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Bacterial Mannitol-1-Phophate Dehydrogenase (*mtlD*) Transgene, Confers Salt Tolerance in the Fourth Generation Transgenic Maize (*Zea Mays. L*) Plants

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Abstract

About 15% of global agricultural lands are exposed to high salinity, resulting in low crop yields and reduced food supplies. Attempts to develop salinity tolerant crops via selection and breeding have not been sufficient. Bacterial mannitol-1-phophate Dehydrogenase (*mtlD*) has been known for its tolerance to salinity. Maize is the third cereal crop (after wheat and rice) that is severely affected by soil salinity. We have genetically engineered maize plants with the bacterial *mtlD* gene, confirmed the integration and expression of this transgene in upto forth progenies, and have confirmed that transgenic plants transcribing the *mtlD* gene have higher rate of photosynthesis and are more tolerant to different concentrations of NaCl (especially at 200 mM level) as compared with their wild-type non-transgenic control plants.

Keywords: *mtlD*; Corn; maize; transgenic plant; Zea mays; Salinity

Abbreviations

Act1: Actin Rice Promoter; BAP: Benzylaminopurine; CaMV35S: Cauliflower Mosaic Virus (CaMV) 35S Promoter; IBA: Indole-3-butyric Acid; JS101: plasmid containing bar and *mtlD* gene; MS: Murashige and Skoog; Nos: nopaline synthase terminator; PCR: Polymerase Chain Reaction; PinII: potato proteinase inhibitor terminator

Introduction

Salinity occurs due to low rain and high soil water evaporation when water brings up the soluble salts to the top soil at a level that it causes a damaging impact on crops and on economic welfare of the farmers [1]. It is believed [2] that about 15% of the global agricultural lands have high salinity, resulting in drastic effects on crop losses and on reduced global food supplies. Such effects occur when the level of soil salinity reaches above the crops salt tolerance threshold.

Methods of germplasm selection and breeding for development of salinity tolerant varieties have been used for many years [3]. However, screening of crops for salinity tolerance in the field is encountered by spatial heterogeneity of soil Physico-chemical properties and the seasonal rainfall fluctuations [4]. Therefore modern methods, including crop genetic engineering are needed to supplement the crop screening and breeding for salinity tolerance. Maize is considered to be the third major cereal crop (after wheat and rice) that is severely affected by soil salinity [4], and therefore it should be a priority to develop salinity tolerant maize via genetic engineering.

Mannitol-1-phophate dehydrogenase or *mtlD* gene is encoded by an enzyme that can improve morphological and physiological characteristics that are associated with salinity tolerance in crops. For example, Abebe et al. [5] demonstrated that the expression of the *E. Coli mtlD* gene in third generation transgenic (T2) wheat (*Triticum aestivum L. cv* Bobwhite) resulted in an improved tolerance to salinity, enhanced fresh weight, dry weight and plant height as compared with the wild-type non-transgenic wheat plants. Another report, [6], indicates that salinity reduced the growth of non-transgenic tobacco plants by 40%, whereas it had no negative effect on growth of the *mtlD* transgenic tobacco plants.

Another group of researchers reported that *mtlD* transgenic poplar trees (*Populus tomentosa*) survived up to 40 days in a hydroponic culture containing 75 mM NaCl solution, while their non-transgenic plant counterparts only survived 25 mM NaCl [7]. In this experiment, the stomatal conductance and photosynthesis of transgenic poplar were also higher than those of the wild-type control plants under the same salinity condition [7].

Prabhavathi et al. [8] reported that the expression of *mtlD* transgene in the first generation transgenic (T0) eggplant (*Solanum melongena L.*) seedlings grown *in vitro* under 200 mM NaCl resulted in good growth, whereas the wild-type control seeds did not germinate to produce seedlings under the same NaCl condition. Also, canola (*Brassica napus L.*) transgenic seeds, expressing the *mtlD* gene, germinated and their seedlings survived up to 24 days under *in vitro* conditions on Murashige and Skoog medium [9] containing 350 mM of NaCl, whereas their wild-type control seeds failed to germinate under the same NaCl concentration [10].

The expression of the bacterial *mtlD* gene in transgenic potato plants resulted in an increased salt tolerance under both *in vitro* and hydroponic stress conditions. The effect of 100 mM NaCl on potato shoot fresh weight under hydroponic condition was reduced by only 17.3% as compared to 76.5% reduced fresh weight of control non-transgenic plants [11]. Maheswari et al. [12] also reported 1.7 to 2.8-fold shoot and root growth enhancement in the sorghum plants that were expressing the *mtlD* gene. A summary of *mtlD* transgenic crops with enhanced salinity tolerance is shown on Table 1[13-17].

To our knowledge, there has been no report on genetic transformation of maize solely with the bacterial *mtlD* gene. In the

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Species	Improvements	References
Tobacco	Enhanced plant height and fresh weight under salinity stress conditions	[13]
Arabidopsis	Increased germination under salinity stress	[14]
Rice	Salt tolerance	[15]
Wheat	Enhanced cell lines and whole plant salinity tolerance	[5]
loblolly pine	Enhanced plant salinity tolerance	[16]
Poplar	Enhanced plant salinity tolerance	[7]
Cotton	Accumulation of amino acids and enhanced salt tolerance	[17]
Sorghum	Enhanced plant salinity tolerance	[12]
Potato	Enhanced plant salinity tolerance	[11]
Canola	Increased germination under salinity stress	[10]

Table 1: Enhanced salinity tolerance due to the expression of *mtlD* in different crop species.

research presented here, we have explored the possibility of developing salinity (NaCl) tolerant maize plants via transfer of the bacterial *mtlD* gene into the maize genome, testing of transgenic plants for the integration and expression of this heterologous gene, and testing of the T3 plants for photosynthesis rate, stomatal conductance and NaCl tolerance at greenhouse level.

Materials and Methods

Transgene construct

The JS101 plasmid (Figure 1) construct was used in this research. This construct has a cassette containing the bacterial *mtlD* gene regulated by rice actin promoter (*Act1*) and the potato protease inhibitor II terminator. This cassette is linked to a second cassette containing the bar herbicide resistance selectable marker gene regulated by the Cauliflower Mosaic Virus (*35S*) promoter and nopaline synthase (*Nos*) terminator.

Genetic transformation of maize immature embryo-derived cell lines

Highly proliferating, immature-embryo-derived maize calli were co-bombarded via the Biolistic™ gun with the pJS101construct containing the *mtlD* and the herbicide resistance selectable marker genes. The bombarded calli were kept on the same conditioning medium for 24 h, transferred to callus proliferation medium [18] for 5 d, and then placed on a selection medium containing 2.0 mg/L bialaphos, and maintained for 6–8 wk, with 2-wk subcultures into fresh medium. All cultures were maintained in the dark up to this point.

The bialaphos-resistant surviving callus clones were placed on

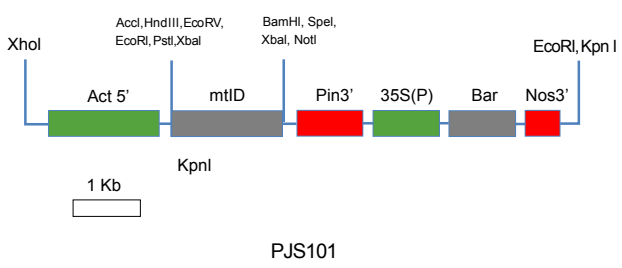

Figure 1: pJS101 containing the bacterial *mtlD* gene is driven by rice actin promoter (Act5') and potato protease inhibitor terminator (Pin3'); and the selectable marker bar gene is driven by 35S promoter and Nos3' end.

our maize regeneration medium [18] and exposed to 60 µmol quanta/m2/s light for 4–6 wk. Plantlets were transferred to a rooting medium containing the MS salts and vitamins and 1 mg/L Indole butyric acid (IBA). *In vitro* cultured bialaphos resistant callus lines were selected and regenerated as per our previous report [18]. Eight to ten centimeters long plantlets were transferred to small pots containing soil mix, and pots were covered with plastic bags and kept under light to mimic their former *in vitro* culture conditions. Small holes were made daily in each plastic bag to acclimate the plants to the greenhouse conditions before they were transplanted into 7.6 L pots, and transferred and grown in a long-day (16 h/d light) greenhouse until maturity.

Leaves of fertile transgenic plants were tested for the integration and expression of the *mtlD*, and mature transgenic plants were self-pollinated towards homozygosity. Seeds were harvested 35-45 days after pollination, when they were dry.

Conformation of the bacterial *mtlD* integration and expression in plants

Polymerase chain reaction (PCR) analyses were performed on the leaves of different putatively transgenic plant progenies to confirm the presence of the *mtlD* transgene in plants. The sequences of primers used for each PCR analysis to confirm the *mtlD* transgene integration included the 5' ATC GGT CGT GGC TTT ATC GG 3' (forward primer) and the 5' TCG ACA AAG CCA ACG TGT TC 3' (reverse primer). The PCR program was set at 94^{0}C for 3 min for one cycle; the following 35 cycles of 30 s at 94^{0}C, 30 s at 55.5^{0}C, 45 s at 720C; one cycle at 72^{0}C for 10 min; and the final cycle at 4^{0}C. The amplified 431bp segment of the *mtlD* gene was also used as DNA probes for Northern blot hybridizations. The PCR products were analyzed by electrophoresis in 0.8% agarose gels containing ethidium bromide, and visualized under ultraviolet light.

Northern blotting was performed to confirm the transcription of the *mtlD* transgene in transgenic plants. Total RNA was isolated from the PCR-positive and from the wild-type control untransformed plants using Trizol reagent following the manufacturer instructions (Invitrogen, CA). RNA gel blot analysis was carried out following modifications of our previous procedure [18]. The Northern blots were exposed to X-ray film and developed in an X-OMAT Processor (Hyblot CL, Denville, Scientific INC, E3018).

Net photosynthesis and stomatal conductance test

The net photosynthetic rate (µmol CO_2 m^{-2} leaf area s^{-1}) and stomatal conductance (mmol H_20 m^{-2} leaf area s^{-1}) of the plants 2nd uppermost leaves were determined using the portable photosynthesis LI-6400XT device as recommended by the manufacturer (LICOR, Lincoln NB).

Salinity tolerance test

A total of 40 seeds of one of the T3 generation lines and a non-transgenic line were sown in small square plastic pots containing BACCTO High Porosity Professional Planting Mix (Michigan Peat Company, Houston, TX). The seedlings were watered daily with normal tap water for two weeks before being salt treated. Salt treatments were performed for 10 days on the seedlings that were at their four-leaf stage of growth. Plant height and the distance from ground level to the tip of the longest leaf were measured in cm for the absolute growth rate (AGR). The formula: AGR = (h2 -h1)/ (t2 -t1) was used, where h2 and h1 represented the final and the initial height of plant; and t2 and t1 represented the final and the initial days [19]. This experiment was replicated in two locations of the same greenhouse for accuracy.

Seedlings were daily treated with equal volume of four different concentrations (0, 100, 200 and 300 mM) of NaCl, while gradually increasing 50 mM per day NaCl to reach their final concentrations within the ultimate 10 days. The commercial 20-20-20 fertilizer (Peter, Salem, OR) was weekly supplemented into the salinity solution for nutritional needs. After 10 days of salt water applications, observations were made on shoot fresh weight, and shoot and root dry weight. Plants were then watered daily for one week in order to allow them to recover from salinity stress injuries.

Results and Discussions

Integration of *mtlD* transgene was confirmed by PCR, and confirmation of the *mtlD* transcription was confirmed via Northern blotting.

PCR analyses of different transgenic progenies confirmed the integration of the bacterial mtlD transgene in maize plants (Figures 2A-2C). The PCR amplification products showed the expected band size of 431 bp in all transformants and in the positive control (JS101 plasmid) as expected. No band was observed in the wild-type control plants.

Northern blot hybridization confirmed the transcription of the *mtlD* transgene in plant progenies (Figure 2D and 2E). The specific PCR amplification product of *mtlD* sequence was used as a probe for Northern blot hybridization.

Salinity tolerance test of fourth generation transgenic plants

The fourth progeny of transgenic (T3) plants were tested for salinity tolerance. Under salinity stress conditions, there was a significant reduction in plant height growth rate (cm growth per day) as salinity levels were increased in both T3 and in the wild-type control plants. However, the wild-type control plants were more retarded due to salinity stress than transgenic plants under the same salinity treatments. The average plant growth rate of the wild-type control plant under 200 mM and under 300 mM NaCl were respectively 1.5 cm per day and 1.1 cm per day, whereas the corresponding data for transgenic plants under the same NaCl concentrations were 1.6 and 1.4 cm per day respectively (Table 2).

Note: in Turkey test, the mean values with the same letter (such as, a and a /b and b, etc) are not significantly different from each other, but mean values with different letters (such as, a and b / b and c) are significantly different (P<0.05).WT: Wild-type control plant.

Figure 3 represents the shoot fresh and dry weight and the root dry weight 10 days after being treated with different concentration of NaCl.

Table 3 confirms that the NaCl treatment had affected the wild-type control seedling growth, resulting in reduced shoot fresh weight, and shoots and root dry weights. However, the *mtlD* transgenic plants were less affected by the NaCl treatments as compared to the wild-type control plants. At 100 mM NaCl treatment, the shoot fresh weight of transgenic plants was reduced by 27%, shoot dry weight by 17%, and root dry weight by 6% as compared to the no salt treated control plants. However, the reduction of shoot fresh weight, shoot and root dry weights in wild-type control plants were 45%, 40% and 19% respectively.

When plants were exposed to 300 mM NaCl, the effect of salinity stress was more severe on wild-type control plants, reducing up to 74% of shoot fresh weight, 62% of shoot dry weight, and 44% of root dry weight. Table 3 also shows that T3 plants exhibited greater salt tolerance at the same salt stress condition than the wild-type plants, as they reduced shoot fresh weight, shoot dry weight and root dry weight by 52%, 40%, and 24% respectively. The effect of salinity stress also reduced the absolute plant height rate of both transgenic and wild-type plants. The plant height rates of transgenic and wild-type plants were not significantly different under no-salt stress condition. Overall, the

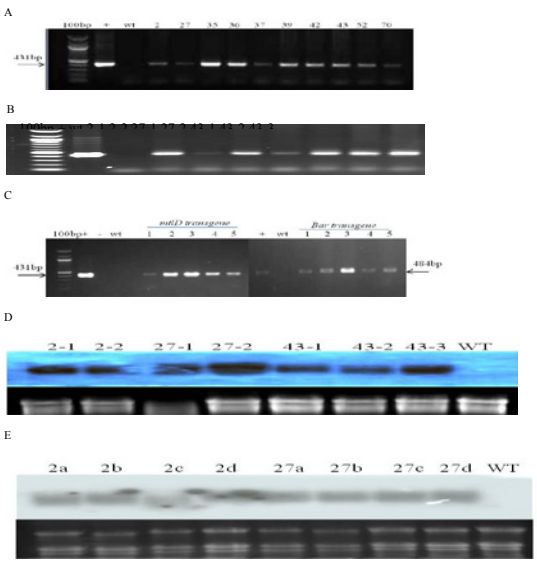

Figure 2: Molecular analyses of *mtlD* transgenic plants. A. PCR of T0 plants confirming the expected 431bp band size of the *mtlD* integration in plants. B. PCR of T1 plants also confirming the expected 431bp band size of the *mtlD* integration in plants. C. PCR of T2 progeny confirming the stability of both *mtlD* and the *bar* transgenes in plants. WT: Wild-type control; +: Plasmid DNA; -: water as negative control. D. Northern blot showing the transcription of the *mtlD* transgene in T1 plant progeny. E. Northern blot showing the transcription of the *mtlD* in T2 plant progeny.

Genotype	NaCl treatment	Mean value
mtlD	0mM	2.6a
WT	0mM	2.6a
mtlD	100mM	2.0b
WT	100mM	2.0bc
mtlD	200mM	1.6dc
WT	200mM	1.5d
mtlD	300mM	1.4d
WT	300mM	1.1d

Table 2: The effect of different salinity treatments on the absolute plant growth rate in T3 *mtlD* plants.

	Percentage of fresh and dry biomass reduction (%)			
Treatment (NaCl)	Genotypes	Shoot fresh wt	Shoot dry wt	Root dry wt
100 mM	WT	45.7	40.4b	19.0bc
	mtlD	27.5e	17.4d	5.8d
200 mM	WT	67.4b	61.1a	36.8a
	mtlD	46.8	33.4c	12.9cd
300 mM	WT	74.7a	62.3a	43.7a
	mtlD	51.7c	39.8b	23.7b

Means within columns followed by the same letter are not significantly different (P<0.05)

Table 3: Effect of *mtlD* expression on growth reduction of the T3 progeny plants versus the wild-type control plants treated with different NaCl concentrations for 10 days followed by seven days of water recovery.

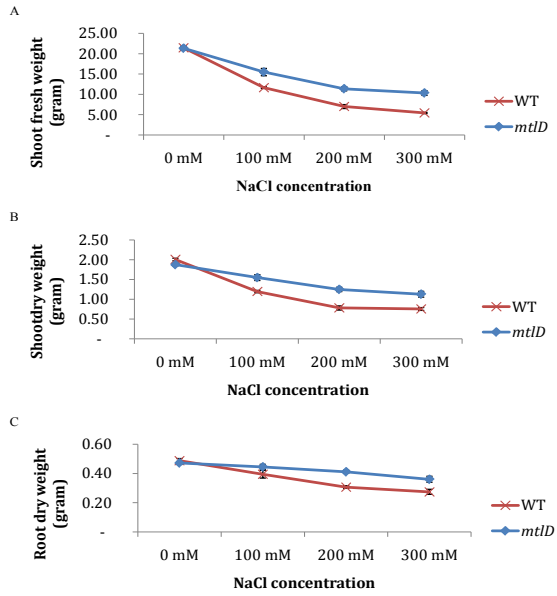

Figure 3: The effect of different NaCl concentrations on shoot's fresh and dry weight and root's dry weight. Data are expressed as the means ± SEs (n=3). A. Shoot fresh weight of transgenic and non-transgenic control plants. B. Shoot dry weight of transgenic and non-transgenic control plants. C. Root dry weight of transgenic and non-transgenic control plants.

increase in salt concentration had more negative effects on the wild-type control plants as compared to the T3 plants.

Figure 4 represents samples of the T3 *mtlD* versus the wild-type control plants after 10 days of 200 mM NaCl treatment followed by seven days of water treatment.

The work presented here agrees with the work of other scientists indicating that the expression of *mtlD* gene conferred salinity tolerance, and enhanced fresh and dry biomass of transgenic plants in sorghum [12] and potato [11].

In our studies, we did not find any abnormal phenotypes such as dwarfed or stunted growth of transgenic plants that expressed the bacterial *mtlD* transgene as compared to their wild-type control plants under no-salt condition. The same result was reported for the *mtlD* transgenic eggplant [8].

In the research presented here, salt stress experiments demonstrated significant differences in shoot fresh and dry weight, and root dry weight of transgenic as well as the wild-type control plants under high salinity concentrations. Also the *mtlD* transgene plants showed greater plant height growth rate and less shoot and root reduced growth under salt stress than the wild-type control plants. Similar results were reported in *mtlD* transgenic wheat [5], eggplant [8] and tobacco [19].

The effects of salinity stress on photosynthesis and stomatal conductance of T3 *mtlD* and wild-type control plants are shown in Table 4 and Figure 5. Table 4 shows that the photosynthesis rate and stomatal conductance declined with increased salinity concentrations in the wild-type non-transgenic plants, and were significantly lower as compared to transgenic plants at 100 mM NaCl. However, the photosynthesis rate and stomatal conductance were not significantly different in transgenic plants that were treated with 100 mM and 200 mM of NaCl followed by seven days of water treatment recovery.

Figure 5 shows that the effect of salinity on photosynthesis in the wild-type control plants was more severe than on those of transgenic plants that were treated with 100 mM of NaCl for 10 days, followed by seven days of water treatment recovery. All wild-type control plants were dead with shrunken and dried leaves after 7 days of water treatment, not being able to recover from 10 days of 200 mM of NaCl treatment. The data presented in table 4 confirm that the *mtlD* transgenic plants exhibited higher photosynthesis and stomatal conductance than the wild-type control plants that were treated with 100 mM NaCl for 10 days, agreeing with the report from Hu et al. [7] indicating that there

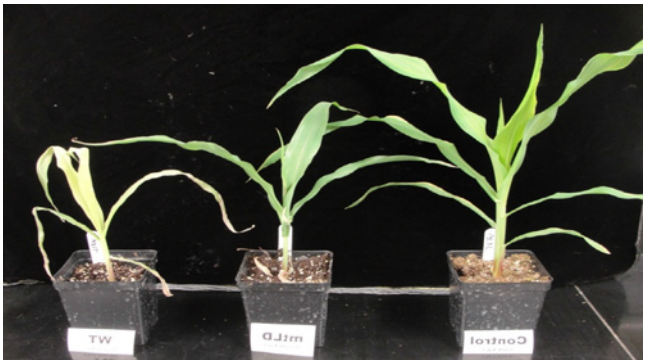

Figure 4: A control plant watered regularly (left), a T3 *mtlD* plant (middle) and a wild-type control plant (right) exposed to 200 mM NaCl treatment for 10 days followed by seven days of watering.

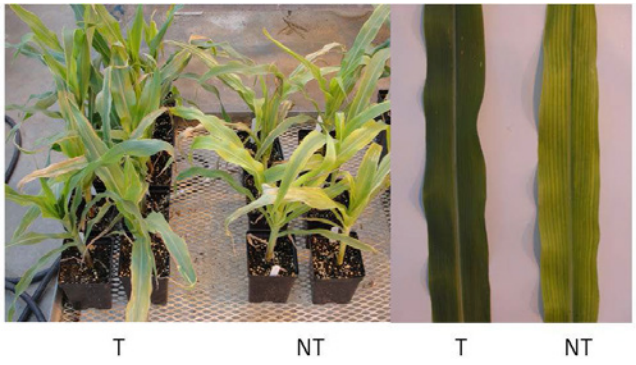

Figure 5: Effect of NaCl treatment on photosynthesis of T3 versus that of the wild-type non-transgenic control plants after 10 days of 100 mM NaCl treatment followed by seven days of water treatment recovery.
T: fourth generation transgenic plants, NT: non-transgenic control plants.

Genotype/Treatment (NaCl)	Photosynthesis (μmol CO^2 $m^{-2}s^{-1}$)	Stomatal conductance (mol H^20 $m^{-2}s^{-1}$)
mtlD transgenic plants		
0 mM	23.60 ± 1.18a	0.147 ± 0.014a
100 mM	19.12 ± 0.6b	0.124 ± 0.011a
200 mM	18.06 ± 0.37b	0.113 ± 0.004ab
Wild-type control plants		
0 mM	22.63 ± 1.03a	0.148 ± 0.009a
100 mM	14.90 ± 0.55c	0.086 ± 0.005b
200 mM	-	-

Means within columns followed by the same letter are not significantly different (P<0.05)

Table 4: Effect of NaCl treatment on photosynthesis and stomatal conductance in T3 plants. Values are means ± SEs (n=3).

were higher stomatal conduction and photosynthetic rates in poplar transgenic plants as compared with their wild-type control plants treated with 50 mM of NaCl for 21 days.

The authors hope that the research presented here will be one step closer to developing of a maize genotype that can tolerate the salinity in parts of the world that farmers suffer from losses of this abiotic stress factor.

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