The freshwater cyanobacterium *Anabaena doliolum* transformed with *ApGSMT-DMT* exhibited enhanced salt tolerance and protection to nitrogenase activity, but became halophilic

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Glycine betaine (GB) is an important osmolyte synthesized in response to different abiotic stresses, including salinity. The two known pathways of GB synthesis involve: 1) two step oxidation of choline (choline → betaine aldehyde → GB), generally found in plants, microbes and animals; and 2) three step methylation of glycine (glycine → sarcosine → dimethylglycine → GB), mainly found in halophilic archaea, sulphur bacteria and the cyanobacterium *Aphanothecae (Ap.) halophytica*. Here, we transformed a salt-sensitive freshwater diazotrophic filamentous cyanobacterium *Anabaena (An.) doliolum* with *N*-methyltransferase genes (*ApGSMT-DMT*) from *Ap. halophytica* using the triparental conjugation method. The transformed *An. doliolum* synthesized and accumulated GB in cells, and showed increased salt tolerance and protection to nitrogenase activity. The salt responsiveness of the transformant was also apparent as GB synthesis increased with increasing concentrations of NaCl in the nutrient solution, and maximal [12.92 μmol (g dry weight)] in cells growing at 0.5 M NaCl. Therefore, the transformed cyanobacterium has changed its behaviour from preferring freshwater to halophily. This study may have important biotechnological implications for the development of stress tolerant nitrogen-fixing cyanobacteria as biofertilizers for sustainable agriculture.

**INTRODUCTION**

Salinization of soil and water is one of the major factors adversely affecting the global productivity and biodiversity (Epstein & Bloom, 2005). Low rainfall, poor irrigation water quality, excessive use of fertilizers and rising global temperatures are major factors responsible for increasing the salinity. High salinity causes both ionic and osmotic stresses resulting in growth retardation and ultimately death of the organism. A great diversity of organisms, mostly micro-organisms, survive and grow well in high salt environments (Oren, 2011). To do so, they have to balance the osmolarity of their cytoplasm with that of their surrounding medium.

There are two fundamental cellular responses used by micro-organisms against salt stress. One is accumulation of K+ ions and counter extrusion of Na+. Alternatively, they synthesize de novo and/or accumulate compatible solutes such as polyols (glycerol), sugars (sucrose, trehalose) and amino acid derivatives (glycine betaine, ectoine), which are highly soluble uncharged or zwitterionic low molecular mass molecules with no adverse effect on cellular enzymic machinery. If these compounds are available in the external medium, cells can take them up through membrane bound transporters and accumulate them. The strategy adapted by organisms under a particular saline condition depends upon the level of salinity and energetic cost of a reaction to complete minus energy available through various metabolic processes (i.e. energetic balance) (Oren, 2011).

Glycine betaine (N,N,N-trimethylglycine, GB) is one of the most studied compatible solutes (Chen & Murata, 2011) that organisms synthesize and accumulate under different abiotic (salt, drought, cold), oxidative and organic (abscisic acid, polymixin B, salicylic acid, etc.) stresses (Gorham *et al.*, 1985; Reed *et al.*, 1986; Lai *et al.*, 1991; Klähn & Hagemann, 2011). In bacteria, plants and animals, GB is synthesized by a two step oxidation of choline to betaine aldehyde, and then to GB. However, a different pathway
exists in methanogenic archaea that involves methylation of glycine mediated by N-methyltransferases (Lai et al., 1999). In *Ectothiorhodospira halochloris* and *Actinopolyspora halochloris*, Nyúngsöla et al. (2000, 2001) characterized the enzymes as glycine sarcosine methyltransferase (GSMT), which catalyses the methylation of glycine to sarcosine (N-monomethylglycine) and subsequently to dimethylglycine, and sarcosine dimethylglycine methyltransferase (SDMT), which catalyses the methylation of sarcosine to dimethylglycine and then to GB.

In comparison with the methylation of glycine, the choline oxidation pathway could be expensive under natural conditions and was low when choline-oxidizing enzymes were expressed (Nomura et al., 1995). Furthermore, glycine is one of the major amino acids produced in vivo, while cyanobacteria lack endogenous choline and have to rely on its exogenous supply. Hence, GB synthesis in transformed cyanobacteria expressing the choline oxidation pathway could be expensive under natural conditions and is not a suitable option. Moreover, the level of GB production was low when choline-oxidizing enzymes were expressed (Chen & Murata, 2002; Rontein et al., 2002). Plants transformed with choline monooxygenase accumulated GB in roots (Hibino et al., 2002), leaves and flowers (Holmström et al., 2000; Sulpice et al., 2003), while expression of the *ApGSMT-DMT* genes in *Arabidopsis* resulted in high accumulation of GB in all organs (Waditee et al., 2005).

Cyanobacteria are unique prokaryotic photoautotrophs that possess plant type metabolism and oxygenic photosynthesis (photosystem I and II), and fix dinitrogen. They are closely related to plant chloroplasts and happen to be an appropriate system for studying plant responses to various stresses. In nitrogen-deficient paddy soils or soils with low nitrogen input, *N*₂-fixing cyanobacterial forms often account for half or more of the species present, and play a significant role in sustaining and improving the rice field productivity (Roger & Kulasooriya, 1993).

*Aphanothece* (Ap.) *halophytica* is a unicellular halophilic cyanobacterium, which synthesizes GB through methylation of glycine and can grow at 3 M NaCl and up to pH 11. Two N-methyltransferases encoding *Ap. halophytica* GSTM (ApGSMT) and *Ap. halophytica* dimethylglycine transferase (ApDMT) have been isolated and characterized from *Ap. halophytica* strain 7418 (Waditee et al., 2005). Waditee-Sirisattha et al. (2012) attempted to transform *Anabaena* 7120 with N-methyltransferase genes with little success. The reported amount of GB synthesized was too low (~40 nmol (mg fresh weight)⁻¹), and the transformant exhibited a marginal increase in NaCl tolerance (120 mM NaCl) compared to that of the wild (100 mM NaCl). This prompted us to transform a freshwater filamentous and diazotrophic cyanobacterium *Anabaena* (*An.*) *doliolum*, an isolate of local paddy fields with N-methyltransferase genes (*ApGSMT-DMT*) from *Ap. halophytica* and to study the GB synthesis and accumulation, growth under salt stress and dinitrogen fixation.

### METHODS

**Strains and culture conditions.** The unicellular halophilic cyanobacterium *Ap. halophytica*, originally isolated from Solar Lake, Sinai, by Dr Y. Cohen (Garlick et al., 1977), was a kind gift from Professor T. Akazawa (Nagoya University, Japan). The cyanobacterium is maintained axenically in our laboratory in BG11 medium (initial pH 7.4) (Rippka et al., 1979) containing 18 mM NaNO₃ and Turk Island Salt solution (Garlick et al., 1977; Codd et al., 1979) as described by Rai (1990).

*An. doliolum* Bhardwaja is a filamentous freshwater diazotrophic cyanobacterium isolated from paddy fields of Uttar Pradesh, India. The cyanobacterium was grown and maintained in combined nitrogen free Allen–Arnon (AA) medium (Allen & Arnon, 1955), at 28±1 °C, illuminated with a combination of daylight and white fluorescent lamps, giving an average light intensity of 70 μE m⁻² s⁻¹ for 14 h per day. Liquid cultures were shaken manually twice a day.

*An. doliolum* cells transformed with empty vector (+pRL488) and *ApGSMT-DMT* (pRL488 + *ApGSMT-DMT*) were grown in AA + 10 μg kanamycin ml⁻¹ (Km10) and AA + Km10 + 0.3 M NaCl, respectively. The different nitrogenous media for growing the *ApGSMT-DMT* transformant contained nitrate (KNO₃, 5 mM), glycine (1 mM) and ammonium (NH₄Cl, 0.5 mM) at final concentrations in the nutrient solution (AA + Km10 + 0.3 M NaCl). Growth of the homogeneous cyanobacterial suspension was measured turbidimetrically at 650 nm.

**Isolation of N-methyltransferase genes (ApGSMT and ApDMT) and their expression in E. coli.** The *ApGSMT-DMT* genes were amplified from the genomic DNA of *Ap. halophytica* using *ApGSMT-DMT* Fp and *ApGSMT-DMT* Rp primers (Table 1). The PCR product was cloned in vector pRL488 (Elhai, 1993) using SalI and KpnI. The plasmid pRL488 is designed to facilitate promoter luxAB transcriptional fusions and can replicate in *E. coli* and *Anabaena* using an origin of replication from pBR322 and pDU1 (Reaston et al., 1982; Wolk et al., 1984; Elhai, 1993). The resulting construct pRL488 + *ApGSMT-DMT* was cloned in *E. coli* DH5α. Transformants were selected on LB + Km90 agar plates. From the colonies that appeared on the plate, transformed bacterial colonies were picked, inoculated in the same medium and grown overnight. To check the transformation, plasmids isolated from *E. coli* pRL488 and *E. coli* containing pRL488 + *ApGSMT-DMT* were separated on a 1% agarose gel. To confirm the expression of the genes, RNA was isolated using TRI Reagent (Sigma–Aldrich).

**Table 1. List of the specific primers used in the study**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td><em>ApGSMT-DMT</em> Fp</td>
<td>5’ ATGTGCGACAAGTTGCGACGATTGCCGTCG 3’</td>
</tr>
<tr>
<td><em>ApGSMT-DMT</em> Rp</td>
<td>5’ ATTGTTACGAGTTGACCGCTCAGTTCAT 3’</td>
</tr>
<tr>
<td>rpoD Fp</td>
<td>5’ GAAAGCCTTATCCGCGCGCAAC 3’</td>
</tr>
<tr>
<td>rpoD Rp</td>
<td>5’ CGGCGTGCTGATGCGGTTGTTG 3’</td>
</tr>
<tr>
<td><em>ApGSMT</em> Fp</td>
<td>5’ ACGCCCTTACGCGACGCAAA 3’</td>
</tr>
<tr>
<td><em>ApGSMT</em> Rp</td>
<td>5’ TCAGCGACGGAGGTTCGGCGAGC 3’</td>
</tr>
<tr>
<td><em>ApDMT</em> Fp</td>
<td>5’ CCTCAGATCGGGAATCGTCTG 3’</td>
</tr>
<tr>
<td><em>ApDMT</em> Rp</td>
<td>5’ TTCCCCAACCAGTCTTCTCCG 3’</td>
</tr>
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according to the manufacturer’s protocol. Good quality RNA was used to generate cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). cDNA was used as a template to amplify rpoD, ApGSMT and ApDMT in the wild-type (WT), transformed E. coli (E. coli + pRL488; E. coli + pRL488 + ApGSMT-DMT) and Ap. halophytica using primers listed in Table 1.

Construction of transgenic An. doliolum containing pRL488 and pRL488+ApGSMT-DMT. Lethal doses of kanamycin and NaCl for WT An. doliolum were determined by growing the cells in graded concentrations of the drug (0–25 μg ml⁻¹) and NaCl (0–0.5 M). Kanamycin was lethal at 2 μg ml⁻¹, while the cyanobacterium could not grow/survive beyond 0.2 M NaCl. Triparental conjugation was used to transform An. doliolum (Elhai & Wolk, 1988; Rai & Tiwari, 1999). Accordingly, the construct pRL488 + ApGSMT-DMT was inserted into E. coli DH5α containing pRL623, which acted as Parent I, and was screened on LB agar plates containing Km™ + Ch™. Transformation was confirmed by isolating the plasmids from E. coli containing pRL488, pRL623 and pRL488 + ApGSMT-DMT, and Parent I, and subsequently separating on an agarose gel. Simultaneously, E. coli DH5α containing pRL623 was transformed with empty vector pRL488, and used to construct transgenic An. doliolum + pRL488. To construct exconjugants of An. doliolum + pRL488, WT An. doliolum was mixed with E. coli containing RP4, pRL623 and pRL488 (A), while for An. doliolum + pRL488 + ApGSMT-DMT, WT An. doliolum was mixed with E. coli containing RP4, pRL623 and pRL488 + ApGSMT-DMT (B). The resulting mixtures (A and B) were spread over a nucleopore membrane filter (Axiva) lying on agar nutrient containing AA (95 %) plus LB medium (5 %), and incubated under light at 28 ± 1 °C for 48 h. For screening An. doliolum + pRL488 and An. doliolum + pRL488 + ApGSMT-DMT, the nucleopore membrane filters were transferred to another plate containing AA + Km™. After 48 h, the membrane containing An. doliolum + pRL488 + ApGSMT-DMT was transferred to another agar plate containing AA + 0.3 M NaCl + Km™. After 7 days, green pinhead colonies appearing on the plates were picked and inoculated in their respective media. Transformation was confirmed by PCR analysis. Growth of the transformants was measured at NaCl concentrations ranging from 0 to 0.5 M.

Estimation of GB at different salinity. Homogeneous suspensions of An. doliolum WT, transformed with empty vector (+ pRL488), and ApGSMT-DMT (pRL488 + ApGSMT-DMT) grown for 10 days at different NaCl concentrations (<0.5 M) and nitrogen sources, and E. coli WT, + pRL488 and pRL488 + ApGSMT-DMT grown overnight in LB, LB + Km™, LB + 0.5 NaCl + Km™, respectively were used to estimate GB as described by Wall et al. (1960).

Estimation of nitrogenase activity. Nitrogenase activity was measured by using the acetylene reduction assay (Stewart et al., 1968) using a gas chromatograph (Series 5765; Nucon Engineers) fitted with Porapak-R column and hydrogen flame-ionization detector. An. doliolum WT, and transformants containing empty vector, and ApGSMT-DMT were grown in AA, AA + Km™ and AA + 0.3 M NaCl + Km™, respectively, for 10 days. Cultures were harvested and transferred to their respective fresh media (in which they were pre-grown) except that the NaCl concentration in all the cultures was kept at 0.1 M, and the cultures were incubated under standard growth conditions for 24 h. The cyanobacterial cells (60–70 μg protein) thus treated were taken in calibrated stoppered vials of about 15 ml capacity, and acetylene concentration was kept at 10 %. Nitrogenase activity was measured by incubating the cells under standard growth conditions with intermittent shaking. After 3 h incubation, the reaction was terminated by injecting 0.8 ml 15 % (w/v) trichloroacetic acid. A known amount of gas (200 μl) was withdrawn from the scaled vials and injected into the gas chromatograph, and ethylene peak area was recorded. All the experiments were performed in triplicates.

RESULTS

Amplification of the N-methyltransferase genes (ApGSMT-DMT) and their expression in E. coli

N-Methyltransferase genes of Ap. halophytica consisted of 798 bp ApGSMT and 834 bp ApDMT, which on amplification gave a fragment of 1632 bp (Fig. S1a, available with the online version of this paper). Fig. S1(b) confirmed the proper construction of pRL488 + ApGSMT-DMT. PCR amplification was done from the cDNA for ApGSMT and ApDMT genes along with rpoD as internal control. Ap. halophytica was used as a control to check the expression of N-methyltransferase genes. While rpoD expression was seen in all of the samples, ApGSMT and ApDMT expression was observed only in Ap. halophytica and E. coli + pRL488 + ApGSMT-DMT (Fig. S1c). The amount of GB accumulated in E. coli transformed with ApGSMT-DMT was 80.62 μmol (g dry weight)⁻¹ (Fig. S1d).

Transformation and growth estimates of An. doliolum

Fourteen clones were selected to check the transformation of An. doliolum with ApGSMT-DMT genes. All the clones grew well at 0.3 M NaCl. Expression of ApGSMT and ApDMT was observed only in Ap. halophytica and An. doliolum transformed with ApGSMT-DMT, confirming the proper insertion of the genes as well as their expression (Fig. 1a). Increasing salt concentration of the nutrient solution reduced the growth of WT An. doliolum (Fig. 1b). For WT cells, 0.3 M NaCl in the nutrient solution was found to be lethal, whereas An. doliolum transformed with ApGSMT-DMT (pRL488 + ApGSMT-DMT) grew profusely up to 0.5 M NaCl (Fig. 1c).

GB synthesis by transformed An. doliolum

Transformed An. doliolum cells (pRL488 + ApGSMT-DMT) failed to grow on standard freshwater AA medium (Fig. 1d), but grew well when the medium was supplemented with NaCl (Fig. 2a), indicating an obligate requirement of NaCl (at least 0.1 M) for their growth. Repeating the experiment with other ApGSMT-DMT transformed clones confirmed the result. The amount of GB in the transformed cells increased with increasing concentration of NaCl in the medium (Fig. 2b). This is indicative of the increased activity of the promoter with increasing concentration of salt, i.e. the salt-responsive nature of the promoter (luxAB). Maximal accumulation of GB [12.92 μmol (g dry weight)⁻¹] was recorded at 0.5 M NaCl concentration. There was no GB synthesis by An. doliolum WT and the strain transformed with empty vector (+ pRL488).
Nitrogenase activity

Since the ApGSMT-DMT-transformed strain was unable to grow in the absence of NaCl, we selected an NaCl concentration of 0.1 M, which WT and transformed cells could tolerate. Salinity reduced the nitrogenase activity of An. doliolum (data not shown). Incubation of cyanobacterial cells in nutrient solution with 0.1 M NaCl for 3 h showed 2.0–2.65 times higher nitrogenase activity in the ApGSMT-DMT transformant (Fig. 3) than that of WT and empty vector cells. This indicated that GB accumulation in the ApGSMT-DMT-transformed cells protected the nitrogenase activity.

Growth and GB accumulation by ApGSMT-DMT transformants grown with different nitrogen sources

The data on nitrogenase activity prompted us to study whether nitrogen is limiting for GB synthesis. We observed growth and GB accumulation by transformants on different nitrogen sources. When ApGSMT-DMT-transformed An. doliolum cells were grown in medium supplemented with different forms of nitrogen, nitrate supported the maximal growth rate and GB accumulation, followed by dinitrogen, glycine and ammonium (Fig. 4).

DISCUSSION

An. doliolum is a salt-sensitive, N₂-fixing cyanobacterium growing profusely in paddy fields of eastern Uttar Pradesh, India. Application of this cyanobacterium (12.5 kg hectare⁻¹) in paddy fields resulted in higher yields (increased the number of panicles per hill and the number of seeds per ear), and reduced the use of chemical N-fertilizer by 25% (Dubey & Rai, 1995). The agronomic importance of the rice field cyanobacterium and increasing salinization of paddy fields prompted us to transform An. doliolum with ApGSMT-DMT genes from Ap. halophytica to introduce salt tolerance and allow the N₂-fixing ability of the transformant to be examined. The native organism Ap. halophytica showed higher levels of N-methyltransferase.
genes expression than that of the ApGSMT-DMT-transformed An. doliolum. Nyysölä et al. (2001) also reported that the specific activity of recombinant GSMT was lower than that of native GSMT.

The amount of GB accumulated [80.62 µmol (g dry weight)^{-1}] by E. coli transformed with ApGSMT-DMT was identical to that reported for E. coli cells transformed with GSMT and SDMT from Ectothiorhodospira halochloris [78 µmol (g dry weight)^{-1}] (Nyysölä et al., 2000). The ApGSMT-DMT-transformed An. doliolum cells accumulated a high amount of GB when subjected to NaCl stress, which increased with increasing concentration of NaCl, and was maximal [12.94 µmol (g dry weight)^{-1}] at 0.5 M NaCl. This value is almost 1/6 of the transformed E. coli cells, possibly due to slow growth of cyanobacteria (compared to E. coli) and unavailability of substrate. Cyanobacterial growth solely depended upon elemental nitrogen, while E. coli grew on nitrogen-sufficient organic nutrient. Moreover, the transcription level of, particularly, ApDMT in An. doliolum was lower than that of E. coli (Figs 1 and S1). However, the value is almost 1/6 of the transformed ApGSMT-DMT. Further, the increase in the level of NaCl tolerance of the transformed Anabaena sp. PCC 7120 was less (120 mM) than that of the WT (100 mM), while the transformed An. doliolum grew in up to 0.5 M NaCl. In the study with Anabaena sp. PCC 7120, the whole operon with promoter was used (Waditee-Sirisattha et al., 2012), while plasmid pRL488 used in the present study was designed to facilitate promoter luxAB transcriptional fusions (Elhai, 1993). Therefore, high GB accumulation in the An.
The cyanobacterium Ap. halophytica grows in nutrient solution devoid of NaCl (Nyysölä et al., 2000). The studies indicate that archaea, sulphur bacteria and anoxygenic phototrophic bacteria synthesizing GB through methylation are all halophilic and unable to grow in nutrient solution devoid of NaCl (Nyysölä et al., 2000). The cyanobacterium Ap. halophytica essentially requires Na ions (Oren, 2000), and the strain Ap. halophytica used in the current study needed a minimal concentration of 0.1 M NaCl for its growth. It is possible that the constitutive accumulation of GB in the cells constrains their survival below some osmolarity threshold level as a compensatory physiological mechanism.

Accumulation of GB in transformed An. doliolum (pRL488+ApGSMT-DMT) protected nitrogenase activity under saline conditions (Fig. 3). Antibiotic resistance conferred by vector pRL488, methylation by ApGSMT-DMT and nitrogen fixation seemed to have highly burdened the cyanobacterial metabolic process for energy, reductant and the photosynthates. Both methylation and nitrogen fixation are energetically costly processes. With perfect energy transfer, in addition to reductants (8 electrons), 16 molecules of ATP must be expended to fix one molecule of N₂. Simultaneously, generation of the methyl donor SAM (S-adenosylmethionine) in An. doliolum expressing ApGSMT-DMT costs the cells 12 ATP molecules per active methyl group of AdoMet (Atkinson, 1977). Furthermore, halophilic life is also energetically expensive (Oren, 2011). Moreover, the dinitrogenase active site contains the most complex [Fe–S] cluster, an iron and molybdenum cofactor (FeMo-co). The synthesis of FeMo-co, in addition to NifH, NifEN and NifF proteins, inorganic iron, sulphur, molybdenum and organic acid homocitrate, also requires SAM and Mg-ATP. Since SAM is required for the biosynthesis of FeMo-co, forced diversion of SAM towards GB synthesis could potentially be detrimental for nitrogenase maturation, as well as other pathways requiring SAM (Curatti et al., 2006, 2007; Rubio & Ludden, 2008). However, the cyanobacterium still managed to grow on elemental nitrogen, and it synthesized GB and acquired salt tolerance. Maximal growth rate and GB accumulation by the ApGSMT-DMT transformant in nitrate-supplemented nutrient solution (Fig. 4) further confirms the fact that nitrogen is one of the major limiting factors for the transformants growing on elemental nitrogen. The slow growth rate and poor yield of cyanobacterium on ammonium may be attributed to the low level of GB synthesis in ammonium-grown cells. Though ammonium is the preferred and less energy-demanding nitrogen source for most cyanobacteria, it often supports poorer growth (Allen & Arnon, 1955). Almost all nutrient media use nitrate as the nitrogen source, since nitrate is the preferred nitrogen source for the growth of cyanobacteria in culture (Fogg et al., 1973).

This study will open the way to transform symbiotic and free-living N₂-fixers to increase their salt tolerance and productivity. The use of salt-tolerant N₂-fixers can also increase the availability of nitrogen to the crops under saline conditions and reduce the use of chemical fertilizers, which are deteriorating the quality of soil.

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REFERENCES


glycine methylation genes from *Aphanotohece halophytica* synthesized glycine betaine showing increased tolerance to salt. *Arch Microbiol* **194**, 909–914.


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