Increased salt stress tolerance and modified sugar content of bread wheat stably expressing the *mtlD* gene

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Abstract: The bacterial *E. coli mtlD* gene (encoding mannitol-1-phosphate dehydrogenase) under the control of maize ubiquitin promoter was used in transforming bread wheat cv. Giza 163 *via* biolistic device. The presence and expression of the transgene in two selected T_0 transgenic lines were confirmed at the molecular level. The tolerance of transgenic T_2 families to salt (8 g/l, 3 NaCl: 1 $CaCl_2$) stress was evaluated at the greenhouse over two consecutive seasons. In either season, grain yield per plant of transgenic family 235/3 was significantly the highest under salt stress, while that of the wild-type control was the lowest. These results coincided with the rate of *mtlD* transgene expression of the two T_1 transgenic lines. The results of mean total biomass per plant and plant height perfectly contradicted those of grain yield per plant across treatment and seasons. Differences in total seed storage protein concentrations among different genotypes were non-significant within or across environmental conditions. Mannitol was detected in grains and plantlets of the two transgenic families. The soluble sugars significantly increased in the transgenic plantlets and grains as compared to the wild-type control. On the contrary, the total sugar level significantly decreased in the transgenic plantlets. Fructose, glucose and galactronic acid highly increased in the transgenic plantlets and grains, while sorbitol, mannose and galactose decreased. In conclusion, the results indicated that *mtlD* gene confers salt stress protection in transgenic wheat through the induction of mannitol and reducing sugars accumulation in plant tissues.

[A.M. Ramadan, H.F. Eissa, S.E. Hassanein, A.Z. Abdel Azeiz, O.M. Saleh, H.T. Mahfouz, F.M. El-Domyati, M.A. Madkour and A. Bahieldin. **Increased salt stress tolerance and modified sugar content of bread wheat stably expressing the *mtlD* gene.** *Life Sci J* 2013;10(2):2757-2770] (ISSN:1097-8135). http://www.lifesciencesite.com. 384

Key Words: Southern, Northern, PAT assay, Herbicide resistance, Genetic transformation, Abiotic stress

1. Introduction

Increased salinization of arable lands has severe damaging effects that are expected to result in as high as 50% loss by the year 2050 (Wang *et al*., 2003). Accordingly, attention should be given towards the development of salt-tolerant crop plants *via* plant breeding and/or genetic transformation (Bohnert *et al*., 2006; Cherian *et al*., 2006; Cattivelli *et al*., 2008, Maheswari *et al*., 2010). In addition, it has been suggested that metabolic engineering of osmoprotectant synthesis pathways could be one of the best strategies for enhancing abiotic stress tolerance of plants (Bhatnagar-Mathur *et al*., 2008).

In response to osmotic stress, plants usually accumulate low-molecular-weight compatible solutes to maintain cell turgor (osmoregulation). Accumulation of these compatible solutes does not interfere with cell's metabolism and can function as oxygen free radical scavengers or chemical chaperones, hence, protect macromolecules, enzymes and proteins. The most common compatible osmolytes in plants include sugar alcohols (e.g., mannitol, sorbitol, etc.). Overexpression of these polyols was described as a potential route for improving abiotic stress tolerance in plants (Yokoi *et al*., 2002; Wang *et al*., 2003; Almeida *et al*., 2007). Mannitol is considered as the most abundant sugar alcohol. It is synthesized as a primary photosynthetic product in nature in which it is represented in trace amounts in more than 100 plant species, including many crops such as celery, olive and carrot (Conde *et al*., 2007; Prabhavathi and Rajam, 2007). Mannitol has multiple functions in bacteria, fungi, algae and plants. Because of these functions, many attempts are required in order to generate highly salt-tolerant transgenic plants.

Tobacco and Arabidopsis, like many other plants, do not normally accumulate mannitol. They exhibit biosynthesis of mannitol and an increased salinity tolerance when they were transformed with the *E. coli* mannitol-1-phosphate dehydrogenase

(*mtlD*) gene (Tarczynski *et al*., 1993; Thomas *et al*., 1995; Karakas *et al*., 1997). An increased resistance to oxidative stress was also found in tobacco plants accumulating mannitol in the chloroplasts (Stoop *et al*., 1996). Abebe *et al.* (2003) reported an improved tolerance to salinity in bread wheat expressing the *mtlD* gene. Similar results have been reached *via* the genetic transformation of rice (Huizhong *et al*., 2000), eggplant (Prabhavathi *et al*., 2002), petunia (Chiang *et al*., 2005), loblolly pine (Tang *et al*., 2005), poplar (Hu *et al*., 2005), sorghum (Maheswari *et al*., 2010), tomato (Khare *et al*., 2010) and potato (Rahnama *et al*., 2011) with the *mtlD* gene. In these plants, mannitol biosynthesis and accumulation increased, while its catabolism decreased under stress conditions (Williamson *et al*., 2002; Abebe *et al*., 2003; Sickler *et al*., 2007).

In this study, we report the successful transfer and expression of *E. coli mtlD* gene in bread wheat (cv. Giza 163) under the control of maize ubiquitin promoter. Expression of *mtlD* gene resulted in the biosynthesis of mannitol and a consequent improvement in salt stress tolerance of the generated transgenic wheat plants. Additionally, levels of soluble sugars were also studied. The results might scope the light on the possible different reactions of sugar metabolism in bread wheat.

2. Materials and Methods

Plant expression vector

The genetic construct pAB_4 (8.53 kb, Figure 1), containing the *E. coli mtlD* gene (encoding mannitol-1-phosphate dehydrogenase) (Tarczynski *et al*., 1992), was used as plant expression vector. The *mtlD* gene was functioning under the control of maize ubiquitin (*ubi*) promoter (Christensen *et al*., 1992) and *NOS* terminator. The plasmid contains *bar* gene (encoding the phosphinothricin acetyl transferase) as a selectable marker for Basta herbicide resistance (De Block *et al*., 1987) driven by *CaMV35S* promoter, with maize *Adh1* intron in the 5`non-translated region, and terminated by *NOS* terminator.

A number of intermediate construction steps were done in order to obtain the plasmid pAB_4. The *P-cab* promoter of pcabMTLD plasmid was replaced by maize ubiquitin promoter (*P-ubi*) and *ubi1* intron of pAHC17 plasmid to obtain pAB_3. *Hin*DIII fragment of *bar* gene cassette (2.09 kb), obtained from pAB_1 (Bahieldin *et al*., 2005), was integrated into the unique *Hin*DIII site of pAB_3 to obtain pAB_4 (8.53 kb).

Wheat transformation

Immature embryos were isolated from field-grown bread wheat (*Triticum aestivum* L.) cv. Giza 163 (G163) and pre-cultured for 1-4 days in the dark on modified MS medium (Weeks *et al*., 1993). The protocol used in transforming wheat plants was performed according to Sivamani *et al.* (2000). Primary transformants were transferred to the biocontainment greenhouse of AGERI, ARC, Egypt and tested using leaf painting assay with a 0.1% aqueous solution of Glufosinate 200™ (AgrEvo USA, NJ, USA) containing 20% glufosinate ammonium.

Molecular analysis of putative *mtlD* transgenics

Genomic DNAs were extracted from two selected putative transgenics, resistant during leaf painting to the herbicide Basta, as well as the wild-type control, using DNeasy™ Plant Mini kit (Qiagen Inc., cat. no. 69104). We also required that transgenics possess undisturbed overall performance as compared to the wild-type control. PCR was performed by the amplification of the partial-length *bar* (400 bp) and full-length *mtlD* (1.08 kb) genes using specific primers with the following sequences:

bar-F	5`TACATCGAGACAAGCACGGT3`
bar-R	5`GTGCCCTTGACCGTACTGCA3`
mtlD-F	5`CGAGATCTAACAATGAAAGCAT TACATTTTGGCGC3`
mtlD-R	5`GGGATATCTTATTGCATTGCTTT ATAAGCGG3`

The reaction conditions were optimized and mixtures (50-ul total volume) composed of dNTPs (0.2 mM), $MgCl_2$ (1.5 mM), 1x buffer, primer (0.2 µM), DNA (100 ng), Taq DNA polymerase (2 units). Amplification was carried out in a Hybaid PCR Express programmed for 40 cycles as follows: 94°C/4 min for primary denaturation (1 cycle); 94°C/1 min for denaturation, 55°C/1 min for annealing with *bar* gene and 58°C/1.2 min for annealing with *mtlD* gene, 72°C/2 min for extension (38 cycles); 72°C/8 min (1 cycle); 4°C (infinitive). Agarose (1.2%) was used for resolving PCR products. A Lambda phage DNA/ *Bst*EII digest was used as a standard DNA (8.45, 7.24, 6.37, 5.69, 4.82, 4.32, 3.68, 2.32, 1.93, 1.37, 1.26, 0.70, 0.12 kb). The run was performed at 80 V in Bio-Rad submarine (8 cm X 12 cm). Bands were detected on UV-transilluminator and photographed.

Genomic Southern analysis (Southern, 1975) was carried out for the two selected T_0 transgenics. The analysis was performed to ensure the presence of the introduced genes in the wheat genome and to estimate the copy number of the *bar* gene by reconstructing one and five copies as compared to the control. Reconstruction was made by mixing genomic DNA of wild-type plant with pAB_4 plasmid according to the following formula (size of plasmid X 30/size of wheat genome = µg of plasmid/30 µg of genomic DNA) to obtain one copy of the construct in wheat genome. Amount of plasmid was multiplied by five to reconstruct five-copy number of the construct. Leaf samples of different genotypes were digested with *Hin*DIII to liberate the *bar* gene cassette (2.09 kb). Probe used was prepared by the digestion of pAB_4 with *sal*I/*Kpn*I to obtain 200-bp fragment of *bar* gene.

Phosphinothricin acetyltransferase (PAT) assay was carried out for the two putative T_0 transgenic plants to confirm the presence and expression of the *bar* gene. Protein was extracted from the leaves of the transgenic plants and PAT assay was carried out according to Spencer *et al.* (1990). Northern blotting was performed to detect the expression of *mtlD* transgene in the two transgenic plants at T_1 generation. Total RNA was extracted from transgenic as well as wild-type control plants using Total RNA Isolation system (Promega, cat. no. Z3100). Probe used was prepared by digesting pAB_4 with *Sph*I/*Not*I to release 400 bp-fragment of *mtlD* gene.

Salt stress experiment

T_3 and T_4 grains, collected from progenies of the two homozygous transgenic bread wheat families (79/8 and 235/3) with the highest *mtlD* gene expression, were utilized in salt stress experiments in two consecutive seasons 2009/2010 and 2010/2011, respectively. Transgenic and wild type grains were sown and grown in sand culture in a randomized complete blocks design with four replicates (10 plants per replicate). The two experiments were conducted at the biocontainment greenhouse of AGERI, ARC, Giza, Egypt. Plastic dishes of 45 cm in height, 50 cm in diameter and capacity of 50 kg were filled up to 7 cm from the top with pre-washed fine sand. Modified Hoagland solution (Johnson *et al*., 1957) was used as the nutrient supplement. Internal parts of 15-day-old transgenic plant leaves were sprayed with 1 g/l Basta (half the recommended dose) to prove *bar* gene expression. Then, 21-day-old transgenic and wild-type seedlings were divided into two groups; one was irrigated with salt-free water, while the other was irrigated with salt solution (8 g/l salt, 3 NaCl : 1 $CaCl_2$). Data were collected at maturity for yield-related traits; i.e., plant height (cm), total biomass per plant (g) and grain yield per plant (g). To prove substantial equivalence of transgenic versus wild-type plants, seed storage protein was extracted by suspending 250 mg of fine powdered seed in 0.5 ml of protein extraction buffer (0.125 M tris-HCl, pH 6.8, 2.5% SDS, 10% glycerol) in a microfuge tube. The extract was centrifuged at 13,000 g for 5 min at 4°C and the supernatant was collected. The total protein content was estimated in four replicates for the two transgenic T_4 families and their wild-type control, following the Bradford method (Bradford, 1976) with bovine serum albumin as a protein standard.

Sugar analysis

Total and soluble sugars were extracted from dried 21-day-old plantlets and grains of transgenic T_4 families 79/8 and 235/3 as well as the wild-type control. Total sugars were extracted by digesting 100 mg of the samples in 5-ml 1 N HCl for 1 h at 100°C. Soluble sugars were extracted by mixing 100 mg of the ground plantlets or grains with 5 ml ethanol (80%) in screw-capped tubes for 1 h at 100°C. The samples were centrifuged at 1000 xg for 10 min at room temperature. The extraction was repeated three times and the supernatants were combined (Masuda *et al*., 1996). The total and soluble sugar levels were determined by phenol sulphuric method (Dubois *et al*., 1956). The soluble sugars were analyzed by using HP-5890 GC equipped with HP-5972 mass spectrometer. One-ml of the soluble sugar extract was evaporated to dryness at 40°C under stream of nitrogen. Half-ml of isopropanol (HPLC grade) was added to remove water residue, shaken gently and evaporated to dryness under a stream of nitrogen at 40°C. The remaining residue was silylated as described by Kirk and Sawyer (1991). Then, 250 µl of hydroxyl amine hydrochloride (2.5%) in anhydrous pyridine were added and the reaction was incubated in an oven at 80°C for 30 min. After cooling, 0.5 ml of silylation reagent (trimethylchlorosilane [TMS], N, N-O bis-[trimethylsilyl] acetamide, 1:5 by volume) was added and incubated in an oven at 80°C for 30 min. The GC separation conditions were; inlet temperature (250°C), mobile phase (helium), flow rate (1 ml/min), oven temperature program (initial temperature 80°C, 10°C/min, up to 200°C for 10 min), HP-innowax column (30 m X 0.25 mm ID) and MS detector temperature (300°C). The obtained mass spectra were analyzed by Wiley7N mass library. Individual and mixture of the trimethylchlorosilane (TMS) derivatives of standard monosaccharides and sugar alcohols (ribose, xylose, glucose, mannose, galactose, fructose, mannitol, sorbitol and galacturonic acid) were injected into the GC to ensure the retention time of each sugar. Experiments of all sugars analyses were performed twice in three replicates.

Two-way analysis of variance (ANOVA) with two factors (salt treatment and genotype) for the two salt stress experiments in seasons 2009/2010 and 2010/2011, while non-factorial statistical analysis for protein analysis in season 2010/2011, were conducted. Multiple comparisons (based on the least significant differences, or LSD, at 5%) were performed following the procedure outlined in MSTATC program (Michigan State University) for yield and attributes and protein analysis, while Duncan's New Multiple Range test (Duncan 1955) for sugar analysis. Practices at the biocontainment greenhouse complied with the biosafety guidelines and regulations in Egypt with regard to growing and testing transgenic plants.

3. Results and Discussion

The bacterial *E. coli mtlD* gene (Tarczynski *et al*., 1992), under the control of ubiquitin promoter, was used for genetic transformation of bread wheat plants cv. Giza 163 using biolistic bombardment. A

number of ten independent transgenic plants, based on resistance to the herbicide Glufosinate™ or Basta (1 g/l), were generated from three transformation experiments (efficiency of 1.1%), as indicated by leaf painting followed by phosphinothricin acetyltransferase (PAT) enzyme assays to demonstrate the expression of the herbicide-resistance gene (*bar*). Of these primary transgenic lines, two lines (79 and 235) were selected for further characterization and homozygous families were grown up to T_4 generation. Selection was based on the normal plant appearance, fertility, uniform herbicide-resistance by leaf painting followed by PAT enzyme assays (data provided upon request).

Molecular analysis of putative transgenics

The presence and expression of the transgene(s) in the two putative T_0 transgenic lines were tested. PCR was primarily carried out using primers specific for *bar* and *mtlD* genes. The two T_0 transgenic lines 79 and 235 showed the expected amplicon sizes for the *bar* (400 bp, Figure 2a) and *mtlD* (1.08 kb, Figure 2b) genes. Similar amplicons were recovered for the positive control (pAB_4), while no amplicons were recovered for the wild-type control (cv. Giza 163). Gene presence was confirmed by PCR in subsequent generations to insure inheritance of the *mtlD* gene (data provided upon request), while spraying with the herbicide for testing the *bar* gene expression.

Southern blot analysis was performed to confirm the transgene presence and to estimate insert copy number in the genome of the two T_0 transgenic plants (Figure 3). A single band of the expected size (2.09 kb) was recovered from genomic DNAs of the two transgenic lines 79 and 235 when digested with *Hin*DIII. A band with a similar size was also recovered from the positive control (pAB_4), while no such band was recovered from the wild-type control. The band intensity for the two transgenics indicated that the insert was integrated at one-copy number, only. These results were confirmed at subsequent T_1 generation of both transgenic lines through Mendelian segregation of 3 herbicide-resistant: 1 non-resistant after plants are sprayed with the herbicide Basta (data provided upon request).

PAT assay was used to detect the activity of phosphinothricin acetyl transferase enzyme encoded by *bar* gene in the two T_0 transgenic lines 79 and 235. PAT activity was detected by thin layer chromatograph (TLC) in leaf protein extracts. Figure 4 shows the occurrence of PAT activity in the two selected transgenic lines, while no such activity was detected in wild-type control. Expression of *mtlD* transgene in the T_1 progeny of the two transgenic lines was verified by northern blot analysis. Seedlings of the two events were grown and sprayed with 1 g/l Basta to select three resistant transgenic plants from each line. A distinct 400-bp band was observed in all six samples (Figure 5) as well as in the positive control (total RNA isolated from *mtlD*-transformed *E. coli* cells). These data also indicate no transgene silencing at T_1 generation. From the results of leaf painting (data provided upon request), PAT assay and northern blotting, it was concluded that transgenic line 235 had higher *bar* as well as *mtlD* gene expression than line 79. T_3 and T_4 families of these two transgenic lines were generated for subsequent testing under salt stress, while T_4 families for protein and sugar analyses.

Evaluation of *mtlD*-transgenic plants under salt stress

The tolerance of the two transgenic families (79/8 and 235/3) at T_3 and T_4 to salt (8 g/l, 3 NaCl : 1 $CaCl_2$) stress was studied at the biocontainment greenhouse over two consecutive seasons (2009/2010 and 2010/2011). The results of yield and its attributes generated in the two seasons are presented in Table 1. In either season, the mean grain yield per plant of 235/3 family was significantly the highest under salt stress as it over-yielded those of 79/8 family and the wild-type control. These results coincided with the rate of *mtlD* transgene expression of the two events. The records of the reduction percentages indicated that 79/8 family was slightly affected by salt stress in 2009/2010, while 235/3 family in 2010/2011. The reduction percentages in the two seasons indicate that the wild-type control was severely affected by salt stress. The results of the mean total biomass per plant and mean plant height perfectly contradicted those of the mean grain yield per plant. The mean total biomass per plant of wild-type control in either season was significantly higher than those of the two transgenic families within and across treatment. However, the results of the reduction percentage indicated that the wild-type control was severely affected by salt stress. Transgenic family 79/8 was slightly affected by salt stress as the reduction percentages of the mean total biomass per plant in either season was the lowest. It was evident that transgenic plants were slower in growth as compared to the wild-type control. This phenomenon indirectly affected the total biomass per plant of transgenic events. The mean plant height of wild-type control in either season was significantly higher than those of the two transgenic families within and across treatment. The reduction percentage of this trait showed no consistent trend of results. We speculate that plant height also indirectly affects total biomass per plant. Differences in the mean total seed storage protein concentrations between plants of the two transgenic families and the wild-type control within or across environmental conditions were non-significant. This trait is an important indicator of substantial

equivalence of transgenic lines and the wild-type control.

However, the two transgenic events were characterized by the appearance of long root hairs, a feature that is absent in the wild-type control (data provided upon request). No explanation can be given for such phenomenon, except that it possibly allows for the higher influx capacity of water and minerals. Hu *et al.* (2005) indicated that the fast-growing large roots of the transgenic poplar plants indicate that the *mtlD* gene induces an increase in the root : shoot ratio, which may account for the dwarfism of the transgenic plants. The present results indicated that plant height of the two transgenics significantly reduced in accordance with the results of Hu *et al.* (2005). However, the results of Maheswari *et al.* (2010) indicated that *mtlD*-transgenic sorghum maintained higher shoot growth under NaCl stress when compared to untransformed controls.

We speculate that the phenomena of slow growth and dwarfism of the two transgenic plants indirectly justify the reduced biological yield as compared to the wild-type control. This result is consistent with the response of tobacco and poplar to transformation with the *mtlD* gene (Karakas *et al.*, 1997; Hu *et al.*, 2005, respectively). Also, Xiong and Zhu (2002) indicated that plants with high stress resistance have a dwarf form, which can reduce water and energy consumption and facilitate energy redistribution. Thus, the slow growth of the transgenic plants might be the only cause of the improved salt tolerance. This suggestion is supported by the finding that plants sprayed with the growth retardant paclobutrazol show enhanced salt tolerance (Hu *et al.*, 2005). The latter study indicated another possible reason for the slow growth of *mtlD*-transformed poplar, which is the use of the *NPTII* selective marker gene. This conclusion seems unlikely because other authors in earlier studies (Arnoldo *et al.*, 1992; Dale and McPartlan, 1992; Tarczynski *et al.*, 1993) indicated that the growth rate was unaffected by transformation with empty vector with *NPTII* gene. Besides, in the present study, *bar*, not *NPTII*, was the selectable marker gene utilized in the transformation procedure and during growth in subsequent generations. Other earlier studies (Goddijn *et al.*, 1997; Romero *et al.*, 1997) indicated that stunted growth occurs in plants transformed with trehalose synthetase (*ost*A) or *TPS*1 gene when these two genes are driven by the *CaMV35S* promoter, while other studies indicated the occurrence of stunted growth due to other promoters (Holmström *et al.*, 1996; Jaglo-Ottosen *et al.*, 1998; Kasuga *et al.*, 1999). However, Kasuga *et al.* (1999) provided evidence that the *CaMV35S* promoter might be one of the causes of dwarfism in transgenic plants. Although we have utilized this promoter in driving the *bar* gene, the latter explanation of *CaMV35S* promoter possible action seems unlikely, where in one of our earlier work (Bahieldin *et al.*, 2005) to introduce the barley *HVA1* gene into wheat to confer drought tolerance, the same *bar* gene cassette, with the *CaMV35S* promoter, was utilized and plant height of all transgenic plants increased as compared to the wild-type control. However, the results of total biomass in our earlier study (Bahieldin *et al.*, 2005) are in harmony with that in the present study. Therefore, the effect of this promoter on growth rate, not plant height, is likely. This conclusion is supported by the performance of bread wheat (cv. Hi-Line) plants transformed with *bar* gene cassette only (transgenic line 84, see Sivamani *et al.*, 2000).

Sugar analysis of mannitol-accumulating plants

The results of total and soluble sugar levels also indicate the influence of introducing the *mtlD* gene in modifying/adding some reactions in the sugar metabolism in wheat. Total and soluble sugar levels were measured in both grains and 21-day-old plantlets of *mtlD*-transgenic T_4 families 79/8 and 235/3 and wild-type control (cv. G163) (Table 2). The mean soluble sugar levels significantly increased in the plantlets and grains of transgenic families as compared to the wild-type control. On the contrary, the mean total sugar level significantly decreased in the transgenic plantlets, while the decrease was non-significant in the transgenic grains.

The mean soluble sugar levels were identified by GC/MS in grains and 21-day-old plantlets of *mtlD*-transgenic T_4 families 79/3 and 235/3 and wild-type control (cv. G163) (Table 3 and Figure 6). The results of wild-type control almost indicated no mannitol accumulation, while trace amount was detected in its grains (0.036 mg/100 g dry weight). Elevated levels of mannitol were found in grains and plantlets of transgenic families 79/8 and 235/3. The presence of mannitol was confirmed by comparing the retention time and compatibility of the mass spectra of the standard mannitol with that obtained from the samples (Figure 7). The levels of fructose, glucose and galactronic acid highly increased in the plantlets and grains of the two transgenic families as compared to those of the wild-type control. On the other hand, the levels of sorbitol, mannose and galactose dramatically decreased in both plantlets and grains of the two transgenic families as compared to those of the wild-type control.

Based on the results of soluble sugars, we were able to draw the possible pathway of sugar biosynthesis in bread wheat as a result of introducing the *mtlD* gene (Figure 8). We can't ignore the fact that the plant adapts itself to overcome any malfunction of a biosynthesis pathway by activating another one. The

reduction of fructose-6-phosphate (F6P) to mannitol-1-phosphate (Mt1P), which is catalyzed by mannitol-1-phosphate 5-dehydrogenase, is concomitant with NAD^+ accumulation. The latter affects the redox balance in several organisms (Loesche and Kornman, 1976; Edwards *et al*., 1981; Ezra *et al*., 1983; Rosenberg *et al*., 1984; Rager *et al*., 1999; Neves *et al*., 2000).

$$\text{D-fructose-6-phosphate} + \text{NADH} + \text{H}^+ \xrightarrow[\text{5-dehydrogenase}]{\text{Mannitol 1-phosphate}} \text{D-mannitol-1-phosphate} + \text{NAD}^+$$

In the present study, *mtlD* gene participates in the occurrence of major changes in the soluble sugars level of the transgenic plant as compared to the wild-type control. First, it highly increased the overall soluble sugars levels. Second, as it resulted in the accumulation of mannitol, it consequently induced the pathway towards the biosynthesis of fructose and glucose. The latter induction counteracts the biosynthesis of mannose and galactose or enhances the conversion of these sugars to other sugars in both grains and plantlets. Introduction of the *mtlD* gene into bread wheat plantlets led to direct conversion of F6P to Mt1P and accumulation of NAD^+. The increased amount of NAD^+ affects the redox reactions balance in the *mtlD*-transgenic wheat plant, as it may result in feedback inhibition of several NAD^+ catalyzed enzymatic reactions or stimulate other enzymatic reactions. Fructose increased in both plantlets and grains of the transgenic line as a result of activation of a mannitol dehydrogenase enzyme (EC: 1.1.1.67) that catalyzes conversion of mannitol to fructose, utilizing NAD^+ as a co-enzyme. The elevated levels of fructose can also be explained by the possible conversion of sorbitol from the other direction of the pathway utilizing NAD^+ as a co-enzyme of sorbitol dehydrogenase (E.C. 1.1.1.14). This might justify the decrease of sorbitol in transgenic lines as glucose is still produced by the conversion of fructose. Alternatively, sorbitol can be directly converted to glucose by aldose reductase (E.C. 1.1.1.21), which also utilizes NAD^+ as a co-enzyme. It is also suggested that disturbed sugar levels induces activities of hexokinase (E.C. 2.7.1.1) and galactokinase (E.C. 2.7.1.6) in plantlets and grains of the transgenic plants to enhance the conversion of mannose and galactose through several steps towards the production of F6P and G6P, respectively. In conclusion, these reactions justify the dramatic decrease in mannose and galactose levels, while increase in fructose and glucose levels.

Increasing of the soluble sugars level (monosaccharides) of both plantlets and grains of the transgenic lines should, consequently, result in the decrease in total carbohydrates (polysaccharides). This conclusion is supported by the accumulation of galacturonic acid, the building unit of pectin, and glucose, the building unit of cellulose, in the transgenic grains and plantlets. Pectin and cellulose are the main polysaccharides that might be affected. Reduction in the levels of these two polysaccharides, which are the first defense system towards plant pathogens, might lead to a more susceptible plant to several pathogens. On the other hand, plantlets and grains of the transgenic line must have a sweeter taste than any other wheat grains, due to its higher level of fructose. This high level of soluble sugars is more suitable for baking industry as they are fermentable and can easily be utilized by yeast during bread making. The unexpected presence of trace amounts of mannitol in the non-transgenic grains suggests the possible presence of a mannitol biosynthesis pathway in bread wheat.

Variation in mannitol content in individual lines has been reported in transgenic tobacco (Tarczynski *et al*., 1993; Rupérez and Toledano, 2003), Arabidopsis (Thomas *et al*., 1995; Zhifang and Loescher, 2003), petunia (Chiang *et al*., 2005), loblolly pine (Tang *et al*., 2005), and eggplant (Prabhavathi and Rajam, 2007). It was suggested that variations in mannitol content is a result of the different copy number and the positional effects of the transgene (Hobbs *et al*., 1990; Celebi-Toprak *et al*., 2005; Tang *et al*., 2005; Waditee *et al*., 2007). Our results only agree with the suggestion of the positional effects as the two mannitol-accumulting transgenics have one copy of the insert.

Additional information about the mechanism underlying the salt tolerance is required before we can conclude the role(s) of *mtlD* gene; as mannitol was not the only soluble sugar whose level was elevated. Abebe *et al*. (2003) suggested that the performance of mannitol-accumulating transgenic plants (0.6-2.0 umol g^{-1} FW) improved because of the scavenging of reactive oxygen, rather than osmoregulatory effects, as the plant did not accumulate sufficient mannitol to sustain the osmotic potential. In our study, we can indicate that mannitol also does not contribute to the osmoregulatory effects on our transgenic plantlets under salt stress as its level, in dry weight sample, reached about 0.4 mg/100 g DW (~20 umol g^{-1} DW). Chiang *et al*. (2005) indicated that two transgenic petunia lines overexpressing mannitol-1-phosphate dehydrogenase accumulate as low as 3.39 umol g^{-1} dry weight (DW) in average under normal condition. Therefore, levels of other carbohydrates in all petunia transgenic lines

were not changed. Hu *et al.* (2005) indicated that *mtlD* gene expression and/or mannitol accumulation may result in disturbed carbohydrate metabolism. Similar conclusions were reached in transgenic tobacco containing the *gutD* (Sheveleva *et al*., 1998) or *IMT1* (Sheveleva *et al*., 2000) gene. Hu *et al.* (2005) also indicated that the low levels of glucose and sucrose in tissue-cultured transgenic poplar plants might result in decreased energy available for other metabolic processes, thus leading to reduced biosynthesis and growth. The results of the present study indicate that glucose and fructose levels increased. We speculate that these increases help providing energy necessary for the plant to cope with abiotic stress conditions. In agreement with our speculation, Chiang *et al.* (2005) indicated that fructose, glucose and sucrose are important substrates in plant metabolism, hence, increase of these soluble sugars may enhance tolerance of the high-mannitol containing plants to abiotic stress.

Hu *et al.* (2005) indicated that the expression of the *mtlD* gene and accumulation of mannitol were unaffected by NaCl stress in transgenic plants. This may be due to the use of the constitutive *CaMV35S* promoter, which likely drives the transgene at similar expression levels regardless of external stimuli. In our finding, *mtlD* gene was driven by the constitutive maize *ubi* promoter. Therefore, we expect that levels of mannitol in *mtlD*-transgenics, estimated under normal condition, will likely be similar to those under stress condition. However, it might be worthwhile to measure other soluble sugars under salt stress in order to get a better picture on the indirect effect of the transgene as well as the environmental condition on the levels of indigenously expressed soluble sugars in wheat. However, Hu *et al.* (2005) indicated that the transgenic plants maintain higher cell membrane integrity under salt stress, which supports the hypothesis that mannitol might serve as a protective function. The reason for this speculation is that mannitol participates in water-enforced hydrophobic interactions and combines with high-molecular-weight compounds in biological membranes and, thus, maintains their biological activity.

Table 1. Means (a) and LSD values (at 5%) (b) for plant height (cm), total biomass per plant (g), grain yield per plant (g) and grain protein (%) of the two transgenic families 79/8 and 235/3 at T_3 and T_4 generation plus the wild-type Giza 163 (G163) at seasons 2009/2010 and 2010/2011, respectively, under normal (0 ppm salt, C) as well as salt stress (8000 ppm salt, T) condition.

(a) Means

Season	09/10				10/11			
Trait/genotype	C	T	Average	Reduction %	C	T	Average	Reduction %
Plant height (cm)								
79/8	54.3	49.4	51.9	9.0	51.6	50.8	51.2	1.6
235/3	50.1	51.5	50.8	-2.7	53.7	50.0	51.9	6.9
G163	71.2	68.2	69.7	4.2	74.9	70.4	72.7	6.0
Average	58.3	56.4			60.1	57.1		
Total biomass/plant (g)								
79/8	23.1	20.7	21.9	10.4	22.2	19.3	20.8	13.1
235/3	24.4	19.3	21.9	20.9	24.0	20.0	22.0	16.7
G163	29.6	21.5	25.6	27.4	28.6	22.8	25.7	20.3
Average	25.7	20.5			24.9	20.7		
Grain yield/plant (g)								
79/8	4.44	2.93	3.69	34.0	4.23	2.63	3.43	37.8
235/3	4.89	3.06	3.98	37.4	4.68	2.95	3.82	37.0
G163	5.12	2.61	3.87	49.0	5.00	2.31	3.66	53.8
Average	4.82	2.87			4.64	2.63		
Grain protein (%)								
79/8	-	-	-		14.2	13.5	13.9	5.0
235/3	-	-	-		13.0	14.5	13.8	-10.3
G163	-	-	-		14.4	13.9	14.2	3.5
Average	-	-			13.9	14.0		

(b) LSD (at 5%)

	Plant height		Total biomass/plant		Grain yield/plant		Grain protein	
Season	09/10	10/11	09/10	10/11	09/10	10/11	09/10	10/11
Treatment (T)	6.1	4.6	3.0	4.9	1.33	1.62	NS	NS
Genotypes (G)	11.2	13.1	2.6	3.1	0.38	0.46	NS	NS
T x G	13.4	15.5	2.1	2.3	0.30	0.52	NS	NS

Table 2. Means for total and soluble sugars contents (g/100 g dry weight) in grains and 21-day-old plantlets of the *mtlD*-transgenic T_4 families 79/8 and 235/3 and wild-type control (cv. G163).

	Grains		Plantlets	
	Total	Soluble	Total	Soluble
G163	90.880^{A}	3.100^{B}	28.170^{A}	3.063^{C}
79/8	85.970^{A}	4.577^{A}	25.440^{B}	8.870^{A}
235/3	87.410^{A}	4.187^{A}	26.290^{B}	7.450^{B}

Means within columns followed by the same letter are not significantly different by Duncan's New Multiple Range test (P<0.05).

Table 3. Means for soluble sugar levels (mg/100 g DW) in grains and 21-day-old plantlets of *mtlD*-transgenic T_4 families 79/3 and 235/3 and wild-type control (cv. G163).

	Grains			Plantlets		
Sugar	G163	79/3	235/8	G163	79/3	235/8
Mannitol	0.036^{B}	0.205^{A}	0.290^{A}	0.007^{C}	0.219^{B}	0.402^{A}
Sorbitol	0.570^{A}	0.107^{B}	0.179^{B}	0.082^{A}	0.077^{B}	0.013^{C}
Fructose	1.342^{C}	2.657^{A}	1.747^{B}	0.159^{C}	3.569^{A}	2.554^{B}
Mannose	0.154^{A}	0.078^{B}	0.088^{B}	0.148^{A}	0.052^{C}	0.123^{B}
Galactose	0.190^{A}	0.003^{C}	0.076^{B}	0.201^{A}	0.088^{C}	0.145^{B}
Glucose	0.701^{C}	0.813^{B}	0.988^{A}	1.809^{B}	4.409^{A}	3.726^{A}
Galacturonic acid	0.107^{C}	0.214^{B}	0.419^{A}	0.185^{B}	0.456^{A}	0.437^{A}

Means within rows followed by the same letter are not significantly different by Duncan's New Multiple Range test (P<0.05).

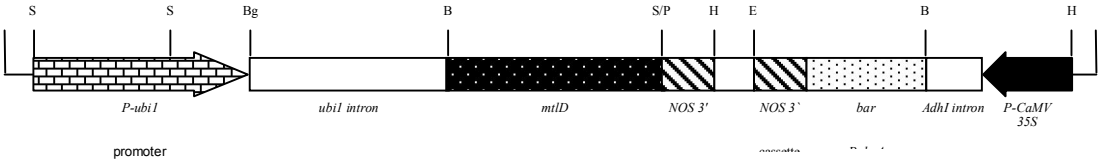

Figure 1. Map of the plant expression vector pAB_4 harboring *mtlD* and *bar* genes. S: *Sac*I, Bg: *Bgl*II, B: *Bam*HI, P: *Pst*I, E: *Eco*RI, H: *Hin*DIII.

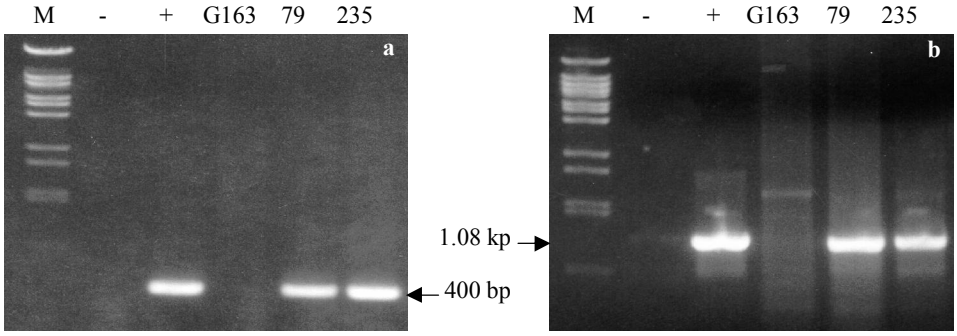

Figure 2. PCR products of partial-length *bar* gene (a) and full-length *mtlD* gene (b) of the two T_0 transgenic lines 79 and 235. Lambda DNA/*Bst*EII digest was used as a DNA standard (M) with MW shown in M & M section. -: No DNA, +: positive control (pAB_4), G163: negative control.

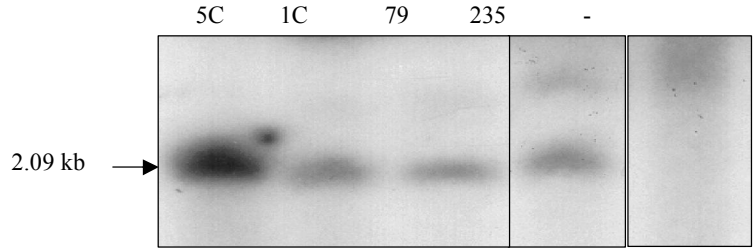

Figure 3. Southern blot of genomic DNA from T_0 transgenic lines 79 and 235 as well as wild-type (WT) control (cv. Giza 163). The DNA was digested with *Hin*DIII. *Sal*I/*Kpn*I *bar* gene fragment (200 bp) was used as a probe. -: WT, 1C: reconstructed one-copy, 5C: five-copy.

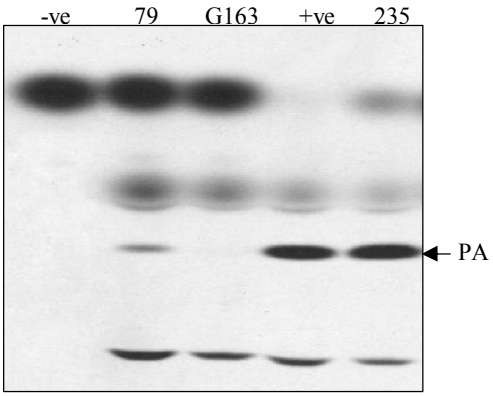

Figure 4. Thin layer chromotograph showing PAT activity in leaf protein extracts of the two *mtlD*-transgenic lines 79 and 235. +ve: *HVA1*-transgenic wheat cv. Hi-Line with *bar* gene (Bahieldin *et al*. 2005), G163: wild-type control, -ve: negative control (no protein).

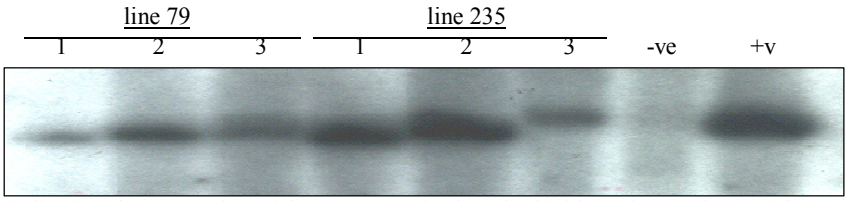

Figure 5. Northern blot indicating the expression of the *mtlD* gene in three herbicide-resistant plants of the T_1 progenies (1, 2 and 3) of the two transgenic lines (79 and 235) against a 400-bp *mtlD* fragment used as a probe. -ve: total RNA isolated from wild-type control (cv. Giza 163), +ve: total RNA isolated from transformed *E. coli* cells with mannitol-1-phosphate dehydrogenase gene.

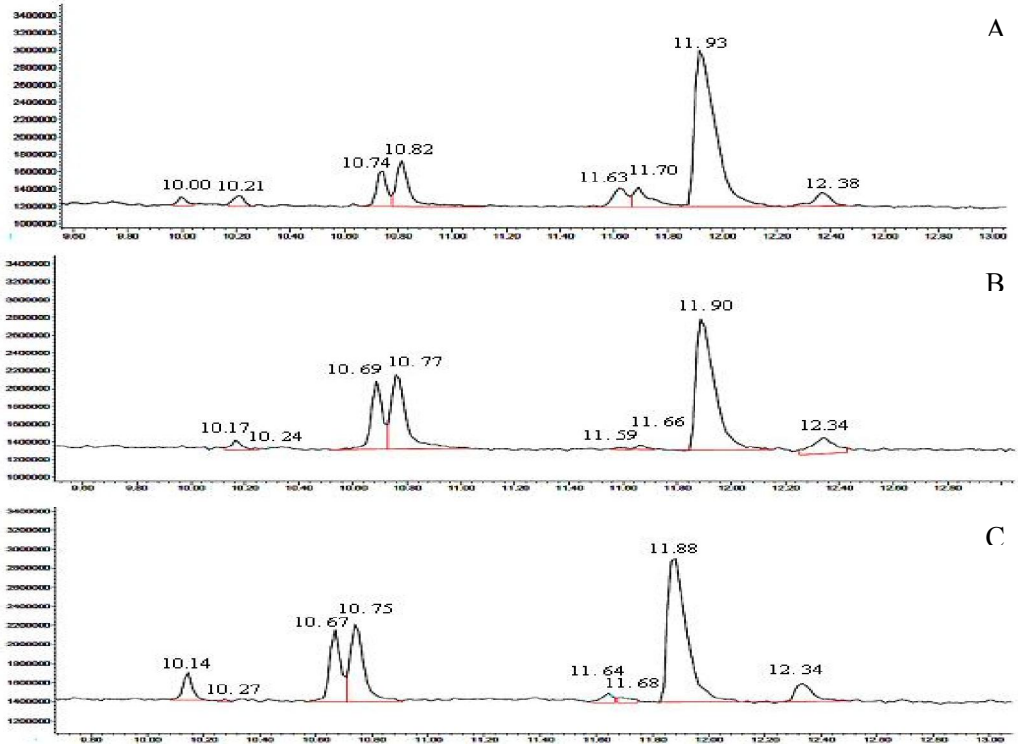

Figure 6. GC/MS chromatograms of soluble sugar contents extracted from 21-day-old plantlets of wild-type control G163, *mtlD*-transgenic wheat families 79/8 and 235/3 (A, B and C, respectively). GC/MS chromatograms (D, E and F) illustrate the soluble sugars extracted from grains of the same plants, respectively.

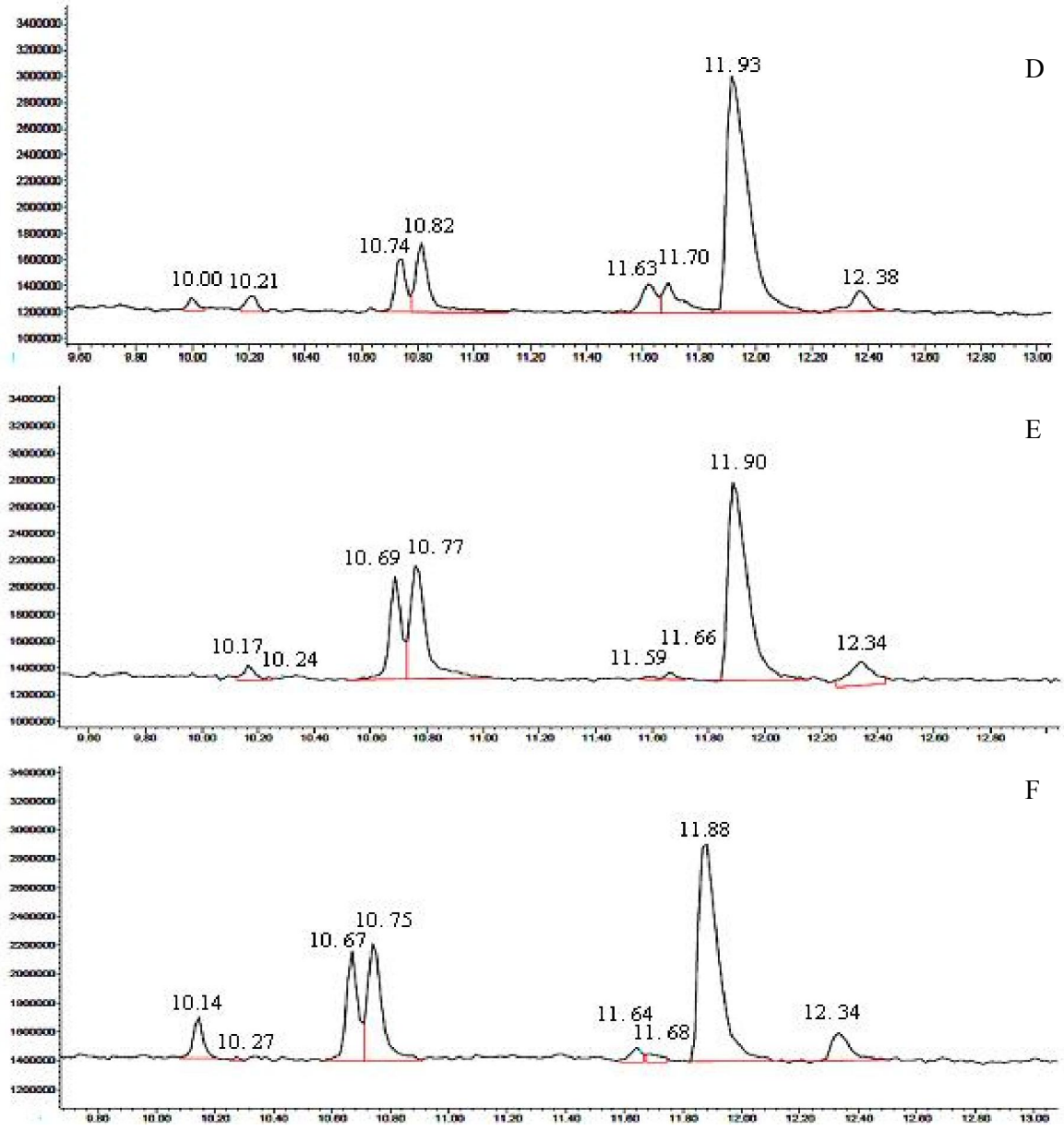

Figure 6. Continued

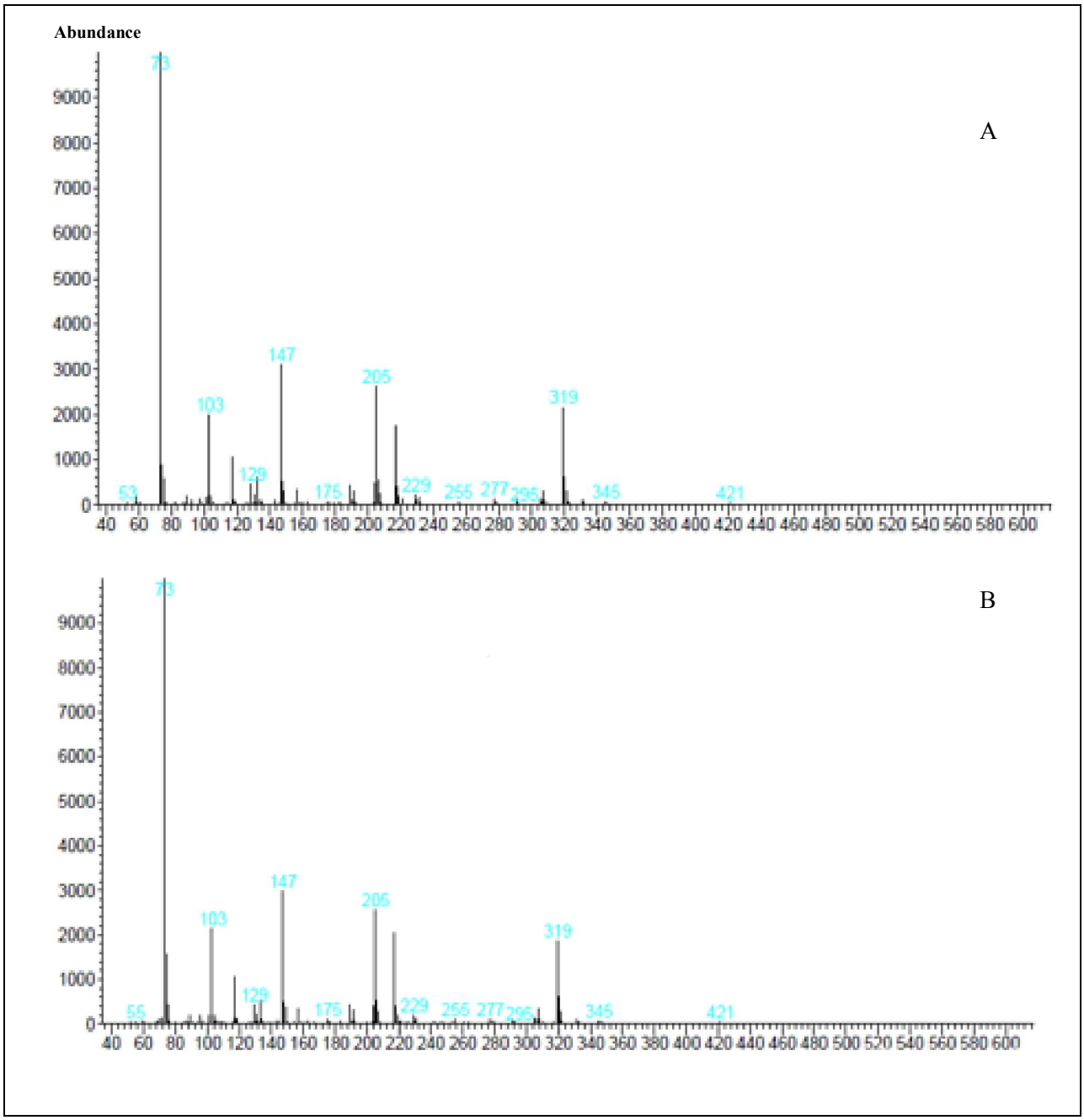

Figure 7. Mass spectrum of mannitol-hexa-O-trimethylsilyl obtained from the MS-library (A) and from the *mtlD*-transgenic wheat plant. The standard mannitol into the GC, with the same retention time and compatibility of the mass spectra of the mannitol peaks for all the analyzed samples, with the mannitol mass spectrum of the GC/MS library by a quality of 98%.

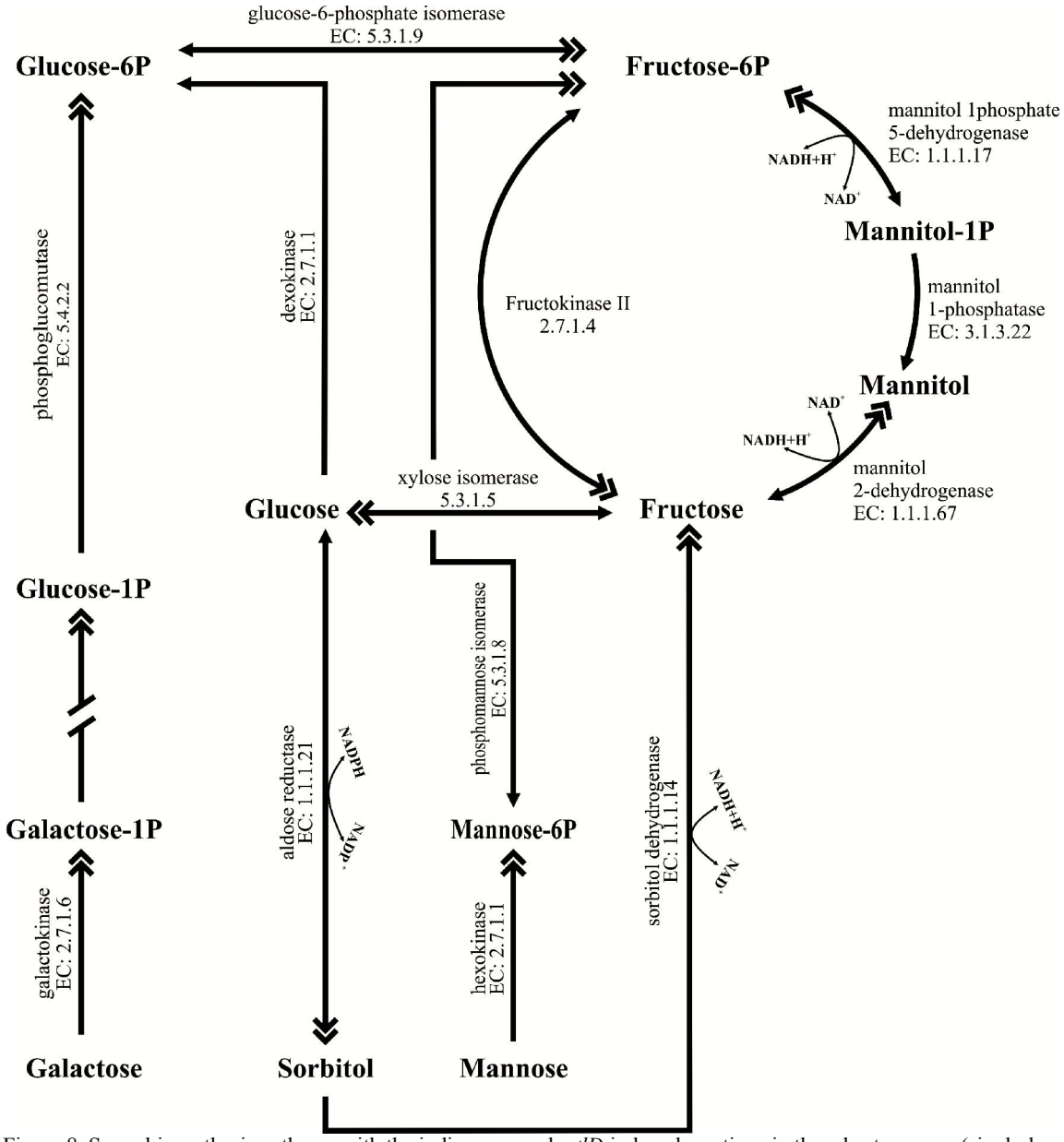

Figure 8. Sugar biosynthesis pathway with the indigenous and *mtlD*-induced reactions in the wheat genome (single-headed arrows). Double-headed arrows indicate further reactions possibly induced due to the expression of *mtlD* transgene.

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4/26/2013