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Choline metabolism in glycinebetaine accumulating and non-accumulating near-isogenic lines of *Zea mays* and *Sorghum bicolor*

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ABSTRACT

Glycinebetaine (GB) is a compatible solute that is accumulated by some plant species, especially under conditions leading to tissue osmotic stress. Genetic modification for accumulation of GB in an attempt to produce more stress tolerant plants has been a focus for several groups in recent years. However, attempts to increase tissue GB concentrations have been unsuccessful, with many transgenic lines accumulating far lower concentrations than naturally-occurring GB accumulators. A better understanding of the metabolic regulation of GB synthesis is necessary for successful molecular breeding and biotechnology. We utilized previously developed near-isogenic lines for GB accumulation to characterize the biochemical basis for GB deficiency in maize and sorghum. Salinity resulted in increased accumulation of choline in both accumulating and non-accumulating lines. When grown in the presence of NaCl, GB-non-accumulating lines had increased concentrations of choline and phosphocholine, but not GB. Decreased GB synthesis can be explained from the increased concentrations of phosphocholine *in planta* and the strong inhibition of N-phosphoethanolamine methyltransferase by phosphocholine observed *in vitro*. The lack of GB accumulation in GB−/− homozygous NILs was not due to the lack of the putative choline monooxygenase (the enzyme responsible for choline oxidation to betaine aldehyde) gene or protein that we describe. The previously identified bet1 locus does not appear to be choline monooxygenase. However, the lack of GB synthesis does affect the synthesis and turnover of choline moieties in GB non-accumulating lines, which may lead to alterations in overall 1-carbon metabolism in plants.

1. Introduction

One mechanism that plants have developed to survive stressful conditions, especially those involving drought or saline environments, is the synthesis and accumulation of osmolytes (or osmoprotectants). Osmolytes can be classed in one of three main categories: amino acids such as proline and glutamine; sugar alcohols such as pinitol and mannitol; and methylated metabolites such as 3-dimethylsulfoniopropinate (DMSP), choline-*O*-sulfate (CS), and glycinebetaine (GB) (**6**) (see Fig. 1) (Rhodes and Hanson, 1993). All of these osmolytes are thought to act as compatible solutes, in that they are small, uncharged, molecules (at physiological pH) and do not inhibit normal cellular processes. Their exact protective roles in plants are still undetermined. It is thought that when these compounds accumulate (>5 μmol g^{-1} dry weight) they act to lower the cellular water potential, allowing for the continued uptake and translocation of water. There are also several reports describing the ameliorative effects of GB (**6**) and proline on stress-associated damage to cell membranes and proteins (Brady et al., 1984; Paleg et al., 1984; Arakawa and Timasheff, 1985; Incharoensakdi et al., 1986; Ashihara et al., 1997; Mansour, 1998). The exogenous application of GB (**6**) has been shown to alleviate salt and dehydration stresses in non-accumulating plant and bacterial species, further re-enforcing the theory that GB (**6**) plays a role in cellular water balance (Ahmad et al., 1987; Harinasut et al., 1996; Makela et al., 1998, 1999).

GB, (**6**), and proline have been detected in nearly all plant families as well as in numerous bacterial, algal, and animal species (Cook and Wagner, 1984; Landfald and Strom, 1986; Blunden et al., 1992, 1996a,b, 1999; Rhodes and Hanson, 1993). However, not all members of a family are able to synthesize GB (**6**), most notably members of the Gramineae differ in their ability and level of GB (**6**) synthesis. Certain lines of maize and sorghum are able to produce and accumulate significant amounts of GB (**6**) while the ability is absent in rice (Rathinasabapathi et al., 1993).

In *Escherichia coli*, the synthesis of GB (**6**) from choline (**4**) is accomplished by a two enzyme system: choline (**4**) is first oxidized by choline dehydrogenase (CDH, E.C. 1.1.99.1) to betaine aldehyde, and then betaine aldehyde (**5**) is oxidized to GB (**6**) via betaine aldehyde dehydrogenase (BADH, E.C. 1.2.1.8). Glycinebetaine (**6**) synthesis in plants, as in *E. coli*, is a two step oxidation of choline

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(**4**). The first step is catalyzed by a unique ferredoxin-dependent choline monooxygenase (CMO, E.C. 1.14.15.7), first purified and characterized from spinach (*Spinacia oleracea*) (Burnet et al., 1995). It is located in the chloroplast stroma, requires light, uses ferredoxin as a cofactor, and contains an unusual Rieske-type [2Fe–2S] active site (Rathinasabapathi et al., 1997a). CMO homologs have been identified in most plants that accumulate GB (**6**); these include *Beta vulgaris*, *Amaranthus caudatus* (Russell et al., 1998), *Suaeda liaotungensis* (Li et al., 2003), *Atriplex prostrata* (Wang and Showalter, 2004), and *Atriplex hortensis* (Shen et al., 2002). All of these CMO homologs contain the same Rieske-type center and seem to be targeted to the chloroplast stroma. However, in members of the Gramineae that accumulate GB (**6**), only partial sequences showing similarity to CMO have been reported. Some non-accumulators, notably rice and Arabidopsis, also have CMO genes but no associated activities.

The second enzyme involved in GB (**6**) synthesis, BADH, has likewise been found in all natural accumulators. Unlike CMO, BADH is localized to the cytosol and chloroplast (Weigel et al., 1986), and possibly the peroxisome (Ishitani et al., 1995). BADH shows high similarity to many non-specific aldehyde dehydrogenases. In fact, many GB non-accumulators can oxidize exogenously supplied betaine aldehyde (**5**) to GB (**6**) (Weretilnyk et al., 1989). In amaranth and beets, both CMO and BADH expression are inducible by salt, drought, and abscisic acid treatments (McCue and Hanson, 1992; Russell et al., 1998).

Since GB (**6**) synthesis requires only two metabolic steps and uses a relatively abundant metabolite (choline), transformation of non-accumulators with GB biosynthetic genes has been the focus of several studies. Most of these attempts have employed either choline oxidase (codA) genes from Arthrobacter or CDH/BADH (betB) genes from *E. coli* (Holmstrom et al., 1994, 2000; Hayashi et al., 1997, 1998; Alia et al., 1998a, 1998b, 1999; Nomura et al., 1998; Sakamoto et al., 1998, 1999, 2000; Sulpice et al., 2003; Bhattacharya et al., 2004). In transgenic tobacco plants harboring the spinach CMO gene, the protein was properly targeted to the chloroplasts and was active in crude extracts (Nuccio et al., 1998). The transformed species gained stress tolerance to cold, salt, and water stress, even though the levels of free GB (**6**) in the transformed plants ($\sim$1–3 μmol g FW^{-1}) were at least 10-fold lower than most natural accumulators.

A possible explanation for the low levels of GB (**6**) produced in these plants could be a matter of substrate availability. Serine is the initial source of choline (**4**), which is synthesized from ethanolamine (**1**). The synthesis of choline (**4**) from ethanolamine (**1**) can occur via one of three possible routes, all of which require an S-adenosylmethionine dependent methyltransferase (Fig. 1) (Datko and Mudd, 1988b). Ethanolamine (**1**) can undergo direct methylations to choline (**4**); alternatively ethanolamine (**1**) can be phosphorylated then undergo a series of methylation reactions. Finally, ethanolamine (**1**) can be conjugated with phosphatidate, then undergo methylations to phosphatidylcholine (Ptd-Cho; **14**) from which choline (**4**) can be released. It should be noted that the routes are not mutually exclusive and that there may be exchange between the three pathway intermediates. In soybean, the initial methylation occurs at the phospho-base level with phosphoethanolamine (P-EA; **7**) undergoing methylation to phosphomonomethylethanolamine (P-MME; **8**), after which a conversion to phosphatidylmonomethylethanolamine (Ptd-MME; **12**) occurs via a citidyl intermediate. The Ptd-MEA is then converted to phosphatidylcholine (Ptd-Cho; **14**) by two more methylations. Free choline (**4**) is supplied by the release of phosphocholine (**10**) from Ptd-choline (**14**) followed by dephosphorylation. This route is thought to supply the required choline (**4**) in barley. In spinach, another GB accumulator, the phosphobase route is thought to be predominant (Giddings and Hanson, 1982). The first step in the phosphorylated pathways is catalyzed by N-phosphoethanolamine methyltransferase (PEAMT, E.C. 2.1.1.103). Nuccio et al. (2000) were able to clone the spinach PEAMT through a complementation experiment utilizing a mutant of *Schizosaccharomyces pombe* which was deficient in choline (**4**) synthesis. The cloned gene product catalyzed not only the first methylation reaction (P-EA (**7**) → P-MME (**8**)), but also the two subsequent methylations. They also found that the enzyme was inhibited by the final end-product P-choline (**10**), as well as by S-adenosylhomocysteine (SAH). The enzyme was exclusively active on P-EA (**7**), showing no activities with EA (**1**) or Ptd-EA (**11**), further confirming the observation that choline (**4**) synthesis occurs predominantly along the phosphobase route in spinach. More recently, PEAMT has been cloned and characterized from wheat (Charron et al., 2002). The wheat PEAMT was isolated following a screen of cold induced genes, and was found to have similar kinetic and substrate specificities to those of the spinach form. Further examination also showed that salt, water stress, and ABA treatments increased the expression and accumulation of PEAMT transcripts and protein in both spinach and wheat.

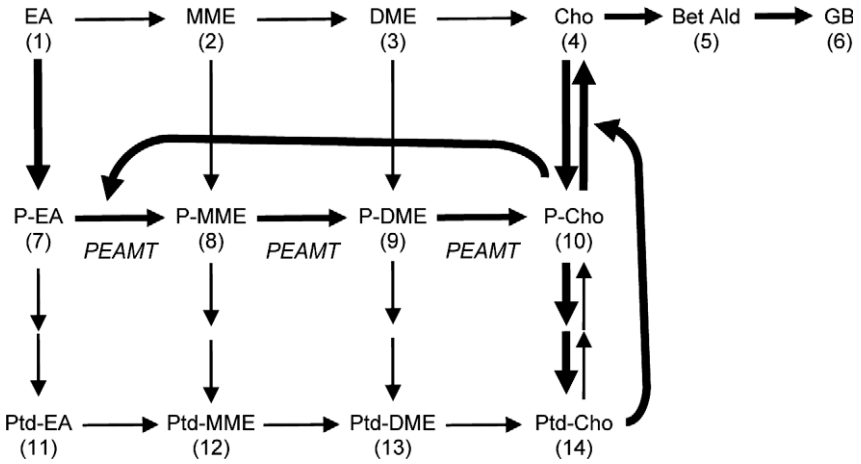

Fig. 1. Proposed regulation of choline (**4**) metabolism in maize and sorghum leaves. The *bet* mutation leads to increased levels of choline (**4**) and P-choline (**10**), which leads to inhibition of phosphoethanolamine methyltransferase (PEAMT) activity and expression, resulting in decreased *de novo* synthesis of choline. EA, ethanolamine (**1**); MME, monomethylethanolamine (**2**); DME, dimethylethanolamine (**3**); Cho, choline (**4**); BetAld, betaine aldehyde (**5**); GB, glycinebetaine (**6**); P-EA (**7**), phosphoethanolamine; P-MME (**8**), phosphomonomethylethanolamine; P-DME (**9**); phosphocholine (**10**) phosphodimethylethanolamine; Ptd-EA (**11**), phosphatidylethanolamine; Ptd-MME (**12**), phosphatidylmonomethylethanolamine; Ptd-DME (**13**), phosphatidyldimethylethanolamine (**14**).