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Salt Tolerance of Glycinebetaine-Deficient and -Containing Maize Lines[1]

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Pairs of homozygous near-isogenic glycinebetaine-containing (*Bet1/Bet1*) and -deficient (*bet1/bet1*) F_8 lines of *Zea mays* L. (maize) were tested for differences in salt (150 mM NaCl or 127.25 mM NaCl plus 22.5 mM $CaCl_2$) tolerance. The *Bet1/Bet1* lines exhibited less shoot growth inhibition (as measured by dry matter accumulation, leaf area expansion rate and/or, plant height extension rate) under salinized conditions in comparison to their near-isogenic *bet1/bet1* sister lines. These growth differences were associated with maintenance of a significantly higher leaf relative water content, a higher rate of carbon assimilation, and a greater turgor in *Bet1/Bet1* lines than in *bet1/bet1* lines under salinized conditions. These results strongly suggest that a single gene conferring glycinebetaine accumulation (and/or a tightly linked locus) plays a key role in osmotic adjustment in maize.

Yancey (1994) has recently discussed the roles of betaines and their sulfonio analogs as compatible solutes and in cell volume regulation. These solutes are excluded from the hydration sphere of proteins and tend to stabilize the tertiary structure of proteins (Yancey, 1994). They also prevent or reverse the disruption of the tertiary structure caused by noncompatible (perturbing) solutes such as urea (Bateman et al., 1992). It is probable that these compounds have similar functions in higher plants (Wyn Jones and Storey, 1981; Grumet and Hanson, 1986; Robinson and Jones, 1986; Hanson and Gage, 1991; Hanson et al., 1991; Rhodes and Hanson, 1993), but rigorous genetic experiments with higher plant mutants defective in betaine synthesis are needed to verify this point.

Genetic tests for the role of glycinebetaine in osmotic stress resistance in *Zea mays* L. (maize) are now possible because of the development of a series of near-isogenic F_8 pairs of glycinebetaine-containing and glycinebetaine-deficient lines (Yang et al., 1995). Here we report the growth, water relations, gas-exchange characteristics, and solute compositions of these glycinebetaine-containing and glycinebetaine-deficient near-isogenic lines in salinized and nonsalinized environments, including environments in which salinization was conducted with NaCl:$CaCl_2$ (molar ratio 5.7:1) to eliminate the potentially confounding variable of sodium-induced calcium deficiency (Maas and Grieve, 1987).

MATERIALS AND METHODS

Growth Experiment 1

Plant Material and Cultural Conditions

An initial growth experiment was conducted with three F_8 sister lines that were either homozygous glycinebetaine-containing *Bet1/Bet1* (PUD7), homozygous glycinebetaine-deficient *bet1/bet1* (PUD4), or heterozygous *Bet1/bet1* (PUD2) (i.e. a mixture of *Bet1/Bet1*, *Bet1/bet1*, and *bet1/bet1* plants in an approximately 1:2:1 ratio; Yang et al. [1995]). Growing conditions and soil fertility management procedures used were similar to those described by Premachandra et al. (1993). Seeds were sown on February 17, 1993, into flats containing a 2:2:1 (v/v/v) mixture of perlite, peat moss, and top soil, respectively, amended with 680 g of $Ca(H_2PO_4)_2$, 454 g of KNO_3, 454 g of $MgSO_4$, 3.6 g of ground limestone, and 57 g of Peter Frit Industries (Allentown, PA) trace elements No. 555, all per m^3 of mix. The plants were fertilized at each watering with 200 mg L^{-1} each of N and K supplied from 517 mg L^{-1} KNO_3 and 367 mg L^{-1} NH_4NO_3 with 46 mg L^{-1} P supplied via 75% technical grade phosphoric acid in the irrigation system. Seedlings were grown on benches in the Horticulture greenhouse at Purdue University, under approximately 28°C daytime maximum and 23°C nighttime minimum temperatures. On March 5, 1993, 10 plants of uniform size of each line were selected and transplanted into 21.5-cm (diameter) × 22-cm (height) pots containing the same soil mixture. Plants were grown under well-irrigated, nonsalinized conditions for a further 2 weeks. At this time (March 18, 1993; 28 d after planting) one-half of the plants from

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Abbreviations: **A**, net CO_2 assimilation rate; AWC, apoplastic water content; *bet1*, recessive allele conferring glycinebetaine deficiency; *Bet1*, wild-type allele conferring glycinebetaine accumulation; *E*, transpiration rate; g_s, stomatal conductance to water vapor; ψ_w, leaf water potential; ψ_π, osmotic potential; $\psi_{\pi(100)}$, osmotic potential at full turgor; RWC, relative water content.

each population were maintained under nonsalinized conditions for the subsequent 2 weeks and the other half were salinized beginning 28 d after planting by irrigation daily to soil capacity (500 mL d^{-1}) with nutrient medium containing 50 mM NaCl (5 d), followed by 100 mM NaCl (3 d), and finally 150 mM NaCl (5 d).

Estimation of Leaf Area and Leaf Rolling

Plants were monitored for leaf area and leaf area expansion rate by measuring daily maximum leaf length and width of each leaf, assuming leaf area = maximum length × maximum width × k (Sanderson et al., 1981). (k was empirically determined to equal 0.75–0.76 for these maize lines by measuring leaves of known maximum length and width with a leaf area meter following harvest; see below.) After 4 d at 150 mM NaCl, the uppermost expanded leaf of each plant was scored for leaf rolling at hourly intervals (from 8 AM to 6 PM) by measuring actual (rolled) leaf width and fully unrolled leaf width at half-length, expressing the degree of leaf rolling as (100 − [(rolled leaf width/unrolled leaf width) × 100])%.

Gas-Exchange Measurements

After 5 d at 150 mM NaCl (i.e. 13 d after initiating the salt treatment), **A**, g_s, and E were measured between 10 AM and 12 PM on the first and second uppermost expanded leaves, using a Li-Cor LI-6200 portable photosynthesis system (Li-Cor Instruments, Lincoln, NE) with a 1-L leaf chamber. Average photosynthetic photon flux during the treatment period was approximately 30 mol m^{-2} d^{-1}. Temperature and RH during gas-exchange measurement were 28 ± 2°C and 45 ± 5%, respectively, and ambient CO_2 concentration was 350 to 370 μL L^{-1}. Incident photosynthetic photon flux to the sample leaves was approximately 1400 μmol m^{-2} s^{-1}.

Leaf Water Relations

ψ_w was measured by use of a pressure chamber (PMS Instruments Co., Corvallis, OR) on the same leaves used to measure gas-exchange characteristics. After water potentials were measured, the leaves were frozen in liquid nitrogen in sealed polyethylene bags. Leaf samples were later thawed and centrifuged at 1000g for 20 min at 6 to 8°C to extract cell sap, and the ψ_π of the sap was measured using a Wescor 5100C vapor pressure osmometer (Wescor Inc., Logan, UT). Samples of the same leaf tissue used for water potential and solute analysis were also taken for estimation of leaf RWC as described by Premachandra et al. (1993). Leaf discs were excised, weighed, floated on water for 4 h, reweighed to determine rehydrated weight, and then dried in an oven at 70°C to a constant dry weight. ψ_π was corrected for the dilution of symplastic sap by apoplastic water that occurs when sap is expressed from frozen and thawed tissue (Tyree, 1976); a constant apoplastic fraction of 0.15 was assumed (Wenkert, 1980). Turgor was calculated by subtracting the corrected ψ_π from ψ_w. $\psi_{\pi(100)}$ was calculated as $\psi_{\pi(100)} = (\psi_\pi\ [\text{RWC} - \text{AWC}]/[1.0 - \text{AWC}])$ (Wilson et al., 1979). After sap was extracted and gas exchange, water potential, and RWC were measured, the remaining leaves were harvested for leaf area determination with a CI-2O1 leaf area meter (CID Inc., Moscow, ID). The shoot tissue was bulked and weighed to estimate total shoot fresh weight and then dried in an oven at 80°C for 2 d and 65°C for 4 d and reweighed to determine total shoot dry weight.

Solute Analyses

Aliquots of cell sap were used for total sugar determination using the anthrone method (Yoshida et al., 1976), for nitrate determination by the method of Cataldo et al. (1975), for determination of total amino acid levels by the ninhydrin method of Yemm and Cocking (1955), and for glycinebetaine determination by stable isotope dilution plasma desorption MS as described by Yang et al. (1995). Na^+, K^+, Ca^{2+}, and Mg^{2+} concentrations were determined in extracted cell sap diluted 100-fold with distilled water using a Perkin Elmer Spectra 1100 atomic absorption spectrophotometer. Chloride was determined by the mercury thiocyanate method (Adriano and Doner, 1982).

Growth Experiment 2

A second growth experiment was conducted using essentially the same procedures as described for experiment 1 but with the following specific modifications. Only two genotypes were compared: *Bet1/Bet1* (PUB3) and *bet1/bet1* (PUB1) (Yang et al., 1995). Seeds were planted on April 19, 1993, and transplanted into large pots (26 cm [diameter] × 30 cm [height]) on April 30, 1993, with two plants per pot. Soil fertility management was as described above except that, in addition, Hoagland solution was applied every 5 d (in the case of salt treatments, Hoagland solution was prepared in the appropriate concentration of NaCl plus $CaCl_2$ [see below] before application). Salinization was initiated 22 d after planting (on May 11, 1993). Salt treatment was with 42.5 mM NaCl plus 7.5 mM $CaCl_2$ for 6 d (May 11, 1993–May 16, 1993), 85 mM NaCl plus 15 mM $CaCl_2$ for 15 d (May 17, 1993–May 31, 1993), and 127.5 mM NaCl plus 22.5 mM $CaCl_2$ for 7 d (June 1, 1993–June 6, 1993). Plants were watered daily to soil capacity (1 L) with salt solutions prepared in nutrient medium and supplemented with Hoagland solution every 5th d. One plant from each pot was monitored for changes in ψ_w by excising leaves during the stress treatment, and the second plant from each pot was used for nondestructive growth measurements (leaf area and plant height) during the stress treatment and for determination of ψ_π, ψ_w, RWC, solute composition, and shoot dry weight determination at the termination of the experiment on June 6, 1993. RWC at the time of final harvest was determined by excising and weighing approximately 1 g fresh weight of leaf tissue, immersing leaf tissue in 25 mL of water for 20 h, reweighing to determine rehydrated fresh weight, and drying in an oven at 70°C for 72 h to determine dry weight. (An independent test verified that RWC values obtained with this 20-h rehydration procedure were very similar to those obtained with the leaf disc flotation, 4-h rehydration procedure used in experiment 1. Thus, the r^2

value for RWC measurements determined by the 4- and 20-h rehydration procedures was 0.859 [n = 74; range 0.65–0.98), and the slope of the regression equation of best-fit was not significantly different from 1.0.) Samples were taken from both developed leaves and developing leaves for solute analysis of cell sap and for RWC determination at the time of final harvest. However, because ψ_w was only determined on the developed leaves in this experiment, it was not possible to calculate turgor of the developing leaves. Glycinebetaine levels of cell sap were determined by a colorimetric periodide method (Lerma et al., 1991).

Growth Experiment 3

A third growth experiment was conducted as a replicate of experiment 2, using the same two genotypes, *Bet1/Bet1* (PUB3) and *bet1/bet1* (PUB1), and identical soil fertility management. Seeds were planted on October 1, 1993, and transplanted on October 16, 1993. Salinization was initiated 30 d after planting (on November 1, 1993). Salt treatment was as follows: 42.5 mM NaCl plus 7.5 mM $CaCl_2$ for 7 d (November 1, 1993–November 6, 1993), 85 mM NaCl plus 15 mM $CaCl_2$ for 10 d (November 7, 1993–November 16, 1993), and 127.5 mM NaCl plus 22.5 mM $CaCl_2$ for 5 d (November 17, 1993–November 21, 1993). Plants were harvested for determination of ψ_π, ψ_w, RWC (20-h rehydration procedure), solute composition, and shoot dry weight at the termination of the experiment on November 21, 1993 (21 d after salt treatment was initiated) as described above. ψ_w was determined on both the developed and developing leaves.

RESULTS

Growth Experiment 1

Leaf Water Relations

Table I is a summary of leaf water relations of three contrasting F_8 sister lines grown under nonsalinized (control) and salinized conditions (5 d at 50 mM, 3 d at 100 mM, and 5 d at 150 mM NaCl). This particular stress treatment was moderate and of relatively short duration (i.e. only 5 d at the 150 mM NaCl level) and resulted in a maximum decrease in ψ_w from −0.96 to −1.43 MPa and a maximum decrease in leaf RWC from 0.99 to 0.93. No significant differences between lines within treatments were observed for ψ_w, turgor, $\psi_{\pi(100)}$, or osmolality. However, the *Bet1/Bet1* line (PUD7) exhibited a significantly higher leaf RWC than did the *bet1/bet1* line (PUD4) (Table I).

Leaf Rolling

The three genotypes exhibited a striking phenotypic difference in degree of leaf rolling, as illustrated in Figure 1. The *Bet1/Bet1* line (PUD7) exhibited the least leaf rolling of all three genotypes. The heterozygous *Bet1/bet1* population (PUD2) was intermediate in its leaf rolling characteristics and the *bet1/bet1* (PUD4) line exhibited the most severe leaf rolling (Fig. 1). Although leaves of these *bet1/bet1* plants did not exhibit lower overall turgor than *Bet1/Bet1* plants in this experiment (Table I), the leaf RWC data and leaf rolling scores (Fig. 1) point to significant effects of the *Bet1* allele on leaf water relations and osmotic adjustment, since leaf rolling is known to be determined by the turgor of specific leaf cells (in rice, leaf rolling is associated with loss of turgor of leaf bulliform cells, and leaf osmotic adjustment can markedly influence this overt symptom of stress [Hsiao et al., 1984]).

The results of Figure 1 and Table I represent means of five individual plants of each genotype. In the case of the heterozygous population (PUD2), it should be emphasized that this population in fact comprises approximately 25% *bet/bet1* individuals, approximately 25% *Bet1/Bet1* individuals, and approximately 50% *Bet1/bet1* individuals (Yang et al., 1995). Consistent with this, it was found that one of the five plants in the salinized heterozygous population was glycinebetaine deficient and that this individual plant had the highest midday leaf rolling score in this heterozygous population (30.8 ± 2.5%, averaged over the 11 AM to 1 PM period). In contrast, the mean leaf rolling score for the four

Table I. *ψ_w, RWC, turgor, $\psi_{\pi(100)}$, and osmolality of sap from developed leaves of three F_8 families of maize grown under nonsalinized (control) or salinized conditions (experiment 1)*

Each value is the mean of five plants.

Family[a]	Genotype	ψ_w	RWC	Turgor	$\psi_{\pi(100)}$	Osmolality
		MPa		*MPa*	*MPa*	*mmol L^{-1}*
Control						
PUD4	*bet1/bet1*	−0.99	0.99	0.38	−1.36	467
PUD2	*Bet1/bet1*	−0.96	0.99	0.46	−1.41	487
PUD7	*Bet1/Bet1*	−0.96	0.99	0.45	−1.39	481
LSD (0.05)		0.09	0.01	0.11	0.05	17
Salinized						
PUD4	*bet1/bet1*	−1.43	0.93	0.14	−1.46	541
PUD2	*Bet1/bet1*	−1.43	0.93	0.15	−1.47	540
PUD7	*Bet1/Bet1*	−1.43	0.95[b]	0.13	−1.49	535
LSD (0.05)		0.04	0.02	0.05	0.06	18

[a] See Yang et al. (1995). [b] Mean of *Bet1/Bet1* plants is significantly different at the P = 0.05 level from mean of sister line *bet1/bet1* plants in same treatment.

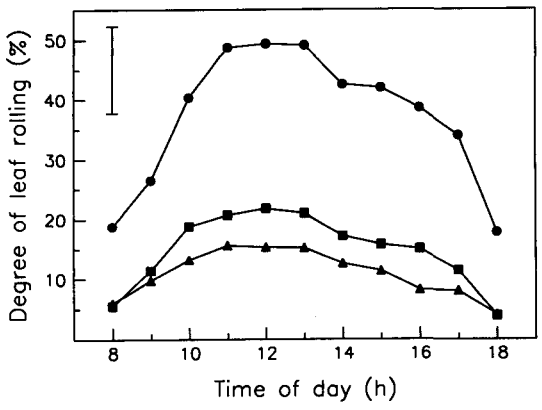

Figure 1. Degree of leaf rolling (100 − [(rolled leaf width/unrolled leaf width) × 100]) percentage between 8 AM and 6 PM for salinized plants of lines PUD4 (*bet1/bet1*) (●), PUD2 (*Bet1/bet1*) (■), and PUD7 (*Bet1/Bet1*) (▲) 1 d before gas-exchange measurements (Table II) and harvest for leaf water relations and dry weight measurements (Table I; experiment 1). Each value is the mean of five plants. The vertical bar represents the LSD (P = 0.05).

glycinebetaine-containing plants in this segregating population for the same period was 18.76 ± 6.4%.

Gas-Exchange and Growth Parameters

The *Bet1/Bet1* line showed a significantly higher mean **A**, g_s, and *E* than did the *bet1/bet1* line (Table II). Again, the heterozygous population was intermediate for all three physiological parameters measured (Table II). Mean plant height at the time of harvest and mean plant height extension rate during the stress period showed significant genotypic differences, with the *Bet1/Bet1* line exhibiting less severe height growth inhibition than the homozygous *bet1/bet1* line. However, no significant differences were observed between genotypes within treatments for total shoot dry weight at harvest or total leaf area at harvest in the stress treatment (Table II). Nevertheless, when plant height, leaf area, and shoot dry weight of the salinized plants are expressed as a percentage of their corresponding nonsalinized controls, a consistent growth advantage of plants containing the *Bet1* allele under salinized conditions becomes apparent (Table II). *Bet1/Bet1* plants exhibited less percentage of inhibition of growth than did *bet1/bet1* plants for all growth parameters measured (Table II).

Solute Composition

Although the three families did not differ significantly for total osmolality of cell sap (Table I), significant differences in solute composition of cell sap were observed between *Bet1/Bet1* and *bet1/bet1* plants. Not surprisingly, these lines differed significantly for glycinebetaine content under both nonsalinized and salinized conditions (Table III) (Yang et al., 1995). Although total amino acid levels were not significantly different, as discussed previously the *bet1/bet1* plants maintained a significantly higher pool of Ser in both salinized and nonsalinized environments (Yang et al., 1995). Moreover, the salinized *bet1/bet1* plants exhibited more extensive depletion of Ala and more extensive accumulation of Pro than did the salinized *Bet1/Bet1* plants (Yang et al., 1995). Under salinized conditions the *Bet1/Bet1* plants maintained significantly higher concentrations of Cl^-, Mg^{2+}, and total sugars than *bet1/bet1* plants (Table III). It is possible that the more severe inhibition of **A** in *bet1/bet1* plants (Table II) contributed to the more extensive depletion of the total sugar pool in these plants under saline conditions. Consistent with this, the single glycinebetaine-deficient plant in the salinized segregating population (PUD2) exhibited the lowest sugar level (93.49 mmol L^{-1}) of all plants in this population (in contrast, the mean ± SD sugar level of the four glycinebetaine-containing plants in this salinized segregating population was 106.6 ± 6.2 mmol L^{-1}).

Growth Experiment 2

A second experiment was conducted to explore further the differential responses of *Bet1/Bet1* and *bet1/bet1* plants to salinity stress with emphasis on clarifying genotypic differences in growth and leaf water relations in response to salinization. Although the above experiment revealed no

Table II. *A (μmol m^{-2} s^{-1}), g_s (mol m^{-2} s^{-1}), and E (mmol m^{-2} s^{-1}) of developed leaves of three F_8 families of maize grown under nonsalinized (control) or salinized conditions (experiment 1)*

Leaf area expansion rates (cm^2 d^{-1}) for the period 7 to 9 d (LER1) and 9 to 12 d (LER2) after salinization was begun, total leaf area at harvest (LA) (cm^2), plant height (PH) (cm), plant height extension rates (PHER) (cm d^{-1}) during the entire stress period, and total shoot dry weight (g) at harvest are also shown. Each value is the mean of five plants. Values in parentheses represent growth as a percentage of control.

Family	Genotype	A	g_s	*E*	LER1	LER2	LA	PH	PHER	Dry Wt
Control										
PUD4	*bet1/bet1*	38.5	0.30	8.05	279	381	3971	33.3	1.50	20.8
PUD2	*Bet1/bet1*	37.3	0.29	7.79	325	393	4219	33.9	1.47	18.4
PUD7	*Bet1/Bet1*	38.0	0.32	8.28	313	392	4038	34.5	1.58	17.8
LSD (0.05)		2.5	0.04	0.60	51	31	364	3.8	0.26	3.1
Salinized										
PUD4	*bet1/bet1*	9.9	0.05	1.26	183 (66)	129 (34)	2657 (67)	24.6 (74)	0.68 (45)	16.1 (77)
PUD2	*Bet1/bet1*	10.8	0.06	1.49	230 (71)	160 (41)	2864 (68)	26.3 (78)	0.82 (56)	17.6 (96)
PUD7	*Bet1/Bet1*	13.0[a]	0.07[a]	1.67[a]	218 (70)	172[a] (44)	2914 (72)	27.3[a] (79)	0.87[a] (58)	16.9 (95)
LSD (0.05)		2.5	0.01	0.30	43	42	357	2.2	0.21	2.2

[a] Mean of *Bet1/Bet1* plants is significantly different at the P = 0.05 level from mean of sister line *bet1/bet1* plants in same treatment.

Table III. *Solute compositions of sap from developed leaves of three F_8 families of maize grown under nonsalinized (control) or salinized conditions (experiment 1)*

Each value is the mean of five plants and is expressed as mmol L^{-1}. Bet, Glycinebetaine; AA, total amino acids.

Family	Genotype	Organic Solute			Inorganic Solute					
		Bet	AA	Sugars	K^+	Na^+	Ca^{2+}	Mg^{2+}	Cl^-	NO_3^-
Control										
PUD4	*bet1/bet1*	0.03	23.3	165.3	101	0.24	10.7	13.5	46.8	31.8
PUD2	*Bet1/bet1*	0.77	23.4	177.6	100	0.17	10.5	13.3	44.6	32.7
PUD7	*Bet1/Bet1*	3.51[a]	22.4	172.8	101	0.21	11.1	13.4	39.8	32.8
Salinized										
PUD4	*bet1/bet1*	0.04	20.5	94.4	108	37.1	10.0	12.6	157.0	28.6
PUD2	*Bet1/bet1*	5.72	22.8	104.0	102	36.5	10.5	12.9	152.6	26.8
PUD7	*Bet1/Bet1*	7.28[a]	20.7	119.0[a]	100	38.4	11.4	15.0[a]	197.9[a]	27.0

[a] Mean of *Bet1/Bet1* plants is significantly different at the P = 0.05 level from mean of sister line *bet1/bet1* plants in the same treatment.

evidence for any marked differences in total Ca^{2+} levels between treatments and between genotypes within treatments, it was considered possible that these responses might have been confounded by salt-induced calcium deficiency (Maas and Grieve, 1987). To avoid this potentially confounding problem, the second experiment was conducted so that a constant Na^+:Ca^{2+} molar ratio (5.7:1) was maintained (Maas and Grieve, 1987). The following additional modifications to the experimental procedures were made: (a) a different pair of homozygous glycinebetaine-containing and -deficient lines were used and the heterozygous population was excluded, (b) the salinity stress treatment was prolonged, (c) plants were grown in larger containers and planted at a density of two plants per pot, and (d) fertilization procedures were amended to supplement the nutrient medium with Hoagland solution every 5 d.

Table IV is a summary of ψ_w, RWC, turgor, $\psi_{\pi(100)}$, and osmolality values observed for developed and developing leaves of the homozygous *bet1/bet1* and *Bet1/Bet1* lines at the end of the second growth experiment. In this experiment, developed leaves of *Bet1/Bet1* plants showed significantly higher RWC, turgor, $\psi_{\pi(100)}$, and osmolality than did *bet1/bet1* plants. Developing leaves also showed significantly higher RWC, $\psi_{\pi(100)}$, and osmolality values (Table IV). At the time of final harvest the *Bet1/Bet1* plants were not significantly different from *bet1/bet1* plants with respect to total leaf area, plant height, and dry weight under nonsalinized conditions (Table V). Under salinized conditions, however, the *Bet1/Bet1* plants exhibited significantly greater leaf area, leaf area expansion rate, plant height, and dry weight in comparison with *bet1/bet1* plants (Table V).

As in the previous experiment, developed leaves of *Bet1/Bet1* plants exhibited significantly greater pools of glycinebetaine, total sugars, Cl^-, and Mg^{2+} than *bet1/bet1* plants under salinity stress (Table VI). In this second experiment, developed leaves also showed significantly greater K^+ and Ca^{2+} levels and significantly lower nitrate pools under salinity stress. It is not clear whether these latter differences in solute levels (not observed in experiment 1) are a function of the different pair of lines used or a function of the different fertilization regimes and growth conditions used in this second experiment (see "Materials and Methods").

Table IV. *ψ_w, RWC, turgor, $\psi_{\pi(100)}$, and osmolality of sap from developed and developing leaves of two F_8 families of maize grown under nonsalinized (control) or salinized conditions (experiment 2)*

Each value is the mean of five plants.

Family[a]	Genotype	ψ_w	RWC	Turgor	$\psi_{\pi(100)}$	Osmolality
		MPa		*MPa*	*MPa*	*mmol L^{-1}*
Control (developed)						
PUB1	*bet1/bet1*	−0.94	0.99	0.20	−1.18	467
PUB3	*Bet1/Bet1*	−0.99	0.99	0.20	−1.21	481
Salinized (developed)						
PUB1	*bet1/bet1*	−1.43	0.88	0.04	−1.35	522
PUB3	*Bet1/Bet1*	−1.43	0.93[b]	0.14[b]	−1.56[b]	575[b]
LSD (0.05)		0.04	0.03	0.05	0.13	19
Control (developing)						
PUB1	*bet1/bet1*	ND[c]	0.98	ND	−1.06	372
PUB3	*Bet1/Bet1*	ND	0.99	ND	−1.09	376
Salinized (developing)						
PUB1	*bet1/bet1*	ND	0.73	ND	−1.14	537
PUB3	*Bet1/Bet1*	ND	0.80[b]	ND	−1.32[b]	577[b]
LSD (0.05)		ND	0.04	ND	0.12	37

[a] See Yang et al. (1995). [b] Mean of *Bet1/Bet1* plants is significantly different at the P = 0.05 level from mean of sister line *bet1/bet1* plants in the same treatment. [c] ND, Not determined.

Table V. *Leaf area expansion rate (LAER) ($cm^2\ d^{-1}$) for the stress period, total leaf area at harvest (LA) (cm^2), plant height (PH) (cm), and total shoot dry weight (g) at harvest (June 6, 1993) for two F_8 families of maize grown under nonsalinized (control) or salinized conditions (experiment 2)*

Each value is the mean of five plants.

Family	Genotype	LAER	LA	PH	DW
Control					
PUB1	*bet1/bet1*	331	8867	102	68.3
PUB3	*Bet1/Bet1*	340	9031	107	67.4
LSD (0.05)		17	452	6	4.1
Salinized					
PUB1	*bet1/bet1*	206	5796	53	34.9
PUB3	*Bet1/Bet1*	228[a]	6364[a]	61[a]	40.7[a]
LSD (0.05)		17	452	6	4.1

[a] Mean of *Bet1/Bet1* plants is significantly different at the P = 0.05 level from mean of sister line *bet1/bet1* plants in the same treatment.

Growth Experiment 3

A third experiment was conducted with the same genotypes as used in experiment 2 primarily to test the reproducibility of the difference in leaf water relations associated with salinization with 150 mM Na^+:Ca^{2+} (5.7:1). As summarized in Table VII the *Bet1/Bet1* line again exhibited significantly greater RWC, turgor, $\psi_{\pi(100)}$, and osmolality than did the *bet1/bet1* line in both developed and developing leaves. As in experiment 2, these water relations differences were associated with a significant difference in leaf area expansion rate, especially during the later part of the stress treatment (Table VIII).

DISCUSSION

The above data with near-isogenic maize lines differing for glycinebetaine level lend support to the idea that glycinebetaine accumulation offers partial protection from the inhibitory effects of salinity stress on shoot growth. This is true whether plants are salinized with NaCl alone or with NaCl:$CaCl_2$ (5.7:1), suggesting that this effect is unrelated to salt-induced calcium deficiency (Maas and Grieve, 1987). The results presented here demonstrate that, as osmotic stress develops, leaf rolling occurs more rapidly in the *bet1/bet1* lines and that **A**, g_s, and *E* all appear to be more markedly inhibited by the stress treatment in the *bet1/bet1* lines. Eventually, as leaf turgor is lost more rapidly in the *bet1/bet1* lines, shoot growth (leaf area expansion, dry matter accumulation, and plant height extension) is more severely impaired in these lines. Together, these results strongly suggest a role for glycinebetaine in maintaining a favorable water status and shoot growth under salinity stress in maize.

Grumet and Hanson (1986) have developed barley isopopulations differing 2-fold for glycinebetaine accumulation. The high-glycinebetaine concentration population maintained a ψ_π of 0.1 MPa lower than the low-glycinebetaine concentration population at all salt levels. Grumet and Hanson (1986) concluded that there is coordinated genetic control of glycinebetaine level and ψ_π, implying that glycinebetaine accumulation is a mandatory component of osmoregulation in barley. Grumet and Hanson (1986) noted that the differences in solute potential between low-glycinebetaine concentration and high-glycinebetaine concentration barley isopopulations are not accounted for by glycinebetaine per se, and they suggested that glycinebetaine levels in barley are controlled by osmoregulatory genes that have pleiotropic effects and whose alleles confer various ψ_π levels by influencing solute levels as a whole. In contrast to barley, significant differences in ψ_π become apparent between low- and high-glycinebetaine concentration maize genotypes only under salinized conditions, implying that the genetic basis for the quantitative variation in glycinebetaine level in maize is fundamentally different from that described by Grumet and Hanson (1986) for barley. In maize, the single gene determining glycinebetaine presence or absence specifically affects glycinebetaine synthesis at the level of choline oxidation (Lerma et al., 1991; Yang et al., 1995). However, as in barley, differences in ψ_π levels between salinized *bet1/bet1* and *Bet1/Bet1* maize plants cannot be attributed to glycinebetaine alone but rather to other solutes (most notably sugars and Cl^-). These results might be explained if it is assumed that glycinebetaine is localized primarily in the chloroplast (Robinson and Jones, 1986), where it stabilizes the photosynthetic apparatus and hence the rate of photosynthesis under saline conditions (Rhodes and Hanson, 1993). This might be expected to affect total carbohydrate status, including total hexose and/or Suc concentration, which might in turn pleiotropically influence the accumulation

Table VI. *Solute compositions of sap from developed leaves of two F_8 families of maize grown under nonsalinized (control) or salinized conditions (experiment 2)*

Each value is the mean of five plants and is expressed as mmol L^{-1}. Bet, Glycinebetaine; AA, total amino acids.

Family	Genotype	Organic Solute			Inorganic Solute					
		Bet	AA	Sugars	K^+	Na^+	Ca^{2+}	Mg^{2+}	Cl^-	NO_3^-
Control										
PUB1	*bet1/bet1*	0.2	17.0	156.9	97	0.21	11.2	13.5	48.3	54.7
PUB3	*Bet1/Bet1*	3.2[a]	21.0[a]	149.5	102	0.17	11.4	14.0[a]	43.9	46.8[a]
Salinized										
PUB1	*bet1/bet1*	0.2	22.4	88.1	112	13.3	10.9	13.0	194	32.7
PUB3	*Bet1/Bet1*	10.9[a]	23.4	110.9[a]	120[a]	13.6	11.1[a]	14.4[a]	231[a]	24.0[a]

[a] Mean of *Bet1/Bet1* plants is significantly different at the P = 0.05 level from mean of sister line *bet1/bet1* plants in the same treatment.

Table VII. *ψ_w, RWC, turgor, $\psi_{\pi(100)}$, and osmolality of sap from developed and developing leaves of two F_8 families of maize grown under nonsalinized (control) or salinized conditions (experiment 3)*

Each value is the mean of five plants.

Family	Genotype	ψ_w	RWC	Turgor	$\psi_{\pi(100)}$	Osmolality
		MPa		*MPa*	*MPa*	*mmol L^{-1}*
Control (developed)						
PUB1	*bet1/bet1*	−0.75	0.98	0.36	−1.09	383
PUB3	*Bet1/Bet1*	−0.72	0.97	0.40	−1.09	384
Salinized (developed)						
PUB1	*bet1/bet1*	−1.37	0.85	0.06	−1.21	490
PUB3	*Bet1/Bet1*	−1.31	0.88[a]	0.20[a]	−1.33[a]	516[a]
LSD (0.05)		0.20	0.02	0.08	0.05	14
Control (developing)						
PUB1	*bet1/bet1*	−0.69	0.98	0.37	−1.03	363
PUB3	*Bet1/Bet1*	−0.68	0.98	0.40	−1.06	370
Salinized (developing)						
PUB1	*bet1/bet1*	−1.43	0.80	0.03	−1.14	488
PUB3	*Bet1/Bet1*	−1.38[a]	0.84[a]	0.12[a]	−1.26[a]	514[a]
LSD (0.05)		0.04	0.02	0.06	0.05	18

[a] Mean of *Bet1/Bet1* plants is significantly different at the P = 0.05 level from mean of sister line *bet1/bet1* plants in the same treatment.

and/or compartmentation of other solutes, including inorganic ions.

Glycinebetaine at relatively low concentrations (10–25 mM) has been shown largely to prevent the inhibition of cell proliferation and cell volume reduction associated with hyperosmotic shock in animal cells (Petronini et al., 1992). The precise mechanism by which glycinebetaine mediates these effects is unknown. In *Escherichia coli* low (1 mM) exogenous concentrations of glycinebetaine greatly stimulate growth in medium of high osmolarity; the accumulation of glycinebetaine is accompanied by its replacement of other cytoplasmic solutes (e.g. trehalose and some K^+ and glutamate) but has little effect on the total amount of cytoplasmic solutes (Cayley et al., 1992). However, the volume of cytoplasmic water per unit of cell dry weight increases greatly (20–50%), suggesting that glycinebetaine accumulation may limit growth rate inhibition by increasing the volume of free (i.e. cytoplasmic) water (Cayley et al., 1992). A similar phenomenon in maize might explain the higher leaf RWC of *Bet1/Bet1* plants than *bet1/bet1* plants in salinized environments.

The present results are somewhat analogous to the results of Tarczynski et al. (1993) with respect to the role of mannitol in stress protection of tobacco. In tobacco a single gene (introduced to tobacco by transformation) conferring the ability to accumulate a compatible osmolyte, mannitol, has been shown to confer increased salt tolerance (Tarczynski et al., 1993). In maize, however, the more conventional breeding strategy used to develop near-isogenic glycinebetaine-containing and -deficient lines (Yang et al., 1995) leaves open the possibility that the difference in salt tolerance associated with alternative alleles of a single gene conferring glycinebetaine presence or absence is due to some other locus tightly linked to *bet1*. For example random amplified polymorphic DNA markers that discriminate between homozygous *Bet1/Bet1* and *bet1/bet1* near-isogenic F_8 lines have been identified (Yang et al., 1995). It remains plausible that the growth and water relations differences between homozygous *Bet1/Bet1* and *bet1/bet1* near-isogenic F_8 lines reported here are due to one or more of these tightly linked loci rather than to *bet1* per se. Resolution of this question awaits isolation of a *Bet1* cDNA clone suitable for transformation experiments with *bet1/bet1* maize lines.

As yet, nothing is known about the effects of *bet1* on drought resistance of maize. Ideally, drought resistance

Table VIII. *Leaf area expansion rates of two F_8 families of maize grown under nonsalinized (control) or salinized conditions (experiment 3)*

Each value is the mean of five plants.

Family	Genotype	Leaf Area Expansion Rate ($cm^2\ d^{-1}$)			
		Nov 5–10	Nov 10–15	Nov 15–19	Nov 19–22
Control					
PUB1	*bet1/bet1*	289.4	267.9	232.9	174.6
PUB3	*Bet1/Bet1*	260.3	260.0	223.7	183.5
Salinized					
PUB1	*bet1/bet1*	206.5	168.2	82.0	83.8
PUB3	*Bet1/Bet1*	217.9	189.5	121.0[a]	121.0[a]
LSD (0.05)		38.8	44.1	28.1	34.5

[a] Mean of *Bet1/Bet1* plants is significantly different at the P = 0.05 level from mean of sister line *bet1/bet1* plants in the same treatment.

studies should be conducted with heterotic hybrids rather than inbreds, so that the contribution of the wild-type *Bet1* allele to agronomically important yield characteristics (yield potential and yield stability across multiple drought-prone locations) of maize can be evaluated (Jensen and Cavalieri, 1983). This will require mating near-isogenic pairs of *Bet1/Bet1* and *bet1/bet1* lines derived from the presently described germplasm (Yang et al., 1995) with pairs of near-isogenic *Bet1/Bet1* and *bet1/bet1* lines derived from a population from a different genetic background (e.g. glycinebetaine-containing and -deficient selections from inbred P77; Lerma et al., 1991). This will generate potentially heterotic F_1 near-isogenic hybrids carrying zero, one, or two doses of the *Bet1* allele suitable for multiple-location yield testing (work in progress). Such yield trials will be essential to identify possible yield penalties associated with the *Bet1* allele (e.g. lower yield potential under optimum conditions [cf. Grumet et al., 1987]) or the possibility of higher susceptibility of *Bet1/Bet1* genotypes to leaf pathogens whose growth is stimulated by glycinebetaine (Strange et al., 1974; Pearce et al., 1976).

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