Choline and Osmotic-Stress Tolerance Induced in *Arabidopsis* by the Soil Microbe *Bacillus subtilis* (GB03)

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Submitted 4 May 2009. Accepted 19 April 2010.

Choline (Cho) is an essential nutrient for humans as well as the precursor of glycine betaine (GlyBet), an important compatible solute in eukaryotes that protects cells from osmotic stress caused by dehydrating conditions. The key enzyme for plant Cho synthesis is phosphoethanolamine *N*-methyltransferase (PEAMT), which catalyzes all three methylation steps, including the rate-limiting *N*-methylation of phosphoethanolamine. Herein, we report that the beneficial soil bacterium *Bacillus subtilis* (strain GB03) enhances *Arabidopsis* Cho and GlyBet synthesis associated with enhanced plant tolerance to osmotic stress. When stressed with 100 mM exogenous mannitol, GB03-exposed plants exhibit increased transcript level of *PEAMT* compared with stressed plants without bacterial exposure. Endogenous Cho and GlyBet metabolite pools were elevated by more than two- and fivefold, respectively, by GB03 treatment, consistent with increased stress tolerance. Moreover, in the *xipol* mutant line with reduced Cho production, a loss of GB03-induced drought tolerance is observed. Osmotic-stressed plants with or without GB03 exposure show similar levels of abscisic acid (ABA) accumulation in both shoots and roots, suggesting that GB03-induced osmoprotection is ABA independent. GB03 treatment also improves drought tolerance in soil-grown plants as characterized by phenotypic comparisons, supported by an elevated accumulation of osmoprotectants. These results provide a biological strategy to enhance Cho biosynthesis in plants and, in turn, increase plant tolerance to osmotic stress by elevating osmoprotectant accumulation.

Osmotic stress caused by drought, salinity, or cold results in dehydration that limits plant productivity and geographical distribution. In fact, most flowering plants do not survive a loss of greater than 5 to 15% of their relative water content (Gaff 1971). Although abscisic acid (ABA)-mediated stomata closing is a common response in plants to decreased water loss through transpiration during water deficit (Zhu 2002; Verslues and Zhu 2005), dehydration also leads to synthesis and accumulation of certain low molecular weight compounds, which enhance plant stress tolerance through increasing cellular osmotic pressure and, thereby, lower the free water potential of

cells for preventing water loss, or through stabilization of proteins and membrane structures (Yancey 1994). Such molecules are termed osmoprotectants or compatible solutes because they protect cells from osmotic stress without inhibition of cytosol enzyme activities (Rhodes and Hanson 1993).

Glycine betaine (GlyBet) is an important osmoprotectant that confers tolerance to drought, salinity, and cold stress (Rhodes and Hanson 1993; Gorham 1995; Sakamoto and Murata 2000). In higher plants, GlyBet is synthesized in chloroplast through a two-step oxidation of the primary metabolite choline (Cho) (Fig. 1A) (Rhodes and Hanson 1993). The two oxidation steps are catalyzed by a ferredoxin-dependent Cho monooxygenase (CMO) and an NAD⁺-dependent betaine aldehyde dehydrogenase (BADH), respectively (Rhodes and Hanson 1993; Sakamoto and Murata 2000). Many bacteria also have enzymes that oxidize Cho to GlyBet (Rhodes and Hanson 1993; Sakamoto and Murata 2000). Genetic engineering using bacterial or plant Cho oxidase genes has been shown to increase GlyBet accumulation and stress tolerance in plants such as *Arabidopsis* and tobacco that lack an identified Cho oxidase (Hayashi et al. 1997; Alia et al. 1998; Sakamoto et al. 2000; Sakamoto and Murata 2001). However, GlyBet production in such transgenic plants is limited by inadequate Cho biosynthesis (Nuccio et al. 1998, 1999; Huang et al. 2000), thus triggering efforts to enhance Cho synthesis in plants.

Plant Cho is synthesized in the cytosol via stepwise methylation of ethanolamine derivatives (Fig. 1) (Zeisel and Blusztajn 1994). Conversion from phosphoethanolamine (P-EA) to phosphor-monomethyl-ethanolamine (P-MME) is the first methylation step as well as the rate-limiting step (Datko and Mudd 1988; Nuccio et al. 2000; McNeil et al. 2001). The cytosolic enzyme phosphoethanolamine *N*-methyltransferase (PEAMT) catalyzes all three methylation steps in the Cho biosynthetic pathway, eventually converting P-EA to phosphocholine (PC). Free Cho is then generated either through direct dephosphorylation of PC, as in spinach (Summers and Weretilnyk 1993), or from phosphatidylcholine that derived from PC, as in tobacco (McNeil et al. 2000). Whereas the second and third methylation can also at the phosphatidyl-base level (Summers and Weretilnyk 1993; McNeil et al. 2000), the majority of Cho production is mediated by PEAMT (Nuccio et al. 1998, 2000; Mou et al. 2002). In fact, overexpressing *PEAMT* in transgenic tobacco significantly increases biosynthesis of Cho and GlyBet (McNeil et al. 2001), whereas silencing of *PEAMT* results in a drastic reduction of Cho production and hypersensitivity to salinity in *Arabidopsis* (Mou et al. 2002). Therefore, PEAMT is critical for enhancing osmotic-stress tolerance in plants via genetic manipulation of Cho biosynthesis.

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In addition to metabolic engineering, beneficial soil microorganisms provide another plausible approach for enhancing osmoprotectant biosynthesis in osmotic-stressed plants. In soils, a significant number of bacteria or fungi are found to colonize roots and stimulate plant growth. Such microorganisms have been applied to various agricultural crops for increased seed germination, plant weight, harvest yields, and disease resistance (Kloepper et al. 1980; Glick 1999; Van Wees et al. 2008). Fungal symbiosis by arbuscular mycorrhizae increased accumulation of the osmoprotectant proline in soybean plants under drought stress (Porcel and Ruiz-Lozano 2004). In contrast, plant growth-promoting rhizobacteria (PGPR), which include a wide variety of beneficial soil bacteria, have not been identified in regulating plant osmoprotectant accumulation. *Bacillus subtilis* GB03 is a PGPR strain that enhances plant vegetable growth, photosynthesis, and iron uptake, as well as disease resistance via emission of volatile chemicals (Ryu et al. 2003, 2004; Zhang et al. 2007, 2008b, 2009). Unidentified components in such complex blends of bacterial volatiles also confer plant salt tolerance through tissue-specific gene regulation of the Na⁺ transporter HKT1 (Zhang et al. 2008a). DNA microarray studies revealed *PEAMT* as one of the GB03-

induced genes in *Arabidopsis* grown without osmotic stress (Zhang et al. 2007), indicating that GB03 may be capable of increasing osmoprotectant accumulation in plants under dehydration stress. To test this hypothesis, GB03's impact on plant osmotic-stress tolerance was investigated, focusing on osmoprotectant regulation at the levels of gene expression, metabolite accumulation, and phenotypic response.

RESULTS

GB03 regulation of plant *PEAMT* expression was first confirmed by real-time polymerase chain reaction (PCR) analysis in *Arabidopsis* grown without osmotic stress. Consistent with previous microarray studies, gene expression of *PEAMT* was induced by GB03 treatment, as indicated by the greater *PEAMT* transcript accumulation observed at 2 and 4 days post GB03 treatment, compared with untreated controls (Fig. 1B). More importantly, GB03-induced gene expression of *PEAMT* by quantitative reverse-transcription PCR was observed in plants grown with 100 mM mannitol that imposed osmotic stress (Fig. 2A). Exposure to GB03 resulted in an approximately threefold increase in *PEAMT* gene expression for osmotic-

Fig. 1. Phosphoethanolamine N-methyltransferase (*PEAMT*) gene expression in nonstressed *Arabidopsis* is induced by GB03. **A**, *PEAMT* catalyzes all methylation steps in the biosynthetic pathway of choline (Cho) and glycine betaine in plants. CMO: cho monooxygenase; BADH: NAD⁺-dependent betaine aldehyde dehydrogenase. **B**, GB03 increases *PEAMT* mRNA levels in plants without osmotic stress. Three biological replicates of RNA were extracted and used for polymerase chain reaction analysis. Representative gel electrophoresis images are shown. Ubiquitin 10 (*UBQ10*) expression was used as a control.

stressed plants with 4 days of treatment. This transcriptional induction was accompanied by elevated metabolic pools of Cho and GlyBet in osmotically stressed plants (Fig. 2B). Fourteen days after treatment, a 127% increase in Cho content was

observed in GB03-exposed plants compared with nonexposed control plants. Meanwhile, GlyBet levels in these osmotic-stressed plants were elevated from 0.46 $\mu\text{g/g}$ dry weight in control plants to 2.41 $\mu\text{g/g}$ dry weight in GB03-treated plants

Fig. 2. GB03 increases phosphoethanolamine *N*-methyltransferase (PEAMT) transcription and biosynthesis of choline (Cho) and glycine betaine (GlyBet) in osmotic-stressed plants. **A**, PEAMT transcription of plants grown with 100 mM mannitol was elevated with exposure to GB03. Values detected by quantitative real-time polymerase chain reaction are relative to water-treated values observed on day 1; black and white bars represent GB03 and water controls, respectively, with an asterisk (*) indicating $P \leq 0.05$ for treated versus controls (t test; $n = 3$, mean $\pm$ standard deviation). **B**, GB03 increases Cho and GlyBet accumulation in osmotic-stressed plants. Two-day-old plants were transferred from nonstress condition to growth media with 100 mM mannitol and treated with or without GB03. Fourteen days after treatment, Cho and GlyBet content were measured. Black and white bars represent GB03 and water control treatments, respectively. An asterisk (*) indicates statistical difference by t test analysis with P value ≤ 0.05 between treatments; $n = 3$, mean $\pm$ standard deviation.

Fig. 3. GB03 enhances plant tolerance to osmotic stress. **A**, Representative images of plants with exposure to GB03 or water as control for 14 days. GB03 triggers enhancement of plant growth as characterized by **B**, total leaf surface area per plant and **C**, whole-plant fresh weight. Black and white bars represent GB03 and water control treatments, respectively. An asterisk (*) indicates statistical difference by t test analysis with P value ≤ 0.05 between treatments; $n \geq 4$, mean $\pm$ standard deviation.

(Fig. 2B). Together with the gene induction of *PEAMT*, metabolite accumulation of Cho and GlyBet indicate that GB03 enhances osmoprotectant levels in osmotic-stressed plants through regulation of *PEAMT* transcription.

To examine whether GB03 is capable of enhancing plant osmotic-stress tolerance with increased osmoprotectant accumulation, plants grown with 100 mM mannitol and treated with or without GB03 were monitored for growth vigor. Fourteen days after treatments, GB03 exposure increased plant tolerance to mannitol-induced osmotic stress, as evidenced by the improved plant growth compared with the stressed plants without GB03 treatment (Fig. 3). Specifically, total leaf surface area (per plant) and whole-plant fresh weight in plants under osmotic stress were increased by more than two- and fivefold, respectively, when treated with GB03. To examine the contribution of *PEAMT* in GB03-mediated increases in drought tolerance, a previously characterized *xipot1* mutant line with reduced Cho production (Cruz-Ramirez et al. 2004) was assayed under low and elevated osmotic-stress conditions. In the absence of media mannitol, the *xipot1* mutant (*peamt*) line was larger with GB03 treatment versus a water control ($P < 0.05$); however, with mannitol present in the media, induced drought tolerance was lost in the *peamt* line with no significant difference in weight between GB03 and water treatments (Fig. 4). Therefore, GB03 activation of osmotic-stress tolerance is linked with induction of Cho biosynthesis mRNAs and elevated accumulation of Cho and GlyBet metabolites.

The phytohormone ABA is known to regulate many stress-induced genes as well as stomata closing in response to water deficit. Although GB03-treated plants exhibit enhanced tolerance to osmotic stress, GB03 has also been shown to down-regulate shoot ABA levels in plants without osmotic stress (Zhang et al. 2008b). Therefore, ABA levels in osmotic-stressed plants with or without GB03 exposure were compared to determine whether ABA mediates GB03-enhanced plant osmotic-stress tolerance. Organ-specific measurements of ABA contents revealed that plants accumulated similar levels of ABA in both

Fig. 4. Phosphoethanolamine *N*-methyltransferase (*peamt*) mutant *xipot1* that reduces choline accumulation disrupts GB03-induced plant drought tolerance. *PEAMT* is not necessary for GB03-induced growth promotion in plants without mannitol in the medium whereas *peamt* mutation reduces plant drought tolerance induced by GB03. An asterisk (*) indicates $P < 0.05$ for treated versus controls (t test; $n = 3$, mean $\pm$ standard deviation).

shoots and roots when grown under osmotic stress, independent of GB03 treatment (Fig. 5), indicating that GB03-triggered plant osmo-protection is not mediated through ABA action. Under osmotic stress, ABA levels range from 632 to 875 pmol g^{-1} fresh weight in shoots and 189 to 229 pmol g^{-1} fresh weight in roots, both of which are significantly higher than those in nonstressed plants (Fig. 5).

The ability of GB03 in enhancing plant tolerance to osmotic stress was subsequently examined in soil-grown conditions with drought stress. Leaf water potential of plants without GB03 treatment dropped from -0.18 ± 0.04 to -4.8 ± 0.05 Mpa, recorded at 0 and 12 days after being withheld from watering. In comparison, leaf water potential of GB03-treated plants dropped from -0.21 ± 0.05 to -2.61 ± 0.05 MPa after 12 days of drought treatment, indicating an improved desiccation tolerance in GB03-treated plants. Consistently, 15 days after being withheld from watering, plants without GB03 treatment severely shriveled, whereas GB03-treated plants exhibited greater resistant to dehydration stress (Fig. 6). Consistent with this phenotypic drought tolerance, Cho and GlyBet contents in GB03-treated plants were 260 and 58% greater than in control plants without added GB03 (Table 1). A 19% increase in whole-plant dry weight with GB03 treatment was observed compared with control plants (Table 1). GB03-induced drought tolerance was confirmed by the fact that soil-water activities were similar for both treated and control plants (Table 1).

DISCUSSION

Abiotic stress afflicts plant growth and, consequently, productivity in agricultural crops. Recently, several reports have started to establish the role of beneficial soil bacteria in improving plant tolerance to abiotic stress, including drought and salinity (Cho et al. 2008; Zhang et al. 2008a; Belimov et al. 2009). Among these PGPR strains, *Pseudomonas chlororaphis* O6 induces stomatal closure to decrease water loss in drought-stressed plants (Cho et al. 2008), *Variovorax paradoxus* 5C-2 reduces plant accumulation of the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) triggered by drought stress (Belimov et al. 2009), and *B. subtilis* GB03 confers plant salt tolerance via organ-specific regulation of plant Na^+ importer HKT1 (Zhang et al. 2008a). In addition to these mechanisms, GB03 is demonstrated in this study to enhance osmoprotectant biosynthesis and accumulation in plants under mannitol- and drought-induced dehydration stress.

Gene expression of *PEAMT* is induced by salinity or light (Weretilnyk et al. 1995). Although salinity induces osmoprotectant accumulation in plants, it is not included as an experimental approach for studying GB03-triggered plant osmoprotection because plant stress tolerance in such cases can be attributed to GB03 regulation of plant Na^+ homeostasis, as reported previously (Zhang et al. 2008a). Nonetheless, the *PEAMT* mRNA level was increased in salt-stressed plants

Table 1. GB03 increases plant drought tolerance^a

Parameter	GB03 (T)	Control (C)	P value	T/C (%)
Soil water activity	0.84 $\pm$ 0.02	0.82 $\pm$ 0.01	9E-04	102.4
Dry weight (mg)	49.4 $\pm$ 0.8	41.4 $\pm$ 1.2	2E-05	119.4
Choline (μ g/g DW)	7.2 $\pm$ 0.3	2.0 $\pm$ 0.2	4E-06	362.4
Glycine betaine (μ g/g DW)	2.1 $\pm$ 0.3	1.4 $\pm$ 0.1	5E-03	158.1

^a Three-week-old plants were treated with GB03 or water as control 2 days before starting drought treatment. Fifteen days after drought treatment, plants were collected and soil water activities were measured to confirm similar drought stress to both GB03-treated and control plants. Statistical difference is indicated by t test analysis with P value < 0.05 between treatments; $n \geq 3$, mean $\pm$ standard deviation.

when exposed to GB03, indicating that GB03 is capable of enhance plant osmoprotection under such condition (H. Zhang and P. W. Paré, *unpublished data*). We also observed that addition of 10 mM Cho to plant growth media not only failed to enhance plant stress tolerance to 100 mM mannitol without GB03 treatment but also impaired GB03-induced stress tolerance (data not shown). This may be explained by the fact that exogenous supplementation of Cho to growth media inhibits *PEAMT* expression (Mudd and Datko 1989a and b). In addition, although exogenous Cho may be taken up by roots to improve endogenous osmoprotection, it also increases osmotic stress to the plant because the exogenous osmolyte concentration is immediately elevated with the presence of Cho in the media.

To further confirm the role of *PEAMT* in GB03-induced osmotic stress tolerance, the *PEAMT* mutant line *xipot1* with reduced root and shoot levels of Cho, phospho-Cho, and phosphatidyl-Cho compared with the parental line (Col-0) (Cruz-Ramirez et al. 2004) were grown under osmotic stress. Loss of drought tolerance between GB03 and water treatments in the *peamt* mutant with elevated mannitol in the medium indicated that *PEAMT* induction is, at least in part, necessary for GB03-induced drought tolerance.

How GB03 regulates gene expression of *PEAMT* for the subsequent accumulation of Cho and GlyBet remains unclear. Higher plants accumulate GlyBet in response to osmotic stress

(Jones and Story 1981). Most eukaryotic biosynthetic pathways for GlyBet formation are a two-step Cho oxidation, though direct methylation of glycine to GlyBet has been identified in several bacteria (Nyyssola et al. 2000; Waditee et al. 2005). In addition to being a key constraint for GlyBet biosynthesis, Cho production is also critical for synthesizing the major plant membrane lipid phosphatidylcholine (PC) (Fig. 1) (Moore 1990; Bolognese and MacGraw 2000). Although auxin increases PC biosynthesis (Price-Jones and Harwood 1983), auxin homeostasis in plants is regulated by GB03 (Zhang et al. 2007). Moreover, GB03 increases GlyBet content by 2.5-fold in plants under normal growth condition (data not shown); such elevated accumulation of cytosol osmolytes may increase cellular water content, resulting in greater turgor pressure that favors cell expansion, which has been observed in GB03-treated plants (Zhang et al. 2007). Further investigation of a possible link between Cho biosynthesis and auxin homeostasis may shed light on the mechanism for GB03-regulated Cho production.

In the potted-plant study, plants are grown in a nonsterile system with the addition of GB03 to the soil. A green fluorescent protein (GFP)-labeled GB03 strain served as a positive control so that bacterial growth could be confirmed by visual inspection of the fluorescence (data not shown). This is important in case some condition compromised bacterial growth either due to some abiotic soil condition or another bacterium that out com-

Fig. 5. Absciscic acid (ABA) levels in osmotic-stressed plants are not affected by GB03. Under osmotic stress caused by 100 mM exogenous mannitol, accumulation of ABA in **A**, shoots or **B**, roots is not regulated by GB03. Black and white bars represent GB03 and water control treatments, respectively. An asterisk (*) indicates statistical difference by *t* test analysis with *P* value ≤ 0.05 between treatments; *n* = 4, mean $\pm$ standard deviation.

petes GB03 so that the plant is not actually exposed to the GB03 treatment. In the petri dish assays, GB03 growth could be visualized whereas, in soil, the presence of GB03 could be confirmed only by visualization of the GFP signal.

A major function of ABA in regulating plant stress responses is to prevent water loss caused by osmotic stress. Osmotic-stressed plants with or without GB03 exposure accumulated similar levels of ABA, which were significantly higher than those in plants without osmotic stress. Such observation provides two implications: i) ABA does not mediate GB03-induced plant tolerance to osmotic stress, because ABA levels in stressed plants were not affected by GB03 treatment; or ii) in addition to GB03-induced osmoprotection, ABA functioned in osmotic stress protection, because stressed plants accumulated higher ABA than nonstressed controls. Such increases in ABA levels were consistent with the observation that plants rapidly increase ABA biosynthesis under osmotic stress (Ikegami et al. 2009). Indeed, drought-stressed *Atriplex prostrata* biosynthesizes GlyBet from Cho independently of ABA regulation although ABA can initiate global physiological changes in response to osmotic stress (Wang and Showalter 2004).

In addition to Cho's critical role in plant stress tolerance, it is an essential human nutrient. Cho is necessary for the structural integrity and signaling of cell membranes, including cholinergic neurotransmission and muscle function, as well as being the major dietary source of methyl groups (Zeisel 2006). In particular, Cho is vital for fetal brain development, in that it reduces the risk of neural tube defects and increases lifelong memory function (Zeisel 2000, 2006). Therefore, metabolic engineering for enhanced plant Cho biosynthesis has been suggested to improve dietary Cho supply from plants (McNeil et al. 2001). In addition

to genetic manipulation, the ability of GB03 in regulating plant Cho biosynthesis demonstrates a novel strategy for increasing crop nutrition with respect to Cho supply.

MATERIALS AND METHODS

Plant growth and treatments.

Arabidopsis thaliana (Col-0) seed were surface sterilized and then planted on one side of specialized plastic petri dishes (100 by 15 mm) that contain a center partition (I plates; Fisher Scientific, Pittsburgh); both sides contained one-half-strength Murashige and Skoog (MS) solid media with 0.8% (wt/vol) agar and 1.5% (wt/vol) sucrose. Before being placed in the growth room, seed were vernalized for 2 days at 4°C in the absence of light. *Arabidopsis* were grown under sterile conditions with a light and dark cycle of 16 and 8 h, respectively, at 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ light, $21 \pm 4^\circ\text{C}$ temperature, and $40 \pm 10\%$ relative humidity.

One day before plant experiments, bacterial strains were streaked onto tryptic soy agar (TSA) plates and incubated at 28°C in the absence of light for 24 h. PGPR cells were harvested from TSA plates in double-distilled water (DDW) to yield 10^9 CFU ml^{-1} , as determined by optical density and serial dilutions with plate counts. Two-day-old *Arabidopsis* seedlings were then transferred to new growth media with or without 100 mM mannitol and inoculated with 20 μl of bacterial suspension culture or DDW applied by drop to the nonplant side of the petri dish. Leaf surface area was quantified as described by Zhang and associates (2007).

For drought treatments, 8-day-old seedlings were transferred from half-strength MS media to soil which had already been

Fig. 6. GB03 enhances drought tolerance in plants. Fifteen days after drought treatment, plants with GB03 treatment show improved stress tolerance compared with control plants. Three-week-old plants were treated with GB03 or water (control) 2 days before starting drought treatment ($n = 3$).

watered, having a water activity of 0.999 to 1.002. Plants were then allowed to grow and adapt in the soil. Three-week-old plants were inoculated with either GB03, by adding a bacterial suspension culture of 1.2×10^3 CFU/ml, 50 µl/plant near the roots, or an equal volume of water as control. At 2 days after bacteria treatment, plants were subjected to drought stress by withholding water for 15 days.

Leaf water potential measurements.

Plants were drought treated as previously described. At the beginning and 12 days after withholding water, the water potential (Ψ_w) of fully expanded leaves was measured. Four 0.50-cm² discs were collected from different plants with or without GB03 treatment and sealed in sample chambers with end-window thermocouple psychrometers (J. R. D. Merrill Specialty Equipment Company, Logan, UT, U.S.A.). Detailed procedures and theory for leaf water potential measurements by thermocouple psychrometry was provided by Oosterhuis (2003).

RT-PCR analysis.

Total RNA from whole plants was extracted by using RNease mini kits (Qiagen, Valencia, CA, U.S.A.). First-strand cDNA was synthesized from 5 µg of total RNA using MuMLV-RT (Fisher Scientific, Houston). The following primers were used (5' to 3'): [*PEAMT*] TTCCGAAATGGCTGCATCG and CCTT GATCCAACCGACCAT; [*UBQ10*] CGATTACTCTTGAGGT GGAG and AGACCAAGTGAAGTGTGGAC. The optimized linear range for *PEAMT* amplification was determined as 25 cycles and for *UBQ10* as 24 cycles.

Quantitative real-time PCR analysis.

Total RNA from whole plants was extracted by using RNease mini kits (Qiagen). Reverse transcription of all the RNA samples was carried out using random hexamers. An ABI TaqMan Reverse Transcription Reagent Kit (Applied Biosystems, Foster City, CA, U.S.A.) was used for first-strand synthesis in 50-µl reactions (containing 1 µg of total RNA) at 37°C for 60 min. Gene expression levels were detected by an ABI PRISM 7000 Sequence Detection System. The primers were designed by using PrimerQuest program (Integrated DNA Technology, Coralville, IA, U.S.A.). 18S rRNA primers were used for RNA normalization. SYBR green PCR master mix (Applied Biosystems) was used for 50-µl PCR reactions as follow: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 60 s. The relative expression levels of all the samples were calculated and analyzed (User Bulletin no. 2, ABI PRISM 7700 Sequence Detection System). The threshold cycle (Ct value) of the target genes and 18S RNA in different samples were obtained after quantitative real-time PCR reaction. In brief, the normalizer 18S RNA Ct value was subtracted from the gene of interest Ct (target gene) to produce the dCt (delta Ct) value of the sample. The dCt value of the calibrator (the sample with the highest dCt value) was subtracted from every other sample to produce the ddCt value. Two to the -ddCt power (2^{-ddCt}) was taken for every sample as the relative expression levels.

Cho and GlyBet determination.

Cho and GlyBet extraction and measurements were performed as described in Grieve and Grattan (1983). Briefly, Cho and GlyBet were cold precipitated as periodide crystals from aqueous plant extracts aliquoted into two fractions. To one fraction, H₂SO₄ (2 N, pH 0.3) was added at a ratio of 1:1 for co-precipitation of Cho and GlyBet while potassium phosphate buffer (0.2 M, pH 6.8) was added to the other extract in a ratio of 1:1 for precipitation of Cho alone. Cho and GlyBet concen-

trations were determined spectrophotometrically at 365 nm. Plants grown with mannitol were collected 14 days after GB03 or DDW treatments. Drought-stressed plants were collected 15 days after drought treatment.

ABA assay.

ABA was extracted as described by Zhang and associates (2008b). Ground tissue was extracted in 1 ml of a 90% methanol solution, containing acetic acid at 20 ml/liter and butyl hydroxytoluene (Sigma-Aldrich) at 10 mg/liter for 24 h at 4°C. The ABA contents in the solution were determined using a Phytodetek ABA immunoassay kit (Agdia Inc., Elkhart, IN, U.S.A.). Mixed ABA isomers (Sigma-Aldrich, St. Louis) were used as standards.

Statistical analysis.

All measurements were performed with at least three biological replicates, each containing multiple plants. Numbers of independent biological replicates are indicated for each experiment in the figure and table legends. Student's *t* test *P* values were obtained for statistical analysis by using SAS software.

ACKNOWLEDGMENTS

We thank C. Bednarz for expert training in taking leaf water potential measurements. PEAMT mutant seed were kindly provided by L. Herrera-Estrella (Instituto Politecnico Nacional Unidad Irapuato; Guanajuato, Mexico). This research was funded in part by the Texas Tech University Research Development Fund and the Robert A. Welch Foundation (D-1478).

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