Salinity Tolerance of Tomato Infected with *Agrobacterium* Containing a Bacterial *mtlD* Gene Encoding Mannitol-1-Phosphate Dehydrogenase

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Abstract: *Putative transgenic plants of two tomato varieties (Castle Rock and Super Strain B) were obtained using* Agrobacterium*-mediated gene transfer procedures. The* mtlD *gene encoding mannitol-1-phosphate dehydrogenase that originally isolated from* E. coli *was the target gene. Percentages of produced shoots from* Agrobacterium *infected and non-infected explants grown on MS_1 medium containing 200 mg/l cefatoxime or 200 mg/l cefatoxime + 50 mg/l kanamycin were scored. No significant differences were observed between* Agrobacterium*-infected and non-infected explants when cultured on MS_1 medium containing cefatoxime only. Non-transformed explants cultured on medium with kanamycin and cefatoxime have turned yellow and neither developing callai nor regenerating shoots were produced from those explants. However, under the same selective conditions, some shoots were developed from cotyledonary leaves which, co-cultivated with* Agrobacterium *containing the* mtlD *gene. The percentages of* Agrobacterium*-infected explants that could produce shoots on MS_1 medium containing cefatoxime and kanamycin were 9.6% and 7.2% for Castle Rock and Super Strain B cultivars, respectively. Following micropropagtion on the selective medium containing kanamycin, most of these shoots showed further growth and have developed roots.*

Under NaCl stress, shoot-apexes of the Agrobacterium*-infected regenerates could develop further shoots whereas, those from non-infected regenerates showed no additional growth of shoots. In the absence of salt-stress, there were no significant differences in fresh weight of shoots between the non-infected (control) and Agrobacterium-infected regenerates. The mean fresh weight of the putative transgenic shoots increased about 2.4 (Castle Rock) and 2.8 (Super Strain B) folds higher than that of the control when cultured on MS medium supplemented with 1% NaCl. Concerning root formation, shoot-apexes of the putative transgenic plants produced roots, however, salt stress inhibited root formation of the non-transgenic shoots. The present results demonstrate that, infecting explants of tomato with* Agrobacterium *containing a bacterial* mtlD *gene encoding mannitol-1-phosphate dehydrogenase could improve growth of* Agrobacterium*-infected regenerates under salinity stress for both cultivars.*

INTRODUCTION

Water deficit and salinity are the most important abiotic factors that depress crop productivity in drought-prone areas. Efforts to improve yield through classical breeding have met with limited success primarily because tolerance to such stresses are controlled by many genes and their simultaneous selection is difficult (Richards, 1996; Yeo, 1998 and Flowers *et al.*, 2000). Unlike classical breeding, genetic engineering is a faster and more precise means of achieving improved tolerance (Cushman and Bohnert, 2000) because it avoids the transfer of unwanted chromosomal regions. Through genetic engineering resistance genes isolated from any organism can be selectively transferred to a target organism without the need for sexual reproduction.

Arrillaga *et al.*, (1998) improved tomato progeny for salt stress by transferring *hal2* gene to tomato tissue cultures and their regenerating plantlets. A significant progress was achieved by developing transgenic tomato plants over expressing AtNHX1, a single-gene controlling vacuolar Na^+/H^+ antiport protein, introduced from *Arabidopsis thaliana* (Zhang and Blumwald, 2001). The over expression of this gene was shown to improve salt tolerance in *Arabidopsis* (Apse *et al.*, 1999). Transgenic tomato plants over expressing this gene were able to grow, set flower and produce fruit in the presence of 200 mM NaCl in greenhouse hydroponics, whereas the non-transgenic plants did not survive under the saline

conditions. The transfer and over expression of the same AtNHX1 gene into canola (*Brassica napus*), resulted in salt-tolerant transgenic plants that were able to grow and produce seeds in the presence of 200 mM NaCl (Zhang *et al.*, 2001).

One major molecule that has been a target for engineering resistance to water deficit and salinity stress is mannitol which protects plants through osmotic adjustment, osmoprotection of macromolecules and serves as a sink for reducing power and storage of carbon. The *E. coli mtlD* gene encodes for mannitol-1-phosphate dehydrogenase which catalyses the reversible conversion of fructose-6-phosphate to mannitol-1-phosphate was expressed and translated into a functional enzyme in tobacco, resulting in the synthesis and accumulation of mannitol (Tarczynski *et al.*, 1992). Transgenic tobacco plants expressing the bacterial mannitol-1-phosphate dehydrogenase gene were tolerant to high concentrations (250 mM) of salt (Tarczynski *et al.*, 1993). It has been concluded that, accumulation of mannitol is associated with protection of plant cells from drought and salt stress (Karakas *et al.*, 1997 and Shen *et al.*, 1997a). *Arabidopsis* (Thomas *et al.*, 1995) transformed by *mtlD E. coli*-derived showed tolerance for osmotic and salt stress. Using the biolistic technique, Abebe *et al.*, (2000) have transformed wheat plants (*Triticum aestivum* cv. Bobwhite) with the *E. coli mtlD* gene. Tolerance to water stress and salinity was evaluated using calli and T_2 plants transformed with or without *mtlD* (Abebe *et al.*, 2003). The results showed that ectopic expression of the *mtlD* gene for the biosynthesis of mannitol in wheat improves tolerance to water stress and salinity. Similarly, Bahieldin, *et al.*, (2003) could develop Egyptian bread wheat with improved tolerance to salt stress using the bacterial *mtlD* gene. Transformed *Saccharomyces cerevisiae* with multicopy plasmids encoding the mannitol-1-phosphate dehydrogenase of *E. coli*, produced mannitol which restored the ability of a glycerol-defective osmosensitive mutant to grow in the presence of high NaCl concentrations (Chaturvedi *et al.*, 1997).

This study aimed at introducing *mtlD* gene into cotyledonary leaves of tomato and investigating the ability of regenerating transgenic plants to tolerate salt stress.

MATERIALS AND METHODS

Plant materials and target tissue for transformation

The seeds of the two tomato cultivars (Castle Rock and Super Strain B) were surface sterilized and germinated on MS medium. After 14 days, cotyledonary leaves of the grown seedlings were aseptically sectioned transversely in two parts and used as explants source for genetic transformation.

Agrobacterium strain

A. tumefaciens strain LBA 4404, which contains pCaMVMTLDL cassette sub-cloned into the *Hind*III site of the binary vector pBin19, was kindly provided by Professor B. J. Hans (Dept of Biochemistry, Univ of Arizona, Tucson, AZ 85721, USA). The structure of the HindIII fragment as described by Mitchell *et al.*, (1992) is shown in Figure (1).

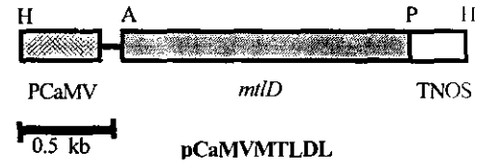

Figure 1: The structure of the *Hind*III fragment (sub-cloned into the *Hind*III site of the binary vector pBin19) contained a 35S promoter of CaMV virus (P CaMV), the *mtlD* structural gene with a 5 leader, and a nopaline synthase termination signal (T NOS).

Transformation

Culture of *A. tumefaciens* was maintained on solid LB medium (1% Bacto trypton, 0.5% Bacto yeast extract, 1% NaCl and 1.5% agar, pH 7.0) containing 50 mg /l kanamycin at 28 °C. Single colonies of a new culture were picked up and cultured in 50 ml of liquid LB medium containing 50 mg /l kanamycin with continuous shaking (200 rpm) at 28 °C to the mid-log phase (OD_{600} 1.2-1.5). *A. tumefaciens* bacterial cells were collected by centrifugation at 4000 rpm for 10 minutes and re-suspended in liquid MS medium supplemented with 40 mg/l acetosyrengone. The *A. tumefaciens* cells density was adjusted to give an OD_{600} of 0.5. Under aseptic conditions, the cotyledonary leaves of 14-days old seedlings were cut transversally and transferred to Petri dishes. MS medium with the bacterium was poured over the explants and incubated for 10 min then the cotyledonary leaves were gently dried on sterile filter papers.

The infected cotyledon discs were transferred to solid MS1 (Mahmoud *et al.*, 2004) supplemented with 40 mg/l acetosyringone to improve the transformation efficiency in plants through the co-cultivation time. The co-cultivation was performed at 20 °C in darkness for two days. Following co-cultivation, explants were washed with liquid MS medium containing 500 mgl^{-1} cefatoxime, dried on blotting paper and cultured on solid MS_1 medium supplemented with 200-mg/l of cefatoxime for five days to inhibit *A. tumefaciens* growth and for recovering without selection pressure. For selection of transgenic tissue and regenerated shoots, explants were transferred to selective MS_1 medium supplemented with 200 mg/l cefatoxime and 50 mg/l kanamycin.

After infection with *Agrobacterium* strain, 125 explants were cultured on MS_1 medium containing 50 mg/l kanamycin and 200 mg/l cefatoxime. There were 5 Petri dishes each with 25 explants per treatment. Two Petri dishes with 50 explants infected with *Agrobacterium* were cultured on MS_1 medium containing 200 mg/l cefatoxime. As a control, 50 non-treated explants, were cultured on MS_1 medium with the same concentration of kanamycin and cefatoxime and another 50 explants were cultured on kanamycin free medium. Shoots developed from transformed explants were micro-propagated on half-MS medium with 50 mg/l kanamycin.

Salt tolerance test

Shoot-apexes of control plants and mtlD-transformed plants, grown on MS_1 medium containing kanamycin, were cultured on MS_1 containing 0, or 10 gl^{-1} NaCl. After one-month, shoot-apexes showed further shoot and root growth were recorded and fresh weights of individual six plants were determined.

RESULTS AND DISCUSSION

In a previous work (Mahmoud *et al.*, 2005), the results indicate that all cotyledonary leaves of Castle Rock and Super Strain B tomato cultivars become necrotic and died within two weeks when cultured on medium with 50 mg/l kanamycin. On medium without kanamycin all explants for both cultivars survived and showed further shoot growth from cotyledonary leaves. Therefore, medium with 50 mg/l kanamycin is used as a selective medium following transformation in the present experiment.

Percentages of *Agrobacterium* infected and non-infected explants that produced shoots on MS_1 medium containing only 200 mg/l cefatoxime or 200 mg/l cefatoxime and 50 mg/l kanamycin are shown in Table 1. No significant differences were observed between infected and non-infected explants when cultured on MS_1 medium containing only 200 mg/l cefatoxime. These results indicate no negative effect of treating with *Agrobacterium* or the presence of cefatoxime on shoot regeneration of both cultivars. Non-infected explants cultured on medium with kanamycin and cefatoxime have turned yellow and no callus or shoots could develop from those explants under these conditions (Fig. 2). However, on this medium, some shoots were developed from cotyledonary leaves co-cultivated with *Agrobacterium* containing the *mtlD* gene (Table 1 and Fig. 2). The percentages of *Agrobacterium*-infected explants which produced shoots on MS_1 medium containing 200 mg/l cefatoxime and 50 mg/l kanamycin were 9.6% (12 explants out of 125) and 7.2% (9 explants out of 125) for Castle Rock and Super Strain B cultivars, respectively. These shoots were maintained in a similar medium for two months. Following micropropagtion on half-MS medium containing kanamycin, most of these shoots showed further growth and developed roots.

Table 1: Percentages of *Agrobacterium* infected and non-infected explants produced shoots on MS_1 medium containing 200 mg/l cefatoxime (Cef) or 200 mg/l cefatoxime + 50 mg/l kanamycin (K).

	NaCl (%)	Cultivar	
		Castle Rock	Super Strain B
Non-infected (Control)	Cef	88.0	92.0
	Cef + K	00.0	00.0
Agrobacterium -infected	Cef	85.0	87.0
	Cef + K	9.6	7.2

Some of these shoots did not show any further growth following micropropagtion on medium containing kanamycin. It seems that these shoots were developed as a chimera from transformed and non-transformed cells. Chimeric shoots that are composed of antibiotic-resistant and antibiotic-sensitive shoots following *Agrobacterium*-mediated transformation have already been observed (Firoozabady and Kuehnle, 1995). This was explained in Kohleria plants by Geier and Sangwan, (1996) as escaping shoots that could

have been protected from the inhibition effect of kanamycin by the aid of the transformed shoots. *Agrobacterium*-mediated transformed flax, which selected on medium containing kanmycin showed lower transgenic segregation than expected (Dong and Mchughen, 1993). It was suggested that the plants have developed from transformed as well as non- transformed tissue as a result of multi-cellular origin of shoot organogenesis. The failure of further growth following micropropagtion on medium containing kanamycin could also be attributed to the loss of the selectable marker or the whole *Agrobacterium* plasmid during the bacterial culture.

The restricted development of shoots under the stress of kanamycin and cefatoxime, only following *Agrobacterium* treatment, might confirms that the plants regenerated from treated explants are putative genetically transformed with target DNA. Consequently, transformation efficiency of tomato explants by *A. tumefaciens* strain was evaluated as a regeneration frequency on selective medium. However, PCR and/or DNA hybridization may be required in further studies to confirm the integration of transferred foreign DNA into the genome of such putative transgenic tomato regenerates.

The present results, is in agreement with many of those reported in other comparable studies in tomato where transformation efficiencies was [9 %, Van Roekel *et al.*, (1993); 11%, Ling *et al.*, (1998); 6%, Vidya *et al.*, (2000); and 20%, Park *et al.*, (2003)]. Variation in the efficiency of transformation was attributed to a number of factors such as plant variety (Ling *et al.*, 1998 and Ellul *et al.*, 2003), explants material (Ohki *et al.*, 1978; Fillati *et al.*, 1987 and Bird *et al.*, 1988), growth regulators (Ohki *et al.*, 1978; McCormick *et al.*, 1986 and Pfitzner, 1998), bacterial concentration (Shanin *et al.*, 1986) and *Agrobacterium* virulent genes inducers (Stachel *et al.*, 1986).

Salt tolerance test of *mtlD*-putative transgenic shoots

Salt tolerance was tested by evaluating the response of shoot-apexes of the regenerated shoots grown on MS_1 medium containing kanamycin to salt stress. This test was accomplished by culturing shoot-apexes of the plantlets produced from the *Agrobacterium* co-cultivated explants on Murashige and Skoog medium (1962) supplemented with 1% NaCl. In control, MS medium free from NaCl, all cultured shoot-apexes showed further shoot growth and root formation. However, in MS medium containing 1% NaCl, shoot-apexes from explants infected with *Agrobacterium* developed shoots and roots whereas, those from non-transformed plants showed no roots

The main fresh weight of shoots developed from shoot-apexes after one month of culturing on MS medium containing 0.0 or 1% NaCl are shown in Table 2. In the absence of salt-stress, there were no significant differences in fresh weight of shoots between the wild and transformed plants (Table 2– and Fig.3). However, under NaCl stress, the *mtlD* putative-transformed plants have had a growth advantage over the control in terms of higher fresh-weight and root production. The main fresh weight of the putative-transgenic shoots of Castle Rock cultivar increased about 2.3 fold higher than that of the control when cultured on MS medium supplemented with 1% NaCl. Regarding Super Strain b cultiver, under NaCl stress the main fresh weight of the putative-transgenic shoots increased to be about 2.7 fold higher than that of the control. These results indicate that *mtlD* gene do provide protection against salt stress. Concerning root formation, it was observed that the shoot-apexes of the putative transgenic plants produced roots, however, salt stress inhibited root formation of the non-transgenic shots (Fig.3).

Table 2. The main fresh weight (g) of *Agrobacterium*-infected tomato shoots grown under NaCl stresses. Stress was applied to shoot-apexes of the regenerated shoots grown on MS_1 medium containing kanamycin by supplementing the Murashige and Skoog medium with 1% NaCl. Measurements were taken one month after stress. Data are means of 6 shoot-apexes *.

	NaCl (%)	Cultivar	
		Castle Rock	Super Strain B
Non-transgenic (Control)	0.0	2.567 a	4.047 a
	1.0	0.842 c	1.140 c
Putative transgenic	0.0	2.220 ab	3.590 a
	1.0	1.950 b	3.032 b
$LSD_{0.05}$		0.395	0.493

*Means followed by the same letter in a column are not significantly different at $P < 0.05$ as determined by Fisher's protected LSD test.

The present results demonstrate that, co-cultivating explants of tomato with *Agrobacterium* containing a bacterial *mtlD*

gene of *E. coli*, encoding mannitol-1-phosphate dehydrogenase, could improve growth of *Agrobacterium*-infected regenerates under salinity stress for both cultivars. These findings are in agreement with earlier studies that used the same *mtlD* gene in tobacco (Tarczynski *et al.*, 1992, 1993; Karakas *et al.*, 1997; Shen *et al.*, 1997a) and wheat (Abebe *et al.*, 2003). Similarly, Su *et al.*, (1999) obtained three rice transgenic lines with bacterial *mtlD* and demonstrated that biosynthesis and accumulation of mannitol in plants correlated with salt-stress tolerance of

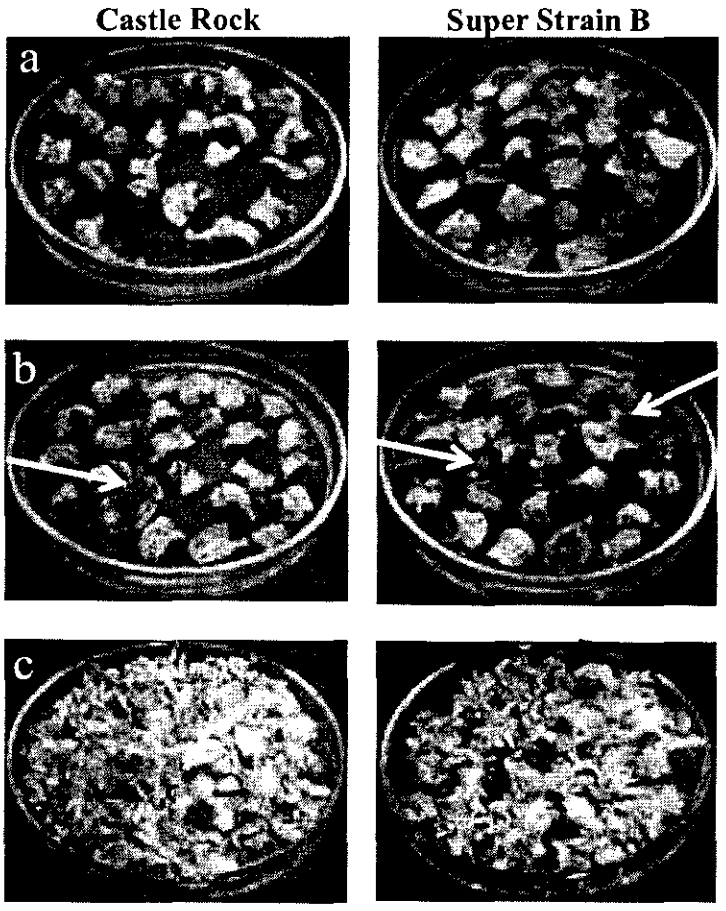

Figure 2: *Agrobacterium tumefaciens*-mediated transformation in tomato.
a: Non infected cotyledonary leaves on selective medium (MS_1+ 50 mg/l km)
b: Putative transgenic shoots (arrows) developed from *Agrobacterium*-infected cotyledonary leaves on MS_1 medium supplimented with 50 mg/l km.
c: Non-infected cotyledonary leaves on MS_1 medium without km

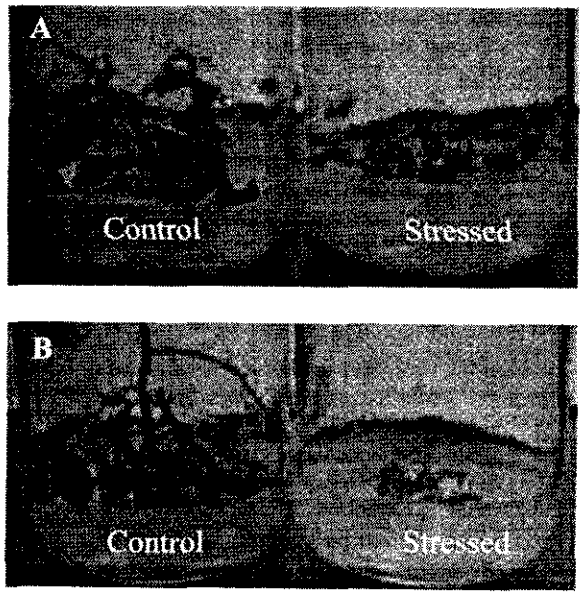

Figure 3: Effect of salinity on the growth of shoots regenerated from *Agrobacterium* infected and non-infected explants. Shoot-apexes of the regenerated shoots developed on MS_1 medium containing kanamycin were stressed by supplementing the MS medium with 1% NaCl for one month. (A), Castle Rock and (B), Super Strain B.

plants. *Arabidopsis thaliana* plants transformed with bacterial *mtlD* encoding mannitol-1-phosphate dehydrogenase have higher mannitol content and were able to withstand NaCl salinity up to 400 mM, whereas the wild type seeds ceased to germinate at 100 mM NaCl (Thomas *et al.*, 1995).

The enhancement of salt tolerance of the *Agrobacterium*-infected plants might be due to mannitol production result in *mtlD* gene expression. Mannitol has been proposed to enhance tolerance to water deficit stress primarily through osmotic adjustment (Loester *et al.*, 1992). Besides its function in osmotic adjustment, mannitol improves tolerance to stress through scavenging of hydroxyl radicals and stabilization of macromolecular structures (Smirnoff and Cumbes, 1989; Crowe *et al.*, 1992; Shen *et al.*, 1997a, 1997b). The importance of mannitol as a scavenger of the hydroxyl radical has been demonstrated in vitro (Smirnoff and Cumbes, 1989) and *in vivo* using transgenic tobacco (Shen *et al.*, 1997a).

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تحمل الملوحة فى الطماطم التي تم عدوتها بالاجروبكتريم الحاملة للجين البكتيري *mtlD* الذى يشفر للمنيتول-1-فوسفات ديهيدروجينيز

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تم في هذه الدراسة إنتاج نباتات من الطماطم من منفصلات نباتية تم عدوتها بالأجروبكتريم (*Agrobacterium*) والتى تحمل الجين*mtlD* المعزول من بكتريا القولون (*Escherichia coli*) واستخدم في هذه الدراسة الأوراق الفلقية كمنفصلات نباتية (Explants) لإجراء التحول الوراثى وقدرت النسبة المئوية للمنفصلات النباتية التى أمكنها تكوين أفرع خضرية على البيئة المحتوية علي 200 مللجم /لتر من السيفوتكس أو 200 مللجم /لتر السيفوتكس + 50 مللجم /لتر من الكاناميسين. ولم تظهر النتائج فروق معنوية بين المنفصلات النباتية المعاملة بالاجروبكتريم والغير معاملة (الكنترول) عند الزراعة علي البيئة المحتوية على السيفوتكس فقط أما عند الزراعة علي البيئة الإنتخابية المحتوية علي السيفوتكس والكاناميسن فقد حدث اصفرار وعدم قدرة على تكوين كالوسات أو أفرع خضرية وذلك بالنسبة للمنفصلات النباتية الغير معاملة بالأجروبكتريم في حين أن بعض المنفصلات النباتية المعاملة بالأجروبكتريم التي تحمل الجين*mtlD* أمكنها تكوين أفرع خضرية تحت نفس الظروف. وبلغت النسبة المئوية للمنفصلات النباتية التي حدث بها تكشف وكونت بعض الأفرع الخضرية 9.6 % و 7.2 % وذلك للصنفين كاسل روك و سوبر أسترين بى المستخدمين في هذه الدراسة على التوالى ، وأمكن لمعظم هذه الأفرع الخضرية مواصلة النمو وتكوين جذور علي البيئة الانتخابية المحتوية على الكاناميسين

وتحت ظروف الإجهاد الملحى الناتج عن إضافة ملح كلوريد الصوديم أمكن للقمم النامية المأخوذة من الأفرع الخضرية المتكشفة من المنفصلات النباتية المعاملة بالأجروبكتريم والنامية علي البيئة المحتوية علي الكاناميسين مواصلة النمو وتكوين المزيد من الأفرع الخضرية بينما القمم النامية المأخوذة من نباتات الكنترول (الغير معاملة بالأجروبكتريم) لم تتمكن من إعطاء المزيد من الأفرع الخضرية. وفي غياب الإجهاد الملحى لم تكن هناك فروق معنوية بين متوسطات الأوزان الطازجة للأفرع الخضرية لنباتات المقارنة (الكنترول) الغير معاملة بالإجروبكتريم وتلك المحولة وراثياً. وبلغت الزيادة فى متوسط وزن الأفرع الخضرية 2.4 مرة بالنسبة للصنف كاسل روك و 2.8 مرة بالنسبة للصنف سوبر أسترين بى بالمقارنة بنباتات الكنترول وذلك عند زراعتها علي البيئة المحتوية على 1% من كلوريد صوديم. أما بالنسبة لتكوين الجذور فقد أمكن للأفرع الخضرية النامية عن القمم النامية المأخوذة من النباتات المحولة وراثياً تكوين جذور تحت ظروف الإجهاد الملحى (1% كلوريد صوديوم) إلا أن هذا الإجهاد أدى إلى تثبيط كامل لتكوين الجذور من نباتات الكنترول. وتشير هذه النتائج إلى أن العدوى بالأجروبكتريم التي تحمل الجين *mtlD* المأخوذ من بكتريا القولون يمكن أن يحسن من قدرات النمو لنباتات الطماطم تحت ظروف الإجهاد الملحى.