DISSERTATION

ROLE OF THE CELL WALL IN FREEZING TOLERANCE OF PLANTS

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY RUSSELL L. WEISER ENTITLED ROLE OF THE CELL WALL IN FREEZING TOLERANCE OF PLANTS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

ROLE OF THE CELL WALL IN FREEZING TOLERANCE OF PLANTS

Acoustic emissions were observed during freezing of woody supercooling (Fraxinus americana, Malus x 'Dolgo', Pyrus communis, and Fraxinus pennsylvanica) and non-supercooling species (Pinus edulis, Pinus ponderosa, and Cornus sericea). Observations of hundreds of samples revealed several consistent responses. Acoustic emissions were in no case associated with the high temperature exotherm (extracellular freezing) and are therefore not a result of ice formation per se. The emissions just preceded the low temperature exotherm in supercooling species and stopped at the same time intracellular freezing was completed (near -40°C). Although some acoustic emissions occurred during freezing of non-supercooling species the quantity was considerably less. The concept of negative turgor as a cause of cavitation of cellular water, detected as acoustic emissions, is proposed to explain these observations.

When etiolated pea (Pisum sativum) cv. Alaska) seedlings were acclimated at 2°C, changes in several cell wall properties were observed. The weight of the cell wall increased by 40% and arabinosyl content doubled while other

glycosyl residues and cellulose remained essentially unchanged. The level of hydroxyproline increased by 80%. Arabinose and especially hydroxyproline are indicators of the glycoprotein extensin. The increase in these components translates to a significant increase in this cell wall structural protein. Measurement of the mRNA for extensin by Northern blot analysis revealed a more than 3-fold increase in the total of all transcripts found (6.0, 4.5, 3.5, 2.6, 2.3, and 1.5 kilobases), while increases in certain transcripts (6.0, 2.6, and 1.5 kilobases) appeared to be especially promoted. The possible structural role of extensin in freezing tolerance and its regulation at the gene level are examined.

Pea epicotyls grown in osmotic stress up to -1.15 MPa imposed by growth in polyethylene glycol solutions were visibly stunted and became 10°C more tolerant to freezing. During this period extensin level in the cell wall increased significantly. When water was withheld from etiolated seedlings the water potential dropped from -0.42 to -1.14 MPa and was coincident with a 7°C increase in freezing tolerance, a 44% increase in cell wall extensin, and a dramatic drop in extensin mRNA. In both water stress treatments there was no specific increase in extensin mRNA transcripts identified at 6.0, 4.5, 3.5, 2.6, 2.3, 1.8, and 1.5 kilobases.

Pea epicotyls that were either wounded or exposed to ethylene showed no clear change in freezing tolerance, or total extensin mRNA, but had a significant rise in cell wall extensin. The same mRNA transcripts were found with the addition of a band at 1.2 kilobases. Interestingly, the 1.5 kilobase transcript was upregulated significantly more than other bands in both wounded and ethylene treated peas.

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DEDICATION

To my parents who always believed in me, and to Tere who showed me how to believe in myself.

TABLE OF CONTENTS

INTRODUCTION

Environmental stresses on plants limit the efficiency of food production and the utilization of marginal agricultural lands. Of the major climatic variables causing stress on plants, temperature and water are the least predictable (Christiansen, 1979), and freezing stress results in particularly spectacular losses. The physiological and meteorological aspects of this problem are such that relatively minor improvements in plant cold hardiness, or the timing of its development, would significantly increase crop productivity (Weiser, 1978). The evolved ability of plants to cold acclimate provides substantial protection to many temperate zone species, but capitalizing on this requires mechanistic understanding (Boyer, 1982). The extent to which this ability is expressed is a composite trait involving coordinated molecular, cellular, and whole plant responses. However, the specific changes responsible for genotype or induced differences in freezing stress resistance are not known. Therefore, more research on plant cell modifications during cold acclimation - especially if focused on specific gene products with potential adaptive significance - is urgently needed. There are recent indications (Guy et al., 1985; Meza-Basso, 1986; Tseng & Li, 1987) that "cold hardiness

genes" may be preferentially expressed under acclimating conditions, but these are unidentified and no clear relationship to acclimation has been shown.

Certain aspects of freezing stress have been effectively described and predominant general features of the freezing process and cell injury seem well accepted (Burke et al., 1976; Levitt, 1980; Lyons et al., 1979; Steponkus, 1984). For example, under usual conditions it appears that initial ice formation occurs extracellularly and thus lowers the water potential there as the temperature continues to decline; the resulting water potential gradient provides the driving force for cell dehydration; and the volumetric contraction that occurs involves cytorrhysis rather than plasmolysis because ice crystals do not readily penetrate the cell wall. Evidence from many studies also supports the general conclusion that the plasma membrane is the primary site of freezing stress injury (Levitt, 1980; Lynch & Steponkus, 1987; Lyons et al., 1979; Steponkus, 1984; Uemura & Yoshida, 1984). However, the nature of plasma membrane damage, how it occurs, and how it is minimized in hardy cells are more widely debated issues.

One view is that freezing stress produces specific membrane lesions as components of sublethal cell injury (Arora & Palta, 1986; Hellergren et al., 1985; Iswari & Palta, 1987; Palta & Li, 1980). Perhaps similarly, Uemura and Yoshida (1986) proposed that injury includes the

decomposition of specific frost susceptible membrane proteins. However, observations of plasma membrane behavior in isolated protoplasts have enabled Steponkus and his coworkers (Dowgert & Steponkus, 1984; Gordon-Kamm & Steponkus, 1984a; Gordon-Kamm & Steponkus, 1984b; Steponkus, 1984) to conclude that freezing injury involves cataclysmic membrane failure. They suggested that non-acclimated cells are either lysed during thawing because plasma membrane vesicles deleted during volumetric contraction are not reincorportated during reexpansion (Dowgert & Steponkus, 1984; Gordon-Kamm & Steponkus, 1984b; Steponkus, 1984); or at lower temperatures, suffer loss of osmotic responsiveness because of dehydration-induced membrane destablization (Dowgert & Steponkus, 1984; Gordon-Kamm & Steponkus, 1984a; Steponkus, 1984).

Since there is little doubt that membranes are the critical sites of cell damage, changes responsible for acclimation should occur in membranes and/or their immediate environment in the cell. In plant cells, this environment includes the cell wall, but there has been very little consideration of wall changes relative to acclimation. This is true despite many classic and contemporary efforts to define the essential cellular changes that minimize the impact of freezing stress. Most of these efforts are well summarized in comprehensive reviews (Levitt, 1980; Steponkus, 1984).

Adaptive changes in the immediate environment of the membrane may alter the stress that occurs or protect membrane sites directly (Lyons et al., 1979). For example, non-specific colligative dilution by neutral sugars and other innocuous solutes is thought to prevent dehydration-induced toxic accumulation of potentially damaging compounds (Lineberger & Steponkus, 1980; Meryman et al., 1977). Another possible type of indirect protection is the freezing inhibition proposed by Olien and Smith (1977, 1981). They suggested that specific polysaccharides in cold hardy plants interfere with ice crystal growth, thereby minimizing injury to membrane sites. Evidence for direct membrane protection through non-colligative action is provided by the work of Volger and Heber (1975) and, more recently, Rosas et al. (1986). Both groups showed that proteins extracted from cold acclimated plants protected thylakoid membranes from damage during <u>in vitro</u> freeze tests. Santarius (1982) concluded that both colligative and specific molecular interactions were responsible for the membrane cryoprotection effects he observed using dextrans. The contents of many protoplasmic constituents have been extensively studied (Levitt, 1980; Steponkus, 1984) relative to cold acclimation and extracellular freezing tolerance. Sugars increase with acclimation in many plants, and sorbitol is typically accumulated during hardening in certain fruit trees. The contents of proline and other

amino acids, soluble protein, and starch have also been related to freezing resistance. However, in spite of these efforts, little is known about specific means by which intracellular components contribute to stress resistance.

Adaptive changes in membranes per se may increase their tolerance of various stresses, and alterations in lipid components have been considered most often in this regard. The acclimation-correlated changes most commonly reported include increased fatty acid unsaturation, increased phospholipid content, reduced phase transition temperature, and general augmentation of membrane systems (Steponkus, 1984). One of the prevailing concepts is that membrane composition - especially fatty acid saturation - changes in a way that confers greater stress resistance by modifying fluidity. However, conflicting results have been obtained and there remains considerable controversy about the occurrence and role of fluidity changes in acclimation (Lynch & Steponkus, 1987; Pomeroy & Raison, 1981; Vigh et al., 1987).

Critical interpretation of membrane acclimation research has been limited because most such analyses have been conducted with mixed membrane preparations. Recent research with purified plasma membrane fractions indicated relatively little change in lipid composition during acclimation (Yoshida & Uemura, 1984). However, results with other plants (Yoshida, 1984b) and from more detailed

molecular species analysis (Lynch & Steponkus, 1987) revealed substantial plasma membrane lipid changes during acclimation. Yoshida and his colleagues (Uemura & Yoshida, 1984) have also found "remarkable" polypeptide changes in the plasma membrane and suggested that glycoprotein-mediated alteration in cell surface functions may be involved in cold acclimation. Furthermore, Yoshida (1984a) concluded that proteins are a major determinant of plasma membrane thermotropic behavior. Although it is clear that major plasma membrane lipid and protein changes accompany cold acclimation, the challenge of "...elucidating the roles of these observed changes..." (Lynch & Steponkus, 1987) remains unmet. Clearly, this is true for all changes that have been described; i.e., the mechanisms of cold acclimation are still not known. However, the multitude and variety of acclimation-related changes are consistent with the notion that a freeze-thaw cycle is a "sequential series of potentially lethal stress barriers which are overcome by individual facets of the cold acclimation process..." (Steponkus, 1984). In other words, the stress of extracellular freezing poses many problems (Olien & Smith, 1981) for the plant cell, and survival (cold hardiness) requires a corresponding set of adaptive changes ("facets of cold acclimation").

There is rationale and some evidence for the idea that one of these "facets" is an altered cell wall. One aspect

of the rationale for this is simply the juxtaposition of the wall and plasma membrane. The cell wall is well-situated to influence the outcome of extracellular freezing vis a vis the plasma membrane. Furthermore, in plant cells, the plasma membrane shares close structural and functional relationships with the cell wall (Darvill & Albersheim, 1984; Johnson-Flanagan & Singh, 1986; Roland, 1973; Yamazaki et al., 1983). The physical association of wall and membrane raises the possibility that these two cell components may not respond independently to such stimuli as the stresses encountered during a freeze-thaw cycle.

There are a number of conceivable ways in which the cell wall and/or its components could contribute to cold hardiness, i.e. help overcome "lethal stress barriers". One of these involves the potential to alter ice crystal formation such that adhesion stress (Olien & Smith, 1977; Olien & Smith, 1981) is reduced. Wall polymers in situ may interact with ice as do soluble plant polysaccharides and fish glycoproteins (Olien & Smith, 1977; Olien & Smith, 1981). Another possibility is that changes in the extent or type of wall-membrane attachments may stabilize this region against the effects of stress. Wallner et al. (1986) observed a cold-induced deposition of callose (ß-1,3-glucan) in suspension cultured pear cells, which typically occurs at the wall-membrane interface. Singh and his co-workers (Johnson-Flanagan & Singh, 1986) showed that the physical

association between the wall and membrane changed during cold acclimation. The number of sites of membrane attachment to the cell wall increased, and they concluded that the wall alters plasma membrane behavior during stress.

The most well-founded rationale for a cell wall role in freezing resistance centers on alteration of its physical properties per se. If - as generally agreed - cell dehydration causes much of the freezing injury, then increased wall rigidity (elastic modulus) could contribute to cold hardiness by resisting, and/or moderating the effects of, freeze-induced cell collapse. As pointed out by Steudle et al. (1977), elastic properties are as important to controlling water relations as the water permeability of the cell membrane. Such a role for increased elastic modulus has been invoked to explain how some plant cells avoid both freezing and dehydration in the presence of ice at -40°C (George & Burke, 1977; George & Burke, 1984; Rajashekar & Burke, 1982). This increase in elastic modulus is thought also to require wall capillary (pore) size small enough to retain water in the liquid state (George & Burke, 1984; Rajashekar & Burke, 1982). The explanation is that cell wall rigidity prevents collapse (and the accompanying dehydration) of ray parenchyma cells in acclimated trees of some species. Hypothesized large negative turgor maintains a metastable equilibrium state and intracellular ice

nucleation temperature of water (George & Burke, 1977; George & Burke, 1984; Rajashekar and Burke, 1982).

Evidence has also been presented for a similar, though less pronounced phenomenon (i.e. freeze-induced negative turgor), in non-woody plant tissues (Anderson et al., 1983; Rajashekar, personal communication). These NMR-based comparisons of freezing curves for tissues exposed to intra- and extracellular freezing indicate that dehydration resistance - probably involving cell wall rigidity - is one component of cold hardiness. Earlier NMR data were thought to show that the cells of cold acclimated tissues lose as much water during extracellular freezing as do those in non-acclimated tissues (Gusta et al., 1975). However, subsequent analysis of NMR freezing curves (especially as obtained for intra - vs. extracellular freezing) has been more precise and indicates clear differences in the kinetics of freeze-induced cell dehydration (Anderson et al., 1983; Rajashekar & Burke, 1982). Therefore, in the range of temperatures which may cause injury via expansion-induced lysis (Steponkus, 1984), increased wall rigidity may protect cells from freezing stress (Anderson et al., 1983; George & Burke, 1977; George & Burke, 1984; Rajashekar and Burke, 1982). That is, if the walls of cold acclimated cells are sufficiently rigid, negative turgor may develop and permit equilibration with extracellular ice at higher cell water content. Clearly, however, a predominant feature of

acclimated cells is an increased capacity to withstand water loss (Gusta et al., 1975; Levitt, 1980; Steponkus, 1984). Thus, any role of the wall must be viewed as only one of possibly many components contributing to the general acclimated condition in plant cells.

In addition to resisting cell collapse, a uniformly rigid wall may also beneficially modify the pattern of freeze-induced volumetric contraction that does occur. The cell collapse which accompanies freezing (Iljin, 1933; Johnson-Flanagan & Singh, 1986; Pushkar et al., 1980; Tao et al., 1983) and drought (Pearce & Beckett, 1987) stress is non-uniform, and may thus cause injurious differential mechanical stress on the membrane (Iljin, 1933; Pushkar et al., 1980; Tao et al., 1983). Such observations indicate that intact "...cells behave differently from isolated protoplasts during... an extracellular freeze-thaw cycle" (Singh & Johnson-Flanagan, 1987). Acclimation-related modifications of the wall, and of its relationship to the plasma membrane, may minimize the injurious mechanical stresses exerted on the membrane during volumetric contraction.

The dynamic nature and structural complexity of the plant cell wall are consistent with its apparently diverse functional roles (Darvill & Albersheim, 1984; Esquerre-Tugaye et al., 1979; Johnson-Flanagan & Singh, 1986; Lee-Stadelmann et al., 1984; Olien & Smith, 1981; Roland, 1973;

Sadava et al., 1973; Stafstrom & Stahelin, 1986; Tierney & Varner, 1987; Wilson & Fry, 1986; Yamazaki et al., 1983). However, structure-function relationships for various wall components are not well understood, and this has probably restricted full consideration of this part of the cell in such processes as stress acclimation. Yet, the influence of the cell wall on water relations and other aspects of stress response have led numerous researchers (Bolanos & Longstreth, 1984; George et al., 1974; Kikuta & Richter, 1986; Pavlik, 1984) to point out the specific need for more information on wall changes as part of acclimation. Although very limited, there have been some relevant findings. For example, numerous reports indicate that not only osmotic, but also elastic adjustment contributes to salt and drought stress acclimation. Increased volumetric modulus of elasticity (probably related to increased wall rigidity) has been detected during acclimation to salt (Bolanos & Longstreth, 1984), drought (Ike & Thurtell, 1981; Melkonian et al., 1982), and freezing (Rajashekar, personal communication) stress. Furthermore, it is known that cell walls undergo thickening during cold acclimation (Levitt, 1980; Li & Palta, 1978). Another related observation is that the walls of cold acclimated cells are much more resistant to the action of polysaccharides than are those of non-acclimated cells (Bartolo et al., 1987; and various personal communications).

The research presented in this dissertation was designed and conducted to further our understanding of the role of the cell wall in freezing tolerance. The results are divided into three sections. The first set of experiments in Chapter 1 is a study of the ultrasonic acoustic emissions emitted by woody stem sections during freezing. The goal of this work was to provide further evidence that negative turgor exists as a result of extracellular ice formation. That is, the work was intended to determine if the dehydrating force from extracellular ice causes cavitation of intracellular water that may be measured as ultrasonic acoustic emissions. Chapter 2 is a study of the cell wall composition changes during cold acclimation. In these experiments peas were cold acclimated at 2°C, and various cell wall components that could have a structural impact in terms of cell wall rigidity were measured to determine if any consistent changes occurred during acclimation. Chapter 3 is a follow up study from the results of Chapter 2. The consistent change observed during cold acclimation was an increase in the amount of extensin. The experiments in Chapter 3 were designed to test the relationship between these two responses. Peas were exposed to treatments designed to increase freezing tolerance and/or extensin content and observe changes in the other factor. If an increase in freezing tolerance can be stimulated by some sublethal stress other than growth in the

cold, does the extensin level also increase? Conversely, if an increase in the level of extensin occurs, does the freezing tolerance increase?

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CHAPTER 1

FREEZING WOODY PLANT STEMS PRODUCES ACOUSTIC EMISSIONS

Summary

Freezing woody stem segments of supercooling and non-supercooling species resulted in acoustic emissions in characteristic reproducible patterns. In the supercooling species examined (*Fraxinus americana*, *Malus* x 'Dolgo', *Pyrus communis*, and *Fraxinus pennsylvanica*), large numbers of acoustic emissions began after extracellular freezing, but before freezing of the supercooled fraction, and ended near -40°C. Acoustic emissions also occurred in species that did not supercool (*Pinus edulis*, *Pinus ponderosa* and *Cornus sericea*), but to a much lesser extent. Cavitation of water within the cells during freezing is discussed as a source of acoustic emissions and possible cause of freezing injury.

Introduction

Plants have numerous mechanisms for avoiding injury when exposed to subfreezing temperatures and consequent extracellular ice formation. Freezing tolerance usually requires tolerance of the cell dehydration which occurs in response to the low water potentials of extracellular ice. The cell collapse which accompanies this dehydration strain is cytorrhysis rather than plasmolysis, i.e. the plasma membrane and cell wall remain in contact during volume

reduction (Levitt, 1980). However, in xylem ray parenchyma of cold hardy supercooling species, both cell dehydration (collapse) and intracellular freezing are avoided despite the formation of extracellular ice (Burke et al., 1976; George & Burke, 1977; George & Burke, 1984).

The mechanism which permits hardy supercooling species to retain liquid water in ray parenchyma cells in metastable equilibrium, and resist collapse, to temperatures as low as the homogeneous nucleation point is not known. However, it is clear that major cell modifications occur during acclimation since species such as apple, although hardy to -40°C in midwinter, may be injured at -2 to -3°C during active summer growth (Burke et al., 1976; Levitt, 1980).

Freezing resistance of supercooling plants has been attributed to lack of nucleating substances for intracellular freezing and to formation of ice growth barriers (Burke et al., 1976). These ideas explain this behavior in fully hardy tissues that show a low temperature exotherm (LTE) at the homogeneous nucleation point, but fail to explain the gradual shift of that LTE to lower temperatures during cold acclimation. Another possible explanation is that equilibrium between supercooled cell water and extracellular ice is achieved through the development of negative turgor in the supercooled cell (George & Burke, 1977; George & Burke, 1984). For this explanation to be valid, fully supercooled (-40°C) cells

would have to withstand a very large dehydrating force since $\psi_{ice} = 1.16(T^{\circ}C)$. Therefore, cell wall rigidity would be critically important in determining whether deep supercooling or dehydration occurs in the cells of extracellularly frozen plant tissue.

Rigid cell walls, however, can prevent cell dehydration only as long as the cohesion of cell water and its adhesion to cell components can also resist this force. When limiting tensions are exceeded, cavitation occurs (Tyree et al., 1984b; Tyree & Dixon, 1986), resulting in expansion of gas bubbles. In a supercooled cell, water itself could cavitate or its adhesion to cellular components could fail; both of these events would reduce the effectiveness of supercooling as a cold hardiness mechanism. Cavitation is a rapid event and would probably result in cell water migrating to extracellular ice since no apparent kinetic barrier to water movement exists based on D_2O studies of shagbark hickory (George & Burke, 1977), and the chemical potential gradient is in that direction.

Numerous studies of primary water stress have led researchers to believe that negative pressures in xylem water cause vapor cavitations which can be detected by the accompanying acoustic emissions (Crombie et al., 1985; Milburn, 1973; Tyree & Dixon, 1983; Tyree et al., 1984a; Tyree et al., 1984b; Tyree & Dixon, 1986). Acoustic emissions (AEs) were observed by Tyree and Dixon (1983) in

water stressed Thuja occidentalis. They concluded that vapor cavitations in xylem vessels were the source of the AEs because: they occurred only when xylem pressure potential (ψ_{xp}) was below -1 MPa; the rate of AEs increased as ψ_{xp} was decreased; and AEs stopped when ψ_{xp} was raised above -1 MPa.

Water under the tensions proposed (40 MPa) for supercooled ray parenchyma cells would also be subject to cavitation, and we reasoned that this might be detected as acoustic emissions during extracellular freezing. In this study, ultrasonic acoustic emissions (cavitations?) in stems of various woody species were monitored during freezing, with simultaneous differential thermal analysis (DTA). The results were consistent with the idea that negative turgor develops after extracellular freezing, supporting the theory that hydrostatic tensions help maintain the supercooled state.

Materials and Methods

One year old stems of white ash (Fraxinus americana), dolgo crabapple (Malus x 'Dolgo'), Bartlett pear (Pyrus communis), pinyon pine (Pinus edulis), and redosier dogwood (Cornus sericea) were collected from trees growing outdoors at the Colorado State University campus on 29 January 1986. Marshall's seedless green ash (Fraxinus pennsylvanica) was harvested in Minnesota on 27 September, 1985. Samples were wrapped in moist paper towels and stored at -20°C until

needed; they were then thawed at 5°C prior to use. The bark was removed from tissue pieces (7 mm x 25 mm), which were then split longitudinally. The acoustic emission mini-transducer was affixed to the flat side of the split section with a rubber band, and the differential thermocouple was similarly attached to the opposite, outer side of the twig section. A reference thermocouple was placed in the freezing chamber, isolated from the sample (Fig. 1.1). Differential thermal analysis (DTA) was used to detect exotherms that resulted from freezing of stem tissue water during cooling. Exotherms were recorded as the difference in temperature between the differential and reference thermocouples as described by others (George & Burke, 1977). Reference temperature (RT), differential temperature (DT), and cumulative acoustic emissions (AEs) were recorded on strip charts during cooling at approximately -1°C/min. The acoustic emissions were detected with equipment supplied by Acoustic Emission Technology Corporation. A model 204B AE testing system was used with a model 140 preamplifier. The preamplifier contained a model FL35 bandpass filter so that only emissions in the 350-700 KHz frequency range were detected. The filter response in this frequency range is very uniform (± 0.75 dB). A model MC500L mini-transducer (5 mm diameter and 3 mm thick) was used to reduce thermal mass and improve differential thermal analysis sensitivity. The

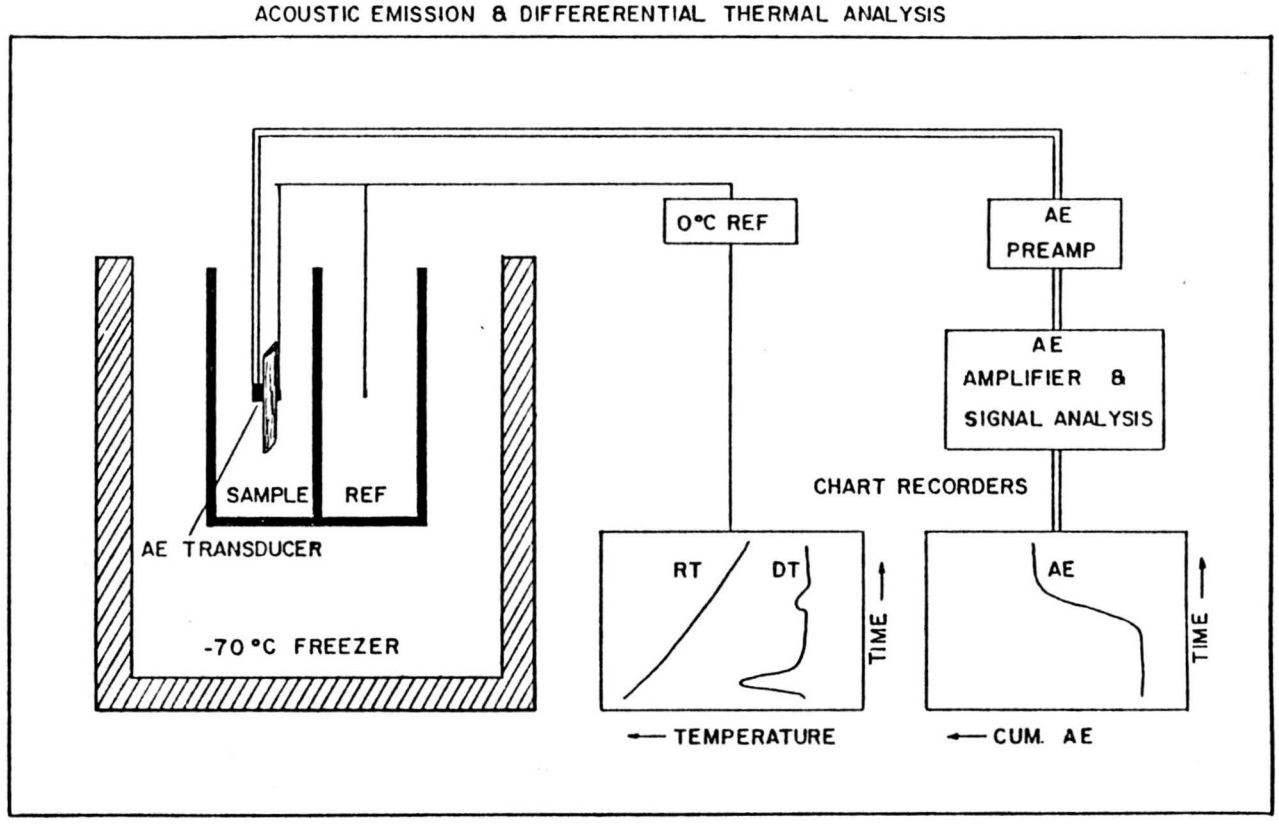

Figure 1.1. Schematic diagram of experimental system for measuring and recording acoustic emissions with simultaneous differential thermal analysis of woody stem sections. RT = reference temperature, DT = differential temperature, and AE = cumulative acoustic emissions.

frequency response of this sensor is shown in Figure 1.2. The overall system gain used was 78 dB.

Results and Discussion

During freezing of supercooling woody stem sections, AEs consistently began after extracellular freezing, marked by the high temperature exotherm (HTE), but before freezing of deep supercooled water, indicated by the LTE (Fig. 1.3 a,b,c). The AEs continued through the LTE and ceased at near -40°C (Table 1.1). The temporal correlation between AE and LTE (Fig. 1.3) was more firmly established by the data in Table 1.2. In these experiments, wood samples were subjected to repeated freeze thaw cycles. The effect of the first freeze-thaw treatment was to substantially lower the temperature at which supercooled water in green ash began to freeze, i.e. the LTE in the second freeze cycle. The green ash stems were only partially acclimated (September 27 in Minnesota), with a LTE at -27°C. However, the LTE occurred at near -40°C during subsequent freezing exposure (freeze cycle 2 and 3, Table 1.2). A corresponding reduction in the temperature at which AEs began during freezing was also observed after the first freeze-thaw treatment. The effect of the first freeze-thaw (i.e. the decline in the temperature at which AE and LTE began) was essentially the same for the two parameters. In contrast, although some AEs occurred during the freezing of non-supercooling species (Fig. 1.3 d,e,f), the initial freeze-thaw treatment did not

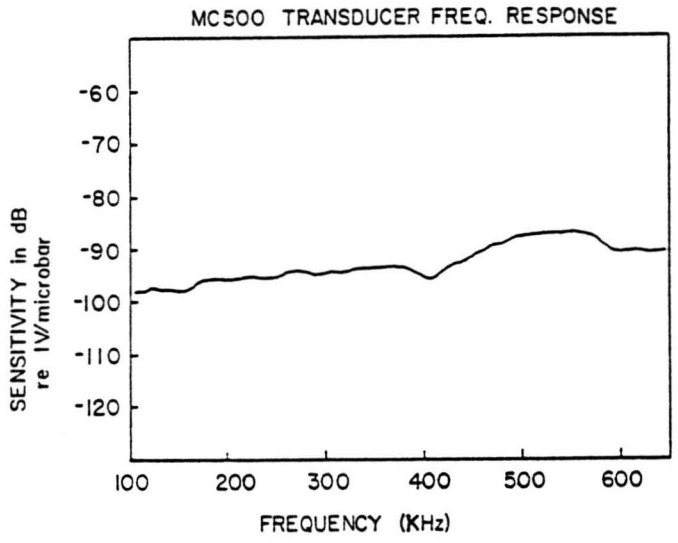

Figure 1.2. Frequency response of MC500 transducer.

Figure 1.3. Representative patterns of acoustic emissions and differential thermal analysis on stem sections of supercooling species (a,b,c) and nonsupercooling species (d,e,f) collected 29 January 1986 from the Colorado State University campus grounds. RT = reference temperature, DT = differential temperature, and AE = cumulative acoustic emissions.

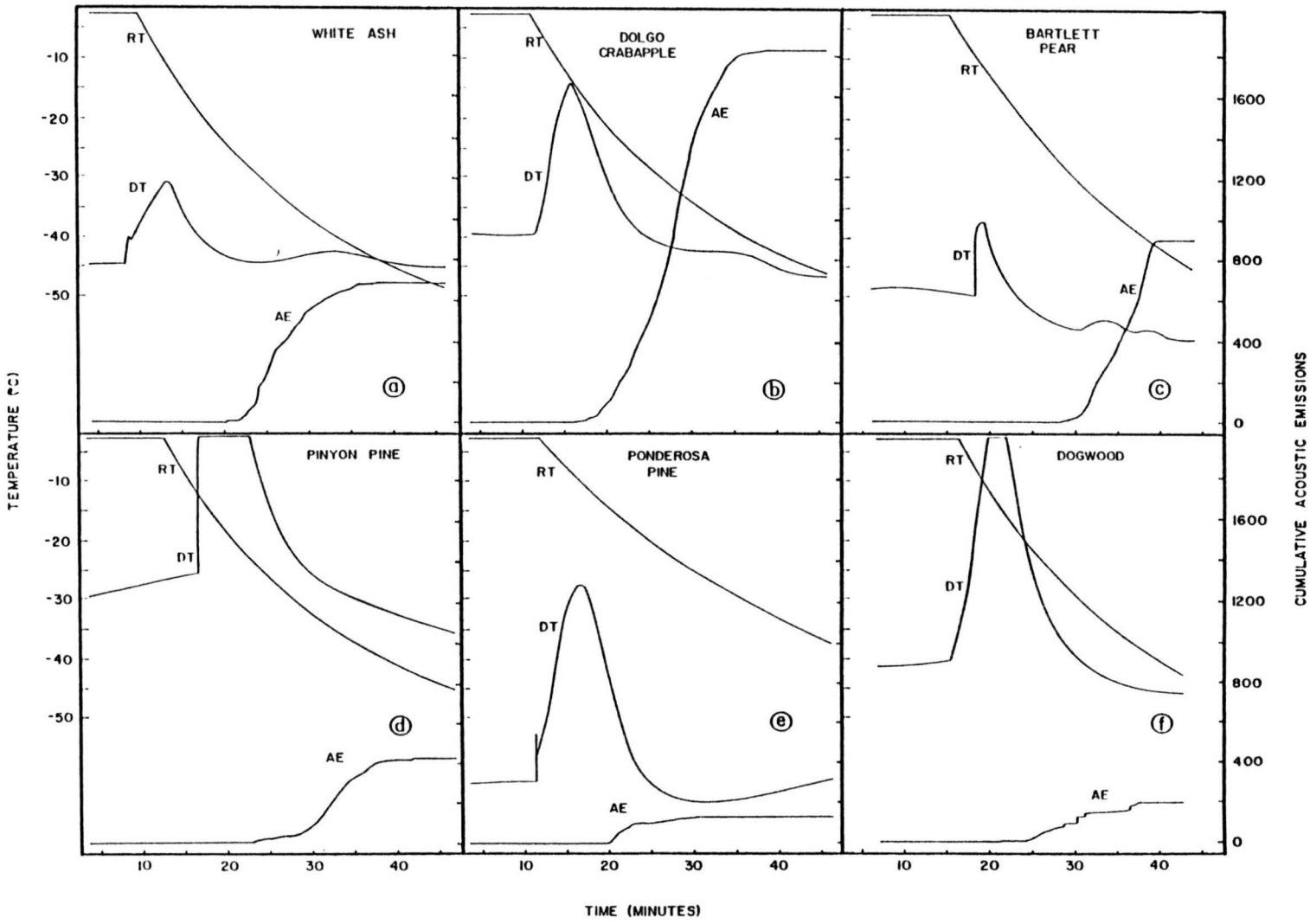
WHITE ASH
DOLGO CRABAPPLE
BARTLETT PEAR
PINYON PINE
PONDEROSA PINE
DOGWOOD
RT
DT
AE
a
b
c
d
e
f
TEMPERATURE (°C)
-10
-20
-30
-40
-50
CUMULATIVE ACOUSTIC EMISSIONS
0
400
800
1200
1600
10
20
30
40
TIME (MINUTES)

Table 1.1 Temperatures at which exotherms and acoustic emissions occurred during the cooling of 'Dolgo' crabapple stem sections. Samples were collected on 29 Jan. 1986. Freezing was initiated at various temperatures (indicated by HTE) by controlling external nucleation.

Starting, ending, or peak temperatures (°C) for exotherms and acoustic emissions

High-Temp Exotherm (HTE)	Low-Temp Exotherm (LTE)		Acoustic Emissions (AEs)	
Start	Start	Peak	Start	End
-2.8	-32.8	-37.6	-13.6	-39.0
-4.2	-33.5	-39.4	-13.9	-39.2
-7.2	-33.1	-38.2	-13.6	-37.0
-8.0	-35.2	-39.9	-17.3	-40.2
-11.2	-34.8	-40.1	-19.3	-38.2
-12.6	-36.0	-40.5	-18.3	-39.5

Table 1.2 The effect of freeze-thaw treatment on the low temperature exotherm (LTE) and acoustic emission (AE) behavior of green ash and red-osier dogwood stems during subsequent freezing cycles.

Starting and peak temperatures (°C) for exotherms and acoustic emissions

Tissue	Freeze Cycle	Low-Temp Exotherm (LTE) Start	Low-Temp Exotherm (LTE) Peak	Acoustic Emissions (AEs) Start	Acoustic Emissions (AEs) Peak
Green ash	1	-27.4	-29.4	-19.7	-43.9
	2	-38.5	-40.5	-28.1	-44.3
	3	-37.2	-39.7	-31.2	-42.9
Dogwood	1	---	---	-21.3	-37.9
	2	---	---	-20.0	-37.5
	3	---	---	-19.3	-38.5

affect the temperature at which AEs occurred in dogwood stems (Table 1.2). The pattern of AE accumulation in all species was similar, i.e. it began after extracellular freezing and was completed near -40°C. However, although the pattern was similar for non-supercooling and supercooling species, the total accumulated counts were consistently and substantially higher for the supercooling stems (Fig. 1.3, Table 1.3).

The observation that initial extracellular freezing (i.e., the HTE) was not accompanied by AEs (Fig. 1.3) was of interest because it indicated that ice formation per se was not a major source of AEs. Many species were examined, but in no case did AEs coincide with initiation of the HTE. Even relatively more energetic freezing (initiated at -10 to -12°C rather than -2 to -4°C) was not accompanied by simultaneous AEs (Table 1.1). Although ice formation may not produce AEs directly, oven-dried stems produced no AEs during freezing to -60°C (data not shown). This indicated that the events producing AEs were in some way associated with the presence of water. If, as suggested (Crombie et al., 1985; Milburn, 1973; Tyree & Dixon, 1983; Tyree et al., 1984a; Tyree et al., 1984b; Tyree & Dixon, 1986), AEs are evidence of cavitation, then data shown in Figure 1.3 is consistent with the idea (George & Burke, 1977; George & Burke, 1984) that negative turgor contributed to maintenance of the supercooled state in ray parenchyma cells of frozen

Table 1.3 Total cumulative acoustic emissions for supercooling and nonsupercooling species collected 29 Jan. 1986.[z]

Supercooling Species			Nonsupercooling Species		
White Ash	'Dolgo' Crabapple	'Bartlett' Pear	Pinyon Pine	Ponderosa Pine	Dogwood
620	1840	920	270	140	90
520	3920	760	110	50	180
680	2560		580	90	70
	1290		400		
	1540				
	1200				
$\bar{x}$ = 607	2058	840	340	93	113

[z]Each datum represents the total acoustic emissions detected during the freezing of one stem segment.

wood. Since AEs began before the LTE but after the HTE, we speculate that the ability to resist cavitation may be important in the cold hardiness of supercooling species. This interpretation is problematic, however, because some AEs also occurred during the freezing of woody stems in which cell water did not deep supercool (Fig. 1.3). This showed that the source(s) of AE also likely included events other than the cavitation of ray parenchyma cell water with consequent ice formation.

One possible source of AEs in both types of wood is the cavitation of water in cell wall capillaries which may resist freezing by development of substantial curvature-induced tensions (Burke et al., 1976; Levitt, 1980). Although this possibility exists throughout the wood (not just in ray parenchyma cell walls), the amount of water involved is likely to be relatively small. Accurate quantitative interpretation of AE data was not possible because so little is known about the nature and propagation of the ultrasonic signal in wood. However, the typically low level of accumulated AEs in non-supercooling species (Fig. 1.3 and data not shown) was consistent with the small amounts of unfrozen water that may exist after the freezing which produces the HTE. The greater accumulation of AEs in wood of the supercooling species represented in Figure 1.3 a,b, and c was probably related to the presence of more liquid water (supercooled ray parenchyma cells) at sub-

freezing temperatures. Previous workers (Burke et al., 1976; George & Burke, 1977) proposed that water in these cells remains in a metastable equilibrium with extracellular ice because large hydrostatic tensions (negative pressure potentials) are imposed. If this is true, then water in xylem ray parenchyma cells is subject to cavitations which could account for the larger numbers of AE counts which were recorded during the freezing of woody stems of deep supercooling species compared to non-supercooling ones. Furthermore, AEs (cavitations?) began before the freezing of supercooled water (i.e., the LTE) begins. Water released by cavitation may migrate to extracellular ice and contribute to the LTE. Although extracellular freezing at temperatures as low as -14°C was not accompanied by simultaneous AEs, we cannot exclude the possibility that some of the AEs associated with the LTE were a direct consequence of rapid ice formation.

It seems unlikely that cells would survive violent cavitation, so the capacity to resist cavitation may be a critical characteristic which develops during cold acclimation. Perhaps the tissue injury sometimes observed prior to the initiation of the LTE is caused by cavitation of a fraction of supercooled cells. In this regard, it is worth noting that the correlation between tissue "killing points" and the LTE are not always consistent or precise. The association of acoustic emissions with water freezing in

woody stems has not been previously reported. The possibility that AEs arise from the cavitation of deep supercooled water merits further study, because cavitation could conceivably limit supercooling and/or be a direct cause of cell destruction.

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CHAPTER 2

CELL WALL AND EXTENSIN mRNA CHANGES DURING COLD ACCLIMATION OF PEA SEEDLINGS

Summary

During exposure to 2°C, pea (Pisum sativum) seedlings cold acclimated to a killing temperature of -6°C. Associated with this increase in freezing resistance was an increase in the weight of cell walls and changes in wall composition. Arabinosyl content increased by 100%, while other cell wall glycosyl residues and cellulose increased by no more than about 20%. The cell wall hydroxyproline content increased by 80%. Arabinose and hydroxyproline are both major components of the structural cell wall glycoprotein, extensin. The increase in these components indicates that the level of extensin in the cell wall increases during cold acclimation. Northern blot analysis, using the pDC5A1 genomic clone as a probe, revealed a more than 3-fold increase in total extensin mRNA during exposure to cold temperature. While specific extensin transcripts of 6.0, 4.5, 3.5, 2.6, 2.3, 1.8, and 1.5 kilobases were identified, those at 6.0, 2.6, and 1.5 kB were especially promoted by low temperature treatment. The rise in extensin during cold acclimation may be regulated, at least in part, at the gene level. The possible structural role of this protein in freezing protection is discussed.

Introduction

During extracellular freezing, plant cells are exposed to stresses associated with dehydration and consequent volume reduction, direct effects of low temperature, and mechanical effects of extracellular ice. The plasmalemma response to protoplast freeze-thaw exposure has been elegantly characterized by Steponkus (1984) and his coworkers, who attribute injury to the effects of dehydration. These characterizations have relied upon the use of isolated protoplasts for ready observation of ideal osmometric behavior. However, others have proposed that cells of intact tissues do not exhibit ideal osmometric behavior and that the cell wall has a role in freezing stress response. For example, Anderson et al. (1983) presented evidence that freeze-induced cell water loss is slowed in a way related to the bulk mechanical properties of the tissue. In this case, and in both salt (Bolanos and Longstreth, 1984) and drought (Ike and Thurtell, 1981; Melkonian, Wolfe, and Steponkus, 1982) stress acclimation, altered cell wall elasticity was considered an important component of the adaptive response. Reports of cell wall thickening during cold acclimation (Huner et al., 1981) are consistent with this idea. In woody plants which deep supercool in the acclimated state, xylem ray parenchyma cell walls appear to resist collapse against extreme dehydrating forces (George and Burke, 1977). Evidence that these forces

may be strong enough to induce cavitation in cells of woody stems was presented in Chapter 1.

Cell wall changes which could significantly alter relevant mechanical properties include increased deposition of the glycoprotein extensin, a change known to be stress-induced (Wilson and Fry, 1986). A structural role for extensin was first proposed by Lamport and Northcote (1960). The glycoprotein they discovered, later named extensin for its role in cessation of extension growth, was found to contain virtually all the hydroxyproline in the cell. Currently, extensin is viewed to contribute to the strength and rigidity of the cell wall by forming an interpeptide-linked network that is separate from, but complementary to the cellulose mesh.

It was hypothesized that if structural changes in the cell wall increase its rigidity during acclimation, thereby limiting freeze-induced cell water loss and resulting injury, an increase in freezing resistance should occur. In this chapter, data are presented that quantify cell wall weight, total glucan, noncellulosic glucan, cellulose, and glycosyl content during acclimation. A pronounced increase in arabinose content led us to suspect an increase in extensin. Cell wall hydroxyproline content and extensin mRNA were measured during acclimation to test this theory.

Materials and Methods

Unless otherwise indicated, the conditions for growth, acclimation, and freezing tolerance measurement were as follows. Experiments were conducted with seedlings of Pisum sativum, cultivars Alaska and Melrose. In most experiments seeds were germinated and seedlings grown in the dark at 22°C, using vermiculite moistened with deionized water. In certain cases peas were germinated and grown in moist paper towels at 26°C. For cold acclimation, seedlings which had grown to a stem length of 2 to 3 cm (4 to 6 days after germination) were transferred to a cold room at 2°C. There was only minimal elongation growth at 2°C. In some experiments, cotyledons were removed to deprive the seedling of energy reserves immediately prior to low temperature exposure. All experiments were done with epicotyl tissues only. The experiments that provided the data shown in Figures 2.1 to 2.5 were conducted by John Waddell from our laboratory who generously allowed me to include it in this dissertation.

Freezing Tolerance

Freeze stress tests were conducted with 1.0 cm or 1.5 cm sections of epicotyl cut just below the apical hook, depending on the experiment. One or two sections were cut from each seedling in a treatment, placed in deionized water, and then randomly distributed to test tubes.

Each tube contained three or four sections and 0.5 ml of deionized water. Either four or eight replicate tubes were used for each test test temperature of each treatment. After equilibration at -2°C within a methanol bath, freezing was initiated by nucleating the water. One hour later the temperature was reduced at a rate of 5°C per hour. After reaching selected stress temperatures, frozen samples were held for 30 minutes to equilibrate, then allowed to thaw at 2°C for 18 hours, after which 3 ml deionized water were added. Estimates of freezing injury were based on measurement of electrolyte leakage using a Radiometer CDM3 conductivity meter. Conductivity data for unfrozen controls and freeze-stressed tissues, both before and after boiling, were used to calculate the temperature at which 45 or 50% injury (LT_{45} or LT_{50}, respectively) occurred.

Cell Wall Isolation

Epicotyl cell walls were obtained using a procedure similar to that described for other tissues (Gross and Wallner, 1977). Following Polytron homogenization in 20 mM PO_4 buffer (pH 6.9) and filtration through Miracloth, the filter residue was thoroughly washed with excess cold buffer. Crude cell walls were then extracted with chloroform: methanol (1:1) and finally with acetone; the cell wall preparations were dried *in vacuo* over P_2O_5 to a constant weight.

Cell Wall Neutral Sugar Composition

The non-cellulosic neutral sugar composition of cell walls was determined using the method of Albersheim et al. (1967). This method includes trifluoroacetic acid (TFA) hydrolysis of cell walls; reduction of monosaccharides to their respective alditols; acetylation to the volatile alditol acetates; and separation/quantification via GLC with inositol as an internal standard. To determine total neutral sugar composition, cell walls were hydrolyzed with 72% v/v H_2SO_4, which yields glucosyl from cellulose.

The alditol acetates were separated on a column of 3% ECNSS-M on Gas-chrom Q (Applied Science Lab). The initial oven temperature was 180°C with a programmed linear increase to 225°C; injection port and FID temperatures were 225°C. The carrier gas was N_2, used at a flow rate of 20 ml/min.

All results described here are representative of experiments which were conducted in essentially the same way a minimum of three times. All replicate experiments gave comparable results. The responses of the two cultivars used ('Alaska' and 'Melrose') were essentially the same.

Cell Wall Hydroxyproline Content

Cell wall content of hydroxyproline was determined using a gas chromatographic method adapted from that of Kaiser et al. (1974) by McNeil (personal communication). Amino acids from wall hydrolysates were converted to the N-heptafluorobutyryl isobutyl ester derivatives and separated

on a DB-1 capillary column (J and W Scientific) with a temperature program of 4 min. at 100°C, then increased at 8°C/min. to 250°C. Injection port and FID temperatures were 300°C. The carrier was helium at a pressure of 100 MPa (corresponding to a flow rate of approximately 0.5 ml/min.).

Extensin mRNA Content

The extensin genomic DNA clone pDC5A1 described by Chen and Varner (1985) was generously provided by Mary Tierney, Biotechnology Center, Ohio State University. Plasmids were isolated using alkaline lysis (Maniatis et al. 1982) and purified by banding two separate times in CsCl-ethidium bromide gradients.

Total RNA from 7 g of epicotyl tissues was isolated by the phenol:chloroform method (Dean et al., 1985) and separated by electrophoresis through 1.2% agarose gels containing formaldehyde (6.5%), 0.2 M MOPS pH 7.0, 50 mM NaOAc, and 5 mM EDTA, pH 8.0. The RNA was then blotted onto nitrocellulose and fixed by 2 minute UV irradiation 12 cm under a germicidal UV light. Prehybridization of bound RNA was carried out at 65°C for 4 h in 6x SSC, 5x Denhardt's solution, 0.1% SDS, and 10 ug/ml denatured herring sperm DNA. Hybridization was accomplished after 18h at 65°C with ^{32}P-labelled pDC5A1 probe added to the prehybridization solution. Nick-translation of plasmids was performed with a kit from Amersham using alpha ^{32}P-dCTP (>800 Ci/mmol).

Following incubation, the filters were washed in 1x SSC with 0.1% SDS at 55°C three times, one hour each as described by Showalter et al. (1985). Autoradiography was performed at -80°C with dual intensifying screens. Quantification was by scanning densitometry of the autoradiograms (Showalter et al. 1985).

Many months were spent solving the technique problems encountered in performing Northern blot analysis of pea extensin using the pDC5A1 probe from carrot. After the failure of numerous experiments because the ^{32}P-labelled probe bound too tightly to the nitrocellulose paper, I visited Dr. Avtar Handa's laboratory at Purdue University where the protocol for prehybridization, hybridization, and washing of the Northern blot were successful and were adopted in all further experiments.

Results and Discussion

Pea epicotyls from seedlings which were germinated and grown at 22°C for 4 to 6 days, and then exposed to 2°C in the dark, gradually cold acclimated to a LT_{50} of -6°C (Fig. 2.1). This level of hardiness was achieved by 20 days at 2°C and was accompanied by an increase in the content of dry matter relative to fresh weight. Non-acclimated epicotyls contained 5.3% dry matter compared to 8.1% for those from acclimated seedlings of comparable size. The accumulation of solutes, membrane augmentation, etc. are typical cellular changes associated with cold acclimation (Levitt, 1980)

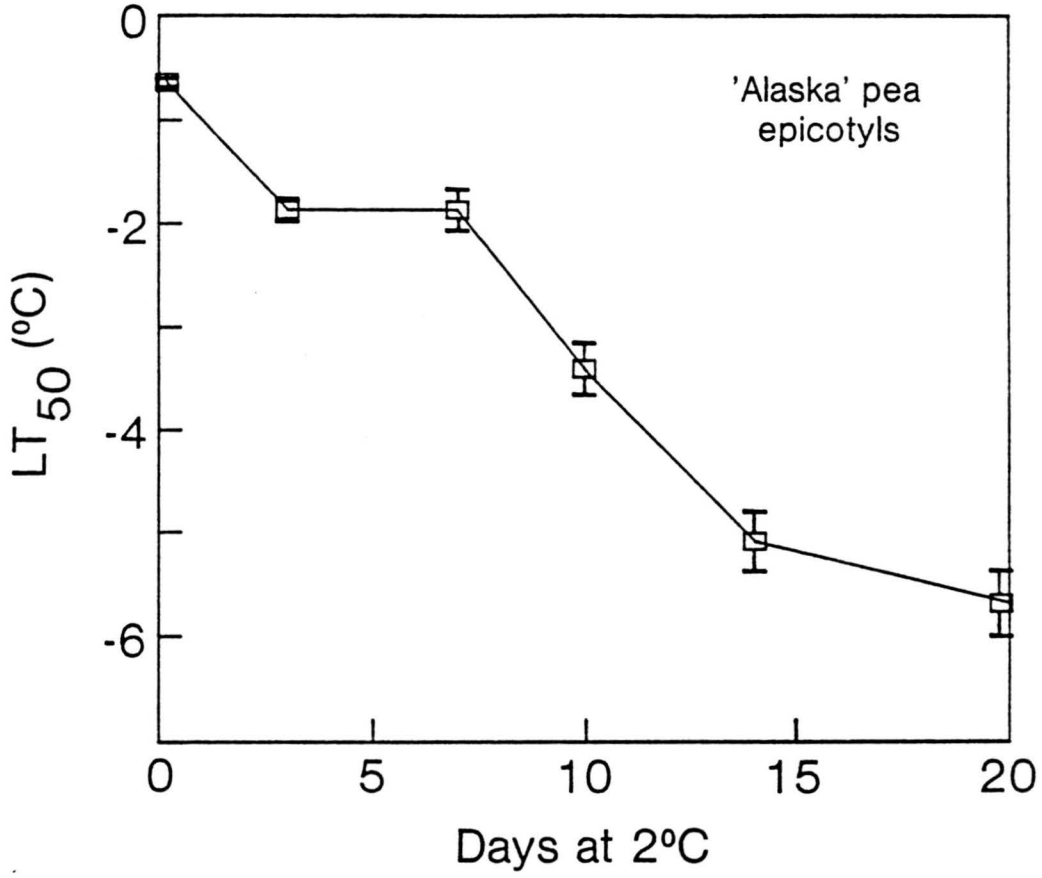


Figure 2.1 Cold acclimation of pea (cv. Alaska) seedlings at 2°C in the dark. Seedlings were grown at 22°C in the dark for 5 days and then transferred to 2°C. The LT_{50} was the freezing temperature which resulted in 50% electrolyte leakage from epicotyl sections after freeze-thaw treatment. Error bars represent ± standard error, n=3. (Source: John Waddell)

which contribute to increased dry matter content. It was observed that some of this increase in pea epicotyls was cell wall material; wall weight per gram fresh weight increased from 12 mg to 17 mg during 20 days at 2°C in the dark. This is consistent with anatomical observations of cell wall thickening during cold acclimation of other tissues (Huner et al., 1981).

During cold treatment there appeared to be an increase in total wall glucan in both cultivars examined (Fig. 2.2). This trend was consistent in three separate experiments. Since non-cellulosic (TFA-hydrolysable) glucan content was relatively low and constant (Fig. 2.2), any increase in total glucan must have been cellulose. The glucan data in Figure 2.2 do not indicate an increase in the β-1,3-glucan, callose. Extracellular callose deposition (observed with aniline blue staining) is a common response to temperature stress (Smith and McCully, 1977). Callose deposition occurred during cold acclimation of cultured pear cells (Wallner, Wu, and Anderson-Krengel, 1986). Either callose deposition was not substantially increased in acclimating pea epicotyls, or it did not remain with the analyzed wall fraction during sample preparation.

The apparent increase in cellulose (Fig. 2.2) was accompanied by a comparable increase in cell wall pectic polysaccharides. Galacturonosyl residues were not measured in this study, but changes in rhamnosyl and galactosyl

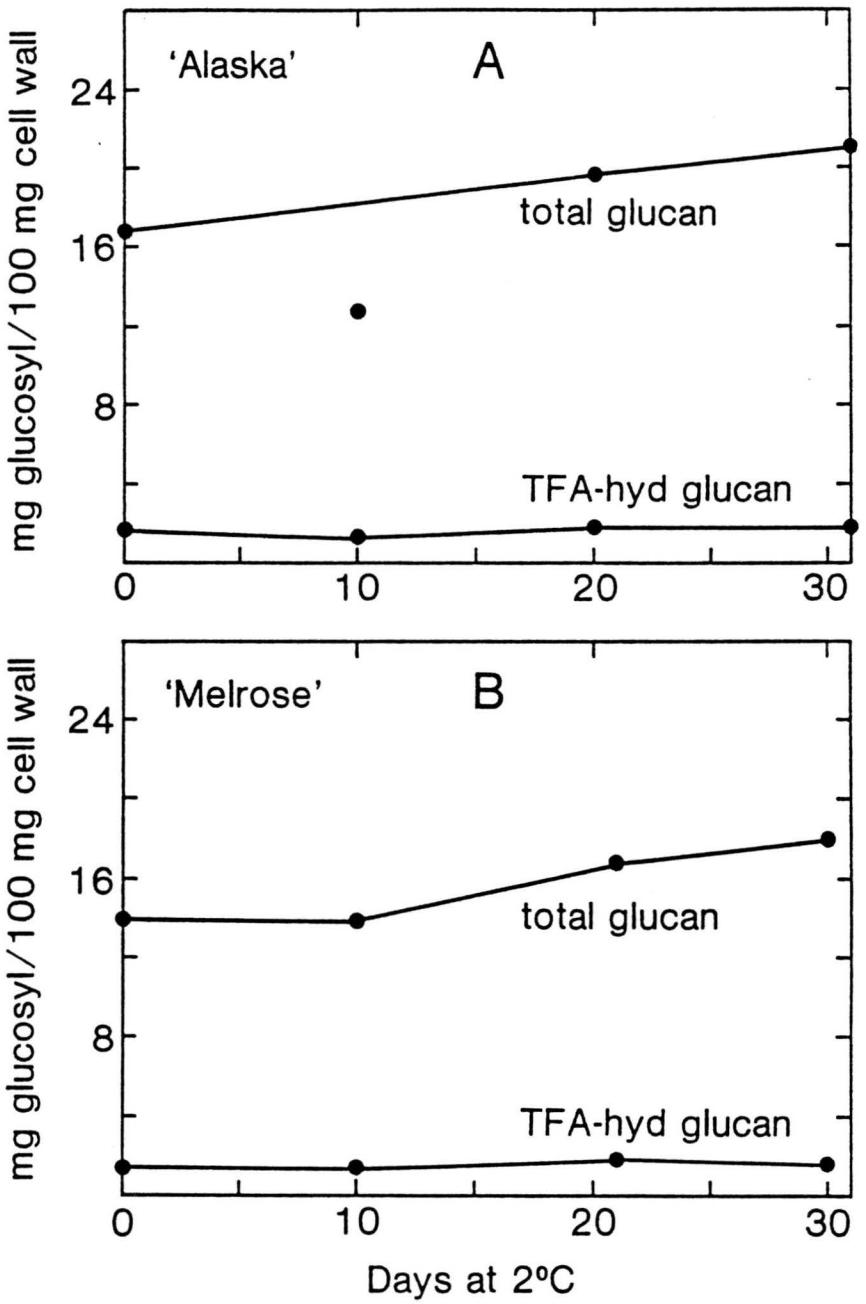


Figure 2.2. Glucan content of 'Alaska' (A), and 'Melrose' (B) pea epicotyl cell walls during cold acclimation. Total glucan was determined from H_2SO_4 hydrolysates; noncellulosic glucan was determined from TFA hydrolysates. In both cases, glucosyl residues were measured by GLC of alditol acetate derivatives. (Source: John Waddell)

content (Fig. 2.3) reflect changes in the pectic polysaccharide fraction (Talmadge et al., 1973). The increases in total wall glucan (Fig. 2.2) and galactosyl content (Fig. 2.3) were both about 20% during 20 days at 2°C, indicating that neither change (cellulose nor pectic polysaccharides) was particularly specific.

In contrast, there was a more pronounced increase in arabinosyl content of cell walls in cold acclimating pea epicotyls (Fig. 2.3). After 20 days at 2°C, the amount of arabinosyl residues in both 'Alaska' and 'Melrose' cell walls doubled. Possible involvement of the low temperature-induced increase in wall arabinosyl content in acclimation is shown by the effect of a treatment which inhibits the development of freezing tolerance (Fig. 2.4). Siminovitch and Cloutier (1982) showed that endosperm removal from rye seedlings inhibited cold acclimation in the dark, perhaps by limiting the "augmentation of protoplasm." Cotyledon removal from pea seedlings prior to low temperature exposure prevented both cold acclimation (Fig. 2.4) and the increased wall arabinosyl content (Fig. 2.5) in epicotyls.

Wall arabinose occurs as side branches to the rhamnogalacturonosyl main chain of pectic polysaccharides (Lamport et al., 1973; Talmadge et al., 1973). As such, some of the large increase in arabinosyl residues can be accounted for in the same way as the increased amounts of galactosyl and rhamnosyl residues, i.e. in pectic

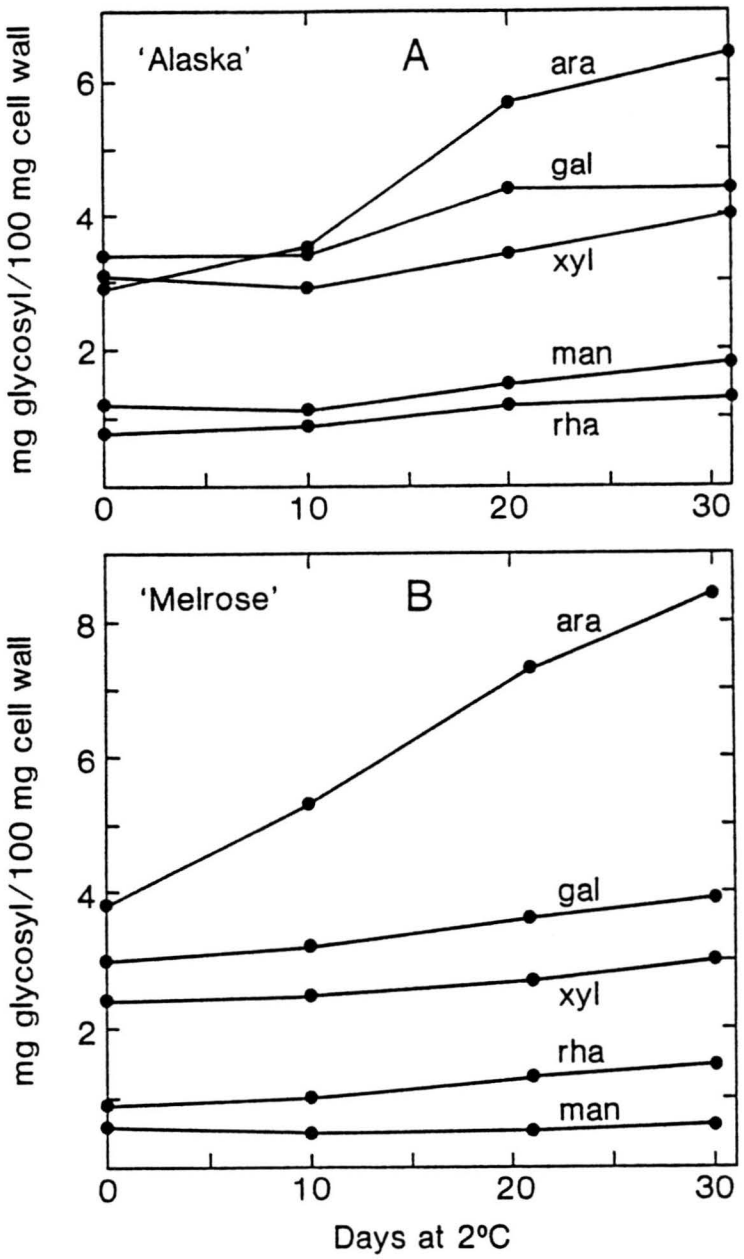


Figure 2.3. Neutral sugar (except glucose) composition of epicotyl cell walls of 'Alaska' (A), and 'Melrose' (B) pea seedlings during cold acclimation. Glycosyl residues were determined by GLC of alditol acetate derivatives. Wall polysaccharide hydrolysis was accomplished with H_2SO_4 (A) or TFA (B). (Source: John Waddell)

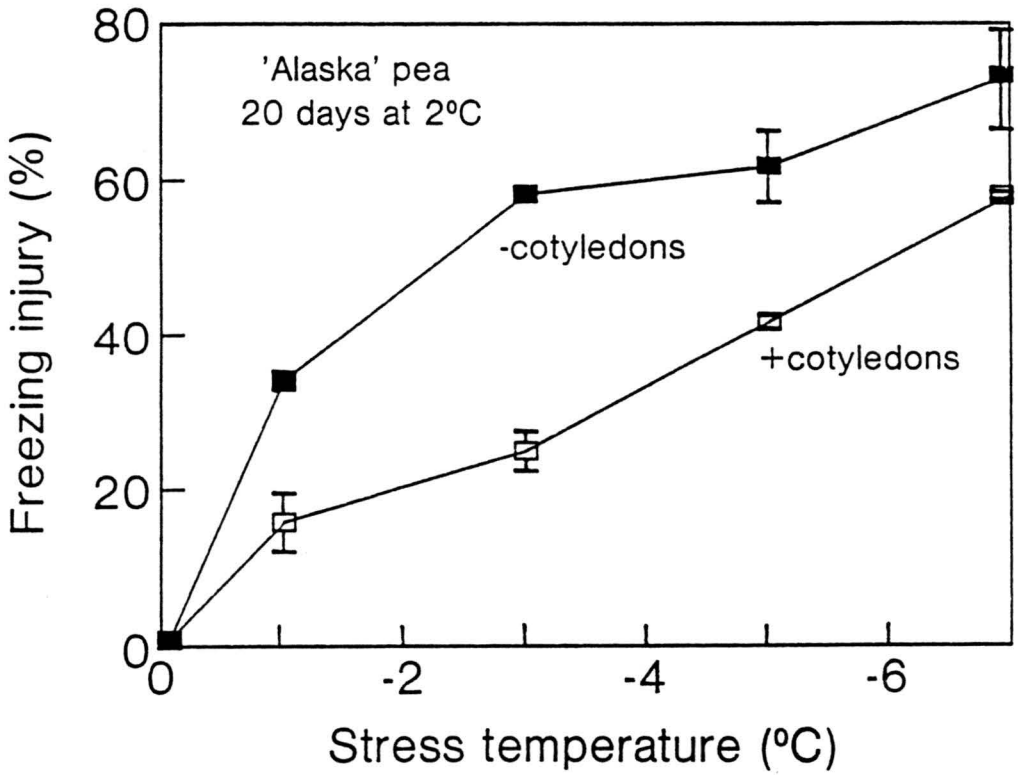


Figure 2.4. Effect of cotyledon removal on cold acclimation of 'Alaska' pea seedlings in the dark. Seedlings were grown in the dark at 22°C for 5 days, cotyledons were then carefully excised and seedlings placed at 2°C for 20 days. Freezing injury at the indicated temperatures was determined by measuring electrolyte leakage from epicotyl sections after freeze-thaw treatment. Error bars represent ± standard error, n=3. (Source: John Waddell)

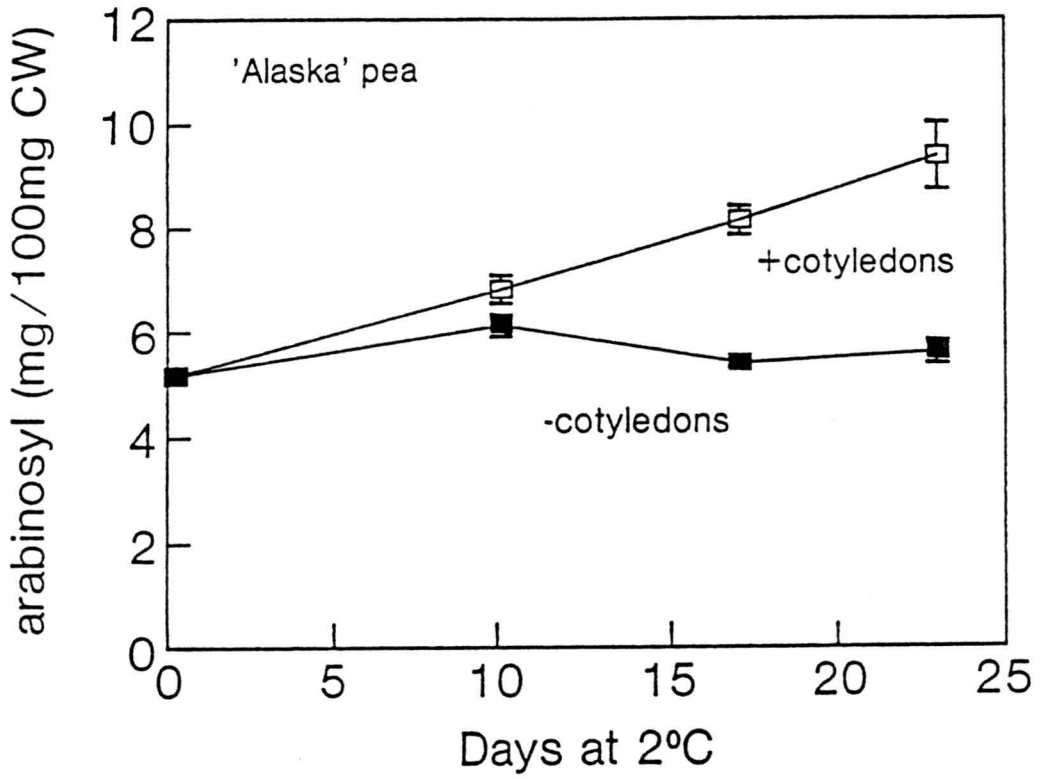


Figure 2.5. Effect of cotyledon removal on the change in arabinosyl content of 'Alaska' pea epicotyls at 2°C in the dark. Seedling treatment was as described for Figure 2.4. Arabinosyl content was determined by GLC of alditol acetate derivatives prepared from TFA-hydrolyzed cell walls. Error bars represent ±standard error, n=3. (Source: John Waddell)

polysaccharides. However, because the increase was specifically greater, much of the arabinosyl increase must be as part of a wall polymer(s) in which it is the predominant neutral sugar component. In addition to pectic polysaccharides, arabinose also occurs in glucuronoarabinoxylan (Darvill et al., 1980), but this polymer is not abundant in dicot walls and contains far more xylose than arabinose, by about 4:1. For these reasons, and since xylosyl residue content increased less than did arabinosyl (Fig. 2.3), it seems that the increase probably occurred in some other wall polymer.

A fraction which could account for a specific increase in cell wall arabinosyl content is the hydroxyproline-rich glycoprotein extensin, since its predominant carbohydrate is arabinose (Lamport, 1967). Extensin is roughly one third peptide, 45% of which is hydroxyproline (Wilson and Fry, 1986). The remaining two thirds is carbohydrate composed largely of arabinose bound to hydroxyproline and a small amount of galactose attached to serine (Van Holst and Varner 1984; Lamport et al., 1973). Galactose occurs as single molecules while arabinose is bound to the peptide backbone as short side chains that interact by hydrogen bonding. The resulting polyproline II helical structure gives extensin its rigid linear nature (Van Holst and Varner, 1984). Isodityrosine linkages formed inter and intra-molecularly bind extensin to the cellulose microfibrils of the wall

(Fry, 1982). The specific increase in cell wall arabinosyl content observed during acclimation suggested that an increase in extensin occurred. To test this hypothesis duplicate samples of pea epicotyls grown at 26°C and acclimated at 2°C were tested for freezing tolerance, cell wall hydroxyproline content, and extensin mRNA level. Three separate experiments were conducted and will hereafter be referred to as experiment 1, experiment 2, and experiment 3.

After 28 days at 2°C peas acclimated an average of 8°C. Cell wall hydroxyproline and extensin mRNA contents increased in all experiments by an average of 75 and 230%, respectively (Table 2.1). Pea epicotyls in experiment 2 had the lowest increase in extensin mRNA and hydroxyproline and also developed the least freezing resistance. A correlation between extensin mRNA and hydroxyproline was also apparent during the acclimation time course (Fig 2.6). Total extensin mRNA more than doubled after 7 days, while hydroxyproline lagged behind showing a larger increase during the second week at low temperature. Lower levels of both were observed in the third week samples. The close temporal correlation between cell wall hydroxyproline and extensin mRNA indicates that cell wall extensin is regulated at the transcriptional level, and suggests the extensin level depends directly upon the mRNA level. As described in the Materials and Methods, 7 grams of epicotyl sections were used to obtain each extensin mRNA data point shown in Figure 2.6, but it is only a single experiment. Two other

Table 2.1 Change in freezing tolerance (LT_{45}), cell wall hydroxyproline content, and amount of extensin mRNA in etiolated 'Alaska' pea epicotyls after 28 days at 2°C for three separate experiments.

Experiment	LT_{45} (°C)	Hyp (% Increase)	Extensin mRNA (% Increase)
1	-8.1	78	245
2	-6.7	69	178
3	-9.1	78	268
$\bar{x}$	-8.0	75	230

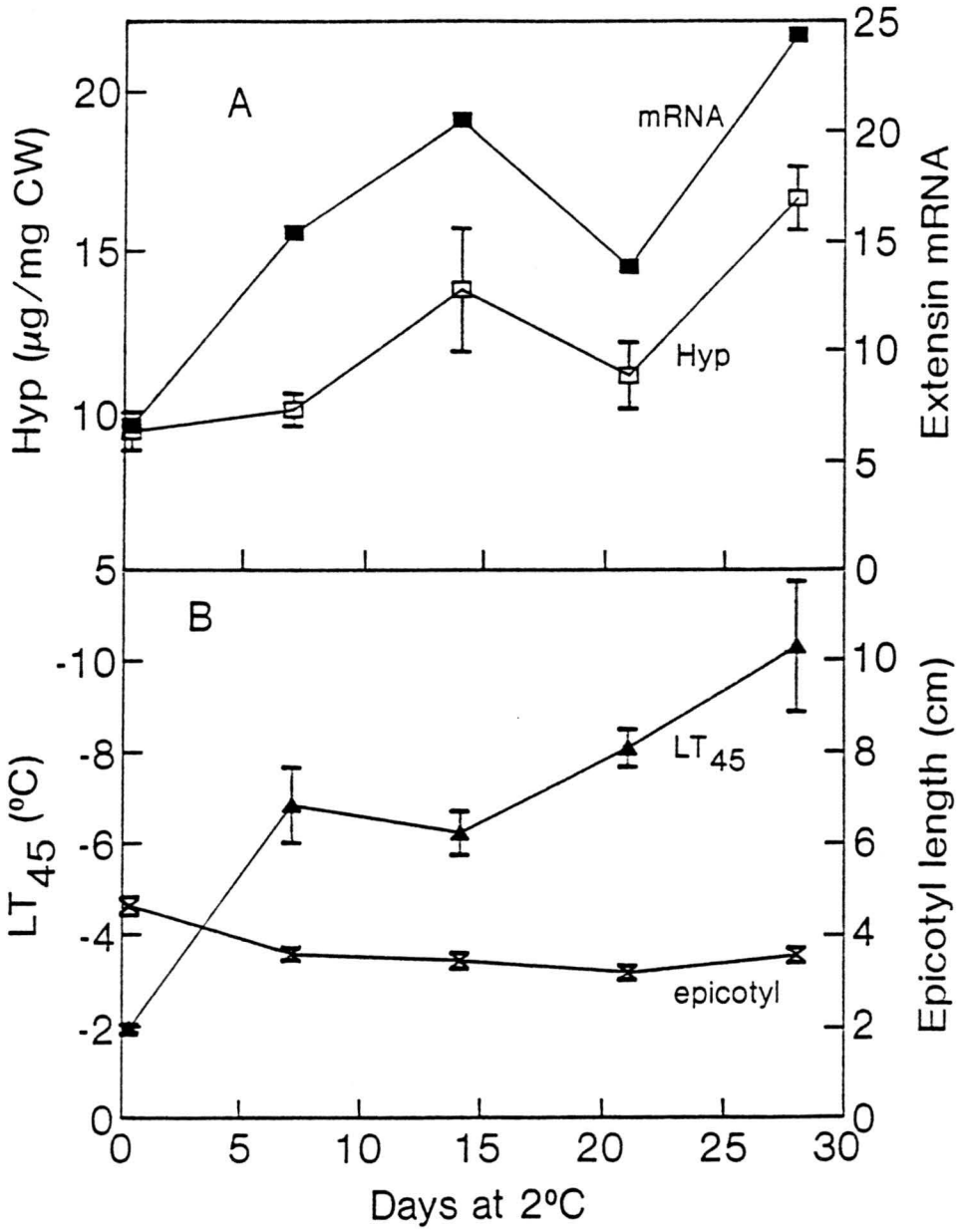


Figure 2.6. Duplicate samples of etiolated 'Alaska' peas from experiment 1 germinated at 26°C and acclimated at 2°C were tested for: (A) Extensin mRNA from Northern blot analysis (values are totals of all the different size bands);cell wall hydroxyproline level measured by gas chromatography of N-heptafluorobutyryl isobutyl ester derivatives (± standard error, n=3); and (B) Freezing tolerance given as lethal temperature for 45% of tissue (LT_{45}) as measured by electrolyte leakage (± standard error, n=8); and average epicotyl length (± standard error, n ≥ 50).

experiments it were conducted and the means for each parameter (epicotyl length, LT_{45}, extensin mRNA, and cell wall hydroxyproline) during the time course were normalized. These results are shown in Figures 2.7, 2.8, 2.9, and 2.10 and clearly strengthen the relationship between these responses.

Since total RNA was used in the Northern blot analyses the amount of poly A^+ RNA was not known. If the ratio of poly A^+ to total RNA increased during acclimation at 2°C, then the increase in mRNA for extensin could be the result of a higher fraction of poly A^+ RNA. However, it was found that the amount of total RNA per gram fresh weight was constant (data not shown) and the ratio of poly A^+ to total RNA decreased slightly (results from one experiment, data not shown). Therefore, the increase in extensin mRNA apparent in Fig. 2.11 is, if anything, less pronounced than it should be.

Northern blot analysis revealed a maximum of 10 different size mRNA molecules that hybridize with the extensin gene (Fig. 2.11). Other researchers (Chen & Varner, 1985) have found multiple bands for extensin occur in carrot roots. The origin and function of the different size extensin transcripts is not known, but Tierney and Varner (1987) speculated that individual genes may be regulated by different environmental signals. Clearly, one of these signals for pea seedlings is low temperature. The

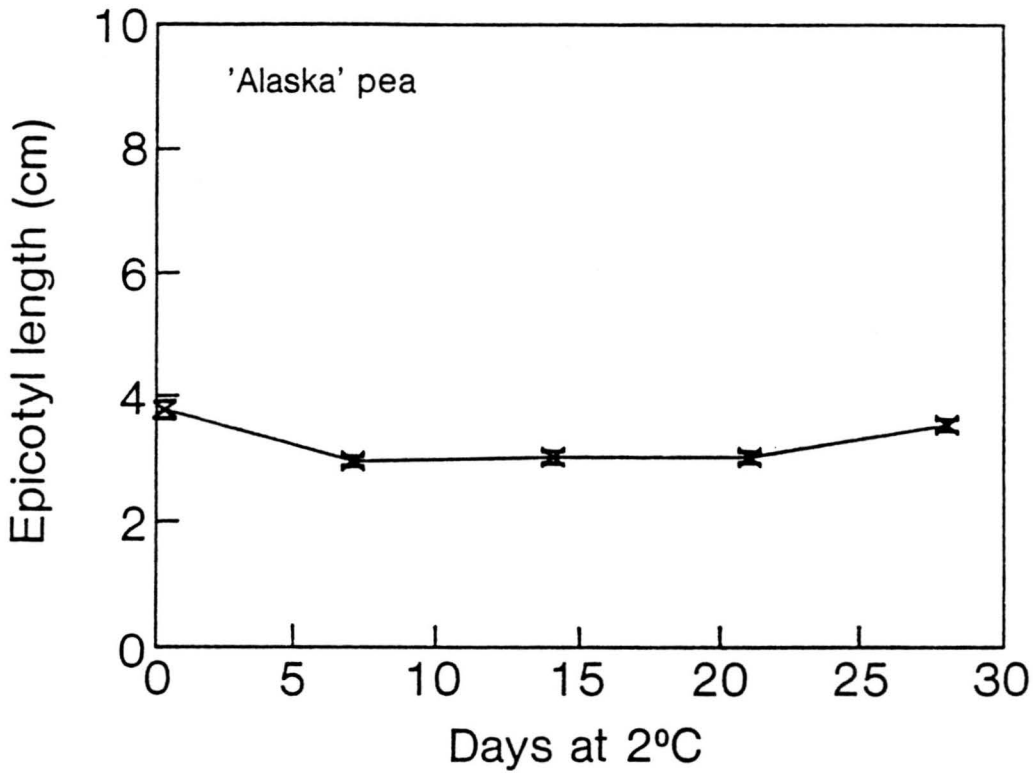


Figure 2.7. Mean of 'Alaska' pea epicotyl length for experiments 1-3. Epicotyls were measured from cotyledon to apical hook (± standard error, n ≥ 125).

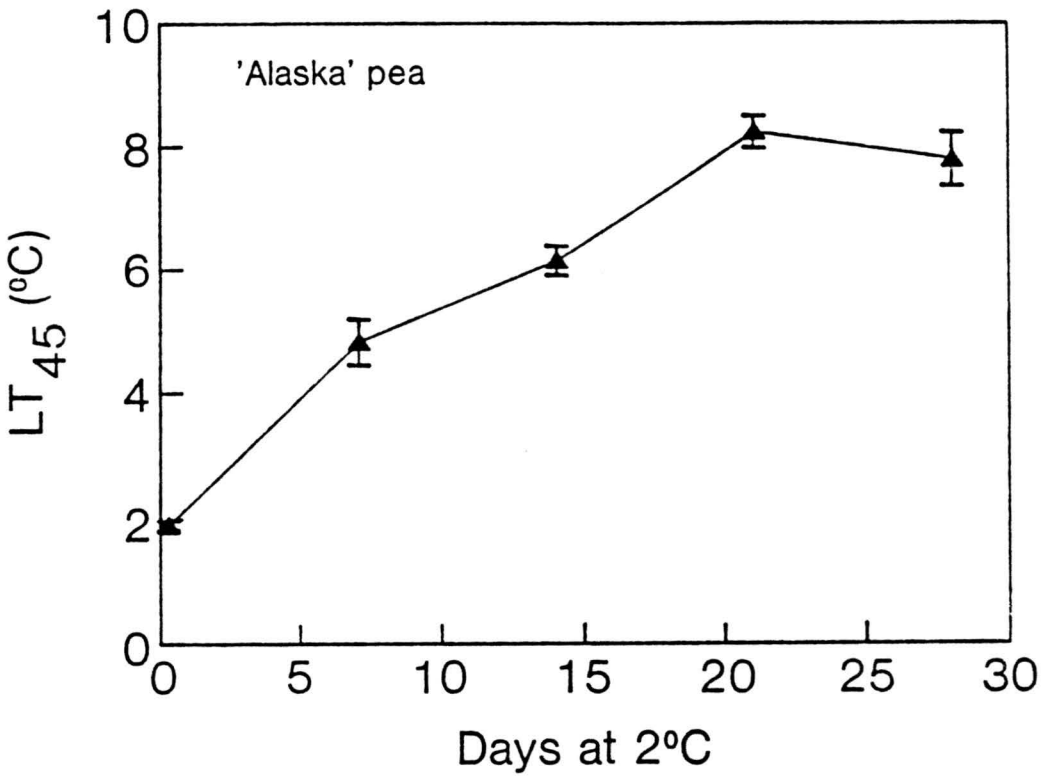


Figure 2.8. Freezing injury (based on electrolyte leakage) of 'Alaska' peas germinated at 26°C and acclimated at 2°C. Leakage was measured at 4 test temperatures (unfrozen, -3, -6, and -9°C); The LT_{45} value (in °C) was calculated by linear extrapolation between the test temperatures that bracket 45% injury. Mean of LT_{45} from three separate experiments whose replicates are treated as paired samples is presented (± standard error, n=20).

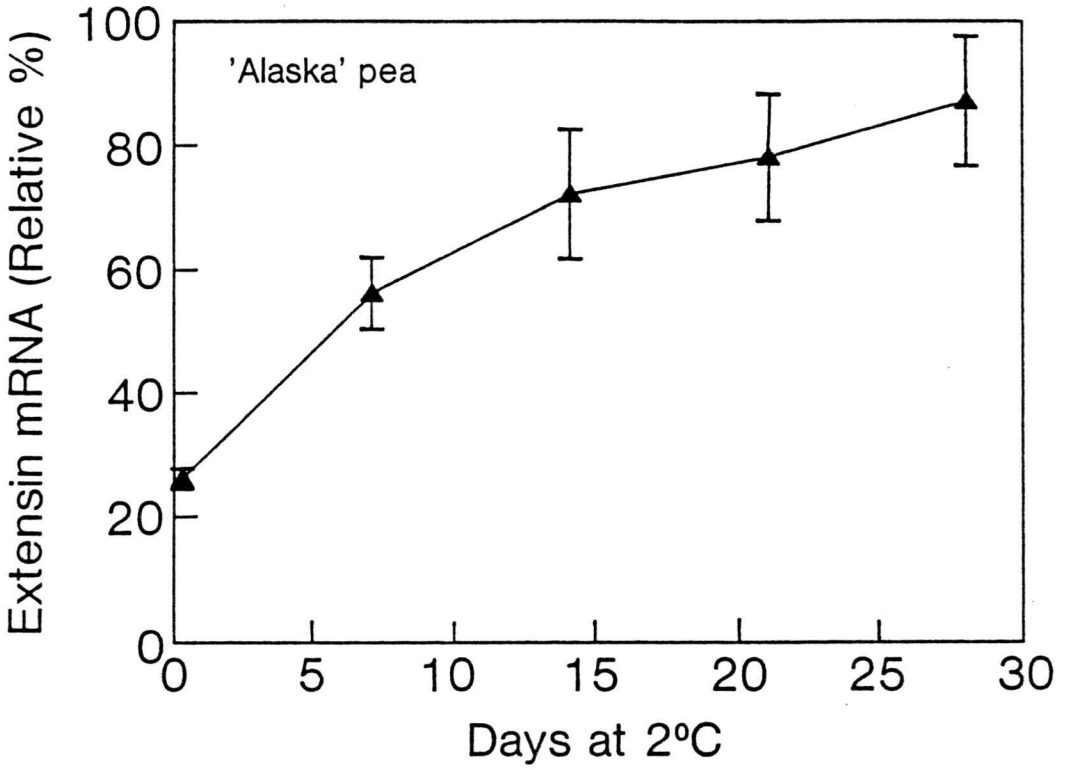


Figure 2.9. Extensin mRNA from etiolated 'Alaska' peas germinated at 26°C and acclimated at 2°C was visualized by Northern blot analysis (^{32}P genomic clone pDC5A1) and quantified by scanning densitometry of autoradiograms. Densitometer scans from experiments 1-3 were normalized for comparison (mRNA totals from each treatment were calculated as a percent of the treatment value with the highest amount of mRNA); Mean of normalized values are presented (± standard error, n=3)

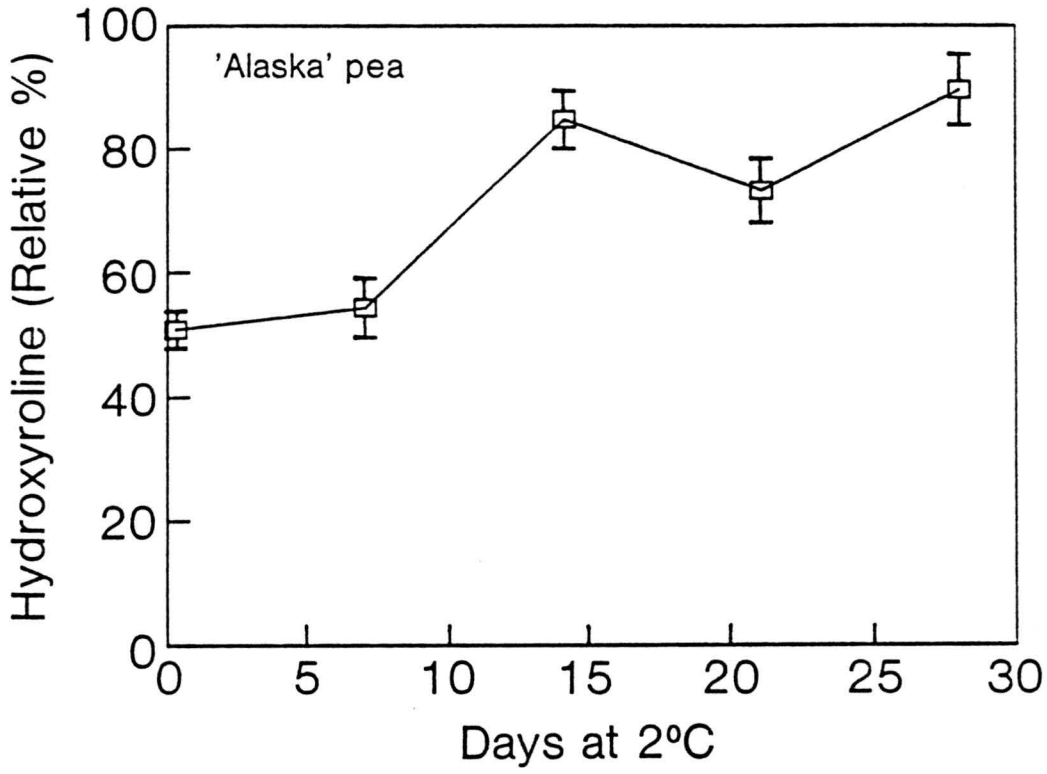
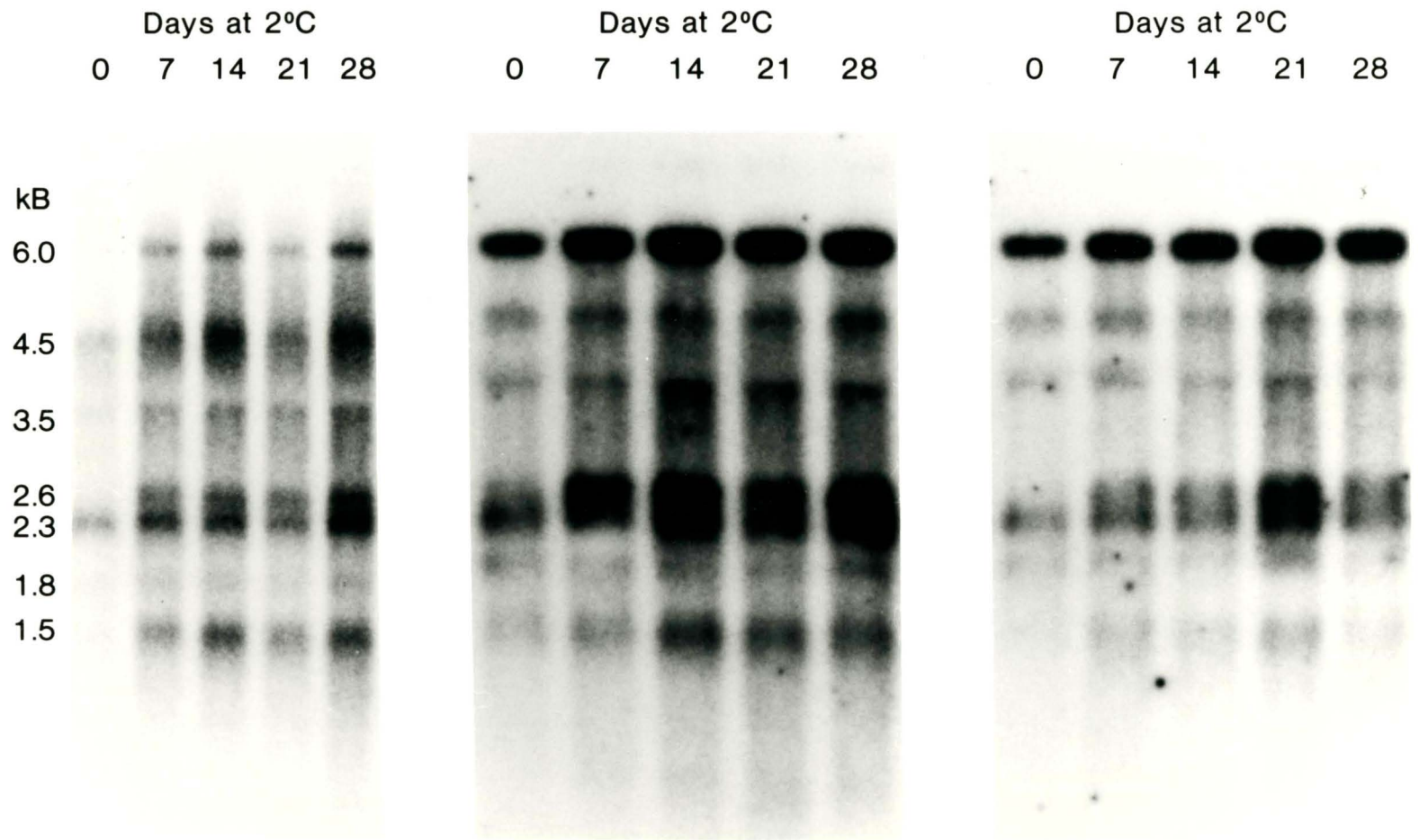


Figure 2.10. Cell wall hydroxyproline from etiolated 'Alaska' peas germinated at 26°C and acclimated at 2°C. Hydroxyproline was measured by gas chromatography of n-heptafluorobutyryl isobutyl ester derivatives from experiments 1-3. Three replicates from each experiment made a total of nine replicates used. Data from each replicate was normalized (calculated as a percent of the treatment with the highest value) before calculation of the overall mean (± standard error, n=9).

Figure 2.11. Northern blot analysis from (A) experiment 1, (B) experiment 2, and (C) experiment 3 of 'Alaska' peas germinated at 26°C and acclimated at 2°C. Total RNA hybridized with the extensin genomic DNA clone pDC5A1 labelled with ^{32}P and visualized by autoradiography. Mean size (kilodaltons ± standard error) of the individual bands were: 6.0 ± 0.15, 4.5 ± 0.15, 3.5 ± 0.11, 2.6 ± 0.02, 2.3 ± 0.05, 1.8 ± 0.05, 1.5 ± 0.04.


Days at 2°C
0 7 14 21 28
kB
6.0
4.5
3.5
2.6
2.3
1.8
1.5
(a)
Days at 2°C
0 7 14 21 28
(b)
Days at 2°C
0 7 14 21 28
(c)

inductive effect of the 2°C treatment, especially on the 1.5, 2.6, and 6.0 kB transcripts, was most striking in the Northern blot of experiment 1 (Fig 2.11 A). Experiments 2 & 3 also showed that low temperature enhanced the amount of the 1.5, 2.6, and 6.0 kB transcripts (Fig 2.11 B and C).

Because extensin mRNA and the amount of hydroxyproline in the cell wall both show a similar increase during a time course of cold acclimation and among experiments, it is reasonable to suggest that the amount of extensin in the cell wall is largely regulated at the gene level.

Although cold acclimation occurs during this same time period, the pattern is not the same as the increase in extensin. Specifically, much of the acclimation occurs during the first week of cold temperature exposure while cell wall extensin does not significantly increase. This result is not inconsistent with an adaptive role for extensin since cold acclimation is a complex series of events that may include membrane changes, osmotic adjustment, etc. (Levitt, 1980). Also, Lamport (1967) pointed out that a small amount of extensin could crosslink to a large amount of cell wall polysaccharide, so small changes induced early in acclimation may have a disproportionately large effect.

In conclusion, when pea epicotyls were acclimated at 2°C both extensin gene transcription and product incorporation in the cell wall increased. When cotyledons

were removed, exposure to low temperature did not increase freezing resistance or arabinose content. Increased wall extensin appeared to closely follow the increase in extensin mRNA and freezing resistance increased during this time. These results suggest that extensin may play an important structural role in the acclimation of pea, perhaps by increasing rigidity of the cell wall and thereby increasing resistance to collapse caused by freeze-induced dehydration.

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CHAPTER 3

CHANGES IN EXTENSIN IN RESPONSE TO ETHYLENE, WATER STRESS, AND WOUNDING OF PEA EPICOTYLS AS RELATED TO FREEZING INJURY

Summary

Pea epicotyls grown in osmotic stress up to -1.15 MPa (imposed by growth in polyethylene glycol solutions) were visibly stunted and became 10°C more tolerant to freezing. During this period extensin level in the cell wall increased. When water was withheld from etiolated seedlings, the water potential dropped from -0.42 to -1.14 MPa and was coincident with a 7°C increase in freezing tolerance, a 44% increase in cell wall extensin, but a dramatic drop in total extensin mRNA. In both types of water stress treatment, there was no specific increase in extensin mRNA transcripts identified at 6.0, 4.5, 3.5, 2.6, 2.3, 1.8, and 1.5 kB.

Pea epicotyls that were either wounded or exposed to ethylene showed no clear change in freezing tolerance or total extensin mRNA, but had a substantial rise in cell wall extensin. The same mRNA transcripts were found and there was also a band at 1.2 kB. Interestingly, the 1.5 kB transcript was upregulated significantly more than other bands in both wounded and ethylene treated peas.

Introduction

Most research on freezing stress tolerance of plant cells has focused on membrane behavior during a freeze-thaw

cycle. Membranes must remain intact and functional during freezing, when extracellular ice formation challenges plant cells by exposure to extreme desiccating forces (George & Burke, 1977). Steponkus (1984) has shown that the plasmalemma of acclimated protoplasts are better adapted to expansion and contraction than non-acclimated protoplasts. Many investigators have demonstrated the critical role of the plasmalemma in the cell's ability to resist the effects of freezing stress. In contrast, the importance of cell walls to freezing stress resistance has received limited attention. However, various approaches indicate that cell wall elasticity modification may be an important component of adaptation to various stresses (Bolanos & Longstreth, 1984; Ike & Thurtell, 1981; Melkonian et al., 1982). The rigid cell walls of xylem ray parenchyma cells may provide an example of a stress-related structural role. George and Burke (1977) found that these cells, in certain species, resist collapse even upon exposure to the -46 MPa chemical potential imposed by extracellular ice at -40°C. As shown in Chapter 2, acoustic emissions occur during lethal freezing treatment of supercooled xylem parenchyma. Since acoustic emissions indicate cavitation (Tyree et al., 1984; Tyree & Dixon, 1986), these results are consistent with the idea that large negative turgor is a characteristic of deeply supercooled cells in woody plant tissue.

Anderson et al. (1983) have also suggested that increased resistance to collapse may be a component of cold acclimation for herbaceous plant tissues. Under this assumption, cell wall alterations are likely to contribute to the acclimation process. Wall changes that could influence mechanical properties include increased deposition of the glycoprotein extensin, a change known to be stress induced (Wilson & Fry, 1986). The glycoprotein that Lamport and Northcote (1960) discovered and later named extensin contains nearly all the hydroxyproline in the cell. Extensin is thought to contribute to cell wall strength and rigidity through crosslinks in an interpeptide-linked network among the cellulose microfibrils (Wilson & Fry, 1986).

The results of Chapter 2 show that extensin content increased in pea epicotyls during cold acclimation. Extensin mRNA and cell wall extensin both increased, indicating extensin regulation at the gene level. The increase in extensin coincident with improved freezing tolerance is only suggestive of a role in acclimation; obviously, the increase may simply be coincident with, not essential for, the development of freezing tolerance.

Therefore, our subsequent experiments, described in this chapter, were conducted to help clarify the relationship between cold acclimation and cell wall extensin. The approach was to take advantage of possible

cross-adaptive effects of various treatments other than low temperature. Treatments known to induce extensin production and/or increased stress tolerance were used to test the correlation between extensin content and freezing tolerance.

Desiccation stress has been found to increase freezing tolerance of winter cereals (Siminovitch & Cloutier, 1982; Cloutier & Andrews, 1984) to a similar degree as that induced by several weeks of cold acclimation. Red-osier dogwood (Cornus sericea Michx.) (Chen et al., 1977), and cabbage leaves (Brassica capitata L.) (Cox & Levitt, 1976) can also be cold acclimated at room temperature by imposing water stress. We proposed to determine if pea epicotyls could be cold acclimated by water stress, and if so, whether the same changes in extensin content occur as with the low temperature acclimation response.

Ethylene exposure causes pea cell wall extensin content to increase (Eisinger, 1983), and ethephon treatment was shown to improve the freezing tolerance of sweet cherry buds (Proebsting & Mills, 1976). Thus, ethylene was also selected as a treatment for the study reported here.

A third treatment chosen was mechanical wounding because it stimulates ethylene production in a variety of plant tissues. Also, previous work in our laboratory (Bartolo, 1987) showed that wounding caused correlative effects on freezing tolerance and cell wall properties.

We hypothesized that if structural changes in the cell wall increase its rigidity during acclimation, thereby limiting freeze-induced cell water loss and related injury, some increase in freezing resistance should occur, regardless of the acclimation treatment.

Materials and Methods

Plant materials

Pea (Pisum sativum, cv. Alaska) seeds were germinated on moist paper towels in a dark growth chamber at a constant 26°C. After germination, seedlings were exposed to one of the following sublethal stress treatments and then held in darkness at 26°C, unless otherwise indicated. All experiments were done with epicotyl tissues only.

Osmotic stress was provided by growing seedlings in polyethylene glycol (PEG) with a molecular weight of 6000-7500. Concentration of PEG was 150, 200, or 250 g/1000 g water to provide water potentials of -0.45, -0.73, or -1.15 MPa, respectively, as measured by freezing point depression (Steuter et al., 1981).

Dehydration stress was imposed by withholding water from seedlings germinated and grown in vermiculite. Dehydration became progressively more severe and samples were taken at 3, 5, 7, or 9 days after water was withheld. A portion of the data (water potential, LT_{50}, and hydroxyproline) in Figure 3.2 was from experiments conducted

by Boyang Chu from our laboratory who kindly allowed inclusion of them here.

Wounding was accomplished by piercing the uppermost 1.5 cm of the epicotyls at a density of 190 holes per cm^2 with a device made from straight pins. The seedlings were left otherwise intact so that the cotyledons provided a carbohydrate source while the seedlings were incubated for 12, 24, or 36 h prior to sampling.

Ethylene treated seedlings were grown at 22°C in airtight glass jars filled with 50 ppm ethylene in air. The ethylene/air mixture was replaced every 24 h to limit changes in the CO_2/O_2 ratio. Seedlings were grown for either two or four days after the ethylene was added.

Water Potential

Water potential of epicotyl tissues was measured by thermocouple psychrometry. Calibration was by vapor equilibration against NaCl solutions. Temperature was maintained constant at 25°C (in a water bath) for both equilibration and measurement. The equilibration and cooling times for measurement of samples were 3 hours and 5 seconds, respectively. Water potential was measured for eight epicotyl segments (using the 1.5 cm region just below the apical hook) simultaneously.

Freezing Tolerance

Freeze stress tests were conducted as described previously (see Chapter 2), using 1.5 cm sections of epicotyl. One section was cut from each seedling in a treatment, placed in deionized water, and then randomly distributed to test tubes.

Each tube contained four sections and 0.5 ml of deionized water. After equilibration at -2°C, freezing was initiated by nucleating the water. One hour later, the temperature was reduced at a rate of 5°C per hour; after reaching selected stress temperatures, frozen samples were held for 30 minutes to equilibrate, then allowed to thaw at 2°C for 18 hours, after which 3 ml deionized water were added. Estimates of freezing injury were based on measurement of electrolyte leakage using a Radiometer CDM3 conductivity meter. Conductivity data for unfrozen controls and freeze-stressed tissue, both before and after boiling, were used to calculate the temperature at which 45 or 50% injury (LT_{45} or LT_{50}, respectively) occurred.

Cell Wall Isolation

Epicotyl cell walls were obtained using a procedure similar to that described for other tissues (Gross and Wallner, 1979). Following Polytron homogenization in 20 mM PO_4 buffer (pH 6.9) and filtration through Miracloth, the homogenate was thoroughly washed with excess cold buffer. Crude cell walls were then extracted with chloroform:

methanol (1:1) and finally with acetone; the cell wall preparations were dried *in vacuo* over P_2O_5 to a constant weight.

Cell Wall Hydroxyproline Content

Cell wall hydroxyproline content was determined using a gas chromatographic method adapted by McNeil (personal communication) from that of Kaiser et al. (1974). Amino acids from wall hydrolysates were converted to the N-heptafluorobutyryl isobutyl ester derivatives and separated on a DB-1 capillary column (J and W Scientific) with a temperature program which included 4 minutes at 100°C, then an increase of
8°C/min. to 250°C. Injection port and FID temperatures were each 300°C. The carrier was helium at a pressure of 100 MPa (corresponding to a flow rate of approximately 0.5 ml/minute).

Extensin mRNA Content

These methods were essentially the same as those used in Chapter 2. The extensin genomic DNA clone pDC5A1 described by Chen and Varner (1985) was generously provided by Mary Tierney. Plasmids were isolated using alkaline lysis (Maniatis et al. 1982) and banded twice in CsCl-ethidium bromide gradients.

Total RNA from 7 g samples of epicotyl tissues was isolated by the phenol:chloroform method (Dean et al., 1985)

and electrophoresed through 1.2% agarose gels containing formaldehyde (6.5%), 0.2 M MOPS pH 7.0, 50 mM NaOAc, and 5 mM EDTA pH 8.0. RNA was then blotted onto nitrocellulose and fixed by 2 minute UV irradiation 12 cm below a germicidal UV light. Prehybridization of bound RNA was carried out at 65°C for 4h in 6x SSC, 5x Denhardt's solution (Maniatis et al., 1982), 0.1% SDS, and 10 ug/ml denatured herring sperm DNA. Hybridization was accomplished after 18h at 65°C with ^{32}P labelled pDC5A1 probe added to the prehybridization solution. Nick-translation of plasmids was performed with a kit from Amersham using alpha ^{32}P-dCTP (>800 Ci/mmol).

Following incubation, the filters were washed in 1x SSC with 0.1% SDS at 55°C three times, one hour each as described by Showalter et al. (1985). Autoradiography was performed at -80°C with dual intensifying screens. Quantification was by scanning densitometry of the autoradiograms (Showalter et al. 1985)

Results and Discussion

Water Stress

Pea seedlings grown under osmotic stress in solutions of PEG were visibly stunted in comparison to the controls. The level of osmotic stress appeared to be related to subsequent freezing tolerance (Fig. 3.1). The temperature at which 50% injury occurred (LT_{50}) was -12.5°C for the -1.15

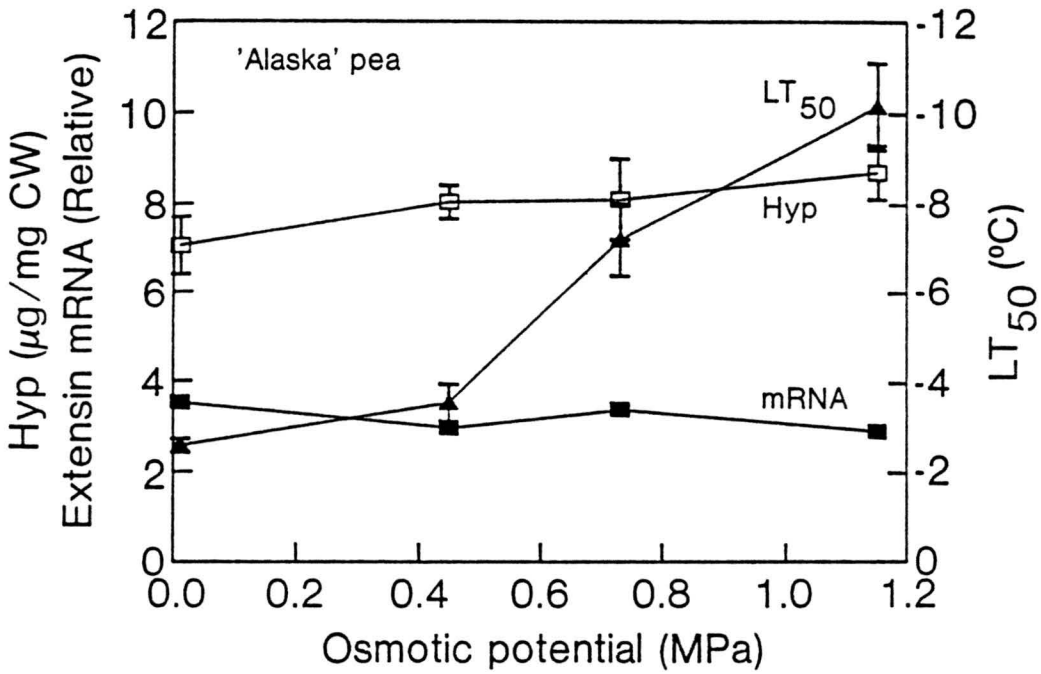


Figure 3.1. 'Alaska' peas germinated in water were transferred to polyethylene glycol (PEG) solutions for 2 to 3 days. The concentration of PEG in the solutions corresponded to osmotic potentials of 0, -0.45, -0.73, and -1.15 MPa. Measurements were made of: cold tolerance (LT_{50} mean ± standard error, n=8) based on electrolyte leakage; cell wall hydroxyproline (mean ± standard error, n=3) based on gas chromatography of N-heptafluorobutyryl isobutyl ester derivatives; and extensin mRNA from Northern blot analysis quantified by scanning densitometry of autoradiographs (Fig.3.3 A) from total RNA of pea probed with the ^{32}P labelled genomic clone (pDC5A1).

MPa treatment, a 10°C increase in tolerance compared to the controls. The amount of hydroxyproline in the cell wall rose slightly (about 18%). When water was withheld from peas grown in vermiculite, the tissue water potential decreased substantially (-.42 to -1.14 MPa) after 9 days (Fig. 3.2). The freezing tolerance (LT_{50}) and hydroxyproline level both increased (-3.8 to -10.9°C, and 4.5 to 6.5 ug/mg cell wall, respectively). However, the total mRNA level for extensin declined by a very substantial amount.

The water stress-induced decline in total extensin mRNA did not appear to mask any substantial increase in amount of a particular transcript (Figs. 3.3 A & B). Unlike the low temperature response, no bands showed increased binding for tissue that was previously exposed to either osmotic or drought stress. Indeed, drought-stressed pea epicotyls showed rather pronounced decreases in several extensin transcripts (Figs. 3.3 A & B). Rewatering the drought-stressed peas returned tissue water potential, hydroxyproline, and freezing tolerance to values almost identical to those of the controls (Fig. 3.2). The loss of stress resistance which followed rewatering was not surprising, but the apparent decline in hydroxyproline content was, since extensin is typically incorporated as a structural component of the cell wall. It may be that

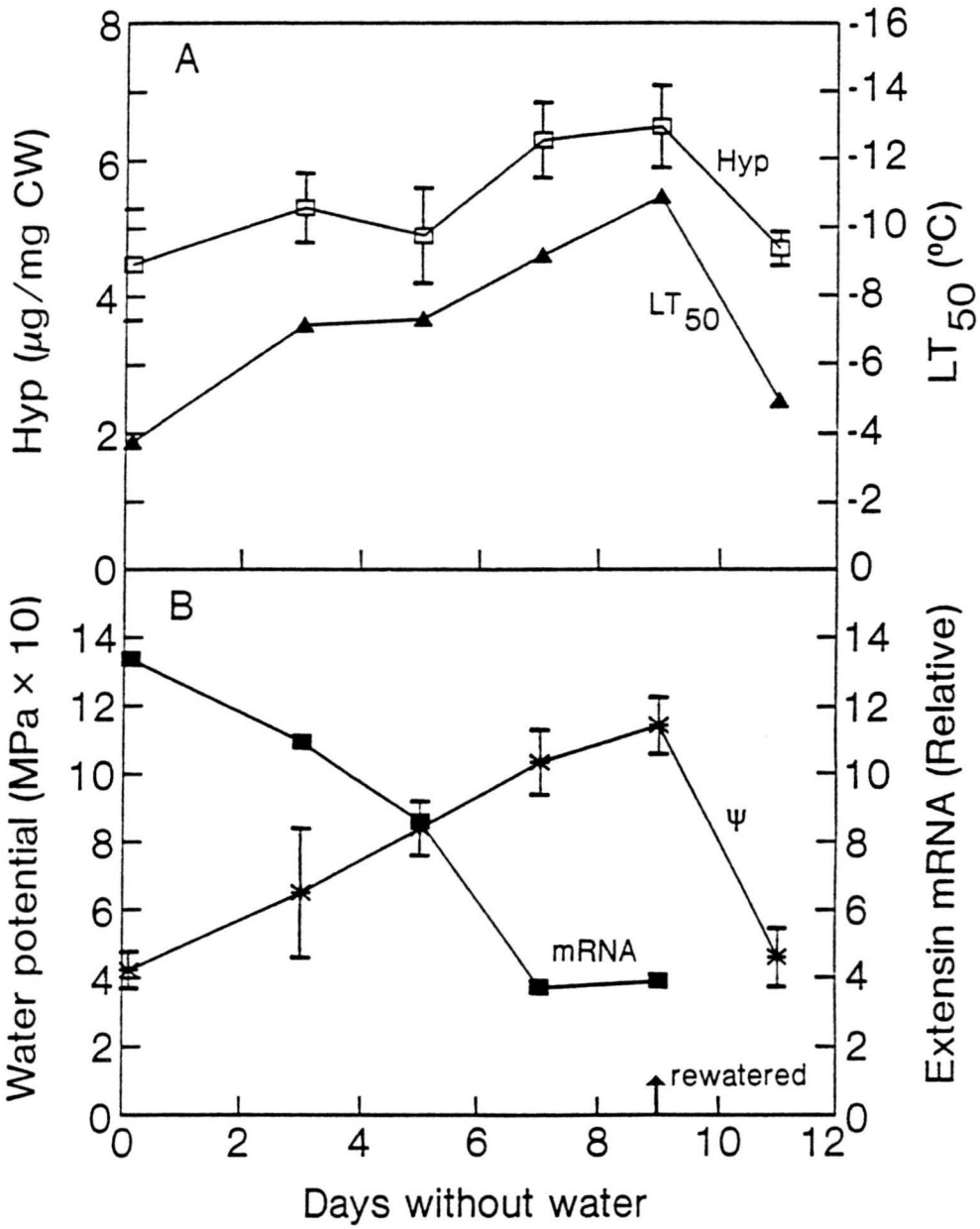
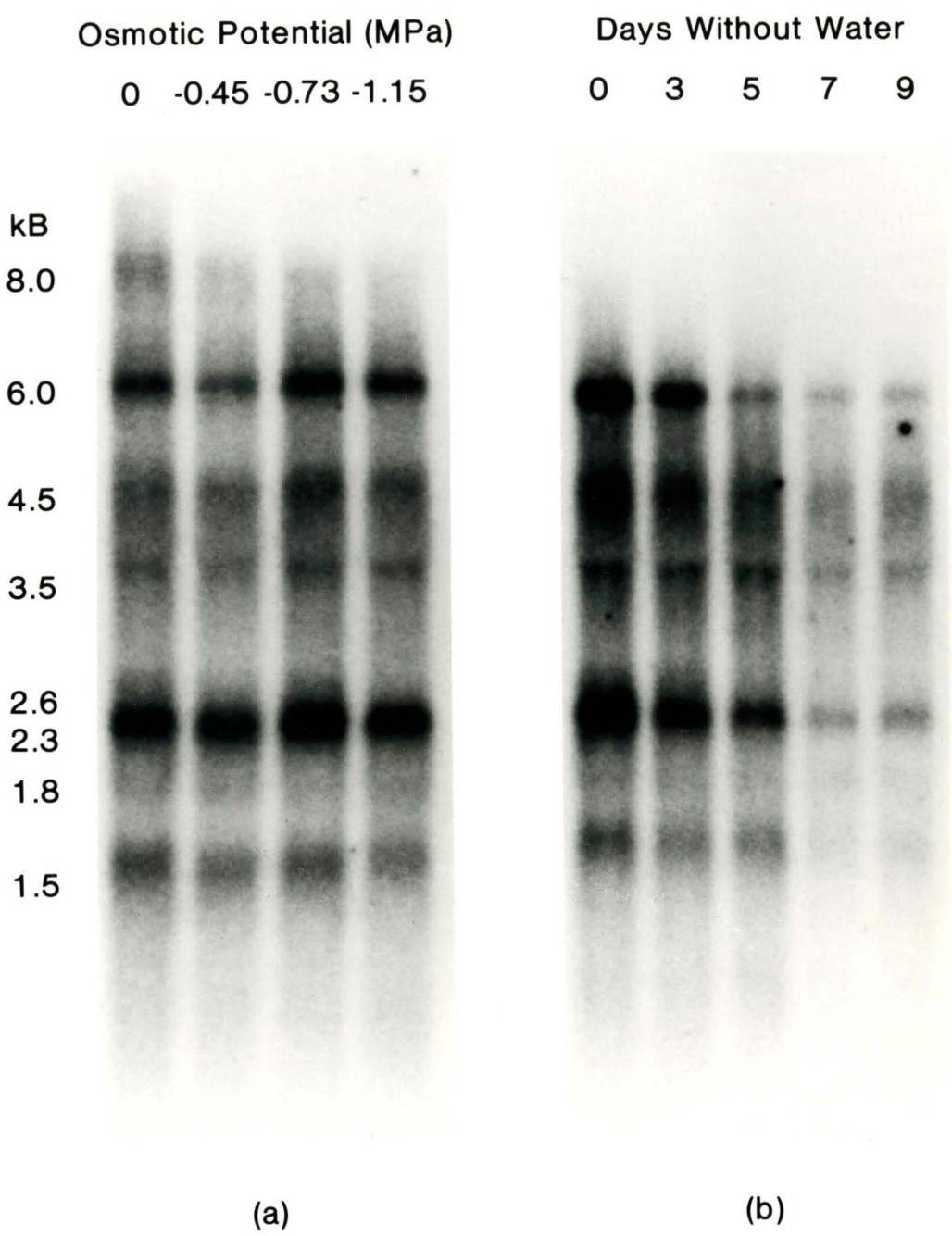


Figure 3.2. 'Alaska' peas germinated in moist vermiculite were allowed to dehydrate 9 days before rewatering. Measurements were made of: (A) cold tolerance (LT_{50}, mean, n=8) and cell wall hydroxyproline (mean ± standard error, n=3) (gas chromatography of N-heptafluorobutyryl isobutyl ester derivatives); and (B) water potential of epicotyls by thermocouple psychrometry (mean ± standard error, n=8) and extensin mRNA from Northern blot analysis quantified by scanning densitometry of autoradiographs from total RNA of pea probed with the ^{32}P labelled genomic clone (pDC5A1).

Figure 3.3. Northern blots of 'Alaska' pea total RNA probed with the ^{32}P labelled genomic clone (pDC5A1) and visualized by autoradiography for the following treatments: (A) peas germinated in water and transferred to polyethylene glycol (PEG) solutions for 2 to 3 days; and (B) peas germinated in moist vermiculite that were allowed to dehydrate 9 days before rewatering.


Osmotic Potential (MPa)
0 -0.45 -0.73 -1.15
Days Without Water
0 3 5 7 9
kB
8.0
6.0
4.5
3.5
2.6
2.3
1.8
1.5
(a)
(b)

synthesis of other new wall material after rewatering was more rapid than synthesis of extensin.

In summary, a water deficit imposed either by osmotic or dehydration stress can result in a substantial increase in freezing tolerance of etiolated pea epicotyls (Figs. 3.1 & 3.2). This change was in all cases accompanied by a significant rise in the amount of extensin in the cell wall, but the extensin gene activity did not appear to be upregulated. If extensin synthesis is regulated at the gene level one would expect the mRNA for extensin to increase rather than decrease. A possible alternative explanation is that an extensin precursor is stored in an inactive pool until an environmental signal stimulates a sequence of events that lead to its incorporation in the wall.

Because the amount of hydroxyproline increased concomitantly with freezing tolerance in peas acclimated by cold temperature (Figs. 2.6 A & 2.10) and water stress (Figs. 3.1 & 3.2) the contribution of extensin deposition to acclimation cannot be ruled out. However, in some treatments there is no increase in freezing tolerance associated with the increase in cell wall hydroxyproline (Figs. 3.4 & 3.5). The amount of hydroxyproline accumulation was greater in the drought than in the osmotically stressed samples, but the degree of acclimation was less in the drought treatment. If the amount of extensin in the cell wall is related to the freezing

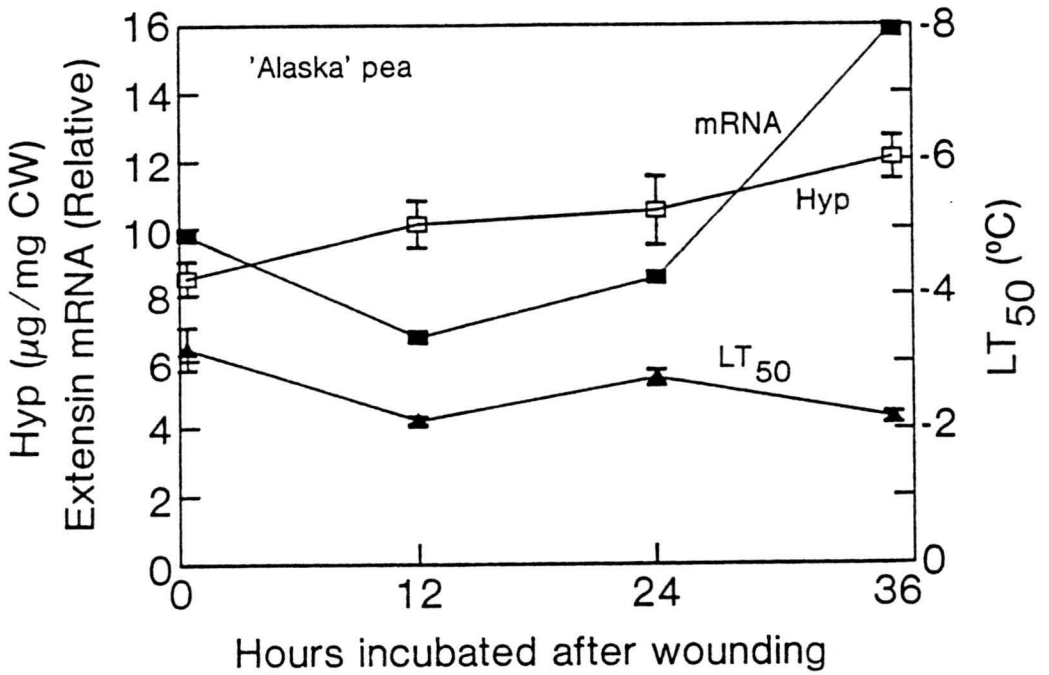


Figure 3.4. 'Alaska' peas germinated at 26°C were wounded by puncturing with pins and incubated up to 36 hours before harvesting for measurement of: cold tolerance (LT_{50} mean ± standard error, n=8) based on electrolyte leakage; cell wall hydroxyproline (mean ± standard error, n=3) based on gas chromatography of N-heptafluorobutyryl isobutyl ester derivatives; and extensin mRNA from Northern blot analysis quantified by scanning densitometry of autoradiographs from total RNA of pea probed with the ^{32}P labelled genomic clone (pDC5A1).

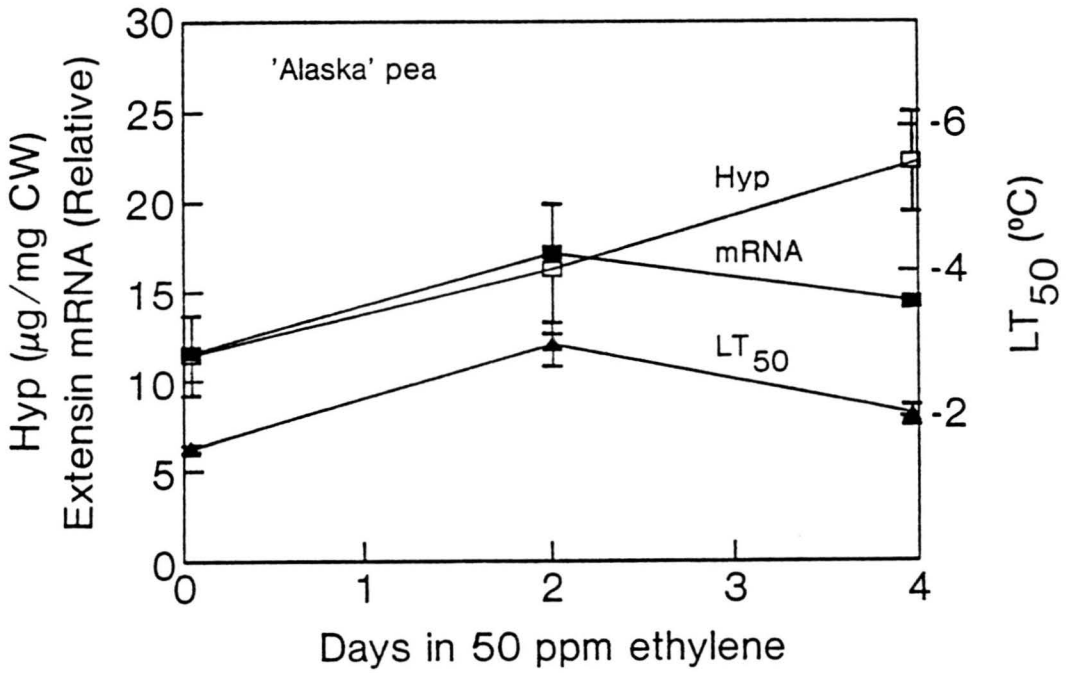


Figure 3.5. 'Alaska' peas germinated 5d at 26°C in an air atmosphere were transferred to 50ppm ethylene for up to 4 days before measuring cold tolerance (LT_{50} mean ± standard error, n=4) based on electrolyte leakage; cell wall hydroxyproline (mean ± standard error, n=3) based on gas chromatography of N-heptafluorobutyryl isobutyl ester derivatives; and extensin mRNA from Northern blot analysis quantified by scanning densitometry of autoradiographs from total RNA of pea probed with the ^{32}P labelled genomic clone (pDC5A1).

tolerance, one would expect the drought treated samples to be more freezing tolerant. However, it could be that the age of the seedling makes a difference. In the osmotically stressed peas, the -1.15 MPa treatment was harvested 6 days after planting while the controls were 4 days old, an age difference of 2 days. The drought treatment, however, resulted in an age difference of 9 days between the control and most severe treatment.

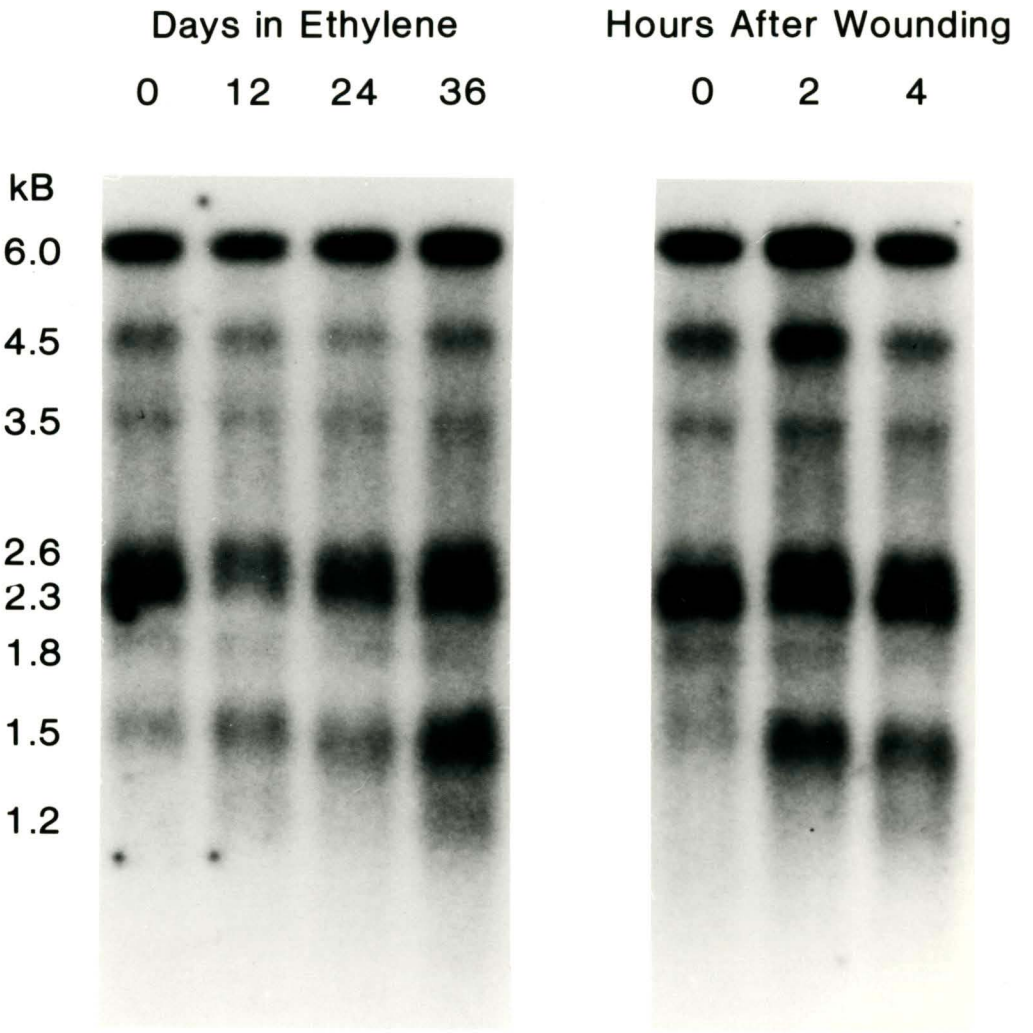
Wounding

During incubation after wounding the level of hydroxyproline in the cell wall increased by about 30% but no change in freezing tolerance occurred (Fig. 3.4). The level of total mRNA for extensin began increasing 24 hours after wounding and by 36 hours had almost doubled. Since the level of cell wall extensin had begun to rise prior to the increase in total extensin mRNA, it seems that there must be another point of regulation besides gene expression. However, it should be noted that there was a substantial and early increase in the 1.5 kB extensin transcript (Fig. 3.6 A). Regardless of the effect of wounding on extensin mRNA, it was clear that there was no substantial influence in freezing tolerance.

Ethylene Exposure

Peas germinated and grown in 50 ppm ethylene showed the characteristic thickening of the epicotyl just below the

Figure 3.6. Northern blots of 'Alaska' pea total RNA probed with the ^{32}P labelled genomic clone (pDC5A1) and visualized by autoradiography for the following treatments: (A) peas germinated at 26^{o}C, wounded by puncturing with pins, and incubated up to 36 hours before harvesting; and (B) peas germinated 5d at 26^{o}C in an air atmosphere then transferred to 50ppm ethylene for up to 4 days before harvest.


Days in Ethylene
0
12
24
36
Hours After Wounding
0
2
4
kB
6.0
4.5
3.5
2.6
2.3
1.8
1.5
1.2
(a)
(b)

hook as seen in other studies (Eisinger, 1983). The ethylene treatment also consistently increased cell wall hydroxyproline, but total extensin mRNA level was not consistently affected (Fig. 3.5). However, exposure to ethylene did substantially increase the expression of the 1.5 kB extensin transcript (Fig. 3.6 B). Thus, as for the wounding treatment, the increased wall extensin may be the result of more 1.5 kB mRNA. However, also as observed for wounding, ethylene exposure did not consistently increase freezing tolerance (Fig. 3.5). After 4 days of ethylene exposure the LT_{50} was less than half a degree C lower, while hydroxyproline content increased by about 50%.

Several of the observations from these experiments have shed new light on the relationship between extensin and freezing tolerance, and the regulation of extensin at the gene level. In all cases of increased freezing tolerance the level of hydroxyproline in the cell wall also increased significantly. This included responses to osmotic stress (Fig. 3.1), drought (Fig. 3.2), and low temperature (Figs. 2.6 A & 2.10). However, there were other cases (wounding and ethylene treatments) in which hydroxyproline content increased, but freezing tolerance did not (Figs. 3.4 & 3.5).

The point of regulation of the extensin level in the cell wall is unclear. The strong correlation between extensin mRNA and cell wall extensin found previously for peas acclimated at 2°C (Figs. 2.6 & 2.10) does not hold true

for water stress (Figs. 3.1 & 3.2), wounding (Fig. 3.4), or ethylene (Fig 3.5) treatments.

One possibility is that extensin precursors are stored in pools at some point in the regulation between transcription and wall incorporation. Another possibility is that the stress treatments may reduce degradation of the protein in the cell wall so that even though synthesis of new protein is rapidly declining, a cumulative increase in the wall occurs.

Another point to consider is the proposal by Tierney and Varner (1987) that different size extensin mRNA transcripts may be members of a multigene family, each of which could be regulated by different environmental signals and have distinct products which function differently. Thirty-six hours after wounding the total extensin mRNA level increased 1.6-fold, while the level of the 1.5 kB transcript increased by 5.7-fold (Fig. 3.6 A), as measured with scanning densitometry. A similar response occurs in the ethylene treatment in which, after two days of exposure, the total extensin mRNA increased 1.5-fold and the 1.5 kB band increased 3.6-fold. This differential activation of genes may be important in subsequent effects on wall properties and related cell traits. That is, low temperature-induced extensin incorporation may be quite different from that induced by water stress, wounding, and ethylene.

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CONCLUSIONS

From this study of acclimation and freezing tolerance in relation to cell wall properties, several conclusions may be drawn. Ultrasonic acoustic emissions were observed in woody stems during freezing. Characteristics of these emissions included: their absence during the high temperature exotherm; absence in oven dried stems; occurrence before the low temperature exotherm; and more emissions in supercooling than in non-supercooling species. These characteristics are consistent with the idea that negative turgor imposed on xylem ray parenchyma cells by extracellular ice leads to cavitation of cell water because the rigid cell wall does not collapse under these forces. Furthermore, it is probable that these cavitation events are cataclysmic and lead to cell death. One may further speculate that the low temperature exotherm observed by many researchers in hundreds of studies (attributed to intracellular freezing) may be a result of cell water that exits after cavitation and then freezes extracellularly.

Examination of etiolated pea cell wall changes during acclimation at 2°C revealed specific increases in arabinose and hydroxyproline content. Both are components of the glycoprotein, extensin. Northern blot analyses revealed that the messenger RNA for extensin increased in response to

cold. Given these results and the well documented structural role of extensin, one can infer that the glycoprotein extensin that accumulates in the cell wall may help protect the freezing cell against dehydration.

It was also found that water stress, imposed by either osmotic or drought treatments, induced a substantial increase in freezing tolerance and also resulted in higher levels of extensin in the cell wall. Ethylene and wounding were treatments that induced increases in cell wall extensin, but produced no change in freezing tolerance. Although the higher level of hydroxyproline did not result in an increase in freezing tolerance in these cases, in treatments for which freezing tolerance did increase, there was also a significant increase in hydroxyproline.

In contrast to acclimation at 2°C, water stress-treated seedlings displayed an increase in hydroxyproline while the mRNA level for extensin dropped. These results suggest points of regulation alternative to the gene level and could be a fruitful area for further research. Another such area that was touched upon by this work but not explained is the occurrence of the multiple transcripts for extensin and especially the apparent specific increase of the bands at 6.0, 2.6, and 1.5 kB for the cold treatment and 1.5 and 1.2 kB bands that are upregulated significantly more than other bands by both wounding and ethylene treatment. The role of the cell wall has been somewhat neglected in studies of

resistance to freezing temperatures. Although the membrane is ultimately the site of injury, the cell wall, by its rigid nature and proximity to the membrane certainly may modify such injury.