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Cell Wall Proteins at Low Water Potentials[1]

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ABSTRACT

We investigated the proteins extractable from cell walls of stem tissues when plants were subjected to low water potentials (low ψ_w). Dark-grown soybean seedlings (*Glycine max* [L.] Merr.) showed decreased stem growth when the roots were exposed to vermiculite having low water content (ψ_w = −3 bar). After a time, growth resumed but at a reduced rate relative to the controls. The extractable protein increased in the cell walls as ψ_w decreased, especially a 28-kilodalton protein in the young tissue. In contrast, a 70 kilodalton protein, mainly extractable from mature cell walls, appeared to decrease slightly at low ψ_w. No hydroxyproline was present in either protein, which shows that neither protein is related to extensin. The level of the 28 kilodalton protein increased in the cell wall of the dividing region soon after the initial growth inhibition, and it appeared in the elongating tissue at about the time growth resumed. The correlation between growth and these protein changes suggests that the two events could be related.

The enlargement of plant cells is often affected adversely by low ψ_w.[3] Developing floral tissues, expanding leaves and elongating stems respond to a decrease in ψ_w prior to the roots, which may grow unabated (23, 26, 31). This differential response can be used to study the effects of low ψ_w on growth. In dark-grown soybean seedlings, roots grow rapidly while stem growth is inhibited at low ψ_w. The inhibition is followed by an accumulation of solutes that maintains turgor at a near constant value (7, 22). The solutes are metabolic substrates derived from cotyledonary reserves (23). Because they are present in high amounts together with high turgor, factors other than substrate availability and turgor must cause the inhibition of stem growth. One possibility is that biochemical changes occur in the cell walls because wall extensibility is metabolically controlled and undergoes modification after long periods of low ψ_w (8, 21, 29, 30).

The constituents of cell walls are altered when tissues are wounded (25, 27) or their growth rate changes (16, 28). Extensin, a hydroxyproline-rich glycoprotein that crosslinks with wall carbohydrates, accumulates in a soluble extractable form (25, 27) when tissue is excised (9). Hydrolytic enzymes are reported to change in activity as a result of changes in growth (10, 15, 17, 20, 28).

This indicates that, because stem growth is inhibited at low ψ_w, changes might occur in cell wall proteins. This possibility has not been investigated. We therefore used soybean seedlings growing in vermiculite having differing water contents to examine the proteins in the cell walls of stem tissues at low ψ_w. Extractable proteins accumulated under these conditions, particularly a 28 kD protein.

MATERIALS AND METHODS

Plant Materials. Soybean seeds (*Glycine max* [L.] Merr. cv Williams) were surface sterilized in 1% NaOCl, rinsed in running tap water for 2 h, planted in vermiculite wetted to runoff with 0.1 mM $CaCl_2$ (about 5.0 ml $CaCl_2$/g of vermiculite), and kept in the dark at 100% RH and 29°C. After 48 h, seedlings were selected for uniformity and transplanted to vermiculite containing the same amount of solution (1× seedlings, control) or 1/8 the amount of solution (1/8× seedlings, treatment). The ψ_w was −0.1 bar in the 1× vermiculite and −3.0 ± 0.2 bar in the 1/8× vermiculite and was constant throughout the experiment. All seedling manipulations were done at 100% RH under a green safelight (green fluorescent bulb wrapped in cellophane with negligible transmission below 475 nm and above 575 nm).

Growth Rates. The growth rate was determined by measuring total stem length at 24 h intervals after transplanting.

Water Potential Measurements. The ψ_w was determined isopiestically using thermocouple psychrometers according to Boyer and Knipling (4) and Boyer (2). For the dividing region, ten 5-mm segments were used per sample. For the elongating and mature regions, five 15-mm segments were used per sample.

Cytoplasmic Protein and Cell Wall Protein. Stems were harvested 24, 48, 72, and 96 h after transplanting and divided into 3 regions: the 5 mm section just below the cotyledons (dividing region); the next 15 mm (elongating region); and the remainder of the stem (mature region).

Samples of 3 to 8 stem segments were ground at 0°C in 62.5 mM Tris-HCl (pH 7.2), 1% mercaptoethanol, and protease inhibitors: 0.5 mM phenyl methyl sulfonyl fluoride, 0.5 mM ε-amino-*n*-caproic acid, and 0.1 mM benzamidine. After centrifuging at 1,000*g* for 3 min, the supernatant was decanted and recentrifuged at 13,000*g* for 3 min. The resulting supernatant was the cytoplasmic extract. The pellet from the 1,000*g* centrifugation was rinsed 10 times by resuspending in fresh grinding buffer, recentrifuging, and discarding the supernatant. The washed pellet was the cell wall fraction. After the final rinse, the pellet was resuspended in 62.5 mM Tris-HCl (pH 7.2), 0.5 M $CaCl_2$, and 5 mM DTT and was incubated at 5°C from 3 to 12 h with occasional mixing. The suspension was centrifuged at 13,000*g* for 15 min and the supernatant (cell wall extract) was decanted from the cell wall pellet and desalted on a miniature Sephadex G-50-80 column using the spun column technique of Helmerhorst and Stokes (13). Protein determinations were according to Bradford (5).

Protein Separation. SDS-Polyacrylamide gel electrophoresis

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[3] Abbreviation: ψ_w, water potential.

(SDS-PAGE) was performed on the cell wall extracts according to Laemmli (18) on 12.5% gels except the acrylamide:bis-acrylamide was 30:0.6. Constant amounts of protein were loaded (20 μg/lane). Gels were stained with Coomassie blue R250, destained, and dried between two pieces of porous cellophane. The lanes on the gels were scanned at a wavelength of 550 nm in a Beckman DU8 scanning spectrophotometer. The relative areas under the peaks were determined by weighing the appropriate areas of paper on which the peaks were displayed.

Amino Acid Analysis. The 28 kD and 70 kD protein bands from the cell wall extract on an SDS-PAGE gel were made visible with 4 M sodium acetate (14). The bands were then excised, washed, and pulverized in 50 mM ammonium bicarbonate and 0.1% SDS. After overnight incubation at 4°C, the acrylamide was removed by centrifugation. The supernatants (containing protein) were concentrated and dialyzed in Amicon Centricon 10 microconcentrators. After the initial concentration, the proteins were rinsed in the microconcentrators five times with 50 mM ammonium bicarbonate and five times with water. The resulting solution was lyophilized and used for amino acid analyses performed by the Biotechnology Support Laboratory at Texas A & M University.

RESULTS

Growth and Tissue Protein Contents at Low ψ_w. Stem growth was inhibited after transplanting the seedlings to 1/8× vermiculite. Stem length remained almost constant for 48 h (Fig. 1A) and eventually began to increase, but at a reduced rate (1.1 mm/h after 72 h) compared to the controls (2.2 mm/h).

The ψ_w of the 1× stems remained high throughout the experiment, but the ψ_w of the 1/8× stems decreased rapidly after transplanting to the 1/8× vermiculite (Fig. 1B). During the first 24 h, ψ_w decreased to −6.5 bars in the elongating and mature tissue and −8.0 bars in the dividing tissue. After reaching the lowest ψ_w, a recovery of 2 or 3 bars began in all of the tissues. Meyer and Boyer (23) and Cavalieri and Boyer (7) found similar decreases in ψ_w of various regions of the stem, and solutes accumulated in the stem tissues. A similar accumulation should have occurred in the present experiments because the conditions were identical. Therefore, turgor should have been high in the elongating tissues.

If turgor was high, the water content of the tissues should have remained high as ψ_w decreased. To test this idea, the fresh weight of stem tissue from seedlings selected for uniform stem diameters was measured. The fresh weight per unit length did not decrease upon transplanting to 1/8× vermiculite (Fig. 2). Likewise, the fresh weight of the 1× stem tissue remained constant except in the dividing region where the fresh weight increased after 48 h (Fig. 2A). The increase in fresh weight of the 1× dividing region was correlated with an increase in its ψ_w (*cf.* Figs. 1B and 2A). The similarity in fresh weights of the 1× and 1/8× tissues confirmed the turgor maintenance in these stems and allowed us to conveniently express the data in succeeding experiments on a fresh weight basis.

The exposure of the seedlings to the drier vermiculite resulted in much shorter stems than in controls by the end of the experiment. Because this lag in development could in itself cause differences in stem characteristics, it was necessary to compare seedlings on both a developmental scale (stem length) and a time scale (hours after transplant). It is important to note that in the developmental comparisons, no corrections were necessary for fresh weight differences because only 1× tissue younger than 48 h (Figs. 1 and 2) was included and the fresh weight of this tissue was the same as in the 1/8× tissue. The time comparison included older tissue, however, and a correction for fresh weight was necessary in the dividing region.

The total protein in the cytoplasm decreased with age in all

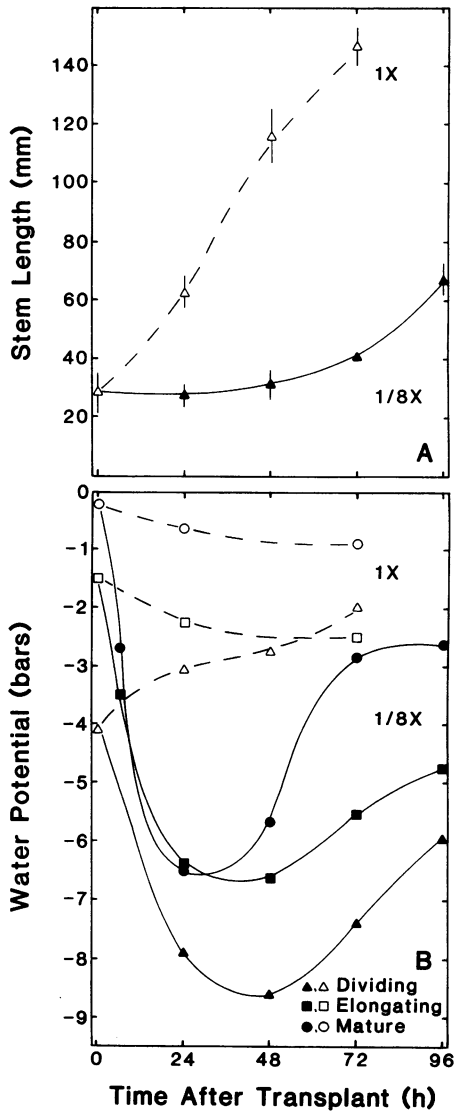

FIG. 1. Growth (A) and water potential (B) of stems of soybean seedlings transplanted to 1× or 1/8× vermiculite. Water potential data in (B) are representative of three replicate experiments.

the stem tissues (Fig. 3), but the decrease was delayed in the 1/8× stems (Fig. 3A–C). When this comparison was made on a developmental scale, the differences largely disappeared in the statistical variability between treatments (Figs. 3D–F). In contrast, the protein contents of cell wall extracts were considerably higher in 1/8× seedlings than in 1× seedlings regardless of whether the data were expressed on a time (Fig. 4A–C) or stem length basis (Fig. 4D–F).

Protein Separation. When proteins in the cell wall extracts were separated, a 28 kD protein was apparent and accumulated at low ψ_w (Fig. 5). The increase was greatest in dividing region and could be observed after 24 h (Fig. 5A). The 28-kD protein became the major extractable protein in the dividing region at low ψ_w. In the elongating region, a similar accumulation occurred, but was delayed for 48 to 72 h (Fig. 5B). In the mature region, the 28 kD protein was only a minor band even 96 h after transplanting into 1/8× vermiculite, and accumulation was slight (Fig. 5C).

In contrast, a 70 kD protein became more abundant as the stem tissue matured (Fig. 5A–C) and was a major extractable protein in the mature tissue (Fig. 5C). The protein appeared to

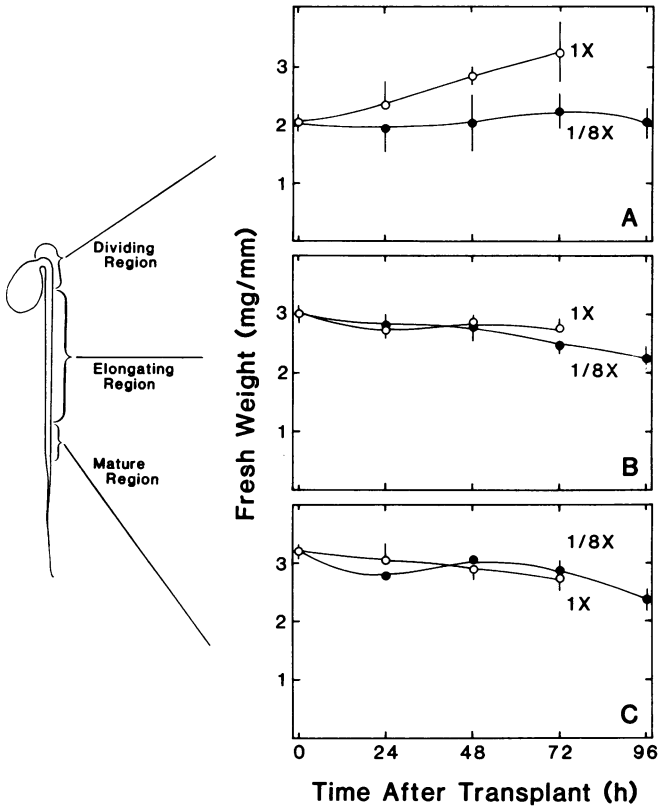

FIG. 2. Effects of transplanting on the fresh weight/unit length of dividing (A), elongating (B), and mature (C) tissue segments from stems of soybean seedlings. The regions indicated on the representative seedling (left) are those sampled in all of the succeeding experiments. Note that the dividing (5 mm) and elongating (15 mm) regions were always harvested relative to the cotyledons as indicated in the diagram; thus only the mature region increased in length throughout the experiment.

decrease in the cell walls of 1/8× stems compared to controls (Fig. 5), although the effect was small.

These changes in wall protein were not caused by differences in stem development because they were also apparent when comparisons were made on a length basis. Figure 6 shows that the stem tissues contained approximately equal amounts of the 28 kD protein at a 28 mm stem length but the level became greatly elevated in 1/8× stems after they had elongated to 68 mm. The increase was greatest in the dividing region (Fig. 6B) and became progressively less in the elongating (Fig. 6D) and mature regions (Fig. 6F). The 70 kD protein contrasted with this behavior. The progressive increases in the elongating (Fig. 6C) and mature tissues (Fig. 6E) were accompanied by a slight suppression of the relative amount of this protein in the 1/8× stems compared to the controls.

In the cytoplasmic fraction, we detected a 28 kD protein but not a 70 kD protein. This raised the possibility that the 28 kD protein in the cell wall was a contaminant from the cytoplasm. We tested whether the association of the 28 kD protein with the wall was the result of fortuitous binding during the extraction procedure (10) by adding excess 28 kD protein to the grinding medium during the homogenization of the tissue. Table I shows that the 28 kD protein did not increase in the cell wall fraction (Table 1). There is no doubt some cytoplasmic contamination of the cell wall extract, since heavily overloading the gels with the cell wall extract showed minor banding corresponding with the cytoplasmic banding patterns. However, the results of Table I together with the low level of contamination by other cytoplasmic proteins indicated that the 28 kD protein is normally associated with the cell wall.

Amino Acid Composition of the 28 kD and 70 kD Cell Wall Proteins. Table II shows that the 28 kD and 70 kD proteins were composed of significant amounts of all the protein amino acids except hydroxyproline, which was absent, and methionine, which was present in low amounts (about 2 residues per protein molecule). Tryptophan and cystine/cysteine were not determined. The composition of the 28 kD protein differed somewhat from that of the 70 kD protein. The 28 kD protein had less aspartate/asparagine, serine, glycine, threonine, and alanine than the 70 kD protein but more glutamate/glutamine, tyrosine, and lysine.

DISCUSSION

The treatments used in these experiments caused the rates of stem growth to differ widely. Consequently, the effects of low ψ_w could have been simply to delay stem development, and the protein differences could have resulted from the different developmental states of the tissues. On the other hand, the protein differences could have resulted from ψ_w-induced alterations in cell metabolism that would be visible at the same degree of development. It was necessary to separate these effects by considering seedling responses at comparable times and at comparable stem development. We used stem length to indicate the stage of development because the anatomy of the elongating tissue was similar in the control and low ψ_w treatments (22). Thus, developmental differences were associated mostly with the amount of mature tissue, which was measurable in terms of stem length.

The results show that salt extractable proteins accumulated in the cell walls of soybean stems at low ψ_w regardless of whether the comparisons were made at the same time or at the same length. This indicates that ψ_w-induced differences in cell metabolism were involved in the accumulation. In the cytoplasm, on the other hand, there was an effect only if comparisons were made at the same time. In this case, the differences are attributable to the delay in stem development and not to specifically altered metabolism. Consequently, the accumulation of wall proteins was specific for the walls and was not an indirect result of an overall accumulation of protein in the cell.

The accumulation was not the same for all wall proteins. When constant amounts of protein were separated on gels so that comparisons could be made on a relative basis, a 28 kD protein increased dramatically in the dividing and elongating regions. Low ψ_w brought about the increase regardless of whether the comparison was made on a time or length basis, again indicating that wall metabolism had been altered by these conditions. As a result, the 28 kD protein became the major wall protein on the gels from the dividing region. The lack of a significant accumulation in the mature tissue suggests that the 28 kD wall protein may have been involved in new wall development.

In contrast, the 70 kD protein was scarcely present in the dividing region but was the major protein extractable from the mature walls. It showed slightly less relative abundance at low ψ_w. This effect is consistent with an involvement of the 70 kD protein in cell maturation, the rate of which was slowed under these conditions (23).

These responses to low ψ_w could not be attributed to wounding (1, 12), because the experiments were conducted with intact seedlings. External osmotica were not used and thus the protein changes could not have resulted from indirect effects of high concentrations of exogenous solute. The roots were exposed only to a limited water supply and the effects were transmitted internally to the stem in a fashion likely to occur under natural conditions.

It is important to consider how these changes might occur in an intact plant. Changes in the protein of the walls could reflect altered amounts of wall enzymes. The amino acid composition

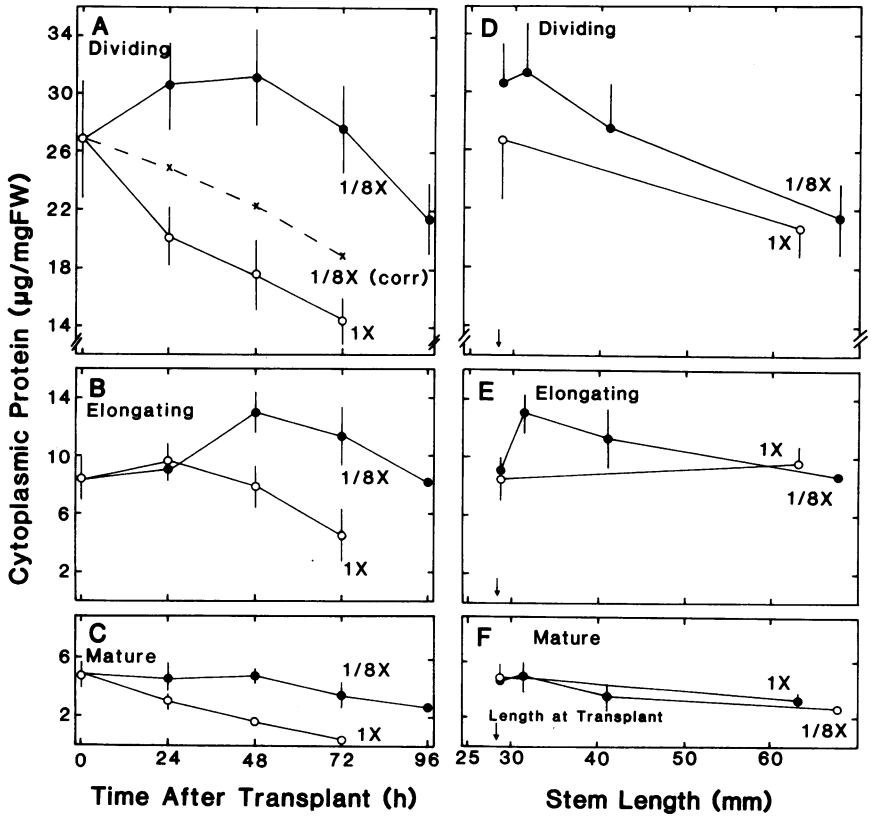

FIG. 3. Content of protein in the cytoplasm of dividing (A), elongating (B) and mature (C) regions of soybean stems at various times after transplanting to 1× or 1/8× vermiculite. The same data compared on the basis of stem length are shown for the dividing (D), elongating (E) and mature (F) regions. The 1/8× (corr) values shown in (A) were calculated from the 1/8× data on the basis of the 1× (control) fresh weights (Fig. 2A). No correction was necessary for the analogous measurement in (D) (see text). Three segments were ground for each extraction. Data represent means ± SD of three replicates.

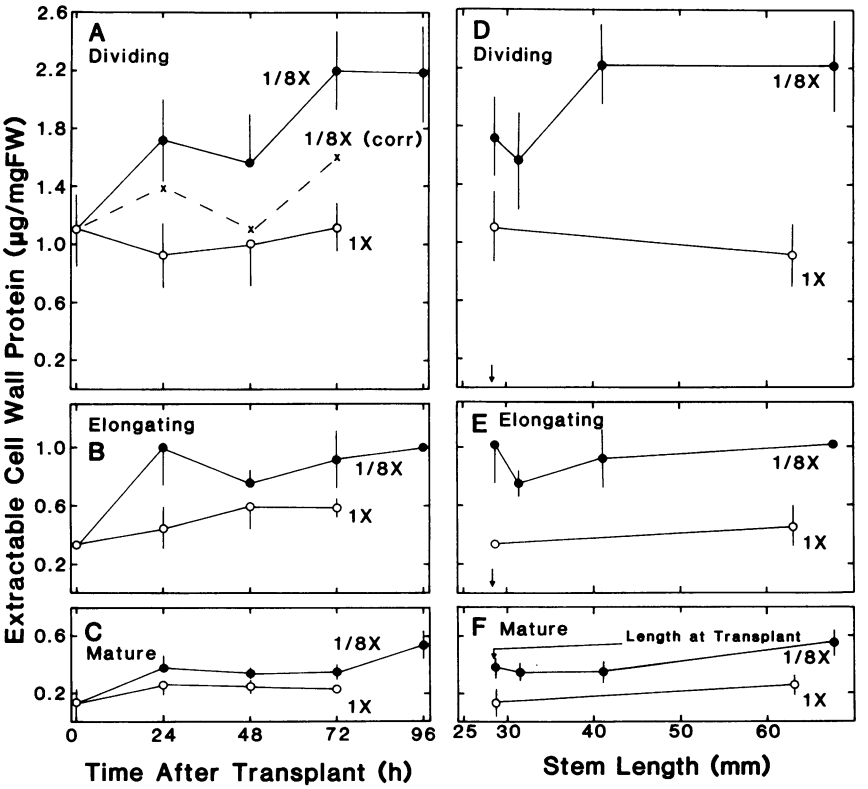

FIG. 4. Content of extractable protein from cell walls of dividing (A), elongating (B) and mature (C) regions of soybean stems at various times after transplanting to 1× or 1/8× vermiculite. The same data compared on the basis of stem length are shown for the dividing (D), elongating (E) and mature (F) regions. Note that the protein values on the vertical axes are 1/10 those of Figure 3. The 1/8× (corr) values shown in (A) were calculated as described in Figure 3. No correction was necessary for (D) (see text). Three segments were ground for each extraction. Data represent means ± 1 SD of three replicates.

of both the 28 kD and 70 kD protein is similar to that of many enzymes and had no extremes often associated with structural proteins. Cell wall loosening may involve cell wall hydrolases, which change in activity as growth changes (10, 15, 17, 19, 20). Such a role is possible for the 28 kD protein in particular although the molecular mass as determined by SDS-PAGE does not correspond with studied cell wall hydrolases. Wall peroxidases also change in activity but their molecular masses are larger than 28 kD (11) and they are more likely to be prevalent in mature tissue where secondary wall formation would be occurring.

A structural role for these proteins is also possible. Although the amino acid compositions of the 28 kD and 70 kD proteins

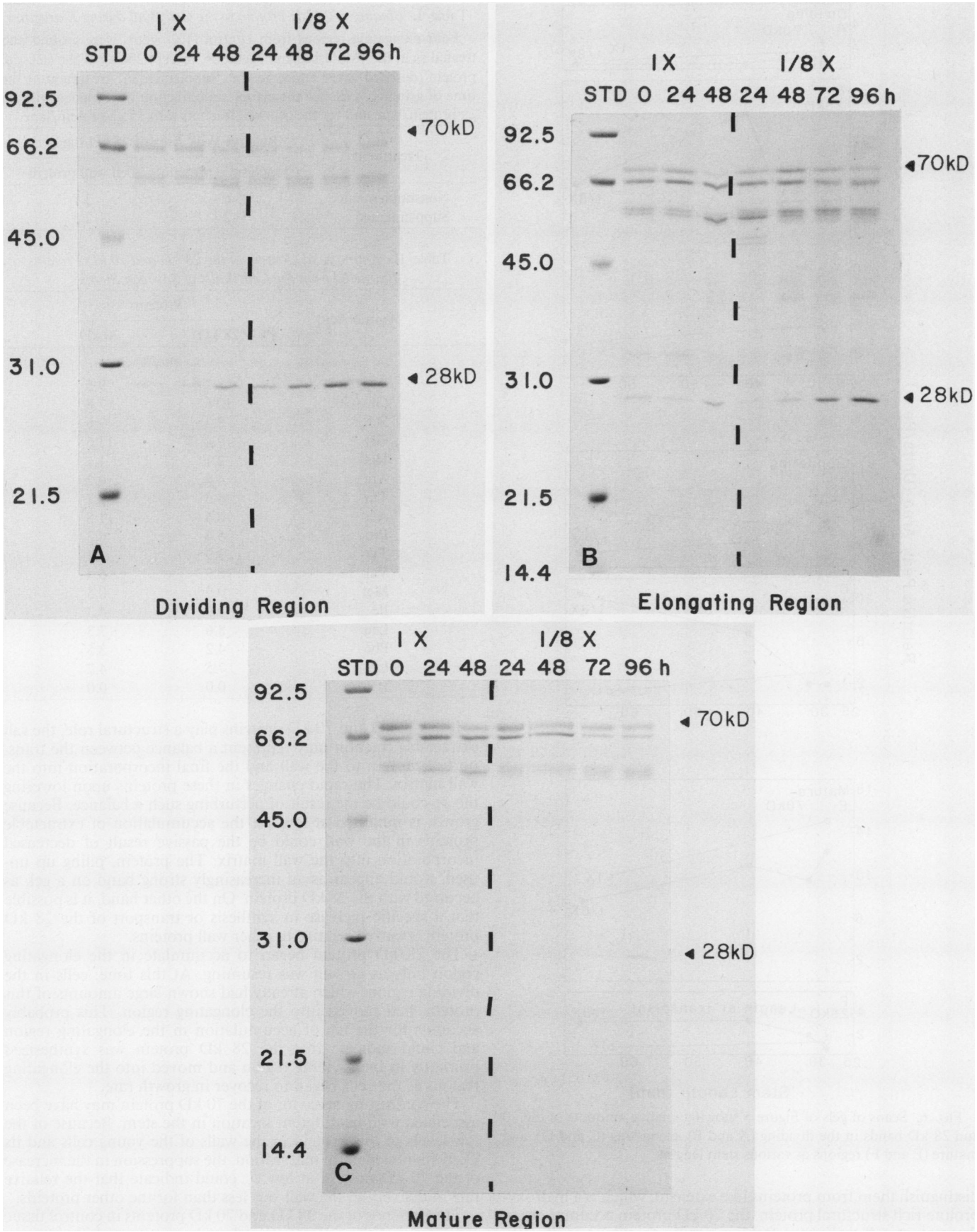

FIG. 5. Comparison of proteins from cell walls of 1× and 1/8× stems of soybean seedlings at 24 h intervals after transplanting. SDS-PAGE gels (20 μg protein/lane) show salt-extractable proteins from the dividing (A), elongating (B), and mature (C) regions. Arrows to the right of the gels indicate the 70 and 28 kD bands.

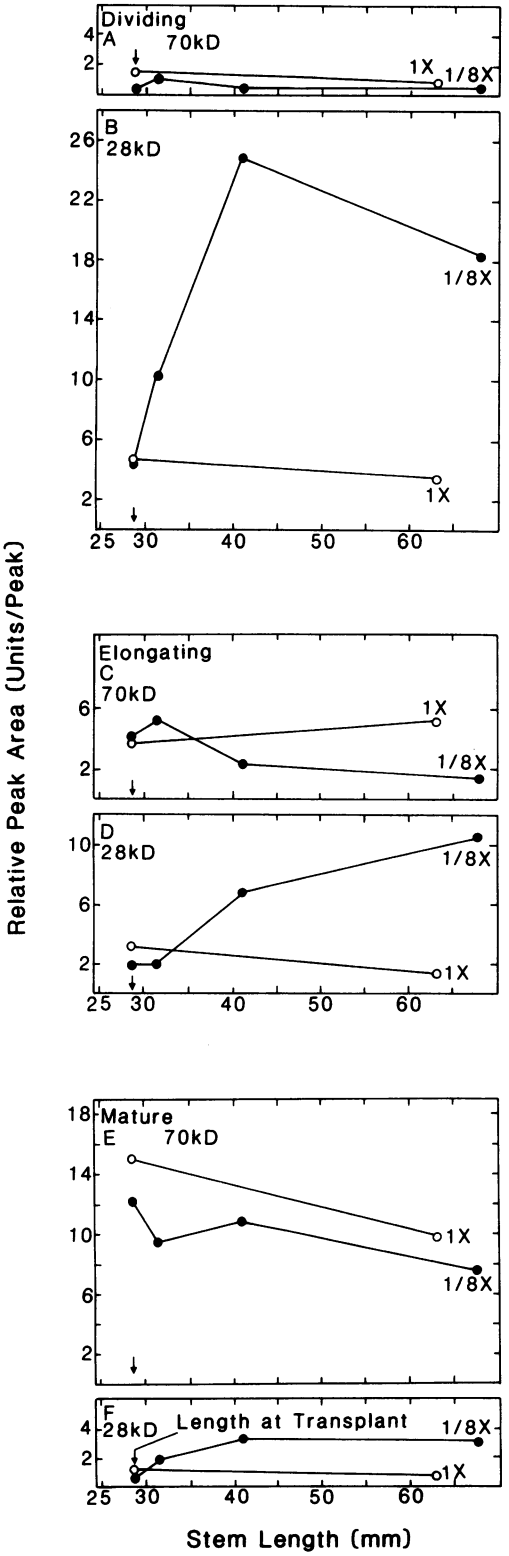

FIG. 6. Scans of gels of Figure 5 showing relative amounts of the 70 and 28 kD bands in the dividing (A and B), elongating (C and D), and mature (E and F) regions at various stem lengths.

distinguish them from proteins like extensin, which is a hydroxyproline-rich structural protein, the 70 kD protein accumulates in the mature cell walls in a fashion similar to extension (6, 19). Extensin was not detected in the current study because this protein is not resolvable with standard SDS-PAGE (27).

Table I. *Binding of 28 kD Protein to the Cell Wall during Extraction*

Four elongating regions from control (1X) stems were ground and treated as in the "Materials and Methods," except 200 μg of the cell wall protein (desalted) were added to the "supplemented" treatment at the time of grinding. Gels for the cytoplasmic fraction were loaded with 25 μg protein/lane and for the cell wall fraction with 15 μg protein/lane.

Treatment	Relative Peak Area of 28 kD Band	
	Cytoplasmic protein	Cell wall protein
Nonsupplemented	5.6	3.1
Supplemented	6.2	2.5

Table II. *Amino Acid Content of the 28 kD and 70 kD Proteins Extracted from the Cell Walls of Soybean Stems*

Amino Acid	Protein	
	28 kD	70 kD
	mol%	
Asp/Asn	8.2	9.5
Glu/Gln	10.6	7.8
Ser	8.3	9.6
Gly	9.4	13.6
His	2.1	1.1
Arg	4.4	4.2
Thr	6.3	8.7
Ala	8.4	11.3
Pro	5.4	6.3
Tyr	3.9	2.1
Val	6.9	6.4
Met	0.9	0.5
Ile	4.9	4.3
Leu	8.6	7.3
Phe	4.2	3.5
Lys	7.5	4.2
HPro	0.0	0.0

If the 28 kD and 70 kD proteins play a structural role, the salt extractable fraction may represent a balance between the transport of protein to the wall and the final incorporation into the wall matrix. The rapid changes in these proteins upon lowering the ψ_w could be the result of perturbing such a balance. Because growth is inhibited at low ψ_w, the accumulation of extractable proteins in the wall could be the passive result of decreased incorporation into the wall matrix. The protein, piling up unused, would appear as an increasingly strong band on a gel, as occurred with the 28 kD protein. On the other hand, it is possible that a specific increase in synthesis or transport of the 28 kD protein occurred relative to other wall proteins.

The 28 kD protein began to accumulate in the elongating region only as growth was resuming. At this time, cells in the dividing region, which already had shown large amounts of this protein, had moved into the elongating region. This probably accounts for the lag of accumulation in the elongating region and could indicate that the 28 kD protein was synthesized primarily in the dividing region and moved into the elongating regions as the cells began to recover in growth rate.

The contrasting behavior of the 70 kD protein may have been associated with its different location in the stem. Because of the low levels of this protein in the walls of the young cells and its likely involvement in maturation, the suppression in the increase of the 70 kD protein at low ψ_w could indicate that the relative rate of delivery to the wall was less than for the other proteins.

The presence of the 28 kD and 70 kD proteins in control tissue indicates that the differences at low ψ_w represent shifts in regulation and not an appearance or disappearance of proteins. Growth involves a combination of both physical and metabolic

components and, in soybean stems exposed to low ψ_w, the initial inhibitory event appears to be a physical disruption of the ψ_w gradient from the xylem to the outer cortex (3, 24). It is likely that cell wall extensibility decreases sometime afterward (21, 30), but turgor often remains high due in part to rapid osmotic adjustment (7, 22, 23). The modest resumption of growth that occurred late in the experiment implies that the water potential gradient was reestablished and the metabolic changes were somewhat reversed. The fact that the recovery of growth did not occur until the elongating region showed enhanced levels of the 28 kD protein suggests that the accumulation may have been important for the growth recovery.

We propose that the 28 kD protein is normally associated with early wall growth, either in a catalytic or structural role. The regulation of this protein indicates that early wall metabolism is modified greatly in seedlings whose growth is modified by low ψ_w. The 70 kD protein also shows evidence of regulation but its association with the mature tissue implies a role in the normal maturing of the cell walls.

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