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GLYCINEBETAINE IS A MAJOR NITROGEN-CONTAINING SOLUTE IN THE MALVACEAE

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Key Word Index—*Gossypium*; Malvaceae; cotton; glycinebetaine; compatible solute; salinity.

Abstract—The zwitterionic quaternary ammonium compound glycinebetaine was detected in all but three of over 100 species of various genera in the family Malvaceae. In a more limited range of species, particularly of *Gossypium*, glycinebetaine accumulated to concentrations sometimes in excess of 100 mM water in response to water deficit or salinity stress. Glycinebetaine concentrations were highest in young tissues and accounted for about 10% of the total nitrogen. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Glycinebetaine has been identified as a stress metabolite in bacteria, algae and higher plants [1, 2]. It occurs widely in some families such as the Chenopodiaceae and the Poaceae, but is almost absent in others [2, 3]. Many taxa have not been systematically investigated for their ability to produce glycinebetaine.

One large family in which there are very few reports of glycinebetaine is the Malvaceae. Glycinebetaine was detected by TLC and Dragendorff's reagent in two *Abutilon* species, and a Dragendorff-positive substance was detected in *Lavatera plebeja* [2]. High concentrations of methylated onium compounds were found in *Hibiscus tiliaceus* from mangrove swamps in Northern Queensland [4]. Seeds of the American salt marsh plant *Kosteletzkya virginica* contain about 1% dry weight of betaine (assayed as Dragendorff-positive substances) before germination [5].

RESULTS AND DISCUSSION

Extracts of cotton (*Gossypium hirsutum* L.) applied to TLC plates and sprayed with Dragendorff's reagent exhibited characteristic orange–purple spots [6] at the same R_f as glycinebetaine. The extract, purified on ion-exchange resins, gave a precipitate with the periodide reagent [7, 8], co-eluted with glycinebetaine on a Sarasep Na^+-form carbohydrate HPLC column and gave a major ^1H NMR signal at 3.25 ppm (9 methyl group protons of the quaternary ammonium group) and a minor signal corresponding to the CH_2 group at 4.13 ppm [9, 10]. Glycinebetaine has also been detected in field-grown cotton by FAB-mass spectrometry (A. Hanson, personal communication).

The distribution of glycinebetaine in other members of the Malvaceae was determined by TLC. All species of *Gossypium* examined (over half of all species) contained large amounts of glycinebetaine (>20 mmol kg^{-1} fresh weight). Smaller amounts of choline were detected in nearly all species. Glycinebetaine was also detected in other genera of the Malvaceae including *Abutilon* (6 spp), *Althaea* (5 spp), *Hibiscus* (13 spp), *Kosteletzkya* (10 spp), *Lavatera* (6 spp), *Malva* (6 spp) and *Sida* (6 spp). Glycinebetaine was found in over 100 taxa, together with variable amounts of choline and, in some cases, other Dragendorff-positive compounds.

The effect of salt stress on glycinebetaine concentrations in four members of the Malvaceae grown in a flood-bench facility is shown in Table 1. Glycinebetaine concentrations increased with increasing salt stress in all four species, with the highest concentration (over 100 mM plant sap) being found in *G. herbaceum africanum* at 200 mM NaCl. This species

Table 1. Glycinebetaine concs in expressed sap of young, fully expanded leaves of members of the Malvaceae. Plants were grown in pots filled with baked clay granules on a flood bench and irrigated daily with nutrient soln +0, 125 or 200 mM NaCl (+ 1/20th $CaCl_2$). Values are means of four replicates±s.e.

Species	0 mM NaCl	125 mM NaCl	200 mM NaCl
Kosteletzkya virginica	24.4±5.93	39.7±1.12	60.0±7.42
Althaea rosea	6.5±0.75	23.4±2.64	37.1±6.95
Gossypium stocksii × *G. hirsutum*	10.1±2.16	19.2±1.37	46.6±5.53
G. herbaceum africanum	39.4±0.89	63.4±3.16	99.7±4.06

Table 2. Glycinebetaine concs in expressed sap of leaves of different ages (positions on stem – lowest numbers are the oldest leaves) of *Gossypium herbaceum* M18. Plants were grown in aerated hydroponic culture. Values are means of four replicates ± s.e.

	Glycinebetaine (mM)	
Leaf position	0 mM NaCl	200 mN NaCl
4	2.9±1.70	23.2± 5.64
8	8.2±1.18	40.4±10.76
10		78.9±12.24
12	16.9±9.31	
16	35.9±6.20	

had the highest content of glycinebetaine at all salt concentrations. Glycinebetaine concentrations are determined not only by the level of salt stress but also by the age of the tissue. This is shown for hydroponically-grown *G. herbaceum* M-18 in Table 2 (the other four accessions in this experiment gave similar results). Glycinebetaine concentrations were much higher in the younger leaves (higher positions on the stem) than in the older ones, and again higher in the salt-treated plants than in the controls. In another hydroponic experiment, with the *G. hirsutum* variety Acala SJ-2, the glycinebetaine concentration in mature cotton seeds remained constant (at 20 mmol kg^{-1} dry weight) in external NaCl concentrations ranging from 0 to 300 mM.

The increase in glycinebetaine concentrations in the sap of *Gossypium* species subjected to cyclical drought stress is shown in Table 3. The highest concentrations (over 100 mM) were found in the drought stressed plants of the commercial cotton variety Acala SJ-2. A significant increase in the concentration of glycinebetaine is also apparent if the results are expressed on a dry weight basis (i.e. the increase in concentrations is not due solely to dehydration of the leaves). Similar results have been obtained with other lines of *G. hirsutum* (data not shown).

The occurrence of large concentrations of glycinebetaine in cotton and other members of the Malvaceae has several interesting implications. Since this compound is associated with resistance to abiotic stresses in other species, it may have a similar role here (as a compatible or protective solute), but the high concentrations suggest that it is largely located in the vacuoles. There are reports which suggest that glycinebetaine attracts or sustains insect pests [11]. Another consideration is the amount of nitrogen 'tied up' in a metabolically inert end product. The methylated onium compounds in *Hibiscus tiliaceus* [4] accounted for 10% of the nitrogen in young leaves, and 6% in older leaves. In cotton the accumulation of 300 mmol kg^{-1} dry weight (found in drought-stressed cotton) would represent about 8–10% of the total nitrogen.

Table 3. Glycinebetaine concentrations in the expressed sap of young, fully expanded leaves of members of the Malvaceae. Plants were grown in 1.5 m × 15 cm diameter drainpipes filled with John Innes No. 3 compost and either watered daily or subjected to cyclical H_2O stress (watered about once each week). Values are means of four replicates ± s.e.

Species	Control	H_2O stressed
G. hirsutum cv. Acala SJ2	28.7±0.69	107.2± 5.5
G. sturtianum	25.4±1.60	58.6±11.7
G. herbaceum africanum	24.7±1.99	67.8±10.0
G. davidsonii	23.3±2.24	58.6±16.6

If the synthesis of glycinebetaine in cotton could be eliminated, for example by genetic engineering, the physiologist would have a useful tool with which to study the role of this compound in a commercially important agricultural crop.

EXPERIMENTAL

Plant material. Material used in this investigation was grown from seeds obtained in habitat, or from The Royal Botanic Gardens, Prof. J. M. Stewart, Mr Anwar Mirza, Dr Iftikhar Khan, Dr A. H. D. Brown, Prof. O. J. Blanchard, or from commercial sources. Unless otherwise stated, plants were grown in potting compost in glasshouses in Bangor.

Salinity treatment. Plants of the wild *Gossypium* species were used to provide softwood cuttings for the experiments. Seeds of hexaploid hybrids and other Malvaceae were sown in plugtray segments (P84, Plantpak, Maldon, U.K.) and transplanted to the experimental system as seedlings.

Four rooted cuttings or seedlings per treatment were transplanted to 1.65 l square plant pots filled with 'Seramis' baked clay granules. After 2 weeks in a greenhouse maintained at a minimum of 28° and with supplementary sodium vapour lighting, the pots were moved to a flood-bench facility made from plastic containers 35 × 80 × 60 cm fitted with a section of 15 mm hosepipe connected to a pump which was immersed in 300 l storage tanks of treatment soln. Nutrients were supplied as 'Phostrogen' (1 g l^{-1}, Phostrogen, Corwen, Wales, U.K.) supplemented with micronutrient solution (0.5 ml l^{-1}) [12]. Macronutrient concns were 5 mM K^+, 6 mM NO_3^-, 1.8 mM PO_4^{3-}, 0.7 mM Ca^{2+} and 0.6 mM Mg^{2+}. Salt ($NaCl + CaCl_2$ at a Na:Ca ratio of 20:1) was added after 5 days in daily increments of <40 mM $days^{-1}$ to final concns of 125 and 200 mM NaCl. The electrical conductivities of the treatment solns were checked on the last day of salt addition and salt concns were adjusted to the required values. During the experiment the concns of salts and nutrients were checked by ion chromatography, and H_2O or nutrients added to maintain constant conditions. Treatment solns were pumped into the flood benches for 15 min each morning to a level 5 mm below the surface of the Seramis granules.

Table 2 is taken from the results of an experiment in which 5 cotton varieties (one *G. hirsutum* variety (NIAB 78), two *G. arboreum* lines (Chitagang Hill and

Barmenicum) and two *G. herbaceum* lines (M-3 and M-18) were grown in aerated hydroponic (liquid) culture, with or without 200 mM NaCl and 10 mM $CaCl_2$.

Water deficit stress. Seedlings or cuttings of four *Gossypium* species were planted singly in John Innes No. 3 compost in plastic drainpipes (15 cm diam × 1.5 m). All plants were well watered until 6 weeks after transplanting, after which 6 replicates were subjected to cyclical H_2O deficit. This was achieved by withholding water for about 7 days, or until wilting was observed. Another 6 replicates continued to be watered every day. Leaf samples were taken 6 weeks after the start of the imposition of H_2O deficits, i.e. after several cycles of stress.

Extraction and purification. Fresh leaf material was either placed in microcentrifuge tubes and frozen for sap extraction [13] or rapidly frozen in liquid air, crushed and extracted in a sealed glass vial with hot (95°) MeOH for 2 hr. The MeOH was evapd in a stream of air and the glycinebetaine dissolved in H_2O. The aq. extract was purified by passing through C_{18} and anion solid-phase extraction columns. These procedures were necessary to remove polysaccharides and salts prior to TLC or HPLC. For TLC the aq. extract was dried and redissolved in MeOH.

Aq. extracts were purified on ion-exchange resins prior to qualitative analysis. After passage through Dowex 1 (OH-form) and Amberlite CG50 (H^+ form), glycinebetaine was retained on a column of Dowex 50 (H^+ form) and eluted with 4 M MH_4OH. The eluate was dried *in vacuo*.

Chromatography. TLC was performed on silica gel eluted with MeOH–Me_2CO–conc HCl (45:5:2) or MeOH–NH_4OH (3:1) [6]. Detection was with Dragendorff's reagent [14]. Full colour development was observed after 4 hr at 4°. Quantitative analysis was performed by HPLC on a 250 × 4 mm (i.d.) Sarasep Na^+-form carbohydrate column eluted with 25 mM Na_2SO_4 at 80°. The flow rate was 0.6 ml min^{-1} and glycinebetaine was detected with a Shodex refractive index detector.

Modified periodide assay. The periodide assay for quaternary ammonium compounds [7, 8] was modified by dissolving the periodide ppt in MeOH instead of CH_2Cl_2.

NMR. Extracts purified on ion exchange resins were dissolved in D_2O containing 4 μmol 2-methylpropan-2-ol as int. standard and examined in a Bruker NMR spectrometer at 250 MHz.

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