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THE ADAPTIVE SIGNIFICANCE OF GLYCINEBETAINE ACCUMULATION IN WATER- OR SALT-STRESSED BARLEY

By

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ABSTRACT

THE ADAPTIVE SIGNIFICANCE OF GLYCINEBETAINE ACCUMULATION IN WATER- OR SALT-STRESSED BARLEY

By

Rebecca Grumet

Betaine (glycinebetaine) accumulates in certain plants during osmotic stress and may be central to cytoplasmic osmoregulation. To test the effect of altered betaine levels on response to stress, I developed two barley (Hordeum vulgare L.) isopopulations differing in betaine content. First, inheritance of betaine level was studied. Neither maternal nor cytoplasmic effects were significant for four pairs of reciprocal crosses. General combining ability accounted for 74.6% of the genetic effects from an incomplete 13 X 13 diallel. Generation mean analysis showed that betaine level was a predominantly additive trait. Narrow-sense heritabililty values were 0.53 and 0.63 from midparent-offspring regression and generation mean analysis, respectively. Since the betaine trait in barley is nuclear and highly heritable, an isopopulation approach was possible.

To minimize linkage effects the isopopulation procedure included several parents and two rounds of crossing. The two isopopulations had significantly different betaine

levels in unstressed conditions (22.8 vs. 32.4 umol·g-1 dry weight). Several morphological and developmental characters indicated that the populations were otherwise comparable.

When the isopopulations were salinized, 300 mM NaCl caused an 8-fold increase in betaine and a 5 bar drop in solute potential (ψ_s). The absolute difference in betaine between the populations did not change with salinization. Although selected only for betaine level, high betaine isopopulations and parents maintained a 1 bar lower ψ_s at all salt levels. Betaine level was perfectly coordinated with ψ_s (r^2=0.99); all genotypes fell on the same line. These observations are readily explained if: betaine accumulation is a mandatory component of osmoregulation in barley, and osmoregulation as a whole is under genetic control.

The isopopulations were compared for response to water deficits in greenhouse trials. The low betaine-high ψ_s population had a higher rate of leaf production, and in optimal environments accumulated up to 35% more above-ground dry matter. When water stressed, the dry matter differences disappeared. The populations did not differ for water use efficiency or assimilate partitioning to the leaves. Although selection for high betaine-low ψ_s produced a more stable population with respect to water stress (regression response technique, b=0.84), growth in optimal environments was reduced.

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AOV	analysis of variance
BC	backcross
CI	cereal introduction
cv	cultivar
DAP	days after planting
df	degrees of freedom
GCA	general combining ability
h	heritability
LSD	least significant difference
P	probability level
PPFD	photosynthetic photon flux density
rep	replicate
R.H.	relative humidity
RWC	relative water content
SCA	specific combining ability
SD	standard deviation
SE	standard error
SS	sum of squares
USDA	United States Department of Agriculture
WUE	water use efficiency
uE	microEinsteins
ψ_s	solute potential

INTRODUCTION

Betaine (glycinebetaine) accumulates in several plant species in response to water- or salt-stress and is hypothesized to be central to osmoregulation by acting as a compatible, cytoplasmic solute (Wyn Jones et al., 1977). A good deal of circumstantial evidence supports this view (Wyn Jones and Storey, 1981; Hanson and Hitz, 1982), but the effect of differing levels in-vivo has never been tested directly. For this dissertation I set out to test the effect of genetically altered betaine level on the response of barley plants to water- or salt-stress by creating two isopopulations differing primarily in betaine level.

Osmoregulation and Compatible Solutes.

Osmoregulation, the active accumulation of solutes within the cell in response to a reduction in external water potential, is an important adaptive response that enables an organism to establish equilibrium between the osmotic strength of the cell and that of the environment (Brown, 1964; Yancey et al., 1982). The solutes accumulated may either be salts that are readily absorbed from the environment, organic solutes that are synthesized in

response to osmotic stress, or some combination of the two (Flowers et al., 1977; Jeffries, 1981). Because proteins are susceptible to conformational changes in response to ionic influences, most macromolecular function is extremely sensitive to the cellular ionic environment (Yancey et al., 1982). From an evolutionary perspective there are two possible adaptive responses to the conflicting demands of osmotic equilibrium and biochemical activity (Yancey and Somero, 1979): (a) The evolution of enzymes capable of functioning in high salt conditions, or (b) the evolution of a concentrated intracellular environment that is compatible with biochemical activity. Examples of both sorts of adaptations exist in nature, but the latter is more common.

The evolution of different sets of enzymes seems to be limited to the extremely halophilic bacteria (Yancey et al., 1982). When various citrate cycle enzymes were isolated from several bacterial species and studied in-vitro, they were found to have NaCl optima of 1-4 M (Brown, 1964). This is in direct contrast to inhibition by NaCl at concentrations greater than 200 mM for enzymes from most other organisms (Flowers et al., 1977; Brown, 1964). Amino acid sequence data showed there to be extensive substitution in the enzymes from the halophilic bacteria (Brown, 1964). Thus these bacteria have evolved a rather drastic form of adaptation requiring the modification of hundreds, or possibly thousands, of proteins (Yancey et al., 1979).

Alteration of the cellular ionic environment is more commonly observed. In most systems the enzymes from salt-tolerant and salt-sensitive species do not differ in-vitro (Flowers et al., 1977). Many species actively exclude inorganic salts and, instead, achieve osmotic balance with low molecular weight, compatible, organic solutes (Munns et al., 1982; Flowers et al., 1977). The concept of compatible solutes was introduced by Brown and Simpson (1972) to describe molecules that protect enzymes against inhibition in conditions of low water potential. In fact, there is evidence to suggest that there has been stringent evolutionary selection for the solute composition of cells (Jeffries, 1981; Yancey et al., 1979). Throughout nature the major osmolytes for water- or salt-stressed eukaryotes have been restricted to a few classes of low molecular weight organic compounds including: polyols, reducing sugars, amino acids, urea, and methylamines (Jeffries, 1981; Yancey et al., 1979).

Timasheff et al. (1982) studied the relation of protein hydration and structural stability and found that all structure-stabilizing solutes were preferentially excluded from the domain of the protein molecule. Protein self-association was promoted by molecules such as sucrose, glycerol, hexylene glycol, and amino acids and was linked to preferential hydration of the protein. The opposite was true for denaturants, these all interacted preferentially

with functional groups of the proteins and induced unfolding.

The accumulation of urea in many salt-water fish is surprising because it is a destabilizing solute, but Yancey and Somero (1979) found that it was always accompanied by the accumulation of stabilizing methylamines and amino acids in a constant 2:1 ratio (urea: methylamines and amino acids). At these relative concentrations the methylamines and amino acids were found to counteract the destabilizing effect of urea on the melting temperature of ribonuclease and the renaturation of lactate dehydrogenase. This implies that both the type and relative concentrations of solutes are important aspects of osmoregulation.

Osmoregulation in Plants.

In the past decade a good deal of experimental work with higher plants has shown osmoregulation to be a mechanism of adaptation to both drought- and salt-stress (Hsiao et al., 1976; Turner and Jones, 1980; Morgan, 1984). The water relations of a plant cell may be summarized as $\psi_w = \psi_s + \psi_p$, where ψ_w, ψ_s, and ψ_p, are water, solute, and turgor potentials, respectively (Boyer, 1985). Since the water potential of pure water is defined to be zero and water moves down a water potential gradient, ψ_w, and ψ_s, are negative terms; ψ_p is positive. A drop in solute potential caused by the accumulation of solutes will promote water

movement into the cell. This process may enable a plant to maintain turgor, and thereby growth, in environments of low external water potential (Hsiao et al., 1976; Turner and Jones, 1980; Morgan, 1984a).

To maintain turgor, any solute may be used, and studies have shown that despite known toxicity, Na^+ ions can be major contributors to decreased solute potential in several plant species (Munns et al., 1982). However, reports comparing enzymes isolated from salt-tolerant and salt-sensitive plant species have failed to show in-vitro differences among the enzymes. Greenway and Osmond (1972b) studied malate dehydrogenase, aspartate transaminase, glucose-6-phosphate dehydrogenase, and isocitrate dehydrogenase from the halophytes, Atriplex spongiosa and Salicornia australis and from the glycophyte Phaseolus vulgaris. NaCl concentrations in excess of 200 mM were inhibitory to enzymes from all three species; the sensitivity existed whether or not the plants had been growing in high salt conditions. Mannitol, however, did not inhibit the enzymes and so the inhibition was not attributable to low osmotic potential per se.

Similarly, Ben Amotz and Avron (1972) studied the halophilic alga Dunaliella which accumulates high levels of NaCl within the cell and has a high NaCl requirement for in-vivo photosynthesis. The requirement for high salt, however, was not observed for photosynthesis and enzyme

activity in-vitro; in fact, salt was inhibitory. They further reported that the requirement for salt in-vivo could be replaced by glucose or glycine indicating that the NaCl had a non-specific osmoregulatory function.

The apparent contradiction of high internal salt levels and the inhibitory effects of NaCl on enzyme function led both groups of investigators to suggest that intracellular compartmentation may play a important role in osmoregulation for plants. Mature plant cells are highly vacuolated and so it is possible that potentially toxic levels of inorganic ions may be tolerated by sequestration in the vacuole (Flowers et al., 1977; Hellebust, 1976).

Several experimental approaches have indicated non-uniform distribution of ions within the cell. X-ray dispersive spectra (Jeschke and Stelter, 1976) and electron probe x-ray microanalysis (Pitman et al., 1981) indicated preferential accumulation of Na in the vacoule. The K/Na ratio was approximately 1 in the vacuole and 10 in the cytoplasm. X-ray microanalysis of the intertidal alga Porphyra umbilicalis also showed Na levels to be low in the cytoplasm and high in the vacuoles (Wiencke, et al., 1983).

The differential distribution of ions within the cell presumably requires active transport across the tonoplast (Jeffries, 1981; Kirst and Bisson, 1982). The tonoplast is unable to withstand hydrostatic pressure differences (Jeffries, 1981) and so it is necessary to maintain osmotic

balance between the vacuolar and cytoplasmic compartments. This led Wyn Jones et al. (1977) to suggest that in plant cells, compatible solutes serve as cytoplasmic osmolytes and thereby maintain both cytoplasmic, biochemical function, and osmotic balance within the cell. Wyn Jones and Gorham (1983) note that many low molecular weight organic compounds that accumulate in osmotically stressed plant cells (e.g. glycerol, proline, and betaine), are not present in sufficient quantity to be osmotically important unless they are selectively accumulated within the cytoplasm.

Thus plant cells may utilize both inorganic and organic solutes for osmoregulation (Munns et al., 1982). The ability to tolerate large quantities of Na^+ within the cell varies among species and is thought to be one differentiating feature between halophytes and glycophytes (Greenway and Munns, 1980; Munns et al., 1982). Other solutes that can make a major contribution to osmoregulation in expanded leaves include sugars, sugar alcohols, amino acids, organic acids, K^+ and Cl^- (Morgan, 1984; Ford and Wilson, 1981).

In summary, the importance of osmoregulation for all forms of life is increasingly recognized (Strom et al., 1983). It should be noted, though, that direct evidence for a biological role of individual compatible solutes in plant or animal cells is very limited; evidence has yet to be produced verifying that accumulation of these compounds is

adaptive and not a reflection of impaired metabolism (Strom et al., 1983; Yancey et al., 1982; Hanson and Grumet, 1985). Strom et al. (1983) emphasize that hard experimental evidence for the function of these compounds in plant cells should be of highest priority.

Evidence that Betaine is a Compatible, Cytoplasmic Osmoticum.

Quaternary ammonium compounds such as betaine are found in many microbial, plant, and animal species (Mackay et al., 1984; Yancey et al., 1982; Wyn Jones and Storey, 1981). Within the plant kingdom betaine accumulates in members of the Gramineae, Chenopodiaceae, Amaranthaceae, and Compositae in response to water- or salt-stress (Wyn Jones and Storey, 1981). Betaine is hypothesized to function as a compatible, cytoplasmic osmoticum.

Ecological and physiological evidence supports an osmoregulatory role for betaine in plants: the species that accumulate the most betaine are generally halophytes (Wyn Jones and Storey, 1981) the amount of betaine accumulated is directly proportional to the external stress level (Hanson and Wyse, 1982; Coughlan and Wyn Jones, 1982; Storey and Wyn Jones, 1978) and steady accumulation of betaine occurs in barley and tropical pasture grasses during long-term water deficits in the field (Hitz et al., 1982; Ford and Wilson, 1981).

To determine whether or not betaine is compatible with biochemical function, many investigators have tested the effect of betaine on enzymatic activity and various metabolic processes in-vitro. Betaine at 0.5 M was shown not to inhibit the activity of malate dehydrogenase, glutamate oxaloacetate transaminase, and pyruvate kinase (Pollard and Wyn Jones, 1979). Paleg et al. (1981) found that thermal inactivation of several enzymes was reduced in a concentration dependent manner if the enzymes were heated in the presence of betaine. Paleg et al. (1984) also tested the effect of betaine on polyethylene glycol induced precipitation of glutamine synthase and found the effectiveness of 1 M betaine in inhibiting precipitation to vary with pH. Nash et al. (1982) studied the effects of betaine on the thermostability of enzymes in intact, isolated organelles. They reported that at physiological levels, (20 mM) there was no effect, but at 0.5 M betaine, heat inactivation of NAD isocitrate dehydrogenase and malate dehydrogenase was retarded in intact mitochondria. This level of betaine had no inhibitory effect on these enzymes from lysed mitochondria.

High betaine concentrations (0.5 or 1 M) were also found to be compatible with in-vitro polysome stabililty (Brady et al., 1984) and protein synthesis (Gibson et al., 1984). Larkum and Wyn Jones (1979) found betaine to be superior to sorbitol for the stability of isolated

chloroplasts. A range of betaine levels (10 - 500 mM) also had protective effects on bacteria growing in high salt conditions (LeRudulier and Valentine, 1982). Thus betaine has the properties of a compatible solute.

Support for a cytoplasmic location of betaine in plants comes from electron microscopy studies (Hall et al., 1978) and studies with isolated vacuoles (Leigh et. al, 1981) which indicate a disproportionate amount of betaine is located in the cytoplasm relative to the vacuole.

All of this evidence is consistent with the hypothesis that betaine functions as a compatible, cytoplasmic osmoticum; it accumulates during stress, is probably localized in the cytoplasm, and is non-toxic. It has been suggested that betaine accumulation is an adaptive response to osmotic stress (Wyn Jones et al., 1977). It should be noted, though, that this evidence is circumstantial; there is not yet direct evidence for the function of betaine in plant cells in-vivo, nor has the effect of directly altering physiological levels of betaine in-vivo been examined.

Potential Agricultural Signficance of Betaine Accumulation.

The possibility that increased betaine level is an adaptive response to water- or salt-stress has agricultural implications. Lack of water and/or poor quality water are major factors limiting crop productivity throughout the world (Boyer, 1982). Forty percent of the land surface is

estimated to be arid or semi-arid (Fisher and Turner, 1978), and additional acreage is being continually lost to salinization as a result of high fertilizer use and poor quality irrigation water (Flowers et al., 1977). But if world food production is to continue to meet expanding demand, it will almost certainly be necessary to cultivate increasingly marginal land (Lewis and Christiansen, 1981). Although the need to breed for stress-resistant crops has long been recognized (Gauss, 1910), it is becoming increasingly important.

A focus of plant breeders, physiologists, and more recently molecular biologists, has been to identify specific traits that confer stress resistance. The goal is to enhance their expression in crop plants, or transfer them to other cultivars or species (Blum, 1979; LeRedulier, et al., 1984; Quarrie, 1980; Rick, 1978). Betaine accumulation is one stress resistance candidate that has been investigated in recent years (LeRudulier et al., 1984; Hanson and Grumet, 1985; Wyn Jones and Gorham, 1983). A potentially adaptive biochemical trait such as betaine accumulation is of particular interest because metabolic traits are probably the most suitable for emerging molecular genetic technologies (Hanson et al., 1985; Hanson and Grumet, 1985). On the other hand, metabolic traits have not yet been exploited to any great extent. This may, in large part, be due to the difficulty in extrapolating from changes on a

biochemical level to effects on whole plant growth, and ultimately, crop performance (Hanson and Grumet, 1985).

As Reitz (1974) cautions, many traits have been correlated with plant stress responses, but it is a long step from correlation to establishing a determining influence in stress resistance. Furthermore, traits that confer stress resistance, or enhance survival, are not necessarily the same as those that increase yield (Atsmon, 1973). With regard to betaine accumulation, many important questions remain unanswered. For example: will increasing the level of betaine in a crop species that normally accumulates moderate amounts of betaine enhance stress resistance? Will introducing betaine into a species that does not normally accumulate this compound increase its stress resistance? Will altering betaine levels independently of other aspects of osmoregulation affect the solute balance within the cell? Will increasing betaine have an effect on productivity in favorable environments?

Testing Adaptive Value Using a Physiological-Genetic Approach.

One of the best methods to begin to answer the above sorts of questions is to "measure the worth" of a trait by isogenic analysis (Eslick and Hockett, 1974; Reitz, 1974; Quarrie, 1980). The aim is to produce genotypes that differ in known genetic ways for use in physiological studies.

This sort of approach has recently been used by Morgan (1984b) to study osmoregulation and Quarrie and Henderson (1982) to study the role of ABA in drought resistance.

I therefore set out to test the effect of altered betaine levels using a physiological-genetic approach. I utilized naturally occurring variability for betaine content (Ladyman et al., 1983) to develop two barley isopopulations that differed in betaine level but were otherwise genetically comparable. Because betaine accumulation is a metabolic trait and possibly overshadowed by other morphological or phenological characters that confer stress resistance, special care was taken when developing the isopopulations to minimize potentially confounding effects of linkage. The two populations were studied for growth patterns and osmoregulatory behavior in well-watered, salt-stressed and water-stressed environments.

In assessing the usefulness of a potentially adaptive trait, it is also important to determine inheritance of the trait (Townley-Smith and Hurd, 1979; Blum, 1979). In conjunction with isopopulation development, I also performed genetic analyses of betaine level in barley.

The dissertation is divided into three chapters: (1) genetic control of glycinebetaine level in barley, (2) genetic evidence for an osmoregulatory function of glycinebetaine accumulation, and (3) growth studies of two

barley isopopulations differing in betaine level and osmoregulation.

Literature Cited

Atsmon, D. 1973. Breeding for drought resistance in field crops. p. 157-176. In R. Moav (ed.) Agricultural Genetics: Selected Topics. John Wiley and Sons, New York.

Ben-Amotz, A. and M. Avron. 1972. Photosynthetic activities of the halophilic alga Dunaliella parva. Plant Physiol. 49:240-243.

Blum, A. 1979. Genetic improvement of drought resistance in crop plants. A case for sorghum. p.429-445. In H. Mussel and R. C. Staples (ed.) Stress Physiology in Crop Plants. John Wiley and Sons, New York.

Boyer, J.S. 1982. Plant productivity and environment. Science 218:443-448.

Boyer, J.S. 1985. Water transport. Ann. Rev. Plant Physiol. 36:473-516.

Brady, C.J., T.S. Gibson, E.W.R. Barlow, J. Speirs, and R.G. Wyn Jones. 1984. Salt tolerance in plants. I. Ions, compatible organic solutes, and the stability of plant ribosomes. Plant, Cell and Environ. 7:571-578.

Brown, A.D. 1964. Aspects of bacterial response to the ionic environment. Bacteriol. Rev. 28:296-329.

Brown, A.D. and J.R. Simpson. 1972. Water relations of sugar-tolerant yeasts: the role of intracellular polyols. J. Gen. Mircrobiol. 72:589-591.

Coughlan, S.J. and R.G. Wyn Jones. 1980. Some responses of Spinacia oleracea to salt stress. J. Exp. Bot. 31:883-893.

Eslick, R.F. and F.A. Hockett. 1974. Genetic engineering as a key to water use efficiency. Agric. Meterol. 14:13-23.

Fischer, R.A. and N.C. Turner. 1978. Plant productivity in the arid and semiarid zones. Ann. Rev. Plant Physiol. 29:277-317.

Flowers, T.J., P.F. Troke, and A.R. Yeo. 1977. The mechanisms of salt tolerance in halophytes. Ann. Rev. Plant Physiol. 28:89-121.

Ford, C.W. and J.R. Wilson. 1981. Changes in the level of solutes during osmotic adjustment to water stress in four tropical pasture species. Aust. J. Plant Physiol. 8:77-91.

Gauss, R. 1910. Acclimitization in breeding drought resistant cereals. American Breeders Magazine. 1:209-217.

Greenway, H. and C.B. Osmond. 1972. Salt responses of enzymes from species differing in salt tolerance. Plant Physiol. 49:256-259.

Gibson, T.S., J. Speirs and C.J. Brady. 1984. Salt tolerance in plants II. In-vitro translation of m-RNAs from salt-tolerant and salt-sensitive plants on wheat germ ribosomes. Responses to ions and compatible organic solutes. Plant, Cell and Envrion. 7:579-587.

Greenway, H. and R. Munns. 1980. Mechanisms of salt tolerance in nonhalophytes. Ann. Rev. Plant Physiol. 31:149-190.

Hall, J.L., D.M.R. Harvey and T.J. Flowers. 1978. Evidence for the cytoplasmic localization of betaine in leaf cells in Suaeda maritima. Planta 140:59-62.

Hanson, A.D. and R. Grumet. 1985. Betaine accumulation: metabolic pathways and genetics. p.71-92. In J.L. Key and T. Kosuge (ed.) Cellular and Molecular Biology of Plant Stress. UCLA Symposia on Cellular and Molecular Biology. Vol. 22. Alan R. Liss, New York.

Hanson, A.D. and W.D. Hitz. 1982. Metabolic responses of mesophytes to plant water deficits. Ann. Rev. Plant Physiol. 33:163-203.

Hanson, A.D., N.E. Hoffman and C. Samper. 1985. Identifying and manipulating metabolic stress resistance traits. Hort Science (in press)

Hanson, A.D. and R. Wyse. 1982. Biosynthesis, translocation, and accumulation of betaine in sugarbeet and its progenitors in relation to salinity. Plant Physiol. 70:1191-1198.

Hellebust, J.A. 1976. Osmoregulation. Ann. Rev. Plant Physiol. 27:485-505.

Hitz, W.D., J.A.R. Ladyman and A.D. Hanson. 1982. Betaine synthesis and accumulation in barley during field water stress. Crop Sci. 22:47-54.

Hsiao, T.C., E. Acevedo, E. Fereres, D.W. Henderson. 1976. Stress metabolism. Water stress, growth, and osmotic adjustment. Phil. Trans. R. Soc. Lond. Ser. B. 273:479-500.

Jeffries, R.L. 1981. Osmotic adjustment and the response of halophytic plants to salinity. BioScience 31:42-46.

Jeschke, W.D. and W. Stelter. 1976. Measurement of longitudinal ion profiles in single roots of Hordeum and Atriplex by use of flameless atomic absorption spectroscopy. Planta 128:107-112.

Kirst, G.O. and M.A. Bisson. 1982. Vacuolar and cytoplasmic pH, ion composition, and turgor pressure in Lamprothamnium as a function of external pH. Planta 155:287-295.

Ladyman, J.A.R., K.M. Ditz, R. Grumet and A.D. Hanson. 1983. Genotypic variation for glycinebetaine accumulation by cultivated and wild barley in relation to water stress. Crop Sci. 23:465-468.

Larkum, A.W.D. and R.G. Wyn Jones. 1979. Carbon dioxide fixation by chloroplasts isolated in glycinebetaine. Planta 145:393-394.

Leigh, R.A., N. Ahmad and R.G. Wyn Jones. 1981. Assessment of glycinebetaine and proline compartmentation by analysis of isolated beet vacuoles. Planta 153:34-41.

LeRudulier, D., A.R. Strom, A.M. Dandekar, L.T. Smith, and R.C. Valentine. 1984. Molecular biology of osmoregulation. Science 224:1064-1068.

LeRudulier, D. and R.C. Valentine. 1982. Genetic engineering in agriculture: osmoregulation. TIBS 7:431-433.

Lewis, C.F. and M.N. Christiansen. 1981. Breeding plants for stress environments. p.151-178. In K.J. Frey (ed.) Plant Breeding II. Iowa State Univ. Press. Ames, Iowa.

Mackay, M.A., R.S. Norton and L.J. Borowitzka. 1984. Organic osmoregulatory solutes in cyanobacteria. J. Gen. Microbiol. 130:2177-2192.

Morgan, J.M. 1984a. Osmoregulation and water stress in higher plants. Ann. Rev. Plant Physiol. 35:299-319.

Morgan, J.M. 1984b. Osmoregulation as a selection criterion for drought tolerance in wheat. Aust. J. Agric. Res. 34:607-614.

Munns, R., H. Greenway and G.O. Kirst. 1982. Halotolerant eukaryotes. p.59-135. In O.E. Lange, C.B. Osmond, H Zeiger (ed.) Vol. 12C. Physiological Plant Ecology. Encyclopedia of Plant Physiology. Springer-Verlag, Heidelberg.

Nash, D., L.G. Paleg and J.T. Wiskich. 1982. Effect of proline, betaine and some other solutes on the heat stability of mitochondrial enzymes. Aust. J. Plant Physiol. 9:47-57.

Paleg, L.G., T.J. Douglas, A. van Daal, D.B. Keech. 1981. Proline, betaine and other organic solutes protect enzymes against heat inactivation. Aust. J. Plant Physiol. 8:107-114.

Paleg, L.G., G.R. Stewart, and J.W. Bradbeer. 1984. Proline and glycinebetaine influence protein solvation. Plant Physiol. 79:974-978.

Pitman, M.G., A. Lauchli and R. Stelzer. 1981. Ion distribution in roots of barley seedlings measured by electron probe x-ray microanalysis. Plant Physiol. 68:673-679.

Pollard, A. and R.G. Wyn Jones. 1979. Enzyme activities in concentrated solutions of glycinebetaine and other solutes. Planta 144:291-298.

Quarrie, S.A. 1980. Cereal yields and drought resistance. Nature 285:612-613.

Quarrie, S.A. and I.E. Henderson. 1982. Biparental inheritance of drought-induced accumulation of abscisic acid in wheat and pearl millet. Annals of Botany 49:265-269.

Rick, C.M. 1978. The tomato. Sci. Amer. 239:76-87.

Reitz, L.P. 1974. Breeding for more efficient water use - is it real or a mirage? Agric. Meterol. 14:3-11.

Storey, R. and R.G. Wyn Jones. 1978. Salt stress and comparative physiology in the Gramineae. III. Effect of salinity upon ion relations and glycinebetaine and proline levels in Spartina townsendii. Aust. J. Plant Physiol. 5:817-829.

Strom, A.R., D. LeRudulier, M.W. Jacowec, R.C. Bunnell, and R.C. Valentine. 1983. Osmoregulatory genes and osmoprotective compounds. p.39-59. In T. Kosuge, C.P. Meredith, and A. Hollaender (ed.) Genetic Engineering of Plants - An Agricultural Perspective. Plenum Press, New York.

Timasheff, S.N., T. Arakawa, H. Inoue, K. Gekko, M.J. Gorbunoff, J.C. Lee, G.C. Na, E.P. Pittz, and V. Prakesh. 1982. The role of solvation in protein structure stability and unfolding. p.48-50. In F. Franks and S.F. Mathias (ed.) Biophysics of Water. John Wiley and Sons, New York.

Turner, N.C. and M.M. Jones. 1980. Turgor maintenance by osmotic adjustment. p.87-104. In N.C. Turner and P.J. Kramer (ed.) Adaptation of Plants to Water and High Temperature Stress. John Wiley and Sons, New York.

Townley-Smith, T.F. and E.A. Hurd. 1979. Testing and selecting for drought resistance in wheat. p.447-464 In H. Mussel and R.C. Staples (ed.) Stress Physiology in Crop Plants. John Wiley and Sons, New York.

Wiencke, C., R. Stelzer, and A. Lauchli. 1983. Ion compartmentation in Porphyra umbilicalis determined by electron probe x-ray microanalysis. Planta 159:336-341.

Wyn Jones, R.G. and J. Gorham. 1983. Aspects of salt and drought tolerance in higher plants. p.355-370. In T. Kosuge, C.P. Meredith and A. Hollaender. (ed.) Genetic Engineering of Plants - An Agricultural Perspective. Plenum Press, New York.

Wyn Jones, R.G. and R. Storey. 1981. Betaines. p.171-204. In L.G. Paleg and D. Aspinall (ed.) Physiology and Biochemistry of Drought Resistance. Academic Press, Sydney.

Wyn Jones, R.G., R. Storey, R.A. Leigh, N. Ahmad and A. Pollard. 1977. A hypothesis on cytoplasmic osmoregulation. p.126-136. In E. Marre and O. Ciferri (ed.) Regulation of cell membrane activities in plants. Elsevier Press, Amsterdam.

Yancey, P.H., M.E. Clark, S.C. Hand, R.D. Bowlus and G.N. Somero. 1982. Living with water stress: evolution of osmolyte systems. Science 217:1212-1222.

Yancey, P.H. and G.N. Somero. 1979. Counteraction of urea destabilization of protein structure by methylamine osmoregulatory compounds in Elasmobranch fish. Biochem J. 183:317-323.

CHAPTER I

GENETIC CONTROL OF GLYCINEBETAINE LEVEL IN BARLEY

1.1 Abstract

The accumulation of betaine (glycinebetaine, N,N,N-trimethylglycine) in water- or salt-stressed barley (Hordeum vulgare L.) plants may be a metabolic adaptation to stress. We investigated the inheritance of betaine level using genotypes varying in betaine content. Shoots of unstressed plants grown in growth chambers were assayed for betaine at the three-leaf stage. Analyses of four pairs of reciprocal crosses indicated that neither maternal nor cytoplasmic effects were significant but that there may be some dominance of the low betaine trait. An incomplete diallel made with eight low- and five high-betaine parents indicated that general combining ability accounted for the majority (74.6%) of genetic effects, suggesting a largely additive trait. Additive, dominance, and epistatic components of heritability were estimated by generation mean analysis of the parental, F_1, F_2, and backcross

generations of a single high- X low-betaine cross. This analysis also showed that betaine accumulation was a predominantly additive trait. Narrow-sense heritability values for the trait were 0.53 and 0.63, from mid-parent-offspring regression and generation mean analysis, respectively. The level of betaine accumulation in barley is a nuclear, predominantly additive trait of relatively high narrow-sense heritability and so evaluation of the adaptive significance of betaine in water- and salt-stressed plants using an isopopulation approach should be possible.

1.2 Introduction

Betaine (glycinebetaine, N,N,N-trimethylglycine) accumulates in chenopods and several grasses in response to water- or salt-stress. In recent years, much circumstantial physiological and biochemical evidence has supported the view that this is a metabolic adaptation to stress (Wyn Jones and Storey, 1981; Hanson and Hitz, 1982). The amount of betaine accumulated by various salt-tolerant plants is proportional to the external salt concentration (Hanson and Wyse, 1982; Coughlan and Wyn Jones, 1980; Storey and Wyn Jones, 1978, 1979), and steady accumulation of betaine occurs during long-term water deficits in the field with barley (Hordeum vulgare L.) and tropical pasture grasses (Hitz et al., 1982; Ford and Wilson, 1981). Wyn Jones et al. (1977) have proposed that betaine acts as a non-toxic, and possibly

protective, cytoplasmic osmoticum in stressed plants. Some experimental evidence exists for a predominantly cytoplasmic location of betaine (Hall et al., 1978; Leigh et al., 1981) and for protective effects of betaine in vitro (Larkum and Wyn Jones, 1979; Pollard and Wyn Jones, 1979; Jolivet et al., 1982). Moreover, applied betaine protected various bacteria against the inhibitory effects of high salt concentrations (Strom et al., 1983).

Although these observations are consistent with an adaptive role for betaine during water- and salt-stress, they do not eliminate the possibility that betaine accumulation in plants is the result of stress injury. To test the adaptive value of stress-induced betaine accumulation, and to evaluate the possibility of breeding for high betaine levels, it is desirable to develop genetically comparable lines differing in betaine content. Ladyman et al. (1983) showed significant genetic variability for betaine content among barley genotypes. Accordingly, we are developing barley isopopulations differing primarily in betaine content for evaluation under water- and salt-stress. Although stress conditions cause a large increase (3-10 fold) in betaine content, the relative betaine contents of a range of genotypes were similar whether measured in young, well-watered plants grown in controlled environments or in older plants under water-stress conditions in the field (Ladyman et al., 1983). It can therefore be considered valid to evaluate the genetic potential for betaine accumulation by testing young,

unstressed plants grown in controlled conditions.

To determine the suitability of a trait for physiological-genetic study, as well as its potential usefulness in plant breeding, knowing its mode of inheritance is useful. In this study three questions were asked about the betaine trait as measured in young, unstressed plants: Are the genes controlling betaine level nuclear or cytoplasmic? Are the genetic effects additive, dominant, or epistatic? How heritable is the trait?

1.3 Materials and Methods

Four experiments were used to answer the questions above. (1) The significance of maternal and cytoplasmic effects was tested using four pairs of reciprocal high- X low-betaine F_1 crosses and their parents. (2) A partial diallel cross of 13 parents (eight low-betaine accumulators and five high-betaine accumulators) was made, and relative contributions of general (GCA) vs. specific combining abilities (SCA) estimated. (3) Generation mean analysis was performed to determine additive, dominance, and epistatic components of gene action using a high-betaine parent, a low-betaine parent, the F_1, the F_2, and two backcross generations. (4) Heritability estimates were made using midparent-offspring regression, ratios of genetic variances, and the separation of environmental from genetic variance among sets of diverse genotypes.

1.3.1 Selection of Parental Genotypes

Selection of barley genotypes used as parents for the inheritance studies was based on the following criteria: (1) high- or low-betaine content as measured under water-stressed and nonstressed conditions in the growth chamber and field (Ladyman et al., 1983), (2) compatibility of flowering dates for crossing, and (3) ease of working with cultivated (rather than wild) accessions. [Accessions of both *Hordeum vulgare* and its wild relative, *H. spontaneum* C. Koch, were evaluated (Ladyman et al., 1983); since the range in betaine content for both groups was very similar, *H. vulgare* accessions were chosen]. Seeds were obtained from the USDA Small Grains Collection (Beltsville, Md.); all were spring barleys. Cereal Introduction (CI) numbers of the low-betaine genotypes were: 709, 5199, 9309, 10064, 11806 (cv. 'Proctor'), 12456, 13057, and 15293; the high-betaine genotypes were 3480, 6577, 10138, 13626 (cv. 'Arimar'), and 14936.

1.3.2 Plant Culture

Seeds were stratified on moist filter paper in the dark at 4 C for 1 week; they were then kept in darkness at room temperature for 1 day to allow radicle emergence before planting in a mix of peat, sand, and loam (1:2:1). The plants for crossing were grown in the greenhouse and watered with half-strength Hoagland's solution. Powdery mildew (*Erysiphe graminis* DC. f. sp. *hordei* Em. Marshal) was controlled with sulfur pots, aphids (*Macrosipnum avenae*

Fabricius) by occasional spraying with Pirimore [2-(dimethylamino)-5,6-dimethyl-4-pyrimidinyl dimethylcarbamate, 50% wettable powder, ICI Americas Inc., Wilmington, Del]. Plants analyzed for betaine content were grown in the soil mix given above in controlled environment chambers (16-hour day, 21 C, R.H. 70%; 8-hour night, 16 C, R.H. 85%) and were watered daily with half-strength Hoagland's solution.

1.3.3 Betaine Assay

Shoots were harvested 14-17 days after planting, frozen in liquid nitrogen, freeze-dried, and ground in a Wiley Mill to mesh size 40. Betaine content of the shoots (umol betaine·g dry wt^{-1}) was measured using a modification of the spectrophotometric assay of Wall et al. (1960); an ion exchange step was added to eliminate interfering compounds, and the periodide precipitate was rinsed to lower blank readings. Accurately weighed 30-50 mg subsamples were extracted in 5 ml of water for 30 min or 1 h at 100 C. A 4-ml portion of the aqueous leaf extract (or 4 ml of water containing 50-600 ug betaine standards) was shaken with 0.2 ml of a slurry of the H^+ form of AG-50 resin (Bio-Rad Laboratories, Richmond, CA; 2 vol settled resin: 1 vol water) to bind betaine and other cations. The supernatant was aspirated off the settled resin and discarded, and the resin was washed twice with 2 ml of water. Betaine was eluted from the resin with 3 ml 4N NH_4OH, which was drawn off and evaporated to dryness under an infrared lamp in a

stream of air. The dried eluate was taken up in 0.6 ml of 1 N H_2SO_4 of which 0.5 ml was transferred to a 1-ml vial (ReactiVials, Pierce Chemical Company, Rockford, IL) and cooled to 4 C. Then 0.2 ml of cold KI-I_2 reagent (Wall et al., 1960) was added, and the contents of the vial were mixed and left overnight at 4 C to allow crystallization of the periodide. The vials were centrifuged at 4 C in a clinical centrifuge for 5 min, the supernatant was drawn off using a Pasteur pipet with a very fine tip, taking care not to disturb the pellet. The pellet was then rinsed with 0.2 ml cold 1 N H_2SO_4, recentrifuged at 4 C, and the supernatant was drawn off as above. The periodide in the pellet was dissolved by agitating with 0.2 ml dichloroethane; in plant samples, a light brown residue remained after the periodide had dissolved. A 30 ul aliquot of the periodide solution was diluted with a further 4 ml dichloroethane, and absorbance at 365 nm was read. With standards, the relationship between absorbance (y) and betaine (x) was linear in the range 50-600 ug (typical standard curve with triplicate samples: $y = 0.0019x - 0.039$, $r^2 = 0.98$), and spikes of betaine (200 ug) added to barley shoot samples were recovered quantitatively. The spectrophotometric assay (y) was checked against the pyrolysis - gas chromatography assay (x) (Hitz and Hanson, 1980) by using both methods to analyze 10 barley shoot samples spanning a range of betaine levels; agreement between methods was very good ($y = 1.28x - 6.47$, $r^2 = 0.99$).

1.3.4 Test for Maternal and Cytoplasmic Inheritance

Four pairs of reciprocal F_1 crosses were made using CI 3480 and CI 14936 (high betaine) and CI 13057 and CI 15293 (low betaine) as parents in a high vs. low mating design. The eight F_1 hybrids and their four parents were grown in a randomized, complete block design with nine replicates; each block was a 20.5-cm pot divided into 12 sections. Reciprocal differences were tested using orthogonal contrasts. Degree of dominance was assigned to the betaine trait if the F_1 was significantly different from the midparent value ($LSD_{0.05}$); partial dominance was assigned if the F_1 value was intermediate between the low parent and midparent value, complete dominance was assigned if the F_1 was not significantly different from the low parent.

1.3.5 Partial Diallel Cross and Analysis

Crosses were made using all 13 selected genotypes (8 low in betaine, 5 high) as parents. Seeds were obtained for 68 of the 78 possible F_1 combinations (Fig. 1). Reciprocal crosses were not included. The 13 parents and 68 F_1 hybrids were grown for betaine analysis in a 9 X 9 triple lattice design. Each block was a 16-cm pot containing nine entries.

Adjusted means from the analysis of variance of the lattice design were used to partition the adjusted genotypic SS into components for GCA and SCA. The partitioning was performed according to Griffing's (1956) Model I, method 2, using multiple regression. The SCA effects for missing F_1

hybrids were set equal to zero for purposes of analysis. (Estimates and t-tests of SCA effects for other crosses showed this to be reasonable.) The relative contribution of GCA to total genetic variation was calculated as the ratio of SS GCA to SS entries. A similar statistic was calculated for SCA.

1.3.6 Generation Mean Analysis

The F_1, F_2, and two backcross generations of a cross between CI 11806 'Proctor' (P, low betaine) and CI 13626 'Arimar' (A, high betaine) were made. The F_1 population tested for betaine content included equal numbers of P X A and A X P; the F_2 and backcross generations were derived from P X A F_1 plants. Because the large, segregating generations could not be blocked, pots were rotated within the growth chamber daily. The generations were grown in four groups at different times in the growth chamber: parents with F_1's, parents with F_2's, parents with the backcross to Proctor, and parents with the backcross to Arimar. Generation mean analysis was performed using weighted least squares regression (Mather and Jinks, 1977; Rowe and Alexander, 1980). The adequacy of models containing environmental, additive, dominance, epistatic, and genotype X environmental interaction effects was evaluated using chi-square tests.

1.3.7 Heritability Estimates

Narrow sense heritability was estimated using the midparent-offspring regression value for the 68 F_1 hybrids (Falconer, 1981). Narrow-sense heritability was also calculated from generation variances as $h = [2\sigma^2_{F_2} - (\sigma^2_{BC_1} + \sigma^2_{BC_2})]/\sigma^2_{F_2}$ using the generations derived from the Proctor X Arimar cross (Warner, 1952). Broad-sense heritability was estimated by separating genetic from environmental variation in replicated trials. The data used for this estimate were those reported by Ladyman et al. (1983) for 145 diverse H. vulgare and H. spontaneum genotypes entered in replicated screening trials. The plants were grouped into three experiments, each containing 49 genotypes; check plants of Proctor and Arimar were included in each trial. Estimates of genetic and environmental components of variance were calculated for each experiment using the expected mean square for treatments [$MS(T) = \sigma^2_e + r\sigma^2_g$] and the expected mean square for error [$MS(E) = \sigma^2_e$]. Heritability was calculated as $h = \sigma^2_g/(\sigma^2_g + \sigma^2_e)$ (Allard, 1960).

1.4 Results

1.4.1 Maternal and Cytoplasmic Inheritance

Comparisons among means for betaine content of the genotypes used in the maternal and cytoplasmic inheritance study are listed in Table 1. The analysis of variance of these data showed highly significant differences for betaine

Table 1. Betaine content of four pairs of reciprocal F_1 crosses and their parents

Genotype	Betaine content† u mol·g dry wt^{-1}		Midparent value	low parent
Parents				
CI 3480 (A)	31.2	a		
CI 14936 (B)	28.4	a		
CI 13057 (C)	19.2	bc		
CI 15293 (D)	14.3	d		
F_1 crosses				
A X C	16.2	cd	25.2	complete
C X A	17.1	bcd		complete
A X D	18.5	bc	22.8	partial
D X A	20.8	b		none
B X C	18.1	bcd	23.8	complete
C X B	16.9	bcd		complete
B X D	17.0	bcd	21.4	complete
D X B	19.2	bc		none

†Each value is the mean of 9 replicates, SE = 1.45.

$LSD_{0.05}$= 4.08 $LSD_{0.01}$= 5.42.

Means followed by the same letters are not significantly different by $LSD_{0.05}$.

content among genotypes (Table 2). The division of genotypic variance into orthogonal contrasts indicated that the majority of variation was due to the difference between the high- and low-betaine parents. When the members of the four pairs of reciprocal crosses were contrasted with each other, no significant differences were noted, nor was a significant difference detected when the set of F_1 crosses with a high betaine female parent was contrasted with those with a low-betaine female parent. However, the F_1 values, were generally lower than would be predicted from midparent values (Table 1).

1.4.2 Partial Diallel Analysis - Estimation of GCA and SCA

Betaine contents of the 13 parents and their 68 F_1 hybrids are shown in Figure 1. The range of betaine levels in the hybrids (range = 9.5 to 26.8 u mol·g dry wt^{-1}) resembled that of the parents (range = 9.1 to 28.9 u mol·g dry wt^{-1}). High-betaine X high-betaine crosses generally gave high F_1's and low X low crosses generally gave low F_1's, although there were cases where the F_1 means were significantly different from their midparent values (Fig. 1). Two of the low- X low-betaine crosses were higher than their respective midparent values ($LSD_{0.05}$). When those F_1's and their parents were retested in a separate replicated trial for possible complementation, no significant differences were found among the parental and F_1 genotypes.

Significant variation for betaine content was detected

Table 2. Analysis of variance and orthogonal comparisons for betaine content of four pairs of reciprocal F_1 crosses and their parents.

Source	df	Mean squares
Genotype	11	225.1**
High- vs. low parents	1	1501.5**
(A X C) vs. (C X A)†	1	4.3
(A X D) vs. (D X A)	1	24.5
(B X C) vs. (C X B)	1	6.1
(B X D) vs. (D X B)	1	21.6
High females vs. low females	1	21.1
Error	87	19.0

**Significant at the 0.01 probability level.

†Parents A,B,C, and D were CI nos. 3480, 14936, 13057 and 15293, respectively. Parents A and B are high in betaine content, C and D are low (Table 1).

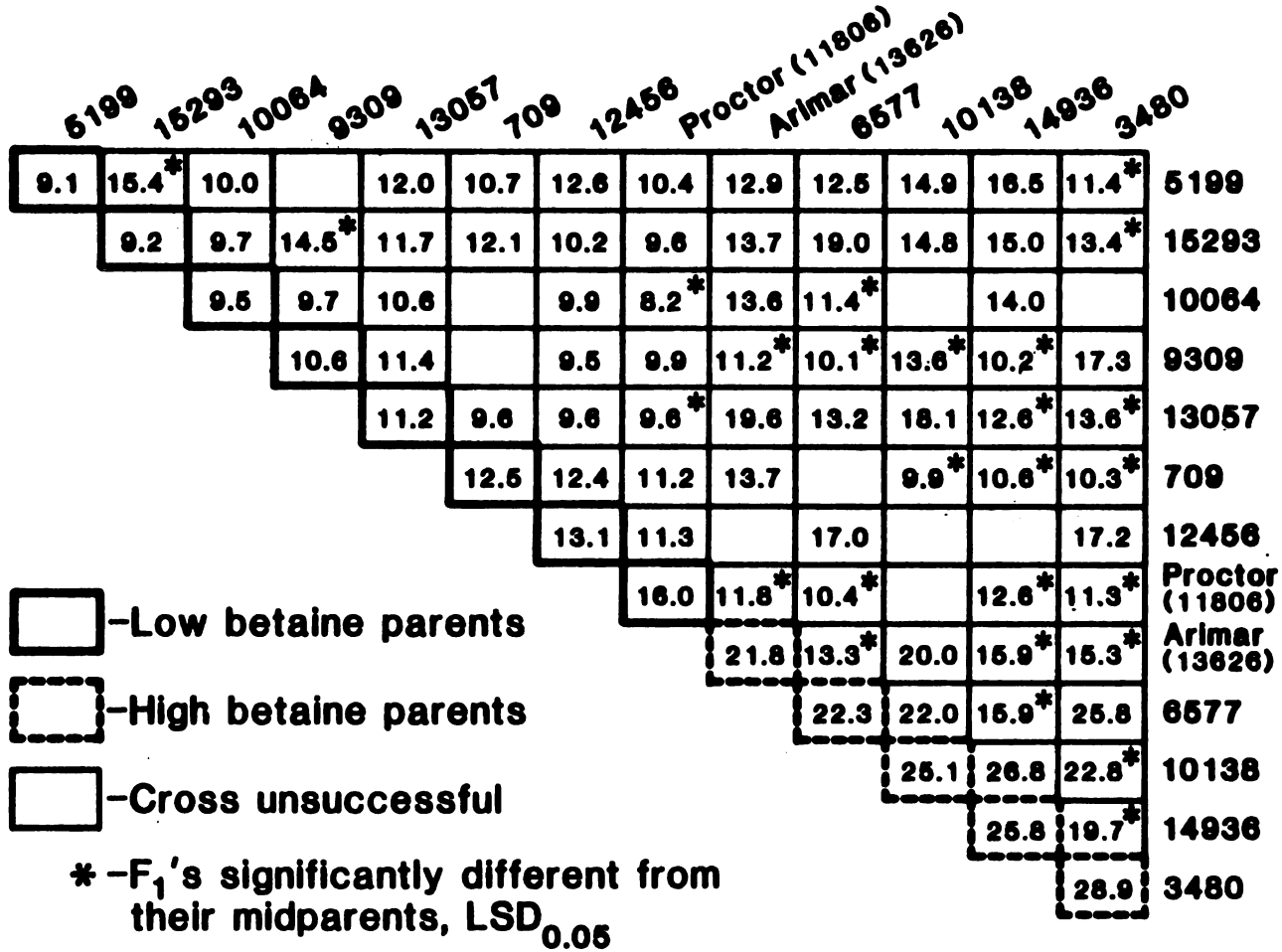


Figure 1. Betaine levels (umol g^{-1} dry wt) of the 13 parents and the 68 F_1 hybrids used in the partial diallel.

among the genotypes entered in the partial diallel (Table 3). Both GCA and SCA effects were significant; the relative contributions to total genetic effects were 74.6% and 25.4% respectively.

1.4.3 Generation Mean Analysis - Estimation of Genetic Effects

The distribution of betaine content for the six generations analyzed showed that a simple additive-dominance model was not sufficient to describe the betaine trait (Fig. 2, Table 4). The additive-dominance model was significantly improved by including epistatic effects, but not by including genotype X environment interactions. The linear regression coefficients for the genetic component of a model containing environmental, additive, dominance and epistatic effects (model 5) showed that when tested individually, only the coefficient for additive gene action was significantly different from zero. It may be noted, however, that the distribution of betaine content in the F_2 generation is skewed to the low end (Fig. 2).

1.4.4 Heritability Estimates

Agreement was noted among heritability estimates based on the three groups of genotypes tested and the three different methods used. The narrow-sense estimate based on midparent-offspring regression was 0.53 while the narrow-sense estimate based on generation variances was 0.63. The broad-sense heritability calculated from the replicated trial data of Ladyman et al. (1983) was 0.53.

Table 3. Analysis of variance for GCA and SCA of betaine content and relative contributions of GCA and SCA effects.

Source	df	Mean squares	Contribution to genetic effects
Entries	80	45.5**	
General combining ability (GCA)	12	226**	74.6%
Specific combining ability (SCA)	68	11.8**	25.4%
Error	136	5.67	

**Significant at the 0.01 probability level.

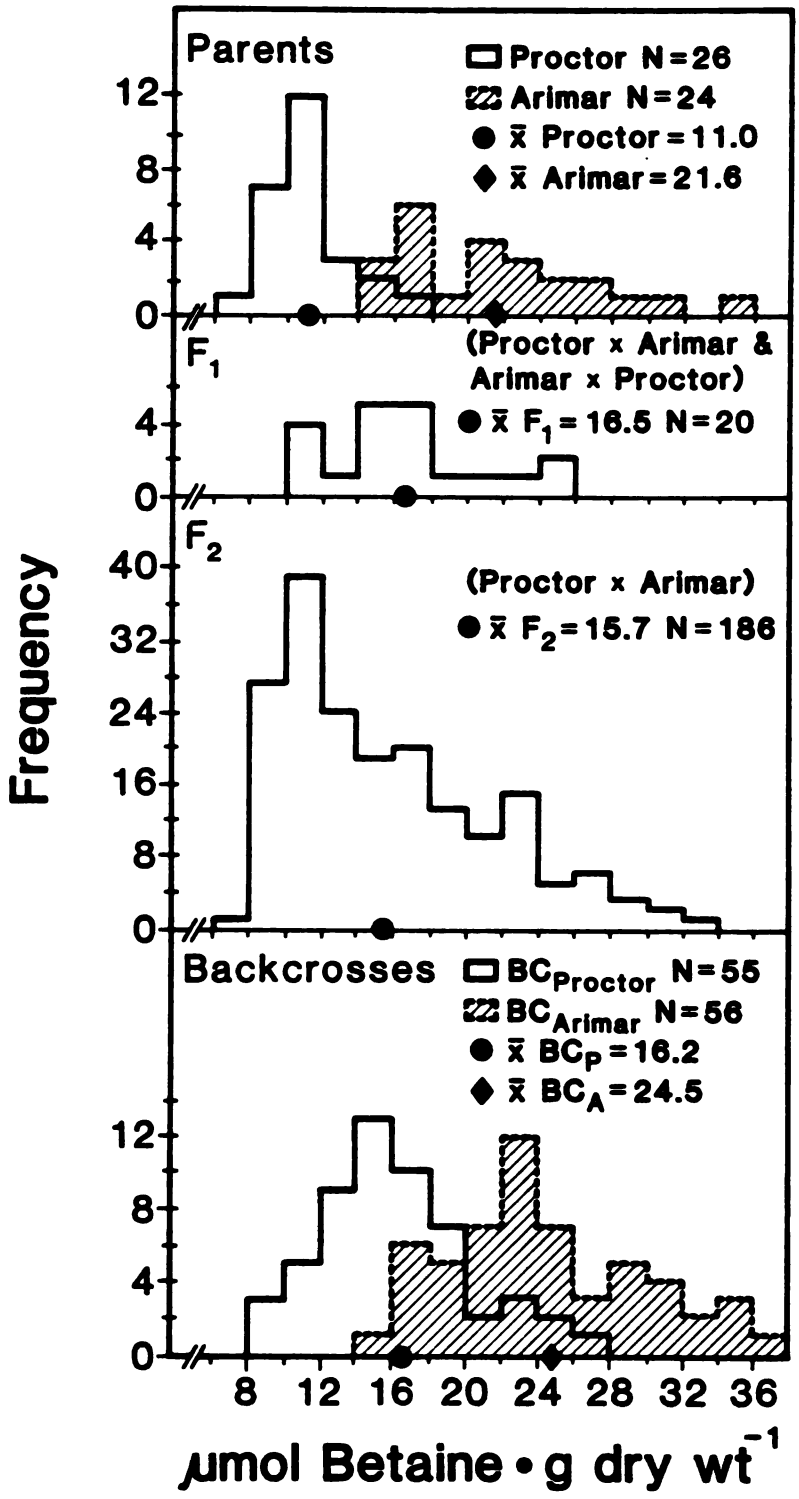


Figure 2. Distribution of betaine content in Proctor, Arimar, and their F_1, F_2, and two backcross generations.

Table 4. Generation mean analysis of Proctor, Arimar, and their F_1, F_2, and two backcross generations for betaine content.

I. Sequential modeling, weighted least squares regression.

Model	Description	df	X^2	% Variability accounted for
1.	Environment	8	<0.001	46.9
2.	Environment + additive	7	<0.005	95.6
3.	Environment + additive + dominance	6	<0.005	95.9
4.	Environment + additive + dominance + genotype X environment interaction	3	<0.005	97.0
5.	Environment + additive + dominance + epistatic	3	0.203	99.0

II. Genetic effects estimated from model 5.

Effect		Regression coefficient ± S.E.	t
Additive		-4.94 ± 0.58	-8.47**
Dominance		15.21 ± 6.56	2.32
Epistatic:	A X A	15.40 ± 6.48	2.38
	A X D	33.49 ± 12.27	2.73
	D X D	-6.08 ± 5.86	-1.04

**Significantly different from zero at the 0.01 probability level by a t-test with 3 df.

1.5 Discussion

Four pairs of reciprocal F_1 high- X low- and low- X high-betaine crosses were used to test for maternal and cytoplasmic inheritance of the betaine trait. Because no significant reciprocal differences were observed among the F_1's, we conclude that maternal and cytoplasmic effects do not influence the betaine trait in these genotypes and that inheritance of betaine content is likely to be controlled by nuclear genes.

In the partial diallel, GCA effects were larger than SCA effects for the genotypes entered. GCA effects were approximately 75% of the total genetic effects, indicating that the betaine trait in these genotypes is primarily attributable to additive genetic effects. Consistent with an additive trait was the lack of overall transgressive variation and the lack of complementation among the diallel F_1's. Additive genetic effects were also the most important contributor to the genetic model obtained by generation mean analysis. Environmental and additive effects accounted for 95.6% of the total variability and the estimation of genetic effects showed that additive genetic effects were the only individually significant factor. We conclude from the generation mean analysis and the partial diallel analysis that betaine content is predominantly an additive trait.

Although additive genetic effects are more important than non-additive effects for betaine accumulation, the

presence of significant SCA effects, and the occurrence of many F_1's in the partial diallel whose betaine levels were below the midparent values implicate some dominance effects. That the F_1 values obtained from the reciprocal high X low crosses were generally lower than the midparent values is also consistent with dominance of the low betaine character, as is the skewed distribution of betaine in the F_2 generation from the Proctor X Arimar cross (Fig. 2).

Heritability estimates suggest that variation for betaine content in barley has a relatively large genetic component (>50%). When evaluating the heritability estimate based on midparent-offspring regression, the following considerations should be taken into account. Firstly, heritability estimates are based on the assumption that the parents are a random sample from the population at large (Falconer, 1981), but in this experiment, parental lines had been chosen for high- or low-betaine content. It has been demonstrated, however, that although selection of parents may reduce precision, it does not bias the estimate from midparent-F_1 regression (Falconer, 1981). Secondly, because parents and offspring were grown in the same environment, there is a genotype X environment bias which may inflate the estimate of heritability (Casler, 1982). Despite these reservations, the midparent-offspring value (0.53 $\pm$ 0.08) was consistent with the other two heritability estimates and had a low standard error.

A more important consideration relevant to all three heritability values, is that because any heritability estimate is a ratio of genotypic and environmental variance, high values may be obtained by a large genetic component, by a low environmental component, or both. The plants used in these experiments were grown in controlled environment chambers, so it is reasonable to assume that the heritability estimates are higher than would be found in field trials. We therefore conclude that 50-60% is an upper limit for the heritability of betaine content in barley. We note that the attributes of the betaine trait (nuclear, predominantly additive, high heritability) make it amenable to plant breeding efforts--should physiological-genetic studies establish its adaptive worth.

1.6 Literature Cited

Allard, R.W. 1960. Principles of plant breeding. John Wiley & Sons, Inc. New York. 485 p.

Casler, M.D. 1982. Genotype X environment interaction bias to parent-offspring regression heritability estimates. Crop Sci. 22:540-542.

Coughlan, S.J., and R.G. Wyn Jones. 1980. Some responses of Spinacea oleracea to salt stress. J. Exp. Bot. 31:883-893.

Falconer, D.S. 1981. Introduction to quantitative genetics. Longman, New York. 340 p.

Ford, C.W., and J.R. Wilson. 1981. Changes in the level of solutes during osmotic adjustment to water stress in leaves of four tropical pasture species. Aust. J. Plant Physiol. 8:77-91.

Griffing, B. 1956. Concept of general and specific combining ability in relation to diallel crossing systems. Aust. J. Biol. Sci. 9:463-493.

Hall, J.L., D.M.R. Harvey, and T.J. Flowers. 1978. Evidence for the cytoplasmic localization of betaine in leaf cells in Suaeda maritima. Planta 140:59-62.

Hanson, A.D., and W.D. Hitz. 1982. Metabolic responses of mesophytes to plant water deficits. Annu. Rev. Plant Physiol. 33:163-203.

Hanson, A.D., and R. Wyse. 1982. Biosynthesis, translocation, and accumulation of betaine in sugarbeet and its progenitors in relation to salinity. Plant Physiol. 70:1191-1198.

Hitz, W.D. and A.D. Hanson. 1980. Determination of glycinebetaine by pyrolysis - gas chromotography in cereals and grasses. Phytochemistry 19:2371-2374.

Hitz, W.D., J.A.R. Ladyman, and A.D. Hanson. 1982. Betaine synthesis and accumulation in barley during field water-stress. Crop Sci. 22:47-54.

Jolivet, Y., F. Larher, and J. Hamelin. 1982. Osmoregulation in halophytic higher plants: The protective effect of glycinebetaine against the heat destabilization of membranes. Plant Sci. Lett. 25:193-201.

Ladyman, J.A.R., K.M. Ditz, R. Grumet, and A.D. Hanson. 1983. Genotypic variation for glycinebetaine accumulation by cultivated and wild barley in relation to water stress. Crop Sci. 23: 465-468.

Larkum, A.W.D., and R.G. Wyn Jones. 1979. Carbon dioxide fixation by chloroplasts isolated in glycinebetaine. Planta 145:393-394.

Leigh, R.A., N. Ahmad, and R.G. Wyn Jones. 1981. Assessment of glycinebetaine and proline compartmentation by analysis of isolated beet vacuoles. Planta 153:34-41.

Mather, K., and J.L. Jinks. 1977. Introduction to biometrical genetics. Cornell Univ. Press, Ithaca, N.Y. 231 p.

Pollard, A., and R.G. Wyn Jones. 1979. Enzyme activities in concentrated solutions of glycinebetaine and other solutes. Planta 144:291-298.

Rowe, K.E., and W.L. Alexander. 1980. Computations for estimating the genetic parameters in joint-scaling tests. Crop Sci. 20:109-110.

Storey, R., and R.G. Wyn Jones. 1978. Salt stress and comparative physiology in the Gramineae. III. Effect of salinity upon ion relations and glycinebetaine and proline levels in Spartina townsendii. Aust. J. Plant Physiol. 5:817-829.

Storey, R., and R.G. Wyn Jones. 1979. Response of Atriplex spongiosa and Suaeda monoica to salinity. Plant Physiol. 63:156-162.

Strom, A.R., D. LeRudulier, M.W. Jacowec, R.C. Bunnell, and R.C. Valentine. 1983. Osmoregulatory genes and osmoprotective compounds. p. 39-59. In T. Kosuge, C.P. Meredith, and A. Hollaender (ed.) Genetic engineering of plants - An agricultural perspective. Plenum Press, New York.

Wall, J.S., D.D. Christianson, R.J. Dimler, and F.R. Senti. 1960. Spectrophotometric determination of betaines and other quaternary nitrogen compounds as their periodides. Anal. Chem. 32:870-874.

Warner, J.N. 1952. A method for estimating heritability. Agron. J. 44:427-430.

Wyn Jones, R.G., and R. Storey. 1981. Betaines. p. 171-204. In L.G. Paleg and D. Aspinall (ed.) Physiology and biochemistry of drought resistance. Academic Press, Sydney.

Wyn Jones, R.G., R. Storey, R.A. Leigh, N. Ahmad, and A. Pollard. 1977. A hypothesis on cytoplasmic osmoregulation. p. 126-136. In E. Marre and O. Ciferri (ed.) Regulation of cell membrane activities in plants. Elsevier Press, Amsterdam.

CHAPTER II

GENETIC EVIDENCE FOR AN OSMOREGULATORY FUNCTION OF GLYCINEBETAINE ACCUMULATION IN BARLEY

2.1 Abstract

Betaine (glycinebetaine) accumulates in several plant families in response to water- or salt-stress. Although betaine is hypothesized to be central to cytoplasmic osmoregulation, there is no direct evidence for this. We therefore genetically altered betaine level in barley by creating two isopopulations differing significantly in mean betaine level in unstressed condtions (22.8 and 32.4 umol·g^{-1} dry wt, respectively). To minimize linkage effects, the isopopulation procedure included several parents and two rounds of crossing. Measurements of various morphological and developmental characters indicated that the two populations were otherwise genetically comparable.

Response to salinization (from 0 - 300 mM) of the high- and low-betaine isopopulations was compared with that of the high- and low-betaine parents. NaCl at 300 mM caused an 8-10-fold increase in betaine level in the two populations and a 5 bar drop in solute potential (ψ_s). The difference in

betaine level between the two populations was constant with salinization. Although selected only for differing betaine level, the parents and isopopulations differed also for ψ_s; the high betaine genotypes maintained a ψ_s 1 bar lower than the low betaine genotypes at all salt levels. Furthermore, in both populations and parents, betaine level was linearly related to ψ_s (r^2=0.99) implying co-ordinate regulation of the two traits. These observations are most readily explained if betaine accumulation is a mandatory component of osmoregulation in barley.

2.2 Introduction

Betaine (glycinebetaine, N,N,N-trimethylglycine) accumulates in a number of plant families in response to water- or salt-stress and is hypothesized to function as a non-toxic or compatible cytoplasmic osmoticum (Wyn Jones et al., 1977). Several lines of evidence support the idea that betaine has an osmoregulatory function: many halophytes accumulate betaine (Wyn Jones and Storey, 1981); the amount of betaine accumulated by various salt-tolerant plants is directly proportional to the external salt concentration (Hanson and Wyse, 1982; Coughlan and Wyn Jones, 1982; Storey and Wyn Jones, 1978); and steady accumulation of betaine coincides with a decline in solute potential during

long-term water deficit in the field in barley and tropical pasture grasses (Hitz et al., 1982; Ford and Wilson, 1981).

A cytoplasmic location for betaine is supported by electron microscopy studies (Hall et al., 1978) and analyses of isolated vacuoles (Leigh et al., 1981), which indicate a disproportionate amount of betaine is in the cytoplasm relative to the vacuole. Experiments with isolated chloroplasts (Larkum and Wyn Jones, 1979), enzymes (Pollard and Wyn Jones, 1979; Paleg et al., 1981), and ribosomes (Brady et al., 1984) confirm that betaine is compatible with metabolic functions. Also, exogenous betaine has been shown to protect bacteria grown in high salt conditions (LeRudulier et al., 1984).

Although this evidence is consistent with the hypothesis that betaine acts as a cytoplasmic osmoticum, it is circumstantial. There is not yet direct evidence for any such beneficial function of betaine in plant cells, nor can the possibility that betaine accumulation is an injury response be eliminated (Strom et al., 1983; Hanson and Grumet, 1984). We therefore chose a physiological-genetic approach to investigate the effect of altered betaine levels on the response of barley (Hordeum vulgare L.) plants to osmotic stress.

In this work, we used naturally occurring variability for betaine level within the barley gene pool (Ladyman et al., 1983) to select several high- and low-betaine

genotypes. Since the betaine trait in barley is nuclear and highly heritable (Grumet et al., 1985), these genotypes were then used to create two isopopulations differing in betaine level. Isopopulations have the advantages over isogenic lines that they can be used for multigenic traits and require a relatively small number of generations to create (Quizenberry, 1982; Burton,1968).

Salt stress experiments provide an opportunity to measure osmoregulation in defined, steady state conditions and so the parental genotypes and high- and low-betaine isopopulations were tested for their response to salinity. In this paper we describe the process of isopopulation development and present results of salt stress trials.

2.3 Materials and Methods

2.3.1 Isopopulation development.

In the simplest case isopopulation development involves divergent selection among F_2 progeny of high- X low-betaine crosses for high- or low-betaine individuals that are then used to establish isopopulations (Burton, 1968; Eslick and Hockett, 1974). Ideally, all non-betaine traits are segregating randomly, but in practice, linkage associations between betaine and other stress-related, non-betaine traits could bias performance of the isopopulations (Burton, 1968; Eslick and Hockett, 1974; Rasmusson and Gengenbach, 1983). Because betaine accumulation is a metabolic trait, it could

be overshadowed by morphological or phenological characaters that confer stress resistance. We therefore took additional steps to minimize potentially confounding effects of linkage: (a) Several high and several low parents of diverse origins were crossed in order to increase the range of phenotypes for all non-betaine traits and to vary the combinations of alleles at linked loci; (b) A second round of crossing was included to further mix the parental genotypes and break linkages.

The isopopulation development procedure is summarized in Figure 3. Since betaine content of young, unstressed plants can be used to predict genetic potential for betaine accumulation during stress (Ladyman et al., 1983), all screening for betaine content was of 2- to 3-week old plants grown in well-watered conditions in the growth chamber. Plant growth conditions for crossing and screening were as in Grumet et al. (1985). Betaine content of the shoots was measured using the periodide assay described in Chapter 1.

Criteria for the choice of parents were: (1) High or low betaine content as measured in replicated trials in well-watered and water-stressed conditions in the growth chamber and field (Ladyman et al., 1983); (2) Compatibililty of flowering dates for crossing. Seventy accessions of H. vulgare and 269 of its wild relative H. spontaneum C. Koch were evaluated (Ladyman et al., 1983); since the range in betaine contents for both groups was very similar, only H.

PROCEDURE FOR MODIFIED ISOPOPULATION DEVELOPMENT

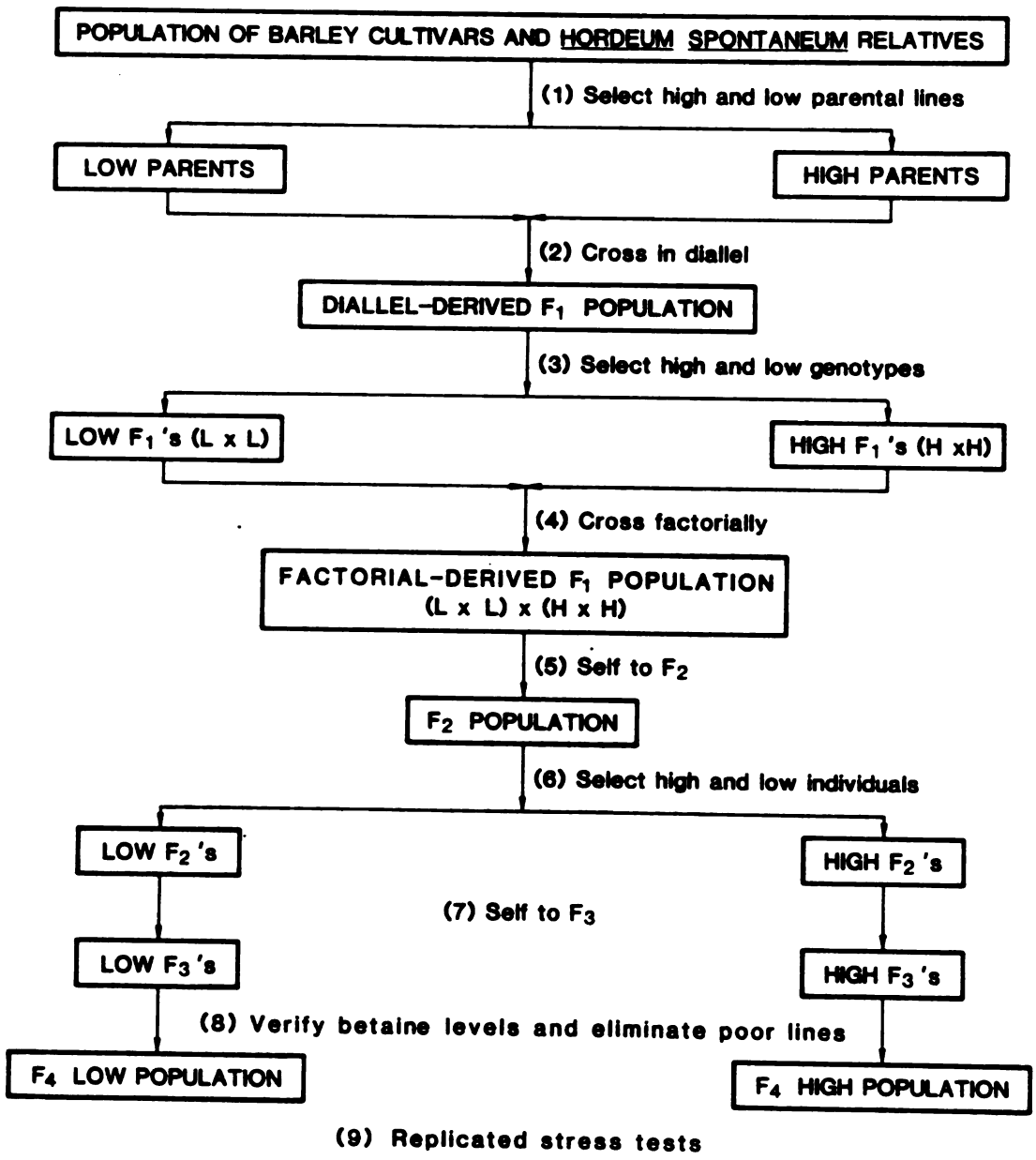


Figure 3. The modified isopopulation development procedure.

vulgare genotypes were chosen. Eight low betaine accessions (CI# 709, 5199, 9309, 10064, 11806, 12456, 13057, 15293) and five high betaine accessions (CI# 3480, 6577, 10138, 14936) were used to initiate the populations; all were spring barleys. Seed was obtained from the USDA Small Grains Collection (Beltsville, MD) and multiplied in the greenhouse.

All possible crosses among the 13 parents were attempted. Seed was obtained for 68 of the possible 78 hybrid combinations. The 68 F_1 genotypes and 13 parental genotypes were grown in a 9 X 9 triple lattice design and analyzed for betaine content. Each block was a 16 cm diam. pot containing 9 entries. The seven hybrids highest in betaine, and the seven lowest, were selected for the second, factorial round of crossing. Thirty-eight of the 49 possible double-cross hybrid combinations were successful. One plant per hybrid was selfed to give the double-cross F_2 population. Ten individuals per double cross F_2 family (380 total) were screened for betaine; the top and bottom 10% were selected. The selected F_2 individuals were selfed to form F_3 families.

To enable screening, the 76 high or low F_3 families were split into 9 planting sets. Each pot contained one representative of each family in a given set. Five replicates of each set were grown in a random design. The five F_3 plants per family were bulked at harvest and

analyzed for betaine. The number of high and low families was cut approximately in half. Distribution of betaine levels was checked in the F_4 generation using seed derived from 30 plants per selected F_3 family. Seed from the F_4 families were randomly assigned into 5 planting sets and grown in a randomized design with 3 replicates.

2.3.2 Salt stress experiments with isopopulations and parent mixes.

Plant material and growth conditions. Parent or population mixes were prepared with an equal number of seeds per high or low parental genotype or F_4 family, respectively. The stratification and growth chamber conditions were as previously described (Grumet et al., 1985) with a PPFD of ca. 150 uE m^{-2} sec^{-1}. A split plot design with 5 replicates was used. The main effect, salt level (0, 100, 200, 300 mM NaCl in 50% Hoagland's solution), was assigned using a randomized complete block design; the isopopulations or parent mixes were the subtreatment. Clay pots (21 cm diam.) filled with vermiculite were marked in half; 5 high or 5 low plants were grown in each half. The plants were irrigated daily for 10 days with 50% Hoagland's solution, then daily with stepwise increases of 50 mM NaCl every second day. Each pot was supplied with 1 liter/day to allow for leaching of salts. They were maintained at their respective salt levels until 8 days after the highest level was reached at which time they were harvested.

Growth measurements and betaine determinations. Shoot fresh weight of the 5 plants per sample was measured immediately at harvest. One youngest, fully expanded leaf per sample was removed for solute potential (ψ_s) measurements (see below). The remaining shoot material was reweighed, frozen in liquid N_2, and freeze-dried. Dry weights were corrected for the removed leaf. The dried samples were ground in a Wiley mill to mesh size 40 for betaine and ash weight determinations. For comparisons with osmotic potential, betaine levels were expressed on a fresh weight basis using the appropriate fresh weight/dry weight ratios. Ash content was determined by incinerating 400-mg samples in ceramic crucibles at 480 C for 8 h.

In one experiment root dry weights were estimated as follows. The roots were freed from the vermiculite by washing with water, frozen in liquid N_2, oven dried at 70 C for 2 d, and weighed. The samples were ground and ashed as described above to correct for trapped vermiculite.

Solute potential measurements. To distinguish solute accumulation from passive concentration, all ψ_s measurements were corrected to 100% relative water content (RWC) using a similar method to that of Ludlow et al. (1983). One youngest, fully expanded leaf blade per sample was put into a test tube with 5 ml distilled H_2O, covered with a plastic bag and kept in a dark cabinet overnight at 20 C to hydrate before sampling for ψ_s. One 5 mm diameter

punch was removed approximately 5 cm from the base of the blade, sealed in a foil envelope, and frozen in liquid N_2. Tests showed that betaine levels of the portion of the shoot sampled for ψ_s were representative of the shoot as a whole (data not shown). The disks were stored at -20 C in a sealed container for up to one week before ψ_s determination. No trend indicating dehydration during storage was observed when ψ_s values of replicates determined throughout the week were compared. Two more punches, one on each side of the first, were removed and pooled with the other reps of a given treatment (10 punches/treatment). They were fresh-weighed and then floated on distilled H_2O for 3-4 h, blotted and reweighed (floated weight), and then dried in an oven for 1 d at 70 C to obtain dry weight. A correction factor (Barrs, 1968) [(fresh weight - dry weight)/(floated weight - dry weight)] was used to adjust ψ_s to 100% RWC; all samples used for ψ_s measurements were at or above 90% RWC.

A Wescor HR-33T Dewpoint Microvoltmeter equipped with C-52 sample chambers was used to determine ψ_s. Leaf disks were thawed just before placing in the sample chambers. After equilibration (1 h), 2 or 3 consecutive readings/sample were taken at 15-min intervals using the dewpoint mode. All chambers were checked regularly to be sure they read within 5% of the predicted value of a 0.61 M sucrose standard.

2.3.3 Characterization of individual parental genotypes.

A randomized complete block design with 4 or 6 replicates was used for the 13 genotypes. Each 21 cm diam. clay pot contained 1 plant/genotype planted in vermiculite; the shoots were harvested at 17 or 27 d. Fresh weight and ψ_s were determined as above. Because each sample consisted of only one plant, the leaf used for ψ_s measurement was frozen in liquid N_2 after removing the punches and pooled with the rest of the sample for dry weight and betaine measurements.

2.3.4 Statistical analyses.

The salt stress experiments with the isopopulations and the parent mixes were repeated 3 times. The first experiment with the parent mixes had a maximum salt level of 200 mM NaCl; all other experiments included a 300 mM NaCl treatment. Pooled analyses of variance were calculated using the data from all 3 experiments for the isopopulations and for the two experiments including 300 mM NaCl for the parent mixes. There were no significant treatment X experiment interactions.

Determination of ψ_s of the individual parental genotypes was repeated 3 times. The dry weight/fresh weight ratios were measured in two of these experiments. The pooled data for the ψ_s and ratios were analyzed using an unweighted analysis of variance.

2.3.5 Exogenous betaine experiments.

Seeds (CI# 11806, multiplied Mesa, Az, 1978) were dehusked and surface sterilized by soaking with continuous stirring for 7 min in 70% ethanol and 10 min in 5.25% (w/v) Na-hypochlorite with 0.5% (v/v) Tween 20 (Sigma Chemical Co., St. Loius, Mo.). All subsequent work was done in sterile conditions until the plants were transplanted into vermiculite. The seeds were rinsed with distilled H_2O and germinated on wet filter paper in Petri dishes in the dark at 20 C for 3 d.

Twenty-two germinated seedlings were placed between halves of sponge stoppers, inserted into 25-ml vials containing 10 ml of 50% Hoagland's solution, and transferred to the growth chamber for 4 d. 450 umol of betaine then was added to 9 of the vials to give ca. 50 mM betaine solution. The plants were returned to the growth chamber for 4 d (2-leaf stage) at which time volume uptake was noted (average uptake in the control and betaine treated samples was 4.3 and 3 ml, respectively). The plants were removed and the solution checked for contamination by plating 100 ul subsamples on nutrient broth agar. Plants from contaminated vials were discarded.

The plants were individually transplanted into vermiculite in 250 ml pots, kept in the growth chamber, and watered daily with 50% Hoagland's solution for 18 d (5 leaf stage). Any plants with morphological abnormalities were

discarded at this stage. Fresh and dry weights, ψ_s, and betaine were determined as above for the individual parental genotypes.

2.4 Results

2.4.1 Characterization of the parents.

In unstressed conditions, the high and low betaine parents differed approximately two-fold in mean betaine level (Fig. 4a). They did not differ with respect to average values and ranges for the following morphological and phenological characters: plant height, leaf color and shape, stem color, number of rows/head, head length and shape, and heading date. However, the high betaine genotypes had systematically lower ψ_s and higher dry weight/fresh weight ratios (Fig. 4 b,c).

High- and low-betaine parents were tested for their response to salt stress as high or low mixtures in which all genotypes were equally represented. Plants in all stress treatments were turgid and showed no injury symptoms. Salinization inhibited growth as measured by shoot fresh and dry weight (Fig. 5 a,c) to a similar extent in both the high and low parent mixes. In both mixes, salinization increased the dry weight/fresh weight ratio (Fig. 5e); the ca. 5 mg dry wt/g fresh wt difference between highs and lows remained constant with increasing salt.

Figure 4. Betaine levels (A), solute potentials (B), and mg dry weight/g fresh weight (C) for the 13 parental genotypes used to initiate the isopopulations.

A. Data are the mean of 4 reps.
B. Data are pooled from 3 experiments (4 or 6 reps/experiment). The difference between the highs and lows is significant by AOV (P=0.01).
C. Data are pooled from two experiments (4 and 6 reps/experiment). The difference between highs and lows is significant by AOV (P=0.01).

No low betaine parent had a significantly lower ψ_s or higher dry weight/fresh weight ratio than any high betaine parent by LSD (P=0.05).

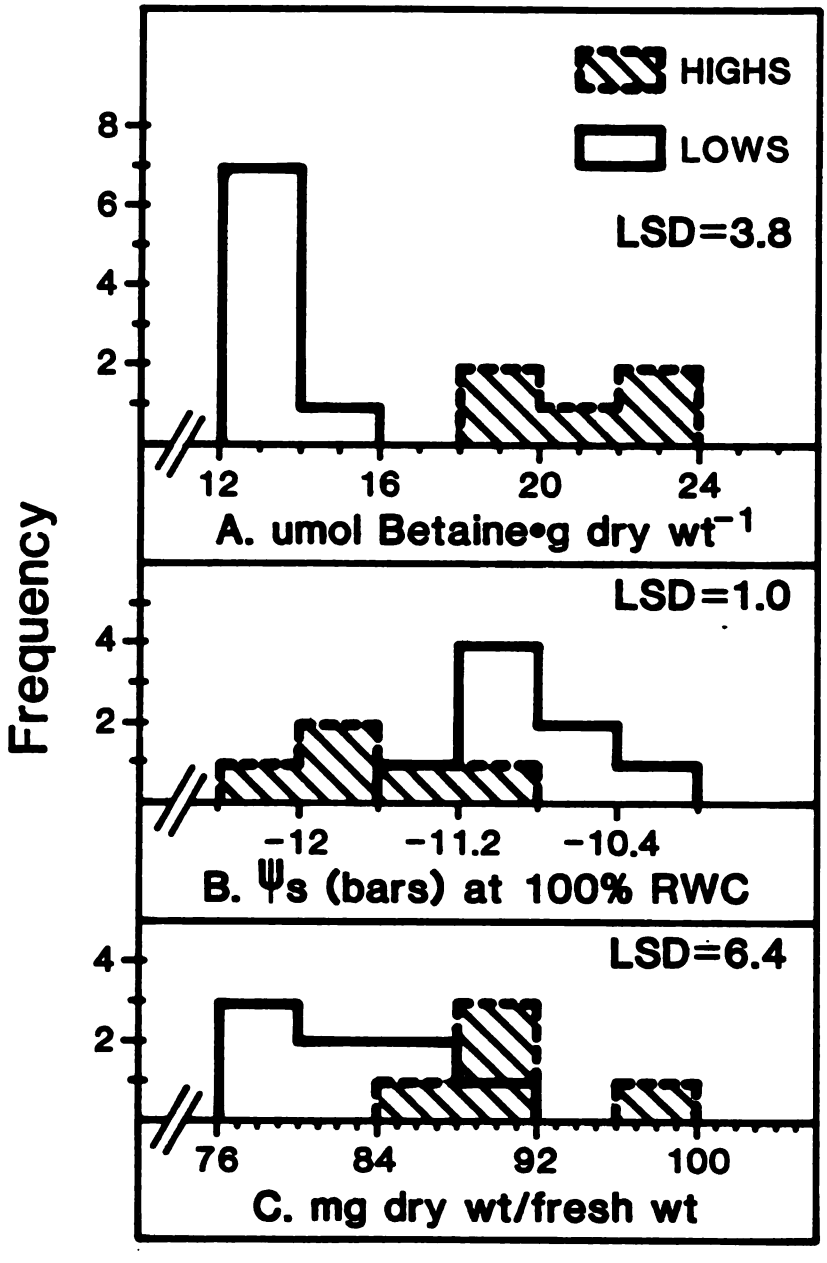

HIGHS
LOWS
LSD=3.8
8
6
4
2
12
16
20
24
A. umol Betaine•g dry wt^{-1}
LSD=1.0
4
2
-12
-11.2
-10.4
B. Ψs (bars) at 100% RWC
LSD=6.4
4
2
76
84
92
100
C. mg dry wt/fresh wt
Frequency

Figure 4

Figure 5. Growth responses: fresh weight (A,B), dry weight (C,D), and mg dry weight/g fresh weight (E,F), of the parent mixes and isopopulations to increasing salt.

Each sample consists of 5 plants. Each point is the pooled data from 3 experiments (5 reps/ experiment).

Salt level had a highly significant effect (AOV; P=0.01) on all 3 parameters. The highs and lows were only significantly different for mg dry weight/g fresh weight (AOV; P=0.01).

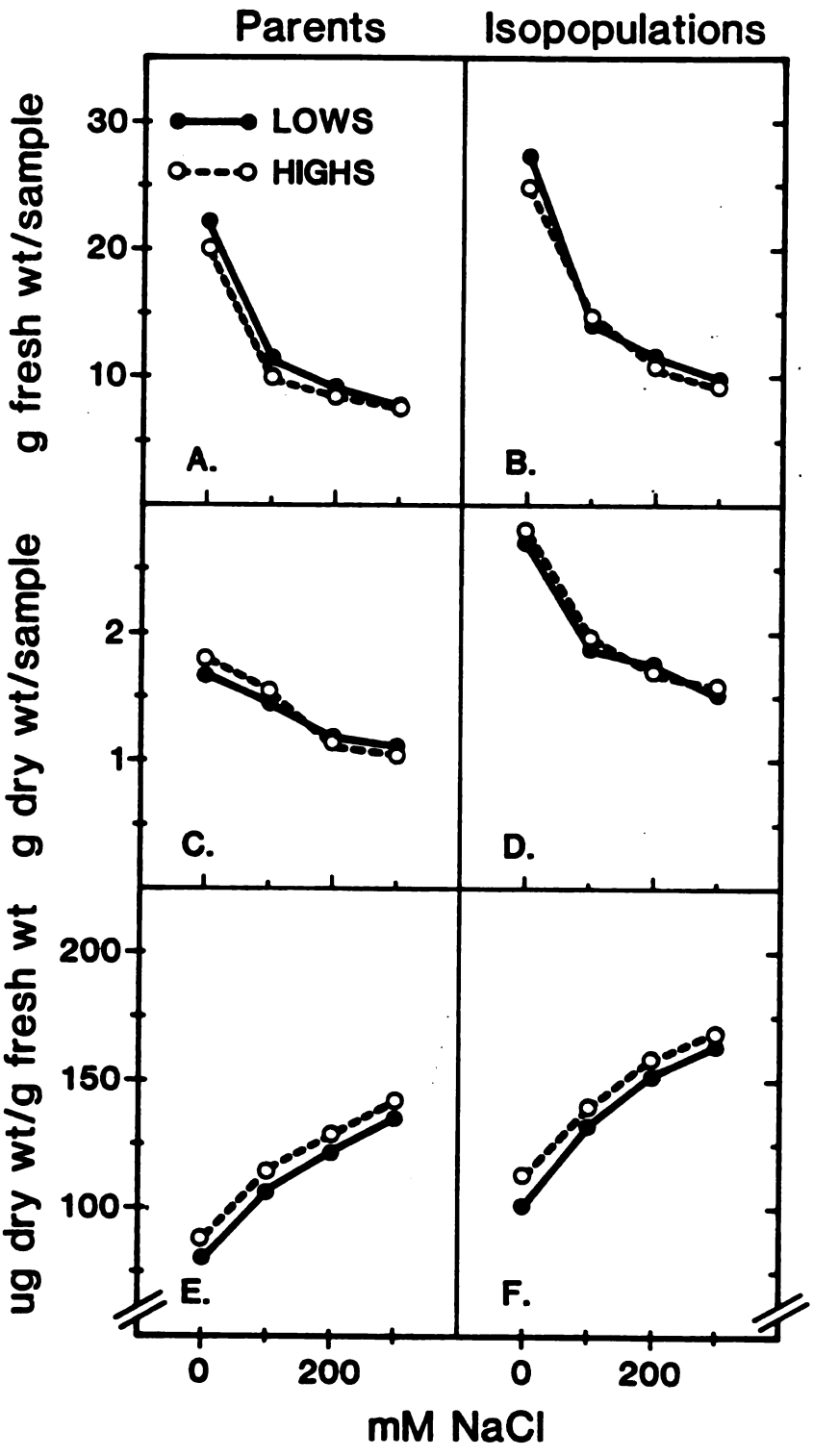

Parents
Isopopulations
LOWS
HIGHS
g fresh wt/sample
g dry wt/sample
ug dry wt/g fresh wt
30
20
10
2
1
200
150
100
A.
B.
C.
D.
E.
F.
0
200
0
200
mM NaCl

Figure 5

As expected, salinization also caused a decrease in ψ_s at 100% RWC in both groups of parents (Fig. 6a). This osmotic adjustment was linear (r^2=0.99), but partial. There was an approximately 1.7 bar drop/100 mM NaCl vs. a predicted 4.4 bar drop based on osmotic potential of the salt solutions (Wyn Jones and Gorham, 1983). In an experiment where ψ_s was measured using non-rehydrated leaves (not shown) there was a total drop in ψ_s of 4.3 bars/100 mM NaCl for both highs and lows; the difference between highs and lows persisted. There was a highly significant difference (AOV; P=0.01) between the high and low parent mixes for ψ_s (Fig. 6a). The high betaine genotypes maintained a consistently lower ψ_s at all salt levels by approximately 1 bar. The total adjustment with respect to salt (ca. 5 bars with 300 mM NaCl) was equivalent for the two groups.

Betaine levels rose in salinized plants (Fig. 7a); the absolute difference between the high and low betaine groups persisted at all salt concentrations but the relative difference decreased.

2.4.2 Isopopulation development.

Distribution of betaine contents measured in unstressed conditions throughout isopopulation development is shown in Figure 8. There was no transgressive variation in any generation. The seven highest and seven lowest diallel-derived hybrids (8b, shaded) were selected for the

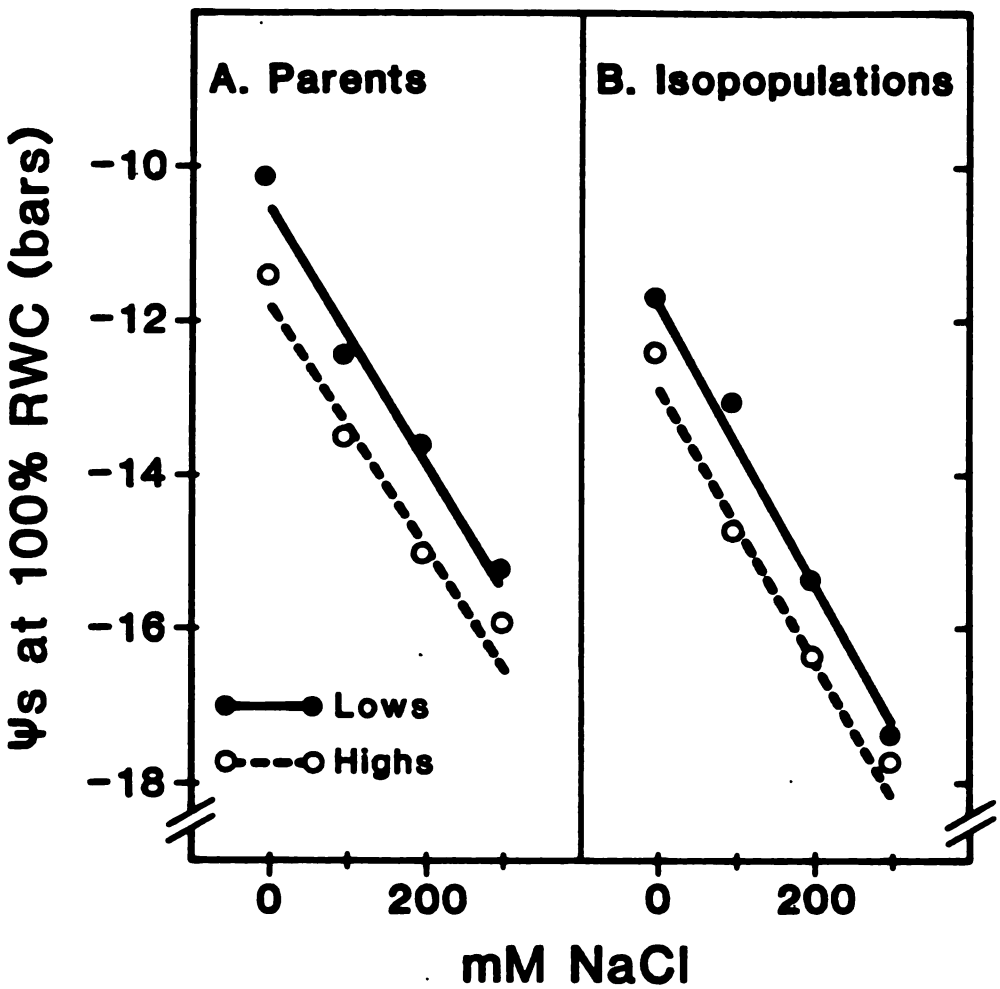


Figure 6. Solute potentials of the parent mixes (A) and isopopulations (B) in response to salt stress.

Data are pooled results of 3 experiments (5 reps/experiment).

The salt and genotype effects were highly significant (AOV; P=0.01).

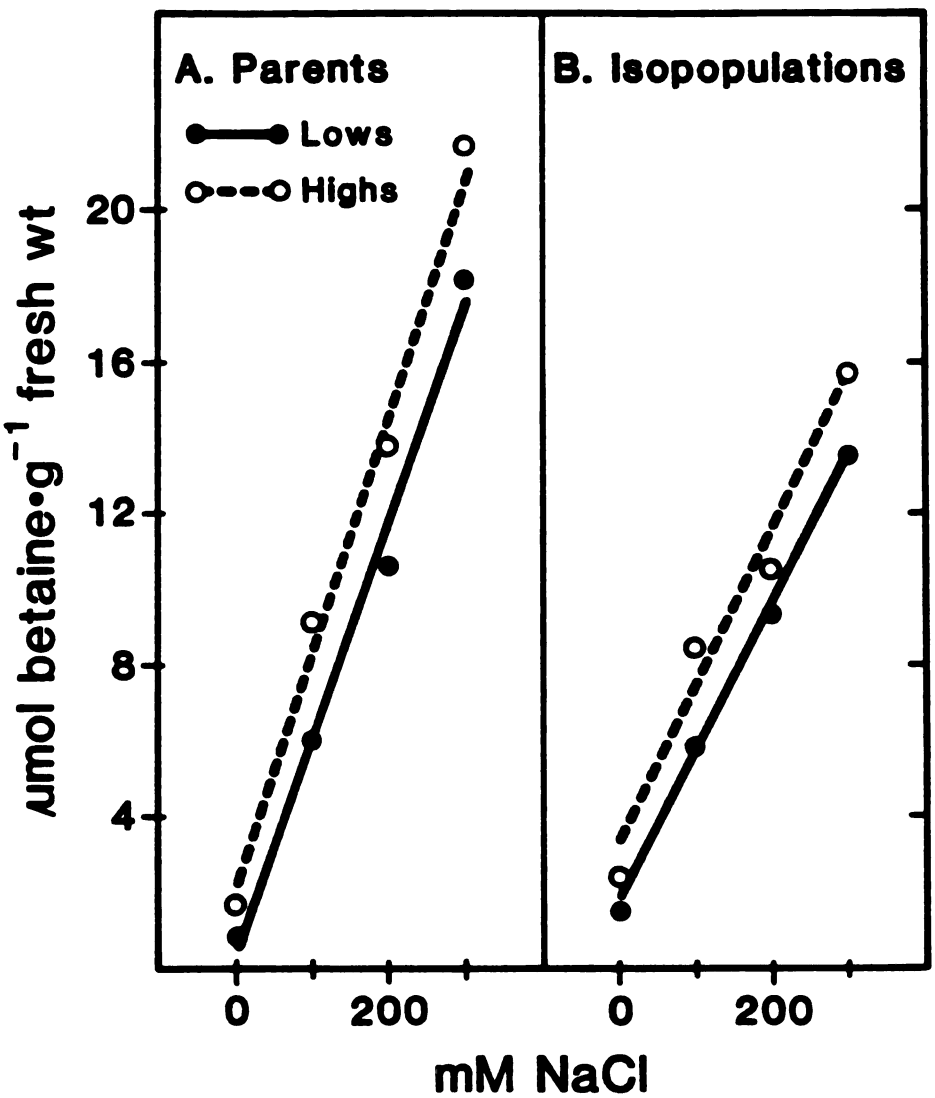
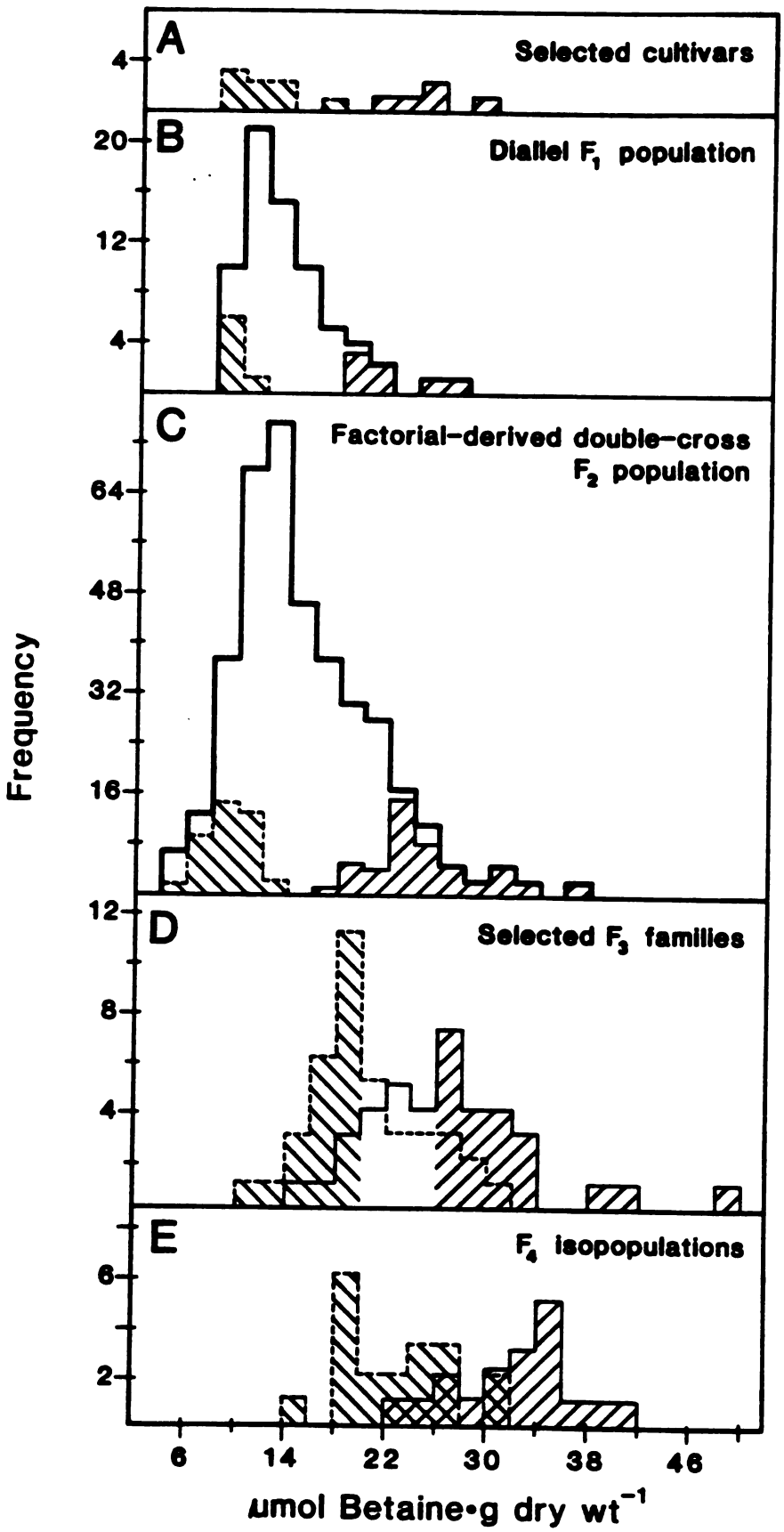


Figure 7. Betaine levels of the parent mixes (A) and isopopulations (B) in response to salt stress.

Data are pooled results of 3 experiments (5 reps/experiment).

The salt and genotype effects were highly significant (AOV; P=0.01).

Figure 8. Distribution of betaine levels during the isopopulation development procedure.


A
Selected cultivars
B
Diallel F1 population
C
Factorial-derived double-cross
F2 population
D
Selected F3 families
E
F4 isopopulations
Frequency
4
20
12
4
64
48
32
16
12
8
4
6
2
6
14
22
30
38
46
µmol Betaine•g dry wt-1

second round of crossing; they differed two-fold in mean betaine level (21.6 and 9.7 umol·g^{-1} dry wt, respectively). Pedigrees of the selected F_1's indicated that all 7 of the highest F_1's were derived from high X high crosses and that 5 of the 7 lowest F_1's were derived from low X low crosses.

To force a mixture of the high and low genetic backgrounds the selected F_1 hybrids were crosssed factorially (only highs X lows and lows X highs). The double-cross F_2 individuals were screened for betaine (Fig. 8c) and the lowest and highest 10% selected (Fig. 8c, shaded). These formed two distinct groups with a two-fold difference in mean betaine level ($\bar{x}$ lows = 9.0 umol betaine·g^{-1} dry wt and $\bar{x}$ highs = 23.9 umol·g^{-1} dry wt). Each of the 13 parents was represented in the pedigrees of both groups.

The high and low F_2 individuals were selfed and the resulting F_3 families tested for betaine (Fig. 8d). The number of F_3 families carried to the F_4 generation was cut in half (Fig. 8d, shaded) to reduce misclassification errors that occur at the F_2 generation due to environmental effects or heterozygosity. The mean betaine contents of the selected F_3 families were 17.3 and 31.4 umol·g^{-1} dry wt, respectively. All 13 of the parents were represented among the selected low F_3 families; 12 of the 13 parents were represented among the selected high F_3 families. The selected F_3 selected families were selfed to the F_4 generation.

The mean betaine values of the high- and low-betaine F_4 families were 22.8 and 32.4 umol·g^{-1} dry wt, respectively; this difference was highly significant (t-test; P=0.01). Root/shoot ratio, shoot ash content, seed yield, heading date, plant height, head length, number of tillers, seed weight and percent germination varied within each population but the mean values and ranges were the same for the two populations (Table 5). Several qualitative characters, stem color, susceptibility to mildew and aphids, onset of senescence of older leaves, and leaf angle, were also not noticeably different between the two populations.

2.4.3 Responses of the isopopulations to salt stress.

Although plants were selected only for betaine level, the process of isopopulation development did not eliminate differences in ψ_s and dry weight/fresh weight ratio between the highs and lows. The high betaine isopopulation had significantly greater dry weight per unit fresh weight (P=0.01), and at all salt levels, a consistently lower ψ_s by approximately 1 bar (P=0.01). Thus the isopopulations responded to salinity in a very similar way to the parent mixes (Fig. 5 b,d,f; 6b; 7b).

One of several possible explanations for the failure to eliminate the association between betaine and ψ_s would be that betaine level per se, governs ψ_s. To test this idea, barley seedlings (low betaine CI# 11806) were preloaded with betaine to determine whether raising betaine level would

Table 5. Mean values ± standard deviation for several characteristics of the selected F_3 families forming the isopopulations.

Trait	highs	lows
Betaine level (umol·g dry wt^{-1})	32.4 ± 4.8**	22.8 ± 4.5**
Root/shoot ratio (unstressed)	0.18 ± 0.05	0.16 ± 0.02
Root/shoot ratio (300 mM NaCl)	0.26 ± 0.05	0.24 ± 0.02
% Ash weight (unstressed)	16.9 ± 0.8	15.4 ± 2.5
%Ash weight (300 mM NaCl)	16.5 ± 0.5	16.4 ± 3.1
# tillers (at 3 weeks)	3 ± 0.8	3 ± 0.8
Age at flowering (days)	53 ± 4	55 ± 4
Flag leaf width (cm)	1.3 ± 0.3	1.2 ± 0.2
Mature plant height (cm)	65 ± 10	68 ± 8
Head length (cm)	5.9 ± 1.4	6.5 ± 1.4
Seed yeild (g·pot^{-1})	13.6 ± 4.5	16.2 ± 4.7
Seed weight (mg)	43.4 ± 5.6	42.1 ± 6.3
% germination	86 ± 12	83 ± 14

**highs and lows significantly different (t-test; P=0.01).

lower ψ_s in unstressed conditions. Although the shoot betaine level was raised from 1.3 to 5.5 umol·g^{-1} fresh wt, ψ_s was unaffected. There was also no significant effect of preloading betaine on fresh weight, dry weight and dry weight/fresh weight ratio (Table 6).

2.5 Discussion

The two groups of parental genotypes were equally variable for several morphological and phenological characteristics showing that a range of non-betaine alleles was introduced into the isopopulations in differing combinations. If these traits may be considered representative of less easily measured characters, bias due to non-betaine traits should have been minimized at the outset. Notable exceptions to the random distribution, however, were ψ_s and dry weight/fresh weight ratio. For both traits the high betaine genotypes had a phenotype generally associated with more stressed environments.

The 1-2 umol·g^{-1} fresh wt difference in betaine level between the highs and lows is far too small to account for either the 1 bar difference in ψ_s, or the 5 mg dry wt/g fresh wt difference. Note, though, that a 5 mg dry wt/g fresh wt difference is consistent with a 1 bar difference in ψ_s. If the difference in osmoregulation was acheived either completely with inorganic ions, e.g. KCl, or completely with organic compounds with an average molecular weight of 200,

Table 6. Effect of applied betaine on unstressed barley plants.

Treatment	betaine level	ψ_s	fresh weight	dry weight	dry wt/ fresh wt
	umol/g fresh wt	bars	g	g	mg/g
Control	1.32 ±0.12	-10.1 ± 0.2	1.50 ±0.16	0.16 ± 0.01	110 ±8
+ Betaine	5.50 ±1.06**	-10.5 ± 0.4	1.30 ±0.15	0.15 ± 0.02	116 ±4

Betaine levels were determined using the whole shoot; ψ_s using leaf disks from the midblade position of the youngest fully expanded leaf.

Data are means of 6 or 7 replicates ± S.E.

**Betaine treated significantly different from the controls (t-test; P=0.01)

the predicted dry weight difference between the two groups would be approximately 2 and 8 mg/g fresh wt, respectively. The observed 5 mg difference falls comfortably within this range.

Pedigree analysis and evaluation of several morphological and developmental characters during and after the isopopulation development process indicated that the 13 parental genotypes were successfully mixed to produce two genetically comparable populations differing primarily in betaine level. It is striking, though, that despite two hybridization steps, selection only on the basis of betaine level, and theoretical random assortment of all non-betaine traits, the high betaine population had lower osmotic potentials and higher dry weight/fresh weight ratios.

The inability to dissociate differences in betaine from differences in ψ_s is even more evident when betaine level is plotted against ψ_s (Fig. 9). There is an excellent linear relationship ($r^2 = 0.99$; $P<0.001$) in which both high and low genotypes fall on the same line. Three possible explanations for the inability to disrupt this association are: (1) Betaine itself governs ψ_s; (2) Linkage between gene(s) for betaine and gene(s) for osmoregulation; or (3) Indirect selection for osmoregulatory genes with pleiotropic effects.

The first possibility, that betaine regulates ψ_s, is unlikely since preloading unstressed barley plants with

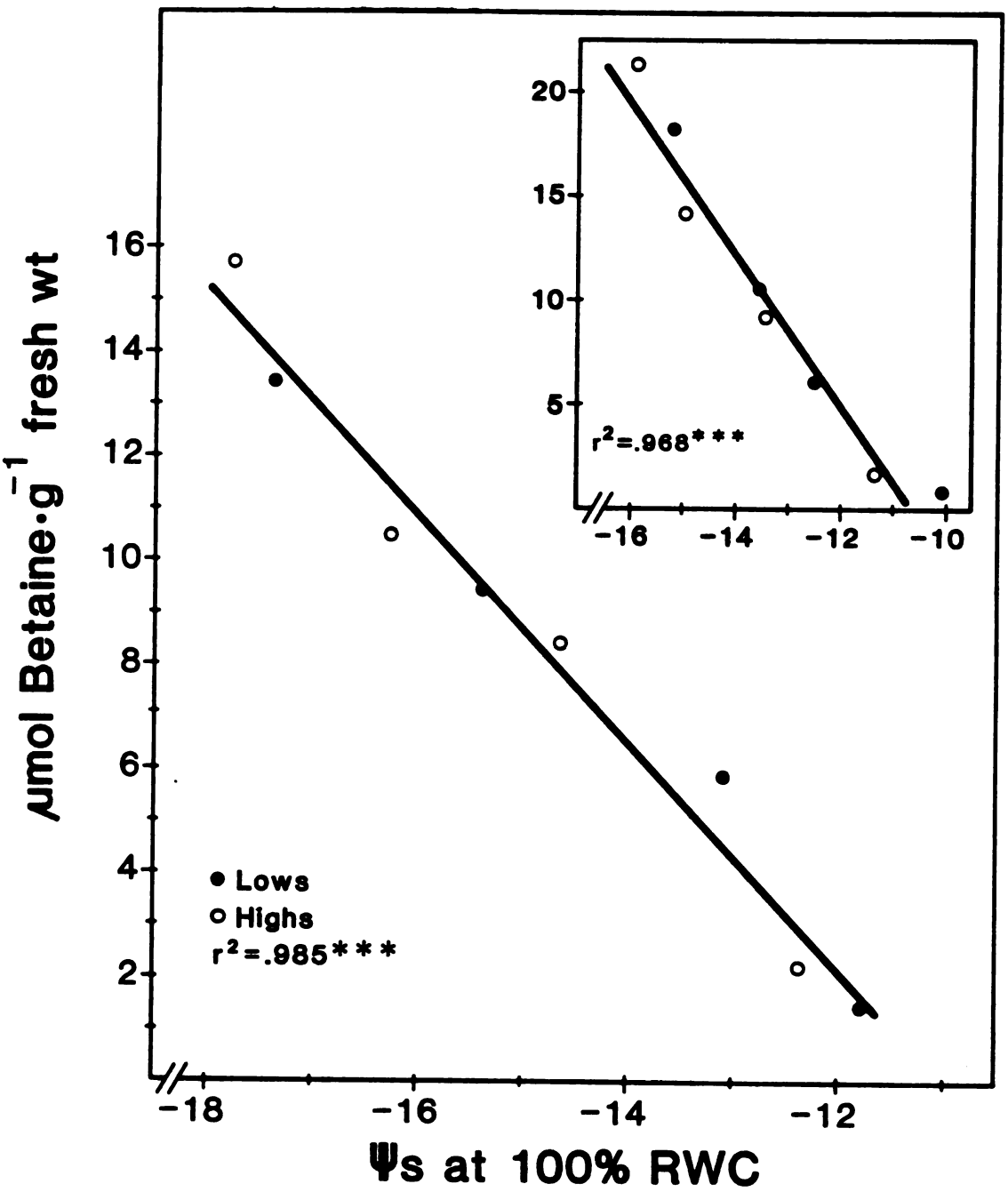


Figure 9. Betaine level vs. solute potential for the isopopulations and parent mixes (inset).

*** significant at P<0.001.

Data are pooled from 3 experiments (5 reps/experiment).

betaine did not alter ψ_s. According to the relationship shown in Figure 9, the 4 umol·g^{-1} fresh wt increase in betaine level acheived in the experiment of Table 6 should have lowered ψ_s by approximately 2 bars; a drop of 0.9 bar or greater would have been detected in this experiment (LSD; P=0.05).

The interpretation of this experiment is, of course, contingent upon the assumption that supplied betaine was distributed among and within shoot cells in the same way as endogenously produced betaine. This assumption was checked by autoradiography of $^{[14]}$C-betaine fed tissues which showed the $^{[14]}$C-betaine to be evenly distributed along the length of leaf blades. It is also supported by results showing that supplied betaine behaves like endogenous betaine in that it is readily loaded into the phloem and translocated from mature leaves to growing organs (Ladyman et al., 1980).

The second possibility, failure to break tight linkages during isopopulation development, cannot be discarded on genetic grounds alone (Burton, 1968; Eslick and Hockett, 1974). However, physiological considerations argue strongly against this explanation. Osmoregulation involves coordinated changes in many processes (Morgan, 1984a) and so it is necessary to either postulate close linkage of many different traits that contribute to osmotic adjustment, or alternatively, to suppose that there exists a single gene with major effects on osmoregulation closely linked to a

betaine gene. Even if these genes were linked, this would still not fully explain the observed behavior of the isopopulations. Betaine level was always perfectly coordinated with total ψ_s. Thus, it would also be necessary to invoke coordinate regulation of the betaine and osmoregulatory genes.

A simpler explanation for our results is that the high- and low-betaine parents did not differ genetically for betaine accumulation per se, but for osmoregulation as a whole, and that betaine levels are controlled by osmoregulatory genes with pleiotropic effects. In this case, differences in betaine would act as a marker for genetic differences in osmoregulation. This possibility, indirect selection for osmoregulatory genes, is supported by the demonstration of such genes in wheat (Morgan, 1984b) and Arabidopsis (Langridge, 1958).

Although a lower ψ_s might cause an increase in betaine levels via a direct effect on the biosynthetic enzymes, this seems unlikely as such effects have not been observed in in-vitro studies of betaine biosynthesis in spinach (Hanson et al., 1985; Pan et al., 1981). On the other hand, our results can be very readily explained in light of Wyn Jones' hypothesis that betaine is a cytosolic osmolyte (Wyn Jones et al., 1977). In this case, betaine accumulation would be one of several coordinately regulated components of osmoregulation.

As genetic analyses of the betaine trait in barley show it to be under nuclear control and highly heritable (Grumet et al., 1985) these conclusions may also be extended to osmotic behavior. Furthermore, the difference in solute potential between the two populations has two implications bearing on osmoregulation. First, because the two populations had equal total dry matter production in all conditions, it is unlikey that the decreased ψ_s of the high betaine-low ψ_s population arose from a back up of unused assimilates resulting from decreased growth. Second, in accordance with observations by McCree et al., (1984) and Schwarz and Gale (1981), since the two populations acheived equal dry matter production in the set of environments tested, the metabolic cost of osmoregulation at a 1-bar lower ψ_s was either minimal, or offset by an increase in assimilation.

In conclusion, the inability to genetically dissociate low ψ_s from high betaine level, and the almost perfect correlation between ψ_s and betaine content regardless of salt level or genotype, is the first genetic evidence that betaine accumulation in higher plants has an osmoregulatory function.

2.6 Literature Cited

Barrs, H.D. 1968. Determination of water deficits in plant tissues. p. 236-268. In T.T. Kozlowski (ed.) Water Deficits and Plant Growth. Vol I. Academic Press, New York.

Brady, C.J., T.S. Gibson, E.W.R. Barlow, J. Speirs, and R.G. Wyn Jones. 1984. Salt tolerance in plants. I. Ions, compatible organic solutes, and the stability of plant ribosomes. Plant, Cell and Environ. 7:571-578.

Burton, G.W., J.B. Gunnels, and R.S. Lowrey. 1968. Yield and quality of early and late maturing, near-isogenic populations of pearl millet. Crop Sci. 8:431-434.

Coughlan, S.J. and R.G. Wyn Jones. 1980. Some responses of Spinacia oleracea to salt stress. J. Exp. Bot. 31:883-893.

Eslick, R.F. and F.A. Hockett. 1974. Genetic engineering as key to water use efficiency. Agric. Meterol. 14:13-23.

Ford, C.W. and J.R. Wilson. 1981. Changes in the level of solutes during osmotic adjustment to water stress in four tropical pasture species. Aust. J. Plant Physiol. 8:77-91.

Grumet, R., T.G. Isleib, and A.D. Hanson. 1985. Genetic control of glycinebetaine level in barley. Crop Sci. 25:618-622.

Hall, J.L., D.M.R. Harvey and T.J. Flowers. 1978. Evidence for the cytoplasmic localization of betaine in leaf cells in Suaeda maritima. Planta 140:59-62.

Hanson, A.D. and R. Grumet. 1985. Betaine accumulation: metabolic pathways and genetics. p.71-92. In J.L. Key and T. Kosuge (ed.) Cellular and Molecular Biology of Plant Stress. UCLA Symposia on Cellular and Molecular Biology. Vol. 22. Alan R. Liss, New York.

Hanson, A.D., A.M. May, R. Grumet, J. Bode, G.C. Jamieson and D. Rhodes. 1985. Betaine synthesis in chenopods; localization in chloroplasts. Proc. Natl. Acad. Sci. USA. 82:3678-3682.

Hanson, A.D. and R. Wyse. 1982. Biosynthesis, translocation, and accumulation of betaine in sugarbeet and its progenitors in relation to salinity. Plant Physiol. 70:1191-1198.

Hitz, W.D., J.A.R. Ladyman and A.D. Hanson. 1982. Betaine synthesis and accumulation in barley during field water stress. Crop Sci. 22:47-54.

Ladyman, J.A.R., K.M. Ditz, R. Grumet, and A.D. Hanson. 1983. Genotypic variation for glycinebetaine accumulation by cultivated and wild barley in relation to water stress. Crop Sci. 23:465-468.

Ladyman, J.A.R., W.D. Hitz, and A.D. Hanson. 1980. Translocation and metabolism of glycinebetaine in barley plants in relation to water stress. Planta 150:191-196

Langridge, J. 1958. An osmotic mutant of Arabidopsis thaliana. Aust. J. Biol. Sci. 11:457-470.

Larkum, A.W.D. and R.G. Wyn Jones. 1979. Carbon dioxide fixation by chloroplasts isolated in glycinebetaine. Planta 145:393-394.

Leigh, R.A., N. Ahmad, and R.G. Wyn Jones. 1981. Assessment of glycinebetaine and proline compartmentation by analysis of isolated beet vacuoles. Planta 153:34-41.

LeRudulier, D., A.R. Strom, A.M. Dandekar, L.T. Smith, and R.C. Valentine. 1984. Molecular biology of osmoregulation. Science 224:1064-1068.

Ludlow, M.M., A.C.P. Chu, R.J. Clements and R.G. Kerslake. 1983. Adaptation of species of Centrosema to water stress. Aust. J. Plant Physiol. 10:119-130.

McCree, K.J., C.E. Kallsen, and S.G. Richardson. 1984. Carbon balance of sorghum plants during osmotic adjustment to water stress. Plant Physiol. 76:898-902.

Morgan, J.M. 1984a. Osmoregulation and water stress in higher plants. Ann. Rev. Plant Physiol. 35:299-319.

Morgan, J.M. 1984b. Osmoregulation as a selection criterion for drought tolerance in wheat. Aust. J. Agric. Res. 34:607-614.

Paleg, L.G., T.J. Douglas, A. van Daal, and D.B. Keech. 1981. Proline, betaine and other organic solutes protect enzymes against heat inactivation. Aust. J. Plant Physiol. 8:107-114.

Pan, S., R.A. Moreau, C. Yu, and A.H.C. Huang. 1981. Betaine accumulation and betaine aldehyde dehydrogenase in spinach leaves. Plant Physiol. 67:1105-1108.

Pollard, A. and R.G. Wyn Jones. 1979. Enzyme activities in concentrated solutions of glycinebetaine and other solutes. Planta 144:291-298.

Quizenberry, J.E.. 1982. Breeding for drought resistance and plant water use efficiency. p.193-212. In M.N. Christiansen and C.F. Lewis (ed.) Breeding Plants for Less Favorable Environments. John Wiley and Sons, New York.

Rasmusson, D.C. and B.G. Gengenbach. 1983. Breeding for physiological traits. p.231-254. In D.R. Wood, K.M. Rawal, and N.M. Wood (ed.) Crop Breeding. Amer. Soc. of Agron., Inc. Madison, Wisc.

Schwarz, M. and J. Gale. 1981. Maintenance respiration and carbon balance of plants at low levels of sodium chloride salinity. J. Exp. Bot. 32:933-941.

Strom, A.R., D. LeRudulier, M.W. Jacowec, R.C. Bunnell, and R.C. Valentine. 1983. Osmoregulatory genes and osmoprotective compounds. p.39-59. In T. Kosuge, C.P. Meredith, and A. Hollaender (ed.) Genetic Engineering of Plants - An Agricultural Perspective. Plenum Press, New York.

Storey, R. and R.G. Wyn Jones. 1978. Salt stress and comparative physiology in the Gramineae. III. Effect of salinity upon ion relations and glycinebetaine and proline levels in Spartina townsendii. Aust. J. Plant Physiol. 5:817-829.

Wyn Jones, R.G. and J. Gorham. 1983. Osmoregulation. p.35-58 In O.E. Lange, C.B. Osmond, H. Zeiger (ed.) Vol. 12C. Physiological Plant Ecology. Encyclopedia of Plant Physiology. Springer-Verlag, Heidelberg.

Wyn Jones, R.G. and R. Storey. 1981. Betaines. p. 171-204. In L.G. Paleg and D. Aspinall (ed.) Physiology and Biochemistry of Drought Resistance. Academic Press, New York.

Wyn Jones, R.G., R. Storey, R.A. Leigh, N. Ahmad and A. Pollard. 1977. A hypothesis on cytoplasmic osmoregulation. p. 126-136. In E. Marre and O. Ciferri (ed.) Regulation of Cell Membrane Activities in Plants. Elsevier Press, Amsterdam.

CHAPTER III

GROWTH STUDIES OF TWO BARLEY ISOPOPULATIONS DIFFERING IN GLYCINEBETAINE LEVEL AND OSMOREGULATION

3.1 Abstract

Betaine (glycinebetaine) is thought to act as a cytosolic osmolyte in water- or salt-stressed plants and so high betaine levels have been suggested to be of adaptive value during osmotic stress. We previously developed two barley isopopulations differing in betaine level and solute potential (ψ_s) (Grumet and Hanson). In this work, the isopopulations were compared for growth responses to water stress in greenhouse trials. The low betaine-high ψ_s population had a higher rate of leaf production, and in optimal environmental conditions (adequate water, high irradiance, warm temperatures) accumulated up to 35% greater total above-ground dry matter production than the high betaine-low ψ_s population. The difference in dry matter production disappeared in less favorable environments. The behavior of the populations could not be accounted for by differences in partitioning of dry matter to the leaves or in water use efficiency. Thus, although selection for high

betaine-low ψs resulted in a population with more stability in water stressed environments as judged by regression analysis (31 environments, b=0.84), it also reduced growth in optimal environments.

3.2 Introduction

Plants subjected to environments of decreasing external water potential are often able to maintain turgor by the process of osmotic adjustment (Hsiao et al., 1976; Morgan, 1984). Osmotic adjustment is the active accumulation of solutes within the cell (Turner and Jones, 1980) and betaine, which accumulates in response to osmotic stress in many grasses and chenopods, is thought to contribute to this process by acting as a non-toxic cytoplasmic osmoticum (Wyn Jones et al., 1977). Two barley (Hordeum vulgare L.) isopopulations differing primarily in betaine level and solute potential (ψ_s) were previously developed and characterized for their response to salt stress in controlled environment conditions (Grumet and Hanson). Although the two populations differed consistently for betaine level and ψ_s at all salt levels, no differences in growth were observed between the two populations.

In this investigation we sought to determine the effect of differences in ψ_s and betaine on plant growth in response to relatively long-term water stress. The isopopulations are highly suited to this type of study because they are

genetically comparable for traits other than ψ_s and betaine level (Grumet and Hanson). Greenhouse experiments were used to measure total above-ground dry matter production at mid-anthesis in well-watered and water-stressed conditions, water use efficiency, specific leaf weight, and rate of leaf production for the two isopopulations.

3.3 Materials and Methods

Greenhouse water stress experiments. Greenhouse water stress experiments were performed using large, 40-l plastic pots (36 cm diam.) with drainage holes and a planting density of 300 plants/m^2 (30 plants/pot). Seeds were prepared as described in Grumet et al. (1985) and planted in alternate rows (5 plants/row) of the two isopopulations in a soil mix of peat:sand:loam, 1:2:1. The water holding capacity of the soil (g H_2O/g dry soil) was 35%. The seedlings were irrigated daily with 50% Hoagland's solution for 10-12 days before stress regimes were imposed; at this time the pots were mulched with ca. 2 cm of Perlite to limit evaporation.

Environments ranging from well-watered (irrigated daily with 50% Hoagland's solution to field capacity) to severely water-stressed were established. In Experiment 1, stressed plants were either surface irrigated with 0.5, 0.75, 1, 2, 3, or 4 l every 3 d, or allowed to wilt before rewatering to field capacity (wilt-rewater cycles were 7-11 days). In

Experiments 2 and 3, stressed plants were subsurface irrigated by supplying 50% Hoagland's solution through drip irrigation lines (Chapin Watermatics Inc., Watertown, NY) into three 10-12 cm deep plastic tubes (2.8 cm diameter) per pot at a rate of 20-60 ml/min. The tubes were embedded in pea gravel to facilitate percolation. Watering levels were: once per week until run-through, 3 l/week, or wilt-rewater. In Experiment 4 all plants were well-watered.

All water stress experiments were performed between mid-April and late September. Daily high temperatures at canopy level ranged from 24 to 43 C; nighttime lows ranged from 14 to 20 C. Supplemental lighting was provided with high intensity Na lamps for 16 h/d. On a sunny day, mid-day PPFD at canopy height was 1500-2000 uE m^{-2} sec^{-1}. The mean percent sunny days in the 4 experiments was 66%. Powdery mildew and aphids were controlled as in Grumet et al. (1985). Above-ground dry matter was harvested at mid-anthesis by row in order to separate the two populations. The plants were frozen in liquid N_2 and oven dried at 60 C. The dry weight data was analyzed by the regression-response technique (Finlay and Wilkinson, 1963) using pot mean yield as an indicator of the yield potential of the environment.

Water use efficiency. Conditions for the water use efficiency (WUE) experiments were as above, except for the following changes. Three, 2 cm long, plastic tubes (1 cm

diam.) were inserted into drainage holes and secured with silicone. The pots were placed onto wooden platforms with a 1-l tray underneath. A measured amount of 50% Hoagland's solution was supplied each day to allow for 50-1000 ml run-off. Daily water use was determined by ml supplied - ml of run-off after 24 h (complete run-through took ca. 20 h). Non-competitive, single population plantings were used (30 plants/pot); WUE was calculated as total above-ground dry matter production at mid-anthesis (g)/ kg water supplied. WUE experiments were done in the spring and fall (April and October plantings). Daily high and nighttime low temperatures for the October experiment were 24-40 C and 16-24 C, respectively; the percent sunny days was 45%.

Specific leaf weight. To determine specific leaf weight (mg dry wt/cm^2), 10 youngest, fully expanded leaves from main tillers were removed from each pot in the October experiment. Leaf areas were estimated by weighing cut-out photocopies of the leaf blades. Leaf weight was determined after oven drying at 60 C.

3.4 Results and Discussion

The two isopopulations were studied for growth responses to a range of irrigation levels in the greenhouse in the spring and summer (Fig. 10). The populations were planted in alternate rows to force competition and thereby maximize differences between them. Using the regression

Figure 10. Yield at mid-anthesis (total above-ground dry matter) of the high betaine-low ψ_s population or the low betaine-high ψ_s population vs. pot mean yield.

n = 31 environments

The difference in slope for the two populations is highly significant (t=6.75***).

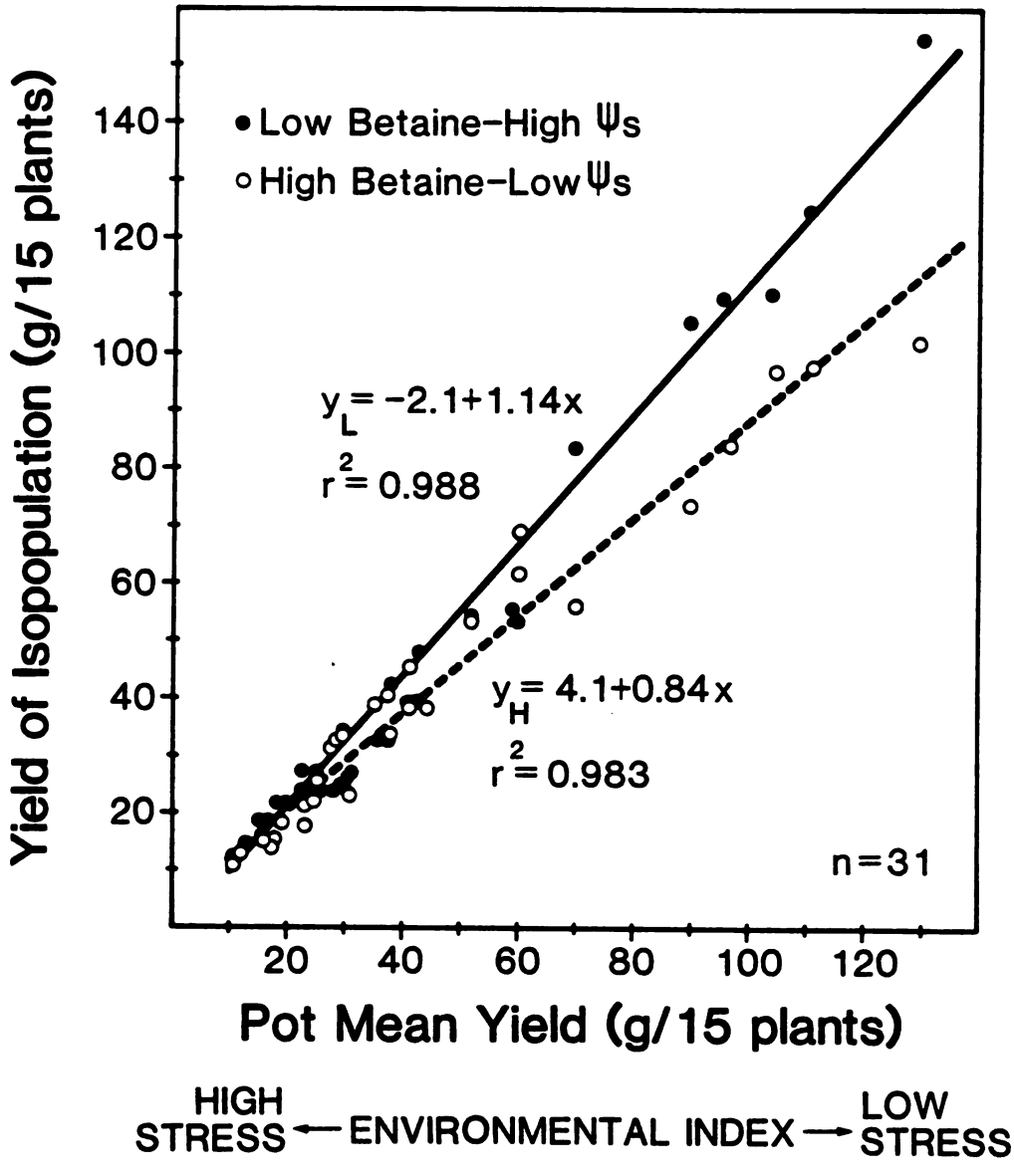

Yield of Isopopulation (g/15 plants)
140
120
100
80
60
40
20
• Low Betaine-High Ψs
○ High Betaine-Low Ψs
$y_L = -2.1+1.14x$
$r^2 = 0.988$
$y_H = 4.1+0.84x$
$r^2 = 0.983$
n=31
20
40
60
80
100
120
Pot Mean Yield (g/15 plants)
HIGH STRESS ← ENVIRONMENTAL INDEX → LOW STRESS

Figure 10

response technique to analyze yield in the 31 greenhouse "environments", the high betaine-low ψ_s population showed a more stable response to increasing water stress (b=0.84) than did the low betaine-high ψ_s population (b=1.14). The difference in stability, however, was due to growth differences in the favorable rather than stressed conditions.

At mid-anthesis the low betaine-high ψ_s population had more total above-ground dry matter than did the high betaine-low ψ_s population when the two populations were grown in competitive plantings in well-watered, optimal conditions (high irradiance, warm temperatures). This difference disappeared when growth conditions were less favorable, either by reduced water or in fall plantings (shorter, cloudier days) (Table 7). The absence of growth differences between the isopopulations in the earlier, salt stress experiments (Grumet and Hanson) was probably due to sub-optimal conditions in the growth chamber (e.g. PPFD of ca. 200 uE m^{-2} sec^{-1} in the growth chamber vs. 1500-2000 uE m^{-2} sec^{-1} for the greenhouse on sunny days). The difference between the isopopulations was also not apparent in non-competitive plantings in either optimal or sub-optimal conditions (Table 7).

The low betaine-high ψ_s population produced leaves faster than the high betaine-low ψ_s population in well-watered environments (Fig. 11). The difference was

Table 7: Average plant weight at mid-anthesis (43 DAP) for the well-watered isopopulations in competitive and non-competitive plantings.

Planting arrangement	Planting time	Average plant weight (g) Low betaine-high ψs	High betaine-low ψs
competitive	spring[1]	8.3 ± 1.5	6.1 ± 0.9 *
	fall	2.1 ± 0.6	1.9 ± 0.4
non-competitive	spring[2]	7.5 ± 0.5	6.9 ± 0.6
	fall[3]	1.8 ± 0.2	1.8 ± 0.2

* significantly different from the low betaine-high ψs population (AOV; P=0.05).

1. mean of 4 reps ± S.E.; 15 plants/rep
2. mean of 3 reps ± S.E.; 30 plants/rep
3. mean of 4 reps ± S.E.; 30 plants/rep

Figure 11. Rate of leaf production for the two isopopulations in a competitive fall planting.

Each point is the mean of 4 reps; 15 plants/rep.

The difference between the populations is significant (AOV; P=0.05).

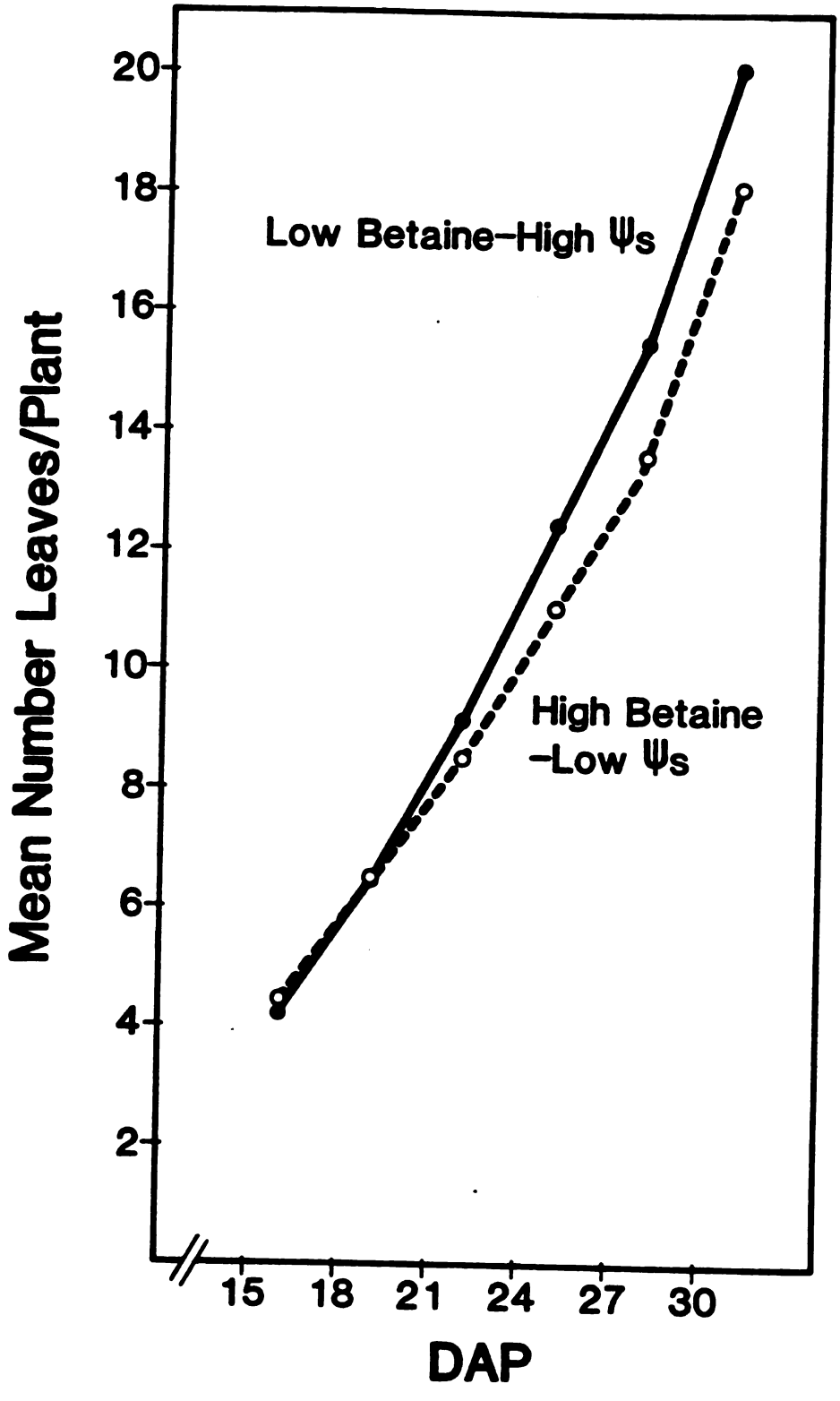

Mean Number Leaves/Plant
20
18
16
14
12
10
8
6
4
2
Low Betaine-High Ψs
High Betaine
-Low Ψs
15
18
21
24
27
30
DAP

Figure 11

significant in the fall (AOV;P=0.05) and highly significant in the spring (AOV; P=0.01) for both competitive and non-competitive plantings. The consistently observed difference in rate of leaf production, like the differences in betaine and ψ_s, suggests that it was a genetic effect largely independent of environment. It is likely that the difference in the rate of leaf production would enhance dry matter gain of the low betaine-high ψ_s population when growing in competition by giving these plants an advantage in light interception that is compounded throughout the growing season. The competitive advantage would likely be most important in conditions where total growth rate is not limited by other environmental factors.

The convergence of the curves for dry matter production as water stress increased (Fig. 10) is noteworthy. Possible explantions based on competition for light and for water were considered. Competition for light when water is not limiting could arise from differences between the isopopulations for dry matter partitioning between photosynthetic and non-photosynthetic structures, e.g. root/shoot partitioning, specific leaf weight, or leaf/stem production. Previous studies indicated that the two populations did not differ for root/shoot partitioning (Grumet and Hanson) and the present work showed that the isopopulations also did not differ for specific leaf weight (values for the low betaine-high ψ_s and high betaine-low ψ_s

population were 2.5 and 2.6 mg/cm^2, respectively). Although leaf to stem ratio was not quantified, there were no evident differences in growth habit between the isopopulations.

A difference in water use efficiency is also unlikely to account for the observed behavior of the isopopulations. In well-watered environments, the two populations had equal water use efficiencies (2.8 g dry matter/kg H_2O). However, if the populations maintained their ψ_s differences in response to water stress as they did when salinized (Grumet and Hanson), the lower solute potentials of the high betaine-low ψ_s population may have enabled these plants to extract an additional increment of water at lower soil water potentials (Turner and Jones, 1980). This would allow for additional growth by the high betaine-low ψ_s population in the water-limited environments thereby narrowing the gap between the populations.

Thus the advantages of the high betaine-low ψ_s genotypes may outweigh disadvantages as the stress level increases. Although a distinct crossover point where the high betaine-low ψ_s population outgrew the low betaine-high ψ_s population was not observed in these experiments, there may be conditions where the high betaine-low ψ_s population would perform better. Jensen (1981) reports, that despite consistent trends, the crossover points of regression response curves for maize genotypes with respect to drought stress vary greatly with the set of environments. It should

also be emphasized that the greenhouse barley trials measured total above-ground dry matter production, and not grain yield.

The cause of the difference between the isopopulations for dry matter production in optimal environments is unclear. While it is possible that the difference in growth is a result of differences in ψ_s or betaine, it may also be due to effects of linked genes, or it may be caused by highly pleiotropic genes which also influence osmoregulation, betaine level, and dry weight/fresh weight ratio (Simmonds, 1979). Although the latter two possibilities cannot be ruled out, it is interesting to speculate about the mechanistic basis by which differences in ψ_s or betaine could cause differences in growth.

Lower solute potentials or higher betaine levels may be metabolically expensive in terms of carbon use for maintenance processes. McCree et al. (1984) and Schwarz and Gale (1981), however, studied the energy demand of osmoregulation and considered it to be trivial. The failure to observe dry matter differences between the isopopulations in light-limited, well-watered conditions, where the relative cost of osmoregulation as a portion of total carbon available should be higher than in optimal conditions, supports these observations.

It is also possible that in the highest yielding environments nitrogen became a limiting factor. Since

betaine is a nitrogen containing molecule, the high betaine genotypes could be more affected than the low betaine genotypes. However, as for the costs of osmoregulation, the difference in betaine nitrogen between the isopopulations is a very small fraction of the total nitrogen demand. Given total N = 10-20 $mg \cdot g^{-1}$ dry wt (Janick et al., 1974), and the betaine difference between the highs and lows is 10-20 $umol \cdot g^{-1}$ dry wt (Grumet and Hanson), or ca. 0.2 mg $N \cdot g^{-1}$ dry wt, the difference in betaine N is only 1-2% of total N.

Another explanation for the difference in growth rate might be that metabolic function and cell growth at low ψ_s are still in some way impaired, even when the toxic osmolytes are excluded from the cytoplasm. Or perhaps, the populations differ primarily in cell wall properties so that the threshhold turgor for wall expansion is altered. Osmoregulation and growth would, in turn, be affected.

It is interesting that Quisenberry et al. (1984) also observed a similar apparent penalty of low solute potential on shoot dry matter production for several cotton lines. Although the lines that had lower solute potentials had higher turgor pressures, there was a strong, negative correlation between low solute potential and growth (shoot dry matter production). They concluded that selection for enhanced osmotic adjustment would result in decreased growth.

The connection between reduced growth potential in optimal environments and stress-resistance traits has been frequently observed (Blum, 1979; Begg and Turner, 1976; Jensen, 1981) and ecological studies have suggested that slow growth itself may be a mechanism of adaptation to stressful environments (Parsons, 1968). This view has been extended to agricultural systems where it has been demonstrated for grain crops growing on stored soil moisture, that reduced growth may confer an advantage by conserving more water for the grain-filling period (Richards, 1983; Passioura, 1972).

In contrast to the reduced shoot dry matter production observed for the low ψ_s genotypes in this study and by Quisenberry et al. (1984), Morgan (1984) found no deleterious effects of low ψ_s on the grain yield of F_4 lines of wheat differing in osmoregulation. Data for total biomass were not reported. It will therefore be very interesting to see how the barley isopopulations compare for grain yield when tested in the field. Multi-site dryland and irrigated trials with our barley isopopulations are currently being performed by Dr. Albrechtsen at Utah State University.

3.5. Literature Cited

Begg, J.E. and N.C. Turner. 1976. Crop water deficits. Adv. Agron. 28:161-217.

Blum, A. 1979. Genetic improvement of drought resistance in crop plants. A case for sorghum. p.429-445. In H. Mussel and R.C. Staples (ed.) Stress Physiology in Crop Plants. John Wiley and Sons, New York.

Finlay, K.W. and G.N. Wilkinson. 1963. The analysis of adaptation in a plant breeding program. Aust. J. Agric. Res. 14:742-754.

Grumet, R. and A.D. Hanson. (in preparation) Genetic evidence for an osmoregulatory function of glycinebetaine accumulation in barley.

Grumet, R.G., T.G. Isleib, and A.D. Hanson. 1985. Genetic control of glycinebetaine level in barley. Crop Sci. 25:618-622.

Hsiao, T.C., E. Acevedo, E. Fereres, and D.W. Henderson. 1976. Water stress, growth, and osmotic adjustment. Phil. Trans. R. Soc. Lond. 273:479-500.

Janick, J., R.W. Schery, F.W. Woods, and V.W. Ruttan. 1974. Plant Science - An Introduction to World Crops. W.H. Freeman and Co., San Francisco.

Jensen, S.D. 1981. Breeding plants for stress environments - Discussion. p.167-168. In K.J. Frey (ed.) Plant Breeding II. Iowa State Univ. Press, Ames, Iowa.

McCree, K.J., C.E. Kallsen, and S.G. Richardson. 1984. Carbon balance of sorghum plants during osmotic adjustment to water stress. Plant Physiol. 76:898-902.

Meyer, R.F. and J.S. Boyer. 1981. Osmoregulation, solute distribution, and growth in soybean seedlings having low water potentials. Planta 151:482-489.

Morgan, J.M. 1984a. Osmoregulation and water stress in higher plants. Ann. Rev. Plant Physiol. 35:299-348.

Morgan, J.M. 1984b. Osmoregulation as a selection criterion for drought tolerance in wheat. Aust. J. Agric. Res. 34:607-614.

Parsons, R.F. 1968. The significance of growth rate comparisons for plant ecology. Amer. Naturalist 102:595-597.

Passioura, J.B. 1972. The effect of root geometry on the yield of wheat growing on stored water. Aust. J. Agric. Res. 23:745-752.

Quisenberry, J.E., G.B. Cartwright, and B.L. McMichael. 1984. Genetic relationship between turgor maintenance and growth in cotton germplasm. Crop Sci. 24:470-483.

Richards, R.A. 1983. Manipulation of leaf area and its effect on grain yield in droughted wheat. Aust. J. Agric. Res. 34:23-31.

Schwarz, M. and J. Gale. 1981. Maintenance respiration and carbon balance of plants at low levels of sodium chloride salinity. J. Exp. Bot. 32:933-941.

Simmonds, N.W. 1979. Priciples of Crop Improvement. Longman Group Limited, New York.

Turner, N.C. and M.M. Jones. 1980. Turgor maintenance by osmotic adjustment: a review and evaluation. p.87-104 In N.C. Turner and P.J. Kramer (ed.) Adaptation of Plants to Water and High Temperature Stress. John Wiley and Sons, New York.

Wilson, J.R. and M.M. Ludlow. 1983. Time trends of solute accumulation and the influence of potassium fertilizer on osmotic adjustment of water-stressed leaves of three tropical pasture grasses. Aust. J. Plant Physiol. 10:523-537.

Wyn Jones, R.G., R. Storey, R.A. Leigh, N. Ahmad, and A. Pollard. 1977. A hypothesis on cytoplasmic osmoregulation. p. 126-136. In E. Marre and O. Ciferri (ed.) Regulation of Cell Membrane Activities in Plants. Elsevier Press, Amsterdam.

DISCUSSION

The objective of this research was to assess the adaptive significance of stress-induced betaine accumulation by determining the potential for, and effect of, genetically altering betaine levels in barley. Genetic studies and isopopulation development showed that the betaine trait in barley is nuclear and highly heritable, and that it is possible to select and breed for lines with differing betaine levels. The results of salt- and water-stress trials, however, raise general issues about the types of differences that were found, the method by which they were obtained, and if there are inherent constraints imposed upon such a study by using only natural variability. Each issue will be dealt with in turn.

(a) What type of variability was found, and at what level did selection act?

The salinization and growth studies showed that the differences in betaine content were associated with differences in several possibly interrelated traits: solute potential (ψ_s), dry weight/fresh weight ratio, total dry matter production in optimal environments, and rate of leaf

production. In general, high betaine genotypes resembled mildly stressed plants. This leads to speculation about the organizational level at which selection occurred (Fig. 12).

In light of the observed relationship between ψ_s and betaine with response to salt stress in the isopopulations and parents, and the failure of applied betaine to cause changes in ψ_s or the dry weight/fresh weight ratio, it is unlikely that selection was at the level of genes coding for enzymes in the betaine biosynthetic pathway (Level C). It is possible that selection was for gene(s) with major effects on osmoregulation (Level B), or alternatively, selection may have acted at an even higher level, for some sort of highly pleiotropic gene(s) that confer the phenotype of a mildly stressed plant (Level A).

If selection was at the level of osmoregulation, the selected genes could either be regulatory (i.e. for coordination of the interdependent osmotically related traits), or structural (e.g. for differences in cell wall components so that different turgor pressures are required for cell expansion). Differences in growth could either result from differences in ψ_s, or alternatively, be controlled by genes closely linked to the ψ_s genes.

Selection for highly pleiotropic gene(s) is also not unreasonable; plant breeders have often found that the traits they select for have pleiotropic effects (Simmonds, 1979). It is possible that the high betaine-low ψ_s

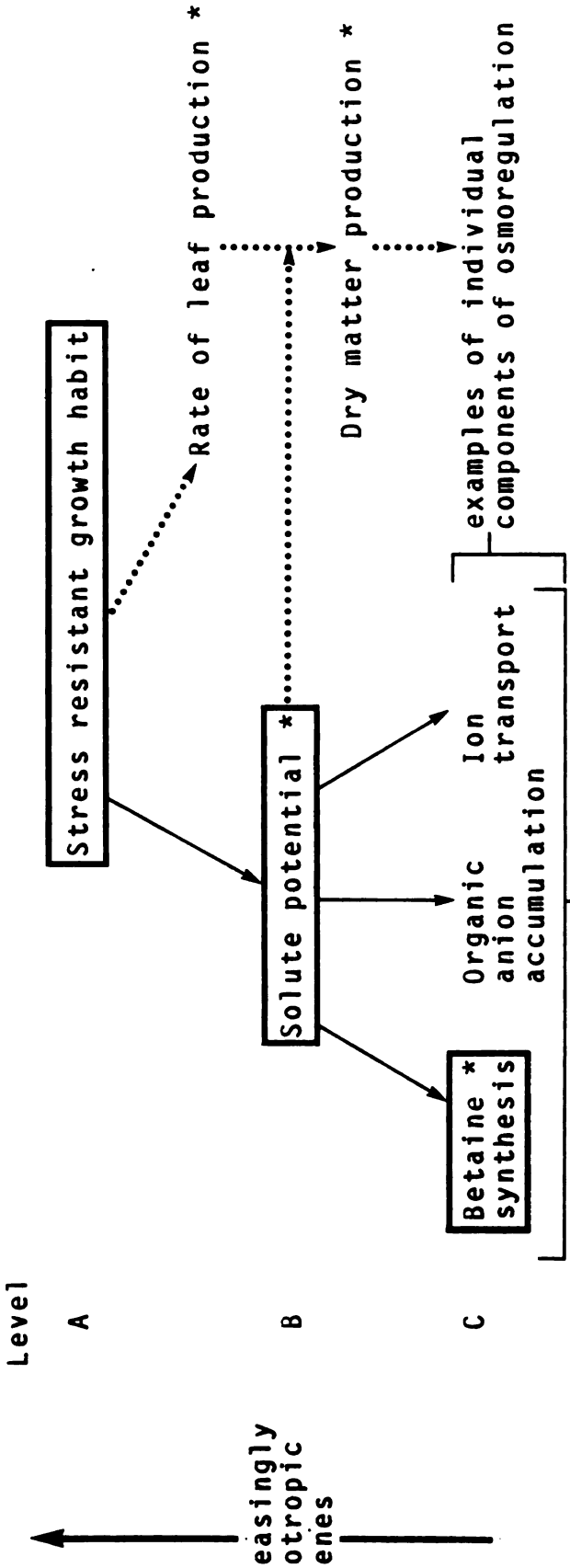


Figure 12. Possible relationship of observed differences between the isopopulations.

Circled traits represent possible levels of selection.

* Traits that differed in the two populations.

genotypes have a limit imposed upon their growth rate that is not either a direct cause or consequence of the differences in ψ_s and betaine. It is of interest that several other studies have indicated that reduced yield potential in optimal environments is associated with stress resistance traits (Jensen, 1981; Blum, 1979; Begg and Turner, 1976). Since it has been suggested that slow growth, per se, is an adaptation to adverse environments (Parsons, 1968), differences in betaine may be part of a larger complex of stress resistance characters that are under the control of highly pleiotropic genes.

(b) Was the type of variability found constrained by the method of selection?

The results of the salt stress experiments indicated that selection for high or low betaine content in well-watered conditions did not provide genotypes that produced different amounts of betaine in response to stress, i.e. there were no genotype X environment interactions. Instead, the absolute difference in betaine content between the highs and lows was constant at all salt concentrations; at high salt the relative difference between the two groups was quite modest.

It is possible that selection for betaine content in stressed conditions, or for the difference in betaine level between stressed and unstressed conditions, would uncover

variability for response to stress. It should be noted that although all screening during isopopulation development was done with well-watered plants, the parental genotypes used to intiate the isopopulations were selected on the basis of betaine level in both water-stressed and well-watered trials. Since the parental genotypes also did not differ in response to salinization, the additional labor required for screening using stress trials is probably not justifiable. Furthermore, among the genotypes tested by Ladyman et al. (1983), the selected parents represented the full range (25-50 $umol \cdot g^{-1}$ dry wt) for absolute increase of betaine in response to water stress. This suggests, that at least among the genotypes screened by Ladyman et al., there was not a great deal of variability for response to stress.

The stressed/unstressed betaine ratios of the selected parental genotypes ranged from 2.0 - 3.2. There were, however, a few accessions screened by Ladyman et al. (1983) with somewhat more extreme ratios (1.6 - 3.8). If verified, these lines may be of interest for future work. They may be cases where the association between high betaine and low ψ_s is broken, or alternatively, the genotypes with a very low stressed/unstressed betaine ratio may fail to osmoregulate. Either result could provide further information about the osmoregulatory role of betaine.

(c) Are there inherent limitations of natural variability for physiological-genetic studies?

There was only a 2-3 fold range in betaine content in unstressed conditions among the H. vulgare and H. spontaneum genotypes tested; stress did not increase the difference. This relatively narrow range, the absence of betaine nulls, and the lack of transgressive variation in crossing studies, all suggest that variation for betaine level is for some reason, constrained. This is consistent with an adaptive trait. For traits that are subject to selection pressure there is a range of levels that confer adaptedness. To vary a great deal in either direction from that range would have negative effects, and the levels found within successful genotypes fall within the adapted range (Simmonds, 1979). In contrast, a non-adaptive trait, or one that is not subject to selection pressure, would be more likely to vary widely (Hartl, 1980).

In future work additional variation might be achieved by isolating betaine-deficient or betaine-overproducing mutants by conventional means, or by altering betaine synthesis using molecular genetic techniques. The use of a betaine null could answer the question: does eliminating betaine have a neutral or deleterious effect? If it were deleterious, this would support the view that normal levels of betaine are of adaptive value. A betaine overproducer

could be used to ask: would increasing the levels of betaine have positive or negative effects? The answer to this question is likely to vary with the environment.

Another limitation to using natural variability is that, although the isopopulations provided useful insight into the relationship between betaine and ψ_s, and possibly other growth phenomena, it was not possible to test the effect of differing betaine levels per se. The alternative approaches of isolating mutants or molecular genetic manipulation, might provide a better system for addressing questions about the betaine trait itself.

An equally important question, however, is: is it desirable to separate differences in betaine from differences in ψ_s? The evidence that betaine is a protective cytoplasmic osmolyte has become increasingly convincing in recent years, but there is no evidence that betaine levels are a limiting factor in osmoregulation. In fact, the results presented in this dissertation imply that the level of betaine is a closely regulated component of ψ_s in balance with other contributing solutes. If adaptation is of interest, a more relevant question for crop improvement may be: are differences in ψ_s desirable? It is only recently that investigators have begun to examine the effects of osmoregulatory differences within a species (Morgan, 1984; Quisenberry, 1984); the results to date are promising.

Literature Cited

Begg, J.E. and N.C. Turner. 1976. Crop water deficits. Adv. Agron. 28:161-217.

Blum, A. 1979. Genetic improvement of drought resistance in crop plants. A case for sorghum. p.429-445. In H. Mussels and R.C. Staples (ed.) Stress Physiology in Crop Plants. John Wiley and Sons, New York.

Hartl, D.L. 1980. Principles of Population Genetics. Sinauer Associates, Inc., Sunderland, MA.

Ladyman, J.A.R., K.M. Ditz, R. Grumet, and A.D. Hanson. 1983. Genotypic variation for glycinbetaine accumulation by cultivated and wild barley in relation to water stress. Crop Sci. 23:465-468.

Jensen, S.D. 1981. Breeding plants for stress environments - Discussion. p.168-168. In K.J. Frey (ed.) Plant Breeding II. Iowa State University Press, Ames, Iowa.

Morgan, J.M. 1984. Osmoregulation as a selection criterion for drought tolerance in wheat. Aust. J. Agric. Res. 34:607-614.

Parsons, R.F. 1968. The significance of growth rate comparisons for plant ecology. Amer. Naturalist 102:595-597.

Quisenberry, J.E., G.B. Cartwright, and B.L. McMichael. 1984. Genetic relationship between turgor maintenance and growth in cotton germplasm. Crop Sci. 24:479-483.

Simmonds, N.W. 1979. Principles of Crop Improvment. Longman Group Ltd., New York.

APPENDIX

APPENDIX

Protocol for colorimetric betaine assay. (For written description, see Chapter 1 p. 25-26)

freeze-dry sample, grind to mesh size 40 (Wiley Mill)
oven dry at 70 C
weigh 30-50 mg sample into small testtube
+ 5 ml H_2O, vortex
boil 30-60 min
* cool (at least 1 hr., 4 C)
↓ → pellet
supernatant
remove 4 ml
+ 200 ul AG-50 (H^+) slurry (2:1 resin:water) (begin standards)
vortex, let settle
↓ → aspirate supernatant
resin
wash with 2 ml H_2O
vortex, let settle
↓ → aspirate supernatant
resin
+ 3 ml 4 N NH_4OH
vortex and let settle
↓ → resin
supernatant
remove to 30 ml beaker
* dry with infared lamp and fan (ca. 3 h)
↓
redissolve in 0.6 ml 1 N H_2SO_4
remove 0.5 ml to 1 ml reaction vials, chill 1 h, 4 C
+ 0.2 ml cold KI-I_2 reagent
cover with parafilm, vortex gently
* refrigerate overnight

centrifuge (in the cold)

↓ → aspirate supernatant (with fine-tipped glass needle)

pellet
+ 0.2 ml 1 N cold H_2SO_4
centrifuge at 4 C

↓ → aspirate supernatant

pellet
+ 200 ul dichloroethane
dissolve thoroughly (mechanically disrupt pellet)

↓

30 ul to small testtube + 4 ml dichloroethane
read in spectrophotometer at 365 nm

↓

* stopping points in the protocol

Notes:

1. KI-I_2 reagent: 15.7 g I_2 + 20.0 g KI into 100 ml H_2O
   shake to dissolve
   store at 4 C

2. dichloroethane: reagent grade

3. AG-50 H^+ Resin: BioRad Laboratories 2:1 settled resin:H_2O, vol/vol

4. calculation of umol betaine/g dry weight from standard curve of betaine-HCl (y=mx+b):

$$\frac{(\text{absorbance} - b)/m \times 0.8 \times 1/153.6 \text{ umol/ug} \times 10^3}{\text{mg dry wt/sample}}$$

5. range of standards for unstressed samples: 50 - 200 ug

6. reagent blanks have no absorbance at 365 nm

7. soak vials in chromic acid after each use