Plant Physiol. (1986) 82, 1102–1106
0032-0889/86/82/1102/05/$01.00/0

Effects of NaCl and $CaCl_2$ on Cell Enlargement and Cell Production in Cotton Roots[1]

Received for publication June 10, 1986 and in revised form August 13, 1986

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ABSTRACT

In many crop species, supplemental Ca^{2+} alleviates the inhibition of growth typical of exposure to salt stress. In hydroponically grown cotton seedlings (*Gossypium hirsutum* L. cv Acala SJ-2), both length and weight of the primary root were enhanced by moderate salinities (25 to 100 millimolar NaCl) in the presence of 10 millimolar Ca^{2+}, but the roots became thinner. Anatomical analysis showed that the cortical cells of these roots were longer and narrower than those of the control plants, while cortical cells of roots grown at the same salinities but in the presence of only 0.4 millimolar Ca^{2+} became shorter and more nearly isodiametrical. Cell volume, however, was not affected by salinities up to 200 millimolar NaCl at either 0.4 or 10 millimolar Ca^{2+}. Our observations suggest Ca^{2+}-dependent effects of salinity on the cytoskeleton. The rate of cell production declined with increasing salinity at 0.4 millimolar Ca^{2+} but at 10 millimolar Ca^{2+} was not affected by salinities up to 150 millimolar NaCl.

Most crop plants suffer a decline in growth when exposed to saline conditions. The deleterious effects of salinity are thought to result from water stress, ion toxicities, ion imbalance, or a combination of these factors. One of the requirements for growth is maintenance of cell turgor above a threshold level; under saline conditions, osmotic withdrawal of water from enlarging cells may cause their turgor pressure to drop below the threshold. Unless the plant can generate a sufficiently negative osmotic potential to reverse the flow of water, either by uptake of ions from the medium or by synthesis of organic osmotica, growth will stop. Ion imbalances in plants can, for example, occur when high concentrations of Na^+ in the soil reduce the amounts of available K^+, Mg^{2+}, and Ca^{2+} (10) or when Na^+ displaces membrane-bound Ca^{2+} (7). Sometimes Na^+ has direct toxic effects, as when it interferes with enzyme structure and function. It may also interfere with the function of potassium as a cofactor in various reactions. Many of the deleterious effects of Na^+, however, seem to be related to the structural and functional integrity of membranes. Swelling, distortion, and other structural changes have been observed in various membranes of salt-stressed plants, and the plasmalemma has been shown to lose its selective permeability (4, 13, 27). Sodium also inhibits K^+ and Ca^{2+} uptake in salt-stressed cotton roots (5).

In many but not all salt-sensitive species, supplemental Ca^{2+} partly reverses the deleterious effects of Na^+. For example, in bean plants exposed to 50 mM NaCl, elevated Ca^{2+} levels minimized Na^+ uptake and restored growth (24, 25). Cotton, a rather salt-resistant crop species, is, nonetheless, fairly salt-sensitive during the seedling stage (20). Supplemental Ca reduced Na^+ influx, improved K^+/Na^+ selectivity and actually stimulated root growth at salinities up to 150 mM NaCl (GR Cramer, unpublished data; 6, 14); furthermore, it seemed to improve the resistance of the roots to microbial attack (20 and references therein). The Ca^{2+} level of the medium also influenced the morphology of salt-stressed roots: roots grown in the presence of 10 mM Ca^{2+} were not only longer but also thinner than roots grown in 0.4 mM Ca^{2+} (6). This raised the question whether the observed changes in growth were mediated through changes in cell size, the rate of cell production, or both (29, 34). We here report that salinities up to 150 mM NaCl affect cell production only when Ca^{2+} levels are relatively low. The three-dimensional shape of root cells, but not cell volume, is also differentially affected by salinity depending on the external Ca^{2+} concentration, which suggests direct or indirect effects of Na^+ and Ca^{2+} on the cytoskeleton.

MATERIALS AND METHODS

Seeds of *Gossypium hirsutum* L. cv Acala SJ-2 were imbibed in aerated deionized H_2O for 24 h and then "planted" between strips of germination paper kept upright by insertion into plastic racks with vertical slots. The racks were placed over 3.7 L plastic containers, which were filled with the various treatment solutions so that the lower edges of the paper strips were just immersed. Evaporation was minimized by covering the containers with Saran Wrap. One-tenth concentration modified Hoagland solution (10) served as the basic nutrient medium. Treatments consisted of 25, 50, 100, 150, and 200 mM NaCl superimposed on the basic medium, either without additional Ca^{2+} (that is, the final Ca^{2+} concentration was 0.4 mM) or with enough supplemental $CaCl_2$ to raise the final Ca^{2+} concentration to 10 mM. All solutions had a pH of 6.5 and were aerated. The temperature was kept constant by setting all of the containers into a large water bath maintained at 27°C.

The seedlings were harvested on the 6th d after imbibition when the cotyledons were beginning to expand. Freehand cross and longitudinal sections were prepared from root segments 10 to 12 mm proximal to the root tip. The only exceptions were the roots obtained from the 150 mM NaCl, 0.4 mM Ca^{2+} treatment, which were so short that sections had to be taken 3 to 5 mm proximal to the tip. In all cases, preliminary observations showed that the cells of the cortex had attained maximal length in the region chosen for analysis. Both the tissue and the edge of the razor blade were kept wet during sectioning to avoid shrinkage and distortion of cells. The unstained sections were immediately mounted in water and examined with a Zeiss microscope equipped with a light tube, and the cross-sectional areas of the root, stele, and 20 cortical cells, and the lengths of 20 cortical cells (excluding endodermal cells) were measured in each root using a Zeiss MOP-3 digitizing pad. The cross-sectional area of

[1] Supported by National Science Foundation grant DMB84-04442 to A. L. and E. E.

the cortical cells varied greatly among the outer, middle, and inner regions of the cortex of any given cross-section; cells were chosen at random from all three regions for measurement. The mean number of cortical cells intercepted by five randomly chosen radii was also determined. Five roots of each treatment were analyzed, and the experiment was repeated three times. The 95% confidence intervals for each set of data were calculated with Minitab (Minitab Project, Pennsylvania State University, 215 Pond Laboratory, University Park, PA 16802).

RESULTS

Root and Tissue Dimensions. The effects of NaCl at two Ca^{2+} concentrations on the length and fresh weight of cotton roots were described by Cramer *et al.* (6; Fig. 1). The corresponding cross-sectional areas 10 to 12 mm proximal to the root tip are shown in Figure 2A. The roots generally reached their maximum diameter within 10 mm of the tip; thus, their diameter was constant within the segment chosen for analysis. The only exception occurred in roots grown in 0.4 mM Ca^{2+}/150 mM NaCl. These roots were quite short and conical throughout their length, and cell differentiation and maturation occurred very close to the apex. As the NaCl concentration increased, the low Ca^{2+} roots gradually thickened, and in 150 mM NaCl they attained more than twice the cross-sectional area of the controls. The high Ca^{2+} roots, on the other hand, became much thinner with increasing salinity. The differences in cross-sectional area between the low and high Ca^{2+} roots were highly significant statistically at 50 mM and higher NaCl concentrations.

The cross-sectional area of the stele (Fig. 2B) showed similar trends with increasing salinity at both calcium levels, but the changes were less pronounced than the changes in total cross-sectional area of the root, especially under low Ca^{2+} conditions. Consequently, there was a significant decline in the ratio of the cross-sectional area of the stele to that of the root with increasing salinity at 0.4 mM Ca^{2+} (Fig. 2C). In other words, the cortex became disproportionately thick. Under high Ca^{2+} conditions, the ratio of stelar area to root area also decreased slightly with increasing salinity, but the differences between salt-stressed and control roots were not significant at the 95% confidence level.

Numbers and Dimensions of Cortical Cells. The cortex (excluding the endodermis) was chosen for detailed analysis of cellular dimensions because, unlike the stele, it consists of a single cell type. The coefficient of variation for the cross-section area of individual cortical cells within each root was typically close to 0.5, reflecting the considerable differences between the relatively thin cells of the inner and outer regions of the cortex, which under control conditions sometimes had a cross-sectional area of only 250 μm^2, and the much stouter cells characteristic of the middle layers of the cortex, which occasionally exceeded 3500 μm^2 in cross-sectional area. As in many seed plants, cell division stopped sooner in the cortical parenchyma than in either the epidermis or endodermis (11, p. 231). Consequently, cortical cells elongate more than epidermal or endodermal cells and were therefore considered *a priori* to be more likely to show whether the various salt treatments affected cell elongation.

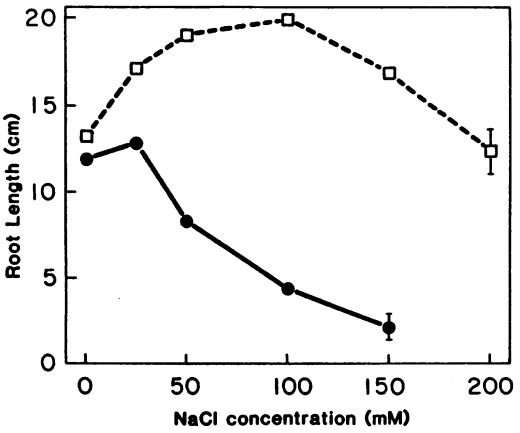

FIG. 1. Effect of NaCl and supplemental Ca^{2+} on the length of the tap root of 6-d-old cotton seedlings. Symbols used in this and all subsequent figures: (——), seedlings grown in 0.4 mM Ca^{2+}; (– – –), seedlings grown in 10 mM Ca^{2+}. Error bars represent 95% confidence intervals. (Redrawn from Cramer *et al.* [6]).

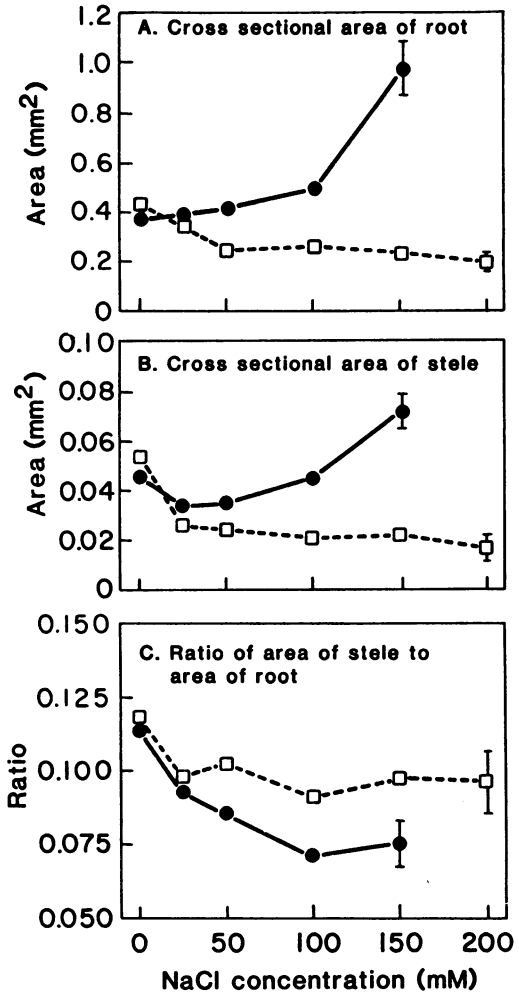

FIG. 2. Effect of NaCl and supplemental Ca^{2+} on the cross-sectional area of the entire root (A), cross-sectional area of the stele (B), and ratio of stelar cross-sectional area to root cross-sectional area (C).

There was no evidence of any shrinkage or distortion of cells during sectioning. The cells looked turgid, and cyclosis was evident in many cells in the longitudinal sections.

Despite the marked changes in root diameter and in the proportion of the root occupied by the stele elicited by certain treatments, the number of concentric layers of cortical cells remained roughly constant regardless of the Na^+ or Ca^{2+} concentration of the medium. Roots grown in 0.4 mM Ca^{2+} contained an average of 7.68 layers of cortical cells while those grown in 10 mM Ca^{2+} had 7.30 layers. The differences between treatments were insignificant at the 95% confidence level (data not shown). Thus, the changes in the width of the cortex must have predominantly been the result of changes in the width of the cells of the cortex, and measurements of the cross-sectional areas of individual cortical cells confirmed that this was indeed the case at

salinities above 25 mM NaCl (Fig. 3). At 0.4 mM Ca^{2+}, the cells became wider with increasing salinity. At 10 mM Ca^{2+}, they became narrower; in particular, the ordinarily rather stout cells of the middle layers of the cortex became much thinner, the cross-sectional area of individual cortical cells ranging from about 120 μm^2 to about 1000 μm^2.

Although the Ca^{2+} concentration of the medium did not affect the length of cortical cells at 0 or 25 mM NaCl, it did have an effect at higher salinities (Fig. 4). In the presence of 10 mM Ca^{2+}, mean cell length increased about 50% at 50 mM NaCl, compared to the controls, but did not undergo any further changes with increasing salinity. In 0.4 mM Ca^{2+}, cell length declined as salinity increased beyond 50 mM NaCl, and at 150 mM NaCl, the low Ca^{2+} cells were only a third as long as the high Ca^{2+} ones.

The mean volume and surface areas of cortical cells were calculated from the data on cell length and cell cross-sectional area. At NaCl concentrations up to 150 mM, cell volume remained constant at both Ca^{2+} levels, the changes in cross-sectional area and length that were elicited by the various treatments cancelling each other (Fig. 5). There were, however, significant differences in cell surface area between the high and the low Ca^{2+} treatments in the presence of 100 mM or higher NaCl concentrations, cell surface area only being about 75% of control at low Ca^{2+} levels (Fig. 6).

Relative Rate of Cell Production. Comparison of Figures 1 (root length) and 4 (length of cortical cells) reveals that the curves are not quite parallel. In 0.4 mM Ca^{2+}, root length diminishes more sharply with increasing salinity than does cell length. In 10 mM Ca^{2+}, both root growth and cell elongation are enhanced by moderate salinities, but the maxima occur at different NaCl concentrations: root length attains a maximum of 150% of control at 100 mM NaCl, while the cortical cells attain their maximum length, 143% of control, at 50 mM NaCl. Furthermore, although cell length does not decline significantly at higher salinities, root length does decline at salinities above 100 mM NaCl. These differences suggest that salinity affects the rate of cell production and that its effects differ under low and high Ca^{2+} conditions.

When the seeds were transplanted to the various treatment solutions after 24 h imbibition in distilled-deionized H_2O, the radicle was just beginning to pierce the seed coat. Hence, essentially all growth (that is, cell division and cell elongation after emergence of the radicle) occurred during exposure to a control or a treatment solution, and differences in the total number of cells per root at the time of harvesting may be assumed to reflect differences in the rate of cell production caused by the various treatments. Using the reciprocals of cell length and the data on root length presented by Cramer *et al.* (6), the total numbers of cortical cells per cell file can be determined for a representative section of the mature part of the root, for example, a segment equivalent to 10% of the total length of the root. This procedure eliminates the need to take the shorter cells of the root tip into account, which, in any case, constitute only a relatively small portion of total root length. These values, in turn, should be proportional to the mean relative rate of cell production. The method does not, of course, give any information on instantaneous rates of cell production or whether the rate of cell production remained constant throughout the 5 d treatment period, nor can it be used to distinguish between the rate of cell division and the influence of the number of meristematic cells on the rate of cell production. Nonetheless, it provides a useful way of quantitatively accounting for the differences of the shapes of the curves in Figures 1 and 4. Thus, under nonsaline conditions, the difference in root length observed in plants grown in 0.4 or 10 mM Ca^{2+} can be explained by a roughly 20% greater rate of cell

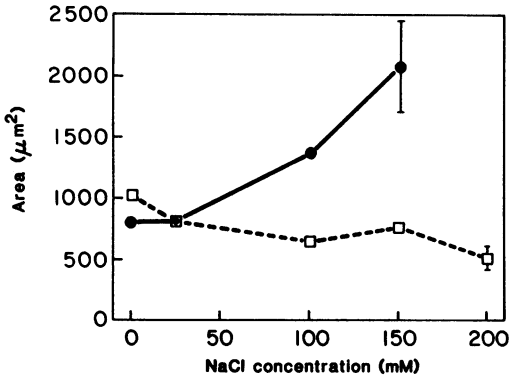

FIG. 3. Effect of NaCl and supplemental Ca^{2+} on the cross-sectional area of individual cortical cells.

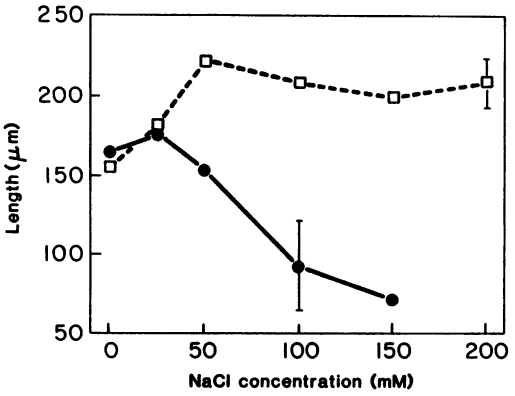

FIG. 4. Effect of NaCl and supplemental Ca^{2+} on the length of individual cortical cells.

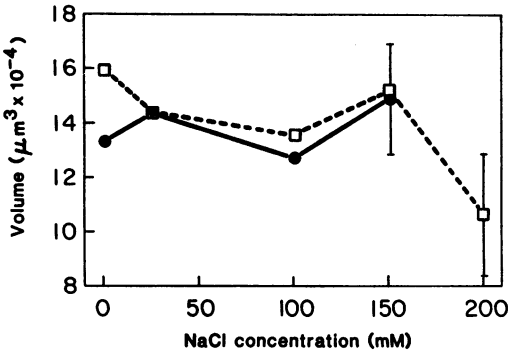

FIG. 5. Effect of NaCl and supplemental Ca^{2+} on the volume of individual cortical cells.

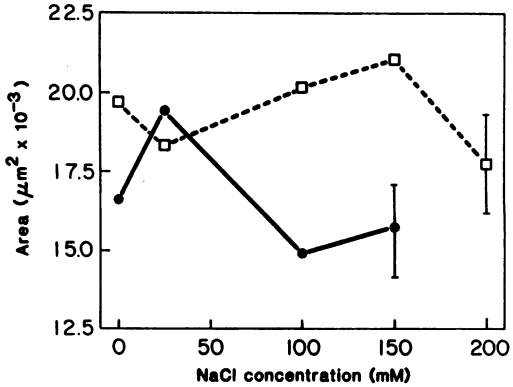

FIG. 6. Effect of NaCl and supplemental Ca^{2+} on the surface area of individual cortical cells.

Table I. *Number of Cortical Cells per File per Root Segment*

Each segment consists of 10% of the total length of the root and is assumed to consist entirely of mature, fully elongated cells. The relative rate of cell production is assumed to be proportional to the total number of cells per segment.

External Concentration		Number of Cells	Relative Rate of Cell Production
Ca^{2+}	Na^+		
mM			
0.4	0	72.2	100.0
	25	72.9	101.0
	50	54.1	75.0
	100	47.6	65.9
	150	29.3	40.6
10	0	85.0	117.7
	25	94.2	130.4
	50	85.5	118.4
	100	95.4	132.1
	150	84.3	116.7
	200	58.9	81.5

production in high Ca^{2+} roots than in low Ca^{2+} roots (Table I). This higher rate, in the presence of 10 mM Ca^{2+}, is maintained with increasing salinities up to 150 mM, and only at 200 mM NaCl does the rate of cell production begin to decline. At 0.4 mM Ca^{2+}, cell production appears to be adversely affected by NaCl concentrations as low as 50 mM.

DISCUSSION

Differentiation has been reported to occur closer to the root apical meristem in several halophytes (16) and nonhalophytes (33) when the plants are exposed to salinity. Early maturation of the Casparian strip may be advantageous by minimizing movement of ions through the apoplast into the root xylem. Faster growth of salinized cotton roots, at least at high external Ca^{2+} concentrations, may also be beneficial by allowing roots to reach quickly the deeper layers of the soil, which are often less saline than the soil surface. The slow growth of roots in high Na^+/low Ca^{2+} solutions may well be the result of damage to the plasmalemma, with consequent disruption of several processes related to growth and cell production.

Interaction of Ca^{2+} and Na^+ with the Cell Wall and Plasmalemma. It is widely accepted that Ca^{2+} increases the rigidity of plant cell walls by complexing with wall matrix polysaccharides. Protons and, in certain cases, K^+ and Na^+ have been shown to displace Ca^{2+} from cell walls, thereby increasing wall extensibility (17 and references therein; 32). Thus, one might expect increased growth under saline conditions. At low Ca^{2+} levels, however, salinity diminished root growth not only in cotton but also in *Phaseolus vulgaris* (23, 24), *Elytrigia intermedia* and *E. pontica* (35), and several other species (GR Cramer, unpublished data). Conversely, at moderate salinities, supplemental Ca^{2+} increased both root length (Fig. 1) and weight (6). Furthermore, at high salinities, cell surface area tended to be smaller at low Ca^{2+} concentrations than at high ones (Fig. 6). These observations do not support the idea that Na^+ behaves in a manner analogous to H^+ in displacing Ca^{2+} from the cell wall.

An alternative hypothesis is possible, however. The difference in osmotic potential between the low and high Ca^{2+} media may be considered negligible, and as cell volumes were similar under low and high Ca^{2+} conditions, it seems unlikely at first glance that there were any major differences in cell turgor. Low Ca^{2+} roots grown at 100 and especially 150 mM NaCl, however, were softer in texture, which suggests that cell wall Ca^{2+} may indeed have been displaced by Na^+, resulting in increased wall extensibility. (Several authors [19, 22] have postulated that changes in the rheological or metabolic properties of cell walls allow growth to continue under various osmotic conditions.) Cell turgor, though, may have been lower, so that net cell enlargement was approximately the same as in high Ca^{2+} roots. Adequate Ca^{2+} levels are necessary for membranes to function normally (5, 7, 17, 26). Under high Na^+/low Ca^{2+} conditions, the plasmalemma of cotton roots may have become too leaky to maintain normal cell turgor (27). Indeed, Cramer (5) demonstrated that supplemental Ca^{2+} improved K^+/Na^+ selectivity in salt-stressed cotton roots. Relatively undifferentiated tissues from several halophytes grown under saline conditions have high K/Na ratios, indicating that such plants retain a high degree of membrane selectivity (16).

In addition to weakening of the middle lamella by temporary displacement of Ca^{2+}, cell growth requires secretion of new wall materials into the apoplast. Both plant and animal cells require Ca^{2+} for fusion of secretory vesicles with the plasmalemma (17 and references therein). Observations on wall thickness suggest that at low Ca^{2+} levels, cell wall deposition proceeds rather slowly in salt-stressed cotton (15). Our data do not allow distinction among the possible interactive effects of Na^+ and Ca^{2+} on cell wall extensibility, cell wall synthesis, plasmalemma function, and cell turgor.

Effects of Ca^{2+} and Na^+ on the Cytoskeleton. Calcium interaction with cytoskeletal proteins and the role of Ca^{2+} in cell division are well documented (8, 17). The presence of free Ca^{2+} at concentrations greater than 0.1 μm raises the concentration of tubulin necessary for microtubule assembly, and micromolar concentrations cause depolymerization of existing microtubules (17). Furthermore, ultrastructural and experimental studies of a number of plants have revealed a close correlation between the orientation of microtubules subjacent to the plasmalemma (often termed cortical microtubules) and the orientation of cellulose microfibrils in the innermost, that is, the most recently deposited layer of the cell wall (28, 36). Our observations on cell shape clearly point to direct or indirect effects of Ca^{2+} and Na^+ on microtobule organization. It is conceivable that in cotton roots grown in high Na^+/low Ca^{2+} media, intracellular Ca^{2+} concentrations might rise enough to inhibit microtubule assembly or even promote depolymerization. This, in turn, would result in more random deposition of new wall material and a more nearly isodiametrical cell shape.

The cortical cells of cotton roots exposed to high Na^+/high Ca^{2+} concentrations were much longer and narrower than those of the controls. As cell volume remained constant at all but the highest Na^+ concentration tested, it appears that these conditions promote rigidity of the cytoskeleton, resulting in relatively little radial expansion of cells. The presence of helical arrays of cortical microtubules in plant cells has repeatedly been demonstrated by indirect immunofluorescence (28 and references therein; 30). We envision the cortical cells in our control plants as containing low pitched helices of microtubules that can stretch somewhat, allowing cells to widen as well as elongate; in plants grown under high Na^+/high Ca^{2+} conditions, these helices may lose some of their plasticity. The causes of the increased rigidity of the cytoskeleton merit further study.

Relative Rate of Cell Production and Plane of Cell Division. Comparison of the numbers of cells per file per unit length in the cortex with root length suggested that supplemental Ca^{2+} enhanced the rate of cell production by approximately 20 to 30% of salinities ranging from 25 to at least 100 mM NaCl, while salinity diminished the rate of cell production considerably at low Ca^{2+} levels. Hepler (18) demonstrated that agents that reduce Ca^{2+} influx into the cell nearly double the duration of metaphase in *Tradescantia* staminal hairs. Salinization of nutrient media

sharply reduces Ca^{2+} activity in the medium (6) as well as Ca^{2+} transport and content in cotton plants, especially at low Ca^{2+} concentrations (5, 20), and it seems quite possible that these changes may affect intracellular Ca^{2+} concentration in such a way as to increase the duration of metaphase. Metaphase, of course, is quite brief compared to the total cell cycle duration, and other factors too presumably contribute to the decreased rate of cell production in salinized, low Ca^{2+} roots, such as inability to maintain appropriate K^+ concentrations or K^+/Na^+ ratios in the cytoplasm of meristematic cells (5, 16, 19). In any case, the differences between plants grown in 0.4 and 10 mM Ca^{2+} in the absence of NaCl imply that Ca^{2+} levels play as important a role in cell cycle kinetics in plants as they do in animals (8). It is also worth noting that salinity and osmotic stress are not always inhibitory to cell production, which proceeds at a normal rate in severely salt-stressed tomato embryos (21) and incompletely hydrated carrot embryos (1).

While Na^+ and Ca^{2+} appeared to have interactive effects on cell shape and the rate of cell production within files of cortical cells, they did not seem to affect the basic organization of the cortex, as indicated by the essentially constant number of layers of cortical cells. Similarly, when the halophyte *Suaeda maritima* is grown under saline conditions, root diameter and the cross-sectional area of cortical cells become much larger than in nonsalinized plants, but the number of layers of cells in the cortex and total number of cortical cells remain constant (16). Changes in the number of layers of cortical cells would require a more profound morphogenetic change, namely an increase in the number of T-divisions in the meristem, than changes in the rate of cell production within a file of cells. The slightly greater number of cortical cells per radius in salinized, low Ca^{2+} roots may reflect some disorganization of the microtubules involved in mitosis and cytokinesis. A few excess T-divisions might account for the small increase in number of cortical cells per radius. It is also possible that the combination of salt stress and low external Ca^{2+} activity might be lethal to a few meristematic cells in cotton roots and lead to a minor reorganization of the root apex, as suggested by observations on cold-stressed corn roots (3) and osmotically stressed sunflower leaves (12).

Role of Hormones. Hormones may be involved in the observed changes in cell shape. Ethylene production is often stimulated by stressful conditions, but apparently it is not known whether ethylene production increases in salt-stressed roots. Nonetheless, the changes in cell shape in our high Na^+/low Ca^{2+} treatments were remarkably similar to those occurring in cells exposed to ethylene (9) and are consistent with the observations of Roberts *et al.* (30) on ethylene-induced microtubule orientation. Bangerth (2) noted that Ca^{2+}-deficiency and ethylene symptoms were quite similar and that Ca^{2+} deficiency stimulated ethylene production. This information, combined with the observations that Na^+ can displace Ca^{2+} from membranes (7) and that under saline conditions, Ca^{2+} tissue concentrations are reduced to a significantly greater extent at lower external Ca^{2+} concentrations than at higher ones (20), suggests that ethylene may be involved in the changes in cell shape that we observed. Increased ethylene production resulting from Ca^{2+} deficiency would also explain the decline in the rate of cell production, relative to the controls (31). Other hormones, in particular, auxins, may be involved as well (25). Further work in this area seems warranted.

Conclusion. Our data show that the enhancement of root elongation observed in salt-stressed cotton seedlings provided with supplemental Ca^{2+} is the result of two distinct processes: cell elongation is favored at the expense of radial cell expansion, and high rates of cell production are maintained. These processes are probably mediated by interactions of Na^+ and Ca^{2+} with the cell wall, plasmalemma, and cytoskeleton.

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