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Near-Isogenic Lines of Maize Differing for Glycinebetaine[1]

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A series of near-isogenic glycinebetaine-containing and -deficient F_8 pairs of *Zea mays* L. (maize) lines were developed. The pairs of lines differ for alternative alleles of a single locus; the wild-type allele conferring glycinebetaine accumulation is designated *Bet1* and the mutant (recessive) allele is designated *bet1*. The near-isogenic lines were used to investigate whether glycinebetaine deficiency affects the pool size of the glycinebetaine precursor, choline, using a new method for glycinebetaine and choline determination: stable isotope dilution plasma desorption mass spectrometry. Glycinebetaine deficiency in maize was associated with a significant expansion of the free choline pool, but the difference in choline pool size was not equal to the difference in glycinebetaine pool size, suggesting that choline must down-regulate its own synthesis. Consistent with this, glycinebetaine deficiency was also associated with the accumulation of the choline precursor, serine. A randomly amplified polymorphic DNA marker was identified that detects the *bet1* allele. In 62 F_8 families tested the 10-mer primer 5′-GTCCTCGTAG produced a 1.2-kb polymerase chain reaction product only when DNA from *Bet1/bet1* or *bet1/bet1* lines was used as template. All 26 homozygous *Bet1/Bet1* F_8 families tested were null for this marker.

It is now well established that betaines and their sulfonio analogs can play important roles in osmotic adjustment and/or osmoprotection in bacteria (Csonka and Hanson, 1991), cyanobacteria (Borowitzka, 1986), marine algae (Blunden and Gordon, 1986), and mammals (Garcia-Perez and Burg, 1991) (see Yancey [1994] for a recent review of the role of betaines and their sulfonio analogs as compatible solutes). It is probable that these compounds have similar functions in higher plants (Robinson and Jones, 1986; Hanson and Gage, 1991; Hanson et al., 1991; Rhodes and Hanson, 1993). Toward the goal of genetically testing the role of glycinebetaine in osmotic stress resistance in maize (*Zea mays* L.), we have developed a series of near-isogenic F_8 pairs of glycinebetaine-containing and glycinebetaine-deficient lines. Here we report how these near-isogenic lines were derived and describe some of their molecular and biochemical characteristics.

The F_8 lines were derived from a hybrid between glycinebetaine-containing and glycinebetaine-deficient maize inbreds (inbreds 1146 and 1506, respectively) described by Rhodes and Rich (1988). Glycinebetaine deficiency in inbred 1506 is attributable to a recessive allele of a single locus. At least 13 other sources of glycinebetaine-deficient maize lines share a deficiency at the same locus (Lerma et al., 1991). In the homozygous condition the recessive allele confers a lesion (X) in choline oxidation to betaine aldehyde in the glycinebetaine biosynthetic pathway (Lerma et al., 1991): choline — X → betaine aldehyde → glycinebetaine.

We designate the recessive allele of this locus *bet1* and the wild-type allele *Bet1*. The F_8 families were obtained by selecting and selfing heterozygotes (*Bet1/bet1*) from the F_2 generation through to the F_7 generation. At the F_7 generation homozygous *Bet1/Bet1* and *bet1/bet1* individuals were then selected and selfed.

In the process of deriving and testing these lines we developed a new method for routinely quantifying glycinebetaine and its precursor, choline, using stable isotope dilution PD-MS (Harrison and Cotter, 1990). This method differs from the FAB-MS method we have previously used for glycinebetaine determination (Rhodes et al., 1987, 1989) in that it does not require prior derivatization of glycinebetaine. PD-MS proved to be particularly useful for choline determination, showing several advantages over alternative MS methods that have been described, including FAB-MS of *O*-heptafluorobutyryl choline derivatives (Lerma et al., 1988; Rhodes, 1990) and monitoring of thermal degradation products of choline by DCI-MS (Lerma et al., 1991). The PD-MS method was applied to the advanced progeny from this breeding program to test whether lack of glycinebetaine accumulation under salinity stress is associated with an elevated choline pool, as initially observed by Lerma et al. (1991) in a separate maize population.

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Abbreviations: DCI-MS, desorption chemical ionization mass spectrometry; FAB-MS, fast atom bombardment mass spectrometry; m/z, mass to charge ratio; PD-MS, plasma desorption mass spectrometry; RAPD, random amplified polymorphic DNA.

In the process of directly testing the degree of isogenicity of these lines by scoring a pair of homozygous *bet1/bet1* and *Bet1/Bet1* F_7 sister lines for polymorphisms using random DNA primers, we identified a RAPD marker capable of detecting the *bet1* allele in advanced progeny from this cross.

MATERIALS AND METHODS

Plant Material

Families of *Zea mays* L. used in this study were derived from a Northrup King Seed Co. F_1 hybrid (1146 × 1506) initially described by Rhodes and Rich (1988). Inbred 1146 contains glycinebetaine, whereas inbred 1506 is glycinebetaine deficient (Rhodes and Rich, 1988). In Figure 1 is a summary of the pedigrees of all of the families that we have derived from F_1 hybrid 1146 × 1506 and a compilation of data acquired during the last 8 years concerning the frequency of glycinebetaine-deficient individuals observed in each family from analyses of both greenhouse- and field-grown plants. The numbers in parentheses adjacent to each line refer to the number of glycinebetaine-deficient plants observed among the total number of plants of each family analyzed (Fig. 1). Thus, for the F_2 population, 15 of 45 plants tested were glycinebetaine deficient, as determined by stable isotope dilution FAB-MS of field- and greenhouse-grown plants (Rhodes and Rich, 1988). Two heterozygous F_3 families gave rise to all of the F_8 families shown (Fig. 1).

During the breeding program (Fig. 1), emphasis was placed on selecting and advancing heterozygotes (*Bet1/bet1*) to the F_7 generation. This was possible because heterozygotes exhibit approximately one-half of the glycinebetaine level of homozygous *Bet1/Bet1* individuals in the same environment (Rhodes and Rich, 1988; Rhodes et al., 1989). At the F_6 and F_7 generations pairs of homozygous (*Bet1/Bet1* and *bet1/bet1*) lines were derived for test purposes (e.g. homozygous selections A and B of Fig. 1 were used primarily for PD-MS method development; homozygous selections C and D were used primarily for tests of isogenicity and identification of RAPD markers associated with *bet1*; see below). At the F_8 generation emphasis was placed on selection for putative homozygotes from each of the 19 heterozygous F_7 populations advanced. At the F_8 generation, the lines were coded (PUA1 to PUS2) as indicated in Figure 1, sampled, and sib-selfed, except for three deliberately advanced heterozygous lines, PUD1, PUD2, and PUD3, in which 20 individual plants were sampled for glycinebetaine and selfed. Only 3 of the 31 putative homozygous *Bet1/Bet1* selections advanced later proved to be heterozygous (*Bet1/bet1*) as determined by progeny analysis and the detection of glycinebetaine-deficient segregants: PUA4, PUC3, and PUP4 (Fig. 1).

Isolation and Quantification of Glycinebetaine in Field-Grown Populations

Samples of approximately 1 to 1.5 g fresh weight of young expanding leaf tissue were generally taken from 10 to 25 individual plants of each of the families grown under rain-fed conditions in the summers of 1986 (F_1), 1987 (F_2), 1988 (F_3's), 1989 (F_4's), 1990 (F_5's), 1991 (F_6's), 1992 (F_7's), and 1993 (F_8's) at the Purdue University, Horticulture Department O'Neall Farm (Lafayette, IN). Typically seeds were planted in mid- to late May, and samples were taken for glycinebetaine analysis in late July/early August of each year. (Plants were tagged at the time of sampling and then selfed at the time of flowering, except at the F_8 generation when putative homozygous selections were sib-selfed.) Samples of leaf tissue for glycinebetaine determination were immediately placed in preweighed vials containing 10 mL of methanol at the field site. After reweighing to determine fresh weight of tissue sampled, samples were subsequently stored at 4°C in the dark. The methanol extracts were phase separated with chloroform (5 mL) and water (6 mL), and the upper, aqueous phase was dried and redissolved in 2 mL of water. The aqueous extract was passed through a column of Dowex-1-OH^-, and the aqueous eluant from this column was then applied to a column of Dowex-50-H^+ as described previously (Rhodes and Rich, 1988). Glycinebetaine was eluted with 6 mL of 6 M NH_4OH and quantified by a periodide method as described previously (Lerma et al., 1991), except for F_1, F_2, and F_3 families, which were analyzed by stable isotope dilution FAB-MS as described previously (Rhodes and Rich, 1988), and certain F_6 and F_7 families, which were analyzed for glycinebetaine level by stable isotope dilution PD-MS (see below). The field-grown families analyzed by PD-MS included F_6 families A and B and F_7 families C, D, and E (Fig. 1).

Isolation and Quantification of Glycinebetaine and Choline in Greenhouse-Grown, Salinized, and Nonsalinized Populations

Plants of various homozygous (*Bet1/Bet1* and *bet1/bet1*) F_6, F_7, and F_8 families were grown initially under well-irrigated, nonsalinized conditions in the greenhouse for 3 to 4 weeks, using the pot, soil, and culture conditions described by Saneoka et al. (1995; growth experiment 1). Generally after 3 to 4 weeks under nonsalinized conditions one-half of the plants from each population were maintained under nonsalinized conditions for the subsequent 3 to 4 weeks. The other half were salinized by irrigation daily with nutrient medium containing 50 mM NaCl (1 week), followed by 100 mM NaCl (up to 1 week), and finally 150 mM NaCl (for up to 2 weeks). Representative plants of salinized F_6 families were used for mea-

Figure 1. (Figure appears on facing page.) Summary of breeding program used to generate 65 F_8 families of maize differing for glycinebetaine level (see text for details). Values in parentheses refer to the number of glycinebetaine-deficient plants observed of the total number of plants analyzed for each line. The penultimate column refers to the RAPD score obtained using UBC primer 306 (see legend for Fig. 3 and text). ND, Not determined.

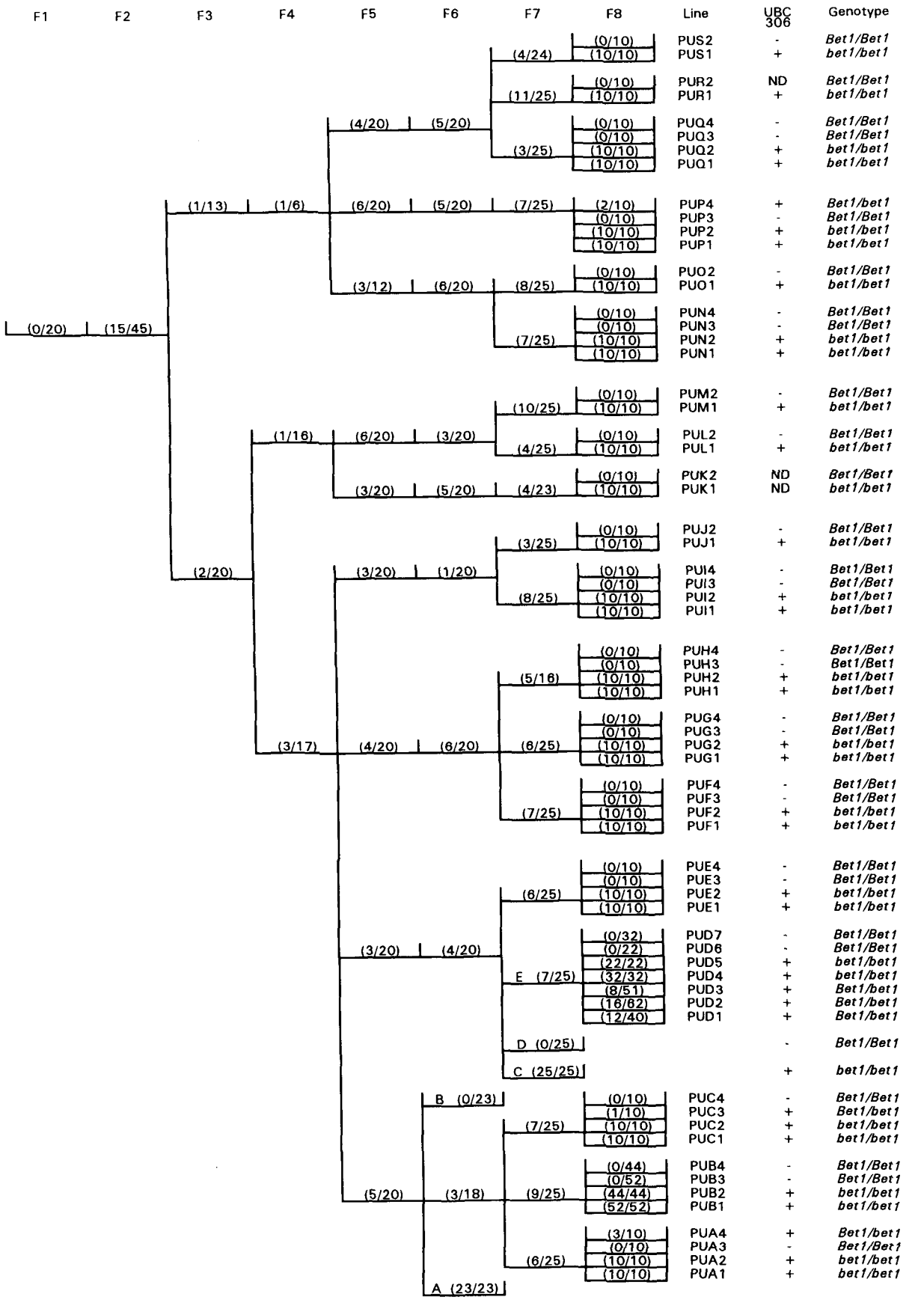

Figure 1. (Legend appears on facing page.)

surements of rates of oxidation of [$^2H_3$]betaine aldehyde to [$^2H_3$]glycinebetaine after 1 week at 150 mM NaCl (see below).

Samples (1–1.5 g fresh weight) of greenhouse-grown populations were taken from young expanding (developing) leaves after 1 or 2 weeks of treatment with 150 mM NaCl by extracting leaf tissue in 10 mL of methanol as described above for field-grown plants. In some experiments with F_8 lines cell sap was derived from frozen and thawed developed leaves as described by Saneoka et al. (1995). Cell sap was treated similarly to the aqueous phase following phase separation of methanol extracts.

Betaines and amino acids were purified by ion-exchange chromatography from cell sap as described for aqueous phase extracts of maize by Rhodes et al. (1989). Glycinebetaine and choline levels of methanol extracts or cell sap were determined by the colorimetric periodide assay (as above for field-grown populations) or by PD-MS as follows. When choline was also to be determined by PD-MS both 1528 nmol of [$^2H_9$]glycinebetaine and 1400 nmol of [$^2H_9$]choline chloride were added as internal standards prior to phase separation of the methanol extracts or by adding these standards directly to cell sap ([$^2H_9$]choline chloride was omitted when glycinebetaine alone was to be determined). After the samples were subjected to Dowex-1-OH^- ion-exchange chromatography (as described above for samples from field-grown plants), half of the aqueous eluant was applied to a column of Dowex-50-H^+ and subsequently eluted with 6 mL of 6 M NH_4OH to specifically recover glycinebetaine free of choline. When choline was not to be determined, the entire aqueous eluant from Dowex-1-OH^- was applied to Dowex-50-H^+. The remainder of the aqueous eluant was applied to a column of Dowex-50-H^+ and subsequently eluted with 6 mL of 2.5 M HCl to recover choline plus glycinebetaine. The Dowex-50-H^+ eluants were dried under a stream of air. In the case of the dried HCl eluants these were further extracted with 1 mL of acetonitrile:methanol (20:1, v/v) (to remove inorganic ions). The acetonitrile:methanol extracts of the dried HCl eluants from Dowex-50-H^+ were brought to dryness under a stream of air prior to PD-MS analysis. Total amino acid levels of cell sap were determined by GC of *N(O,S)*-heptafluorobutyryl isobutyl esters using α-amino-*n*-butyrate as an internal standard (Rhodes et al., 1989).

PD-MS

PD-MS analyses were performed using a Bioion 20 plasma desorption mass spectrometer (Bioion Nordic, Uppsala, Sweden). Samples were typically dissolved in 50 μL (for samples derived from plant tissues) or 200 μL (for authentic standards) of methanol, and 2-μL aliquots were applied to a sample target (a 1-mg/mL solution of nitrocellulose in acetone electrosprayed onto an aluminized-Mylar PD-MS foil). After drying in a stream of nitrogen, samples were inserted into the Bioion 20 sample carousel, and spectra were obtained for 15 min at an acceleration voltage of 17,000 V. Initial method development was with authentic mixtures of unlabeled glycinebetaine, choline chloride (Sigma), and their $^2H_9$ homologs (Rhodes et al. [1987] for methods of preparation of [$^2H_9$]glycinebetaine). [$^2H_9$]Choline chloride was obtained from Cambridge Isotope Laboratories (St. Louis, MO). Glycinebetaine was quantified from the signal intensity at m/z 118 relative to the [$^2H_9$]glycinebetaine internal standard (m/z 127) in the dried 6 M NH_4OH Dowex-50-H^+ eluants. Experiments with authentic standard mixtures of [$^2H_0$]glycinebetaine and [$^2H_9$]glycinebetaine indicated that the m/z 118:127 ion intensity ratio was highly correlated (r^2 = 0.99; n = 16) with the molar ratio of [$^2H_0$]glycinebetaine:[$^2H_9$]-glycinebetaine. Choline was quantified from the signal at m/z 104 relative to the signal at m/z 113 from the [$^2H_9$]choline internal standard in the acetonitrile:methanol extracts of the dried 2.5 M HCl eluants from Dowex-50-H^+. Experiments with authentic standard mixtures of [$^2H_0$]choline and [$^2H_9$]choline indicated that the m/z 104:113 ion intensity ratio was highly correlated (r^2 = 0.98; n = 8) with the molar ratio of [$^2H_0$]choline:[$^2H_9$]choline.

Metabolism of $^2H_3$-Betaine Aldehyde to [$^2H_3$]Glycinebetaine

[$^2H_3$]Betaine aldehyde chloride (1.6 mM) feeding experiments on leaf discs of salinized F_6 plants (150 mM NaCl; 1 week) and procedures used for isolation of betaine pools from these tissues were exactly as described previously (Lerma et al., 1991), except that the betaine fractions derived from Dowex-50-H^+ ion-exchange chromatography after elution with 6 M NH_4OH were not derivatized and were analyzed by PD-MS directly (see above). The endogenous pool of [$^2H_0$]glycinebetaine and the [$^2H_3$]glycinebetaine derived from oxidation of [$^2H_3$]betaine aldehyde in vivo were quantified from the signal intensities at m/z 118 and 121, respectively, relative to the [$^2H_9$]glycinebetaine internal standard (m/z 127; 764 nmol/sample).

Identification of RAPD Markers

DNA was initially isolated from two homozygous F_7 sister lines C and D (Fig. 1) (in which C is of genotype *bet1/bet1* and D is of genotype *Bet1/Bet1*), using the modified cetyltrimethylammonium bromide method (Bernatzky and Tanksley, 1986). Seeds were treated with 70% ethanol for 5 min, followed by 50% bleach for 30 min with gentle shaking. The seeds were then rinsed four times with distilled water and germinated at room temperature for 4 to 5 d in moist paper towels until the coleoptiles were approximately 5 to 8 cm in length. Coleoptiles from 10 to 15 seeds of each line were harvested with a razor blade and combined for DNA extraction (Bernatzky and Tanksley, 1986). DNA was dried under vacuum and dissolved in 400 μL of 10 mM Tris-HCl, 1 mM disodium EDTA (pH 8.0). Working stock solutions of the maize DNA were prepared at a concentration of 10 μg/mL.

RAPD reactions were prepared according to standard protocols (Williams et al., 1992) and amplified using modified thermocycling conditions (Wittwer and Garling, 1991). DNA amplifications were performed in 25-μL vol-

umes containing 50 mM KCl, 10 mM Tris-HCl (pH 8.8 at 25°C), 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.1 mM deoxyribonucleotide triphosphates, approximately 0.2 mM primer (optimized depending on primer source), 1 unit of *Taq* DNA polymerase, and 50 ng of maize template DNA. Primer kits tested against DNA template prepared from lines C and D included 80 primers from Operon (primer kits A, B, C, and E, 20 primers per kit; Operon Technologies Inc., Alameda, CA) and 400 primers from UBC RAPD primer sets 2, 3, 4, and 5 (100 primers per set; UBC RAPD Primer Synthesis Project, University of British Columbia, Vancouver). Reaction mixtures were overlayed with one drop of mineral oil. Amplifications were performed in a Perkin Elmer-Cetus DNA Thermal Cycler (Fairfield, OH) or in an Ericomp Twin Block System Easy Cycler (San Diego, CA) programmed for 45 cycles of 20 s at 94°C, 40 s at 37°C, and 1 min at 72°C. Temperatures were cycled at the fastest possible ramp times between each temperature. Reaction products were analyzed by electrophoresis in 1.4% agarose gels run at 100 V in 0.04 M Tris, 0.005 M sodium acetate, 0.001 M disodium EDTA, pH 7.9, for approximately 2.5 h. Gels were stained in 1 μg/mL ethidium bromide solutions for 2 to 4 h and photographed under UV light. Individual reactions were prepared according to a standard protocol. Variation in reaction conditions between samples was minimized by preparing large-scale reaction mixtures containing the reaction components that did not vary. This reaction mixture was pipetted into a number of tubes, and the template DNA and primer were then added separately into each tube.

Five of the 480 primers tested were found to consistently detect polymorphisms between template DNA from lines C and D: Operon primer B1 (5'-GTTTCGCTCC), UBC primer 113 (5'-ATCCCAAGAG), UBC primer 136 (5'-TACGTCTTGC), UBC primer 163 (5'-CCCCCCAGAT), and UBC primer 306 (5'-GTCCTCGTAG). The primer UBC 306 was used to test DNA extracted from 62 F_8 lines shown in Figure 1. DNA extraction procedures and RAPD reactions for the F_8 lines were as described above for lines C and D.

RESULTS

Determination of Glycinebetaine and Choline Pools in Maize Leaf Tissue by PD-MS

Figure 2, A and B, shows typical PD-MS spectra of the betaine fractions (NH_4OH eluants from Dowex-50-H^+) of young expanding leaves from individual plants of a homozygous glycinebetaine-deficient F_6 family (Fig. 1A) (Fig. 2A) and a homozygous glycinebetaine-containing F_6 family (Fig. 1B) (Fig. 2B) grown under nonsalinized conditions in the greenhouse. (Note that choline is not eluted from Dowex-50-H^+ with NH_4OH, and therefore, these betaine fractions are essentially choline free.) A strong signal at m/z 118 corresponding to the protonated molecular ion of glycinebetaine (M plus H^+) [where M = $(CH_3)_3$-N^+-CH_2-COO^-] is observed only in B (Fig. 2B); the glycinebetaine-deficient plant extract A exhibits an extremely low signal at m/z 118 relative to the internal standard, [$^2H_9$]glycinebetaine (m/z 127) (Fig. 2A). The

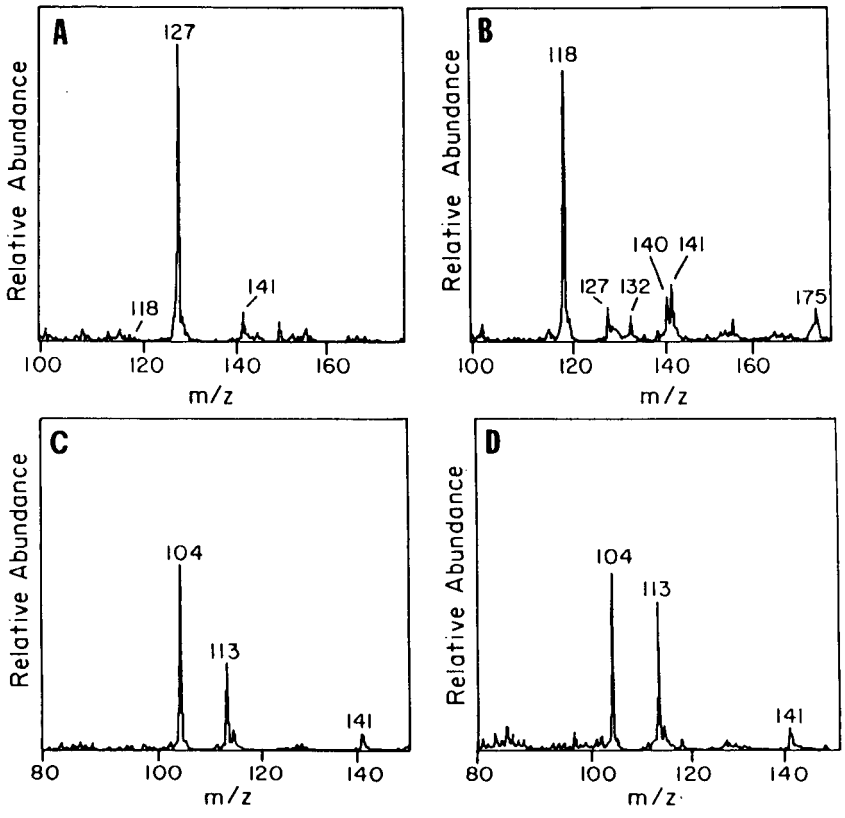

Figure 2. A and B, PD-MS spectra of betaine fractions of young expanding leaf tissue from individual plants of a glycinebetaine-deficient F_6 family (A) and a glycinebetaine-containing F_6 family (B), displayed over the mass range 100 to 180 atomic mass units. C and D, PD-MS spectra of the corresponding choline fractions of young expanding leaf tissue of the same individual plants of the glycinebetaine-deficient F_6 family (C) and the glycinebetaine-containing F_6 family (D), whose betaine spectra are shown in A and B, respectively, displayed over the mass range 80 to 150 atomic mass units. See text for interpretation of spectra.

ions at m/z 132, 140, and 175 in the glycinebetaine-containing sample (Fig. 2B) represent adduct ions of [$^2H_0$]glycinebetaine that are also observed with authentic standards of [$^2H_0$]glycinebetaine (not shown). The ion at m/z 132 represents a *trans*-methylation product of [$^2H_0$]glycinebetaine (M plus CH_3^+) and the ion at m/z 140 represents a sodium adduct of [$^2H_0$]glycinebetaine (M plus Na^+). The (M plus CH_3^+) and (M plus Na^+) ions are also observed in FAB-MS analyses of underivatized [$^2H_0$]glycinebetaine (Hougaard et al., 1991). The ion at m/z 175 represents a glycinebetaine adduct with a [$^2H_0$]glycinebetaine fragment ion; thus, (M + [$(CH_3)_2$-N^+ =CH_2]). The glycinebetaine-deficient sample lacks the glycinebetaine adduct ions at m/z 132, 140, and 175 (Fig. 2A). The ion at m/z 141 in both spectra represents an ion of unknown structure originating from the nitrocellulose matrix and/or solvent, i.e. this ion is the main background ion observed when the solvent (methanol) alone is applied to the matrix (data not shown).

Figure 2, C and D, shows corresponding PD-MS spectra of the choline fractions from the same plants of each family. The molecular cation of choline gives a signal at m/z 104; the [$^2H_9$]choline internal standard gives a signal at m/z 113. Note that the choline fractions were derived by HCl elution of the Dowex-50-H^+ ion-exchange resin ("Materials and Methods") and would be expected to contain both glycinebetaine and choline. A useful feature of the PD-MS method is that the signal from choline is far more intense than the signal from glycinebetaine, so that [$^2H_0$]glycinebetaine (m/z 118) and [$^2H_9$]glycinebetaine (m/z 127) remain virtually undetectable in the choline plus glycinebetaine ion-exchange fraction even in plant extracts from the glycinebetaine-containing family (Fig. 2D). (Similar results have been obtained with authentic standard mixtures of choline and glycinebetaine; the signal intensity from the molecular cation of choline is in great excess of that of the protonated molecular cation of glycinebetaine when present in equimolar mixtures [data not shown].) It is notable that the individual from the glycinebetaine-deficient F_6 family A (Fig. 2C) exhibits a slightly higher choline pool as judged from the ratio of ion intensities at m/z 104:113 relative to the individual from the glycinebetaine-containing sister line B (Fig. 2D; equivalent fresh weights of leaf tissue were used for the two samples illustrated).

The mean choline and glycinebetaine pool sizes of the two F_6 families and their glycinebetaine:choline ratios when grown under nonsalinized or salinized (150 mM NaCl, 2 weeks) conditions are summarized in Table I. These results are derived from analyses of five plants of each genotype for each metabolite in each treatment by the PD-MS method. (Glycinebetaine levels determined by PD-MS in the populations illustrated in Table I were compared with values determined in the same plants by the colorimetric, periodide assay described by Lerma et al. [1991]; excellent agreement between the two methods was obtained [$r^2 = 0.982$; $n = 20$].) Analysis of variance of the data summarized in Table I indicated that the two families differ significantly not only for glycinebetaine pool size but also choline pool size in both environments. The glycinebetaine-deficient family A exhibits a significantly greater choline pool size relative to the glycinebetaine-containing family B in both treatments. This would not be unexpected if glycinebetaine deficiency were due to a metabolic block that impairs choline utilization in betaine aldehyde synthesis (Lerma et al., 1991) and where the accumulated choline may then down-regulate its own synthesis (Mudd and Datko, 1989a, 1989b). Whereas the glycinebetaine pool of the glycinebetaine-containing F_6 family increased from 10.9 to 17.1 μmol/g fresh weight in response to salinity stress, no equivalent expansion of the choline pool was observed in the glycinebetaine-deficient sister line (Table I).

PD-MS evidence that these two F_6 families do not differ for the capacity to metabolize betaine aldehyde to glycinebetaine is summarized in Table II. When leaf discs of salinized plants (150 mM NaCl, 1 week) were supplied with [$^2H_3$]betaine aldehyde, both genotypes accumulated [$^2H_3$]glycinebetaine at similar rates. [$^2H_3$]Glycinebetaine was quantified by PD-MS from the signal at m/z 121 relative to the internal standard at m/z 127. The results of Table II are highly comparable to those obtained with a pair of near-isogenic lines of maize derived from an inbred population (P77) found to be segregating for the glycinebetaine trait, using FAB-MS of betaine esters to quantify [$^2H_3$]glycinebetaine and [$^2H_0$]glycinebetaine pools (Lerma et al., 1991). The results of Table II are consistent with a metabolic block at a step proximal to betaine aldehyde dehydrogenase in the glycinebetaine biosynthesis pathway

Table I. *Choline and glycinebetaine pool sizes (as determined by PD-MS) of young expanding leaf tissues of two F_6 families of maize grown under nonsalinized or salinized (150 mM NaCl, 2 weeks) greenhouse conditions*

F_6 Family[a]	[NaCl]	Choline[bc]	Glycinebetaine[bc]	Glycinebetaine:Choline Ratio[b]
	mM	*μmol/g*	*μmol/g*	
A	0	2.466 ± 0.142	0.016 ± 0.002	0.0066 ± 0.0008
B	0	1.322 ± 0.111[d]	10.908 ± 1.340[d]	8.306 ± 1.148[d]
A	150	2.493 ± 0.231	0.022 ± 0.001	0.0087 ± 0.0009
B	150	1.573 ± 0.269[d]	17.102 ± 1.546[d]	10.982 ± 0.857[d]

[a] A, *bet1/bet1*; B, *Bet1/Bet1* (see Fig. 1). [b] Means ± SD of five plants of each family in each treatment. [c] Pool sizes are expressed on a per g fresh weight basis. [d] Mean of *Bet1/Bet1* plants (B) is significantly different at the P = 0.05 level from mean of sister line *bet1/bet1* plants (A) in the same treatment.

Table II. *Time course of incorporation of [$^2H_3$]betaine aldehyde into [$^2H_3$]glycinebetaine (as determined by PD-MS) in two F_6 families of maize grown under salinized (150 mM NaCl, 1 week) greenhouse conditions*

F_6 Family[a]	[$^2H_0$]Glycinebetaine[bc]	[$^2H_3$]Glycinebetaine after Time (h) of Incubation in 1.6 mM [$^2H_3$]Betaine Aldehyde[c]			
		1	2	3	4
	nmol/g	*nmol/g*			
A	42 ± 11	171	314	597	910
B	15,127 ± 1147	199	367	512	716

[a] A, *bet1/bet1*; B, *Bet1/Bet1* (see Fig. 1). [b] Means ± SD of five determinations of each family. [c] Pool sizes are expressed on a per g fresh weight basis.

in the *bet1/bet1* plants. Glycinebetaine-deficient plants probably have a metabolic lesion at the step catalyzed by choline monooxygenase (Lerma et al., 1991). As in the F_6 families shown in Table I, glycinebetaine-deficient selections from P77 also showed an elevated choline pool relative to glycinebetaine-containing selections (Lerma et al., 1991). However, in the latter case only single determinations of choline pool size by DCI-MS were performed on the two genotypes, and statistical significance of the observed difference in choline titer was not established (Lerma et al., 1991).

To test whether an elevated choline pool co-segregates with *bet1*, three contrasting F_7 families (C, D, and E of Fig. 1) were sampled for both glycinebetaine and choline determination by PD-MS from field-grown plants in the summer of 1992 (Table III). Glycinebetaine levels observed in these field-grown plants were low in comparison to nonsalinized greenhouse grown plants (cf. Table I). However, it should be noted that the summer of 1992 was unusually wet; more than 12 inches of rain were received in the 4 weeks preceding sampling of the plants. In grasses, glycinebetaine level can reflect the water-stress history of the plants sampled (Ladyman et al., 1980; Hitz et al., 1982). We presume that glycinebetaine levels in the homozygous *Bet1/Bet1* family (Table III) were low because of lack of water stress preceding sampling. Nevertheless, despite a 3-fold smaller glycinebetaine pool size than in nonsalinized greenhouse-grown plants (cf. Table I), the homozygous glycinebetaine-accumulating F_7 family D exhibited a statistically significantly smaller choline pool than the homozygous glycinebetaine-deficient F_7 family C (Table III). Furthermore, individuals in the segregating F_7 family E could be partitioned into three discrete groups [E(i), E(ii), and E(iii) in the ratio 7:11:7, respectively] exhibiting significantly different glycinebetaine:choline ratios (Table III). The glycinebetaine-deficient group E(i) showed a significantly greater choline pool than the putative homozygous *Bet1/Bet1* group E(iii) of the same segregating population in the same environment (Table III). Selections from group E(i), exhibiting glycinebetaine:choline ratios of 0.008 ± 0.001, gave rise to F_8 lines PUD4 and PUD5, which were both *bet1/bet1* (Fig. 1). Selections from group E(iii), exhibiting glycinebetaine:choline ratios of 2.005 ± 0.283, gave rise to F_8 lines PUD6 and PUD7, which were both *Bet1/Bet1* (Fig. 1). Selections from group E(ii), exhibiting intermediate glycinebetaine:choline ratios of 1.167 ± 0.142, gave rise to F_8 lines PUD1, PUD2, and PUD3, which were all *Bet1/bet1* (Fig. 1), as expected if heterozygotes carrying one dose of the *Bet1* allele exhibit approximately one-half the glycinebetaine:choline ratio of homozygotes carrying two doses of the *Bet1* allele.

Developed leaves of homozygous *bet1/bet1* (PUD4) and *Bet/Bet1* (PUD7) F_8 families derived from the segregating F_7 family E continued to show significant differences in their free choline pools under nonsalinized and salinized conditions in the greenhouse (Table IV), much as the developing leaves of homozygous F_6 families shown in Table I. The results of Table IV were derived using a different method of extraction of choline and glycinebetaine, i.e. isolation from cell sap rather than by methanol extraction, indicating that the difference in choline titer between *bet1/bet1* and *Bet1/Bet1* plants is not an artifact of the methanol extraction procedure.

The results of Tables I, III, and IV strongly suggest that *bet1* is a modifier of free choline level in maize. To our

Table III. *Choline and glycinebetaine pool sizes (as determined by PD-MS) of young expanding leaf tissues of three F_7 families of maize grown under field conditions (summer 1992)*

F_7 Family[a]	No. of Plants	Choline[b]	Glycinebetaine[b]	Glycinebetaine:Choline Ratio
		μmol/g	*μmol/g*	
C	10	1.645 ± 0.080	0.018 ± 0.006	0.011 ± 0.004
D	10	1.412 ± 0.138[c]	3.416 ± 1.092[c]	2.459 ± 0.943[c]
E	25	1.861 ± 0.204	1.929 ± 1.421	1.077 ± 0.786
E(i)	7	2.050 ± 0.248	0.016 ± 0.001	0.008 ± 0.001
E(ii)	11	1.767 ± 0.138	2.057 ± 0.258	1.167 ± 0.142
E(iii)	7	1.822 ± 0.119[d]	3.642 ± 0.495[d]	2.005 ± 0.283[d]

[a] C, *bet1/bet1*; D, *Bet1/Bet1*; E, *Bet1/bet1* (see Fig. 1); E(i), homozygous *bet1/bet1* glycinebetaine-deficient plants of family E; E(ii), putative heterozygous *Bet1/bet1* plants of family E; E(iii), putative homozygous *Bet1/Bet1* plants of family E. [b] Pool sizes are expressed on a per-g fresh weight basis. [c] Mean of *Bet1/Bet1* plants (D) is significantly different at the P = 0.05 level from mean of sister line *bet1/bet1* plants (C). [d] Mean of putative *Bet1/Bet1* plants (E(iii)) is significantly different at the P = 0.05 level from mean of *bet1/bet1* plants (E(i)).

Table IV. *Choline and glycinebetaine pool sizes (as determined by PD-MS) of cell sap derived from developed leaf tissues of two F_8 families of maize grown under nonsalinized or salinized (50 mM NaCl, 5 d; 100 mM NaCl, 3 d; 150 mM NaCl, 5 d) greenhouse conditions*

F_8 Family[a]	[NaCl]	Choline[b]	Glycinebetaine[b]	Glycinebetaine:Choline Ratio[b]
	mM	*μmol/mL*	*μmol/mL*	
PUD4	0	1.529 ± 0.102	0.033 ± 0.008	0.022 ± 0.005
PUD7	0	1.179 ± 0.172[c]	3.513 ± 0.618[c]	3.038 ± 0.763[c]
PUD4	150	2.266 ± 0.413	0.043 ± 0.015	0.019 ± 0.004
PUD7	150	1.666 ± 0.192[c]	7.277 ± 1.328[c]	4.444 ± 1.116[c]

[a] PUD4, *bet1/bet1*; PUD7, *Bet1/Bet1* (see Fig. 1). [b] Means ± SD of five plants of each family in each treatment. [c] 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.

knowledge, this is the first gene known to influence free choline level in maize. However, because the choline pool does not expand to the same extent that the glycinebetaine pool is diminished in the *bet1/bet1* lines, this implies that choline may down-regulate its own synthesis. Choline is derived from ethanolamine, which is in turn derived from Ser (Rhodes and Hanson, 1993). If choline down-regulates its own synthesis, it would be expected to cause accumulation of its precursors. Preliminary evidence to indicate that glycinebetaine deficiency results in expansion of the free Ser pool is shown in Table V. The *bet1/bet1* line (PUD4) showed a significantly greater Ser pool (expressed as a percentage of the total free amino acid pool) than the *Bet1/Bet1* sister line (PUD7) under both nonsalinized and salinized conditions (Table V). Under salinity stress, as leaf relative water contents decline, Ser and Pro levels tend to increase at the expense of Ala (Rhodes et al., 1989). The *bet1/bet1* line showed more extensive accumulation of Ser and Pro and more extensive depletion of Ala under salinity stress than the *Bet1/Bet1* line (Table V). In part this may be a function of the more rapid decline in leaf relative water content in *bet1/bet1* lines (Saneoka et al., 1995).

Table V. *Amino acid composition of sap from developed leaves of of two F_8 families of maize grown under nonsalinized (Cont) or salinized (Salt) conditions as described in Table IV*

Each value is the mean of five plants, and is expressed as percentage of total amino acid pool (total amino acid levels [μmol/mL of sap] are also shown). Amino acids are listed in order of their GC retention time (Rhodes et al., 1989). GABA, γ-aminobutyrate; Asx, Asn + Asp; Glx, Glu + Gln.

Amino acid	PUD4 (*bet1/bet1*)		PUD7 (*Bet1/Bet1*)		LSD(0.05)
	Cont	Salt	Cont	Salt	
	% Total		*% Total*		
Ala	66.4	32.3	69.1	46.3[a]	3.0
Gly	4.8	4.5	4.4	5.2	1.2
Val	1.5	1.6	1.4	1.4	0.2
Thr	3.0	3.3	3.1	3.1	0.3
Ser	12.8	40.0	8.3[a]	29.7[a]	2.3
Leu	1.0	1.2	0.9	0.9	0.2
Ile	0.4	0.6	0.4	0.4	0.1
GABA	1.5	1.5	1.7	1.2	0.4
Pro	0.8	5.7	0.8	3.2[a]	0.9
Asx	2.2	3.4	2.7	3.3	0.8
Phe	0.2	0.2	0.2	0.2	0.1
Glx	4.5	4.8	6.4[a]	4.3	1.3
Tyr	0.1	0.2	0.1	0.2	0.1
Total (μmol/mL)	22.7	21.1	21.7	17.4	4.8

[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.

Identification of a RAPD Marker Associated with the *bet1* Allele

Homozygous F_7 families C and D were used to test for isogenicity between representative sister lines and to simultaneously screen for 10-mer oligonucleotide primers that gave reproducible RAPD polymorphisms between DNA from *bet1/bet1* and *Bet1/Bet1* plants. Of 480 primers tested, only 5 have been found to give consistent polymorphisms between F_7 families C and D (see "Materials and Methods"). Each primer tested gave at least two (often three or four) distinct, scoreable PCR products. Assuming, conservatively, that each of the 480 different primers tested yielded on average two PCR products from distinct loci, then among the resulting 960 loci tested only five polymorphic loci were observed. Thus, less than 0.53% of the total loci can be assumed to be different between the F_7 *bet1/bet1* and *Bet1/Bet1* pair of lines (C and D) tested. F_8 pairs of lines can, therefore, be estimated to be more than 99.5% identical (cf. theoretically they should be 99.2% identical if the F_1 hybrid was assumed to be heterozygous at all loci).

One of the five 10-mer primers (UBC primer 306 [5'-GTCCTCGTAG]) that consistently distinguished between lines C and D was selected for further study. As illustrated by the arrow in Figure 3 this primer yields a 1.2-kb PCR product only when the *bet1* allele is present in the DNA used as template. The 1.2-kb product is detected only in homozygous *bet1/bet1* (C, PUD4, PUD5) and heterozygous *Bet1/bet1* (PUD1, PUD2, PUD3) F_8 sister lines derived from the same F_7 segregating population (Fig. 3). Sixty-two F_8 lines were tested with UBC primer 306. The results obtained are summarized in the penultimate column of Figure 1, where a score of "−" indicates lack of the 1.2-kb PCR product (cf. lane D of Fig. 3), and "+" indicates the occurrence of the 1.2-kb PCR product (cf. lane C of Fig. 3). An exact match between RAPD polymorphism and

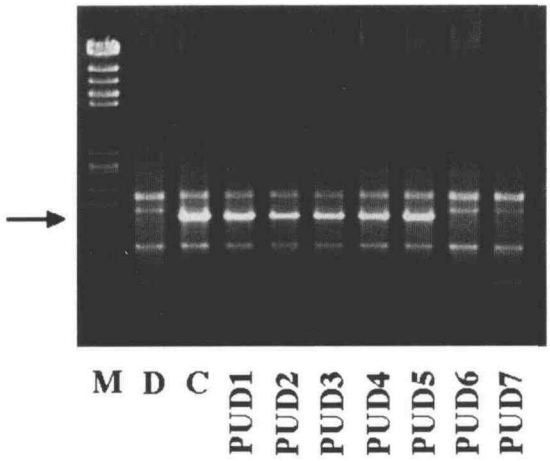

Figure 3. RAPD bands generated using UBC primer 306 with DNA template from F_7 families D and C and from F_8 families PUD1 to PUD7 (see Fig. 1). The arrow indicates the 1.2-kb RAPD band amplified from *Bet1/bet1* and *bet1/bet1* genotypes. *Bet1/Bet1* genotypes (C, PUD6, and PUD7) are null for this marker. M, Mol wt markers (an equimolar mixture of λ-phage digested with *Hin*dIII and λ-phage digested with *Hin*dIII plus *Eco*RI).

independently scored glycinebetaine polymorphism was obtained. Thus, all of the *bet1/bet1* F_8 selections tested, the three deliberately advanced heterozygous populations (PUD1, PUD2, and PUD3), and the three inadvertently advanced heterozygous populations (PUA4, PUC3, and PUP4) revealed the presence of the RAPD marker associated with the *bet1* allele. None of the 26 putative *Bet1/Bet1* F_8 selections tested (i.e. those selections exhibiting no glycinebetaine-deficient progeny) was found to exhibit the polymorphism associated with the *bet1* allele (Fig. 1). Presumably the marker detected by UBC primer 306 must be very tightly linked to the *bet1* allele to account for the lack of recombination between *bet1* and marker among the 62 F_8 lines advanced (Fig. 1). Amplification of a slightly larger 1.3-kb fragment is detected in D, PUD6, and PUD7 (Fig. 3). However, amplification of this fragment is not reliable and there is no evidence that this marker is associated with the *Bet1* allele.

DISCUSSION

The results presented here illustrate the utility of PD-MS for routinely quantifying glycinebetaine and choline in plant extracts. This method has advantages over alternative methods such as DCI-MS (Lerma et al., 1991; Wood et al., 1991) and FAB-MS (Rhodes and Rich, 1987; Rhodes, 1990; Hanson and Gage, 1991; Hanson et al., 1991) in that derivatization of the quaternary ammonium compounds is not required and that choline can be readily detected and quantified even in the presence of a large (more than 10-fold) excess of glycinebetaine. These data suggest that a recessive allele (*bet1*) of a single locus conferring glycinebetaine deficiency may simultaneously confer a significantly elevated free choline pool in maize. These findings are pertinent to a discussion of the regulation of flux to choline in response to salinity stress in glycinebetaine-accumulating and -nonaccumulating plants presented by Weretilnyk and Summers (1992). A genetic block in choline oxidation to betaine aldehyde in maize results in nearly complete inhibition of salinity stress-induced flux to glycinebetaine. This mutation produces only a modest expansion of the choline pool, indicating that mechanisms must exist to coordinately regulate the amount of choline made by maize plants in relation to the amount of choline oxidized to glycinebetaine, as postulated by Weretilnyk and Summers (1992). In the absence of such mechanisms, the choline pool of the glycinebetaine-deficient families would have been expected to increase in proportion to the amount of glycinebetaine accumulated by the glycinebetaine-containing sister lines. Clearly, choline (or some product of choline metabolism other than glycinebetaine) must down-regulate its own synthesis to prevent such excessive choline accumulation. Mechanisms to achieve such feedback control over choline synthesis have been described and involve down-regulation of *N*-methylation of phosphorylethanolamine by choline (Mudd and Datko, 1989a, 1989b). This down-regulation of flux via the ethanolamine → choline pathway may in turn cause accumulation of the ethanolamine precursor, Ser (Table V).

The RAPD marker that is associated with the *bet1* allele will likely be of great utility in rapidly confirming genotypes of any additional germplasm derived from the F_8 pairs of lines described here (including hybrids obtained from crosses with glycinebetaine-deficient and glycinebetaine-containing lines derived from inbred P77; Lerma et al. [1991]). However, UBC primer 306 does not appear to detect an equivalent RAPD polymorphism in segregating populations derived from a glycinebetaine-containing inbred (B73) and a glycinebetaine-deficient inbred (A188) that is allelic with the presently described germplasm (W.-J. Yang and D. Rhodes, unpublished data). Thus, this primer may be of limited use in other maize populations segregating for *bet1*. We are currently testing whether this RAPD marker can be converted to a restriction fragment length polymorphism or a sequence-characterized amplified region (Paran and Michelmore, 1993) that can then be mapped to a specific chromosomal location in recombinant inbred families (cf. Burr and Burr, 1991) or can be shown to detect polymorphisms co-segregating with *bet1* in other maize germplasm. Preliminary results indicate that *bet1* is localized near the centromere on the short arm of chromosome 3 (Rhodes et al., 1993). Consistent with this location, the tightly linked marker, *bnl13.05B* (Rhodes et al., 1993), has been found to detect a restriction fragment length polymorphism that co-segregates with *bet1* in the F_8 populations described here (W.-J. Yang and D. Rhodes, unpublished data).

The primary purpose of generating the presently described near-isogenic F_8 lines has been to test whether or not glycinebetaine accumulation influences osmotolerance in maize. Evidence is presented in the companion paper (Saneoka et al., 1995) that F_8 *Bet1/Bet1* plants exhibit greater salt tolerance than do *bet1/bet1* sister lines.

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