant Physiol. Plant Molec. Bio. 50: 571-599.

Tinker, P.B. 1976. Roots and water. Transport of water to plant roots in soil. Philos. Trans. R. Soc. London, Ser. 273:445-461.

Westwood, M.N. 1993. Temperature-Zone Pomology Physiology and Culture. Third Edition. Timber Press, Portland Oregon.

Weiser, C.J. 1970. Cold resistance and injury in woody plants. Sci. 169:1299-1278.

Winberg, O.F.E. 1948. The Satsuma, p.1-2. In: Fairhope Courier (daily periodical) Nov.1948. Fairhope Ala.

Wisiniewski. M., C. Bassett, and L.V, Gusta. 2003. An overview of cold hardiness in woody plants: seeing the forest through the trees. HortScience 38:952-959.

Wolfe, J. and P. L. Steponkus. 1981. The stress-strain relation of the plasma membrane of isolated plant protoplasts. Biochem. Biophys. Acta 643:663-668.

Wolfraim, L.A., R. Langis, H. Tyson, and R.S. Dhindsa. 1993. cDNA sequence, expression, and transcript stability of a cold acclimation-specific gene, *cas18*, of alfalfa (*Medicaga falcata*) cells. Plant Physiol. 101:1275-1282.

Yelenosky, G. and G. Horanic. 1969. Supercooling in wood of citrus seedlings. Cryobiology 5:281-283.

Yelenosky, G. 1975. Cold hardening in citrus stems. Plant Physiol. 56:540-543.

Yelenosky, G. 1978a. Coldhardening of 'Valencia' orange trees to tolerate -6.7C without injury. J. Amer. Soc. Hort. Sci. 103:449-452.

Yelenosky, G. 1978b. Freeze survival of citrus trees in Florida p. 297-311. In: P.H. Li and A. Sakai (eds.) Plant Cold Hardiness and Freezing Stress. Academic Press, New York.

Yelenosky, G. 1982. Indicators of citrus cold-hardening in the field. Proc. Fla. State Hort. Soc. 95:7-10.

Yelenosky, G., C.J. Hearn, and D.J. Hutchison. 1984. Nonhardening temperatures-major factors in freeze damage to citrus trees in December 1983. Proc. Fla. State Hort. Soc. 97:33-36.

Yelenosky, G. 1985. Cold hardiness in citrus. Hort. Rev. 7:201-238.

Yelenosky, G. 1991. Minireview: Responses and adaptions of citrus trees to environmental stresses. Israel J. Bot. 40:239-250.

Young, R. and A. Peynado. 1965. Changes in cold hardiness and certain physiological factors of Red Blush grapefruit seedlings as affected by exposure to artificial hardening temperatures. Proc. Amer. Soc. Hort. Sci. 86:244-252.

Young, R. 1966. Freezing points and lethal temperatures of citrus leaves. Proc. Amer. Soc. Hort. Sci. 88:272-279.

Young, R. and A. Peynado. 1967. Freezing and water-soaking in citrus leaves. Proc. Amer. Soc. Hort. Sci. 91:157-162.

Young, R. 1970. Induction of dormancy and cold hardiness in citrus. HortScience. 5:411-413.

Young, R. and W.D. Bell, 1974. Photosynthesis in detached leaves of cold-hardened citrus seedlings. J. Amer. Soc. Hort. Sci. 99:400-403.

Young, R., and M. Mann. 1974. Freeze distribution of sour orange leaf cells. J. Amer. Soc. Hort. Sci. 99:403-407.

Yubero-Serrano, E., E. Moyano, N. Medina-Escobar, J. Munoz-Blanco, and J. Caballero. 2003. Identification of a strawberry gene encoding a non-specific lipid transfer protein that responds to ABA, wounding and cold stress. J. Exp. Bot. 54:1865-1877.

Zhang, C. and P. Lang, F. Dane, R.C. Ebel, N.K. Singh, R. Locy, and W.A. Dozier. 2005. Cold acclimation induces genes of trifoliate orange (*Poncirus trifoliata)*. Plant Cell Reports 23:764-76.

# CHAPTER II

## PLANT WATER RELATIONS OF *PONCIRUS TRIFOLIATA* (TRIFOLIATE ORANGE) AND *CITRUS UNSHIU* (SATSUMA MANDARIN) DURING COLD ACCLIAMTION


### Abstract

*P. trifoliata* is a very cold tolerant citrus species that is capable of surviving freezes to -26˚C, whereas *C. unshiu* cannot survive below -10˚C. It is not known if the difference in cold tolerance of these two species is related to differences in water relations during cold acclimation, even when drought stress is minimized. *C. unshiu* and *P. trifoliata* trees were kept unacclimated by holding day/night air temperatures at 25˚C/20˚C, or acclimated by dropping day/night air temperatures weekly from 25˚C/20˚C to 20˚C/15˚C to 15˚C/10˚C, and then to 10˚C/5˚C for 9 weeks. During cold acclimation, stomata closed similarly for both species. Stomata never completely closed, which allowed low levels of transpiration and $CO_2$ assimilation. Root resistance was higher for *C. unshiu* than *P. trifoliata*, and was higher for acclimated than unacclimated plants. Plant water deficits, as measured by stem water potential, declined similarly for both species during cold acclimation, while remaining similar and constant when unacclimated. The similarity in plant water deficits between species during cold acclimation and the responses of stomata and root resistance cannot explain the contrasting cold hardiness of these two species.

## Introduction

Cold temperatures promote citrus cold tolerance (Ebel et al., 2005; Yelenosky et al., 1984; Yelenosky, 1978, 1985, 1996; Young, 1961), which is often characterized by development of plant water deficits (Yamada et al., 1985; Yelenosky, 1982, 1985). Plant dehydration during cold acclimation may be an important adaptive mechanism that enhances survival during freeze events. Drought stress has been shown to enhance cold tolerance in addition to promoting plant water deficits (Davies et al., 1981, Yelenosky, 1978, 1979), but whether cold acclimation promotes plant water deficits under well-watered conditions is not well understood.

Plant water deficits are an important component for survival during freeze events. It is commonly held that freezes cause ice to form in the apoplast (Meryman, 1956, 1966; Yelenosky, 1985, 1996). Water expands by 9% as it turns into ice and whether the expanding ice crystals penetrate the symplast, which is lethal, depends on the extent of apoplastic hydration. Unacclimated citrus have fully turgid apoplasts especially when soil moisture is not limiting such that freeze events cause apoplastic ice to directly penetrate the symplast (Young and Mann, 1974). The most obvious example of excessive apopolastic water of *Citrus sp.* during freeze events is the outward expansion of bark that develops cracks (Ebel et al., 2004; Nesbitt et al., 2002). Dehydration during cold acclimation reduces apoplastic water content, which results in more air space for ice crystals to grow without penetrating the symplast. Death occurs during severe freezes of fully cold acclimated plants when the symplast dehydrates (Steponkus, 1984; Yelenosky, 1985) because the matric potential of the ice declines below the osmotic potential of the symplast.

It is not clear if species with contrasting cold tolerance, such as *C. unshiu* and *P. trifoliata*, develop different levels of dehydration during cold acclimation. *C. unshiu* has a maximum cold hardiness of about -10˚C (Yelenosky, 1985) whereas *P. trifoliata*, a close relative of commercial *Citrus sp.* within the family Rutaceae*,* can survive -26˚C (Spiegel-Roy and Goldschmidt, 1996). Considering that there is considerable overlap in gene expression during cold acclimation and drought stress of these plants (Xiong et al., 2002; Zhang et al., 2004), it is conceivable that cold temperatures induce differences in plant water deficits that may in turn induce differential gene expression. *P. trifoliata* is currently being used as a model plant for *Citrus sp.* to understand differential gene expression that lends itself to their contrasting cold tolerance (Cai et al., 1994, 1995; Champ et al., 2007; Durham et al., 1991; Lang et al., 2005; Moore et al., 2000; Porat et al., 2002; Sahin-Cevik, 2006a, 2006b, 2006c; Weber et al., 2003; Yelenosky et al., 1993; Zhang et al., 2004, 2005).

Understanding how plants with contrasting cold tolerance might differentially alter plant water deficits requires an understanding of how plants regulate water movement. Water movement in the soil-plant-atmosphere continuum can be described using an analogy of Ohm's law (Landsberg and Jones, 1981; Van den Honert, 1948), which describes flow as being proportional to driving force (the water potential gradient) and inversely proportional to the resistances in the flow path:

**Equation 1:** $$\mathbf{T = (\rho_{vs} - \rho_{va}) / (r_{vs} - r_{va}) = (\Psi_{soil} - \Psi_{stem}) / (R_{root} + R_{soil})}$$

where T is transpiration, $\rho_{vs}$ is vapor pressure at the leaf surface, $\rho_{va}$ is vapor pressure of the atmosphere, $r_{vs}$ is stomatal resistance and the inverse is stomatal conductance, $r_{va}$ is boundry layer resistance, $\Psi_{soil}$ is soil water potential, $\Psi_{stem}$ is stem water potential, $R_{root}$ is

root resistance, and $R_{soil}$ is resistance of water movement from the soil to the root surface. Transpiration is driven by evaporative demand ($\rho_{vs} - \rho_{va}$), which is controlled by stomata at the leaf-air-interface and accelerated by wind which reduces $r_{va}$. Water evaporation from the substomatal cavity causes plant water deficits to develop as indicated by a decrease in $\Psi_{stem}$. As $\Psi_{stem}$ declines below $\Psi_{soil}$, water is pulled into the plant from the soil. $R_{root}$ represents about 2/3 of total plant hydraulic resistance (Landsberg and Jones, 1981; Passioura, 1988) and therefore is an important control point for water movement in the soil-plant-atmosphere continuum. Plants regulate water movement and plant water deficits through $r_{vs}$ and $R_{root}$ (Alphalo and Jarvis, 1993; Ball et al., 1987; Jarvis and Morrison, 1981; Kramer, 1985).

Dehydration of citrus during cold acclimation is partly due to an increase in $R_{root}$, which inhibits water uptake (Kadoya et al., 1981; Kramer, 1969; Nielson, 1974; Syvertsen et al., 1983). Although maintaining open stomata would promote dehydration, stomata close during cold acclimation (Bialoglowski, 1937; Cameron, 1941; Young and Bell, 1974), which may be a result of higher $R_{root}$ (Kadoya et al., 1981). The objective of this study was to determine whether there are differences in $\Psi_{stem}$, $r_{vs}$, and $R_{root}$ between *P. trifoliata* and *C. unshiu* during cold temperature exposure when soil moisture is not limiting. The results are discussed with respect to how differences may explain the contrasting cold hardiness of these two species.

## Materials and Methods

*Plant material and culture.* Two year-old Satsuma mandarin [*Citrus unshui* (Marc.) 'Owari'] budded on trifoliate orange rootstocks [*Poncirus trifoliata* (L.) Raf.

‘Rubidoux’] and ungrafted *P. trifoliata* ‘Rubidoux’ plants were used in this experiment. Shoot growth of test plants varied from 60 to 90 cm in total length above the graft union for Satsuma mandarin and single, staked trifoliate orange were 31 to 85 cm high at the time of the experiment. Plants were grown in 3.8-L pots in a 1 pine bark : 1 sand substrate by volume amended with 4.45 $kg \cdot m^{-3}$ of dolomitic lime, 8.9 $kg \cdot m^{-3}$ of 18-6-12 Polyon 12-14 month controlled release fertilizer (Pursell Technologies, Inc, Sylacauga, Ala.), and 0.89 $kg \cdot m^{-3}$ a micronutrient package (Micromax, The Scotts Co., Marysville, Ohio). All plants were supplemented with water soluble $20N\text{-}20P_2O_5\text{-}20K_2O$ with micronutrients, which were applied at 234 $mg \cdot L^{-1}$ N when watered. The plants in this experiment were watered when soil moisture dropped below 85% of the water holding capacity of the substrate except on days where physiological measurements were made.

*Acclimation treatments.* Acclimation treatments were conducted in two environmental growth chambers (Model E15; Conviron, Winepeg, Canada) with fluorescent lighting (320 $\mu mol \cdot m^{-1} \cdot s^{-1}$ at midtree height) and ambient relative humidity. Acclimation treatments were chosen based on their relationship to temperature, growth, and acclimation. Citrus growth ceases below 12˚C, plants cold acclimate below 10˚C, and are fully cold-hardened when exposed to 10˚C or less for about 3 weeks (Bain, 1949; Cooper et al., 1954; Yelenosky et al., 1984; Yelenosky, 1985; Young, 1961; Young and Peynado, 1962, 1965). Plants in the unacclimated treatment were exposed to air temperature of 25˚C day/20˚C night. Plants in the acclimated treatment were exposed to air temperatures of 25˚C day/20˚C night for 7 d; 20˚C day/15˚C night for 7 d; 15˚C day/10˚C night for 7 d; and 10˚C day/5˚C night for 35 d. Both acclimation treatments were subjected to a 12/12h light/dark photoperiod throughout the experiment. The cold

acclimation treatment was previously shown to produce a fully cold hardened state for *C. unshiu* (Ebel et al., 2004; Nesbitt et al., 2002).

*Data Collected.* Data were collected 1x/week for 9 weeks.

On the day data was collected, plants were watered 3-4 hours after the start of the light period. Twenty minutes after watering, gas exchange measurements were performed on three leaves per plant using a portable photosynthesis system (Model 6400, LiCor, Lincoln, Nebraska). The most recently mature leaves; approximately the fifth from the terminal were measured. All plants in both chambers were watered again and left for 20 minutes to ensure that they were well watered before $\Psi_{stem}$ measurements were collected.

$\Psi_{stem}$ measurements were performed using two leaves from each plant, which included the fifth to tenth leaf from the shoot tip. Leaves were initially wrapped in plastic then aluminum foil the night before data was collected to allow $\Psi_{leaves}$ to equilibrate to $\Psi_{stem}$ (Garnier and Berger, 1985). Wrapped leaves were severed at the base of the petiole with a razor blade and $\Psi_{stem}$ measured using a pressure chamber (Model 1000, PMS Instrument Co., Corvallis, Oregon) that was pressurized at 1 MPa/20 s using compressed air. Following $\Psi_{stem}$ measurement, leaf area of each leaf was measured, which was used to calculate whole plant transpiration as described below (LI-3100 Area Meter, LiCor Inc., Lincoln, Nebraska).

After the $\Psi_{stem}$ measurement, which was 2 hr after the last watering, each pot was wrapped in a plastic bag to minimize evaporation from the substrate and weighed. The plants were returned to the chamber and whole plant transpiration was allowed to occur for 4 hr after which plastic bags were removed and pots were reweighed.

After the pots were weighed a second time, soil moisture was measured using a portable soil moisture meter (Type HH2, Delta T Devices Ltd., Burwell Cambridge, UK) on each plant. Three readings per plant were measured. After soil moisture readings were performed, the temperature in the acclimated chamber was decreased according to the previously stated regimen for temperature decline.

After data were collected at the end of the $9^{th}$ week, all leaves were removed and leaf area measured. Leaf area of new growth since the start of the experiment was measured separately from that of leaves present at the start of the experiment. New leaf growth was identified by a tag that had been wrapped around the stem at the start of the experiment. Total leaf area from each week was determined by adding the final leaf area with the leaf area of leaves removed to measure $\Psi_{stem}$, and adding leaf area of new growth assuming a linear growth rate for the entire treatment period for unacclimated plants, and for the first 3 weeks for the acclimated plants after which growth had stopped.

*Theoretical Approach for Determining $R_{root}$.* $R_{root}$ was estimated by utilizing the variables from the first and third quantities of Equation 1, which can be rearranged to solve for $R_{root}$:

**Equation 2:** $$R_{root} = [(\Psi_{soil} - \Psi_{stem}) / T] - R_{soil}$$

Before and during the day of data collection, the soil was maintained in a well watered state. We assume that $\Psi_{soil}$ was equal to zero. $\Psi_{soil}$ for soil at water holding capacity is generally considered to be about -0.3 MPa such that assuming that it is zero in our study introduces a small error. We also assume that $R_{soil}$ was equal to zero, which is a reasonable assumption for well-watered soils (Reicosky and Richie, 1976; Reid and Hutchinson, 1986). These assumptions simplify Equation 2 to:

**Equation 3:** $R_{root} = -\Psi_{stem} / T$

$\Psi_{stem}$ was measured, and T was determined by:

**Equation 4:** $T = (PW_0 - PW_t) / A_{leaf} / t$

where PW was pot weight (g) measured 2 hrs after watering ($PW_o$) and about 4 hrs later ($PW_t$), $A_{leaf}$ is total leaf area of the plant ($m^2$), and t was the time elapsed between weight measurements (s). To determine $R_{root}$ in units of $MPa \cdot s \cdot m^{-1}$, units that are the most commonly reported in the literature for liquid resistance, T was multiplied by $1 \times 10^{-6}$ $m^3 \cdot g^{-1}$ to convert transpiration unit from weight ($g \cdot m^{-2} \cdot s^{-1}$) to volume ($m^3 \cdot m^{-2} \cdot s^{-1}$).

*Statistical Analysis.* Data were analyzed as a 2 (species) x 2 (acclimation treatment) factorial, split plot over time (week), blocked by location within each growth chamber (5 blocks, one plant of each genotype/block), and blocked by experimental run. The experiment was conducted twice.

## Results and Discussion

The lack of a species by acclimation by week (S*A*W) interaction for $\Psi_{stem}$ ($P > F = 0.4787$, Table 2.1) but a significant A*W interaction ($P > F = <0.0001$) indicates that $\Psi_{stem}$ decreased similarly for both species during cold acclimation but remained nearly constant at about -0.3 MPa for the control trees (Fig. 2.1). The decrease in $\Psi_{stem}$ indicates cold acclimated plants had higher plant water deficits. These results concur with other studies on *C. unshiu* that show a decrease in leaf water potential during cold acclimation (Yamada et al., 1985; Yelenosky, 1982) and less water content (Yelenosky, 1982), but vary with other studies on *Citrus sp.* where leaf water potential of cold acclimated citrus

was higher than unacclimated controls (Vu and Yelenosky, 1987). The different effect of cold acclimation on plant water potential in Vu and Yeleonsky's (1987) study may relate to temperatures and possibly to evaporative demand the trees were exposed to. They used substantially higher light period temperatures for the acclimated (15.6˚C) and unacclimated (32.2˚C) treatments and higher dark period temperatures for the acclimated (5.4˚C) and unacclimated (21.1˚C) treatments than in the current study. Their higher temperatures may have resulted in higher evaporative demand for unacclimated trees that may have had a larger impact on plant water potential than in our study. The difference in water relations was probably not due to drought stress since their watering approach was similar to ours.

Leaf conductance decreased for both species during cold acclimation (Fig. 2.2). Although there was a significant S*A*W interaction ($P > F = 0.0190$), the differences were due to higher conductance for *C. unshiu* controls compared to *P. trifoliata* controls since leaf conductance for both species was similar for acclimated trees. As with leaf conductance, there was a significant S*A*W interaction for transpiration ($P > F = 0.0426$), however, unlike leaf conductance, transpiration rates for *P. trifoliata* were lower than *C. unshiu* on some dates for both temperature treatments. *C. unshiu* had higher $CO_2$ assimilation rates compared to *P. trifoliata* for both acclimation treatments, as indicated by the significant S*W interaction ($P > F = <0.0001$). $CO_2$ assimilation rates were lower for acclimated plants compared to the controls as indicated by the significant A*W interaction ($P > F = <0.0001$). There was a slight increase in $CO_2$ assimilation rate towards the end of the experiment, which probably indicates metabolic adjustment to lower temperatures. In summary, the light period temperature of 10˚C for cold treated

plants was apparently high enough to open stomata slightly to allow some gas exchange including transpiration and assimilation of $CO_2$. Nevetheless, the stomata closed substantially for cold treated plants that suppressed transpiration and assimilation of $CO_2$ compared to the control plants.

$R_{root}$ increased for both species during cold treatment but remained nearly constant for control plants (Fig. 2.3). There was a significant S*A*W interaction for $R_{root}$ ($P > F$ = <0.0001) and data transformed to a $log_{10}$ ($P > F = 0.0017$). $R_{root}$ was higher for *C. unshiu* compared to *P. trifoliata* for each acclimation treatment. Although several studies have demonstrated that $R_{root}$ increases during cold acclimation in general (Kriedemann and Barrs, 1981; Landsberg et al., 1981), it is not clear if the increase is due to changes in cold-induced changes of the root system, or a decrease in flow rate. The route of water uptake into root systems is most likely controlled by water channel proteins, most of which appear down-regulated during cold acclimation (Jang et al., 2004). However, there is a substantial body of evidence that $R_{root}$ is indirectly related to flow rate (Kriedemann and Barrs, 1981; Landsberg and Jones, 1981). Nevertheless, the higher $R_{root}$ for acclimated plants would inhibit water uptake and help promote plant water deficits.

The two assumptions made in this study, that $\Psi_{soil}$ and $R_{soil}$ were zero during the period used to measure whole plant transpiration, introduced some error in estimating $R_{root}$, but were considered small in this study. The plants were watered 2 hrs before the pots were weighed the first time for determining whole plant transpiration and transpiration was allowed to occur for 4 hrs before the plants were weighed again. The 6 hr period between measurements would tend to promote a decrease in $\Psi_{soil}$ and an

increase in $R_{soil}$. The changes in $\Psi_{soil}$ and $R_{soil}$, if significant, would have increased the hydraulic strain in the plant which would have lowered $\Psi_{stem}$. To test whether $\Psi_{stem}$ decreased during the transpiration period, $\Psi_{stem}$ was measured at the end of the transpiration period on the last day of the experiment and compared to $\Psi_{stem}$ measurements right before the beginning of the transpiration period. Only $\Psi_{stem}$ measurements for the controls are reported here since any differences would likely be higher for control plants, which had much higher transpiration rates than acclimated plants. $\Psi_{stem}$ before the first and after the second pot measurements averaged -0.25 and -0.30 MPa for *P. trifoliata*, respectively, and -0.30 and -0.33 MPa, for *C. unshiu*, respectively. The means within each species were not statistically different between the first and second measurements ($P > F = <0.0001$).

The decrease in $\Psi_{stem}$ for acclimated plants in this study could not be explained by leaf conductance since the closure of stomata would have promoted higher $\Psi_{stem}$. $R_{root}$ would have enhanced plant water deficits that would suppress $\Psi_{stem}$, but the change in $R_{root}$ likely had a minor contribution to declining $\Psi_{stem}$. By the end of the experiment, $\Psi_{stem}$ of acclimated plants was more than 4x lower than controls, whereas $R_{root}$ was only about 15% higher for acclimated plants than the controls. Rather, the lower $\Psi_{stem}$ was more likely due to accumulation of osmotically active substances which have been shown to be inversely correlated with plant water potential (Burchett et al., 2006; Yelenosky, 1982).

The results of this experiment do not support our hypothesis that cold temperatures develop greater plant water deficits in *P. trifoliata* compared to *C. unshiu*

that would help explain its greater freeze tolerance. $\Psi_{stem}$ and leaf conductance were similar during cold acclimation for both species, despite the higher $R_{root}$ for *C. unshiu* compared to *P. trifoliata*.

## Literature Cited

Aphalo, P.J. and P.G. Jarvis. 1993. The boundary layer and the apparent responses of stomatal conductance to wind speed and to the mole fractions of $CO_2$ and water vapour in the air. Plant, Cell and Environ. 16:771-783.

Bain, F.M. 1949. Citrus and climate. Calif. Citrograph. 34:382-449.

Ball, J.T., I.E. Woodrow, and J.A. Berry. 1987. A model predicting stomatal conductance and its contribution to the control of photosynthesis under different environmental conditions. Progress in Photo. Res. 4:221-228.

Bialoglowski, J. 1937. Effect of extent and temperature of root on transpiration of rooted lemon cuttings. Proc. Amer. Soc. Hort. Sci. 34:96-102.

Burchett, S., S. Niven, and M.P. Fuller. 2006. The effect of cold-acclimation on the water relations and freezing tolerance of *Hordeum vulgare* L. CryoLetters 27:295-303.

Cai, Q., C.L. Guy, and G.A. Moore. 1994. Extension of the linkage map in citrus using random amplified polymorphic DNA (RAPD) markers and RFLP mapping of cold-acclimation-responsive loci. Theor. Appl. Genet. 89:606-614.

Cai, Q., G.A. Moore, and C.L. Guy. 1995. An unusual Group 2 LEA gene family in citrus responsive to low temperature. Plant Mol. Biol. 29:11-23.

Cameron, S.H. 1941. The influence of soil temperature on the rate of transpiration of young orange trees. Proc. Amer. Soc. Hort. Sci. 38:75-79.

Champ, K.I., V.J. Febres, and G.A. Moore. 2007. The role of CBF transcriptional activators in two Citrus species (*Poncirus* and *Citrus*) with contrasting levels of freezing tolerance. Physiol. Plant. 129:529-541.

Cooper, W.C., B.S. Gorton, and S. Tayloe. 1954. Freezing tests with small trees and detached leaves of grapefruit. Proc. Amer. Soc. Hort. Sci. 63:167-172.

Davies, F.S., B.W. Buchanan, and J.A. Anderson. 1981. Water stress and cold hardiness in field-grown citrus. J. Amer. Soc. Hort. Sci. 106:197-200.

Durham, R.E., G.A. Moore, D. Haskell, and C.L. Guy. 1991. Cold-acclimation induced changes in freezing tolerance and translatable RNA content in *Citrus grandis* and *Poncirus trifoliata*. Phys. Plant. 82:519-522.

Ebel, R.C., B.L. Campbell, M.L. Nesbitt, W.A. Dozier, J.K. Lindsey, and B.S. Wilkins. 2005. A temperature index model to estimate long-term freeze-risk of Satsuma mandarins grown on the northern coast of the Gulf of Mexico. J. Amer. Soc. Hort. Sci. 130:500-507.

Ebel, R.C., P.A. Carter, W.A. Dozier, D.A. Findley, M.L. Nesbitt, B.R. Hockema, and J.L. Sibley. 2004. Pattern of exotherm and electrolyte leakage measured at high frequency of Satsuma mandarin exposed to subfreezing temperatures. HortScience 39:1614-1616.

Garnier, E. and A. Berger. 1985. Testing water potential in peach trees as an indicator of water stress. J. Hort. Sci. 60:47-56.

Jang, J.Y., Kim, D.G., Y.O. Kim, J.S. Kim, and H.S. Kang. 2004. An expression analysis of a gene family encoding plasma membrane aquaporins in response to abiotic stresses in Arabidopsis thaliana. Plant Mol. Biol. 54:713-725.

Jarvis, P.G. and J.I.L Morrison. 1981. Stomatal control of transpiration and photosynthesis, p. 247-279. In: P.G. Jarvis and T.A. Mansfield (eds.) Stomatal Physiology, Cambridge Univ. Press, Cambridge.

Kadoya, K., F. Mizutani, and A. Hino. 1981. Effects of root environmental conditions on stomatal behavior and translocation of photosynthates. Proc. Intl. Soc. Citriculture. 2:498-501.

Kramer, P.J. 1969. Plant and soil water relationships. McGraw-Hill, Inc. p. 482.

Kramer, P.J. 1985. An early discussion of cell water relations in thermodynamic terminology. Plant Cell Environ. 8:171-172.

Kriedemann, P.E. and H.D. Barrs. 1981. Citrus orchards, p. 325-417. In: T.T. Kozlowski (ed.) Water Deficits and Plant Growth, Vol. 6, Academic Press, New York.

Landsberg, J.J. and H.G. Jones. 1981. Water deficits and plant growth. Chapter 6, Apple Orchards, Academic Press, New York, NY.

Lang, P., C.K. Zhang, R.C. Ebel, F. Dane, and W.A. Dozier. 2005. Identification of cold acclimated genes in leaves of *Citrus unshiu* by mRNA differential display. Gene 359:111-118.

Meryman, H.T. 1956. Mechanics of freezing in living cells and tissues. Sci. 124:515 521.

Meryman, H.T. 1966. Review of biological freezing, p. 2-114. In: H.T. Meryman (ed.) Cryobiology, Academic Press, New York.

Moore, G.A., I. Tozlu, C.A. Weber, and C.L. Guy. 2000. Mapping quantitative trait loci for salt and cold tolerance in *Citrus grandis* (L.) Osb. X *Poncirus trifoliata* (L.) Raf. hybrid populations. Acta Hort. 535:37-45.

Nesbitt, M.L., R.C. Ebel, D. Findley, B. Wilkins, F. Woods, and D. Himelrick. 2002. Assays to Assess Freeze Injury of Satsuma mandarin (*Citrus unshiu*). HortScience 37:871-877.

Nielsen, K.F. 1974. Roots and root temperatures. In: E.W. Carson (ed.) The Plant Root Environment, Carson Univ. Press, Virginia.

Passioura, J.B. 1988. Water transport in and to roots. Ann. Rev. Plant Physiol. Plant Mol. Biol. 39:245-265.

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

Reicosky, D.C. and J.T. Richie. 1976. Relative importance of soil resistance and plant resistance in root water absorption. Soil Sci. Soc. Amer. J. 40:293-297.

Reid, J.B. and B. Hutchinson.1986. Soil and plant resistances to water uptake by *Vicia faba* L. Plant Soil 92:431-441.

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

Sahin-Cevik, M. and G.A. Moore. 2006b. Two AP2 domain containing genes isolated from the cold-hardy Citrus relative *Poncirus trifoliata* are induced in response to cold. Funct. Plant Biol. 33:863-875.

Sahin-Cevik, M. and G.A. Moore. 2006c. Isolation and characterization of a novel RING-H2 finger gene induced in response to cold and drought in the interfertile Citrus relative *Poncirus trifoliata*. Physiol. Plant. 126:153-161.

Spiegal-Roy, P. and E.E. Goldschmidt. 1996. Biology of citrus. Cambridge University Press Cambridge, UK p.30.

Steponkus, P.L. 1984. Role of the plasma membrane in freezing injury and cold acclimation. Annu. Rev. Plant Physiol. 35:543-584.

Syvertsen, J.P., R.M. Zablotowicz, and M.L. Smith, JR. 1983. Soil Temperature and flooding effects on two species of citrus. Plant and Soil 72(3):3-12.

Van den Honert, T.H. 1948. Water transport in plants as a catenary process. Discuss. Faraday Soc. 3:146-153.

Vu, J.C.V. and G. Yelenosky. 1987. Photosynthetic characteristics in leaves of 'Valencia' orange (*Citrus sinensis* (L.) Osbeck) grown under high and low temperature regimes. Env. Exp. Bot. 27:279-287.

Weber, C.A., G.A. Moore, Z. Deng, and F.G. Gimmiter, Jr. 2003. Mapping freeze tolerance quantitative trail loci in a *Citrus grandis* x *Poncirus trifoliata* $F_1$ pseudo-testcross using molecular markers. J. Amer. Soc. Hort. Sci. 128:508-514.

Xiong, L., K.S. Schumaker, and J. Zhu. 2002. Cell signaling during cold, drought, and salt stress. The Plant Cell Supplement 2002:S165-S183.

Yamada, H., H. Mukai, N. Utsunomiya, A. Sugiura, and T. Tomana. 1985. The effect of low root temperature on the cold hardiness of citrus species and avocado. J. Jap. Soc. Hort. Sci. 53:419-426.

Yelenosky, G. 1978. The effect of withholding water on cold hardiness of ‘Valencia’ orange and ‘Star Ruby’ grapefruit trees in controlled freezes. Proc. Fla. State Hort. Soc. 91:18-20.

Yelenosky, G. 1979. Water-stress-induced cold hardening of young citrus trees. J. Amer. Soc. Hort. Sci. 104:270-273.

Yelenosky, G. 1982. Indicators of citrus cold-hardening in the field. Proc. Fla. State Hort. Soc. 95:7-10.

Yelenosky, G. 1985. Cold hardiness in citrus. Hort. Rev. 7:201-238.

Yelenosky, G. 1996. An overview of Florida citrus freeze survival. Proc. Fla. State Hort. Soc. 109:118-123.

Yelenosky, G., C.J. Hearn, and D.J. Hutchison. 1984. Nonhardening temperatures – Major factor in freeze damage to citrus trees in December 1983. Proc. Fla. State Hort. Soc. 97:33-36.

Yelenosky, G., D. Hutchison, and H. Barrett. 1993. Freezing resistance of progeny from open-pollinated pummel x trifoliate orange citrus hybrids. HortScience 28:1120-1121.

Young, R.G. 1961. Influence of day length, light intensity, and temperature on growth, dormancy, and cold-hardiness of Red Blush grapefruit trees. Proc. Amer. Soc. Hort. Sci. 78:174-180.

Young, R.H. 1969. Influence of day length, light intensity, and temperature on growth, dormancy, and cold-hardiness of Red Blush grapefruit trees. Proc. Amer. Soc. Hort. Sci. 78:174-180.

Young, R. and W.D. Bell. 1974. Photosynthesis in detached leaves of cold-hardened citrus seedlings. J. Amer. Soc. Hort. Sci. 99:400-403.

Young, R. and M. Mann. 1974. Freeze disruption of sour orange leaf cells. J. Amer. Soc. Hort. Sci. 99:403-407.

Young, R.H. and A. Peynado. 1962. Growth and cold-hardiness of citrus and related species when exposed to different night temperatures. Proc. Amer. Soc. Hort. Sci. 81:238-243.

Young, R.H. and A. Peynado. 1965. Changes in cold hardiness and certain physiological factors of Redblush grapefruit seedlings as affected by exposure to artificial hardening temperatures. Proc. Amer. Soc. Hort. Sci. 86:244-252.

Zhang, J.Z., R.A. Creelman, and J. Zhu. 2004. From laboratory to field. Using information from Arabidopsis to engineer salt, cold, and drought tolerance in crops. Plant Physiol. 135:615-621.

Zhang, C.K., P. Lang, R.C. Ebel, F. Dane, N.K. Singh, R.D. Locy, and W.A. Dozier. 2004. Can. J. Plant Sci. Canadian J. Plant Sci. 23:417-424.

Zhang, C., P. Lang, F. Dane, R.C. Ebel, N.K. Singh, R. Locy, and W.A. Dozier. 2005. Cold acclimation induces genes of trifoliate orange (*Poncirus trifoliata)*. Plant Cell Reports 23:764-769.

Table 2.1. *P* > *F* of the ANOVA for dependent variables measured during cold acclimation of *C. unshiu* and *P. trifoliata*.

| Significance | $\Psi_{stem}^{y}$ (MPa) | $C_{leaf}$ (mole/m$^2$/s) | Transpiration (mmole/m$^2$/s) | $ACO_2$ ($\mu$mole/m$^2$/s) | $R_{root}$ (MPa s/m) | $Log_{10}$ $R_{root}$ (MPa s/m) |
|---|---|---|---|---|---|---|
| S*A*W$^z$ | 0.4787 | 0.0190 | 0.0426 | 0.3573 | <0.0001 | 0.0017 |
| A*W | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 |
| S*W | 0.0386 | 0.4202 | 0.0128 | <0.0001 | <0.0001 | 0.8649 |
| W | <0.0001 | 0.0005 | 0.0037 | <0.0001 | <0.0001 | <0.0001 |
| S*A | 0.9109 | 0.3687 | 0.7193 | 0.9372 | 0.0168 | 0.0722 |
| A | <0.0001 | 0.0776 | 0.0172 | 0.5205 | 0.0005 | 0.0063 |
| S | 0.0861 | 0.2771 | 0.9642 | 0.0024 | 0.1625 | <0.0001 |

$^z$S = specie, A = acclimation treatment, W = week

$^y\Psi_{stem}$ = stem water potential, $C_{leaf}$ = leaf conductance, $ACO_2$ = net $CO_2$ assimilation rate, $R_{root}$ = root resistance, and $Log_{10}$ $R_{root}$ = root resistance for values transformed to the $log_{10}$.

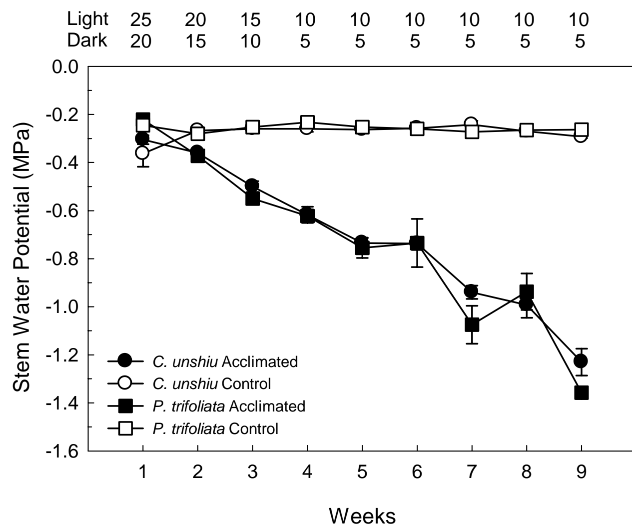


Fig. 2.1. Stem water potential during cold acclimation of *C. unshiu* and *P. trifoliata*. The numbers above the graph indicate temperatures ($^{o}$C) during the light and dark periods, which were 12 hr each. Each data point is a mean of 10 plants from two experiments. Vertical bars indicate 2x the standard error of the mean. Vertical bars not visible are within the height of the data symbol.

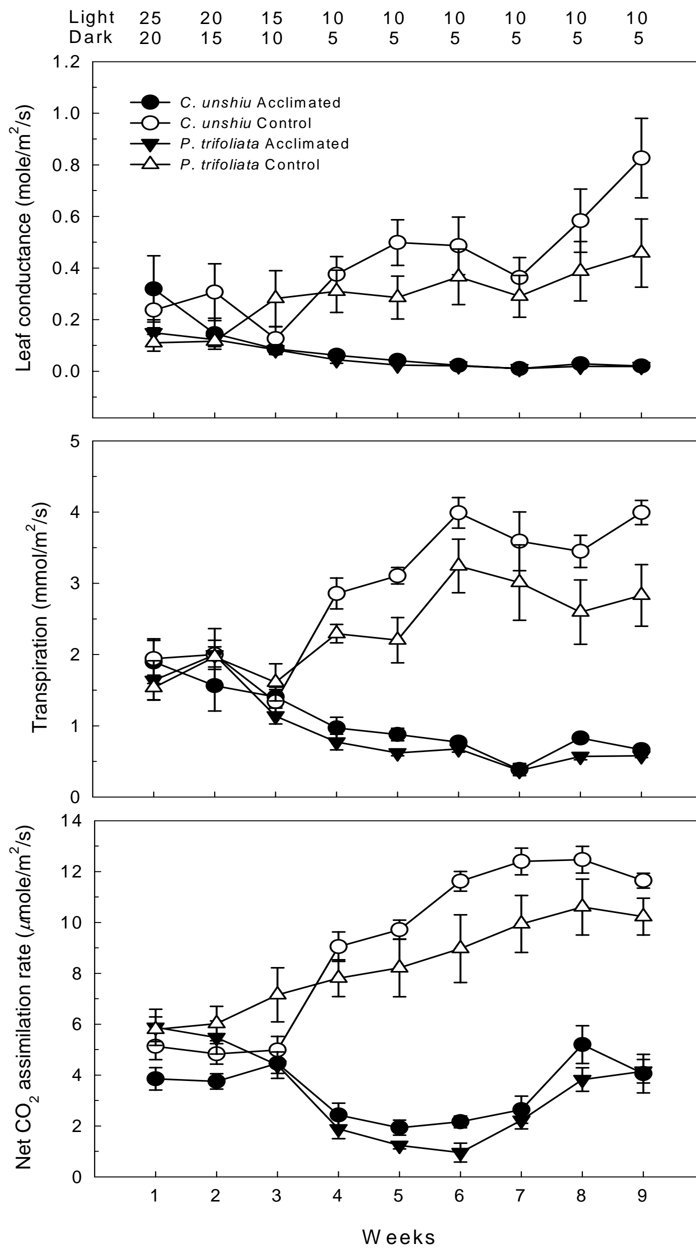


Fig. 2.2. Decline in leaf conductance, transpiration and net $CO_2$ assimilation rate during cold acclimation of *C. unshiu* and *P. trifoliata*. The numbers above the graph indicate temperatures ($^oC$) during the light and dark periods, which were 12 hr each. Each data point is a mean of 10 plants from two experiments. Vertical bars indicate 2x the standard error of the mean. Vertical bars not visible are within the height of the data symbol.

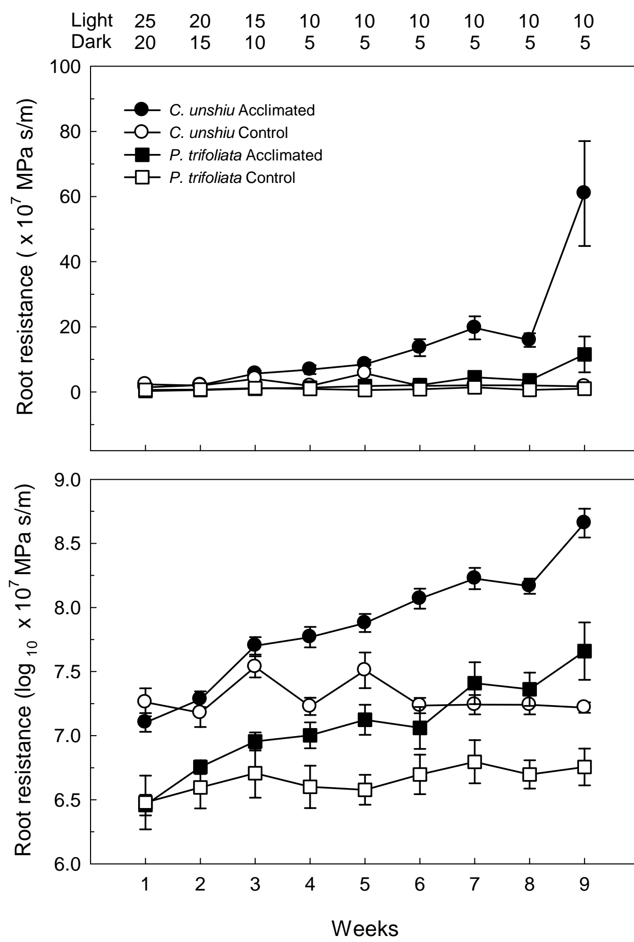


Fig. 2.3. Increase in root resistance during during cold acclimation of *C. unshiu* and *P. trifoliata*. The lower graph shows the data transformed by converting the values to the $\log_{10}$. The numbers above the graph indicate temperatures ($^{o}$C) during the light and dark periods, which were 12 hr each. Each data point is a mean of 10 plants from two experiments. Vertical bars indicate 2x the standard error of the mean. Vertical bars not visible are within the height of the data symbol.

# CHAPTER III

## GENE EXPRESSION OF 14-3-3 PROTEIN IN *CITRUS UNSHIU* AND *POCIRUS TRIFOLIATA* WHEN FULLY COLD-HARDENED


### Abstract

*Citrus unshiu*, a cold hardy commercial *Citrus* species, is able to withstand temperatures to -10˚C; whereas *Poncirus trifoliata*, commonly used as a rootstock for *C. unshiu*, can withstand temperatures as low as -26˚C. Accumulation of 14-3-3 protein has been shown to induce changes in gene expressed in *C. unshiu* during acclimating temperatures. It is not known whether this occurs in *P. trifoliata*, and if both *Citrus* species undergo the same accumulation of 14-3-3 proteins when fully acclimated or cold hardened. *C. unshiu* and *P. trifoliata* trees were kept unacclimated by holding day/night air temperatures at 25˚C/20˚C, or acclimated by dropping day/night air temperatures weekly from 25˚C/20˚C to 20˚C/15˚C to 15˚C/10˚C, and then to 10˚C/5˚C for 9 weeks. Quantitative relative RT-PCR was used for differential expression of cDNA fragments isolated from unacclimated control and cold-acclimated plants. Down regulation of 14-3-3 protein was shown in both acclimated species when fully cold-hardened to 5˚C. Gene expression of 14-3-3 proteins may be an important factor during mid to late-acclimation, but may no longer be needed when plants are fully acclimated. Further investigation needs to be conducted for duplication and replication of cDNA fragments for sequence analysis and full length clones.

## Introduction

*Citrus* is a cold-sensitive genus and most commercially important varieties of *citrus* are susceptible to freezes. Yet, there is significant variation among citrus species and relatives for cold tolerance ranging from very cold-sensitive types, such as *Citrus grandis* (L.) Osbeck (pummelo), to the cold-hardy interfertile citrus relative *Poncirus trifoliata* (L). Raf., which can withstand temperatures of -26˚C when cold-acclimated (Yelenosky, 1985; Spiegel-Roy and Goldschmidt, 1996). In the previous century, *P. trifoliata* was used in breeding programs mainly in Florida (Soost and Cameron, 1975) and in other countries such as Japan and Russia (Gmitter et al., 1992) in efforts to produce cold-tolerant commercial species. Despite considerable effort, cold-tolerant rootstocks produced by crossing *Poncirus* and *Citrus*, having good fruit quality, acceptable tree characteristics, and cold hardiness has not been successful to date. Long juvenility periods, polyembryony (apoxmis), heterozygosity, sterility, self- and cross-incompatibility, inbreeding depression, and the quantitative inheritance of cold tolerance are biological problems that are limiting production of cold-hardy citrus varieties (Soost and Cameron, 1975; Soost and Roose, 1996).

Many species of higher plants can increase their freezing tolerance when exposed to low, nonfreezing temperatures (Guy, 1990). This adaptive process, known as cold acclimation, involves several cellular and metabolic changes including increased levels of sugars, soluble proteins, proline, and organic acids as well as the appearance of new isoforms of proteins and altered lipid membrane composition (Hughs and Dunn, 1990). Studies have shown that many of these biochemical and physiological changes are regulated by low temperature through changes in gene expression. Changes in gene

expression between cold-acclimated and non-acclimated plants have been studied extensively using differential screening of cDNA libraries (Chauvin et al., 1993; Gilmour et al., 1992; Monroy et al., 1993; Weretilnyk et al., 1993), differential display, and subtractive cDNA libraries (Horvath and Olson, 1998). A number of cold-regulated genes have been identified and characterized in several herbaceous plants, such as *Arabidopsis thaliana* (L.) Heynh. (Gilmour et al., 1992; Horvath et al., 1993); and recently a number of low-temperature-responsive (LTR) genes, mostly dehydrins, have been cloned from a range of both dicotyledon and monocotyledon species (Hughes and Dunn, 1996).

Of the LTR genes cloned, the contenders for a role in signal transduction is limiting. Jarillo et al. (1994) identified the LTR genes *RC1*and *RC2* in *Arabidopsis*, which have homology to a small highly conserved family of eukaryotic proteins, known as 14-3-3 proteins. 14-3-3 proteins are involved in the regulation of multifunctional cellular enzymes, such as, plant calcium dependent protein kinases (Aitken, 1996; Ferl, 1996; Camoni et al., 1998), and plant plasma membrane $H^+$-ATPase (Baunsgaard et al., 1998; Camoni et al., 1998). In *Arabidopsis*, the binding of 14-3-3 protein to the calcium dependent protein kinase (CDPK) can activate the enzyme, and thus, likely further activates CDPK signal transduction pathways in this plant (Camoni et al., 1998). Camoni et al. (2000) demonstrated in maize that 14-3-3 proteins interact with the $H^+$ -ATPase and their interaction is dependent on the phosphorylation status of the proton pump. The two genes encoding 14-3-3 proteins found in *Arabidopsis* were induced in response to low temperature, and the accumulation of 14-3-3 proteins was associated with an increase in cold tolerance. The genes expressed were thought to have an adaptive role in this process (Jarillo et al., 1994). 14-3-3 proteins were identified and induced 2.3 fold in cold-

acclimated versus non-acclimated *C. unshiu* (Ping et al., 2005). It was suggested that, considering 14-3-3's 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 flow (Ping et al., 2005).

*C. unshiu* is considered one of the most cold hardy commercial *Citrus* species (Yelenosky, 1985), and is able to withstand temperatures to -10˚C; whereas *P. trifoliata*, used in breeding programs and commonly as a rootstock for *C. unshiu*, can withstand temperatures as low as -26˚C (Spiegel-Roy and Goldschmidt, 1996). There must be a significant genetic overlap among these species, yet differences in freeze tolerance likely result from significant differences in gene expression during cold acclimation (Lang et al. 2004, Zhang et al., 2005). Understanding a plant's response to a stress requires a comprehensive evaluation of stress-induced changes in gene expression. It has been recognized that the accumulation of 14-3-3 proteins induces genes expressed in *C. unshiu* during acclimating temperatures (Ping et al., 2005); it has not been determined whether this occurs in *P. trifoliata*, and if both *citrus* species undergo the same accumulation of 14-3-3 proteins when fully acclimated or cold-hardened. Quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR) has been used extensively to study gene expression because of its high sensitivity and reproducibility regardless of the abundance of mRNA transcript (Okamoto et al., 2003; Pires-alves et al., 2003; Taylor and Harrier, 2003). The purpose of this study was to evaluate the difference in gene expression of 14-3-3 proteins using quantitative RT-PCR when *C. unshiu* and *P. trifoliata* are fully cold-acclimated and unacclimated.

## Materials and Methods

*Plant material and culture.* Two year-old Satsuma mandarin [*Citrus unshui* (Marc.) 'Owari'] budded on trifoliate orange rootstocks [*Poncirus trifoliata* (L.) Raf. 'Rubidoux'] and ungrafted *P. trifoliata* 'Rubidoux' plants were used in this experiment. Shoot growth of test plants varied from 60 to 90 cm in total length above the graft union for Satsuma mandarin and single, staked trifoliate orange were 31 to 85 cm high at the time of the experiment. Plants were grown in 3.8-L pots in a 1 pine bark : 1 sand substrate by volume amended with 4.45 $kg \cdot m^{-3}$ of dolomitic lime, 8.9 $kg \cdot m^{-3}$ of 18 N-6 $P_2O_5$-12 $K_2O$, (Polyon 12-14 month controlled release fertilizer, Pursell Technologies, Inc, Sylacauga, Ala.), and 0.89 $kg \cdot m^{-3}$ a micronutrient package (Micromax, The Scotts Co., Marysville, Ohio). All plants were supplemented with water soluble 20N-20P-20K and micronutrients, which were applied at 234 $mg \cdot L^{-1}$ N when watered. Additional fertilizer was applied to promote growth since the fertilizer regime that had been applied was not. Soil moisture readings were conducted daily using a portable soil moisture probe (Type HH2, Delta T Devices Ltd., Burwell Cambridge, UK). Three readings per plant were taken for all plants in both acclimation treatments, and plants were watered individually when soil moisture dropped below 85% of the water holding capacity of the substrate; except on the day of data collection when plants were watered more frequently to maintain water holding capacity near 100%.

*Acclimation treatments.* Acclimation treatments were conducted in two environmental growth chambers (Model E15; Conviron, Winepeg, Canada) with fluorescent lighting (320 $\mu mol \cdot m^{-1} \cdot s^{-1}$ at midtree height) and ambient relative humidity. Acclimation treatments were chosen based on there relationship to temperature, growth,

and acclimation. Citrus growth ceases below 12˚C, plants cold acclimate below 10˚C, and plants become fully cold-hardened when exposed to 10˚C or less for about 3 weeks (Bain, 1949; Cooper et al., 1954; Yelenosky et al., 1984; Yelenosky, 1985; Young, 1961; Young and Peynado, 1962, 1965). Plants of the unacclimated treatment were held at air temperature 25˚C day/20˚C night throughout the duration of the experiment. Plants in the acclimated treatment were exposed to sequential air temperatures of 25˚C day/20˚C night for 7 d; 20˚C day/15˚C night for 7 d; 15˚C day/10˚C night for 7 d; and 10˚C day/5˚C night for 21d. Both acclimated treatments were subjected to a 12/12h light/dark photoperiod throughout the experiment. The cold acclimation treatment was previously shown to produce a fully cold hardened state for *C. unshiu* (Ebel et al., 2004; Nesbitt et al., 2002). Five plants per genotype were used for in both acclimation treatments.

*Data Collected.* Data were collected once per week for 4 weeks. On the day data were collected, plants were watered 3-4 hours after the start of the light period. To reduce risk of mechanical damage to the plants, after watering, and before all other measurements were performed; three to four fully expanded leaves from each plant in both acclimation treatments were collected and immediately immersed in liquid nitrogen and stored at -80˚C for RNA extraction, which was performed after the termination of the experiment.

Gas exchange measurements were then performed on three leaves per plant using a portable photosynthesis system (Licor Model 6400 Lincoln, Nebraska). The most recently mature leaves; approximately the fifth from the terminal were measured. All plants in both chambers were watered again and left for 20 minutes to ensure plants were well watered before $\Psi_{stem}$ measurements were collected.

$\Psi_{stem}$ were determined using three leaves from each plant, using the fifth to tenth leaf from the shoot tip. Leaves were initially wrapped in plastic then aluminum foil, the night before data was collected to allow $\Psi_{leaves}$ to equilibrate to $\Psi_{stem}$ (Garnier and Berger, 1985). Wrapped leaves were severed at the base of the petiole with a razor blade and $\Psi_{stem}$ was measured using a pressure chamber (Model 1000, PMS Instrument Co., Corvalis, Oregon) that was pressurized at 1 MPa/20 s using compressed air.

$\Psi_{leaf}$ measurements were taken using three leaves from each plant, using the leaves that were present beneath the leaves used for $\Psi_{stem}$ measurements. Leaves were severed at the base of the petiole with a razor blade and $\Psi_{leaf}$ measured using a pressure chamber (Model 1000, PMS Instrument Co., Corvalis, Oregon) that was pressurized at 1 MPa/20 s using compressed air.

Soil moisture was measured for each container to ensure water holding capacity was at or above 85% and the temperature in the acclimated chamber was decreased according to the previous stated regimen for acclimated temperature decline.

At the end of the 4$^{th}$ week, plants of the unacclimated chamber were held at 25˚C day/20˚C night and plants of the cold-acclimated chamber were held at 10˚C day/5˚C for an additional 14d; measurements of water potential and gas exchange rates were not performed during this period. Well-watered conditions were maintained for both chambers. Plants remained in chambers for the collection of leaf tissue for RNA extraction performed after the termination of the experiment. Leaf tissue was collected just as before at the end of the 5$^{th}$ and 6$^{th}$ week.

*RNA Extraction.* RNA was extracted from fully cold-acclimated *C. unshiu* and *P. trifoliata* 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 $\mu L^{-1}$) and incubated for 30 min at 37˚C to digest the remaining genomic DNA. The resulting preparation was treated with DNase inactivation reagent (20% volume) for 2min, followed by centrifugation for 1 min at 14 000 x g. The resulting supernatant was transferred to a new tube, and the concentration of isolated RNA was measured using an Eppendorf Biophotometer (Brinkmann Instruments, NY). Total RNA prepared from leaves at the end of the $6^{th}$ week of treatment was used as the unacclimated control and RNA from leaves harvested at the end of the $6^{th}$ week of cold acclimation was used as the treatment. mRNA differential display and quantitative RT-PCR were used to evaluate the differences in response and gene expression of 14-3-3 protein in both cold-hardened and non-acclimated species. Primer pairs were used and cDNA was amplified exponentially using PCR.

*Quantitative Relative Reverse Transcriptase Polymerase Chain Reaction* (RT-PCR) Quantitative relative RT-PCR was used for differential expression of DNA fragments isolated from unacclimated control and cold-acclimated plants. Total RNA (2.5µg) was reversed transcribed with 0.5mM dNTP, 5mM oligo(dT)-primers, 10mM Tris-HCL, pH 8.3, 50mM 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 h. 1µL of the reverse transcriptase reaction was amplified in a 25µL reaction with 10mM Tris-HCl, pH 8.4, 50mM KCL, 1.5mM $MgCl_{2,}$ 2.5mM dNTPs, 0.3 µM actin primer, 0.6 µM actin competitor, 0.5mM of gene specific primer and *Taq* polymerase (Qiagen). The PCR cycle program was as followed: 30s at 94˚C, 30s at 55˚C, and 30s at 72˚C for 32 cycles. The specific oligonucleotide primer was designed

for the differential display (DD) product for RT-PCR. The optimal PCR annealing temperature and cycle number is shown in Table 3.1. 10 μl of the amplification products were separated using 1.8% agarose gel electrophoresis and stained with ethidium bromide. The stained gel was used for quantification 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

The species by acclimation by week interaction (S*A*W) was not significant for $\Psi_{stem}$ ($P > F$ = 0.6044, Table 3.2), whereas there was a significant A*W interaction ($P > F$ = <.0001). These results suggest both species decreased similarly throughout the cold acclimation treatment, whereas plants of the non-acclimated treatment remained nearly constant at about -0.3 MPa (Fig. 3.1). The decrease in $\Psi_{stem}$ during cold acclimation was not related to soil moisture. On the day data was collected plants were well watered and water holding capacity was maintained near 100%. Soil moisture measurements demonstrated well-watered conditions (data not shown). Results also concur with results from the previous water relations study.

$\Psi_{leaf}$ measurements decreased similarly for both species during cold acclimation compared to $\Psi_{leaf}$ for control trees, which averaged from -0.4 MPa to -0.5 MPa respectively. There was a significant A*W interaction ($P > F$ = <0.0001), but a non-significant S*A*W interaction ($P > F$ = 0.4351) which indicates the acclimation plants had lower $\Psi_{leaf}$ than the control non-acclimated plants; and at each level of acclimation there were no species difference.

Leaf conductance decreased for both species during cold acclimation and was lower than the controls by the 4th week (Fig. 3.2). Results were not significant between acclimated and unacclimated species over time (S*A*W [$P > F$ = 0.5775]). Even though leaf conductance approached zero, there was still some transpiration during the cold period. There was a significant A*W interaction for transpiration ($P > F$ = <0.0001) and stomatal conductance ($P > F$ = 0.0002) in the acclimated species. The pattern of leaf transpiration of the controls was similar to leaf conductance of the controls for both species (Fig. 3.2). Transpiration rates for both species were lower than the controls, but above zero by the end of the acclimation period.

cDNA fragments amplified by one primer group is shown in Fig. 3.3; both cold-acclimated *C. unshiu* and *P. trifoliata* show down regulation of 14-3-3 protein when fully cold hardened to 5˚C as indicated by the lower luminosity of the bands (Fig. 3.3). These results differ from a study on *C. unshiu*, showing up-regulation of 14-3-3 protein when cold-acclimated to 7˚C (Ping et al., 2005). The contrasting results are likely due to the different temperatures and duration of those temperatures that the plants were exposed to in both experiments. In the present study, leaf tissue used for RNA extraction was removed from the plant at the end of the 6th week, where plants had been exposed to 10˚C day/5˚C night temperatures for 21d. In the previous study, RNA was extracted from leaf tissue at the end of the 5th week, where plants had been exposed to 18˚C day/7˚C night temperatures for 7d (Ping et al., 2005). A possible conclusion would be that gene expression of 14-3-3 proteins is an important factor during mid to late-acclimation, but the gene is no longer needed when plants are fully acclimated or cold-hardened.

Further investigation of this experiment needs to be conducted. Duplication and replication of cDNA fragments need to be analyzed for sequencing and development of full length clones, to determine if the genes expressed in both species are identical. mRNA differential display needs to be conducted for the expressed bands between acclimated and non-acclimated species to analyze relative differences in intensity of cDNA amplifications, which may confirm predicted function of gene expression of 14-3-3 protein in *C. unshiu* is the same in *P. trifoliata.* Relative RT-PCR only provides an estimate of the relative changes in gene expression between samples. Competitive quantitative RT-PCR is a technique that can provide a near exact value, but all measurements of amplification rate are estimates, and the most reliable estimates are based upon repeated measurements (Pallansch et al., 1990). Due to the extensively reported high false positive rate characteristic of the differential display procedure, cDNA amplified fragment length polymorphism (AFLP) can be performed as a comparison of differential display (Blears et al., 1999; Breyne et al., 1997; Savelkoul et al., 1999) to further explore the alteration of gene expression in citrus and other related species exposed to cold acclimation temperatures (Ping, 2005; Zhang, data unpublished). cDNA-AFLP has been found to be a more reliable transcriptome profiling technique, in order to get a more complete understanding of the molecular mechanisms (Savelkoul et al., 1999). More detailed characterization of the signal transduction important 14-3-3 gene identified in *C. unshiu* and *P. tifoliata* will shed light on the understanding of the responsive mechanisms under cold acclimation.

## Literature Cited

Aitken, A. 1996. 14-3-3 and its possible role in co-ordinating multiple signaling pathways. Trends. Cell. Biol. 6:341-347.

Bain, F.M. 1949. Citrus and climate. Calif. Citrograph 34:382-449.

Baunsgaard, L., A.T. Fuglsang, T. Jahn., H.A.J. Korthout, B. De Boer, and M.G. Palmgren. 1998. The 14-3-3 proteins associate with the plant plasma membrane $H^+$-ATPase to generate a *Fusicoccin* binding complex and a *Fusicoccin* responsive system. Plant J. 13:661-667.

Blears, M.J., S.A. De Grandis, H. Lee, and J.T. Trevors. 1999. Amplified fragment length polymorphism (AFLP): review of the procedure and its applications. J. Ind. Microbiol. Biotech. 21:99-114.

Breyne, P., W. Boerjan, T. Gerats, M. van Montagu, and A. Vangysel. 1997. Applications of AFLP$^{TM}$ in plant breeding, microbiology, and genetics. Belg. J. Bot. 129:107-117.

Camoni, L., J.F. Harper, and M.G Palmgren. 1998. 14-3-3 proteins activate a plant calcium-dependent protein kinase (CDPK). FEBS Lett. 430:381-384.

Camoni, L., V. Iori, M. Marra, and P. Aducci. 2000. Phosphorylation-dependent interaction between plant plasma membrane $H^+$-ATPase and 14-3-3 proteins. J. Biol. Chem. 275:9919-9923.

Chauvin, L.P., M. Houde, and F. Sarhan. 1993. A leaf-specific gene stimulated by light during wheat acclimation to low temperature. Plant Mol. Biol. 23:255-265.

Cooper, W.C., B.S. Gorton, and S. Tayloe. 1954. Freezing tests with small trees and detached leaves of grapefruit. Proc. Amer. Soc. Hort. Sci. 63:167-172.

Ebel, R.C., P.A. Carter, W.A. Dozier, D.A. Findley, M.L. Nesbitt, B.R. Hockema, and J.L. Sibley. 2004. Pattern of exotherm and electrolyte leakage measured at high frequency of Satsuma mandarin exposed to subfreezing temperatures. HortScience 39:1614-1616.

Ferl, R.J. 1996. 14-3-3 proteins and signal transduction. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47:49-73.

Garnier, E. and A. Berger. 1985. Testing water potential in peach trees as an indicator of water stress. J. Hort. Sci. 60:47-56.

Gilmour, S.J., N.N. Artus, and M.F. Thomashow. 1992. cDNA sequence-analysis and expression of two cold-regulated genes of *Arabidopsis thaliana*. Plant Mol. Biol. 18:13-21.

Gmitter, F.G., J.W. Grosser, and G.A. Moore. 1992. Citrus, p. 335-369. In: F.A. Hammerschlag and R.E. Litz (eds.) Biotechnology of Perennial Crops. CAB International, Wallingford, UK.

Guy, C.L. 1990. Cold acclimation and freezing stress tolerance: role of protein metabolism. Annu. Rev. Plant Physiol. Plant Mol. Biol. 41:187-223.

Hughes, M.A. and M.A. Dunn. 1990. The effect of temperature on plant growth and development. Biotech. Genet. Eng. Rev. 8:161-188.

Hughes, M.A. and M.A. Dunn. 1996. The molecular biology of plant acclimation to low termperature. J. Exp. Bot. 47:291-305.

Horvath, D.P., B.K. McLarney, and M.F. Thomashow. 1993. Regulation of *Arabidopsis thaliana* L. (Heyn) cor78 in response to low temperature. Plant Physiol. 103:1047-1053.

Horvath, D.P., and P.A. Olson. 1998. Cloning and characterization of cold-regulated glycine-rich RNA-binding protein genes from leafy spurge (*Euphorbia esula* L.) and comparison to heterologous genomic clones. Plant Mol. Biol. 38:531-538.

Jarillo, J.A., J. Capel, A. Leyva, J. Miguel, J.M., Martinez-Zapater, and J, Salinas. 1994. Two related low-temperature-inducible genes of *Arabidopsis* encode proteins showing high homology to 14-3-3 proteins, a family of putative kinase regulators. Plant Mol. Biol. 25:693-704.

Lang, P., C.K. Zhang, R.C. Ebel, F. Dane, and W.A. Dozier. 2005. Identification of cold acclimated genes in leaves of *Citrus unshiu* by mRNA differential display. Gene 359:111-118.

Monroy, A.F., Y. Castonguay, S. Laberge, F. Sarhan, L.P. Vezina, and R.S. Dhindsa. 1993. A new cold-induced alfalfa gene is associated with enhanced hardening at subzero temperature. Plant Physiol. 102:873-879.

Nesbitt, M.L., R.C. Ebel, D. Findley, B. Wilkins, F. Woods, and D. Himelrick. 2002. Assays to Assess Freeze Injury of Satsuma mandarin (*Citrus unshiu*). HortScience 37:871-877.

Okamoto, M., J.J. Vidmar, and A.D.M. Glass. 2003. Regulation of NRT1 and NRT2 gene families of *Arabidopsis thaliana*: responses to nitrate provision. Plant Cell Phsiol. 44:304-317.

Pallasch, L., H. Beswick, J. Talian, and P. Zelenka. 1990. Use of an RNA folding algorithm to choose regions for amplification by the polymerase chain reaction. Analyt. Biochem. 185:57-62.

Pires-Alves, M., M.F. Grossi-de-Sa, G.B.S. Barcellos, C.R. Carlini, and M.G. Moraes. 2003. Characterization and expression of a novel member (JBURE-II) of the urease gene family from jackbean [*Canavalia ensiformis* (L.) DC]. Plant Cell Physiol. 44:139-145.

Savelkoul, P.H.M., H.J.M. Aarts, J. de Haas, L. Dijkshoorn, B. Duim, M. Otsen, J.L.W. Rademaker, L. Schouls, and J.A. Lenstra. 1999. Amplified-fragment length polymorphism analysis: the state of an art. J. Clinic. Microbiol. 37:3083-3091.

Soost, R.K. and J.W. Cameron. 1975. Citrus, p. 507-540. In: J. Janick and J.N. Moore (eds.) Advances in Fruit Breeding, Purdue University Press, West Lafayette, IN.

Soost, R.K. and M.L Roose. 1996. Citrus, p. 257-323. In: J. Janick and J.N. Moore (eds.) Advances in Fruit Bredding, Purdue University Press, West Lafayette, IN.

Spiegal-Roy, P. and E.E. Goldschmidt. 1996. Biology of Citrus. Cambridge University Press, Cambridge, UK p.30.

Taylor, J. and L.A Harrier. 2003. Expression studies of plant genes differentially expressed in leaf and root tissues of tomato colonized by arbuscular mycorrhizal fungus *Glomus mosseae*. Plant Mol. Biol. 51:619-629.

Weretilnyk, E., W. Orr, T.C. White, B. Iu, and J. Singh. 1993. Characterization of three related low-temperature-regulated cDNAs from winter *Brassica napus*. Plant Physiol. 101:171-177.

Yelenosky, G. 1985. Cold hardiness in citrus. Hort. Rev. 7:201-238.

Yelenosky, G., C.J. Hearn, and D.J. Hutchison. 1984. Nonhardening temperatures – Major factor in freeze damage to citrus trees in December 1983. Proc. Fla. State Hort. Soc. 97:33-36.

Young, R.G. 1961. Influence of day length, light intensity, and temperature on growth, dormancy, and cold-hardiness of Red Blush grapefruit trees. Proc. Amer. Soc. Hort. Sci. 78:174-180.

Young, R.H. and A. Peynado. 1962. Growth and cold-hardiness of citrus and related species when exposed to different night temperatures. Proc. Amer. Soc. Hort. Sci. 81:238-243.

Young, R.H. and A. Peynado. 1965. Changes in cold hardiness and certain physiological factors of Redblush grapefruit seedlings as affected by exposure to artificial hardening temperatures. Proc. Amer. Soc. Hort. Sci. 86:244-252.

Zhang, C., P. Lang, F. Dane, R.C. Ebel, N.K. Singh, R. Locy, and W.A. Dozier. 2005. Cold acclimation induces genes of trifoliate orange (*Poncirus trifoliata)*. Plant Cell Rep. 23:764-769.

Table 3.1. Oligonucleotide primer sequence, PCR conditions, and cycle numbers for the differential display (DD) product with quantitative relative reverse transcription (RT)-PCR

| DD Product | Forward Primer (5'-3') | Reverse Primer (5'-3') | PCR annealing temperature (C°) | Cycle number |
|---|---|---|---|---|
| 010 | ttgcaccatggccaaacag | ggttgccagaattggtatgtag | 55 | 32 |

Table 3.2. *P* > *F* of the ANOVA for dependent variables measured during cold acclimation of *C. unshiu* and *P. trifoliata*.

| Significance | $\Psi_{stem}$[y] (MPa) | $\Psi_{leaf}$ (MPa) | $C_{leaf}$ (mole/m$^2$/s) | Transpiration (mmole/m$^2$/s) | $ACO_2$ ($\mu$mole/m$^2$/s) |
|---|---|---|---|---|---|
| S*A*W[z] | 0.6044 | 0.4351 | 0.5775 | 0.6511 | 0.4230 |
| A*W | <0.0001 | <0.0001 | 0.0002 | <0.0001 | <0.0001 |
| S*W | <0.0001 | 0.8313 | 0.8268 | 0.4640 | 0.0056 |
| W | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 |
| S*A | 0.7153 | 0.6644 | 0.3719 | 0.8020 | 0.5862 |
| A | <0.0001 | <0.0001 | 0.0008 | <0.0001 | 0.0060 |
| S | <0.0001 | 0.6208 | 0.1770 | 0.0040 | <0.0001 |

[z]S = specie, A = acclimation treatment, W = week
[y]$\Psi_{stem}$ = stem water potential, $\Psi_{leaf}$ = leaf water potential, $C_{leaf}$ = leaf conductance, and $ACO_2$ = net $CO_2$ assimilation rate.

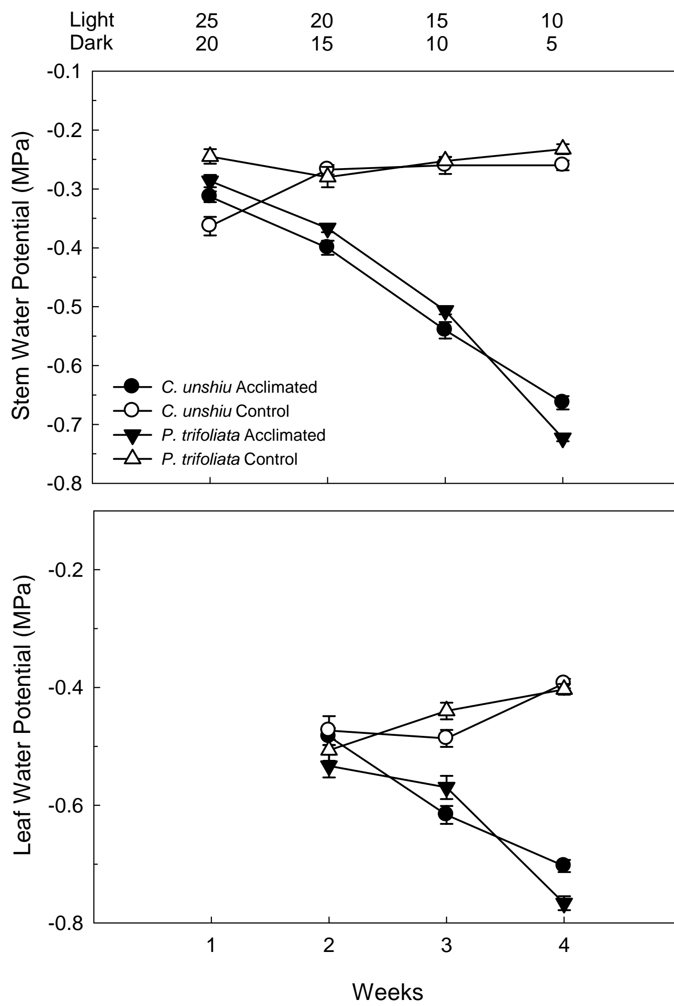


Fig. 3.1. Stem water potential and leaf water potential during cold acclimation of *C. unshiu* and *P. trifoliata*. The numbers above the graph indicate temperatures (˚C) during the light and dark periods, which were 12 hr each.

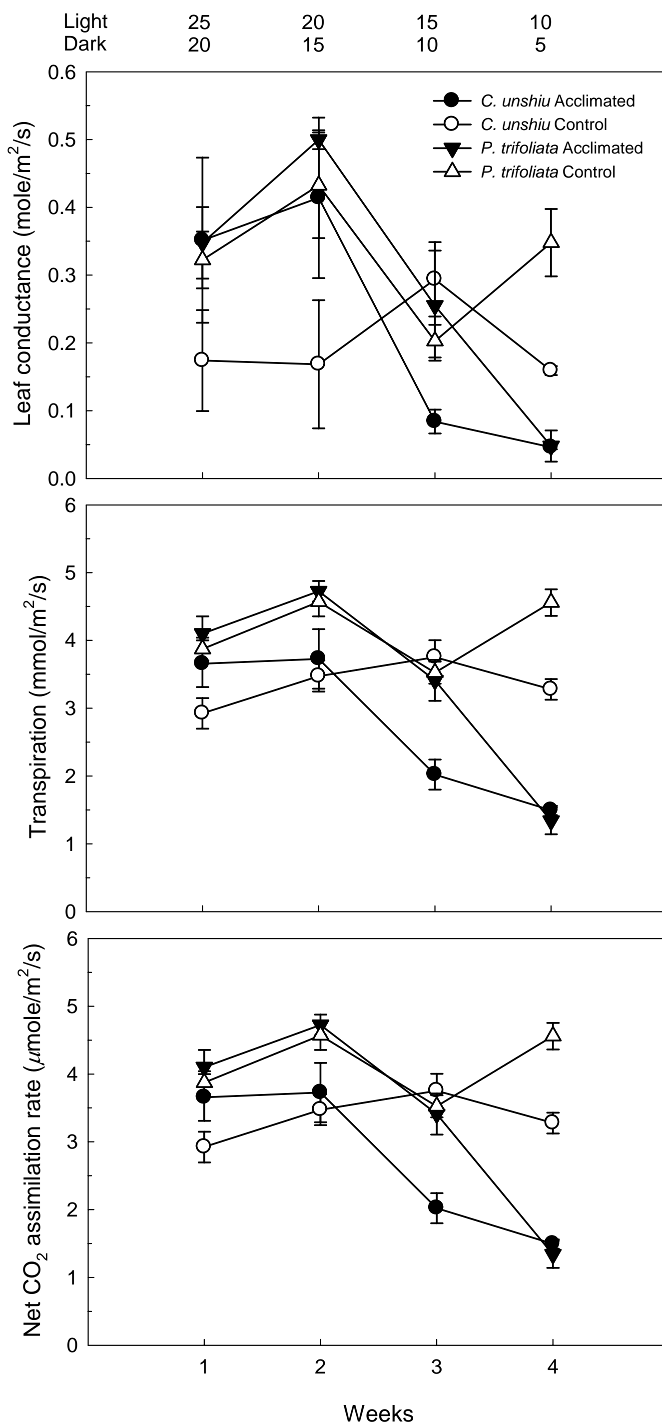


Fig. 3.2. Decline in leaf conductance, transpiration, and net $CO_2$ assimilation rate during cold acclimation of *C. unshiu* and *P. trifoliata*. The numbers above the graph indicate temperatures (C˚) during the light and dark periods, which were 12 hr each.

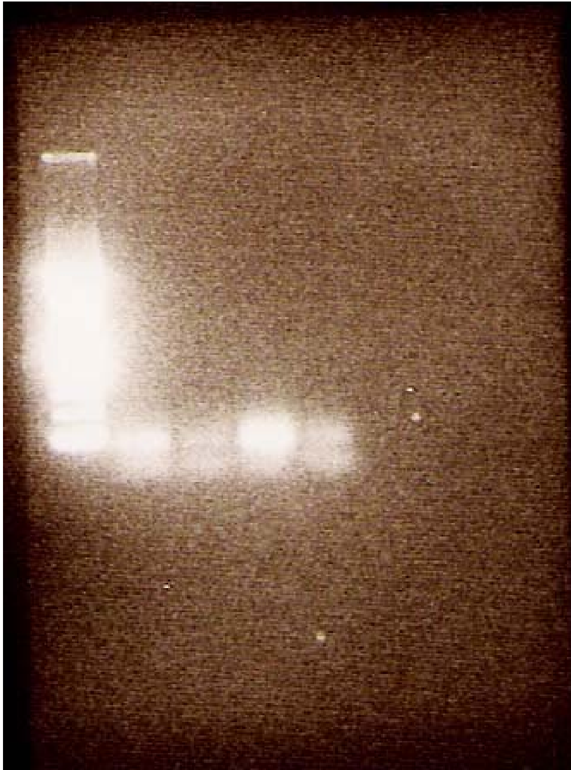

Fig. 3.3. Differential display (DD) products separated using a 1.8% agarose gel electrophoresis. Lanes (left to right) DNA ladder bp 100, unacclimated control *P. trifoliata*, cold-acclimated *P. trifoliata*, unacclimated control *C. unshiu*, cold-acclimated *C. unshiu*.

# CHAPTER IV

## SUGGESTIONS FOR FUTURE RESEARCH

Research has provided evidence that environmental temperature exerts a profound influence on cellular metabolism, affects the integrity of cell structure, and influences the direction of specific developmental events. It is also known that plants that exhibit high tolerance to cold temperatures undergo significant changes in ultrastructure during cold acclimation (Burke and Stushnoff, 1979; Gordon-Kamm and Steponkus, 1984a, 1984b; Kratsch, 2000; Singh, 1979; Steponkus, 1984). Because *P. trifoliata* is able to withstand lower temperatures compared to *C. unshiu*, it needs to be determined if *P. trifoliata* demonstrates these same ultrastructural changes. Ultrastructure of membranes and how they may differ between *P. trifoliata* and *C. unshiu* during cold acclimation is one important consideration and line of potential research.

There has been extensive research conducted on gene expression in citrus and a number of important findings have been documented. 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), while other genes and enzymes related to secondary metabolism, cell-wall modification, and oxidative tolerance have been identified form citrus fruits treated by heat or plant hormones (Lafuente et al., 2004; Sanchez-Ballesta et al., 2003). Cold-acclimation induced gene expression was examined on *P. trifoliata* (Zhang, 2005) and resulted in the up-regulation of several genes involved in the maintenance of osmotic

balance, scavenging of reactive oxygen species, and photo-oxidative protection of plants. Genes identified were: *Betaine/proline transporter*, *Water-channel protein*, *Aldo-keto reductase*, *Early-light-inducible protein*, *Nitrate transporter NRT1*, *Tetratricopeptide repeat-containing protein*, *F-box protein family*, and *Ribosomal protein L15* (Zhang et al, 2005). Zhang et al. (2004) also examined down-regulated gene expression of cold acclimated *P. trifoliata* and found three of the six down-regulated genes were associated with photosynthesis, one gene with cell wall biogenesis and two genes with biotic defense. Genes found were: *Chlorophyll a/b binding protein* (*LHC*), *Photosystem II OEC 23*, *Carbonic anhydrase*, *Tumor related protein*, *Pyrrolidone-carboxylate peptidase*, and *Beta-galactosidase*. Another study was conducted to identify cold acclimated genes in *C. unshiu*, six up-regulated genes and two down-regulated genes were confirmed. Cold – acclimation induced genes included: *14-3-3 d-2 protein*, *ribosomal protein L15*, *Nucleoside diphosphate kinase III*, *regulator of chromosome condensation-like protein*, *amino acid permease*, *miraculin-like protein 2*, and *beta-galactosidase* (Lang et al., 2005). Similar genes were identified in both *C. unshiu* and *P. trifoliata* in response to cold acclimation, but further research needs to be conducted to examine specific function of genes within species. Osmotic modulation, photo-oxidative protection and photosynthesis adjustment were three responses for *P. trifoliata* to low temperature (Zhang et al., 2004, 2005); whereas, the identified genes in *C. unshiu* were mainly related to signal transduction, protein synthesis, amino acid transport, adjustment of chromosome structure, plant defense, and cell wall metabolism (Lang, 2005).

The cold acclimation process requires the 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 the activation of ion channels (Knight et al., 1996) through gene expression. *P. trifoliata*, the most cold hardy of citrus species, is used in breeding programs and as a rootstock for *C. unshiu*. However, *P. trifoliata* is considerably more cold hardy. There must be a significant genetic overlap among these species, yet differences in freeze tolerance likely result from significant differences in gene expression during cold acclimation (Lang et al. 2005, Zhang et al. 2005).Comparing the differences of composition of soluble, membrane protecting structures and gene regulation of these two different areas in *C. unshiu* and *P. trifoliata* are also important considerations and lines of potential research.

Depending on specific genetic features and characteristics of temperature stress, plants during cold acclimation produce cells of specific ultastructure (Levitt, 1980) which induce gene expression to increase cold tolerance (Thomashow, 1999). Determining ultrastructural changes in *P. trifoliata* during cold acclimation and comparing the ultrastructure of membranes between *P. trifoliata* and *C. unshiu* will further assist in isolation of genes expressed during cold acclimation, and explain the difference in cold tolerance between these two citrus species. Findings may potentially lead to the genetic modification of *C. unshiu* to increase cold hardiness, and ultimately revitalize the Satsuma mandarin industry in the Southeast region of the U.S.

## Literature Cited

Bohnert, H.J. D.E. Nelson, and R.G. Jensen. 1995. Adaption to environmental stresses. Plant Cell. 7:1099-1111.

Burke, M.J. and C. Stushnoff. 1979. Frost hardiness: a discussion of possible molecular causes of injury with particular refernce to deep supercooling of water, p.197-225. In: H. Mussell and R.C. Staples (eds.) Stress Physiology in Crop Plants. Wiley-Interscience, New York.

Cai, Q., G.A. Moore, and C.L. Guy. 1995. An unusual group 2 *LEA* gene family in citrus responsive to low temperature. Plant Mol. Biol. 29:11-23.

Gordon-Kamm, W.J and P.L. Steponkus. 1984a. The behavior of the plasma membrane following osmotic contraction of isolated protoplasts: implications in freezing injury. Protoplasma 123:83-94.

Gordon-Kamm, W.J. and P.L. Steponkus. 1984b. The influence of cold acclimation on the behavior of the plasma membrane following osmotic contraction of isolated protoplasts. Protoplasma 123:161-173.

Hara, K., M. Yagi, N. Koizumi, T. Kusano, and H. Sano. 1999. Screening of wound-responsive genes identifies an immediate-early expressed gene encoding a highly charged protein in mechanically wounded tobacco plants. Plant Cell Physiol. 41:684-691.

Knight, H., A.J. Trewavas, and M.R. Knight. 1996. Cold calcium signaling in *Arabidopsis* involves two cellular pools and a change in calcium signature after acclimation. Plant Cell 8:489-503.

Kratsch, H.A., and Wise R.R. 2000. The ultrastructure of chilling stress. Plant Cell Environ. 23:337-350.

Lafuente, M.T., J.M. Sala, and L. Zacarias. 2004. Active oxygen detoxifying enzymes and phenylalanine ammonia-lyase in the ethylene-induced chilling tolerance in citrus fruit. J. Agric. Food Chem. 52:3606-3611.

Lang, P., C. Zhang, R.C. Ebel, F. Dane, and W.A. Dozier. 2005. Identification of cold acclimated genes in leaves of *Citrus unshiu* by mRNA differential display. Gene 359:111-118.

Levitt, J. 1980. Responses of plants to environmental stresses. Second Edition, Vol.1. Chilling, freezing, and high temperature stresses. Academic Press, New York.

Lynch, D.V. and P.L. Steponkus. 1987. Plasma membrane lipid alterations associated with cold acclimation of winter rye seedlings (*Secale cereale* L.cv *Puma*). Plant Physiol. 83:761-767.

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

Sanchez-Ballesta, M.T., Y. Lluch, M.J. Gosalbes, L. Zacarias, A. Granell, and M.T. Lafuente. 2003. A survey of genes differentially expressed during long-term heat-induced chilling tolerance in citrus fruit. Planta 218:65-70.

Singh, J. 1979. Ultrastructural alterations in cells of hardened and non-hardened winter rye during hyperosmotic and extracellular freezing stresses. Protoplasma 98:329-341.

Steponkus, P.L. 1984. Role of the plasmamembrane in freezing injury and cold acclimation. Annu. Rev. Plant Physiol. 35:543-584.

Thomashow, M.F. 1994. *Arabidopsis thaliana* as a model for studying mechanisms of plant cold tolerance, p. 807-834. In: E. Meyeowitz and C. Sommervile (eds.) In Arabidopsis. Cold Spring Harbor Laboratory, Cold Spring Harbor.

Thomashow, M.F. 1999. Plant cold acclimation: Freezing tolerance genes and regulatory mechanisms. Annu. Rev. Plant Physiol. Plant Molec. Bio. 50: 571-599.

Tseng, J. and P.H. Li. 1991. Changes in protein synthesis and transductible messenger RNA populations associated with ABA-induced cold hardiness in potato (*Solanum commersonii*). Physiol. Plant 81:349-358.

Zhang, C., P. Lang, R.C. Ebel, F. Dane, N.K. Singh, R.D. Locy, and W.A. Dozier. 2004. Down-regulated gene expression of cold acclimated *Poncirus trifoliata*. Can. J. Plant Sci. 85:417-424.

Zhang, C., P. Lang, F. Dane, R.C. Ebel, N.K. Singh, R.D. Lacy, and W.A. Dozier. 2005. Cold acclimation induced genes of trifoliate orange (*Poncirus trifoliata*). Plant Cell Rep. 23:764-769.