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Over-expression of bacterial *mtlD* gene confers enhanced tolerance to salt-stress and water-deficit stress in transgenic peanut (*Arachis hypogaea*) through accumulation of mannitol

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Abstract

Previous work on a number of transgenics having *mtlD* has established the role of mannitol accumulation in the alleviation of abiotic stresses like salinity and drought. In the present study we have characterized the peanut (cv. GG 20) plants transformed with *mtlD* (from *Escherichia coli*) for its tolerance to abiotic stresses. Salinity and water-deficit stress tolerance were evaluated using different physio-biochemical and growth parameters in transgenic and wild-type plants both at seedling and full-growth stage. Here we demonstrate that biosynthesis of mannitol in transgenic peanut lines due to the over-expression of *mtlD* gene improves its tolerance for salinity and water-deficit stress over WT. This was revealed by better growth and physio-biochemical parameters like mannitol content, proline levels, total chlorophyll content, osmotic potential, electrolytic leakage and relative water content in transgenics over WT. It is concluded that the better performance of mannitol-synthesizing transgenic plants was due to the stress-shielding role of mannitol. However we are not ruling out the possibility of induction of a series of signal- transductions in transgenic plants in response to the *mtlD* expression, which may activate other protective reactions against salinity and drought stresses.

Keywords: Groundnut; mannitol 1-phosphate dehydrogenase; abiotic-stress tolerance; drought-stress; salinity-stress.
Abbreviations: ANOVA_Analysis of variance; DMSO_dimethylsulfoxide; EC_Electrical conductivity; HPLC_High performance liquid chromatography; LSD_Least significance difference; PEG_Polyethylene glycol; RWC_Relative water content; T_transgenic; WT_wild type.

Introduction

Globally peanut (*Arachis hypogaea* L.) is economically one of the important oil and food crop and it ranks third and fourth as a source of protein and edible-oil respectively. In about 120 countries under different agro-climatic zones between latitudes 40 °S and 40 °N peanut is grown on around 21-24 M ha of land annually. Though a native of South America, peanut is presently cultivated mainly in Asian (11.82 M ha), African (7.6 M ha) and American (1.1 M ha) countries in semi- arid regions and also in India, China, Nigeria, USA, Myanmar, Senegal, Sudan, Indonesia, Argentina and Vietnam (USDA, 2013). Peanut is generally grown across a wide range of environments under rain-fed conditions especially where frequent drought is a major limiting factor to the productivity with very low-inputs. Drought is the foremost constraint to peanut production in the semi-arid areas, which accounts for about 70% of the peanut growing area. Globally it has been estimated that approximately 830 M ha area is salt-affected (Martinez-Beltran and Manzur, 2005) whereas in India it is around about 7.61 M ha (Singh et al., 2007). Therefore it is pertinent to develop salinity and drought stress-tolerant peanut genotypes to exploit and utilize vast drought-prone and salinity affected areas of the world (Abebe et al., 2003; Akcay et al., 2010).

During osmotic stress there is induced accumulation of different osmolytes, which not only act as osmoprotectants but also function for osmotic adjustment. Among many approaches possible in the development of transgenic for abiotic stress tolerance; overexpression of the genes involved in the biosynthesis of osmolytes, such as mannitol (Tarczynski et al., 1992), trehalose (Garg et al., 2002) etc. has showed increased abiotic-stress tolerance. Under abiotic stresses many plants accumulate mannitol (Khare et al., 2010) which is regulated by inhibition of mannitol-competing pathways and also due to the reduced consumption and catabolism of mannitol (Stoop et al., 1996). Role of mannitol in the alleviation of salinity and osmotic-induced stresses in many plants was demonstrated in different plants (Prabhavathi et al., 2002; Chan et al., 2011; Khare et al., 2010) but it is not naturally synthesized in peanut.

The *mtlD* is a bacterial gene which encodes an enzyme (mannitol 1-phosphate dehydrogenase; EC 1.1.1.17) that converts fructose 2 with 6-phosphate to mannitol 1-phosphate. In transgenic plants, this gene converts mannitol 1-phosphate to mannitol via nonspecific phosphatases. Using the *mtlD* gene from *Escherichia coli*, we have developed transgenic peanut which may prove an alternative means to expedite the development of water-deficit stress and salinity-stress tolerant peanut. Here we report that under experimental conditions enhanced mannitol-accumulation in transgenic peanut plants overexpressing *mtlD* improves the tolerance for salinity and water-deficit stresses.

Table 1. Effect of imposing different levels of salt-stress on mannitol content in the peanut seedlings after 12 days of stress imposition

.Plant No.	Mannitol Content (µg g^{-1} FW)				
	NaCl Concentration (mM)				
	0	50	100	150	200
MTD.1	1.26±0.130 b [z]	1.28±0.121 c	2.89±0.074 a	3.41±0.126 c	7.63±0.202 b
MTD.2	2.30±0.155 a	2.17±0.143 ab	2.59±0.189 a	2.91±0.081 c	5.75±0.213 c
MTD.3	1.72±0.213 ab	1.70±0.077 bc	2.81±0.120 a	7.82±0.161 a	12.29±0.652 a
MTD.4	1.71±0.113 ab	2.37±0.180 a	3.16±0.208 a	5.54±0.216 b	7.52±0.239 b
WT	ND	ND	ND	ND	ND
LSD	0.63	0.54	0.61	0.61	1.52

The data are mean of three replicates±SE; ND: Not detected; [z]Means followed by the same lower case letters within a column are not significantly different (P≤0.05).

Results and Discussion

Accumulation of mannitol in transgenic plants having *mtlD* gene is expected to impart abiotic-stress tolerance (Punji et al., 2007; Khare et al., 2010). Therefore peanut *mtlD* transgenic lines and WT (cv. GG 20) were evaluated for various physio-biochemical and growth parameters against salinity and water-deficit stress. Results of each parameter are discussed below:

Accumulation of mannitol

Under salt-stress (in seedlings)

Mannitol which is naturally synthesized in many plant species but absent in peanut, is a six-carbon, non-cyclic sugar-alcohol having its role in the storage of energy, regulation of coenzymes, free- radical scavenging and osmoregulation (Stoop et al., 1996). Mannitol levels were estimated in WT and transgenic lines exposed to different levels of salt-stress (i.e. 0, 50, 100, 150, and 200 mM) through HPLC. A peak in the peanut transgenic plants was recorded at a retention time (4.2 min) identical to the mannitol standard. No peak was observed in the WT samples indicating the absence of mannitol (Table 1).

With increasing levels of salt-stress, an increasing trend in the mannitol concentration in transgenic lines was observed which is to the tune of 2.5 (MTD.2) to 7.1 (MTD.3) times than its initial concentration (Table 1). As compared to the un-stressed plants, 3-10 folds mannitol accumulation in transgenic plants has already been reported earlier (Prabhavati et al., 2002). Thus increase in mannitol content, with increasing salinity stress in the transgenic indicates the salinity-stress tolerance capacity in transgenic peanut. These observations were same as observed by Punji et al. (2007) where at 200 mM NaCl, WT plants withered whereas the transgenic plants do withstand the stress condition in Indica rice.

Under water-deficit stress (in seedlings)

With increasing levels of drought stress, an increasing trend in the mannitol accumulation in different transgenic lines was observed. Mannitol levels at different extent of drought generated by assorted PEG concentrations (i.e. 0, 5, 10, 15 and 20% PEG) in transgenic lines ranged from 0.30 to 5.577 µg g^{-1} FW of tissue whereas no mannitol was detected for WT (Table 2). This indicated that the transgene has expressed and may result in the enhanced tolerance capacity of the transgenics for water-deficit stress.

Under water-deficit stress (at full-growth stage)

In all the transgenic lines, *mtlD* gene expression was witnessed under PII containment facility. Approximately 1.3-1.8 folds increase in mannitol content was witnessed in stressed peanut transgenics compared to the un-stressed transgenics (Table 3) which corresponded with the previous report of Punji et al. (2007). It means, accumulation of higher levels of mannitol in the transgenic peanut lines indicates its tolerance capacity for water-deficit stress.

Overall increase in accumulation of mannitol in the transgenic peanut lines indicates its tolerance capacity for both salinity and drought stress. This also confirms the expression of the transgene *in-planta*. Even between different transgenic lines, significantly different levels of mannitol accumulation, was detected under both abiotic stress (both salt and drought) and non-stress conditions. This variation in the levels of mannitol in various transgenic lines might be caused because of the positional difference of integration of the transgene (Prabhavathi et al., 2002; Prabhavathi and Rajam, 2007).

In the past reports, various transgenic plant species with varying levels of mannitol accumulation in its tissues have been shown to be tolerant to different type of abiotic stresses including salinity and drought (Huizhong et al., 2000; Abebe et al., 2003; Khare et al., 2010). This advocates that the level of mannitol synthesized and accumulated in the transgenic tissues was ample enough for imparting osmoprotection via compatible solute mechanism (Stoop et al., 1996).

Proline estimation Under salt-stress (in seedlings)

Under salinity- and drought- stress, enhanced accumulation of proline in *mtlD* derived transgenic plants is reported in many studies which may play a role in neutralizing the negative effect of these stresses (Ramanjulu and Sudhakar, 2000; Kumar et al., 2010). An index for determining salinity-tolerance was proposed by Ramanjulu and Sudhakar (2000), after finding positive correlation, between amount of free proline accumulation, and salinity-tolerance in mulberry cultivars.

On the similar note, a steady increase in proline-accumulation was observed both in transgenic and WT lines, but a drastic change was noticed at 200 mM NaCl concentration, indicating a very high level of proline accumulation. Proline content in transgenics at different levels of salt-stress varied from 33.3 (MTD.3) to 12.29 (MTD.1) µg g-1 FW of tissue (Fig. S1A). More pronounced increase in proline content was observed for all the transgenic lines compared to WT. Similar trend have been reported under salt-stress in other plants too (Koca et al., 2007; Ahmad et al., 2007).

Table 2. Effect of imposing different levels of PEG induced water-deficit stress on mannitol content in the peanut seedlings after 12 days of stress imposition.

Plant No.	Mannitol Content (µg g^{-1} FW)				
	PEG level (%)				
	0	5	10	15	20
MTD.1	0.857±0.029 c [z]	1.820±0.026 c	2.553±0.154 b	3.587±0.098 b	4.483±0.098 b
MTD.2	0.363±0.026 d	2.107±0.070 c	2.803±0.075 b	3.290±0.064 c	4.503±0.109 b
MTD.3	0.300±0.015 d	2.273±0.066 b	2.780±0.078 b	3.077±0.083 c	5.393±0.212 a
MTD.4	1.407±0.127 b	2.150±0.096 ab	3.387±0.150 a	3.633±0.065 b	4.597±0.182 b
MTD.7	1.753±0.035 a	2.407±0.170 ab	3.460±0.134 a	4.377±0.122 a	5.577±0.162 a
WT	ND	ND	ND	ND	ND
LSD	0.174	0.276	0.347	0.251	0.446

The data are mean of three replicates±SE; ND: Not detected; [z]Means followed by the same lower case letters within a column are not significantly different (P≤0.05).

Under water-deficit stress (in seedlings)

Till 15% of PEG, increase in the proline-content was found gradual both in transgenic and WT but, a drastic increase was observed at 20% PEG which was more distinct in transgenics over WT. Proline content in transgenics at different levels of PEG varied from 18.6 (MTD.2 and MTD.7) to 337.6 (MTD.1) µg g^{-1} FW of tissue. Increase in proline content was 16.5 times in transgenic (MTD.1) compared to WT which has recorded only 6.4 folds increase at 20% PEG (Fig. S1B). Similar observations were also recorded by Nanjo et al. (1999) and Rai et al. (2012) for Arabidopsis and tomato respectively.

Under water-deficit stress (at full-growth stage)

Proportionately faster increase of proline content over any other amino acid in peanut under water-deficit stress was observed by Mekhari et al. (1977). Further they suggested its potential to be used as an evaluating parameter for selecting drought tolerant genotype. In our study also, substantial increase was observed for proline accumulation in both transgenic and WT peanut lines under drought-stress over well-watered conditions (Fig. S1C). Significantly higher proline accumulation was observed for the transgenic line MTD.7 under water-deficit stress over other transgenic or WT counterparts. Compared to the non-stress situation, proline accumulation was 2.1 folds in the WTs *vis-á-vis* 8.6 folds in transgenics under water-deficit stress.

The results of both *in vitro* and greenhouse conditions are in agreement with Bates et al. (1973) and Mekhari et al. (1977). Since proline is also known to be associated with several functions during stress *viz*. osmotic adjustment (Voetberg and Sharp, 1991), osmo-protection (Kishor et al., 2005), free radical scavenger and antioxidant activity (Sharma and Dietz, 2006). Therefore it could be concluded that, proline accumulation is an indicator for stress, and *mtlD* peanut transgenics responded with higher proline accumulation, which probably resulted in tolerance of transgenic peanut lines for different abiotic stresses.

Total-chlorophyll content Under salt-stress (in seedlings)

Under abiotic stress conditions, like salinity and drought the chlorophyll content of the leaves is usually disturbed resulting in altered photosynthetic activity (Rai et al., 2013). In our study, no significant difference for total-chlorophyll in peanut *mtlD* transgenic and WT was recorded before stress-imposition. But at different levels of salinity-stress, mean chlorophyll content was found significantly higher in all the transgenic lines over WT (Fig. S2A). Moreover, a gradual

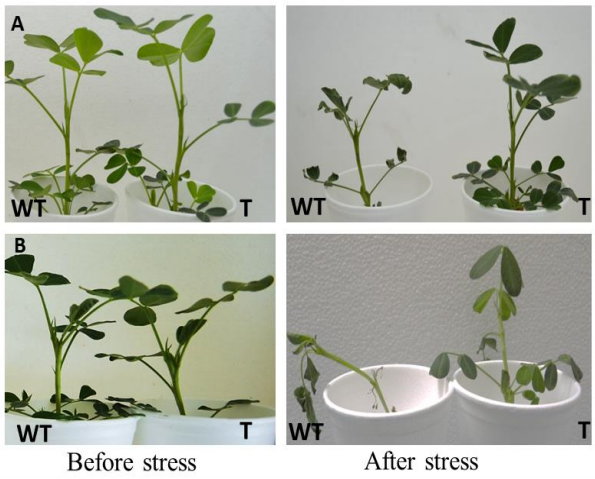

Fig 1. Response of 22 days old WT and transgenic (T) seedlings after 12 days of (A) NaCl induced (150 mM NaCl) salt-stress in hydroponics system and (B) PEG –induced (20% PEG-6000) water-deficit stress.

decrease in the total chlorophyll of both transgenic and WT were recorded with increasing levels of salinity-stress. Husaini and Abdin (2008) also reported the reduction in chlorophyll content of the transgenic strawberry plantlets for osmotin gene when treated with NaCl which could be because of the interference of Na^+ and Cl^- ions with chlorophyll biosynthetic pathway associated enzymes. The decrease in chlorophyll content could also be due to the formation of unstable complex because of disturbance in the integration of chlorophyll molecules (Husaini and Abdin, 2008). This may perhaps be one of the reasons for decrease in the total chlorophyll content with increasing levels of salinity- stress.

Under water-deficit stress (in seedlings)

In peanut seedlings, a gradual reduction in the total-chlorophyll content was recorded with increasing levels of water-deficit stress. This reduction was significantly higher in WT over transgenic lines. The rate of decrease in total-chlorophyll in transgenic was comparatively less (i.e. 1.25 times in MTD.2 to 1.69 times in MTD.4) compared to the WT which recorded 2.36 times reduction over non-stress conditions. The mean chlorophyll content at 20% PEG was found 1.93 times higher in MTD.2 transgenic (0.86 µg g^{-1} FW) compared to the WT (0.45 µg g^{-1} FW) (Fig. S2B).

Table 3. Effect of imposing PEG induced water-deficit stress on mannitol content in the full-grown peanut plants after 24 days of stress imposition.

Plant No.	Mannitol Content (µg g^{-1} FW)	
	Water Deficit Stress	Well Watered
MTD.1	3.02±0.311 c[z]	2.25±0.296 ab
MTD.2	3.30±0.421 c	1.81±0.219 b
MTD.3	4.74±0.413 a	2.98±0.378 a
MTD.4	3.72±0.104 bc	2.83±0.159 a
MTD.7	3.58±0.172 bc	2.71±0.293 a
MTD.9	4.23±0.154 ab	3.03±0.272 a
WT	ND	ND
LSD (0.05)	0.816	0.779

The data are mean of three replicates±SE; ND: Not detected; [z]Means followed by the same lower case letters within a column are not significantly different (P≤0.05).

Prabhavathi et al. (2002) also reported 1.5-2.0 fold increase in the chlorophyll content for few *mtlD* transgenic brinjal lines under water- deficit stress.

Under water-deficit stress (at full-growth stage)

Significantly higher total- chlorophyll was recorded in transgenic lines compared to WT under water-deficit stress. However no significant difference in the total- chlorophyll values was observed under well-watered condition between transgenic and WT except for MTD.9 line which showed significantly higher chlorophyll over other lines. For both transgenic and WT, reduction in chlorophyll content was observed under water-deficit stress compared to well-watered conditions (Fig. S2C). Slight reduction in the chlorophyll-fluorescence under salinity- and drought- stress conditions was also recorded by Fedina et al. (2006) in barley.

In the present study the transgenic peanut lines overexpressing mannitol were able to maintain higher chlorophyll content under both salinity and drought stresses probably due to its ability in enhancing their proline content, which in turn significantly lowers the level of reactive oxygen species and alleviates salt-stress induced enhancement in ribulose oxygenase activity (Sivkumar et al., 2000; Husaini and Abdin, 2008). As low chlorophyll-content has been reported to directly limit the photosynthetic potential and primary productivity in plants therefore maintenance of elevated chlorophyll-content in transgenic lines over WT under stress conditions is of special significance (Curran et al., 1990; Fiella et al., 1995).

Cell membrane leakage Under salt-stress (in seedlings)

Cell membrane leakage has its roles in protecting plants against different stresses and rate of injury to cell membrane is usually used to measure the tolerance of plant for various stresses (Akcay et al., 2010). The mean injury was found significantly more in the WT over transgenic at different salinity levels including under no salt treatment. Genome-wide expression analyses in *Arabidopsis* has shown substantial alteration (up and down) in the expression levels of various genes when celery M6PR transgene was introduced even when no stress was imposed (Chan et al., 2011). Under high salt concentration (200 mM NaCl) the electrolytic leakage was significantly lower in the transgenic line MTD.2 compared to other transgenic and WT. Maximum cell membrane leakage was exhibited by WT at various salt concentrations which are suggestive of better membrane stabilization property in the *mtlD* transgenics.

The cell membrane leakage showed increasing trend with rise in NaCl stress (Fig. S3A). Blum et al. (1989) in barley also reported the role of cell membrane stability under salt-stress conditions as a major component of electrolyte leakage from the cell. Zhao and Harris (1992) in halophyte and non-halophyte plants and Gadallah (1999) in *Vicia faba* observed the role of cell membrane stability under salt-stress conditions as a major component of electrolyte leakage from the cell.

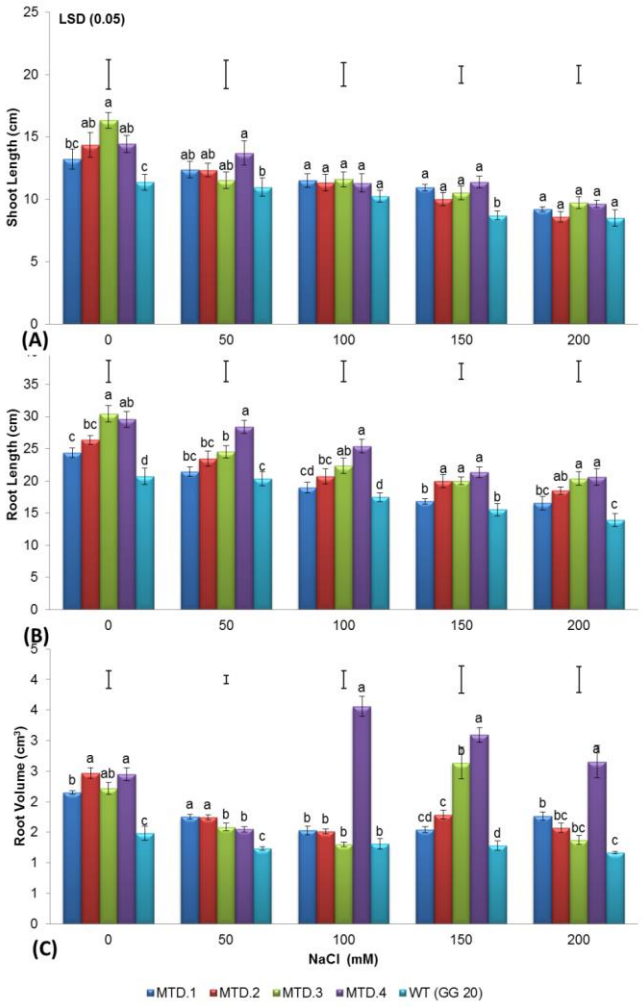

Fig 2. Effect of imposing different levels of salt-stress on seedlings of transgenics (MTDs) and WT on (A) Shoot-length, (B) Root-length and (C) Root-volume. Values are mean of three replicates and bars indicate ±SE; bars on the top represent the $LSD_{0.05}$.Bars having same lower case letters within a treatment are not significantly different (P≤0.05).

Under water-deficit stress (in seedlings)

Maximum cell membrane leakage was exhibited by WT over transgenic at different water- deficit stress levels including no-stress. At 20% PEG, the electrolytic leakage was significantly lower in transgenics over WT which might be due to the expression of *mtlD* gene in the transgenics. On the whole, cell membrane leakage showed increasing trend with the increase in water-deficit stress (Fig. S3B) which means, cell membrane stability under drought had a major role in conferring stress tolerance (Tyagi et al., 1999). Significantly lower electrolyte leakage was also observed in transgenic tomato lines over-expressing *BcZAT12* gene when exposed to drought stress which is indicative of restored membrane integrity in the transgenic plants (Rai et al., 2013). However no significant change in the ion leakage was observed with increasing concentration of PEG in peanut cultivar Gazipasa (Akcay et al., 2010).

Under water-deficit stress (at full-growth stage)

Taken as a whole, cell membrane leakage showed increasing trend with the imposition of drought compared to the well-watered conditions. However maximum cell membrane leakage was exhibited by WT over transgenics both under drought and well- watered conditions. Under drought significantly lower values for electrolytic leakage was recorded in transgenics over WT (Fig. S3C). This is suggestive of better membrane stabilization property in the *mtlD* peanut transgenics. Sugar-alcohols like mannitol is also known to function as scavengers of reactive oxygen species (ROS), thus averting peroxidation of lipids and consequent cell damage (Stoop et al., 1996). Significantly lower electrolytic leakage in the *mtlD* transgenics over WT even when no stress is imposed was observed for both salinity and water-deficit stress which could be due to the accumulation of mannitol in the transgenic peanut lines which is otherwise absent in the WT.

Relative water content (RWC)

Under salt-stress (in seedlings)

It is a parameter often used to assess the water retention capacity of plants (Rai et al., 2013). During non-stress conditions, no significant change in RWC was recorded in either WT or transgenic lines but under salt- stress, WT recorded a significant lowering in its RWC. Moreover with increasing levels of salinity, *mtlD* transgenics peanut lines retained higher RWC than WT. Among transgenics, highest RWC was recorded in the line MTD.1 whereas, WT exhibited lowest RWC at different levels of salinity stress (Fig. S4A). Similar results were obtained in transgenic tobacco where salinity stressed plants maintained higher RWC compared to the WT (Karakas et al., 1997).

Under water-deficit stress (in seedlings)

With increasing levels of water-deficit stress, transgenic *mtlD* peanut lines always exhibited higher RWC than WT, indicating greater tolerance capacity of transgenics for drought-stress tolerance. However under non-stress, no significant change in RWC was recorded for either WT or transgenic peanut lines. Akcay et al. (2010) also noted decrease in RWC with increase in the PEG concentration in peanut. Among the transgenics, significantly higher RWC was observed for the transgenic line MTD.4, whereas WT exhibited lowest RWC for different concentrations of PEG (Fig. S4B). This is in agreement with the reports of Karakas et al. (1997) in tobacco, and Rai et al. (2013) in tomato where transgenics lines performed better than the WT under drought-stress, with minimum reduction in RWC.

Under water-deficit stress (at full-growth stage)

Both under well-watered and water-deficit stress, *mtlD* transgenic peanut lines preserved significantly higher RWC over WT which could be because of accumulation of mannitol in its tissues. Under drought conditions, transgenic peanut lines for *mtlD* gene showed less reduction of RWC over WT which was indicative of greater tolerance of transgenics for drought stress (Fig. S4C). Similar results were also reported by previous workers for other plants *viz*. tobacco and wheat (Karakas et al., 1997; Abebe et al., 2003).

Osmotic potential

Under salt-stress (in seedlings)

NaCl typically forms majority of the salts and high salt levels exhibits a water- deficit or osmotic- stress due to the decreased osmotic potential in the soil (Zhu, 2007). Compared to the WT, significantly high osmotic potential was observed for *mtlD* peanut transgenic lines even when no-stress was imposed. Among transgenic lines, significantly higher osmotic potential was recorded for MTD.2 (up to 150 mM NaCl) whereas at 200 mM NaCl concentration, MTD.4 showed significantly higher osmotic potential over all other lines (Fig. S5A). Karakas et al. (1997) while studying the *mtlD* transgenic in tobacco also observed better osmotic adjustment in fully expanded leaves. Whereas Guo et al. (2013) in lentil recorded reduced growth under salt-stress mainly due to ion injury, rather than to the associated low osmotic potential.

Under water-deficit stress (in seedlings)

Significantly high osmotic potential was recorded for the transgenic lines over WT even when no stress was imposed. It could be due to the fact that abiotic stress may result in changes which consists of both protective (adaptive) responses and damage effects which can involve altered expression of distinctly different genes (Chan et al., 2011). Among different transgenic lines, MTD.4 and MTD.7 recorded significantly higher osmotic potential at different levels of water-deficit stress (Fig. S5B). These outcomes are in concurrence with that of Brini et al. (2007) in *Arabidopsis thaliana* where, wheat derived sodium/hydrogen ion (Na^+/H^+) antiporter *TNHX1* and H^+ pyrophosphatase *TVP1* enhanced the osmotic potential of transgenic plants.

Under water-deficit stress (at full-growth stage)

Significantly higher leaf osmotic potential was recorded in the transgenic lines, both under well-watered and water-deficit stress conditions over WT (Fig. S5C). This is suggestive of the fact that transformed peanut lines were able to uphold more water in its cells under drought compared to WT. In the expanding leaves of drought-stress tobacco transgenic plants for *mtlD* gene, Karakas et al. (1997) also observed better osmotic potential than WT plants.

It has been reported that sugar-alcohols can maintain an artificial sphere of hydration around the macro-molecules due to its water-like hydroxyl (-OH) group and thus imitate the

structure of water (Schobert, 1977). Therefore significantly higher osmotic potential of transgenic lines, over WT even when there is no stress (both salinity and drought) could be because of presence of mannitol in the transgenic lines which is otherwise absent in the WT plants.

Growth parameters

Under salt-stress (in seedlings)

After 12 days of salt-stress imposition, in the peanut seedlings, growth parameters like shoot-length, root-length and root-volume of both transgenic and WT plants were found significantly affected. Root-length and root-volume was found more in the transgenic lines over WT at different levels of salt-stress including no-stress conditions. With the increasing concentration of salt, a gradual decrease in the shoot-length was recorded (Fig 1A). Transgenic line MTD.4 showed better root-length, shoot-length and root-volume over any other transgenic line under various salinity stress levels (Fig. 2A-C). Overexpression of *mtlD* gene in other crop plants like rice (Huizhong et al., 2000), brinjal (Prabhavathi et al., 2002) and potato (Askari et al., 2012) has also resulted in transformed plants with increased salinity tolerance.

Under water-deficit stress (in seedlings)

With the increasing levels of drought-stress, different growth parameters showed overall decreasing trend for both transgenic and WT lines. However all the parameters were found significantly higher in the transgenic lines over WT. It was found that, at high PEG concentrations (15 and 20%) transgenic line MTD.4 performed significantly better over other transgenic lines (Fig. 1B; Fig. 3A-C). Similar observations were recorded by Prabhavathi et al. (2002) in transgenic seedlings of brinjal which grew well under 10% PEG stress when compared to the WT seedlings. Superior performance of *mtlD* transgenics *vis-à-vis* WT was also observed by Abebe et al. (2003) and Rahname et al. (2011) in wheat and potato respectively.

Under water-deficit stress (at full-growth stage)

The effect of water- deficit stress, on the shoot-length of the transgenic and WT plants under PII containment facility in plastic pots was recorded, both before and after imposing the drought stress (Fig. 4). Under well-watered condition, the plant growth was more in all the transgenic lines compared to WT. An apparent reduction in the shoot-length was observed under water-deficit stress, and difference was prominent between different transgenic lines as well. In water-deficit stress situation, increase in plant- height was significantly more in the transgenic lines over WT. However three transgenic lines viz. MTD.3, MTD.4 and MTD.7 performed significantly better over other three transgenic lines (Fig. 5). This clearly indicated the tolerance of transgenics for shoot-elongation to drought stress conditions.

Materials and Methods

Plant materials

The de-embryonated cotyledons from the dry seeds of the popular cultivar GG20 was subjected to ***Agrobacterium*** mediated genetic transformation. The putative transgenics

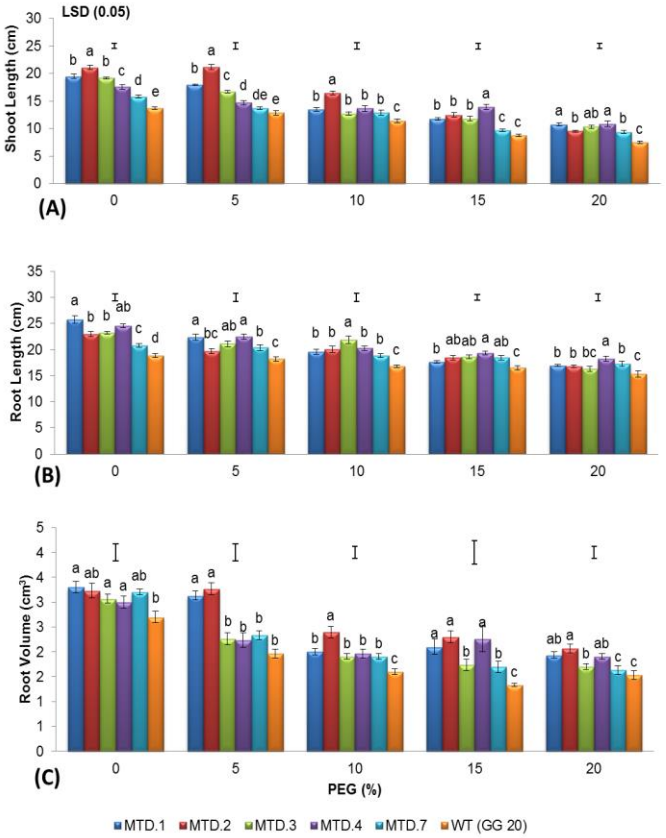

Fig 3. Effect of imposing different levels of water-deficit stress on 22 days old seedlings of transgenics (MTDs) and WT on (A) Shoot-length, (B) Root-length and (C) Root-volume. Values are mean of three replicates and bars indicate ±SE; bars on the top represent the $LSD_{0.05}$. Bars having same lower case letters within a treatment are not significantly different ($P \leq 0.05$).

were confirmed by *mtlD* gene specific PCR and Southern hybridisation (Bhauso, 2012). Homozygous transgenic lines (T_2 generation) which were confirmed for integration of *mtlD* transgene were used for the present investigation.

Seedling evaluation for salt and water-deficit stress

To test the response to salt-stress (NaCl-mediated) and water-deficit stress (Polyethylene Glycol or PEG-mediated), the seeds (T and WT) were surface sterilized and inoculated in the conical flask containing Hoagland's solution and kept at 28 °C in the culture-room for germination. After 05 days, seedlings were transferred to the disposable-cups containing Hoagland's solution. Ten days old plants were again transferred to another set of disposable-cups containing Hoagland's solution mixed with 0, 50, 100, 150 and 200 mM NaCl for salt -stress experiments and 0, 10, 15, 20, 25% PEG for water-deficit stress experiments. The data was recorded after 12 days of stress induction when seedlings were 22 days old.

These lines were evaluated for their tolerance for salinity in 4 lines (MTD.1, 2, 3 and 4) and drought stress in 5 lines (MTD.1, 2, 3, 4 and 7). Physiological and biochemical parameters like mannitol, proline, total chlorophyll, osmotic potential, electrolytic leakage and RWC were analyzed from the uppermost fully expanded leaves collected before stress

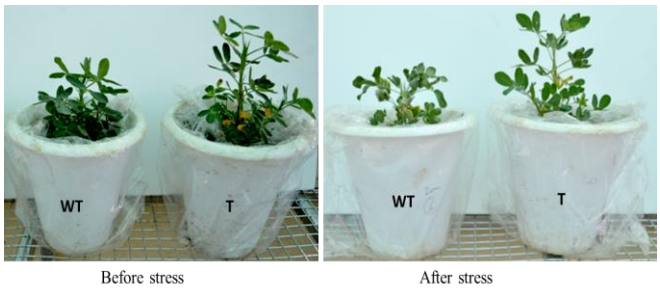

Fig 4. Response of on 45 days old WT and transgenic (T) plants after 24 days of water-deficit stress under containment facility in plastic-pots.

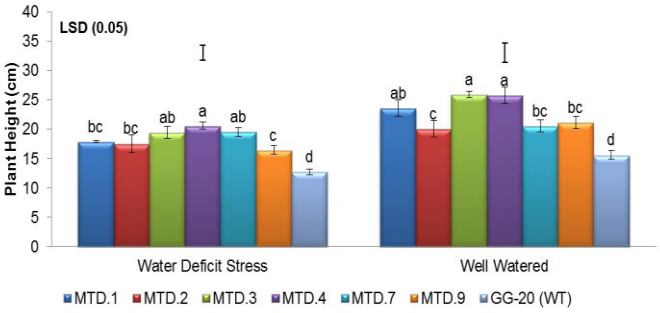

Fig 5. Effect of imposing water-deficit stress on 45 days old peanut transgenics (MTDs) and WT after 24 days of withholding the irrigation on shoot-length. Values are mean of three replicates and bars indicate ±SE; bars on the top represent the $LSD_{0.05}$. Bars having same lower case letters within a treatment are not significantly different ($P \leq 0.05$).

was released. For growth parameters data on shoot-length, root-length and root-volume was recorded.

Evaluation at full growth stage for water-deficit stress

The 45 days old *mtlD* transgenic (in T_2 generation) and WT lines were used for water-deficit stress tolerance studies after withholding the irrigation for 24 days. Six independent transgenic lines (MTD.1, 2, 3, 4, 7 and 9) along with WT were evaluated for all those physio-biochemical parameters which were used for seedling experiments. For growth parameters data on shoot-length was scored.

Estimation of mannitol content

Mannitol was estimated in the leaves of seedlings (WT and T_2 generation transgenic) and quantified as described by Tarczynski et al. (1992). Fresh leaves were used for sample preparation and mannitol content was quantified by binary gradient HPLC (Shimadzu LC 10 series). Ten µL of sample was injected per run (operated at 1 mL min^{-1}) with acetonitrile: water (80:20) as mobile phase using 5µ Luna, NH2 100 Å column at 40°C for the separation. RI detector was used for the detection. Mannitol (SRL; 10 mg L^{-1}) was used as the standard and peak area of each sample was quantified and the area of standard was used to determine the amount of mannitol in our samples.

Estimation of proline content

Proline content in the leaf sample was estimated as per Bates et al. (1973). Leaves (500 mg) from the seedling were homogenized using a mortar and pestle with 5 mL sulfosalicylic acid (3%) and centrifuged (5000 g; 10–15 min) and supernatant was collected. The supernatant volume was adjusted to 5 mL (with distilled water), then Glacial acetic acid (5 mL) and Ninhydrin (1 mL) was added and mixture was boiled (in water bath) for 1 h and cooled to room temperature. Then toluene (10 ml) was added and allowed to stand (2-3 min) for the colour development and absorbance was recorded spectrophotometrically at 520 nm. The proline content was calculated from proline standard (10 mg 100-1 mL in 3% sulfosalicylic acid).

Estimation of total chlorophyll

Total chlorophyll content in leaf-tissue was determined through dimethylsulfoxide (DMSO) method (Hiscox and Israelstam, 1979). Leaf tissue (100 mg) was placed in culture tubes (15 mL) containing DMSO (4 mL) and incubated in a water bath (65 °C for 12 h). Absorbance of the extract was read at 645 and 663 nm in the spectrophotometer and total-chlorophyll content was calculated. Total chlorophyll (mg g^{-1} fresh weight) = 7[(20.2 × OD645) + (8.02 × OD663)] × V/(1000 × W), where 'V' is the volume of extract and 'W' is the weight of tissue in g.

Estimation of osmotic potential

Leaf tissue (1 g) frozen in liquid N_2 and thawed in Eppendorf tubes (1.5 mL) with a pore at the bottom were placed in another tube and centrifuged (6000 rpm; 5 min) so as to collect the cell-sap. Then osmotic potential was measured in the collected sap using Vapor Pressure Osmometer (Lycor).

Estimation of electrolyte leakage

Membrane electrolyte leakage was analyzed as per Hoekstra et al. (2001). Fresh leaf disc (1 cm diameter) washed with distilled water and dried on Whatman filter paper was incubated in distilled water (25 mL) with continuous shaking (2 h). Initial electrical conductivity (EC) is measured using EC-TDS analyzer (ELICO-CM183), after which the leaf-discs were boiled (in water-bath for 30 min) and final EC is taken. The cell leakage (%) is computed using following formula. Leakage (%) = [(Final EC- Initial EC)/ Final EC] x 100.

Estimation of relative water content (RWC)

The fresh leaf discs were used to measure RWC as per Barrs and Weatherey (1962). Initial fresh weight (FW) of leaf-discs were taken and were floated in petri-plates containing water (8 h) for hydration and weighed again to measure the turgid weight (TW). It is then dried in a hot air oven (80 °C for 72 h) and weighed till a consistent dry weight (DW) was obtained. RWC was calculated as RWC= [(FW-DW)/(TW-DW)] x 100.

Statistical analysis

Statistical analysis was done with three replicates per analysis and significance of the treatment effects was determined by one-way ANOVA of SPSS 11.0 (Statistical Package For Social Sciences, SPSS Inc., Illinois) at 5% probability level using Tukey's test.

Conclusion

Based on the physiological characterization of *mtlD* transgenic and WT peanut (cv. GG 20), we have clearly

demonstrated that the overexpression of *mtlD* gene in the transgenic peanut has improved its salt- and drought-stress tolerance. This enhances abiotic-stress tolerance in the *mtlD* transformed peanut lines is possibly because of the accumulation of mannitol in its tissues.

However the actual reasons for abiotic stress tolerance could be much more complex than what might be anticipated of an osmoticum or osmoprotectant like mannitol. In fact, sugar-alcohols not only acts as an osmoregulator, but it can enhance the salinity-tolerance of a plant via other means like by keeping the enzyme- activity in a cell through steadying the surface bound water of protein and keeping its conformation in solution. It is very much possible that synthesis and accumulation of mannitol in the *mtlD* transgenic peanut plant tissues might induce a series of signal transduction pathways. This in turn may activate various tolerance responses against abiotic stresses like salinity and drought, which ultimately end up in increasing stress tolerance of transgenic peanut lines. Till date many transgenic peanut lines have been developed by different labs for various genes across the world with degrees of improved abiotic- and biotic-stress tolerance. But to our knowledge, this is the first report of *mtlD* transgenic characterization for abiotic stress tolerance in the peanut. Further analyses of these transgenic lines needs to be done, so as to determine how the synthesis of mannitol results in change in the expression of numerous genes possibly by separating the primary and secondary effects of mannitol. Then only we will be able to pin-point the exact mode of action of *mtlD* gene, in imparting abiotic stress tolerance to the transgenic lines.

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