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Increased Tolerance to Salinity and Drought in Transgenic Indica Rice by Mannitol Accumulation

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The osmolytes, including mannitol have been shown to be very important in abiotic stress tolerance. Thus, the present study was undertaken with the aim to enhance abiotic stress tolerance in basmati indica rice by introduction of the *E. coli* mannitol-1-phospho dehydrogenase (*mtl*D) gene, which is involved in mannitol synthesis in plants. Several putative transgenic rice plants were generated by *Agrobacterium*-mediated transformation. The presence of the transgene in the primary transformants was confirmed by PCR using hygromycin phosphotransferase (*hpt*) and *mtl*D gene specific primers. Southern hybridization also revealed the integration of the transgene. Transgenic lines exhibited mannitol accumulation, which was correlated to the increased tolerance of the transgenics against salinity and drought stress. The T_1 transgenic seed germination and seedlings growth showed better performance than that of wild type during abiotic stresses under *in vitro* and *in vivo* growth conditions.

Key words: abiotic stress tolerance, mannitol, mannitol-1-phosphodehydrogenase, transgenic rice.

Plant productivity is adversely affected by environmental factors such as salinity, drought, temperature extremes, etc. (1-5). To overcome these limitations and to meet the demands of the increasing population of the world, it is imperative to make crops tolerant to these stresses. Though conventional plant breeding approaches are being undertaken, the progress made in this area has been very slow. Thus genetic engineering methods have been adopted as they have been shown to be fast means of achieving stress tolerance (5-9). Moreover by using genetic engineering approaches it is possible to introduce genes from any heterologous source and also multiple genes can be introduced simultaneously.

Abiotic stresses are known to disturb the intracellular water balance of the biological organisms. To counteract such conditions, plants accumulate various low molecular weight compounds such as sugars, sugar alcohols, amino acids and quaternary ammonia compounds (2, 4, 6, 7, 10-12). These metabolites, widely known as compatible solutes or osmolytes are neutral, non-toxic and do not interfere with normal metabolic reactions even at high concentrations (13).

Several sugar alcohols are known to act as osmolytes and mannitol is one of such examples. Mannitol is a six-carbon non-cyclic sugar alcohol widely distributed in bacteria, fungi, algae and at least 100 species of vascular plants (14). It serves an important role in the storage of carbon and energy, regulation of co-enzymes, free-radical scavenging, osmoregulation and serves as compatible solute (14-17). In celery, mannitol is the major product of leaf photosynthesis and constitutes as much as 50% of the phloem-translocated photoassimilate (18). Therefore, over-expression of a gene involved in mannitol synthesis would result in the redirection of carbon and energy flow from metabolites recognised by the host organism to produce compounds.

The *E. coli mtl*D gene, which encodes for mannitol-1-phosphodehydrogenase has been introduced into plants to synthesise mannitol. Tarczynski *et al* (19) reported for the first time the over-expression of *mtl*D gene into tobacco, which led to the accumulation of mannitol and such transgenics had an increased ability to withstand salt stress (20). Thereafter, the *mtl*D gene was introduced into plants like *Arabidopsis* (21), tobacco (22), eggplant (23), non-basmati rice (24) and wheat (25) and has been found to be useful in conferring tolerance to salinity and drought stress. Shen *et al* (26) targeted the *mtl*D gene into the chloroplast genome of tobacco and the transgenics showed increased tolerance to oxidative stress. Further, *mtl*D gene is involved

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in the synthesis of mannitol in plants from fructose-6-phosphate, which is in contrast to its role in *E. coli* where this gene is involved in the catabolism of mannitol to form fructose or fructose-6-phosphate (19). In plants, the reduced carbon and cellular energy (i.e. NADH) have been diverted to produce mannitol.

In the present study, we have developed transgenic indica basmati using rice cultivar Pusa Basmati-1 (PB-1) with *E. coli mtl*D gene, and such transgenics exhibited enhanced tolerance to salinity and drought which can be correlated with mannitol accumulation.

Materials and Methods

Plant material and vector construction — The seeds of basmati indica rice (*cv* PB-1) used for the present study were obtained from Indian Agricultural Research Institute, New Delhi. The *E. coli mtl*D gene was procured from Prof Hans J Bohnert, (University of Illinois, USA). The binary cloning vector pCAMBIA 1380 was provided to us by Prof RJ Jefferson (Centre for Application of Molecular Biology in Agriculture - CAMBIA, Australia).

A 1.5 kb *Xba*I and *Hin*dIII fragment containing *E. coli mtl*D gene was isolated from plasmid pUC 18 and ligated into the same sites in the vector pBinAR. The plasmid DNA containing *mtl*D expression cassette in pBinAR was restricted with *Eco*RI and *Hin*dIII and ligated to the same sites of binary vector pCAMBIA 1380. The recombinant plasmid pCAMBIA 1380-*mtl*D (Fig. 1A) was checked for the presence of the transgene by PCR and DNA restriction enzyme digestion analysis and then mobilized into *Agrobacterium tumefaciens* (strain LBA4404) for use in rice transformation.

Rice transformation — *Agrobacterium*-mediated transformation was carried out as described by Kumria *et al* (27). The scutellum-derived embryogenic calli from mature seeds of rice were used for co-cultivation with *A. tumefaciens* carrying pCAMBIA 1380-*mtl*D. The calli were selected on regeneration medium (MS + 2.5 mg l^{-1} kinetin + 0.5 mg l^{-1} napthalene acetic acid + 3% maltose) containing 50 mg l^{-1} hygromycin and 500 mg l^{-1} cefotaxime and incubated at 26 ± 2 °C, 16 h photoperiod for one month with one sub-culture. The proliferating calli were transferred to the regeneration medium (MS + 2.5 mg l^{-1} kinetin + 0.5 mg l^{-1} napthalene acetic acid + 3% maltose + 250 mg mg l^{-1} cefotaxime) for two months with one sub-culture. The regenerated plants were transferred to the pots with soil:vermiculite mix (1:1) in the culture room and then to the contained green-house facility for flowering and seed set.

Molecular analysis — The putative transgenics were analyzed for the integration of the transgenes by PCR and Southern hybridization. The leaves of the primary transformants and the untransformed control were used for the isolation of genomic DNA by the CTAB method (28). About 100 ng of the total genomic DNA was used to amplify *hpt* and *mtl*D genes by PCR using the oligonucleotides- 5'-AGCTGCGCCGATGGTTTCTACAA-3' and 5'-ATCGCCTCG CTCCAGTCAATG-3' as forward and reverse primers for *hpt* gene and 5'-TTGGCGCAGGTAATATCGGTCGTG-3' and 5'-GCAGCGGCAATACCTTCAATCAGGT-3' as forward and reverse primers for *mtl*D gene. The PCR programme included DNA denaturation at 94 °C for 5 min followed by 40 cycles of amplification (Denaturation at 94 °C for 1 min, primer annealing at 52 °C (*hpt* gene) and 50 °C (*mtl*D gene) for 1 min, and the extension at 72 °C for 2 min) and finally 10 min at 72 °C. The amplified products were visualized on 1% agarose gel.

To determine the integration and copy number of the transgenes, 10 µg genomic DNA was digested with *Xho*I (which gives a fall out of the *hpt* gene) and *Eco*RI (which is a unique site in the T-DNA and gives transgene copy number), respectively, overnight at 37 °C. The restricted DNA was electrophoresed on 0.8% agarose gel. The DNA was transferred on nylon membrane (Hybond-N, Phamacia, USA). The probe used was 1 kb *hpt* gene fragment. It was prepared by labelling the DNA with [α-^{32}P] dCTP (Board of Radiation and Isotope Technology, India) using the nick translation kit (Bangalore Genei, India) as per the manufacturer's guidelines. The blot was hybridized and washed according to the protocol of Sambrook *et al* (29). The membrane was exposed to X-ray film (XK-5 Kodak) in the dark and incubated in -70 °C for a suitable time period before developing.

Segregation analysis — To study the segregation of the transgene, the seeds from the T_1 transgenic as well as untransformed control (regenerated plants) plants were dehusked, surface-sterilized with 0.1% $HgCl_2$ for 20 min, washed thoroughly with sterile water and soaked overnight in sterile water in dark. The seeds were germinated on MS basal for 2 days in light at 26 ± 1 °C and 16 h photoperiod, and then transferred on to MS basal medium containing 50 mg l^{-1} of hygromycin-B. The segregation was scored on

the basis of hygromycin resistance or sensitivity after 10 days of seed inoculation.

Extraction and determination of mannitol content — The sugar alcohol was extracted from the leaves of T_1 transgenic and wild type seedlings by grinding 50 mg fresh weight of the frozen tissue in methanol:chloroform:water (12:5:3) at 4 °C. An equal volume of water (4 °C) was then added to the extract. The extract was centrifuged at 1000 x g for 10 min and supernatant was collected. The collected supernatant (100 µl) was vacuum dried. The resulting pellet was dissolved in 1 ml water. The sample was purified by passing through 0.2 micron filter before being used for the analysis (19).

Mannitol levels were analysed and quantified by binary gradient High Performance Liquid Chromatography (Shimadzu LC 10 series). Ten microlitre sample was injected per run. The system was operated at 1 ml min^{-1} with acetonitrile:water in the ratio of 80:20 as the mobile phase. The separation was done by using 250 x 4.6 mm Luna, NH_2 column at 40 °C and the detection was done using the RID detector. The 10 mg l^{-1} purified mannitol from Sigma was used as the standard. The area of the peak of each sample was quantified using the area of the standard to determine the amount of mannitol.

Abiotic stress tolerance assays — The transgenics were checked for their tolerance towards salinity and drought stresses at seed germination and seedling growth stage.

To test the response to salt stress, seeds from the transgenic lines as well as the control plants (non-transformed plants), after surface-sterilization, were inoculated on MS basal medium fortified with 150 mM NaCl and kept in light at 26 ± 1 °C in the culture room. The percent seed germination and seedling height, fresh and dry weight of the wild-type and transgenics was taken after 15 days of seed inoculation as the wild-type seedlings have become pale yellow and could not grow further after about a month of stress application. A few of the seedlings from transgenic lines and the control were also kept under unstressed conditions.

The one-month-old transgenic seedlings (raised *in vitro*) were transferred to culture tubes containing soil : vermiculite (1:1) and watered with one tenth MS to allow the seedlings to establish. After about a week, five seedlings from each line were watered once a day with 2 ml of one-tenth MS containing 150 mM NaCl for a month. Few seedlings from each transgenic line as well as wild type were also watered with one-tenth MS without salt, which served as control (unstressed condition). Salt tolerance of the transgenic seedlings was based on the growth performance of the seedlings in the presence of the stress agent as compared to the wild-type seedlings. Similar assay was also performed with 200 mM NaCl, which was found to be lethal for wild-type seedlings, while the transgenic seedlings could withstand the stress.

For drought stress tolerance assay, the seeds from transgenic lines as well as control plants after surface-sterilization were inoculated into Petriplates lined with Whatman No. 1 paper discs. About 3 ml of one-tenth MS medium supplemented with 400 mM and 600 mM mannitol was poured on paper discs daily to observe the effect of mannitol-mediated drought stress on seed germination in transgenics for 10 days.

Six-week-old transgenic seedlings were also used for the drought tolerance assay. They were transferred into soil:vermiculite (1:1) in small plastic pots with holes at the bottom. The pots were kept in flat bottom plastic trays containing water. The seedlings were grown for another 15 days. The seedlings were then imposed to drought stress by withdrawing water for 7 days after which they were allowed to recover by adding water for 4 days. Few of the seedlings were watered regularly and maintained as unstressed control.

Statistical analysis — Four transformation experiments were done to obtain about 47 putative rice transgenic plants. The control seedlings used for the abiotic stress studies were derived from the seeds of non- transformed plants regenerated from callus cultures.

Results and Discussion

Rice transformation — Large number of transformants (about 47) were obtained from about 638 co-cultivated calli and after hygromycin selection as described in Materials and Methods. The primary transformants were transferred to plastic pots in the culture room and then shifted to the green-house where they were grown to maturity. The transformants were observed to be morphologically normal and fertile.

Molecular analysis — The putative transformants were analysed for transgene integration by PCR and Southern blot analysis. PCR was performed using *hpt* specific gene

primers, and 7 out of 13 plans tested showed presence of an expected amplified product of 1 kb (Fig. 1B). The PCR was also performed using *mtl*D gene specific primers and an amplification product of 1 kb was obtained in 62% of the transgenics. (Fig. 1C). The transgenics which were found to be positive for *hpt* gene were also positive for *mtl*D gene.

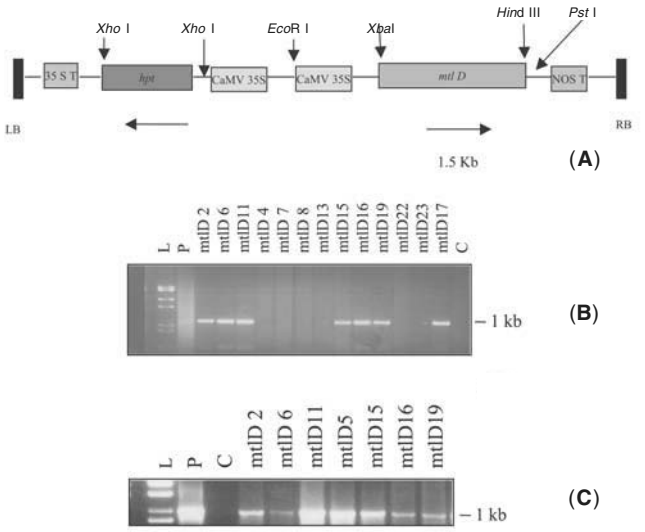

Fig. 1. (**A**) The T-DNA map of pCAMBIA 1380-*mtl*D binary plasmid construct *mtl*D- mannitol-1-phosphate dehydrogenase**;** CaMV-CaMV 35S promoter; *hpt* - hygromycin phosphotransferase; 35S T- 35 S terminator; NOS T- Nopaline synthase terminator; LB-Left Border; RB- Right Border, (**B**) PCR analysis with *hpt* gene primers: L-λ *Eco*RI–*Hin*dIII ladder; P- Plasmid DNA; DNA from transgenic lines *mtl*D 2, 6, 11, 4, 7, 8, 13, 15, 16, 19, 22, 23, and 17; C- DNA from untransformed control, and (**C**) PCR analysis with *mtl*D gene primers: L-1 kb ladder; P-Plasmid DNA; DNA from transgenic lines *mtl*D 2, 6, 11, 5, 15, 16 and 19; C- DNA from untransformed control.

The PCR positive transgenics were further confirmed for transgene integration by Southern blot hybridization. For transgene integration, genomic DNA was digested with *Xho*I and hybridized with *hpt* gene probe. All the plants analysed were positive for transgene integration (Fig. 2A). In Southern analysis for copy number of the transgene, which was done by restricting genomic DNA with *Eco*RI and *hpt* gene as a probe, the transgenic lines *mtl*D 2, 6, 11 and 16 showed single copy of the transgene and *mtl*D 15 and 19 showed presence of two copies (Fig. 2B).

***Segregation analysis* —** The T_1 seeds obtained after selfing of the primary transformants were tested for segregation of the transgene on the basis of their resistance towards hygromycin. The transgenic lines *mtl*D 2*, mtl*D 6,

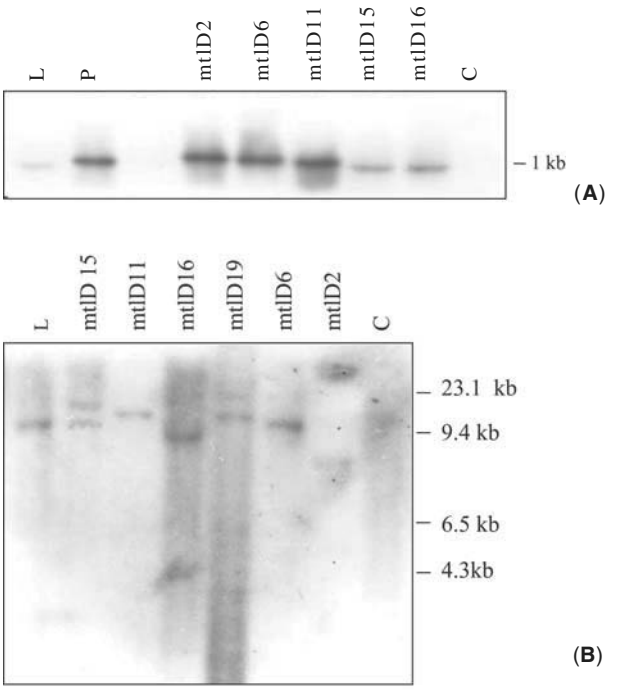

Fig. 2. (A) Southern analysis for transgene integration using *hpt* gene as probe: L – 1 kb ladder; P- Plasmid DNA; C- DNA from untransformed control; DNA from transgenic lines *mtl*D-2, 6, 11, 15 and16, and **(B)** Southern analysis for copy number using *hpt* gene as probe: L- λ *Hin*d III ladder; DNA from transgenic lines *mtl*D 15, 11, 16, 19, 6 and 2 ; C- DNA from untransformed control.

*mtl*D 11 and *mtl*D 16 showed a 3:1 segregation ratio confirming the presence of single copy of the transgene. The transgenic line *mtl*D 19 did not follow the Mendelian ratio as the line possessed two copies of the transgene. The transgenic line *mtl*D 15 showed a segregation ratio of 3:1, inspite of being a double copy transgenic line. The possible explanation for the same could be integration of two transgenes at a single locus. Similar observations have been made earlier (30, 31).

***Mannitol content in transgenic lines* —** When the mannitol levels were estimated in the transgenic lines by HPLC, a peak in the transgenic lines was seen at a retention time (5.8 min) identical to that for mannitol as standard. The profile from the control (non-transformed) plant showed no peak for mannitol indicating the absence of mannitol in wild type rice plants. The quantitative analysis showed that the mannitol concentration in the transgenic lines ranged from 0.14 to 0.96 μmol g^{-1} fresh weight of tissue (Table 1). The variation in the levels of mannitol may be due to differential expression of the transgene, depending on the position of its integration in the genome (32).

Table 1. Mannitol content in leaf tissues of the T_1 transgenic lines

Transgenic line	Mannitol concentration (µmol g^{-1} fr wt)	% Seed germination in 150 mM NaCl	% Seed germination in 400 mM mannitol
Control	Not detected	13*	0
*mtl*D2	0.14 ± 0.005	86	NT
*mtl*D 6	0.96 ± 0.03	87	73
*mtl*D15	0.71 ± 0.28	76	NT
*mtl*D 16	0.18 ± 0.015	62	NT
*mtl*D 19	0.72± 0.18	78	60

Note: Values represent means ± SE (n= 3); NT, not tested
*Although the control seeds germinated but they did not grow further.

Abiotic stress response of transgenics — The seeds from transgenic and control plants were inoculated on MS medium with and without 150 mM NaCl. On MS basal medium (minus salt), 90% of the transgenic and control seeds germinated with no difference in their seedling growth (data not shown). The seeds from transgenic lines could withstand the imposed salt stress (150 mM NaCl) and showed higher seed germination percentage, while the germination of wild-type seeds was dramatically affected (Fig. 3; Table 1). The percentage of seed germination in various transgenic lines ranged from 62 to 87 %. On basal MS medium plus 150 mM NaCl, both the wild type and the transgenic seeds germinated however, the wild type seedlings could not grow after about a month. Under salt stressed conditions, the growth performance of transgenic seedlings was much better than wild-type seedlings as transgenic seedlings showed significant

Fig. 3. Salt tolerance, based on the germination of T_1 seed of transgenic lines *mtl*D 19 and *mtl*D 16 on MS basal medium supplemented with 150 mM NaCl. Picture was taken after one month of seed inoculation.

increase in seedling height, and fresh and dry weight as compared to the control seedlings (Table 2). However, the seeds from both transgenic lines and wild-type plants did not germinate on medium containing 200 mM NaCl. The variation in percent seed germination and seedling growth could be due to the variation in transgene expression and mannitol accumulation in different transgenic lines.

When one-month old T_1 seedlings (in test tubes containing soil:vermiculite mix) were exposed to salt stress (150 mM NaCl) for 20 days, it was observed that the transgenic seedlings were healthy, grew well and showed significantly higher growth as compared to the wild type seedlings, which exhibited significant reduction in growth (Table 2). However, 200 mM NaCl was found to be lethal for wild-type seedlings, while transgenic seedlings could withstand the stress.

Our results demonstrate that rice transgenics expressing *mtl*D gene showed enhanced tolerance to salt stress both at seed germination and seedling growth stage. It thus corroborates the earlier reports that the expression of *mtl*D gene in transgenic crops result in improved tolerance against salt stress (19-25).

For checking drought tolerance, the T_1 transgenic and wild type control seeds were germinated in one-tenth MS medium containing 400 and 600 mM mannitol. While the transgenic seeds could tolerate up to 600 mM mannitol, wild type seeds failed to germinate even at 400 mM mannitol after 10 days of inoculation (Table 1).

The T_1 seedlings from the transgenic lines were also studied for drought tolerance under *in vivo* growth conditions by withdrawing water from the plastic pots for seven days. It was observed that the leaves of the control seedlings started wilting from the fourth day, while the leaves of transgenic seedlings exhibited wilting from the sixth day of water stress. The wilting in case of the transgenics was less severe and they could recover on being re-watered, while the wild type plants could not. Similar results have also been documented earlier wherein *mtl*D transgenic crops showed increased tolerance to drought stress (22, 23, 25).

Though the transgenic lines *mtl*D 2 and *mtl*D 16 containing 0.14 and 0.18 M mannitol levels showed the same level of stress tolerance as the *mtl*D 6, 15 and 19 transgenic lines with 0.96, 0.71 and 0.72 mannitol levels, respectively, suggesting the minimum threshold level of mannitol required for stress tolerance.

Table 2. Effect of salt (150 mM NaCl) on the growth of transgenic seedlings

Transgenic line	Seedling height (cm)	Fresh weight (g)	Dry weight (g)
Control	4.80 ± 0.42 [#]((21.45 ± 1.15) [$]	0.055 ± 0.009 (0.29 ± 0.09)	0.012 ± 0.002 (0.090 ± 0.005)
*mtl*D 2	5.60 ± 0.42 (26.01 ± 0.21)	0.080 ± 0.016 (0.36 ± 0.06)	0.020 ± 0.004 (0.099 ± 0.05)
*mtl*D 6	7.37 ± 0.47 (28.07 ± 0.47)	0.070 ± 0.007 (0.49 ± 0.03)	0.015 ± 0.002 (0.122 ± 0.075)
*mtl*D 15	NT (25.01 ± 0.19)	NT (0.31 ± 0.024)	NT (0.108 ± 0.005)
*mtl*D 16	6.50 ± 0.45 (24.62 ± 0.28)	0.080 ± 0.012 (0.37 ± 0.03)	0.016 ± 0.005 (0.117 ± 0.002)
*mtl*D 19	6.00 ± 0.35 (29.00 ± 1.37)	0.057 ± 0.004 (0.50 ± 0.04)	0.015 ± 0.002 (0.126 ± 0.02)

Note: Values represent mean ± SEM of 5 replicates.
[#]Salt tolerance was checked by raising seedlings from seeds germinated on MS basal medium (in Petriplates) fortified with 150 mM NaCl. Data was taken after 15 days of seed inoculation.
[$]Values in parentheses represent the salt tolerance assay of T_1 seedlings of *mtl*D transgenics grown in tubes containing soil:vermiculite (1:1) and watered with one-tenth MS medium containing 150 mM NaCl.
Data were recorded after 20 days of salt stress.
NT - Not tested.

Thus, we conclude that the introduction of mannitol biosynthesis gene in rice leads to enhance tolerance to salinity and drought stress although the exact mechanism by which *mtl*D gene confers tolerance is not clear. The proposed mechanisms include osmotic adjustment, stabilization of macromolecules and membranes and scavenging of hydroxyl radicals (2, 16, 21, 26, 33). Interestingly, though mannitol accumulation in T_1 transgenic lines was marginal as reported earlier in other transgenic crops (16, 19-22, 24, 25), still they could show increased tolerance to abiotic stresses. Previously, it was suggested that the amount of mannitol accumulation in transgenics was not adequate to provide protection through osmotic adjustment, and thus the involvement of mannitol in stress tolerance may be through its anti-oxidant property as small amounts of mannitol is sufficient for this (19-22, 25). Further, it was also shown that marginal accumulation of other osmolytes like glycine-betaine (31, 34, 35), fructans (35) and proline (36) also impart tolerance to abiotic stresses. However, as the expression of the osmolyte-encoding genes individually only confer marginal tolerance to abiotic stress, which is under the control of several genes, it may be necessary to stack more genes of the abiotic stress tolerance in transgenic plants to make them more effective for withstanding various abiotic stresses (2, 37). In fact, the simultaneous expression of the genes encoding *mtl*D and glucitol-6-phospho dehydrogenase in transgenic loblolly pine resulted in enhanced tolerance to salt stress (38).

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