Cloning of a functional mannose-6-phosphate reductase (M6PR) gene homolog from Egyptian celery plants (*Apium graveolens*): overexpression in non-mannitol producing plants resulted in mannitol accumulation in transgenic individuals

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Abstract Salinity is a major limiting factor affecting crops production, survival and distribution worldwide. Engineering dehydration stress tolerance in commercial crops is a trait of economic importance, especially in saline-affected areas. In this work, we are reporting the cloning of the *M6PR* gene homolog (encoding a key enzyme, mannose-6-phosphate reductase, for mannitol biosynthesis in celery) from Egyptian celery plants. Using RACE technique, the full-length Egyptian-*M6PR* gene (1333 bp) was cloned into pRI-201AN plant expression vector. Analysis of the cloned gene revealed that both American and Egyptian clones had both start and stop codons in frame and was found to be 930 base long. The newly cloned *EM6PR* gene was found to be 126 base longer than its American counterpart at the non-coding region. Six differences at nucleotide level between the Egyptian and American sequences were observed, three of which in the coding region resulting in three polymorphic amino acids differences (tryptophan vs. leucine, glutamine vs. histidine and isoleucine vs. leucine). The newly cloned gene was introduced to tobacco via *Agrobacterium* and PCR analysis

of T₀ plants indicated the presence of the *EM6PR* gene into 10 out of 38 tobacco individuals. Moreover, RT-PCR analysis confirmed the presence of *EM6PR* transcripts in 9 out of the 10 PCR positive plants. GC/MS analysis of some RT positive individuals indicated the accumulation of mannitol in transgenics tobacco, while mannitol was absent in non-transgenic controls.

Keywords Mannose-6-phosphate reductase · M6PR gene · Celery · Mannitol production · RACE technique

Introduction

Dehydration-inducing conditions as in salinity, drought and low-freezing temperatures are limiting factors affecting plant productivity and distribution worldwide (Chinnusamy et al. 2005). All those conditions impose water deficit on plant cells and, if prolonged, cause drastic effects on plant productivity and survival (Zhu 2001). Salinity, on its own, causes hyperosmotic and hyperionic effects; the hyperosmotic effect is exerted when outside-cell solute osmotic pressure's increases to a point where cells are no longer able to acquire their needs of water, resulting in water flux outside cells and cell-turgor loss; hyperionic effect results from the presence of high concentration of sodium ions in the root-growing zone, leading to sodium-toxicity (Zhu 2001).

Several strategies have been investigated to engineer salinity tolerance in economically important crops (Turan et al. 2012; Gupta and Huang 2014). Genes that were discovered to play important roles in plant adaptation and survival under extreme saline conditions could be grouped into five major groups. The 1st group includes genes that are involved in Na⁺ sequestration and compartmentation

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(Shi et al. 2000; Sottosanto et al. 2007; Pasapula et al. 2011). The 2nd group involves genes responsible for the detoxification of reactive oxygen species (ROS) (Bowler et al. 1991; Gupta et al. 1993; Aono et al. 1993; Foyer et al. 1995; Aono et al. 1995; Roxas et al. 1997; Wang et al. 1999; Kwon et al. 2002). The 3rd group includes genes coding for transcription activation of downstream genes needed for adaptation to salinity stress (Jaglo-Ottosen et al. 1998; Kasuga et al. 1999; Gilmour et al. 2000; Haake et al. 2002; Liu et al. 2014; Xiong et al. 2014; Rong et al. 2014). The 4th group includes genes coding for proteins and chaperons that are upregulated under saline condition (Hong et al. 1988). The 5th group includes genes coding for compatible solutes and osmoprotectant as in glycine-betaine (Le Rudulier et al. 1984), mannitol (Tarczynski et al. 1993; Everard et al. 1997), and proline synthesis (Hu et al. 1992).

Compatible solutes (e.g., proline, sugar alcohols, fructans, trehalose, quaternary ammonia compounds, and tertiary sulfonic compounds) are non-toxic organic metabolites of low-molecular weight that accumulate to high concentration at the cytosol without interfering/disrupting the normal cellular metabolism, and causing a decrease in osmotic potential of cells (Gorham et al. 1985; Hinch and Hagemann 2004). Therefore, many of the compatible solutes have been the subject of extensive studies by different groups to improve salinity tolerance in economically important crops (Sickler et al. 2007; Goel et al. 2011; Guerzoni et al. 2014). Sugar alcohols, such as mannitol, galactitol and sorbitol, represent the chemically reduced form of aldoses or ketose sugars in plants (Loescher and Everard 1996). Mannitol is naturally produced in numerous plant species and bacteria (coffee plant, celery, olive tree and avocado), and it has been estimated that about 30% of all photosynthetically fixed carbon in higher plants is transported in a sugar alcohol form (Bielecki 1982). Although sugar alcohols are the predominant form of translocated carbohydrates (mainly, in the form of sorbitol, mannitol, and galactitol) in higher plants, they are always translocated in association with a sugar, such as sucrose and raffinose (Williamson et al. 2002). Usually a single type of sugar alcohol is present or dominant (in a plant species), and sugar alcohols are characteristic to certain families as in sorbitol that is characteristic to the *Rosacea* family (prunes, plums, cherry and apple).

Mannitol is one of the most widespread sugar alcohol in plants; it is found in more than 100 plant species including many crops such as carrot, parsley, celery, green beans, cabbage, pumpkins, coffee and olive trees (Williamson et al. 2002). Plant species that produce mannitol as their primary photosynthetic products tend to have a substantial dehydration stress tolerance as in celery, coffee and olive trees (Loescher et al. 1992; Stoop et al. 1996); and because

of its water-like hydroxyl groups, it was suggested that it may mimic the structure of water (Galinski and Truper 1994) and, therefore, maintains an artificial sphere of hydration around macromolecules; it has also been suggested that mannitol serves as a scavenger of reactive oxygen species and preventing peroxidation of lipids which can lead to membrane damage (Smirnoff and Cumbes 1989; Loescher et al. 1992; Tarczynski et al. 1993; Bohnert and Jensen 1996; Stoop et al. 1996; Bohnert and Shen 1999). Moreover, synthesis of mannitol was also suggested to work as a supplemental mechanism to dissipate reducing power (NADPH) accumulated during the light reactions of photosynthesis. Therefore, mannitol production on its own is a defensive mechanism (Loescher 1987).

Mannitol biosynthesis gene was first isolated and studied in *E. coli*. The *mtlD* gene, which was cloned from *E. coli*, encodes for mannitol-1-phosphate dehydrogenase enzyme, an enzyme which is responsible for synthesis and catabolism of the mannitol (Tarczynski et al. 1992). It was reported that enhancement in tolerance to salinity was evident in many transgenic plants (tobacco, eggplant, wheat, poplar, sorghum, and potato) expressing the *mtlD* gene (Tarczynski et al. 1992; Karakas et al. 1997; Prabhavathi et al. 2002; Abebe et al. 2003; Hu et al. 2005; Maheswari et al. 2010; Rahnama et al. 2011; Hema et al. 2014); transgenic individuals expressing the *mtlD*-gene were better adapted to higher salinity (ranging from 75 up to 200 mM NaCl) (Tarczynski et al. 1993; Karakas et al. 1997; Prabhavathi et al. 2002; Hu et al. 2005; Rahnama et al. 2011). On the other hand, mannitol accumulation was minor in sorghum and potato transgenic individuals, suggesting that the role of mannitol in stress tolerance could be as a scavenger of ROS but not as an osmolyte (Maheswari et al. 2010; Rahnama et al. 2011). Similarly, expression of the *E. coli* GutD gene encoding glucitol-6-phosphate dehydrogenase, a key enzyme for the biosynthesis of the sugar alcohol sorbitol in maize plants, also increased sorbitol accumulation and enhanced salt tolerance compared to non-transgenic controls (Liu et al. 1999). Rice plants (*Oryza sativa* L.) expressing the *E. coli* GutD and the *mtlD* genes accumulated both sorbitol and mannitol in their vegetative tissue and were more salt tolerant than their non-transgenic counterparts (Huizhong et al. 2000).

Loescher et al. (1992) presented an evidence for the important role of the *M6PR* gene and its role in mannitol production in celery (*A. graveolens* L.). Photosynthetic-¹⁴C-radiolabeling studies in celery showed that mannose 6-phosphate (M6P) and mannitol-1-phosphate were among the early photosynthetic products in celery leaves, and the enzymatic assays revealed that an NADPH-dependent *M6PR* activity was detected in leaves. Everard et al. (1997) went on to purify the enzyme from celery leaf extracts; the enzyme had the ability to reduce the early photosynthetic

product M6P, aldo-keto sugar, into mannitol-1-phosphate which is converted to mannitol via non-specific phosphatase. Zhifang and Loescher (2003) compared the mode of action of *M6PR* gene in higher plants with *mtlD* in bacteria. They clearly stated that “the reaction of mannitol biosynthesis in bacteria is a reversible reaction: fructose 6-phosphate $\leftrightarrow$ Mannitol, while in higher plants mannitol is accumulated in irreversible manner”, therefore, leading to higher accumulation of mannitol. Later work by Chan et al. (2011) on sequence analysis of mannitol dehydrogenase (*MTD*)-cDNA gene in celery revealed that *MTD* gene certainly encodes the catabolic enzyme which oxidizes mannitol to mannose.

Zhifang and Loescher (2003) introduced the *M6PR* gene into *Arabidopsis*, and transgenic *Arabidopsis* plants showed normal growth patterns and developments in soil irrigated with 300 mM NaCl. Further study on *Arabidopsis* by Sickler et al. (2007) revealed that plants transformed with celery's mannose-6-phosphate reductase (*M6PR*) gene produce mannitol and grow normally in the absence of stress. Following a 12-day treatment of the wild type and transgenic plants with 100 or 200 mM NaCl, the total shoot fresh weight, leaf number, and leaf area were significantly greater in transgenic individuals versus wild-type plants. Also, the presence of the *M6PR* helped in maintaining photosystem II and carboxylation efficiencies in transgenic plants. Chan et al. (2011) reported that *Arabidopsis* plants overexpressing the *M6PR* gene had elevation in transcripts of genes downstream of ABA pathway, which was also coupled with transcripts of genes related to redox and cell wall-strengthening pathways.

In the current work, we sought of cloning a functional *M6PR* gene homolog (a key mannitol biosynthesis

enzyme) from Egyptian local variety and test the newly cloned gene in non-mannitol producing model plant (tobacco) to confirm mannitol production in transgenic tobacco individuals.

Materials and methods

Seed germination

Celery seeds (Egyptian and American) were surface sterilized with 70% ethanol for 30 s followed by submerging in 20% commercial bleach solution for 10 min. Seeds were washed with sterilized distilled H₂O 4–5 times and then germinated on hormone-free MS medium supplemented with 10.0 g/l sucrose and 7.0 g/l agar.

Induction of *M6PR* production in celery and RNA isolation

In vitro growing 3-week-old celery seedlings were submersed with 100 ml of 150 mM NaCl solution for 2 days. Seedlings were harvested and a 100 mg seedling tissue was used for RNA isolation. Tissues were grinded in liquid nitrogen to fine powder. Qiagen Rneasy Mini Kit (Cat #74904) was used following the standard protocol provided by the manufacturer.

Generation of cDNA and cloning of *M6PR* homolog

(1) Generating cDNA Degenerate forward and reverse primers were specially designed to amplify a 580 bp partial *M6PR* fragment (ParFor and ParRev, Table 1). RNA was

Table 1 Primers and oligos used in the amplification of partial *EM6PR* sequence and full-length amplification of cDNA ends (RACE) of the *M6PR* gene

Primer	Sequence (5'-3')	Conc.pmol	Length
ParFor	CGGCTTYAARATGCC	10	15
ParRev	GCCATACTCTKRCAGAA	10	18
CodFor	ATGGCAATAACTCTTAACACGGCT	10	25
CodRev	TCAAGCATAAACATCTATTCCCCAAGC	10	27
UFor	TTAGGCTACACAACACAGTTCT	12	22
URev	TAGAGAATGAGGGGAAGTGGT	12	21
Smarter IIA Oligonucleotide	AAGCAGTGGTATCAACGCAGAGTACXXXXX (X = undisclosed base in the proprietary SMARTer oligo sequence)	12	30
3'-RACE CDS Primer A	AAGCAGTGGTATCAACGCAGAGTAC(T) ₃₀ (N = A, C, G or T; V = A, G or C)	12	57
3'-RACE GSP1	TCGAGACGCACCCTTACTTCCAAA	10	24
5'-RACE CDS Primer A	(T) ₂₅ VN (N = A, C, G or T; V = A, G, or C)	12	27
5'-RACE GSP2	TGGTTGCATCAACCTCCCAAACAC	10	24
10× Universal Primer A Mix (UPM)	Long CTAATACGACTCACTATAGCAAGCAGTGGTATCAAACGCAGAGT	0.4	45

first treated with TURBOTM DNase enzyme (ThermoFisher sci., cat no. Am2238) by treating 5.0 µg of RNA with 2.0 U of the enzyme in a 50 µl reaction, following the standard protocol provided by the manufacturer. cDNA was generated using the M-MuLVTM enzyme (NEB, Cat #M0253S); the reaction was carried out as followed; in an eppendorf tube, 11.0 µl RNA from previous step was mixed with 2.0 µl degenerate ParRev primer and 4.0 µl of 2.5 mM dNTPs. The tube was incubated at 70 °C for 5 min, then immediately placed in ice for further 10 min; 1.0 µl of the M-MuLV enzyme was added to the reaction along with 0.25 µl RNase inhibitor and 2.0 µl of 10X buffer, and the tube was incubated at 42 °C for 90 min and then for 10 min at 90 °C. (2) PCR amplification of the partial sequence 2.0 µl of generated cDNA was used to amplify a 580 bp M6PR partial sequence using the following program (95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 60 s; this was followed by a 5 min cycle at 72 °C). (3) Amplification of the 3' and 5' ends of M6PR amplification was carried out using the SMARTer RACE cDNA Amplification Kit from Clontech (Cat #634923) following the manufacturer's protocol; a complete list of primers used for the cloning is provided in Table 1. (4) Sequencing of amplified sequence and GeneBank submission the purified 5' and 3' RACE products were cloned into pGEM-T Easy Vector system (Promega, Cat #A1360) for sequencing. Depending on the sequence results of 3' and 5' ends of the gene, two sets of primers were newly designed; the 1st were designed to clone the coding region of M6PR (CodFor and CodRev), the 2nd set was designed to isolate the 5' UTR with the coding region (UFor and URev). Full-length gene products were cloned into pGEM-T Easy Vector system, and the sequence was submitted into the GeneBank (Accession no KY652164).

Cloning into plant expression vector

E.coli clones carrying the full-length open reading frame with no stop codons at the right orientation were selected. Plasmid DNA isolated from the selected clones was digested with *Sac* II (NEB, Cat #R0157S) enzyme, and Klenow fragment enzyme (NEB, Cat #M0210S) was employed to create blunt ends, then the linear plasmids were digested with *Sal* I (NEB, Cat #R0138S) enzyme to release the gene from the pGEM T-easy vector. The product was run on 1% agarose gel and the full-length gene band was purified. Expression vector pRI 201-AN DNA (Takara, Cat #3264-3267; Fig. 4) was digested with *Nde* I (NEB, Cat #R0111S). Blunt ends were produced by Klenow enzyme. The linear plasmid was digested with *Sal* I

enzyme. The purified gene fragment was ligated into the pRI 201-AN expression vector and transformed into *E. coli* for amplification. After performing plasmid mini prep for positive colonies, followed by double digestion with *Sal* I and *Xba* I (NEB, Cat #R0145S) enzymes to confirm insert ligation, the plasmid was transformed into *Agrobacterium* strain LBA4404.

Tobacco transformation

Tobacco leaves from aseptically growing tobacco plants were dissected with scalpel and placed into a clean sterile petri dish. Using a sharp scalpel, the leaves were dissected into 0.5 cm discs. *Agrobacterium* harboring M6PR gene was grown overnight to an O.D = 0.5–0.7. Tobacco leaf segments were then soaked in bacterial culture for 15–20 min (the dissection of leaves was carried out in the presence of *Agrobacterium* cultures). Explants were then placed on sterile filter paper for a few min before being placed on initiation medium {MS basal salts mixture supplemented with Gamborg's B₅ vitamins + 2.0 mg/l BA + 0.2 mg/l NAA + 30 g/l sucrose and 6.5 g/l agar} for 2 days. Explants were removed and placed on establishment medium {initiation medium supplemented with 500 mg/l Cefotaxime for 3 weeks; explants were subcultured at least twice on the same medium with 3-weeks interval}. Shoots (0.5–2.0 cm long) were dissected and placed on the rooting medium {1/2 strength MS basal salts mixture + Gamborg's B₅ vitamin + 10.0 g/l sucrose + 0.1 mg/l NAA}.

Acclimatization

Healthy plantlets with well-developed roots were excised, washed from media debris and transferred into pots containing a mixture of clay, sand and peat moss (1:1:1) in a Conviron[®] unit at 25 ± 2 °C and 16/8 h light/dark photoperiod. For the first 7–10 days, plantlets were covered with plastic bags, and then the bags were gradually removed. Plantlets were watered every 2–3 days and fertilization was conducted with 2.0 g/l KristalonTM once a week.

PCR and RT-PCR screening

Leaf discs from 38 putative transgenic tobacco plants were excised for DNA and RNA isolation, using DNeasy[®] Plant Mini Kit (Cat # 69104) and Qiagen RNeasy Mini Kit, respectively, following the manufacturer provided protocol.

Fig. 1 RT-PCR in celery plants. **a** Amplification of 580 bp partial *M6PR* gene: lane 1: RT from American celery; lane 2: negative control for American celery reaction. lane 3: RT from Egyptian

celery; lane 4: negative control for Egyptian celery reaction **b** sequence alignment with published sequence

GC/MS analysis

GC/MS analysis was conducted to determine the presence or absence of mannitol. 250 mg of leaves tissue was grinded in liquid nitrogen followed by the addition of 250 μ l 80% ethanol. Samples were incubated at 90–95 $^{\circ}$ C for 2 h, then centrifuged at 14,000 rpm and supernatant was transferred into fresh tube. The supernatants were evaporated in steam bath by rotary evaporator at 80–85 $^{\circ}$ C.

150 μ l of NH₂OH-premedine 2.5% was added to the evaporated samples and placed in oven at 80 $^{\circ}$ C for 30 min. A 300 μ l TMCS (N,o-Bis(trimethyl-silyl)-acetamid:Chlorotrimethyl-silan in a ratio of 5:1) was added to the samples and placed in oven at 80 $^{\circ}$ C for 30 min. 2.0 ml of the derivatized samples was injected into GC instrument (Hewlett Packard 5890, Mass detector 5972) using HpInno-Wax Column (30 m $\times$ 0.25 mm $\times$ 0.25 μ m), with a Mobile phase Helium at flow rate of 1.0 ml/min. The

Fig. 2 The 3' and 5' reaction steps for cloning of the *M6PR* gene from Egyptian celery plants. **a** Lane M: 1 kb; lanes 1 and 2 American RNA; lanes 3 and 4 Egyptian RNA. **b** Lane 1: American 3' reaction,

lane 2 Egyptian 3' reaction. **c** 3' cloning and *EcoRI* digestion results. **d** Lanes 1 and 3 American 5' reaction; lanes 2 and 4 Egyptian 5' reaction. **e, f** 5' cloning and *EcoRI* digestion results

injection chamber temperature was set at 250 °C, where the temperature started at 100 °C and increased to 150 °C (at a rate of 7 °C/min) followed by raising temperature to 200 °C (at a rate of 4 °C/min) and the flow was detected against Wilay 7 N library. Mannitol calculation in samples was conducted according to the following formula: (Mannitol area/total area of extracted sugars) × 100 = % of mannitol in the sample.

Results and discussion

The first report on cloning of *M6PR* gene from mannitol producing plant came from the Loescher's group at Michigan State University (Everard et al. 1997), which was followed by further confirmation of mannitol production in *Arabidopsis* plants overexpressing the *M6PR* gene (Zhi-fang and Loescher 2003).

In the present study, a reverse transcriptase-based method was employed to clone a full-length *M6PR* gene homolog from Egyptian local variety of Celery, with the aim of testing the functionality of the newly cloned gene in a non-mannitol producing plant. This was performed using the SMARTer (Switching Mechanism At RNA Termini) RACE technology (Clontech, USA). In 5' SMART cDNA synthesis strategy, the polyA RNA is annealed with a modified oligo (dT) primer (5'-CDS primer A), and with the presence of amodified MMLV-reverse transcriptase enzyme that possess a terminal transferase activity (SMARTScribe RT) the enzyme uses this primer to

synthesize cDNA from the transcript. So, upon reaching the 5' end of the transcript it adds a 3–5 residue tail to the newly synthesized cDNA strand that could be recognized by a terminal series of modified bases in the SMARTer IIA Oligonucleotide. The newly synthesized cDNA would have a sequence tag attached to it and used as a template for PCR, with one primer (UPM) complementary to the sequence tag and the second primer (GSP2) specific to the gene of interest (Table 1). Similarly, for 3' RACE a modified oligo (dT) primer containing a 3' sequence tag (3'-CDS primer A) is used as a primer for reverse transcription with SMARTScribe RT; only then a gene-specific primer and (UPM) primer complementary to the sequence tag would be needed for 3' PCR amplification (SMARTer RACE cDNA Amplification kit user manual).

Therefore, the first step in the isolation of a full-length gene is to have a partial sequence of the gene. Total RNA was isolated from the Egyptian and American celery plants to generate cDNAs. Using sequence alignment of previously published *M6PR* homologs from dicotyledon plants, new degenerate primers were designed to amplify a 580 bp partial fragment of the targeted *M6PR* gene (Table 1; Fig. 1). Sequence analysis of the amplified partial sequence revealed a high sequence homology (98%) when compared to the published American celery sequence (Fig. 1).

In the 3' reaction, the 3'-CDS primer A (provided with the kit) was used for cDNA synthesis, and based on the previously amplified fragment, a gene-specific primer was designed, and both were used for 3' end amplification; a 750 bp fragment was amplified (Fig. 2). In the 5' reaction,

Fig. 3 **a** M: 1 kb marker, lane 1: –ve, lane 2: coding region 930 bp, lane 3: long fragment 1208 bp and lane 4, 5: lane 2 and 3 after gel purification. **b** pGEM plasmids carrying 930 and 1208 bp fragments digested with EcoRI. Lanes 1 and 3 (930 bp), lanes 5 and 6 (1208 bp), **c** Lane 1: pGEM plasmid carrying the 1208 bp fragment, lane 2: digested with SACII, lane 3: pGEM plasmid carrying the 930 bp fragment, Lane 4: digested with SACII. **d** Lane 1: Purified and

digested PRI 201-AN expression vector with SalI. Lane 2: Purified and digested 1208 bp fragment with SalI. Lane 3: Purified and digested 930 bp fragment with SalI. **e** Colony PCR for white colonies. Lane 1, 4, and 5 has the expected 1208 bp fragment, **f** XbaI and SalI digestion for the 1208 bp fragment, **g** NdeI digestion for PRI 201-AN carrying the 1208 bp fragment

5'-CDS primer A and SMART IIA oligo were employed in cDNA synthesis. Then, a gene-specific primer, based on the previously cloned partial sequence, was used for the 5' end amplification, and a 550 bp fragment was amplified (Fig. 2). The amplified fragments (580, 750 and 550 bp) were gel purified and cloned into pGEM T-easy vector (Fig. 3). Sequence analysis of the 750 and 550 bp fragments revealed that the Egyptian sequences were 90 and 36 bases longer than the American sequences at the 3' end and the 5' end, respectively (Fig. 4).

Analysis of the initial partial sequence (580 bp), and the sequences of the 3' and 5' (750 and 550 bp, respectively) versus the American published *M6PR* gene (1207 bp), revealed several differences that could be summarized as follows: (1) the full-length *EM6PR* was 1333 bp long (GenBank KY652164), about 126 base longer than its American homolog (using MAFFT program) (Fig. 5). Similarly, sequence analysis study of two cloned *M6PR* homologs from *Orobancha ramosa* L. and *O. aegyptiaca* L., revealed an identical sequence similarity of the coding region between the two sequences, with a 10 bp deletion at the 3' UTR of *O. aegyptiaca* L. (Delavault et al. 2002; Aly et al. 2009); (2) open reading frames analysis revealed that both genes had the start and stop codons in frame and were

Fig. 4 Schematic representation of the pRI 201-AN expression vector

930 bases long; (3) six changes at nucleotide level were observed between the Egyptian and American sequences, and out of those six changes, three nucleotides were found in the coding region resulting in 3 polymorphic amino

Fig. 5 Full-length gene sequencing of the Egyptian M6PR gene aligned to the American M6PR gene sequence available in the GeneBank

M6PR	-----tagagaaagaaggaggatagtttt
KY652164	ggcagcaagcaaaacagaagcaacacaaggagag tagagaaagaaggaggatagtttt *****
M6PR	ttaggctacacacacagttctaaaaatcttttatcgtttgttaggtttgacaatggcaa
KY652164	ttaggctacacacacagttctaaaaatcttttatcgtttgttaggtttgacaatggcaa *****
M6PR	taactcttaacagcggctttaaaaatgccggttctgggtctcggcgtctggcgtatggacc
KY652164	taactcttaacagcggctttaaaaatgccggttctgggtctcggcgtctggcgtatggacc *****
M6PR	gtaatgaaatcaagaatctcctcctttccgcgattaaccttgggttatcgtcactttgact
KY652164	gtaatgaaatcaagaatctcctcctttccgcgattaaccttgggttatcgtcactttgact *****
M6PR	gtgctgtgactacaagaatgagttagaagtagggaggcatttaagaggcgttttgata
KY652164	gtgctgtgactacaagaatgagttagaagtagggaggcatttaagaggcgttttgata *****
M6PR	ctgatcttgtcaagaggaggatctgtttattactaccaagctctggaactcagaccatg
KY652164	ctgatcttgtcaagaggaggatctgtttattactaccaagctctggaactcagaccatg *****
M6PR	gacatgtaattgaggcatgcaaaaacagctctcaagaagcttcagctagaatatcttgatc
KY652164	gacatgtaattgaggcatgcaaaaacagctctcaagaagcttcagctagaatatcttgatc *****
M6PR	tttacctcattcacttcccaatggcttctaaacattccggaattggtactactcgaagta
KY652164	tttacctcattcacttcccaatggcttctaaacattccggaattggtactactcgaagta *****
M6PR	tcttgatgatgaaggtgtttgggagggtgatgcaaccatttctggaagctacatggc
KY652164	tcttgatgatgaaggtgttt gg gaggtgatgcaaccatttctggaagctacatggc *****
M6PR	atgagatggagaagctggttgaatgggcttagtccgtagcataggaatcagcaactatg
KY652164	atgagatggagaagctggttgaatgggcttagtccgtagcataggaatcagcaactatg *****
M6PR	atgtttacttgaccagagatatctgtcatattccaagatcaagcctgctgtaaatcaga
KY652164	atgtttacttgaccagagatatctgtcatattccaagatcaagcctgctgtaaatcaga *****
M6PR	tcgagacgcacccttacttccaaagagattctctgatcaaatctgtcagaagtatggca
KY652164	tcgagacgcacccttacttccaaagagattctctgatcaaatctgtcagaagtatggca *****
M6PR	ttgctatcacagcacacacaccactaggcggcgcatggcctaactgagcgatttgat
KY652164	ttgctatcacagcacacacaccactaggcggcgcatggcctaactgagcgatttgat *****
M6PR	cagtttctgtgcttagatgatccagttcttaagaaattatctgacaaacacaacaagtcac
KY652164	cagtttctgtgcttagatgatccagttcttaagaaattatctgacaaacacaacaagtcac *****
M6PR	cagctcagattgttctccgttggggtgtgcagcgcaacacaattgtaattccaagtcac
KY652164	cagctcagattgttctccgttggggtgtgcagcgcaacacaattgtaattccaagtcac *****
M6PR	cgaaaactaaaagactcgaggaaaacatcaacatttttgactttgagttgagcaaggag
KY652164	cgaaaactaaaagactcgaggaaaac ct caacatttttgactttgagttgagcaaggag *****
M6PR	atatggagctcatcaaaacatggagcgcaaccaaaggagtaacacacctgctaaagctt
KY652164	atatggagctcatcaaaacatggagcgcaaccaaaggagtaacacacctgctaaagctt *****
M6PR	ggggaatagatgtttatgcttgatggcataaacacattcttactgtattttatcattgt
KY652164	ggggaatagatgtttatgcttgatggcataaacacatt ct tactgtattttatcattgt *****
M6PR	tattccacaattcagagtgtgtgtcatttttacttgctattgtgtgtggagggaatgtg
KY652164	tattccacaattcagagtgtgtgtcatttttacttg ct tattgtgtgtggagggaatgtg *****
M6PR	tgttgagttgttagtaattgtacaaggcataaagcctttaaataaaccatcatatgta
KY652164	tgttgagttgttagtaattgt ta aaggcataaagcctttaaataaaccatcatatgta *****
M6PR	aatgggaaatgccatgatttggtca-----
KY652164	aatgggaaatgccatgatttggtca ctttgtattttatttattcaaccactccctca *****
M6PR	-----aaaaa
KY652164	ttctctattgtttgtattgtttatttcttaacatttgagcaatttatatcac aaaaa *****
M6PR	aaaaaaaaaaaaa
KY652164	aaaaaaaaaaaaa *****

Fig. 6 The coding region translation of *EM6PR* alignment with *M6PR*. Changes in amino acid sequences is represented via red color letters

M6PR	MAITLNSGFKMPVLGLGVWRMDRNEIKNLLLSAINLGYRHFDCAADYKNELEVGEAFKEA
KY652164	MAITLNSGFKMPVLGLGVWRMDRNEIKNLLLSAINLGYRHFDCAADYKNELEVGEAFKEA
M6PR	FDTDLVKREDLFITTKLWNSDGHVIEACKNSLKKLQLEYLDLYLIHFPMASKHSGIGTT
KY652164	FDTDLVKREDLFITTKLWNSDGHVIEACKNSLKKLQLEYLDLYLIHFPMASKHSGIGTT
M6PR	RSILDDEGVWEVDATISLEATWHEMEKLVEMGLVRSIGISNYDVYLTRDILSYSKIKPAV
KY652164	RSILDDEGVLEVDATISLEATWHEMEKLVEMGLVRSIGISNYDVYLTRDILSYSKIKPAV
M6PR	NQIETHPYFQRDSLIFKFCQKYGIAITAHTPLGGALANTERFGSVSCLDDPVLKKLSDKHN
KY652164	NQIETHPYFQRDSLIFKCHKYGIAITAHTPLGGALANTERFGSVSCLDDPVLKKLSDKHN
M6PR	KSPAQIVLRWGVQRNTIVIPKSSKTKRLEENINIFDFELSKEDMELIKTMERNQRSNTPA
KY652164	KSPAQIVLRWGVQRNTIVIPKSSKTKRLEENLNIFDFELSKEDMELIKTMERNQRSNTPA
M6PR	KAWGIDVYA
KY652164	KAWGIDVYA

Fig. 7 Different stages of transgenic tobacco shoots development. **a** Regeneration of tobacco shoots on regeneration medium. **b** Well-developed shoots on rooting medium. **c** Transformed shoots acclimatized in small pots in growth chamber. **d** Fully developed tobacco plants with flowers

(a)

(b)

Fig. 8 **a** PCR screening of putative transgenic tobacco shoots, white arrows indicating positive samples. **b** RT-PCR of the positive tobacco plants indicating the presence of *M6PR* transcripts in 9 out of the 10 plants tested

acids differences W (tryptophan) versus L (leucine), Q (glutamine) versus H (Histidine) and I (isoleucine) versus L (leucine) (Fig. 6).

The full-length fragment was cloned and transformed into competent *E. coli* cells, insert size and sequence was determined via EcoRI digestion and sequence analysis. The

Fig. 9 **a** Chromatogram for the soluble sugars in the negative control sample. **b** Chromatogram of mannitol in the transgenic tobacco sample. In **b** the mannitol peak is indicated with a red circle

pGEM-T Easy vectors carrying the insert were isolated, and digested to release the insert, which was ligated into pRI 201 AN plant expression vector (Fig. 4). The pRI-201 AN vector carrying the EM6PR was then used to transform *Agrobacterium*, strain LBA 4404, for tobacco transformation (Fig. 7).

Well-developed shoots were screened via PCR for the presence of the EM6PR gene. A total of 38 putatively transgenic tobacco plantlets were screened using EM6PR-specific primers; ten plantlets were found to be PCR positives and when RT-PCR was performed to confirm the presence of EM6PR transcript, nine plantlets were positive (Fig. 8). Further analysis was conducted by selecting two well-established transgenic tobacco plants expressing the EM6PR gene, along with non-transformed tobacco plants for GC/MS analysis. Mannitol assessment data revealed that mannitol peak was only observed in total soluble sugars extract derived from transgenic tobacco plants (only one sample is showed in Fig. 9b), while this peak was completely absent in non-transgenic tobacco extract (Fig. 9a); mannitol represented about 51.6% of the total soluble sugars extracts in transgenic tobacco plants (an average of two samples), indicating that the newly cloned EM6PR gene (GenBank KY652164) could be used for further work to improve dehydration stress tolerance in economically important crops.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interests. The current work was partially funded through a Science and Technology Development Fund (STDF) Grant No. 400, Egyptian Ministry for Scientific Research.

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