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Differential Solute Regulation in Leaf Blades of Various Ages in Salt-Sensitive Wheat and a Salt-Tolerant Wheat × *Lophopyrum elongatum* (Host) A. Löve Amphiploid[1]

Timothy David Colmer[2,3], Emanuel Epstein*, and Jan Dvorak

Department of Land, Air and Water Resources (T.D.C., E.E.) and Department of Agronomy and Range Science (J.D.), University of California, Davis, California 95616-8627

Leaf blades of different ages from a salt-tolerant wheat × *Lophopyrum elongatum* (Host) A. Löve (syn. *Agropyron elongatum* Host) amphiploid and its salt-sensitive wheat parent (*Triticum aestivum* L. cv Chinese Spring) were compared for their ionic relations, organic solute accumulation, and sap osmotic potential (π_{sap}). The plants were grown for 18 d in nonsaline (1.25 mM Na^+) and salinized (200 mM NaCl) nutrient solutions. The response of leaf blades to NaCl salinity depended greatly on their age or position on the main stem. Na and proline levels were highest in the oldest leaf blade and progressively lower in younger ones. Glycine betaine and asparagine levels were highest in the youngest blade. The π_{sap} was similar for corresponding leaf blades of both genotypes, but contributions of various solutes to the difference in π_{sap} between blades from control and 200 mM NaCl treatments differed greatly. The NaCl-induced decline in π_{sap} of the youngest leaf blade of Chinese Spring was predominately due to the accumulation of Na and to a lesser extent asparagine; in the amphiploid, it was due to a combination of glycine betaine, K, Na, and asparagine. Proline contributed little in the youngest blade of either genotype. In the older blades Na was the major solute contributing to the decline in π_{sap}. Thus, the maintenance of low Na and high K levels and the accumulation of glycine betaine in the young leaf tissues contributed to the NaCl tolerance of the amphiploid. No such role was evident for proline.

Soil salinity, usually NaCl, may reduce plant growth by ion toxicity and water deficits (Epstein, 1980; Greenway and Munns, 1980) and by affecting mineral nutrition (Ball et al., 1987; Lazof and Läuchli, 1991). Plants growing in saline media may diminish internal water deficits by the absorption of inorganic ions and the synthesis of organic solutes, both of which contribute to osmotic adjustment (Epstein, 1980). Many salt-tolerant nonhalophytes tend to restrict Na uptake and take up more K than do the less tolerant ones (Epstein, 1980; Greenway and Munns, 1980; Shah et al., 1987). In addition, salinity tolerance of cereals may be related to the accumulation of Na in old leaves and the continued transport of K to young leaves (Greenway et al., 1965; Yeo et al., 1985; Yeo and Flowers, 1986; Wolf et al., 1991). However, although Na "exclusion" has often been implicated as one of the mechanisms of salt tolerance in nonhalophytes, this conclusion cannot be generalized (Cramer et al., 1994, and refs. therein).

Limited accumulation of Na (and Cl) in the shoots of salt-tolerant nonhalophytes diminishes the role of these solutes in osmotic adjustment (Greenway and Munns, 1980). The accumulation of K and low-molecular-weight organic solutes may therefore be particularly important for osmotic adjustment in leaves of salt-tolerant nonhalophytes. Gly betaine and Pro are two organic solutes that may function as osmotically active solutes (osmolytes) in members of the Gramineae (Stewart et al., 1979; Rhodes and Hanson, 1993). Some nonhalophytic crops (e.g. barley, wheat, and maize) accumulate Gly betaine, whereas others (e.g. rice) do not (Grumet and Hanson, 1986; McDonnell and Wyn Jones, 1988; Brunk et al., 1989; Rathinasabapathi et al., 1993). Its accumulation in salinized plants, presumably for osmotic adjustment of the cytoplasm (Hall et al., 1978; Matoh et al., 1987), is considered to be an important physiological trait contributing to the maintenance of growth under salinity (Greenway and Munns, 1980; Rhodes and Hanson, 1993). However, direct experimental evidence to support this claim is scant. Furthermore, one study of barley cultivars showed that Gly betaine levels were higher in the shoots of a salt-sensitive than a salt-tolerant cultivar (Wyn Jones and Storey, 1978). The role of Pro in the adaptation of nonhalophytes to salinity is even less clear than that of Gly betaine (Greenway and Munns, 1980; Rabe, 1990).

The use of crosses between crop species and related wild species as a source of novel germplasm for improving salinity tolerance provides an opportunity to investigate the genetic and physiological basis of plant tolerance of salinity (Rush and Epstein, 1981; Storey et al., 1985; Omielan et al., 1991). The enhanced salt tolerance of a

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[3] Present address: Department of Plant Sciences, The School of Agriculture, The University of Western Australia, Nedlands 6009, WA, Australia.

* Corresponding author; e-mail eqepstein@ucdavis.edu; fax 1-916-752-1552.

Abbreviations: FID, flame ionization detector; π, osmotic potential; π_{sap}, sap osmotic potential.

wheat (*Triticum aestivum* L. cv Chinese Spring) × tall wheatgrass (*Lophopyrum elongatum* [Host] A. Löve; syn. *Agropyron elongatum* [Host]) amphiploid is associated with a low rate of Na accumulation and the maintenance of K levels in its youngest leaf, compared to its wheat parent (Schachtman et al., 1989; Omielan et al., 1991). In contrast, the amphiploid cross of wheat (cv Chinese Spring) × *Thinopyrum bessarabicum* (Savul and Rayss) A. Löve (syn. *Agropyron bessarabicum* [Savul and Rayss]), which is closely related to *L. elongatum* and is often considered congeneric with it, had only a moderately lower Na level in its youngest leaf than did its wheat parent and no differences in their K levels when grown under NaCl salinity (Gorham et al., 1986). Furthermore, the Gly betaine level in the youngest leaf of the wheat × *T. bessarabicum* amphiploid was similar to that of its wheat parent, despite the fact that leaves of the *T. bessarabicum* parent contain high levels of Gly betaine (Gorham et al., 1986). These findings are surprising, since the amphiploid is considerably more NaCl tolerant than the parental wheat (Gorham et al., 1986).

In view of these conflicting findings concerning the role of Na "exclusion" and compensatory osmolytes such as K, Gly betaine, and Pro in salinity tolerance in the Triticeae, the present work deals with the relations of these solutes to the NaCl tolerance of the wheat × *L. elongatum* amphiploid and its wheat parent. All leaf blades of these two genotypes were individually compared, since the inconsistencies noted above may have been caused by differential partitioning of solutes among leaves (Greenway et al., 1965; Ahmad and Wyn Jones, 1982; McDonnell and Wyn Jones, 1988; Madan et al., 1994). Furthermore, the possibility exists that solutes other than those most commonly assayed may play a role in salt tolerance. Therefore, a comprehensive assessment of the accumulation of numerous solutes in the wheat and wheat × *L. elongatum* amphiploid was made by use of atomic emission spectroscopy, GLC, and ^{1}H NMR spectroscopy. The results of this study show that the maintenance of low Na and high K levels and the accumulation of Gly betaine in the young leaf tissues contribute to the NaCl tolerance of the amphiploid, a finding not apparent from some earlier work on this topic (Gorham et al., 1986).

MATERIALS AND METHODS

Plant Culture

Bread wheat (*Triticum aestivum* L. cv Chinese Spring; $2n = 6x = 42$; genome AABBDD) and the octaploid amphiploid from a cross between Chinese Spring × tall wheatgrass (*Lophopyrum elongatum* [Host] A. Löve; $2n = 2x = 14$; genome EE; syn. *Agropyron elongatum* [Host]) were used in this study. Amphiploid seeds were germinated 1 d before seeds of the faster growing Chinese Spring in all experiments except in the experiment used to determine relative growth rates, in which seeds of the amphiploid were germinated 3 d before those of Chinese Spring, since this resulted in plants of a similar developmental stage at the commencement of the NaCl treatment. Seeds were germinated in the dark at 25.6 ± 1.0/22.1 ± 1.1°C day/night temperature on cheesecloth-covered polypropylene mesh over nutrient solution. The nutrient solution contained (mM) K^+, 1.5; Ca^{2+}, 4.0; Mg^{2+}, 0.25; NH_4^+, 0.5; Na^+, 1.25; NO_3^-, 3.5; SO_4^{2-}, 3.85; HPO_4^{2-}, 0.050; H_4SiO_4, 0.25; Cl^-, 0.45; Fe-EDTA, 0.050; and micronutrients of one-quarter-concentration modified Hoagland solution (Epstein, 1972). Regarding the inclusion of silicate, see Epstein (1994).

At 3 d after imbibition the solutions were aerated, and 4 d after imbibition the shoots of the seedlings were exposed to sunlight in a temperature-controlled greenhouse (25.6 ± 1.0/22.1 ± 1.1°C day/night temperature; midday photon flux density was about 1100–1200 μmol m^{-2} s^{-1}). At 7 d after imbibition of the amphiploid, 24 seedlings of each genotype were transferred to the same 100-L solution culture tank (12 tanks in total) containing the same aerated solution described above, except that 0.020 mM $Fe(NH_4)_2(SO_4)_2$ was added. The pH of these solutions was adjusted with KOH to 5.5 ± 0.05 every 2nd d prior to the initial harvest and every 1 d after the NaCl treatments were imposed. The concentration of solution P in each tank was determined every 3 d using the spectrophotometric phosphomolybdate blue method of Murphy and Riley (1962), and $NH_4H_2PO_4$ was added to maintain P at 0.050 mM. The nutrient solution in each 100-L tank was renewed every 7 d throughout the treatment period.

NaCl Treatments

At 14 d after imbibition of the amphiploid, 6 plants of each genotype were harvested from each tank, leaving 18 plants of each genotype per tank. At this time, the amphiploid had 2.3 to 2.4 leaves and Chinese Spring had 2.9 to 3.0 leaves. Treatments of control (1.25 mM Na^+), 100, 150, and 200 mM NaCl were imposed, three replicate tanks of each treatment being arranged randomly in the greenhouse. The treatment period was 18 d.

Growth Analysis

Root and shoot growth of the two genotypes was assessed by measuring dry weights at the start and end of the 18-d treatments and calculating relative growth rates (RGR), using the formula: RGR = (ln $dweight_2$ − ln $dweight_1$)/($t_2 - t_1$), where $dweight_1$ = dry weight (g) at time 1; $dweight_2$ = dry weight (g) at time 2; and t_1 and t_2 = time 1 and time 2 in d.

In addition, the rate of increase in length of the youngest leaf on the main stem of both genotypes was measured between d 15 and 17 of exposure to the NaCl treatments. For each replicate tank, three plants of each genotype that had the youngest leaf lengths between 5 to 10 cm on d 15 were selected for these leaf growth studies.

Harvest

After 18 d of NaCl treatments the plants were harvested. To minimize any effects of time of day at harvest on genotype or treatment comparisons, all plants in replicate 1 were harvested, then those in replicate 2, and finally those in replicate 3. The roots and stem base of the plants were rinsed three times, for 10 s each time, in double deionized

water. The leaf blades on the main stem were excised and separated according to age, the oldest leaf blade being number 1, the second oldest leaf blade number 2, etc. After the 18 d of exposure to 200 mM NaCl, Chinese Spring had 5.1 to 5.2 leaves and the amphiploid had 5.0 leaves; the sixth leaf of the amphiploid was just starting to emerge but was too small for analysis. The control Chinese Spring had 7.3 to 7.6 leaves and the amphiploid had 6.2 to 6.5 leaves. One leaf blade of each age class for each replicate was sealed immediately after its excision in an air-tight glass vial and frozen at −70°C for subsequent measurement of leaf π_{sap}. The "sheaths," which were the base of the main stem with sheaths and young leaves not yet emerged, were also kept for mineral analysis. In addition, the roots were excised and blotted to remove surface water. Fresh weights of all tissues were recorded before they were frozen in liquid N_2 and lyophilized, after which dry weights were recorded.

Mineral Analysis

K and Na were extracted from lyophilized and ground tissue samples by shaking in 0.5 M HCl for 2 d (Hunt, 1982). La-Cs reagent was added to dilutions of the extraction solutions to give final La and Cs concentrations of 2000 and 1000 μg mL^{-1}, respectively, and K and Na were determined by atomic emission flame photometry (Sotera and Stux, 1979).

Perchloric Acid Extraction of Metabolites

The concentrations of at least 36 metabolites, including Gly betaine, Suc, Glc, amino acids, and organic acids, in perchloric acid extracts of leaf blade tissue were determined using the combined ^{1}H NMR spectroscopy and GLC procedure described by Fan et al. (1993).

Lyophilized leaf tissues were pulverized to 1- to 3-μm particles with an agate ball in a Teflon chamber using a Braun Mikro-Dismembrator II (Melsungen, Germany). Low-molecular-weight, water-soluble solutes were extracted twice from 100-mg samples of leaf tissue with 3 mL of 5% (w/v) perchloric acid at 4°C as described by Fan et al. (1993). Aliquots of the extracts were put into GC vials and lyophilized for subsequent GLC analysis. The remaining extract was prepared for ^{1}H NMR spectroscopy according to the procedure of Fan et al. (1993).

GLC

Lyophilized samples for GLC analysis of 24 amino acids and 9 organic acids (for a complete list, see Fan et al., 1993) were derivatized with 400 μL of *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide:acetonitrile (1:1, v/v) by sonication at 60°C for 3 h. The samples were kept in the dark at room temperature (approximately 25°C) for approximately 24 h before GLC analysis using a Varian 3300 gas chromatograph (Varian Instruments, Palo Alto, CA) equipped with an FID and a DB-1 open tubular column of 0.18 mm i.d. and 40 m length (J & W Scientific, Folsom, CA). The injector, FID, and column temperature programs were as described by Fan et al. (1993). FID signals were acquired and processed using Peaksimple III software (SRI Instruments, Torrance, CA). All peak assignments were verified manually, and compounds were quantified by comparing peak areas from the sample extracts with those of standards.

^{1}H NMR Spectroscopy

^{1}H NMR spectroscopy was used to determine the concentrations of Gly betaine, Glc, and Suc in leaf extracts that were redissolved in 0.7 mL of ^2H_2O. One-dimensional ^{1}H NMR spectra were acquired at 7 T on a General Electric QE-300 spectrometer at 25°C. Acquisition parameters were a 90° pulse with gated solvent suppression, a 1.0-s interpulse delay, a 1.36-s acquisition time, a 3003-hertz spectral width, 8,192 sampling points, and 128 passes. The data were zero-filled to 16,384 points, and the free induction decays were apodized using a line broadening of 1 hertz prior to Fourier transformation. Chemical shift scales were calibrated by assigning the anomeric proton resonance of Suc to 5.42 ppm, which was originally calibrated by Fan et al. (1993) against the methyl protons of 2,2′-dimethylsilapentane-5-sulfonate assigned to a chemical shift value of 0 ppm. Gly betaine, Suc, and Glc concentrations in the extracts were quantified from the ^{1}H NMR spectra according to the procedure of Fan et al. (1993). Briefly, the peak areas of the N^+-CH_3 of Gly betaine (3.27 ppm), the anomeric proton of Suc (5.42 ppm), and the anomeric protons for α- and β-Glc (5.24 and 4.64 ppm, respectively) and the β-CH_3 of Ala (1.49 ppm) were determined. The peak areas were corrected for their number of protons and saturation factors, and the ratios of the corrected peak areas for Gly betaine, Suc, and Glc to that of Ala were determined. The concentrations of these solutes were then calculated by multiplying this ratio by the Ala concentration as determined by GLC-FID.

Expressed Leaf Blade π_{sap}

π_{sap} measurements expressed from freeze/thawed leaf blades were made at 25°C with a Wescor (Logan, UT) model 5110 C vapor pressure osmometer. Eight-microliter subsamples of sap expressed from whole leaf blades were analyzed. The osmometer was calibrated with new 100-, 290-, and 1000-mmol/kg osmolality standards (Wescor Inc.), and the calibration was checked after every 10 measurements.

Estimated Contributions of Various Solutes to Differences in Leaf Blade π_{sap}

The π of a solution is given by $\pi = -nRT/V$, where n = number of solute molecules; R = the universal gas constant; T = temperature in °K; and V = volume in L. Both solute accumulation and a lower water content may cause the π_{sap} expressed from leaf blades to become more negative. The contributions of various solutes to the π_{sap} were calculated from the data on tissue solute levels (μmol g^{-1} dry weight) and tissue water contents (mL g^{-1} dry weight) for leaf blades grown in control and 200 mM NaCl solutions. The difference in the contribution of a given solute to

the π_{sap} of control and NaCl-treated leaf blades was then calculated by subtracting the control value from that in the corresponding 200 mM NaCl-treated leaf blade.

Statistical Analysis

Genotype and treatment comparisons of growth, tissue inorganic ion, organic solute, and π data were examined statistically by analysis of variance for a split plot design. Genotype and tissue means were compared using LSD. Leaf blade means were compared according to their position on the main stem counting from the oldest leaf and also according to their relative age from the youngest leaf (see figure legends).

RESULTS

Growth of Plants Exposed to Low and High NaCl Concentrations

The higher salt tolerance of the amphiploid than that of Chinese Spring was demonstrated by a higher relative growth rate (dry weight basis) of its roots (18%) and shoots (27%), a 33% faster elongation rate of its youngest leaf, and a smaller percentage reduction in relative growth rate, when exposed to 200 mM NaCl (Table I; Fig. 1). In contrast, the control (1.25 mM Na^+) Chinese Spring plants grew faster than those of the amphiploid (Table I; Fig. 1).

Distribution of K and Na in Plants Exposed to Low and High NaCl Concentrations

At 1.25 mM Na^+, tissue Na levels were 2 orders of magnitude lower than those of K, and the levels of these solutes were similar in the two genotypes (Fig. 2, A and C). Eighteen days of exposure to 200 mM NaCl greatly increased the Na levels in both genotypes (Fig. 2, A and B). Na levels in the roots of the two genotypes were similar (Fig. 2B). However, the levels of Na in the "sheaths" and leaf blades of the amphiploid were significantly lower than those in Chinese Spring (Fig. 2B). In both genotypes, the Na level increased progressively with leaf age; the Na levels were, however, lower in the amphiploid than in Chinese Spring, the percentage differences between the two genotypes being highest in the youngest leaf blade (Fig. 2B).

The levels of K in the roots and "sheaths" of both genotypes were decreased to approximately 50 and 65% of the values in the controls, respectively, by 18 d of exposure to 200 mM NaCl (Fig. 2, C and D). In the leaf blades, however, the K levels in the amphiploid were decreased less, to 64 to 86% of the values in controls, in comparison to K levels in Chinese Spring, which were 44 to 60% of the values in controls (Fig. 2, C and D).

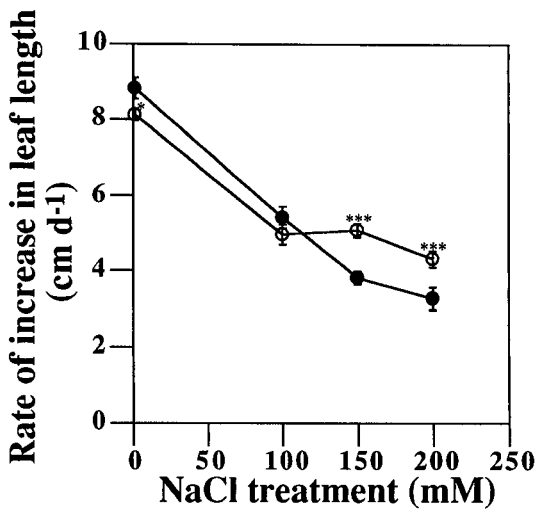

Figure 1. Rate of increase in length of the youngest leaf of Chinese Spring and Chinese Spring × *L. elongatum* amphiploid grown for 15 to 17 d in NaCl treatments. Plants were raised for 13 d (Chinese Spring) or 14 d (amphiploid) in modified one-quarter-concentration Hoagland solution with 4.0 mM Ca^{2+} and then exposed to NaCl treatments in this solution. Youngest leaf lengths at the start of the measurements were between 5 and 10 cm. Values are means ± SE of three replicates, with three plants per replicate. Statistical significance of differences between genotype means at a particular level of external NaCl is indicated by * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.001$). ●, Chinese Spring; ○, Chinese Spring × *L. elongatum* amphiploid.

Table I. *Root and shoot relative growth rates for Chinese Spring and Chinese Spring × L. elongatum amphiploid grown at 1.25 mM Na^+ (control) or 200 mM NaCl*

Plants were raised for 11 d (Chinese Spring) and 14 d (amphiploid) in modified one-quarter-concentration Hoagland solution with 4.0 mM Ca^{2+} and then NaCl was added to the nutrient solution for an additional 18 d. Relative growth rates were calculated from dry weight measurements at the start and end of the 18-d treatments. Values are means of three replicates, and the values followed by a different letter are statistically different at the 1% level.

Genotype	Control	200 mM NaCl	Percent of Control
Root relative growth rate (d^{-1})			
Chinese Spring	0.203[a]	0.110[c]	54.2
Amphiploid	0.176[b]	0.130[d]	73.9
Shoot relative growth rate (d^{-1})			
Chinese Spring	0.220[e]	0.070[g]	31.8
Amphiploid	0.178[f]	0.089[h]	50.0

Distributions of Organic Solutes in the Shoots of Plants Exposed to Low and High NaCl Concentrations

The combined ^1H NMR and GLC analysis of leaf blade extracts showed significant increases in the levels of Gly betaine, Pro, and Asn in the leaf blades of the two genotypes when exposed to 200 mM NaCl (Fig. 3). The high NaCl treatment did not significantly affect the levels, in terms of a potential role in osmotic adjustment, of the other amino acids assayed (data not shown; for a complete list of solutes assayed, see Fan et al., 1993). Organic acid levels were also determined; the levels of ascorbate, citrate, fumarate, malate, lactate, oxalate, pyruvate, and succinate in the leaf blades of both genotypes were not significantly affected by the high NaCl treatment (data not shown).

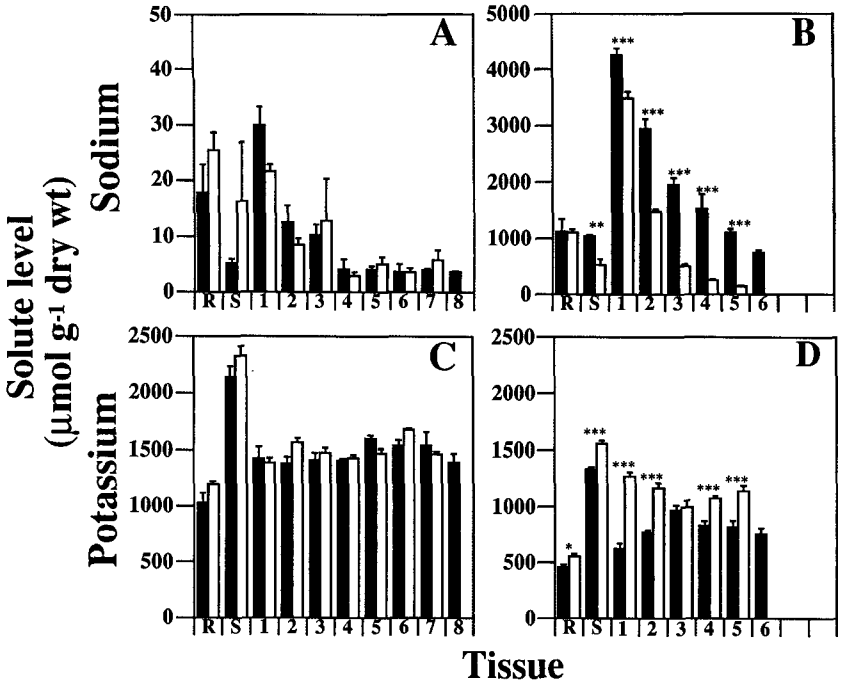

Figure 2. Levels of sodium (A and B) and potassium (C and D) in various tissues from Chinese Spring and Chinese Spring × *L. elongatum* amphiploid grown for 18 d with 1.25 mM Na^+ (control) (A and C) or 200 mM NaCl (B and D). Plants were grown as described in Figure 1. At harvest, plants were separated into main stem leaf blades of various ages (number 1 was the oldest leaf blade), "sheath" (S) (main stem base with sheaths and unemerged leaves), and roots (R). Values are means ± SE of three replicates, with 17 plants per replicate. Statistical significance of differences between genotype means of a given leaf number is indicated by * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.001$). ■, Chinese Spring; □, Chinese Spring × *L. elongatum* amphiploid.

At 1.25 mM Na^+ the levels of Gly betaine, Pro, and Asn were comparatively low (Fig. 3, A, C, and E); only Gly betaine had an appreciable level, viz. 45 μmol g^{-1} dry weight, in the youngest leaf blade of both genotypes (Fig. 3A).

The NaCl-induced increases in Gly betaine, Pro, and Asn levels were highly dependent on leaf blade age or the position of the leaf blades on the main stem. At 200 mM NaCl, the Gly betaine level was highest in the youngest leaf blade of both genotypes, and in the amphiploid it was twice that in Chinese Spring (Fig. 3B). In addition, the high NaCl treatment increased the level of Pro by 2 orders of magnitude in the older leaf blades of both genotypes (Fig. 3, C and D). However, in contrast to the shoot distribution of Gly betaine, Pro levels were highest in the oldest leaf blade and progressively decreased in the younger leaf blades of both genotypes (Fig. 3, B and D). Furthermore, the Pro levels in the leaf blades of the amphiploid were lower than those in Chinese Spring, except for the oldest leaf blade, in which the opposite was the case (Fig. 3D). Asn levels were also generally increased by the high NaCl treatment (Fig. 3, E and F). Asn levels were highest in the youngest leaf blade and lowest in the oldest leaf blade of both genotypes, which was the opposite of the shoot distribution of Pro (Fig. 3, D and F). When the youngest leaf blades of the two genotypes were compared there was no difference in their Asn levels (cf. leaf 5 of the amphiploid with leaf 6 of Chinese Spring, Fig. 3F).

The 200 mM NaCl treatment, however, decreased the Suc level in the youngest leaf blade of both genotypes by 20 to 30%, relative to the values in the controls (data not shown). For the older leaf blades, the Suc levels in the amphiploid were lowered from 190 to 93 μmol g^{-1} dry weight (50% reduction, $P < 0.05$) and in Chinese Spring from 165 to 65 μmol g^{-1} dry weight (60% reduction, $P < 0.05$). The Glc levels in the leaf blades of both genotypes were generally not affected by the 200 mM NaCl treatment (data not shown), with the exception of the youngest leaf blade of Chinese Spring (see below). The Glc levels were between 75 and 180 μmol g^{-1} dry weight in the two youngest leaf blades and 25 and 50 μmol g^{-1} dry weight in all other leaf blades of both genotypes.

NaCl-Dose Response for Na, K, Gly Betaine, Pro, Asn, and Glc Levels in the Youngest Leaf Blades

The Na level in the youngest leaf blade of the amphiploid increased from 110 to 140 μmol g^{-1} dry weight between the 100 and 200 mM NaCl treatments, whereas in Chinese Spring it increased exponentially with the external NaCl dose to the high level of 740 μmol g^{-1} dry weight at 200 mM NaCl (Fig. 4A). The level of K in the youngest leaf blade of both genotypes was decreased in a similar manner by the 100 and 150 mM NaCl treatments; however, at 200 mM NaCl the K level in Chinese Spring continued to decrease to 60% of the control value, whereas in the amphiploid this trend was reversed and the K level at that salinity was 80% of the control value (Fig. 4B).

The Gly betaine level in the youngest leaf blade of the amphiploid increased almost linearly with NaCl dose, but in Chinese Spring it only increased significantly at 200 mM NaCl (Fig. 4C). The level of Pro in the youngest leaf blade of both genotypes increased significantly only when plants were exposed to 200 mM NaCl ($P < 0.01$); in the amphiploid it was one-third of the value in Chinese Spring (Fig. 4D). In contrast to Pro, Asn accumulated in the youngest leaf blade of the two genotypes at all levels of NaCl salinity; however, the levels did not differ statistically between

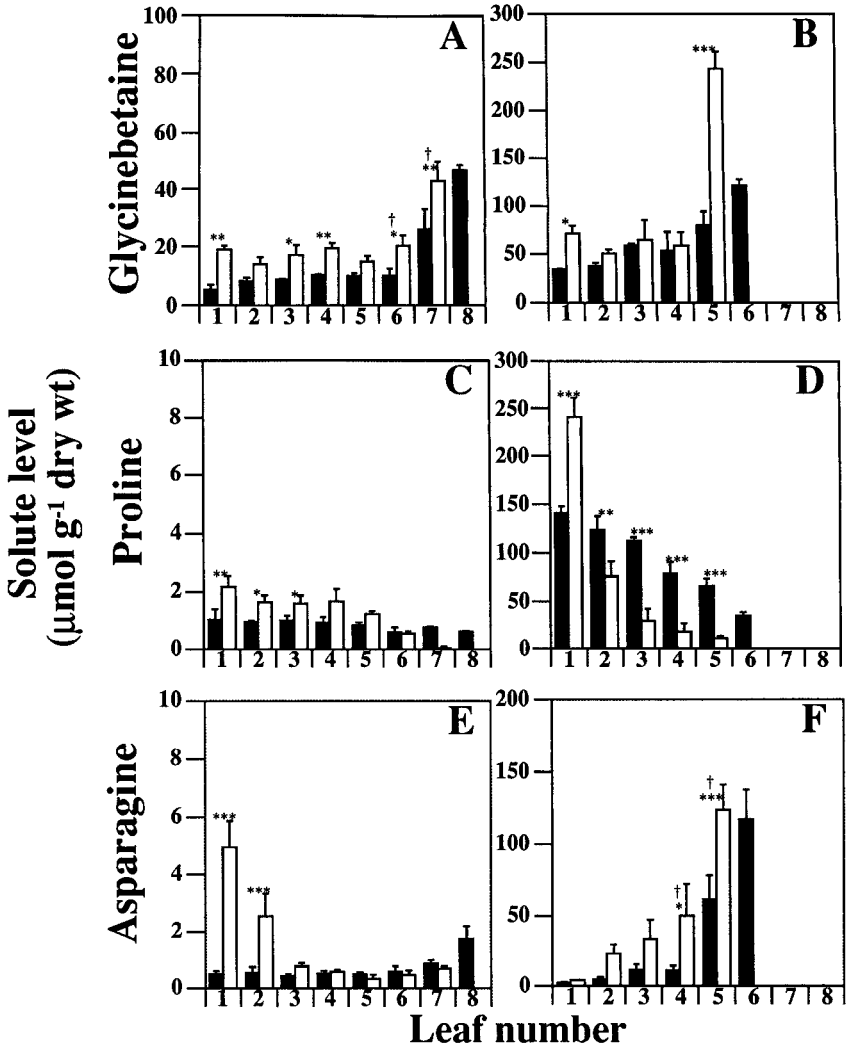

Figure 3. Levels of Gly betaine (A and B), Pro (C and D), and Asn (E and F) in leaf blades of various ages from Chinese Spring and Chinese Spring × *L. elongatum* amphiploid grown for 18 d with 1.25 mM Na^+ (control) (A, C, and E) or 200 mM NaCl (B, D, and F). Plants were grown as described in Figure 1. The oldest leaf blade was assigned number 1. Values are means ± SE of three replicates, with 17 plants per replicate. Statistical significance of differences between genotype means of a given leaf number is indicated by * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.001$). † indicates that there was no significant difference if leaf blades were compared as the youngest, second youngest, and so on. ■, Chinese Spring; □, Chinese Spring × *L. elongatum* amphiploid.

the two genotypes (Fig. 4E). The Glc level in the youngest leaf blade of the amphiploid was virtually constant, whereas it declined significantly with NaCl dose in Chinese Spring (Fig. 4F).

Water Content in Leaf Blades of Plants Exposed to Low and High NaCl Concentrations

At 1.25 mM Na^+, leaf blade water contents were between 5 and 6.3 mL g^{-1} dry weight, except for the oldest leaf blade of the amphiploid, which had a water content of 3.9 mL g^{-1} dry weight. Eighteen days of exposure to 200 mM NaCl resulted in water contents in the three youngest leaf blades of both genotypes that were 50 to 74% of the values in the controls (Table II). In the two oldest leaf blades of Chinese Spring and the oldest one of the amphiploid, the water contents were only 10% of the values in the controls (data not shown). Thus, with the exception of leaf blade 2, the high NaCl treatment had a similar effect on water contents in both genotypes (Table II; data not shown).

π_{sap} of Leaf Blades of Plants Exposed to Low and High NaCl Concentrations

Under control conditions, the π_{sap} of both genotypes was between −0.98 and −1.19 MPa for leaf blades 6 and younger and between −1.13 and −1.54 MPa for leaf blades 5 and older. The 200 mM NaCl treatment resulted in more negative π_{sap} values for all leaf blades, but there were no significant differences between the two genotypes (Table II; data not shown). In both genotypes, the differences in π_{sap} of the youngest leaf blade between the control and NaCl-treated (100, 150, and 200 mM) plants approximately corresponded to the changes in π of the external solution (data not shown).

Estimated Contributions of Various Solutes to the Differences in π_{sap} from Control and NaCl-Treated Leaf Blades

The contributions of various solutes to the difference in π_{sap} between the control and 200 mM NaCl-treated leaf blades were calculated as described in "Materials and

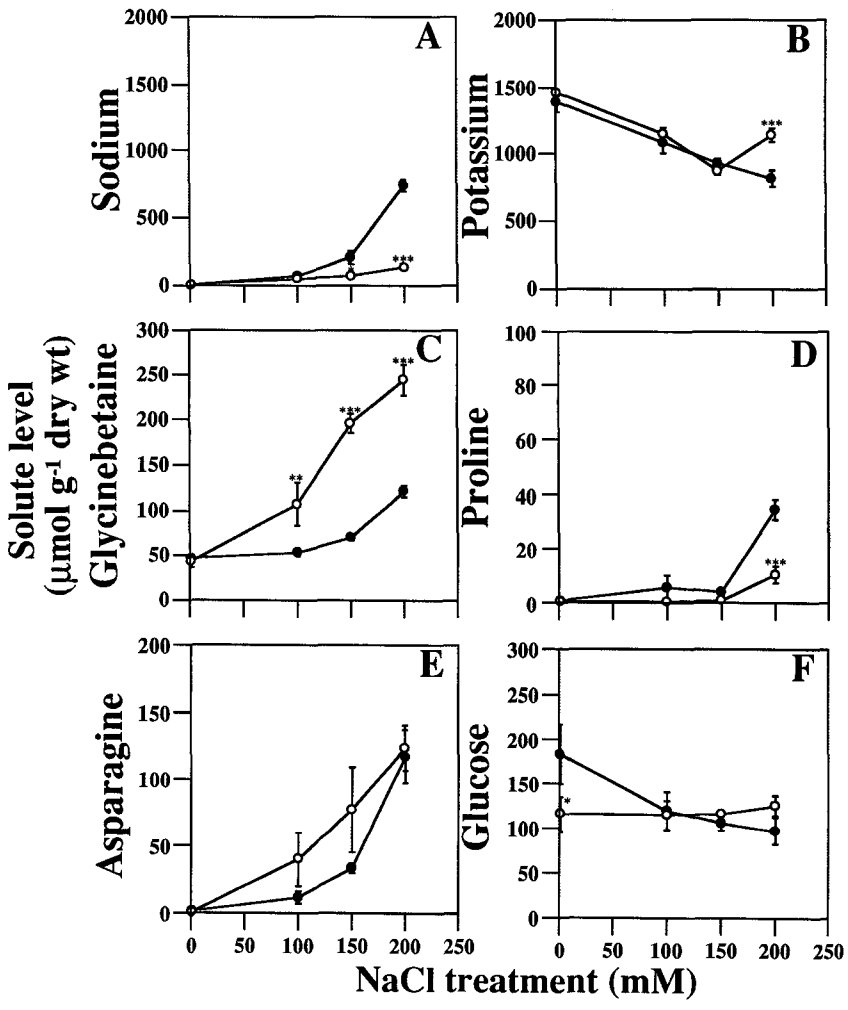

Figure 4. The effect of NaCl dose on Na (A), K (B), Gly betaine (C), Pro (D), Asn (E), and Glc (F) levels in the youngest leaf blade of Chinese Spring and Chinese Spring × *L. elongatum* amphiploid. Plants were grown as described in Figure 1. Values for control and 200 mM NaCl treatments are those that appear in Figures 2 and 3. Values are means ± SE of three replicates, with 17 plants per replicate. Statistical significance of differences between genotype means at a particular level of external NaCl is indicated by * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.001$). ●, Chinese Spring; ○, Chinese Spring × *L. elongatum* amphiploid.

Methods." This approach did not distinguish between the contributions of lower tissue water contents or solute accumulation to the more negative π_{sap}. However, lower water contents may have been associated with 30 to 50% of the NaCl-induced declines in π_{sap} of the three youngest leaf blades of both genotypes (Table II). The remaining declines in π_{sap} were due to increases in solute content per unit of tissue, which has been termed "osmotic adjustment" (Hsiao et al., 1976).

Although both genotypes had similar differences in leaf blade π_{sap} (control minus 200 mM NaCl treatment) for leaf blades at the same position on the main stem, the contributions of several solutes to the leaf blade π_{sap} varied greatly (Table II). The contributions of selected solutes to the NaCl-induced decline in π_{sap} of the youngest leaf blade were: Gly betaine, 19% in the amphiploid and only 6% in Chinese Spring; Asn, about 10% in both genotypes; Pro, less than 3% in both genotypes; and Glc, 4% in the amphiploid and −4% in Chinese Spring. In the older leaf blades the contributions of Gly betaine or Asn to the decline in π_{sap} were less than 3.5% in either genotype.

K and Na made contrasting contributions to the decline in π_{sap} of the leaf blades of the amphiploid and Chinese Spring (Table II). K contributed 11% in the youngest leaf blade of the amphiploid and −16% in that of Chinese Spring. In contrast, Na in the youngest leaf contributed only 12% to the decline in π_{sap} of the amphiploid but 54% in Chinese Spring. Furthermore, Na was the major solute that contributed to the decline in π_{sap} of the older leaf blades of both genotypes. The contribution of Cl to the decline in π_{sap} was not determined because there was not enough tissue sample for analysis. However, if the Cl level was similar to that of Na it would account for the remaining decline in π_{sap} of the youngest leaf blade of Chinese Spring but not in that of the amphiploid (Table II).

Correlations between Leaf Blade Organic and Inorganic Solute Levels

The relationships between the levels of organic and inorganic solutes in the leaf blades of all ages of both genotypes were investigated using regression analysis. There was a positive linear correlation between the Pro and Na levels in the leaf blades of the two genotypes ($r^2 = 0.91$) (Fig. 5A). In contrast, there was a negative exponential relationship between leaf blade Asn and Na levels in both

Table II. *Leaf blade water contents, π_{sap}, and estimated percentage contributions of various solutes to the differences in π_{sap} between control and 200 mM NaCl-treated leaf blades of Chinese Spring (CS) and Chinese Spring × L. elongatum amphiploid (Am.)*

Treatment	Leaf Blade Age and Genotype					
	Youngest[a]		Second youngest		Third youngest	
	CS	Am.	CS	Am.	CS	Am.
Water content (mL g^{-1} dry weight)						
Control	5.88	5.71	6.19	5.77	6.32	5.47
200 mM NaCl	4.35	4.03	4.18	3.36	3.12	2.95
π_{sap} (MPa)						
Control	−0.98	−1.15	−1.07	−1.19	−1.05	−1.31*
200 mM NaCl	−1.78	−1.85	−2.34	−2.38	−2.73	−2.64
Difference in π_{sap}	−0.80	−0.70	−1.27	−1.20	−1.68	−1.33
Estimated contribution of the following solutes to the difference in π_{sap}[b] (%)						
Gly betaine	6.4	19.4***	2.9	2.9	2.4	3.7
Asn	8.9	11.4	2.8	3.2	0.6	2.2
Pro	2.6	1.0	3.1	1.1*	3.9	1.9*
Glc	−4.5	4.3***	−1.3	−1.7	0.2	0.5
Na^+	54.5	12.2**	52.2	16.1***	73.6	30.2***
K^+	−15.8	11.5***	−12.7	4.4**	4.1	11.2
Difference in π_{sap} accounted for (%)	52.1	59.8	47.0	26.0	84.8	49.7

[a] The youngest leaf blades of the controls were the eighth and seventh and of the 200 mM NaCl-treated the sixth and fifth emerged leaves, for CS and Am., respectively. [b] The contributions of various solutes to the difference in π_{sap} between control and 200 mM NaCl-treated leaf blades were calculated as described in "Materials and Methods." Statistical significance of differences between genotype means is indicated by * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.001$).

genotypes (Fig. 5B) and between leaf blade Gly betaine and Na levels for Chinese Spring (Fig. 5C). The relationship between Gly betaine and Na was not clear for the amphiploid (Fig. 5C). There were no significant relationships between the leaf blade levels of Pro, Asn, or Gly betaine and those of K (data not shown).

DISCUSSION

This comprehensive survey of potential osmolytes in salinized wheat (cv Chinese Spring) and a wheat × *L. elongatum* amphiploid showed that solute accumulation in individual leaf blades was highly dependent on their age or position on the main stem. Na and Pro levels were highest in the oldest leaf blade and decreased progressively in younger leaf blades, whereas those of Gly betaine and Asn were highest in the youngest leaf blade. The π_{sap} was similar for leaf blades at the same position on the main stem for both genotypes, but the relative contributions of several solutes to the difference in π_{sap} between leaf blades grown under the control and 200 mM NaCl treatments differed very much. Gly betaine, K, and Glc made a larger and Na a smaller contribution to the decline in π_{sap} of the youngest leaf blade of the amphiploid than in that of Chinese Spring. If the Cl level in the youngest leaf blade of the two genotypes was similar to that of Na, as shown previously by Schachtman et al. (1989) for these two genotypes, then the above-mentioned solutes can account for the NaCl-induced decline in π_{sap} of the youngest leaf blade of Chinese Spring. In the amphiploid, however, these solutes can only account for 70% of the decline in π_{sap}. The possibility thus remains that at least one other solute, as yet unidentified, plays a role in the decline in π_{sap} of the youngest leaf blade of the amphiploid. Nevertheless, the present findings provide strong evidence that the combination of Gly betaine accumulation in the youngest leaf blade, a low rate of Na accumulation, and the maintenance of a high K to Na ratio contribute to the NaCl tolerance of the amphiploid. In contrast, no such role in NaCl tolerance is evident for Pro.

The finding that Gly betaine accumulation contributed 19% of the decline in π_{sap} of the youngest leaf blade of the wheat (cv Chinese Spring) × *L. elongatum* amphiploid, but only 6% in that of Chinese Spring, contrasts with the results of Gorham et al. (1986). They reported that a wheat (cv Chinese Spring) × *T. bessarabicum* amphiploid exposed to 250 mM NaCl had youngest leaf Gly betaine levels about equally as low as those of its wheat parent, and yet the amphiploid was found to be superior in salt tolerance to the wheat parent. Their findings are surprising, since the *T. bessarabicum* parent of the amphiploid, and tall wheatgrasses generally, contains high levels of Gly betaine (Gorham et al., 1986; Weimberg and Shannon, 1988). The findings of Gorham et al. (1986) notwithstanding, the present finding of an association between high Gly betaine accumulation and salt tolerance in the amphiploid is consistent with other research (Rhodes and Hanson, 1993, and refs. therein) implicating Gly betaine as an important osmolyte contributing to the salinity tolerance of numerous organisms.

Pro accumulation did not appear to play a role in the enhanced salt tolerance of the amphiploid relative to that of Chinese Spring. The levels of Pro were higher in leaf blades of the salt-sensitive Chinese Spring than in those of the more salt-tolerant amphiploid (except in the oldest leaf blade). This finding and similar results with drought-

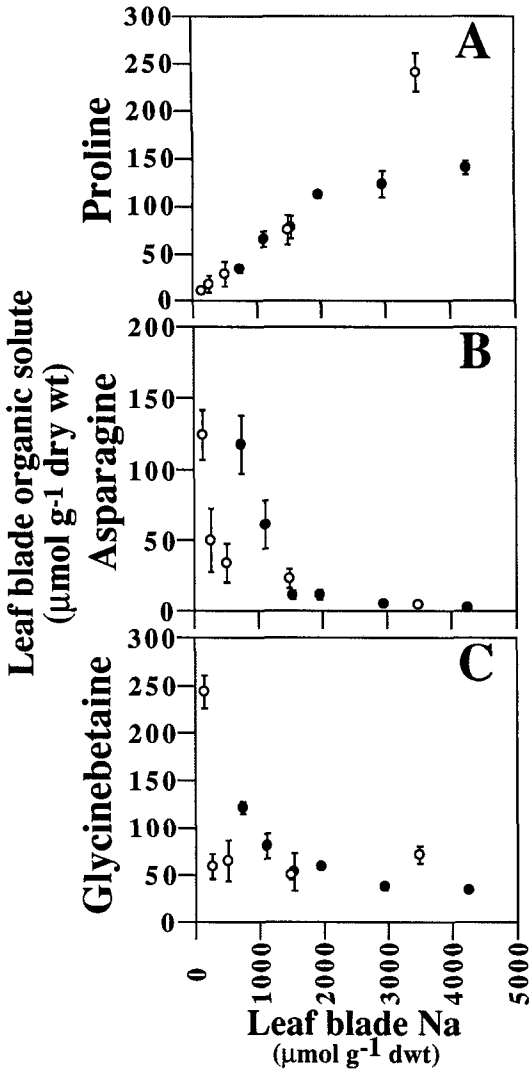

Figure 5. Relationships between Pro (A), Asn (B), and Gly betaine (C) accumulation and the level of Na in leaf blades of various ages from Chinese Spring and Chinese Spring × *L. elongatum* amphiploid grown for 18 d at 200 mM NaCl. Plants were grown as described in Figure 1. The oldest leaf blade was assigned number 1. Values are means ± SE of three replicates, with 17 plants per replicate. Organic solute values were taken from Figure 3, B, D, and F, and the Na values were taken from Figure 2B. ●, Chinese Spring; ○, Chinese Spring × *L. elongatum* amphiploid.

stressed barley cultivars (Hanson et al., 1977, 1979) and salinized tomato lines (Tal et al., 1979) raise doubt regarding the adaptive role of leaf Pro accumulation. Furthermore, the tight correlation between leaf blade Na and Pro levels suggests that Na accumulation promotes Pro synthesis. Thus, Pro accumulation in leaves of salt-stressed nonhalophytes may be a survival response to NaCl-induced cellular water deficits (Wyn Jones and Storey, 1978; Greenway and Munns, 1980), which would be particularly severe if ions build up in the extracellular spaces of leaves, as shown by Flowers et al. (1991).

In contrast to Pro, Asn did make a significant (9–11%) contribution to the decline in π_{sap} of the youngest leaf blade of both genotypes. Asn, like Gly betaine, accumulated mostly in the youngest leaf blade, whereas Pro accumulated mostly in the old leaf blades of both genotypes. However, since Asn may be stored in vacuoles (Miflin and Lea, 1977; Dietz et al., 1990), its role in osmotic adjustment of the cytoplasm remains to be demonstrated. Asn and Pro may be involved in the storage of reduced nitrogen in NaCl-stressed plants (Jefferies, 1980; Stewart and Larher, 1980; Rabe, 1990), in addition to their possible role in osmotic adjustment.

These findings should be helpful in crop improvement programs dealing with plant tolerance of salinity. Enhanced Gly betaine accumulation and maintenance of a high K to Na ratio in young leaves are traits to be targeted. Furthermore, the pronounced differences and gradients in the levels of various solutes in different-age leaf blades show the importance of leaf-by-leaf analysis versus bulk analyses of shoots when comparing responses among genotypes. Finally, those using biotechnological approaches to improve plant tolerance of salinity by increasing the synthesis of organic solutes should developmentally target organic solute accumulation in the youngest leaves, rather than the whole shoots, to gain substantial improvements in whole plant tolerance of salinity with a minimal diversion of carbon and nitrogen resources from growth.

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