Metabolic Engineering for Betaine Accumulation in Microbes and Plants

Plants accumulate a variety of osmoprotectants that improve their ability to combat abiotic stresses. Among them, betaine appears to play an important role in conferring resistance to stresses. Betaine is synthesized via either choline oxidation or glycine methylation. An increased betaine level in transgenic plants is one of the potential strategies to generate stress-tolerant crop plants. Here, we showed that an exogenous supply of serine or glycine to a halotolerant cyanobacterium *Aphanothece halophytica*, which synthesizes betaine from glycine by a three-step methylation, elevated intracellular accumulation of betaine under salt stress. The gene encoding 3-phosphoglycerate dehydrogenase (PGDH), which catalyzes the first step of the phosphorylated pathway of serine biosynthesis, was isolated from *A. halophytica*. Expression of the *Aphanothece* PGDH gene in *Escherichia coli* caused an increase in levels of betaine as well as glycine and serine. Expression of the *Aphanothece* PGDH gene in *Arabidopsis* plants, in which the betaine synthetic pathway was introduced via glycine methylation, further increased betaine levels and improved the stress tolerance. These results demonstrate that PGDH enhances the levels of betaine by providing the precursor serine for both choline oxidation and glycine methylation pathways.

Salinity is a major problem affecting world overall agricultural production. Approximately 20% of the cultivated land worldwide is impaired by high salinity which causes ion imbalance and hyperosmotic stress in plants (1). Plants have evolved various strategies to cope with salinity, including the accumulation of low molecular weight organic compatible solutes (osmoprotectants) such as sugars, some amino acids, and quaternary ammonium compounds (2–4). Glycine betaine (N,N,N-trimethylglycine, hereafter betaine) is a major osmoprotectant to protect plants from high salinity (2–4). Most known biosynthetic pathways of betaine include a two-step oxidation of choline: choline → betaine aldehyde → betaine. The first step is catalyzed by choline monooxygenase (CMO) in plants (5), choline dehydrogenase (CDH) in animals and bacteria (6, 7), and choline oxidase in some bacteria (8, 9). The second step is catalyzed by betaine aldehyde dehydrogenase (BADH) in all organisms (6, 10, 11). Introducing the betaine synthetic pathway into betaine non-accumulating plants has been applied to improve salt tolerance of plants (8, 12–15). However, the accumulation levels of betaine in the transformed plants are relatively low. Supplying betaine precursors such as choline, ethanolamine, and serine to the transformed plants enhance betaine accumulation. Thus, the availability of betaine precursors limits the betaine biosynthesis (15, 16–18).

Recently, we showed that a halotolerant cyanobacterium, *Aphanothece halophytica* (*A. halophytica*), synthesizes betaine from glycine by a three-step methylation, which is catalyzed by two *N*-methyltransferases (ApGSMT and ApDMT). ApGSMT is responsible for the two-step methylation reactions of glycine to sarcosine and then sarcosine to dimethylglycine. ApDMT is responsible for the specific methylation of dimethylglycine to betaine (19). Co-expression of ApGSMT and ApDMT in fresh water *Synecochoccus* cells accumulated significant amounts of betaine and conferred sufficient salt tolerance so the cells were capable of growth in sea-water. *Arabidopsis* plants that were transformed with ApGSMT and ApDMT accumulated substantial amounts of betaine and increased tolerance to salt stress (20). However, in this case, the exogenous supply of glycine enhanced the accumulation level of betaine.

Serine and glycine are readily interconvertible by serine hydroxymethyltransferase (SHMT) (21), thus enhancement of serine biosynthesis would eventually lead to the increased level of glycine. Serine biosynthesis in plants proceeds by two pathways: the so-called phosphorylated pathway and the photorepiratory pathway (21, 22). In the photorepiratory pathway, serine and glycine are the intermediates produced by ten different enzymes and are subsequently recycled as 3-phosphoglycerate (3-PGA) to the Calvin cycle (21). In the phosphorylated pathway, serine is derived from 3-PGA, d-3-Phosphoglycerate dehydrogenase (PGDH; EC 1.1.1.95), which is responsible for the first step in this pathway, catalyzes the oxidation of 3-PGA.

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‡ To whom correspondence should be addressed: Research Institute of Meijo University, Tenpaku-ku, Nagoya, Aichi 468-8502, Japan. Tel.: 81-52-838-2277; Fax: 81-52-832-1545; E-mail: takabe@ccmfs.meijo-u.ac.jp.

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Rungaroon Waditee‡, Nazmul H. Bhuiyan‡, Emi Hirata§, Takashi Hibino¶, Yoshito Tanaka‡, Masamitsu Shikata‡, and Teruhiro Takabe‡§†

From the ‡Research Institute of Meijo University, Nagoya 468-8502, Japan, the §Graduate School of Environmental and Human Sciences, Meijo University, Nagoya 468-8502, Japan, and the ¶Shimadzu Co., Nakagyou-ku, Kyoto 604-8511, Japan
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to form phosphohydroxyypyruvate (PHP) by utilizing NAD as a cofactor. PHP is subsequently transaminased by phosphoserine aminotransferase to yield phosphoserine, which is then dephosphorylated to serine by phosphoserine phosphatase (23). 3-PGA is the reaction product catalyzed by ribulose-1,5-bisphosphatase carboxylase/oxygenase (RuBisCO). Moreover, 3-PGA is produced from sucrose and starch via the glycolysis pathway. Therefore, 3-PGA would be produced in significant amounts in photosynthetic organisms. Although an increase in transcript level of Arabidopsis PGDH (AtPGDH) has been mentioned (23), the role of PGDH on abiotic-stress tolerance has never been examined in plants. In cyanobacteria, there is evidence indicating that cyanobacteria are capable of synthesizing serine directly from 3-PGA (24). Thus, the route for serine biosynthesis via 3-PGA in higher plants remains largely unexplored.

In the present study, the Aphanothece PGDH gene (ApPGDH) was isolated and functionally characterized. A. halophytica was originally isolated from the Dead Sea and is known to accumulate significant amounts of betaine at high salinity (19). The ApPGDH gene was transferred into Escherichia coli, which has a betaine synthetic pathway via choline oxidation; it was also transferred into Arabidopsis plants, in which the betaine synthetic pathway was introduced via glycine methylation. The results manifested that the heterogeneous expression of ApPGDH significantly enhanced betaine levels in both betaine accumulating and non-accumulating organisms.

EXPERIMENTAL PROCEDURES

Strains, Culture Conditions, and Chemicals—E. coli strains DH5α and BL21 (DE3) were used for the cloning and expression of protein, respectively. E. coli cells were grown at 37 °C in LB or MMA media containing 50 μg/ml ampicillin. The growth of E. coli cells was monitored by measuring the optical density at 620 nm (A620) with an Erma AE-22 photoelectric colorimeter. A. halophytica cells were grown photoautotrophically in BG11 liquid medium plus 18 mM NaNO3 and Turk Island salt solution at 28 °C, as previously described (25). A Hitrap-Q Sepharose column was purchased from Amersham Biosciences. PHP was prepared from the hydroxypyruvic acid amingroup (cyclohexylammonium) salt, as described previously (26). Other chemicals of analytical grade were purchased from Katayama (Tokyo, Japan).

Cloning and Expression of Recombinant ApPGDH—The genomic DNA of A. halophytica was partially sequenced by a shotgun method using a Shimadzu multicapillary DNA sequencing system (Shimadzu Co., Kyoto, Japan). A homology search was made using the BLAST program, as previously described (27). The ApPGDH gene, homologous to several PGDHs from the genome data base of bacteria, plants, and animals, was found. The coding region of the ApPGDH gene was amplified by PCR from A. halophytica genomic DNA using the primer set, ApPGDH-NcoF and ApPGDH-BamR. The sequences of all the primers are shown in Table 1. The amplified fragments of ApPGDH were ligated into the EcoRV restriction site of pBlueScript SK+. The resulting plasmid, pApPGDH-His6 (BamHI), was transferred into E. coli DH5α cells. The DNA sequence of ApPGDH in the plasmid was determined using a 310-genetic analyzer (Applied Biosystems, Foster City, CA) and analyzed with the DNASIS program (Hitachi Software Engineering Co., Kanagawa, Japan).

The expression vector of ApPGDH was constructed by digestion of the plasmid pApPGDHSK (BamHI) with NcoI and BamHI, and then ligated into the corresponding sites of the pET3d plasmid. The resulting plasmid pApPGDH-PET3D was transferred first into E. coli DH5α cells and then into E. coli BL21 (DE3) cells. E. coli BL21 (DE3) cells harboring pApPGDH-PET3D were grown at 37 °C in LB medium containing ampicillin (50 μg/ml) until absorbance at 620 nm (A620) reached 0.5–0.6. Then, 1 mm isopropyl-β-D-thiogalactopyranoside (IPTG) was added, and the cells were grown for another 3 h. Cells from 0.5-liter cultures were then harvested by centrifugation at 5,000 × g for 10 min and washed twice with Buffer A (200 mM Tris-Cl, pH 8.0, 200 mM KCl, 1 mM EDTA, 2 mM 2-mercaptoethanol). Total cell extracts were obtained by sonication with a Kubota Insonator 201 m at 180 watts for 15 min. Unbroken cells were removed by centrifugation at 10,000 × g for 10 min. The soluble ApPGDH protein was precipitated by the addition of ammonium sulfate (25% of saturation). The precipitated fraction was dissolved in Buffer A and dialyzed against the same buffer. The dialyzed soluble fraction was applied onto a Hitrap-Q Sepharose column (4.6 mm × 5.0 cm). The ApPGDH protein was eluted with a gradient of 0–1000 mM NaCl. Fractions containing ApPGDH were pooled, dialyzed against buffer A, and applied to the same column. After two rounds of column chromatography, the purified ApPGDH protein was obtained.

Construction of Transgenic Arabidopsis Plants Expressing ApPGDH—The ApPGDH gene was amplified from genomic DNA of A. halophytica by the primer set, ApPGDH-NcoF and ApPGDH-SalR. The amplified ApPGDH gene was ligated into the EcoRV restriction site of pBlueScript SK+. The resulting plasmid, pApPGDH-His6 (Sall), was digested with Ncol and Sall, and ligated into the corresponding sites of the pcTcHis2C plasmid, generating pApPGDH-HIS6. The ApPGDH coding region with a C-terminal His6 tag was amplified by the primer set, ApPGDH-NcoF and HisBamH-R using the plasmid pApPGDH-His6 as a template. The amplified fragment was ligated into the EcoRV site of pBsk+ to generate pApPGDH-His6- SK+. Then, the fragment was prepared by double digestion with XbaI and KpnI and ligated into the corresponding sites of the pBI101H.35S binary vector. The resulting plasmid, pBI101H-ApPGDH-His6, was transferred into Agrobacterium tumefaciens strain LBA4404 by electroporation. Arabidopsis plants were transformed using the floral dip method (28). Transformed plants were selected on Murashige and Skoog (MS) (29) agar media with 50 μg/ml kanamycin and 25 μg/ml

| Table 1: Primers for isolation and expression of the ApPGDH gene |
|-----------------|-----------------|------|
| Primers | 3′-5′ | Base pairs |
| ApPGDH-NcoF | 5′-ACCCATAGCATTTTGTTTCAAG-3′ | 25mer |
| ApPGDH-SalR | 5′-GCGGTGCAATTTGAATGGCTCACT-3′ | 29mer |
| ApPGDH-BamR | 5′-CTGTTACCTTTCAATGTTTAAATACTCAG-3′ | 29mer |
| preApPGDH-NcoF | 5′-GACCATGATCTTCCCTTCAATTCTTCTGT-3′ | 25mer |
| preApPGDH-SalR | 5′-GGCTGATCTTCCCTTCAATTCTTCTGT-3′ | 25mer |
| HisBamH-R | 5′-GTGGAATCCCTTCAATGATGATGTATG-3′ | 26mer |
hygromycin, and later transferred to soil for seed production. Transformants with empty vector, pBI101H.35S were also produced. Homozygous transgenic plants (T3 generation) showing high expression levels of ApPGDH protein were used for further analysis.

To construct transgenic Arabidopsis plants expressing ApPGDH, which is targeted into the chloroplast, the fragment encoding a 60-amino acid transit peptide for chloroplast targeting was amplified from the Arabidopsis PGDH gene (22) by PCR using the primer set, preAtPGDH-NcoF and preAtPGDH-NcoR. The amplified fragment was ligated into the Ncol site of the corresponding sites of pBI101H.35S binary vector. The resulting plasmid, pBI101H-AtTP-ApPGDH-His6, was transformed into E. coli strain LBA4404 by electroporation.

Transformants with empty vector, pBI101H.35S were also produced and grown on MS agar medium in a growth chamber at 20 °C until use. At the time of germination test, surface-sterilized seeds were stratified in the dark for 3 days and then plated on the MS agar medium and incubated in the growth chamber. For salt-stress treatment, seeds were sown on the MS agar medium containing various NaCl concentrations. For low temperature treatment, plants were grown on the MS agar medium and incubated at 5, 10, and 22 °C. For dark treatment, three- or four-week-old plants grown on the MS agar medium were transferred into the dark for 3 days, and the samples were then immediately collected for further analysis.

The Arabidopsis plants expressing ApPGDH, ApGSMT, and ApDMT were generated by crossing between the transgenic plants expressing ApPGDH and the transgenic plants expressing ApGSMT and ApDMT (20). F1 progeny plants were screened for the presence of the genes by PCR and confirmed by Western blotting. Progeny plants that accumulated high expression levels of ApPGDH, ApGSMT, and ApDMT were used for further analysis.

**Plant Material, Growth Conditions, and Stress Treatments**—A. thaliana (ecotype Columbia) was used as the wild type in this study. The seeds of wild type and transgenic plants were germinated and grown on MS agar medium in a growth chamber at 22 °C with a 16-h light (200 μE m⁻² s⁻¹)/8-h dark cycle and 60% relative humidity, unless otherwise stated. For the germination test, surface-sterilized seeds were stratified in the dark for 3 days and then plated on the MS agar medium and incubated in the growth chamber. For salt-stress treatment, seeds were sown on the MS agar medium containing various NaCl concentrations. For low temperature treatment, plants were grown on the MS agar medium and incubated at 5, 10, and 22 °C. For dark treatment, three- or four-week-old plants grown on the MS agar medium were transferred into the dark for 3 days, and the samples were then immediately collected for further analysis.

The quantum yield in photosystem II (PSII) was measured with a MiniPAM chlorophyll fluorescence system (Walz Effeltrich, Germany), as previously described (20).

**Amino Acid Analysis**—E. coli cells or plant tissues were homogenized in absolute methanol. The supernatant was collected, and the pellet was re-extracted with 90% methanol. Methanol extracts were dried in a vacuum rotary evaporator at 45 °C. The dry residues were re-extracted with a mixture of water and chloroform (1:1, v/v). The upper aqueous phase was filtered through a 0.22-μm membrane filter. The filtrate was dried in a vacuum and stored at −20 °C until use. At the time of analysis, samples were dissolved in mobile phase solution (pH 2.6) containing 14.1 g of trilithium citrate tetrahydrate, 70 ml of 2-methoxyethanol, and 13.3 ml of 60% HClO₄ per liter and injected into a shim-pack Li column of the amino acid analyzer (Shimadzu, Japan).

**Enzyme Assay**—PGDH activity was assayed as previously described with some modifications (22). Enzymatic activity of PGDH in the direction of NADH oxidation (the reverse reaction) was carried out at 30 °C. The assay mixture in a volume of 0.5 ml contained 10 μl of purified enzyme, 25 mM HEPES (pH 7.5), 100 μM NADH, 400 mM KCl, and 90 μM PHP. The reaction was started at addition of PHP. One unit of enzyme activity is defined as the amount that oxidizes 1 μmol of NADH per min under the indicated conditions. PGDH activity for the forward direction was also assayed at 30 °C. The assay mixture (500 μl) contained 10 μl of purified enzyme, 200 mM Tris-HCl (pH 9.0), 25 mM EDTA, 5 mM 3-PGA, 2.5 mM dithiothreitol, and 0.5 mM NAD⁺. The reaction was initiated by the addition of 3-PGA. One unit of enzyme activity is defined as the amount that reduces 1 μmol of NAD⁺ per min under the indicated conditions. For the measurement of PGDH activity in plants, crude extracts were prepared by homogenizing plant tissues with buffer A and then centrifuged at 10,000 rpm for 5 min. The supernatants were used for the enzyme assay.

**Other Methods**—SDS-PAGE and Western blot analyses were carried out according to standard protocol as described previously (20). Antibody raised against His₆ was obtained from R&D systems (Minneapolis, MN). Protein content was determined by the Lowry method as previously described (15). The molecular weight of native ApPGDH protein was estimated by using gel filtration chromatography with a Superdex 200 column (Amersham Biosciences). Chloroplast was prepared as described (30). Betaine was measured as described previously (15).

**RESULTS**

Exogenous Supply of Glycine or Serine Enhances the Accumulation of Betaine in A. halophytica—A. halophytica synthesizes betaine from glycine via a three-step methylation (19). Because the betaine levels in betaine-accumulating organisms are very high, it has been suggested that the genes involved in betaine synthesis are well-organized so that the supply of precursors for betaine synthesis is not limited. To investigate whether the precursor glycine is a limiting factor for betaine synthesis in A. halophytica, the levels of free amino acids were measured. Under salt-stress conditions, the levels of free glycine and serine in the cells markedly decreased (data not shown). An exogenous supply of glycine did not alter the betaine level in A. halophytica at low salinity (0.5 M NaCl), but it strikingly increased the betaine level at high salinity (1.5 M NaCl) (Fig. 1A). Similar results were observed when serine was supplied (data not shown). These results indicate the level of glycine is a limiting factor for the accumulation of betaine, even for the betaine-accumulating organism A. halophytica. In cyanobacteria, the importance of serine biosynthesis via a phosphorylated pathway has been suggested (24). We therefore measured the PGDH activity in A. halophytica. As shown in Fig. 1B, PGDH activity of A. halophytica cells increased about 1.5–2.3-fold upon the increase of salinity from 0.5 to 2.5 M NaCl.
Cloning and Biochemical Characterization of ApPGDH

The PGDH gene has not yet been isolated from cyanobacteria. Shot-gun cloning of the A. halophytica genome revealed the presence of a single candidate gene that has high homology to the PGDHs. The putative gene was isolated and sequenced. It revealed that the product for ApPGDH consists of 526 amino acids with a molecular mass of 56,410 Da. The deduced amino acid sequence of ApPGDH is highly homologous to the putative PGDH from Synechocystis sp. PCC6803 (GenBank accession no. NP441198) (~79% identity). ApPGDH is closely related to the PGDHs from Bacillus (31), Arabidopsis (22), and rat (32) (38–44% identity), but forms a family distinct from E. coli (33) and yeast PGDHs (GenBank P40054) (34) (32–36% identity). The ApPGDH protein consists of three distinct domains: nucleotide binding, substrate binding, and regulatory domains as well as a long C-terminal domain like the Arabidopsis and mammal PGDHs (22, 32).

ApPGDH could be expressed in E. coli BL21 (DE3) cells in an active form. The recombinant enzyme was purified by ammonium sulfate fractionation and ion exchange column chromatography. The polypeptide chain of ApPGDH has a molecular mass of 57 kDa, calculated by SDS-PAGE (data not shown), which is consistent with the molecular mass deduced from the DNA sequence. Gel filtration chromatography suggests that ApPGDH associates as a tetrameric protein (data not shown).

The substrates of ApPGDH were 3-PGA and PHP. Although the E. coli PGDH has also been shown to catalyze the oxidation/reduction reaction between α-ketoglutarate and hydroxyglutarate (37), ApPGDH did not catalyze this reaction. Kinetic parameters for the ApPGDH together with five other PGDHs from different organisms are shown in supplemental Table S1. The Km values of ApPGDH for 3-PGA and PHP were 0.90 and 0.15 mM, respectively. These values were lower than those of PGDHs from bacteria (33, 35), animals (32, 36), and a plant (22). The activity of ApPGDH was not inhibited by substrate (0–200 μM PHP) when sufficient salt was added to the reaction mixture. Serine and 13 other amino acids at concentrations up to 20 mM in the reaction did not reduce or inhibit PGDH activity. Effects of various salts on the ApPGDH activity were carried out, and the results are shown in Fig. 2. A and B. Addition of salts, such as NaCl, KCl, MgCl2, and CaCl2, increased the ApPGDH activities in both directions, although further addition of salt decreased the activities. ApPGDH activities were increased severalfold when the reaction mixtures contained 0.1–0.3 M salt for the forward reaction and 0.3–0.5 M salt for the reverse reaction. High PGDH activity at these high concentra-
tions of salt has not been reported in any PGDHs. The pH optimum was around pH 9.0 and 7.5 for the forward and reverse reactions, respectively (Fig. 2, C and D). The activity for the forward reaction changed significantly upon the changes of pH, while the activity for the reverse reaction was relatively independent of pH. Effect of various temperatures on the stability of enzyme activity was also tested (Fig. 2E). The activity remained stable after incubation at 40 °C for 7 min, but decreased to 50% when the enzyme was incubated at 50 °C for 7 min.

**Overexpression of ApPGDH in E. coli Leads to an Increase in the Endogenous Levels of Serine and Glycine**—*E. coli* has the phosphorylation pathway for serine biosynthesis (33). We examined whether the overexpression of ApPGDH in *E. coli* increases the endogenous levels of glycine and serine. As shown in Fig. 3, A and B, *E. coli* cells expressing ApPGDH exhibited elevated levels of serine and glycine. Their levels were up to 2.5–4.0-fold higher than in non-transformed cells. Addition of NaCl to the growth medium resulted in higher levels of serine and glycine in both wild type and transformed *E. coli* cells. However, their levels in the *E. coli* cells expressing ApPGDH were always notably higher than those in non-transformed control cells. The *E. coli* cells expressing ApPGDH grew faster than the control cells (Fig. 3, C and D). The PGDH activity in the ApPGDH-expressing cells was 5–6-fold higher than that in the control cells (data not shown).

*E. coli* can synthesize betaine by oxidation of choline (6). The effects of precursor supply on the accumulation of betaine have never been examined. We measured the betaine content in both the control and ApPGDH-expressing cells. As shown in Fig. 3E, betaine levels in *E. coli* cells expressing ApPGDH were about 2-fold higher than those in the control cells at all salinity conditions tested.

**Overexpression of ApPGDH, ApGSMT, and ApDMT in E. coli Lead to an Increase in the Endogenous Level of Betaine under Saline Conditions**—We further examined the effect of overexpression of ApPGDH, ApGSMT, and ApDMT on the accumulation of betaine in *E. coli* cells. As shown in Fig. 4, overexpression of ApGSMT and ApDMT increased the accumulation of betaine in *E. coli* at all salinity conditions tested. The overexpression of ApPGDH, ApGSMT, and ApDMT further enhanced the betaine level. The levels of betaine in the cells expressing ApPGDH, ApGSMT, and ApDMT were 1.2–1.8-fold higher than those in the cells expressing ApGSMT and ApDMT.

Levels of serine and glycine slightly decreased in the cells expressing ApGSMT and ApGSMT compared with the control cells (data not shown). However, their levels in the cells expressing ApPGDH, ApGSMT, and ApDMT were about 2.5–3- and 3–4-fold higher than those in the control cells or in the cells expressing ApGSMT and ApDMT, respectively (data not shown).

**Overexpression of ApPGDH in Arabidopsis Plants Leads to an Increase in Levels of Free Amino Acids**—We addressed the question of whether the plants overexpressing ApPGDH could increase the levels of serine and glycine. Here, we generated two kinds of transgenic plants harboring the *ApPGDH* gene targeted to the chloroplast and cytosol. Fifty independent lines of transgenic plants expressing ApPGDH in cytosol (PG1–50) and ten independent lines of transgenic plants expressing ApPGDH
in the chloroplast (chPG1–10) were analyzed. Western blotting of 15 individuals from the T3 generation was shown in Fig. 5A. This result indicated that ApPGDH could be expressed in Arabidopsis plants. To prove the subcellular localization of the chloroplast-targeted ApPGDH, the chloroplast fraction was prepared from the wild type and transgenic plants. Only the chloroplast-targeted ApPGDH; the chloroplast fraction was collected.

Levels of free amino acids in the wild-type, transgenic controls plants were similar to wild-type plants (supplementary Fig. S1). Transgenic plants expressing ApPGDH exhibited 2–8-fold higher levels of serine and glycine than those in the wild-type plants. Transgenic plants also exhibited elevated levels of glutamine and cysteine. The increased levels of glycine and serine were varied among the transgenic lines. We analyzed the correlation of PGDH activity and the levels of amino acids. A good correlation was observed between PGDH activities and levels of glycine or serine (Fig. 5B).

The transgenic plants expressing ApPGDH exhibited higher PGDH activity than that of wild-type plants. Interestingly, the PGDH activity in the stems and root tissues was relatively high in both the wild-type and transgenic plants. In the dark, PGDH activity was retained at relatively high levels in all tissues of transgenic Arabidopsis plants (Fig. 5C).

Arabidopsis Plants Expressing ApPGDH Exhibit an Improved Tolerance to Salt and Cold Stresses—We examined stress tolerance of transgenic plants at various developmental stages. When seeds of the wild type and transgenic plants were germinated on the MS agar medium, almost all seeds germinated at the same rate (Fig. 6A). However, the germination of the wild-type plants was severely inhibited at high salinity (100 mM NaCl). A very similar result was observed when the seeds were germinated at low temperature (10 °C) (data not shown). When 10-day-old plants were transferred to the MS medium containing 120 mM NaCl and allowed to grow for five more days, the sizes of the wild-type plants were significantly shorter than the transgenic plants as shown in Fig. 6B. Photosynthetic yield of transgenic plants was higher than the wild-type plants under these conditions (data not shown). When the plants were germinated and allowed to grow 15 more days at the same low temperature (10 °C), the sizes of wild-type plants were smaller than the transgenic plants expressing ApPGDH as shown in the left panel of Fig. 6C. Similar phenotypes were observed at the vegetative and reproductive stages (right panel of Fig. 6C).

Under salt-stress conditions, PGDH activity of transgenic plants was much higher than that of wild-type plants (Fig. 6D). Co-expression of ApPGDH, ApGSMT, and ApDMT in Arabidopsis Increased Betaine Levels and Improved the Tolerance for Salt and Cold Stresses—We generated the transgenic Arabidopsis plants manipulated with three genes, ApPGDH, ApGSMT, and ApDMT, by crossing. Western blotting revealed that ApPGDH, ApGSMT, and ApDMT could be expressed in these plants (Fig. 7A). The PGDH activity in crude extracts of the transgenic plants (PGxMT26 and PGxMT28) showed about 2-fold higher activity than the wild-type plants (Fig. 7B). The N-methyltransferase activities could be detected only in the transgenic plants (data not shown). The level of glycine in the transgenic plants were about 2–3-fold higher than that in the wild-type plants (data not shown). Levels of glutamic acid and glutamine were also higher in the transgenic plants than in the wild-type plants (data not shown).

Fig. 7C showed that level of betaine in transgenic plants was 0.5 μmol/gFW when the plants were grown under normal conditions, but that the level increased up to 1.2 μmol/gFW when the plants were exposed to 100 mM NaCl. The betaine level was 1.8 and 0.4 μmol/gFW, respectively. Contents of these amino acids in the transgenic controls plants were similar to wild-type plants (supplementary Fig. S1). Transgenic plants expressing ApPGDH exhibited 2–8-fold higher levels of serine and glycine than those in the wild-type plants. Transgenic plants also exhibited elevated levels of glutamine and cysteine. The increased levels of glycine and serine were varied among the transgenic lines. We analyzed the correlation of PGDH activity and the levels of amino acids. A good correlation was observed between PGDH activities and levels of glycine or serine (Fig. 5B).

The transgenic plants expressing ApPGDH exhibited higher PGDH activity than that of wild-type plants. Interestingly, the PGDH activity in the stems and root tissues was relatively high in both the wild-type and transgenic plants. In the dark, PGDH activity was retained at relatively high levels in all tissues of transgenic Arabidopsis plants (Fig. 5C).
the root tissues of the transgenic Arabidopsis plants expressing ApGSMT, ApDMT, and ApPGDH was about 1.5-fold higher than that of the transgenic plants expressing ApGSMT and ApDMT (MT2, SG2 in a previous report (20)). The levels of betaine in the transgenic plants expressing ApGSMT, ApDMT, and ApPGDH were similar to those in the plants expressing ApGSMT and ApDMT that were exogenously supplied with glycine.

The increased amount of betaine in the transgenic plants expressing ApGSMT, ApDMT, and ApPGDH led us to investigate the stress tolerance of these plants at various growth stages. Under salt-stress conditions, the transgenic plants expressing ApGSMT, ApDMT, and ApPGDH exhibited an increased yield of germination compared with the transgenic plants expressing ApGSMT and ApDMT (data not shown). When 10-day-old seedlings were transferred to MS agar medium containing 200 mM NaCl and allowed to grow for five additional days, wild-type plants exhibited severe inhibition of growth and subsequently died whereas the transgenic plant could survive (Fig. 8A, right panel). The degree of growth inhibition in transgenic plants expressing ApGSMT and ApDMT (MT2) was much more severe than that of the transgenic plants expressing ApGSMT, ApDMT, and ApPGDH (PGxMT28) during the vegetative stage (Fig. 8B, left). Further experiments were carried out during the reproductive stage. High salinity conditions resulted in severe damage in the wild-type plants (data not shown). The transgenic plants expressing ApGSMT, ApDMT, and ApPGDH exhibited more tolerance for salt stress than the transgenic plants expressing ApGSMT and ApDMT (Fig. 8B, right).

**DISCUSSION**

The results presented here demonstrate the importance of the ApPGDH gene in serine or glycine engineering for the enhancement of betaine accumulation through choline oxidation or the glycine methylation pathway in microbes and plants, respectively. Overexpression of ApPGDH in *E. coli* resulted in a large increase of serine and glycine levels (Fig. 3, A and B).
PGDH for the Accumulation of Betaine

These results demonstrate that ApPGDH efficiently catalyzes the reaction in the forward direction in E. coli, namely in the direction of serine biosynthesis, although the activity for reverse direction is much higher than the forward direction in vitro. Interestingly, the levels of free serine and glycine in the E. coli cells expressing ApPGDH markedly increased with increasing salinity, although the promoter of ApPGDH was salt-independent (Fig. 3, A and B). One of reasons for the increase in serine and glycine could be due to the increase of PGDH levels by salt. Another reason for this could be due to the activation of ApPGDH enzyme by salt as shown in Fig. 2A. However, the in vitro activation for the forward reaction of ApPGDH was higher at 0.1 M NaCl than at 0.3 M NaCl (Fig. 2A). Therefore, in addition to the increase in \( V_{\text{max}} \) of ApPGDH by NaCl, other factors, such as the increased activities of the second and third reactions in serine biosynthesis, the enhanced supply of 3-PGA and the decreased activity of serine catabolic reactions, would also contribute to the increased level of serine at high salinity. Regardless of the molecular mechanisms, our data indicate that overexpression of ApPGDH alone or together with ApGSMT and ApDMT increased free serine and glycine contents upon the increase of salinity.

Overexpression of ApPGDH in E. coli increased the betaine levels about 2-fold compared with non-transformed cells (Fig. 3E). Hitherto, it was thought implicitly that betaine-accumulating organisms would change the metabolism to accumulate betaine in an optimum way under abiotic-stress conditions. However, the present data indicate that the adaptation was not optimized. To our knowledge, this is the first example showing the increase in betaine level by genetic engineering in betaine-accumulating organisms. It will also be interesting to introduce the ApPGDH gene into the transgenic plants expressing the choline oxidizing enzymes such as COX or CMO (8, 15) to enhance the betaine levels.

Levels of betaine in A. halophytica were increased by exogenous addition of glycine or serine at high salinity (Fig. 1A). The ApPGDH activity also increased with increasing concentrations of NaCl (Fig. 1B). These results indicate that ApPGDH is a salt-inducible protein and the increase of ApPGDH under high salinity would contribute to the increase in the levels of serine. Salt induction of PGDH, as observed in A. halophytica, has not been described in other organisms.

ApPGDH can be expressed in various organs of the Arabidopsis plants such as the leaves, stems, and the root tissues (data not shown). The transgenic Arabidopsis plant expressing ApPGDH accumulated high levels of amino acids in both the leaves and the root tissues. The serine level in their root tissues was about 3-fold higher than that in the wild-type plants (data not shown). Interestingly, the increased accumulation of serine and glycine in the transgenic plants expressing ApPGDH conferred tolerance for cold and salt stresses (Fig. 6). Elevated accumulation of serine and glycine has been reported in plants grown at high salinity, or low temperature, or under anaerobic condition (23). Serine is the major source for C1 metabolism (39), and glycine is involved in the synthesis of purines. Serine is the precursor of phosphatidylglycerol (phospholipid), which has been shown to be involved in adaptive responses to abiotic stresses in plants (40). Serine is also a precursor of strong antioxidant compounds like cysteine, which has been shown to be involved in stress tolerance in plants (41). Thus, serine may play important roles either directly and/or indirectly in the responses of plants to various environmental stresses, although their molecular mechanisms are not clear.

Arabidopsis plants have both phosphorylation and photorespiratory pathways for serine biosynthesis (22, 23). The present results of Figs. 5 and 6 show that the phosphorylation pathway for serine biosynthesis plays an important role under environmental stresses. This is compatible with a report showing the increased accumulation of mRNAs involved in the phosphorylation pathway under abiotic stress conditions (23). It is also compatible with the fact that SHMT was not induced upon salt stress in Arabidopsis (42). During stress condition, the photosynthesis would be inhibited due to the closure of the stomata. It is not surprising that the plant has to activate alternative route for amino acid synthesis under abiotic stress.

Fig. 7A shows that ApPGDH, ApGSMT, and ApDMT could be expressed in Arabidopsis plants. Betaine levels in the transgenic plants expressing ApPGDH, ApGSMT, and ApDMT were notably higher than those in the transgenic plants expressing ApGSMT and ApDMT (Fig. 7C). The right panel of Fig. 7C shows that the level of betaine in the root tissues of transgenic plants expressing ApPGDH, ApGSMT, and ApDMT (PGxMT28) was significantly higher than that in the transgenic plants expressing ApPGDH and ApGSMT (MT2). This might be due to a high activity of PGDH, which, in turn, produces more serine in the root tissues. Indeed, levels of serine and glycine in the root tissues were much higher than those in the leaves (data not shown). These facts support a view-point
that the simultaneous introduction of the glycine methylation pathway and ApPGDH to non-betaine accumulating plant Arabidopsis relieves the limitation of the supply of the precursor glycine.

Fig. 8 shows that the transgenic Arabidopsis plants expressing ApPGDH, ApGSMT, and ApDMT exhibited an enhanced tolerance for salt stress compared with those of the wild-type or transgenic plants expressing ApGSMT and ApDMT. This was caused by the elevated levels of betaine because of the overexpression of ApPGDH. This is the first report on betaine engineering using the PGDH gene. In conclusion, the present study clearly demonstrates that the in vivo supply of serine and glycine by overexpression of the ApPGDH gene could enhance the betaine levels in both the choline oxidation pathway and the glycine methylation pathway.

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