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Synthesis and oxidative insolubilization of cell-wall proteins during osmotic stress

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Abstract. The cell walls in the new white roots of jack pine (*Pinus banksiana* Lamb.) were observed to constrict around the shrinking protoplast of osmotically stressed roots, and pressure was maintained via an apparent adjustment of cell-wall size and elasticity. These elastic alterations of the cell wall permitted the root cells to maintain full turgor despite the loss of most of the water in the tissue. The constriction of the root cell wall around the dehydrating protoplasts to maintain turgor may reflect changes in cell wall structure. We found that these shrinking root cells synthesize and secrete into the intercellular fluid a set of proteins. These proteins become tightly associated (i.e. guanidine HCl- and sodium dodecyl sulfate-insoluble) with the cell wall but can be released from the matrix, after briefly boiling in 0.1% sodium dodecyl sulfate, by the combination of guanidine HCl, $CaCl_2$ and dithiothreitol. However, these cell-wall proteins became insoluble with time. The proteins could subsequently be destructively extracted from the wall with acid $NaClO_2$ treatments. After these proteins were incorporated into the cell walls, the roots adopted a new, smaller maximal tissue volume and elastic coefficients returned to normal levels.

Key words: Cell wall – Osmotic stress – Oxidative insolubilization – *Pinus* (root turgor) – Root polypeptides – Turgor

Introduction

The cell walls of higher plants have been observed to contract somewhat during osmotic dehydration prior to plasmolysis (Carpita et al. 1979; Carpita 1982). Although, the concept that the extracelluar matrix can elastically constrict around the protoplast to regulate pressure has been proposed as a mechanism to explain the apparent maintenance of turgor in shrunken tissue, the phenomenon has never been convincingly demonstrated at the cellular level (Buxton 1985; Eze et al. 1986; Levitt 1986; Marshall et al. 1991; Marshall and Dumbroff 1999). There is, however, a wealth of experimental evidence that plant tissues elastically expand and contract by large amounts over the course of typical diurnal variation in moisture availability (Huck et al. 1970; Kozlowski 1972).

Plants regulate plasticity of the cell wall during expansion and growth via the action of cell wall proteins (Sadava and Chrispeels 1973; McQueen-Mason et al. 1992). The strengthening of the extracellular matrix in response to fertilization, pathogen signalling or development occurs in concert with oxidative activity and seems to be shared by other eukaryotes (Whitmore 1978a, b; Shapiro 1991; Bradley et al. 1992). In plants, structural proteins secreted into the intercellular spaces presumably bind to the matrix via weak electrostatic interactions and thus may be washed-out from the intercellular spaces by dilute salts (Hon et al. 1994). Subsequently, these proteins are rendered insoluble by post-translational modification(s) and cannot be extracted by detergents (Fry 1982), salts (Stuart and Varner 1980), alkalis or acids (Mort and Lamport 1977) or organic solvents (Briggs and Fry 1990), but can be released by sequential treatment with hydrolases followed by HF (Qi et al. 1995).

Here we have considered the relationship between tissue elasticity and cell-wall proteins. We measured the average elasticity of the whole roots in response to osmotic stress and collected the proteins secreted in the intercellular fluid (IF), the salt- and SDS-insoluble cell-wall proteins that non-covalently bind the wall, and the

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Abbreviations: GCD = 6 M guanidine hydrochloride, 0.1 M $CaCl_2$ and 50 mM dithiothreitol; GHCl = guanidine hydrochloride; IF = intercellular fluid; PEG = polyethyleneglycol; PVDF = polyvinylidene difluoride; rpHPLC = reversed-phase high-pressure liquid chromatography; TFA = trifluoroacetic acid

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proteins held by acid-$NaClO_2$-labile bonds that are presumed to be covalent. Our results suggest that the regulation of turgor, cell size, elasticity and diameter of jack pine roots is associated with the secretion and subsequent insolubilization of cell wall proteins.

Materials and methods

Plant materials. Jack pine (*Pinus banksiana* Lamb.) seeds were germinated in RIGI-POT plastic forms filled with peat with a 16/8 h day/night regime at 24/20 °C, under 212 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) from mixed fluorescent and incandescent lamps, and 80% relative humidity. The seedlings were watered weekly with 1.0 g l^{-1} of commercial fertilizer (20-20-20 NPK). Upon emergence of the first mature needles, the seedlings (ca. 5 cm tall) were lifted from the plastic forms, washed free of soil, their brown roots removed leaving the newlygrown white roots, and the intact seedlings transferred to 1.5-ml micro-centrifuge tubes set in an aerated holding tank. The tank was filled 0.8 cm above the mouth of the tubes with 1/16-strength Murashige and Skoog's (M&S) salt mixture (Gibco/BRL Life Technologies) dissolved in deionized water. The plants were held in these cultures on a laboratory bench at 25 °C for 72 h under 300 μmol m^{-2} s^{-1} of PAR composed of light from mixed fluorescent and incandescent lamps, plus some daylight, on a 12/12 h light/dark cycle prior to experimental treatments. The holding tank was covered with a cardboard box at night. Osmotic stress was imposed after microtubes were removed from the hydroponic tank and set into perspex holders with individual aeration tubes. The dilute hydroponic buffer was replaced with a fresh volume or osmotic stress imposed with polyethylene glycol (PEG) 8000 (0.29 g ml^{-1}) dissolved in 1/16 M&S. The diameter of the white roots was measured with a caliper.

Pressure-volume analysis. After 8 h of hyperosmotic stress the roots were rapidly rinsed in water, the excess moisture was blotted with kraft paper and fresh weights recorded. The roots were wrapped in saran wrap, and turgor pressure was determined using a PMS (PMS Instrument Co., Corvallis, Oregon) model 600 pressure chamber (Scholander et al. 1964) following the method of Buxton et al. (1984). Extruded sap was collected on absorbent paper contained in a series of capped, pre-weighed microtubes and the sap weighed to five decimal places. The volumetric elastic coefficient (Broyer 1952) or bulk elastic modulus (Phillip 1958) of the roots, ε, was calculated as P/(dv/v) (Dainty 1976) where v is the water volume per unit dry weight.

Microscopy. Control and PEG-treated seedlings were removed from hydroponic chambers and the living white roots immediately sectioned with a razor blade 1 cm from the growing tip. Sections were stained with 0.1% (w/v) cellufluor dissolved in the corresponding solution, rapidly rinsed in 50% glycerol for 1 min, visualized with ultraviolet light and photographed with a Zeiss microscope.

Extraction of cell wall proteins of jack pine roots during elastic constriction and recovery. All aqueous buffers were ice cold and contained protease inhibitors [1 mM of phenylmethylsulfonylfluoride (PMSF), benzamidine, EDTA and 6-aminohexanoic acid (AHA); 50 μM of pepstatin, leupeptin, chymostatin]. The lyophilized roots of seedlings from each treatment were ground in a ceramic mortar and pestle, washed five times for 2 min in 100 ml of ice-cold neutral wash buffer (50 mM Tris pH 7.0), washed over Miracloth (Calbiochem) with ice-cold ethanol till colourless, rehydrated with 500 ml of neutral buffer, leached for 24 h in 100 ml of ice-cold 6 M guanidinehydrochloride (GHCl) plus 0.1 M $CaCl_2$ and rinsed three times with 500 ml of buffer over Miracloth. Note that GHCl and SDS form a precipitate so the tissue was always washed thoroughly in buffer and ethanol between steps that required these protein-extracting reagents. The purified cell-wall cake was re-suspended in a 250-ml glass flask of swirling 1% SDS that was rapidly brought to the boiling point on a hot plate for the time indicated in the figures, and then immediately washed free of SDS solution with neutral buffer over Miracloth. The salt-leached and detergent-boiled walls (0.1 g) were then extracted for 24 h in 100 ml of ice-cold 6 M GHCl, 5 mM DTT and 0.1 M $CaCl_2$ (GCD). The cell-wall extracts were concentrated to 0.5 ml over a 3000 NMWL AMICON filter, centrifuged at 14,000 rcf for 5 min, the supernatant mixed with an equal volume of 0.05% (v/v) trifluoroacetic acid (TFA; pH 2.75) and subjected to preparative HPLC.

Separation of proteins from carbohydrate and lignin. Frozen root tissue was ground in acetone on ice with a Polytron homogenizer (Model PT20OD; Brinkmann Instruments), centrifuged at 12,000 *g* and the pellet dried under nitrogen. Alternatively, freeze-dried roots were ground with a mortar and pestle. Loosely bound proteins were removed from the cell walls by washing the tissue debris in buffer containing 0.1% SDS, rinsing with dilute wash buffer followed by leaching with 6 M GHCl plus 0.1 M $CaCl_2$ and boiling the cellular debris in 1% SDS for the lengths of time indicated. The cell walls were then rinsed twice in 100 volumes of water and dried on a filter paper with ethanol. The SDS-boiled cell walls were subsequently extracted on ice for 24 h in 100 volumes of GCD with constant agitation. The cell walls were collected on filter paper, rinsed free of guanidine with 100 volumes of water, dehydrated on filter papers with ethanol, air-dried and weighed.

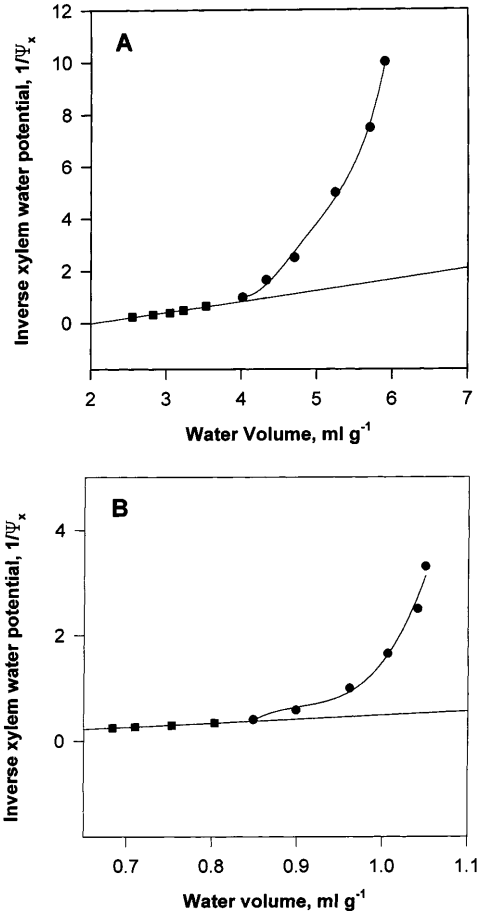


Fig. 1A,B. Representative pressure-volume curves of control and PEG-treated roots of jack pine. **A** Control roots maintained in $1/16^{th}$ M&S medium. **B** Roots stressed for 8 h in −1.0-MPa PEG 8000 dissolved in $1/16^{th}$ M&S medium. ●, Water potential values at positive turgor; ■, osmotic potential values at zero turgor

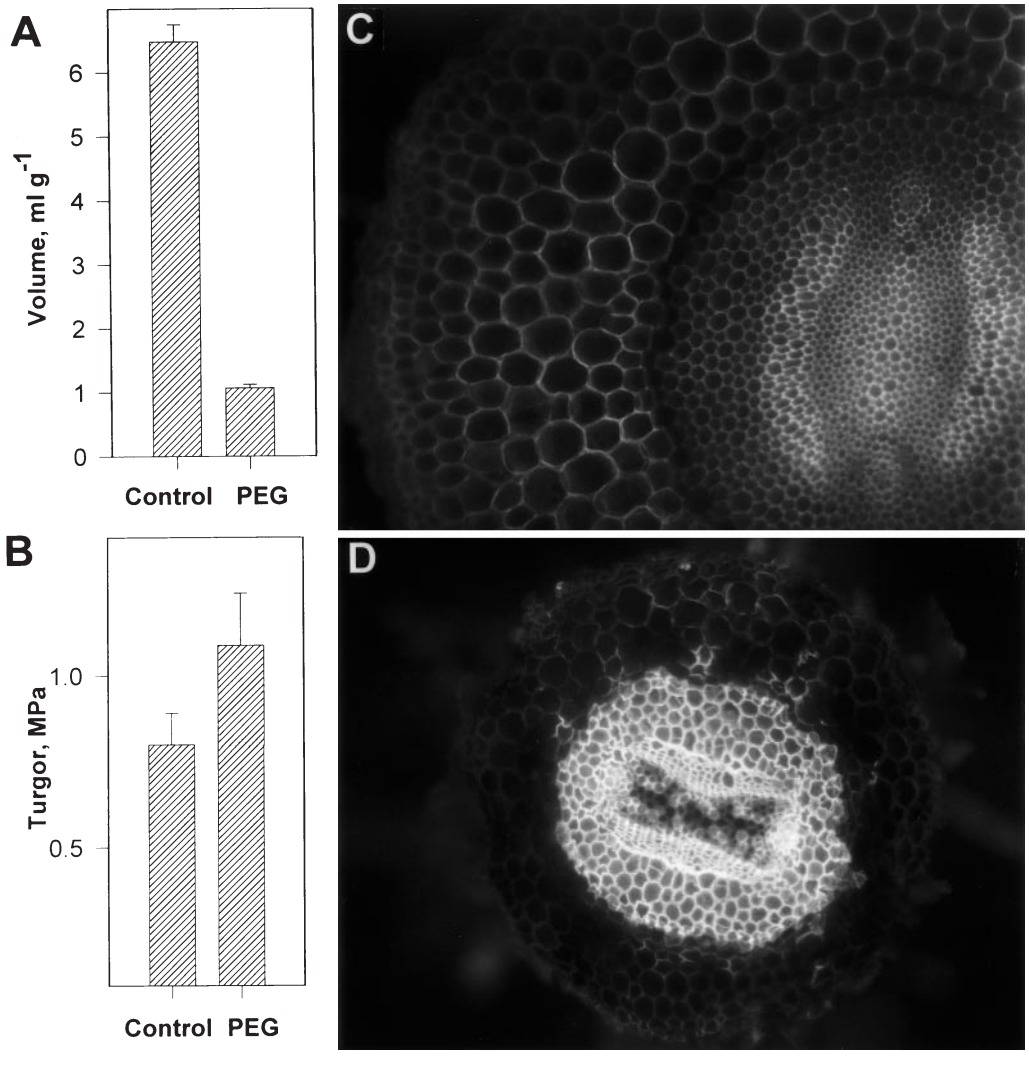
Proteins were separated from the GCD by drop-wise addition of cold ethanol (90% v/v) to extracts followed by centrifugation at 14,000 *g* for 45 min. The pellet was washed three times with 1.5 ml of 80% ethanol, collected at 14,000 *g* for 5 min and the remaining ethanol removed under vacuum.

Preparative reversed phase HPLC. The GCD-cell wall extracts were centrifuged for 5 minutes at 14,000 *g* and the supernatants were mixed with an equal volume of 0.1% TFA and loaded onto to a semi-preparative 7-cm Beckmann C3 Ultrapore column equilibrated with 0.05% TFA in water. After eluting the carbohydrates with the column void volume, proteins suitable for SDS-PAGE analysis were eluted using a single step to 65% acetonitrile plus 0.05% TFA, dried under vacuum and suspended in 125 µl of Laemmli buffer (Laemmli 1970).

Analytical reversed phase (rp) HPLC. Dried pellets were re-suspended in water acidified with 0.05% (v/v) TFA (pH 2.75) and centrifuged for 5 min at 14,000 *g*. Water-soluble supernatants were loaded on a 21-cm analytical, C4 rpHPLC column (Vydak, Hysteria, Calif., USA) and sample components resolved by a 30-min gradient to 70% acetonitrile on a System Gold HPLC column (Beckmann, Mississauga, Ont., Canada). Liquid peaks were dried under vacuum prior to the phenol-sulphuric acid reaction (Hounsell 1993). The void-volume peaks that contained the gelatinous carbohydrate produced the distinctive burnt brown reaction. Liquid peaks were also dot-blotted onto polyvinylidenedifluoride (PVDF) membranes and tested for protein content by the method of Ghosh et al. (1988), or tested for the presence of lignin by staining PVDF dot blots with a saturated solution of phloroglucinol in 18% HCl. Peaks containing lignin produced the distinctive bright red color reaction (Gurr 1968).

Secretion and progressive insolubilization of newly synthesized proteins into the cell wall of jack pine roots in response to osmotic stress. L[^{35}S]Methionine (3.7 MB_2) in Trans^{35}S-label (ICN Biomedicals) was added to the microtubes holding each control or osmotically stressed seedling. Seedlings were labelled for 8 h and then transferred to fresh microtubes, their roots washed three times with 2 mg ml^{-1} of cold L-methionine containing 100 µl l^{-1} Tween 20 and then transferred to a third tube and washed 6 times with 2 mg l^{-1} L-methionine. The roots of 27 intact seedlings were vacuum-infiltrated for 5 min in 50-ml test tubes with nine seedings

Fig. 2A–D. The maintenance of turgor in osmotically dehydrated jack pine roots. **A** Water volume to dry weight ratio of control (1/16 M&S medium) versus PEG 8000 (−1.0 MPa) Values are Mean ± SE (n = 6). **B** Average root turgor measured by pressure chamber of control (1/16 M&S) versus PEG 8000 (−1.0 MPa) Values are Mean ± SE (n = 6). **C** Cross-section of roots maintained in 1/16 M&S medium (control). **D** Cross-section of similar roots after 8 h exposure to PEG 8000. Note that both sections have about seven layers of cells in the cortex and are cut from a developmentally comparable region 1 cm from the root tips. Bar = 100 µM



per tube set in a desiccator attached to a faucet aspirator. The tubes were filled to just above the root collars with intercellular washing fluid containing 5 mM ascorbate, 6 mM $CaCl_2$, 1 mM EDTA with the protease inhibitors described above. Intercellular washing fluid (IF) was collected from fresh roots using 10-ml syringes, without the plungers, set in 50-ml plastic tubes and centrifuged at 1000 *g* for 15 min. Proteins were dried to a powder by lyophilization. The roots were homogenzied with a polytron in 80% acetone and the purified cell walls and GCD extracts prepared as described above. Cell walls were extracted stepwise with 0.04, 0.08, 0.12, 0.24 and 0.48% (w/v) sodium chlorite plus 0.24% (v/v) acetic acid at 60 °C. Insoluble cell-wall materials were pelleted at 1000 *g* and supernatants lyophilized. In the BSA protection assays, dried cell walls were extracted with 0.3% (w/v) sodium chlorite and 0.24% (v/v) acetic acid with and without 0.1 M BSA for 15 min at 70 °C. All lyophilized or precipitated proteins were dissolved in 125 µl of Laemmli buffer.

Analysis by SDS-PAGE and fluorography. Proteins samples of 25 µl were resolved on 10–20%, gradient gels, 12% linear gels or 16% linear gels as indicated in the figure legends using a mini-protean II chamber (Bio-Rad Mississauga, Ontario). Molecular weight standards (Bio-Rad) and cell-wall proteins were visualized by staining gels with Coomasie brilliant blue). Radiolabeled polypeptides were visualized by fluorography using Entensify (NEN, Boston, Mass., USA) and Kodak XAR film.

N-Terminal protein sequencing. Cell-wall proteins from osmotically stressed roots were blotted to PDVF membranes for 2 h at 200 mA in half-strength Towbin buffer (Towbin et al. 1979). Bands were excised from the PVDF membrane and reacted with the manufacturer's reagents in an ABI model 470A gas-phase sequencer (ABI, Foster City, Calif., USA). The phenylthiolhydantoin (PTH) derivatives from each cycle were separated with a gradient from 11% to 90% acetonitrile in 3.5% TFA, 0.1% hexane sulfonic acid on a C-18 ABI/Brownlee PTH column (250 mm long, 2.1 mm i.d.) with 5-µm particle diameter and 80-A pore size, detected at 269 nm with an ABI model 120A on-line PTH analyser, and compared with 5 pmol of PTH-derivitized standards.

Results and discussion

Jack pine roots displayed a loss of water when permitted to equilibrate with a 1-ml volume of hypertonic PEG solution of −1.0 MPa (Marshall 1996). The loss of water from the roots continued for up to 8 h until a steady-state water content in the roots was approached (Marshall 1996). The water potential and osmotic potential of control and PEG-treated jack pine roots were estimated by pressure-volume analyses (Fig. 1A,B). Average root water potential was about −0.1 MPa and −0.6 MPa for the control (Fig. 1A) and PEG-treated (Fig. 1B) roots, respectively, thus indicating that the stressed roots were approaching a water-potential equilibrium with the PEG solution that was partially diluted by the transfer of water from the roots tissues. Osmotic potential values ranged from about −0.6 to −1.6 MPa in the controls and PEG-treated roots, respectively. This method for estimating turgor pressure can be influenced by apoplastic solutes, e.g. penetrating osmotica. Nevertheless, treatments with the non-penetrating PEG-8000 and with the much larger 72,000-Da dextran yielded similar results (not shown). The presence of clear, upward deflections in the inverse pressure-volume plots (Fig. 1B) clearly indicated the presence of turgor pressure. Moreover, similar turgor values were also obtained by the differences between water-potential values measured with the pressure bomb and osmotic-potential values estimated by vapour-pressure osmometry of cell sap samples collected from frozen and thawed roots (not shown).

Jack pine roots rapidly lost most of their water volume (Fig. 2A) but apparently maintained full turgor after 8 h of stress in −1-MPa PEG (Fig. 2B). Subsequent microscopic examination of the newly grown white roots

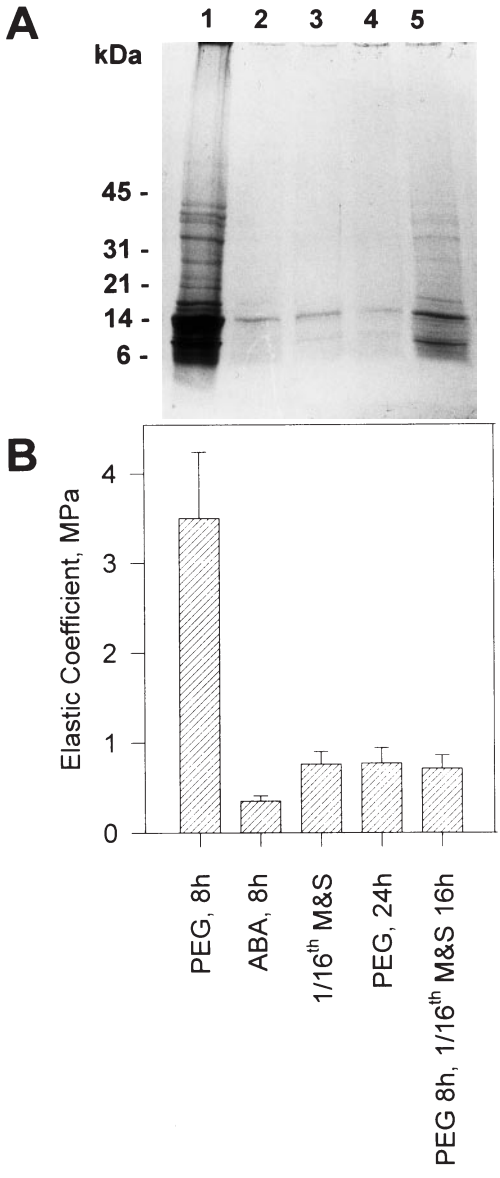


Fig. 3A,B. The GCD-insoluble, cell-wall proteins of jack pine roots during elastic constriction and recovery. **A** Roots were leached in 6 M GHCl, 0.1 M $CaCl_2$, for 24 h, rinsed in ethanol, boiled in 1% SDS, before cell wall proteins were extracted with GCD for 24 h. Proteins from 0.25 g of purified cell walls from each treatment were resolved on 10–20% SDS-PAGE gels after preparative rpHPLC separation from co-extracted carbohydrates. *Lanes*: *1*, −1.0 MPa PEG 8 h; *2*, 10^{-3} abscisic acid (ABA) 8 h; *3*, untreated controls; *4*, PEG 24 h; *5*, PEG 8 h, recovered 16 h. **B** Elastic coefficient of the root cell walls. Values are means ± SE ($n = 5$)

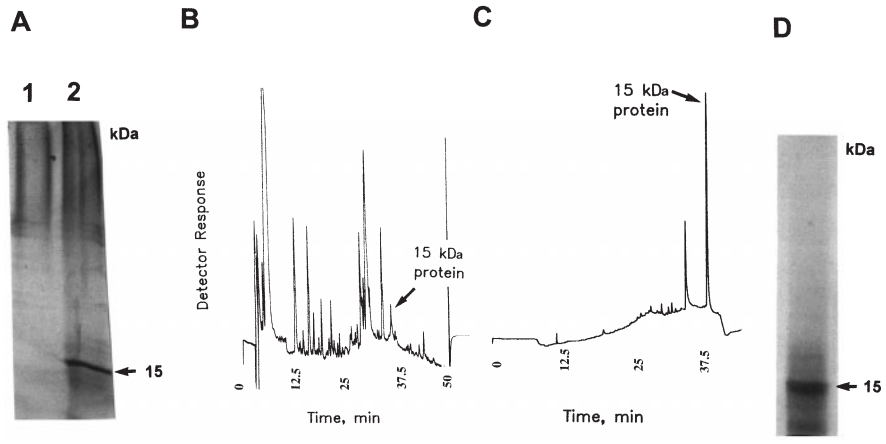


Fig. 4A–D. Separation from carbohydrate and lignin by rpHPLC of GCD-extracted 15-kDa protein that resisted extraction in boiling SDS. **A** Proteins from GCD extracts were separated from carbohydrates by preparative rpHPLC followed by resolution by 12% SDS-PAGE. *Lanes: 1*, control (1/16 M&S medium); *2*, PEG (−1.0 MPa PEG 8000). **B** Analytical separation of a 15-kD protein(s) from carbohydrates and lignins. Void-volume peaks tested positive for carbohydrate by the phenol-sulphuric acid reaction. Retained peaks that eluted before the 15-kD proteins tested positive for the presence of lignins. **C** Collection and recycling of the 15-kDa analytical rpHPLC protein peak. **D** Resolution of the 15-kDa analytical rpHPLC peak by 12% acrylamide SDS-PAGE. *Arrows* indicate the 15-kDa protein(s)

in cross-section (Fig. 2C) showed that the radius of the cortical cells contracted in osmotically stressed tissue (Fig. 2D). Cell-wall proteins from control and osmotically stressed roots were extracted with GCD, purified by HPLC and resolved by SDS-PAGE (Fig. 3). Large amounts (0.1% of dry weight) of these proteins were deposited into the cell walls during elastic constriction (Fig. 3A, lane 1) in response to osmotic stress concurrent with a sharp increase in elastic coefficient (Fig. 3B, lane 1). However, after 24 h of PEG stress these proteins could not be extracted with GCD (Fig. 3A, lane 4) and the elastic coefficient of the cell wall returned to normal (Fig. 3B). Some cell wall proteins could still be extracted after an exposure to −1.0-MPa PEG 8000 for 8 h followed by 16 h exposure to a hypo-osmotic solution (−0.1 MPa) (Fig. 3A, lane 5). Analysis of stress-induced polypeptides with a molecular mass ranging from 6 to 21 kDa indicated the presence of N-terminal amino acid sequences MTNGQTANVS, DDSMQIGTSF, DYANDHDIGL, and GFITSTGTVP which did not show any strong homology to known proteins in this limited region (results not shown).

After boiling the walls in 1.0% SDS for 15 min, a 15-kDa protein was still extracted from the cell wall with GCD (Fig. 4). Following ethanol precipitation of the proteins from GCD, the resulting pellet tested positive for the presence of carbohydrate and lignin by the phenol sulphuric acid and phloroglucinol reactions respectively (results not shown). The ethanol precipitate was soluble in boiling Laemmli buffer, but precipitated on cooling, and no proteins were detected on cellulose or PVDF dot-blots. Nevertheless, smears that did not migrate far into the stacking gel during SDS-PAGE were detected (results not shown). However, the preparative rpHPLC of cell wall proteins from the water-soluble void volume was sufficient to achieve positive protein dot-blots and high-resolution SDS-PAGE gels (Fig. 4A). The proteins were also analytically separated from both carbohydrate and from lignins by rpHPLC (Fig. 4B). The major 15-kDa protein (Fig. 4B) was collected, combined and recycled (Fig. 4C) and subsequently resolved by SDS-PAGE (Fig. 4D). The separation of the cell wall proteins by rpHPLC thus confirmed the absence of covalent attachment to other high-molecular-weight-cell wall components. The rpHPLC separation of the proteins and subsequent electrophoretic resolution (Fig. 4) indicated that these salt- and SDS-resistant proteins were initially insolubilized by a mechanism other than oxidative attachment, perhaps by extensive weak dipole bonds, sometimes termed hydrophobic interactions, previously demonstrated in fungal cell walls (Wösten et al. 1993).

The progressive loss of these proteins by incremental increases in the boiling time of the walls in 0.1% SDS

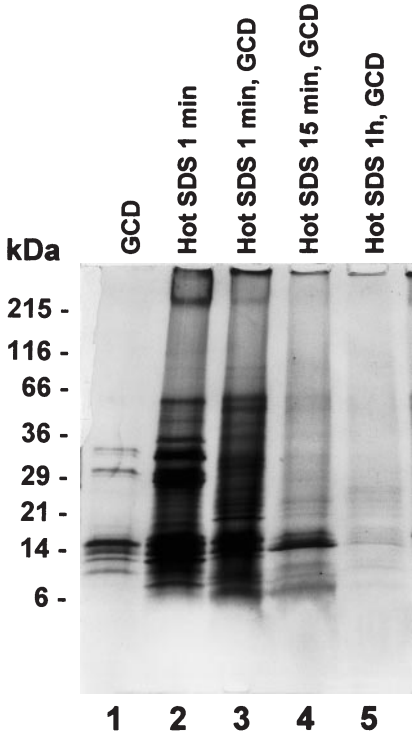


Fig. 5. The GCD-extractable cell wall proteins reside within the fraction solubilized by boiling. Cell walls from roots treated with PEG 8000 for 8 h were washed as described in Fig. 3. *Lanes*: *1*, proteins extracted by GCD before boiling in 0.1% SDS; *2*, proteins extracted by boiling in 0.1% SDS for 1 min; *3*, proteins extracted by GCD after 1 min of boiling in 0.1% SDS; *4*, proteins extracted by GCD after 15 min of boiling in 0.1% SDS; *5*, proteins extracted by GCD after 1 h of boiling in 0.1% SDS

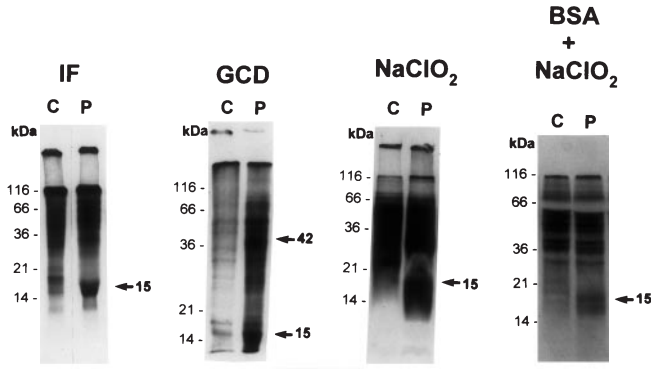


Fig. 6. Secretion and progressive insolubilization of newly synthesized cell wall proteins into the roots from control or osmotically-stressed jack pine seedlings. Fluorographic comparison of methods for extraction of root cell walls: IF proteins were obtained by vacuum infiltration of tissues with 6 mM $CaCl_2$, 1 mM EDTA and 5 mM ascorbate, and the IF collected by centrifugation of the tissue. Proteins extracted by GCD or $NaClO_2$ were obtained from roots that were ground in 80% acetone, washed in 0.1% SDS, leached in 6 M GHCl and 0.1 M $CaCl_2$ and boiled in 1% SDS for 15 min prior to extractions. *IF*, A1-μg sample of protein from the IF was resolved by 12% SDS-PAGE; *GCD*, GCD-soluble proteins from equal dry weights of cell walls were precipitated in ethanol and resolved by 12% SDS-PAGE; *$NaClO_2$*, 2000 cpm per lane of proteins extracted with 0.3% w/v $NaClO_2$ and 0.24% acetic acid were resolved by 15% SDS-PAGE; *$NaClO_2$ + BSA,* 2000 cpm per lane of protein extracted with 0.3 (w/v) $NaClO_2$ and 0.24% acetic acid in the presence of 0.1 M BSA, were resolved on 15% SDS PAGE. *Lanes*: *C*, control, (1/16 M&S medium); *P*, PEG (−1.0 MPa PEG). *Arrows* indicate increased amounts in radiolabeled proteins

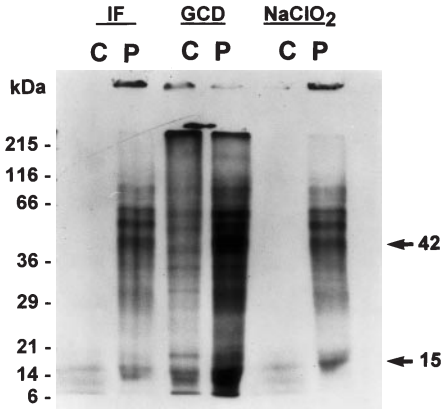


Fig. 7. Fluorographic comparison of sequential extraction of extracellular proteins from the same untreated or osmotically stressed root cell walls. After vacuum infiltration, root tissue was disrupted, boiled in 1% SDS for 15 min and rinsed in ethanol before extraction with GCD, rinsed with water, and subsequently extracted with $NaClO_2$. *IF*, proteins from vacuum infiltration of the tissue; *GCD*, subsequent extraction of GCD-soluble ethanol-precipitable proteins after leaching the tissue in GHCl and boiling 1% SDS; *$NaClO_2$*, progressive extraction of GCD-extracted walls with 0.04%–0.6% (w/v) $NaClO_2$ in the presence of 0.24% acetic acid. *Lanes*: *C*, control (1/16 M&S Medium); *P*, PEG (−1.0 MPa PEG). *Arrows* indicate increases in radiolabeled proteins

(Fig. 5) seems to indicate that the 15-kDa molecules, which were only slightly soluble in 6 M GHCl or 1% SDS at room temperature (Fig. 5, lane 1) were associated with the water-soluble fraction, sometimes referred to as the pectic fraction, of the cell walls (Bacic et al. 1988; Fig. 5, lane 2). These stress-induced 15-kDa proteins were still extracted from the wall by GCD′ presumably after their hydrophobic interaction with the surrounding matrix was destabilized by heating in 0.1% SDS (Fig. 5, lanes 3, 4), thus indicating that these proteins were not yet permanently attached to the wall. Most of the GCD-extractable cell-wall proteins were removed by boiling in 0.1% SDS for 1 h (Fig. 5, lane 5). Hence the oxidative insolubilization of cell-wall proteins in response to dehydration proceeded more slowly than the rapid SDS-insolubilization of proteins observed in response to wounding or elicitors (Bradley et al. 1992).

A comparison of de-novo-synthesized cell wall proteins in the IF with proteins in GCD and $NaClO_2$ extracts (Fig. 6) revealed that both non-destructive and destructive methods of extraction yield a similar set of proteins, particularly proteins of a molecular mass of about 15 kDa. However, destructive extraction with acid $NaClO_2$ released higher-molecular-mass proteins of 25–60 kDa. The de-novo-synthesized and destructively released polypeptides included proteins with molecular masses similar to those of the extensin family of glycoproteins (42 kDa; Stuart and Varner 1980). However, while an increase in the 42-kDa protein was observed in GCD extracts from osmotically stressed roots, only the 15-kDa polypeptide was observed to be increased in destructively extracted cell wall proteins. Protection of these proteins from degradation by BSA (Fig. 6) indicated that the observed molecular masses were not merely proteolytic products. Sequential extraction of the same roots by all three methods, i.e. intercellular washing, followed by GCD extraction of guanidine- and SDS-insoluble proteins, followed by destructive extraction with acid $NaClO_2$ (Fig. 7) revealed a pattern of radiolabelled cell-wall proteins similar to those extracted by the use of each technique alone (Fig. 6).

We interpret the extraction of proteins by $NaClO_2$ to indicate that these proteins may have been oxidatively insolubilized. The preferential release of the protein by acid $NaClO_2$, a reagent that dissolves lignin (Whitmore 1978a, b), is consistent with the disruption of oxidative links between the protein and the extracellular matrix or the hydrolysis of isodityrosine residues (Fry 1982; Varner and Lin 1989; Briggs and Fry 1990; Waffenschimdt et al. 1993). Polypeptides have been also released from cell walls by treatment with cellulase, pectinases and trypsin followed by anhydrous HF, and this comprises the most specific biochemical evidence that cell-wall proteins may have been covalently attached to long-chain carbohydrates (Qi et al. 1995). Our strategy of removing non-covalently attached proteins by progressive extraction in salts, SDS, boiling SDS and then GCD prior to destructive extraction in acid $NaClO_2$ provides evidence supporting the notion that these cell-wall proteins were held by bonds that were preferentially susceptible to oxidative attack and might not have been held by the apparently strong non-covalent interaction with the matrix alone (Fig. 7).

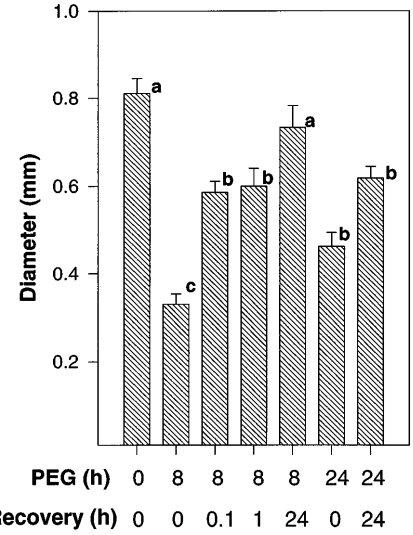


Fig. 8. The effect of PEG 8000 stress and stress relief on the diameter of newly grown white roots of jack pine. The diameter of the roots was measured to the nearest 0.1 mm. The vertical bars represent Means ± SE ($n = 6$). Letters indicate significant differences according to the highly significant difference (HSD) test ($l = 0.05$)

The diameter of growing pine roots decreased visibly by more than 50% during stress, and concomitant insolubilization of these proteins and the complete recovery to original diameter were not observed after 1 h of relief from osmotic stress (Fig. 8). However, roots stressed for 8 h recovered most of their original volume after 24 h of recovery. In contrast, roots stressed for 24 h (when most of the cell wall proteins were oxidatively insolubilized, Fig. 3) did not re-expand to their original diameter (Fig. 8). The capacity of stressed roots to resist re-expansion to their original volumes is consistent with the proposed role of oxidatively insolubilized, cell-wall proteins in the formation of a new network of interlinking molecules that re-define the limit of cellular expansion (Sadava and Chrispeels 1973). The formation of a smaller constricting network of extracellular proteins within the walls of the tissue was thus consistent with the return of the elastic coefficients in the roots to pre-stress levels after 24 h of adjustment to osmotic stress (Fig. 3).

To summarize, in response to osmotic stress, the cell walls of jack pine roots underwent a marked adjustment of elasticity that maintained turgor despite profound dehydration, and these alterations in elasticity were accompanied by the secretion and subsequent insolubilization of cell wall proteins. Hence, these results indicate that the insolubilization of proteins into the cell wall may markedly alter cell wall function and provide a mechanism responsible for the maintenance of turgor in shrunken tissues (Eze et al. 1986; Levitt 1986; Marshall and Dumbroff 1999). Proteins can be held in the fabric of the cell wall by a mechanism other than oxidative attachment (Ye et al. 1991). Hence, non-covalent bonds like those that play a role in plasticity during growth (Virk and Cleland 1990) were apparently crucial to the rapid adjustment of tissue elasticity during stress. These changes were similar to the trends in extractable cell wall proteins that have been observed to accompany the maintenance of turgor in dehydrated coleoptiles (Bozarth et al. 1987; Nonami and Boyer 1989) and the maintenance of turgor in small cells that have adapted to osmotic stress (Handa et al. 1982; Iraki et al. 1989). The putative oxidative attachment of these proteins to the extracelluar matrix seems to accompany the elastic constriction of the cell wall around the shrinking protoplasts in a manner that apparently regulates turgor. With time the roots adjust to the reduced tissue volume and elasticity returns to normal.

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