BIOLOGIA PLANTARUM 54 (4): 647-652, 2010

Metabolic engineering using *mtlD* gene enhances tolerance to water deficit and salinity in sorghum

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Abstract

Sorghum bicolor L. Moench cv. SPV462 was transformed with the *mtlD* gene encoding for mannitol-1-phosphate dehydrogenase from *E. coli* with an aim to enhance tolerance to water deficit and NaCl stress. Transgene (pCAM *mtlD*) integration and expression were successfully confirmed by PCR, Southern, RT-PCR and Western analysis. Segregation analysis based on germination of T_0 seed on hygromycin-supplemented medium revealed an expected Mendelian ratio 3:1 in lines 5, 72 and 75. Retention of leaf water content was remarkably higher in transgenic leaf segments when exposed to polyethylene glycol 8000 (-2.0 MPa), as compared to the untransformed controls. Another significant finding is that the transgenics maintained a 1.7 to 2.8 fold higher shoot and root growth, respectively, under NaCl stress (200 mM) when compared to untransformed controls. These results demonstrate that engineering mannitol biosynthetic pathway into sorghum can impart enhanced tolerance to water deficit and salinity.

Additional key words: biolistics, mannitol, *mtlD* gene, *Sorghum bicolor*, transformation.

Introduction

Water deficit and salinity are major abiotic stresses that limit crop productivity in drought prone areas world over. One possible approach to compliment classical breeding for improving stress tolerance is genetic engineering (Bohnert *et al*. 2006, Cherian *et al*. 2006, Cattivelli *et al.* 2008). The introduction of compatible solute synthesis pathways has emerged as a potential strategy for enhancing abiotic stress tolerance in crop plants (Rathinasabhapathi 2000). Genetic modulation for compatible solutes, however, does not always lead to osmotic adjustment in view of the very low concentrations of these metabolites in transgenic plants (Bohnert and Shen 1999, Valliyodan and Nguyen 2006). The demonstrated protection under stress in over expression studies (Abebe *et al.* 2003, Holmstrom *et al*. 1996, Kishor *et al.* 1995, Tarczynski *et al.* 1993) might be based on other mechanisms such as protection of enzymes and membrane structure, in scavenging of reactive oxygen species and as low molecular mass chaperones (Bohnert and Jensen 1996).

Expression of bacterial mannitol-1-phosphate dehydrogenase (*mtlD*) gene for accumulation of mannitol has been one of the successful transgenic approaches for enhanced tolerance to salt stress in tobacco (Tarczynski *et al.* 1992, 1993), *Arabidopsis* (Thomas *et al.* 1995), rice (Su *et al.* 1999), egg-plant (Prabhavathi *et al.* 2002) and wheat (Abebe *et al.* 2003). In fact, mannitol has been known for long time as free radical scavenger *in vitro* (Smirnoff and Cumbes 1989). Shen *et al.* (1997) developed transgenic tobacco with *mtlD* gene and demonstrated an increased resistance to oxidative stress in the transgenic plants, which indicated a potential role for mannitol in radical oxygen scavenging.

Only few laboratories have reported successful transformation of sorghum using *Agrobacterium* (Howe *et al.* 2006) and particle bombardment (Zhao *et al.* 2000, Jeoung *et al.* 2002, Tadesse *et al.* 2003) until recently. Reports on introduction of agronomically important genes in sorghum are limited to only one on *HT-12* gene for higher grain lysine content (Zhao *et al.* 2003) and

Received 25 August 2008, *accepted* 21 July 2009.
Abbreviations: *mtlD* gene - mannitol-1-phosphate dehydrogenase gene; PCR - polymerase chain reaction.
Acknowledgements: The authors thank Dr. S.V. Rao, NRCS for kindly providing sorghum seed and Dr. Y.S. Ramakrishna, Director CRIDA, for providing the necessary facilities and for encouragement. This work was supported by a grant from the Andhra Pradesh-Netherlands (AP-NL) Biotechnology Programme.
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another on *Cry1Ac* gene for insect resistance (Girijashankar *et al.* 2005).

In this communication, we report for the first time, successful transformation of sorghum with the *E. coli mtlD* gene encoding mannitol-1-phosphate dehydrogenase using biolistic approach for enhanced abiotic stress tolerance.

Materials and methods

Sorghum (*Sorghum bicolor* L. Moench cv. SPV462) seeds were washed with *Tween-20* and then surface sterilized with 0.1 % $HgCl_2$ for 10 min. Subsequently the seeds were rinsed 5 times with sterile distilled water and were germinated in culture bottles in dark for 2 d at 25 ± 1 °C. Shoot tips were excised and cultured on Murashige and Skoog (MS) basal medium supplemented with 0.5 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg dm^{-3} kinetin according to Maheswari *et al.* (2006). Embryogenic calli (12-d-old) were used for transformation experiments. The regeneration, rooting and plant hardening were done according to the protocols described earlier (Maheswari *et al.* 2006).

The fragment containing *mtlD* gene was cloned into TOPO TA cloning vector. *EcoRI* gene fragment was then sub-cloned into pRT101 for mobilization of promoter and terminator. The gene cassette was cloned into plant expression vector pCAMBIA 1305.1 and pCAMBIA 1300 and these constructs, pCAM *mtlD GUS* and pCAM *mtlD,* were used for transformation experiments.

The protocol for particle bombardment was adopted from Rathus *et al.* (2001). About 10 μg of plasmid DNA was precipitated onto 50 mm^3 tungsten particles of 1.1 μm size (*Tungsten M-17*, *BioRad*, Hercules, USA) with 20 mm^3 of spermidine (100 mM) and 50 mm^3 of 2.5 M $CaCl_2$. The cocktail was vortexed for 5 min and centrifuged for 30 s at 13 000 *g*. The supernatant was discarded and pellet was washed thrice with absolute ethanol and finally resuspended in 30 mm^3 of absolute ethanol. Five mm^3 suspension containing 1.6 μg DNA and 417 μg microparticles was loaded onto the 0.1 mm filter holder. Particle inflow gun (*Gene Pro 2000 He*, Hyderabad, India) was used for bombardment using a pressure of 7.85 Pa and a distance of 18.5 cm between filter holder and explant. Embryogenic calli were arranged around the center of the Petri plate and incubated for 4 h on MS medium supplemented with 0.25 M mannitol and 0.25 M sorbitol for osmotic treatment prior to bombardment and 16 h after bombardment in dark. Embryogenic calli were bombarded under partial vacuum (600 Pa).

Histochemical assay for transient GUS expression was performed 24 h after bombardment using GUS-S kit (*Sigma*, St. Louis, MI, USA) according to manufacturer's instructions. The bombarded calli were incubated overnight at 37 °C in a solution containing 50 mM sodium phosphate buffer pH 7.0, 0.1 % *Triton X-100*, 10 mM EDTA and 1 mM 5-bromo-4-chloro-3-indolyl-β-d-glucuronide (Jefferson *et al.* 1987).

Hygromycin lethality test was performed. Embryogenic calli were placed on callusing medium containing different concentrations of hygromycin ranging from 0 to 16 mg dm^{-3} and incubated for 2 weeks under dark at 25 °C. The percentages of killed and surviving calli on selection medium containing different concentrations of hygromycin were recorded. Subsequently the surviving calli on LD_{50} of hygromycin were transferred onto regeneration and rooting medium. The rooted putative transformants were initially transferred to *Soilrite* and subsequently established in pots containing soil. At anthesis the lines were selfed by bagging and generation was advanced.

The inheritance of the transgene (*hpt*) in the T_1 population (obtained by selfing each of the T_0 line) was determined by evaluating the potential of germination on ½ MS basal medium containing 25 mg m^{-3} hygromycin in randomly selected T_0 and untransformed seed samples. Germination was observed after 3 d and germination percentage was scored for segregation analysis after 9 d.

Genomic DNA was isolated from putative transgenic and untransformed control following CTAB method (Doyle and Doyle 1990) for PCR and Southern analysis.

PCR analysis was carried out on putative transgenics obtained as result of bombardment with pCAM *mtlD*. *mtlD* gene specific primers. Forward 5'GTACCGAGC AGTGGCTTGAT 3' and reverse 5' CGGGTATCCAA CTGACGTTT 3' primers were used for detection of *mtlD* coding sequence, using *Gene AmpR* PCR system 9700 (*Applied Biosystems*, Foster city, CA, USA). The conditions were initial denaturation at 94 °C for 5 min followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 30 s and final extension at 72 °C for 5 min. The genomic DNA from the untransformed control plants and pCAM *mtlD* were used as negative and positive controls, respectively.

Genomic DNA of putative T_1 transgenics positive to PCR and untransformed control plants was digested with *EcoRI*. Southern blot analysis was carried out using 10 μg of DNA according to Sambrook and Russel (2001). The digested genomic DNA was separated on 0.8 % agarose gel and blotted onto positively charged nylon membrane (*Hybond*-N, *Amersham*, Buckinghamshire, UK) by capillary method. The coding sequence of *mtlD* gene was used as probe. Labelling and detection was carried out using *Alkaphos* direct labeling and detection kit (*Amersham Biosciences*).

For reverse transcriptase (RT)-PCR RNA was isolated from T_1 plants using *Trizol* method (Chomczynski 1993). About 5 μg of total RNA was used for first strand synthesis according to the manufacturer's instructions

(*Qiagen* first strand cDNA synthesis kit). First strand synthesis was carried out by incubating the reaction mixture at 37 °C for 1 h. Second strand synthesis was carried using the optimized conditions for PCR amplification of *mtlD* gene using gene specific primers.

For Western analysis soluble fractions were isolated from T_1 transgenic plants and untransformed controls using extraction medium containing 50 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 20 mM magnesium acetate, 15 % glycerol and 5 mM β-mercaptoethanol. The homogenate was centrifuged at 20 600 *g* for 15 min at 4 °C. The clear supernatant thus obtained was used for Western blots. The soluble protein content was quantified according to Bradford (1976).

About 20 µg of extracted proteins were resolved on 10 % SDS-PAGE (Laemmli 1970). For Western analysis the proteins were electrophoretically transferred onto PVDF membrane (*Hybond*-P, *Amersham*). These membranes were blocked overnight with 3 % bovine serum albumin. Subsequently, immunological detection was performed with *Streptavidin Biotinylated HRP* complex kit (*Bangalore Genei*, Bangalore, India) according to manufacturer's instructions, using antibodies raised against pure mannitol dehydrogenase (*Sigma*).

The tolerance of the transgenics (T_1) to water deficit (PEG mediated) and NaCl was tested by following methods. Leaf segments were cut from youngest fully expanded leaf at 40-d-old T_1 transgenic and untransformed plants growing in pots under well watered conditions in a glasshouse. These segments were floated on PEG 8000 (0 or -2.0 MPa) for 24 h. The fresh mass of leaf segments was recorded before and after floating on the various solutions. At the end of the experiment dry mass was recorded. Leaf water content was calculated as [(fresh mass - dry mass) / fresh mass] × 100.

The seeds from transgenic (T_0) and untransformed control plants were tested for germination on NaCl. Seeds were surface sterilized and germinated on Whatmann filter paper wicks placed in test tubes containing 0 or 200 mM NaCl. The tubes were kept at 25 ± 1 °C and 12-h photoperiod under irradiance of 85 µmol m^{-2} s^{-1}. Germination percentage was scored after 15 d. The ability to recover from NaCl stress was also monitored by transferring the germinated seedlings into plastic containers containing *Soilrite*. They were irrigated with half strength Hoagland solution and tap water on alternate days. The seedling growth in terms of the root and shoot lengths were measured after 15 d.

There were three replicates for each treatment and the experiment was repeated at least twice. The standard error, *ANOVA* and χ^2-square test were done according to standard statistical procedures.

Results

The presence of characteristic blue spots (Fig. 1*A*) upon histochemical analysis of embryogenic calli suggested the introduction and expression of *GUS* reporter gene in the calli bombarded by the particle inflow gun. Stable *GUS* expression was also observed in florets of unopened cobs (Fig. 1*B*).

Table 1. Frequency of regeneration and transformation of shoot tip derived calli of sorghum bombarded using plasmid pCAM *mtlD*.

Number of calli used	Number of calli in regeneration medium	Number of transgenic plantlets	Regeneration frequency [%]	Transformation efficiency [%]
400	69	18	26.0	17.3
400	96	28	29.1	24.0
400	83	22	26.5	20.8

Results on hygromycin sensitivity of sorghum using untransformed 12-d-old shoot tip calli revealed that LD_{50} was at 6 mg dm^{-3} hygromycin and LD_{100} at 12 mg dm^{-3} hygromycin. Embryogenic calli were bombarded with *mtlD* gene in pCAM *mtlD GUS*. The transformed calli were recovered and proliferated on callusing medium containing hygromycin. The transformation and regeneration efficiencies were 20.67 and 27.20 %, respectively. About 22 shoots were regenerated per callus (Table 1). Efficient rooting was achieved within 2 weeks on media

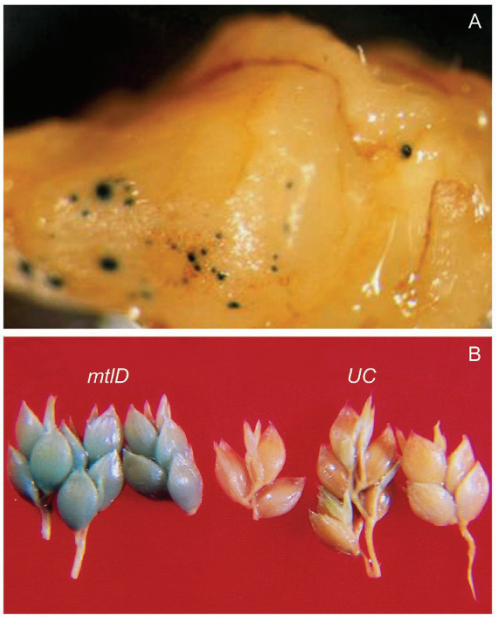

Fig. 1. Genetic transformation of sorghum with *mtlD* gene using particle inflow gun: *A* - transient GUS activity in transformed calli derived from shoot apices; *B* - stable GUS expression in florets.

Table 2. Segregation in progeny of T_0 seed from sorghum *mtlD* transgenic plants based on germination in media containing hygromycin (25 mg dm^{-3}). Tabular χ^2 values for 1 d.f. = 3.841 at 5 % level of significance.

Transgenic (T_0) lines	Number of seeds tested	Number of seeds germinated	Number of seeds not germinated	Segregation ratio	χ^2 value
L5	154	115	39	3:1	0.008
L72	99	78	21	3:1	0.455
L22	80	54	26	2.7:1.3	15.450
L75	100	67	33	3:1	3.413
L110	102	33	69	1:3	59.980

containing 1 mg dm^{-3} naphthaleneacetic acid (NAA). Over 90 % of rooted plantlets grew well and were fertile after transfer to a net house.

The sorghum transgenic T_0 lines with *mtlD* gene were tested for segregation of the *hpt* by germinating the seeds on hygromycin (25 mg dm^{-3}) supplemented medium. Based on the percent germination, it was observed that out of the five plants tested progeny of the plants Nos. 5, 72 and 75 segregated in a ratio of 3:1 while plants Nos. 22 and 110 showed deviation from the expected ratio (Table 2).

PCR analysis was carried out on the genomic DNA isolated from the hygromycin tolerant T_0 transformants and the untransformed control using primers for *mtlD* coding sequence. These transformants showed an amplification product of 800 bp. A similar band was also observed (Fig. 2*A*) in the positive control (pCAM *mtlD*) while no such band was noticed in the untransformed control.

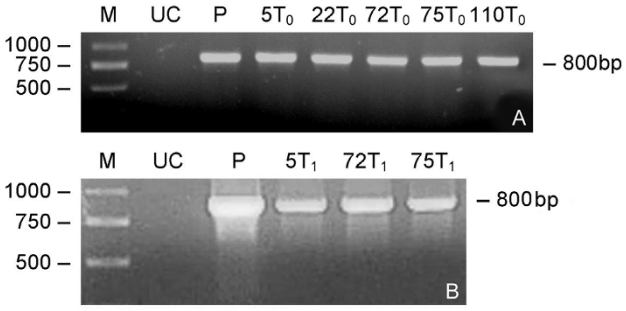

Fig. 2. PCR analysis using *mtlD* gene specific primers: *A* - T_0 transformants; *B* - T_1 transformants; M - 1 kb ladder, UC - untranfrormed control, P - positive control (pCAM *mtlD* plasmid).

PCR analysis and Southern blot hybridization was carried out on T_1 transformants, which segregated in a 3:1 ratio based on their percent germination on hygromycin (Fig. 2*B*). These transformants also showed invariably an amplification product of 800 bp by PCR analysis. *mtlD* coding sequence was used as a probe for Southern blot hybridization. All the three transformants which segregated in a 3:1 ratio, showed a 1200 bp band, which confirmed the integration of the transgene. The untransformed control did not show any such band (Fig. 3*A*).

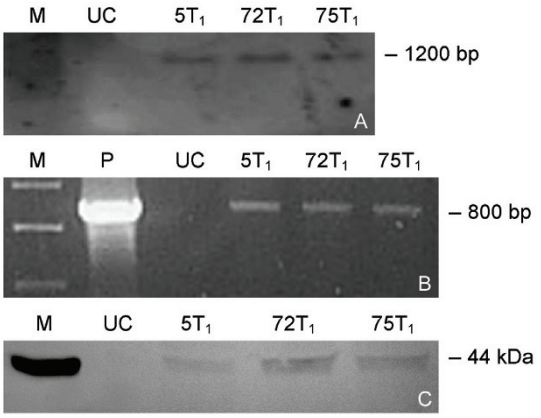

Fig. 3. *A* - Molecular characterization of sorghum transformants by Southern blot analysis of genomic DNA digested with *EcoRI* and probed with *mtlD* coding sequence, M - λDNA/HindIII marker. *B* - RT-PCR analysis of *mtlD* transgenics, M - 1 kb ladder, P - positive control (pCAM *mtlD* plasmid), UC - untranfrormed control. *C* - Immunoblot analysis of mannitol-1-phosphate dehydro-genase in the soluble fraction from leaves of transformed sorghum lines with *mtlD* gene, M - pure mannitol-1-phosphate dehydrogenase (*Sigma*).

Table 3. Leaf water content [%] in *mtlD* transgenic leaf segments incubated in PEG 8000 solution for 24 h. Means ± SE, n = 3. $LSD_{0.05}$: plant lines (G) = 0.998, treatment (T) = 0.705, G × T = 1.411. In parentheses reduction from control [%].

T_1 lines	0 MPa	-2.0 MPa
control	78.3 ± 0.08	56.0 ± 0.87 (29)
$L5T_1$	78.2 ± 0.14	74.6 ± 0.51 (5)
$L72T_1$	79.8 ± 0.17	66.6 ± 0.68 (17)
$L75T_1$	80.4 ± 0.00	68.0 ± 0.00 (15)

Table 4. Root and shoot growth of sorghum *mtlD* T_0 seed under NaCl (200 mM) stress (15 d) and recovery (15 d). Means ± SE, n = 3. $LSD_{0.05}$: G = 1.526, T = 1.079, G × T = 2.158.

T_1 lines	NaCl stress root length [cm]	shoot length [cm]	Recovery root length [cm]	shoot length [cm]
Control	0.97 ± 0.03	0.90 ± 0.15	13.50 ± 0.87	12.33 ± 1.45
$L5T_1$	1.30 ± 0.06	2.07 ± 0.18	37.67 ± 2.19	22.50 ± 0.76
$L72T_1$	1.17 ± 0.12	1.40 ± 0.06	33.67 ± 4.18	20.50 ± 0.29
$L75T_1$	1.67 ± 0.07	1.80 ± 0.12	44.67 ± 0.33	22.33 ± 1.20

RT-PCR analysis was carried out using the total RNA isolated from T_1 transformants and untransformed control. A distinct 800 bp band was observed in all the three transformants, which were Southern positive while the

untransformed plants did not reveal such a band (Fig. 3*B*).

Western blot analysis with antiserum raised against pure mannitol dehydrogenase indicated the presence of a 44 kDa protein band corresponding to the authentic mannitol dehydrogenase in the soluble fraction from the leaves of the T_1 transgenic lines which were positive for Southern and RT-PCR (Fig. 3*C*). No immunoreactive protein band was observed from the untransformed control.

Leaf segment assays for water deficit (PEG 8000) tolerance were done on T_1 plants, which were confirmed positive by molecular analysis. Leaf segments from *mtlD* transgenic plants when incubated on PEG 8000 (-2.0 MPa) maintained significantly higher leaf water content compared to untransformed control, which lost up to 30 % of water (Table 3).

Germination of the transgenic seeds on 200mM NaCl was remarkably higher when compared to untransformed controls. The root and shoot lengths measured at 15 d after germination on NaCl were significantly higher in transgenics when compared to the untransformed controls (Table 4). Interestingly, the *mtlD* transgenics also recorded significantly higher stress recovery in both root and shoot lengths (on an average 2.8 and 1.7 folds).

Discussion

The present investigation described development of transgenic sorghum with enhanced tolerance to water deficit and NaCl stresses. Vegetative as well as reproductive growth of sorghum *mtlD* transgenics was comparable to untransformed controls indicating thereby that the introduction of this gene did not result in any sterility or abnormality. These findings are in agreement with earlier studies that used *mtlD* gene in tobacco (Shen *et al.* 1997, Tarczynski *et al.* 1992, 1993), *Arabidopsis* (Thomas *et al.* 1995), eggplant (Prabhavathi *et al.* 2002) and wheat (Abebe *et al.* 2003).

Based on molecular characterization using PCR the presence of the transgene was confirmed in the T_0 transgenics. Segregation analysis based on germination of T_0 seed in presence of hygromycin revealed a ratio of 3 tolerant to 1 susceptible line, while deviation from this was observed in some of the transformants probably because of variation in transgene copy number (Prasad *et al.* 2000). It has been reported that segregation pattern of the transgene would depend upon the transgene copy number. The Mendelian segregation with single transgene insertion and deviated ratios with multiple transgene integrations have been documented (Hiei *et al.* 1997, Prabhavathi *et al.* 2002, Prasad *et al.* 2000). The T_1 plants that displayed the 3:1 segregation ratio were also found to be positive using PCR, Southern, RT-PCR and Western analyses.

Metabolic engineering for over production of mannitol has been a successful approach to enhance tolerance to water deficit stress (Abebe *et al.* 2003, Tarczynski *et al.* 1993). Our results on leaf segment assay indicated maintenance of higher leaf water content under PEG mediated water stress. Under salt stress, germination potential, root and shoot growth as well as ability of recovery were remarkably higher in the transgenics compared to untransformed controls. However, the individual transgenics differed in their response probably due to the difference in expression levels of the gene. The increment in growth was remarkably higher in case of root as compared to that of shoot. Higher capacity of *mtlD* transgenics to recover from salt stress in terms of higher root growth had been found also in tobacco (Tarczynski *et al.* 1993) and egg plant (Prabhavathi *et al.* 2002). Although it is not clear how mannitol accumulation may lead to new root growth, the metabolite may stimulate processes at cellular level. Stimulatory effects of mannitol in low concentrations on auxin promoted cell and root hair elongation (Jackson 1965, Pike and Richardson 1977) were reported earlier. The tolerance to water deficit and NaCl conferred by *mtlD* gene in sorghum might be due to various mechanisms including stabilization of macromolecules, enzymes, membranes, detoxification of ROS and osmotic adjustment (Bohnert and Shen 1999, Hu *et al*. 2010).

In summary, the results presented in this communication clearly demonstrated the development of transgenic sorghum plants with *mtlD* gene with remarkable tolerance to PEG induced water deficit and NaCl stresses. This is the first report on introduction of gene for abiotic stress tolerance in this crop.

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