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Cell Wall and Extensin mRNA Changes during Cold Acclimation of Pea Seedlings

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ABSTRACT

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 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 three-fold increase in total extensin mRNA during exposure to cold temperature. 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 kilobases 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.

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 has been characterized by Steponkus (22) and his co-workers, 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.* (2) 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 (3) and drought (11, 18) stress acclimation, altered cell wall elasticity was considered an important component of the adaptive response. Reports of cell wall thickening during cold acclimation (10) 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 (8). Evidence that these forces may be strong enough to induce cavitation in cells of woody stems was presented by Weiser and Wallner (28).

A cell wall change which could significantly alter relevant mechanical properties is the increased deposition of the glycoprotein extensin, a change known to be stress induced (27). A structural role for extensin was first proposed by Lamport and Northcote (13). The glycoprotein they discovered, later named extensin for its putative role in cessation of extension growth, was found to contain a great portion of the hydroxyproline in the cell. 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 (14).

It was hypothesized that if structural changes in the cell wall increase its rigidity during acclimation, thereby possibly limiting freeze-induced cell water loss and resulting injury, then an increase in freezing resistance should occur. Data presented here quantify: cell wall weight, total glucan, non-cellulosic 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 (4–6 d 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 before low temperature exposure. All experiments were done with epicotyl tissues only.

Freezing Tolerance

Freeze stress tests were conducted with epicotyl sections cut just below the apical hook to a length of 1.0 cm or 1.5 cm,

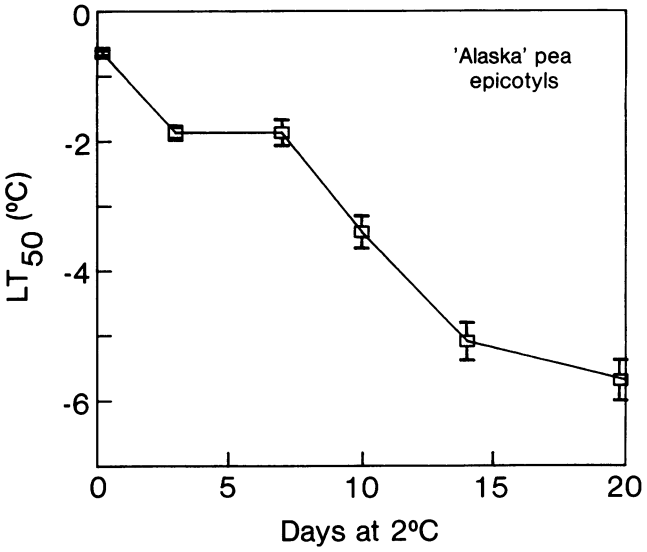

Figure 1. Cold acclimation of pea (cv 'Alaska') seedlings at 2 °C in the dark. Seedlings were grown at 22 °C in the dark for 5 d and then transferred to 2 °C. The LT_{50} was the freezing temperature which resulted in 50% electrolyte leakage from epicotyl sections after freeze-thaw treatment. Error bars represent ± SE.

depending on the experiment. The sections were rinsed with deionized water, and placed in test tubes (1.5 × 10 cm) containing 0.5 mL of deionized water. Four sections were placed in each tube and either four or eight replicate tubes were used, depending on the experiment. After equilibration at −2 °C in a methanol bath, freezing was initiated by nucleating the water with an ice crystal. 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 min to equilibrate. Samples were then thawed at 2 °C for 18 h and 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 (9). Following Polytron homogenization in 20 mM PO_4 buffer (pH 6.9) and filtration through Miracloth, the filtered residue was thoroughly washed with excess cold buffer. Crude cell walls were then further purified by repeated rinsing and filtration first with chloroform:methanol (1:1) and then acetone; the cell wall preparations were dried *in vacuo* over P_2O_5 to a constant weight.

Cell Wall Neutral Sugar Composition

The noncellulosic neutral sugar composition of cell walls was determined using the method of Albersheim *et al.* (1). This method includes 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 flame ionization detector 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 Kaiser *et al.*

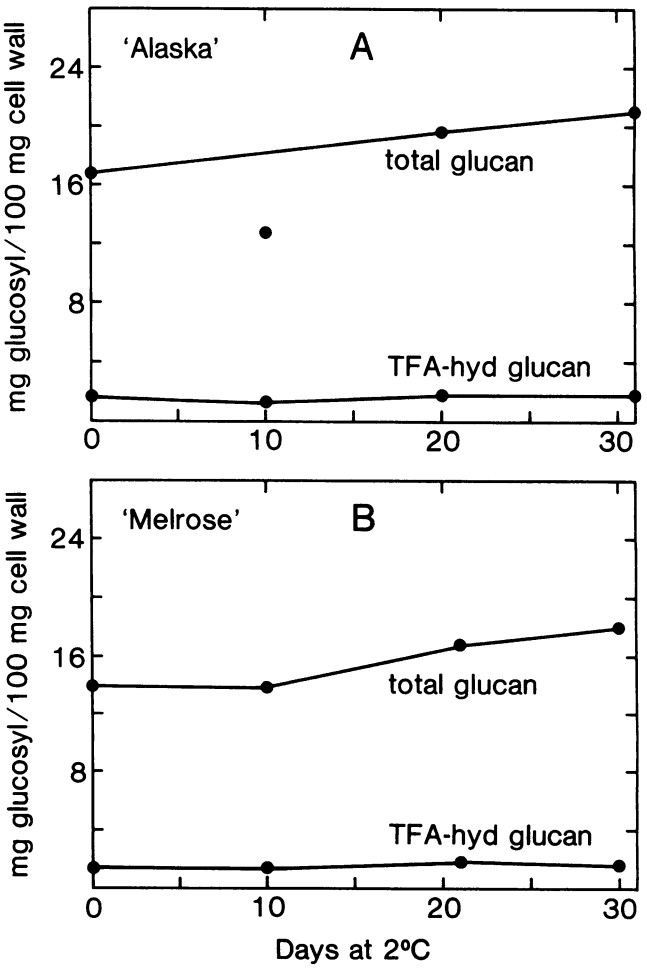

Figure 2. Glucan content of 'Alaska' (A), and 'Melrose' (B) pea epicotyl cell walls during cold acclimation. Total glucan was determined from H_2SO_4 hydrolysates; noncellulosic glucan was determined from TFA hydrolysates. In both cases, glucosyl residues were measured by GLC of alditol acetate derivatives.

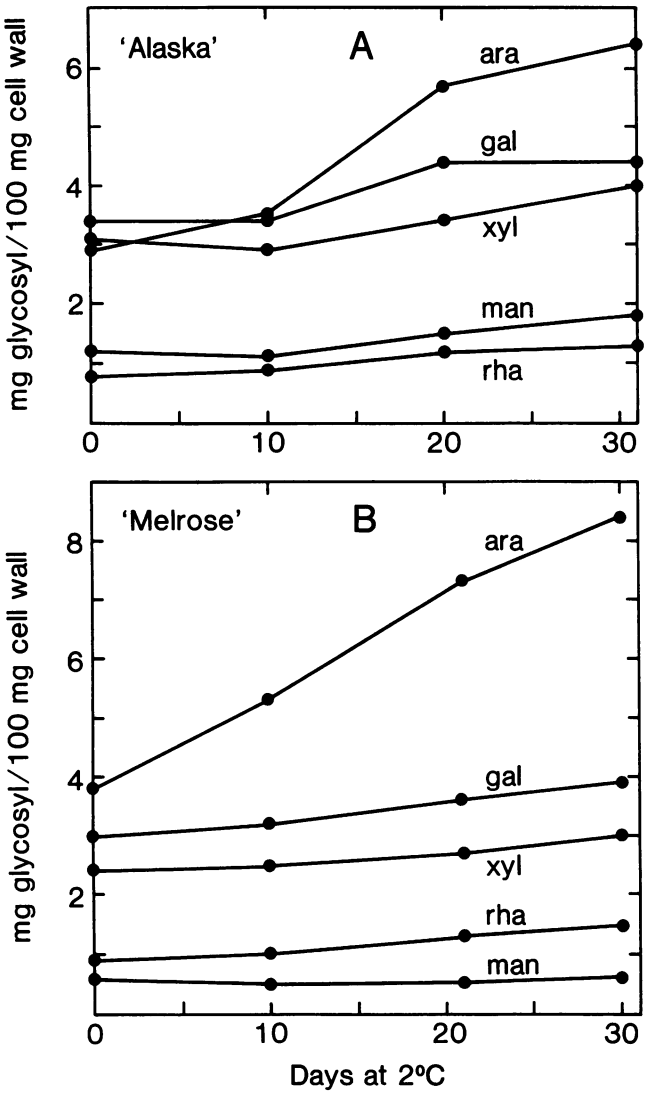

Figure 3. Neutral sugar (except glucose) composition of epicotyl cell walls of 'Alaska' (A), and 'Melrose' (B) pea seedlings during cold acclimation. Glycosyl residues were determined by GLC of alditol acetate derivatives.

(12) by M. 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 flame ionization detector 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 (4) was generously provided by Mary Tierney, Biotechnology Center, Ohio State University. Entire plasmids were isolated using alkaline lysis (17) 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 (6) of which 5 μg from each treatment was separated by electrophoresis through 1.2% agarose gels containing 6.5% formaldehyde, 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 below a germicidal UV light. Prehybridization of bound RNA was carried out at 65 °C for 4 h in 6× SSC, 5× Denhardt's solution, 0.1% (w/v) SDS, and 10 μg/mL denatured herring sperm DNA. Hybridization was accomplished after 18 h at 65 °C with [^{32}P]-pDC5A1 probe added to the prehybridization solution. Nick-translation of entire plasmids was performed with a kit from Amersham using [α-^{32}P]dCTP (>800 Ci/mmol).

Following incubation, the filters were washed in 1× SSC with 0.1% SDS at 55 °C three times, 1 h each, as described by Showalter *et al.* (19). Autoradiography was performed at −80 °C with dual intensifying screens. Quantification was by scanning densitometry of the autoradiograms (19).

RESULTS AND DISCUSSION

Pea epicotyls from seedlings which were germinated and grown at 22 °C for 4 to 6 d, and then exposed to 2 °C in the dark, gradually cold acclimated to an LT_{50} of −6 °C (Fig. 1). This level of hardiness was achieved by 20 d at 2 °C and was accompanied by an increase in dry matter relative to fresh weight. Nonacclimated epicotyls contained 5.3% dry matter compared to 8.1% for acclimated epicotyls of comparable size. The accumulation of solutes, membrane augmentation, etc., are typical cellular changes associated with cold acclimation (16) which contribute to increased dry matter content. It was observed that some of this increase in pea epicotyls was

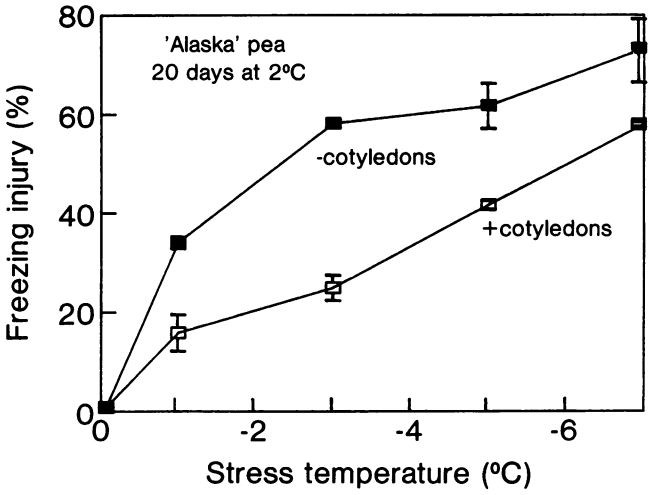

Figure 4. Effect of cotyledon removal on cold acclimation of 'Alaska' pea seedlings in the dark. Seedlings were grown in the dark at 22 °C for 5 d, cotyledons were then carefully excised and seedlings placed at 2 °C for 20 d. Freezing injury at the indicated temperatures was determined by measuring electrolyte leakage from epicotyl sections after freeze-thaw treatment. Error bars represent ±, SE.

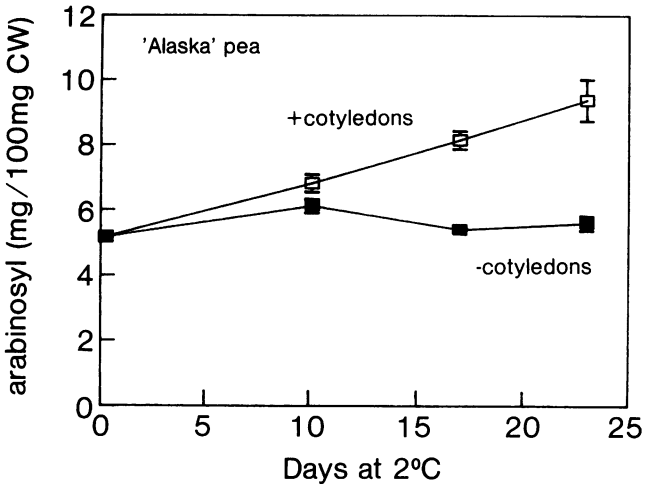

Figure 5. Effect of cotyledon removal on the change in arabinosyl content of 'Alaska' pea epicotyls at 2 °C in the dark. Seedling treatment was as described for Figure 4. Arabinosyl content was determined by GLC of alditol acetate derivatives prepared from TFA-hydrolyzed cell walls. Error bars represent ± SE.

cell wall material; wall weight per gram fresh weight increased from 12 mg to 17 mg during 20 d at 2 °C in the dark. This is consistent with anatomical observations of cell wall thickening during cold acclimation of other tissues (10).

During cold treatment there appeared to be an increase in total wall glucan in both cultivars examined (Fig. 2). This trend was consistent in three separate experiments. Since noncellulosic (TFA-hydrolysable) glucan content was relatively low and constant (Fig. 2), any increase in total glucan must have been as cellulose. The glucan data in Figure 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 (21). Callose deposition occurred during cold acclimation of cultured pear cells (26). Either callose deposition did not substantially increase in acclimating pea epicotyls, or it did not remain with the analyzed wall fraction during sample preparation.

The apparent increase in cellulose (Fig. 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 content (Fig. 3) reflect changes in the pectic polysaccharide fraction (23). The increases in total wall glucan (Fig. 2) and galactosyl content (Fig. 3) were both about 20% during 20 d 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. 3). After 20 days at 2 °C, the amount of arabinosyl residues in both 'Alaska' and 'Melrose' cell walls doubled. The low temperature-induced increase in wall arabinosyl content during acclimation is shown by the effect of cotyledon removal which inhibits the development of freezing tolerance (Fig. 4). Siminovitch and Cloutier (20) showed that endosperm removal from rye seedlings inhibited cold acclimation in the dark, perhaps by limiting the 'augmentation of protoplasm.' This augmentation was described by Levitt (16) as the metabolic accumulation of sugars, proteins, nucleic acids, amino acids, and lipids during cold acclimation. Cotyledon removal from pea seedlings prior to low temperature exposure

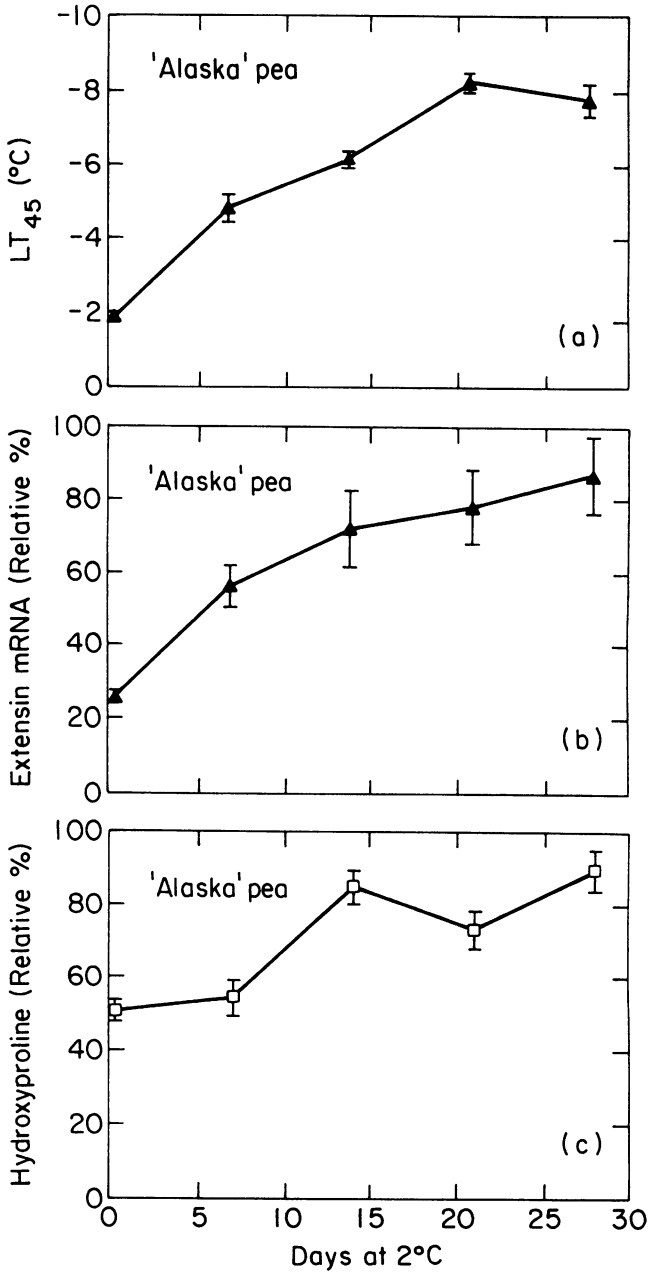

Figure 6. 'Alaska' peas were germinated at 26 °C and acclimated at 2 °C. The data presented are averages of three separate experiments replicated in time. Freezing tolerance **(a)** was measured based on electrolyte leakage at four test temperatures (unfrozen, −3, −6, and −9 °C). The LT_{45} value (in °C) was calculated by linear extrapolation between the test temperatures that bracket 45% injury (± SE). Extensin mRNA **(b)** was visualized by Northern blot analysis (^{32}P genomic clone pDC5A1) and quantified by scanning densitometry of autoradiograms (± SE). Cell wall hydroxyproline **(c)** was measured by gas chromatography of heptafluorobutyryl isobutyl ester derivatives (± SE).

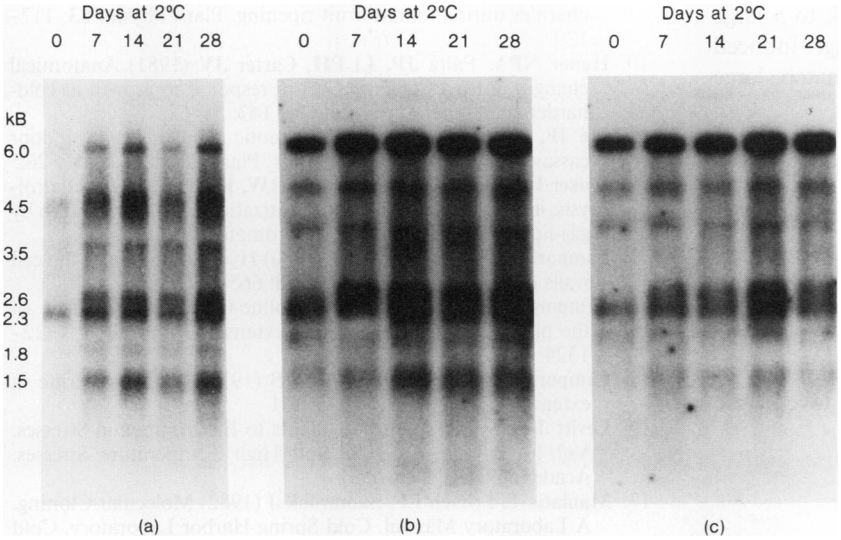

Figure 7. Northern blot analysis from three replications [**(a), (b),** and **(c)**] of 'Alaska' peas germinated at 26 °C and acclimated at 2 °C. Total RNA hybridized with the extensin genomic DNA clone pDC5A1 labeled with ^{32}P and visualized by autoradiography. Mean size (kilodaltons ± SE) of the 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, and 1.5 ± 0.04.

prevented both cold acclimation (Fig. 4) and the increased wall arabinosyl content (Fig. 5) in epicotyls.

Wall arabinose occurs as side branches to the rhamnogalacturonosyl main chain of pectic polysaccharides (15, 23). 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 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 (5), 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. 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, because its predominant carbohydrate is arabinose (14). Extensin is roughly one third peptide, 45% of which is hydroxyproline (27). The remaining two-thirds is carbohydrate composed largely of arabinose bound to hydroxyproline and a small amount of galactose attached to serine (15, 25). 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 (25). Isodityrosine linkages formed intramolecularly may bind extensin molecules among the cellulose microfibrils of the wall (7). 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.

After 28 days at 2 °C peas acclimated an average of 6 °C (Fig. 6a). Extensin mRNA and cell wall hydroxyproline contents increased by an average of 230 and 75%, respectively, during this same period (Fig. 6, b and c). The temporal correlation between cell wall hydroxyproline and extensin mRNA indicates that cell wall extensin is regulated at the level of mRNA accumulation, and suggests that extensin level depends directly upon the mRNA level.

Because 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 and the ratio of poly(A^+) to total RNA decreased slightly (data not shown). Therefore, the increase in extensin mRNA apparent in Figure 7 is 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. 7). Other researchers (4) have found multiple bands for extensin in carrot roots. The origin and function of the different size extensin transcripts are not known, but Tierney and Varner (24) speculated that individual genes may be regulated by different environmental signals. Clearly, one of these signals for pea seedlings is low temperature. The inductive effect of the 2 °C treatment, especially on the 1.5, 2.6, and 6.0 kb transcripts, was evident in all three replications of the Northern blot analysis (Fig. 7).

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. (16). Also, Lamport (14) pointed out

that a small amount of extensin could cross-link 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 mRNA accumulation 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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