Osmoprotective compounds in the Plumbaginaceae: A natural experiment in metabolic engineering of stress tolerance

(betaines/choline *O*-sulfate/compatible solutes/ecological biochemistry)

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ABSTRACT In common with other zwitterionic quaternary ammonium compounds (QACs), glycine betaine acts as an osmoprotectant in plants, bacteria, and animals, with its accumulation in the cytoplasm reducing adverse effects of salinity and drought. For this reason, the glycine betaine biosynthesis pathway has become a target for genetic engineering of stress tolerance in crop plants. Besides glycine betaine, several other QAC osmoprotectants have been reported to accumulate among flowering plants, although little is known about their distribution, evolution, or adaptive value. We show here that various taxa of the highly stress-tolerant family Plumbaginaceae have evolved four QACs, which supplement or replace glycine betaine—namely, choline *O*-sulfate and the betaines of β -alanine, proline, and hydroxyproline. Evidence from bacterial bioassays demonstrates that these QACs function no better than glycine betaine as osmoprotectants. However, the distribution of QACs among diverse members of the Plumbaginaceae adapted to different types of habitat indicates that different QACs could have selective advantages in particular stress environments. Specifically, choline *O*-sulfate can function in sulfate detoxification as well as in osmoprotection, β -alanine betaine may be superior to glycine betaine in hypoxic saline conditions, and proline-derived betaines may be beneficial in chronically dry environments. We conclude that the evolution of osmoprotectant diversity within the Plumbaginaceae suggests additional possibilities to explore in the metabolic engineering of stress tolerance in crops.

Abiotic stresses such as drought and salinity are the major constraints to crop yield (1), and more sources of genes for tolerance to them are needed (2–4). When such genes occur in crops or in closely related wild species, they can be exploited by traditional breeding techniques (3, 4). A much wider potential pool of genes is now available: genetic engineering makes it possible to use any organism as a source of simple adaptations to abiotic stresses (5, 6). This has led to much interest in stress adaptations that may be controlled by one or a few genes.

One such adaptation to dry and saline conditions is the accumulation of osmoprotectants (7). Unlike most solutes, osmoprotectants stabilize proteins and membranes when present at high concentrations and so can be used to raise cytoplasmic osmotic pressure in stressed cells without deleterious effects (7, 8). Betaines and other zwitterionic quaternary ammonium compounds (QACs) are very effective osmoprotectants, and several occur in diverse taxa of flowering plants (Table 1 and ref. 9). Glycine betaine is the most widespread of these; it is the only one for which biosynthetic enzymes and genes have been isolated (10, 11) and the only

one so far targeted for metabolic engineering (17, 18). Biochemical, immunological, and DNA sequence evidence suggests that glycine betaine biosynthesis appeared early in angiosperm evolution (19–21). Less common, and probably more recently evolved in angiosperms, are other osmoprotectants: β -alanine betaine, choline *O*-sulfate, proline betaine, and hydroxyproline betaine (9). We report here that all four of these QACs, as well as glycine betaine, occur in various members of the Plumbaginaceae, a highly stress-tolerant family containing species adapted to a wide range of harsh environments. The pattern of occurrence of these four QACs is consistent with their having a selective advantage over glycine betaine in certain stress environments.

MATERIALS AND METHODS

Plant Samples. Plants were collected from natural habitats or grown under controlled saline (400–450 mM NaCl) conditions in growth chambers as described (12). Their leaves were harvested and freeze dried. Because QACs are generally stable compounds, we also made extensive use of leaves or shoots of herbarium specimens collected from natural habitats. Analyses of herbarium material ranging in age from <1 to >100 yr showed that time-dependent decomposition was appreciable only for β -alanine betaine, which can undergo a β -elimination reaction giving trimethylamine and acrylate. Recent specimens (typically <40 yr) were therefore used and, where necessary, freshly collected, freeze-dried samples were analyzed for confirmation.

Isolation and Analysis of QACs. QACs were extracted from samples (30–100 mg dry weight) by a methanol/chloroform/water procedure and fractionated by ion-exchange chromatography and TLC as described (12, 22). The *n*-butyl esters of betaines were prepared and analyzed by fast atom bombardment/mass spectrometry (FAB-MS) using the methods of Rhodes *et al.* (23). Choline *O*-sulfate was determined by FAB-MS according to Hanson and Gage (22). QACs were quantified relative to internal standards (0.2–1 μ mol) of deuterated glycine betaine, β -alanine betaine, and choline *O*-sulfate, which were synthesized as described (12).

Bacterial Osmoprotection Assays. The bacterial strains were *Escherichia coli* K10 and *Salmonella typhimurium* TL1. Procedures were as described (12) with the following modifications: the minimal medium was that of Neidhardt *et al.* (24); the final NaCl concentration was 0.6 M for *E. coli* and 0.75 M for *S. typhimurium*; cultures were inoculated with cells growing exponentially in the presence of 0.6 or 0.75 M NaCl. Betaines of proline and hydroxyproline were prepared by methylating L-proline with iodomethane and *trans*-4-hydroxy-L-proline with *O*-methyl-*N,N'*-diisopropylisourea.

Table 1. Biosynthetic pathways and structures of zwitterionic QACs in flowering plants

Biosynthetic pathway	Enzyme	Ref(s).
$(\text{CH}_3)_3\text{N}^+\text{CH}_2\text{CH}_2\text{OH} \xrightarrow{-2\text{H}} (\text{CH}_3)_3\text{N}^+\text{CH}_2\text{CHO} \xrightarrow{-2\text{H}} (\text{CH}_3)_3\text{N}^+\text{CH}_2\text{COO}^-$ choline betaine aldehyde glycine betaine	Choline monooxygenase Betaine aldehyde dehydrogenase	10 11
$(\text{CH}_3)_3\text{N}^+\text{CH}_2\text{CH}_2\text{OH} \xrightarrow{+[\text{SO}_4^{2-}]} (\text{CH}_3)_3\text{N}^+\text{CH}_2\text{CH}_2\text{OSO}_3^-$ choline choline-O-sulphate	Choline sulfotransferase	12, 13
$\text{H}_3\text{N}^+\text{CH}_2\text{CH}_2\text{COO}^- \xrightarrow{+3[\text{CH}_3]} (\text{CH}_3)_3\text{N}^+\text{CH}_2\text{CH}_2\text{COO}^-$ β -alanine β -alanine betaine	<i>N</i> -Methyltransferase(s)	12, 14
$\text{Proline} \xrightarrow{+2[\text{CH}_3]} \text{Proline betaine}$	<i>N</i> -Methyltransferase(s)	15
$\text{Proline} \xrightarrow{+[\text{OH}]} \text{hydroxyproline} \xrightarrow{+2[\text{CH}_3]} \text{hydroxyproline betaine}$	Prolyl hydroxylase <i>N</i> -Methyltransferase(s)	16 9

The order of the hydroxylation and methylation steps is not certain.

Biochemical Methods. For immunoblot analyses, proteins were extracted from fresh leaves according to ref. 19 except in the case of *Plumbago zeylanica*, for which an acetone

powder was used. Leaf proteins (100 μg per lane) were separated by SDS/PAGE, transferred to nitrocellulose, and probed with antibodies raised against betaine aldehyde dehydrogenase from spinach (25). Proline was determined colorimetrically in aqueous extracts (26).

Habitat Stresses. The stresses prevailing in typical habitats of the species analyzed were assessed from information given in regional floras and in two monographs (27, 28), from data recorded on herbarium sheets, and from field observations.

RESULTS AND DISCUSSION

QAC Accumulation Patterns. The family Plumbaginaceae consists of ≈ 15 genera and 500–700 species divided into the subfamilies Plumbagoideae and Armerioideae (29–31). The Plumbaginaceae as a whole are tolerant of saline or dry conditions, but there is some specificity in the types of environmental stresses to which different groups in the family are adapted (27, 28). For example, all salt marsh-adapted taxa are members of the Armerioideae, while *Aegialitis* is a genus of mangroves restricted to coastal swamps. We surveyed ≈ 80 species representing all genera and subgeneric sections of the family, using FAB-MS to identify and quantify QACs. All species accumulated choline *O*-sulfate, and, apart from the mangrove *Aegialitis*, at least one betaine. There were four betaine accumulation patterns: glycine betaine alone, β -alanine betaine alone, proline betaine alone, and β -alanine betaine plus the betaines of proline and hydroxyproline (Fig. 1). The distribution of glycine betaine and β -alanine betaine

FIG. 1. FAB-MS of the *n*-butyl derivatives of betaines isolated from shoots of representative Plumbaginaceae. (A) *Plumbago au-*

riculata. (B) *Limonium carolinianum*. (C) *Limonium ferulaceum*. (D) *Limoniastrum monopetalum*. Signals corresponding to the derivatives of the various betaines are as follows: glycine betaine, *m/z* 174; β -alanine betaine, *m/z* 188; proline betaine, *m/z* 200; hydroxyproline betaine, *m/z* 216. Betaines were isolated from freeze-dried tissue. Plants were collected from natural habitats (B–D) or were grown under controlled saline (400 mM NaCl) conditions (A).

Subfamily	Genus	Section	QACs					Main Habitat Stress			
			CS	GB	BA	PB	HB	S/H	S	WD	A
Plumbagoideae	Plumbago (4)		■	■	□	□	□	○	○	●	○
	Plumbagella (1)		■	■	□	□	□	○	○	●	○
	Ceratostigma (2)		■	■	□	□	□	○	○	●	○
	Dyerophytum (2)		■	■	□	□	□	○	○	●	○
Armerioideae	Gladiolimon (1)		■	■	□	□	□	○	○	●	○
	Limonium	Pteroclados (11)	■	■	□	□	□	○	○	●	○
		Limoniendron (1)	■	■	□	□	□	○	○	●	○
		Ctenostachys (3)	■	■	□	□	□	○	○	●	○
		Plathymenium (3)	■	■	□	□	□	○	○	●	○
		Limonium (14)	■	■	□	□	□	○	○	●	○
		Sphaerostachys (1)	■	■	□	□	□	○	○	●	○
		Jovi-barba (1)	■	■	□	□	□	○	○	●	○
		Schizhyemenium (1)	■	■	□	□	□	○	○	●	○
		Circinaria (3)	■	■	□	□	□	○	○	●	○
		Polyarthron (2)	■	■	□	□	□	○	○	●	○
		Myriolepis (2)	■	■	□	□	□	○	○	●	○
		Siphonantha (1)	■	■	□	□	□	○	○	●	○
		Pterolimon (1)	■	■	□	□	□	○	○	●	○
		Arthrolimon (1)	■	■	□	□	□	○	○	●	○
	Armeria	Macrocentron (3)	■	■	□	□	□	○	○	●	○
		Plagiobasis (3)	■	■	□	□	□	○	○	●	○
	Acantholimon	Armeriopsis (2)	■	■	□	□	□	○	○	●	○
		Glumaria (1)	■	■	□	□	□	○	○	●	○
		Staticopsis (3)	■	■	□	□	□	○	○	●	○
	Goniolimon (4)		■	■	□	□	□	○	○	●	○
	Psylliostachys (1)		■	■	□	□	□	○	○	●	○
	Limoniastrum (2)		■	■	□	□	□	○	○	●	○
	Cephalorhizum (1)		■	■	□	□	□	○	○	●	○
	Chaetolimon (1)		■	■	□	□	□	○	○	●	○
	Dictyolimon (2)		■	■	□	□	□	○	○	●	○
	Aegialitis (1)		■	■	□	□	□	○	○	●	○

FIG. 2. Patterns of QAC accumulation and stress adaptation in the family Plumbaginaceae. The taxonomic scheme used is essentially that of Boissier (29). The number of species analyzed from each taxon is given in parentheses. Solid squares indicate the QACs accumulated in each taxon: CS, choline *O*-sulfate; GB, glycine betaine; BA, β -alanine betaine; PB, proline betaine; HB, hydroxyproline betaine. Plant samples included herbarium material collected in natural habitats and freeze-dried shoots of plants grown in controlled saline environments (400–450 mM NaCl). Mean ($\pm$ SD) total QAC content of the 77 species analyzed was $125 \pm 64 \mu\text{mol}\cdot\text{g}^{-1}$ dry weight. Solid circles indicate the main abiotic stresses prevailing in the habitats of the species in each taxon: S/H, saline/hypoxic soils; S, saline soils; WD, water deficit; A, high altitude.

corresponded well to systematic groups based on morphological characters (Fig. 2). Glycine betaine occurred throughout the subfamily Plumbagoideae and in two small taxa from the Armerioideae; most other members of the Armerioideae had β -alanine betaine. Proline betaine and hydroxyproline betaine were restricted to three groups within the Armerioideae. These three groups are not closely related morphologically; one of them (*Limonium* sect. *Arthrolimon*) is native to Australia, while the others (*Limoniastrum* and *Limonium* sect. *Myriolepis*) occur in the Mediterranean region. The hydroxyproline betaine was determined by ^1H NMR analysis to be the *trans*-4-hydroxy-L-proline form (data not shown).

Probable Sequence of QAC Evolution. Given the biochemical and distributional evidence that glycine betaine accumulation is the basal condition among other flowering plants (8, 9, 19), it seems likely that this is so within the Plumbaginaceae and that the betaines of β -alanine, proline, and hydroxyproline are evolutionary novelties. The distribution patterns of these compounds in the Plumbaginaceae (Fig. 2) are consistent with this interpretation. Species in the morphologically unspecialized subfamily Plumbagoideae accumulate glycine betaine, whereas the more advanced elements in the subfamily Armerioideae have produced alternative betaines. This interpretation is further supported by immunoblot analyses (Fig. 3), which show that glycine betaine-accumulating Plumbaginaceae strongly express a glycine betaine synthesis enzyme (betaine aldehyde dehydrogenase) that is immuno-

logically related to those of many other glycine betaine-accumulating angiosperms (19). β -Alanine betaine accumulators have very little or none of this enzyme. Because all members of the family accumulate choline *O*-sulfate, the acquisition of this QAC must have occurred early in the evolution of the Plumbaginaceae.

The replacement of glycine betaine by structurally similar compounds raises the question of the adaptive significance of the alternative QACs. Bacterial osmoprotection bioassays (32, 33) and tests with isolated enzymes (8) indicate that none

FIG. 3. Immunoblot analysis of expression of betaine aldehyde dehydrogenase in species of Plumbaginaceae that accumulate glycine betaine (+GB) or that do not (–GB). Betaine aldehyde dehydrogenase catalyzes the last step in glycine betaine biosynthesis (see Table 1). Lanes: 1, spinach control; 2, *Plumbago zeylanica*; 3, *Limonium sinuatum*; 4, *Limonium latifolium*; 5, *Armeria maritima*.

of the QACs found in the Plumbaginaceae is superior to glycine betaine as an osmoprotectant. However, as we now discuss, a *prima facie* case based on physiological and ecological arguments can be made for other features having led to the replacement of glycine betaine during the evolution of the Plumbaginaceae. While for simplicity we restrict our arguments to abiotic stresses, it should be recognized that the evolution of alternative QACs may also have involved other selective forces—for example, from herbivores and pathogens (14).

Choline O-Sulfate. Choline O-sulfate may serve both as an osmoprotectant and as a means of sequestering SO_4^{2-} ions in harmless form. Choline O-sulfate accumulation occurs throughout the Plumbaginaceae (Fig. 2) and is a unique characteristic of the family. Epidermal salt-secreting glands are also characteristic of the family, occurring in all species regardless of their habitat (27, 34). These glands can secrete massive amounts of Na^+ and Cl^- ions but have little ability to transport SO_4^{2-} (22, 34). Water sources high in NaCl usually also have high SO_4^{2-} levels (2), so that failure to secrete SO_4^{2-} can produce a potentially damaging long-term accumulation within the leaf (35). Conjugating part of the SO_4^{2-} to choline would detoxify it (36). In support of this, SO_4^{2-} salinity leads to higher choline O-sulfate levels than Cl^- salinity (22). Salt glands and choline O-sulfate accumulation may therefore have evolved in concert as complementary components of the family's high basal level of salt tolerance. However, the evolution of choline O-sulfate accumulation adds a biosynthetic demand for choline, which potentially competes with the synthesis of glycine betaine (Table 1) and of phosphatidylcholine. It is therefore noteworthy that the mangrove genus *Aegialitis* accumulates only choline O-sulfate. *Aegialitis* has extremely active salt glands and is the only member of the family adapted to grow in seawater, which contains $\approx 30 \text{ mM SO}_4^{2-}$.

β -Alanine Betaine. Eliminating metabolic competition for choline could be one advantage of replacing glycine betaine with β -alanine betaine. Because this unusual betaine is synthesized via methylation of β -alanine rather than from choline (Table 1), β -alanine betaine accumulators might be expected to have more choline available for choline O-sulfate synthesis, and indeed this is the case (Fig. 4). Biosynthesis of β -alanine betaine is also probably advantageous because it has no direct O_2 requirement. In contrast, the first step in glycine betaine biosynthesis is mediated by a monooxygenase (Table 1) that could not operate in roots under the severe

FIG. 4. Choline O-sulfate content of species of Plumbaginaceae that accumulate glycine betaine (+ Glycine betaine) or that do not (– Glycine betaine). Herbarium samples with total QAC contents of $<20 \mu\text{mol}\cdot\text{g}^{-1}$ dry weight were excluded from the analysis.

hypoxia often prevailing in salt marshes (12, 37). Consistent with this idea, many of the β -alanine betaine-accumulating Plumbaginaceae occur in salt marshes, whereas the glycine betaine accumulators do not, being found mainly in dry environments (Fig. 2). Interestingly, substituting β -alanine betaine for glycine betaine within the subfamily Armerioideae has not narrowed their adaptation, as many β -alanine betaine accumulators are found in dry and dry-saline environments as well as at high altitudes (Fig. 2). β -Alanine betaine thus appears to be effective over a broader ecological spectrum than glycine betaine.

Proline Betaine and Hydroxyproline Betaine. These betaines occur in three taxa that are quite narrowly adapted to very dry or saline environments and provide further evidence of extreme plasticity in the evolution of QAC osmoprotectants in the Plumbaginaceae. In all three taxa, there are marked morphological adaptations to osmotic stress: leaf reduction and development of photosynthetic stems in *Limonium* sections *Myriolepis* and *Arthrolimon*, and fleshy leaves with a highly reflective surface in *Limonium* (27). Dry or saline conditions typically elicit accumulation of free proline in flowering plants, particularly as a short-term response to severe stress (38). In bacterial osmoprotection bioassays, proline is a good osmoprotectant, but proline betaine and hydroxyproline betaine are better (Fig. 5; ref. 33), and both are derived from free proline (Table 1). Plumbaginaceae subjected to particularly harsh osmotic stress may thus have evolved the ability to convert a chronically large pool of proline into more effective osmoprotectants. The reciprocal relationship between levels of free proline and levels of proline-derived betaines within the family fits this suggestion (Fig. 6). The biosynthetic pathways for these betaines may have arisen more than once because the taxa in which they occur are morphologically distinct and geographically isolated. Taken with the evidence for the switch from glycine betaine to β -alanine betaine, this indicates that, despite the large redirections of nitrogen metabolism involved, QAC metabolism is remarkably malleable in evolutionary terms.

FIG. 5. Effect of proline, proline betaine, and hydroxyproline betaine on the growth of *S. typhimurium* (a) and *E. coli* (b) in the presence of NaCl. Compounds (1 mM) were added to cultures of cells growing logarithmically in the presence of 0.75 M NaCl (*S. typhimurium*) or 0.6 M NaCl (*E. coli*).

FIG. 6. Reciprocal relationship in the Plumbaginaceae between accumulation of free proline and that of proline betaine and hydroxyproline betaine. Bars are data for shoots of 20 species chosen to represent diversity within the family, arranged in descending order of proline content. Plant material was from herbarium specimens collected in natural habitats. Key to species (in the order 1–20): *Limonium globuliferum*, *Dyerophytum africanum*, *Limonium pectinatum*, *Limonium mucronatum*, *Limonium puberulum*, *Armeria juniperifolia*, *Plumbago scandens*, *Limonium macrophyllum*, *Plumbago europaea*, *Limonium plumosum*, *Acantholimon talagonicum*, *Limonium ferulaceum*, *Limonium peregrinum*, *Limonium nashii*, *Plumbagella micrantha*, *Cephalorhizum coelicolor*, *Limonium salicorniaceum*, *Limonium monopetalum*, *Limonium diffusum*, *Limonium guyonianum*.

Implications for Metabolic Engineering of Stress Tolerance. The biosynthetic pathways for glycine betaine, choline *O*-sulfate, β -alanine betaine, and proline-derived betaines are relatively simple (Table 1) and so could in principle be engineered into crop plants by recombinant DNA methods (5). The potential for improving general osmotic stress tolerance by engineering glycine betaine accumulation has been apparent for some time (17, 18), and progress has recently been made in this area (39, 40). The data presented here on the versatility of QAC osmoprotectant systems in the Plumbaginaceae, together with the inferences drawn regarding their functional significance, point to some more environment-specific opportunities that can be tested experimentally. These are (i) increasing crop tolerance to SO_4^{2-} salinity, by engineering choline *O*-sulfate accumulation; (ii) improving adaptation to saline, poorly aerated soils by engineering β -alanine betaine accumulation in roots; and (iii) enhancing the effectiveness of proline by engineering its conversion to betaines during severe stress. More generally, our results illustrate the diversity of simple biochemical adaptations to different types of abiotic stresses that have evolved in the angiosperms and that might be drawn on to improve crops.

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