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Effect of Salinity on the Composition, Number and Size of Epidermal Cells along the Mature Blade of Wheat Leaves

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Abstract

Salinity inhibits leaf growth in association with changes in cell size. The objective of this study was to determine the spatial distributions of the composition, number and dimensions of epidermal cells in the mature blades of leaf four of wheat seedlings under saline conditions. Plants were grown in loamy soil either with or without 120 mmol/L NaCl in a growth chamber, and harvested after leaf four was fully developed. The results of the spatial distribution analyses of width along the blade showed that salinity not only reduced the width of the leaf blade, but that it also altered the distribution pattern of blade width along the leaf axis. The reduction in the final size of the leaf blade was associated with a reduction in the total number of epidermal cells and in their widths and lengths. This study also revealed the spatial effects of salinity on the blade and epidermal cell dimensions along the leaf axis. In particular, salinity inhibited the total cell number for interstomatal, sister and elongated cells, implying that cell division in wheat leaves is inhibited by salinity. However, the lengths of interstomatal cells were not affected by salinity (unlike those for the sister and elongated cells), suggesting the relative contributions of cell length and numbers to the reduction in the final length of the blade under salinity is dependent on cell type.

Key words: cell length; cell number; leaf epidermis; salinity; wheat.

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Salinity is one of the major environmental stresses limiting agricultural production worldwide. About 7% of the world's total land area is affected by salinity (Flowers 2004; Munns 2005). There is also a dangerous trend of a 10% annual increase in this area throughout the world. Therefore, the study of salt tolerance in plants has been and remains a major concern.

Wheat is a major food crop in most of the countries where saline soils exist or may develop (Ashraf and McNeilly 1988) and is reported by Maas and Hoffman (1977) to be moderately tolerant to salinity. However, the mechanisms by which salt affects wheat growth are still poorly understood. Leaf growth of wheat is of central importance to the development of the plant. Not only is the expansion of leaves crucial to early seedling establishment by providing a continuous supply of energy and carbon through photosynthesis, it also facilitates the development of other organs such as tillers, ears and grains. More importantly, it has been reported that leaf growth largely determines the rate of plant growth and is the most sensitive to salinity in the early stages of wheat development (Munns and Termaat 1986; Maas and Poss 1989; Hu et al. 2000). Previously, we showed that the final length and width of wheat leaves on the main stem were reduced by about 20–30%, respectively, in plants treated with 120 mmol/L NaCl (Hu et al. 2000). More recently, we observed that the salt-induced reduction in the leaf cross-sectional area of wheat during the steady phase of expansion was associated with a reduction in the number of veins during leaf initiation (Hu et al. 2005). Because final leaf size is determined by the width and number of lateral files and the longitudinal number and length of epidermal cells (Kutschera et al. 1987; Kutschera 1989), and because the epidermis is considered the main factor affecting the rate of growth by restraining expansion of the internal tissues (Taize 1984; Kutschera 1992; Wenzel et al. 1997a, 1997b), it is

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necessary to investigate the effect of salinity on the epidermis to achieve a better understanding of leaf growth under saline conditions.

Studies on the relationship between the epidermis and leaf size, as associated with contrasting genotypes and other environmental changes, have been conducted in different grass species. Volenec and Nelson (1981) showed that the cells in the longer leaves of a high-yield tall fescue line were both longer and more numerous than those in the shorter leaves of the low-yield line. Reduction in the leaf length of wheat by high light intensity and temperature (Friend and Pomeroy 1970) and of sorghum under drought (McCree and Davis 1974) was associated with a reduction in the epidermal cell length and number. However, Wenzel et al. (1997a, 1997b) reported that, for a given leaf in barley, no consistent correlation existed between changes in epidermal cell size or cell (or file) number with changes in the leaf length or width for different cell types and the nature of the mutation of barley. Similarly, Beemster and Masle (1996) demonstrated that although high soil resistance reduced the leaf size in wheat through a reduction in the width and length of the mature epidermal cells, the relative reduction in cell size depended on the cell type in question. As such, more than one cell type should be examined when attempting to correlate epidermal cell dimensions with leaf dimensions (Wenzel et al. 1997a, 1997b).

Since the effect of salinity on the elongation of the leaves three, four and five on the mainstem of wheat plants was reported in our previous study (Hu et al. 2000), the objective of this study was to determine: (i) the effect of salinity on the spatial distribution of epidermal cell dimensions and number; (ii) the literal changes in cell files and number of veins in mature blades of wheat leaf four; and (iii) the association between the changes in the dimensions of the leaf and cell. Our results will also help clarify if the inhibition of leaf growth in wheat under salinity is attributable to changes in the size and/or the number of the epidermal cells, with there still being no information on the effect of salinity on the anatomy of epidermis in the leaves of this species.

Results

Leaf dimensions

The mature blade of leaf four was 35 cm long on average for the control treatment and 26 cm long for the salinized treatment, for a reduction in the length of leaf blade by salinity of about 26%. For the control plants, the width of the leaf blade increased from the leaf base, reached a maximum at the middle of the leaf, and then decreased rapidly. By contrast, under saline conditions, the leaf width increased from the leaf base to 10% relative leaf length, remained almost unchanged to 30% relative leaf length,

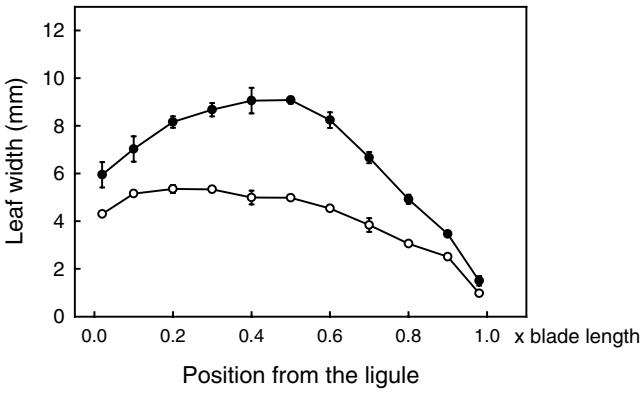

Figure 1. Spatial distribution of the width of the mature blade of leaf four of wheat seedlings grown in soil with either no added NaCl (•) or 120 mmol/L NaCl (○). Error bars ($n = 5$) represent standard errors and fit within the plot symbol if not visible.

and then decreased slightly with distance thereafter (Figure 1). The maximum average leaf width was about 9.1 mm for the control plants and 5.4 mm for the saline plants, whereas the respective average leaf widths were about 6.6 mm and 4.1 mm. Thus, from the base to the middle of the leaf, the reduction in the blade width by salinity was about 28–45%. The average reduction in width along the whole leaf by salinity was about 35%, which was greater than the reduction observed in the length of the blade.

Number and width of veins

The distribution pattern of the number of veins across the leaf width was similar to that of the width of the leaf blade for the control plants, increasing from the value at the base (25) to reach a maximum in the middle of the leaf (32) and decreasing thereafter (15 at 90% relative leaf length). By contrast, this variable showed a different pattern to that of leaf width in the saline treatment: the number of veins was virtually unchanged from the leaf base to a position of about 70% relative leaf length (21–22 in both cases) before decreasing with distance from the base after this point to match the control value at 90% relative leaf length (Figure 2). The total number of veins is closely correlated with leaf width in both treatments ($r = 0.98$), although the number was reduced under saline conditions by 15% at the leaf base and 36% at the middle of the leaf blade.

By contrast, the distribution patterns of the average width of the veins were similar in both treatments (Figure 2), where the average width increased from the leaf base, remained unchanged or increased slightly up to 60% of the leaf length, and then decreased with distance thereafter. The average width per vein was about 0.21–0.29 mm for the control treatment and 0.15–0.24 mm for the salinized treatment. The reduction in the

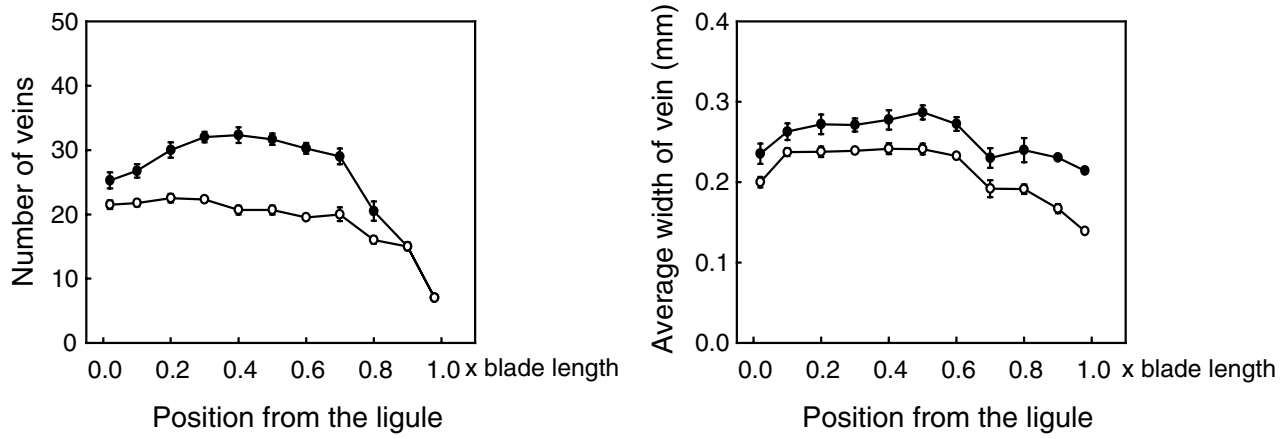

Figure 2. Spatial distribution of the width and number of veins in the mature blade of leaf four of wheat seedlings grown in soil with either no added NaCl (•) or 120 mmol/L NaCl (○). Error bars ($n = 5$) represent standard errors and fit within the plot symbol if not visible.

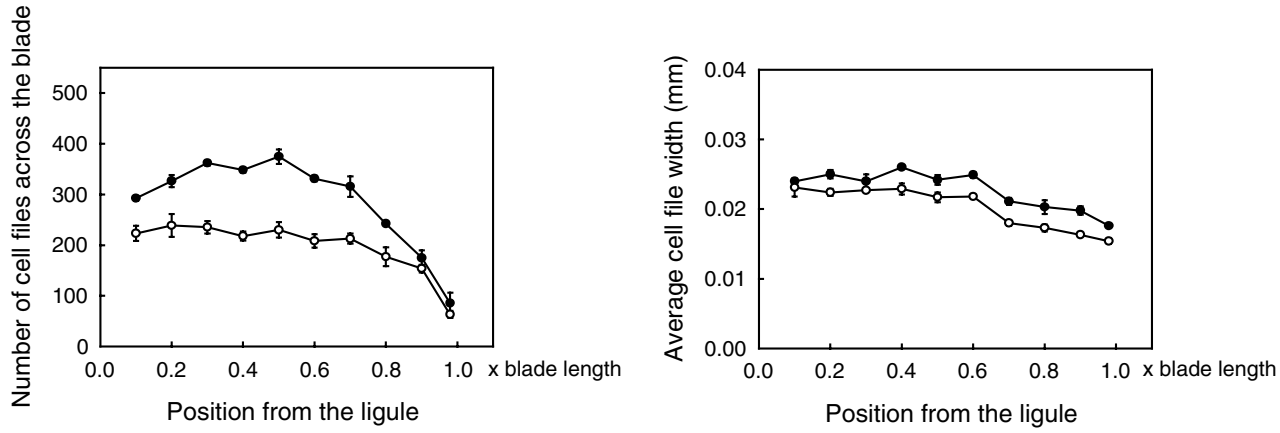

Figure 3. Spatial distribution of the width and number of cell files in the mature blade of leaf four of wheat seedlings grown in soil with either no added NaCl (•) or 120 mmol/L NaCl (○). Error bars ($n = 5$) represent standard errors and fit within the plot symbol if not visible.

average width of the veins in the saline plants was about 10–15% between the leaf base and 70% relative leaf length and about 20–35% in the distalmost part of the leaf blade.

Number of cell files and cell width

The spatial distribution of the number of cell files across the blade along the leaf axis was also similar to that of the blade width regardless of the treatment (Figure 3). For the control treatment, the number of cell files increased from the leaf base (300) to the middle of the leaf (370) and decreased thereafter with distance (85 at the distal part), whereas, for the salinized treatment, it remained almost unchanged between 10 and 30% of the leaf length (about 240–210 between the base and 70% of leaf length) and then decreased with distance (65 at the leaf tip). Overall, salinity reduced the file number by an average of 30% in the whole leaf. In contrast to the different distribution patterns of the file number between the two treatments along the leaf axis (but ones that match those for leaf width in each case), the results for the width of the cell file in Figure 3 show a similar pattern in both treatments: the width in the first half of leaf was greater than that in second half. The treatments did differ, however, in that, except at the leaf base, salinity reduced the width of cell file by an average of 11%.

Because the density of the cell files is the reciprocal of their width, the number of cell files per mm were accordingly lower in the first half leaf than that in the second half and was increased by salinity (Figure 4). Interestingly, there was less change in the number of cell files per vein within the leaf, and no effect of salinity was observed in most locations (Figure 3), with the number of cell files per vein being about 10–13 regardless of the location and treatment.

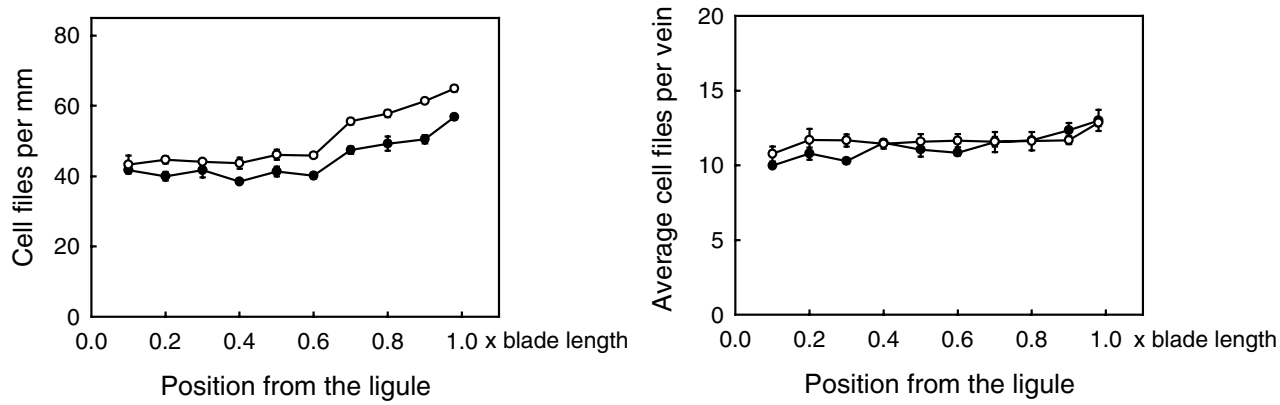

Figure 4. Spatial distribution of the density of cell files per millimetre and per vein in the mature blade of leaf four of wheat seedlings grown in soil with either no added NaCl (●) or 120 mmol/L NaCl (○). Error bars ($n = 5$) represent standard errors and fit within the plot symbol if not visible.

Cell length, cell density and number of cells along a file

The mature cell length for the three cell types present in the cell file is given in Figure 5. Elongated cells were about 0.7–1.3 mm long, whereas sister and interstomatal cells were 0.2–0.3 mm and 0.1–0.2 mm long, respectively. Thus, the elongated cells were 4–6 times longer than interstomatal and sister cells. The distribution patterns of the cells along the leaf axis also varied with cell type. The length of the interstomatal cells remained unchanged up to the middle of the leaf, increased with distance and then decreased at the distalmost part of the leaf for both treatments. From the base to the middle of the leaf, sister cells either decreased in length slightly for control treatment or increased for the salinized treatment. Thereafter, their lengths remained almost constant up to 60–70% relative leaf length, increased up to 80% relative leaf length, and then decreased with distance. By contrast, there was a very slight decrease in the length of the interstomatal cells between the blade base and 80% or 90% relative leaf length, with a rapid decrease thereafter. As such, there was no general effect of salinity on their lengths along the leaf axis. For example, salt stress did not affect their lengths in most locations, whereas it reduced the lengths at relative locations of 30% and 70–80% and at the leaf tip, and increased the lengths at the relative position of 90%. The lengths of sister cells in the saline treatment were reduced at both the basal and distal parts of leaves compared to the control, whereas the lengths of elongated cells between the leaf base and 80% were generally smaller under the control treatment.

Again, cell density (cells per mm length) displayed the opposite trend to cell length, with salinity increasing the average density of cells within the leaf by 12% for the elongated cells, 11% for the sister cells and 3% for interstomatal cells.

The total cell number within a file was reduced by salinity regardless of cell type (Table 1), although there was a greater reduction in cell number for the interstomatal cells (23%) than for the sister and elongated cells (16%) (Table 1).

Discussion

Although the results of our previous studies (Hu et al. 2000; Hu and Schmidhalter 2001; Hu et al. 2005), which revealed that salinity reduces both the length and width of wheat leaves, are confirmed here, the present study provides additional information on some of the underlying anatomical changes and the association between changes in the dimensions of the mature leaf and of the epidermis in wheat seedlings. For example, our results show that salinity not only reduced the width of the leaf blade, but it also altered the spatial distribution pattern of blade width along the leaf axis (Figure 1). This indicates that salinity affects leaf width differentially during leaf development. The reduction in the width is attributable to reductions in both vein number and average vein width (Figures 1 and 2) although, the reduction in the former varied with location. The maximum reduction in the number of veins in leaf four observed under salinity (36%) agrees with the value reported for the same leaf number under the same level of salinity (120 mM NaCl) by Hu et al. (2005). Their study on the effect of salinity on the cross-sectional area in the expanding wheat leaf four also demonstrated that the reduction in vein number occurred mainly at the leaf base during leaf initiation. It is known that the development of the veins involves the formation of a primordium that encircles the shoot apical stem before undergoing elongation growth (Sharman 1942; Sylvester et al. 1990; Timmermans et al. 1998), with cell division being distributed uniformly throughout the primordium (Sharman 1942; Sylvester et al. 1990). Thus, the fact that the observed trends of a reduction in the cell files across the blade and a lack of change in cell files per vein at a

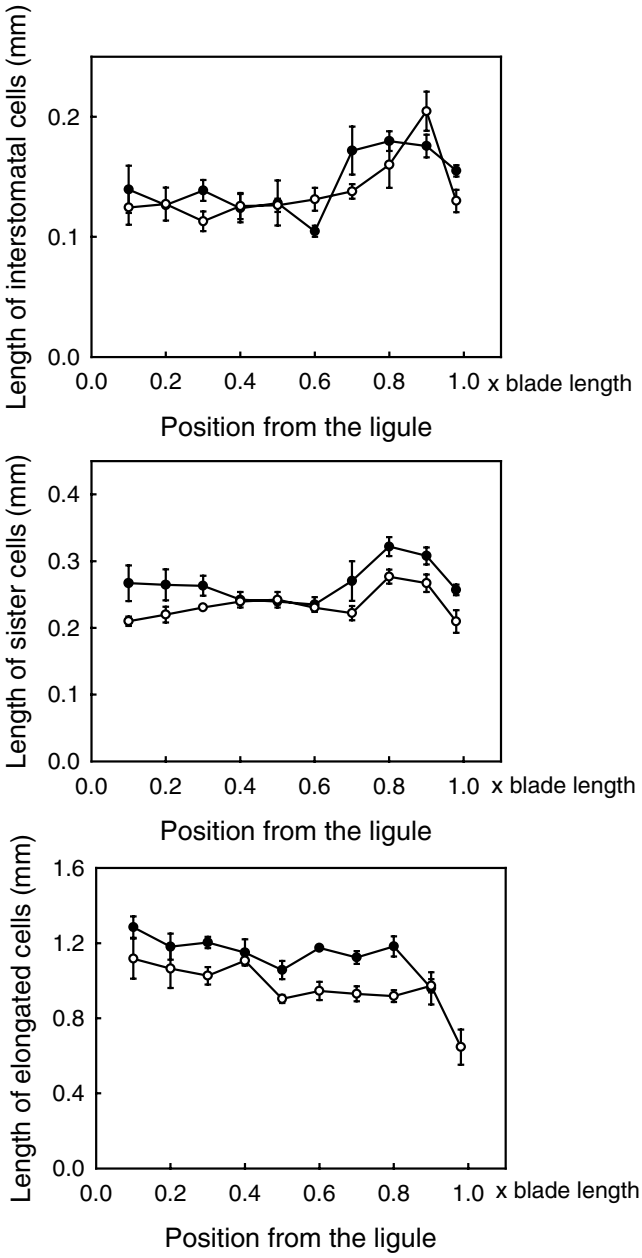

Figure 5. Spatial distribution of the lengths of interstomatal, sister and elongated cells in abaxial of the mature blade of wheat leaf four grown in soil with either no added NaCl (•) or 120 mmol/L NaCl (○). Error bars ($n = 5$) represent standard errors and fit within the plot symbol if not visible.

salinity level of 120 mmol/L NaCl match that in the number of veins in Figures 2 and 3 suggests that salinity must affect the cell dividing for the files during the formation of the veins, but not the composition of cell types.

Table 1. Total number of cells counted along single files from the ligule to the blade on the abaxial epidermis

	Cell number		
Cell types	Control	120 mmol/L NaCl	Reduction (%)
Interstomatal cells	2494 ± 184.5†	1932 ± 158.2†	23%
Sister cells	1324 ± 77.4†	1116 ± 43.7†	16%
Elongated cells	331 ± 16.3†	276 ± 15.6†	16%

†Mean ± *SE*; $n = 5$.

Salinity caused a reduction in the sizes of the mature cells with respect to both their widths and lengths (Figures 3 and 5), with the reduction in cell length under saline conditions being dependent on the cell type. The different epidermal cell types in a leaf of barley or wheat vary greatly in final length according to the genotype of the plant and environmental conditions (e.g. Beemster and Masle 1996; Wenzel et al. 1997a, 1997b). Here, the elongated cells of wheat leaf four were reduced the most by salinity, whereas the interstomatal cells were reduced the least, showing that it is important to examine more than one cell type in mature leaves of wheat to accurately assess the effects of saline conditions.

Furthermore, the reduction observed in total leaf length under saline conditions is a function of the combination of cell lengths and numbers, which also vary according to the cell type. For example, although there was no change in the length of the insterstomatal cells under salinity, their total number along a file was reduced 23% (and 16% for both the sister and elongated cells). Thus, data here reveal that the inhibition of cell division for interstomatal cells in abaxial cells was essential for growth in the saline treatment. For the sister and elongated cells, however, salinity restricted both their length and number. The differential effects of salinity on the different cell types observed here thus explains the fact that other studies investigating the growth of plants under saline conditions have variously reported that either reduced cell numbers or length, or both, were associated with the reduction in growth (e.g. Friend and Pomeroy 1970; McCree and Davis 1974; Keyes et al. 1989; Beemster and Masle 1996).

Cell division and elongation occur during the period of leaf growth. The growth of wheat leaves, like other grass leaves, is limited to a small region near the leaf base that is enclosed in the older leaf sheath. Cells divide and elongate only within the leaf growth zone. Because salt stress is caused by high salt concentrations (such as of Na and Cl in soils), one would expect that ionic toxicity and/or ion deficiency (e.g. K and Ca) causes the inhibition of leaf growth. However, analyses of mineral elements carried out on the same scale as the growth analyses for the growing leaves of sorghum, wheat and maize rule out Na and Cl toxicity as a direct cause of the inhibition of leaf growth in grass (Bernstein et al. 1995; Hu and Schmidhalter 1998a; Hu et al. 2000; Neves-Piestun and Bernstein 2005). Potassium and

Ca deficiency can be similarly discounted (Hu and Schmidhalter 1998a).

Thus, other explanations for the reduction in growth must be sought. One productive line of enquiry might revolve around the fact that cell division is probably controlled by signalling and candidate genes (Zhu 2001). For example, the study by Cramer and Quarrie (2002) showed that abscisic acid (ABA) concentrations in the leaf growth zone of maize were increased significantly by salinity, suggesting that the cell division may be inhibited by an increase in ABA under saline conditions. Additionally, the reduction in the final cell length can also be influenced by any of the changes in cell extensibility, turgor pressure, and the duration of cell elongation. A review by Munns (1993) suggested that turgor was unlikely to play a role in the reduction of elongation in the growing leaf. Similarly, recent studies found that no decrease in turgor pressure (as measured with a pressure probe) occurred in the elongating cells of wheat under saline conditions (Arif and Tomos 1993; Fricke and Peters 2002), probably because the turgor pressure of the elongating tissues was being maintained via sufficient osmotic adjustment for the saline conditions (Hu and Schmidhalter 1998b).

Duration of cell elongation remains an important potential explanation. First, a shortened duration of individual cell expansion might reduce the leaf elongation rate (LER) without there being any changes in cell division and elongation rate (MacAdam et al. 1989). The duration of cell elongation may be determined by the chemical composition and properties of the cell wall, which is deposited after cell elongation, thereby preventing further cell expansion (MacAdam et al. 1989). A modified capacitance of cell walls such that they yield irreversibly was suggested to be the major factor limiting growth under salt stress (Cramer and Bowman 1991; Neumann et al. 1994). Other factors that may contribute to cessation of cell elongation have also been investigated. Extensibility of the cell wall is thought to be reduced by formation of covalent bonds between phenolic residues of pectins, hemicelluose, and structural proteins of cell wall (Fry 1986). Peroxidase promotes this bonding by catalyzing the formation of free radicals of the residues (de Souza and McAdam 2001). Finally, apoplastic pH is considered to play an important role in cell wall loosening and tissue growth. However, Neves-Piestun and Bernstein (2001) reported that salinity-induced inhibition of leaf elongation in maize is not mediated by changes in the cell wall acidification capacity in the growing tissues of the leaves. Altogether, it is clear that much more research is needed in this area.

In conclusion, this study revealed the spatial effect of salinity on blade and epidermal cell dimensions along the leaf axis in wheat seedlings. The reduction in the final size of leaf blade is associated with a reduction in the total number of epidermal cells and in their width and length. In particular, salinity inhibited the total cell number for interstomatal, sister and elongated cells. However, the length of interstomatal cells was not affected by salinity, suggesting that the relative contributions of cell length and numbers to the reduction in the final length of blade at a salinity level of 120 mmol/L NaCl is dependent on the cell type.

Materials and Methods

Growth conditions

Six seeds of spring wheat (*Triticum aestivum* L. cv. Thasos) that were pre-germinated for 2 days on filter paper wetted by tap water at 20 °C were sown in 1.5-L pots (10 cm in diameter and 20 cm high) containing loam soil. The soil was initially watered to 0.25 g H_2O g^{-1} dry soil (which allowed for optimal aeration) with 0.2 g NH_4NO_3 kg^{-1} dry soil. The test salt level of 120 mmol/L NaCl was obtained by adding NaCl to the nutrient solution. The soil was mixed thoroughly and kept in tightly closed plastic boxes for one week to facilitate equilibration. Thereafter, the soil was sieved and put into the pots. Soil moisture content was maintained at the initial level by watering with tap water. To minimize water loss through evaporation, the pots were covered with a perforated plastic film that allowed the plants to grow through the small holes. One week after sowing, the seedlings were thinned to four plants per pot. The experiment was conducted in a growth chamber with a 16-h photoperiod. The light intensity was approximately 550 μmol photon^{-2} s^{-1} (PPFD). The air temperature was 20 °C (day/night) and the relative humidity was maintained at 55–65%.

Leaf preparation and morphometric analysis of the blade epidermis

The fully developed leaf four in the seedlings was removed from four plants that had similar lengths for leaf four in both the control and 120 mmol/L NaCl treatments. The leaves were cut into 3-cm lengths, cleared in absolute methanol at room temperature for 1 or 2 days until the chlorophyll was completely removed, and then subsequently transferred to 90% lactic acid for further clearing and storage until analysis (modified from Wenzel et al. 1997a, 1997b). The lengths of the leaf segments were measured after clearing in methanol and no shrinkage was observed. The cleared leaves were mounted on a Zeiss microscope (Zeiss Axioscope, Germany) at a magnification of 2.5–5.0 times with the objectives being connected to a PC-based image processing system. Images were captured using a Zeiss Axio Vision system mounted on top of the microscope. An image analyzer, SigmaScan Pro 5 (SYSTAT Software, Point Richmond, CA, USA), was used to measure the relevant morphometric parameters.

The epidermis of a wheat leaf contains a variety of cells, with the files of the different cell types being arranged in a regular pattern (Stebbins and Shah 1960; Esau 1977; Beemster and

Masle 1996). The classification used here was described in detail by Beemster and Masle (1996). Briefly, there are usually two stomatal rows between two adjacent veins, comprising stomatal complexes separated by interstomatal cells. Adjacent to these files are ones of wider "sister cells". The two inner files of sister cells, which are located between two adjacent stomatal rows, are separated by several files of unspecialized, long and narrow "elongated" cells on the blade of the abaxial side, and enlarged and shorter "bulliform" cells on the adaxial side. One or two other files above the larger veins are composed of sclerenchymatous cells. Because abaxial composition of cell types is characterised by most epidermal cell types in mature leaves of wheat (Beemster and Masle 1996), the morphometric analysis of the elongated, sister and interstomatal cells was conducted on the abaxial side only in this study.

Because salinity affects the leaf length and width in wheat (Hu et al. 2000), the sampling positions along the leaves of the different plants and treatments were standardized for the analyses using a scale of 0–100% of blade length with a 10% interval (for a total of 10 locations). Blade width, the number of veins, the total number of files across the blade, and the length of elongated, sister and interstomatal cells on the abaxial side were measured at each of the 10 locations along each leaf. The numbers of cells along the files were also counted. The cell files per vein and the average widths of the vein and cell file were calculated as the ratios of the cell file number across the blade to the vein number, the blade width to the vein number, and the blade width to the total number of cell files, respectively.

Statistical analysis

A completely randomized design was used with five replicates for all treatments. Data were analyzed using an analysis of variance (ANOVA) to test the significance of the main effects using JMP 4.02 (SAS 2000).

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