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Changes in freezing tolerance, abscisic acid concentraion, and gene expression during cold acclimation of Acer rubrum fine roots /

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CHANGES IN FREEZING TOLERANCE, ABSCISIC ACID CONCENTRAION, AND GENE EXPRESSION DURING COLD ACCLIMATION OF ACER RUBRUM FINE ROOTS

A Thesis Presented

By

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DEDICATION

To Amy Stukuls, whose endless support and encouragement were essential to the completion of this work

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ABSTRACT

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Fine roots (i.e. diameter < 1 mm) of 1 year-old Acer rubrum were studied during the autumn and early winter of 1997 to determine if this root type is capable of cold hardening, and if cold hardening is associated with changes in abscisic acid (ABA) concentration and gene expression. An electrolyte leakage assay was employed to determine the freezing tolerance of fine roots every two weeks throughout a natural period of cold acclimation, and results showed that freezing tolerance increased markedly during cold acclimation (e.g. from a killing temperature of -2 °C on September 7, 1997, to a killing temperature lower than -10 °C by January 6, 1998). Through the use of an enzyme linked immunosorbent assay (ELISA), it was seen that patterns of ABA accumulation in fine roots varied significantly throughout cold hardening, and reverse transcription – polymerase chain reaction (RT-PCR) showed that modifications in gene expression occur during the acclimation period.

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CHAPTER 1

LITERATURE REVIEW

Introduction

The survival of root tissue in trees during the winter has been shown to markedly affect overall tree recovery from winter freezing injury in subsequent months. Since this is the least hardy tissue found in woody plants, it is of paramount concern to overall plant survival that roots are capable of adapting to freezing temperatures during the winter. While there is great variability in size and age of roots within an individual plant, the few previous studies focusing on root cold acclimation in woody plants have often failed to explain which roots were examined. There is evidence that fine roots are more sensitive to freezing stress than larger and older root types in woody plants (Columbo et al. 1995; Mityga and Lanphear 1971; Wiest and Steponkus 1976). For this reason, the study of cold acclimation in fine roots would provide greater insight into the adaptive responses in woody plants during the acquisition of freezing tolerance.

Since its discovery over 30 years ago, the role of abscisic acid (ABA) as a stress hormone has been widely studied (Skriver and Mundy 1990). ABA is ubiquitous in higher plants, but its involvement in cold acclimation in deciduous tree roots has received little attention. While it is known that ABA accumulates in many plant tissues during cold acclimation, it has not been determined what effects ABA concentration has on freezing tolerance of roots throughout the hardening process (Zeevart and Creelman 1988).

Previous studies have not fully explained what physiological responses accompany the acquisition of freezing tolerance during cold acclimation in woody plant roots. For example, the effects of temperature during cold acclimation on ABA concentration and gene expression in deciduous tree root tissue is in need of further study. This information could lead to the establishment of molecular markers indicating levels of cold tolerance during acclimation. It would then be possible to identify families of trees that would be better able to respond to decreasing temperatures during acclimation with a heightened state of tolerance.

Freezing Injury in Plants

As stationary inhabitants of a constantly changing environment, plant survival in temperate regions is dependent upon their ability to adapt to a diverse range of growing conditions. The seasonal variability in temperature in these regions throughout the year necessitates that changes in plant physiology and biochemistry must occur for extremes in temperature to be endured.

Freezing imposes many strains on plant tissues. Exposure to low temperature is not the cause of freezing injury per se, but rather it is the molecular changes that accompany ice formation which are most injurious to plant tissues. The primary site of freezing injury is the cell membrane, which is ruptured when water freezes intracellularly (Levitt 1980). This is the most direct form of freezing injury. Tolerant plants are able to resist injury due to intracellular ice formation by displacing cellular water into extracellular spaces where it then freezes. While this spares the membrane from rupturing, the protoplasm is left

dehydrated. For this reason, freezing imposes a dehydration stress on the cell that is similar to dehydration from drought, and freezing tolerance is primarily a function of tolerance to extracellular ice formation and the accompanying dehydration of the cell (Levitt 1980).

Low temperatures also alter interactions between different macromolecules, particularly those responsible for membrane properties. The lipid composition of the membrane leaves it particularly vulnerable to freezing injury in part because lipids are the only substances aside from water that exist as a liquid at non-freezing temperatures, and can undergo a phase transition from the liquid crystalline to the solid gel state at lower temperatures (Levitt 1980). This transition can markedly lower membrane permeablility, preventing the escape of water into extracellular spaces before freezing occurs (Levitt 1980). Therefore, the plasticity of the membrane during freezing events is a determining factor in the ability of a cell to tolerate and subsequently recover from the stress. This fact is reflected by the use of the electrolyte leakage test (Flint et al. 1967), which quantifies the efflux of ions from the protoplast as a means of assessing damage to membrane transport functions and loss of semipermeability during freeze thaw cycles.

The loss of water from the protoplasm during extracellular ice formation also corresponds with a rise in concentration of dissolved substances in the cell, such as salt ions and organic acids. This factor places an osmotic stress on the cell, compromising the function of membrane-bound systems (including those

involved in ATP formation and protein phosphorylation (Thomashow 1990)). In addition, the high concentration of certain molecules resulting from water loss will hasten particular reactions, while the kinetics of other reactions will be impeded by low temperature (Palva 1994).

A second form of injury resistance is avoidance of extracellular ice formation through supercooling. Water within plant tissues often supercools below 0 °C before freezing, which spares plant cells from the stress of ice formation. This ability is dependent on the absence of ice-nucleating agents and is variable among species as well as individuals within a species (Levitt 1980). Since the presence of ice-nucleators is invariably high in soil, root tissue does not undergo extensive supercooling (Levitt 1980). While supercooling is an effective means of stress resistance in the short term, if freezing temperatures persist, water will eventually freeze intracellularly.

Acclimation and Tolerance

The ability of a plant to adapt to progressively colder temperatures is known as cold acclimation, a process which leads to a greater tolerance of low temperature stress. In freezing tolerant species, the acclimation process involves changes in plant biochemistry, metabolism, and physiology so that the strain induced by extracellular freezing can be tolerated.

Acclimation in woody perennials is usually first stimulated by the initiation of dormancy during short day photoperiods. Tolerance develops by exposure to

low non-freezing temperatures, and increases in response to falling temperature, until a maximum state of hardiness is reached (Levitt 1980).

The degree to which a plant may resist low temperature injury is variable among species of plants as well as between different tissues within a plant. In woody plants, root tissue is less freezing resistant than shoot tissue (Pellet 1971; Pellet and White 1969; Mityga and Lanphear 1971; Smit-Spinks et al. 1985). For example, in Acer saccharum (sugar maple), stems survived to -37 °C whereas roots were injured by -12 °C (Bertrand et al. 1997). Since both stem and root tissue are essential to plant processes, the overall capacity of the plant to endure freezing stress is largely determined by the survival strategies of its least hardy tissue (i.e. root tissue). In support of this notion, Bertrand et al. (1997) related the ability of sugar maple to recover from low temperatures to the state of hardiness of its roots.

There are significant differences in freezing tolerance among types of roots within an individual plant. Fine roots (also referred to as non-woody or feeder roots and typically with a diameter of < 1 mm) are responsible for the majority of nutrient and water uptake in trees (Marshall and Waring 1985). Fine roots are less suberized and do not have the secondary thickening associated with woody roots (i.e. diameter > 1mm). The degree of winter hardiness has been correlated with greater lignification and suberization of cortical and epidermal cells, which is characteristic of woody roots (Hendrick and Pregitzer 1993). Consequently, fine roots are more susceptible to low temperature injury

than woody roots. While dramatic differences exist between these root types, very few freezing tolerance studies have distinguished between fine and woody roots. However, one study questioned the ability of fine roots to achieve acclimation at all (Havis 1976), but preliminary work in this laboratory has disproved this misconception (unpublished data).

The survival of roots during the winter is crucial for plant recovery from freezing stress during the following months. It was observed in Quebec that winters with thin snow cover and very low temperatures resulted in sugar maple dieback in 1932 (Pomerleau as cited by Bertrand et al. 1994) and in 1981 (Auclair 1996) as well as yellow birch dieback in 1936 (Braathe as cited by Bertrand et al. 1994). Since snow cover protects and insulates roots from low temperatures, it is likely that these incidences of dieback were a consequence of freezing damage to roots (Auclair 1996). Additionally, xylem embolisms can result in an irreversible loss of conductivity and eventual branch dieback if the damaged root system is unable to refill the xylem (Cox and Malcolm 1997).

Acclimation is not a static condition, as tolerance can be readily lost by increases in temperature. The seasonal variation in freezing tolerance of plants has been well documented in the past. For example, Calme et al (1994) found that roots of A. saccharum could endure a temperature of -30 °C during the winter, but a -6 °C frost in August was lethal to the same type of roots. While this is certainly indicative of changes in physiology and biochemistry, this study did

not explain what these changes might have been, nor did they distinguish which type of roots were examined.

Since we are currently experiencing an increase in the average global annual temperature resulting in erratic winter temperatures, sporadic increases in temperature during the winter can induce a loss of acclimation in hardened tissue. Warming trends also diminish snow cover and when thawing cycles are followed by rapid freezing, there is the potential for considerable injury in dehardened root tissue. Despite the ecological importance of this phenomenon, few studies have focused on understanding the implications of periodic dehardening episodes on plant roots during the winter. Such information could lead to the identification of families of trees that would be more prone, as well as resistant, to episodes of thawing and freezing during the winter.

Exposure to low temperatures is not the only method of stimulating cold acclimation. In several species, freezing tolerance can be artificially induced by exogenous application of the plant hormone abscisic acid (ABA) to cell cultures or whole plants at non-acclimating temperatures (Zeevart and Creelman 1988; Palva 1994). The degree of freezing tolerance achieved by exogenous ABA is often comparable with that achieved by low temperatures (Chen et al. 1983; Palva 1994). In addition, the concentration of endogenous ABA increases during the acclimation process (Chen et al. 1983; Palva 1994). These findings led Chen

et. al (1983) to propose that endogenous ABA stimulates the induction of freezing tolerance during cold acclimation.

Endogenous ABA concentration has also been shown to correspond with the level of freezing tolerance in trees. Bertrand et al. (1997) reported that when the concentration of endogenous ABA in the xylem of sugar maple reached its peak, the maximum level of root freezing tolerance was attained. In addition, acclimated sugar maple contained a tenfold greater concentration of xylem ABA than unacclimated sugar maple (Bertrand et al. 1997).

Through the use of Arabidopsis thaliana mutants, additional evidence for the role of ABA in the acclimation process has been provided. Heino et al. (1990) showed that mutants incapable of synthesizing ABA could not achieve freezing tolerance, but exogenous applications of ABA could restore this capacity. ABA insensitive Arabidopsis mutants could not acclimate in response to low temperature or exogenous ABA, demonstrating that ABA must be perceived for freezing tolerance induction (Palva 1994).

ABA has been implicated in a variety of stress responses, all of which involve a change in water status, including high osmoticum, drought, freezing and salinity stress. At the cellular level, these stresses are quite similar in that they leave the protoplast dehydrated, which appears to be the signal initiating ABA synthesis. Guerrero and Mullet (1986) found that during drought stress, transcription and translation were required for ABA biosynthesis. The physical

phenomena of cellular water loss is believed to stimulate the production of mRNAs and proteins involved in ABA accumulation (Bray 1993). The cell might recognize water loss through changes in turgor or cytoplasmic volume, thereby triggering the production of ABA biosynthetic gene products (Bray 1993). The fact that multiple stresses all result in ABA production supports this theory since each of these stresses cause similar changes in water status. Moreover, exposure to one stress such as drought has been shown to promote an increase in freezing tolerance, presumably by stimulating ABA synthesis (Chandler and Robertson 1994).

Molecular Aspects of Acclimation

Among the biochemical changes that have been observed in plants during cold acclimation are alterations in membrane composition (more specifically, an increased phospholipid to protein ratio and elevated membrane lipid content), increases in sugars and soluble proteins, and elevated concentrations of proline and other organic acids (Levitt 1980). While many of the molecular changes that occur in plant cells during acclimation could be brought about by pre-existing macromolecules (e.g. enzymes, structural proteins, lipids) that undergo physical changes in their properties in response to low temperatures, it is also likely that cold acclimation involves changes in protein synthesis as originally proposed by Weiser in 1970. According to this theory, exposure to low temperatures stimulates gene transcription which results in the synthesis of new polypeptides

involved in freezing tolerance acquisition. Likewise, during non-acclimating temperatures, genes involved in freezing tolerance are inactive.

Weiser's theory was substantiated when it was shown that cycloheximide, a protein synthesis inhibitor, prevented cold acclimation in several species including Solanum commersonini (Chen et al. 1983), Brassica napus, and wheat (Thomashow 1990). That protein synthesis and altered gene expression are components of cold acclimation was further evidenced by in vitro translation studies (Thomashow 1990).

Many cold induced proteins are also synthesized in response to ABA, suggesting that ABA is a mediator of cold acclimation at the level of gene expression (Chandler and Robertson 1994). The accumulation of ABA during cold acclimation could be just a step in the signal transduction pathway that ultimately leads to biochemical and physiological adjustments (due in part to altered gene expression) that harden the plant against stress. The identification and characterization of gene products appearing during instances of increased ABA biosynthesis would be useful in identifying which species or families of trees are better able to tolerate freezing induced desiccation.

Over 70 different genes are believed to be regulated by ABA (Chandler and Robertson 1994). A number of mRNAs induced during water-deficit are thought to encode gene products that preserve cellular function during water loss. Such genes, specifically termed lea (late embryonic abundant gene), were first identified by amino acid sequencing during seed development but it has

since been deduced that lea genes appear in vegetative tissues during stress (including drought, low temperature, and osmotic stress (Bray 1993)). These genes have been predicted to function by sequestering ions (so that cellular function is not destroyed by the increase in ion concentration during dehydration), in the protection of proteins (as well as renaturing unfolded proteins), and in further maintenance of membrane structures by binding water within the cell (Bray 1993). If the appearance of specific genes during acclimation were linked with the degree of freezing tolerance, it might then be possible to infer which genes aid in injury resistance during low temperatures. Additionally, the expression of such genes may be able to serve as an indicator of the capacity of tree species to harden.

The mechanism by which ABA induces changes in gene expression during acclimation is poorly understood, in part because an ABA receptor has yet to be identified. ABA might serve as a regulator of transcriptional events, producing gene products beneficial to stress tolerance (Bray 1993). ABA has been found to stimulate overall transcription including rRNA, tRNA, as well as mRNA. Alternatively, ABA may function in post-transcriptional events by processing transcripts, stabilizing mRNA, or through the regulation of translation and protein activity (Chandler and Robertson 1994).

In other cases, exogenous ABA does not induce the same alterations in gene expression in unstressed plants as in stressed plants, suggesting that ABA might interact with another factor present only during stress. Other studies have

shown that ABA results in expression independent of stress events, however the presence of the stress may enhance the expression of these genes (Chandler and Robertson 1994). Alternatively, this may be due to the presence of substances in unstressed plants that might antagonize the effects of ABA, thus impeding ABA induced gene expression. For example, unstressed plants may contain a higher level of gibberellin (GA) than stressed plants. Since GA and ABA exert opposing effects with respect to gene expression, the factor of stress may be necessary to inhibit GA production in order for ABA induced genes to appear (Chandler and Robertson 1994).

The identification and characterization of genes that are involved in cold acclimation is important to our understanding of freezing tolerance in plants. While current techniques in the genetic analysis of freezing tolerance have shown that in many species cold acclimation is associated with altered gene expression, it is not clear what ramifications these differentially expressed genes have on the state of hardiness of plants. A time-course study relating altered gene expression with the physiological ability of the plant to endure freezing would be beneficial to our comprehension of cold acclimation. From an ecological standpoint, this information would be most valuably applied to forest species, which have been neglected (as has root tissue) compared to other experimental systems used in this area of research.

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CHAPTER 2

PATTERNS IN FREEZING TOLERANCE DURING COLD ACCLIMATION OF ACER RUBRUM FINE ROOTS

Abstract

An electrolyte leakage assay was employed to determine the freezing tolerance of Acer rubrum fine roots (i.e. diameter < 1mm) throughout a period of natural cold acclimation. Root-zone temperatures were monitored throughout the sampling period, and cold hardiness appeared to be affected by trends in daily minimum root-zone temperatures.

Introduction

Various studies have established that roots are the least hardy tissues found in woody plants (Pellet 1971; Pellet and White 1969; Mityga and Lanphear 1971; Smit-Spinks et al. 1985) and evidence also indicates that tree recovery from winter freezing stress is directly affected by the ability of roots to acclimate and endure low temperatures (Auclair et al. 1996; Bertrand et al. 1997). While root freezing tolerance has been extensively studied in conifers, studies of this process in deciduous trees have been limited. Since freezing tolerance varies among plant species and ecotypes, the information attained in these studies may not be relevant to freezing tolerance in deciduous trees.

Because of its importance in wetland mitigation and forest reclamation, red maple (Acer rubrum) has become valuable as a landscape tree. In nature it covers a wide geographic range in a multitude of environments from south-

eastern U.S. to as far north as Nova Scotia, Canada. Despite its ecological and economic importance, the acquisition of freezing tolerance in tissues of A. rubrum has not been previously investigated. As major episodes of deciduous tree dieback occur throughout northern U.S. and Canada, questions are raised about the susceptibility of tree roots to freezing injury during the winter (Auclair et al. 1996). Moreover, erratic winter temperatures brought on by trends in global warming are suspected to play a role in forest tree decline by affecting root freezing tolerance (Auclair et al. 1996).

As a result of varying developmental stages in tree root systems, different morphologies of roots exist within a plant. There is evidence that non-woody fine roots (i.e. diameter < 1mm) are more sensitive to freezing stress than older roots in woody plants (Pellet and White 1969, Mityga and Lanphear 1971, Smit-Spinks et al. 1985). Studies by Mityga and Lanphear (1971) with Taxus cuspidata and Wiest and Steponkus (1976) with Pyracantha coccinea showed that young roots could not acquire cold tolerance, while Colombo et al. (1995) demonstrated that young root tips of Picea mariana could acquire tolerance to -16 °C. These studies, while demonstrating differences in freezing tolerance between older and younger roots, focused on evergreen species. Freezing tolerance in deciduous tree fine roots has received little attention.

Fine roots account for the majority of nutrient and water uptake in trees, and often serve as sinks for large quantities of photosynthate (Fredericksen and Zedaker 1995). Since the capacity for water and nutrient uptake decreases with

root age, fine roots are critical for tree viability (Hendrick and Pregitzer 1993). A study by Grier et al. (1980) found that fine roots may be responsible for as much as two-thirds of the annual biomass production in trees. Thus, their functional role in trees is paramount, and the loss of fine roots during winter freezing events could greatly compromise tree survival during the following growing seasons.

An understanding of fine root cold acclimation would be as much of a value practically as scientifically, since landscape trees are predominantly overwintered in above-ground containers where temperatures can reach lower extremes than below ground. The loss of the absorptive fine roots in containerized plants could be detrimental to regrowth after overwintering. In containerized T. cuspidata, slow growth following the winter was attributed to the failure of young roots to acclimate in response to environmental temperatures (Mityga and Lanphear 1971). A better understanding of temperature effects on cold acclimation of fine roots could lead to an effective hardening regime for containerized plants.

In this study, the cold hardening ability of fine roots of 1-year old A. rubrum seedlings was evaluated. Freezing tolerance was assessed every two weeks throughout a natural period of cold-acclimation. During this period, air and root-zone temperatures were measured to determine the relationship between temperature and cold-acclimation of fine roots.

Materials and Methods

Plant Material

A. rubrum (red maple) grown from seed was used as experimental material for this study. Seeds were collected on the Holyoke Range near Amherst, MA and sown in June, 1996. Seedlings were planted in one-gallon nursery containers and grown throughout the 1996 and 1997 growing seasons at the South Deerfield Research Facility using standard nursery practices. The seedlings were overwintered (1996/1997) in an unheated polyhouse under a thermal blanket. Sampling began on September 5, 1997, and continued through January, 1998. On November 1, 1997, the remaining plants to be sampled were left outside and covered with a thermal blanket to maintain root-zone temperatures as close as possible to natural below ground temperatures (preliminary studies have indicated that root-zone temperatures under a thermal blanket are comparable to in-ground temperatures).

Temperature Measurement

Root-zone temperatures were measured throughout the experiment with copper-constantan thermocouples connected to a datalogger (21X, Campbell Scientific, Inc., Logan, UT) by inserting thermocouples at various depths in pots of two separate plants grown at the nursery. In addition, copper-constantan thermocouples were also used to measure freezer air temperatures in freezing tolerance tests as described below. Two other thermocouples were positioned

under an aluminum shield to measure air temperature 180 cm above ground. In-ground temperatures were measured with three thermocouples placed 20 cm below the soil in each of two plots adjacent to the nursery.

Laboratory Freezing Tests

Five plants were harvested from the overwintering blocks once every two weeks throughout a period of cold acclimation from September 7 to December 3, 1997 and once on January 6, 1998. Shoots were removed and the root systems were then washed under cold water to remove all medium. Fine root tissue (non-woody root tissue less than 1 mm in diameter) was removed from the root system and placed immediately into a refrigerator at 2 $\pm$ 2 °C in a petri dish containing moist filter paper until all samples had been prepared in this manner.

Six individual samples, each consisting of approximately 500 mg of root tissue cut into 1 cm-long pieces, were prepared from each plant. Each sample was then placed into a 9.5 x 1.5 cm polypropylene test tube containing 4.0 ml of frozen (-2 °C) 0.4 mM $CaSO_4$. Care was taken to ensure that samples were in contact with the ice before adding an additional 0.5 ml of 0.4 mM $CaSO_4$. This procedure has been proven to avoid supercooling by allowing ice nucleation to occur in the tissue (Iverson et al. 1980).

The tubes were then placed in a programmable freezer (ScienTemp, Adrian, MI) set at -2 °C, and left overnight before beginning the freezing test. A separate set of similarly prepared samples, serving as the non-frozen controls,

remained in petri dishes in the refrigerator at 2 ± 2 °C during the freezing test period.

Each freezing test consisted of 6 test temperatures (non-frozen control, -2, -4, -6, -8 and -10 °C), with one sample per plant used for each temperature. Each sample was held at the designated temperature for approximately 2 hr, and then placed into the refrigerator to thaw.

Measurement of Freezing Injury

Injury was assessed by an electrolyte leakage assay (Columbo 1994; Columbo et al. 1995). Thawed samples (with a standard thawing time of 12 h) were infiltrated under partial vacuum (0.17 MPa) for 20 min and then returned to the refrigerator (2 ± 2 °C) to incubate for 12 h. Samples were then shaken at room temperature for 8 h on a rotary shaker before reading the initial conductivity (IC) of the $CaSO_4$ bathing solution with an electrical conductivity meter (Model 1054, VWR Scientific, Boston, MA). Samples then were frozen overnight at -30 °C to kill the tissue, thawed in the refrigerator (2 ± 2 °C), and shaken for 2 h at room temperature before the final conductivity (FC) of the bathing solution was read. An index of injury (It) was used to calculate injury sustained by each sample according to the following formula (Flint et al. 1967):

$$It = \frac{RC_{frozen} - RC_{control}}{1 - RC_{control}}$$

where RC (relative conductivity) = IC/FC.

Statistical Analysis

An analysis of variance was performed on index of injury data collected for each test temperature among all test dates (SAS Institute Inc., Cary, NC). A second-order linear regression model (Sigma Stat, Jandel Scientific Inc.) was used to assess the effects of date on trends of decreased injury sustained at each test temperature.

Results

Figure 2.1 shows the daily minimum air, ground and root-zone temperatures. Mean root-zone temperature did not fall below 0 °C until October 23 when ground and air temperatures were just above freezing (.42 and .46 °C respectively). The lowest root-zone temperature of -5.84 °C was experienced on November 17 at which point ground temperature was 0 °C and air temperature was -0.19 °C.

The effect of sample date on index of injury (It) at each test temperature proved to be highly significant (P=.01), as presented in Table 1. Figure 2.2 shows the mean index of injury for each test temperature as a function of date. An It of .50 (It_{50})represents the leakage of 50% of tissue electrolytes, and this degree of injury is generally considered to be fatal (as well as values above .50; Flint et. al 1967)). These data were analyzed by a second-order linear regression model to assess the relationship of date on trends in hardiness, as reflected by a lower index of injury at lower test temperatures. For this analysis, the linear

components of each regression line were highly significant (P=.01) and the quadratic components were significant for all lines except for -6 °C.

Figure 2.3 presents the highest temperature at which 50% of tissue electrolytes were lost (lt_{50}) represented by bars extending from the upper x-axis over each test date. The lt_{50} is considered to be a fatal level of injury and this value is also located within each bar. For comparative purposes, the root-zone temperature is presented as in Figure 2.1 with the temperature scale along the y-axis.

Fine roots first became acclimated to -2 °C on the October 22 test. Up until this point, the lowest temperature reached was 2.3 °C two days prior to the freezing test date.

On the following test date of November 2, fine roots could survive to -4 °C. This gain in acclimation followed the first root-zone exposure to freezing temperature, with the lowest minimum temperature being -1.4 °C nine days prior to this freezing test.

On November 16 fine roots could survive to -8 °C. This test date was preceded by 5 consecutive days at below 0 °C temperatures. Although temperatures remanded between 2.3 and -1.6 °C for the week before the December 3rd test, a loss of freezing tolerance to -8 °C was observed. However, the following test date was marked by a tolerance beyond the lowest test temperature of -10 °C.

Discussion

In regions where climate varies seasonally, perennial plants undergo cold acclimation, enabling them to survive the formation of ice within tissues during winter freezing events. This process follows an annual cycle, where cold tolerance is minimal during the growing season, develops gradually in the fall to a maximum level in the winter, and is subsequently lost in the spring. Seasonal variations in plant cold hardiness have been well noted in the past. For example, Calme et al. (1994) found that in sugar maple, roots could survive to -37 °C during the winter, but a -6 °C frost in August was lethal. In the herbaceous plant winter rape (Brassica napus var. oleifera), tissues could increase in cold tolerance from -5 to -17 °C from September to December (Kacperska-Palacz 1978). Likewise, a temperature of -2 °C was lethal to A. rubrum fine roots in September, while a temperature of less than -10 °C in January was not.

The hardening process proceeds in stages, with each stage preparing for the next (Levitt 1980). Harvey (1922) reported that there is a "threshold temperature" above which hardening does not occur. The range of temperatures which stimulate hardening varies among species, varieties and ecotypes (Levitt 1980). In cabbage, a temperature of 12 °C was sufficient to stimulate cold hardiness, while no hardening occurred in response to 18 °C (Le Saint 1966). Wiest and Steponkus (1976) found that mature roots of P. coccinea acclimated to -26 °C when exposed to 4 °C for 5 weeks, while young roots sustained lethal

injury at -5 °C under the same conditions. In this study, A. rubrum fine roots did not become acclimated to -2 °C until temperatures fell below 5 °C for five consecutive days.

The first stage of cold hardening usually occurs when the plant is exposed to low non-freezing temperatures for several days or weeks (Levitt 1980). During this time, sugars and other protective substances accumulate in the protoplasm, intracellular water content decreases, and the central vacuole divides into many smaller vacuoles (Levitt 1980). These cellular modifications give the protoplasm the capacity for even greater hardening when temperatures regularly stay between -3 ° and -5 °C (Levitt 1980).

In fine roots of A. rubrum, the first increase in tolerance to -2 °C was measured on October 22 when temperatures fell within the range of 2.3 ° and 4.4 °C for 5 consecutive days. Prior to this date, root-zone temperatures did not reach below 5 °C except for 2 occasions separated by several days of higher temperatures (between 5 ° and 14 °C). This finding implies that 5 consecutive days of exposure to temperature in the low above 0 °C range is sufficient to acclimate fine root tissue to -2 °C.

In the period between October 22, when the first gain in tolerance was measured, and the following test date on November 2, roots were first exposed to freezing temperatures, though just on two occasions and separated by several

days of warmer temperatures. Consequently, the November 2nd test was met by a heightened freezing tolerance to -4 °C.

A reorganization of biomembrane structure occurs in acclimating plants when temperatures fall below 0 °C, including changes in phospholipid content and increased unsaturation of lipids (Levitt 1980). These membrane alterations allow cellular water to readily flow into extracellular spaces, so that intracellular ice is not formed (which is invariably lethal) when temperatures reach lower extremes (Levitt 1980). The protoplasm achieves its maximum level of tolerance during the terminal stage of cold hardening when temperatures are no higher than -5 °C to -15 °C. At this point, the extent to which a cell can tolerate freezing is genetically determined (Levitt 1980).

A. rubrum fine roots gained tolerance to -8 °C by the November 16th test, at which point minimum daily root-zone temperatures remained between 0 °C and -5.9 °C for 5 consecutive days. A subsequent loss in tolerance was measured on the following test on December 3rd when roots were killed by -8 °C. During the period between these two tests, minimum temperatures never reached higher than 2.5 °C and fell as low as -5.9 °C. However, in the 13 days immediately before this loss in tolerance was measured, temperatures only reached freezing three times, and these minimums were only slightly below 0 °C. This finding suggests that the changes that enabled fine roots to survive to -8 °C

on November 16th were brought about by the consistent exposure to temperatures of at least -2 °C, and above this limit, tolerance is lost.

This loss in tolerance calls into question the plasticity of the plasma membrane of fine roots during winter freezing events. Levitt (1980) explained that the plasma membrane of hardened plants may return to a lower state of tolerance when severe cold spells have ended, however they retain the ability to regain tolerance when temperatures fall again. In New England as well as other temperate regions, it is not uncommon for temperatures to rapidly fluctuate within a short duration of time. If root-zone temperatures between 2.5 ° and -5.9 °C (which was the range between November 16 and December 3) are not sufficient to maintain hardiness to -8 °C, fine roots stand the threat of death should root-zone temperatures fall quickly. The loss of absorptive fine root tissue during the winter would not only limit the plant's ability for water uptake in the following growing season, but xylem embolisms may also lead to an irreversible loss of conductivity and eventual branch dieback if the damaged root system is unable to refill the xylem (Cox and Malcolm 1997).

In a study by McEnvoy and McKay (1997), root frost tolerance of common ash (Fraxinus excelsior L.) seedlings was shown to decrease in response to short term fluctuations in temperature. When the mean weekly temperature rose from approximately 2 °C to 4 °C, seedlings suffered a loss in tolerance form -2 °C to 0 °C. This study was carried out in Britain where a loss in tolerance may

not be as potentially threatening to survival as it is in Northern U.S. and Canada, since environmental temperatures do not typically reach 0 °C.

Another possible explanation for the loss in tolerance from November 16th to December 3rd is implied by the results of the January 6th test. Minimum temperatures prior to this date never reached below -1.89 °C, but a tolerance greater than -10 °C was measured. It is possible that the decrease in tolerance on December 3rd was a result of cellular and molecular alterations that might have ultimately led to a heightened state of tolerance (as was observed on the next test date), but at that time, left tissue in a fragile metabolic state. It could be that up to this point, tolerance was a result of biophysical readjustments to the cold, whereas the changes leading to a tolerance greater than -10 °C involved molecular alterations, such as changes in gene expression. These molecular changes may have been transduced, but not yet manifested to bring about an increase in tolerance until the January 6th test date. Only genetic analyses of root tissue at varying points of acclimation could make this determination.

In conclusion, this study showed that A. rubrum fine root tissue follows a progression in cold tolerance that is closely influenced by root-zone temperature. While root tissue was killed by -2 °C from September until mid-October, a temperature of -10 °C was not lethal in January. In addition, it was seen that increases in cold hardiness in A. rubrum fine roots are not permanent, and

tolerance can be lost if temperatures rise for relatively brief periods during the acclimation period.

Table 1. Levels of statistical significance for the effect of date on index of injury at each test temperature. ** represents statistical significance at the 0.01 probability level, and test temperatures are represented by T followed by the appropriate temperature.

Effect	Significance
Date: T_{-2}	**
Date: T_{-4}	**
Date: T_{-6}	**
Date: T_{-8}	**
Date: T_{-10}	**

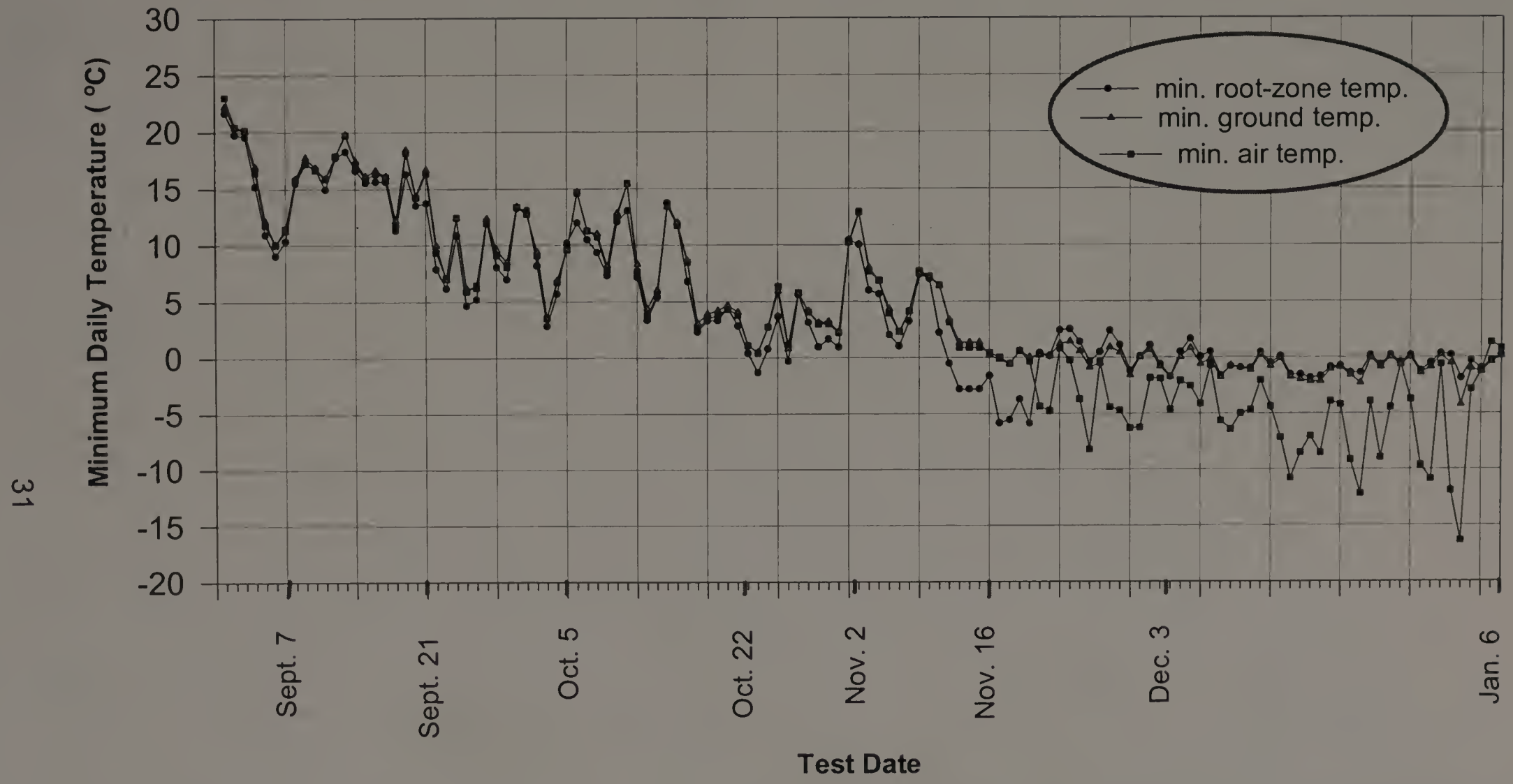

Figure 2.1 Average daily minimum root-zone, air, and ground temperatures during the acclimation period. The root-zone temperature is an average of 3 thermocouples placed at the top, middle, and bottom of the container. Dates in which freezing tolerance was measured are labeled along the x-axis, and each tick represents one day.

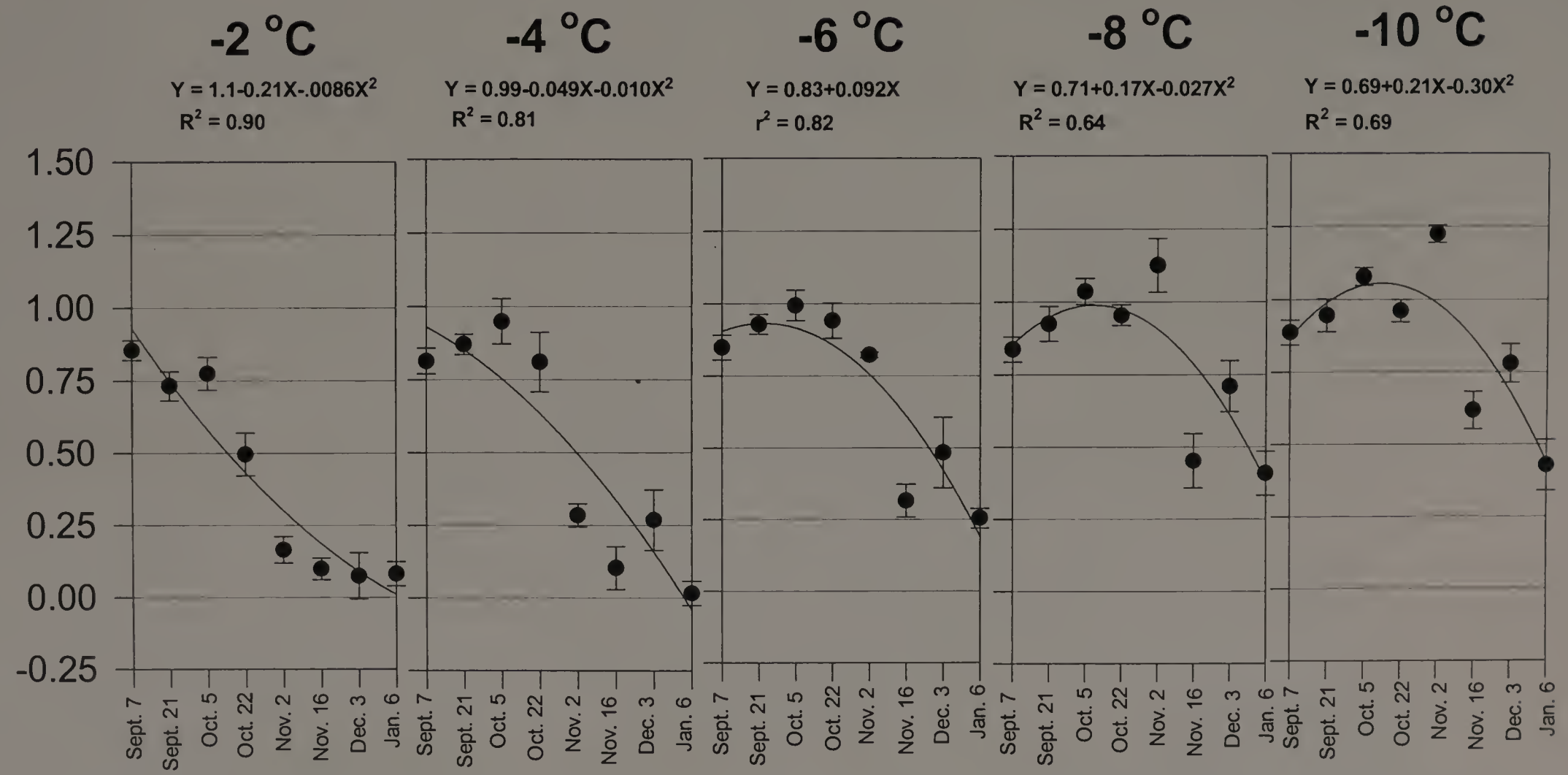

Figure 2.2 Mean index of injury presented as a function of date. Five plants were used to calculate each mean value. The second order linear regression line is shown, as well as the regression equation and coefficients of determination (R^2 or r^2). Standard error bars are shown for mean index of injury.

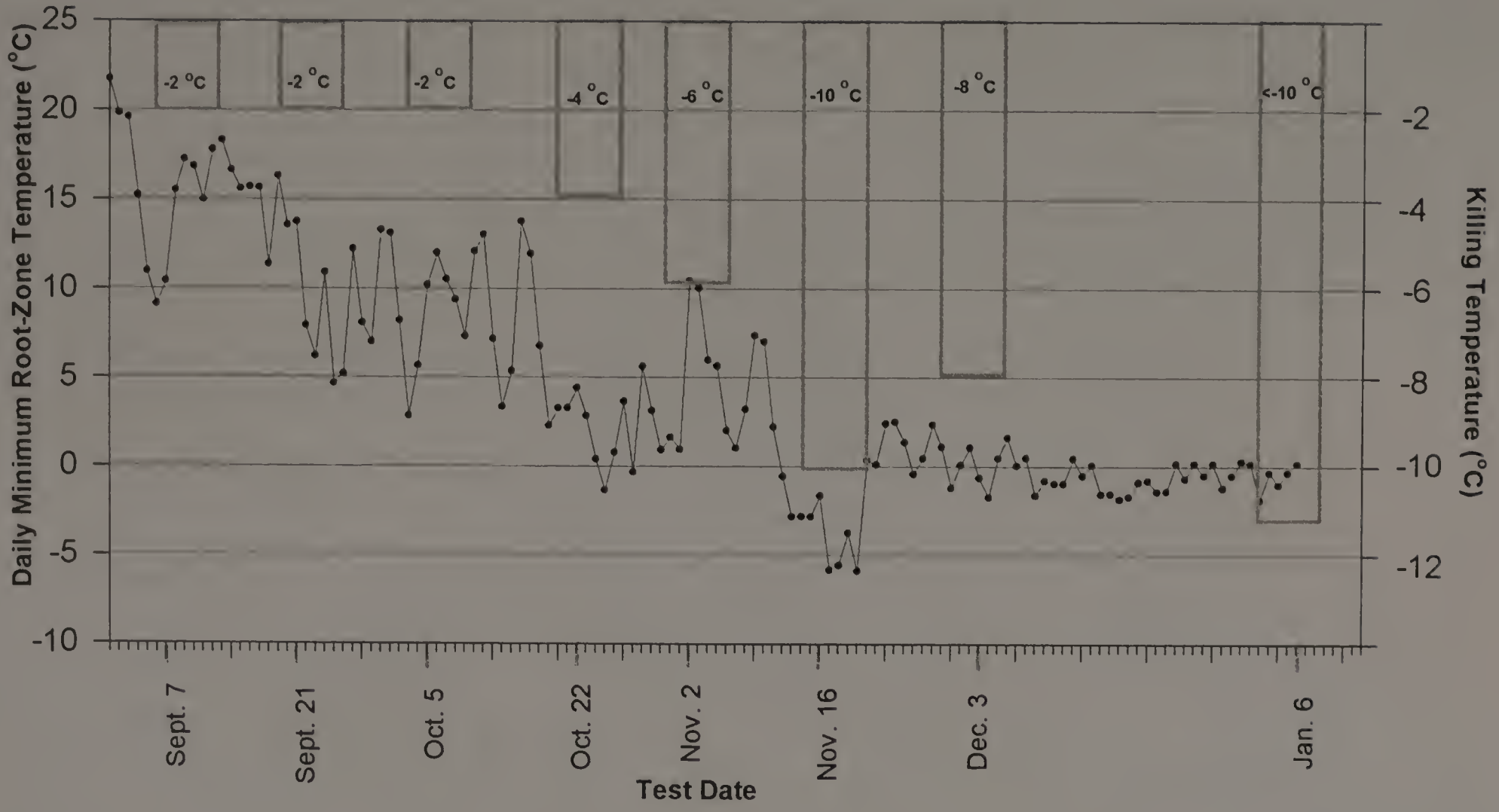

Figure 2.3 Killing temperature as a function of date. The killing temperatures at each date are represented by bars extending from the upper x-axis with the scale on the right y-axis. LT_{50} values are presented within each bar. For comparative purposes, the daily minimum root-zone temperatures are also presented as in Figure 2.1.

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CHAPTER 3

ACCUMULATION OF ABA IN FINE ROOTS OF ACER RUBRUM DURING COLD ACCLIMATION

Abstract

Abscisic acid (ABA) concentration was measured in fine roots (i.e. diameter < 1mm) of A. rubrum throughout a period of natural cold acclimation. During this period ABA concentration differed significantly with date, and fine roots increased in freezing tolerance from a killing temperature of -2 °C in September, 1997 to a killing temperature of less than -10 °C in January, 1998. Concentrations of ABA did not show a close correlation with the level of freezing tolerance in fine roots throughout cold acclimation.

Introduction

Investigators studying climactic mechanisms of forest tree dieback have implicated the death of tree roots during winter freezing events as a component of tree decline in northern hardwood forests (Auclair et al. 1996; Bertrand et al. 1997). Roots are known to be less freezing tolerant than shoots, and roots also deharden faster than shoots (Pellet 1971; Pellet and White 1969; Mityga and Lanphear 1971; Smit-Spinks et al. 1985). These factors place roots in a position of vulnerability to winter freezing stress injury when root-zone temperatures exceed the level of root freezing tolerance as often happens during severe freezing events (Auclair et al. 1996). The loss of roots during freezing events can markedly effect overall tree recovery in later growing seasons by limiting the

plant's capacity for water and nutrient uptake (Frederickson and Zedaker 1995). In addition, xylem embolisms can result in an irreversible loss of conductivity and eventual branch dieback if the damaged root system is unable to refill the xylem (Cox and Malcolm 1997). Despite the importance of this process of environmental adaptation, little is known about the physiological changes that accompany cold acclimation in deciduous tree roots.

The plant hormone abscisic acid (ABA) has been postulated to play a role in cold acclimation of both herbaceous and woody plant tissues. In many plant species including Acer negundo L. (Thomashow 1990), Arabidopsis thaliana (Heino et al. 1990), and Solanum commersonii (Chen et al. 1983), exogenous application of ABA can stimulate an increase in freezing tolerance in the absence of acclimating temperatures. Furthermore, analyses of endogenous levels of ABA have revealed that concentrations often reach a peak in the early stages of cold acclimation (Chen et al. 1983). Based on these observations, it has been suggested that ABA serves as a trigger that initiates cold acclimation (Chandler and Robertson 1994).

The mechanism by which ABA brings about an increase in cold hardiness is believed to be at the level of gene expression. It has been shown that protein synthesis is a vital component of cold acclimation (Heino et al. 1990), and the observation that changes in endogenous ABA concentrations are often followed by the appearance of novel gene products and increased stress resistance strengthens the argument that ABA is a critical component of cold acclimation

(Chandler and Robertson 1994). The accumulation of ABA during cold acclimation could be just a step in the signal transduction pathway that is initiated by declining temperatures and ultimately leads to biochemical and physiological adjustments (due in part to altered gene expression) that harden the plant against stress (Bray 1993).

In this study, the accumulation of ABA during the acquisition of cold tolerance in fine roots of Acer rubrum (red maple) was investigated. The freezing tolerance and endogenous concentration of ABA in fine roots of A. rubrum was measured every two weeks throughout a period of cold acclimation. Root-zone temperatures were also measured to monitor possible relationships between temperature and ABA content and freezing tolerance of fine roots.

Materials and Methods

The materials and methods for plant material, temperature measurement, laboratory freezing tests, and measurement of freezing injury are the same as described in Chapter 2 of this thesis (separate plants were used to test freezing tolerance and to measure ABA).

ABA Extraction and Measurement

At each of the eight sample dates (Sept. 7 and 21, Oct. 5 and 22, Nov. 2 and 16, and Dec. 3, 1997, and Jan. 6, 1998), three plants were harvested for ABA measurement. Fine roots (i.e. width of < 1mm) were removed from each plant, and washed under cold tap water to remove media. Tissue was then frozen in liquid nitrogen and stored at -80 °C until extraction.

Samples of fine roots from each test date were ground to a fine powder in liquid nitrogen in a cold mortar and pestle. Approximately 1.0 g of the ground root tissue was transferred into a 15 ml polypropylene test tube containing 5 ml of distilled and deionized water with 200 mg/l butylated hydroxytoluene as an antioxidant (Cahill and Ward 1989). Samples were then incubated in the dark at 4 °C for 20 hours on a rotary shaker. Preliminary tests revealed that 20 hours was an efficient extraction period as compared with 36 and 48 hours. After incubation, .25 g of polyvinylpolypyrollidone was added to each tube, and samples were placed on a rotary shaker for 30 min at 4 °C in the dark. Samples were then centrifuged at 2500 g for 30 min at room temperature. One milliliter of the aqueous extract was then transferred into a 1.5 ml microcentrifuge tube.

After extraction, ABA concentration was measured in 100 ul of the liquid extract by an ELISA method (enzyme linked immunosorbent assay; Sigma , St. Louis, MO). This assay uses a highly specific ABA monoclonal antibody (ABA-15-I-C-%) bound to each well of a microtitration plate. A known amount of tracer (ABA labelled with phosphatase) and the unknown amount of ABA in the sample were then added to each plate, setting up a competitive binding reaction between the hormone in the sample and the tracer. Once this competitive binding reaction was complete, the unbound tracer and sample ABA were washed away, and the substrate p-nitrophenylphosphate (PNPP) was added. A yellow color is produced when the tracer enzymatically reacts with PNPP. The intensity of the color is inversely proportional to the amount of ABA in the sample

(which is determined by a standard curve). The sample was then added to a spectrophotometric cuvette and absorbance of the sample was measured at 405 nm (Milton Roy Co. Spectronic 1201).

Tissue Dry Weight Analysis

In order to determine whether trends in measured ABA concentration were a result of changes endogenous ABA levels or fluctuations in tissue fresh weight, the dry weight of tissue at each test date was measured. This was achieved by measuring the mass of 3 portions of ground fine root tissue from one plant per sample date, and then drying the tissue in a forced air oven for 1 week at 75 °C. Samples were then placed into a dessicator for 3 days before dry weight was measured.

Statistical Analysis

Analyses of variance were performed on index of injury (It_{50}) data (as described in Chapter 2 of this thesis), fresh weight: dry weight data, and ABA concentrations across all 8 test dates (SAS Institute Inc., Cary, NC). A second-order linear regression model (Sigma Stat, Jandel Scientific Inc.) was used to assess the effects of date on trends of injury sustained at each test temperature, as well as mean ABA concentrations over time. To assess any possible relationships between ABA concentration and freezing tolerance, a correlation statistic was calculated (Peterson correlation coefficient, SAS Institute Inc., Cary, NC).

Results

Analysis of variance showed that ABA concentration varied significantly (P = .01) among all eight test dates (Table 3.1; for reference, mean ABA values are shown). However, the results of the linear regression analysis indicated that there was not a strong linear relationship between levels of mean ABA over time (R^2 = 0.341). Since the primary objective of this study was to evaluate changes in ABA accumulation patterns rather than to make assessments of absolute ABA amounts, the units used to describe data is the amount of ABA (in pmol) measured in 100 ul of the 5 ml of liquid extracted from 1 g of ground root tissue.

Correlation analysis between ABA concentration and lt_{50} data revealed that there was not a strong correlation between these two variables (r^2 = 0.297; this indicates that only 29.7% of the variation in lt_{50} can be explained by variation in mean ABA concentration at each test date). Tissue dry weight analysis revealed that the ratio of fresh weight : dry weight did not differ significantly during the experimental period (non-significance at the .05 level; data not shown).

Figure 3.1 shows the mean endogenous concentration of ABA (an average of 3 plants), the killing temperature (as determined in Chapter 2 of this thesis), and the daily minimum root-zone temperatures throughout the experimental period. On the first three test dates, root tissue was killed by -2 °C. Endogenous ABA concentrations during the first three test dates were between .56 and 1.67 pmol.

The first increase in freezing tolerance on October 22nd was accompanied by an increase in the mean ABA concentration from 1.11 pmol on the second test date (October 5) to 3.93 pmol. The lowest temperature experienced up to this point was 2.3 °C two days prior to this test date.

Freezing tolerance increased from a killing temperature of -2 °C to a killing temperature of -4 °C on the November 2nd test date. This gain in freezing tolerance followed the first root-zone exposure to freezing, with the lowest minimum temperature of -1.4 °C occurring nine days prior to this test date. Endogenous ABA concentration fell from 3.93 pmol on October 22nd to 0.74 pmol on November 2nd.

The highest concentration of ABA (4.13 pmol) was measured on November 16th. This test date was preceded by 5 consecutive days of freezing temperatures, and the killing temperature decreased to -10 °C.

On the following test date (December 3), root-zone temperatures climbed slightly above zero, the killing temperature fell from -10 °C to -8 °C, and ABA concentrations declined from 4.13 pmol on the previous test date, to 1.2 pmol. An increase in freezing tolerance from a killing temperature of -8 °C to a killing temperature lower than -10 °C was measured on the last test date, January 6th. At this point temperatures fell within the range of 1.63 °C and -1.88 °C since the previous test date, and concentration of endogenous ABA declined to .52 pmol.

Discussion

While concentrations of endogenous ABA differed significantly among all eight test dates, the relationship between ABA concentration and date was not a linear one. Furthermore, ABA concentration did not show a close correlation to the level of freezing tolerance at each date.

There is a great deal of evidence that ABA found in the xylem sap is produced by water-stressed roots and then moves into above-ground plant parts via the xylem stream (Zhang and Davies 1987; Zeevaart and Creelman 1988). Bertrand et al. (1997) showed that ABA concentration in the xylem sap of A. saccharum rose steadily throughout cold hardening, and when xylem ABA reached its peak, the greatest root freezing tolerance was attained. In contrast, Schill et al. (1996) showed that in young trees of A. platanoides, ABA fluctuated seasonally in the xylem sap, and did not increase linearly during the months of cold acclimation from September to January.

Bertrand et al. (1994) found that increases in xylem sap ABA of A. saccharum also correlated with cold stress at the root level. Their study did not quantify ABA concentrations in roots, but evidence supports that ABA production occurs in the roots then moves into the xylem sap (Liang et al. 1997; Cornish and Zeevart 1985).

Due to their relative inaccessibility, few studies have explored the role of ABA in roots, while the majority of studies quantify ABA levels in the xylem sap.

In exception, Cornish and Zeevart (1985) showed that endogenous ABA levels increased 100 times their pre-stressed levels in detached roots of osmotically stressed Xanthium strumarium L. While these authors did provide evidence that detached roots have the ability to synthesize and accumulate ABA, their findings may not be applicable to ABA accumulation in intact plant roots during stress.

Fort et al. (1997) found that water stressed oak seedlings (Quercus robur L.) showed no increases of ABA in intact root tips as compared with controls, but yet there was a threefold increase in xylem sap ABA, suggesting that ABA is exported from stressed roots, and that xylem sap ABA concentration may be a better indicator of stress at the root level. Additionally, Liang et al. (1997) found that in drought stressed Zea mays L., roots showed an increase in ABA concentration, and an increased export into the xylem.

The experiments of Zhang and Tardieu (1996) established that the ability of maize root tissue to synthesize ABA decreased with age. They suggested that the increased deposition of cell wall material in older roots decreased protoplasmic volume, and since ABA is believed to be produced in the protoplasm, the capacity for synthesis becomes limited with age (Zhang and Tardieu 1996).

While it was not the aim of the present study to evaluate the ABA biosynthetic properties of different root types in A. rubrum, the results of Zhang and Tardieu (1996) showing that young roots are greater producers of ABA than older roots coupled with the results of Fort et al. (1997) and Liang et al (1997)

showing that intact roots readily export ABA into the xylem lend a possible explanation for the non-linear patterns of ABA concentration observed in this study. If fine roots of A. rubrum, like maize, are the greatest producers of ABA in the root system (since they are among the youngest roots), and if stressed roots rapidly export ABA into the xylem, then it should not be anticipated that levels of fine root ABA should increase throughout cold hardening, as root ABA may have been exported into the xylem.

Although many studies have shown that root ABA is often exported into the xylem stream, the loss of leaves during the acclimation period inevitably caused a reduction in transpirational xylem flow. Therefore, any fluxes in ABA from root tissue must have been a function of root pressure, which is often quite minimal during the winter as a result of low ion uptake. For this reason, rapid root export of ABA may not account for the inconsistent patterns in ABA accumulation observed in this study.

Alternatively, other aspects of ABA metabolism may account for levels of ABA accumulation in fine roots. There are various other metabolites in the ABA biosynthetic pathway, and these forms may ultimately become ABA, but they would be unmeasurable using this immunoassay. For example, the glucose ester form of ABA is often referred to as "bound" ABA, and this form can undergo transesterification into the active form of ABA (Walton 1980). Since the immunoassay used in this study is highly specific for 2-cis-(S)-ABA, other forms of ABA, including the glucose ester form, go undetected.

There are relatively few studies dealing with ABA accumulation during cold acclimation in woody plant root tissue, and there is a scarcity of information on how environmental temperatures (which are obviously not constant) affect endogenous ABA concentrations in roots. Consequently, it is impossible to know how transient the effects of temperature are on ABA levels in roots during a natural period of cold acclimation, and how ABA affects the level of cold tolerance in woody plant roots. Moreover, without the benefit of a controlled laboratory environment, it is impossible to determine the precise temperatures that result in the acquisition of cold tolerance, and changes in ABA concentration. However, it was the intent of this study to observe any changes that may occur in fine root ABA concentration during a natural period of cold acclimation, rather than in a laboratory setting. Even still, this may not be possible since ABA synthesis is a function of freezing-induced dehydration, and many variables are no doubt present in such a response.

In conclusion, the results of this study suggest that endogenous ABA concentration does not serve as an indicator of the level of freezing tolerance in A. rubrum fine roots. It does show, however, that ABA does accumulate in fine root tissue in a non-linear fashion throughout the acquisition of cold tolerance in A. rubrum. While ABA concentrations fluctuated significantly throughout the cold hardening period, there does not appear to be a direct relationship between ABA accumulation in fine roots and minimum root-zone temperatures.

Table 3.1 Levels of statistical significance for the effect of date on ABA concentration. Concentration means are listed for reference (pmol * 100ul extract^{-1}) . ** represents statistical significance at the 0.01 probablity level.

Effect	Mean [ABA]	Significance
Sept. 7	0.557	**
Sept. 21	1.67	**
Oct. 6	1.11	**
Oct. 19	3.93	**
Nov. 2	0.743	**
Nov. 16	4.13	**
Dec. 2	1.21	**
Jan. 6	0.517	**

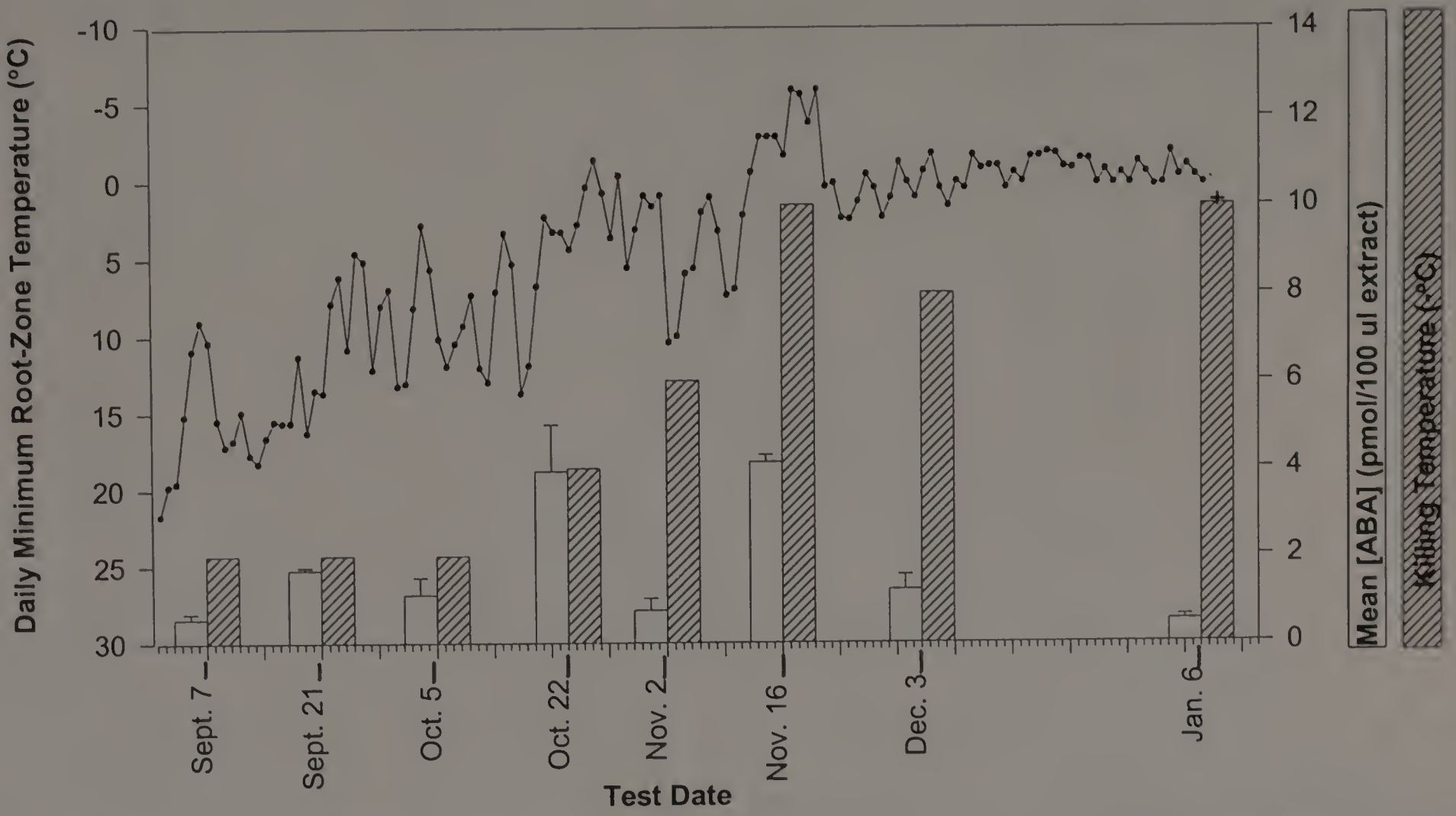

Figure 3.1 ABA concentration and freezing tolerance as a function of date. ABA concentrations (white bars) and killing temperatures (shaded bars) are shown at each sample date, with ranges along the right y-axis. Daily minimum root-zone temperatures throughout the experimental period are plotted as a line, with the range along the left y-axis. On Jan. 6, killing temperature was greater then the lowest test temperature (-10 °C). Error bars represent standard deviation of the mean.

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CHAPTER 4

CHANGES IN GENE EXPRESSION DURING COLD ACCLIMATION IN FINE ROOTS OF ACER RUBRUM

Abstract

Reverse transcription-polymerase chain reaction (RT-PCR) was used to observe if cold acclimation in fine roots of Acer rubrum is associated with modifications in gene expression. Analyses revealed that fine roots acquired considerable cold tolerance, and gene expression changes occurred during the acclimation period.

Introduction

A variety of molecular and biochemical changes have been observed in plants during cold acclimation. Among these are alterations in membrane composition (more specifically, an increased phospholipid to protein ratio and elevated membrane lipid content), increases in sugars and soluble proteins, and elevated concentrations of proline and other organic acids (Levitt 1980). While many of the molecular changes that occur in plant cells during acclimation could be brought about by pre-existing macromolecules (e.g. enzymes, structural proteins, lipids etc.) that undergo physical changes in their properties in response to low temperatures, it is also likely that cold acclimation involves changes in protein synthesis as originally proposed by Weiser in 1970. Weiser postulated that exposure to low temperature stimulates the transcription of a particular set of genes that result in the synthesis of polypeptides involved in cold

tolerance. Likewise, during non-acclimating temperatures, genes involved in freezing tolerance are not transcribed.

Weiser's theory was substantiated when it was shown that cycloheximide, a protein synthesis inhibitor, prevented cold acclimation in several species including Solanum commersonini (Chen et al. 1983), Brassica napus, and wheat (Thomashow 1990). In vitro translation studies provided additional evidence that protein synthesis and altered gene expression are components of cold acclimation (Thomashow 1990).

While current techniques in the genetic analysis of freezing tolerance have shown that in many species cold acclimation is associated with altered gene expression, it is not clear what ramifications these differentially expressed genes have on the state of hardiness of plants. A time-course study relating altered gene expression with the physiological ability of the plant to endure freezing could provide greater insight into physiological facets of cold acclimation. From an ecological standpoint, this information would be most valuably applied to forest species, which have received little attention compared to herbaceous plants.

Although it has been well established that many plants undergo changes in gene expression during cold acclimation, there is a general paucity of information on this subject in relation to tissues of woody plants. This study was aimed at determining if gene expression changes occur during cold acclimation of fine root tissue of A. rubrum.

Methods and Materials

The materials and methods for plant material, laboratory freezing tests, and measurement of freezing injury are the same as described in Chapter 2 of this thesis (separate plants were used to test freezing tolerance and to analyze gene expression).

RNA Extraction

Various methods of RNA extraction were performed (De Vries et al. 1988, Bertrand et al. 1997, Logemann et al. 1987, Pawlowski et al. 1994), all of which were unsuccessful in yielding RNA. It was deduced that the high concentration of phenolic compounds characteristic of many woody plant species (Glenn et al. 1972) may have served to irreversibly bind with RNA and remove it from solution during the phenol extraction step (a step which is common to most RNA extraction procedures). To account for this problem, the following revised method of RNA extraction from woody plant roots was developed.

Approximately 3 g of fine root tissue was excised from each of two plants on each sample date (September 8 and 22, October 6 and 19, and November 2 and 16) and then frozen in liquid nitrogen and stored at -80 °C until extraction. Each 3 g sample was ground to a powder with a pre-chilled mortar and pestle with liquid nitrogen and total RNA was extracted by the following method. Ten ml of acetone (pre-chilled at -80 °C) was added to the powdered tissue, and the mixture was shaken for 30 sec, and then centrifuged at 10,000 g at 0 °C for 5 min. The acetone supernatant was then decanted and discarded. This step was

repeated a second time and then the tissue was transferred into a 50 ml centrifuge tube containing 5 ml of RNA extraction buffer [7 M urea, 100 mM Tris (pH 8.0), 10 mM EDTA (pH 7.0), 1% sodium lauryl sulfate, 1% betamercaptoethanol, and 0.5 g polyvinylpolypyrrolidone] and 5.0 ml phenol: chloroform: isoamyl alcohol (PCI; 25:24:1). The mixture was then incubated for 10 minutes on a rotary shaker at room temperature before centrifugation at 800 g at room temperature.

The aqueous layer was then transferred into a 15 ml tube containing 5 ml PCI. The samples were shaken on the rotary shaker for 3 min and then centrifuged at 800 g at room temperature for 15 min. The aqueous layer was then transferred into new tubes containing an equal volume of chloroform: isoamyl alcohol (24:1), shaken for 3 min, and centrifuged at 10,000g for 15 min at 4 °C. The aqueous phase was then transferred into microcentrifuge tubes and nucleic acids were precipitated at -20 °C for 30 min with 1 volume of 95% ethanol.

The samples were then centrifuged at 7000g at 4 °C and the supernatant was decanted and the pellets were left to air dry. The pellets were resuspended in 0.75 ml diethylpyrocarbonate (DEPC) treated water and 0.75 ml 8M LiCl and then were placed at -20 °C for 1-16 h. After incubation, the tubes were centrifuged at 7000g for 25 min at 4 °C and the supernatant was discarded. The pellets were resuspended in 300 ul DEPC treated H_2O, 100 ul 10M NH_4Ac, and 1

ml 95% ethanol. The tubes were centrifuged at 10,000g for 15 min at 4 °C, the supernatant was discarded, the pellet was air dried, and then dissolved in 50 ul DEPC treated H_2O. Samples were stored at -20 °C until further analysis.

Reverse Transcription Polymerase Chain Reaction

Changes in gene expression were analyzed by differential display reverse transcription polymerase chain reaction (RT-PCR; GenHunter Corp., Nashville, TN). RNA samples (1 sample per plant, 2 plants per each sample date) were first treated with DNase to remove any contamination of DNA from the RNA samples (MessageClean Kit, GenHunter Corp. Nashville, TN). DNA-free mRNA was anchored with an oligo-dt primer at the beginning of the poly (A) tail, followed by polymerase chain reaction using one arbitrary primer. This technique resulted in amplified cDNA subpopulations which were then separated on a 6% denaturing polyacrylamide DNA sequencing gel prepared as described below.

6% Denaturing Polyacrylamide Gel Electrophoresis

A 6% polyacrylamide gel solution mix was prepared in the following manner. To make one 50 ml gel solution (which is approximately the amount needed for 1 polyacrylamide sequencing gel), 2.85 g of acrylamide and 0.15 g of N,N' - methylene bisacrylamide was stirred until dissolved in 23 ml of DI H_2O and then 21.02 g of urea (molecular biology grade) was added and dissolved. Five ml of 10 x TBE buffer (100 mM Tris, 90 mM boric acid, 1 mM Na_2EDTA) was heated to 37 °C and added to the acrylamide solution mixture along with enough DI H_2O to bring the final volume to 50 ml. The solution was stirred until fully dissolved,

filtered through a 0.22 micron Corning filter, and then 22.7 ul of N,N,N',N',-tetra-methylethylenediamine was added. Prior to use, 0.32 ml of a 10% (w/v) ammonium persulfate solution (freshly prepared) was added to the 50 ml gel solution. The gel solution was poured into a glass plate sequencing gel mold and allowed to polymerize for 1 hour.

Prior to loading samples, the gel was prerun for 30 min in 1 x TBE buffer (prepared as described above for 10 x TBE buffer, then diluted to 1 x) at constant watts (40 W, volts and milliamps consistently stayed at 1500 and 25, respectively). The samples were then heated at 80 °C for 2 minutes and then approximately 5 ul of cDNA from each PCR sample (2 samples per date) and 2.8 ul of loading dye (95% formamide, 10 mM EDTA (pH 8.0), 0.09% xylene cyanole, and 0.09% bromophenol blue) was loaded into each well and run for approximately 2 h. The gel was then removed from the glass plates onto 3M chromotagraphy paper and covered with Saran Wrap, and then dryed at 80 °C for 40 min under vacuum. The dryed gel was then placed in an autoradiography cassette (Lightning Box, DuPont) with one sheet of x-ray film (Fujifilm medical x-ray film) at -80 °C for 1 week before developing. The amplified mRNA samples were analyzed side by side to allow differentially expressed genes to be identified.

Results and Discussion

Freezing tolerance in fine roots increased from less than -2 °C on September 8, 1997 to greater than -10 °C on January 6 (data shown in Chapter 2 of this thesis). Comparison of amplified mRNA samples from the 6 sample dates (from September to November) showed that numerous changes in gene expression occur during the aclimation period (Figure 4.1). This study is the first to show that fine roots of A. rubrum have the capacity to acclimate to the cold, and that modifications in gene expression occur during this process.

It has been established that in many plants, some of the alterations in polypeptide synthesis during cold acclimation involve changes in mRNA populations. In their studies of spinach, Guy et al. (1985) first provided evidence for this. By using in vitro translation, they showed that poly (A) RNA from 2-day acclimated plants synthesized two polypeptides that were not produced in nonacclimated plants. After 8 days of acclimation, four additional polypeptides were produced, and decreased synthesis of three polypeptides was detected. The fact that these studies showed that many changes occur during so short a duration of time during cold acclimation makes it not surprising that fine root acclimation is accompanied by so many changes in mRNA populations since sampling was on a two week basis.

Similiar results were found in Arabidopsis thaliana, where increases in translatable mRNAs encoding four novel polypetides were detected in acclimated, but not non-acclimated plants (Thomashow 1990). Likewise, in cold-

acclimated Brassica napus, Meza-Basso et al. (1986) found increases in mRNAs encoding nine polypeptides. Mohapatra et al. (1987a; 1987b) reported increases in translatable mRNAs for seven polypeptides in cold-acclimated Medicago falcata and five polypeptides in cold acclimated M. sativa. Each of these studies dealt with molecular changes at the whole-plant level (Thomashow 1990; Meza-Basso et al. 1986; Mohapatra et al. 1987a; Mohapatra et al. 1987b).

While many of the early studies on the process of cold acclimation dealt with woody plant species, virtually all the current research on molecular aspects of cold acclimation involve herbaceaous species. As an exception, Bertrand et al. (1997) found that during a natural acclimation period, some in vitro translation products in A. saccharum buds and roots (not specifically fine roots) were up-regulated while others were down-regulated. This is consistent with results observed in this study where some reverse transcribed cDNA products appeared during the course of cold acclimation, while other products dissapeared.

Many studies have characterized the function of differentially expressed mRNA species during cold acclimation. A number of mRNAs synthesized during freezing-related drought stress are believed to encode polypeptides that preserve cellular function during freezing events. These genes are believed to play a role in the sequestration of ions, in the protection of proteins, and in the maintenance of membrane structures by binding water within the cell (Bray 1993). However, definitive roles for many cold-induced proteins have yet to be reported (Chandler and Robertson 1990)

While this study showed that numerous changes in gene expression occur in A. rubrum fine roots during a natural period of cold acclimation, there is no conclusive evidence that these alterations are related to freezing tolerance. Further analysis directed at characterizing these genes would provide greater understanding of cold tolerance in woody plants. In addition, it would be useful if this work was undertaken in a controlled setting so that possible interactions between temperature, gene expression, and cold tolerance could be observed.

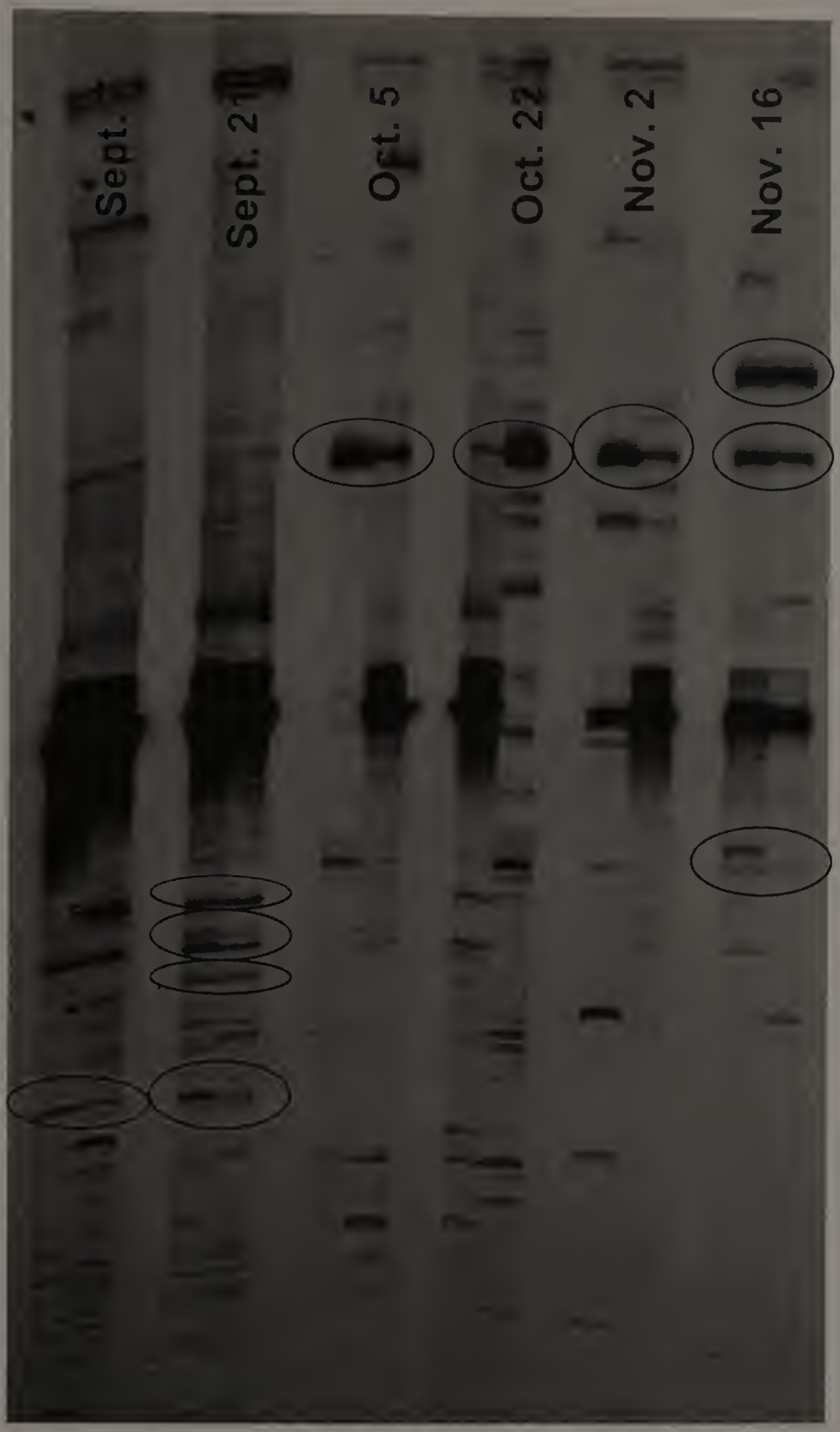

Figure 4.1 Changes in reverse transcribed mRNA products during cold acclimation. Changes in cDNA bands are represented by black ovals, and sample dates are labelled along the tops of lanes. Two replicates per date are shown, and only the differentially expressed bands shown in both replicated are circled.

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