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Analysis of cell wall hardening and cell wall enzymes of salt-stressed maize (*Zea mays*) leaves

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Abstract. It has been indicated that salinity inhibits maize (*Zea mays* L.) leaf growth and leaf cell expansion by increasing the apparent yield threshold of the cell wall. We tested whether this increase in the apparent yield threshold was a physical property of cell walls, using *in vitro* creep-type assays. Salinity had no significant effects on cell wall structural properties based upon several different *in vitro* assays. In support of these results, there were no differences between control and salt-stressed plants in their total apoplastic concentration of cell wall proteins, in the activity of apoplastic peroxidases or xyloglucan endotransglycosylase. We conclude that short-term salinity does not appear to inhibit maize leaf elongation by hardening the physical structure of the cell walls of the growing zone.

Keywords: apoplastic protein, cell wall extensibility, creep assay, peroxidase, salinity, *Zea mays* L.

Introduction

When salinity is applied to the root medium of maize (*Zea mays* L.) plants, leaf elongation is immediately inhibited (Cramer and Bowman 1991*a*, *b*; Cramer 1992). There is a rapid hydraulic signal (lowered water potential), which reduces the effective turgor of the growing zone. Within an hour or two, osmotic adjustment occurs in the growing zone and turgor recovers. Leaf elongation also recovers to a new steady-state rate, but this rate is below the control rate. The apparent yield threshold is increased (verified by two independent methods), causing a reduced effective turgor force for cell elongation (Cramer and Bowman 1991*a*; Cramer and Schmidt 1995). During these early events, photosynthesis does not appear to limit growth (Mladenova 1990; Cramer *et al.* 1994*a*).

There is no single mechanism for the control of cell expansion under stressed conditions (Cramer and Bowman 1993) that can explain responses in a variety of environmental stresses and genotypes. Although in the past, attention has focused on the effects of salinity on turgor, almost all reports indicate that growing cells of salt-stressed plants maintain normal turgor (Cramer and Bowman 1993). There is some evidence to support the hypothesis that salinity affects the cell walls of the growing zone of grasses (Lynch *et al.* 1988; Cramer and Bowman 1993; Neumann 1995).

The mechanisms for cell wall hardening during osmotic stress are not completely known, but there is considerable evidence that enzymes rather than turgor regulate cell wall extension (Cosgrove 1997*a*, *b*). The biochemistry of the cell wall is very complex and the field of cell wall biochemistry is still young (Carpita 1996; Cassab 1998). It is known that loosening or hardening processes control cell wall extension. Loosening refers to the rearrangement or breakage of load-bearing bonds in the cell wall, whereas hardening refers to the rearrangement or addition of load-bearing bonds. A number of enzymes have been identified as potential regulators of cell wall extension.

Peroxidases appear to be involved in cell wall hardening. Cell wall peroxidases can cause lignification and stiffening of the cell wall (van Huystee 1987). Cell walls may become lignified when cell expansion ceases (Macadam *et al.* 1992*a*, *b*), when the cell is under biotic attack (Kristensen *et al.* 1999), and when it differentiates to particular specialization, most notably the xylem (Christensen *et al.* 1998). Peroxidases act by forming phenolic cross-links in the cell wall.

Biochemical regulation of cell wall loosening is less clear. It has been suggested that xyloglucan endotransglycosylase (XET) may loosen cell walls (Fry *et al.* 1992; Passioura and Fry 1992). Drought and abscisic acid (ABA) appear to affect XET activity in maize root apices (Wu *et al.* 1994). Other possible enzyme activities could affect cell wall loosening, such as glucosidases or enzymes affecting the H-bonding of glucoarabinoxylans (GAX) in the cell wall (Carpita and Gibeaut 1993). A direct involvement of expansins in cell wall loosening has been elucidated

Abbreviations used: ABA, abscisic acid; AGP, arabinogalactan protein; GAX, glucoarabinoxylans; *L*, hydraulic conductance; *m*, cell wall extensibility; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; XET, xyloglucan endotransglycosylase.

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(McQueen-Mason *et al.* 1992). Furthermore, expansin activity is correlated to root elongation responses to osmotic stress (Wu *et al.* 1996).

Wall creep-type assays have been widely used in the analysis of growth, and measure the viscoelastic properties of the cell wall (Cosgrove 1993). The validity of comparing this *in vitro* method to *in vivo* methods has been questioned, particularly in the case of measuring cell wall extensibility (Hohl and Schopfer 1992). Nevertheless, the method is useful in determining if there are changes in structural properties of the cell wall (Cosgrove 1993).

In some cases, results from wall creep-type assays have confirmed *in vivo* observations (Cosgrove 1993). There are two such studies particularly relevant to the experiments presented in this paper, because they use techniques similar to the ones used in this study and because they found positive correlations with *in vivo* responses. In one wall creep-type assay (Okamoto and Okamoto 1994), a series of weights was applied to the tissue to distinctly detect alterations in the yield threshold and the extensibility of the cell wall by cell wall pH. In another wall creep-type assay (Neumann 1993), a hardening of the cell wall of growing leaf cells was detected within 20 min of exposure to saline solutions using a method with a single application of weight. Thus, it seemed highly likely that similar changes in cell wall properties could be detected in our experimental system.

In this paper, two hypotheses are tested: (1) that salinity inhibits growth by hardening the physical structure of the cell wall of the leaf growing zone; and (2) that cell wall enzymes are involved in this apparent cell wall hardening during salinity stress. The first hypothesis is tested by *in vitro* wall analysis with a wall creep-type assay. The second hypothesis is tested by assaying a number of enzymes thought to be involved in cell wall hardening.

Materials and methods

Plant material and growth conditions

Maize caryopses (*Zea mays* L. Pioneer hybrid 3906) were germinated and grown as described previously (Cramer and Bowman 1991*a*). Salinity treatments consisted of hydroponically grown plants (0.25-strength Hoagland's solution) treated with 80 mM NaCl for 4 h when leaf elongation reached steady-state conditions, unless specified otherwise. All measurements were performed on the growing zone of the third leaf.

Determination of in vitro *cell wall properties of salt-stressed plants*

Method 1 (3–12-g weight for 50 min)

The growing zone of the third leaf (basal 2-cm section) was excised from the plant. To adequately measure *in vitro* properties in this tissue, turgor must be removed. The optimum method for turgor removal was determined: several methods were tried that would effectively yield isolated cell walls, including vacuum suction, 50% glycerol, dimethyl sulfoxide, 2–20% Triton-X, and 95% ethanol. None of these treatments were satisfactory. In fact, the latter three treatments completely eliminated cell wall creep (data not shown). A more detailed comparison was performed (Table 1) on live, boiled (2 min), microwaved (on high for 30 s) or freeze-thawed (in liquid N_2) tissue. Leaf elongation data were collected for 1 h using 0, 3, 6, 9 and 12 g of weight in a similar method of analysis as Cramer and Bowman (1991*a*). The analysis of the plant growth parameters using the *in vivo* method on intact plants (Cramer and Bowman 1991*a*) has been previously verified using the pressure block technique (Cramer and Schmidt 1995).

One end of the tissue was fixed with a fast-drying cyanoacrylate glue to a small bath (cut piece of syringe) and the other end was attached to a clip on a displacement transducer (Cramer and Bowman 1991*a*). The bathing solution for the segments was 0.5 mM $CaCl_2$. Weights were applied to the transducer for 50 min to cause tissue extension. Tension was applied to the tissue only once; there were no repeat measurements on the same tissue. A linear extension phase (creep) was measured after plastic and elastic extensions were subtracted. A similar method of applied force was used to study the effects of auxin on long-term linear creep (Cleland *et al.* 1987).

Method 2 (0.1–6-g weight for 3 min)

Turgor was eliminated by freeze-thawing the basal 2-cm section of growing zone tissue. Tissues were fixed in the displacement transducer as in method 1. Tissues were bathed with 1 mM $CaCl_2$, and weights were applied with increasing mass (0.1–6 g). The weight was applied for 3 min then removed, and the next heaviest weight was applied 10 s later to the same tissue and left on for 3 min, similar to the method of Neumann (1993). Plastic and elastic components were calculated according to Neumann (1993). The only difference between this method and Neumann's is that he used only one weight of 3 g, whereas we used 13 different weights (0.1–0.5 g in 0.1-g increments, 0.5–3 g in 0.5-g increments, and 3–6 g in 1-g increments). This method combines the method of Neumann (1993) with the multiple weights analysis of Okamoto and Okamoto (1994) in order to detect the yield threshold of the cell wall. Note that this analysis does not subtract out the creep (linear phase) from the plastic component (exponential phase) as in method 1.

Method 3 (growing zone not detached)

A method similar to another approach of Neumann's was followed, because of the possibility that excision could increase the variability of plastic and elastic measurements (Chazen and Neumann 1994). In their method, the growing zone was freeze-thawed with a 30-s exposure to aerosol gas *in situ*. Method 3 is similar to method 2 except that isolated excised tissue segments were not analysed. Instead, the growing zones of the intact leaves were freeze-thawed with liquid N_2 *in situ* before weight analysis. Freezing was restricted to only the 2-cm basal section of the growing zone by placing liquid N_2 in a 5-mL section of a 50-mL plastic centrifuge tube. The tube had a 1-cm hole cut out of the bottom in order for it to be fitted snugly around the basal section of the plant.

Enzymatic and protein analyses of the apoplastic free space of the leaf growing zone

Protein analysis in the apoplastic free-space of the cell wall

The rationale of analysing the cell wall free space for proteins rather than extract ionically bound cell wall proteins was that the proteins of the apoplastic free space would represent proteins that were newly secreted, and therefore might be a more accurate assessment of proteins regulating growth. The apoplast extraction method was modified from Shinkle *et al.* (1992). A 1-cm segment of the basal region of the leaf growing zone was excised under 50 mM $CaCl_2$ at 4°C and vacuum infiltrated for 3 min. $CaCl_2$ (50 mM) was used to reduce the possibility of leakage from the cytoplasm. Approximately 40 growing zone segments were placed apical side down and one layer thick into 30-mL syringes for centrifugation. Samples were initially

spun at 240 *g* for 4 min at 4°C to remove adhering surface solution. This extract was discarded. Samples were recentrifuged at 650 *g* for 10 min at 4°C, the extract was collected and recentrifuged at 1300 *g* for 10 min at 4°C and this second extract was also collected. Cytoplasmic leakage was determined by assaying for malate dehydrogenase activity. No activity was detected indicating that there was no cytoplasmic leakage into the apoplast. Protein concentration was determined using the BCA standard assay from Pierce (Rockford, IL, USA).

In preparation for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis, protein extracts were precipitated with 90% acetone, pelleted by centrifugation, the acetone decanted by air-drying, and the pellet resuspended in distilled deionized water. SDS–PAGE analysis was performed with a 3% polyacrylamide stacking layer (pH 6.9) and a 12% polyacrylamide separating layer (pH 8.9) with a running buffer of 1.5 M Tris base, 1.92 M glycine and 1% SDS. Extracted apoplastic protein (150 µg) in sample buffer (4 M urea, 100 mM dithiothreitol, 60% (v/v) glycerol, 2% (w/v) SDS and 0.01% (w/v) bromophenol blue) was loaded and electrophoresed for 3 h at a constant 40 A using a Hoefer Scientific PS 1500 DC power supply (Hoefer Scientific, San Francisco, CA, USA). The gel was placed in staining solution (50% (v/v) methanol, 12% (v/v) acetic acid, 0.06% (w/v) Coomassie R-250) for 2 h and destained in 10% (v/v) acetic acid for 18–24 h. Gel results were recorded by photography and densitometry (Hoefer Scientific GS 300 Transmittance/Reflectance Scanning Densitometer).

In vivo cell wall peroxidase assay

Peroxidase activity was assayed using guaiacol (8.25% solution). Peroxidase activity was measured by absorbance at 470 nm in a spectrophotometer. Approximately 1 g of 2-cm-long excised growing zones of the third leaf was placed in a test tube with 4 mL of solution. Four different solutions were used: (1) 0.5 mM $CaSO_4$; (2) 0.5 mM $CaSO_4$ + 150 mM NaCl; (3) treatment 1 + guaiacol; and (4) treatment 2 + guaiacol. Treatments 1 and 2 served as blanks to be subtracted from treatments 3 and 4. No H_2O_2 was added, so that peroxidase activity was dependent upon native production of H_2O_2. Solutions were assayed hourly for 4 h. Absorbance increased linearly with time. Therefore, only final absorbance values at 4 h are given for estimates of *in vivo* peroxidase activity.

Total apoplastic peroxidase activity

Total apoplastic peroxidase activity was assayed in a similar manner to that by Shinkle *et al.* (1992). Apoplastic solutions were extracted from freshly excised growing zones from intact plants previously treated with control or 80 mM NaCl solutions for 4 h in a similar manner to that for protein assays described above (650-*g* spins). Peroxidase activity was assayed in a mixture containing 3 mL of 10 mM sodium phosphate buffer (pH = 6.0), 30 µL of 33% H_2O_2 and 8.4 µL of 0.2 M guaiacol. The reaction was initiated by adding 10 µL of enzyme extract. Absorbance was measured at 470 nm and activity was expressed as ΔOD (mg protein)$^{-1}$ min^{-1}.

Cell wall peroxidase isozyme activity

Cell wall peroxidases were assayed according to Lagrimini and Rothstein (1987). Each aliquot of enzyme extract (containing 20 µg of protein) was applied across the center of a precast polyacrylamide gel for analytical isoelectric focusing, pH 3.5–9.5 (Ampholine PAGplate, Code No. 80-1124-80, Pharmacia LKB, Biotechnology AB, Uppsala, Sweden). The samples were subjected to electrophoresis for 1.5 h at 0.125 W cm^{-2} at 10°C. The gel was then soaked in 10 mM sodium phosphate buffer (pH = 6.0) for 30 min to remove the ampholines and equalize the pH throughout the gel. The gel was stained for peroxidase activity with 0.01% (w/v) 3-amino-9-ethyl carbazole, 0.16% (v/v) H_2O_2 and 0.2% (v/v) guaiacol in 10 mM sodium phosphate buffer (pH 6.5), and photographed.

XET assay

After 4 h of salinity treatment, growing zones were excised from the third leaf, frozen in liquid N_2 and ground with a mortar and pestle. Approximately 400 mg of tissue (fresh weight) was used for each assay. XET activity in the growing zone was determined according to the procedures by Fry and co-workers (Fry *et al.* 1992; Wu *et al.* 1996). Dr Stephen Fry kindly provided the (1-^{3}H)XG7-ol.

Statistics

All statistical analyses were performed using ANOVA (StatView, v. 5.01, SAS Institute, Inc.). Results were considered significant when $P \leq 0.05$.

Results

In vitro *wall analysis*

Distinct differences were found between the turgor-removing techniques. Heating the tissue by boiling or microwaving increased tissue variability and appeared to artificially increase the plasticity of the wall (a 6- to 9-fold increase of the plastic component, P). When turgor is removed, the elasticity of the tissue is expected to increase but not the plasticity. In freeze-thawed tissues, P increased 2- to 3-fold compared to the controls. The creep rate (slope in Table 1) was increased substantially in microwaved tissues, but was unaffected by the other turgor-removing treatments. We concluded that freeze-thawing the tissue was the best technique to analyse the cell walls *in vitro*, because this treatment's characteristics (Table 1) most closely resembled live tissue.

The primary purpose and design of the following wall creep-type experiments was to test if changes in the apparent yield threshold of living plants was reflected in structural changes in the cell wall. Such changes in the yield threshold of the cell wall have been clearly detected *in vitro* (Okamoto and Okamoto 1994).

In vitro wall creep analysis of salt-stressed maize leaves was first performed using method 1 (Fig. 1). Leaf elongation of excised tissues exhibited elastic, plastic and linear extension (creep) phases in response to applied tension (data not shown), in a similar manner to that described previously for intact maize leaves (Cramer and Bowman 1991*a*) and

Table 1. Comparison of turgor-removing techniques analysed according to method 1

The slope of the linear phase is the rate of cell wall creep. Data are means ± s.e.; *n* = 17–23

Turgor-removing technique	Elastic component (µm)	Plastic component (µm)	Slope (µm min^{-1})
Live	237 ± 34	126 ± 30	0.837 ± 0.205
Freeze-thawed	360 ± 63	351 ± 59	1.132 ± 0.306
Boiled	415 ± 79	715 ± 100	0.851 ± 0.173
Microwaved	552 ± 178	945 ± 128	3.353 ± 0.681

freeze-thawed *Avena* coleoptiles and *Cucumis* hypocotyls (Cleland *et al.* 1987). However, leaf elongation rates (without applied tension) of excised leaves (12.8 μm min^{-1}) were reduced by 60% compared to intact leaves (32 μm min^{-1}). Freeze-thawed control tissue had a similar rate of creep (Table 1) and slope of the line (Fig. 1) to live tissue. In live tissue the slope of the line in Fig. 1 represents the growth coefficient, $mL/(m + L)$, where m is cell wall extensibility, and L is hydraulic conductance. When freeze-thawed tissues of salinity-treated plants were compared to that of control plants, no differences were found and no yield threshold was detectable (x-intercept), and the intercepts of the lines went through 0 g of weight (Fig. 1).

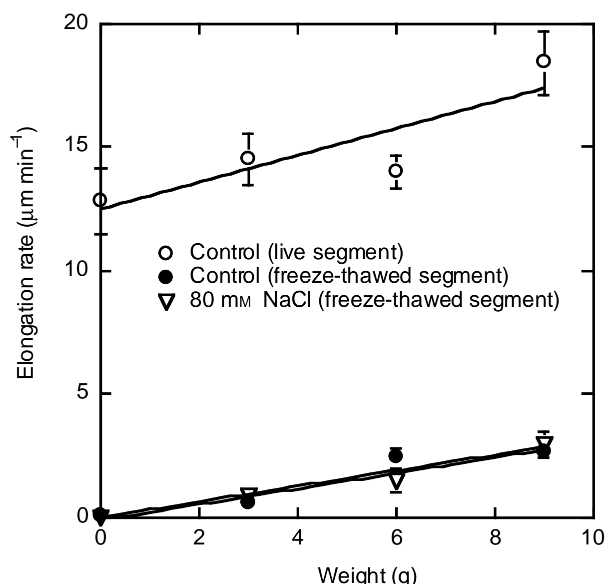

Fig. 1. *In vitro* wall creep analysis (linear phase in method 1) of segments of leaf growing zones of maize. Segments were stretched by different amounts of weights and analysed as before (Cramer and Bowman 1991*a*). Elongation rate represents the amount that extension increased over time. Plastic and elastic extension was subtracted out for samples with weights applied to them. Each datum is the mean ± s.e.; n was between 6 and 14 replicates. A replicate was the application of a single weight to a new tissue that had not previously had weight applied to it. Note that freeze-thawed segments do not have turgor.

These results were not expected, particularly the lack of yield threshold. It was decided to repeat this experiment with a new, more sensitive method of analysis using a large number of smaller weights to accurately determine the yield threshold (Fig. 2).

A very small yield threshold was clearly detected by method 2. The results were consistent with the findings in Fig. 1. No differences in cell wall properties were detected between control and salinity-treated plants. Elastic extension increased linearly with increasing weight. Plastic extension did not increase linearly. This is most likely due to some of the plastic component (irreversible exponential extension) being partially used up by the previous application of weight. This affects the absolute accuracy of the cell wall extensibility. Nevertheless, the relative changes between treatments can be assessed. Furthermore, these experiments were primarily designed to detect the absolute value of the yield threshold of the cell wall. Note that the plastic extension component (exponential phase) also has a much higher variability than the creep component (linear phase) and the elastic component in live tissues (Cramer and Bowman 1991*a*).

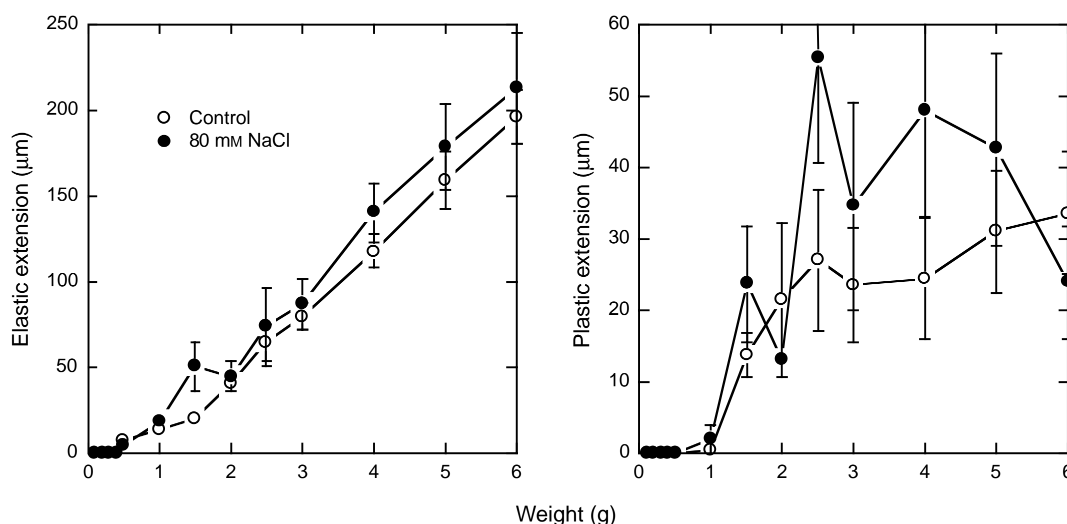

Fig. 2. *In vitro* wall analysis using method 2. Data are means ± s.e., n was between 2 and 14 replicates. The yield threshold is the x-intercept. The cell wall extensibility is represented by the slope of the line in the plot of plastic extension *vs* weight.

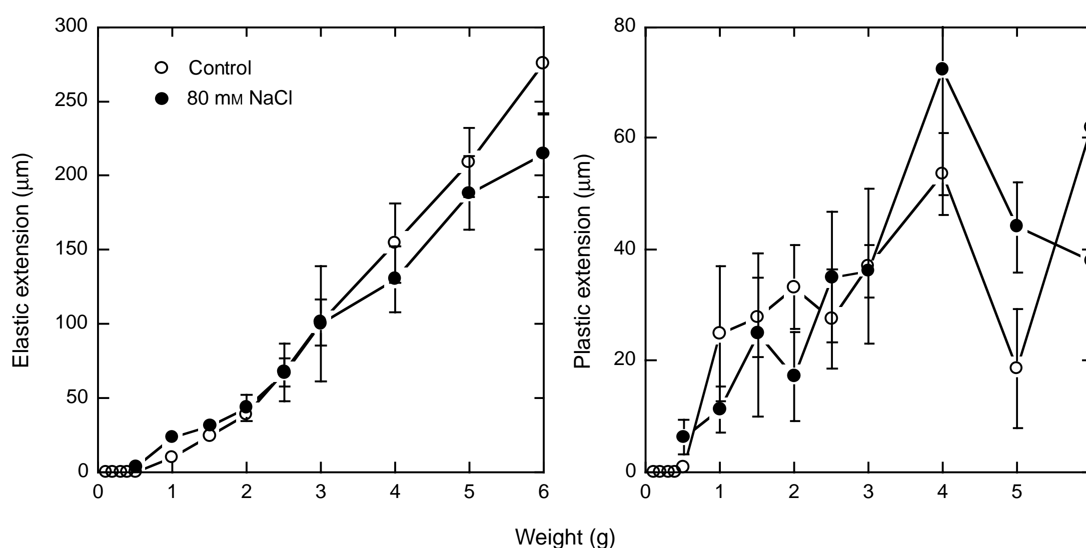

Fig. 3. *In vitro* wall analysis using method 3. Data are means ± s.e., *n* was between four and seven replicates.

Because variability was high in plastic extension of excised tissues, method 2 was modified in an attempt to reduce this variability. Some of this variability might result from the way the tissue was fixed in the displacement transducer apparatus. Isolated excised tissue segments were not analysed as before; instead, the growing zones of the intact leaves were freeze-thawed with liquid N_2 *in situ* before weighing. The results were consistent with previous results, indicating that the yield threshold of this tissue without turgor is very small, and that salinity has no effect on either the yield threshold of the cell wall or cell wall extensibility (Fig. 3). Similar results were found with maize plants treated with 80 mM NaCl for 24 h and with barley plants treated with 80 mM NaCl for 4 h (data not shown).

These findings are in contrast to previous *in vivo* findings where the apparent cell wall yield threshold was found to increase with salinity (Cramer and Bowman 1991*a*; Cramer and Schmidt 1995), and contrast with another report (Neumann 1993) that used *in vitro* analysis. However, these results were obtained with three different very rigorous methods, with consistent results for all three methods. These data indicate that the plant may need to be a living tissue to adequately determine the nature of the salinity effect. In living tissues, salinity may have effects on secretion or biosynthesis of polymers that are targeted to the cell wall, on the pH in the apoplast, or cause ABA accumulation in the apoplast, which could then trigger solute leakage from the cell into the cell wall apoplast, disturbing water relations.

Role of cell wall proteins in the growth response of salt-stressed plants

Protein analysis of the apoplastic extracts indicates that salinity (4 or 24 h later) had no significant effect ($P \geq 0.19$) on the protein concentration in the apoplast (Table 2). Note that extract volumes were significantly reduced by salinity after 24 h.

Analysis of 1-dimensional SDS–PAGE assays of low-speed (650 *g*) protein extractions also confirm that there were no significant differences in different protein classes (Fig. 4). SDS–PAGE gels of high-speed (1300 *g*) protein extractions were identical to low speed protein extractions (data not shown). Densitometer scans of all gels confirmed that there were no differences in any of the bands (data not shown).

Salinity did not significantly affect *in vivo* cell wall peroxidase activity ($P \geq 0.48$) as assayed on an *in vivo* basis (control = 0.038 ± 0.003 absorbance g^{-1} FW tissue; salinity = 0.042 ± 0.002 absorbance g^{-1} FW tissue; n = 10). Note that this assay does not include addition of H_2O_2 in order to rely on native production of H_2O_2, and more realistically simulates *in vivo* conditions in the apoplast. Similarly, no significant differences were found between salinity and

Table 2. Protein concentrations in apoplastic extracts of salt-stressed growing zones of the third leaf of maize
Data are means ± s.e.; *n* = 20; gz = growing zone segment

Treatment	Spin force (*g*)	Extract volume (µL gz^{-1})	Apoplastic protein (µg μL^{-1})
Control	650	12.3 ± 1.12	1.33 ± 0.149
	1300	15.5 ± 0.66	1.33 ± 0.197
Salinity 4 h	650	12.1 ± 0.24	1.20 ± 0.138
	1300	14.8 ± 1.00	1.52 ± 0.172
Salinity 24 h	650	7.94 ± 1.00	1.58 ± 0.168
	1300	9.18 ± 0.59	1.52 ± 0.199

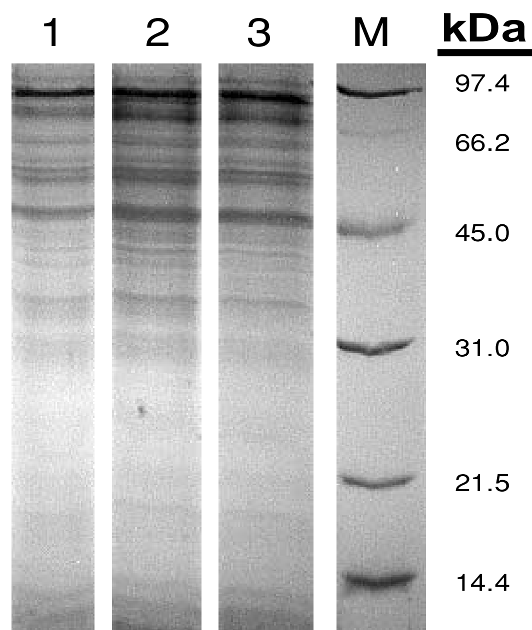

Fig. 4. Representative example of a 1-dimensional SDS–PAGE of apoplastic proteins from the growing zone of the third leaf of maize. Protein was stained with Coomassie Blue. Lane 1 is a low-speed extract (650 *g*) of 24-h salt-stressed plants; Lane 2 is a low-speed extract of 4-h salt-stressed plants; Lane 3 is a low-speed extract of control plants; Lane M contains low weight SDS–PAGE molecular weight standards from Bio-Rad. All lanes were loaded with 150 μg of protein.

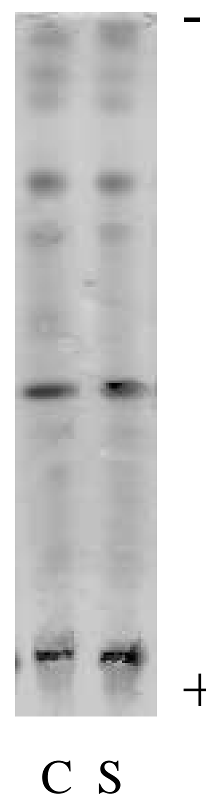

Fig. 5. Effect of salinity on cell wall peroxidase isozymes of the growing zone of the third leaf of maize. C = control; S = 80 mM NaCl treatment for 4 h.

control treatments for total cell wall peroxidase activity (data not shown) and cell wall peroxidase isozymes (Fig. 5).

Salinity did not have any significant effects ($P \geq 0.92$) on XET activity in the growing zone of the third leaf after 4 h (99.2 ± 6.8%) or 24 h (102 ± 2.0%) of treatment as compared to controls (100 ± 3.4%). XET activity is expressed as a % of control (means ± s.e.; $n = 5$; control treatments averaged 10 403 counts per minute assay^{-1}).

Discussion

We have found no support for the hypothesis that the wall of cells in the maize leaf growing zone is structurally hardened by short-term (less than 1 d) salinity. The *in vitro* cell wall assay results contrast with those of Neumann (1993), in which 100 mM NaCl reduced the irreversible plastic extension of the primary leaves of maize by about one-third. Because our results differed from that of Neumann (1993), we approached the problem with different methods, including mimicking the method (Figs 2 and 3) of Neumann (1993). Note that Neumann's method uses only a single application of weight of 3 g.

We do not have a good explanation for the differences in our results from that of Neumann (1993), other than that it is possible that the different genotypes used have different responses to salinity. Species differences to osmotic stress have been found (Lu and Neumann 1998); leaf cell wall extensibility was reduced by osmotic stress in maize and barley, but not in rice. Likewise, species differences within *Hordeum* have been observed in salt-stressed plants (G. R. Cramer, unpublished results).

Changes in cell wall properties were found in the leaf elongation zone of salt-stressed barley (Lynch *et al.* 1988). However, the response was dependent on salinity level, genotype and time. In all cases, no significant differences in cell wall properties of the growing zones were found between control and salt-stressed plants within the first couple of days of salinity.

Consistent with our *in vitro* cell wall assay results, we have not found any effects of salinity on cell wall enzyme activities. Several different assays for peroxidase activity were assessed, including an *in vivo* assay, none of which detected any differences in salt-stressed plants. In addition, the total XET activity was unaffected by salinity. However, it was recently shown that there are multiple transcripts in the leaf growing zone of barley (Schünmann *et al.* 1997). Each isozyme could have different functions within the growing zone. Measurements of total XET activity may mask a differential response of the individual XET isozymes to

salinity. Thus, the XET results from this study are inconclusive, and more detailed XET assays are needed to fully explore the involvement of XET in the response of salt-stressed plants. Note, however, that the XET assay used in this paper is the same as the method used to detect increased activity in drought-stressed root tips (Wu *et al.* 1994).

In an apparent contradiction to what is stated above about wall hardening, salinity was found to affect secretion and the properties of the cell wall of tobacco cells, (Iraki *et al.* 1989*a–c*; Zhu *et al.* 1993). However, the nature of these experiments is considerably different. Salinity treatments were long-term and very high (428 mM NaCl). In addition, the cells were bathed in the salinity medium, whereas only the roots and not the elongation zone of the grass leaves were exposed to the saline medium. Thus, we do not think that the data from experiments on tobacco cells are directly comparable to experiments on the elongation zone of salt-stressed grass leaves.

What could be the cause of growth inhibition in salt-stressed maize? There are a number of possibilities. One possibility is that the increase in the apparent yield threshold by salinity in intact, live plants may be the result of a decrease in the biosynthesis and/or secretion of oligosaccharides, non-cellulosic polysaccharides or arabinogalactan proteins (AGPs) to the cell wall. These molecules may continuously lubricate the cell wall or act as apoplastic signals that contribute to loosening of the cell wall. Reduction of AGPs in the apoplast by binding with various compounds or by mutation inhibits cell expansion (reviewed in Majewska-Sawka and Nothnagel 2000), and salinity reduces the secretion of AGPs in the apoplast (Zhu *et al.* 1993). By these mechanisms, the effect of salinity on the cell wall would only be apparent in live tissues and would not be observed with *in vitro* assays of the cell wall, consistent with the results found in this study.

An alternative hypothesis for the inhibition of leaf growth in salt-stressed maize is that ABA, which increases in the growing zone during salinity stress (Cramer *et al.* 1998), may act as a regulator of the apparent yield threshold in salt-stressed plants by increasing solute leakage from growing cells into the apoplast. This would result in a reduced turgor that would not be detected by previous methods (Cramer and Bowman 1991*a*; Cramer and Schmidt 1995). Again, *in vitro* assays would not be able to detect these differences. However, this alternative hypothesis is doubtful, because apoplastic solute concentrations (K^+ and Na^+) measured by two independent techniques in salt-stressed maize leaves are quite low and cannot account for the growth inhibition (A. Läuchli, pers. comm.).

Passioura, Munns and co-workers (Munns *et al.* 2000*a*, *b*; Passioura and Munns 2000) have reported that shoot water status may regulate leaf expansion of salt-stressed grasses in the light. When plants are under high evaporative demand, pressurization of the roots is able to fully restore growth rates of salt-stressed plants over time periods similar to that in the present study (Munns *et al.* 2000*a*). This occurs whether pressure is added 4 h after the salinity is imposed, or the pressure is continuously applied. The full recovery of leaf elongation rates after pressure is applied is immediate, and implies that there are no permanent effects of salinity (e.g. cell wall hardening, ion toxicity). In the dark, pressurization is not capable of restoring growth rates of salt-stressed plants (Munns *et al.* 2000*a*, *b*). These data indicate that water relations may limit growth during periods of high transpiration, while at other times hormonal signals may be more important (Munns *et al.* 2000*a*, *b*).

There is evidence that apoplastic pH is affected in drought-stressed plants (Bacon *et al.* 1998; Bogoslavsky and Neumann 1998). The changes in apoplastic pH could have important changes in the distribution of ABA. ABA concentrations in the apoplast would increase, and this in turn could affect turgor as described above. In addition, the pH change could affect cell wall extensibility by altering the activity of expansins (Cosgrove 1998).

It doesn't appear that pH is an important factor in salt-stressed plants, since all evidence (reviewed in Cramer 1997) indicates that salinity decreases apoplastic pH in contrast to an increase in apoplastic pH caused by water deficit. In roots of salt-stressed plants, apoplastic acidification is not inhibited by salinity (Schubert and Läuchli 1986; Zidan *et al.* 1990). In nutrient solutions, acidification is a response to an excess uptake of cations relative to anions.

Na concentrations are comparatively low in leaves of salt-stressed maize (Cramer *et al.* 1994*b*), so it is possible that an increase in apoplastic pH may occur in the leaf growing zone. However, the rate and the capacity of acidification of the apoplast in the growing zone of maize leaves, either in the presence or absence of NaCl in the apoplast, is unaffected in salt-stressed plants compared to controls (N. Bernstein, pers. comm.).

In summary, moderate salinity does not appear to inhibit maize leaf elongation by physically hardening the cell wall. This conclusion is in part based upon *in vitro* wall analysis and is supported by the lack of effect of salinity on apoplastic protein concentrations and enzyme assays thought to be involved in cell wall hardening. Furthermore, in addition to the present study, no changes in cell wall extensibility were detected in salt-stressed plants compared to controls using the *in vivo* methods of the applied tension technique (Cramer and Bowman 1991*a*) or the pressure block technique (Cramer and Schmidt 1995). The salinity-induced increase in the apparent yield threshold previously detected (Cramer and Bowman 1991*a*; Cramer and Schmidt 1995) is not reflected by changes in the physical properties of cell walls *in vitro*. The apparent yield threshold appears to be detectable only in living tissues. It is clear that plant responses are complex and will depend on the level and

duration of salinity, the type of salt, the developmental stage at exposure, the time of day, and other factors. The mechanism by which salinity inhibits maize leaf elongation remains an enigma.

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