Enhancement of salt tolerance of the tomato cultivar Edkawy under saline conditions using genetic transformation with the AtNHX1 gene

El-Awady A.M. Mohamed[1,2], El-Tarras A.E Adel [1,2], El Dessoky S. Dessoky [1, 3], Essam El-Den M. Khaled [1,3] and Ibrahim N. E. [4,5]

[1]Scientific Research Deanship, Biotechnology Research Unit, Taif University, Taif, KSA
[2]Genetics Department, Faculty of Agriculture, Cairo University, Giza, Egypt
[3]Agriculture Genetic Engineering Research Institute (AGERI), Agriculture Research Center (ARC), Giza, Egypt
[4]Department of Biotechnology, Faculty of Science, Taif University, Taif, KSA
[5]Bioinformatics Department, Genetic Engineering and Biotechnology Institute, Sadat University, Sadat City, Egypt

ABSTRACT

Tomato (*Solanum lycopersicum* L.), is considered one of the most important vegetable crops grown in KSA. Recently, salinity and using of the costly desalinized water decrease the production and increase its cost. Development of high salt tolerance cultivars are important demand for expansion of tomato cultivation in the kingdom.

The present study was aimed to optimize a transformation protocol that can be used efficiently with the valuable Egyptian salt-tolerant cultivar Edkawy as well as to explore the further potentials improvement of its salt tolerance. Simultaneously, we optimized the transformation protocol and used one of the safe, plant origin and effective salt tolerance gene, AtNHX1 for transformation. Hypocotyls explants exceeded from 8 days seedlings were co-cultivated with agrobacterium for 48 h before transferring to shoots induction medium. Adventure shoots were successfully obtained directly from the explants or from callusing explants using MS medium supplemented with 1 mg L^{-1} 2IP and 0.1 mg L^{-1} naphthaleneacetic acid (NAA). The regeneration frequency was calculated as more than 90 %. Number of shoots/ explants was increased after subculture in MS medium containing 1 mg L^{-1} IBP and 0.3 mg L^{-1} Kinetin. Rooting was successfully performed in MS medium containing 0.18 mg L^{-1} indolacetic acid (IAA) and 2% sucrose. After confirming the transformation events in the F_0 plants by PCR analysis and prove the expression of the reporter GUS gene, the transformation efficiency was calculated as a

24%. F1 plants expressed the BAR gene were selected after germination of F_1 seeds in MS medium containing the herbicide, pasta as a selection agent for 3 weeks.

These data indicates the successful transformation of the local tomato cultivar, Edkawy with suitable transformation efficiency using 2IP that replaces the costly plant growth hormone Zeatin. The present reported protocol can be used for further improvement of the Edkawy cultivar and the obtained transgenic F_1 plants will be evaluated for their salt tolerance capabilities.

Key wards: Tomato, Edkawy, transformation, 2IP, Zeatin, Salt tolerance

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Introduction

In KSA, tomato (*Solanum lycopersicum* L., formerly *Lycopersicon esculentum* L.), is considered one of the most important vegetable crops grown as the tomato cultivated aria represent 13.5% of the total vegetables cultivated aria. Tomatoes are cultivated in KSA in both open field and greenhouse. Recently, salinity intolerance is one of the most important constraints to tomato production in KSA. Increased water and soil salinity produces tomatoes with improved taste, yet concentrations as low as 25 mM NaCl will initially impact fruit yield and size while higher concentrations will eventually prevent growth. Most of tomato production is obtained using the greenhouse and using the costly desalinized water for irrigation. Development of high salt tolerance cultivars are important demand for expansion of tomato cultivation using the reused water or the relatively saline underground water and hence decrease the production cost.

High salinity in soil solution inhibits plant growth. Accumulation of salts in irrigated soil is a primary factor depressing yield in crop production. When the electrical conductivity (EC) of a soil solution is over 4 dS m^{-1}, the yield of most crops is reduced (Munns, 2005). This EC level is equivalent

to 40 mmol L^{-1} NaCl. Among salts, Na^+ is the major cause of ion-specific damage to plants. High concentrations of Na^+ in the rhizosphere and apoplast impose osmotic stresses on plants, resulting in a reduction in water uptake or in the removal of water from plant cells. High salinity causes ion imbalance and hyperosmotic stress in plants. Elevated concentration of Na^+ in the cytosol can cause a range of metabolic toxicities by competing with K^+ for binding to active sites of various enzymes (Munns, 2005).

One of the most important mechanisms for plant adaptation to high salinity is the exclusion of Na^+ ion from cells, which has been proposed as a function of a Na^+/H^+ antiporter and Na^+-ATPase (Foolad and Jones, 1993). Among the known antiporter genes, the Arabidopsis thaliana AtNHX1 was consistently used in several plants such as Arabidopsis (Apsa et al., 1999), Canola (Zhang, et al, 2001), cotton (He et al., 2005) and wheat (Xuea et al., 2005) resulted in significantly enhancement of their salt tolerance. In tomato plants, Blumwald and coworkers (Zhang and Blumwald 2001) reported that likewise allowed those plants to grow in the presence of 200 mM NaCl that is equivalent to 40% of the salt concentration of seawater and will inhibit the growth of almost all crop plants. The growth of the wild-type plants in this study was severely inhibited by the presence of 200 mM NaCl in the growth solution, and most of the plants died or were severely stunted. However, the transgenic plants grew, flowered, and produced fruit. The high sodium and chloride content in the leaves of transgenic plants grown in salty water demonstrated that enhanced vacuolar accumulation of Na^+ ions, mediated by the Na^+/H^+ antiport, allowed transgenic plants to ameliorate the toxic effects of Na^+. Most notable was the production of fruit by these transgenic plants grown in the presence of 200 mM NaCl. While the transgenic leaves accumulated Na^+ to almost 1% of their dry weight, the fruits displayed only a marginal increase in Na^+ content and a 25% increase in K^+ content.

The Egyptian tomato cultivar Edkawy was developed using traditional selection by the Egyptian farmers in Edko region, El-Beherah Governorate, Egypt. This cultivar showed remarkable salt tolerance capability (Saker and Rady, 1999). However, the productivity and value of this cultivar could be greatly increased by the introduction of stably inherited traits such as natural salt tolerance and auto-resistance against fungal diseases using genetic transformation. During the last two decades *in vitro* regeneration and transformation of tomato was well established worldwide. Several reports on genetic transformation of tomato have been published (Velcheva et al. , 2005; Roy et al. , 2006; Shahriari et al. , 2006; Koc et al. , 2007, Lilian et al, 2010) in which the transformation efficiencies ranging from 10 to 33% were obtained for several cultivars. However, it was shown that the most

parameters affecting transformation efficiency is the *in vitro* regeneration ability, which is basically determined by the explants and the genotype.

Here, we report the optimization of genetic transformation protocol for the Egyptian salt-tolerance tomato cultivar and explore the further potentials improvement of its salt tolerance. Therefore, one of the safe, plant origin and effective salt tolerance gene, AtNHX1 was used for transformation.

Materials and Methods

Agrobacterium–mediated transformation of the tomato with salt tolerant gene

1-Plant material

Seeds of the Egyptian tomato cultivar, Edkawy were obtained from the Agriculture Research Center (ARC), Giza, Egypt and were germinated to obtain the hypocotyls explants.

2-Construct

The construct containing the b-glucuronidase (GUS), Bar and *AtNHX1* genes is provided by the Department of Genetics, Faculty of Agriculture, Cairo University, Egypt (Fig. 1).

Figure 1. Plasmid map of the transformation vector 35S ATNHX1.

Media

The constitution of different MS medium used in the study is as the following:

Co-cultivation medium

MS basal medium (Murashige & Skooge 1962) sublimated with 1 mg L^{-1} BAP and 1 mg L^{-1} kinetin.

-shoot induction medium

MS basal medium sublimated with 1 mg L^{-1} 2IP or 1 mg L^{-1} Zeatin and 0.1 mg L^{-1} naphthalene acetic acid (NAA), 8 g L^{-1} agar and 500 mg L^{-1} claforan.

-Shoot multiplication medium

MS media supplemented with 1 mg L^{-1} BAP and 0.3 mg L^{-1} kinetin and 500 mg L^{-1} claforan.

-Shoot Elongation medium

Hormone free -MS medium+ 500 mg L^{-1} claforan.

-Rooting medium

MS medium (2% sucrose) supplemented with 0.18 mg L^{-1} indolacetic acid (IAA) and 250 mg L^{-1} claforan.

Methods

Tomato plant Transformation

For the *Agrobacterium*- mediated transformation of tomato plants, the protocol described by Hamza and Chupeau 1993 and El-Awady et al 2003 was modified as the following: hypocotyls explants were exceeded from 8-day-old-in vitro grown seedlings. After co-cultivation for 48 h with Agrobacterium strain LB4404 harboring the transformation vector, the explants were transferred to two different medium for callus and shoots induction medium (containing 1 mg L^{-1} Zeatin or 1 mg L^{-1} 2IP) with 0.1 mg L^{-1} naphthaleneacetic acid (NAA). Three weeks after infection, micro shoots were initiated directly from explants or from callusing explants and were transferred to shoot multiplication medium. Subsequently, multiple shoots were removed individually and transferred to the MS medium without growth regulators for elongation. Regenerated plants (2–3 cm in height) were transferred to bottles containing 25 ml of rooting medium. All cultures were incubated in a growth chamber at 25 °C, for 16 h of light. Rooted shoots (8–10 cm in height) were transferred to soil and grown in the green house. Transformed and non-transformed tomato plants (based on PCR confirmation) were selfed to obtain the T_1 seeds. Tomato fruits were collected after 4 months of culture. Subsequently, seeds were sterilized and placed on germination medium containing Pasta for further selection of the transformed seedlings. Surface sterilized untransformed seeds were germinated in the same medium as a control. Rooted plantlets were noticed and scored for the resistance for the herbicide, pasta after 3 weeks of culture. The inheritance of transgene into the resistance seedlings will be confirmed by the PCR analysis.

Molecular verification of transformed plants

1- PCR analysis

Genomic DNA from leaves (0.5 to 1 g FW) of wild type and transgenic plants was prepared by the Plant DNA preparation Kit, gena (Jean Bioscience, Germany) and used for PCR analysis. Two sets of primers were used for the detection of the bar and ATNHX1 genes by PCR in the transgenic tomato plants with the 35s-ATNHX1 construct. The sequence of the specific primers for the AtNHX1 gene was F 5'/ TTT TGGCTTAAATTCATATTCAA /3' and R 5'/ GGCTTAAAGTGTCCATG 3'/. While the sequence of the specific primers for the bar gene is F 5'/ GAGGAGTGG ACG GACGAC /3' and R 5'/ GAAGTCCAGCTGCCAGAAAC /3'. The volume was completed up to 20 µl with sterilized distilled water. The PCR temperature profile used for the amplification consists of initial denaturation cycle at 94°C/ 5 min followed by 35 cycle of 94°C/1min, 53°C/1min, 72°C/1min and terminal extension cycle at 72°C /7min for AtNHX1 gene. The PCR program for bar gene is similar to that of AtNHX1 gene except the annealing temperature which was 56°C. Finally, the PCR products were electrophoresed on 1% agarose.

2- Histochemical GUS assay

The histochemical assay to screen for the expression of beta glucurodinase (GUS) activity in transgenic tomato plants was carried out according to the method of Jefferson et al. 1987. For analysis, callus and leaves were incubated in a reaction buffer containing 12.5 mM K3Fe (CN) 6, 12.5 mM K4Fe (CN) 6, 20% methanol, 1% Triton X-100 and 38.3 mM 5-bromo-4-chloro-3-indolyl glucuronide as a substrate for the enzyme. The explants incubated in staining solution at 37°C for 24 h and the developed blue spots were recorded.

Results and Discussion

In the present study, an optimized protocol for transformation and regeneration of the Egyptian tomato (cv. Edkawy) has been developed. As Edkawy is relatively salt-tolerant, the Na/H^+ antiporter

gene AtNHX1 was used for transformation to explore the further possibilities to enhance its salt tolerance.

Unfortunately, the application of such safe plant origin genes rendering plants tolerant against advised conditions, is still far from routine applications in tomato, particularly when it comes to commercial varieties (Horsch et al., 1985).

Transformation of tomato plants with the desired gene were successfully performed in different stages as illustrated in Fig. 2. As recommended in most tomato transformation protocols, the hypocotyls were exceeded from 8 days old tomato seedlings (Dan et al, 2006; Sun et al, 2006; Frary et al. 1996). More than 90% of the explants developed micro adventure shoots either from the callusing explants using the medium containing (1 mg L^{-1} 2IP or 1 mg L^{-1} Zeatin) with, 0.1 mg L^{-1} NAA (Fig. 2-A). Direct shoot initiation, rather than the callus, was observed at the edge of the hypocotyls proximal end. Similar observations were reported by Raj, et al. (2005), Marmar El-Siddig et al. (2009.) and Peres, et al. (2001) who attributed this to be due to hormonal metabolism and/or genetic background of organogenetic competence. The regeneration frequency was calculated as a percentage of the number of regenerated explants/ total number of explants (Ling et al., 1998). Using of 2IP, a cytokinin that is much less expensive than Zeatin and obtaining almost the same results is an advantage for our developed protocol.

Number of shoots/ explants was calculated as 2-3 shoots and interestingly, it was increased to 5-6 shoots/ explants after the subculture in shoot multiplication medium containing 1 mg L^{-1} BAP and 0.3 mg L^{-1} kinetin for two weeks. Marmar El-Siddig et al.(2009.) reported the occurrences of higher frequency of shoot initiation from both transformed and untransformed plantlets in medium containing BA comparing with that containing Zeatin during the transformation of tomato (cv. Castel rock). Moreover, Zeatin is more expensive and less chemically stable than BAP (Letham and Palni, 1983) Shoot elongation was performed in free hormone MS medium, while rooting was successfully performed in MS medium containing 0.18 mg L^{-1} indolacetic acid (IAA) and 2% sucrose (Fig. 2-B).

Based on the PCR analysis of the regenerated plants (Fig.3), 24% of them were transformed and accordingly the transformation efficiency was calculated as 24%. High frequency of shoot induction in the untransformed tissues may be attributed to the competence of the cells for regeneration (Velcheva et al., 2005). Similar reports showed that transformation efficiencies ranging from 10 to 33% were obtained for several tomato cultivars (McCormick et al, 1986; Frary et al. 1996; Ling et al. 1998; Cortina and Culiáñez-Macià, 2004). For Edkawy cultivar, Saker and Rady, 1999, reported a

transformation frequency of 20% using hypocotyls explants and medium containing 2 mg L^{-1} l Zeatin and 0.2 mg L^{-1} IAA.

Figure 2. Agrobacterium-mediated transformation of tomato plants with the AtNHX1 construct. A showed the callus and shoots initiation, B is shoots elongation stage and C is acclimatization and growing of whole plants.

In acclimatization stage, regenerated and transformed tomato plantlets were cultivated in pots containing petmoss and soil and covered with plastic packages under thermal lights for hardening and growing in the green house until obtaining the tomato fruits (after self pollination) that contain the F_1

seeds (Fig. 2-C). All regenerated plants exhibit normal morphological characters. Although, some transgenic plants were sterile, eleven transgenic plants were fertile and set viable seeds

As the transformation vector (Fig. 1) consisting of three genes, GUS and the Bar gene downstream of one promoter and terminator and AtNHX1 gene under another Nos. promoter and terminator. The molecular confirmation of transformation event was performed using PCR analysis with specific primers for BAR and AtNHX1 genes. The results confirmed stable integration of the bar and AtNHX1 genes in the transformed plants (F_0) as shown in Fig. (3-a and b). While the expected amplification bands with molecular weight of 350 and 500 bp (corresponding to bar and AtNHX1 genes, respectively) were detected in positive control (lane P) and the putative transgenic plants (lane 1,2,3 and 4), they were not detected in the negative control (lane N).

In addition, the expression of GUS gene was confirmed in the callus and leaves of T_0 plants (Fig. 4). Expression of GUS gene in the F_0 plants indicated the possible expression of Bar gene in these plants as the two genes are under the control of the same promoter.

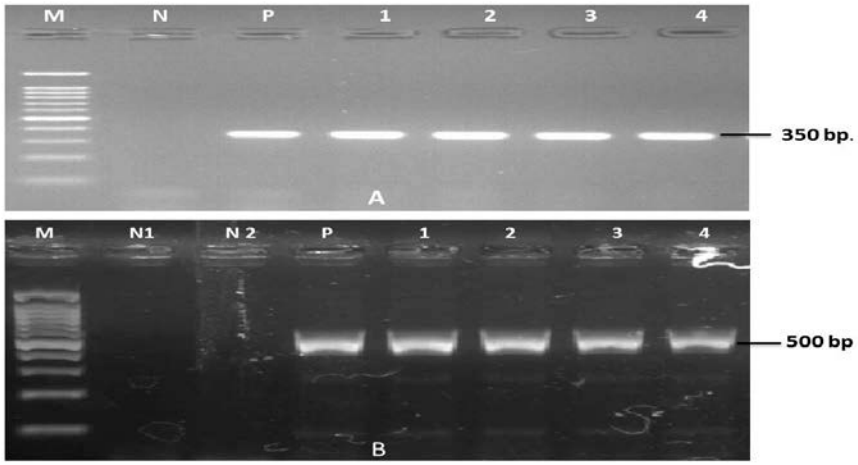

Figure 3. Molecular confirmation of the transformation event of bar and AtNHX1 genes in T_0 plants.

The PCR amplification of the 350 bp. fragment corresponding to bar gene (A) and the 500 bp. fragment corresponding to the AtNHX1 gene (B) in putative transgenic tomato plants; M: 100 Kb DNA ladder; N: non transgenic plant P: Positive control; 1, 2, 3 and 4 are positive tested plant samples.

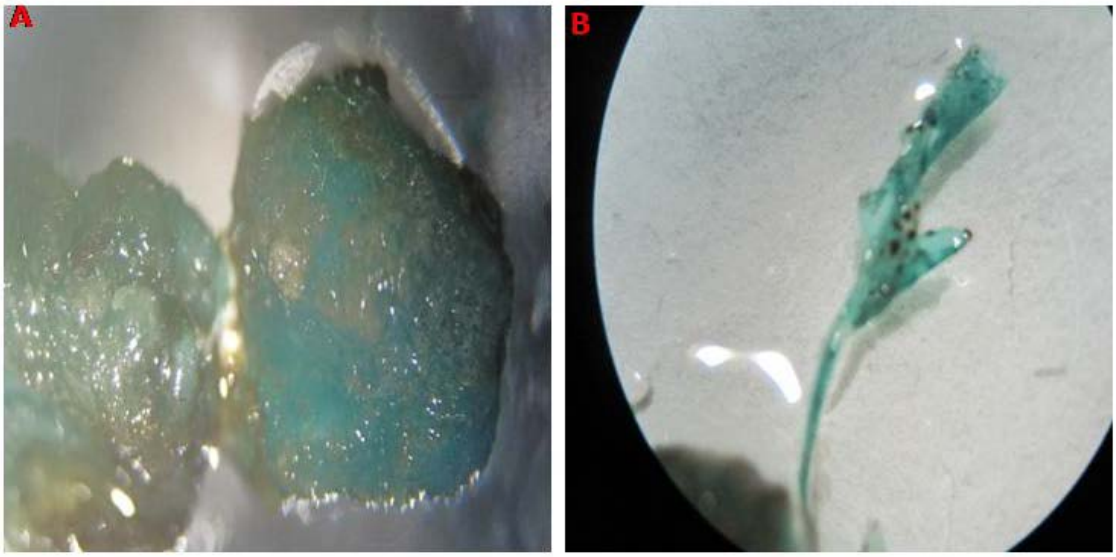

Figure 4. Expression of reporter marker GUS gene in the Callus (A) and leaves (B) of the T_0 plant (T_0-9)

Moreover, inheritance of the transgenes into the F_1 plants was checked by the detection of the expression of bar gene. Surface sterilized untransformed seeds were germinated in the MS medium containing the herbicide, pasta as a selection agent. After 3 weeks of culture while, rooted plantlets (27%) were noticed and scored as pasta-tolerant, non-tolerant plants (73%) could not grow and turned white (data not shown). Furthermore, the inheritance and expression of other transgenes into the tolerant seedlings will be confirmed by the PCR analysis.

As a conclusion, based on the data of the present study we can conclude that an optimized protocol for transformation of the Egyptian tomato local cultivar, Edkawy was obtained. In this protocol, the cytokinin 2IP was used in instead of the much expensive, Zeatin that was used in the most previous reports of tomato transformation. Improvement of shoot multiplication step using MS medium containing 1 mg L^{-1} BAP and 0.3 mg L^{-1} kinetin is another advantage of our protocol. The reported regeneration system is repeatable and can be easily used for further improvement of the important local tomato cultivar, Edkawy. In addition, we obtained transgenic F_0 tomato plants of Edkawy cultivar and the presence of transformed Bar and AtNHX1 genes in their genome was confirmed by the PCR analysis. Moreover, the expression of GUS gene in these T_0 plants and

inheritance of the transgene(s) into the second generation plants (F_1) was proved. Further selection and evaluation of salt-tolerance will be performed later in the transgenic homozyogous F_1 plants.

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