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Update on Abiotic Stress

Betaines and Related Osmoprotectants. Targets for Metabolic Engineering of Stress Resistance[1]

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Osmoprotectants (also termed compatible solutes) occur in all organisms from archaebacteria to higher plants and animals. They are highly soluble compounds that carry no net charge at physiological pH and are nontoxic at high concentrations. Osmoprotectants serve to raise osmotic pressure in the cytoplasm and can also stabilize proteins and membranes when salt levels or temperatures are unfavorable. Osmoprotectants therefore play important roles in the adaptation of cells to various adverse environmental conditions (Yancey, 1994). Chemically, there are three types: betaines and allied compounds, polyols and sugars (e.g. mannitol and trehalose), and amino acids such as Pro. This *Update* will focus on betaines and their biosynthetic pathways. Advances in polyol and Pro research are covered elsewhere (Delauney and Verma, 1993; Stoop et al., 1996).

Betaines are amino acid derivatives in which the nitrogen atom is fully methylated, i.e. they are quaternary ammonium compounds. Figure 1 shows the structures of the three best-known betaines from plants, Gly betaine, Pro betaine (stachydrine), and β-Ala betaine, as well as choline-*O*-sulfate and DMSP (Rhodes and Hanson, 1993). The last two compounds are strictly speaking not betaines because DMSP has a tertiary sulfonium in place of a quaternary ammonium group, and choline-*O*-sulfate has a sulfate ester instead of a carboxyl group—but they are close structural analogs of betaines and have similar chemical and physiological properties.

The compounds in Figure 1 differ in their taxonomic distribution (Blunden and Gordon, 1986; Rhodes and Hanson, 1993). For instance, Gly betaine is widespread among both flowering plants and algae, whereas DMSP is rare in higher plants but common in algae. Certain crop plants such as rice, soybeans, and potatoes lack significant amounts of betaines or any other osmoprotectant. This deficiency is the rationale for recent interest in using metabolic engineering technology to install the synthesis of osmoprotectants in such crops in order to improve their tolerance to drought, salinity, and other stresses.

The levels of betaines and other osmoprotectants typically rise during exposure to stresses such as salinity, water deficit, and low temperature because the biosynthetic enzymes are stress induced. Osmoprotectants are largely confined to the cytoplasm (including organelles) and are almost absent from the vacuole, which generally occupies about 90% of the cell volume. For example, the halophyte *Atriplex gmelini* was found to have 320 mM Gly betaine in the cytoplasm, but only 0.24 mM in the vacuole (Matoh et al., 1987). Isolated chloroplasts of various species have also been shown to contain high concentrations of Gly betaine or DMSP, particularly when isolated from salt-stressed plants; Figure 1B illustrates this point with data from three species.

MAGNITUDE AND MECHANISM OF PROTECTIVE EFFECTS

The protective properties of betaines were first recognized in experiments in which they were supplied to bacteria whose growth was inhibited by high salt concentrations (Le Rudulier et al., 1984). Typical data for Gly betaine and DMSP are shown in Figure 2: In media containing 0.6 M NaCl, the bacteria grow very slowly unless supplied with one of these compounds, which they take up from the medium and accumulate to intracellular levels of >1 M. The physicochemical basis for this striking osmoprotective effect is not fully understood, but there is good evidence that it lies partly in the exclusion of osmoprotectant molecules from the water layer in contact with protein surfaces (Timasheff, 1992). This creates a situation in which native (i.e. folded) protein structures are thermodynamically favored because they present the least possible surface area to the water. Most other solutes such as NaCl or $MgSO_4$ interact directly with protein surfaces and favor unfolding, which leads to denaturation. Osmoprotectants also have cryoprotectant and heat-protectant properties, and exclusion from protein surfaces is probably part of the protective mechanism in these cases as well (Carpenter and Crowe, 1988; Winzor et al., 1992).

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Abbreviations: BADH, betaine aldehyde dehydrogenase; CMO, choline monooxygenase; DMSP, 3-dimethylsulfoniopropionate; DMSP-aldehyde, 3-dimethylsulfoniopropionaldehyde; DMSP-amine, 3-dimethylsulfoniopropylamine; SMM, *S*-methylmethionine.

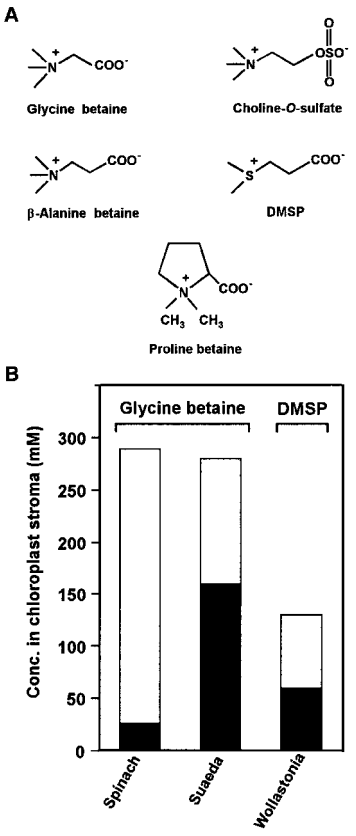

Figure 1. A, Chemical structures of betaines and related compounds that occur in flowering plants and marine algae. B, Concentrations of Gly betaine or DMSP measured in chloroplasts isolated from leaves of salt-tolerant plants grown in nonsaline (black bars) or saline (white bars) conditions. Data are from Robinson and Jones (1986), Génard et al. (1991), and Trossat et al. (1998). Conc., Concentration.

BIOSYNTHETIC PATHWAYS, ENZYMES, AND GENES

Detailed knowledge of biochemical pathways is a prerequisite for metabolic engineering. Biosynthetic routes have now been established for all of the compounds shown in Figure 1. Most of the enzymes participating in these pathways have been identified, and genes for some of them have been cloned. The following is a summary of the current status of knowledge for each compound.

Gly Betaine

Gly betaine occurs in diverse marine algae and at least 10 flowering plant families, including Chenopodiaceae, Amaranthaceae, Gramineae, Compositae, and Malvaceae (Blunden and Gordon, 1986; Rhodes and Hanson, 1993). Its synthesis has been studied mainly in species of Chenopodiaceae, and to some extent in Amaranthaceae and Gramineae. In these cases, Gly betaine is synthesized via a two-step oxidation of choline (Fig. 3). In Chenopodiaceae and Amaranthaceae, the first step (choline → betaine aldehyde) is mediated by CMO, an unusual Fd-dependent monooxygenase with a Rieske-type iron-sulfur cluster, which has been characterized and cloned. The active enzyme is a dimer or trimer of identical 43-kD subunits and is localized in the chloroplast stroma (Burnet et al., 1995; Rathinasabapathi et al., 1997). Because reduced Fd is generated by photosynthetic electron transport, CMO activity in vivo is strongly light dependent. Both drought and salinity induce CMO expression (Rathinasabapathi et al., 1997; Russell et al., 1998). As CMO has only been found in Chenopodiaceae and Amaranthaceae (Russell et al., 1998), other families may have different choline-oxidizing enzymes that may not be located in plastids. Could these families have choline dehydrogenases or choline oxidases similar to the enzymes known to catalyze choline oxidation in bacteria (Le Rudulier et al., 1984; Hayashi et al., 1997)?

The second step of Gly betaine synthesis is catalyzed by BADH (Fig. 3), an NAD-dependent dehydrogenase that has been characterized and cloned from Chenopodiaceae, Amaranthaceae, and Gramineae. BADH is not unique to Gly betaine synthesis; it also attacks DMSP-aldehyde (see below) and seems to be identical to ω-aminoaldehyde dehydrogenase, a ubiquitous enzyme of polyamine metabolism (Trossat et al., 1997). This probably explains why plants with no Gly betaine have some BADH activity (Holmstrom et al., 1994; Rathinasabapathi et al., 1994). BADH is a dimer of identical 54-kD subunits. It is a stromal enzyme in Chenopodiaceae, and, surprisingly, lacks a typical transit peptide. Nonetheless, BADH is efficiently targeted to chloroplasts in transgenic tobacco (Rathinasabapathi et al., 1994). In Gramineae, however, most of the BADH may be peroxisomal (Nakamura et al., 1997), which reinforces the idea that the choline-oxidizing enzyme in this family may not be CMO, as CMO could not function in peroxisomes where there is no reduced Fd. BADH is induced by osmotic stress in both Chenopodiaeae and Gramineae (Rhodes and Hanson, 1993).

Choline-*O*-Sulfate

Choline-*O*-sulfate occurs throughout the Plumbaginaceae (Hanson et al., 1994) and in some marine algae (Blunden

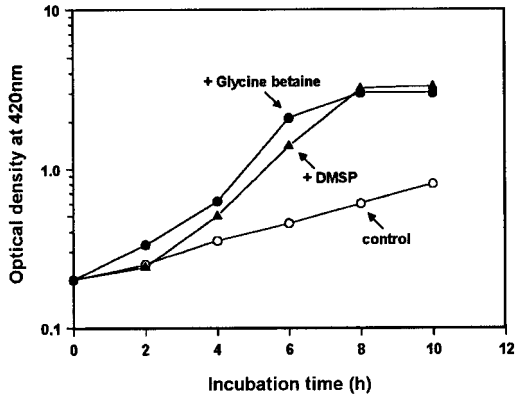

Figure 2. An illustration of the osmoprotective effect of Gly betaine or DMSP on cells of the bacterium *Escherichia coli* cultured in a highly saline medium (0.6 M NaCl). Growth in the presence of 1 mM Gly betaine or DMSP was far more rapid than in their absence (control). These compounds accumulated to intracellular levels of >1 M. Bacterial growth was measured by the optical density of the cultures at 420 nm. Data are from Paquet et al. (1994).

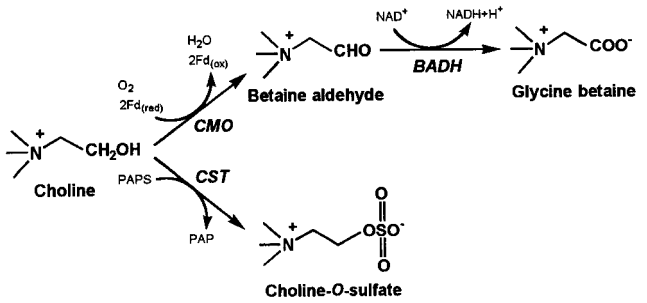

Figure 3. Biosynthetic pathways of Gly betaine in Chenopodiaceae and of choline-*O*-sulfate in Plumbaginaceae. The product of the CMO reaction is the hydrate form of betaine aldehyde. CST, Choline sulfotransferase; PAP, 3′-phosphoadenosine-5′-phosphate; PAPS, 3′-phosphoadenosine-5′-phosphosulfate.

and Gordon, 1986). In addition to being an osmoprotectant, it also provides a means of detoxifying sulfate, which is often abundant in saline environments (Hanson et al., 1994). The conversion of choline to choline-*O*-sulfate is catalyzed by a 3′-phosphoadenosine-5′-phosphosulfate-dependent choline sulfotransferase (Fig. 3) (Rivoal and Hanson, 1994). This sulfotransferase is a soluble enzyme with an extremely high affinity for choline (K_m = 5.5 μM), which probably reflects the low cytoplasmic choline levels that prevail in plants (Nuccio et al., 1998). Saline conditions induce sulfotransferase enzyme activity in both leaves and roots (Rivoal and Hanson, 1994).

Pro Betaine and β-Ala Betaine

β-Ala betaine and Pro betaine are found in some species of Plumbaginaceae; Pro betaine also occurs in Rutaceae, Leguminosae, Compositae, and other families (Rhodes and Hanson, 1993; Hanson et al., 1994; Nolte et al., 1997). Both compounds are also found in marine algae (Blunden and Gordon, 1986) and are known to be formed by methylation of the corresponding amino acid; however, the enzyme(s) involved have yet to be identified (Essery et al., 1962; Hanson et al., 1994). For Pro betaine, in vivo radiolabeling and phytochemical data suggest that the two methylations are catalyzed by different enzymes (Essery et al., 1962; Naidu et al., 1987).

DMSP

DMSP biosynthesis is important not only in relation to osmoprotection but also because the DMSP produced by marine algae is the precursor of atmospheric dimethylsulfide gas. This gas has a key role in the global sulfur cycle and influences climate (Malin and Kirst, 1997). DMSP is present in diverse marine algae (Blunden and Gordon, 1986; Malin and Kirst, 1997), but has so far been found in only a few flowering plants: *Spartina* spp. and sugarcane from the Gramineae and *Wollastonia biflora* from the Compositae. In all cases DMSP is synthesized from Met. However, conversion of Met to DMSP seems to have evolved independently three times, once in algae and twice in flowering plants, because there are three different pathways (Fig. 4).

DMSP Synthesis in Algae

The steps involved in DMSP synthesis have been demonstrated in the green macroalga *Enteromorpha intestinalis*, and appear to be the same in diverse microalgae (Gage et al., 1997). They are: transamination of Met to give the corresponding 2-oxo acid, followed by reduction to a 2-hydroxy acid, then *S*-methylation and oxidative decarboxylation (Fig. 4). The enzymes catalyzing the first three steps have been demonstrated in vitro (Summers et al., 1998); in vivo $^{18}O_2$-labeling data indicate that the final step is mediated by an oxygenase (Gage et al., 1997).

DMSP Synthesis in Flowering Plants

DMSP synthesis in flowering plants has been investigated in *W. biflora* and *S. alterniflora*, which have somewhat different pathways. In both species, the first step is conversion of Met to SMM, catalyzed by Met *S*-methyltransferase, and the last is oxidation of DMSP-aldehyde, catalyzed by

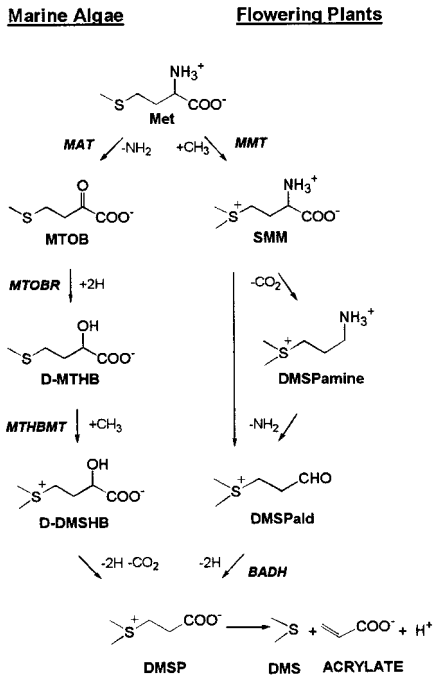

Figure 4. Biosynthetic pathways of DMSP in marine algae and flowering plants. The algal pathway has been found in the green macroalga *E. intestinalis* and various plankton species. Two related pathways have been found in flowering plants, one in the saltmarsh grass *S. alterniflora* and the other in the Pacific coastal plant *W. biflora*. The breakdown of DMSP to give dimethylsulfide (DMS) and acrylate is also shown; this reaction has so far only been demonstrated in marine algae and bacteria. DMSPald, DMSP-aldehyde; D-DMSHB, D-4-dimethylsulfonio-2-hydroxybutyrate; D-MTHB, D-4-methylthio-2-hydroxybutyrate; MAT, Met aminotransferase; MMT, Met *S*-methyltransferase; MTHBMT, 4-methylthio-2-hydroxybutyrate *S*-methyltransferase; MTOB, 4-methylthio-2-oxobutyrate; MTOBR, MTOB reductase.

BADH (Trossat et al., 1996, 1997; Kocsis et al., 1998). Met *S*-methyltransferase has been shown to be cytosolic and has been cloned (Trossat et al., 1996; Bourgis et al., 1999). In *W. biflora*, SMM is apparently converted to DMSP-aldehyde in a single step via an unusual coupled transamination-decarboxylation reaction (Fig. 4) (Rhodes et al., 1997). This conversion is known to occur in the chloroplast (Trossat et al., 1996), but the enzyme(s) involved has not yet been identified. On the other hand, *S. alterniflora* converts SMM to DMSP-aldehyde in two steps via the intermediate DMSP-amine (Kocsis et al., 1998) (Fig. 4). The enzymes have not yet been isolated, but in vivo radiolabeling evidence points to an SMM-specific decarboxylase plus a specific amine oxidase, dehydrogenase or transaminase (Kocsis et al., 1998).

METABOLIC ENGINEERING OF Gly BETAINE SYNTHESIS

Gly betaine accumulation has long been a target for engineering stress resistance (Le Rudulier et al., 1984). The idea that introducing the Gly betaine pathway into plants that lack it will enhance their stress tolerance is based both on comparative physiology (Yancey, 1994) and on genetic evidence from a mutation in maize that abolishes Gly betaine synthesis and reduces salt and heat tolerance (Saneoka et al., 1995; Yang et al., 1996). Wide-crossing work on *Lophopyrum elongatum* and wheat has provided further physiological-genetic evidence that Gly betaine accumulation contributes to salt tolerance (Colmer et al., 1995). Because plants have been found not to catabolize Gly betaine (Rhodes and Hanson, 1993; Nuccio et al., 1998), it has been reasonably assumed that engineering the synthesis of Gly betaine will lead to its accumulation.

Several groups have taken the first step toward this goal by expressing choline-oxidizing enzymes from bacteria (Lilius et al., 1996; Hayashi et al., 1997; Alia et al., 1998; Sakamoto et al., 1998) or spinach CMO (Nuccio et al., 1998) in tobacco and other plants that do not contain Gly betaine ("nonaccumulators"). The transgenic plants produced a little Gly betaine and, in some cases, showed small but significant increases in tolerance to various stresses (for review, see Nuccio et al., 1999). These results are encouraging inasmuch as they confirm that Gly betaine synthesis can be engineered. However, the Gly betaine levels obtained to date in transgenic plants (typically about 0.1–1 μmol g^{-1} fresh weight) are only a small percentage of those in spinach, sugar beet, and other plants that are natural Gly betaine accumulators. The main constraint on Gly betaine production in transgenic plants appears to be the endogenous choline supply, because providing choline exogenously leads to a massive increase in Gly betaine synthesis (Nuccio et al., 1998). It will therefore most likely be necessary to up-regulate the de novo synthesis of choline in order to increase Gly betaine synthesis in nonaccumulators expressing foreign choline-oxidizing enzymes (Nuccio et al., 1998).

CONCLUSIONS AND PROSPECTS

Characterization and cloning of the enzymes of Gly betaine synthesis has enabled us to start using transgenic plants to understand the role of Gly betaine in stress adaptation. This in turn is helping to define the potential of osmoprotectant engineering in crop improvement. Now that pathways to DMSP have been established, a similar approach could be followed for this compound; the same is true for the other osmoprotectants discussed above. There are two reasons to do this. First, some osmoprotectants may be better than Gly betaine in certain environments, which has important implications for engineering crops. We have already indicated that choline-*O*-sulfate could be particularly suitable in high-sulfate conditions because its synthesis can detoxify the sulfate anion. DMSP is another example; since it does not require nitrogen to produce, it may be a better choice than Gly betaine for environments that are poor in nitrogen. The second reason to transgenically express enzymes that produce various osmoprotectants is to explore the in vivo control of metabolism, about which we currently know very little. Introducing novel pathways increases the demand for precursors, and quantitative analysis of how this impacts metabolic fluxes, pool sizes, and gene expression can be highly informative in regard to the control architecture of metabolic networks (Bailey, 1991). For instance, because betaines and related compounds require very large amounts of methyl groups relative to primary metabolism, engineering betaine overproduction has the potential to teach us much about how the supply of one-carbon units is regulated.

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