INDUCTION OF FREEZING TOLERANCE IN JACK PINE SEEDLINGS: CHANGES IN LIPIDS, OXIDATION-REDUCTION AND ANTIOXIDANT ENZYMES DURING COLD ACCLIMATION

by

Sheyun Zhao

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Botany
University of Toronto

© Copyright by Sheyun Zhao 1998
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L’auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L’auteur conserve la propriété du droit d’auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-35380-X
INDUCTION OF FREEZING TOLERANCE IN JACK PINE SEEDLINGS: CHANGES IN LIPIDS, OXIDATION-REDUCTION AND ANTIOXIDANT ENZYMES DURING COLD ACCLIMATION

Sheyun Zhao
Doctor of Philosophy 1998
Graduate Department of Botany
University of Toronto

ABSTRACT

The freezing tolerance of jack pine seedlings (Pinus bankisana Lamb.) was acquired following conditioning at 5°C and a short photoperiod. A 1-week conditioning period significantly increased survival after exposure to -5°C, but was less effective on seedlings exposed to -10°C. Conditioning periods of 2 and 4 weeks resulted in higher levels of survival following exposure to both -5 and -10°C. Changes in two root plasma-membrane-bound activities, H⁺-ATPase and NADH-dependent ferricyanide reductase, were studied in plasma membrane enriched fractions during conditioning and after freezing. Cold conditioning inhibited these two enzyme activities. However, post-freezing activities of both enzymes were enhanced by conditioning.

The changes in major polar lipids and their fatty acid compositions in jack pine roots and needles during cold conditioning were studied. After 4 weeks of cold conditioning, the proportion of phosphatidylcholine (PC) increased in root and needle tissues, while, in roots, monogalactosyldiacylglycerol (MGDG) was decreased during cold conditioning. The proportion of oleic acid (18:1) in roots and linolenic acid (18:3) in needles increased during
cold conditioning. Following 4-weeks of cold conditioning, the resistance of root membranes
to freeze-thaw stress and free radical attack increased as assessed by determining lipid
peroxidation. Lower levels of lipid degradation were found in 4-week conditioned seedlings
following a freeze-thaw event.

Cold conditioning led to gradual increases in sulphydryl (SH) concentration in soluble
proteins and the GSH/GSSG ratios in jack pine roots. *In vivo* and *in vitro* freeze-thaw caused
a decrease in SH content in microsomal proteins; a lower level of SH oxidation was found in
the microsomes of 4-week conditioned seedlings. In this study, we show that GSH has
protective effects on freeze-thaw induced inhibition of H⁺-ATPase activity. The specific
activity of ascorbate regenerating enzymes gradually increased. The activities of the enzymes
involved in the ascorbate-glutathione cycle in jack pine roots were regulated by cold
conditioning. Our results suggest that these enzymes play a protective role following the
exposure of the seedlings to freezing temperatures.
Acknowledgments

I thank Dr. Eduardo Blumwald for his encouragement throughout the course of this work. His patience, enthusiasm and friendship during these last 5 years are deeply appreciated and unforgettable.

I thank Dr. John Williams, a member of my graduate supervision committee, for his guidance on my research and the freedom to work in his lab. I am also grateful to Dr. John Coleman, a member of my graduate supervision committee, for his insight and constructive criticism of my research. I would like to thank the faculty within the Department of Botany.

The fellow students, post-docs, technicians and administrative staff that I have met in the Department of Botany will leave a wonderful lasting impression with me, a special thank to Dr. Mobashsher-uddin Khan who directed me for the techniques on lipid research when I worked in Dr. Williams's lab.

Finally, I would like to express my warmest thanks to my best friend and husband, Jian. His understanding, support and love gave me the strength and inspiration to pursue my goal of a doctoral degree. Thanks to my beautiful daughters, Avrilynn and Maylynn, their love also gave Mummy strength for hard works. They made their contributions in term of times Mummy was at school instead of at home with them. Last but not least, I would like to thank my parents, my grandparents, for all they have given my during my lifetime.
# Table of Contents

**ABSTRACT** i

**ACKNOWLEDGMENTS** iv

**TABLE OF CONTENT** v

**STATEMENT REGARDING PUBLICATIONS** viii

**TABLE OF FIGURES** vii

**TABLE OF TABLES** ix

**ABBREVIATIONS** xii

Chapter 1 **INTRODUCTION** 1

Chapter 2 **LITERATURE REVIEW** 4

2.1. **Intra- and extracellular freezing in plants** 4

2.2. **Plasma membrane is the primary injury site of freezing-thaw stress** 5
   2.2.1. The SH hypothesis of freezing injury 5
   2.2.2. The plasma membrane H^+-ATPase hypothesis of freezing injury 6
   2.2.3. Expansion-induced lysis and loss of osmotic responsiveness 7

2.3. **Freezing induced lipid degradation** 8

2.4. **Freezing resistance and cold acclimation** 12
   2.4.1. Biochemical changes during cold acclimation 14
      2.4.1.1. Compatible solutes 14
      2.4.1.2. Membrane lipid composition 15
      2.4.1.3. Enzyme activity 17
      2.4.1.4. Antioxidant system 19
      2.4.1.5. Oxidation and reduction 25
   2.4.2. Gene expression at low temperatures 27
      2.4.2.1. Alteration in protein composition 27
      2.4.2.2. Isolation and characterization of cold-regulated genes and encoded polypeptides 29

2.5. **Genetics of cold acclimation** 31

2.6. **Cold acclimation in coniferous species** 32

2.7. **Summary** 34

2.8. **Objectives** 34
Chapter 3 THE INDUCTION OF FREEZING TOLERANCE IN JACK PINE SEEDLINGS: THE ROLE OF ROOT PLASMA MEMBRANE H^+-ATPase AND REDOX

3.1. INTRODUCTION

3.2. MATERIALS AND METHODS
3.2.1. Plant material
3.2.2. Cold conditioning of jack pine seedlings
3.2.3. Survival test after freezing
3.2.4. Sampling and experimental design
3.2.5. Plasma membrane isolation and enzyme assays
3.2.6. SDS-PAGE and immunoblotting

3.3. RESULTS
3.3.1. Effect of cold conditioning on frost hardiness of jack pine seedlings
3.3.2. Effect of cold conditioning on plasma membrane-bound enzyme activities

3.4. DISCUSSION

Chapter 4 COLD ACCLIMATION OF JACK PINE SEEDLINGS: EFFECTS ON LIPID COMPOSITION AND THE SUSCEPTIBILITY OF MEMBRANE LIPIDS TO FREEZING INJURY AND PEROXIDATION

4.1. INTRODUCTION

4.2. MATERIALS AND METHODS
4.2.1. Plant material
4.2.2. Extraction and analysis of lipids in jack pine root and needle tissues
4.2.2.1. Extraction of lipids from tissue
4.2.2.2. Separation of polar lipids by TLC
4.2.2.3. Gas chromatography analysis
4.2.3. Isolation of microsomal membranes and the induction of lipid peroxidation
4.2.3.1. Isolation of microsomal membranes
4.2.3.2. Lipid peroxidation induced by a free radical generation system
4.2.3.3. Determination of lipid peroxidation

4.3. RESULTS
4.3.1. Lipid and fatty acid composition of jack pine roots and needles
4.3.2. Effect of cold acclimation on major phospholipids and galactolipids and their fatty acid compositions
4.3.3. In vitro lipid peroxidation in jack pine root microsomes
4.3.4. Lipid peroxidation induced directly from freezing thaw injury
4.3.5. Effects of freezing injury on the fatty acid composition of major root lipids

4.4. DISCUSSION
4.4.1. Lipid composition of jack pine roots and needles.
4.4.2. Effect of cold acclimation on jack pine root and needles lipids, and their fatty acid composition
4.4.3. Effect of cold acclimation on cellular membrane stability assessed by the determination of lipid peroxidation
4.4.4. Effect of freezing injury on major lipids and their fatty acids composition
Chapter 5 CHANGES IN OXIDATION-REDUCTION STATES AND ANTIOXIDANT ENZYMES IN THE ROOTS OF JACK PINE SEEDLINGS DURING COLD ACCLIMATION

5.1. INTRODUCTION

5.2. MATERIALS AND METHODS
   5.2.1. Plant material
   5.2.2. Extraction of soluble and microsomal proteins
   5.2.3. Determination of protein thiol groups
      5.2.3.1. Protein thiol concentrations in soluble and microsomal proteins
      5.2.3.2. In vitro labeling of protein thiol groups
      5.2.3.3. Assessment of freezing damage in protein thiol group
   5.2.4. Extraction and analysis of GSH and GSSG
   5.2.5. Isolation of plasma membrane and measurement of plasma membrane-bound enzyme activities
   5.2.6. Determination of the antioxidant enzyme activity in soluble proteins from root tissue
      5.2.6.1. Assays for measurement of antioxidant enzyme activity
      5.2.6.2. Native PAGE and activity staining

5.3. RESULTS
   5.3.1. Acclimation and susceptibility of protein thiol groups to freezing stress
   5.3.2. Changes in GSH and GSSG levels during cold acclimation
   5.3.3. Regulation of plasma membrane bound enzyme activities by thiol reagents
   5.3.4. Enzymatic changes of ascorbate-glutathione cycle during cold acclimation

5.4. DISCUSSION
   5.4.1. Effect of cold acclimation on glutathione redox status
   5.4.2. The role of glutathione in regulation of plasma membrane bound enzyme activities
   5.4.3. Changes of antioxidant enzymes in jack pine roots during cold acclimation

Chapter 6 SUMMARY AND CONCLUSIONS

6.1. Development of freezing tolerance in the root tissue of jack pine seedlings

6.2. Physiological and biochemical changes during cold acclimation
   6.2.1. Increased phospholipid and lipid fluid in root and needle tissues
   6.2.2. Decreased in NADH-redox associated lipid peroxidation and increased tolerance to free radicals in microsomal membranes isolated from the roots of 4-week conditioned seedlings
   6.2.3. Increased root GSH/GSSG mole ratio and SH group concentrations in soluble proteins during cold acclimation
   6.2.4. GSH plays an important role as an antioxidant to prevent freezing induced thiol oxidation in cellular membranes
   6.2.5. Changes in antioxidant enzymes involved in AsA/glutathione cycle

6.3. A Possible mechanisms of cold acclimation
Statement Regarding Publications

This thesis includes information from a series of papers published or submitted for publications as original articles in referred journals. These include:


All experiments described in this thesis were performed solely by the author.
**TABLE OF FIGURES**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Sequential development of freezing injury</td>
<td>11</td>
</tr>
<tr>
<td>2.2</td>
<td>The formation of active oxygen species in biological systems</td>
<td>23</td>
</tr>
<tr>
<td>2.3</td>
<td>Ascorbate-glutathione cycle pathway</td>
<td>24</td>
</tr>
<tr>
<td>3.1</td>
<td>Effect of cold conditioning (weeks of treatment) on the survival of jack pine seedlings upon exposure to freezing</td>
<td>46</td>
</tr>
<tr>
<td>3.2</td>
<td>Effects of cold conditioning (weeks of treatment) on the growth of jack pine seedlings</td>
<td>47</td>
</tr>
<tr>
<td>3.3</td>
<td>Effect of cold conditioning (weeks of treatment) on root plasma membrane-associated H⁺-ATPase for a range of assay temperatures.</td>
<td>48</td>
</tr>
<tr>
<td>3.4</td>
<td>Immunoblot of the plasma membrane H⁺-ATPase from root tissue.</td>
<td>50</td>
</tr>
<tr>
<td>3.5</td>
<td>Effect of cold conditioning (weeks of treatment) on root plasma membrane-associated NADH-dependent ferricyanide reduction for a range of assay temperatures.</td>
<td>51</td>
</tr>
<tr>
<td>4.1</td>
<td>Time course of the formation of TBARS in jack pine root microsomal membranes by oxygen free radicals generated from a FeSO₄-NADPH system</td>
<td>72</td>
</tr>
<tr>
<td>4.2</td>
<td>NADH-dependent lipid peroxidation in jack pine root microsomal membranes.</td>
<td>73</td>
</tr>
<tr>
<td>4.3</td>
<td>The effects of freeze-thaw on the time course of the formation of TBARS in jack pine root microsomal membranes and the susceptibility to free radicals generated from a FeSO₄-NADPH system.</td>
<td>74</td>
</tr>
<tr>
<td>4.4</td>
<td>The effects of freeze-thaw on the fatty acid composition of PC and PE in jack pine root tissue from non-conditioned (control) and four weeks conditioned (4-wk) seedlings</td>
<td>76</td>
</tr>
<tr>
<td>4.5</td>
<td>The effects of freeze-thaw on the fatty acid composition of DGDG and MGDG in jack pine root tissue from non-conditioned (control) and four weeks conditioned (4-wk) seedlings</td>
<td>77</td>
</tr>
<tr>
<td>5.1</td>
<td>The effects of cold acclimation on protein SH concentrations in soluble and microsomal proteins from jack pine roots.</td>
<td>98</td>
</tr>
<tr>
<td>5.2</td>
<td>SDS-PAGE patterns of soluble proteins from jack pine roots.</td>
<td>99</td>
</tr>
</tbody>
</table>
Figure 5.3 *The effect of in vivo and in vitro freeze-thaw on thiol concentrations in root microsomal proteins.* 101

Figure 5.4. *Changes in the status of glutathione in jack pine roots during cold acclimation.* 103

Figure 5.5. *The effects of pre-incubation with PCMB on plasma membrane H^+-ATPase and ferricyanide reductase activities* 108

Figure 5.6. *The protective effects of GSH on PCMB-treated plasma membrane on H^+-ATPase activity* 110

Figure 5.7 *Effects of in vitro freezing-thaw of plasma membrane and glutathione on H^+-ATPase activity* 112

Figure 5.8 *The effects of cold acclimation on ascorbate and guaiacol peroxidase activities* 116

Figure 5.9 *Native-PAGE of ascorbate and guaiacol peroxidase activity staining in jack pine root soluble proteins* 117

Figure 5.10 *The effect of cold acclimation on monodehydroascorbate reductase (A); dehydroascorbate reductase (B); and glutathione reductase (C) activities.* 119

Figure 6.1 *Summary of the development of freezing tolerance in jack pine seedlings during cold acclimation* 137

Figure 6.2 *A flow chart showing a possible sequence of events leading to cold acclimation as results of increase in freezing tolerance* 141

**TABLE OF TABLES**

Table 2.1 *Changes in enzyme activities during cold acclimation*

Table 4.1 *Lipid composition of root and needle tissue from control and cold-conditioned jack pine seedlings.* 65

Table 4.2 *Fatty acid composition of root lipids.* 66

Table 4.3 *Fatty acid composition from needle lipids.* 67

Table 4.4 *Effect of freeze-thaw stress on major phospholipids and galactolipids extracted from roots of non-conditioned and conditioned seedlings.* 68
Table 4.5 Changes in molecular species in major PL and GL during cold acclimation

Table 5.1 The effects of some thiol reagents on jack pine root plasma membrane H⁺-ATPase.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDHA</td>
<td>monodehydroascorbate</td>
</tr>
<tr>
<td>MDHAR</td>
<td>monodehydroascorbate reductase</td>
</tr>
<tr>
<td>MES</td>
<td>2-[N-Morpholino]ethanesulfonic acid</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>reduced nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NADPH</td>
<td>reduced nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NBT</td>
<td>nitroblue tetrazolium</td>
</tr>
<tr>
<td>N-P-K</td>
<td>nitrogen, phosphorous, potassium mixture</td>
</tr>
<tr>
<td>O₂⁻</td>
<td>superoxide radical</td>
</tr>
<tr>
<td>OH</td>
<td>hydroxyl group</td>
</tr>
<tr>
<td>P</td>
<td>probability</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCMB</td>
<td>p-chloromercuribenzoic acid</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PVP</td>
<td>polyvinyl-polypyrrolidone</td>
</tr>
<tr>
<td>r</td>
<td>correlation coefficient</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SHAM</td>
<td>salicylhydroxamic acid</td>
</tr>
<tr>
<td>Tₖ</td>
<td>transition temperature</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
</tbody>
</table>
TEMED
TLC
Tris
tris(hydroxymethyl)aminomethane
thin layer chromatography
voltage
N,N,N',N'-tetramethylenediamine
Chapter 1 INTRODUCTION

Temperature is an important determinant in the normal distribution of natural plant communities. It defines the range of distribution and the growth of important agricultural crops and forests. Many plants grow at or near, the temperature boundary limits of their genetically determined survival abilities. It has been estimated that only 6.6% of the total continental area of Earth experiences temperatures that do not drop below 15°C and only 25% of the continental surface is considered safe from frost (Sakai and Larcher, 1987). Mean annual temperatures below 0°C, -10°C (survival limit for freezing sensitive plants), -20°C (survival limit of broad-leaved evergreen trees of maritime temperate climates) and -40°C (distribution limit for deep supercooling woody plants) are represented by 64, 48, 35, and 25% of the earth’s landmass, respectively (Sakai and Larcher, 1987). Therefore, over 1/2 of the earth’s landmass is exposed to the temperatures that can damage or kill freezing sensitive plants.

Temperature affects plant in two ways. First, it alters reaction rates and influences the kinetic properties of enzymes leading to changes in plant metabolism. Second, temperature extremes cause damage (Smirnoff, 1995a). At the extremes, the problem for survival is to maintain structural and functional integrity of macromolecules and cells. Unlike animals, land plants are unable to move from adverse environments and thus must rely on physiological and biochemical strategies to allow them to exist within a range of environmental extremes. Therefore, plant adaptation to temperatures occur constantly as temperature patterns modulate diurnally, seasonally, or over centuries. For temperate species, desiccation, frost and heat are often the principal stresses affecting the survival and growth of plants. As noted, resistance to these stresses is comprised of two components: avoidance (the extent to which the plant is able to prevent and reduce the
penetration of a stress into tissue) and tolerance (the ability of a plant to survive a stress with little or no injury) (Levitt, 1980; Nilsen and Orcutt, 1996). In nature, many plant species from temperate regions are able to develop freezing resistance following exposure to low non-freezing temperatures for a short period of time. This complex adaptive process is termed cold acclimation or cold hardiness. During the period of cold acclimation, plants develop different resistance mechanisms to avoid or tolerate freezing stress, including changes in lipid composition; and changes in enzyme activity; increased sugar or amino acid contents and; changes in the level of soluble proteins and gene expressions (Levitt, 1980; Cattivelli and Bartels, 1992; Howarth and Ougham, 1993; Burke, 1995).

The production of coniferous planting stocks is a major component of the Canadian forest industry. Seedlings are lost each year during fall and spring transplanting, or during overwintering storage as a consequence of incomplete hardening and premature dehardening (Lindstrom and Nystrom, 1987; Bigras et al., 1996). The effect of sub-freezing temperatures is considered to be the most critical factor (Colombo, 1990). Therefore, cold hardening of conifer seedlings intended for reforestation is a major concern among forest nursery managers. The roots of the seedlings are more sensitive to freezing temperatures than the shoots since aerial portions of plants are capable of hardening to a far greater degree than are roots (Lindstrom, 1986; Ritchie, 1990).

As shown in the following review of the literature, although biochemical and molecular changes during cold acclimation have been studied extensively in crop species, little information is available concerning the responses of trees to cold acclimation, the root system in particular. The work described here was designed to develop an understanding of the responses of tree seedlings
to cold acclimation, with emphasis on the development of cold hardiness of the roots of jack pine 
(Pinus banksiana Lamb.) seedlings.
Chapter 2

REVIEW OF THE LITERATURE

To date, many studies concerning freezing-injury and cold acclimation have been conducted in order to improve freezing resistance in plants. The following is a brief review of the background of freezing-injury and cold acclimation in plants. The chapter concludes by presenting the objectives of this thesis.

2.1. Intra- and extracellular freezing in plants

In plant tissues freezing can be either intracellular or extracellular. Intracellular freezing has been studied under controlled environmental conditions and induced by rapid freezing and supercooling well below the freezing point of the tissue (Levitt, 1980).

Intracellular freezing is not common in nature (Simionvitch and Scarth, 1938), however, extracellular freezing occurs in all plants in temperate climates (Levitt, 1978; Pearce, 1988). Ice formation begins in the extracellular space because of the low concentration of solutes (as compared to intracellular water) and high prevalence of ice nucleators, such as dust (Palta and Weiss, 1993). Extracellular freezing mainly depends upon two environmental factors, ice nucleation and cooling rates (Steffen et al., 1989; Palta and Weiss, 1993). In nature, during most spring and autumn frosts and winter freezing the maximal cooling rates below 0°C are very slow, usually less than 2°C/hour (Steffen et al., 1989). The gradual growth of extracellular ice withdraws water from the cells. The dehydration and mechanical stresses the cell experiences from extracellular ice contribute largely to freezing injury (Levitt, 1980; Sakai and Larcher, 1987). Several other factors including the duration of exposure to ice, cooling and thawing rates and post-
thaw conditions can also influence the injury (Palta and Weiss, 1993). The process of freezing and extent of freezing injury is also influenced by the anatomy, the age, water content and the hardiness of the particular plants.

2.2. Plasma membrane is the primary injury site of freezing-thaw stress

The cell membrane was recognized over 80 years ago as a site of freeze-thaw injury (Maximov, 1912). However, the nature of this injury at a more fundamental level has been investigated only recently (Levitt, 1980; Hellergren et al., 1983; Palta et al., 1993a; Steponkus, 1990). As the principal barrier between the cytoplasm and the surrounding environment, the semi-permeable characteristics of the plasma membrane are of primary importance in allowing for the efflux/influx of water during a freeze-thaw cycle, while preventing the efflux of intracellular solutes and, most important, precluding the injury of the cytosol by extracellular ice. Maintenance of these structural and functional characteristics during a freeze-thaw cycle is essential to survival. Since cell membranes consist basically of lipids and proteins, membrane damage must be due to either lipid or protein changes, or both. Several mechanisms for plasma membrane injury have been proposed based on the studies of herbaceous plants. The mechanisms include oxidation of protein sulphydryl (SH) groups (Levitt, 1980); decreased plasma membrane $H^+$-ATPase activity (Palta et al., 1993a); and expansion-induced lysis of cell (Gordon-Kamm and Steponkus, 1984).

2.2.1. The SH hypothesis of freezing injury

According to Levitt's SH hypothesis, the freezing injury of plasma membranes could be caused by the aggregation or dissociation of membrane proteins through the formation of
intermolecular disulphide bonds (-S-S-) from thiol groups in adjoining protein molecules as they approach one another when the protoplasm is freeze-dehydrated. This hypothesis was based on the following correlations. (1) An increase in soluble protein -S-S- was found after freezing injury (Levitt, 1967). (2) The thiol concentration of the proteins was proportional to freezing tolerance. (3) An increase in the oxidation of reduced glutathione was correlated with the increase in freezing tolerance, due partly to an increase in ascorbate. The hypothesis was also tested with pure SH-containing enzymes as model systems and it was demonstrated that a number of the enzymes were inactivated by freezing (Levitt, 1966). Even though the hypothesis was postulated specifically for membrane proteins, most the enzymes in the above correlations are soluble proteins in the cytosol.

2.2.2. The plasma membrane H⁺-ATPase hypothesis of freezing injury

Palta proposed that the plasma membrane ATPase was a site of freeze-thaw stress injury (Palta et al., 1977a; Palta et al., 1977b). The following lines of evidence have been provided by independent research groups in support of this hypothesis: (1) Ion leakage is commonly observed from cells that experience freeze-thaw stress and results from membrane rupture or a loss of membrane permeability. The major cation leaking from the freezing injured cells is potassium (K⁺) (Palta et al., 1977b; Pukacki and Pukacka, 1987). Since transport of K⁺ across the cell plasma membrane is coupled with the activity of H⁺-ATPase, these results suggest an alteration in the function of H⁺-ATPase during freeze-thaw stress. (2) A progressive decrease in specific activity of the plasma membrane H⁺-ATPase from Solanum commersonii leaflets was found as the freeze-thaw stress induced injury increased (Iswari and Palta, 1989a). In purified plasma membranes from pine needles, the ATPase activity was totally abolished after lethal freeze-thaw stress (Hellergren et al., 1985). (3) Full recovery of tissue that has undergone freeze-thaw injury required that leaked
ions (primarily K⁺) be pumped back into the cells (Iswari and Palta, 1989b). Furthermore, the recovery from freeze-thaw injury can be prevented by treating the tissue with 100 μM sodium vanadate, an inhibitor of the plasma membrane H⁺-ATPase (Arora and Palta, 1991). Although the molecular mechanisms affecting the plasma membrane H⁺-ATPase following freeze-thaw stress are not clearly understood, Palta and Li (1980) suggested that these changes may result from direct denaturation of membrane transport proteins or changes in the lipid-protein interactions.

2.2.3. Expansion-induced lysis and loss of osmotic responsiveness

Steponkus's research group investigated the cryo-behaviour of plasma membrane in situ by cryo-microscopic studies of protoplasts isolated from winter rye leaves (Secale cereale L. cv. Puma). In these studies, complemented by electron microscopy studies of ultrastructural changes in the plasma membranes, they were able to analyze the phenomenon of freezing injury and they identified the specific "lesions" in the plasma membranes. Comprehensive reviews of these studies have been presented by Steponkus and Lynch (Steponkus and Lynch, 1989), Steponkus (Steponkus, 1990) and Steponkus and Webb (Steponkus and Webb, 1992).

When the protoplasts of rye leaves were cooled to temperatures of -2 to -5°C, destabilisation of plasma membrane was a consequence of osmotic excursions that occur during a freeze-thaw cycle (Steponkus et al., 1993). Dowgert and Steponkus (1984) found that lysis of the protoplasts occurred during osmotic expansion before the initial size was regained following freezing and thawing of the suspending medium. This form of injury, referred as expansion-induced lysis, occurred because the plasma membrane underwent endocytotic vesiculation during osmotic contraction upon freezing (Dowgert and Steponkus, 1984; Gordon-Kamm and Steponkus, 1984). Because intrinsic elastic expansion of the plasma membrane is limited 2 to 3%
(Wolfe and Steponkus, 1983), osmotic expansion requires that additional material be reincorporated into the plasma membrane during osmotic expansion. However, they found that the rate of re-incorporating was not sufficiently rapid to balance the osmotic expansion.

A second form of injury occurred in the plasma membrane of the protoplasts at temperatures below -5°C. This form of injury, termed loss of osmotic responsiveness, was associated with several changes in the plasma membrane ultrastructure following the freeze-thaw cycle. These changes include the appearance of particulate lamellae sub-tending the plasma membrane; the formation of large particulate domains in plasma membrane; and lamellae hexagonal II phase (HII phase) transitions (Gordon-Kamm and Steponkus, 1984). The HII phase is a non-lamellar phase that is a three-dimensional array of inverted cylindrical micelles that have water in the central core of each cylinder. Freeze-fracture EM studies revealed that HII phase was the principal ultrastructural manifestation of freezing injury in protoplasts of A. thaliana (Uemura et al., 1995). The HII phase was observed at a high frequency in the protoplasts frozen at -8 and -10°C, temperatures at which the survival of protoplasts was about 5%. The formation of domains of monolayer configurations following freeze-thaw is attributed to bilayer-bilayer interactions that occur when bilayers are brought into close each other during freeze-induced dehydration.

2.3. Freezing induced lipid degradation

Lipid peroxidation been found in microsomal membranes isolated from wheat plants that were exposed to freezing temperatures (Hetherington et al., 1987; Hetherington et al., 1988; Kendall and McKersie, 1989; Pukacki et al., 1991). Kendall and McKersie (1989) demonstrated that freezing stress accelerated the formation of superoxides in microsomal membranes from crowns of winter wheat as determined by electron spin resonance spectroscopy. When extracellular
water freezes, solutes excluded at the ice-water interface may remain trapped within the tissue (Kendall and McKersie, 1989). As the solubility of oxygen in water increases with lower temperatures, oxygen readily diffuses into the cells during freezing stress. Electron transport chains and enzymes involved in oxidation-reduction reactions may be disrupted by the freezing-dehydration injury. The electrons can not be transferred from their donor to their regular receiver completely. Thus, electrons that leak out from the transport chains can react with oxygen enhancing superoxide or singlet oxygen production (Fridovich, 1970). The production of active oxygen species mediates the degradation of membrane lipids, leading to the accumulation of free fatty acids and/or peroxidation products, the irreversible formation of gel phase domains and loss of membrane functions (McKersie et al., 1990; Shewfelt, 1992). The increase in free fatty acid concentration, with little change in fatty acid unsaturation has been reported in the microsomes isolated from freezing stressed wheat seedlings (Hetherington et al., 1988; Kendall and McKersie, 1989).

Several models have been presented to describe the mechanism of peroxidative degradation in plant membranes. Lipoygenase has been proposed as a primary source of peroxidation (Thompson, 1988) by acting on free fatty acids hydrolyzed either enzymatically (Paliyath and Thompson, 1987) or chemically (Kendall and McKersie, 1989). Alternatively, the critical step may be that rate of free-radical chain propagation reactions of esterified fatty acids in membrane lipids exceeds the capacity of free radical scavenging (Shewfelt and Erickson, 1991). Plant plasma membranes contain redox systems that have the ability to consume oxygen and to generate superoxide by using NAD(P)H as an electron donor (Crane et al., 1991). The redox systems on the plasma membrane play an important role in many physiological processes (Moog and Bruggemann,
1994; Crane et al., 1995; Serrano et al., 1995). However, little is known about the production of active oxygen species that contribute to membrane damage under stress conditions. Recently, Qiu et al. (1995) reported that oxygen consumption and superoxide production was enhanced with an increase in lipid peroxidation in the plasma membrane of wheat roots under water stress. This suggests that the plasma membrane redox system could be an important factor in the production of cellular oxygen radicals and could cause membrane damage through lipid peroxidation under water stress.

In summary, both structure and functions of the plant plasma membrane could be damaged by freeze-thaw as shown in Fig. 2.1. Frost damage is typically associated with (1) loss of semi-permeability, (2) loss of active transport of ions, (3) degradation of phospholipids, (4) redistribution of proteins due to lateral displacement, and (5) a dehydration induced phase transition from lamellar to H_{II} phase (Hallgren and Oquist, 1990). The molecular mechanisms underlying freeze-thaw damage to cellular membrane are certainly complex as pointed out by Steponkus (1990): No single mechanism could explain freezing injury to various tissues or species of different hardiness.
Freezing temperature

Dehydration

Expansion-induced lysis of membrane

Loss of osmotic responsiveness

Thiol oxidation

Aggregation or redistribution of proteins

Loss of active transport

Degradation of phospholipids

Loss of membrane semipermeability

Generation of active oxygen species

Membrane lipid peroxidation

Freezing damage

Fig. 2.1 Sequential development of freezing injury
2.4. Freezing resistance and cold acclimation

For a vast number of species, freezing tolerance is not static, but can change seasonally or when the temperature and other environmental conditions are changed. In nature, the cold acclimation responses of woody plants are usually initiated by short day photoperiod followed by additional freezing tolerance development by low non-freezing temperatures (Weiser, 1970; Sakai and Larcher, 1987).

Cold acclimation is a complex process that can be triggered not only by low non-freezing temperatures but also by different types of stimuli such as drought and abscisic acid (ABA) (Levitt, 1980; Sakai and Larcher, 1987; Palva, 1993; Howarth and Ougham, 1993). Exogenous application of ABA to cell cultures or whole plants at non-acclimating temperatures has been shown to cause rapid induction of freezing tolerance in several plant species including *Solanum commersonii* (Chen et al., 1983), *Arabidopsis thaliana* (Crespi et al., 1991), *Brassica napus* (Orr et al., 1986) and smooth bromegrass (Ishikava et al., 1990). Endogenous ABA content has also been shown to rise in cold-treated plant tissue, including tomato plants (Daie and Campbell, 1981), potato leaves (Chen et al., 1983), the above-ground parts of winter wheat (Taylor et al., 1990), and xylem sap of sugar maple (Bertrand et al., 1997). Direct evidence for the role of ABA in the development of freezing tolerance was provided by *Arabidopsis* mutants, including mutants defective in ABA biosynthesis (*aba*-mutants) and mutants insensitive to ABA (*abi*-mutants) (Heino et al., 1990). By using an ABA-deficient mutant (*aba*-1), Heino et al. (1990) demonstrated that the lack of ABA indeed prevented the development of freezing tolerance which could be restored by exogenous ABA. Nordin et al. (1993) showed that even certain ABA-non-responsive mutants (*abi1*) are
impaired in the cold acclimation process. The exogenous ABA-induced acclimation is completely blocked in ab1 mutants. Taken together, the data indicate that ABA plays a key role in cold acclimation.

Cold acclimation is a highly active process resulting from a combination of physiological and metabolic alterations in plants in response to low temperatures (Levitt, 1980; Graham and Patterson, 1982; Steponkus, 1984). During the cold acclimation period, many events are required to achieve maximum cold hardiness. These include hormonal response to environmental cues, altered gene activity and new gene products, and alteration in metabolism resulting in the accumulation of solutes and changes in lipid composition (Levitt, 1980; Patterson and Graham, 1987; Howarth and Ougham, 1993). In these changes, two basic survival mechanisms have been distinguished: avoidance of freezing (avoidance of ice formation) and tolerance to freezing (formation of ice in plant tissues without lethal consequences) (Levitt, 1980). Supercooling of tissue water and accumulation of antifreeze (cryo-protectant) provide some protection to avoid freeze stress (Levitt, 1980; Sakai and Larcher, 1987). Avoidance mechanisms seem to be more prevalent in herbaceous plants, while tolerance is more characteristic of woody perennials and evergreens (Levitt, 1980). However, both mechanisms may coexist in the same plant but reside in different tissues. For example, vegetative and floral buds can supercool by moving water from the internal tissues to bud scales, where it freezes and causes no injury or reduced injury to those tissues (Colombo, 1990; Nilsen and Orcutt, 1996). Similarly, the ray parenchyma of woody plants supercools; but the bark, including the phloem, tolerates ice formation under freezing conditions (Nilsen and Orcutt, 1996). Needles of pine and spruce can supercool, but survival is primarily by tolerance to ice (Palta and Weiss, 1993).
The ability of plants to survive environmental extremes relates to their ability to adapt morphologically and physiologically to environmental stresses. Small, thick, ericoid pointed leaves, high root/shoot ratio, and the production of roots near the surface all represent important strategies developed by plants to deal with these stresses (Nilsen and Orcutt, 1996). During the early stages of cold acclimation, photosynthesis and respiration increases due to the production of metabolites, such as sugars, polyols and proteins.

2.4.1. Biochemical changes during cold acclimation

In this section, biochemical changes during cold acclimation will be reviewed. Five main areas are addressed including changes (1) compatible solutes, (2) membrane lipid composition, (3) enzyme activity, (4) antioxidant systems and (5) oxidation-reduction status.

2.4.1.1. Compatible solutes

It has been known for many years that plants maintained at low temperatures for long periods accumulate water-soluble carbohydrates and amino acids, and in many cases this is accompanied by an increase in frost hardiness (Levitt, 1980; Smirnoff, 1995a). Solutes such as fructans (Parker, 1959; Livingston III, 1991), sucrose (Parker, 1959; Pollock, 1986; Guy, 1990), polyols (Bohnert et al., 1995; Nilsen and Orcutt, 1996) and proline (Levitt, 1980; Tantau and Dorffling, 1991) accumulate when plants are exposed to low temperature. Neither the cause of accumulation of these solutes nor their function is clear.

The carbohydrates are the primary cryo-protectants present in plants. Sucrose is the most widely found in freezing-tolerant plants; its levels can increase 10-fold during exposure to low temperature (Guy, 1990). Levitt suggested that the accumulation of carbohydrates was a
mechanism for increasing frost resistance, perhaps by depressing the freezing point of cell contents to avoid fatal intracellular freezing (Levitt, 1980). However, Smirnoff pointed out that these solutes do not accumulate to high enough levels to lower the freezing point by more than a few degrees, but they could prepare cells for tolerating freezing by acting as compatible osmolytes to prevent the dehydration of cytoplasm (Smirnoff, 1995a). Guy suggested that these solutes function as cryoprotectants which help to sustain the ordered vicinal water around proteins by decreasing protein-solvent interactions in such a way that subunit association and native conformation are maintained at low water activities. He also pointed out that many of these compounds may also stabilize cellular membranes through interactions with the polar head groups of phospholipids or hydrophobic interactions with the membranes (Guy, 1990).

2.4.1.2. Membrane lipid composition

Qualitative and quantitative changes in plant membrane lipid compositions occurring during cold acclimation are correlated with increased freezing tolerance (Uemura and Steponkus, 1994; Uemura et al., 1995). Several mechanisms could exist in plants for the acclimation of membranes to changing temperatures. These include regulating glycerolipid fatty acid double bond numbers and positions, changes in fatty acyl chain length, attachment positions of saturated and unsaturated fatty acyl chain to glycerol (sn-1 and sn-2), the insertion/removal of sterols and the composition of glycerolipids (Shewfelt, 1992; Ohlrogge and Browse, 1995; Nilsen and Orcutt, 1996). The ability of a plant to change its fatty acid composition could be advantageous in terms of maintaining the cell membrane lipids in a liquid-crystalline phase, thus avoiding a solid-gel state.

In addition to changes in acyl chains during cold acclimation, lipid classes are also affected (Uemura and Yoshida, 1984; Palta et al., 1993b; Uemura et al., 1995). In general, phospholipids
increase relative to sterols with free sterols increasing relative to steryl glucosides. Since the plasma membrane has been implicated as the primary site of freezing injury in plant cells, lipid changes in this membrane have been specifically investigated (Uemura and Yoshida, 1984; Thompson, 1989; Yoshida and Uemura, 1990; Palta et al., 1993b; Uemura et al., 1995). Increases in phospholipids were reported in all of those studies. In woody tissue, such as mulberry bark, plasma membrane phospholipids underwent a dramatic increase during cold acclimation in fall and in winter (Yoshida, 1984). During cold acclimation, the increase in the proportions of phospholipids of winter rye leaf plasma membrane are the consequence of increase in the proportion of di-unsaturated molecular species of PC and PE (Lynch and Steponkus, 1987; Uemura and Yoshida, 1984). Similar changes also have been observed recently in the plasma membrane of Arabidopsis thaliana during cold acclimation (Uemura et al., 1995).

Shewfelt (1992) pointed out that clustering of distinctive molecular species might be sufficient to induce localized phase changes within the membrane. Steponkus et al. (1988) reported that an increase in freezing tolerance of protoplasts isolated from non-acclimated rye leaves was elicited by fusion liposomes composed of either the total phospholipid isolated from plasma membranes of cold-acclimated leaves or mono- (16:0/18:1, 16:0/18:2) or di-unsaturated (18:1/18:1, 18:2/18:2, 18:3/18:3) species of PC. Of the PC species, 18:2/18:2 and 18:3/18:3 liposomes were the most effective, and the 16:0/18:1, 16:0/18:2 and 18:1/18:1 were less effective. Fusion with liposomes containing only saturated PC molecular species (14:0/14:0, 16:0/16:0) provide non-protection. They also demonstrated that the increased freezing tolerance was the result of a transformation in the cryo-behavior of plasma membrane during freezing-induced osmotic contraction. Recently, Uemura et al. (1995) demonstrated that an increase in proportion of PC,
particularly in di-unsaturated species, in plasma membrane isolated from cold acclimated *A. thaliana* leaf tissue contributed to the decreased propensity for the freezing-induced formation of the H$_r$ phase. These changes are consistent with the hypothesis that membrane fluidity plays a role in freezing resistance (Steponkus and Lynch, 1989).

In chloroplast membranes, Uemura and Steponkus (1997) reported that the proportions of monogalactosyldiacylglycerol (MGDG) decreased, but the proportion of digalactosyldiacylglycerol (DGDG) increased in both inner and outer chloroplast membranes in rye leaves after 4-weeks of cold acclimation. Changes in the proportion of MGDG to DGDG may have an effect on the stability of the thylakoid membrane. This is because the galactolipids have very different physical properties (Shipley et al., 1973; Selstam et al., 1990). MGDG is a de-stabilizing lipid and forms a reverse H$_r$ phase with water; while DGDG is a stabilizing lipid and forms a lamellar phase with water (Selstam and Oquist, 1990). Furthermore, an increase in DGDG content might contribute to the survival of an intact thylakoid membrane at very low temperatures since the $Tc$ of DGDG is much lower than that of MGDG (Shipley et al., 1973).

2.4.1.3. Enzyme activity

Enzyme acclimation to temperature occurs constantly as temperature changes. These adaptations entail qualitative and/or quantitative metabolic changes that often provide a competitive advantage, and affect plant survival. Changes in the profiles of isozymes and enzyme concentration, modification by substrate and effectors, and metabolic regulation of enzyme function without changing enzyme composition are all possible strategies for adaptation to temperature stress (Burke, 1995). Activities of many enzymes have been reported to increase during cold acclimation. The changes in enzyme activities during cold acclimation are summarized in Table 2.1.
Table 2.1. Changes in enzyme activities during cold acclimation

<table>
<thead>
<tr>
<th>Enzymes involved in carbohydrate metabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fructose-1,6-diphosphatase</td>
</tr>
<tr>
<td>Sucrose synthase</td>
</tr>
<tr>
<td>Sucrose fructosyl transferase</td>
</tr>
<tr>
<td>Sucrose phosphate synthase</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Enzymes involved in pentose-phosphate pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose-6-phosphate dehydrogenase, hexokinase</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase, Hexokinase</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Enzymes involved in proline metabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamine synthase, proline dehydrogenase</td>
</tr>
<tr>
<td>Ornithine decarboxylase</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Enzymes involved in membrane transport</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tonoplast ATPase</td>
</tr>
<tr>
<td>Plasma membrane ATPase</td>
</tr>
<tr>
<td>Plastos membrane NADH ferricyanide reductase</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antioxidant enzymes: glutathione reductase (GR), ascorbate peroxidase (APX), superoxide dismutase (SOD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GR, APX</td>
</tr>
<tr>
<td>GR, APX, SOD</td>
</tr>
<tr>
<td>GR, APX, SOD</td>
</tr>
<tr>
<td>GR, APX</td>
</tr>
<tr>
<td>Catalase</td>
</tr>
<tr>
<td>GR, APX</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Enzymes involved in phenolic synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylalanine ammonia lyase</td>
</tr>
<tr>
<td>Phenylalanine ammonia lyase</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Enzymes involved in membrane lipid metabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coenzymephosphate transferase</td>
</tr>
<tr>
<td>Galactolipid-galactosyl galactosyl transferase</td>
</tr>
<tr>
<td>UDP-galactosyl transferase</td>
</tr>
<tr>
<td>Desaturases</td>
</tr>
</tbody>
</table>
For most of those enzymes, the increased enzyme activity was correlated with an enhanced gene expression. For example, Crespi et al. (1991) showed that the increase in sucrose accumulation and sucrose synthase activity in wheat seedlings were accompanied by an increase in abundance of both the sucrose synthase subunit and the corresponding mRNA during cold acclimation. Guy et al. (1992) also found an increase in sucrose phosphate synthase activity in spinach at 5°C, concomitant with an increase in sucrose content and the abundance of the enzyme subunit in cold-treated tissue. The accumulation of these enzymes may be the responses and/or adaptations to lower temperature, but not necessary for frost resistance.

2.4.1.4. Antioxidant system

In plants, the auto-oxidation of various electron transport chain components in mitochondria (Cadenas, 1989; Winston, 1990; Smirnoff, 1995b) and in chloroplasts (Asada and Takahashi, 1987; Folyer, 1996) and microsome membrane (Winston, 1990; Ahmad, 1995) cause the formation of superoxide (O$_2^-$). Other active oxygen species, such as hydrogen peroxide and hydroxyl radical (OH$^-$), are derived from O$_2$ by successive univalent reduction. The reactions of formation of active oxygen species are summarized in Fig. 2.2.

Plant cells contain protective and repair systems that, under normal circumstances, minimize the occurrence of oxidative damage. The antioxidants are divided into two types (Smirnoff, 1993). The first type antioxidants react with active oxygen and keep them at a low level. These include superoxide dismutase (SOD), catalase, ascorbate peroxidase (APX), ascorbate and α-tocopherol. The second type reduces oxidized antioxidants. These include reduced glutathione (GSH), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR).
SOD dismutates superoxide to hydrogen peroxide. This maintains a low steady concentration of superoxide and therefore minimizes hydroxyl radical formation by the metal-catalyzed Haber-Weiss reactions (Smirnoff, 1993). Catalase breaks down hydrogen peroxide and mainly exists in peroxisomes and glyoxysomes (Smirnoff, 1995b). However, catalase has a relatively low affinity to hydrogen peroxide (Klapheck et al., 1990; Smirnoff, 1995b) and the activity can be inhibited under stress conditions, such as herbicide (Klapheck et al., 1990) and chilling temperatures (O'Kane et al., 1996; Fadzillah et al., 1996). APX catalyses the oxidation of AsA to monodehydroascorbate (MDHA) using hydrogen peroxide as an oxidant. It exists in chloroplast (Asada, 1992) and cytosol (Mittler and Zilinskas, 1992; Thomsen et al., 1992).

Ascorbate plays a central role in detoxification of activated oxygen. It can directly reduce superoxide, hydrogen peroxide, and hydroxyl radicals (Foyer et al., 1991; Winston, 1990). The α-tocopherol, scavenger of singlet oxygen (Smirnoff, 1993; Smirnoff, 1995b), mainly exists in green photosynthetic tissue located in chloroplast membranes (Hess, 1993). It has an important role in scavenging lipid peroxy radicals and preventing lipid peroxidation (Knox and Dodge, 1985; Winston, 1990).

The regeneration of antioxidants is based on AsA and GSH. Three enzymes have a role in maintaining AsA and glutathione in their reduction forms. These are MDHAR, DHAR and GR. The reactions these enzymes catalyse, known as the ascorbate-glutathione cycle, are shown in Fig. 2.3. MDHAR is active with NADH and NADPH, while GR is NADPH dependent. DHAR uses GSH as a reductant.

The ascorbate-glutathione cycle pathway has been extensively studied in chloroplasts, however, there is good evidence that all of the enzymes involved in the pathway are also located in
the cytosol of both green and non-green tissues (Blumwald et al., 1989; Klapheck et al., 1990; Inze and Montagu, 1995). APXs are thought to be the most important H₂O₂ scavengers in the cytosol and chloroplasts (Asada, 1992; Mittler and Zilinskas, 1994; Inze and Montagu, 1995). The APX in the pea leaf cytosol has been purified to homogeneity and characterized (Mittler and Zilinskas, 1991). Anderson et al. (1983) found that the spinach leaf only 30% of the total leaf DHAR is in chloroplasts. Different isoforms of GR are associated with various sub-cellular compartments: In pea leaves, 70-77% of the total GR activity is in chloroplasts (Anderson et al., 1990; Edwards et al., 1990), 3% is in mitochondria (Edwards et al., 1990) and 20-27% is in the cytosol (Edwards et al., 1990). Ascorbate and glutathione have been found in various tissues of higher plants including roots (Dalton et al., 1986; Foyer, 1993).

As shown in Table 2.1, cold acclimation results in increased enzyme activities of GR, APX, SOD, MDHAR and DHAR (Doulis et al., 1993; Karpinski et al., 1993). Kuroda et al. (1990) reported that during the cold acclimation of apple callus culture, the activities of APX and peroxidase increased immediately and reached maximum levels after 10 days of cold acclimation. The activities of GR and DHAR increased gradually during the cold treatment (Kuroda et al., 1990). Similar increases in GR activities have been reported for other species (de Kok and Oosterhuis, 1983). Increases in ascorbate and/or glutathione concentrations during cold acclimation have been reported in the callus of Arabidopsis (O'Kane et al., 1996), the needles of eastern white pine (Anderson et al., 1992) and Scots pine (Krivoshcheva et al., 1996). However, the changes of antioxidant systems in plant root system during cold acclimation have not yet been reported.
Investigation of antioxidative defences revealed that newly formed needles of Norway spruce contained minimum activities of antioxidant enzymes (Polle et al., 1996). Newly formed leaves of the deciduous tree species, beech, also contain lower antioxidant enzyme activities than mature foliage in summer or buds before bud break (Polle and Morawe, 1995; Polle et al., 1996). These results might suggest that young emerging leaves are especially susceptible to oxidative stress.
\[ O_2 + e^- \rightarrow O_2^- \] (1)
\[ O_2^- + e^- + 2H^+ \rightarrow H_2O_2 \] (2)
\[ H_2O_2 + e^- + H^+ \rightarrow OH^- + H_2O \] (3)
\[ OH^- + e^- + H^+ \rightarrow H_2O \] (4)

Equation 1 represents the univalent reduction of molecular oxygen leading to the formation of superoxide. This reaction is endogenic (through membrane electron transport chains). Further reductions (2), (3) and (4) leading to the formation of hydrogen peroxide, hydroxyl radicals and water are exogenic (through chemical reactions) (Smirnof, 1995b).

The formation of active oxygen species from superoxide in biological systems starts with the dismutation of superoxide to \( H_2O_2 \) in the dependence of SOD [reaction 5].

\[ 2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \] (5)

Further reduction of \( H_2O_2 \) forms the hydroxyl radicals, a major route for this reaction in vivo is the ion-catalysed Haber-Weiss reaction (Fenton reaction).

\[ O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+} \] (6)
\[ Fe^{2+} + H_2O_2 \rightarrow OH^- + OH^- + Fe^{3+} \] (7)

Figure 2.2 The formation of active oxygen species in biological systems
Fig. 2.3 Ascorbate-glutathione cycle
2.4.1.5. Oxidation and reduction

The tripeptide glutathione (γ-glutamyl-cysteinyl-glycine) is the major low molecular weight thiol in most plants and it is the major determinant of the oxidation-reduction state of the cytosol and endoplasmic reticulum (ER) (Lodish et al., 1995). The status of glutathione is a critical factor in the thiol-disulphide exchange reactions. In eukaryotic cells, the GSH/GSSG redox ratio is higher in the cytosol to prevent the formation of disulfide bonds in cytosolic proteins. But the ratio is much lower in ER, which is the site for the formation of disulfide bonds after protein synthesis. In canine pancreatic cells, the GSH/GSSG ratio is about 60 in the cytosol and 2 in ER (Hwang et al., 1992). In plants, cellular GSH concentrations of 1 to 4 mM have been reported for many tissues. However, the actual GSH concentration may vary by more than one order of magnitude between different species, different organs of the same species, and different compartments of the same cells (Rennenberg and Lamoureux, 1990; Hausladen and Alscher, 1993).

Changes in the processes that regulate the GSH concentration in plants under stressful environmental conditions may be considered as adaptive mechanisms. During cold acclimation, an increased GR activity has been reported in many plant species. GR and the NADPH-generating pathway are central in antioxidant defence (Lopez-Barea et al., 1990; Kuroda et al., 1990). Under normal conditions (high NADPH and low GSSG concentrations), GR would be partially inactive (Lopez-Barea et al., 1990; Hausladen and Alscher, 1993). The activity of enzymes involved in the oxidative phase of the pentose-phosphate pathway (PPP) is rather low (Lopez-Barea and Barcena, 1988). One of the main functions of PPP is the production of NADPH that is widely used in biosynthesis (particularly lipids). PPP also plays an important role in maintaining the glutathione pool in the reduced state, essential for against oxidative stress. In animal systems, it has been shown
that enzymes involved in PPP, such as glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) are fully inhibited at the physiological high NADPH/NADP⁺, which is around 100 in rat liver (Nogueira et al., 1986). Dependent on GR, GSSG can overcome the strong inhibition of G6PDH by NADPH (Llobell et al., 1988; Lopez-Barea et al., 1990). In general, when an oxidative stress develops, GSSG concentration increases (Seaz et al., 1990; Kosower and Kosower, 1983; Liu and Thiele, 1996). This activates GR activity and subsequent consumption of NADPH and leads to activation of PPP, thus producing more NADPH to counteract the oxidative stress. In plants, the activation of both GR and PPP have been reported in apple and winter wheat during cold acclimation (Del Rio et al., 1996; Kuroda et al., 1990).

GSH also has an important function in maintaining enzyme SH groups in a reduced form (Ziegler, 1985; Gilbert, 1990; Wells et al., 1993). In addition, GSH may have a role in preventing the formation of intermolecular disulfide bonds in proteins which are usually caused by dehydration during freezing stress (Smith et al., 1990). Oxidation of GSH pool could lead to inactivation of enzymes that depend on SH groups. In animal systems, it has been shown that oxidation or cross-linking of mitochondrial thiol groups induces the membrane permeability transition which is associated with the opening of non-selective channels (Petronilli et al., 1994). The modification of channel protein thiol groups by thiol-reagents has been found to increase the probability of pore opening by shifting its gating potential to higher values. This oxidation-induced mitochondrial damage could be prevented by DTT (Petronilli et al., 1994). Recently, it has been shown that thiol oxidation induced rat liver mitochondrial damage can be reversed by regeneration of the sulphydryl groups (Wudarczyk et al., 1996).
In plants, mitochondria and chloroplasts constitute the major cellular sites for the endogenous formation of active oxygen species (Smirnoff, 1995b; Huang and Philbert, 1996); however they contain a high concentration of GSH (1-5 mM) (Hausladen and Alscher, 1993; Gilbert, 1990). The concentration of GSH in mitochondria is an important factor for regulation of mitochondrial proteins sulphydryl group concentrations against oxidative injury (Wudarczyk et al., 1996).

2.4.2. Gene expression at low temperatures

2.4.2.1. Alteration in protein composition

Weiser (1970) originally proposed that cold acclimation might involve altered gene expression. This hypothesis was substantiated by Chen et al. (1983) who showed that in frost hardy potato species, *Solanum commersonii*, cold acclimation requires protein synthesis and can be inhibited by cycloheximide treatment (Chen et al., 1983). Since then, numerous studies have clearly established that new polypeptides are synthesized in response to low temperatures. Several reviews have examined the link between proteins and the development of freezing tolerance (Graham and Patterson, 1982; Guy, 1990; Palva, 1993). The appearance of these polypeptides seems to correlate with the onset of cold acclimation and freezing tolerance (Palva, 1993). Their role in freezing tolerance is supported by the temporal correlation of their appearance with the onset of freezing tolerance and their disappearance during deacclimation (Guy and Haskell, 1988; Mohapatra et al., 1987; Guy et al., 1992).

The induction of new polypeptides during cold acclimation in different plant species shares several common features. For example, increased synthesis of cold-induced polypeptides is rather
rapid. In *Arabidopsis*, the newly synthesised polypeptides are usually detectable within 12 to 24 hours of low temperature exposure (Kurkela et al., 1988; Lang et al., 1989; Heino et al., 1990). The synthesis of most of these cold-induced polypeptides seems to remain high throughout the acclimation period (Cattivelli and Bartels, 1989; Tseng and Li, 1990). In contrast with heat shock response, during cold acclimation, new polypeptides and mRNAs can be detected. However, the overall protein pattern is not dramatically different from the one observed at normal control temperatures (Lang et al., 1989; Guy, 1990). At low temperatures, the majority of proteins and mRNAs continue to be synthesised. This may be consistent with the need to maintain the basic cellular metabolism during cold acclimation, although at a reduced rate (Cattivelli and Bartels, 1992; Howarth and Ougham, 1993). The synthesis of the majority of these cold-induced proteins is responsive to ABA (Lang et al., 1989; Heino et al., 1990; Mohapatra et al., 1988; Tseng and Li, 1990). In a comparison of cold-induced proteins between different plant species, no specific set of proteins was found and the size of the cold-induced proteins ranged from 10 to 200 kDa (Palva, 1993; Cattivelli and Bartels, 1992).

The accumulation of soluble proteins in the apoplast has been demonstrated in overwintering winter rye leaves during cold acclimation (Griffith et al., 1992; Marentes et al., 1993; Hon et al., 1997). Such proteins extracted from cold acclimated winter rye leaves can modify the normal growth of ice crystals by being adsorbed onto the surface of the ice (Griffith et al., 1992; Jia et al., 1996). These apoplastic proteins called antifreeze proteins (AFPs) are thought to be an important mechanism by which winter rye leaves tolerate ice formation (Griffith et al., 1992; Marentes et al., 1993). Six AFPs, with molecular mass ranging from 15 to 36 kDa, accumulated in winter rye leaves during cold acclimation (Marentes et al., 1993; Hon et al., 1997). Immunogold
localization studies demonstrated that in cold acclimated winter rye leaves, high amounts of AFP accumulated in the cell walls of mesophyll cells that are adjacent to intercellular spaces, and in secondary thickenings of xylem vessels where extracellular freezing occurs (Pihakashi-Maunbach et al., 1996). The AFP accumulated during cold acclimation in frost-tolerant plants can inhibit extracellular ice formation leading to decreased extracellular freezing induced dehydration damage to plant cells (Steponkus and Lynch, 1989; Steponkus and Webb, 1992).

2.4.2.2. Isolation and characterization of cold-regulated genes and encoded polypeptides

The synthesis of cold-induced proteins is due to changes in translation of mRNA. Experiments have shown the appearance or increased relative abundance of individual mRNA in spinach, (Guy et al., 1985; Guy and Haskell, 1988; Neven et al., 1993), alfalfa (Mohapatra et al., 1988; Mohapatra et al., 1987), barley (Hughes and Pearce, 1988; Cattivelli and Bartels, 1989; Cattivelli and Bartels, 1990; Dunn et al., 1990), wheat (Danyluk and Sarhan, 1990; Lin et al., 1990; Danyluk et al., 1991; Houde et al., 1992), potato (Tseng and Li, 1990), and Arabidopsis thaliana (Gilmour et al., 1988; Kurkela et al., 1988; Kurkela and Franck, 1990; Nordin et al., 1991). In each of these cases, a correlation could be made between alteration of mRNA profiles and increasing freezing tolerance. To date, many cold-induced genes from the above mentioned plants have been obtained by differential screening of cDNA libraries with probes from acclimated and non-acclimated plants. The nucleotide sequences of these cDNA clones have been determined, but these data show that most of these isolated genes encode novel polypeptides with unknown functions (Palva, 1993).
Although functions of the corresponding polypeptides are not clear, some interesting common features are starting to emerge, which may be of relevance to their putative functions. Among some protein products of cold-regulated genes from Arabidopsis and wheat (cor genes), the most common structural feature is a conserved lysine-rich motif which occurs in various permutations in about half of these proteins (Palva, 1993). Their biochemical properties are closely related to the dehydrins, which are responsive to ABA (rab) and dehydration (dhn), extremely hydrophilic, glycine-rich and contain two lysine-rich repeating units (Close et al., 1989; Skriver and Mundy, 1990; Piatkowski et al., 1990). It has been postulated that a function of the corresponding RAB and DHN polypeptides could be stabilization of cellular proteins during dehydration stress. The accumulation of dehydrin- or RBA-like and COR polypeptides during cold acclimation may function in protecting against cytoplasmic dehydration upon extracellular freezing (Palva, 1993; Smirnoff, 1995a). Recently, two Arabidopsis thaliana cor genes encoded polypeptides COR6.6 and COR15a have been synthesized in Escherichia coli and purified to near homogeneity (Gilmour et al., 1996). COR6.6 (a 6.6-kDa polypeptides) is thought to be localized in the cytoplasm, where COR15am, a 9.4-kDa polypeptide (the mature polypeptide of COR15a), is localized in the chloroplast. However, Uemura et al. (1996) found that the COR polypeptides had little or no effect on the incidences of either freeze-induced fusion or leakage when the liposomes were suspended in a buffer containing low concentrations of solutes. They concluded that neither COR6.6 nor COR15am had a direct cryo-protective effect on liposomes frozen in vitro. Webb et al. (1996) also found that unlike sucrose, a well-known cryo-protectant, neither COR6.6 nor COR15am had an effect on the dehydration-induced increase in lipid crystalline to gel phase transition temperature (Tc) of phospholipid vesicles.
Since alterations in membrane lipid composition are among the major biochemical modifications that occur during cold acclimation, it would be reasonable to expect some of the enzymes catalyzing membrane lipid biosynthesis or turnover to be cold responsive. So far there is little direct evidence to support this hypothesis (Howarth and Ougham, 1993).

2.5. Genetics of cold acclimation

Genetic analysis of the freezing tolerance and of the ability to cold acclimation has demonstrated the complex polygenic nature of these traits (Sakai and Larcher, 1987). The majority of these studies have been performed with wheat (Stutka and Veize, 1988) and other cereals (Brule-Babel and Fowler, 1989), and have been reviewed by Thomashow (1990). The inheritance of freezing tolerance in cereals, as well as woody plants involves multiple genes (Mishra and Singhal, 1992; Blum, 1988; Thomashow, 1990). The involvement of both dominant and recessive genes with mainly additive effects has been suggested (Palva, 1993). In wheat, 11 of the 21 chromosomes have been implicated in having an effect on freezing tolerance although chromosomes 5A and 5D appear to have major effects (Thomashow, 1990).

Recently, Palta's research group has suggested that in potato, non-acclimated freezing tolerance and acclimation ability are genetically distinct traits that segregate independently (Palta et al., 1993a; Palta and Simon, 1993). Results from lipid analysis of purified plasma membrane obtained from the parents (freezing tolerant and sensitive species of potato), F₁ and the backcross progenies suggest that specific membrane lipids play a role in the genetic ability of plant cells to cold-acclimate (Palta et al., 1993a).
2.6. Cold acclimation in coniferous species

Although several studies have focused on cold acclimation of conifers, most of the studies were interested in physiological responses during cold hardiness, such as bud dormancy (Sakai, 1983; Colombo et al., 1989; Colombo, 1990; Burr et al., 1989; Bigras et al., 1996), photoinhibition of photosynthesis (Martin et al., 1978; Strand and Oquist, 1985; Lundmark and Hallgren, 1988; Hundmark et al., 1988; Krol et al., 1995; Krivosheeva et al., 1996) and root growth potential (Lindstrom and Nystrom, 1987; Burr et al., 1989). Only limited information is available regarding biochemical and molecular changes during cold acclimation in conifers.

One of the most studied biochemical responses during development of frost hardiness in conifers is seasonal variation of antioxidant systems in needle tissue (Smith et al., 1990; Anderson et al., 1992; Hausladen and Alscher, 1994; Polle et al., 1996). Esterbauer and Grill (1978) demonstrated that total glutathione and GR activity in the needles of field-grown Norway spruce (Picea abies L.) was more than 4-fold higher in the winter than in the summer. Douliis et al. (1993) reported that cold-tolerant red spruce (Picea rubens S.) showed significantly higher GR activities than cold-sensitive ones. Increases in AsA and APX activity have been also observed in evergreens during the winter, e.g. in the needles of eastern white pine (Pinus strobus L.) (Anderson et al., 1992). By contrast, DHAR activity was lower in spruce needles in the winter than in the summer suggesting that this enzyme may not be involved in frost-tolerance (Esterbauer et al., 1980). Recently, Polle et al. (1996) reported that MDHAR activity in the needles of Norway spruce (Picea abies L.) was higher in the winter than in the summer. They also reported that when spruce seedlings were exposed to -5 °C for one night in the spring, the activities of antioxidant enzymes (SOD, APX, MDHAR and GR) were inhibited completely in lethal injured needles. While,
surviving needles showed a co-ordinated increase in the activities of these enzymes suggesting an efficient induction of defence systems.

The observations revealed that conifers generally show higher protection from oxidative stress in the winter than in the summer indicates that antioxidants contribute to acclimation of plants to environmental conditions. However, it is not clear, how rapidly antioxidants can be adjusted to meet changing requirements for detoxification under stress conditions.

Needles of evergreen conifers are extremely frost resistant. Hardening of pine in short days and non-freezing low temperatures results in a frost resistance down to about -28°C (Selstam and Oquist, 1985). Frost hardening also resulted in an increase in the levels of phospholipids and galactolipids in both spruce and pine (Senser and Beck, 1984; Selstam and Oquist, 1985; Selstam and Oquist, 1990). Among the galactolipids, DGDG increased to a larger extent than MGDG (Oquist, 1982; Senser and Beck, 1984; Selstam and Oquist, 1985; Selstam and Oquist, 1990).

Other biochemical changes, such as sugar (Parker, 1959) and protein (Jiang et al., 1994) have also been reported in the needles of coniferous trees. An increased plasma membrane bound ATPase activity towards the end of frost hardening has been reported in the needles of Scots pine (*Pinus sylvestris* L.) (Hellergren et al., 1983).

In contrast to the information on shoot frost hardiness, the physiological and biochemical basis for root frost hardiness remains largely unclear. Only a few studies dealing with seasonal root hardiness variations (Smit-Spinks et al., 1985; Lindstrom and Nystrom, 1987), photoperiod and temperature effects on root cold acclimation (Johnson and Havis, 1977; Bigras and D'aoust, 1992) have been reported.
2.7. Summary

In summary, membranes are the main targets of freezing stress and the plasma membrane has attracted most interest. Many plants develop freezing resistance during cold acclimation. Cellular changes that occur during the development of freezing resistance include: increases in the concentration of cryo-protectants (sugars, amino acids, soluble proteins); alteration in enzyme activities and metabolic pathways; changes in membrane phospholipids and proteins; alteration in gene expression; and accumulation of antioxidants. These complex changes indicate the cellular metabolisms and membrane structures undergo considerable modification at low temperature, which may be related to frost survival and/or to improve performance at low temperatures. However, our understanding of coniferous frost hardiness has many gaps, e.g. membrane stability in response to freezing stress, changes in enzyme activities, gene expression, especially in root systems. Thus, the experiments described in this thesis were designed to provide information on the frost hardiness of coniferous root systems.

2.8. Objectives

The production of coniferous planting stock is a major component of the forest industry in North America. Over 2 million coniferous seedlings are transplanted annually in Canada and this number has been increasing at a rate of 10% per year (The State of Canada's Forests, 1992). Seedlings are usually grown in greenhouses under optimal growth conditions and transplanted in the first year or second year following overwintering. In north-western parts of United States and in the most parts of Canada, a large number of containerized seedlings are overwintered outside (Colombo, 1990; Kozlowski and Pallardy, 1997). During the outdoor overwintering, the open-
land-stored container seedlings are exposed to freezing root temperatures since the roots of these seedlings are above the ground (Burr et al., 1989).

A number of investigations have shown that the roots of seedlings are more sensitive to freezing temperature than the shoots (Pellett, 1971; Smit-Spinks et al., 1985). Freezing damage to root systems is concealed because the roots are covered in the growing medium, and the symptoms of root damage are not always immediately manifested as visible symptoms as is the case in shoots. Very often, seedlings with dead root systems appear quite normal, providing no hint of the seedling’s post-planting failure (Ritchie, 1990). In 1987 over 8 million container seedlings died in Thunder Bay, the cause being attributed to a lack of root hardiness (OMNR, 1988). To avoid these losses, it is essential that we understand the mechanisms affecting root freezing tolerances or, alternatively, enhance seedling survival through greenhouse acclimation protocols prior to out-planting and overwintering storage.

Jack pine is an important species as a lumber producer and ranges widely from Nova Scotia and central Quebec to northern British Columbia and the Mackenzie River Valley in Canada (Dominion Forest Service Canada, 1949). In general, jack pine grows in areas characterized by cold winters (average temperature -10°C), and average to warm summer (average temperature 15 to 25°C). (Fowells, 1997).

Cold acclimation of coniferous seedlings has received considerable attention. However, most investigations were carried out with aerial parts of plants. The mechanism of developing freezing tolerance in the root system is largely unknown. Therefore, in this project, we mainly focused our studies on the effects of cold acclimation on the roots of jack pine seedlings. Because the plasma membrane is the primary site of freezing injury (Steponkus, 1990; Arora and Palta,
1991), two plasma membrane associated enzymes (H^+-ATPase and NADH-ferricyanide reductase) can be used as markers to assess freezing damage and to study the effects of cold acclimation on jack pine root plasma membranes.

One of our hypotheses is that freeze-thaw stress may disturb several electron transport chains associated with mitochondria and/or microsomal membranes in jack pine roots. This could lead to the formation of active oxygen species that causes the damage of cellular membranes in roots. Cold acclimation of actively growing young seedlings may elevate the antioxidant levels in root tissue. The antioxidants could then quench active oxygen species during freeze-thaw stress avoiding cellular damage, and increasing seedling survival.

This study has employed cold conditioning treatment (at 5°C) to enhance the ability of jack pine seedlings to survive freezing temperatures and has focused primarily on biochemical changes that occur during the cold conditioning process. The main objectives of this thesis are:

1. To develop an effective cold conditioning procedure for the enhancement of freezing tolerance in jack pine seedlings and to develop a convenient biochemical method to assess freezing damage in jack pine root system.

2. To determine changes in lipid compositions during cold conditioning and to compare root cellular membrane susceptibility to freezing and free radical stresses both before and after cold conditioning treatment.

3. To investigate oxidation-reduction of soluble and membrane proteins and glutathione redox status and to determine their roles in freezing tolerance and in regulation of plasma membrane bound enzyme activities.

4. To determine changes in antioxidant system in roots during cold conditioning process.
Chapter 3

INDUCTION OF FREEZING TOLERANCE IN JACK PINE SEEDLINGS: THE ROLE OF ROOT PLASMA MEMBRANE H⁺-ATPase AND REDOX

3.1. INTRODUCTION

In plant shoots, a series of physiological, biochemical and morphological changes are associated with the development of freezing tolerance. These changes include alteration in lipid composition (Yoshida and Uemura, 1990), increase in soluble sugar and amino acid content, terminal bud formation, and reduction in mitotic activity (Colombo, 1990). In roots, however, the physiological and biochemical basis for freezing tolerance largely remains unclear. An understanding of mechanisms effecting root freezing tolerance is important, since root freezing damage is a serious problem affecting the survival of woody plants (Havis, 1976; Lindstrom, 1986).

Weiser (1970) has stated that roots are probably capable of attaining the same levels of frost hardiness as shoots, but that they do not do so because root temperatures during cold acclimation are usually warmer than temperatures to which shoots are exposed. Alternatively, it cannot be discounted that there are physiological, biochemical and morphological attributes of roots limiting the extent to which frost hardiness can develop. An improved understanding of factors regulating root hardiness may, therefore, provide insights into the frost hardening process in plants in general.

It has been demonstrated that cell membranes are directly involved in cold acclimation and freezing tolerance (Levitt, 1980; Steponkus, 1990) and that plasma membranes are the primary sites of freezing injury (Arora and Palta, 1991). Within the cell membrane, H⁺-ATPase activity has
been implicated in the ability of plants to survive freezing (Bervaes and Kylin, 1972; Hellergren et al., 1983). Iswari and Palta (1989) demonstrated that freezing injury resulted in changes in the inter- and intracellular distribution of particular ions (K⁺), while the permeability of the membrane to water, urea and other organic compounds remained unchanged. Based on this observation, they concluded that freezing injury alters the transport properties of the membranes, and proposed that the alteration of the plasma membrane H⁺-ATPase activity is an early event which occurs following freezing injury. Another important plasma membrane-associated enzyme involved in ion transport and possibly affected by environmental stresses is NADH-ferricyanide reductase (Crane et al., 1991). To our knowledge the relationship of this enzyme to freezing injury has not been examined.

In the present study we have examined the effect of cold-conditioning of containerized jack pine (Pinus banksiana Lamb.) seedlings on their ability to survive freezing temperatures and on root plasma membrane.

3.2. MATERIALS AND METHODS

3.2.1. Plant material

Jack pine seeds from North-western Ontario general seed collection were sown in a 2:1 (v:v) peat and vermiculite mixture filled in plastic container trays. Seedlings were grown in a greenhouse for 8-10 weeks under an 16-hour photoperiod in which natural day lengths were extended with high pressure 400 W sodium lamps (400 μmol m⁻² s⁻¹). Temperature ranged from 20°C to 25°C by day and 15°C by night. The seedlings were watered as needed and fertilized weekly with a solution containing 1 g/L Plant-Prod (N-P₂O₅-K₂O, 20-20-20) (Plant Products, Toronto, ON, Canada). Container trays used to grow seedlings were Spencer-Lemaire "Ferdinand"
trays (Spencer-Lemaire Industries, Edmonton, AB, Alberta, Canada) having square cavities 10 cm tall, 2 cm square and a volume of 40 cm$^3$. All experiments were conducted using Spencer-Lemaire trays because the containers consisted of "books", each book containing six cavities in which individual seedlings were grown. A major advantage of the Spencer-Lemaire system was that individual books could be removed from the trays for seedling freezing treatment without disturbing the root system, and blank cavities could easily be replaced with minimal disturbance to healthy seedlings.

3.2.2. Cold conditioning of jack pine seedlings

Eight or ten-week-old seedlings were transferred directly from a greenhouse to growth chambers (Conviron E7, Controlled Environments Limited, Winnipeg, MB, Canada) for conditioning over 1, 2 and 4 weeks at 5°C under an 8 h photoperiod (150 μmol m$^{-2}$ s$^{-1}$). Lights were provided by cool white fluorescent lamps (40 W) and incandescent lamps (60 W). No visual damage was observed after the seedlings were transferred directly from the greenhouse to 5°C growth chambers. The seedlings were watered as needed.

3.2.3. Determination of growth of jack pine seedlings

The growth of jack pine seedlings was determined based on the changes in plant dry weight. A tray of seedlings (about 100 seedlings) was divided in half, one half of the seedlings were transferred to the cold chamber and another half was kept in the greenhouse as control. Randomly selected seedlings (about 50 seedlings) were used to determine the dry weight.

To determine the dry weight, the roots of the seedlings were washed with running water, the roots and shoots were cut from stems and dried in an oven at 70°C for 24 h and weighted. Three independent experiments (50 seedlings per experiment) were carried out.
3.2.4. Survival test after freezing

The assessment of freezing tolerance in jack pine seedlings developed during cold conditioning was based on the seedling survival test following freezing and thaw events. Briefly, Spencer-Lemaire books containing control (non-conditioned) or cold-conditioned seedlings were placed into polystyrene boxes (100 seedlings/box) and transferred to a freezer (-20°C). A blank book was placed between two books containing seedlings to allow cold air to pass through the blank books in the boxes, so that all seedlings could be frozen at approximately the same rate. Soil temperatures inside the boxes were monitored with a temperature probe (DT-2 Universal Enterprise INC, Beaverton, OR). The soil temperature was gradually decreased. The rates of soil freezing and thawing did not exceed 1°C per hour. When the target temperatures (-5°C and -10°C) were achieved, the boxes containing seedlings were removed from the freezer and kept in the laboratory for a further 24 to 36 hours at room temperature to allow thawing. The rate of thawing was slow, it was less than 2°C per hour.

After thawing, the seedlings were transferred to a growth chamber and grown for 4 weeks under 18-hour photoperiod (250 μmol m⁻² s⁻¹). The temperature ranged from 24°C (light) and 15°C (dark). Seedling survival was estimated at the end of the 4-week period according to Koppenaal et al. (1991). Basically, the percentage of survival was expressed as the number of seedlings that survived the treatment divided by the total number of sampled seedlings after 4-weeks of recovery. One hundred seedlings were used in each experiment.

3.2.5. Sampling and experimental design

For freezing-survival and growth experiments, 100 and 50 seedlings were sampled in each experiment, respectively. Unless otherwise stated, more than 150 seedlings were sampled for the
isolation of root plasma membranes. These seedlings were grown in the greenhouse. Because of the closeness (within an area of about 2 m² on a greenhouse bench) and the high degree of uniformity of the greenhouse environment, seedlings within experiments were visually uniform. For this reason, randomly selected seedlings, trays, or books were used as sampling units.

3.2.6. Plasma membrane isolation and enzyme assays

Plasma membrane-enriched fractions were isolated from root segments of control and conditioned seedlings as previously described by Blumwald et al. (1989) with the following modifications. About 300 seedlings were used in each experiment. The homogenization buffer included final concentrations of 10% insoluble PVP and 1 mM benzamidine. For isolation of plasma membranes, the microsomal pellet was layered onto discontinuous gradients of 28% and 34% (w/v) sucrose and centrifuged in a SW 28 rotor (Beckman, Palo Alto, CA) at 80,000 g for 2.5 h. Enriched plasma membrane fractions were collected from the 28%-34% interface. Contamination of the plasma membrane fraction by mitochondria, tonoplast, Golgi and endoplasmic reticulum was determined as described before (Blumwald et al. 1989). Protein content of the membrane fractions was estimated according to Bradford (1976).

H⁺-ATPase activity was measured according to Ames (1966). Assays were conducted in a 0.5 ml reaction medium containing 1.5 mM Tris-ATP (pH 6.5), 30 mM KCl, 1.5 mM MgSO₄, 100 μM Na-molybdate, 5 μM gramicidin and 15 mM Tris-MES pH 6.5. The reaction solutions were incubated for 1 h and stopped by the addition of 1.5 ml of stop solution containing 10% ascorbic acid and 0.42% Na-molybdate (1:6 v/v). The reduction of ferricyanide by NADH-ferricyanide reductase was measured according to Pantoja and Willmer (1991). Assays were conducted in a 1-ml reaction medium containing 0.25 M mannitol, 1 μM CaCl₂, 0.4 mM ferricyanide, 0.25 mM
NADH and 10 mM Tris-MES pH 8.0. The reactions were started by the addition of 20-25 μg of membrane proteins. The activity was determined by the decrease in absorbance at 340 nm as NADH was oxidised (ε=6.22 mM⁻¹cm⁻¹). Measurements of plasma membrane H⁺-ATPase and NADH-ferricyanide reductase activities were carried out at 5°C intervals from 5°C to 30°C.

3.2.7. SDS-PAGE and immunobloting

Preparation of protein sample for H⁺-ATPase immunoblot was carried out as previously described by Barkla and Blumwald (1991). Briefly, each sample of plasma membranes (25 μg of protein) was incubated for 10 min at room temperature in a 4X SDS sample buffer containing 65 mM Tris (pH 6.5), 4% (w/v) SDS, 10% glycerol, 5% (w/v) β-mercaptoethanol and 0.3% (w/v) bromphenol blue, and then subjected to SDS-PAGE (10% acrylamide) essentially according to Laemmli (1970). The proteins were transferred electrophoretically from the SDS-PAGE to a nitrocellulose membrane (Millipore) using the Mini-Protein II Dual Slab (Bio-Rad, Mississauga, ONT) at 30 mA for 20 h at 4°C. Transfer buffer consisted of 192 mM glycine, 25 mM Tris and 20% methanol.

Membranes were blocked with 50 mM Tris-HCL (pH 7.5), 150 mM NaCl, 0.05% Tween-20 (TBS) and 5% fat-free dry milk (Carnation) for 2 h at room temperature. After blocking, the membranes were incubated with a primary antibody in a dilution of 1:2000 in above blocking buffer overnight. The antiserum was raised against a fusion protein containing the central region of the Arabidopsis plasma membrane H⁺-ATPase (Pardo and Serrano, 1989) and was a kind gift from Professor R. Serrano (Universidad Politecnica, Valencia, Spain). Anti-rabbit Ig G alkaline phosphatase-conjugated secondary antibody (Sigma) with a dilution of 1:7500 in blocking buffer was used to visualize bands in the present of substrate of NBT and BCIP.
3.3. RESULTS

3.3.1. Effect of cold conditioning on frost hardiness of jack pine seedlings

It has been established that the prior exposure of plants to low non-freezing temperatures increases their ability to survive subsequent freezing (Levitt, 1980). We investigated the ability of actively growing, containerized, jack pine seedlings to develop frost-hardening during low temperature treatment and short photoperiod. The exposure of non-conditioned jack pine seedlings to freezing soil temperatures (-5°C and -10°C) resulted in the mortality of the seedlings (Fig. 3.1). Cold conditioning increased seedling survival after freezing (Fig. 3.1). The survival of the seedlings was dependent on the length of the conditioning treatment. A 1-week conditioning treatment significantly increased the survival of the seedlings after exposure to -5°C, but was less effective on seedlings exposed to -10°C. Longer conditioning periods (2 and 4 weeks) resulted in higher survival of seedlings exposed to both -5°C and -10°C (Fig. 3.1). During conditioning for 1, 2 and 4 weeks seedlings did not display visual signs of dormancy (e.g. bud set, cessation of needle elongation). The growth of stem and needles was slower in conditioned seedlings than that in non-conditioned seedlings, as assessed by the determination of root and needle dry weight (Fig. 3.2). However, the elongation of roots and growth of non-lignified immature roots in jack pine was not completely inhibited (data not shown).

3.3.2. Effect of cold conditioning on plasma membrane-bound enzyme activities

To investigate whether increased cold hardiness of jack pine seedlings coincided with changes in root plasma membrane protein functions, the activities of two root plasma membrane-bound enzymes, H⁺-ATPase and NADH-ferricyanide reductase, were monitored in control and
cold-conditioned seedlings. The activities were measured before and after a freeze-thaw event at -5°C and -10°C at increasing temperatures ranging from 5 to 30°C.

The activity of root plasma membrane H⁺-ATPase from jack pine seedlings was temperature dependent. The activity decreased after 1 week of conditioning at 5°C and short photoperiods and a 2- and 4-week conditioning period resulted in further inhibition (Fig. 3.3A). Root plasma membrane H⁺-ATPase activity from control seedlings was greatly inhibited after a freeze-thaw event at -5°C and -10°C (Figs. 3.3B, C). Root plasma membrane H⁺-ATPase activities of seedlings conditioned for 1, 2 and 4 weeks were less affected by a freeze-thaw event (Figs. 3.3B and C). Seedlings conditioned for 1 week displayed a 40 and 60% reduction of H⁺-ATPase activity after exposure to -5°C and -10°C, respectively (Figs. 3.3B and C). The H⁺-ATPase activities of seedlings conditioned for 2 and 4 weeks were not inhibited by a -5°C freeze-thaw event (Fig. 3.3B), were inhibited only 20% after exposure to -10°C (Fig. 3.3C) and the activity displayed the same temperature dependence as controls. Immunoblot of plasma membrane H⁺-ATPase from the root tissue of jack pine showed no difference between control and conditioned seedlings suggesting no modification in protein expressions (Fig. 3.4).

Root plasma membrane redox activity, measured as NADH-dependent ferricyanide reduction, was not affected by the conditioning seedlings for 1 week at 5°C and a short photoperiod, but decreased after continuous conditioning for 2 and 4 weeks (Fig. 3.5A). The inhibition was more marked for activities measured at 15 °C or higher (Fig. 3.5A). Freezing control seedlings at -5°C and -10°C inhibited root plasma membrane redox activity (Figs. 3.5B and C). A freeze-thaw event at -5°C did not affect the activities of the seedlings conditioned for 1, 2 and 4 weeks (Fig. 3.5B). While 1 week conditioning was not effective in preventing inhibition of the
redox activity after exposing the seedlings to -10°C (Fig. 3.5C), conditioning for 2 and 4 weeks resulted in only 20% or no inhibition, respectively (Fig. 3.5C). The addition of 500 μM potassium cyanide (KCN) or 200 μM salicyldihydroxamic acid (SHAM), inhibitors of peroxidase or oxidase activities, respectively (Moller and Crane, 1990), did not affect the levels of redox activities in control or conditioned seedlings, before or after freezing (data not shown).
Figure 3.1 Effect of cold conditioning (weeks of treatment) on the survival of jack pine seedlings upon exposure to freezing. Non-conditioned seedlings (control), one (1wk), two (2wk) and four (4wk) week conditioned seedlings were exposed to a freeze-thaw event and seedling survival was estimated as described in Materials and Methods. Control seedlings were grown for 4 additional weeks in the greenhouse prior to freezing. Based on T-test analyses, the means of the survival were significantly different between control (a) and conditioned seedlings (b) to frozen temperatures (-5 and -10°C) (P<0.01); the means of survival were significantly different between 1-week (c) and 2-, 4-week conditioned seedlings (d) (P<0.05). Values are the means of three independent experiments (100 seedlings each experiment) and error bars represent SDs.
Figure 3.2 Effects of cold conditioning (weeks of treatment) on the growth of jack pine seedlings.

Seedling dry weight was determined as outlined in the Material and Methods. (0) control; (1) one; (2) two; (4) four week conditioned seedlings. Fifty non-conditioned and 50 conditioned seedlings, were randomly sampled per experiment. Bars headed by different letters differ significantly according to T-test analyses (P<0.05). Values are means ± SDs presenting three independent experiments.
Figure 3.3 Effect of cold conditioning (weeks of treatment) on root plasma membrane-associated H⁺-ATPase for a range of assay temperatures. Plasma membranes were isolated from control and conditioned seedlings before and after a freeze-thaw, and H⁺-ATPase activity was measured as described in Materials and Methods. In Figure, 3.3A, asterisks at each temperature indicate a significant difference in the level of ATPase activity between control and conditioned seedlings according to a T-test (P<0.01). In Figures 3.3 B and C, asterisks at each temperature indicate that the levels of ATPase activity in 2- and 4-week conditioned seedlings were significantly higher than that in control and 1 week conditioned seedlings based on T-test analyses (P<0.01). Values are the means of duplicate of three independent experiments and the error bars represent SDs. ●: control; ■: 1 week conditioned; ▲: 2-week conditioned; ▼: 4-week conditioned; A: non-freezing; B: freezing at -5°C; C: freezing at -10°C.
Figure 3.4 Immunoblot of the plasma membrane $\text{H}^+$-ATPase from root tissue. Plasma membranes (25 $\mu$g of protein) isolated from the roots of control (C); one (1); two (2) and four week (4) cold conditioned seedlings were immuno-reacted with anti-$\text{H}^+$-ATPase serum. The figure is representative of five independent experiments.
Figure 3.5 Effect of cold conditioning (weeks of treatment) on root plasma membrane-associated NADH-dependent ferricyanide reduction for a range of assay temperatures. Plasma membranes were isolated from control and conditioned seedlings before and after a freeze-thaw event, and ferricyanide reduction was measured as described in Materials and Methods. T-test analyses were conducted. In Figure 3.5 A, asterisks at each temperature indicate that the levels of ferricyanide reductase activity in control and 1 week conditioned seedlings were significantly higher than that in 2- and 4-week conditioned seedlings (P<0.01). In Figure 3.5 B, asterisks at each temperature indicate that the levels of ferricyanide reductase activity differ significantly between control and conditioned seedlings (P<0.01). In Figure 3.5 C, asterisks at each temperature indicate that the levels of ferricyanide reductase activity in 2- and 4-week conditioned seedlings were significantly higher than that in control and 1 week conditioned seedlings (P<0.01). Values are the means of duplicate of three independent experiments and the error bars represent SDs. ○: control; ■: 1 week conditioned; ▲: 2-week conditioned; ▼: 4-week conditioned: A: non-freezing; B: freezing at -5°C; C: freezing at -10°C.
Temperature (°C)

Ferricyanide reductase activity (µ mol NADH/mg prot.min)

A

B

C
3.4. DISCUSSION

Plants have the ability to acclimate to a variety of environmental stresses. In temperate and northern environments, plants survive extreme freezing temperatures because they develop frost hardiness during the fall when temperatures decline and photoperiods become shorter. In container tree seedling production there is almost always the need to overwinter trees at the nursery prior to shipment to the field for planting. Because the root systems of temperate zone tree species are less frost hardy than shoots, container seedlings are especially susceptible to root damage because of the high sensitivity and risk of exposure of root systems to low temperatures (Lindstrom, 1986).

While development of freezing tolerance in aerial parts of pine seedlings is dependent on both short photoperiods and low temperatures, freezing tolerance of roots is mainly dependent on temperature (Bigras and D'aoust, 1992; Bigras and D'aoust, 1993). Exposure of jack pine seedlings, in the present study, to low non-freezing temperature and short photoperiods increased the ability of the seedlings to survive subsequent freezing. The freezing and thawing rates used in this study (1°C - 1.5°C) simulated the rates at which air temperatures naturally drop (Levitt, 1980; Steffen et al., 1989). It has been postulated that in conifers shoot growth cessation is necessary for maximum cold acclimation (Levitt, 1980). In roots, however, growth cessation is not necessarily correlated with cold hardiness (Timmis and Wornall, 1975; Smit-Spinks et al., 1985; Burr et al., 1989). Moreover, some plants exhibit root growth during the winter (Lyr and Hoffman, 1967). In the present study, roots continued to elongate and at the same time increased in frost hardiness. This indicates that the mechanisms responsible for increased frost hardiness in roots are not dependent on dormancy and are characteristic of growing tissue.
Our results showed that the freezing tolerance of jack pine roots coincided with recovery of the root plasma membrane H⁺-ATPase and redox activities after freezing. Root plasma membrane H⁺-ATPase activity decreased during cold hardening, the decrease in H⁺-ATPase may be due to the decrease in seedling growth rate during the conditioning. In general, ABA concentration increases in response to cold acclimation (Chen et al., 1983; Taylor et al., 1990). The plasma membrane H⁺-ATPase activity can be inhibited by the presence of ABA (Serrano, 1989; Zeevaart and Creelman, 1988). Decreased H⁺-ATPase activity may be caused by an increased ABA concentration in jack pine roots during cold acclimation.

In this study, the plasma membrane H⁺-ATPase activity of control seedling roots was greatly inhibited after freezing to -5 and -10°C. Similar results were obtained for needles from Pinus silvestris (Hellergren et al., 1985), suggesting that the plasma membrane H⁺-ATPase in both needles and roots is sensitive to freezing. Changes in root plasma membrane H⁺-ATPase activities in conditioned seedlings after freezing coincided with an increased survival of seedlings. This suggests that one aspect of the development of root cold hardiness is an ability to maintain the plasma membrane H⁺-ATPase activity during recovery from freezing. Western blot of the root plasma membrane H⁺-ATPase showed no significant difference in the levels of proteins between control and conditioned seedlings (Fig. 3.4), suggesting no modification in the protein expression.

Conditioned seedlings survived freezing temperatures (-5 and -10°C) perhaps in part because of an ability to reverse the injury caused by freezing. Slow freezing causes the gradual formation of extracellular ice, withdrawing water from the cells and collapsing cell walls (Pearce, 1988). Slow thawing allows the plasma membranes and cell walls to expand together, diminishing the possibility of mechanical injury (Palta and Weiss, 1993). Iswari and Palta (1989) demonstrated that there is an
increase in H⁺-ATPase activity after reversible freezing injury. At this level of injury, the increase, or maintenance of the plasma membrane H⁺-ATPase activity may help in the recovery process by generating a protonmotive force that can drive leaked ions back into cells. Water would then move osmotically back into the cells (Palta et al., 1982).

Although cold conditioning inhibited NADH-dependent ferricyanide reduction, the activity was not as drastically inhibited as that of the plasma membrane H⁺-ATPase. The redox activity measured in our experiments was most probably due to the presence of reductases at the plasma membranes of jack pine roots, since these activities were not affected by the presence of 500 μM KCN and 200 μM SHAM (inhibitors of peroxidases and oxidases, respectively). Maintenance of redox activity after a freeze-thaw event could play an important role in the recovery of tissues from freezing. Sustained reductase activities would not only facilitate ion uptake (iron in particular) but would also play a role in preventing the inactivation of many transport proteins. H⁺-ATPases and other membrane transporters contain SH groups that are important for regulation of the activities of these proteins in vivo. A number of studies have suggested that one of the functions of plasma membrane redox systems might be to keep the SH groups of membrane transport proteins in a reduced state, thus preventing their inactivation by oxidation (Kochian and Lucas, 1991; Welch, 1995). The redox system could perform such a function by transferring electrons from NADH to disulfide groups within membrane transport proteins, thereby, regenerating functional SH groups and conserving ion transport processes (Welch, 1995).

In the early spring, pine trees begin to deharden very fast (Glerum, 1973) and bursts of root growth has been observed before bud break (Ritchie and Dunlop, 1980). Following freezing, a comparable increase in the root plasma membrane H⁺-ATPase activity of conditioned seedlings was
only seen at temperatures higher than 20°C. (Figs. 3.3.B,C). The increase of redox activity in conditioned seedlings was observed at temperatures from 5 to 30°C following freezing to -5°C. This elevation in redox activity was only seen in seedlings conditioned for 2 and 4 weeks following freezing to -10°C. Nevertheless, the sustained plasma membrane redox activities observed in conditioned seedlings at low temperatures would ensure cell integrity and the functionality of membrane transport proteins. This would provide the water and nutrient uptake needed for recovery and growth. A vigorous bud break and a high root growth capacity are requirements for successful field performance and plantation establishment.

In summary, this study has demonstrated that the post-freezing activities of plasma membrane bound redox and H⁺-ATPase enzymes are enhanced by conditioning at low temperatures and short photoperiods. These changes may be related to the increase in frost hardiness that is also induced by conditioning. Conditioning enhanced the activity of redox at both cool (e.g. 5 and 10°C) and warm (e.g. 25 and 30°C) temperatures following freezing, but post-freezing stimulation of H⁺-ATPase was only evident at warm temperatures. Elevated activities of ion-transporting plasma membrane proteins after freezing may provide useful markers for frost conditioning or freezing injury in roots of jack pine seedlings.
Chapter 4

COLD ACCLIMATION OF JACK PINE SEEDLINGS: EFFECTS ON LIPID COMPOSITION AND THE SUSCEPTIBILITY OF MEMBRANE LIPIDS TO FREEZING INJURY AND PEROXIDATION

4.1. INTRODUCTION

In temperate and northern climates, plants survive extreme freezing temperatures because they develop frost hardiness in the fall when temperatures decline and photoperiods become shorter. All plant cell membranes may be directly involved in cold acclimation and freezing tolerance (Levitt, 1980; Steponkus, 1990) but plasma membranes are the primary sites of freezing injury (Arora and Palta, 1991). Plant cell membranes are usually disrupted following exposure to lethal environmental stresses including freezing stress. A common symptom of injury is the disruption of membrane integrity as shown by the leakage of cytosolic solutes and the loss of membrane-bound enzyme activities (Levitt, 1980; Palta et al., 1993a). It is generally accepted that freezing-induced injury in plant cells is largely due to mechanical extracellular freezing and freezing-induced dehydration stresses. Several models have been proposed to explain the nature of these injuries to cellular membranes and emphasis is placed on the alteration of water status at the membrane interface due to freezing dehydration (Steponkus, 1990; Shewfelt, 1992; Palta and Weiss, 1993).

The generation of active oxygen radicals under stress conditions causes denaturation of proteins and peroxidation of membrane lipids, leading to damaged cellular membranes during water stress (Quartacci and Navari-Izzo, 1992; Smirnoff, 1993), freezing (Kendall and McKersie, 1989; Polle et al., 1996), chilling ((Hariyda and Parkin, 1993) and air pollution (Osswald et al., 1992; Rao et al.,
Lethal freeze-thaw stress has been shown to produce superoxide in the microsomal membrane fraction of winter wheat, promote loss of microsomal phospholipids, and increase free fatty acids and lipid phase transition temperatures (Kendall and McKersie, 1989; Barlay and McKersie, 1995). Many plant species from temperate regions are able to develop freezing resistance following exposure to low non-freezing temperatures for short periods of time. During the period of cold acclimation, the cryostability of cellular membranes is increased; in part, this is a consequence of changes in their lipid composition including increases in phospholipids and their unsaturated fatty acids (Palta et al., 1993b; Uemura and Steponkus, 1994). Acclimation may also cause plant cell membranes to become increasingly resistant to active oxygen radical attack (Kendall and McKersie, 1989; Senaratna et al., 1985).

We have previously reported that cold acclimation increased the survival of jack pine seedlings after freezing, and that the survival was correlated with the post-freezing activities of plasma membrane bound H⁺-ATPase and NADH-ferricyanide dependent reductase in jack pine roots (Zhao et al., 1995). Here, we study the effect of cold acclimation on the lipid composition of jack pine needles and roots, and investigate whether cold acclimation induces resistance to free radical stress in microsome membranes from jack pine roots.

4.2. MATERIALS AND METHODS

4.2.1. Plant material

Jack pine (*Pinus banksiana* Lamb.) seedlings were grown and cold-conditioned as described previously in Chapter 3.
4.2.2. Extraction and analysis of lipids in jack pine root and needle tissues

4.2.2.1. Extraction of lipids from tissue

Root and needle tissues (2.5 g of fresh weight) from jack pine seedlings were ground into powder in liquid nitrogen. Lipids were extracted with chloroform/methanol extract (2:1; v/v) according to Williams and Merrilees (1970). Briefly, the powder of root or needle tissue was ground in chloroform/methanol quickly and centrifuged at 2,000 g for 5-10 min. The supernatant was collected and the pellet was extracted twice with the chloroform/methanol. The extract was mixed with Sephadex G-25 to remove proteins and dried completely on a rotavapor. Chloroform was added to the dry sample to extract lipids only. The lipid extracts were filtered through a teflon-coated milipore filter and washed thoroughly with chloroform to separate them from Sephadex G-25.

4.2.2.2 Separation of polar lipids by TLC

Individual polar lipids were separated by thin layer chromatography (TLC) on 20 x 20 cm glass plates coated with 0.35 mm of Silica Gel G (EM Science, Gibbstown, NJ) containing ammonium sulphate in the developing solvent mixture of acetone:benzene:water (91:30:8, v/v) according to Khan and Williams (1977). Following the developing, the chromatograms were sprayed with 0.02% 2',7'-dichlorofluoresein (dissolved in dry methanol) and dried quickly. The lipids were visualized under UV light. The lipids separated by TLC, were scraped off for further analysis.

4.2.2.3. Gas chromatography analysis

Individual lipids scraped off from TLC plates were transferred in screw capped glass tubes and methanolyzed in 1.5 N dry methanoic HCl at 80°C for 16 h. Following the methylation, an internal standard, methyl pentadecanoate, was added to each lipids. The fatty acid methyl esters (FAME) were
extracted by hexane for 3 times. FAME from each lipids were analyzed by gas chromatography (GC) 
(Hewlett-Packard, Model 5890) using a 30-meter DB-23 capillary column (J & W Scientific). The 
initial column temperature was maintained at 140°C for 1 min and then increased to 220°C at the rate 
of 3°C/min with a final hold for 7 min. The injector and detector temperatures were at 220 °C.

All experimental procedures were carried out under nitrogen gas protection to prevent lipid 
oxidation.

4.2.3. Isolation of microsomal membranes and the induction of lipid peroxidation

4.2.3.1. Isolation of microsomal membranes

Jack pine roots (15-20 g fresh weight) were homogenized in a buffer containing 25 mM Tris-
MES (pH 7.5), 250 mM sucrose, 3 mM EDTA, 1 mM PMSF and 8% insoluble PVP. The homogenate was filtered through 8 layers of cheesecloth and centrifuged at 15,000 g for 15 min. The 
supernatant was then centrifuged in a TY 35 rotor (Beckman, Palo Alto, CA) at 80 000 g for 45 min. 
The pellet containing the microsomal fraction was resuspended in a buffer containing 5 mM potassium 
phosphate (pH 6.5) and 250 mM sucrose and centrifuged in a Ti 60 rotor (Beckman, Palo Alto, CA) at 
80,000 g for 40 min. The pellet was resuspended in the same buffer.

Control and 4-week conditioned jack pine seedlings were frozen to -10°C as described in 
Chapter 3. Immediately following freezing and thaw, root microsomal membranes were isolated. 
Freezing-thaw induced lipid peroxidation in the microsomes was determined.

4.2.3.2. Lipid peroxidation induced by a free radical generation system

*In vitro* lipid peroxidation was carried out by an NADPH-dependent system as described by 
Buege (1978). Briefly, microsomes (approximately 0.2 mg protein) were subjected to an Fe²⁺/NADPH
dependent lipid peroxidation system composed of 0.012 mM ferrous sulphate, 0.25 mM NADPH and 50 mM Tris-HCl (pH 7.4). The reaction was started by the addition of FeSO₄ and carried out at 30°C in the dark with shaking for different times as indicated in the results.

4.2.3.3. Determination of lipid peroxidation

The extent of lipid peroxidation was estimated from the formation of thiobarbituric acid reactive substances (TBARS) according to Rusterucci et al. (1996) with modifications. Two hundred fifty μg aliquots of microsomal protein were mixed with 500 μl of 10% TCA and 750 μl of 0.67% thiobarbituric acid and heated in a boiling water bath for 15 min. After cooling, the precipitate was removed by centrifugation at 1,000 g for 5 min. The absorbance at 532 nm was measured against a blank that contained all the reagents minus the microsomes. The absorbance for non-specific dissipation was corrected by the subtraction of its absorbance at 600 nm. The extent of lipid peroxidation was expressed as the amount of TBARS formed (ε=156 mM⁻¹cm⁻¹).

4.3. RESULTS

4.3.1. Lipid and fatty acid composition of jack pine roots and needles

In jack pine roots, the phospholipids (PL) were comprised primarily of phosphatidyl choline (PC) (60 mol% of PL) and phosphatidyl ethanolamine (PE) (28 mol% of PL) with small proportions of phosphatidyl inositol (PI) (6.8% of PL) and phosphatidyl glycerol (PG) (4.4% mol% of PL) (Table 4.1). Small amounts of galactolipids and trace amounts of sulfolipids (less than 1.0 mol% total lipids) were also present in root tissues (Table 4.1). In needles, the major lipids were the galactolipids (GL), DGDG and MGDG (Table 4.1). PL from needles were composed of larger amounts of PC (44 mol%
of PL) and equal amounts of PE (30 mol% of PL) and PG (27 mol% of PL). PI was found in trace amounts (less than 1.0 mol% of total lipids) in the needle samples (Table 4.1).

The fatty acid component of phospholipids in root tissue was mainly linoleic acid (18:2), followed by palmitic acid (16:0), with low levels of oleic (18:1) and linolenic acids (18:3) (Table 4.2). A high level of 16:0 (53.8 mol% of PG) was found in the PG fraction. PL also contained a small amounts of eicosatrienic acid (20:3), present mainly in PC and PE. GL in root tissue contained approximately equal amount of 18:2 and 18:3, with a lower amount of 16:0 and 18:1 (Table 4.2).

In needle tissue, fatty acids of PL were 16:0, 18:1, 18:2 and 18:3. This distribution is similar to that observed in root tissue. A high level of trans Δ3 hexadecenoric acid (16:1) (25 mol% of PG) was found in PG from needle. The major fatty acids in needles were 18:3 since MGDG and DGDG had very high levels of 18:3. Hexadecatrienoic acid (16.3) was also detected in MGDG fraction (Table 4.3).

4.3.2. Effect of cold acclimation on major phospholipids and galactolipids and their fatty acid compositions

The quantity of the major PL (PC and PE) in root tissue increased significantly (P<0.01) following 2 weeks of cold acclimation (Table 4.2). However, PC increased and PE decreased significantly (P<0.01) in root tissue after seedlings were held at 5°C for 4 weeks (Table 4.2). As a result, the PC/PE ratio increased from 2 to 4.5 following cold conditioning (Table 4.4). PI increased to its highest level during the first week of cold treatment and then gradually fell to control levels after 4 weeks of cold conditioning. MGDG decreased but DGDG increased after 4 weeks of cold acclimation (Table 4.2). As a consequence, the DGDG/MGDG ratio increased from 0.45 to 0.82 following the cold conditioning (Table 4.4).
In needle tissue, cold acclimation resulted in an increase in total lipid content (Table 4.1). These changes were due to increases in both PL and GL (Table 4.3). The proportion of PC increased progressively from about 44 to 55 mol% during cold acclimation (Table 4.1). Among GL, DGDG increased to a larger extent than MGDG (Table 4.3), leading to an increase in the DGDG/MGDG ratio from 0.61 to 0.85. The proportion of MGDG decreased from 38.1 to 31.2 mol%, but showed no significant difference from 2- to 4-week treatments (Table 4.1).

The major effect of cold acclimation on jack pine root lipids was on their fatty acid composition. Following cold conditioning, the amount of 18:1 increased in most lipid species. The levels of 18:2 remained relatively unchanged in the PC and PE fractions. The levels of 18:2 gradually increased in the PI and PG fractions during the period of conditioning (Table 4.2). The amount of 18:3 increased in all PL in 4-week conditioned seedlings. As a result, the unsaturation of PL increased in roots due to increases in 18:1 and 18:3 in PC and PE which are the predominant root PL.

In needles, the levels of 18:0 and 18:1 decreased following cold conditioning and the amount of 18:3 increased gradually in all lipid species. The proportion of 18:2 also decreased in most lipid species (Table 4.3). Consequently, in needles, the unsaturation of GL increased due to an increase in 18:3 during the period of cold acclimation. The unsaturated to saturated fatty acid ratio in PC also showed a slight increase.

Table 4.5 shows the molecular species of PC and PE in roots, and of DGDG MGDG in needles calculated from data in Table 4.2 and 4.3 by a computer simulation program. In roots, the major mono- and di-unsaturated species, 16:0/18:2 and 18:2/18:2, gradually decreased during cold acclimation. Other mono- and di-unsaturated molecular species containing 18:1 and 18:3 gradually increased during cold acclimation. In needles, DGDG contain higher levels of 16:0/18:3 than MGDG.
and it decreased during cold acclimation in both DGDG and MGDG. Major di-unsaturated molecular species, 18:3/18:3 in GL also increased during cold acclimation.
Table 4.1 Lipid composition of root and needle tissue from control and cold-conditioned jack pine seedlings. Values are mean of duplicates from two independent experiments. Trace: less than 1%.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Tissue</th>
<th>cold-conditioning treatment (weeks)</th>
<th>(mol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>PC</td>
<td>root</td>
<td>48.4±0*</td>
<td>48.9±1</td>
</tr>
<tr>
<td></td>
<td>needle</td>
<td>14.3±1</td>
<td>16.5±1</td>
</tr>
<tr>
<td>PI</td>
<td>root</td>
<td>5.5±1</td>
<td>9.8±0</td>
</tr>
<tr>
<td></td>
<td>needle</td>
<td>trace</td>
<td>trace</td>
</tr>
<tr>
<td>PE</td>
<td>root</td>
<td>22.8±3</td>
<td>22.0±0</td>
</tr>
<tr>
<td></td>
<td>needle</td>
<td>9.5±0</td>
<td>9.5±0</td>
</tr>
<tr>
<td>PG</td>
<td>root</td>
<td>3.5±0</td>
<td>3.8±0</td>
</tr>
<tr>
<td></td>
<td>needle</td>
<td>8.9±1</td>
<td>8.1±0</td>
</tr>
<tr>
<td>DGDG</td>
<td>root</td>
<td>6.2±0</td>
<td>6.9±1</td>
</tr>
<tr>
<td></td>
<td>needle</td>
<td>23.1±1</td>
<td>25.8±1</td>
</tr>
<tr>
<td>MGDG</td>
<td>root</td>
<td>13.5±2</td>
<td>8.5±1</td>
</tr>
<tr>
<td></td>
<td>needle</td>
<td>38.1±2</td>
<td>34.3±0</td>
</tr>
<tr>
<td>SL</td>
<td>root</td>
<td>trace</td>
<td>trace</td>
</tr>
<tr>
<td></td>
<td>needle</td>
<td>5.6±0</td>
<td>5.3±0</td>
</tr>
</tbody>
</table>

(μ mol g⁻¹FW)

Total  | root   | 2.0±0    | 2.3±0  | 2.3±0  | 2.5±0  |
|        | needle | 10.0±0   | 16.5±0 | 14.4±0 | 13.3±0 |

\*±0 indicates values ranging from 0.0 to 0.5
Table 4.2 Fatty acid composition of root lipids.

Values are the means of duplicates from two independent experiments.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Time (weeks)</th>
<th>16:0 (mol%)</th>
<th>18:0 (mol%)</th>
<th>18:1 (mol%)</th>
<th>18:2 (mol%)</th>
<th>18:3 (mol%)</th>
<th>20:3 (mol%)</th>
<th>Total (nmol g⁻¹FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>0</td>
<td>24±1</td>
<td>1±0*</td>
<td>9±0</td>
<td>55±1</td>
<td>7±0</td>
<td>3±0</td>
<td>990±68</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>20±1</td>
<td>1±0</td>
<td>13±1</td>
<td>54±1</td>
<td>7±1</td>
<td>3±0</td>
<td>1130±70</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>20±0</td>
<td>1±0</td>
<td>15±1</td>
<td>53±1</td>
<td>7±1</td>
<td>3±0</td>
<td>1118±53</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>20±1</td>
<td>1±0</td>
<td>15±1</td>
<td>45±2</td>
<td>10±1</td>
<td>4±0</td>
<td>1441±129</td>
</tr>
<tr>
<td>PI</td>
<td>0</td>
<td>55±2</td>
<td>3±1</td>
<td>6±1</td>
<td>30±2</td>
<td>4±0</td>
<td>113±22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>53±2</td>
<td>1±0</td>
<td>7±1</td>
<td>35±1</td>
<td>4±1</td>
<td>226±30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>48±3</td>
<td>1±0</td>
<td>9±0</td>
<td>35±3</td>
<td>5±1</td>
<td>196±11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>40±3</td>
<td>1±0</td>
<td>9±1</td>
<td>36±1</td>
<td>13±2</td>
<td>140±34</td>
<td></td>
</tr>
<tr>
<td>PE</td>
<td>0</td>
<td>28±4</td>
<td>1±0</td>
<td>5±0</td>
<td>45±1</td>
<td>11±1</td>
<td>8±2</td>
<td>463±29</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>26±1</td>
<td>1±0</td>
<td>7±0</td>
<td>44±0</td>
<td>13±1</td>
<td>8±1</td>
<td>510±42</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>24±1</td>
<td>1±0</td>
<td>8±0</td>
<td>44±1</td>
<td>13±1</td>
<td>8±1</td>
<td>514±36</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>21±1</td>
<td>1±0</td>
<td>8±0</td>
<td>39±1</td>
<td>20±2</td>
<td>10±1</td>
<td>361±49</td>
</tr>
<tr>
<td>PG</td>
<td>0</td>
<td>54±0</td>
<td>3±0</td>
<td>7±0</td>
<td>30±0</td>
<td>5±0</td>
<td>72±12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>52±1</td>
<td>2±1</td>
<td>8±0</td>
<td>32±0</td>
<td>6±0</td>
<td>87±10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>51±0</td>
<td>2±0</td>
<td>10±0</td>
<td>32±1</td>
<td>5±0</td>
<td>132±14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>47±1</td>
<td>2±0</td>
<td>10±0</td>
<td>34±2</td>
<td>8±0</td>
<td>107±6</td>
<td></td>
</tr>
<tr>
<td>DGDG</td>
<td>0</td>
<td>15±2</td>
<td>3±0</td>
<td>7±0</td>
<td>33±1</td>
<td>41±0</td>
<td>128±14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>15±1</td>
<td>3±0</td>
<td>9±0</td>
<td>33±1</td>
<td>40±0</td>
<td>161±25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>15±2</td>
<td>3±0</td>
<td>8±0</td>
<td>35±1</td>
<td>39±2</td>
<td>131±18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>10±1</td>
<td>2±0</td>
<td>10±0</td>
<td>36±1</td>
<td>39±2</td>
<td>207±21</td>
<td></td>
</tr>
<tr>
<td>MGDG</td>
<td>0</td>
<td>13±3</td>
<td>1±0</td>
<td>7±1</td>
<td>41±1</td>
<td>37±4</td>
<td>277±50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>12±1</td>
<td>1±0</td>
<td>8±1</td>
<td>37±1</td>
<td>40±4</td>
<td>196±20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>13±3</td>
<td>2±0</td>
<td>9±1</td>
<td>38±2</td>
<td>37±3</td>
<td>188±33</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>13±1</td>
<td>2±0</td>
<td>9±1</td>
<td>39±3</td>
<td>37±4</td>
<td>254±13</td>
<td></td>
</tr>
</tbody>
</table>

* ±0 indicates values ranging from 0.0 to 0.5
Table 4.3 Fatty acid composition from needle lipids.

Values are the means of duplicates from two independent experiments.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Time (weeks)</th>
<th>Fatty acids</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16:0</td>
<td>16:1</td>
<td>16:3</td>
</tr>
<tr>
<td></td>
<td>(Δ(^{-2})trans)</td>
<td>(mol%)</td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>0</td>
<td>29±1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>27±0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>25±1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>26±1</td>
<td></td>
</tr>
<tr>
<td>PE</td>
<td>0</td>
<td>25±0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>28±1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>28±2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>26±1</td>
<td></td>
</tr>
<tr>
<td>PG</td>
<td>0</td>
<td>32±2</td>
<td>25±0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>31±1</td>
<td>25±1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>30±1</td>
<td>27±3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>34±3</td>
<td>24±3</td>
</tr>
<tr>
<td>DG</td>
<td>0</td>
<td>24±1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>21±1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>20±1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>19±0</td>
<td></td>
</tr>
<tr>
<td>MG</td>
<td>0</td>
<td>3±0</td>
<td>5±0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3±0</td>
<td>6±1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3±0</td>
<td>7±1</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3±0</td>
<td>7±1</td>
</tr>
<tr>
<td>SL</td>
<td>0</td>
<td>47±2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>46±1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>47±0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>48±3</td>
<td></td>
</tr>
</tbody>
</table>

* ±0 indicates values ranging from 0.0 to 0.5
Table 4.4 Effect of freeze-thaw stress on major phospholipids and galactolipids extracted from roots of non-conditioned and conditioned seedlings. Values are the mean of duplicates from two experiments.

<table>
<thead>
<tr>
<th>Lipids</th>
<th>Non-conditioned mol%</th>
<th>4-week conditioned mol%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Freeze</td>
<td>Non-freeze</td>
</tr>
<tr>
<td>PC</td>
<td>36.6±2.3</td>
<td>48.4±0.5</td>
</tr>
<tr>
<td>PE</td>
<td>15.8±0.5</td>
<td>22.8±2.6</td>
</tr>
<tr>
<td>DGDG</td>
<td>7.5±1.2</td>
<td>6.2±0.3</td>
</tr>
<tr>
<td>MGDG</td>
<td>24.4±2.9</td>
<td>13.5±1.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mole ratio</th>
<th>Freeze</th>
<th>Non-freeze</th>
<th>Freeze</th>
<th>Non-freeze</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC/PE</td>
<td>2.35±0.1</td>
<td>2.11±0.2</td>
<td>3.79±0.4</td>
<td>4.25±0.5</td>
</tr>
<tr>
<td>DGDG/MGDG</td>
<td>0.30±0.02</td>
<td>0.46±0.04</td>
<td>0.75±0.08</td>
<td>0.82±0.04</td>
</tr>
</tbody>
</table>

*a Expressed as mole percent of total lipids;
*b Calculated base on the nmol/gFW of individual lipids.
Table 4.5 Changes in molecular species in major PL and GL during cold acclimation

The data from Table 4.2 and 4.3 were used in a computer simulation program to estimate the mol% of mono- and di-unsaturated molecular species in PC and PE from roots and in DGDG and MGDG from needles. Molecular species with mol% lower than 1% in control seedlings are not included or shown as t (trace).

<table>
<thead>
<tr>
<th>Molecular species</th>
<th>Time (weeks)</th>
<th>PC</th>
<th>PE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Mono-unsaturated species</td>
<td>mol % of total lipids</td>
<td>mol % of total lipids</td>
<td></td>
</tr>
<tr>
<td>16:0/18:1</td>
<td>6.0</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>16:0/18:2</td>
<td>36.7</td>
<td>40.7</td>
<td></td>
</tr>
<tr>
<td>16:0/18:3</td>
<td>4.7</td>
<td>9.9</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time (weeks)</th>
<th>PC</th>
<th>PE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 1 2 4</td>
<td>0 1 2 4</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Di-unsaturated species</th>
<th>mol % of total lipids</th>
<th>mol % of total lipids</th>
</tr>
</thead>
<tbody>
<tr>
<td>18:1/18:2</td>
<td>9.2 13.7 15.4 13.7</td>
<td></td>
</tr>
<tr>
<td>18:1/18:3</td>
<td>1.2 1.8 2.0 3.0</td>
<td></td>
</tr>
<tr>
<td>18:2/18:2</td>
<td>28.0 28.5 27.2 20.5</td>
<td></td>
</tr>
<tr>
<td>18:2/18:3</td>
<td>7.1 7.4 7.2 9.1</td>
<td></td>
</tr>
<tr>
<td>18:3/18:3</td>
<td>t t t t</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time (weeks)</th>
<th>PC</th>
<th>PE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 1 2 4</td>
<td>0 1 2 4</td>
<td></td>
</tr>
</tbody>
</table>

Di-saturated species

| 16:0/18:0      | 1.9 1.1 1.0 1.4 |

<table>
<thead>
<tr>
<th>Mono-unsaturated species</th>
<th>mol % of total lipids</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0/18:2</td>
<td>3.2 1.1 1.5 1.4</td>
</tr>
<tr>
<td>16:0/18:3</td>
<td>42.2 39.2 37.0 34.7</td>
</tr>
<tr>
<td>18:0/18:3</td>
<td>3.6 2.7 2.8 4.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Di-unsaturated species</th>
<th>mol % of total lipids</th>
</tr>
</thead>
<tbody>
<tr>
<td>18:3/16:3</td>
<td>t t t t</td>
</tr>
<tr>
<td>18:1/18:3</td>
<td>1.2 1.3 1.4 1.4</td>
</tr>
<tr>
<td>18:2/18:3</td>
<td>6.0 2.6 4.1 4.2</td>
</tr>
<tr>
<td>18:3/18:3</td>
<td>39.5 46.1 50.4 51.8</td>
</tr>
<tr>
<td>18:4/18:3</td>
<td>70.5 72.0 73.5 76.5</td>
</tr>
</tbody>
</table>
4.3.3. In vitro lipid peroxidation in jack pine root microsomes

In vitro lipid peroxidation increased rapidly upon treatment of the microsomal membranes with a free radical generating system. TBARS products reached a maximum within 20 min and declined slightly afterwards (Fig. 4.1). This decline could reflect the reaction of these aldehydes to non-TBA-reactive products. Similar results were also reported with microsomal membranes from winter wheat crowns and soybean asolecithin vesicles (McKersie, et al., 1990 and Barclay, et al., 1995). The TBARS levels in the microsomal membranes of 4-week conditioned seedlings were significantly (P<0.01) lower than that from control seedlings (Fig. 4.1). Incubation of the microsomal membranes with exogenous NADH for 1 h prior to TBARS determination showed that redox associated lipid peroxidation of 4-week conditioned seedlings was significantly (P<0.01) lower than that of control seedlings (Fig. 4.2).

4.3.4. Lipid peroxidation induced directly from freezing thaw injury

The initial levels of TBARS in root microsomal membranes isolated from freeze-thaw stressed control and 4-week conditioned seedlings increased from 0.8 and 0.48 n mol/mg protein to 11.4 and 5.7 n mol/mg protein, respectively (Fig. 4.1 and 4.3). Time course experiments revealed that the extent of lipid peroxidation induced by freeze-thaw in vivo was slightly higher than the levels induced by in vitro free radical treatment (Figs. 4.1 and 4.3). After a freeze and thaw event, the microsomal membranes from control and 4-week conditioned seedlings were more susceptible to free radical injury (Fig. 4.3). However, the in vitro lipid peroxidation in microsomal membranes isolated from 4-week conditioned seedlings showed a much lower degree of susceptibility (Fig. 4.3).
4.3.5. Effects of freezing injury on the fatty acid composition of major root lipids

Following a freeze-thaw event, the proportion of PC and PE in roots declined in both control and 4-week conditioned seedlings, while the proportion of MGDG increased, the DGDG remained relatively unchanged (Table 4.4). Fatty acid compositions of PC and PE extracted from roots of freeze-thaw-stressed control and 4-week conditioned seedlings showed less than 15% (P<0.05) decrease in the proportion of 18:2 and increase in the proportion of 18:1, as compared with lipids isolated from non-freezing seedlings (Fig. 4.4). In contrast with PL, GL showed a significant (P<0.01) reduction in the proportion of 18:3 in MGDG and DGDG following the freeze-thaw injury (Fig. 4.5) in both control and 4-week conditioned seedlings. In control seedlings, a freeze-thaw event induced decreases in the proportion of 18:3 levels in DGDG and MGDG by 78 and 67%, respectively. In 4-week conditioned seedlings, the levels of 18:3 composition in DGDG and MGDG decreased by 47%. As a consequence of the reduction in 18:3, the proportion of 16:0, 18:1 and 18:2 increased significantly (P<0.01) following freeze-thaw injury. The increase in the proportion of 16:0 in DGDG and MGDG from control roots was higher than that from 4-week conditioned roots (Fig. 4.5). It should be noted that the fatty acid composition was expressed as mol% instead of mol/gFW, because the fresh weight of the seedlings changed after treatment due to freezing-induced dehydration.
Figure 4.1 Time course of the formation of TBARS in jack pine root microsomal membranes by oxygen free radicals generated from a FeSO₄-NADPH system. Microsomes were isolated from non-conditioned (◆) and four week conditioned (□) seedlings and then treated with 0.012 mM FeSO₄ and 0.25 mM NADPH in a Tris-HCL buffer (pH 7.4) in dark as described in Materials and Methods. Asterisks at each incubation time indicate a significant difference in the level of lipid peroxidation between control and 4-week conditioned seedlings according to a T-test (P<0.01). Values are the means of duplicate of three independent experiments and the error bars represent SDs.
Control 4wk

Figure 4.2 NADH-dependent lipid peroxidation in jack pine root microsomal membranes. Microsomes (0.2 mg proteins) isolated from non-conditioned (Control) and four week conditioned (4-week) seedlings were incubated with 0.5 mM NADH in a 5 mM potassium phosphate buffer (pH 7) for 1 h prior to TBARS determination. Lipid peroxidation in 4-week conditioned seedlings was significantly lower than that in control seedlings, according to a T-test (P<0.01). Values are the means of duplicate of three independent experiments and the error bars represent SDs.
Figure 4.3 The effects of freeze-thaw on the time course of the formation of TBARS in jack pine root microsomal membranes and the susceptibility to free radicals generated from a FeSO₄-NADPH system. Roots of jack pine seedlings were frozen at -10°C and slowly thawed as described in Chapter 3. Microsomes were isolated from freeze-thaw control and four weeks conditioned seedlings and then incubated with a Tris-HCl buffer (pH 7.4) (△ for control; and □ for 4-week conditioned) or with 0.012 mM FeSO₄ and 0.25 mM NADPH in Tris-HCl buffer (pH 7.4) (▲ for control; ■ for 4-week conditioned) in dark before TBARS determination. Asterisks at each incubation time indicate a significant difference in the level of lipid peroxidation between control and 4-week conditioned seedlings according to a T-test (P<0.01). Values are the means of duplicates of three independent experiments and the error bars represent SDs.
Figure 4.4 The effects of freeze-thaw on the fatty acid composition of PC and PE in jack pine root tissue from non-conditioned (control) and four weeks conditioned (4-wk) seedlings. Roots of jack pine seedlings were frozen to -10°C and slowly thawed as described in Chapter 3. Lipids were extracted from roots before (open bars) and after freeze-thaw (hatched bars) events. Based on T-test analyses, the fatty acid compositions of 18:2 and 18:1 in control and 4 week conditioned seedlings differ significantly (P<0.05) before and after a freeze-thaw event. The fatty acid compositions in PC and in PE represent the means of duplicates of two independent experiments and the error bars represent SDs.
Fatty acid composition in MGDG (mol%)

Fatty acid composition in DGDG (mol%)

Error bars represent S.D.

DEPC and in MGDG represent the means of duplicates of two independent experiments and the difference significantly (P<0.01) before and after freeze-thaw events. The fatty acid compositions in L- test analyses, the fatty acid compositions in control and 4-week conditioned seedlings, the lipids were extracted from roots before (open bars) and after freeze-thaw (hatched bars) events. Roots of each pine seedlings were frozen to -10°C and slowly thawed as described in Chapter 3. Pine root tissue from non-conditioned (control) and four weeks conditioned (4-wk) seedlings.

Figure 4.5: The effects of freeze-thaw on the fatty acid composition of MGDG and MGDG in each
4.4. DISCUSSION

4.4.1. Lipid composition of jack pine roots and needles.

The fatty acid composition of PL in jack pine roots reported here is similar to the fatty acid composition reported in microsomal phospholipids from Scots pine (*Pinus sylvestris* L.) roots (Ryyppo et al., 1994). It was previously shown that lipids in conifer needles differ from those of angiosperms in having various C<sub>20</sub> polyunsaturated acids (Jamieson and Reid, 1972). C<sub>20</sub> polyunsaturated acids were also detected in jack pine root lipids. Jamieson and Reid (1972) reported high levels of 18:3 in galactosyl diglycerides. These accounted for 56-80% and 64-73% of total MGDG and DGDG fatty acids, respectively, in *Picea abies*, *Pinus sylvestris*, *Larix decidua* and *Taxus baccata* needles. In jack pine needles, the fatty acids of 18:3 in MGDG and DGDG fell within these ranges. However, the levels of 18:3 in MGDG and DGDG from roots were much lower than that from needles, indicating that not only lipids but also fatty acid composition varies in different organs within the same plant. The proportions of PL and GL from jack pine needles were similar to that from needles of spruce (Senser and Beck, 1984).

4.4.2. Effect of cold acclimation on jack pine root and needles lipids, and their fatty acid composition

An increase in the proportion of PL during cold acclimation is commonly observed in the leaves of many plant species, including herbaceous (Uemura and Yoshida, 1984; Lynch and Steponkus, 1987) and woody species (Yoshida, 1984). Palta (1993b) compared changes in lipids in two genetically related potato species following growth at lower temperatures, and reported an
increase in PL content in the plasma membranes of freezing tolerant species, but not in freezing sensitive species. An increase in PL also occurred in jack pine roots and needles due to the increase in PC fractions (Table 4.2 and 4.3). An increase in PC content has been also reported in cold stressed rye roots (Kinney et al., 1982). Following 4 weeks of cold conditioning, the PC/PE ratio increased more than 2-fold in roots. The distribution of phospholipid head groups in the cellular membranes affects phase transitions from lamellar to $H_\alpha$ phase. PC readily forms bilayer and helps to maintain the lamellar phase. PE is a non-bilayer forming lipid and enhances the transition to the hexagonal phase (Leshem, 1992). Increase in the PC/PE ratio during cold conditioning could prevent the formation of $H_\alpha$ phase upon freezing dehydration which causes the formation of hexagonal II phase ($H_\beta$) lesions on the plasma membranes (Steponkus, 1990; Uemura et al., 1995). PC content rose earlier in needles (after 1 week of cold acclimation) than that in roots (after 4-week of cold acclimation) (Table 4.2 and 4.3), suggesting that a faster cold hardiness could develop in jack pine needles during cold acclimation.

Growth of jack pine seedlings at $5^\circ$C resulted in a decrease in the amount of MGDG relative to both roots and needles. Following cold conditioning for 4 weeks, the DGDG/MGDG ratios increased 2- and 1.4-fold in roots and needles, respectively. The similar changes have also been found in other frost resistant plants, such as Scots pine (Selstam and Oquist, 1990), spruce and ivy (Senser and Beck, 1984). Selstam and Oquist (1990) reported that both low temperature and low light density appeared to be involved in the regulation of DGDG synthesis relative to the amount of MGDG. The changes in the proportion of DGDG to MGDG may affect the stability of plastid and chloroplast membranes. MGDG forms a reversed hexagonal phase with water and is a destabilizing lipid. While DGDG, similar to PC in plasma membranes, forms a lamellar phase with
water and is a stabilizing lipid (Shipley et al., 1973; Selstam and Oquist, 1990). The stabilization of plastid and chloroplast membranes may be an important factor in repairing membrane lipids damaged following freeze-thaw stress because they are the site for fatty acid and/or lipid biosynthesis.

Our results showed that in jack pine seedlings, the regulation of fatty acid metabolism in response to lower growth temperatures differed in roots and needles. Two steps of the desaturation of fatty acids in PL may be involved in roots. Initially, an increase in 18:1 levels was found in most lipid fractions in roots from 1- and 2-week conditioned seedlings, leading to an increase of di-unsaturated molecule species (18:1/18:1, 18:1/18:2 or 18:1/18:3) in roots (Table 4.5). Relatively unchanged levels of 18:2 and 18:3 in control, 1- and 2-week conditioned seedlings indicated that the desaturation of 18:1 to 18:2 and to 18:3 was slow at the early stage of cold acclimation. Further cold conditioning for 2 more weeks, induced an increase in 18:3, indicating the desaturation of 18:2. Decreases in 16:0 and 18:2 and increases in 18:1 and 18:3 in the microsomal phospholipids were also found in Scots pine roots held at 5°C for 19 days (Ryyppo et al., 1994). In jack pine needles, the proportions of 18:1 and 18:2 decreased and the amount of 18:3 gradually increased indicating sequential desaturation of 18:1 to 18:2 and to 18:3 during the period of cold acclimation. A decline in the proportion of 18:2 and an increase in the proportion of 18:3 have been reported in the other woody plants during cold acclimation (Sutinen, 1992; Yoshida, 1986; Senser and Beck, 1984).

Following cold acclimation, di-unsaturated molecular species in major PL and GL increased and contained higher levels of 18:3/18:3 (Table 4.5). The increase in di-unsaturated species of PC and PE and a corresponding decreases in mono-unsaturated species during cold
acclimation has been reported in rye, oat and *Arabidopsis thaliana* (Uemura and Steponkus, 1994; Uemura, 1995). It has been demonstrated that increase in the proportion of di-unsaturated species in liposomes decreases propensity for freezing-induced formation of the H$_{II}$ phase (Sugawara and Steponkus, 1990).

Phase changes within bilayer are postulated to play a role in freezing injury. As the degree of unsaturated acyl chain increases, the phase transition temperature decreases and the plant tissue would be more likely to withstand freezing and post-thaw low temperatures (Shewfelt, 1992). This hypothesis is supported by the survival of conditioned seedlings following a freeze-thaw event (Zhao et al., 1995), and an increase in unsaturated fatty acids of 18:1 and 18:3 in both roots and needles during cold acclimation.

A gradual increase in 18:3 was found in all species of lipids in needles during the period of cold acclimation (Table 4.3). In root tissue, a significant increase in the proportion of 18:3 was found only in 4-week conditioned seedlings (Table 4.2). This suggests that the aerial parts of jack pine seedlings responded faster to cold treatment than the roots. Longer cold conditioning period (4 weeks) may be required to induce a greater freezing tolerance in jack pine roots.

4.4.3. Effect of cold acclimation on cellular membrane stability assessed by the determination of lipid peroxidation

Root microsomal membranes isolated from control seedlings were more susceptible to free radical injury than the membranes isolated from 4-week conditioned seedlings (Fig. 4.1). These results suggest that cold acclimation provided increased the tolerance of root microsomal membranes to free radicals. The lower level of redox-associated lipid peroxidation seen in 4-week conditioned seedlings (Fig. 4.2) would suggest that the production of oxygen radicals generated by
plasma membrane-associated redox systems was lower than that in control microsomes. Alternatively, microsomes from 4 week conditioned seedlings could have an higher capacity for scavenging oxygen radicals.

The rate of oxygen free radical production is a function of the availability of substrate (oxygen) and reducing equivalents. This rate may increase upon freezing of plant cells. Our results show that the freeze-thaw of jack pine seedlings induced lipid peroxidation in root microsomal membranes (Fig. 4.3). The lower degree of lipid peroxidation displayed by the microsomal membranes from 4-week conditioned seedlings supports the notion that the conditioning of the seedlings at lower temperature and short photoperiod increases cellular membrane stability to freeze-thaw stress. This may be due to a) an increased tolerance to free radicals or b) a lower redox activity in the microsomal membranes during the development of cold hardiness.

Cellular membranes can differ in their susceptibility to free radical damage. Following a freeze-thaw event, root microsomal membranes from control seedlings were more susceptible to free radical treatment than that from 4-week conditioned seedlings (Fig. 4.3). The molecular mechanisms conferring a relatively greater tolerance to free radicals are unknown. Nevertheless, it can be suggested that these mechanisms involve membrane-bound components, since microsomal membranes were exposed to free radicals after the removal of cytosolic components. Senaratna, et al. (1985) demonstrated that the microsomal membranes from soybean axes that were susceptible to dehydration injury were also very susceptible to free radical injury. Whereas, similar membrane fractions from dehydration-tolerant axes exhibited quantitatively much less molecular damage when exposed to a similar free radical dose (Senaratna et al., 1985).
4.4.4. Effect of freezing injury on major lipids and their fatty acids composition

The exposure of control seedlings to freezing soil temperatures (-5 and -10°C) resulted in mortality. However, following 4 weeks of cold conditioning, the survival of seedlings increased remarkably (Zhao, et al. 1995). To determine lipids and the fatty acids that were more susceptible to freeze-thaw stress, lipids were extracted from the roots of freeze-thaw stressed seedlings. Our data suggest that PL were susceptible to freeze-thaw injury as indicated by the decrease in PC and PE (Table 4.4). After the freeze-thaw, the mole ratios of PC to PE and DGDG to MGDG in 4-week conditioned seedlings dropped from their original levels but remained significantly (P<0.01) higher than that of controls. This suggests that increase of PC and DGDG played a role in the maintenance of cellular membranes functions in roots after freezing stress. In control seedlings, the mole ratio of DGDG to MGDG decreased significantly (P<0.01) following a freeze-thaw event suggesting that DGDG may be more susceptible to freeze-thawed injury than MGDG. In comparison, the mole ratio of PC to PE remained unchanged suggesting a similar susceptibility of these two PL.

As a comparison of the changes between lipids and their fatty acid composition, our data suggest that different freezing injury mechanisms may occur in PL and GL. Following a freeze-thaw event, the proportions of PC and PE decreased, however their fatty acid compositions remained relatively unchanged (Fig. 4.4). This would suggest that freezing injury may cause a partial degradation of microsomal membrane lipids in jack pine roots, but not affecting their fatty acid composition. The most commonly accepted mechanism of free radical attack on PL involves peroxidation of their fatty acid (Buege and Aust, 1987), and the prediction that fatty acid saturation should increase after exposure to stress. However, the lipid peroxidation of plant membranes may
not follow classical reactions (McKersie et al., 1990; Barlay and McKersie, 1995). When microsomal membranes isolated from winter wheat crowns and from soybean seeds were treated with free radicals, selective degradation of polyunsaturated fatty acids was not observed, even with 40% of degradation of PL (McKersie et al., 1990). Accumulation of free fatty acids and the loss of PL induced by oxygen free radicals have been shown in freezing-injured winter wheat (Kendall and McKersie, 1989) and other environmental stresses (Senaratna et al., 1985; Dhindsa and Matowe, 1981). In contrast with PL, the proportion of GL remained unchanged in roots from freeze-thaw seedlings, but their fatty acid composition changed due to significant degradation of 18:3, which leads to an increase in the level of saturated fatty acid in both DGDG and MGDG. The increase in saturation of DGDG and MGDG could enhance membrane lipid phase transition temperature, and cause a phase transition from liquid-crystal to solid-gel state during post-thaw at low temperatures. As a result, the plastid membrane functions would be disturbed.

The different effects of freeze-thaw stress on PL and GL could be due to differences in their fatty acid compositions. For example, GL contain high levels of 18:3 and they are highly susceptible to free radical attack (Barlay and McKersie, 1995). In 4-week conditioned seedlings, the degradation of lipids caused by freeze-thaw injury was significantly lower than in control seedlings. This could be due to lower levels of free radicals generated by electron transport chains associated with redox system in plasma membrane and intracellular organelles, or an increase in antioxidants in the cytosol and/or plastids during the cold acclimation.

In summary, cold conditioning increased the level of PL in jack pine roots and needles due to an increase in PC. The extent of an increased proportion of PL was higher in needles that than in roots. A considerable shift from saturated to unsaturated fatty acids (increases in di-unsaturated
molecular species) was found in both tissues due to increase in the proportions of 18:1 in roots and of 18:3 in needles. Following 4 weeks of cold conditioning, the mole ratios of PC to PE and DGDG to MGDG significantly increased in root tissue. Cold conditioning increased resistance of root membranes to freeze-thaw stress and free radical attack as assessed by determination of lipid peroxidation. Following freeze-thaw events the proportion of PC and PE decreased, with smaller changes in their fatty acid composition. However, the proportion of MGDG and DGDG in roots showed little change as compared to a major change in their fatty acid compositions. This suggests that different freezing injury mechanisms may occur in PL and GL.
Chapter 5

CHANGES IN OXIDATION-REDUCTION STATE AND ANTIOXIDANT ENZYMES IN THE ROOTS OF JACK PINE SEEDLINGS DURING COLD ACCLIMATION

5.1. INTRODUCTION

The over-production of active oxygen species such as superoxide, hydrogen peroxide and hydroxyl radicals results from the exposure of plants to different environmental stimuli. Increased formation of active oxygen species has been associated with the development of injury symptoms resulting from diverse stress conditions including chilling (Fadzillah et al., 1996; O'Kane et al., 1996), freezing (Kendall and McKersie, 1989), ice encasement (Hetherington et al., 1988; Hetherington et al., 1987), drought (Smirnoff, 1993) and ozone (Rao et al., 1996).

The plasma membrane is the main barrier between the cell and its environment. Levitt (1980) suggested that aggregation or disassociation of membrane proteins causes freezing injury at the plasma membranes by the formation of intermolecular disulphide bonds (-S-S-) from thiol groups when the protoplasm is freeze-dehydrated. Even though the hypothesis is concerned specifically with membrane proteins, no measurements have been made to determine the reduction state of thiol groups in insoluble membrane proteins.

In order to adapt to environmental stresses, plants are equipped with complex antioxidant systems comprised of low molecular weight antioxidants and protective enzymes to protect cellular membranes and organelles from the damaging effects of toxic levels of active oxygen species (Smith et al., 1990; Smirnoff, 1995b; Kocsy et al., 1996). Essentially, antioxidants are divided into three general classes including: (1) lipid soluble and membrane-associated α-tocopherol; (2) water
soluble reductants, AsA and GSH; and (3) enzymes such as SOD, catalase, peroxidases, APX, and GR (Foyer, 1993).

The tocopherol that has been reported mainly exists in green photosynthetic tissue located in chloroplast membranes (Hess, 1993). AsA and GSH have been found in various tissues in higher plants including roots. AsA plays a central role in detoxification of active oxygen species. It can directly reduce superoxide, hydrogen peroxide (H₂O₂), and hydroxyl radicals (Foyer et al., 1991). GSH can also react directly with active oxygen species or it can react with DHA to regenerate AsA. Glutathione also plays a role in the reduction of intermolecular -S-S- bonds in proteins, usually caused by dehydration during freezing stress (Smith et al., 1990).

SOD mediates the conversion of superoxide radicals to molecular oxygen and H₂O₂. Superoxide and H₂O₂ can then react to form highly active hydroxyl radicals through the transition metal-catalyzed Haber-Weiss cycle (Winston, 1990) (Fig. 2.2). Hydroxyl radicals can rapidly attack virtually all macromolecules, leading to serious damage in cellular components (Winston, 1990; Scandolios, 1993). Detoxifying H₂O₂ becomes pivotal in the defence mechanisms against active oxygen species. Catalase and APX can reduce H₂O₂ to water, however, catalase has a very low affinity for H₂O₂ (Graham and Patterson, 1982) and can be inhibited by the exposure to herbicide (Klapheck et al., 1990) and chilling temperatures (O’Kane et al., 1996; Fadzillah et al., 1996). The reaction of AsA with H₂O₂ can occur directly or it can be catalyzed by APX. The regeneration of AsA is catalyzed by MDHAR, DHAR and GR (Smirnoff, 1995b). APX, MDHAR, DHAR and GR together form the AsA-glutathione cycle, a H₂O₂ scavenging pathway (Fig. 2.3). While the AsA-glutathione cycle has been most extensively studied in chloroplasts, there is good evidence
that all the enzymes of the cycle are located in the cytosol or other organelles of both green and non-green tissues (Dalton et al., 1991; Klapheck et al., 1990; Foyer, 1993).

Previous work has shown cold conditioning increased the cryo-stability of root plasma membrane as indicated by the preservation of H^+-ATPase and ferricyanide reductase activities after jack pine seedlings were frozen at -5 or -10°C (Zhao et al 1995). We also demonstrated that freeze-thaw of jack pine roots caused lipid peroxidation that is probably induced by the generation of active oxygen species in frozen tissue (chapter 4). In addition, the levels of lipid peroxidation in 4-week conditioned seedlings were significantly lower than that in control seedlings following freeze-thaw events (chapter 4). The response of AsA-glutathione cycle to chilling temperature in the roots of jack pine seedlings may play an important role in preventing cellular damage by the formation of AsA and GSH before applying freeze-thaw stress. Since the activities of the plasma membrane H^+-ATPase and ferricyanide reductase are dependent on SH groups in membrane proteins (Qian and Murphy, 1993; Serrano et al., 1995), changes in oxidation-reduction in the cytosol during cold conditioning may affect the enzyme activities.

To test our hypothesis, the first objective of the present study was to investigate whether a freeze-thaw event could affect protein thiol groups in microsomal proteins and to determine whether changes in protein thiol groups were correlated to the seedling survival. The second objective was to investigate whether glutathione status in the roots of jack pine seedlings was changed during cold acclimation, and how it regulated plasma membrane bound enzyme activities. Finally, the responses of antioxidant enzymes involved in AsA-glutathione pathway in root soluble proteins to chilling temperature were investigated and their roles in scavenging active oxygen species were discussed.
5.2. MATERIALS AND METHODS

5.2.1. Plant material

Jack pine (*Pinus banksiana* Lamb.) seedlings were grown and cold-conditioned as described previously in Chapter 3.

5.2.2. Extraction of soluble and microsomal proteins

For extraction of soluble proteins, roots of jack pine seedlings (15-20 g) were homogenized in 10 volumes of 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 10% glycerol, 8% insoluble PVP. For the determination of APX activity, roots were homogenized in the above buffer containing 2 mM AsA according to Asada (1992). The homogenate was filtered through eight layers of cheesecloth and centrifuged at 15,000 g for 15 min. The supernatant was centrifuged in a TY35 rotor (Beckman, Palo Alto, CA USA) at 80,000 g for 45 min. The supernatant, (soluble protein fraction) was concentrated with a stirred ultrafiltration cell 8050 (Amicon, Beverly, MA USA) using a regenerated cellulose low binding membrane with a molecular weight cut off at 3 kDa (Millipore, Bedford, MA USA).

Microsomal proteins were isolated as described previously in Chapter 3. Protein concentrations were determined according to Bradford (1976).

5.2.3. Determination of protein thiol groups

5.2.3.1. Protein thiol concentrations in soluble and microsomal Proteins

The assay for determining levels of protein thiol groups was based on the method described by Ravindranath (1994) with modifications. Two hundred micrograms of protein in 500 µl of
extraction buffer were precipitated with 1 ml of cold 10% (v/v) HClO₄ with centrifugation. To remove non-protein-thiol molecules, such as glutathione, the precipitated proteins were mixed with 0.5 ml of 6.5% TCA and then centrifuged at 13,000 g for 10 min. This step was repeated twice. Following the TCA wash, 25 µl of 3 mM DTNB and 1 ml of 0.65 M Tris-HCl (pH 7.6) were added. The samples were centrifuged to remove insoluble proteins. The absorbance in the supernatant was measured at 412 nm. The concentration of thiol groups was determined against a calibration curve using different concentrations of GSH (5, 10, 15, 20, 25 n mol) as a standard.

5.2.3.2. In vitro labeling of protein thiol groups

Bromobimane is a specific fluorescent labelling agent for membrane protein thiol groups (Kosower, et al. 1983). Bands of bromobimane labelled red blood cell membrane protein thiol groups exhibited fluorescence under ultraviolet illumination following SDS-PAGE and corresponded to almost all of those stained with Coomassie blue (Kosower et al. 1983).

A modified assay based on the method described by Kosower et al. (1981) was used for visualizing protein thiol groups in soluble proteins from jack pine root tissue. Bromobimane (from Sigma) was stored as a 60 mM solution in acetonitrile, in the dark, at 4°C. Aliquots of protein were mixed with bromobimane solution (1 µl/20 µg protein), and kept on ice for 60 min. Following labelling, equal amounts of proteins were subjected to SDS-PAGE under non-reducing conditions, essentially as described by Laemmli (1970) except that β-mercaptoethanol was omitted from the sample buffer. After electrophoresis, the gels were fixed in methanol/acetic acid/water (40/10/50; v/v/v) destain solution and photographed under ultraviolet illumination. The gels were then stained with Coomassie blue, destained and photographed again.
5.2.3.3. Assessment of freezing damage in protein thiol group

Control and conditioned seedlings were frozen (-10°C) and thawed as described previously in Chapter 3. Following freeze and thaw events, microsomal membranes were isolated from roots and the concentrations of thiol groups were measured. To determine the effects of in vitro freeze and thaw on the protein thiol groups in microsomal membrane proteins, 200 µg protein was resuspended in 250 µl of resuspension buffer and then frozen in a -20 °C freezer. Samples were frozen for 2 hours then slowly thawed on ice for 30-45 min. The concentrations of protein thiol groups were determined by the assay described above.

5.2.4. Extraction and analysis of GSH and GSSG

Glutathione was assayed according to Griffith (1980). Root tissue (2 grams of fresh weight) was homogenized in 5 ml of 5% sulphosalicylic acid and then centrifuged for 10 min at 13,000 g. Three hundred µl of supernatant was neutralized by the addition of 12 µl of 6.6 M triethanolamine. Half of the sample (150 µl) was used for GSH and GSSG determination. The rest of the sample (150-µl) was treated with 3 µl of 2-vinylpyridine for 60 min at 25°C to mask the GSH by derivatization in order to determine the GSSG concentration. In each case, 50-µl aliquots of the sample were mixed with a reaction solution containing 0.2 mM NADPH, 0.6 mM DTNB, 6.3 mM EDTA and 125 mM K-phosphate buffer (pH 7.5) to 1 ml of final volume. The reaction mixture was incubated at 30°C for 2 min, then the reaction was started by the addition of 10 µl of glutathione reductase (50 Unit/ml, Type III from baker’s yeast, Sigma). The increase of absorbance at 412 nm was monitored for 400 seconds. Quantification of glutathione levels was based on a GSH calibration curve.
5.2.5. Isolation of plasma membrane and measurement of plasma membrane-bound enzyme activities

Plasma membrane-enriched fractions were isolated from root segments of control and conditioned seedlings as previously described in Chapter 3 with some modifications. DTT and β-mercaptoethanol (thiol reducing reagents) were absent in homogenization and resuspension buffers.

H⁺-ATPase activity and the reduction of ferricyanide by NADH-ferricyanide reductase were measured as described previously in Chapter 3. To determine the effects of PCMB on the plasma membrane H⁺-ATPase and NADH-ferricyanide reductase activities, the reactions were carried out at 30 °C. For pre-incubation treatments, the membranes (25 μg of protein) were incubated with individual chemicals (at various concentrations as indicated in the legends to Figures) at room temperature for 15 min and then added into the assay solutions.

To determine the effects of glutathione and in vitro freeze-thaw on the plasma membrane on H⁺-ATPase activity, the plasma membranes (25 μg) resuspended in 150 μl of 30 mM Tris-MES (pH 6.5) with or without 2 mM GSH and GSSG. They were frozen as described previously for the freezing of microsomal membranes in section 5.2.3.3. H⁺-ATPase activity was determined immediately after the treatments.
5.2.6. Determination of the antioxidant enzyme activity in soluble proteins from root tissue

5.2.6.1. Assays for measurement of antioxidant enzyme activity

APX activity was determined from the decrease in absorbance at 290 nm as AsA was oxidized as described by Chen and Asada (ε= 2.8 mM⁻¹cm⁻¹) (1992). The assay was conducted in 1-ml of 50 mM potassium phosphate buffer (pH 7.0) containing 0.5 mM AsA, 0.1 mM H₂O₂. The reaction was started by the addition of H₂O₂. Total peroxidase activity was determined by using guaiacol as substrate. The increase of absorbance due to formation of tetraguaiacol was monitored at 470 nm (ε= 26.6 mM⁻¹cm⁻¹) (Klapheck et al., 1990). The reaction solution contained 50 mM potassium phosphate (pH 7.0), 10 mM guaiacol, and 0.1 mM H₂O₂ in a 1-ml volume. The reaction was initiated by the addition of 10-20 µg soluble protein extract and was followed for 2 min.

The activity of MDHAR was determined by using a coupled assay as described by Zhang and Kirkham (1996), with some modification. The assay was performed in a 1 ml solution containing 0.1 mM NADH, 2.5 mM AsA, 50 mM potassium phosphate buffer (pH 7.6) and 20-40 µg of protein extract. The reaction was started by the addition of 2 units of ascorbate oxidase (EC 1.10.3.3) (Sigma, from cucurbita species). The consumption of NADH was measured at 340 nm for 30 second at 30°C (ε=6.22 mM⁻¹cm⁻¹).

DHAR activity was measured by the rate of increase in the absorbance at 265 nm (ε=14 mM⁻¹cm⁻¹) due to AsA formation (Dalton et al., 1986). The assay mixture contained 50 mM potassium phosphate buffer (pH 7.6), 0.5 mM dehydroascorbate (freshly prepared in N₂-saturated buffer), 0.1 mM EDTA, 5 mM GSH and 20-40 µg of protein extract in a final volume of 1 ml. The
reaction was started by the addition of dehydroascorbate. Correction for non-enzymatic reduction was made by subtracting the values obtained in the absence of protein extracts.

GR activity was measured according to Smith et al. (1988). The assay mixture contained 100 mM potassium phosphate buffer (pH 7.5), 0.75 mM DTNB, 0.1 mM NADPH, 1 mM GSSG and 50 μl of protein extract in a total volume of 2 ml. The reaction was started by the addition of GSSG. The increase in the absorbance at 412 nm was measured from the linear portion of the curve, usually within 5 min. A standard curve was made using commercial glutathione reductase (E.C. 1.6.4.2) (Sigma, type II).

5.2.6.2. Native PAGE and activity staining

Soluble protein from root tissue of control and conditioned seedlings were subjected to discontinuous PAGE under non-denaturing, non-reducing conditions, essentially as described by Laemmli (1970), except that SDS was omitted and the gels were supported by 10% glycerol. Ten percent polyacrylamide gels were used. Electrophoresis was carried out at 4°C with constant current of 15 mA.

The detection of APX activity in native gels was based on the method of Mittler and Zilinskis (1993). Samples were subjected to native PAGE as described above, except that running buffer contained 2 mM AsA. The gels were pre-run for 30 min to allow AsA to enter the gels prior to the application of samples. Following electrophoretic separation, gels were equilibrated with 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM AsA for 30 min. The gels were incubated with 50 mM potassium phosphate buffer (pH 7.0) containing 4 mM AsA and 2 mM H₂O₂ for 20 min and were finally washed with 50 mM potassium phosphate buffer (pH 7.0) for 1 min. The gels were submerged in a solution of 50 mM potassium phosphate (pH 7.8) containing 28
mM TEMED and 2.45 mM nitroblue tetrazolium with gentle agitation. The reaction was continued for 5-10 min until the achromatic bands were observed on a purple blue background. The reaction was stopped by a brief wash in deionized water. The gels were fixed in 10% acetic acid and stored at 4°C.

The detection of typical peroxidase activity in a native gel was also based on the method of Mittler and Zilinskas (1993) and basically was the same as for the detection of APX. Briefly, the gels were equilibrated with 50 mM potassium phosphate buffer (pH 7.0) for 30 min and then incubated for 20 min in a 50 mM potassium phosphate buffer (pH 7.0) containing 4 mM H₂O₂ and 20 mM 4-chloro-1-naphthol. The gels were then washed with deionized water and stored in 10% acetic acid at 4°C.

5.3. RESULTS

5.3.1. Acclimation and susceptibility of protein thiol groups to freezing stress

To investigate whether protein thiol groups changed during the cold conditioning, the concentrations of SH groups were determined in both soluble and microsomal protein fractions isolated from the roots of control and conditioned jack pine seedlings (Fig. 5.1). In control seedlings, the amount of SH groups in soluble proteins was lower than that in microsomal membrane proteins. The level of accessible SH groups in microsomal proteins was about 45.4 nmol/mg protein. This concentration was of the same order of magnitude as that reported in human red blood cell membranes (74-88 nmol/mg protein) (Kosower et al., 1981) and in rubisco isolated from young tomato seedlings (25 mol/mol of rubisco) (Bruggemann, 1995).
During cold conditioning, the amount of SH groups in soluble proteins gradually and significantly (P<0.01) increased, while the levels of SH groups in microsomal proteins changed slightly during the period of conditioning (Fig. 5.1). The banding pattern of SH groups in soluble proteins was determined by a fluorescent labelling SDS-PAGE technique as described in Materials and Methods. Changes in the proteins and the SH concentrations of individual proteins are shown in Fig. 5.2. Only two proteins can be seen in 2- and 4-week conditioned seedlings, with stronger SH group labelling in 4-week conditioned seedlings (Fig. 5.2 A and B). Some proteins did not change in amount and SH concentrations throughout cold acclimation. However, other proteins increased in both of amount and SH group labelling intensity during the period of cold conditioning.

After seedling roots were frozen and thawed in soil, the levels of reduced thiol groups in microsomal proteins from control, 1, 2 and 4-week conditioned seedlings were reduced to 54, 64, 72 and 82% of their original levels (Fig. 5.3), respectively. Rapid freeze and thaw of microsomal membranes isolated from the root tissue that was not exposed to freezing also caused reduction of thiol concentrations in proteins, but showed no difference among the control and conditioned seedlings (Fig. 5.3).

5.3.2. Changes in GSH and GSSG levels during cold acclimation

To investigate whether or not the status of glutathione in the roots of jack pine seedlings changed during cold conditioning the concentrations of GSH and GSSG and the ratio of GSH/GSSG were determined.

The levels of GSH and GSSG concentration were about 90 and 25 nmol/g FW in control jack pine root tissues, respectively (Fig. 5.4). The amount of glutathione varies by more than one
order of magnitude between different species, and different organs within the same species (Smith et al., 1990). In pea seedling roots, the GSH and GSSG concentrations were about 250 and 28 nmol/g FW, respectively (Bielawski and Joy, 1986). Seidenberg et al. (1995) reported earlier that GSH concentration in maize (Zea mays L.) was about 400 nmol/g fwt (Seidenberg et al., 1995). The GSH concentration in Arabidopsis thaliana callus showed a concentration similar to that in jack pine roots (O’Kane et al., 1996).

The level of GSH in jack pine roots increased significantly (P<0.01) during the period of cold acclimation. The concentration of GSH increased to about four times of control level after seedlings were held at low temperature for a week, and reached to a maximum level after seedlings were kept at lower temperature for 2 weeks, about eight-fold higher than the control level. When seedlings were kept for 2 more weeks at 5°C, the GSH concentration declined to the same level as in 1 week conditioned seedlings (Fig. 5.4A). This decline was not due to an age difference, since the control and conditioned seedlings were in the same development stage. GSSG concentrations revealed a similar pattern as the concentration of GSH during cold treatment, except that the GSSG concentration in the roots of 4-week conditioned seedlings dropped close to the level of GSSG in control seedlings (Fig. 5.4B). As a result, the mole ratios of GSH/GSSG were increased significantly (P<0.001) about two times in 1 and 2-week conditioned seedlings, and to more than three fold in 4-week conditioned seedlings (Fig. 5.4C). Similar results have been shown previously for other species. O’kane et al. (1996) demonstrated that the concentration of GSH in A. thaliana callus rose initially after the callus was held at 4°C for 2 days, then declined gradually in the following 6 days at 4°C.
Figure 5.1 The effects of cold acclimation on protein SH concentrations in soluble and microsomal proteins from jack pine roots. Soluble (light bars) and microsomal (black bars) proteins were isolated from non-conditioned (control), one (1wk), two (2wk) and four (4wk) weeks conditioned seedlings as described in Materials and Methods. Two hundred micrograms of proteins were used to determine the thiol concentration. Based on T-test analyses, the means of protein SH concentration in soluble proteins were significantly different between control and conditioned seedlings (P<0.01). Values are the means of the duplicate samples from three independent experiments and the error bars represent SDs.
Figure 5.2 SDS-PAGE patterns of soluble proteins from jack pine roots. Soluble proteins were isolated from non-conditioned (C), one (1wk), two (2wk) and four (4wk) week conditioned seedlings as described in Materials and Methods. A: Coomassie Blue staining; B: fluorescence labelling. One arrow: non-change; Two arrows: gradually increased; Three arrows: only seen in 2- and 4-week conditioned samples. The figure represents three independent experiments.
Figure 5.3 The effect of \textit{in vivo} and \textit{in vitro} freeze-thaw on thiol concentrations in root microsomal proteins. For \textit{in vivo} freeze-thaw experiments, non freeze-thaw non-conditioned (Control), one (1wk), two (2wk) and four (4wk) week conditioned seedlings were frozen and thawed as described previously (Zhao et al, 1995). For \textit{in vitro} freeze-thaw experiments, 200 µg of microsomal proteins from Control, 1-, 2- and 4-week conditioned seedlings were frozen in a -20°C freezer as described in Materials and Methods. Two hundred micrograms of proteins were used to determine the thiol concentration. Light, white and black bars represent the data from \textit{in vivo}, \textit{in vitro} and non-freeze-thaw experiments, respectively. Bars headed by different letters differ significantly (P<0.01) according to T-test analyses. Values are the means of the duplicate of three independent experiments and the error bars represent SDs.
Figure 5.4. Changes in the status of glutathione in jack pine roots during cold acclimation. Total glutathione was extracted from non-conditioned (Control), one (1wk), two (2wk) and four (4wk) week conditioned seedlings, and GSH and GSSG concentrations were determined as described in Materials and Methods. (A) GSH concentrations; (B) GSSG concentrations; (C) GSH/GSSG mole ratios. In each Figure, bars headed by different letters differ significantly (P<0.01) according to T-test analyses. Values are the means of duplicate of four independent experiments and the error bars represent SDs.
5.3.3. Regulation of plasma membrane bound enzyme activities by thiol reagents

In plasma membrane enriched vesicles from control jack pine roots, the addition of 5 mM GSSG to the assay solution slightly inhibited the activity of H⁺-ATPase, while GSH had little effect (Table 5.1). Cysteine showed slight stimulation of the activity of H⁺-ATPase. In the plasma membranes from 4-week conditioned jack pine roots, the addition of thiol chemical slightly inhibited enzyme activity.

P-chloromercuribenzoic acid (PCMB) reacts strongly with protein thiol groups (Qian and Murphy, 1993). Incubation of root plasma membrane from non-conditioned seedlings with 0.05, 0.1 and 0.2 mM PCMB resulted in 60% inhibition of ferricyanide reductase (Fig. 5.5A). The inhibitory effects of PCMB on ferricyanide reductase activity was greatly reduced in the plasma membranes from 4-week conditioned seedlings, with only 14% and 40% inhibition by 0.05 and 0.01 mM PCMB, respectively (Fig. 5.5A). On the other hand, PCMB (0.05 to 0.2 mM) inhibited 50% of the H⁺-ATPase activities in both control and 4-week conditioned seedlings (Fig. 5.5B). However, the addition of 2 mM GSH in the assay solution restored the inhibition caused by pre-incubation of the plasma membranes with 0.1 mM PCMB (Fig. 5.6). Pre-incubation with 2 mM GSH maintained the enzyme activity in the presence of 0.1 mM PCMB in the assay solution.

Rapid freeze-thaw of plasma membrane isolated from the roots of control seedlings inhibited about 40% of control H⁺-ATPase activity (P<0.01) (Fig 5.7). However, this inhibition can be prevented by freezing the membranes in the presence of 2 mM GSH. After a freeze-thaw event, the incubation of the plasma membranes with 2 mM GSH at room temperature for 15 min also
significantly increased (P<0.05) the enzyme activity to near control levels. GSSG has no significant
effect on freeze-thaw stressed the plasma membrane H⁺-ATPase activity (Fig. 5.7).
Table 5.1 The effects of some thiol reagents on jack pine root plasma membrane H⁺-ATPase. All reagents were added to the enzyme assay medium before membranes. Data show means ± SDs of duplicate of three independent experiments. The enzyme activity is expressed as μmole Pi/mg protein.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>non-conditioned</th>
<th>4-week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.7 ± 0.6</td>
<td>6.3 ± 0.5</td>
</tr>
<tr>
<td>GSH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mM</td>
<td>6.9 ± 1.2</td>
<td>5.9 ± 0.4</td>
</tr>
<tr>
<td>5 mM</td>
<td>6.4 ± 1.1</td>
<td>5.5 ± 0.7</td>
</tr>
<tr>
<td>GSSG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mM</td>
<td>6.4 ± 0.6</td>
<td>5.5 ± 0.7</td>
</tr>
<tr>
<td>5 mM</td>
<td>6.0 ± 0.5</td>
<td>4.7 ± 0.7</td>
</tr>
<tr>
<td>Cysteine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mM</td>
<td>7.2 ± 0.6</td>
<td>5.7 ± 0.4</td>
</tr>
<tr>
<td>5 mM</td>
<td>7.4 ± 0.7</td>
<td>5.8 ± 0.6</td>
</tr>
</tbody>
</table>
Figure 5.5 The effects of pre-incubation with PCMB on plasma membrane H⁺-ATPase and ferricyanide reductase activities. Plasma membranes isolated from the roots of non-conditioned (light bars) and four week conditioned (dark bars) seedlings were pre-incubated with 0.05, 0.1 and 0.2 mM PCMB for 15 min at room temperature as described in Materials and Methods. Based on T-test analyses, the means of H⁺-ATPase and ferricyanide reductase activities were significantly different between non-PCMB treated (a) and PCMB treated (b) membranes isolated from non-conditioned seedlings (P<0.01). Values are the means of duplicates of three independent experiments and the error bars represent SDs. A: ferricyanide reductase activity; B: H⁺-ATPase activity.
ATPase activity (μ mol Pi/mg prot.h)

Ferricyanide reductase activity (μ mol NADH/mg prot.min)
Figure 5.6 The protective effects of GSH on PCMB-treated plasma membrane on H\(^+\)-ATPase activity. Plasma membranes isolated from the roots of non-conditioned seedlings were incubated with a normal assay solution (control). Plasma membranes were pre-incubated with 0.1 mM PCMB at room temperature for 15 min, and then incubated with a normal assay solution (PCMB) or an assay solution containing 2 mM GSH (PCMB-GSH). Plasma membranes were pre-incubated with 2 mM GSH and then incubated with an assay solution with 0.1 mM PCMB (GSH-PCMB). All plasma membranes were incubated with the assay solutions at 30°C for 30 min. Bars with different letters differ significantly according to a T-test (P<0.01). Values are the means of duplicates of four independent experiments and the error bars represent SDs.
Figure 5.7 The effects of *in vitro* freeze-thaw of plasma membrane and glutathione on H⁺-ATPase activities. Plasma membranes isolated from the roots of non-conditioned seedlings were frozen and thawed as described in Materials and Methods. (Control) control activity of non-frozen plasma membranes; (Freeze) activity of frozen-thawed plasma membranes; (GSH) activity of plasma membranes that were frozen with 2 mM GSH; (GSSG) activity of plasma membranes that were frozen with 2 mM GSSG; (GSH*) activity of plasma membranes that were incubated with 2 mM GSH for 15 min at room temperature after freeze and thaw; (GSSG*) activity of plasma membranes that were incubated with 2 mM GSSG for 15 min at room temperature after freeze and thaw. Based on T-test analyses, bars headed by letters a and b differ significantly at P<0.01; bars headed by letters a and c differ significantly at P<0.05. Values are the means of duplicates of three independent experiments and the error bars are SDs.
5.3.4. Enzymatic changes of ascorbate-glutathione cycle during cold acclimation

The specific activity of APX in the soluble protein fraction of jack pine roots was 3.8 \( \mu \text{mol AsA/mg prot.min} \). Very different levels of the specific activity of this enzyme have been reported among plants, ranging from 30 \( \mu \text{mol AsA/mg prot.min} \) protein in shoot extracts of rice (Fadzillah et al., 1996); 15.5 \( \mu \text{mol AsA/mg prot.min} \) in extracts from four-day old endosperm of Ricinus (Klapheck, et al., 1990); 250 nmol AsA/mg prot.min in callus tissue of Arabidopsis thaliana to 4 nmol AsA/mg prot.min in the leaves of Arabidopsis thaliana (Rao et al., 1996). The activities of ascorbate regenerating enzymes MDHAR and DHAR in the soluble protein extracts from jack pine roots were 0.64 \( \mu \text{mol NADH/mg prot.min} \) and 0.32 \( \mu \text{mol AsA/mg prot.min} \), respectively. The same levels of enzyme activities were reported for cytosolic extracts of Ricinus endosperm (Klapheck, et al., 1990) and in leaf extracts of spinach (Hossain and Asada, 1984). The specific activity of GR in jack pine roots was about 1.4 nkat/mg protein. This value is comparable to those data reported for the roots of maize seedlings (5 nkat/mg protein) (Kocsy et al, 1996) and pea roots (0.97 nkat/mg protein) (Bielawski and Joy, 1986).

No report has been found that deals with the enzymes involved in AsA-glutathione cycle during the cold acclimation in plant root system. The aim of this investigation was to study the response of the enzymes involved in AsA/glutathione cycle in the jack pine roots to cold acclimation. The activities of APX in the roots of jack pine increased slightly after seedlings were held at 5°C for 2 weeks and rose about 2.3-fold (\( P < 0.01 \)) following 2 more weeks of cold conditioning (Fig. 5.8). Several APX isozymes in root soluble protein extracts were detected in a APX activity stained native PAGE and one of the isomers showed increased APX activity in 4-week conditioned seedlings (Fig. 5.9A). In contrast to APX, guaiacol-dependent peroxidase
activities did not change following 1 and 2 weeks of conditioned seedlings, and increased by 53% (P < 0.01) after 4 weeks of conditioning (Fig. 5.8). However, a peroxidase activity stained native PAGE showed a gradual change in the isomer profile during cold conditioning (Fig. 5.9B). One isomer decreased in its activity and two others increased during cold conditioning (Fig. 5.9B). MDHAR activity (Fig 5.10A) gradually increased during the initial period of cold conditioning and reached to 3.5-fold (P<0.01) of the control activity after 4 weeks of conditioning. DHAR reached a maximum level after 2 weeks of conditioning and then dropped slightly in 4-week conditioned seedlings (Fig. 5.10B). GR activity increased 78% (P<0.01) in 4-week conditioned seedlings (Fig. 5.10C).
Figure 5.8 The effects of cold acclimation on ascorbate and guaiacol peroxidase activities. Soluble proteins were isolated from the roots of non-conditioned (control), one (1wk), two (2wk) and four (4wk) week conditioned seedlings. APX (light bars) and guaiacol-dependent peroxidase (dark bars) activities were determined as described in Materials and Methods. Based on T-test analyses, bars headed by different letters differ significantly (P<0.01). Values are the means of duplicate of three independent experiments and the error bars represent SDs.
Figure 5.9 Native-PAGE of ascorbate peroxidase and guaiacol peroxidase activity staining in jack pine root soluble proteins. Soluble proteins were isolated from the roots of non-conditioned (C), one (1wk), two (2wk) and four (4wk) week conditioned seedlings as described in Materials and Methods. APX and general peroxidases activity stains were carried out according to Mittler and Zilinskas (1993). The results are representative of five independent experiments. A: APX activity staining; B: general peroxidase activity staining. Arrows on the right side of the pictures indicate that isomer activities increased during cold conditioning; an arrow on the left side of picture B indicates that the isomer activity decreased based on visual observations.
Figure 5.10 The effects of cold acclimation on monodehydroascorbate reductase (A); dehydroascorbate reductase (B); and glutathione reductase (C) activities. Soluble proteins were isolated from the roots of non-conditioned (Control), one (1wk), two (2wk) and four (4wk) week conditioned seedlings as described in Materials and Methods. In Figures A and B, bars headed by different letters differ significantly (P<0.01) according to T-test analyses. In Fig. C, bars headed by different letters differ significantly (P<0.01), except that bars headed by a and b differ significantly at P<0.05, according to T-test analyses. Values are the means of duplicates of three independent experiments and the error bars represent SDs.
<table>
<thead>
<tr>
<th>Activity</th>
<th>Control</th>
<th>1wk</th>
<th>2wk</th>
<th>4wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>GR activity (nkat/mg protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHAR activity (μ mol AsA/mg prot.min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDHAR activity (μ mol NADH/mg prot.min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
5.4. DISCUSSION

5.4.1. Effect of cold acclimation on glutathione redox status

The oxidation of specific cysteine sulphydryl groups can play an important role in the regulation of enzyme activity. For some protein, the cysteine SH group must be maintained in the reduced form for activity, while for other proteins the oxidized form (formation of disulphide bond) is essential for function (Ziegler, 1985; Gilbert, 1990). In this study, we found that the levels of SH groups in soluble proteins of the roots were significantly increased following 2 and 4 weeks of conditioned seedlings (Fig. 5.1). The increased number of SH groups in soluble proteins were not only due to the synthesis of new proteins which contain SH groups, but also due to an increase in the number of SH groups in existing proteins (Fig. 5.2). An increase in the levels of SH groups in these proteins indicates that the redox state of the cytosol may be gradually changed during conditioning. It is well known that glutathione is the major regulator of cytoplasmic protein thiol redox status (Ziegler, 1985; Hwang et al., 1992; Hausladen and Alscber, 1993). The increase in the number of SH groups in soluble proteins could be the result of an accumulation of GSH in root cells in response to low temperature.

The concentration of SH groups in microsomal proteins decreased after a freeze-thaw event (Fig. 5.3). In vitro freeze-thaw of isolated microsomal membranes lead to a decrease in SH concentrations, and showed no difference between the control and conditioned seedlings (Fig. 5.3). These results would suggest that maintenance of SH group levels in cellular membranes against thiol oxidation during freezing stresses could be associated with cytosolic thiol-disulphide redox components. The status of glutathione is a critical factor in the thiol-disulphide exchange reactions.
The negative redox potential ($E_0 = -0.34 \, \text{V}$) of GSH allows for efficient reduction of disulfide bonds in proteins (Hausladen and Alschner, 1993). When seedlings were thawed, the protein-S-S-protein formed during the freezing dehydration may be reduced back to the SH groups by the GSH in the cytosol. This may occur during the post-thaw or recovery process, leading to maintain functional conformations of the proteins. Microsomal hydroxymethylglutaryl-CoA (HMG-CoA) reductase, the enzyme involved in isoprenoid biosynthesis, is extremely sensitive to oxidative inactivation by formation of disulfides (Gilbert, 1990), and its activity is dependent on both GSH/GSSG ratio and the GSH concentrations (Gilbert, 1990). Similarly, changes in the thiol-disulfide redox status of glutathione in the cytosol in jack pine roots may be correlated with the stabilizing thiol concentration in microsomal membrane proteins following the freeze-thaw process. In order to prove this hypothesis, we determined the thiol-disulfide redox state of glutathione in jack pine roots during the cold acclimation.

Our results reveal that in jack pine roots, the responses of glutathione to cold temperature were remarkably strong, as the mole ratios of GSH to GSSG increased more than three-fold following 4 weeks of conditioning (Fig. 5.4C). Changes in the mole ratio of GSH to GSSG were in good agreement with the changes in protein thiol concentrations in soluble proteins in conditioned seedlings (Figs. 5.4C and 5.1). These results indicated that status of glutathione was shifted to a more reduced form in conditioned seedlings. This change in glutathione status correlates with the maintenance of SH group levels in microsomal membranes and the survivability of conditioned seedlings after a freezing-thaw event.

Fluctuations of glutathione and glutathione reductase activity in green tissue, high in the winter and low in the summer, were displayed by 21 evergreen winter-hardy plants (Smith et al.,
The processes that influence the cellular glutathione level in plants include biosynthesis and degradation, long distance transport, and the use of glutathione as a substrate in the synthesis of phytochelatins, glutathione conjugates, and mixed disulfides (Rennenberg, 1995; Noctor et al., 1996). In general, glutathione is translocated from leaves to roots through phloem (Rennenberg, 1982; Noctor et al., 1996). Following 4 weeks of cold conditioning, the decline in total glutathione content in jack pine roots after an initial increase may be due to a lower rate of long distance translocation of glutathione due to a decreases in metabolism rates (Fig. 5.4 A and B).

5.4.2. The role of glutathione in regulation of plasma membrane bound enzyme activities

The activity of many enzymes is influenced by the oxidation-reduction state of the cells (Ziegler, 1985; Gilbert, 1990; Qian and Murphy, 1993; Seidenberg et al., 1995). Maintaining or regenerating SH groups in the microsomal proteins during or after oxidative stress can stabilize membrane enzyme proteins, such as channel proteins, pumps, etc. For example, the plasma membrane H⁺-ATPase activity depends on the presence of SH groups in membrane proteins (Elzenga et al., 1989) and is regulated by the oxidation-reduction state of glutathione (Katz and Sussman, 1987; Elzenga et al., 1989; Qian and Murphy, 1993).

The protective effects of GSH on jack pine root plasma membrane H⁺-ATPase may occur before or after the oxidation of SH groups as demonstrated by the maintenance of ATPase activity when GSH was added before or after PCMB treatment (Fig. 5.6). Furthermore, our data indicate that direct freeze-thaw of the plasma membranes inhibited H⁺-ATPase activity (Fig 5.7). This inhibition could be caused by the thiol oxidation of plasma membrane proteins during the freeze-thaw events since the addition of GSH maintained the enzyme activity and the incubation of frozen
and thawed plasma membranes with GSH restored the enzyme activity (Fig 5.7). The lack of an
effect of GSSG on the plasma membrane H⁺-ATPase activity is probably due to the fact that large
GSSG molecules cannot access the plasma membranes. This suggestion is supported by the
requirement of detergent, such as Triton X100, in order to observe any effect of GSSG on the
plasma membrane H⁺-ATPase activity (Qian and Murphy, 1993). The maintenance of H⁺-ATPase
activity by GSH may be caused not only by the prevention of SH oxidation in the enzyme proteins,
but also by the prevention of peroxidation of the plasma membrane lipids through a glutathione-
mediated free radical scavenging system (Seaz et al., 1990; Kumar and Knowles, 1996).

Ferricyanide reductase in root plasma membranes from control jack pine seedlings was
strongly inhibited by PCMB indicating the presence of thiol groups in these proteins. Oxidized thiol
groups in plasma membrane bound redox enzymes may block electron transfer to ferricyanide.
Buckhout and Luster (1991) reported a 70-90% inhibition of ferricyanide reductase after pre-
incubation with 50 mM p-chloromercuriophenyl sulphate (PCMS) in the presence of detergent (0.01
% Triton 100). This indicates that thiol groups are in proteins that face the cytosol. Purified
ferricyanide reductase from spinach leaf plasma membranes was strongly inhibited by 0.1 mM
PCMB (Moller et al., 1995). Unlike the effect on H⁺-ATPase, cold conditioning of jack pine
seedlings for 4 weeks enhanced the tolerance of the plasma membrane-bound redox enzymes to
PCMB (Fig. 5.5). This could be due to an increase in SH groups in these proteins or changes in
protein configuration that could prevent thiol oxidation. Plant growth at low temperatures may
affect the prosthetic groups in the electron transport system that allows the electron transport
system to adapt to oxidative stress. Alternatively, Zinc (Zn) ions may have a role in protecting
membrane from oxidative damage (Cakmak and Marschner, 1990; Rengel, 1995). Those SH
groups that are located on the plasma membrane proteins (possibly at the mouth of ion channels or transport proteins) (Kochian, 1993; Welch, 1995)) can be protected by Zn (Welch and Norvell, 1993; Welch, 1995). However, the effect of Zn on stabilizing root-cell plasma membranes during cold acclimation needs to be studied further in detail.

5.4.3. Changes of antioxidant enzymes in jack pine roots during cold acclimation

Glutathione and GR are important components of the antioxidant system in plants, since the increased levels of GSH could be useful not only in the reduction of disulfide bonds in proteins but also for the metabolism of active oxygen species. The responses of antioxidant enzymes to oxidative and/or chilling stresses have been investigated individually in different plant species, especially for APX and GR (Smith et al., 1990; Anderson et al., 1992; Fadzillah et al., 1996; O'Kane et al., 1996). The study of seasonal variation in the antioxidant system of eastern white pine (Pinus strobus L.) needles showed that the lowest activities of APX and GR were present in the summer and the highest activities in the winter (Anderson et al., 1992). Exposure of A. thaliana to UV-B for 5 days also enhanced APX and GR activities (Rao et al., 1996). A time course study of chilling effects on APX activity in callus tissue of A. thaliana showed a slight decrease of the activity after 1 day at 4°C and a gradual increase in activity during the following 7 days of chilling. However, catalase activities declined after the callus was held at 4°C for 4 days (O'Kane et al., 1996).

Our results suggest that all enzymes involved in the AsA-glutathione cycle in soluble proteins of jack pine roots are regulated by low temperature. Several APX isomers were detected in the root soluble protein fraction (Fig. 5.9A). The presence of different isomers of APX in the roots of Brassica campestris L. cv. Komatsuna was reported (Mormura et al., 1996). Several
isomers of APX were also detected by the same gel assay as used in this study in leaf extracts from many other plant species (Mittler and Zilinskas, 1993). As shown in Figures 5.8 and 5.9A, the increased APX activity in 4-week cold conditioned seedlings was correlated with the enhancement in one of the isomers in root tissue. The increase in this isomer is, perhaps, the result of an expression of increased cytosolic APX transcript level following 4 weeks of cold conditioning. Although several isozymes of APX were detected in the gel assay, only one APX isomer cross-reacted with pea cytosolic APX polyclonal antibodies, and no difference was found between control and cold conditioned seedlings (data not shown).

APXs play an important role in scavenging H$_2$O$_2$. However the mechanism by which APX activity is induced is not known. Stress-induced increases in cytosolic APX transcript levels have been reported in pea (Mittler and Zilinskas, 1994) and in transgenic tobacco plants overexpressing a pea Cu/Zn SOD (Gupta et al., 1993). More recently, Donahue et al (1997) observed that the paraquat-mediated increase in APX mRNA levels were not reflected in correspondingly large increases in APX enzyme activities. The increased APX transcript levels may play an important role in overexpressing APX proteins leading to scavenging of H$_2$O$_2$ in jack pine roots during the recovery from freeze-thaw stress. Further studies are necessary to provide direct evidence.

APX contains four cysteine residues (Asada, 1992). The inhibition of APX by thiol oxidation reagents such as PCMB is one of the characteristic properties that differs from that of guaiacol peroxidase (Asada, 1992). The gradual inactivation of cytosolic APX by thiol, such as GSH, in the present H$_2$O$_2$ and the absence of AsA has been reported (Asada and Chen, 1992). The inhibition was caused by an interaction between H$_2$O$_2$ and GSH leading to the formation of thiyi radicals that inhibited APX activity gradually (Asada and Chen, 1992). In the presence of AsA,
APX activity was protected from H$_2$O$_2$-dependent thiol induced inactivation. The changes in oxidation-reduction of glutathione in jack pine root cells may also affect APX activity in vivo. In general AsA biosynthesis increases under stress conditions (Foyer, 1993), and the increase in AsA in the winter has been reported in coniferous needles (Esterbauer et al., 1980; Anderson et al., 1992). The possible increase in AsA content in jack pine roots could prevent H$_2$O$_2$-dependent thiol induced inactivation.

Peroxidases usually occur as multiple molecular forms (isozymes) and have a number of potential roles in plant growth and development (Rao et al., 1996). Some peroxidases utilize phenolic compounds (coniferyl alcohol) and H$_2$O$_2$ to initiate the chain reactions that leads to lignification (Polle et al., 1994). Increased peroxidase activity (Fig. 5.8) and the changes in isoforms (Fig. 5.9B) suggests that cold conditioning may have enhanced the synthesis of secondary metabolites such as lignin in jack pine roots. During cold conditioning, a gradual decrease in non-lignified white roots was observed.

AsA reacts nonenzymatically with H$_2$O$_2$ at a significant rate (Foyer, 1993). The oxidation of AsA occurs in two sequential steps, forming MDHA first, and subsequently DHA. The MDHA is also produced when AsA is oxidized by superoxide and hydroxyl radicals (Foyer, 1993). To function as an antioxidant, AsA must be maintained in the reduced state. MDHA is directly reduced to AsA by the MDHAR in the presence of NAD(P)H (Hossain et al., 1984). AsA is also regenerated from DHA in a glutathione dependent reaction catalyzed by DHAR. This reaction involves the GR catalized reduction of GSSG to GSH. In 1- and 2-week conditioned seedlings, the activities of two AsA regenerating enzymes in roots were increased but APX remained relatively unchanged (Figs. 5.8 and 5.10A and B). This would suggest that the oxidation of AsA may have
occurred nonenzymatically with active oxygen radicals. The oxidation of AsA may not be efficient to scavenge active oxygen radicals generated during the freezing and thawing processes because 1- and 2-week conditioned seedlings showed lower survival rates to subfreezing temperatures (Zhao et al., 1995). In seedlings conditioned for 4 weeks, the lower DHAR activity would suggest that the regeneration of AsA depended mainly on the increased MDHAR activity.

After seedlings were held at low temperature for 4 weeks, the specific activities of APX and GR in the roots increased 2- and 1.75-fold, respectively. Studies of seasonal variation in the specific activities of APX and GR in the needles of eastern white pine showed that activities of both enzymes in October were 6.9- and 8.5-time higher than that in July, respectively (Anderson et al., 1992). The difference in low temperature-induced APX and GR activities in roots and needles may be a factor contributing to the higher susceptibility of roots to freezing temperatures.

In conditioned jack pine seedlings, the changes in GR activities were not correlated with changes in DHAR but correlated with the changes in the ratios of GSH/GSSG. This may suggest that GR in the cytosol of jack pine roots is not involved in the AsA/glutathione pathway, but plays a role in changing the oxidation-reduction status of glutathione in the conditioned seedlings.

In summary, an increase in GSH and mole ratios of GSH/GSSG in roots was detected during cold conditioning. The changes in oxidation-reduction in root cells may affect the soluble and membrane protein SH concentrations during cold conditioning, and after freeze-thaw stress. In this study, we provided evidence that a freeze-thaw induced SH group oxidation in membrane proteins inhibited plasma membrane H+-ATPase activity. The fact that GSH could protect or restore the enzyme activity suggests that GSH may regenerate SH groups in proteins in vivo under oxidative stress conditions. The activities of the enzymes involved in the AsA-glutathione cycle
could be detected in soluble proteins of jack pine roots, and were regulated by the low temperature. Our results suggest that these enzymes may play a protective role following the exposure of jack pine seedlings to freezing temperatures.
Chapter 6

SUMMARY AND CONCLUSIONS

6.1. Development of freezing tolerance in the root tissue of jack pine seedlings

In this study, a cold conditioning protocol that significantly enhanced survival of jack pine seedlings to subsequent freezing stress was developed. Cold conditioning of actively growing containerized jack pine seedlings increased seedling survival that was partially correlated with biochemical changes in root systems. Cellular membrane cryo-stability of jack pine root cells was gradually increased during the period of cold conditioning. As compared with control roots, conditioned roots a) were able to better maintain plasma membrane bound H+-ATPase and ferricyanide reductase activities (Chapter 3); b) showed low levels of lipid peroxidation (Chapter 4); and c) showed less oxidation of thiol groups in microsomal proteins (Chapter 5) after freeze and thaw events. The increased cryo-stability correlated with a significant increase in seedling survival. The data suggest that the induction of cold hardiness in jack pine root systems may require a longer period of cold conditioning since some of the biochemical changes in root cells took place after 4 weeks of cold conditioning (Chapters 3, 4 and 5).

6.2. Physiological and biochemical changes during cold acclimation

Mechanisms that control stress perception, and gene expression are most likely universal in the plant kingdom, considering the fact that stress-adapted plants are distributed throughout many different families (Bohnert et al., 1995). Under stress, a plant adapts its metabolism and alters its development in order to cope with the severe conditions. Learning about the biochemical and molecular mechanisms by which plants tolerate environmental stresses is necessary for genetic
engineering aimed at improving plant performance under stress. Abiotic stresses, (i.e., drought, high salinity, and low temperatures) affect the processes that are utilized by stress-tolerant plants to cope efficiently with water deficits (Palva, 1993; Bohnert et al., 1995). Under these stress conditions many cellular processes could change, including osmolyte biosynthesis (accumulation of proline; polyols; soluble sugars; ions et etc.), ion uptake (ATPase; potassium channels and transporters) and ion partitioning (tissue-specific transport and storage or excretion), membrane modification (fatty acid unsaturation; membrane turnover), signalling pathways (changes in hormone sensitivity), protein turnover (dehydrins; LEA proteins; protein modification) and transcription control (transcription factors; DNA methylation) (Lee and Chen, 1993; Palva, 1993; Bray, 1993; Smirnoff, 1995a; Bohnert et al., 1995). Most temperate plant species increase their frost tolerance when exposed to non-lethal lower temperatures (Lee and Chen, 1993; Uemura et al., 1995). Complex cellular responses to lower temperatures also allow plants to cope with freezing stress since low and freezing temperatures share common elements (Shewfelt, 1992; Bohnert et al., 1995).

Although many physiological and biochemical changes have been described in crop species, there is a lack of information on the physiological and biochemical responses of conifers (particularly in the root systems) to lower temperatures. To study the biochemical changes in the roots of jack pine seedlings during cold acclimation, we investigated several factors that may affect cellular membrane cryo-stability since cellular membranes are the main targets for freezing damage (Steponkus and Webb, 1992; Palta and Weiss, 1993).
6.2.1. Increased phospholipid and lipid fluidity in root and needle tissues

The increase in phospholipid was mainly due to an increase in PC (Chapter 4). The extent of increased phospholipid concentration was higher in needles than in roots. Changes in phospholipids not only affect membrane phase transition (discussed in chapter 4), but also play an important role in regulation of membrane bound enzyme activities. Modulation of the plasma membrane H+-ATPase depends on the lipid bilayer environment, in particular on the degree of fatty acid unsaturation and the existence of lipid annulus associated with the enzyme (Serrano 1989; Cooke and Burden, 1990). Generally speaking, neutral phospholipids (PC and PE) are associated with the extracellular surface of membranes, while negatively charged phospholipids (PG and PS) are more abundant on the cytosolic side of membranes Thus, lipid asymmetry could affect the activity of membrane-bound redox components. (Leshem, 1992).

6.2.2. Decreased NADH-redox associated lipid peroxidation and increased tolerance to free radicals in microsomal membranes isolated from the roots of 4-week conditioned seedlings

The lower level of NADH-redox associated lipid peroxidation found in 4-week conditioned seedlings (Chapter 4) is probably associated with the decrease in plasma membrane ferricyanide reductase activity during cold conditioning (Chapter 3).

A decrease in proportion of phospholipids suggests that a non-specific degradation of microsomal lipids occur due to the generation of active oxygen radicals following a freeze-thaw event (Chapter 4). The collapse of root plasma membrane ferricyanide reductase activity in control seedlings after freeze-thaw events (Chapter 3) suggests that the electron transport chains may be disrupted due to oxidation of their protein SH groups and/or the peroxidation of membrane lipids
(Chapters 5 and 4). Four-week conditioned seedlings maintained the enzyme activity following freeze-thaw events (Chapter 3) indicating that cold acclimation stabilized the redox systems in the jack pine root plasma membranes. The stabilization of the redox systems could be due to changes in cellular thiol disulfide redox status of glutathione and antioxidant enzymes (Chapter 5). The freezing recovery processes required a sustained redox activity which may be involved in nutrition uptake or lignification of the cell walls (Serrano et al., 1995; Crane et al., 1995).

6.2.3. Increased root GSH/GSSG mole ratio and SH group concentrations in soluble proteins during cold acclimation

During cold acclimation, the GSH/GSSG ratio and SH group concentrations in soluble proteins gradually increased (Chapter 5). The increased SH content in soluble proteins could be due to both synthesis of new cysteine-containing proteins and an increase in SH content in pre-existing proteins. Changes in GSH have regulatory effects on cytosolic enzymes (Ziegler, 1985; Gilbert, 1990). Many cytosolic enzyme activities in chilling-sensitive plants were inhibited, in vivo, by oxidation of their regulatory SH groups (Kingston-Smith et al., 1997). In chilling resistant or cold acclimated plants, the increase or maintenance of SH content may be an important adaptive process to cold temperatures. Antioxidant enzymes, such as APX and DHAR are thiol-regulated enzymes. APX is freezing-labile in vitro (Polle et al., 1990) and also the most frost-sensitive enzyme in non-hardened needles of Norway spruce (Polle et al., 1996), probably due to thiol oxidation. However, an increased APX activity is usually found in cold acclimated plants (Table 2.1). APX activities did not decline in hardened tissue following frost events (Polle et al., 1996). The increase in cellular GSH in jack pine roots could be an important factor for the seedling
survival, since it may contribute to prevent thiol oxidation during freezing stress, thus maintaining APX and other thiol-regulated enzyme activities in cold hardiness tissues.

6.2.4. GSH plays an important role as an antioxidant to prevent freezing induced thiol oxidation in cellular membranes

The *in vivo* freeze-thaw and the oxidation of plasma membrane thiol groups by PCMB inhibited both $\text{H}^+\text{-ATPase}$ and ferricyanide reductase activities (Chapters 3 and 5). A slow freeze-thaw of the plasma membranes *in vitro* inhibited $\text{H}^+\text{-ATPase}$ activity. The inhibition of $\text{H}^+\text{-ATPase}$ could be caused by the oxidation of SH groups in the membranes, since the addition of GSH maintained the enzyme activity and the incubation of the plasma membranes with GSH restored the enzyme activity after a freeze-thaw event (Chapter 5). The maintenance of $\text{H}^+\text{-ATPase}$ and ferricyanide reductase activities in root plasma membranes of 4-week conditioned seedlings, following *in vivo* freeze-thaw events, (Chapter 3) also suggests that GSH may be involved in the stabilization of plasma membrane proteins during or after freezing and thaw events (Chapter 5).

The decrease in SH concentrations in microsomal proteins following freezing also suggests a freezing-induced thiol oxidation. Thiol disulfide redox state changes in proteins are reversible and are affected by the changes in cellular thiol-disulfide redox status (Gilbert, 1990) and depends on GSH concentration and GSH/GSSG ratios (Chapter 5). Freezing-induced irreversible thiol oxidation in the membrane proteins of control seedlings could be due to an irreversible denaturation and precipitation proteins or proteolysis (Gilbert, 1990; Shewfelt, 1992).

The data presented in this thesis provided evidence that suggests that GSH could prevent or repair thiol oxidation induced by freeze-thaw stress. A recent study on effects of late frost in spring on antioxidant system in needle of Norway spruce (*Picea abies* L.) provided evidence for
the notion that freezing stress induced an intensive synthesis of GSH (Polle et al., 1996). Increases in GR activity have been reported in many different plant species including conifers during the cold acclimation (Table 2.1). Glutathione was recently shown to activate the transcription of mRNA coding for the enzymes of Cu/Zn SOD (Herouart et al., 1993; Herouart et al., 1994; Wingsale and Karpinski, 1996) and enzymes involved in phytoalexin and lignin biosynthesis, such as phenylalanine ammonia lyase (PAL) (Wingate et al., 1988). GSH was found to act as an elicitor for these stress genes, while thiol agents had no effects (Inze and Montagu, 1995; Wingsale and Karpinski, 1996).

During the freeze-thaw of jack pine seedlings, GSH may also function as an antioxidant preventing the peroxidation of membrane lipids through a glutathione-mediated free radical scavenging system leading to maintenance of membrane integrity (Seaz et al., 1990; Kumar and Knowles, 1996). Further experiments should be carried out to investigate the direct role of GSH in preventing lipid peroxidation in the jack pine root system. Nevertheless, based on our observations and the evidence accumulated on GSH protective effects on protein thiol oxidation in animal systems, it is clear that GSH plays an important role as an antioxidant to prevent or repair protein thiol oxidation during freezing stress.

6.2.5. Changes in antioxidant enzymes involved in AsA/glutathione cycle

The specific activities of AsA regenerating enzymes (MDHAR and DHAR) gradually increased during cold acclimation indicating that the regeneration of AsA was required due to the formation of active oxygen species during the early stage of cold acclimation (Chapter 5). The response of APX and MDHAR in conditioned seedlings to cold conditioning was correlated to the survival of the seedlings. The increase in APX was probably due to increase in one of the enzyme
isoforms as detected in activity staining native gels. Changes in GR activity were significantly
(r²=0.98; P<0.01) correlated with the changes in the mole ratios of GSH to GSSG but not with
changes in DHAR activity. This suggests that GR in the cytosol of jack pine roots is probably not
involved in the ascorbate/glutathione pathway, but plays a role in the changes in oxidation-
reduction status of glutathione during cold conditioning. The increase in antioxidant enzyme
activities in 4-week conditioned seedlings may contribute to a lower level of lipid peroxidation, and
a lower level of PL and GL degradation following freezing-thaw stress in vivo (Chapter 4).

The balance between the formation and detoxification of active oxygen species is critical to
the cell survival during the periods of stress (Shewfelt and Erickson, 1991). A cold tolerant plant
might use three possible mechanisms to survive low temperatures. The plants may avoid the
production of active oxygen, they may protect themselves from damage by developing antioxidant
systems to efficiently scavenge the activated forms of oxygen, or they might repair the injury after
protein degradation has occurred (Steffen, 1991).

In conclusion, we demonstrated that the root cells of jack pine seedlings developed freezing
resistance during cold conditioning (Fig. 6.1). This could be achieved by both avoidance (decrease
in NADH-redox associated lipid peroxidation) and tolerance (changes in membrane lipid and their
fatty acid compositions; increases in GSH and the ratio of GSH to GSSG; response of antioxidant
system) mechanisms.
Fig. 6.1 Summary of the development of freezing tolerance in jack pine roots during cold acclimation investigated in this study.
6.3. A Possible mechanisms of cold acclimation

Freezing tolerance for any given plant is a multigenic characteristic derived from many factors, such as cell size, cell morphology, tissue structure, plant development stage and dormancy requirements as well as the stage of cold acclimation (Levitt, 1980; Rennenberg, 1982). Cold acclimation is a complex process that involves various physiological, biochemical and molecular changes to achieve maximum freezing tolerance.

The possible biochemical responses to cold acclimation in jack pine seedlings are summarized in Fig. 6.2. When plants are transferred from normal growing temperatures to a lower temperature, the physical properties of membrane lipids change in certain domains (Steponkus, 1990; Uemura et al., 1995). For the plants to adapt to the lowered temperatures, in terms of continued growth, a change in acyl chain compositions of membrane lipids is required to regain a membrane lipid environment (membrane fluidity) similar to that at warmer temperatures (Fig. 6.2). As discussed in Chapter 5, low temperatures also cause changes in cellular redox status. Those changes may affect membrane functions (Cooke and Burden 1990; Chapter 5) and gene expression (Herouart et al., 1993; Wingsle and Karpinski, 1996).

Hetherington and Quatrano (1991) and others (Chandler and Robertson, 1994; Colorado et al., 1995; Mantyla et al., 1995) proposed that ABA serves as a general stress response hormone in plants because desiccation is the common basis for a number of different environmental stresses. The measurement of ABA level was out of the scope of this study, however, several studies have demonstrated that changes in ABA concentration are related to the cold-hardening process in *Acer* species (Irving, 1969; Dumbroff et al., 1979). Recently, it has been reported that ABA concentration increased approximately ten-fold in the xylem sap of sugar maple during winter
acclimation and reached a maximum level before maximum hardiness was reached in bud and roots (Bertrand et al., 1997). ABA not only plays a direct role in response to cell desiccation and as a modulator for growth, but is also involved in the control of gene expression during cold acclimation (Palva, 1993; Chandler and Robertson, 1994).

Calcium is a second messenger that mediates many plant signaling processes (Bush, 1995). The involvement of calcium in low temperature signaling during cold acclimation has been demonstrated from the observed transient changes of cytosolic calcium in response to cold shock (Knight et al., 1991; Monroy et al., 1993) and from electrophysiological studies of the modulation of calcium channel activity by low temperature (Ding and Pickard, 1993). Monroy et al (1993) demonstrated that calcium chelators, calcium channel blockers and inhibitors of calcium-dependent protein kinases prevent cold acclimation. Thus, it has been proposed that calcium functions as a second messenger in response to chilling and cold acclimation (Monroy and Dhindsa, 1995; Knight et al., 1996).

Changes in ABA concentrations (Bertrand et al., 1997) and alteration in cytosolic calcium concentrations (Monroy, 1993) will induce changes in gene expression leading to the synthesis of new enzymatic proteins involved in cryo-protestant synthesis, synthesis of new membrane lipids (Chapter 4), and antioxidant enzymes (Chapter 5) (Fig. 5.6). These adaptive changes in membrane lipids and proteins, and alteration in cytosolic components will allow plants to maintain cell functions at chilling temperatures for a long time, and furthermore increase membrane cryo-stability when plants undergo freezing stress (Fig. 6.2).

The model presented is useful in understanding the mechanisms of cold acclimation. However, this is by no means the only model explaining these results. Further work is needed to
gain a better understanding of how other factors such as mineral nutrition and ABA levels can affect root cold acclimation in tree seedlings.
新膜蛋白

 alterations in membrane lipids
 alterations in redox state (GSH and NAD(P)H)

 Low temperature

 (1) Increase in ABA
 (2) Release Ca^{2+} to cytosol

 Changes in gene expression

 Antioxidant enzymes

 Synthesize new proteins

 Enzymes involved in cryo-protectant synthesis

 New membrane proteins (desaturase)

 Synthesis of new membrane lipids

 Alter membrane protein functions (pump; channel; redox system)

 Tolerance dehydration

 Avoidance dehydration

 FREEZING TOLERANCE

 Fig. 6.2. A flow chart showing a possible sequence of events leading to cold acclimation as results of increase in freezing tolerance. Results presented in this thesis
References:


Dominion Forest Service Canada (1949). Native Trees of Canada (Ottawa: Kins's Printer and Controller of Stationery).


