Rapid Freeze Acclimation of *Poncirus trifoliata* Seedlings Exposed to 10 °C and Long Days

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Abstract. *Poncirus trifoliata* (L.) Raf. seeds were germinated in perlite under intermittent mist at about 25 °C and natural daylight in a greenhouse. Two-week-old seedlings were then transferred into a growth chamber at 25 °C and 16-hour daylength for 1 week. Tissue samples were collected at 0, 6, 24, 168, and 504 hours after temperature equilibration at 10 °C. Freezing tolerance at –6.7 °C, as determined by electrolyte leakage, and stem (leaves attached) water potential (Ψ_x), measured using a pressure chamber, was recorded for a subset of seedlings for each time interval. Red coloration (apparently anthocyanin) developed at the petiole leaflet junction and buds after 48 hours at 10 °C and gradually occurred throughout the leaves during further exposure. Complementary DNA clones for phenylalanine ammonia lyase (PAL), 4-coumarate : coA ligase (4CL), and chalcone synthase (CHS) were used to probe RNA isolated from the leaves. No increase in steady-state messenger RNA level was detected. Increases in freeze hardness occurred within 6 hours in the leaves, and continued for up to 1 week. Water potential initially decreased from –0.6 to –2.0 MPa after 6 hours, then returned to –0.6 MPa after 1 week. Thus, *Poncirus trifoliata* seedlings freeze-acclimate significantly after only 6 hours at 10 °C.

Citrus is a freeze-tender evergreen of tropical and subtropical origin capable of freeze acclimating (Yelenosky, 1985). However, even the hardiest citrus species do not approach the freeze tolerance level of many woody temperate species, some of which can survive freezing in liquid nitrogen when acclimated (George et al., 1974). When fully acclimated, the minimum leaf temperature at which *C. sinensis* can survive is about –6.7 °C (Yelenosky, 1985).

Long-term freeze acclimation of citrus has been studied for many years. Young (1970) used 9- to 12-month-old seedlings of many different cultivars and examined freeze acclimation based on diurnal temperature regimes of 21/10, 16/4, 10/–1, and 7/–3.3 °C. Seedlings were exposed to each temperature regime for 2 weeks successively and degree of acclimation was determined by freezing leaves and then examining the level of watersoaking. His rankings of citrus species in general corre-

lated with field observations following an actual freeze (Cooper, 1965). Citrus trees freeze-acclimated at low temperatures under short (8-h) days were more hardy than those exposed to the same temperatures under long (16-h) days (Young, 1961). Yelenosky (1978) found that the leaves of 'Valencia' oranges on 'Rusk' citrange rootstock would eventually freeze-harden to –6.7 °C after 28 d of exposure to 10 °C. These experimentally determined values agree with the observed low-temperature limit for *C. sinensis*.

Physiological changes occurring during freeze acclimation of citrus include increases in sugar levels, colloid stability, sap concentration, proline, valine, and bound water (Yelenosky, 1985), and decreases in total water, soluble protein, total water content, water potential (Ψ_x), freezing point, other amino acids, and reduced glutathione (Yelenosky, 1978; Young, 1970). Drought (Davies et al., 1981) and salinity stress (Syvertsen and Yelenosky, 1988) also increase freeze tolerance.

Weiser (1970) suggested that cold acclimation might be the result of altered gene regulation. Since then, changes in gene expression related to freeze acclimation have been reported in several crops, including *Pisum sativum* (Weiser et al., 1990), *Bromus inermis* Leyss (Lee et al., 1991), *Arabidopsis thaliana* L. Heynh (Welin et al., 1995), and members of the true citrus group (Cai et al., 1995).

Physiological changes in citrus during

freeze acclimation have been thoroughly examined on a long-term (weekly or monthly) basis. Existing physiological and molecular biology methods now permit examination of early changes in citrus freeze hardness and gene expression. Our objective was to study changes in freeze hardness, Ψ_x , and gene expression of *P. trifoliata* during short-term (hours to weeks) exposure to freeze-acclimating temperatures and long days.

Materials and Methods

Plant material. *Poncirus trifoliata* seeds were purchased from Willits and Newcomb (Arvin, Calif.). Seeds were germinated under mist in the greenhouse in flats using a 100% perlite growing media and held in the greenhouse for 6 weeks under natural daylight and 24 °C day/21 °C nights. Seedlings in flats were then transferred into a Conviron model E15 growth chamber (Winnipeg, Manitoba, Canada) and grown at 25 °C under 16-h days [photosynthetic photon flux (PPF) = 450 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$] for 2 weeks, after which the temperature was decreased to a constant 10 °C.

Assessing freeze hardness. Fully expanded leaves, 2.5-cm stem sections, and 2.5 cm of the root system were harvested from each seedling to be tested using a scalpel. Ten replicates of each tissue sample from separate seedlings were collected at each acclimation (exposure to 10 °C) time of 0, 6, 24, 168, and 504 h. Excised seedling tissues were placed in 16 × 100-mm tubes, and 100 μL of distilled deionized water was added. Tubes were immersed in a precooled (–1 °C) model 2425 glycol bath and circulator (Forma Scientific, Marietta, Ohio) and allowed to equilibrate for 30 min. One subsample of each treatment was removed as a nonfrozen control. Water in the bottom of all tubes was then nucleated homogeneously by adding a small cloud of ice crystals. Temperatures in the bath were lowered 0.1 °C/min until –6.7 °C was reached. Tubes were held for 1 h at –6.7 °C and then removed. Frozen samples were allowed to thaw overnight at 1 °C in a low-temperature incubator to eliminate injury caused by rapid warming. Tubes were removed from the incubator, allowed to reach 20 °C, and initial electrolyte leakage was measured with a model CDM3 conductivity meter (Radiometer, Copenhagen, Denmark). Samples were then autoclaved for 25 min to simulate 100% damage and a second electrolyte leakage measurement was taken. Relative percent electrolyte leakage was determined based on the method of Flint et al. (1967) with minor modifications. Relative electrolyte leakage for each sample was determined using the equation shown below (F = electrolyte leakage of tested sample; NFC = electrolyte leakage of nonfrozen control; BA = before autoclaving; AA = after autoclaving). Percentage of electrolyte leakage is positively correlated with loss of membrane integrity and is generally assumed to be a relative measure of tissue injury. Thus, increased relative electrolyte leakage following exposure to –6.7 °C is associ-

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ated with increased injury due to freezing. This experiment was repeated twice using different sets of seedlings.

% Electrolyte Leakage =

$$1 - \frac{1 - \left(\frac{F_{BA}}{F_{AA}} \right)}{1 - \left(\frac{NFC_{BA}}{NFC_{AA}} \right)} \times 100$$

Ψ_x measurements. Seedlings exposed to the same conditions used for freeze-hardiness measurements were removed from the tray and the lower 0.5 cm below the root stem interface was excised using a sterile scalpel and discarded. The stem with leaflets intact was then placed in a pressure chamber (Soil Moisture Equipment, Santa Barbara, Calif.). Pressure was increased gradually until xylem sap could be seen emerging from the cut end with the use of a 10× hand-held lens (Scholander et al., 1965). Ten replicate seedlings were harvested after 0-, 6-, 24-, 168-, and 504-h exposure to 10 °C. The experiment was repeated three times on different sets of seedlings. Two sets of seedlings were subsets of those harvested for the electrolyte leakage test and the third set was treated identically, but only used for Ψ_x measurements.

Anthocyanin extraction. Five grams of leaf tissue (harvested after 0-, 6-, 24-, 168-, and 504-h exposure to 10 °C) were ground in liquid nitrogen, washed in acetone, and centrifuged, and this process was repeated until the acetone was clear after centrifugation. The supernatant was discarded after each wash. The pellet was then resuspended in TNE buffer (100 mM Tris-HCl pH 8.5, 100 mM NaCl, 10 mM EDTA pH 8.5, 0.1% Triton X-100, by volume, and 15 mM DTT added at use). In some of the samples, a blue-gray color appeared that could be changed to crimson red by reducing the pH to ≈5 by adding 5 N HCl as modified from Neuhaus et al. (1993). This experiment was repeated twice using a subset of seedlings from the electrolyte leakage experiments.

RNA extraction and analysis. Total RNA was extracted from leaves of *P. trifoliata* seedlings exposed to 10 °C for 0, 24, and 168 h, using the method of Schneiderbauer et al. (1991) for plants high in phenolic compounds, except that the RNA was precipitated in 1.7 M LiCl instead of being pelleted through a CsCl cushion. Concentration and integrity of the RNA was verified by electrophoresis through an agarose gel and staining with ethidium bromide. Ten milligrams of total RNA was subjected to electrophoresis in formaldehyde agarose gels (Sambrook et al., 1989) and transferred using a vacuum blotter (Hoefer) to Hybond N+ membranes (Amersham) with 50 mM NaOH as transfer buffer. Complementary DNA (cDNA) clones for phenylalanine ammonia lyase (PAL) and 4-coumarate : coenzyme A ligase (4CL) (from *Populus deltoides*) were provided by Carl Douglas (Univ. of British Columbia). A cDNA clone for citrus chalcone synthase (CHS) was provided by

Diane Luth and Gloria Moore (Univ. of Florida). Clone 24 (Parsons et al., 1989) encoding chlorophyll a/b binding protein (J.M.D., unpublished data) served as a positive control for equal loading and hybridization on the blots. The cDNA inserts were amplified by the PCR using forward and reverse sequencing primers, purified using Qiaex resin (Qiagen, Chatsworth, Calif.), and then labeled with α^{32} dCTP using a random primers DNA labeling kit (Gibco BRL, Gaithersburg, Md.). Hybridization and washing were as previously described (Church and Gilbert, 1984) except that the final wash was performed at 55 °C. Northern analysis was performed on RNA extracted from subsets of seedlings from plants in two independent experiments.

Results and Discussion

Freeze hardiness. Injury to *P. trifoliata* leaves and stems was significantly reduced at -6.7 °C following exposure to 10 °C for 6 h (Fig. 1). Within 6 h of exposure to 10 °C, leaf percent electrolyte leakage was reduced by half compared to leaves harvested at time 0 h, and a similar decrease was observed in stems. Leaf electrolyte leakage reached a plateau at 10% after 24 h of acclimation, but then began to increase again after 168 h. This increase in percent electrolyte leakage coincided with the initiation of leaf drop in the growth chamber. After one week of exposure to 10 °C, stem sections had only 5% electrolyte leakage, eventually showing no relative electrolyte leakage after 504 h when frozen to -6.7 °C. A more rapid decrease in percent electrolyte leakage with acclimation occurred in leaves and stems than in roots. Root tissue showed no significant change in percent electrolyte leakage in 6 h; however, there was a one-third reduction after 24 h. The degree of freeze acclimation of roots was surprising; it is not usually measured because root temperatures rarely fall below 0

°C in commercial citrus-growing regions. Following 504 h of 10 °C, the remaining leaves and the roots showed 15% to 20% electrolyte leakage, but percent electrolyte leakage was not significantly different between the two.

Ψ_x changes. The Ψ_x of the *P. trifoliata* seedlings decreased from -0.75 to -2.0 MPa within 2 h of freeze acclimation, with minimum levels occurring at 6 to 8 h (Fig. 2). After 24 h at 10 °C, Ψ_x levels remained significantly more negative than at time zero. After 1 week of exposure to 10 °C, however, Ψ_x levels approached those of nontreated seedlings.

Young (1970), Cooper (1965), and Yelenosky (1975, 1978) observed that exposure to low temperature regimes reduced freeze injury to citrus trees. These observations were based on trees or seedlings that had been exposed to freeze-acclimating temperatures for 1 week or more. In this study significant reductions in percent electrolyte leakage occurred after as little as 6 h of acclimation. These rapid changes may be due to changes in viscosity of water and membrane changes at low temperatures (Uemura and Yoshida, 1984), but changes in gene expression could also be important.

Similar changes in Ψ_x have been observed in citrus leaves during acclimation. The Ψ_x of 'Valencia' orange leaves on 1-year-old potted plants decreased from -1.8 to -2.0 MPa, but only after 4 weeks of freeze-hardening temperatures (Yelenosky, 1978). Decreases in Ψ_x were also observed in 'Carrizo' citrange leaves after 6 weeks of successively lower temperature exposure to the roots (lowest temperature was 5 °C) (Wilcox et al., 1983). In our work, *P. trifoliata* (the male parent of 'Carrizo' citrange) seedlings showed a similar magnitude of change in Ψ_x after only 6 h of acclimation (Fig. 2). Nevertheless, Ψ_x values approached those of nontreated seedlings after 1 week of acclimation. In previous studies, Ψ_x remained low throughout the acclimation pe-

Fig. 1. Percent electrolyte leakage of roots, stems, and leaves of *P. trifoliata* 'Rubidoux' at -6.7 °C after various freeze-acclimation periods at 10 °C. Arrow indicates where leaf drop began to occur. Each data point represents the mean of 10 seedlings ± se. The experiment was repeated twice, with similar results.

Fig. 2. Ψ_x of *P. trifoliata* seedlings exposed to 10 °C for various time periods using a pressure chamber. Experiments were repeated three times. Representative graphs of each type of experiment are shown; (A) 24-h time course; (B) 1-week time course. Each bar point represents the mean of 10 seedlings $\pm$ SE.

riod (Wilcox et al., 1983; Yelenosky, 1978). The increase in Ψ_x may have occurred because of stomatal closure at low temperatures, which stabilized Ψ_x .

Pigment changes. During exposure to 10 °C, a red pigment became visible first at the junction of the petiole and leaflets of *Poncirus trifoliata* seedlings (48 h) and later on the leaves (168 h) and in the stem (504 h) (data not shown). The pigment was extractable in an aqueous solution and showed a pH-dependent color change from blue (high pH) to pink (low pH) and may, therefore, be an anthocyanin based on previous studies (Neuhaus et al., 1993). Anthocyanin-like compounds were detectable in leaf extracts after 168 h of exposure to 10 °C and in stem extracts after 504 h (data not shown).

RNA changes. In other studies where flavonoid biosynthesis was induced by environmental stresses, messenger RNA levels of genes in the phenylpropanoid pathway (PAL, CHS, 4CL) increased before or simultaneous with pigment accumulation (Christie et al., 1994; Leyva et al., 1995). Christie et al. (1994) showed increased levels of PAL, 4CL, and CHS mRNA when *Zea mays* seedlings were exposed to 10 °C for 24 h, and increased anthocyanin levels in 10 °C treated seedlings as compared to controls at 7 d. Leyva et al. (1995) demonstrated that PAL and CHS transcript levels increased in *Arabidopsis thaliana* when exposed to 4 °C. To determine if this was also occurring in citrus, cDNAs encoding enzymes in the flavonoid biosynthesis pathway were used to probe RNA extracted from *P. trifoliata* seedlings at 0, 24, and 168 h. Hybridization was observed to all three of the probes tested (PAL, CHS, 4CL) as well as to the positive control probe (CAB). However, no increases in steady state RNA levels were observed relative to the 0-h treatment (Fig. 3). This result suggests that processes other than PAL, CHS, and 4CL induction are involved in citrus pigment accumulation, or that transient

increases in mRNA levels occurred that were not detected at 24 and 168 h.

Anthocyanin production often occurs concomitantly with freeze acclimation (Parker, 1963). *Cornus stolonifera* L. (Van Huystee et al., 1967), *Cicer arietinum* L. (Singh et al., 1995), and *Pinus contorta* Dougl. var. *latifolia* Engelm. (Camm et al., 1993) showed increases in anthocyanin production in leaves and/or bark concomitant with increases in freeze hardiness. Anthocyanin accumulation in *Hedera helix* (Steponkus and Lanphear, 1969) and chickpea (Singh et al., 1995) was incidental to increases in freeze hardiness. Parker (1963) suggested that increases in anthocyanin may not be a direct response to acclimating temperatures, but may be an indirect response to increased sugar levels. Whatever the primary cause of anthocyanin accumulation, pigments probably do not promote freezing acclimation in citrus. However, our data clearly show that early biochemical and metabolic changes were taking place in association with acclimation temperatures. Techniques such as differential display of mRNA (Liang and Pardee, 1992) could prove useful for determining the extent to which these changes are due to gene expression, and for identifying the genes involved.

Percentage of electrolyte leakage and Ψ_x change rapidly, within hours, when *P. trifoliata* seedlings are exposed to 10 °C and long days. In addition, there are gross pigment changes that are detectable in crude extracts after 1 week. In an effort to understand citrus freeze acclimation and, thus, possible mechanisms of freeze acclimation in other subtropical crops, short-term changes that occur in hours and even minutes should be examined. Biochemical changes during freeze acclimation may be initiated long before they are manifested at the cell or whole-organ level.

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Fig. 3. Northern blot analysis of total RNA (10 µg/lane) extracted from *P. trifoliata* 'Rubidoux' seedling leaves during a 1-week (168-h) exposure to 10 °C. Ethidium bromide (EtBr) was used to evaluate RNA quantity and quality among lanes. Hybridization probes included: 4-coumarate : coenzyme A ligase (4CL), chalcone synthase (CHS), chlorophyll ab binding protein (CAB), and phenylalanine ammonia lyase (PAL). Blots were washed at moderate stringency. Northern blots were completed on RNA extracted on two separate occasions from two separate experiments with similar results.

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